LIPID NANOPARTICLES FOR DELIVERY OF THERAPEUTIC PAYLOADS TO T CELLS

CROSS REFERNCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of, and priority to, U.S. Provisional Application 63/592,197, filed on October 22, 2023; U.S. Provisional Application 63/598,347, filed on November 13, 2023; U.S. Provisional Application 63/641,887, filed on May 2, 2024; and U.S. Provisional Application 63/677,895, filed on July 31, 2024; the contents of each of which are hereby incorporated herein by reference in their entirety.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

[0002] The content of the electronic sequence listing (252052001640seqlist.xml; Size: 441,952 bytes; and Date of Creation: October 21, 2024) is herein incorporated by reference in its entirety.

BACKGROUND

[0003] The development of lipid nanoparticles (LNPs) has recently made significant advances towards intracellular delivery of payloads such as nucleic acids (e.g., mRNA or siRNA). LNPs are generally comprised of multiple components including an ionizable lipid, a PEGylated lipid, a helper lipid and cholesterol, all of which play important roles in effectively delivering the payload to diseased tissue.

[0004] Despite advances in the development of LNPs, substantial challenges still remain. Specifically, development of LNPs that provide a sufficient quantity of a payload to target cells has been challenging. In particular, development of LNPs that can deliver larger payloads and/or multiple payloads, such as those used in gene editing, is an important goal.

[0005] Additionally, delivery of therapeutic payloads to immune cells such as T cells is currently being investigated but can be challenging. For example, specific delivery of therapeutic agents (e.g., a genetic medicine payload, such as a gene therapy or gene editing agent), to T cells be difficult as any therapeutic agent delivered systemically must evade or overwhelm myriad mechanisms that will prevent its delivery to the relevant microenvironment (for example, liver uptake, phagocytic cell uptake, complement activation, opsonization, phagocytic cell uptake, etc.). Furthermore, after delivery to the target cell, the payload(s) must

be able to disassociate from the endosome so it can be expressed in sufficient quantities in the targeted cell.

[0006] Moreover, LNPs often exhibit unfavorable toxicological profiles, that precludes their use as therapeutic delivery vehicles. LNPs may accumulate and deliver payloads to cells other than the intended target, which results in potential toxicity.

[0007] Accordingly, there exists a need to develop LNPs for safe and effective delivery to immune cells.

SUMMARY OF THE INVENTION

[0008] The disclosure provides lipid nanoparticles (LNPs) or conjugates comprising a lipid nanoparticle (LNP) encapsulating a payload, such as a therapeutic agent (e.g., a gene modifying system) or reporter construct, for delivery to immune cells such as T cells. In some cases, the LNPs and conjugates described herein can deliver the payload to immune cell more effectively than a baseline conjugate or baseline LNP. In some embodiments, the disclosure also provides conjugates comprising an LNP encapsulating a payload, such as a therapeutic agent (e.g., a gene modifying system) or reporter construct, for delivery to immune cells (e.g., T cells) wherein the LNP has at least one targeting moiety on its surface. The conjugates having at least one targeting moiety described herein will also be referred to as targeted LNPs or tLNPs.

[0009] In one aspect, the disclosure provides a lipid nanoparticle (LNP) for delivery of a therapeutic agent to immune cells (e.g., T cells) *in vivo*, *in vitro*, or *ex vivo*, wherein the LNP comprises an ionizable lipid and a helper lipid, wherein the ionizable lipid is selected from V003 or from the lipids in **Table 1**, **Table 2** and **Table 3**, as set forth in the disclosure below, and a therapeutic agent encapsulated within the LNP.

[0010] In another aspect, the disclosure provides conjugates comprising an LNP and a targeting moiety. In some cases, inclusion of a targeting moiety of a targeted LNP enhances the ability of the conjugate or LNP to deliver the payload to immune cells (e.g., T cells). In some embodiments, the targeted LNPs can be formulated in a pharmaceutical composition and can be directly administered to a subject (e.g., patient) in need thereof (e.g., by *in vivo* administration). Once administered, the compositions can deliver significant quantities of a therapeutic agent to the immune cells (e.g., T cells) of the subject.

[0011] Alternatively, the conjugates can be added to isolated immune cells (e.g., T cells) using an *in vitro* or *ex vivo* procedure. Cells that can be derived from the blood, bone marrow, lymphoid organs, or lymph are cells of the immune system, such as cells of the innate or

adaptive immunity, *e.g.*, myeloid or lymphoid cells, including lymphocytes, typically T cells and/or NK cells. In some embodiments, blood is first collected from a subject (e.g., a human patient). In some embodiments, following isolation of the immune cells (e.g., T cells), the immune cells can be activated and expanded. Delivery of the LNPs of the disclosure to the activated T cells results in transduction of the desired payload into the immune cells (e.g., T cells). The activated immune cells (e.g., T cells) can then be administered to the patient. In other embodiments, the LNPs of the disclosure can be delivered to immune cells (e.g., T cells) without activating the immune cells/T cells. T cells that have not been activated prior to mixing with LNPs are referred to herein as rested T cells. In some embodiments, delivery of conjugates of the disclosure are capable of activating the rested immune cells (e.g., T cells).

[0012] In some embodiments, the payload can be one of more nucleic acid molecules. In some embodiments, the one or more nucleic acid molecules can be RNA molecules. In some embodiments, the one or more nucleic acid molecules can be DNA molecules. In some embodiments where the payload comprises two nucleic acid molecules, both nucleic acid molecules can be RNA molecules. In some embodiments where the payload comprises two nucleic acid molecules, both nucleic acid molecules can be DNA molecules. In some embodiments where the payload comprises one nucleic acid molecule can be an RNA molecule and one nucleic acid molecule can be a DNA molecule.

[0013] In some embodiments, the payload can be a nucleic acid encoding a reporter gene, such as green fluorescent protein (GFP). In some embodiments, the payload can be a therapeutic agent, for example, a therapeutic peptide or protein, a nucleic acid comprising a therapeutic agent, or a nucleic acid encoding a therapeutic agent. In some embodiments, the therapeutic agent can be a genetic medicine, wherein the therapeutic agent is capable of modifying, altering or effecting a change in the genomic DNA of a cell (e.g., a cell in the subject). In some embodiments, the therapeutic agent is a gene therapy agent or gene editing agent. In some embodiments, the therapeutic agent is a gene modifying polypeptide. In some embodiments, the therapeutic agent is a gene modifying system.

[0014] In some embodiments, the targeting moiety is an antibody, Fab fragment or single chain variable fragment (scFv), a DARPIN, a VHH domain antibody, a FN3 domain, a nanobody, a single domain antibody or a Centyrin. In other embodiments, the targeting moiety is a folate moiety, an antibiotic mimetic, a polynucleotide (such as a DNA or RNA apatamer), a carbohydrate, a vitamin or a N-Acetylgalactosamine (GalNac). In some embodiments, the targeting moiety is a peptide or protein, such as a ligand or part of a ligand, that binds to a

receptor (e.g., a receptor on the surface of a T cell). In some embodiments, the targeting moiety is a peptide or protein that binds to a receptor or ligand on the surface of an immune cells (e.g., T cells), wherein the affinity of the peptide or protein targeting moiety for the receptor or ligand on the surface of the immune cell (e.g., T cell) is modulated via phage display.

[0015] In certain embodiments, the payload (e.g., therapeutic agent) delivered by the LNP or targeted LNP can be a small molecule, peptide or protein, non-coding RNA (ncRNA), gRNA, siRNA or miRNA, a nucleic acid (e.g., mRNA) encoding a peptide or protein (e.g., a protein for replacement gene therapy, or a protein for modifying or altering the genome or epigenome, e.g., a protein for gene editing), a nucleic acid encoding or comprising one or more components of a system for altering a genome (e.g., one or more components of a ribonucleoprotein (RNP) complex for editing or altering the genome or epigenome (e.g., for introducing insertion-deletion mutations (indels), base editing, epigenetic editing, or target-primed reverse transcription (TPRT), e.g., by a mechanism that requires a recombinase, transposase, retrotransposase, helicase, reverse transcriptase, polymerase, deaminase, methylase, demethylase, or ligase function), or combinations thereof. In certain embodiments, the therapeutic agent (i.e., payload) delivered by the LNP or targeted LNP can be a gene modifying protein, a nucleic acid encoding a gene modifying protein, or a gene modifying system, as described herein.

[0016] In one aspect, the disclosure provides a targeted LNP (conjugate) encapsulating one or more nucleic acids encoding components of a system for modifying or altering DNA (e.g., genomic DNA), wherein the targeted LNP comprises one or more targeting moieties, such as antibodies, Fab fragments, or scFvs, capable of binding to one or more proteins on the cell surface of an immune cell (e.g., T cell). In some embodiments, the targeted LNP is delivered *in vivo* to a subject (e.g., a human patient) in need thereof. In some embodiments, the targeted LNP is delivered using an *ex vivo* or *in vitro* procedure. Following *in vivo*, *ex vivo*, or *in vitro* administration, the nucleic acids encoding components of a system for modifying or altering a genome can be expressed, resulting in altering of the genome (e.g., gene editing) of the immune cell (e.g., T cell). It has surprisingly been discovered that LNPs comprising particular targeting moieties (e.g., antibodies, Fab fragments or scFvs) or particular combinations of targeting moieties (e.g., antibodies, Fab fragments or scFvs), ionizable lipids, and/or helper lipids are capable of enhancing delivery of a therapeutic payload, (e.g., a system capable of altering the genome, such as a gene modifying system) to immune cells (e.g., T cells) following *in vivo* administration to a subject or *ex vivo* delivery, result in enhanced levels of therapeutic activity in the immune cells (e.g., T cells.)

[0017] In some embodiments, an LNP or targeted LNP (conjugate) comprising an ionizable lipid as described herein is capable of delivering a payload (e.g., a therapeutic payload) to 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 80%, 85%, 90%, 95%, or 99% more immune cells (e.g., T cells) than an LNP comprising a baseline lipid (such as V003). In some embodiments, an LNP comprising an ionizable lipid as described herein is capable of delivering a payload (e.g., a therapeutic payload) to 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 80%, 85%, 90%, 95%, or 99% more immune cells (e.g., T cells) than an LNP comprising a baseline lipid (such as V003) in an animal model, as described herein. In some embodiments, the LNP is a targeted LNP.

[0018] In some embodiments, a payload (e.g., a therapeutic payload) delivered to immune cells (e.g., T cells) by an LNP comprising an ionizable lipid as described herein is expressed at levels that are at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% greater than a payload comprising a baseline lipid (such as V003). In some embodiments, a payload (e.g., a therapeutic payload) delivered to immune cells (e.g., T cells) by an LNP comprising an ionizable lipid as described herein is expressed at levels that are at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% greater than a payload comprising a baseline lipid (such as V003). In some embodiments, the LNP is a targeted LNP.

[0019] In some embodiments, at least 2%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, or at least 40% immune cells (e.g., T cells) in a subject comprise a genome that have been modified or altered following *in vivo* administration of an LNP described herein, such as a targeted LNP encapsulating a therapeutic agent (e.g., a system for modifying, altering or editing a genome). In some embodiments, from about 5% to about 50%, from about 5% to about 20%, from about 10% to about 30%, from about 20% to about 50%, from about 20% to about 40%, or from about 20% to about 30% of immune cells (e.g., T cells) in a subject comprise a genome that has been modified or altered following *in vivo* administration of an LNP described herein, such as a targeted LNP.

[0020] In some embodiments, at least 2%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40% or at least 50% of immune cells (e.g., T cells) comprise a genome that have been modified or altered following *ex vivo* incubation of isolated activated or inactivated immune cells (e.g., T cells) with an LNP described herein, such as a targeted LNP encapsulating a therapeutic agent (e.g., a system for modifying, altering or editing a genome). In some embodiments, following *ex vivo* incubation, from about 5% to about

50%, from about 5% to about 20%, from about 10% to about 30%, from about 20% to about 50%, from about 20% to about 40%, or from about 20% to about 30% of immune cells (e.g., T cells) from the isolated immune cell (e.g., T cell) population comprise a genome that has been modified or altered.

[0021] The high level of genome modification or gene editing following *in vivo* administration or *ex vivo* delivery of the targeted LNPs of the disclosure (e.g., tLNPs comprising a gene modifying system configured to insert a CAR into the genome of a T cell) enables effective treatment of particular diseases such as cancer. In some embodiments, the cancer is an advanced leukemia or lymphoma. In some embodiments, the cancer is multiple myeloma. In some embodiments, the cancer is acute lymphoblastic leukemia (ALL), adult ALL, chronic lymphoblastic leukemia (CLL), non-Hodgkin lymphoma (NHL), or Diffuse Large B-Cell Lymphoma (DLBCL).

[0022] The high level of genome modification or gene editing following *in vivo* administration or *ex vivo* delivery of the targeted LNPs of the disclosure (e.g., tLNPs comprising a gene modifying system configured to insert a CAR into the genome of a T cell) also enables effective treatment of autoimmune diseases. In some embodiments, CAR-T cells can be generated using the tLNPs described herein to target B cell markers such as CD19, CD20, and BCMA. In some embodiments, the autoimmune disease is systemic lupus erythematosus, refractory antisynthetase syndrome, myasthenia gravis, systemic scleroderma, and multiple sclerosis.

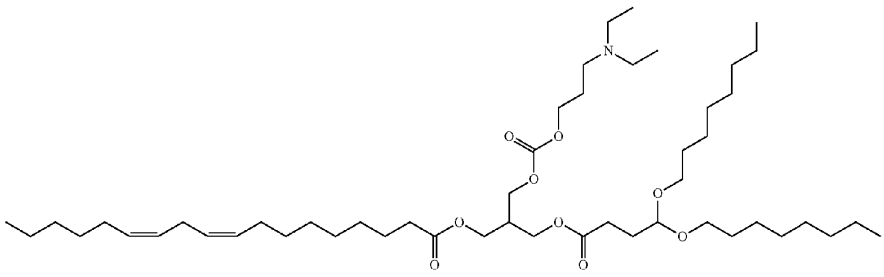
[0023] In some embodiments, the targeted LNPs (conjugates) of the disclosure may be used to modify T cells. In some embodiments, T-cells may include any subpopulation of T-cells, e.g., CD4+, CD8+, gamma-delta, naïve T cells, stem cell memory T cells, central memory T cells, or a mixture of subpopulations. In some embodiments, the conjugates may be used to deliver or modify a sequence encoding a T-cell receptor (TCR) in a T cell. In some embodiments, the conjugates may be used to deliver at least one sequence encoding a chimeric antigen receptor (CAR) to T-cells. For instance, in specific embodiments, the conjugates can be used to deliver an RNA encoding a CAR to T-cells. In some cases, the conjugates are formulated with an mRNA encoding a CAR. Such conjugates can be contacted with T cells to deliver the mRNA molecule encoding the CAR, resulting in cellular expression of the CAR. In some cases, the conjugates are formulated with a DNA molecule encoding a CAR, e.g., a plasmid or linear (e.g., a closed-ended linear DNA) encoding the CAR. Such conjugates can be contacted with T cells to deliver the DNA molecule encoding the CAR, resulting in cellular expression of the CAR. In

some embodiments, the conjugates may be used to deliver at least one sequence encoding a CAR to natural killer (NK) cells. In some embodiments, the conjugates may be used to deliver at least one sequence encoding a CAR to natural killer T (NKT) cells. In some embodiments, the conjugates may be used to deliver at least one sequence encoding a CAR to a progenitor cell, e.g., a progenitor cell of T, NK, or NKT cells.

[0024] In some embodiments, the conjugates can be used to deliver at least one gene modifying system (e.g., a retrotransposon gene modifying system) comprising a heterologous sequence encoding a CAR to an immune cell, e.g., a T cell. In some embodiments, the conjugates can be used to deliver at least one gene modifying system (e.g., a retrotransposon gene modifying system) to insert a heterologous sequence encoding a CAR into the genome of an immune cell, e.g., a T cell. In some embodiments, the conjugates can further be used to deliver at least one gene modifying system (e.g., a heterologous gene modifying system) to the immune cell, e.g., T cell. In some embodiments, the conjugates can further be used to deliver at least one gene modifying system (e.g., a heterologous gene modifying system) to modify or alter the TRAC locus and/or B2M locus in the genome of the immune cell, e.g., T cell. In some embodiments, modification or alteration of the TRAC locus and/or B2M locus results in a reduction or loss of expression of the TRAC and/or B2M locus.

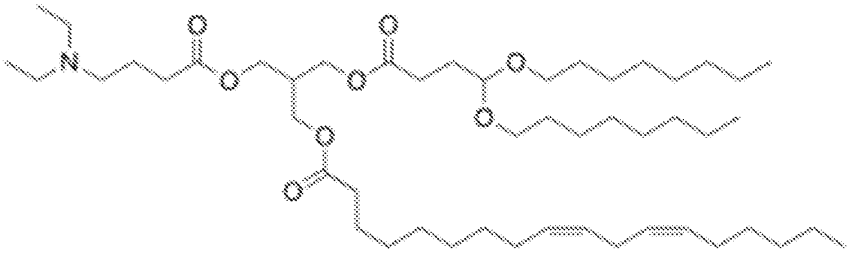
[0025] In some embodiments, the targeted LNPs (conjugates) of the disclosure may be used to simultaneously deliver gene modifying components to T cells that are capable of inserting a sequence encoding a CAR into the genome of the T cells and altering the T cell receptor alpha constant (TRAC) locus and/or the B2M locus in T cells, hence disrupting the TRAC gene and/or B2M gene. In some embodiments, simultaneous insertion of a CAR sequence and knock out of a TRAC and/or B2M gene can be accomplished via LNP delivery of two or more gene modifying systems, e.g., a retrotransposon gene modifying system and a heterologous gene modifying system. In some such embodiments, a single targeted LNP (conjugate) can deliver all of the components of a gene modifying system including (i) a gene modifying polypeptide; (ii) a template RNA (or DNA encoding the template RNA) that binds to the gene modifying polypeptide and a heterologous CAR sequence; and (iii) an additional gene modifying system for modifying DNA to install a mutation (e.g., resulting in knock down or knock out of the TRAC gene and/or B2M gene). Systems for modifying DNA to install a mutation (e.g., resulting in knock down or knock out of a target gene, or a correction or deletion of an aberrant gene) into a human gene are discussed in Section IV. In alternative embodiments, multiple targeted LNPs (conjugates) can be used to deliver the components one or more gene modifying systems. For instance, one conjugate can be used to deliver including (i) a gene modifying polypeptide; and

(ii) a template RNA (or DNA encoding the template RNA) that binds to the gene modifying polypeptide and a heterologous CAR sequence and a second conjugate can be used to deliver an additional gene modifying system for modifying DNA to install a mutation (e.g., resulting in knock down or knock out of the TRAC gene and/or B2M gene). In some embodiments, a first conjugate, as described herein, can be used to deliver a retrotransposon gene modifying system to an immune cell, e.g., a T cell, and a second conjugate, as described herein, can be used to deliver a heterologous gene modifying system to an immune cell, e.g., a T cell. In some embodiments, an LNP of the present disclosure, such as a targeted LNP, comprises the ionizable lipid V003, depicted below.

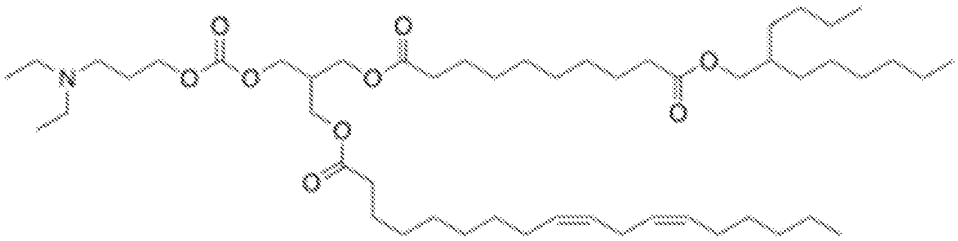


Lipid V003

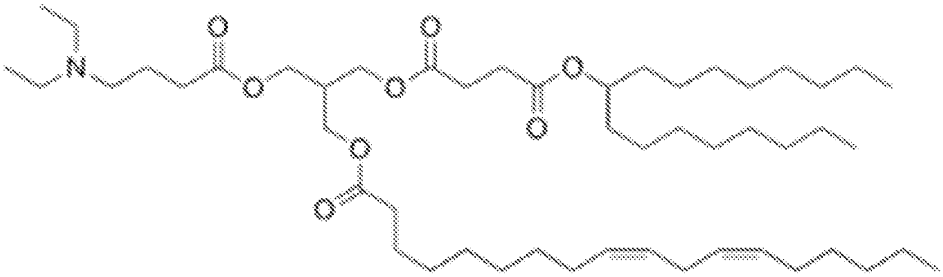
[0026] In some embodiments, an LNP of the present disclosure, such as a targeted LNP, comprises an ionizable lipid in **Table 1**. In some embodiments, the LNP (e.g., a targeted LNP) comprises an ionizable lipid in **Table 2**. In some embodiments, the LNP (e.g., a targeted LNP) comprises an ionizable lipid in **Table 3**. The LNPs and targeted LNPs detailed herein in some embodiments comprise any one or more ionizable lipid as detailed in Tables 1, 2, and 3. In some embodiments, the LNP and targeted LNPs detailed herein comprises 2 or more ionizable lipids, such as any two or more of V003 and the ionizable lipids in Tables 1, 2 and 3. In some embodiments, the LNP and targeted LNPs as detailed herein comprise only one ionizable lipid selected from the group consisting of ionizable lipids in Tables 1, 2 and 3. In some cases, an LNP containing s an ionizable lipid described herein exhibits higher levels of transduction in immune cells (e.g., T cells) and/or higher expression of a payload protein in immune cells (e.g., T cells) relative to LNPs that contain V003 as the ionizable lipid. In some cases, some of the ionizable lipids described herein provide higher levels of transduction and/or protein expression relative to V003 when used as a component of targeted LNP for *in vivo* or *ex vivo* delivery to immune cells (e.g., T cells). In some embodiments, the ionizable lipid has one of the structures depicted below:



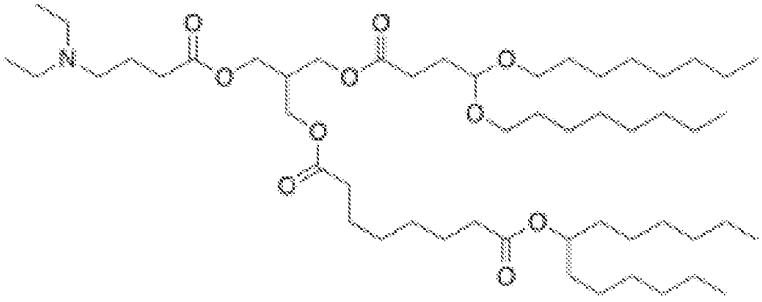
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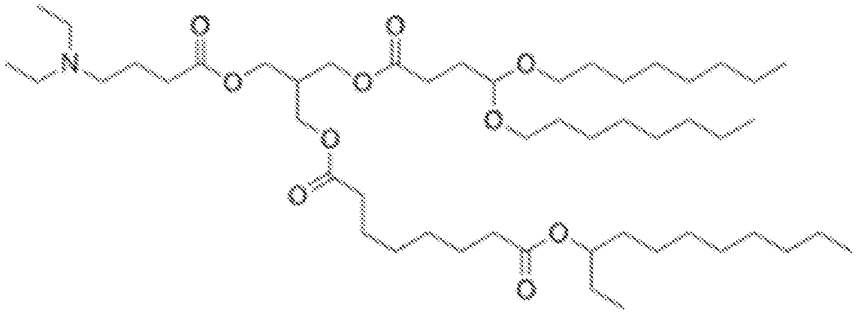
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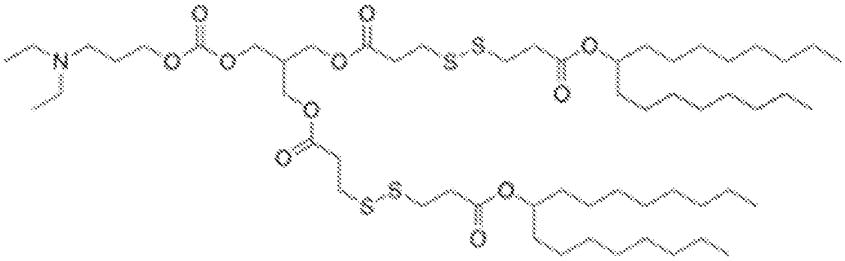
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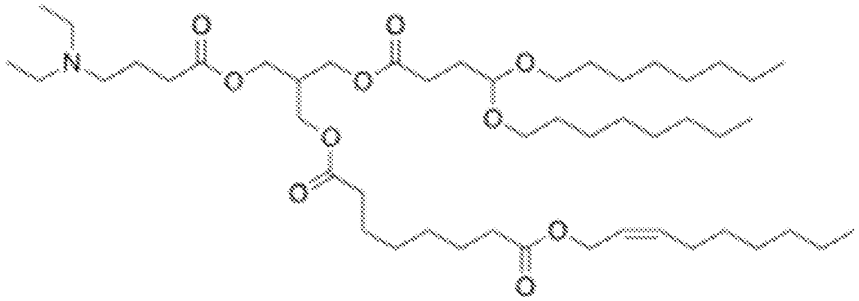
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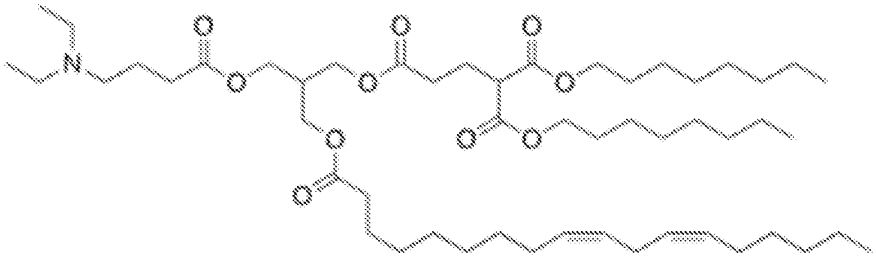
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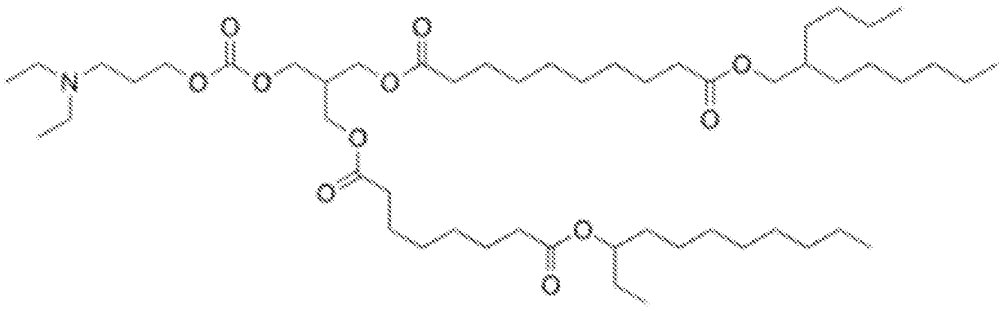
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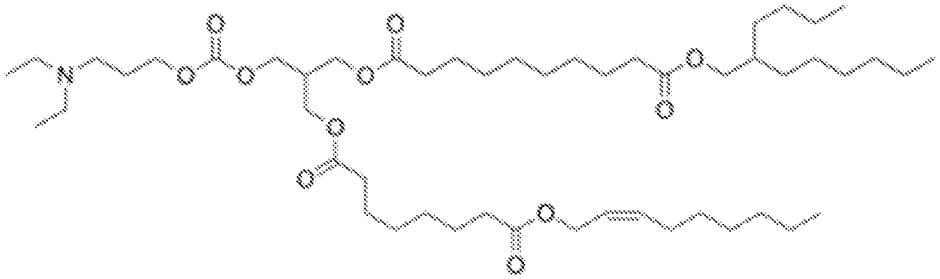
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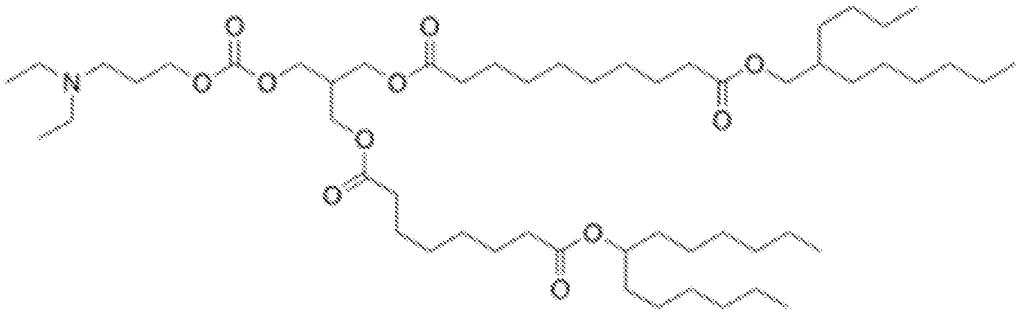
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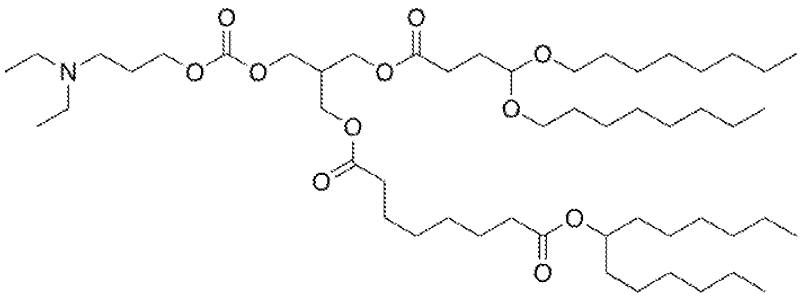
Lipid 176



Lipid 178



Lipid 183



Lipid 232

[0027] In some embodiments, the LNPs of the present disclosure, e.g., targeted LNPs, comprise one or more pegylated lipid molecules. In some embodiments, the targeting moiety

(e.g., antibody, Fab fragment or scFv) of a targeted LNP is associated with or chemically bonded to at least one of the pegylated lipid molecules. In some embodiments, the targeted LNP comprises from about 0.05 mol % to about 2 mol % of the pegylated lipid bonded to the targeting moiety. In some embodiments, the PEG spacer between the lipid and the targeting moiety comprises at least about 5, 10, 20, 30, 50, 50, 60, 70, 80, 90, 200, or 110 ethylene glycol units. In some embodiments, the PEG spacer comprises about 10-120 ethylene glycol units. In some embodiments, the molecular weight of the pegylated lipid bonded to the targeting moiety is from about 500 (i.e., PEG500) to about 5,000 (i.e., PEG5000). In some embodiments, the molecular weight of the pegylated lipid bonded to the targeting moiety is from about 1,000 (i.e., PEG1000) to about 3,000 (i.e., PEG5300). In some embodiments, the lipid component of the pegylated lipid bonded to the targeting moiety is selected from DMG, DPG, DSG, DTA, DOPE, DPPE, DMPE, DSPE, sphingosine, sphingomyelin, and stearic acid.

[0028] Pegylated lipids in the targeted LNPs of the disclosure can imbue different properties to the LNPS, including pharmacokinetics, distribution and endosomal release. Surprisingly, it has been discovered that LNPs comprising different pegylated lipids not covalently bound to a targeting moiety could show different toxicological properties when all other components of the LNPs (e.g., ionizable lipid) and relative proportions are the same. In particular, it has been discovered that LNPs comprising pegylated lipids with PEG lipid anchors having 16 carbon atoms (i.e., C16 PEG lipid anchors) or 18 carbon atoms (i.e., C18 PEG lipid anchors) could potentially reduce or eliminate the toxicity associated with identical LNPs other than the pegylated lipid component. In some embodiments, administration of the LNPs comprising C16 PEG lipid anchors or C18 PEG lipid anchors does not result in substantial liver toxicity. In some embodiments, administration of the targeted LNPs results in no or minimal increases in serum levels of the secreted liver enzyme alanine aminotransferase (ALT).

[0029] In some embodiments, the pegylated lipid has at least one C16 (palmitoyl) PEG lipid anchor. In some embodiments, the pegylated lipid has two C16 PEG lipid anchors (i.e., dialkyl chains of 16 carbons long). In some embodiments, the pegylated lipid is 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE-PEG2000.). In some embodiments, the pegylated lipid is 1,2-Dipalmitoyl-rac-glycero-3-methylpolyoxyethylene (DPG-PEG2000). In some embodiments, the pegylated lipid is C16 PEG ceramide. In some embodiments, the targeted LNPs comprising the C16 pegylated lipids show significantly reduced liver uptake than otherwise identical LNPS comprising C14 pegylated lipids.

[0030] In some embodiments, the pegylated lipid has at least one C18 PEG lipid anchor. In some embodiments, the pegylated lipid has two C18 PEG lipid anchors (i.e., dialkyl chains of 18 carbons long). In some embodiments, the C18 pegylated lipid is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000). In some embodiments, the C18 pegylated lipid is distearoyl-rac-glycerol-PEG2000 (DSG-PEG2000).

[0031] In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the pegylated lipid is DMG-PEG2000.

[0032] In some embodiments, an LNP of the present disclosure, e.g., a targeted LNP, comprises one or more non-pegylated lipids (e.g. non-pegylated phospholipids). Such lipids are often referred to as helper lipids. In some embodiments, the targeting moiety (e.g., antibody, Fab fragment or scFv) is associated with or chemically bonded to at least one of the non-pegylated lipids. In some embodiments, the non-pegylated lipid (helper lipid) is selected from POPC, DOPC, DOPE, and DSPC. In some embodiments, the non-pegylated lipid (helper lipid) is a sphingolipid. In some such embodiments, the non-pegylated lipid is a sphingomyelin. In some embodiments, the sphingomyelin has a head group selected from, phosphocholine, phosphoethanolamine or ceramide. In some embodiments, the sphingomyelin is egg sphingomyelin.

[0033] In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 18% to about 32%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP is from about 20% to about 30%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP is from about 22% to about 28%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 25% to about 30%. some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 30% to about 35%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%,

about 31%, about 32%, about 33%, about 34% or about 35%. As set forth in the examples below, *in vivo* delivery of certain payloads following administration of the disclosed LNPs with these percentages of helper lipids provides enhanced transduction and/or expression of the payloads relative to LNPs with smaller or larger quantities of helper lipid. It will be understood that mol% of the helper lipid as used herein refers to the mol% of the total lipid component of the LNP, which does not include the therapeutic agent (i.e., payload) or the targeting moiety.

[0034] In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP is from about 1:1 to about 7:1. In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 1:1 to about 4:1. In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 1:1 to about 3:1. In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC) is from about 1:1 to about 2.5:1. In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 1:1 to about 2:1. In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC) is from about 1.5:1 to about 2.5:1. In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 2:1 to about 2.5:1.

[0035] In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 20% to about 40% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP is from about 20% to about 40%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 20% to about 30% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 25% to about 40% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 28% to about 32% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 20% to about 40%. In some of the foregoing embodiments, the mol% of cholesterol in the targeted LNP is from about 30%-40% or from about 32% to about 37% (e.g., about 33%, about 34%, about 35% or about 36%).

[0036] In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 20% to about 40% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the

targeted LNP, is from about 25% to about 35%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 20% to about 30% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 25% to about 35%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 25% to about 40% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 25% to about 35%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 28% to about 32% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 25% to about 35%. In some of the foregoing embodiments, the mol% of cholesterol in the targeted LNP is from about 30%-40% or from about 32% to about 37% (e.g., about 33%, about 34%, about 35% or about 36%).

[0037] In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 20% to about 40% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 30% to about 35% (e.g., about 31%, about 32%, about 33%, about 34% or about 35%). In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 20% to about 30% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 30% to about 35% (e.g., about 31%, about 32%, about 33%m about 34% or about 35%). In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 25% to about 40% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 30% to about 35% (e.g., about 31%, about 32%, about 33%, about 34% or about 35%). In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 28% to about 32% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 30% to about 35% (e.g., about 31%, about 32%, about 33%, about 34% or about 35%). In some of the foregoing embodiments, the mol% of cholesterol in the targeted LNP is from about 30%-40% or from about 32% to about 37% (e.g., about 33%, about 34%, about 35% or about 36%).

[0038] In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 35% to about 60% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 35% to about 60% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 30% to about 40%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 35% to about 50% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 35% to about 50% and the mol% of the helper lipid (e.g., DSPC or

sphingomyelin) in the targeted LNP, is from about 30% to about 40%. In some of the foregoing embodiments, the mol% of cholesterol in the targeted LNP is from about 25%-40%,

[0039] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 092. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 092 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 092 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0040] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 093. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 093 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 093 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). PEG2000. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0041] In some embodiments, the targeted LNP comprises Lipid 153. In some embodiments, the targeted LNP comprises Lipid 153 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 153 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). PEG2000. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0042] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 154. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 154 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 154 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). PEG2000. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0043] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 155. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 155 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 155 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). PEG2000. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0044] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 162. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 162 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 162 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). PEG2000. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0045] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 163. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 163 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 163 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). PEG2000. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0046] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 169. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 169 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 169 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). PEG2000. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0047] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 176. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 176 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 176 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). PEG2000. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0048] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 178. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 178 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 178 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). PEG2000. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0049] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 183 In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 183 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 183 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). PEG2000. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0050] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 232. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 232 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 232 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). PEG2000. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0051] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid V003. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid V003 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid V003 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C16 alkyl chain (e.g., two C16 alkyl chains). PEG2000. In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the LNP is DPPE-PEG2000 or DPG-PEG2000.

[0052] In any of the foregoing embodiments, the LNP or conjugate can comprise one or more cholesterol molecules. In some embodiments, the molar ratio between the cholesterol molecule and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 6:1 to about 0.5:1. In some embodiments, the molar ratio between the cholesterol molecule and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 3:1 to about 0.5:1. In some embodiments, the molar ratio between the cholesterol molecule and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 2:1 to about 0.5:1. In some embodiments, the molar ratio between the cholesterol molecule and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 1.5:1 to about 0.5:1. In some embodiments, the molar ratio between the cholesterol molecule and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 1:1 to about 0.5:1. In some embodiments, the molar ratio between the cholesterol molecule and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 1:2 to about 0.8:1.

[0053] In some embodiments, the targeted LNPs (i.e., conjugates) are formed via a Click reaction between a first Click handle on the targeting moiety (e.g., antibody, Fab fragment, or scFv) and a second Click handle on the LNP, thereby generating a Click product. The second Click handle can be on one or more of the lipids comprising the LNP. In some embodiments, the second Click handle is covalently bonded to a pegylated lipid comprising the LNP.

[0054] In one embodiment, the Click product can be formed using a copper-catalyzed Click reaction. One such copper-catalyzed Click reaction is a Huisgen 1,3-dipolar cycloaddition (CuAAC) between an azide and an alkyne. In some embodiments, the first or second Click handle comprises a cyclic derivative of the alkynyl group. In some embodiments, the cyclic

derivative of the alkynyl group is selected from dibenzocyclooctyne. Cyclooctyne, and difluorinated cyclooctyne, In some embodiments, the click chemistry involves strain promoted cycloaddition of azides. In some embodiments, the click chemistry is based upon reaction of strained alkenes. In another embodiment, the Click product can be formed using copper-free Click chemistry. For example, the Click product can be formed between an azide and dibenzocyclooctene (DBCO). Alternatively, the Click product can be formed using a Staudinger reaction between an azide and a phosphine, hence producing an aza-ylide. In some embodiments, the Click product can be formed from an inverse electron demand Diels-Alder reaction between a trans-cyclooctene (TCO) moiety on the first or second Click handle and a tetrazine ring on the first or second Click handle. In some embodiments, the first Click handle comprises a tetrazine (Tz) ring and the second Click handle comprises a TCO moiety. In some embodiments, the tetrazine ring is unsubstituted. In some such embodiments, the tetrazine rung is methyltetrazine. In some embodiments, the tetrazine ring is a 6-methyl substituted tetrazine.

[0055] In some embodiments, the Click product is formed by conjugating an LNP to a targeting moiety that has been modified with an enzyme recognition sequence. For instance, an antibody, Fab fragment or single chain variable fragment (scFv), can be covalently linked to a first Click handle through a linker comprising an enzyme recognition sequence and the LNP is covalently linked to a second Click handle on the LNP, thereby generating a Click product. In some embodiments, the enzyme recognition sequence is a sortase recognition motif or a LplA acceptor peptide. In some embodiments, the antibody Fab fragment or scFv is directly bonded to the enzyme recognition sequence. In some embodiments, the antibody Fab fragment or scFv is bonded to the enzyme recognition sequence via one or more amino acid residues. Particular amino acid residues added that can be covalently attached to the C-terminus of the antibody, Fab fragment or scFv include, but are not limited to $(GGGGS)_v$, $(G)_v$, $(EAAAK)_v$, $(PAPAP)_v$, $(AP)_v$ and $A(EAAAK)_u$ $ALEA(EAAAK)_vA$, wherein u is 1-10 and v is 1-10.

[0056] In certain embodiments, the targeting moiety is a T-cell targeting moiety, for example, an antibody, Fab fragment or scFv that binds to a T-cell antigen selected from the group consisting of CD2, CD3, CD4, CD5, CD7, CD8, CD28, CD137, CD45, T-cell receptor (TCR)β,TCR-α, TCR-α/β, TCR-γ/δ, PD1, CTLA4, TIM3, LAG3, CD18, IL-2 receptor, CD11a, TLR2, TLR4, TLR5, IL-7 receptor, and r IL-15 receptor. In some embodiments the CD80 targeting moiety is a CD80 extracellular domain (ECD).

[0057] In some embodiments, the targeted LNP (conjugate) comprises a targeting moiety that targets (binds) a receptor on the surface of the T cell selected from CD2, CD3, CD4, CD5,

CD6, CD7, and CD8. In some embodiments, the targeting moiety targets a CD3 receptor on the surface of the T cell. In some embodiments, the targeting moiety targets a CD7 receptor on the surface of the T cell. In some embodiments, the targeting moiety targets a CD5 receptor on the surface of the T cell. In some embodiments, the targeting moiety targets a CD2 receptor on the surface of the T cell. In some embodiments, the targeting moiety targets a CD8 receptor on the surface of the T cell.

[0058] In some embodiments, the targeted LNP (conjugate) comprises a targeting moiety that targets CD3 on the surface of the T cell, wherein the targeting moiety is an antibody, Fab fragment or scFv selected from SP34, teclistamab, mosunetuzumab, odronextamab, tebentafusp, tepilizumab, muromonab and visilizumabm, or an antigen-binding portion thereof. In certain embodiments, the targeting moiety is SP34 or an antigen-binding portion thereof. In other embodiments, the targeting moiety is teclistamab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is mosunetuzumab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is odronextamab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is tebentafusp or an antigen-binding portion thereof. In other embodiments, the targeting moiety is muromonab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is visilizumab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is tepilizumab or an antigen-binding portion thereof.

[0059] In some embodiments, a targeted LNP (conjugate) for delivery of a therapeutic agent to immune cells (e.g., T cells) as described herein, comprises:

an ionizable lipid and a helper lipid, wherein the ionizable lipid is selected from V003 or any or the lipids in **Table 1**, **Table 2** and **Table 3**;

a plurality of targeting moieties conjugated to the LNP, wherein the plurality of targeting moieties bind to at least one targeting moiety on a T cell; and

a therapeutic agent encapsulated within the LNP.

[0060] In some embodiments, the plurality of targeting moieties bind to one or more T-cell antigens selected from the group consisting of CD2, CD3, CD4, CD5, CD7, CD8, CD28, CD137, CD45, T-cell receptor (TCR)β,TCR-α, TCR-α/β, TCR-γ/δ, PD1, CTLA4, TIM3, LAG3, CD18, IL-2 receptor, CD11a, TLR2, TLR4, TLR5, IL-7 receptor, and IL-15 receptor. In some embodiments, the targeted LNP (conjugate) comprises a targeting moiety that targets a receptor on the surface of the T cell selected from CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28.

[0061] A targeted LNP in some embodiments comprises two or more different targeting moieties, each of which may bind to the same or a different target. A targeted LNP having two different targeting moieties which bind to the same or different targets may be referred to an a "dual binder" or "dual binding" LNP. In some embodiments, a targeted LNP comprises a plurality of targeting moieties that bind to two or more T-cell antigens selected from the group consisting of CD2, CD3, CD4, CD5, CD7, CD8, CD28, CD137, CD45, T-cell receptor (TCR)β,TCR-α, TCR-α/β, TCR-γ/δ, PD1, CTLA4, TIM3, LAG3, CD18, IL-2 receptor, CD11a, TLR2, TLR4, TLR5, IL-7 receptor, and IL-15 receptor

[0062] In some embodiments, the plurality of targeting moieties can be added to the surface of the LNP by methods described in Section II of the Detailed Description. For instance, in some embodiments, each targeting moiety of the dual binding LNP is conjugated to the lipid nanoparticle through a linker, wherein each linker comprises a Click product formed from a Click reaction between a first Click handle on the targeting moiety and a second Click handle on the LNP. In some such embodiments, the targeting moieties are antibodies or antigen binding fragments thereof. In other such embodiments, the targeting moieties are scFvs. In some embodiments, the targeting moieties are Fab fragments.

[0063] In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein one targeting moiety binds to CD3 and the other targeting moiety binds to CD5. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein one targeting moiety binds to CD3 and the other targeting moiety binds to CD7. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein one targeting moiety binds to CD3 and the other targeting moiety binds to CD8. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein one targeting moiety binds to CD3 and the other targeting moiety binds to CD28. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein one targeting moiety binds to CD5 and the other targeting moiety binds to CD28. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein one targeting moiety binds to CD7 and the other targeting moiety binds to CD28. In some embodiments the CD28 targeting moiety is a CD80 extracellular domain (ECD).

[0064] In some embodiments, the targeted LNP (conjugate) comprises three targeting moieties, wherein one targeting moiety binds to CD3. In some such embodiments, one of the targeting moieties binds to CD7 and the other targeting moiety binds to CD28. In some such embodiments, one of the targeting moieties binds to CD5 and the other targeting moiety binds to

CD28. In some embodiments the CD28 targeting moiety is a CD80 extracellular domain (ECD). In some of the foregoing embodiments, the targeting moiety that binds to CD3 is SP34 or an antigen-binding portion. In some of the foregoing embodiments, the targeting moiety that binds to CD3 is teclistamab or an antigen-binding portion.

[0065] In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein each targeting moiety binds to the same target (e.g., receptor) on the T cell. For instance, in some embodiments, both targeting moieties of the conjugate bind to CD3. In some such embodiments, one of the targets is SP34 or an antigen-binding portion thereof and the other is teclistamab or an antigen-binding portion thereof. In other such embodiments, one of the targets is SP34 or an antigen-binding portion thereof and the other is visilizumab or an antigen-binding portion thereof. In other such embodiments, one of the targets is SP34 or an antigen-binding portion thereof and the other is tepilizumab or an antigen-binding portion thereof. In other such embodiments, one of the targets is visilizumab or an antigen-binding portion thereof and the other is tepilizumab or an antigen-binding portion thereof. In other such embodiments, one of the targets is visilizumab or an antigen-binding portion thereof and the other is teclistamab or an antigen-binding portion thereof. In some embodiments, both targeting moieties of the conjugate bind to CD3. In some embodiments, both targeting moieties of the conjugate bind to CD7.

[0066] In some aspects, the disclosure provides a lipid nanoparticle (LNP) comprising:

(i) a lipid component comprising an ionizable lipid and a helper lipid,;

(ii) one or two targeting moieties conjugated to the LNP, wherein the one or two targeting moieties are configured to target one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; and

(iii) a first mRNA encoding a first gene modifying polypeptide; and

(iv) a first template RNA that binds to the first gene modifying polypeptide and comprises a heterologous object sequence.

[0067] In some embodiments, heterologous object sequence encodes a chimeric antigen receptor (CAR), wherein the CAR comprises an antigen-binding domain, a transmembrane domain, a first intracellular signaling domain, and a second intracellular signaling domain. In some embodiments, the heterologous sequence comprises SEQ ID NO: 7.

[0068] In other aspects, the disclosure provides a targeted lipid nanoparticle (LNP) for *ex vivo* or *in vivo* delivery of a therapeutic agent to immune cells (e.g., T cells), wherein the targeted LNP comprises:

i. an ionizable lipid;

ii. a helper lipid;

iii. two different targeting moieties conjugated to the LNP, wherein each of the two different targeting moieties binds to a target on the surface of the immune cells (e.g., T cells); and

iv. one or more nucleic acids (e.g., two or more nucleic acids) encoding components of a system for modifying or altering genomic DNA (e.g., a gene modifying system), wherein the one or more nucleic acids (e.g., two or more nucleic acids) are RNA molecules.

[0069] In some embodiments, the two different targeting moieties can be added to the surface of the LNP by methods described in Section II of the Detailed Description. For instance, in some embodiments, each of the targeting moieties is conjugated to the lipid nanoparticle through a linker, wherein each linker comprises a Click product formed from a Click reaction between a first Click handle on the targeting moiety and a second Click handle on the LNP. In some such embodiments, the targeting moieties are antibodies or antigen binding fragments thereof. In other such embodiments, the targeting moieties are scFvs. In some embodiments, the targeting moieties are Fab fragments.

[0070] In some embodiments, one of the RNA molecules encoding a component of a system for modifying or altering genomic DNA (e.g., a gene modifying system) is an mRNA encoding a gene modifying polypeptide, as described herein. In some embodiments, one of the components of a system for modifying or altering genomic DNA (e.g., a gene modifying system) is an mRNA encoding a retrotransposon. In some embodiments, one of the components of a system for modifying or altering genomic DNA (e.g., a gene modifying system) is an mRNA encoding a Cas9 nickase fused to a reverse transcriptase (RT) domain. In some embodiments, one of the components of a system for modifying or altering genomic DNA is an mRNA encoding a Cas9-RT fusion protein.

[0071] In some embodiments, one of the RNA molecules comprising a component of a system for modifying or altering genomic DNA (e.g., a gene modifying system) is a guide RNA (gRNA). In some embodiments, one of the RNA molecules comprising a component of a system

for modifying or altering genomic DNA (e.g., a gene modifying system) is a template RNA encoding a heterologous nucleic acid for use with a gene modifying polypeptide to insert the heterologous nucleic acid sequence into a DNA sequence, e.g., the genomic DNA of a cell. In some embodiments, the components of a system for modifying or altering genomic DNA (e.g., a gene modifying system) have nuclease activity, e.g., nickase activity. In some embodiments, the components of a system for modifying or altering genomic DNA (e.g., a gene modifying system) do not have nuclease activity. In some embodiments, the components of a system for modifying or altering genomic DNA (e.g., a gene modifying system) do not elicit a double-stranded break in the genomic DNA. In such embodiments, the system for modifying or altering genomic DNA elicits a single-stranded break in the genomic DNA. In some embodiments, system for modifying or altering genomic DNA induces target-primed reverse transcription (TPRT) to insert a heterologous sequence into the genomic DNA.

[0072] In some embodiments, the ionizable lipid is V003 or a lipid from **Table 1**, **Table 2** or **Table 3**. In some embodiments, the ionizable lipid is Lipid 092. In some embodiments, the ionizable lipid is Lipid 154.

[0073] In some embodiments, the helper lipid of the targeted LNP is DSPC or sphingomyelin. . In some embodiments, the mo1% of the DSPC or sphingomyelin in the targeted LNP is from about 20% to about 40%. In some embodiments, the mo1% of the DSPC or sphingomyelin in the targeted LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the targeted LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%.

[0074] In some embodiments, the targeted LNP further comprises a pegylated lipid. In some embodiments, the pegylated lipid comprises at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the pegylated lipid is DMG-PEG2000. In some embodiments, the pegylated lipid comprises at least one C16 alkyl chain (e.g., two C16 alkyl chains). In some such embodiments, the pegylated is DPPE-PEG2000 or DPG-PEG2000.

[0075] In some embodiments, the targeted delivery (*ex vivo* or *in vivo*) of a system for modifying or altering genomic DNA (e.g., a gene modifying system) with an LNP comprising a T cell specific targeting moiety (e.g., an anti-CD3 targeting moiety) conjugated to the LNP results in gene editing or modification of the target T cells. In some embodiments, the LNPs comprising the targeting moiety are capable of modifying at least 5% of the genomes of the T cells. In some embodiments, the LNPs comprising the targeting moiety are capable of

modifying at least 10% of the genomes of the T cells. In some embodiments, the LNPs comprising the targeting moiety are capable of modifying at least 15% of the genomes of the T cells. In some embodiments, the LNPs comprising the targeting moiety are capable of modifying at least 20% of the genomes of the T cells. In some embodiments, the LNPs comprising the targeting moiety are capable of modifying at least 25% of the genomes of the T cells. In some embodiments, the LNPs comprising the targeting moiety are capable of modifying at least 30% of the genomes of the T cells.

[0076] In some embodiments, the targeted delivery (*ex vivo* or *in vivo*) of a system for modifying or altering genomic DNA (e.g., a gene modifying system) with an LNP comprising two or more T cells specific targeting moieties conjugated to the LNP results in synergistic enhancement of gene editing or modification of the target T cells. In some embodiments, the LNPs comprising two or more T cells specific targeting moieties are capable of modifying at least 5% of the genomes of the T cells. In some embodiments, the LNPs comprising two or more T cells specific targeting moieties are capable of modifying at least 10% of the genomes of the T cells. In some embodiments, the LNPs comprising two or more T cells specific targeting moieties are capable of modifying at least 15% of the genomes of the T cells. In some embodiments, the LNPs comprising two or more T cells specific targeting moieties are capable of modifying at least 20% of the genomes of the T cells. In some embodiments, the LNPs comprising two or more T cells specific targeting moieties are capable of modifying at least 25% of the genomes of the T cells. In some embodiments, the LNPs comprising two or more T cells specific targeting moieties are capable of modifying at least 30% of the genomes of the T cells. In some embodiments, the LNPs comprising two or more T cells specific targeting moieties are capable of modifying at least 30% of the genomes of the T cells. In some embodiments, the LNPs comprising two or more T cells specific targeting moieties are capable of modifying from about 10% to about 30% of the genomes of the T cells. In some embodiments, the LNPs comprising two or more T cells specific targeting moieties are capable of modifying from about 20% to about 30% of the genomes of the T cells. In some embodiments, the LNPs comprising two or more T cells specific targeting moieties are capable of modifying from about 10% to about 20% of the genomes of the T cells.

[0077] In some embodiments, the disclosure provides a method of delivering a therapeutic agent to the T cells of a subject, said method comprising, isolating T cells from the subject; mixing a pharmaceutical composition comprising targeted LNPs of the disclosure with the T cells for a sufficient time to allow for encapsulation of the therapeutic agent in the T cells; and administering the T cells encapsulating the therapeutic agent to the subject. In some

embodiments, the T cells are not activated prior to mixing with the pharmaceutical composition comprising targeted LNPs. In some embodiments, the targeted LNPs include at least one CD3 binder (e.g., antibody or fragment thereof) on their surface. In some embodiments, the targeted LNPs include at least one CD3 binder (e.g., antibody or fragment thereof) on their surface and a CD2, CD3, CD4, CD5, CD7 or CD28 binder (e.g., antibody or fragment thereof) on their surface. In some embodiments, the targeted LNPs include a CD3 binder (e.g., antibody or fragment thereof) and a CD28 binder (e.g., antibody or fragment thereof).

[0078] In some embodiments, the disclosure provides a method for administering a therapeutic composition to a patient, comprising, collecting a blood fraction comprising lymphocytes from the patient; isolating T cells from the blood fraction; contacting the isolated T cells with targeted lipid nanoparticles (LNPs) of the disclosure, thereby producing T cells encapsulating a therapeutic payload; and reinfusing the T cells of step into the patient. In some embodiments, the T cells are not activated prior to mixing with the pharmaceutical composition comprising targeted LNPs. In some embodiments, the targeted LNPs include at least one CD3 binder (e.g., antibody or fragment thereof) on their surface. In some embodiments, the targeted LNPs include at least one CD3 binder (e.g., antibody or fragment thereof) on their surface and a CD2, CD3, CD4, CD5, CD7 or CD28 binder (e.g., antibody or fragment thereof) on their surface. In some embodiments, the targeted LNPs include a CD3 binder (e.g., antibody or fragment thereof) and a CD28 binder (e.g., antibody or fragment thereof).

[0079] In any of the foregoing embodiments, the system for modifying or altering genomic DNA elicits a single-stranded break in the genomic DNA. In some such embodiments, a system for modifying or altering genomic DNA includes a gene modifying polypeptide and a template RNA for a nucleic acid sequence to be inserted at a specific location of the genomic DNA, hence resulting in modification of the genomic DNA. In some embodiments, the gene modifying system comprises a retrotransposon. In some embodiments, the gene modifying system comprises a Cas9 nickase fused to a reverse transcriptase (RT) domain. In some embodiments, a system for modifying or altering genomic DNA induces target-primed reverse transcription (TPRT) to insert a heterologous nucleic acid sequence into a DNA sequence, e.g., genomic DNA.

[0080] In some embodiments, the disclosure provides a lipid nanoparticle (LNP) comprising: (i) a lipid component comprising an ionizable lipid and a helper lipid; (ii) one or two targeting moieties conjugated to the LNP, wherein the one or two targeting moieties are antibodies or fragments thereof configured to bind one or more receptors on the surface of T cells selected

from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; (iii) an mRNA encoding a gene modifying polypeptide; and (iv) a template RNA comprising (a) a sequence that binds to the polypeptide and (b) a heterologous object sequence. In some embodiments, the LNP comprises a CD3 targeting moiety, optionally wherein the CD3 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 19 or Table 32 (or CDR sequences (bolded) selected from Table 19 or Table 32). In some embodiments, the LNP comprises a CD2 targeting moiety, optionally wherein the CD2 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD5 targeting moiety, optionally wherein the CD5 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD7 targeting moiety, optionally wherein the CD7 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD28 targeting moiety. In some embodiments, the LNP comprises a CD4 targeting moiety. In some embodiments, the LNP comprises a CD8 targeting moiety. In some embodiments, the LNP comprises two targeting moieties configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28. In some embodiments, the two targeting moieties are configured to bind to CD3 and CD2, CD3 and CD4, CD3 and CD5, CD3 and CD7, CD3 and CD8 or CD3 and CD28. In some embodiments, the CD3 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 19 or Table 32, and the CD2, CD5, or CD7 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 20. In some embodiments, the ionizable lipid is present at about 20 mol% to about 40 mol% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component. In some embodiments, the ionizable lipid is present at about 20 mol% to about 30 mol% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component. In some embodiments, the ionizable lipid is present at about 20 mol% to about 40 mol% of the lipid component, and the helper lipid is present at about 25 mol% to about 40 mol% of the lipid component. In some embodiments, the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, or about 20 mol% to about 40 mol% of the lipid component, or about 20 mol% to about 30 mol% of the lipid component or about 25 mol% to

about 40 mol% of the lipid component and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component. In some embodiments, the template RNA comprises a sequence present in Table 5B or a sequence selected from SEQ ID NOs: 395-404.

[0081] In some embodiments, the disclosure provides a lipid nanoparticle (LNP), comprising: (i) a lipid component comprising an ionizable lipid and a helper lipid, wherein the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, about 20 mol% to about 40 mol% of the lipid component, about 20 mol% to about 30 mol% of the lipid component or about 25% to about 40% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component; (ii) one or two targeting moieties conjugated to the LNP, wherein the one or two targeting moieties are antibodies or fragments thereof configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; and (iii) a therapeutic agent encapsulated within the LNP, wherein the therapeutic agent comprises one or more nucleic acid molecules (e.g., one or more RNA molecules). In some embodiments, the LNP comprises a CD3 targeting moiety, optionally wherein the CD3 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 19 or Table 32 (or CDR sequences (bolded) selected from Table 19 or Table 32). In some embodiments, the LNP comprises a CD2 targeting moiety, optionally wherein the CD2 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD5 targeting moiety, optionally wherein the CD5 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD7 targeting moiety, optionally wherein the CD7 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD28 targeting moiety. In some embodiments, the LNP comprises a CD4 targeting moiety. In some embodiments, the LNP comprises a CD8 targeting moiety. In some embodiments, the LNP comprises two targeting moieties configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28. In some embodiments, the two targeting moieties are configured to bind to CD3 and CD2, CD3 and CD4, CD3 and CD5, CD3 and CD7, CD3 and CD8 or CD3 and CD28. In some embodiments, the CD3 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 19 or Table

32, and the CD2, CD5, or CD7 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 20. In some embodiments, the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, about 20 mol% to about 40 mol% of the lipid component, about 20 mol% to about 30 mol% of the lipid component or about 25% to about 40% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component. In some embodiments, the therapeutic agent comprises a DNA molecule. In some embodiments, the therapeutic agent comprises an mRNA molecule. In some embodiments, the therapeutic agent comprises two nucleic acid molecules, wherein one nucleic acid is an mRNA and the other nucleic acid is a template RNA. In some embodiments, the therapeutic agent comprises a gene modifying system. In some embodiments, the therapeutic agent comprises a heterologous gene modifying system. In some embodiments, the therapeutic agent comprises a gene modifying system, and the template RNA of the system comprises a sequence present in Table 5B or a sequence selected from SEQ ID NOs: 395-404.

[0082] In some embodiments, the disclosure provides a lipid nanoparticle (LNP), comprising: (i) a lipid component comprising an ionizable lipid and a helper lipid, wherein the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, about 20 mol% to about 40 mol% of the lipid component, about 20 mol% to about 30 mol% of the lipid component or about 25% to about 40% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component; (ii) means for binding a cell surface receptor selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; and (iii) a therapeutic agent encapsulated within the LNP, wherein the therapeutic agent comprises one or more nucleic acid molecules (e.g., one or more RNA molecules). In some embodiments, the means for binding the cell surface receptor is present on the surface of the LNP. In some embodiments, the means for binding a cell surface receptor comprises a targeting moiety, such as an antibody or fragment thereof. In some embodiments, the LNP comprises a CD3 targeting moiety, optionally wherein the CD3 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 19 or Table 32 (or CDR sequences (bolded) selected from Table 19 or Table 32). In some embodiments, the LNP comprises a CD2 targeting moiety, optionally wherein the CD2 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD5 targeting moiety, optionally wherein the CD5 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain

sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD7 targeting moiety, optionally wherein the CD7 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD28 targeting moiety. In some embodiments, the LNP comprises a CD4 targeting moiety. In some embodiments, the LNP comprises a CD8 targeting moiety. In some embodiments, the LNP comprises two targeting moieties configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28. In some embodiments, the two targeting moieties are configured to bind to CD3 and CD2, CD3 and CD4, CD3 and CD5, CD3 and CD7, CD3 and CD8 or CD3 and CD28. In some embodiments, the CD3 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 19 or Table 32, and the CD2, CD5, or CD7 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 20. In some embodiments, the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, about 20 mol% to about 40 mol% of the lipid component, about 20 mol% to about 30 mol% of the lipid component or about 25% to about 40% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component. In some embodiments, the therapeutic agent comprises a DNA molecule. In some embodiments, the therapeutic agent comprises an mRNA molecule. In some embodiments, the therapeutic agent comprises two nucleic acid molecules, wherein one nucleic acid is an mRNA and the other nucleic acid is a template RNA. In some embodiments, the therapeutic agent comprises a gene modifying system. In some embodiments, the therapeutic agent comprises a heterologous gene modifying system. In some embodiments, the therapeutic agent comprises a gene modifying system, and the template RNA of the system comprises a sequence present in Table 5B or a sequence selected from SEQ ID NOs: 395-404.

[0083] In some embodiments, the disclosure provides a gene modifying system comprising a gene modifying polypeptide (or a nucleic acid, e.g., a DNA or mRNA molecule, encoding the gene modifying polypeptide) and a template RNA, wherein the template RNA comprises a sequence present in Table 5B or a sequence selected from SEQ ID NOs: 395-404. In some embodiments, the gene modifying polypeptide comprises an amino acid sequence of Table 4 or Table 5A or a sequence having no more than 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 amino acid differences thereto, or a nucleic acid (e.g., DNA or mRNA) encoding the gene modifying polypeptide. In some embodiments, the gene modifying polypeptide comprises an amino acid

sequence of any one of SEQ ID NOs: 32, 33, 64 or 65, or a sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto. In some embodiments, the template RNA comprises both of a 5'UTR_{retro} and a 3' UTR_{retro}. In some embodiments, the 5'UTR_{retro} and the 3' UTR_{retro} comprise 5' or 3' sequences of Table 4 (e.g., SEQ ID NO: 44 and/or SEQ ID NO: 55) or any of Examples 4-11 or 15-17.

BRIEF DESCRIPTION OF THE DRAWINGS

[0084] **FIG. 1** provides a bar graph showing that higher levels of transfection of targeted LNPs in activated T cells are achieved in the absence of serum.

[0085] **FIGs. 2A** and **2D** provide bar graphs showing the percent of activated T cells that express GFP and the GFP expression levels (MFI), respectively, when the anti-CD3 tLNPs are transfected into activated T cells in culture in the presence of serum (FBS). **FIGs. 2B** and **2C** provide bar graphs showing the percent of T cells that express GFP and the GFP expression levels (MFI), respectively, when the anti-CD3 tLNPs are transfected into activated T cells in culture in the absence of serum (FBS).

[0086] **FIGs. 3A-3D** provide bar graphs showing the percent of rested T cells that express GFP (**FIGs. 3A** and **3B**) and the GFP expression levels (MFI) (**FIGs. 3C** and **3D**) when the anti-CD3 tLNPs are transfected into rested T cells in culture in the presence (**FIGs. 3A** and **3D**) or absence (**FIGs. 3B** and **3C**) of serum (FBS). In rested T cells, all tLNPs conjugated to the anti-CD3 targeting moieties that were screened enhanced transfection efficiency above that of non-targeted base LNPs, both in the presence and absence of serum.

[0087] **FIG. 4** provides a bar graph showing that tLNPs comprising the anti-CD3 targeting moieties induced variable expression (MFI) of the T cell activation marker CD25.

[0088] **FIGs. 5A-5D** provide bar graphs showing the percent of activated T cells that express GFP (**FIGs. 5A** and **5B**) and the GFP expression levels (**FIGs. 5C** and **5D**) when the tested tLNPs are transfected into activated T cells in culture in the presence (**FIGs. 3A** and **3D**) or absence (**FIGs. 3B** and **3C**) of serum (FBS). All of the anti-CD2, anti-CD7, anti-CD3, and anti-CD5 targeted tLNPs outperform non-targeted base LNPs in activated T cells, either when the cells were cultured with serum or without serum.

[0089] **FIGs. 6A-6D** provides bar graphs showing the percentage of rested T cells that express GFP (**FIGs, 6A** and **6B**) and the GFP expression levels (**FIGs. 6C** and **6D**) when the

tested tLNPs are transfected into rested T cells in culture in the presence or **FIGs. 6A** and **6D**) or absence (**FIGs. 6B** and **6C**) of serum (FBS). The targeted LNPs conjugated to anti-CD7, anti-CD5, and anti-CD3 targeting moieties outperformed non-targeted base LNPs, both with and without serum.

[0090] **FIGs. 7A** and **7B** provide bar graphs showing the surface expression levels of the CD25 **(FIG. 7A)** and CD69 **(FIG. 7B)** cell activation markers following administration of the tLNPs comprising an anti-CD3 targeting moiety or dual targeted LNPs conjugated either to the anti-CD3 targeting moiety and the CD80 ECD or to two different anti-CD3 targeting moieties to rested T cells. The dual tLNPs drove increased expression of surface activation markers CD25 and CD69 relative to non-targeted base LNPs, and increased expression of the activation markers by at least two-fold relative to tLNPs with the anti-CD3 targeting moiety alone.

[0091] **FIG. 8A** provides a graph showing the percentage of activated T cells that expressed GFP following transfection with the various tested LNPs comprising different ionizable lipids at different doses. Close to 100% of living activated T cells transfected with LNPs comprising Lipid092 or Lipid154 expressed GFP, starting at the lowest dose. Fewer T cells were transfected with the other LNPs tested across all dose levels. **FIG. 8B** provides a graph showing the GFP expression levels (MFI) in the activated T cells transfected with the various tested LNPs comprising different ionizable lipids at different doses. Transfection with the Lipid154 LNPs resulted in the highest GFP expression levels in the cells, followed by LNPs comprising Lipid092. **FIG. 8C** provides a graph showing the percentage of rested T cells that expressed GFP following transfection with the various tested LNPs comprising different ionizable lipids at different doses. TheLipid092 and Lipid154 LNPs transfected the largest numbers of cells at the 100ng to 400 ng doses. **FIG. 8D** provides a graph showing the GFP expression levels (MFI) in the rested T cells transfect with the various tested LNPs comprising different ionizable lipids at different doses. Transfection with the Lipid092 LNPs resulted in the highest GFP expression levels, followed by LNPs with Lipid154.

[0092] **FIG. 9A** provides a bar graph showing the percentage of activated T cells that expressed GFP at 4 days following transfection with the Lipid 154, Lipid 092 or V003 LNPs at different doses. Substantially more activated T cells expressed GFP at all doses when Lipid092 LNPs or Lipid154 LNPs delivered the gene modifying system compared to activated T cells that were contacted with the V003 LNPs. **FIG. 9B** provides a bar graph showing the GFP expression levels (MFI) in activated T cells at 4 days following transfection with the Lipid 154, Lipid0 092 or V003 LNPs at different doses. Those activated T cells that were transduced with the Lipid092

or Lipid154 LNPs expressed GFP at higher levels (higher MFI) relative to activated T cells transduced with LNPs comprising the V003 ionizable lipid.

[0093] **FIG. 10A** provides a bar graph showing the percentage of activated T cells that expressed GFP at 4 days following transfection with the Lipid 154, Lipid 092 or V003 anti-CD3 tLNPs or the Lipid 092 anti-CD3/CD80 ECD dual tLNPs at different doses. The tLNPs formulated with Lipid092 and Lipid154 both delivered the gene modifying system more effectively to activated T cells than the baseline tLNP formulated with the V003 lipid. **FIG. 10B** provides a bar graph showing the GFP expression levels (MFI) in activated T cells at 4 days following transfection with the Lipid 154, Lipid 092 or V003 anti-CD3 tLNPs or the Lipid 092 anti-CD3/CD80 ECD dual tLNPs at different doses. The activated T cells transduced with the Lipid092 or Lipid154 tLNPs expressed GFP at higher levels (higher MFI) relative to activated T cells transduced with tLNPs comprising the V003 ionizable lipid.

[0094] **FIG. 11** shows that the tLNPs carrying an all-RNA gene modifying system generated human T cells expressing GFP in the engrafted mice, as determined by flow cytometry. **FIG. 11A** provides a bar graph showing the percentage of engrafted human T cells (total T cells, CD4+ T cells or CD8+ T cells) that expressed GFP in mice following administration of Lipid 092 tLNPs comprising an anti-CD3 targeting moiety and an exemplary gene modifying system. **FIG. 11B** shows that engrafted human cells, including CD4+ and CD8+ T cells, expressed GFP at higher levels when tLNPs comprising the gene modifying system were administered to the humanized mice compared to engrafted cells in mice that were administered the negative control tLNP.

[0095] **FIGs. 12A-D** provide bar graphs showing the percentage of activated T cells expressing GFP at 4 days or 7 days following administration of the tested LNPs comprising the exemplary gene modifying system (**FIGs. 12A** and **12C**, respectively) and the GFP expression levels (MFI) in these cells (**FIGs. 12B** and **12D**). Delivery of the gene modifying system payload to activated cells using targeted LNPs formulated with Lipid154 and 22% DSPC generated more cells that expressed GFP (%GFP+) and at higher levels (MFI) relative to the baseline control tLNP that was the identical except that it was formulated with 8% DSPC.

[0096] **FIGs. 13A -C** provide bar graphs showing the percentage of rested T cells expressing GFP at 4 days following administration of the tested LNPs comprising the exemplary gene modifying system (with or without TransAct) (**FIGs. 13A**, respectively) and the GFP expression levels (MFI) at 4 days or 7 days post-administration in these cells (**FIGs. 13B** and **13**, respectively). Delivery of the gene modifying system payload to rested T cells using targeted

LNPs formulated with Lipid154 and a higher percentage of helper lipid (22% DSPC) generated many more cells that expressed GFP (%GFP+) and at higher levels (MFI) relative to the baseline control tLNP ("Std" in **FIG. 13**) that was the identical except that it was formulated with 8% DSPC.

[0097] **FIG. 14A** provides a bar graph showing the percentage of resting T cells that express a CAR at 3 days and 7 days following administration of anti-CD3 tLNPs formulated with either 8% helper lipid or 22% helper lipid and comprising an exemplary gene modifying system (with or without coadministration of TransAct). Delivery of the gene modifying system payload to rested T cells using targeted LNPs formulated with higher helper lipid (22% DSPC) generated many more CAR-T cells (%CAR+) at all dose levelsrelative to the baseline control tLNP that was the identical except that it was formulated with 8% DSPC. **FIGs. 14B** and **14C** provide bar graphs showing the CAR expression levels (MFI) in the rested T cells at 3 days and 7 days following administration of the anti-CD3 tLNPs of **FIG. 14A**. The rested T cells transfected with the tLNPs comprising higher helper lipid exhibited higher CAR expression levels (MFI) relative to the cells transfected with the baseline control tLNPs at most doses at 3 and 7 days, respectively. The coadministration of TransAct ("TA") increased the number of CAR expressing cells and CAR expression levels for both tLNPs formulated with different helper liver percentages.

[0098] **FIG. 15A** provides a bar graph showing the percentages of cells comprising an edit after bulk T cells were co-transfected with tLNPs formulated with a heterologous gene modifying system and a retrotransposon gene modifying system designed to integrate a GFP sequence into the genome of cells. **FIG. 15B** provides a bar graph showing the percentages of cells comprising an edit after bulk T cells were co-transfected with tLNPs formulated with a heterologous gene modifying system and a retrotransposon gene modifying system designed to integrate a CAR sequence into the genome of cells.

[0099] **FIG. 16** is a schematic showing the formation of an LNP conjugated to the C-terminus of a Fab fragment through a sortase mediated ligation followed by a Click reaction.

[0100] **FIG. 17** is a schematic showing the formation of an LNP conjugated to the C-terminus of a Fab fragment through a lipoic acid ligase mediated ligation followed by a Click reaction.

[0101] **FIG. 18** is a schematic showing an enzymatic approach of site-specifically introducing a Tz ring onto a sugar moiety of an antibody.

[0102] **FIG. 19** is a schematic showing an example of a light-induced crosslinking approach of site-specifically introducing a Tz ring onto an antibody.

[0103] **FIG. 20** is a schematic showing an example of using a non-natural amino acid to site-specifically introduce a Tz ring onto an antibody or Fab fragment.

[0104] **FIG. 21** is a schematic showing an example of using a cysteine-maleimide reaction to site-specifically introduce a first Click handle onto a Fab fragment, which subsequently reacts with an LNP bonded to a second Click handle, thereby generating a targeted LNP (conjugate).

[0105] **FIG. 22** is a schematic showing a Fab fragment modified with a free cysteine designed to react with a maleimide group.

[0106] **FIG. 23A** shows a schematic example for producing a site-specific conjugate through the reaction of an anti-CD117 Fab fragment with site-specific sortase recognition motif and an LNP. In the schematic, the squiggly lines represent PEG bonded to a lipid of the LNP. **FIG.23B** shows a schematic of producing a conjugate through the reaction of a Fab fragment that has been modified using a random approach (NHS modification) and an LNP.

[0107] **FIGs. 24A-24F** provide schematics showing the synthesis of Lipid 092.

[0108] **FIGs. 25A-25C** provide schematics showing the synthesis of Lipid 093.

[0109] **FIGs. 26A-26C** provide schematics showing the synthesis of Lipid 162.

[0110] **FIGs. 27A-27C** provide schematics showing the synthesis of Lipid 153.

[0111] **FIGs. 28A-28F** provide schematics showing the synthesis of Lipid 154.

[0112] **FIGs. 29A-29E** provide schematics showing the synthesis of Lipid 155.

[0113] **FIGs. 30A-30F** provide schematics showing the synthesis of Lipid 176.

[0114] **FIG. 31** provides a bar graph showing that a site-specific conjugation process, as described herein, can efficiently conjugate two targeting moieties (anti-CD117 Fab and anti-CD45 Fab, in this case) to the surface of LNPs at specific ratios.

[0115] **FIG. 32** provides a bar graph showing the percentage of human CD3+ T cells comprising indels or a designed edit (via target primed reverse transcription mediated rewriting) at the B2M locus following administration of anti-CD5 tLNPs comprising either a gene editing system or a gene modifying system, respectively, to mice engrafted with human T cells.

[0116] **FIG. 33** provides a bar graph showing the percentage of human T cells that express a CAR after human PBMCs were either transfected with an anti-CD3 tLNP comprising an

exemplary retrotransposon gene modifying system comprising a template RNA encoding a CAR sequence for 4 hours with TransAct (right side) or mock treated as a control (left side).

[0117] **FIG. 34** provides a bar graph showing the percentage of BCMA tumor cells killed in a tumor cell killing assay using the CAR-T cells generated by the exemplary retrotransposon gene modifying system of **FIG. 33** compared to the percentage of tumor cells killed by the mock treated T cells.

[0118] **FIG. 35** provides a bar graph showing the quantification of the IFN-γ cytokine in the human T cells that express a CAR after human PBMCs were either transfected with an anti-CD3 tLNP comprising an exemplary retrotransposon gene modifying system comprising a template RNA encoding a CAR sequence for 4 hours with TransAct (right side) or mock treated as a control (left side).

[0119] **FIG. 36** provides a bar graph showing the quantification of the percentage of edited cells expressing a CAR at 8 days following a 4-hour incubation with tLNPs conjugated to one of four different anti-CD3 targeting moieties (fab fragments) tested. The tLNPs were formulated with an exemplary retrotransposon gene modifying system comprising a template RNA encoding a CAR sequence, and administered at 2 different doses (either 5ug or 2.5ug of LNPs per million cells) .

[0120] **FIG. 37** provides a bar graph showing the quantification of CAR expression levels (MFI) in the CAR-T cells described in **FIG. 36**.

[0121] **FIG. 38** provides a bar graph showing the quantification of the percentage of BCMA tumor cells killed by the CAR-T cells described in **FIG. 36** in a tumor killing assay.

[0122] **FIG. 39** is a schematic showing an exemplary conjugate comprising a Fab fragment and a lipid nanoparticle (LNP).

[0123] **FIG. 40A** is a schematic showing the assembly of an LNP encapsulating a therapeutic payload with a functional group (e.g., maleimide, 2,3-dibromomaleimide, sortase tag, etc.) to be reacted with an antibody or antigen-binding fragment thereof. **FIG. 40B i**s a schematic showing the assembly of an LNP encapsulating a therapeutic payload using a post-insertion technique.

[0124] **FIG. 41** is an exemplary schematic showing the reduction of an interchain disulfide bond in a Fab fragment. After reduction, each cysteine residue is available to react with a thiol-reactive group conjugated to the surface of the LNP.

[0125] **FIG. 42** is an exemplary schematic showing conjugate formation. In **FIG. 42**, a Fab fragment comprising an interchain disulfide bond between the heavy and light chain is contacted with a reducing reagent, whereby the reducing reagent reduces the interchain disulfide to generate two free cysteine residues (step (i)). In step (ii), the Fab fragment is contacted with an LNP comprising a plurality of thiol-reactive groups (e.g., a plurality of maleimide or DBM groups) conjugated to the surface of the LNP, whereby a thiol-reactive group of the plurality reacts with at least one of the two free cysteine residues of the Fab fragment.

[0126] **FIG. 43** is an exemplary schematic showing conjugate formation. In **FIG. 43**, a Fab fragment comprising an interchain disulfide bond between the heavy and light chain is contacted with a reducing reagent, whereby the reducing reagent reduces the interchain disulfide to generate two free cysteine residues (step (i)). In step (ii), the Fab fragment is contacted with an LNP comprising a plurality of maleimide groups conjugated to the surface of the LNP, whereby a maleimide group of the plurality reacts with one of the two free cysteine residues of the Fab fragment.

[0127] **FIG. 44** is an exemplary schematic showing conjugate formation using DBM as a bridging agent.

[0128] **FIG. 45** is an exemplary schematic showing conjugate formation. In **FIG. 45**, a first Fab fragment (Fab1) and a second Fab fragment (Fab2) are each contacted with a reducing reagent, whereby the reducing reagent reduces the interchain disulfide between the heavy and light chain of each Fab. After step (i), each Fab comprises two free cysteine residues with one free cysteine residue on the heavy chain and one free cysteine residue on the light chain. In step (ii), the first Fab fragment and the second Fab fragment are contacted with an LNP comprising a plurality of thiol-reactive groups (e.g., maleimide or DBM) conjugated to the surface of the LNP, whereby a thiol-reactive group of the plurality reacts with at least one of the two free cysteine residues of the first Fab Fragment and another thiol-reactive group of the plurality reacts with at least one of the two free cysteine residues of the second Fab Fragment.

[0129] **FIG. 46** is a schematic showing some examples of a pegylated lipid bonded to a maleimide moiety.

[0130] **FIG. 47** is a schematic showing some examples of non-pegylated lipid bonded to a maleimide moiety.

[0131] **FIG. 48** is a schematic showing some examples of ionizable lipid bonded to a maleimide moiety.

[0132] **FIG. 49** is a schematic showing some examples of sterols bonded to a maleimide moiety.

[0133] **FIG. 50** provides a bar graph showing the percentage of human T cells that express CAR, as assayed by flow cytometry, at 16 hours following the administration of tLNPs formulated with 8% helper lipid and an exemplary all-RNA gene modifying system to mice that were engrafted with human T cells. The tLNPs were conjugated with an anti-CD2, anti-CD3, anti-CD5, or anti-CD7 targeting moiety.

[0134] **FIG. 51** provides a bar graph showing the percentage of human T cells that express CAR, as assayed by flow cytometry, at 16 hours following the administration of tLNPs formulated with 22% helper lipid and an exemplary all-RNA gene modifying system to mice that were engrafted with human T cells. The tLNPs were conjugated with an anti-CD2, anti-CD5 or anti-CD7 targeting moiety.

DETAILED DESCRIPTION

I. DEFINITIONS

[0135] **Antigen binding domain:** The term "antigen binding domain" as used herein refers to that portion of antibody or a chimeric antigen receptor which binds an antigen. In some embodiments, an antigen binding domain binds to a cell surface antigen of a cell. In some embodiments an antigen binding domain binds an antigen characteristic of a cancer, e.g., a tumor associated antigen in a neoplastic cell. In some embodiments, an antigen binding domain binds an antigen characteristic of an infectious disease, e.g. a virus associated antigen in a virus infected cell. In some embodiments, an antigen binding domain binds an antigen characteristic of a cell targeted by a subject's immune system in an autoimmune disease, e.g., a 0-antigen. In some embodiments, an antigen binding domain is or comprises an antibody or antigen-binding portion thereof. In some embodiments, an antigen binding domain is or comprises an scFv or Fab.

[0136] **Expression cassette:** The term "expression cassette," as used herein, refers to a nucleic acid construct comprising nucleic acid elements sufficient for the expression of the nucleic acid molecule of the instant invention.

[0137] **gRNA spacer:** A "gRNA spacer", as used herein, refers to a portion of a nucleic acid that has complementarity to a target nucleic acid and can, together with a gRNA scaffold, target a Cas protein to the target nucleic acid.

[0138] gRNA scaffold: A "gRNA scaffold", as used herein, refers to a portion of a nucleic acid that can bind a Cas protein and can, together with a gRNA spacer, target the Cas protein to the target nucleic acid. In some embodiments, the gRNA scaffold comprises a crRNA sequence, tetraloop, and tracrRNA sequence.

[0139] Gene modifying polypeptide: A "gene modifying polypeptide," and "retrotransposon gene modifying polypeptide" as used herein interchangeably to refer to a polypeptide comprising a retrotransposase reverse transcriptase domain and a retrotransposase endonuclease domain, or a polypeptide comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity to said domains, which is capable of integrating a nucleic acid sequence (e.g., a sequence provided on a template nucleic acid) into a target DNA molecule (e.g., in a mammalian host cell, such as a genomic DNA molecule in the host cell). In some embodiments, the endonuclease domain is a catalytically inactive endonuclease domain. In some embodiments, the retrotransposase reverse transcriptase domain and a retrotransposase endonuclease domain are derived from the same retrotransposase. In some embodiments, the gene modifying polypeptide is capable of integrating the sequence substantially without relying on host machinery. In some embodiments, the gene modifying polypeptide integrates a sequence into a random position in a genome, and in some embodiments, the gene modifying polypeptide integrates a sequence into a specific target site. In some embodiments, a gene modifying polypeptide includes one or more domains that, collectively, facilitate 1) binding the template nucleic acid, 2) binding the target DNA molecule, and 3) facilitate integration of the at least a portion of the template nucleic acid into the target DNA. Gene modifying polypeptides include both naturally occurring polypeptides as well as engineered variants of the foregoing, e.g., having one or more amino acid substitutions to the naturally occurring sequence. Gene modifying polypeptides also include heterologous constructs, e.g., where one or more of the domains recited above are heterologous to each other, whether through a heterologous fusion (or other conjugate) of otherwise wild-type domains, as well as fusions of modified domains, e.g., by way of replacement or fusion of a heterologous sub-domain or other substituted domain. Exemplary gene modifying polypeptides, and systems comprising them and methods of using them, that can be used in the methods provided herein are described, e.g., in WO/2021/178717, which is incorporated herein by reference, including Tables 10, 11, X, 3A, 3B, and Z1 therein. In some embodiments, a gene modifying polypeptide integrates a sequence into a gene. In some embodiments, a gene modifying polypeptide integrates a sequence into a sequence outside of a gene. A "gene modifying system," as used herein, refers to a system comprising a gene modifying polypeptide and a template nucleic acid.

[0140] **Gene modifying system:** A "gene modifying system," as used herein, refers to a system comprising a gene modifying polypeptide, or a nucleic acid (e.g., an mRNA) encoding the gene modifying polypeptide, and a template nucleic acid.

[0141] **Domain:** The term "domain" as used herein refers to a structure of a biomolecule that contributes to a specified function of the biomolecule. A domain may comprise a contiguous region (e.g., a contiguous sequence) or distinct, non-contiguous regions (e.g., non-contiguous sequences) of a biomolecule. Examples of protein domains include, but are not limited to, an endonuclease domain, a DNA binding domain, a reverse transcriptase domain; an example of a domain of a nucleic acid is a regulatory domain, such as a transcription factor binding domain.

[0142] **Exogenous:** As used herein, the term "exogenous," when used with reference to a biomolecule (such as a nucleic acid sequence or polypeptide) means that the biomolecule was introduced into a host genome, cell, or organism by the hand of man. For example, a nucleic acid that is as added into an existing genome, cell, tissue, or subject using recombinant DNA techniques or other methods is exogenous to the existing nucleic acid sequence, cell, tissue or subject.

[0143] **Heterologous:** The term "heterologous", when used to describe a first element in reference to a second element means that the first element and second element do not exist in nature disposed as described. For example, a heterologous polypeptide, nucleic acid molecule, construct or sequence refers to (a) a polypeptide, nucleic acid molecule or portion of a polypeptide or nucleic acid molecule sequence that is not native to a cell in which it is expressed, (b) a polypeptide or nucleic acid molecule or portion of a polypeptide or nucleic acid molecule that has been altered or mutated relative to its native state, or (c) a polypeptide or nucleic acid molecule with an altered expression as compared to the native expression levels under similar conditions. For example, a heterologous regulatory sequence (e.g., promoter, enhancer) may be used to regulate expression of a gene or a nucleic acid molecule in a way that is different than the gene or a nucleic acid molecule is normally expressed in nature. In another example, a heterologous domain of a polypeptide or nucleic acid sequence (e.g., a DNA binding domain of a polypeptide or nucleic acid encoding a DNA binding domain of a polypeptide) may be disposed relative to other domains or may be a different sequence or from a different source, relative to other domains or portions of a polypeptide or its encoding nucleic acid. In certain embodiments, a heterologous nucleic acid molecule may exist in a native host cell genome, but may have an altered expression level or have a different sequence or both. In other embodiments, heterologous nucleic acid molecules may not be endogenous to a host cell or host genome but

instead may have been introduced into a host cell by transformation (e.g., transfection, electroporation), wherein the added molecule may integrate into the host genome or can exist as extra-chromosomal genetic material either transiently (e.g., mRNA) or semi-stably for more than one generation (e.g., episomal viral vector, plasmid or other self-replicating vector). In some embodiments, a domain is heterologous relative to another domain, if the first domain is not naturally comprised in the same polypeptide as the other domain (e.g., a fusion between two domains of different proteins from the same organism).

[0144] Heterologous gene modifying polypeptide: As used herein, the term "heterologous gene modifying polypeptide" refers to a polypeptide comprising a retroviral reverse transcriptase, or a polypeptide comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity to a retroviral reverse transcriptase, which is capable of integrating a nucleic acid sequence (e.g., a sequence provided on a template nucleic acid) into a target DNA molecule (e.g., in a mammalian host cell, such as a genomic DNA molecule in the host cell). In some embodiments, the heterologous gene modifying polypeptide is capable of integrating the sequence substantially without relying on host machinery. In some embodiments, the heterologous gene modifying polypeptide integrates a sequence into a random position in a genome, and in some embodiments, the heterologous gene modifying polypeptide integrates a sequence into a specific target site. In some embodiments, the sequence that is integrated comprises a deletion, substitution, or insertion relative to the target DNA molecule. In some embodiments, a heterologous gene modifying polypeptide includes one or more domains that, collectively, facilitate 1) binding the template nucleic acid, 2) binding the target DNA molecule, and 3) facilitate integration of the at least a portion of the template nucleic acid into the target DNA. Heterologous gene modifying polypeptides include both naturally occurring polypeptides as well as engineered variants of the foregoing, e.g., having one or more amino acid substitutions to the naturally occurring sequence. Heterologous gene modifying polypeptides also include heterologous constructs, e.g., where one or more of the domains recited above are heterologous to each other, whether through a heterologous fusion (or other conjugate) of otherwise wild-type domains, as well as fusions of modified domains, e.g., by way of replacement or fusion of a heterologous sub-domain or other substituted domain. Exemplary heterologous gene modifying polypeptides, and systems comprising them and methods of using them, that can be used in the methods provided herein are described, e.g., in PCT/US2021/020948, which is incorporated herein by reference with respect to heterologous gene modifying polypeptides that comprise a retroviral reverse transcriptase domain. In some embodiments, a heterologous gene modifying polypeptide integrates a

sequence into a gene. In some embodiments, a heterologous gene modifying polypeptide integrates a sequence into a sequence outside of a gene. A "heterologous gene modifying system," as used herein, refers to a system comprising a heterologous gene modifying polypeptide and a template nucleic acid.

[0145] Mutation or Mutated: The term "mutated" when applied to nucleic acid sequences means that nucleotides in a nucleic acid sequence may be inserted, deleted or changed compared to a reference (e.g., native) nucleic acid sequence. A single alteration may be made at a locus (a point mutation) or multiple nucleotides may be inserted, deleted, or changed at a single locus. In addition, one or more alterations may be made at any number of loci within a nucleic acid sequence. A nucleic acid sequence may be mutated by any method known in the art. In some embodiments a mutation occurs naturally. In some embodiments a desired mutation can be produced by a system described herein.

[0146] Nucleic acid molecule: "Nucleic acid molecule" refers to both RNA and DNA molecules including, without limitation, complementary DNA ("cDNA"), genomic DNA ("gDNA"), and messenger RNA ("mRNA"), and also includes synthetic nucleic acid molecules, such as those that are chemically synthesized or recombinantly produced, such as RNA templates, as described herein. The nucleic acid molecule can be double-stranded or single-stranded, circular, or linear. If single-stranded, the nucleic acid molecule can be the sense strand or the antisense strand. Unless otherwise indicated, and as an example for all sequences described herein under the general format "SEQ ID NO:," or "nucleic acid comprising SEQ ID NO:1" refers to a nucleic acid, at least a portion which has either (i) the sequence of SEQ ID NO:1, or (ii) a sequence complimentary to SEQ ID NO:1. The choice between the two is dictated by the context in which SEQ ID NO:1 is used. For instance, if the nucleic acid is used as a probe, the choice between the two is dictated by the requirement that the probe be complementary to the desired target. Nucleic acid sequences of the present disclosure may be modified chemically or biochemically or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those of skill in the art. Such modifications include, for example, labels, methylation, substitution of one or more naturally occurring nucleotides with an analog, inter-nucleotide modifications such as uncharged linkages (for example, methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), charged linkages (for example, phosphorothioates, phosphorodithioates, etc.), pendant moieties, (for example, polypeptides), intercalators (for example, acridine, psoralen, etc.), chelators, alkylators, and modified linkages (for example, alpha anomeric nucleic acids, etc.). Also included are chemically modified bases (see, for example, Table 13), backbones (see, for example 14, **Table**

16), and modified caps. Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of a molecule, e.g., peptide nucleic acids (PNAs). Other modifications can include, for example, analogs in which the ribose ring contains a bridging moiety or other structure such as modifications found in "locked" nucleic acids (LNAs). In various embodiments, the nucleic acids are in operative association with additional genetic elements, such as tissue-specific expression-control sequence(s) (e.g., tissue-specific promoters and tissue-specific microRNA recognition sequences), as well as additional elements, such as inverted repeats (e.g., inverted terminal repeats, such as elements from or derived from viruses, e.g., AAV ITRs) and tandem repeats, inverted repeats/direct repeats, homology regions (segments with various degrees of homology to a target DNA), untranslated regions (UTRs) (5′, 3′, or both 5′ and 3′ UTRs), and various combinations of the foregoing. The nucleic acid elements of the systems provided by the invention can be provided in a variety of topologies, including single-stranded, double-stranded, circular, linear, linear with open ends, linear with closed ends, and particular versions of these, such as doggybone DNA (dbDNA), closed-ended DNA (ceDNA).

[0147] **Primer Binding Sequence:** The term "primer binding site sequence" or "PBS sequence," as used herein, refers to a portion of a template RNA capable of binding to a region comprised in a target nucleic acid sequence. In some instances, a PBS sequence is a nucleic acid sequence comprising at least 3, 4, 5, 6, 7, or 8 bases with 100% identity to the region comprised in the target nucleic acid sequence. In some embodiments the primer region comprises at least 5, 6, 7, 8 bases with 100% identity to the region comprised in the target nucleic acid sequence. Without wishing to be bound by theory, in some embodiments when a template RNA comprises a PBS sequence and a heterologous object sequence, the PBS sequence binds to a region comprised in a target nucleic acid sequence, allowing a reverse transcriptase domain to use that region as a primer for reverse transcription, and to use the heterologous object sequence as a template for reverse transcription.

[0148] It is understood that aspects and embodiments described herein as "comprising" include "consisting of" and "consisting essentially of" embodiments.

II. CONJUGATES

[0149] In one aspect, the disclosure provides an LNP (conjugate) comprising an ionizable lipid as described herein (e.g., in Table 1 or Table 2), wherein the LNP can deliver a payload,

such as a therapeutic agent (e.g., a gene modifying system, such as a retrotransposon gene modifying system and/or a heterologous gene modifying system, as described herein) to an immune cell (e.g., a T cell). In another aspect, the LNP (conjugate) comprises a targeting moiety that binds to a protein (e.g., a protein receptor) on an immune cell (e.g., a T cell), as described herein. In some embodiments, an LNP (conjugate) comprises both an ionizable lipid and a targeting moiety, as described herein.

[0150] In one aspect, the disclosure provides targeted LNPs (conjugates) comprising a targeting moiety and a lipid nanoparticle (LNP) encapsulating a payload (e.g., a therapeutic agent, as described herein, such as a gene modifying polypeptide or a gene modifying system), wherein the targeting moiety binds to a protein (e.g., protein receptor) on an immune cell (e.g., T cell). In some embodiments, the targeting moiety is an antibody or antigen binding fragment thereof. In some instances, the targeting moiety is an antibody, a Fab fragment, a scFv, a DARPIN, a VHH domain antibody, a FN3 domain, a nanobody, a single domain antibody or a Centyrin. In other embodiments, the targeting moiety is a folate moiety, an antibiotic mimetic, a polynucleotide (such as a DNA or RNA apatamer), a carbohydrate, a vitamin or a N-Acetylgalactosamine (GalNac). In some embodiments, the payload (e.g., a therapeutic agent, as described herein, such as a gene modifying polypeptide or a gene modifying system) is capable of modifying one or more genes of the target immune cell (e.g., T cell).

[0151] The conjugates described herein may be used to target and modify immune cells. In some embodiments, the conjugates may be used to modify T cells. In some embodiments, T cells may include any subpopulation of T cells, e.g., CD4+ T cells, CD8+ T cells, gamma-delta T cells, alpha-beta T cells, naïve T cells, stem cell memory T cells, central memory T cells, effector T cells, cytotoxic T cells, helper T cells (e.g., Th1 cells, Th2 cells, Th17 cells, etc.), regulatory T cells, tumor-infiltrating T cells, tissue-resident T cells, or a mixture of subpopulations. In some embodiments, the conjugates may be used to deliver or modify a sequence encoding a T-cell receptor (TCR) in a T cell. In some embodiments, the conjugates may be used to deliver at least one sequence encoding a chimeric antigen receptor (CAR) to T cells. For instance, in specific embodiments, the conjugates can be used to deliver an RNA encoding a CAR to T cells. In some embodiments, the conjugates may be used to deliver at least one sequence encoding a CAR to natural killer (NK) cells. In some embodiments, the conjugates be used to deliver at least one sequence encoding a CAR to natural killer T (NKT) cells. In some embodiments, the conjugates may be used to deliver at least one sequence encoding a CAR to a progenitor cell, e.g., a progenitor cell of T, NK, or NKT cells. In some embodiments, cells modified with at least one CAR (e.g., CAR-T cells, CAR-NK cells, CAR-NKT cells), or a

combination of cells modified with at least one CAR (e.g., a mixture of CAR-T/CAR-NK/CAR-NKT cells) are used to treat a condition as identified in the targetable landscape of CAR therapies in MacKay, et al. Nat Biotechnol 38, 233-244 (2020), incorporated by reference herein in its entirety. In some embodiments, the immune cells comprise a CAR specific to a tumor or a pathogen antigen selected from a group consisting of AChR (fetal acetylcholine receptor), ADGRE2, AFP (alpha fetoprotein), BAFF-R, BCMA, CAIX (carbonic anhydrase IX), CCR1, CCR4, CEA (carcinoembryonic antigen), CD3, CD5, CD8, CD7, CD10, CD13, CD14, CD15, CD19, CD20, CD22, CD30, CD33, CLLI, CD34, CD38, CD41, CD44, CD49f, CD56, CD61, CD64, CD68, CD70, CD74, CD99, CD117, CD123, CD133, CD138, CD44v6, CD267, CD269, CDS, CLEC12A, CS1, EGP-2 (epithelial glycoprotein-2), EGP-40 (epithelial glycoprotein-40), EGFR (HER1), EGFR-VIII, EpCAM (epithelial cell adhesion molecule), EphA2, ERBB2 (HER2, human epidermal growth factor receptor 2), ERBB3, ERBB4, FBP (folate-binding protein), Flt3 receptor, folate receptor-a, GD2 (ganglioside G2), GD3 (ganglioside G3), GPC3 (glypican-3), GPI00, hTERT (human telomerase reverse transcriptase), ICAM-1, integrin B7, interleukin 6 receptor, IL13Ra2 (interleukin-13 receptor 30 subunit alpha-2), kappa-light chain, KDR (kinase insert domain receptor), LeY (Lewis Y), L1CAM (LI cell adhesion molecule), LILRB2 (leukocyte immunoglobulin like receptor B2), MARTI, MAGE-A1 (melanoma associated antigen Al), MAGE- A3, MSLN (mesothelin), MUC16 (mucin 16), MUCI (mucin I), KG2D ligands, NY-ESO-1 (cancer-testis antigen), PRI (proteinase 3), TRBCI, TRBC2, TFM-3, TACI, tyrosinase, survivin, hTERT, oncofetal antigen (h5T4), p53, PSCA (prostate stem cell antigen), PSMA (prostate-specific membrane antigen), hRORl, TAG-72 (tumor- associated glycoprotein 72), VEGF-R2 (vascular endothelial growth factor R2), WT-1 (Wilms tumor protein), and antigens of HIV (human immunodeficiency virus), hepatitis B, hepatitis C, CMV (cytomegalovirus), EBV (Epstein-Barr virus), HPV (human papilloma virus), etc.

[0152] In some embodiments, immune cells, e.g., T cells, NK cells, NKT cells, or progenitor cells are modified *ex vivo* and then delivered to a patient. In some embodiments, a nucleic acid (e.g., RNA, such as mRNA) is delivered by one of the methods mentioned herein, and immune cells, e.g., T cells, NK cells, NKT cells, or progenitor cells are modified *in vivo* in the patient.

[0153] In certain embodiments, the targeting moiety is a T-cell targeting moiety, for example, an antibody, Fab fragment, or scFv that binds to a T-cell antigen selected from the group consisting of CD2, CD3, CD4, CD5, CD7, CD8, CD28, CD137, CD45, T-cell receptor (TCR)-β,TCR-α, TCR-α/β, TCR-γ/δ, PD1, CTLA4, TIM3, LAG3, CD18, IL-2 receptor, CD11a, TLR2, TLR4, TLR5, IL-7 receptor, or IL-15 receptor.

[0154] In certain embodiments, the targeting moiety is a T-cell targeting moiety, for example, an antibody, Fab fragment, or scFv that binds to a T-cell antigen selected from the group consisting of CD2, CD3, CD4, CD5, CD7, CD8, CD28, CD137, CD45, T-cell receptor (TCR)-β,TCR-α, TCR-α/β, TCR-γ/δ, PD1, CTLA4, TIM3, LAG3, CD18, IL-2 receptor, CD11a, TLR2, TLR4, TLR5, IL-7 receptor, and IL-15 receptor. In some embodiments the CD28 targeting moiety is a CD80 extracellular domain (ECD).

[0155] In some embodiments, the targeted LNP (conjugate) comprises a targeting moiety that targets a receptor on the surface of the T cell selected from CD2, CD3, CD4, CD5, CD6, CD7, and CD8. In some embodiments, the targeting moiety targets a CD3 receptor on the surface of the T cell. In some embodiments, the targeting moiety targets a CD7 receptor on the surface of the T cell. In some embodiments, the targeting moiety targets a CD5 receptor on the surface of the T cell. In some embodiments, the targeting moiety targets a CD2 receptor on the surface of the T cell. In some embodiments, the targeting moiety targets a CD8 receptor on the surface of the T cell.

[0156] In some embodiments, the targeted LNP (conjugate) comprises a targeting moiety that targets CD3 on the surface of the T cell, wherein the targeting moiety is an antibody, Fab fragment, or scFv selected from SP34, teclistamab, mosunetuzumab, odronextamab, tebentafusp, tepilizumab, muromonab, visilizumab, Plamotamab, HPN536, Pasotuxizumab, and Flotetuzumab, or an antigen-binding portion thereof. In certain embodiments, the targeting moiety is SP34 or an antigen-binding portion thereof. In other embodiments, the targeting moiety is teclistamab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is mosunetuzumab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is odronextamab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is tebentafusp or an antigen-binding portion thereof. In other embodiments, the targeting moiety is muromonab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is visilizumab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is tepilizumab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is Plamotamab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is HPN536 or an antigen-binding portion thereof. In other embodiments, the targeting moiety is Pasotuxizumab or an antigen-binding portion thereof. In other embodiments, the targeting moiety is Flotetuzumab or an antigen-binding portion thereof.

[0157] In some embodiments, a targeted LNP (conjugate) for delivery of a therapeutic agent to immune cells (e.g., T cells) as described herein, comprises:

an ionizable lipid and a helper lipid, wherein the ionizable lipid is selected from V003 or any or the lipids in **Table 1**, **Table 2** and **Table 3**;

a plurality of targeting moieties conjugated to the LNP, wherein the plurality of targeting moieties bind to at least one targeting moiety on a T cell; and

a therapeutic agent encapsulated within the LNP.

[0158] In some embodiments, the plurality of targeting moieties bind to two or more T-cell antigens selected from the group consisting of CD2, CD3, CD4, CD5, CD7, CD8, CD28, CD80, CD137, CD45, T-cell receptor (TCR)-β,TCR-α, TCR-α/β, TCR-γ/δ, PD1, CTLA4, TIM3, LAG3, CD18, IL-2 receptor, CD11a, TLR2, TLR4, TLR5, IL-7 receptor, and IL-15 receptor. In some embodiments, the targeted LNP (conjugate) comprises a targeting moiety that targets a receptor on the surface of the T cell selected from CD2, CD3, CD4, CD5, CD6, CD7, and CD28. In some embodiments, a targeted LNP comprises two targeting moieties, wherein one targeting moiety binds to CD3 and the other targeting moiety binds to CD7. In some embodiments, a targeted LNP comprises two targeting moieties, wherein one targeting moiety binds to CD3 and the other targeting moiety binds to CD28. In some embodiments, a targeted LNP comprises two targeting moieties, wherein one targeting moiety binds to CD3 and the other targeting moiety binds to CD3. In some embodiments, the one targeting moiety that binds to CD3 comprises a different anti-CD3 antibody, Fab fragment, or scFv than the other targeting moiety that binds to CD3.

[0159] In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein one targeting moiety binds to CD3 and the other targeting moiety binds to CD5. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein one targeting moiety binds to CD3 and the other targeting moiety binds to CD7. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein one targeting moiety binds to CD3 and the other targeting moiety binds to CD28. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein one targeting moiety binds to CD5 and the other targeting moiety binds to CD28. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein one targeting moiety binds to CD7 and the other targeting moiety binds to CD28. In some embodiments the CD28 targeting moiety is a CD80 extracellular domain (ECD). In some embodiments, a targeted LNP comprises two targeting moieties, wherein one targeting moiety binds to CD3 and the other targeting moiety binds to CD3. In some embodiments, the one

targeting moiety that binds to CD3 comprises a different anti-CD3 antibody, Fab fragment, or scFv than the other targeting moiety that binds to CD3.

[0160] In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein each targeting moiety binds to the same target (e.g., receptor) on the T cell. For instance, in some embodiments, both targeting moieties of the conjugate bind to CD3. In some such embodiments, one of the targets is SP34 or an antigen-binding portion thereof and the other is teclistamab or an antigen-binding portion thereof. In other such embodiments, one of the targets is SP34 or an antigen-binding portion thereof and the other is visilizumab or an antigen-binding portion thereof. In other such embodiments, one of the targets is SP34 or an antigen-binding portion thereof and the other is tepilizumab or an antigen-binding portion thereof. In other such embodiments, one of the targets is visilizumab or an antigen-binding portion thereof and the other is tepilizumab or an antigen-binding portion thereof. In other such embodiments, one of the targets is visilizumab or an antigen-binding portion thereof and the other is teclistamab or an antigen-binding portion thereof. In some embodiments, both targeting moieties of the conjugate bind to CD3. In some embodiments, both targeting moieties of the conjugate bind to CD7.

[0161] In certain embodiments, the targeting moiety binds to a CD4+ and/or CD8+ T cell. In other embodiments, the targeting moiety binds to a natural killer (NK) cell. In other embodiments, the targeting moiety binds to a hematopoietic stem cell. In other embodiments, the targeting moiety binds to a lymphoid progenitor cell. In other embodiments, the targeting moiety binds to a myeloid cell. In other embodiments, the targeting moiety binds to a macrophage.

CD2 Targeting Moieties

[0162] In some embodiments, the target molecule is CD2. In some embodiments, the target cell is CD2+. The glycoprotein CD2 is a costimulatory receptor expressed mainly on T cells, NK cells, thymocytes, and dendritic cells that binds to lymphocyte-associated antigen 3 (LFA3; also known as CD58) which is expressed on the surface of B cells, T cells, monocytes, granulocytes, thymic epithelial cells. CD2 also binds to CD48, albeit with a relatively lower affinity. CD2 has an important role in the formation and organization of the immunological synapse that is formed between T cells and antigen-presenting cells upon cell-cell conjugation and associated intracellular signaling. CD2 expression is upregulated on memory T cells as well as activated T cells and plays an important role in activation of memory T cells. See, e.g., Binder et al. (2020) Front. Immunol. 11:1090, hereby incorporated by reference in its entirety.

[0163] In some embodiments, the CD2 targeting moiety includes an antibody or antigen-binding fragment thereof that binds to CD2. In some embodiments, the CD2 targeting moiety is an antibody or antigen-binding fragment thereof (e.g., a Fab, Fab', $F(ab')_2$, Fv fragment, scFv, DARPIN, VHH domain, FN3 domain, nanobody, single domain antibody, or Centyrin). In other embodiments, the CD2 targeting moiety includes a ligand, a folate moiety, an antibiotic mimetic, a polynucleotide (such as a DNA or RNA apatamer), a carbohydrate, a vitamin, a cytokine, or a chemokine. In some embodiments, the CD2 targeting moiety is an anti-CD2 antibody or antigen binding fragment thereof. In some embodiments, the CD2 targeting moiety is an IgA, IgG, IgE, or IgM antibody. In some embodiments, the CD2 targeting moiety is a bispecific or multi-specific antibody or fragment thereof. In some embodiments, the CD2 targeting moiety is a humanized antibody or antigen-binding fragment thereof.

[0164] Exemplary anti-CD2 binders, antibodies, or antigen-binding fragments thereof include Siplizumab (i.e., MEDI-507 or TCD601, ITB-Med LLC), BTI-322 (Lo-CD2a), Alefacept (i.e., a chimeric fusion protein consisting of the CD2-binding portion of human LFA3-Fc, Biogen) CB.219 (e.g., BioXCell), UMCD2 (e.g., Santa Cruz Biotechnology), TS1/8, RPA-2.10, TS1/18, TS1/18.1.1, TS2/18, AB75, and ZR100, as well as anti-CD2 antibodies or antigen-binding fragments thereof disclosed in any of: US 5,730,979; US 5,928,643; US 5,951,983; US 6,764,681; US 7,858,095; US 6,162,432; US 11,732,042; US 12,037,378; US20210032308; US20030068320; US20230365687; WO1999058147; WO2014025198; WO2024180185; WO2023126445; WO2024079046; WO2024079046; etc., each hereby incorporated by reference in its entirety.

[0165] In some embodiments, the CD2 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:269 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:270. In some embodiments, the CD2 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:280 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:281. In some embodiments, the CD2 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:291 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:292. In some embodiments, the CD2 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:302 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:292. SEQ ID NOs:269, 270, 280, 281, 291, 292, and 302 are shown in **Table 20**, with complementary determining regions (CDRs) marked in bold.

[0166] In some embodiments, the CD2 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:269, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:270. In some embodiments, the CD2 targeting moiety comprises a CDR-H1 comprising an amino acid sequence SYWVN (SEQ ID NO:271), a CDR-H2 comprising an amino acid sequence RIDPYDSETHYNQKFTD (SEQ ID NO:272), a CDR-H3 comprising an amino acid sequence SPRDSSTNLAD (SEQ ID NO:273), a CDR-L1 comprising an amino acid sequence RASQSISDYLH (SEQ ID NO:274), a CDR-L2 comprising an amino acid sequence YASQSIS (SEQ ID NO:275), and a CDR-L3 comprising an amino acid sequence QNGHSFPLT (SEQ ID NO:276). In some embodiments, the CD2 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:266 or 267, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:268.

[0167] In some embodiments, the CD2 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:280, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:281. In some embodiments, the CD2 targeting moiety comprises a CDR-H1 comprising an amino acid sequence RYWIH (SEQ ID NO:282), a CDR-H2 comprising an amino acid sequence NIDPSDSETHYNQKFKD (SEQ ID NO:283), a CDR-H3 comprising an amino acid sequence EDLYYAMEY (SEQ ID NO:284), a CDR-L1 comprising an amino acid sequence KSSQSVLYSSNQKNYLA (SEQ ID NO:285), a CDR-L2 comprising an amino acid sequence WASTRES (SEQ ID NO:147), and a CDR-L3 comprising an amino acid sequence HQYLSSHT (SEQ ID NO:287). In some embodiments, the CD2 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least

96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:277 or 278, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:279.

[0168] In some embodiments, the CD2 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:291, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:292. In some embodiments, the CD2 targeting moiety comprises a CDR-H1 comprising an amino acid sequence EYYMY (SEQ ID NO:293), a CDR-H2 comprising an amino acid sequence RIDPEDGSIDYVEKFKK (SEQ ID NO:294), a CDR-H3 comprising an amino acid sequence GKFNYRFAY (SEQ ID NO:295), a CDR-L1 comprising an amino acid sequence RSSQSLLHSSGNTYLN (SEQ ID NO:296), a CDR-L2 comprising an amino acid sequence LVSKLES (SEQ ID NO:297), and a CDR-L3 comprising an amino acid sequence MQFTHYPYT (SEQ ID NO:298). In some embodiments, the CD2 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:288 or 289, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:290.

[0169] In some embodiments, the CD2 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:302, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:292. In some embodiments, the CD2 targeting moiety comprises a CDR-H1 comprising an amino acid sequence EYYMY (SEQ ID NO:293), a CDR-H2 comprising an amino acid sequence RIDPEDGSIDYVEKFKK (SEQ ID NO:294), a CDR-H3 comprising an amino acid sequence GKFNYRFAY (SEQ ID NO:295), a CDR-L1 comprising an amino acid sequence RSSQSLLHSSGNTYLN (SEQ ID NO:296), a CDR-L2

comprising an amino acid sequence LVSKLES (SEQ ID NO:297), and a CDR-L3 comprising an amino acid sequence MQFTHYPYT (SEQ ID NO:298). In some embodiments, the CD2 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:299 or 300, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:290.

CD3 Targeting Moieties

[0170] In some embodiments, the target molecule is CD3. In some embodiments, the target cell is CD3+. CD3 is a multimeric protein complex made up of four polypeptide chains (CD3-epsilon (ε), CD3-gamma (γ), CD3-delta (δ), and CD3-zeta (ζ)) to form a CD3γε–CD3δε–CD3ζζ signaling hexamer that associates with the T cell receptor (TCR). The CD3/TCR complex is critical for T cells to recognize foreign antigens and activate T-cell adaptive immunity. CD3 is expressed by all T cells and is a defining marker of the T lymphocyte lineage. See, e.g., Dong et al. (2019) Nature 573:546-552, hereby incorporated by reference in its entirety.

[0171] In some embodiments, the CD3 targeting moiety includes an antibody or antigen-binding fragment thereof that binds to CD3. In some embodiments, the CD3 targeting moiety is an antibody or antigen-binding fragment thereof (e.g., a Fab, Fab', $F(ab')_2$, Fv fragment, scFv, DARPIN, VHH domain, FN3 domain, nanobody, single domain antibody, or Centyrin). In other embodiments, the CD3 targeting moiety includes a ligand, a folate moiety, an antibiotic mimetic, a polynucleotide (such as a DNA or RNA apatamer), a carbohydrate, a vitamin, a cytokine, or a chemokine. In some embodiments, the CD3 targeting moiety is an anti-CD3 antibody or antigen binding fragment thereof. In some embodiments, the CD3 targeting moiety is an IgA, IgG, IgE, or IgM antibody. In some embodiments, the CD3 targeting moiety is a bispecific or multi-specific antibody or fragment thereof. In some embodiments, the CD3 targeting moiety is a humanized antibody or antigen-binding fragment thereof.

[0172] Exemplary anti-CD3 binders, antibodies, or antigen-binding fragments thereof include SP34 mouse monoclonal antibody (*see*, for example, Pressano, S. The EMBO J. 4:337-344, 1985; Alarcon, B. EMBO J. 10:903-912, 1991; Salmeron A. *et al.*, J. Immunol. 147:3047-52, 1991; Yoshino N. *et al.*, Exp. Anim 49:97-110, 2000; Conrad M L. *et al.*, Cytometry 71A:925-33, 2007; Yang *et al.*, J. Immunol. 137:1097-1100: 1986; US 8,846,042; US 11,013,800; and US 10,870,701), Cris-7 monoclonal antibody (Reinherz, E. L. *et al.* (eds.),

Leukocyte typing II, Springer Verlag, New York, (1986)), BC3 monoclonal antibody (Anasetti *et al.* (1990) J. Exp. Med. 172:1691), OKT3 (Ortho multicenter Transplant Study Group (1985) N. Engl. J. Med. 313:337) and derivatives thereof such as OKT3 ala-ala (Herold *et al.* (2003) J. Clin. Invest. 11:409), visilizumab (Carpenter *et al.* (2002) Blood 99:2712), mosunetuzumab, odronextamab, tebentafusp, teplizumab, teclistamab, muromonab, plamotamab, HPN536, pasotuxizumab, flotetuzumab, and 145-2C11 monoclonal antibody (Hirsch *et al.* (1988) J. Immunol. 140: 3766). Further CD3 binding molecules contemplated herein include UCHT-1 (Beverley, P C and Callard, R. E. (1981) Eur. J. Immunol. 11: 329-334) and CD3 binding molecules described in WO2004/106380; WO2010/037838; WO2008/119567; WO2007/042261; WO2010/0150918; the contents of each of which are incorporated herein by reference in their entirety.

[0173] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:141 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:142. In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:152 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:153. In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:163 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:164. In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:174 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:175. In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:185 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:186. In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:196 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:197. In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:207 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:208. In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:218 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:219. In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:228 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:229. In some embodiments, the CD3

targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:238 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:239. In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:248 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:249. In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:258 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:259. SEQ ID NOs:141-142, 152-153, 163-163, 174-175, 185-186, 196-197, 207-208, 218-219, 228-229, 238-239, 248-249, and 258-259 are shown in **Tables 19, 30,** and **32**, with complementary determining regions (CDRs) marked in bold.

[0174] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:141, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:142. In some embodiments, the CD3 targeting moiety comprises a CDR-H1 comprising an amino acid sequence NYYIH (SEQ ID NO:143), a CDR-H2 comprising an amino acid sequence WIYPGDGNTKYNEKFKG (SEQ ID NO:144), a CDR-H3 comprising an amino acid sequence DSYSNYYFDY (SEQ ID NO:145), a CDR-L1 comprising an amino acid sequence KSSQSLLNSRTRKNYLA (SEQ ID NO:146), a CDR-L2 comprising an amino acid sequence WASTRES (SEQ ID NO:147), and a CDR-L3 comprising an amino acid sequence TQSFILRT (SEQ ID NO:148). In some embodiments, the CD3 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:138 or 139, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:140. In some embodiments, the CD3 targeting moiety is mosunetuzumab or an antigen-binding fragment thereof.

[0175] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence

identity to the amino acid sequence of SEQ ID NO:152, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:153. In some embodiments, the CD3 targeting moiety comprises a CDR-H1 comprising an amino acid sequence DYTMH (SEQ ID NO:154), a CDR-H2 comprising an amino acid sequence GISWNSGSIGYADSVKG (SEQ ID NO:155), a CDR-H3 comprising an amino acid sequence DNSGYGHYYYGMDV (SEQ ID NO:156), a CDR-L1 comprising an amino acid sequence RASQSVSSNLA (SEQ ID NO:157), a CDR-L2 comprising an amino acid sequence GASTRAT (SEQ ID NO:158), and a CDR-L3 comprising an amino acid sequence QHYINWPLT (SEQ ID NO:159). In some embodiments, the CD3 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:149 or 150, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:151. In some embodiments, the CD3 targeting moiety is odronextamab or an antigen-binding fragment thereof.

[0176] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:163, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:164. In some embodiments, the CD3 targeting moiety comprises a CDR-H1 comprising an amino acid sequence GYTMN (SEQ ID NO:165), a CDR-H2 comprising an amino acid sequence LINPYKGVSTYNQKFKD (SEQ ID NO:166), a CDR-H3 comprising an amino acid sequence SGYYGDSDWYFDV (SEQ ID NO:167), a CDR-L1 comprising an amino acid sequence RASQDIRNYLN (SEQ ID NO:168), a CDR-L2 comprising an amino acid sequence YTSRLES (SEQ ID NO:169), and a CDR-L3 comprising an amino acid sequence QQGNTLPWT (SEQ ID NO:170). In some embodiments, the CD3 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:160 or

161, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:162. In some embodiments, the CD3 targeting moiety is tebentafusp or an antigen-binding fragment thereof.

[0177] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:174, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:175. In some embodiments, the CD3 targeting moiety comprises a CDR-H1 comprising an amino acid sequence RYTMH (SEQ ID NO:176), a CDR-H2 comprising an amino acid sequence YINPSRGYTNYNQKVKD (SEQ ID NO:177), a CDR-H3 comprising an amino acid sequence YYDDHYCLDY (SEQ ID NO:178), a CDR-L1 comprising an amino acid sequence SASSSVSYMN (SEQ ID NO:179), a CDR-L2 comprising an amino acid sequence DTSKLAS (SEQ ID NO:180), and a CDR-L3 comprising an amino acid sequence QQWSSNPFT (SEQ ID NO:181). In some embodiments, the CD3 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:171 or 172, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:173. In some embodiments, the CD3 targeting moiety is teplizumab or an antigen-binding fragment thereof.

[0178] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:185, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:186. In some embodiments, the CD3 targeting moiety comprises a CDR-H1 comprising an amino acid sequence NTYAMN (SEQ ID NO:187), a CDR-H2 comprising an amino acid sequence RIRSKYNNYATYYAASVKG (SEQ ID NO:188), a CDR-H3 comprising an amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO:189), a

CDR-L1 comprising an amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO:190), a CDR-L2 comprising an amino acid sequence GTNKRAP (SEQ ID NO:191), and a CDR-L3 comprising an amino acid sequence ALWYSNLWV (SEQ ID NO:192). In some embodiments, the CD3 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:182 or 183, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:184. In some embodiments, the CD3 targeting moiety is teclistamab or an antigen-binding fragment thereof.

[0179] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:196, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:197. In some embodiments, the CD3 targeting moiety comprises a CDR-H1 comprising an amino acid sequence SYTMH (SEQ ID NO:198), a CDR-H2 comprising an amino acid sequence YINPRSGYTHYNQKLKD (SEQ ID NO:199), a CDR-H3 comprising an amino acid sequence SAYYDYDGFAY (SEQ ID NO:200), a CDR-L1 comprising an amino acid sequence SASSSVSYMN (SEQ ID NO:179), a CDR-L2 comprising an amino acid sequence DTSKLAS (SEQ ID NO:180), and a CDR-L3 comprising an amino acid sequence QQWSSNPPT (SEQ ID NO:203). In some embodiments, the CD3 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:193 or 194, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:195. In some embodiments, the CD3 targeting moiety is visilizumab or an antigen-binding fragment thereof.

[0180] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence

identity to the amino acid sequence of SEQ ID NO:207, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:208. In some embodiments, the CD3 targeting moiety comprises a CDR-H1 comprising an amino acid sequence RYTMH (SEQ ID NO:176), a CDR-H2 comprising an amino acid sequence YINPSRGYTNYNQKFKD (SEQ ID NO:210), a CDR-H3 comprising an amino acid sequence YYDDHYCLDY (SEQ ID NO:178), a CDR-L1 comprising an amino acid sequence SASSSVSYMN (SEQ ID NO:179), a CDR-L2 comprising an amino acid sequence DTSKLAS (SEQ ID NO:180), and a CDR-L3 comprising an amino acid sequence QQWSSNPFT (SEQ ID NO:181). In some embodiments, the CD3 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:204 or 205, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:206. In some embodiments, the CD3 targeting moiety is muromonab or an antigen-binding fragment thereof.

[0181] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:218, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:219. In some embodiments, the CD3 targeting moiety comprises a CDR-H1 comprising an amino acid sequence KYAMN (SEQ ID NO:220), a CDR-H2 comprising an amino acid sequence RIRSKYNNYATYYADSVKD (SEQ ID NO:221), a CDR-H3 comprising an amino acid sequence HGNFGNSYISYWAY (SEQ ID NO:222), a CDR-L1 comprising an amino acid sequence GSSTGAVTSGNYPN (SEQ ID NO:223), a CDR-L2 comprising an amino acid sequence GTKFLAP (SEQ ID NO:224), and a CDR-L3 comprising an amino acid sequence VLWYSNRWV (SEQ ID NO:225). In some embodiments, the CD3 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:215 or 216, and a second polypeptide comprising an amino acid

sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:217. In some embodiments, the CD3 targeting moiety is SP34 or an antigen-binding fragment thereof.

[0182] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:228, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:229. In some embodiments, the CD3 targeting moiety comprises a CDR-H1 comprising an amino acid sequence TYAMN (SEQ ID NO:230), a CDR-H2 comprising an amino acid sequence RIRSKYNNYATYYADSVKG (SEQ ID NO:231), a CDR-H3 comprising an amino acid sequence HGNFGDSYVSWFAY (SEQ ID NO:232), a CDR-L1 comprising an amino acid sequence GSSTGAVTTSNYAN (SEQ ID NO:233), a CDR-L2 comprising an amino acid sequence GTNKRAP (SEQ ID NO:191), and a CDR-L3 comprising an amino acid sequence ALWYSNHWV (SEQ ID NO:235). In some embodiments, the CD3 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:226, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:227. In some embodiments, the CD3 targeting moiety is plamotamab or an antigen-binding fragment thereof.

[0183] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:238, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:239. In some embodiments, the CD3 targeting moiety comprises a CDR-H1 comprising an amino acid sequence KYAIN (SEQ ID NO:240), a CDR-H2 comprising an amino acid sequence RIRSKYNNYATYYADQVKD (SEQ ID NO:241), a

CDR-H3 comprising an amino acid sequence HANFGNSYISYWAY (SEQ ID NO:242), a CDR-L1 comprising an amino acid sequence ASSTGAVTSGNYPN (SEQ ID NO:243), a CDR-L2 comprising an amino acid sequence GTKFLVP (SEQ ID NO:244), and a CDR-L3 comprising an amino acid sequence TLWYSNRWV (SEQ ID NO:245). In some embodiments, the CD3 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:236, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:237. In some embodiments, the CD3 targeting moiety is HPN536 or an antigen-binding fragment thereof.

[0184] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:218, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:249. In some embodiments, the CD3 targeting moiety comprises a CDR-H1 comprising an amino acid sequence KYAMN (SEQ ID NO:220), a CDR-H2 comprising an amino acid sequence RIRSKYNNYATYYADSVKD (SEQ ID NO:221), a CDR-H3 comprising an amino acid sequence HGNFGNSYISYWAY (SEQ ID NO:222), a CDR-L1 comprising an amino acid sequence GSSTGAVTSGNYPN (SEQ ID NO:223), a CDR-L2 comprising an amino acid sequence GTKFLAP (SEQ ID NO:224), and a CDR-L3 comprising an amino acid sequence VLWYSNRWV (SEQ ID NO:225). In some embodiments, the CD3 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:246, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:247. In some embodiments, the CD3 targeting moiety is pasotuxizumab or an antigen-binding fragment thereof.

[0185] In some embodiments, the CD3 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:258, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:259. In some embodiments, the CD3 targeting moiety comprises a CDR-H1 comprising an amino acid sequence TYAMN (SEQ ID NO:230), a CDR-H2 comprising an amino acid sequence RIRSKYNNYATYYADSVKD (SEQ ID NO:221), a CDR-H3 comprising an amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO:189), a CDR-L1 comprising an amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO:190), a CDR-L2 comprising an amino acid sequence GTNKRAP (SEQ ID NO:191), and a CDR-L3 comprising an amino acid sequence ALWYSNLWV (SEQ ID NO:192). In some embodiments, the CD3 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:256, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:257. In some embodiments, the CD3 targeting moiety is flotetuzumab or an antigen-binding fragment thereof.

CD5 Targeting Moieties

[0186] In some embodiments, the target molecule is CD5. In some embodiments, the target cell is CD5+. CD5 is a type-I transmembrane glycoprotein with an extracellular region composed of three scavenger receptor cysteine-rich (SRCR) domains. Several CD5 ligands have been reported such as CD72, the IgV(H) frame-work region and several polypeptides (gp40-80, gp150) whose identity remains undetermined. CD5 regulates T cell functions and development, including negative regulation of TCR signaling. CD5 is an activation marker of T cells, wherein the expression of CD5 increases according to the magnitude of the signal delivered by the TCR. Consequently, CD5 expression reflects the heterogeneity of the signal strength associated with each individual TCR within a polyclonal T cell population. See, e.g., Voisinne et al. (2018) Front. Immunol. 9:2900, hereby incorporated by reference in its entirety.

[0187] In some embodiments, the CD5 targeting moiety includes an antibody or antigen-binding fragment thereof that binds to CD5. In some embodiments, the CD5 targeting moiety is an antibody or antigen-binding fragment thereof (e.g., a Fab, Fab', $F(ab')_2$, Fv fragment, scFv, DARPIN, VHH domain, FN3 domain, nanobody, single domain antibody, or Centyrin). In other embodiments, the CD5 targeting moiety includes a ligand, a folate moiety, an antibiotic mimetic, a polynucleotide (such as a DNA or RNA apatamer), a carbohydrate, a vitamin, a cytokine, or a chemokine. In some embodiments, the CD5 targeting moiety is an anti-CD5 antibody or antigen binding fragment thereof. In some embodiments, the CD5 targeting moiety is an IgA, IgG, IgE, or IgM antibody. In some embodiments, the CD5 targeting moiety is a bispecific or multi-specific antibody or fragment thereof. In some embodiments, the CD5 targeting moiety is a humanized antibody or antigen-binding fragment thereof.

[0188] Exemplary anti-CD5 binders, antibodies, or antigen-binding fragments thereof include AFM 16 (e.g., Affimed Therapeutics); AFM 17 (e.g., Affimed Therapeutics), RM354, L17F12, CRIS-1, UCHT2, RM314, SP19, and CD5-5D7, as well as anti-CD5 antibodies or antigen-binding fragments thereof disclosed in any of: US 10,786,549; US20110250203; Dai et al. (2021) Mol Ther. 29(9)2707-2722; etc., each hereby incorporated by reference in its entirety.

[0189] In some embodiments, the CD5 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:357 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:358. SEQ ID NOs:357 and 358 are shown in **Table 20**, with complementary determining regions (CDRs) marked in bold.

[0190] In some embodiments, the CD5 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:357, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:358. In some embodiments, the CD5 targeting moiety comprises a CDR-H1 comprising an amino acid sequence TSGMGVG (SEQ ID NO:359), a CDR-H2 comprising an amino acid sequence HIWWDDDVYYNPSLKS (SEQ ID NO:360), a CDR-H3 comprising an amino acid sequence RRATGTGFDY (SEQ ID NO:361), a CDR-L1 comprising an amino acid sequence QASQDVGTAVA (SEQ ID NO:362), a CDR-L2 comprising an amino acid sequence WTSTRHT (SEQ ID NO:363), and a CDR-L3 comprising an amino acid sequence HQYNSYNT (SEQ ID NO:364). In some embodiments, the CD5

targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:354 or 355, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:356.

CD7 Targeting Moieties

[0191] In some embodiments, the target molecule is CD7. In some embodiments, the target cell is CD7+. CD7 (also known as GP40, LEU-9, Tp40, and TP41) is a transmembrane glycoprotein expressed by T cells, NK cells, and their precursors. It is present in >95% of lymphoblastic T-cell leukemias and lymphomas and a subset of PTCLs. CD7 has a costimulatory role in T-cell activation and cytokine production (e.g., IL-2) upon binding to its ligand, K12/SECTM1.

[0192] In some embodiments, the CD7 targeting moiety includes an antibody or antigen-binding fragment thereof that binds to CD7. In some embodiments, the CD7 targeting moiety is an antibody or antigen-binding fragment thereof (e.g., a Fab, Fab', $F(ab')_2$, Fv fragment, scFv, DARPIN, VHH domain, FN3 domain, nanobody, single domain antibody, or Centyrin). In other embodiments, the CD7 targeting moiety includes a ligand, a folate moiety, an antibiotic mimetic, a polynucleotide (such as a DNA or RNA apatamer), a carbohydrate, a vitamin, a cytokine, or a chemokine. In some embodiments, the CD7 targeting moiety is an anti-CD7 antibody or antigen binding fragment thereof. In some embodiments, the CD7 targeting moiety is an IgA, IgG, IgE, or IgM antibody. In some embodiments, the CD7 targeting moiety is a bispecific or multi-specific antibody or fragment thereof. In some embodiments, the CD7 targeting moiety is a humanized antibody or antigen-binding fragment thereof.

[0193] Exemplary anti-CD7 binders, antibodies, or antigen-binding fragments thereof include SP94 (e.g., Roche Diagnostics), A20153E (e.g., BioLegend), 4H9/CD7 (e.g., BioLegend), 124-1D1, CD7-6B7, B-F12, 4H9, 3A1E, LT7, MEM-186, and MG34, as well as anti-CD7 antibodies or antigen-binding fragments thereof disclosed in any of: US 11,440,958; US 11,390,658; US20240075143; US20230128800; US20230399398; US20230159636; WO2003051926 WO2023185256; Wang et al. (2024) Biomolecules 14(1):106; etc., each hereby incorporated by reference in its entirety.

[0194] In some embodiments, the CD7 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:313 and a light chain variable region

comprising the amino acid sequence of SEQ ID NO:314. In some embodiments, the CD7 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:324 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:325. In some embodiments, the CD7 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:335 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:336. In some embodiments, the CD7 targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:346 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:347. SEQ ID NOs:313-314, 324-325, 335-336, and 346-347 are shown in **Table 20**, with complementary determining regions (CDRs) marked in bold.

[0195] In some embodiments, the CD7 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:313, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:314. In some embodiments, the CD7 targeting moiety comprises a CDR-H1 comprising an amino acid sequence NYGMN (SEQ ID NO:315), a CDR-H2 comprising an amino acid sequence WINTYTGEPTYADDFKG (SEQ ID NO:316), a CDR-H3 comprising an amino acid sequence WAYFYGSSPYFFDY (SEQ ID NO:317), a CDR-L1 comprising an amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO:190), a CDR-L2 comprising an amino acid sequence GTNNRAP (SEQ ID NO:319), and a CDR-L3 comprising an amino acid sequence ALWCSNHLV (SEQ ID NO:320). In some embodiments, the CD7 targeting moiety i5s a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:310 or 311, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:312. In some embodiments, the CD7 targeting moiety is grisnilimab or an antigen-binding fragment thereof.

[0196] In some embodiments, the CD7 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence

identity to the amino acid sequence of SEQ ID NO:324, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:325. In some embodiments, the CD7 targeting moiety comprises a CDR-H1 comprising an amino acid sequence NYAMS (SEQ ID NO:326), a CDR-H2 comprising an amino acid sequence TISGSGGSTYYADSAK (SEQ ID NO:327), a CDR-H3 comprising an amino acid sequence GGLLYFGEFHFDY (SEQ ID NO:328), a CDR-L1 comprising an amino acid sequence RASQGISNYLA (SEQ ID NO:329), a CDR-L2 comprising an amino acid sequence AASSLQS (SEQ ID NO:330), and a CDR-L3 comprising an amino acid sequence QHYNSYPLT (SEQ ID NO:331). In some embodiments, the CD7 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:321 or 322, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:323.

[0197] In some embodiments, the CD7 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:335, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:336. In some embodiments, the CD7 targeting moiety comprises a CDR-H1 comprising an amino acid sequence NAWMS (SEQ ID NO:337), a CDR-H2 comprising an amino acid sequence RIKSKTDGGTTDYAAPVKG (SEQ ID NO:338), a CDR-H3 comprising an amino acid sequence TIEAVAGHFDY (SEQ ID NO:339), a CDR-L1 comprising an amino acid sequence RASQSISSWLA (SEQ ID NO:340), a CDR-L2 comprising an amino acid sequence KASSLES (SEQ ID NO:341), and a CDR-L3 comprising an amino acid sequence QQYNNYSPT (SEQ ID NO:342). In some embodiments, the CD7 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:332 or 333, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at

least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:334.

[0198] In some embodiments, the CD7 targeting moiety comprises a heavy chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:346, and/or a light chain variable region comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:347. In some embodiments, the CD7 targeting moiety comprises a CDR-H1 comprising an amino acid sequence RYAMS (SEQ ID NO:348), a CDR-H2 comprising an amino acid sequence SISASGATTFYADPVKG (SEQ ID NO:349), a CDR-H3 comprising an amino acid sequence DQDFDILTGYLNWFDP (SEQ ID NO:350), a CDR-L1 comprising an amino acid sequence RVSQSVSSYLA (SEQ ID NO:351), a CDR-L2 comprising an amino acid sequence DTSNRAT (SEQ ID NO:352), and a CDR-L3 comprising an amino acid sequence QQRRNWPLT (SEQ ID NO:353). In some embodiments, the CD7 targeting moiety is a Fab fragment comprising a first polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:343 or 344, and a second polypeptide comprising an amino acid sequence having at least 90% (e.g., at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%) sequence identity to the amino acid sequence of SEQ ID NO:345.

CD28 Targeting Moieties

[0199] In some embodiments, the target molecule is CD28. In some embodiments, the target cell is CD28+. CD28 is a T-cell costimulatory molecule. It is a homodimeric glycoprotein member of the Ig gene superfamily and has a single IgV domain. It is expressed on T cells where it is activated upon binding to its ligands B7-1 or B7-2 (CD80 or CD86), which are expressed on professional antigen-presenting cells. CD28 does not affect T cell activation unless the T-cell receptor is first engaged by cognate antigen. Upon antigen recognition, CD28 signaling strongly amplifies T-cell receptor signaling to activate T cells, and CD28 co-stimulation of T cells increases glucose uptake and glycolysis during an immune response.

[0200] In some embodiments, the CD28 targeting moiety includes an antibody or antigen-binding fragment thereof that binds to CD28. In some embodiments, the CD28 targeting moiety is an antibody or antigen-binding fragment thereof (e.g., a Fab, Fab', $F(ab')_2$, Fv fragment, scFv,

DARPIN, VHH domain, FN3 domain, nanobody, single domain antibody, or Centyrin). In other embodiments, the CD28 targeting moiety includes a ligand, a folate moiety, an antibiotic mimetic, a polynucleotide (such as a DNA or RNA apatamer), a carbohydrate, a vitamin, a cytokine, or a chemokine. In some embodiments, the CD28 targeting moiety is an anti-CD28 antibody or antigen binding fragment thereof. In some embodiments, the CD28 targeting moiety is an IgA, IgG, IgE, or IgM antibody. In some embodiments, the CD28 targeting moiety is a bispecific or multi-specific antibody or fragment thereof. In some embodiments, the CD28 targeting moiety is a humanized antibody or antigen-binding fragment thereof.

[0201] Exemplary anti-CD28 binders, antibodies, or antigen-binding fragments thereof include Theralizumab (i.e., TGN1412, TAB08, or CD28-SuperMAB, e.g., TeGenero), davoceticept (i.e., ALPN-202, e.g., Alpine Immune Sciences, Inc.), FPT155 (Five Prime Therapeutics, Inc.), 10F3, RM404, 15E8, CD28.3, Leu-2, 9.3, EX5.3D10, YTH913.12, S20013F, S20013B, and QA17A12, as well as anti-CD28 antibodies or antigen-binding fragments thereof disclosed in any of: US 7,175,843; US 8,168,759; US 8,785,138; US 8,785,604; US 10,273,281; US 11,117,949; US20180112000; US20230227530; US20230348600; US20230382972; WO1994029436; WO2002051871; Tan et al. (2002) J Immunol 169:1119-1125; Elsyed et al. (2023) Mabs 15(1):2220839; etc., each hereby incorporated by reference in its entirety.

[0202] In some embodiments, the CD28 targeting moiety is a CD28 receptor ligand. In some embodiments, the CD28 receptor ligand is CD80. Accordingly, in some embodiments, the CD28 targeting moiety is CD80. CD80 is a costimulatory molecule known for its role in T-cell activation and also in regulating the activity of normal and malignant B cells. Surface CD80 is expressed transiently on activated B cells, macrophages, and DCs. In certain embodiments, the CD28 targeting moiety is a CD80 extracellular domain (ECD), for example a CD80 ECD comprising an amino acid sequence of SEQ ID NO:365 or comprising at least about 80% (such as about any of 81%, 82%, 83%, 84%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or more) sequence identity to the amino acid sequence of SEQ ID NO:365.

Exemplary Dual Targeting Moieties

[0203] In some embodiments, the targeted LNP (conjugate) comprises two or more targeting moieties, wherein each targeting moiety independently binds to a T-cell antigen selected from the group consisting of CD2, CD3, CD4, CD5, CD7, CD8, CD28, CD80, CD137, CD45, T-cell receptor (TCR)-β,TCR-α, TCR-α/β, TCR-γ/δ, PD1, CTLA4, TIM3, LAG3, CD18, IL-2 receptor, CD11a, TLR2, TLR4, TLR5, IL-7 receptor, and IL-15 receptor. In some embodiments, the

targeted LNP (conjugate) comprises two or more targeting moieties, wherein each targeting moiety independently targets a receptor on the surface of the T cell selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, and CD28. In some embodiments, the targeted LNP (conjugate) comprises two or more targeting moieties, wherein at least a first targeting moiety targets CD3, and wherein at least a second targeting moiety targets a receptor on the surface of the T cell is selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, and CD28. In some embodiments, the first targeting moiety comprises a CD3-binding domain comprising a VH comprising a CDR-H1, a CDR-H2, a CDR-H3 of any one of mosunetuzumab, odronextamab, tebentafusp, teplizumab, teclistamab, visilizumab, muromonab, SP34, plamotamab, HPN536, pasotuxizumab, and flotetuzumab, and a VL comprising a CDR-L1, a CDR-L2, and a CDR-L3 of any one of mosunetuzumab, odronextamab, tebentafusp, teplizumab, teclistamab, visilizumab, muromonab, SP34, plamotamab, HPN536, pasotuxizumab, and flotetuzumab. In some embodiments, the first targeting moiety comprises a CD3-binding domain comprising the VH and the VL of any one of mosunetuzumab, odronextamab, tebentafusp, teplizumab, teclistamab, visilizumab, muromonab, SP34, plamotamab, HPN536, pasotuxizumab, and flotetuzumab. Exemplary sequences for the first targeting moiety and/or the second targeting moiety can be found, e.g., in **Tables 19, 20, 30,** and **32.**

[0204] In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein a first targeting moiety binds to CD3, and a second targeting moiety binds to CD5. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein a first targeting moiety binds to CD3, and a second targeting moiety binds to CD7. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein a first targeting moiety binds to CD3, and a second targeting moiety binds to CD28. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein a first targeting moiety binds to CD5 and a second targeting moiety binds to CD28. In some embodiments, the targeted LNP (conjugate) comprises two targeting moieties, wherein a first targeting moiety binds to CD7, and a second targeting moiety binds to CD28. In some embodiments the CD28 targeting moiety is a CD80 extracellular domain (ECD). In some embodiments, a targeted LNP comprises two targeting moieties, wherein a first targeting moiety binds to CD3, and a second targeting moiety binds to CD3. In some embodiments, the first targeting moiety that binds to CD3 comprises a different anti-CD3 antibody, Fab fragment, or scFv than the second targeting moiety that binds to CD3.

[0205] In some embodiments, a targeted LNP comprises two targeting moieties, wherein a first targeting moiety binds to CD3, and a second targeting moiety binds to CD5. In some

embodiments, the first targeting moiety that binds to CD3 comprises a VH comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:141, 152, 163, 174, 185, 196, 207, 218, 228, 238, and 258, and a VL comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:142, 153, 164, 175, 186, 197, 208, 219, 229, 239, 249, and 259, and the second targeting moiety that binds to CD5 comprises a VH comprising the amino acid of SEQ ID NO:357, and a VL comprising the amino acid sequence of SEQ ID NO:358.

[0206] In some embodiments, a targeted LNP comprises two targeting moieties, wherein a first targeting moiety binds to CD3, and a second targeting moiety binds to CD2. In some embodiments, the first targeting moiety that binds to CD3 comprises a VH comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:141, 152, 163, 174, 185, 196, 207, 218, 228, 238, and 258, and a VL comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:142, 153, 164, 175, 186, 197, 208, 219, 229, 239, 249, and 259, and the second targeting moiety that binds to CD2 comprises a VH comprising the amino acid of SEQ ID NO:269, and a VL comprising the amino acid sequence of SEQ ID NO:270. In some embodiments, the first targeting moiety that binds to CD3 comprises a VH comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:141, 152, 163, 174, 185, 196, 207, 218, 228, 238, and 258, and a VL comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:142, 153, 164, 175, 186, 197, 208, 219, 229, 239, 249, and 259, and the second targeting moiety that binds to CD2 comprises a VH comprising the amino acid of SEQ ID NO:280, and a VL comprising the amino acid sequence of SEQ ID NO:281. In some embodiments, the first targeting moiety that binds to CD3 comprises a VH comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:141, 152, 163, 174, 185, 196, 207, 218, 228, 238, and 258, and a VL comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:142, 153, 164, 175, 186, 197, 208, 219, 229, 239, 249, and 259, and the second targeting moiety that binds to CD2 comprises a VH comprising the amino acid of SEQ ID NO:291, and a VL comprising the amino acid sequence of SEQ ID NO:292. In some embodiments, the first targeting moiety that binds to CD3 comprises a VH comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:141, 152, 163, 174, 185, 196, 207, 218, 228, 238, and 258, and a VL comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:142, 153, 164, 175, 186, 197, 208, 219, 229, 239, 249, and 259, and the second targeting moiety that binds to CD2 comprises a VH comprising the amino acid of SEQ ID NO:301, and a VL comprising the amino acid sequence of SEQ ID NO:292.

[0207] In some embodiments, a targeted LNP comprises two targeting moieties, wherein a first targeting moiety binds to CD3, and a second targeting moiety binds to CD7. In some

embodiments, the first targeting moiety that binds to CD3 comprises a VH comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:141, 152, 163, 174, 185, 196, 207, 218, 228, 238, and 258, and a VL comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:142, 153, 164, 175, 186, 197, 208, 219, 229, 239, 249, and 259, and the second targeting moiety that binds to CD7 comprises a VH comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 313, 324, 335, and 346, and a VL comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 314, 325, 336, and 347. In some embodiments, the first targeting moiety comprises a CD3-binding domain comprising the VH and the VL of any one of mosunetuzumab, odronextamab, tebentafusp, teplizumab, teclistamab, visilizumab, muromonab, SP34, plamotamab, HPN536, pasotuxizumab, and flotetuzumab, and the second targeting moiety comprises a CD7-binding domain comprising the VH and the VL of grisnilimab.

[0208] In some embodiments, a targeted LNP comprises two targeting moieties, wherein a first targeting moiety binds to CD3, and a second targeting moiety binds to CD28. In some embodiments the CD28 targeting moiety is a CD80 extracellular domain (ECD). In some embodiments, the first targeting moiety that binds to CD3 comprises a VH comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:141, 152, 163, 174, 185, 196, 207, 218, 228, 238, and 258, and a VL comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:142, 153, 164, 175, 186, 197, 208, 219, 229, 239, 249, and 259, and the second targeting moiety that binds to CD28 comprises the amino acid sequence of SEQ ID NO:365. In some embodiments, the first targeting moiety comprises a VH comprising the amino acid sequence of SEQ ID NO:218 and a VL comprising the amino acid sequence of SEQ ID NO:219, and the second targeting moiety that binds to CD28 comprises the amino acid sequence of SEQ ID NO:365.

[0209] In some embodiments, a targeted LNP comprises two targeting moieties, wherein a first targeting moiety binds to CD3, and a second targeting moiety binds to CD3. In some embodiments, the first targeting moiety that binds to CD3 comprises a different anti-CD3 antibody, Fab fragment, or scFv than the second targeting moiety that binds to CD3. In some embodiments, the first targeting moiety and the second targeting moiety each independently comprises a CD3-binding domain comprising the VH and the VL of any one of mosunetuzumab, odronextamab, tebentafusp, teplizumab, teclistamab, visilizumab, muromonab, SP34, plamotamab, HPN536, pasotuxizumab, and flotetuzumab, wherein the first targeting moiety comprises a different a CD3-binding domain than the second targeting moiety. In some embodiments, the first targeting moiety and the second targeting moiety each independently

comprises a CD3-binding domain comprising a VH comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:141, 152, 163, 174, 185, 196, 207, 218, 228, 238, and 258, and a VL comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:142, 153, 164, 175, 186, 197, 208, 219, 229, 239, 249, and 259, wherein the first targeting moiety comprises a different a CD3-binding domain than the second targeting moiety. In some embodiments, the first targeting moiety comprises a VH comprising the amino acid sequence of SEQ ID NO:196 and a VL comprising the amino acid sequence of SEQ ID NO:197, and the second targeting moiety comprises a VH comprising the amino acid sequence of SEQ ID NO:218 and a VL comprising the amino acid sequence of SEQ ID NO:219.

Methods of Making Targeted LNPs (Conjugates)

[0210] Different approaches can be used to introduce a targeting moiety onto the surface of an LNP. For example, one approach relies on functionalizing a preformed LNP with a targeting moiety. The LNP generally includes a lipid that has polyethylene glycol (PEG) spacer functionalized with a reactive moiety such as a thiol, amine, maleimide or carboxylic acid group. The functionalized lipid of the LNP reacts with a complementary group that is covalently bonded to a targeting moiety, hence generating a conjugate of the LNP and the targeting moiety.

[0211] In some embodiments, the targeting moiety is conjugated to the lipid nanoparticle through a linker, and wherein the linker comprises a Click product formed from a Click reaction between a first Click handle on the targeting moiety and a second Click handle on the LNP. In some such embodiments, the targeting moiety is an antibody or antigen binding fragment thereof. In other such embodiments, the targeting moiety is a scFv. In some embodiments, the targeting moiety is a Fab fragment.

[0212] In one embodiment, the Click product can be formed using a copper-catalyzed Click reaction. One such copper-catalyzed Click reaction is a Huisgen 1,3-dipolar cycloaddition (CuAAC) between an azide and an alkyne. In some embodiments, the first or second Click handle comprises a cyclic derivative of the alkynyl group. In some embodiments, the cyclic derivative of the alkynyl group is selected from dibenzocyclooctyne. cyclooctyne, and difluorinated cyclooctyne. In some embodiments, the click chemistry involves strain promoted cycloaddition of azides. In some embodiments, the click chemistry is based upon reaction of strained alkenes.

[0213] In another embodiment, the Click product can be formed using copper-free Click chemistry. For example, the Click product can be formed between an azide and

dibenzocyclooctene (DBCO). Alternatively, the Click product can be formed using a Staudinger reaction between an azide and a phosphine, hence producing an aza-ylide.

[0214] In some embodiments, the Click product can be formed from an inverse electron demand Diels-Alder reaction between a trans-cyclooctene (TCO) moiety on the first or second Click handle and a tetrazine ring on the first or second Click handle. In some embodiments, the first Click handle comprises a tetrazine (Tz) ring and the second Click handle comprises a TCO moiety. In some embodiments, the tetrazine ring is unsubstituted. In some such embodiments, the tetrazine rung is methyltetrazine. In some embodiments, the tetrazine ring is a 6-methyl substituted tetrazine.

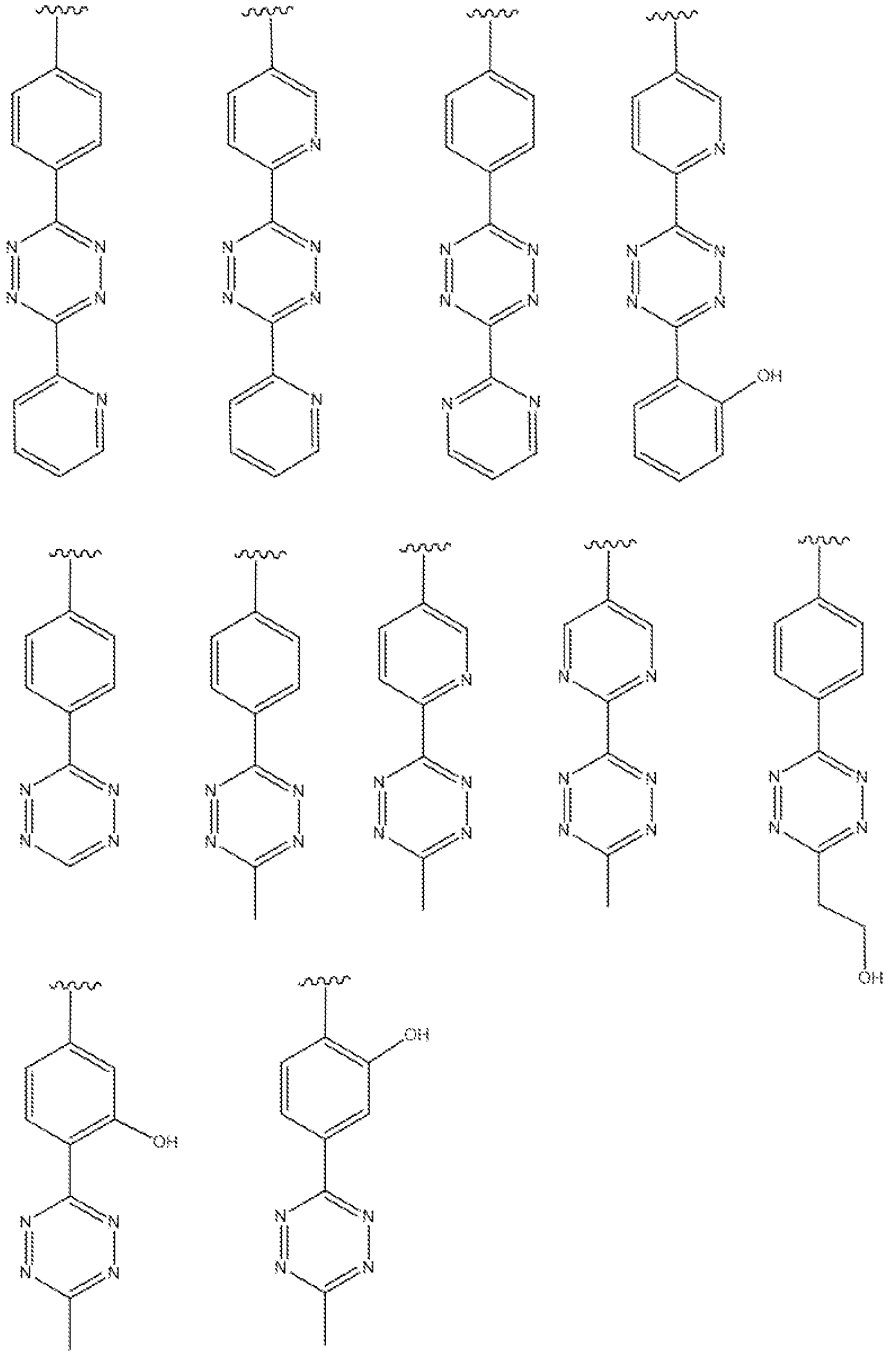
[0215] In another embodiment, the targeting moiety (e.g., antibody, Fab fragment or scFv) is first selectively modified with an enzyme recognition sequence. An enzyme recognizing the enzyme recognition sequence can site-specifically introduce the first Click handle onto the targeting moiety through covalent attachment. The first Click handle can next react with the second Click handle on the LNP to produce the targeted LNP. Hence, in one embodiment, an antibody, Fab fragment or single chain variable fragment (scFv) that is covalently linked to a first Click handle through a linker comprising an enzyme recognition sequence is reacted with an LNP comprising a second Click handle, thereby forming a Click reaction product that conjugates the antibody, Fab fragment or scFv to the LNP. In some embodiments, the antibody Fab fragment or scFv is directly bonded to the enzyme recognition sequence. In some embodiments, the targeting moiety (e.g., antibody Fab fragment or scFv) is bonded to the enzyme recognition sequence via one or more amino acid residues. Particular amino acid residues added that can be covalently attached to the C-terminus of the targeting moiety (e.g., antibody, Fab fragment or scFv) include, but are not limited to $(GGGGS)_v$ (SEQ ID NO:1), $(G)_v$, $(EAAAK)_v$ (SEQ ID NO:3), $(PAPAP)_v$ (SEQ ID NO:4), $(AP)_v$ and $A(EAAAK)_u$ $ALEA(EAAAK)_vA$ (SEQ ID NO:2), wherein u is 1-10 and v is 1-10.

[0216] In some embodiments, the enzyme recognition sequence is a sortase recognition motif or an LplA acceptor peptide. In some embodiments where the LNP is conjugated to an antibody, the C-terminus of one or more of the heavy or light chains of the antibody is covalently bonded to the enzyme recognition sequence (e.g., sortase recognition motif or LplA acceptor peptide) either directly or through a linker comprising one or more amino acid residues, a set forth herein. In some embodiments where the LNP is conjugated to a Fab fragment, the C-terminus of the heavy or light chain of the Fab fragment is covalently bonded to the enzyme recognition sequence (e.g., sortase recognition motif or LplA acceptor peptide). In some

embodiments where the LNP is conjugated to s scFv, the C-terminus of the scFv is covalently bonded to the enzyme recognition sequence (e.g., sortase recognition motif or LplA acceptor peptide). In some of the foregoing embodiments, the first Click handle comprises a tetrazine ring or TCO moiety and the second Click handle comprises a tetrazine ring or TCO moiety. In some such embodiments, the first Click handle comprises a tetrazine ring and the second Click handle comprises a TCO moiety. In some embodiments, the tetrazine ring is a methyltetrazine. In some embodiments, the conjugation efficiency achieved by the disclosed method is greater than 50%, greater than 60%, greater than 70%, greater than 80%, or greater than 90%. In some embodiments, the conjugation efficiency achieved by the disclosed method is from about 60% to about 95%. In some embodiments, the conjugation efficiency achieved by the disclosed methods are from about 70% to about 85%.

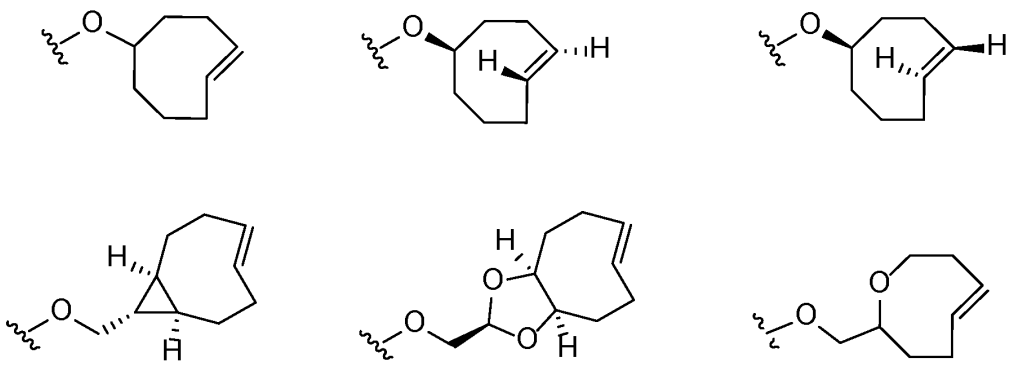
[0217] In some embodiments, the linker further comprises a spacer between the targeting moiety and the Click product. The spacer can include additional functional groups that indirectly link the targeting moiety to the Tz group. The spacer may also include additional amino acid residues that indirectly links the targeting moiety to the Tz group.

[0218] In some embodiments, the first or second Click handle is a tetrazine derivative having one of the following structures:



wherein ~~~ represents the point of attachment to the linker of the conjugate or to the LNP.

[0219] In some embodiments, the first or second Click handle is a TCO derivative having one of the following structures:



[0220] In some embodiments, the spacer that links the targeting moiety to the Tz ring is an enzyme recognition sequence. Accordingly, the disclosure provides methods of conjugating an LNP to a targeting moiety that has been modified with an enzyme recognition sequence, wherein said conjugating is accomplished via a Click reaction between a Tz ring covalently bound to the targeting moiety and a TCO moiety bound to the LNP. For instance, the disclosure provides methods of conjugating an LNP to an antibody, Fab fragment or single chain variable fragment (scFv), wherein the antibody, Fab fragment or scFv is covalently linked to a first Click handle (Tz ring) through a linker comprising an enzyme recognition sequence and the LNP is covalently linked to a second Click handle (TCO moiety), said method comprising contacting the LNP with an antibody, Fab fragment or scFv such that first Click handle reacts with the second Click handle to form a Click reaction product (dihydropyridazine) that conjugates the antibody, Fab fragment or scFv to the LNP. In some embodiments, the antibody, Fab fragment or scFv is directly bonded to the enzyme recognition sequence. In some embodiments, the antibody Fab fragment or scFv is bonded to the enzyme recognition sequence via one or more amino acid residues. Particular amino acid residues added that can be covalently attached to the C-terminus of the antibody, Fab fragment or scFv include, but are not limited to $(GGGGS)_v$ (SEQ ID NO:1), $(G)_v$, $(EAAAK)_v$ (SEQ ID NO:3), $(PAPAP)_v$ (SEQ ID NO:4), $(AP)_v$ and $A(EAAAK)_u$ $ALEA(EAAAK)_vA$ (SEQ ID NO:2), wherein u is 1-10 and v is 1-10.

[0221] In some embodiments, the enzyme recognition sequence is a sortase recognition motif or a LplA acceptor peptide. In some embodiments where the LNP is conjugated to an antibody, the C-terminus of one or more of the heavy or light chains of the antibody is covalently bonded to the enzyme recognition sequence (e.g., sortase recognition motif or LplA acceptor peptide) either directly or through a linker comprising one or more amino acid residues, a set forth herein. In some embodiments where the LNP is conjugated to a Fab fragment, the C-terminus of the heavy or light chain of the Fab fragment is covalently bonded to the enzyme recognition sequence (e.g., sortase recognition motif or LplA acceptor peptide). In some

embodiments where the LNP is conjugated to s scFv, the C-terminus of the scFv is covalently bonded to the enzyme recognition sequence (e.g., sortase recognition motif or LplA acceptor peptide). In some embodiments, the conjugation efficiency achieved by the disclosed method is greater than 50%, greater than 60%, greater than 70%, greater than 80%, or greater than 90%. In some embodiments, the conjugation efficiency achieved by the disclosed method is from about 60% to about 95%. In some embodiments, the conjugation efficiency achieved by the disclosed methods are from about 70% to about 85%.

[0222] In one embodiment, lipid nanoparticles conjugated to an antibody, Fab fragment or scFv in a site-specific manner are produced according to the following steps:

(i) covalently bonding a peptide with a sortase recognition site to one or more C-terminus of the antibody or Fab fragment, or to the C-terminus of the scFv;

(ii) contacting the product of step (i) with a first Click handle comprising a Tz ring covalently bound to three or more glycine residues in the presence of a sortase enzyme under conditions suitable for the sortase enzyme to ligate the antibody, Fab fragment or scFv to the first Click handle;

(iii) contacting the product of step (ii) with a lipid nanoparticle (LNP), wherein one or more lipids comprising the LNP include a second Click handle capable of reacting with the first Click handle, wherein the second Click handle comprises a TCO moiety, thereby generating a Click product.

[0223] In some embodiments, step (i) involves covalently bonding the peptide with the sortase recognition site to a linker covalently bonded to one or more C-terminus of the antibody or Fab fragment, or to the C-terminus of the scFv. In some embodiments, the linker comprises one or more amino acid residues. Particular amino acid residues added that can be covalently attached to the C-terminus of the antibody, Fab fragment or scFv include, but are not limited to $(GGGGS)_v$ (SEQ ID NO:1), $(G)_v$, $(EAAAK)_v$ (SEQ ID NO:3), $(PAPAP)_v$ (SEQ ID NO:4), $(AP)_v$ and $A(EAAAK)_u$ $ALEA(EAAAK)_vA$ (SEQ ID NO:2), wherein u is 1-10 and v is 1-10.

[0224] In some embodiments, the sortase recognition motif bonded to the C-terminus of the antibody, Fab fragment or scFv is bonded to one or more amino acid residues. For instance, in some embodiments, the glycine (G) of the LPXTG motif (SEQ ID NO:8) can be bonded to one or more (e.g., between 1-10) histidine (H) residues (see **FIG. 16**). The glycine and the histidine residues are removed during the transpeptidation reaction.

[0225] In some embodiments, step (ii) (i.e., the sortase-catalyzed reaction) is carried out in the presence of sortase A5. Sortase A5 is an engineered pentamutant variant of the wild-type sortase from *Staphylococcus aureus* that is significantly more active than the wild-type sortase. See U.S. patent No. 9,267,227. In such embodiments, the sortase recognition site bond to the antibody, Fab fragment or scFv in step (i) has the sequence LPXTG (SEQ ID NO:8), where X is any amino acid residue. In step (ii), sortase A catalyzes the transpeptidation reaction between the LPXTG recognition motif (SEQ ID NO:8) and a glycine residue bound to the first Click handle by cleaving the sortase recognition site between the threonine and the glycine residues.

[0226] In other embodiments, step (ii) is carried out in the presence of the sortase enzyme *Streptococcus pyogenes* sortase A (SpSrtA WT). In such embodiments, the sortase recognition site bond to the antibody, Fab fragment or scFv in step (i) has the sequence LPXTA (SEQ ID NO:9), where X is any amino acid residue. In step (ii), SpSrtA catalyzes the transpeptidation reaction between the LPXTA recognition motif (SEQ ID NO:9) and a glycine residue bound to the first Click handle by cleaving the sortase recognition site between the threonine and the alanine residues.

[0227] **FIG. 16** shows the formation of an LNP conjugated to the C-terminus of a Fab fragment through a sortase mediated ligation followed by a Click reaction. In the schematic, a C-terminus of the Fab fragment is first covalently modified with a sortase recognition motif covalently bonded to six histidine (H) residues. The sortase modified Fab fragment is then reacted with a first Click handle (Click handle 1) covalently bonded to glycine (G) residues. The resultant Fab fragment modified with the first Click handle is then reacted with an LNP that comprises at least one lipid that is covalently bonded to a second Click handle (Click handle 2), thus affording an LNP site-specifically conjugated to the Fab fragment.

[0228] In one embodiment, the conjugate comprises a protein targeting moiety as set forth above (e.g., antibody, Fab fragment or scFv) and a lipid nanoparticle (LNP), wherein a C-terminus of the targeting moiety is conjugated to the lipid nanoparticle through a linker, and wherein the linker comprises a sortase recognition motif and a Click product formed from a Click reaction between a first Click handle on the targeting moiety and a second Click handle on the LNP. In some embodiments, the C-terminus of one or more of the heavy or light chains of the antibody, the C-terminus of the heavy or light chain of the Fab fragment, or C-terminus of the scFv is conjugated to the lipid nanoparticle through a linker, and wherein the linker comprises a sortase recognition motif and a Click product formed from a Click reaction between a first Click handle on the antibody, Fab fragment, or scFv and a second Click handle on the

LNP. In particular embodiments, the sortase recognition motif comprises an LPXT motif, wherein X is any amino acid residue. In some such embodiments, the sortase recognition motif comprises an LPET motif (SEQ ID NO:10). In some embodiments, the linker comprises 3 or more glycine residues between the sortase recognition motif and the Click product. In some embodiments, the linker comprises between 3 and 50 glycine residues. In some embodiments, the linker comprises between 3 and 10 glycine residues. In some such embodiments, the linker comprises 3, 4, 5, or 6 glycine residues.

[0229] In some embodiments, the conjugates have the structure Antibody-LPXT$(G)_n$-Click product-LNP, Fab fragment- LPXT$(G)_n$-Click product-LNP, or scFv- LPXT$(G)_n$-Click product-LNP, wherein X is any amino acid residue and n is between 1 and 20 (e.g., between 3 and 10 or between 3 and 6). In these embodiments, the leucine (L) residue of the sortase recognition motif is bonded to at least one C–terminus of the antibody or Fab fragment or to the C-terminus of the scFv. In some such embodiments, X is E. In some embodiments, the linker between the antibody (or Fab fragment or scFv) and the Click includes the following amino acid residues: LPETGGG (SEQ ID NO:12), LPETGGGG (SEQ ID NO:13), LPETGGGGG (SEQ ID NO:14), LPETAGGG (SEQ ID NO:15), or LPETGGGGGG (SEQ ID NO:16).

[0230] In some embodiments, the linker comprises additional amino acid residues between the antibody, Fab fragment or scFv and the sortase recognition motif. In such embodiments, a C-terminus of the antibody, Fab Fragment or scFv can be covalently modified with one or more amino acid residues prior to covalently linking the sortase recognition motif. For instance, in particular embodiments, the conjugates have the structure Antibody-Z-LPXT$(G)_n$-Click product-LNP, Fab fragment-Z-LPXT$(G)_n$-Click product-LNP, or scFv-Z-LPXT$(G)_n$-Click product-LNP, wherein Z is a linker between the antibody (or Fab fragment or scFv) and the leucine of the sortase recognition motif. In some embodiments, Z comprises one or more amino acid residues. In some embodiments Z is $(GGGGS)_v$ (SEQ ID NO:1), $(G)_v$, $(EAAAK)_v$ (SEQ ID NO:3), $(PAPAP)_v$ (SEQ ID NO:4), $(AP)_v$ and $A(EAAAK)_u$ $ALEA(EAAAK)_vA$ (SEQ ID NO:2), wherein u is 1-10 and v is 1-10. In some embodiments, Z is GG, GGG, GGGG (SEQ ID NO:18), GGGGG (SEQ ID NO:19), GGGGGG (SEQ ID NO:20), and GGGGGGG (SEQ ID NO:21) or GGGGGS (SEQ ID NO:22).

[0231] In some embodiments, the conjugates have the structure Antibody-LPXT$(G)_n$-dihydropyridazine-LNP, Fab fragment- LPXT$(G)_n$- dihydropyridazine -LNP, or scFv-LPXT$(G)_n$- dihydropyridazine -LNP, Antibody-Z-LPXT$(G)_n$- dihydropyridazine-LNP, Fab fragment-Z-LPXT$(G)_n$- dihydropyridazine -LNP, or scFv-Z-LPXT$(G)_n$- dihydropyridazine -

LNP, wherein variables n and Z are defined as above. In particular embodiments, the dihydropyridazine moiety is a 1,4- dihydropyridazine.

[0232] In another embodiment, the LNP lipid is conjugated to an antibody, Fab fragment or scFv in a site-specific manner, comprising

(i) covalently bonding a LplA acceptor peptide site to the antibody, Fab fragment or scFv;

(ii) contacting the product of step (i) with a carboxylic acid compound that includes a first Click handle comprising a Tz ring in the presence of a lipoic acid ligase under conditions suitable for lipoic acid ligase to ligate the antibody, Fab fragment or scFv to the first Click handle; and

(iii) contacting the product of step (ii) with a lipid nanoparticle (LNP), wherein one or more lipids comprising the LNP includes a second Click handle capable of reacting with the first Click handle, wherein the second Click handle comprises a TCO moiety, thereby generating a Click product.

[0233] In some embodiments of step (i) the Lp1A acceptor peptide is covalently bonded to one or more C-terminus of the antibody or Fab fragment. In other embodiments of step i) the Lp1A acceptor peptide is covalently bonded to the C-terminus of the scFv.

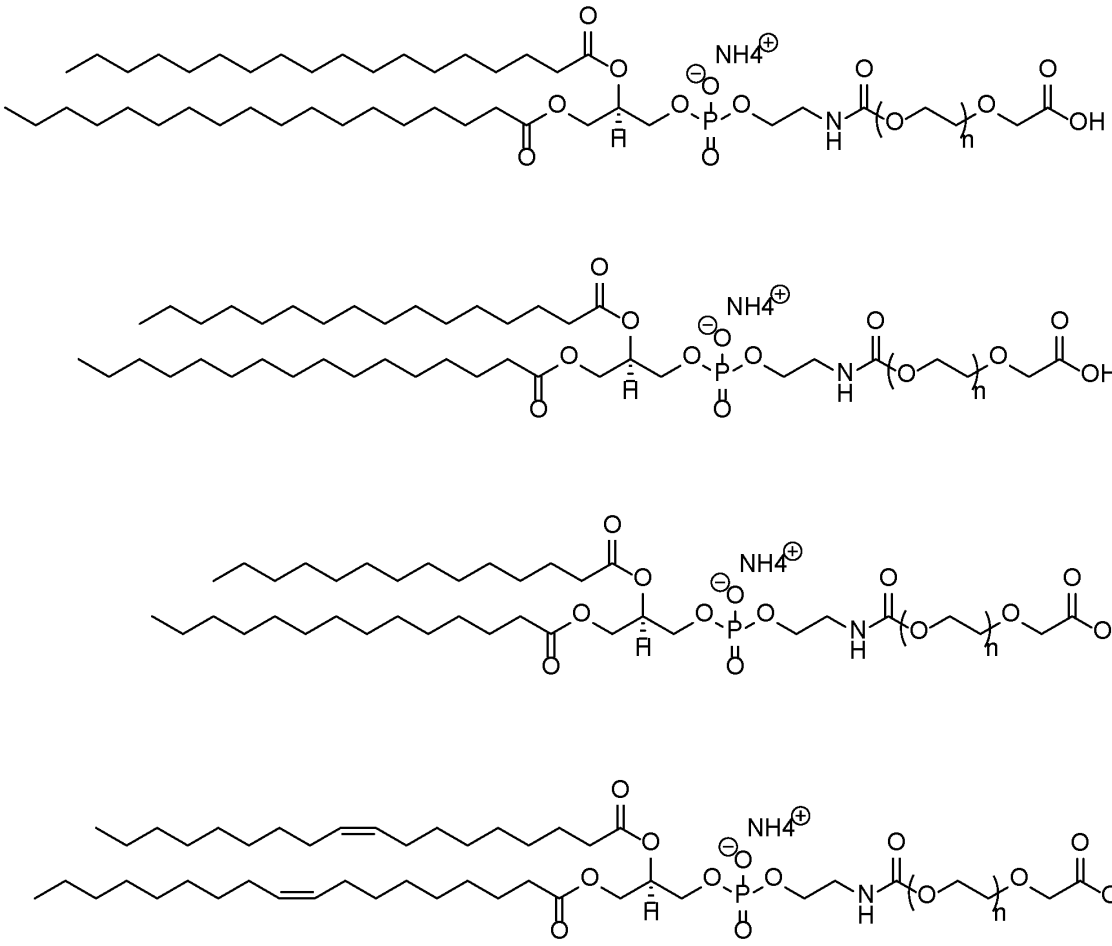
[0234] In some embodiments, step (i) involves covalently bonding the peptide with the Lp1A acceptor peptide to a linker covalently bonded to one or more C-terminus of the antibody or Fab fragment, or to the C-terminus of the scFv. In some embodiments, the linker comprises one or more amino acid residues. Particular amino acid residues added that can be covalently attached to the C-terminus of the antibody, Fab fragment or scFv include, but are not limited to $(GGGGS)_v$ (SEQ ID NO:1), $(G)_v$, $(EAAAK)_v$ (SEQ ID NO:3), $(PAPAP)_v$ (SEQ ID NO:4), $(AP)_v$ and $A(EAAAK)_u$ $ALEA(EAAAK)_vA$ (SEQ ID NO:2), wherein u is 1-10 and v is 1-10.

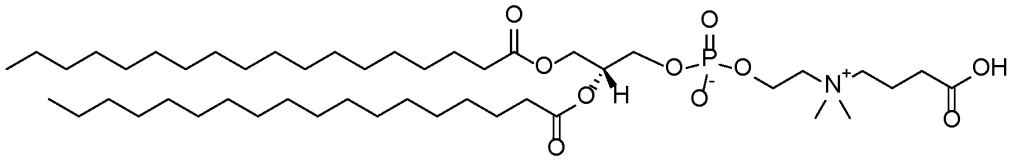
[0235] In some embodiments, step (ii) (i.e., the lipoic acid ligase catalyzed reaction) is carried out in the presence of a mutated lipoic acid ligase, wherein the tryptophan reside (W) at position of the lipoic acid ligase is mutated. Specific W37 mutants are described in Cohen *et al.*, 2012, *ChemBioChem*, 13, 888-894. In some embodiments, the mutant lipoic acid ligase is selected from W37V, W37I, W37T, W37L, W37C, W37A, and W37G.

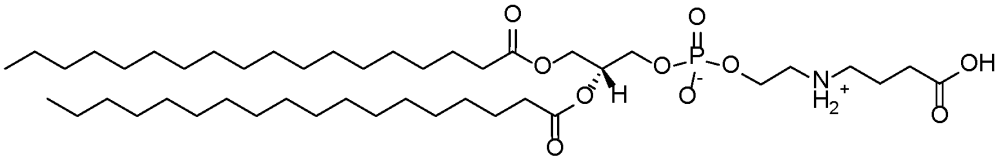
[0236] In some embodiments, the carboxylic acid in step (ii) (i.e., the lipoic acid ligase catalyzed reaction) is a C_3-C_{20} carboxylic acid. In some embodiments, the carboxylic acid in

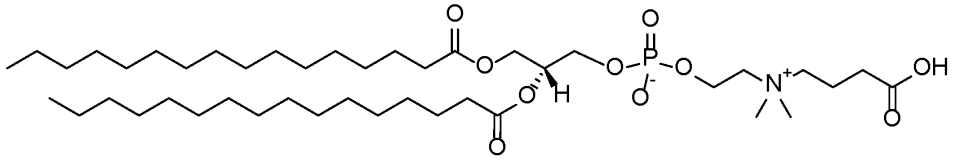
step (ii) is a fatty acid, for instance a C_7-C_{19} fatty acid. In some such embodiments, the fatty acid is selected from decanoic acid, palmitic acid, lauric acid or octanoic acid.

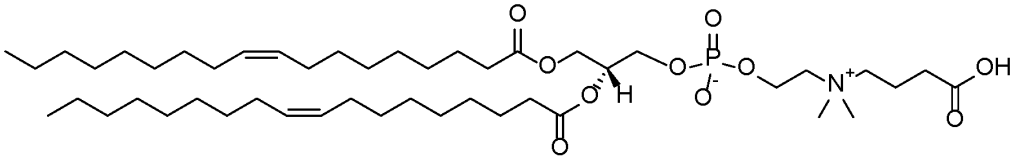
[0237] In other embodiments, the carboxylic acid in step (ii) has the formula COOH-R-Click handle, where R includes two lipid tales. In some such embodiments, the carboxylic acid as shown below, wherein n = 1—120.

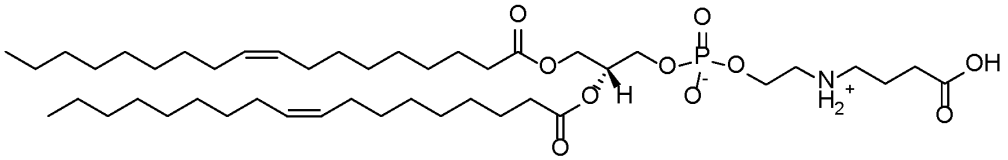


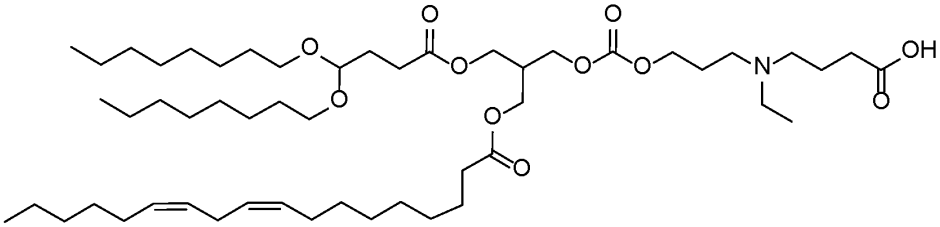


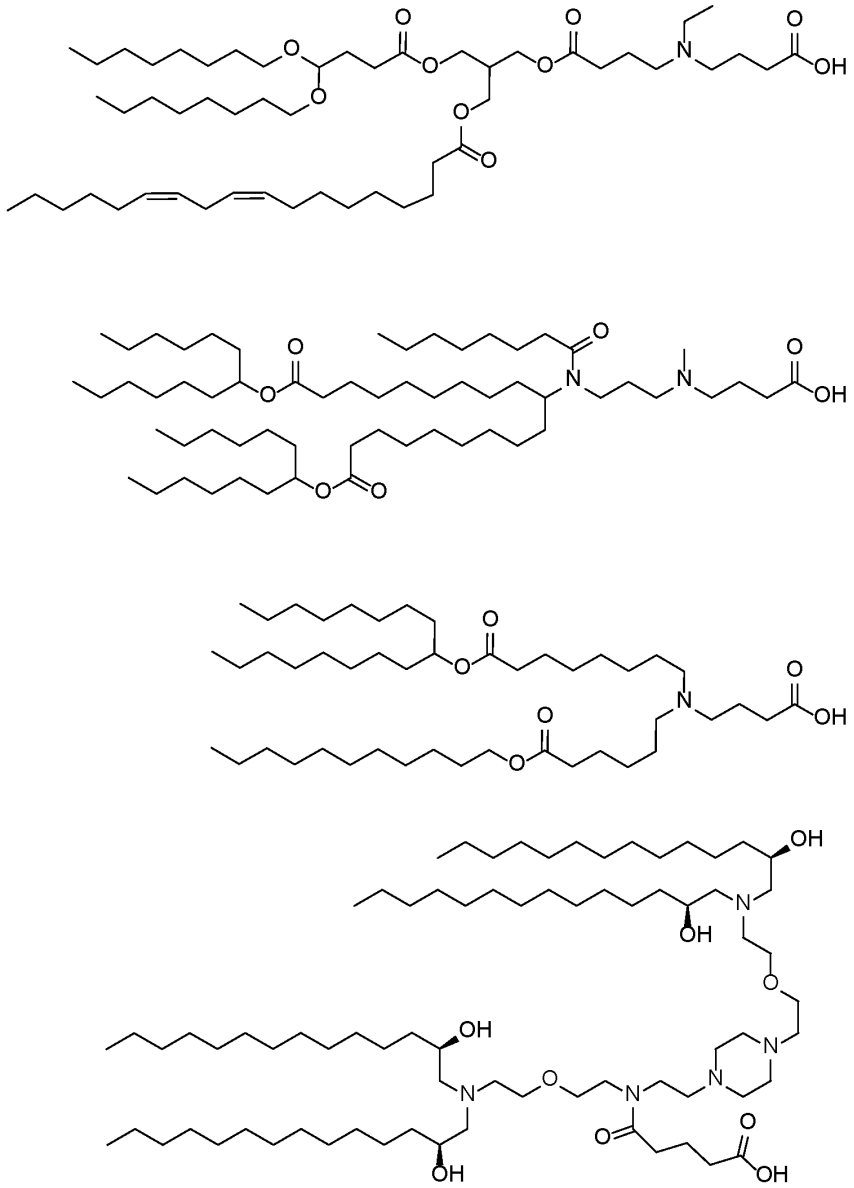












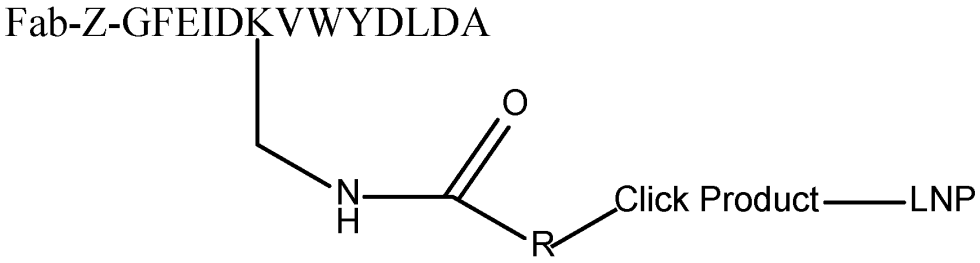
[0238] **FIG. 17** depicts the formation of an LNP conjugated to the C-terminus of a Fab fragment through a LplA ligation followed by a Click reaction. In the schematic, a C-terminus of the Fab fragment is first covalently modified with a LplA acceptor peptide. Specifically, a first Click handle (Click handle 1) comprising a Tz ring is first introduced by chemically reacting a carboxylic acid to the lysine residue of the LplA acceptor peptide. The resultant Fab fragment modified with the first Click handle is then reacted with an LNP that comprises at least one lipid that is covalently bonded to a second Click handle comprising a TCO moiety (Click handle 2), thus affording an LNP site-specifically conjugated to the Fab fragment.

[0239] In some embodiments, the conjugate produced by methods disclosed herein comprises a protein targeting moiety as set forth above (e.g., antibody, Fab fragment or scFv), conjugated to the lipid nanoparticle through a linker, and wherein the linker comprises a lipoic acid ligase (LplA) acceptor peptide and a Click product formed from a Click reaction between a first Click handle on the targeting moiety and a second Click handle on the LNP. In some embodiments, the linker further comprises one or more additional amino acid residues between the protein targeting moiety (e.g., antibody, Fab fragment or scFv) and the LplA acceptor peptide. In some embodiments, the LplA acceptor peptide has the sequence GFEDKVWYDLDA (SEQ ID NO:23). In some embodiments, the conjugate comprises an antibody, wherein the C-terminus of one or more of the heavy or light chains of the antibody is bonded to the linker. In some embodiments, the conjugate comprises a Fab fragment, the C-terminus of the heavy or light chain of the Fab fragment is bonded to the linker. In some embodiments, the conjugate comprises a scFv, wherein the C-terminus of the scFv is bonded to the linker.

[0240] In some embodiments, the linker comprises additional amino acid residues between the targeting moiety (e.g., antibody, Fab fragment or scFv) and the LplA acceptor peptide. In such embodiments, a C-terminus of the targeting moiety (e.g., antibody, Fab Fragment or scFv) can be covalently modified with one or more amino acid residues prior to covalently linking the LplA acceptor peptide. For instance, in particular embodiments, the conjugates have the structure targeting moiety-Z-LplA acceptor peptide-Click product-LNP (e.g., Antibody-Z- LplA acceptor peptide -Click product-LNP, Fab fragment-Z- LplA acceptor peptide -Click product-LNP, or scFv-Z- LplA acceptor peptide -Click product-LNP), wherein Z is a linker between the antibody (or Fab fragment or scFv) and the glycine residue of the LplA acceptor peptide. In some embodiments, Z comprises one or more amino acid residues. In some embodiments Z is $(GGGGS)_v$ (SEQ ID NO:1), $(G)_v$, $(EAAAK)_v$ (SEQ ID NO:3), $(PAPAP)_v$ (SEQ ID NO:4), $(AP)_v$ and $A(EAAAK)_u$ $ALEA(EAAAK)_vA$ (SEQ ID NO:2), wherein u is 1-10 and v is 1-10. In some embodiments, Z is GG, GGG, GGGG (SEQ ID NO:18), GGGGG (SEQ ID NO:19), GGGGGG (SEQ ID NO:20), and GGGGGGG (SEQ ID NO:21) or GGGGGS (SEQ ID NO:22).

[0241] It will be understood that in the forgoing embodiments, the lysine (K) residue of the LplA acceptor peptide is covalently linked to Click product, which is covalently linked to the LNP. Specifically, to generate conjugates, the side chain lysyl group reacts with the carboxylic acid compound that includes the first Click handle. The resultant modified targeting moieties (antibodies or Fab fragments or scFvs) are reacted with an LNP that has been modified with a second Click handle, as disclosed herein, thereby generating a Click product. An LNP surface

modified with a Fab fragment is depicted below, where R is a lipid group (e.g., C_2-C_{30} alkyl group) and the LplA accepter peptide comprises the sequence of GFEIDKVWYDLDA (SEQ ID NO:24).



[0242] In other embodiments, the enzyme recognition sequence is a transglutaminase enzyme recognition sequence (LLQG, SEQ ID NO:25). The transglutaminase enzyme recognition sequence (LLQG, SEQ ID NO:25) is also referred to as Q-tag. The Q-tag may be present on or can be inserted at one or more locations of targeting moiety, (e.g., antibody, Fab fragment or scFv), for instance at a C-terminus. The transglutamine enzyme catalyzes the reaction between a side-chain amide group on the Q-tag and an alkyl-primary amine on a component of the LNP (e.g., a lipid), thus linking the antibody to the LNP through an amide bond.

[0243] In other embodiments, the enzyme recognition sequence is a sequence recognized by formylglycine generating enzyme, specifically CXPXR, wherein each X is any amino acid. In such embodiments, the CXPXR sequence can be inserted at one or more locations of the targeting moiety (e.g., antibody, Fab fragment or scFv), for instance at a C-terminus. The formylglycine generating enzyme converts the cysteine thiol of CXPXR into an aldehyde group, which can be reacted with an aminooxy or hydrazine group covalently bonded to a component of the LNP.

[0244] In another embodiment, the lipid nanoparticle is conjugated to the targeting moiety, e.g., antibody, Fab fragment or scFv, in a site-specific manner through a sugar moiety on a glycosylated antibody. **FIG. 18** shows one particular embodiment of introducing a Tz Click handle through a sugar moiety on an antibody. In step 1, an azide moiety is site-specifically introduced into a sugar moiety of the targeting antibody catalyzed by the enzymes galactosidase and transferase. The azide functionality then reacts with a dibenzocyclooctene (DBCO)-MeTz via a Click reaction. The Click reaction is used to introduce the Tz ring, in this case MeTz onto the antibody, which can then be reacted with a TCO moiety on an LNP (not shown) in a second Click reaction, hence affording a conjugate.

[0245] In another embodiment, the LNP is conjugated to a targeting moiety (e.g., antibody, Fab fragment or scFv) in a site-specific manner through a light-induced crosslinking reaction. **FIG. 19** shows one particular embodiment using oYo-Link Tz. In **FIG. 19**, a Tz ring (e.g., MeTz) is covalently bound to a site specific antibody label (oYo link) that specifically reacts with residues on the heavy chain of the antibody. The oYo-Link tetrazine covalently bound to the antibody reacts with TCO (not shown) bound to an LNP, hence generating the surface-modified LNP.

[0246] In another embodiment, the lipid nanoparticle is conjugated to the targeting moiety (e.g., antibody, Fab fragment or scFv) in a site-specific manner by mutating an amino acid residue on the targeting moiety and subsequently reacting the mutated targeting moiety with a compound that includes a Tz ring. **FIG. 20** shows particular embodiments where an azide functionality is introduced during antibody or Fab fragment production. The azide group then undergoes a Click reaction with a DBCO group that is linked to a tetrazine ring. The tetrazine ring bound to the antibody reacts with TCO (not shown) bound to an LNP, hence generating the surface modified LNP.

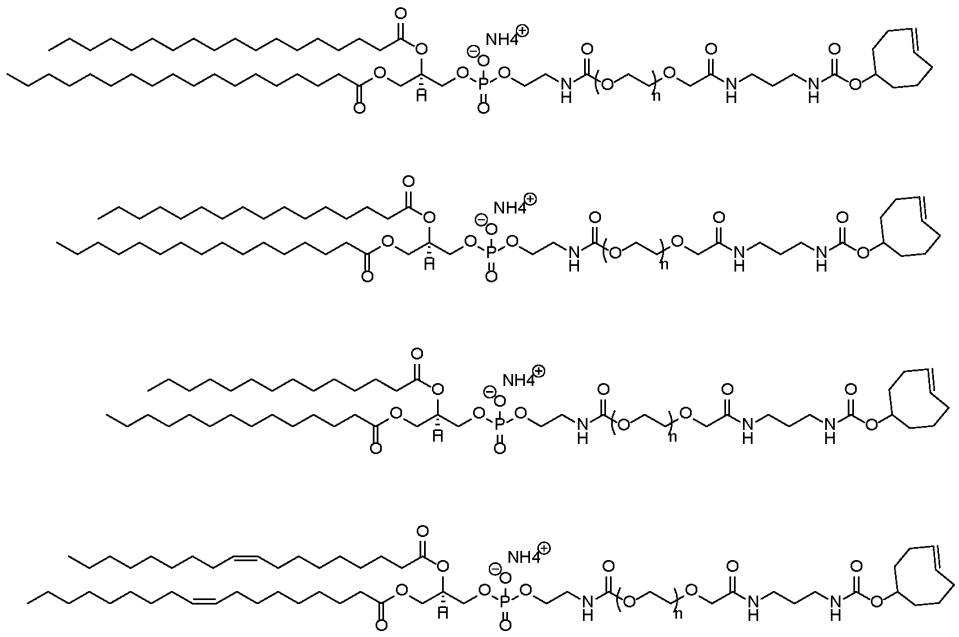
[0247] In another embodiment, the lipid nanoparticle is conjugated to the targeting moiety (e.g., antibody, Fab fragment or scFv) in a site-specific manner by using a cysteine-maleimide reaction to introduce the first Click handle onto the targeting moiety (antibody, Fab fragment or scFv). **FIG. 21** shows one particular embodiment of introducing a first Click handle (e.g., Tz) onto a Fab fragment. In **FIG. 21**, a hinge with a free cysteine is first introduced at a C-terminus of the Fab fragment. In some such embodiments, an engineered inter-chain disulfide bound away from the C-terminus and buried in the CL-CH1 interface is introduced for improved stability (**FIG. 22**). The resultant modified Fab fragment is reacted with a maleimide moiety with a first Click handle (Click handle 1) covalently bonded to the maleimide nitrogen. The first Click handle than reacts with a second Click handle (Click handle 2) on the LNP, hence generating the surface modified targeted LNP. In some embodiments, Click handle 1 is a TZ and Click handle 2 is a TCO moiety,

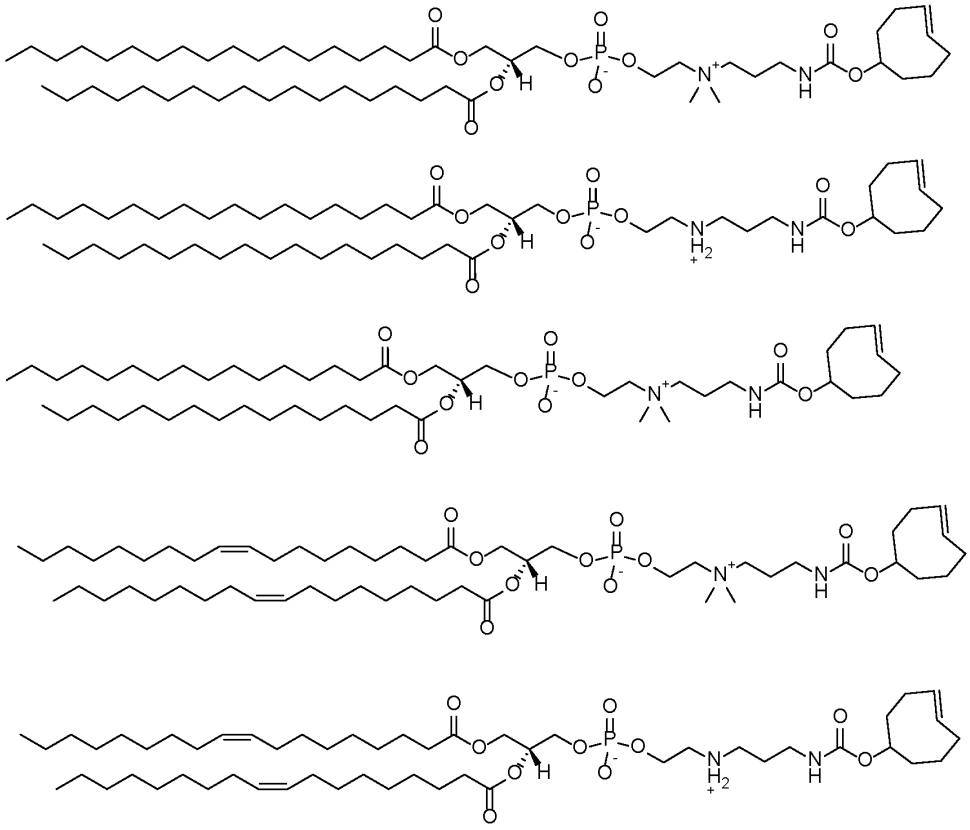
[0248] In some embodiments, the Click product can be formed using any suitable photo-induced Click chemistry reaction. In some embodiments, the Click product can be formed using photoinducible 1,3-dipolar cycloaddition reaction between a tetrazole and an alkene (*see*, e.g., Song et al., *Angew. Chem., Int. Ed.* 2008, 47 (15), 2832-2835).

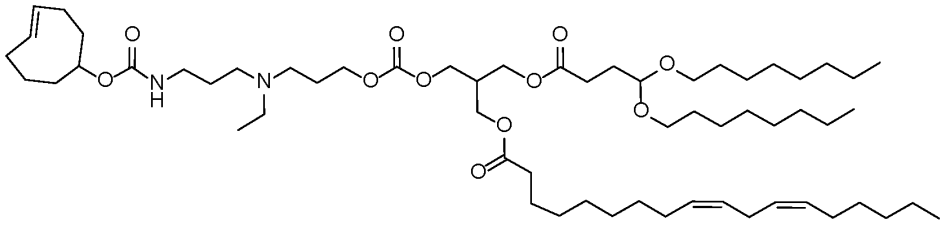
[0249] In some embodiments, the Click product can be formed using oxime and hydrazone ligations. In some embodiments, a ketone or aldehyde can react with α effect amine, such as

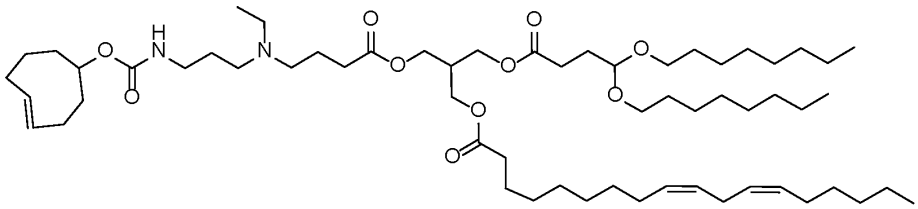
hydroxylamine, hydrazine and hydrazide (*see*, e.g., Agten et al., *ChemBioChem* 2013, 14 (18), 2431-2434 and Dirksen et al., *J. Am. Chem. Soc.* 2006, 128 (49), 15602-15603).

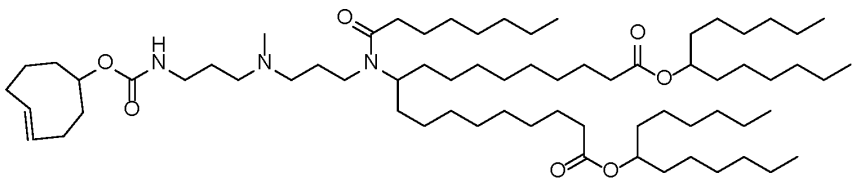
[0250] Exemplary lipids and cholesterol molecules bonded to Click handles that can be used to make targeted LNPs are depicted below.

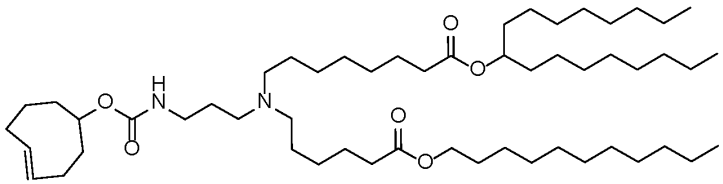


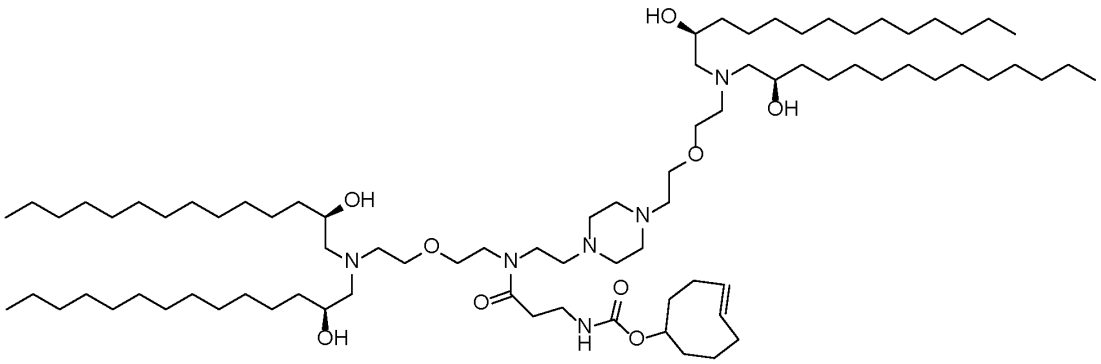


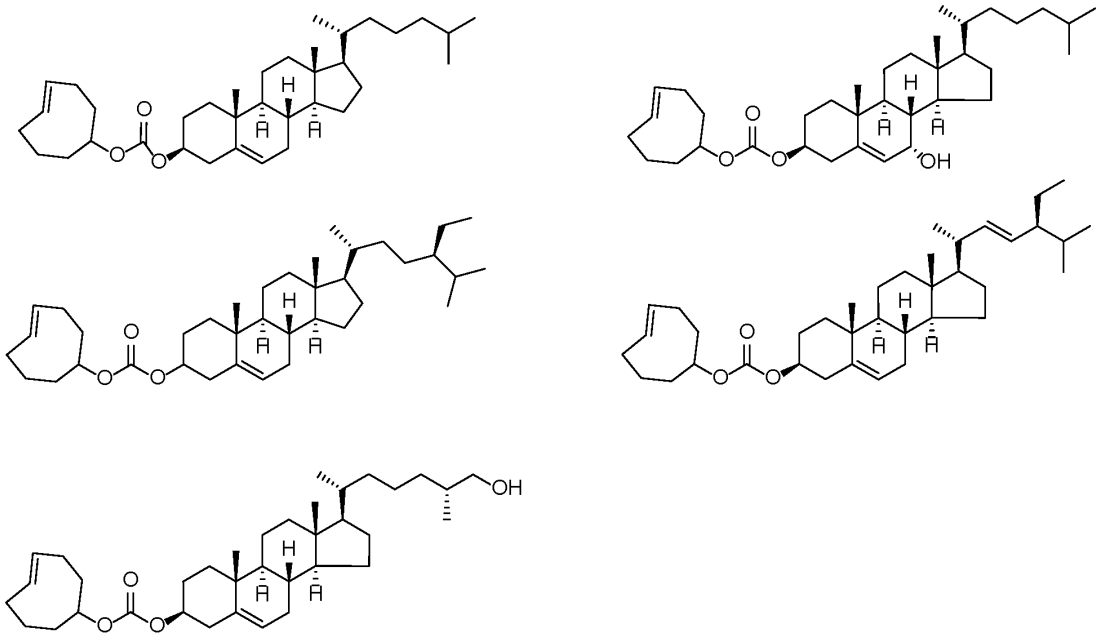












Interchain C_L-C_H1 disulfide reduction followed by conjugation

[0251] IgG antibodies consist of four polypeptide chains linked by disulfide bonds. The two polypeptide chains of low molecular weight are call light chains (L). The light chains consist of a variable light chain domain (V_L) and a constant light chain domain (C_L). The heavy chains consist of a variable heavy light domain (V_H) and three constant heavy chain domains (C_H1, C_H2, and C_H3). The Fab region of the antibody includes the V_L, C_L, V_H, and C_H1 domains. The Fc region includes the constant heavy chain domains C_H2, and C_H3. A hinge region of the IgG antibody covalently links the C_H1 domain to the C_H2 domain. The two heavy chains of IgG antibodies are connected in the hinge region by a variable number of disulfide bonds depending on the IgG subclass. Different subclasses of IgG antibodies have varying numbers of interchain disulfide bonds. Additionally, the light chain is covalently linked to the heavy chain via a disulfide bond between the light chain and the heavy chain. Using standard IgG nomenclature, this natural interchain disulfide bond is also referred to as the C_L-C_H1 disulfide bond to distinguish it from disulfide bonds present in the hinge region. Therapeutic antibodies of type IgG1 possess an intermolecular disulfide bond between Cys233 (Kabat numbering) of the heavy domain and Cys214 (Kabat numbering) of the light domain. Therapeutic antibodies of type IgG4 possess an intermolecular disulfide bond between Cys127 (Kabat numbering) of the heavy domain and Cys214 (Kabat numbering) of the light domain. Therapeutic antibodies of type IgG2 possess an intermolecular disulfide bond between Cys135 (Kabat numbering) of the heavy domain and Cys214 (Kabat numbering) of the light domain.

[0252] Proteolytic cleavage of an IgG antibody results in the formation of a Fab fragment known as a $F(ab')_2$ fragment. The $F(ab')_2$ fragment does not include the C_H2 domain or the C_H3 domain. However, the hinge region of the antibody is retained in a $F(ab')_2$ fragment. The $F(ab')_2$ fragment includes disulfide bonds that covalently link two Fab fragments. Reduction of the disulfide bond in the $F(ab')_2$ generates two F(ab') fragments. The sulfhydryl (thiol) groups of the F(ab') could potentially react with a thiol-reactive group on the surface of an LNP, hence generating a conjugate. However, owing to the presence of multiple sulfhydryl groups in the hinge region of the F(ab') fragment, site-specific conjugation is challenging. Moreover, the reduction of the $F(ab')_2$ to the F(ab') fragments could also disrupt the natural interchain disulfide bonds between the C_L and C_H1 regions of the Fab fragments, hence further compromising site-specific conjugation.

[0253] As set forth herein, Fab fragment (e.g., a Fab fragment that binds to CD164) can be site-selectively conjugated to the surface of a precursor lipid nanoparticle (LNP) through the natural interchain disulfide bond between the heavy chain and the light chain (i.e., the C_L-C_H1 disulfide bond) of the Fab fragment to make a targeted LNP (conjugate).

[0254] The term "precursor LNP" or "base LNP" refers to an LNP that has been functionalized with a reactive moiety (e.g., thiol-reactive group or polyglycine) prior to reacting with the Fab fragment. The process for conjugating a targeting moiety, as disclosed herein, involves reducing the natural interchain disulfide bond between the C_L and C_H1 domains of a Fab fragment, and reacting the reduced Fab fragment with a thiol-reactive group (e.g., a maleimide or DBM group) covalently bonded to the surface of a precursor LNP, thus forming a conjugate. Alternatively, the reduced Fab fragment can be reacted with a lipid that has been chemically modified (functionalized) with a thiol-reactive group (e.g., maleimide or DBM group). The resultant lipid can then be inserted into a preexisting LNP, thus generating a conjugate. As described herein, despite the removal of the natural interchain disulfide bond linking the heavy and light chains of the Fab fragment, the resulting conjugates are able to effectively target specific cell types depending on the nature of the Fab targeting moiety. For instance, specific Fab fragments for targeting immune cells (e.g. T cells) as disclosed herein. A schematic of an LNP site-specifically conjugated to a Fab fragment is shown in **FIG. 39**.

[0255] In one embodiment, Fab fragments used for conjugation may be used by recombinant methods. In particular embodiments, the Fab fragments generated recombinantly are designed not to include a hinge region at the C-terminus. Therefore, the recombinantly generated Fab fragments include only one disulfide bond between the C_L-C_H1 and domains. As set forth

herein, the C_L-C_H1 can then be reduced and the resultant free thiol groups can be used as anchors to conjugate the Fab fragment to the surface of an LNP.

[0256] In some embodiments, the Fab fragment is of the IgG class, the IgM class, or the IgA class. In some embodiments, the Fab fragment is of the IgG class and has an IgGl, IgG2, IgG3, or IgG4 isotype. In some embodiments, the Fab fragment is a native protein. In some embodiments, the Fab fragment is an engineered protein.

[0257] In one aspect, the disclosure provides methods of making a targeted LNP, said method comprising:

(i) contacting a composition comprising a Fab fragment with a reducing reagent, wherein the Fab fragment comprises a heavy chain and a light chain and an interchain disulfide bond linking the constant light chain domain (C_L) and the constant heavy chain domain 1 (C_H1), whereby the reducing reagent reduces the interchain disulfide bond of the Fab fragment to generate two free cysteine residues; and

(ii) contacting the product of step (i) with a precursor LNP comprising a plurality of thiol-reactive groups covalently bonded to one or more lipids of the precursor LNP, thereby forming a targeted LNP.

[0258] In some aspects, the disclosure provides a conjugate produced by a method comprising:

(i) contacting a composition comprising a Fab fragment with a reducing reagent, wherein the Fab fragment comprises a heavy chain and a light chain and an interchain disulfide bond linking the constant light chain domain (C_L) and the constant heavy chain domain 1 (C_H1), whereby the reducing reagent reduces the interchain disulfide bond of the Fab fragment to generate two free cysteine residues; and

(ii) contacting the product of step (i) with a precursor LNP comprising a plurality of thiol-reactive groups covalently bonded to one or more lipids of the precursor LNP, thereby forming a targeted LNP.

[0259] In some embodiments, the thiol-reactive group (e.g., maleimide, pyridyl disulfide, 2,3-dibromomaleimide, or haloacetyl) is chemically reacted with a lipid molecule to create a modified lipid wherein the thiol-reactive group is covalently attached to the lipid where it is capable of reacting with at least one free cysteine residue of the reduced Fab fragment (either on the heavy or light chain of the Fab fragment). The reaction between the thiol-reactive group and the at least one free cysteine residue can be completed prior to or after formation of the LNP

with the modified lipid. For instance, as shown in **FIG. 40A**, the various components (e.g., lipids) comprising the LNP and a therapeutic payload can be mixed with lipid molecules, including one or more lipids that comprise a thiol-reactive group, thus generating an LNP that comprises a plurality of thiol-reactive groups (each thiol-reactive group shown schematically as a "functional group" in **FIG. 40A**). The thiol-reactive group can then be reacted with at least one free cysteine residue of the Fab fragment, hence generating a conjugate. Alternatively, a lipid that has been modified with the thiol-reactive group can be directly reacted with at least one free cysteine residue of a Fab fragment. As depicted in **FIG. 40B**, **t**he resultant modified lipid attached to the Fab fragment can then be inserted into a pre-formed LNP that has not yet been surface modified. This procedure allows for the reaction to be performed on an individual lipid molecule rather than on the surface of the LNP.

[0260] Any suitable reducing reagent can be used to reduce the interchain disulfide bond of the Fab fragment. Examples of reducing reagents include, but are not limited to, 2-mercaptoethanol, 2-mercaptoethylamine, dithiothreitol (DTT), dithioerythritol (DTE), and tris(carboxyethyl)phosphine (TCEP), and combinations thereof. In some embodiments, the reducing reagent is a mild reducing reagent. Examples of mild reducing reagents include, e.g., DTT, TCEP, and DTE. In some embodiments, the reducing reagent is TCEP. Any suitable reaction conditions can be used for the reduction of the interchain disulfide bond in step (i). In some embodiments, the reduction reaction can occur in water, aqueous buffer, or cell culture media. In some embodiments, the reduction reaction is performed at physiological pH (*e.g.*, about 7.4). In some embodiments, the reduction reaction is performed at physiological temperature (*e.g.*, about 37° C). In some embodiments, the reduction reaction is performed between 0°C and 40°C, e.g., between 10°C and 35°C, between 15°C and 30°C, between 20°C and 30°C, or between 20°C and 25°C. In some embodiments, the reduction reaction is performed at ambient temperature (*e.g.*, about 23 to about 25° C). In some embodiments, the reduction reaction is performed at about 0° C to about 4° C.

[0261] In some embodiments, excess reducing agent is removed following step (i), prior to conjugation to the precursor LNP. In some embodiments, excess reducing agent is not removed following step (i), prior to conjugation to the precursor LNP.

[0262] In some embodiments of the method or process, the thiol-reactive groups on the LNP (or lipid to be post-inserted into an LNP) comprises any suitable reactive group, including but not limited to, maleimide, pyridyl disulfide, 2,3-dibromomaleimide, or haloacetyl.

[0263] In some embodiments, the thiol-reactive group is maleimide. In some embodiments, maleimide reacts with one of the two free cysteine residues of the Fab fragment (either on the heavy or light chain) to form a thiosuccinimide moiety. In some embodiments, maleimide reacts with a free cysteine residue on the heavy chain of the Fab fragment. In some embodiments, maleimide reacts with a free cysteine residue on the light chain of the Fab fragment. In some embodiments, two maleimide groups each react with the Fab fragment, wherein one maleimide reacts with a free cysteine residue on the light chain and the other maleimide reacts with a free cysteine residue on the heavy chain.

[0264] Any suitable conditions can be used for the reaction between the thiol-reactive group and at least one of the two free cysteine residues of the Fab fragment in step (ii). In some embodiments, the reaction can occur in water, aqueous buffer, or cell culture media. In some embodiments, the reaction is performed at physiological pH (*e.g.*, about 7.4). In some embodiments, the reaction is performed at physiological temperature (*e.g.*, about 37° C). In some embodiments, the reduction reaction is performed between 0°C and 40°C, e.g., between 10°C and 35°C, between 15°C and 30°C, between 20°C and 30°C, or between 20°C and 25°C. In some embodiments, the reaction is performed at ambient temperature (*e.g.*, about 23 to about 25° C). In some embodiments, the reaction is performed at about 0° C to about 4° C.

[0265] A schematic showing the reduction of an interchain disulfide bond in a Fab fragment is depicted in **FIG. 41**. In some embodiments, the Fab fragment depicted in **FIG. 41** can be produced recombinantly without a hinge region at the C-terminus. Accordingly, in such embodiments, the Fab fragment only comprises a single interchain disulfide bond, which is located in the C_L-C_H1 interface of the Fab fragment. As shown in **FIG. 41**, the interchain disulfide bond is located between the heavy chain and the light chain of the Fab fragment. The reduction reaction breaks the covalent linkage forming the disulfide bond, thereby generating two free cysteine residues that can react in a subsequent step with a thiol-reactive group.

[0266] **FIG. 42** shows an exemplary schematic of conjugate formation, as described herein. In **FIG. 42**, a Fab fragment comprising an interchain disulfide bond between the heavy and light chain is contacted with a reducing reagent, whereby the reducing reagent reduces the interchain disulfide to generate two free cysteine residues (step (i)). In step (ii), the reduced Fab fragment is reacted with an LNP comprising a plurality of thiol-reactive groups (e.g., maleimide or DBM) conjugated to the surface of the LNP, whereby the thiol-reactive groups react with the free cysteine residues of the reduced Fab fragment. The Fab fragment is site-specifically conjugated

to the surface of the LNP via a linkage through at least one of the free cysteine residues of the Fab fragment.

[0267] Following reaction of the reduced Fab fragment with the thiol-reactive group of the LNP, either the heavy chain, light chain or both the heavy chain and light chain of the Fab fragment are conjugated to the surface of the LNP. The various orientations are depicted in **FIG. 43**. In some embodiments, the conjugates formed by methods disclosed herein include at least one, at least two or all three orientations shown in **FIG. 43**. The concentration of the thiol-reactive group (e.g., maleimide) will likely determine which orientation is dominant. In some embodiments, increasing the number of thiol-reactive groups on the LNP increases the number of Fab fragments conjugated to two thiol-reactive groups. In some embodiments, decreasing the number of thiol-reactive groups on the LNP decreases the number of Fab fragments conjugated to two thiol-reactive groups. Regardless of the orientation, the heavy chain and the light chain remain intact on the surface of the LNP, thus forming a functional Fab fragment that is capable of engaging with a receptor on a targeted cell.

[0268] In some embodiments, the thiol-reactive group is maleimide, as shown in **FIG. 43**. In some embodiments, maleimide reacts with one of the two free cysteine residues of the antibody or antigen-binding fragment thereof. In some embodiments, maleimide reacts with one of the two free cysteine residues of the Fab fragment to form a thiosuccinimide moiety. In some embodiments, maleimide reacts with a free cysteine residue on the heavy chain of the Fab fragment. In some embodiments, maleimide reacts with a free cysteine residue on the light chain of the Fab fragment. In some embodiments, two maleimide groups on the LNP each react with the Fab fragment, wherein one maleimide reacts with a free cysteine residue on the light chain and the other maleimide reacts with a free cysteine residue on the heavy chain.

[0269] In some embodiments, the thiol-reactive group is 2,3-dibromomaleimide (DBM) as shown in **FIG. 44**. Following reduction of the disulfide bond, the reduced Fab fragment is added to DBM covalently bonded to a lipid (represented by squiggly line in **FIG. 44**). As set forth above, the lipid may be part of an LNP or may be post-inserted into an LNP following reaction with the Fab fragment. Both of the free cysteine residues displace the two bromine groups of DBM, hence generating a dithiomalemide. The dithiolmalemide can be converted to the corresponding maleamic acid via hydrolysis. DBM reacts with a free cysteine residue on the heavy chain and a free cysteine residue on the light chain of the Fab fragment to form a bridge between the cysteine residues. Accordingly, the heavy and light chain of the Fab fragment are effectively bridged together following reaction with DBM.

[0270] In all embodiments discussed above, the thiol-reactive group can be introduced onto any of the lipids comprising the LNP. In some embodiments, the conjugate can comprise one or more pegylated lipid molecules. In some embodiments, the thiol-reactive group is covalently bonded to at least one of the pegylated lipid molecules, hence generating the structure Lipid-PEG_x-thiol-reactive group, wherein x is 2-120 ethylene glycol units. In such embodiments, at least one free cysteine residue of a Fab fragment reacts with the thiol-reactive group bonded to the one or more of the pegylated lipids comprising the LNP. In some embodiments, the LNP comprises from about 0.05 mol % to about 2 mol % of the pegylated lipid bonded to the thiol-reactive group. In some embodiments, the PEG spacer between the lipid and the thiol-reactive group comprises at least about 5, 10, 20, 30, 50, 50, 60, 70, 80, 90, 200, or 110 ethylene glycol units. In some embodiments, the PEG spacer comprises about 10-120 ethylene glycol units. In some embodiments, the molecular weight of the pegylated lipid bonded to the thiol-reactive group is from about 500 (i.e., PEG500) to about 5,000 (i.e., PEG5000). In some embodiments, the molecular weight of the pegylated lipid bonded to the thiol-reactive group is from about 1,000 (i.e., PEG1000) to about 3,000 (i.e., PEG5300). In some embodiments, the thiol-reactive group is bonded to at least one of the non-pegylated phospholipids comprising the LNP. In some embodiments, the thiol-reactive group is bonded to at least one of the ionizable lipids comprising the LNP. In some embodiments, the thiol-reactive group is bonded to at least one of the sterol molecules comprising the LNP. In some embodiments, the lipid portion of the pegylated lipid bonded to the thiol-reactive group is selected from DMG, DPG, DSG, DTA, DOPE, DPPE, DMPE, DSPE, sphingosine, sphingomyelin, and stearic acid. **FIG. 45** shows some examples of a pegylated lipid bonded to a thiol-reactive group, in this case a maleimide moiety.

[0271] In some embodiments, the thiol-reactive group is bonded to at least one of the non-pegylated phospholipids (helper lipids) comprising the LNP. **FIG. 46** shows some examples of a non-pegylated lipid bonded to a maleimide moiety.

[0272] In some embodiments, the thiol-reactive group is bonded to at least one of the ionizable lipids comprising the LNP. **FIG. 47** shows some examples of an ionizable lipid bonded to a maleimide moiety.

[0273] In some embodiments, the thiol-reactive group is bonded to at least one of the sterol molecules comprising the LNP. **FIG. 48** shows some examples of sterols bonded to a maleimide moiety.

[0274] The disclosed methods provide stable conjugates that display excellent ability to transduce specific targeted cells. In some aspects, the disclosure provides a conjugate

comprising an LNP and a Fab fragment, wherein the LNP is covalently bonded to either or both a first cysteine residue in the constant region of the heavy chain of the Fab fragment and a second cysteine residue in the constant region of light chain of the Fab fragment. In some embodiments, the Fab fragment does not comprise a disulfide bond linking the constant region of the heavy chain of the Fab fragment and the constant region of the light chain of the Fab fragment. In some embodiments, both the constant region of the heavy chain constant region of the heavy chain and the constant region of the light chain of the Fab fragment are covalently bonded to the LNP. In some embodiments, only the constant region of the heavy chain of the Fab fragment is covalently bonded to the LNP. In some such embodiments, the light chain remains associated with the covalently bond heavy chain on the surface of the LNP. In some embodiments, only the constant region of the light chain of the Fab fragment is covalently bonded to the LNP. In some such embodiments, the heavy chain remains associated with the covalently bond light chain on the surface of the LNP. In some of the foregoing embodiments, the Fab fragment is linked to the LNP through a thiosuccinimide moiety. In other of the foregoing embodiments, the Fab fragment is linked to the LNP through a dithiomalemide moiety In still other of the foregoing embodiments, the Fab fragment is linked to the LNP through a maleamic acid moiety.

[0275] In some embodiments, the Fab fragment conjugated to the LNP is an IgG1 Fab fragment. In some such embodiments, the cysteine at position 214 (Kabat numbering) of the light chain of the IgG1 Fab fragment is covalently bonded to the LNP. In other such embodiments, the cysteine at position 233 (Kabat numbering) of the heavy chain of the IgG1 Fab fragment is covalently bonded to the LNP. In other such embodiments, the cysteine at position 233 (Kabat numbering) of the heavy chain of the IgG1 Fab fragment and the cysteine at position 214 (Kabat numbering) of the light chain of the IgG1 Fab fragment are covalently bonded to the LNP.

[0276] In some embodiments, the Fab fragment conjugated to the LNP is an IgG2 Fab fragment. In some such embodiments, the cysteine at position 214 (Kabat numbering) of the light chain of the IgG2 Fab fragment is covalently bonded to the LNP. In other such embodiments, the cysteine at position 127 (Kabat numbering) of the heavy chain of the IgG2 Fab fragment is covalently bonded to the LNP. In other such embodiments, the cysteine at position 127 (Kabat numbering) of the heavy chain of the IgG2 Fab fragment and the cysteine at position 214 (Kabat numbering) of the light chain of the IgG2 Fab fragment are covalently bonded to the LNP.

[0277] In some embodiments, the Fab fragment conjugated to the LNP is an IgG4 Fab fragment. In some such embodiments, the cysteine at position 214 (Kabat numbering) of the light chain of the IgG4 Fab fragment is covalently to the LNP. In other such embodiments, the cysteine at position 127 (Kabat numbering) of the heavy chain of the IgG4 Fab fragment is covalently to the LNP. In other such embodiments, the cysteine at position 127 (Kabat numbering) of the heavy chain of the IgG4 Fab fragment and the cysteine at position 214 (Kabat numbering) of the light chain of the IgG4 Fab fragment is covalently to the LNP.

[0278] The processes described herein also enable the ability to conjugate two or more different Fab fragments to the surface of an LNP. An embodiment showing the conjugation of two different Fab fragments (which can bind to the same or different antigen) to the surface of an LNP is shown in **FIG. 45**. In **FIG. 45**, two Fab fragments (Fab1 and Fab2) are reduced (step (i)) and reacted (step (ii)) with a precursor LNP comprising a thiol-reactive group (e.g., maleimide or DBM). Following the reaction in step (ii), both Fab1 and Fab2 are conjugated to the surface of the LNP. Despite reduction of the disulfide bonds in Fab1 and Fab2, the heavy chain and light chain in Fab1 and the heavy and light chain in Fab2 remain together on the surface of the LNP. In other words, neither the heavy chain or light chain of Fab1 associate with the heavy or light chain of Fab2 on the surface of the LNP.

[0279] In some embodiments involving conjugating two Fab fragments (i.e., a first Fab fragment and a second Fab fragment) to the surface of an LNP, the first Fab fragment and the second Fab fragment can be reduced in the same reaction (e.g., the first and second Fab fragments are mixed in a reaction vessel and contacted with the same reducing reagent). In some embodiments, the first Fab fragment and the second Fab fragment are reduced separately (e.g., the first and second Fab fragments thereof are each contacted with a reducing reagent in separate reaction vessels). In some embodiments, the first Fab fragment is contacted with the reducing reagent prior to step (ii) (wherein the reduced first Fab fragment is conjugated to the LNP surface). In some embodiments, the second Fab fragment is contacted with the reducing reagent after step (ii) (wherein the reduced second Fab fragment is conjugated to the LNP surface). In some embodiments, the reduced first Fab fragment and the reduced second Fab fragment are contacted with the LNP simultaneously. In some embodiments, the reduced first Fab fragment thereof and the reduced second Fab fragment are contacted with the LNP sequentially (in either order). It can be contemplated that any number of Fab fragments thereof can be implemented in the method or process (e.g., a third, fourth, fifth, etc. Fab fragment). In some embodiments, a total of three different Fab fragments can be conjugated to the surface of the LNP. In some

embodiments, a total of four different Fab fragments can be conjugated to the surface of the LNP.

[0280] In some embodiments, the reaction between at least one of the two free cysteine residues of the Fab fragment and the thiol-reactive group of the LNP forms at least one covalent bond. In some embodiments, the formation of at least one covalent bond between at least one of the two free cysteine residues of the Fab fragment and the thiol-reactive group of the LNP is reversible. In some embodiments, the formation of at least one covalent bond between at least one of the two free cysteine residues of the Fab fragment and the thiol-reactive group of the LNP is irreversible. In some embodiments, the reaction efficiency between at least one of the two free cysteine residues of the Fab fragment and the thiol-reactive group of the LNP is greater than 5%, greater than 10%, greater than 25%, greater than 50%, greater than 60%, greater than 70%, greater than 80%, or greater than 90%. In some embodiments, the reaction efficiency between at least one of the two free cysteine residues of the Fab fragment and the thiol-reactive group of the LNP is from about 5% to about 30%, about 10% to about 20%, about 25% to about 50%, about 30% to about 40%, about 50% to about 80%, about 60% to about 70%, about 70% to about 95%, or about 80% to about 90%. In some embodiments, the conjugate product of the disclosed method can be purified from remaining intermediate product using any suitable technique such as, but not limited to, ultrafiltration and diafiltration.

[0281] In some embodiments, conjugates prepared by the method or process disclosed herein have a high density of the Fab fragment on the surface of the LNP. For instance, the conjugate can comprise a plurality of Fab fragments conjugated to the LNP surface. In some embodiments, the conjugate can comprise more than 10 Fab fragments per LNP. In some embodiments, the conjugate can comprise more than 20 Fab fragments per LNP. In some embodiments, the conjugate can comprise more than 30 Fab fragments. In some embodiments, the conjugate can comprise more than 50 Fab fragments per LNP. In some embodiments, the conjugate can comprise more than 75 Fab fragments per LNP. In some embodiments, the conjugate can comprise more than 100 Fab fragments. In some embodiments, the conjugate can comprise from about 50 to about 200 Fab fragments per LNP. In some embodiments, the conjugate can comprise from about 100 to about 200 Fab fragments per LNP. In some embodiments, the conjugate can comprise from about 100 to about 230 Fab fragments per LNP. In some embodiments, the conjugate can comprise from about 10 to about 150 Fab fragments per LNP. In some embodiments, the conjugate can comprise from about 10 to about 30 Fab fragments per LNP.

[0282] Conjugates prepared by site-specific methods (e.g., **FIGs. 16-23** and **39-49**) have a high density of the targeting moiety on the surface of the LNP. For instance, the conjugate can comprise more than one targeting moiety (e.g., antibody, Fab fragment or scFv) per LNP. In some embodiments, the conjugate can comprise more than 10 targeting moieties (e.g., antibodies, Fab fragments or scFvs) per LNP. In some embodiments, the conjugate can comprise more than 20 targeting moieties (e.g., antibodies, Fab fragments or scFvs) per LNP. In some embodiments, the conjugate can comprise more than 30 targeting moieties (e.g., antibodies, Fab fragments or scFvs). In some embodiments, the conjugate can comprise more than 50 targeting moieties (e.g., antibodies, Fab fragments or scFvs) per LNP. In some embodiments, the conjugate can comprise more than 75 targeting moieties (e.g., antibodies, Fab fragments or scFvs) per LNP. In some embodiments, the conjugate can comprise more than 100 targeting moieties (e.g., antibodies, Fab fragments or scFvs). In some embodiments, the conjugate can comprise from about 50 to about 200 targeting moieties (e.g., antibodies, Fab fragments or scFvs) per LNP. In some embodiments, the conjugate can comprise from about 100 to about 200 targeting moieties (e.g., antibodies, Fab fragments or scFvs) per LNP. In some embodiments, the conjugate can comprise from about 100 to about 230 targeting moieties (e.g., antibodies, Fab fragments or scFvs) per LNP. In some embodiments, the conjugate can comprise from about 10 to about 150 targeting moieties (e.g., antibodies, Fab fragments or scFvs) per LNP. In some embodiments, the conjugate can comprise from about 10 to about 30 targeting moieties (e.g., antibodies, Fab fragments or scFvs) per LNP.

[0283] In some embodiments, the weight ratio between a targeting moiety on the surface of the LNP and the payload (e.g., RNA) encapsulated in the LNP can be 1:20, 1:15, 1:10, 1:9, 1:8, 1:7. 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1, 20:1.

[0284] In some embodiments, the conjugates prepared by site-specific methods (e.g., **FIGs. 16-23** and **39-49**) can have two or more different targeting moieties on the surface of the LNP. For example, the conjugate can have two different targeting moieties. In some embodiments, the conjugate can have three different targeting moieties. In some embodiments, the conjugate can have two different targeting moieties, wherein the mass ratio of the two targeting moieties on the surface of the LNP is 20:1, 15:1, 10:1, 9:1, 8:1, 7:1. 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:15, or 1:20. In some embodiments, the weight ratio between the one or more targeting moieties on the surface of the LNP and the payload (e.g., RNA) encapsulated in the LNP can be 1:20, 1:15, 1:10, 1:9, 1:8, 1:7. 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1, 20:1.

III. LIPID NANOPARTICLES

[0285] Lipid nanoparticles, in some embodiments, comprise one or more ionic lipids, such as non-cationic lipids (e.g., neutral or anionic, or zwitterionic lipids), also referred to herein as helper lipids; one or more conjugated lipids (such as PEG-conjugated lipids or lipids conjugated to polymers described in Table 5 of WO2019217941; incorporated herein by reference in its entirety); one or more sterols (e.g., cholesterol); and, optionally, one or more targeting molecules (e.g., conjugated receptors, receptor ligands, antibodies); or combinations of the foregoing.

[0286] Lipids that can be used in nanoparticle formations (e.g., lipid nanoparticles) include, for example, those described in Table 4 of WO2019217941, which is incorporated by reference—e.g., a lipid-containing nanoparticle can comprise one or more of the lipids in table 4 of WO2019217941. Lipid nanoparticles can include additional elements, such as polymers, such as the polymers described in table 5 of WO2019217941, incorporated by reference.

[0287] In some embodiments, conjugated lipids, when present, can include one or more of PEG-diacylglycerol (DAG) (such as l-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG)), PEG-dialkyloxypropyl (DAA), PEG-phospholipid, PEG-ceramide (Cer), a pegylated phosphatidylethanoloamine (PEG-PE), PEG succinate diacylglycerol (PEGS-DAG) (such as 4-0-(2',3'-di(tetradecanoyloxy)propyl-l-0-(w-methoxy(polyethoxy)ethyl) butanedioate (PEG-S-DMG)), PEG dialkoxypropylcarbam, N-(carbonyl-methoxypoly ethylene glycol 2000)- 1 ,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt, and those described in Table 2 of WO2019051289 (incorporated by reference), and combinations of the foregoing.

[0288] In some embodiments, sterols that can be incorporated into lipid nanoparticles include one or more of cholesterol or cholesterol derivatives, such as those in W02009/127060 or US2010/0130588, which are incorporated by reference. Additional exemplary sterols include phytosterols, including those described in Eygeris et al (2020), dx.doi.org/10.1021/acs.nanolett.0c01386, incorporated herein by reference.

[0289] In some embodiments, the lipid particle comprises an ionizable lipid, a non-cationic lipid, a conjugated lipid that inhibits aggregation of particles, and a sterol. The amounts of these components can be varied independently and to achieve desired properties. For example, in some embodiments, the lipid nanoparticle comprises an ionizable lipid is in an amount from about 20 mol % to about 90 mol % of the total lipids (in other embodiments it may be 20-70% (mol), 30-60% (mol) or 40-50% (mol); about 50 mol % to about 90 mol % of the total lipid present in the lipid nanoparticle), a non-cationic lipid in an amount from about 5 mol % to about

35 mol % of the total lipids, a conjugated lipid in an amount from about 0.5 mol % to about 20 mol % of the total lipids, and a sterol in an amount from about 20 mol % to about 50 mol % of the total lipids. The ratio of total lipid to nucleic acid (e.g., comprising the therapeutic agent and/or encoding the gene modifying polypeptide, template nucleic acid, or gene modifying system) can be varied as desired. For example, the total lipid to nucleic acid (mass or weight) ratio can be from about 10: 1 to about 30: 1.

[0290] In some embodiments, the average LNP diameter of the targeted LNP formulation may be between 10s of nm and 100s of nm, e.g., measured by dynamic light scattering (DLS). In some embodiments, the average LNP diameter of the targeted LNP formulation may be from about 40 nm to about 150 nm, such as about 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm. In some embodiments, the average LNP diameter of the targeted LNP formulation may be from about 50 nm to about 100 nm, from about 50 nm to about 90 nm, from about 50 nm to about 80 nm, from about 50 nm to about 70 nm, from about 50 nm to about 60 nm, from about 60 nm to about 100 nm, from about 60 nm to about 90 nm, from about 60 nm to about 80 nm, from about 60 nm to about 70 nm, from about 70 nm to about 100 nm, from about 70 nm to about 90 nm, from about 70 nm to about 80 nm, from about 80 nm to about 100 nm, from about 80 nm to about 90 nm, or from about 90 nm to about 100 nm. In some embodiments, the average LNP diameter of the targeted LNP formulation may be from about 70 nm to about 100 nm. In a particular embodiment, the average LNP diameter of the targeted LNP formulation may be about 80 nm. In some embodiments, the average LNP diameter of the targeted LNP formulation may be about 100 nm. In some embodiments, the average LNP diameter of the targeted LNP formulation ranges from about l mm to about 500 mm, from about 5 mm to about 200 mm, from about 10 mm to about 100 mm, from about 20 mm to about 80 mm, from about 25 mm to about 60 mm, from about 30 mm to about 55 mm, from about 35 mm to about 50 mm, or from about 38 mm to about 42 mm.

[0291] An LNP described herein, e.g., a targeted LNP, may, in some instances, be relatively homogenous. A polydispersity index may be used to indicate the homogeneity of a LNP, e.g., the particle size distribution of the lipid nanoparticles. A small (e.g., less than 0.3) polydispersity index generally indicates a narrow particle size distribution. A LNP may have a polydispersity index from about 0 to about 0.25, such as 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.20, 0.21, 0.22, 0.23, 0.24, or 0.25. In some embodiments, the polydispersity index of a LNP may be from about 0.10 to about 0.20.

[0292] The zeta potential of an LNP may be used to indicate the electrokinetic potential of the composition. In some embodiments, the zeta potential may describe the surface charge of a LNP. Lipid nanoparticles with relatively low charges, positive or negative, are generally desirable, as more highly charged species may interact undesirably with cells, tissues, and other elements in the body. In some embodiments, the zeta potential of a LNP may be from about -10 mV to about +20 mV, from about -10 mV to about +15 mV, from about -10 mV to about +10 mV, from about -10 mV to about +5 mV, from about -10 mV to about 0 mV, from about -10 mV to about -5 mV, from about -5 mV to about +20 mV, from about -5 mV to about +15 mV, from about -5 mV to about +10 mV, from about -5 mV to about +5 mV, from about -5 mV to about 0 mV, from about 0 mV to about +20 mV, from about 0 mV to about +15 mV, from about 0 mV to about +10 mV, from about 0 mV to about +5 mV, from about +5 mV to about +20 mV, from about +5 mV to about +15 mV, or from about +5 mV to about +10 mV.

[0293] The efficiency of encapsulation of a protein and/or nucleic acid (e.g., an mRNA encoding a polypeptide), describes the amount of protein and/or nucleic acid that is encapsulated or otherwise associated with an LNP after preparation, relative to the initial amount provided. The encapsulation efficiency is desirably high (e.g., close to 100%). The encapsulation efficiency may be measured, for example, by comparing the amount of protein or nucleic acid in a solution containing the lipid nanoparticle before and after breaking up the lipid nanoparticle with one or more organic solvents or detergents. An anion exchange resin may be used to measure the amount of free protein or nucleic acid (e.g., RNA) in a solution. Fluorescence may be used to measure the amount of free protein and/or nucleic acid (e.g., RNA) in a solution. For the lipid nanoparticles described herein, the encapsulation efficiency of a protein and/or nucleic acid may be at least 50%, for example 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%. In some embodiments, the encapsulation efficiency may be at least 80%. In some embodiments, the encapsulation efficiency may be at least 90%. In some embodiments, the encapsulation efficiency may be at least 95%.

[0294] An LNP of the disclosure may optionally comprise one or more coatings. In some embodiments, an LNP may be formulated in a capsule, film, or tablet having a coating. A capsule, film, or tablet including a composition described herein may have any useful size, tensile strength, hardness or density.

[0295] Additional exemplary lipids, formulations, methods, and characterization of LNPs are taught by WO2020061457, which is incorporated herein by reference in its entirety.

[0296] In some embodiments, *in vitro* or *ex vivo* cell lipofections are performed using Lipofectamine MessengerMax (Thermo Fisher) or TransIT-mRNA Transfection Reagent (Mirus Bio). In certain embodiments, LNPs are formulated using the GenVoy_ILM ionizable lipid mix (Precision NanoSystems). In certain embodiments, targeted LNPs of the disclosure are formulated using 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA) or dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA or MC3), the formulation and *in vivo* use of which are taught in Jayaraman et al. Angew Chem Int Ed Engl 51(34):8529-8533 (2012), incorporated herein by reference in its entirety.

[0297] Additional specific LNP formulations useful for delivery of nucleic acids are described in US8158601 and US8168775, both incorporated by reference, which include formulations used in patisiran, sold under the name ONPATTRO.

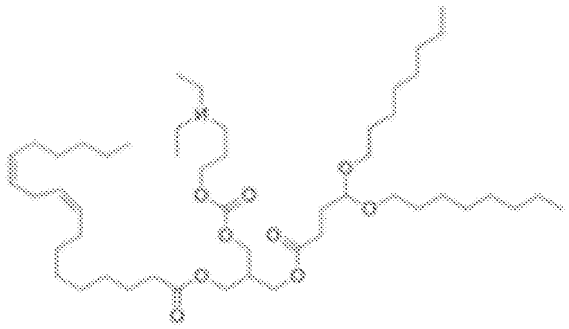
Ionizable Lipids

[0298] The LNPs of the disclosure (e.g., targeted LNPs) comprise one or more ionizable lipids. In some embodiments, an ionizable lipid may be a cationic lipid, an ionizable cationic lipid, e.g., a cationic lipid that can exist in a positively charged or neutral form depending on pH, or an amine-containing lipid that can be readily protonated. In some embodiments, the cationic lipid is a lipid capable of being positively charged, e.g., under physiological conditions. Exemplary cationic lipids include one or more amine group(s), which bear the positive charge. In some embodiments, the lipid particle comprises a cationic lipid in formulation with one or more of neutral lipids, ionizable amine-containing lipids, biodegradable alkyn lipids, steroids, phospholipids including polyunsaturated lipids, structural lipids (e.g., sterols), PEG, cholesterol and polymer conjugated lipids. In some embodiments, the cationic lipid may be an ionizable cationic lipid. An exemplary cationic lipid as disclosed herein may have an effective pKa over 6.0. In embodiments, a lipid nanoparticle may comprise a second cationic lipid having a different effective pKa (e.g., greater than the first effective pKa), than the first cationic lipid. A lipid nanoparticle may comprise between 40 and 60 mol percent of a cationic lipid, a neutral lipid, a sterol, a polymer conjugated lipid, and a therapeutic agent as described herein (e.g., one or more nucleic acids (e.g., RNA) comprising a gene modifying system) encapsulated within or associated with the lipid nanoparticle. In some embodiments, the therapeutic agent (e.g., one or more nucleic acids) is co-formulated with the cationic lipid. The therapeutic agent (e.g., one or more nucleic acids) may be adsorbed to the surface of an LNP, e.g., an LNP comprising a cationic lipid. In some embodiments, the therapeutic agent (e.g., one or more nucleic acids) may be encapsulated in an LNP, e.g., an LNP comprising a cationic lipid. In some embodiments, the

lipid nanoparticle may comprise a targeting moiety, e.g., coated with a targeting agent. In embodiments, the LNP formulation is biodegradable. In some embodiments, a lipid nanoparticle comprising one or more lipid described herein encapsulates at least 1%, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 92%, at least 95%, at least 97%, at least 98% or 100% of an RNA molecule (e.g., an mRNA molecule).

[0299] In some embodiments, the lipid to nucleic acid ratio (mass/mass ratio; w/w ratio) can be in the range of from about 1 : 1 to about 25: 1, from about 10: 1 to about 14: 1, from about 3 :1 to about 15: 1, from about 4: 1 to about 10: 1, from about 5: 1 to about 9: 1, or about 6: 1 to about 9: 1. The amounts of lipids and nucleic acid can be adjusted to provide a desired N/P ratio, for example, N/P ratio of 3, 4, 5, 6, 7, 8, 9, 10 or higher. Generally, the lipid nanoparticle formulation's overall lipid content can range from about 5 mg/ml to about 30 mg/mL.

[0300] In some embodiments, the LNP (e.g., targeted LNP) comprises the ionizable lipid V003, depicted below. V003 is described in U.S. Patent No. 10,059,655.

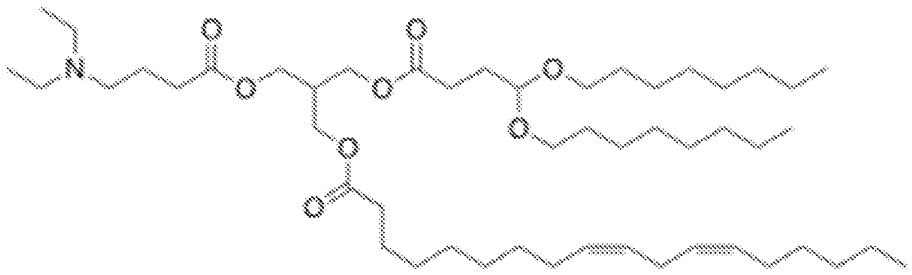
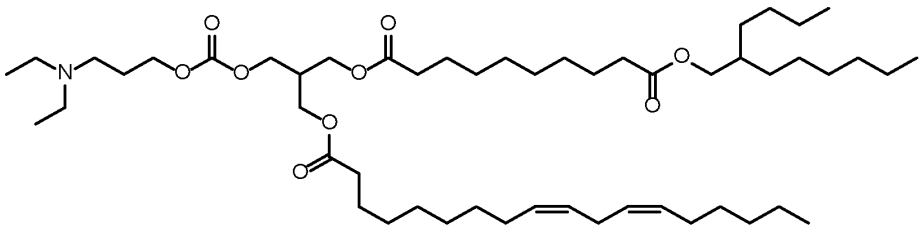
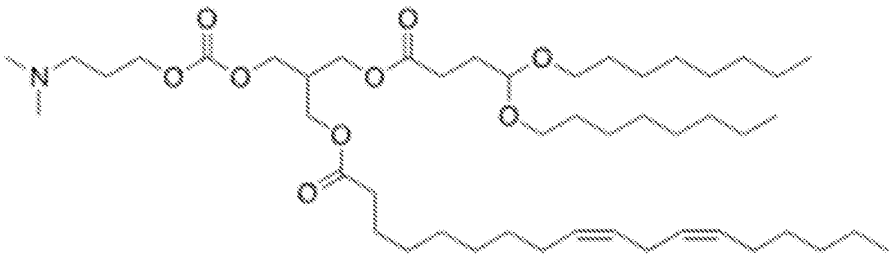
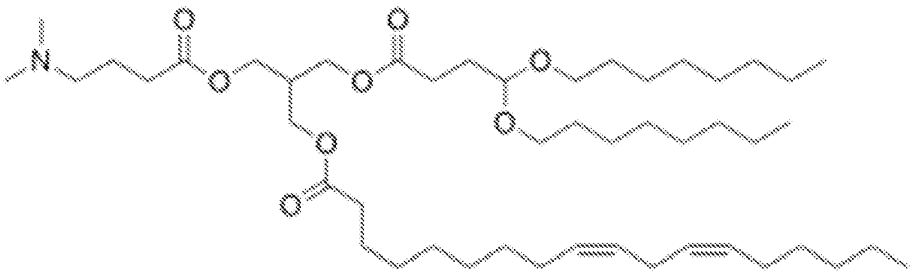


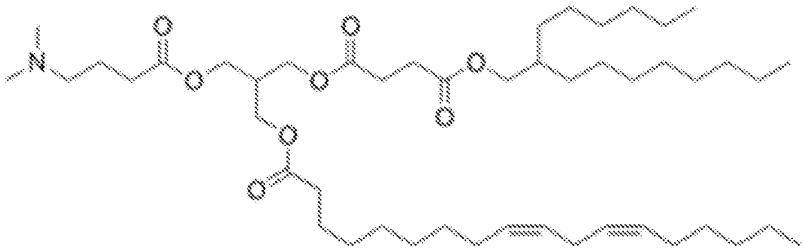
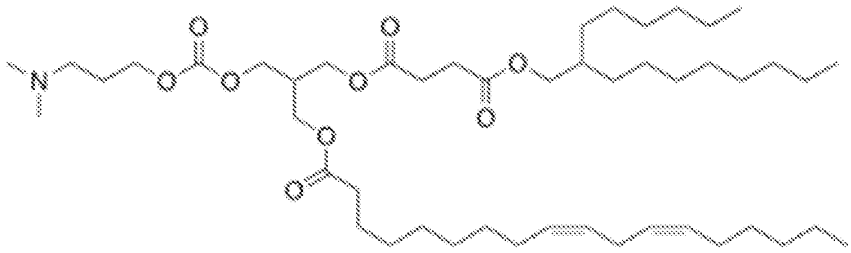
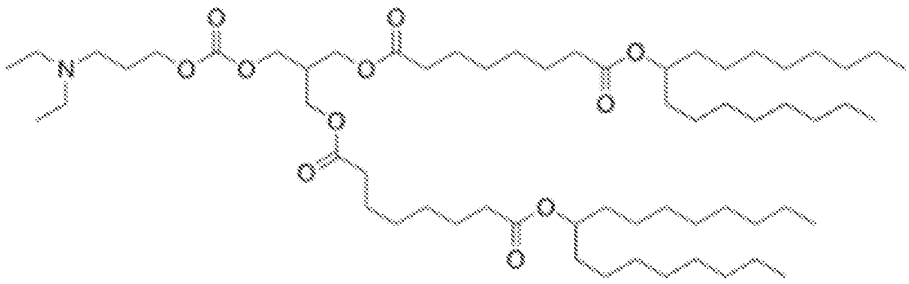
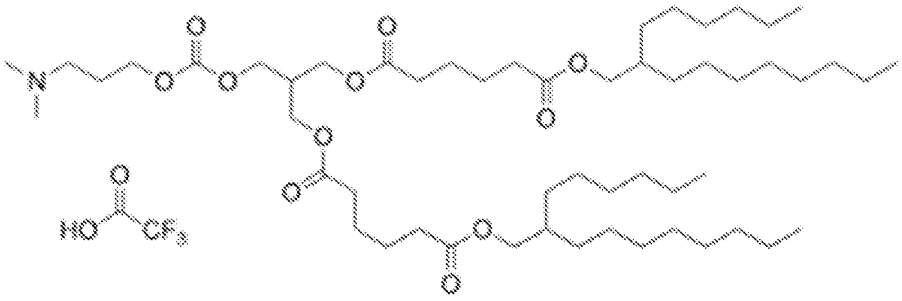
Lipid V003

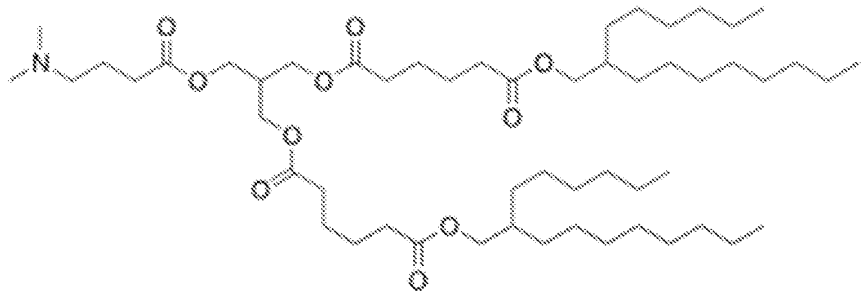
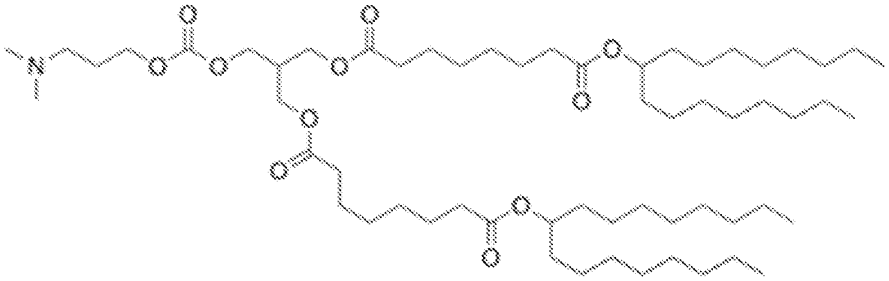
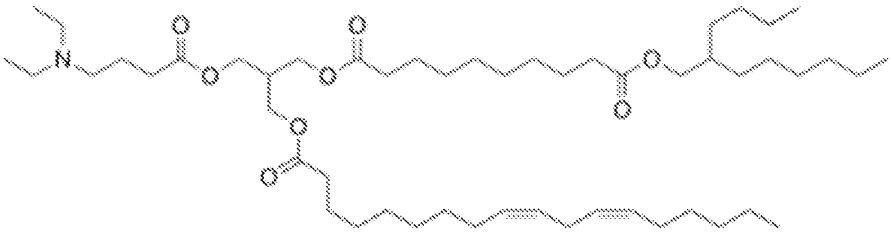
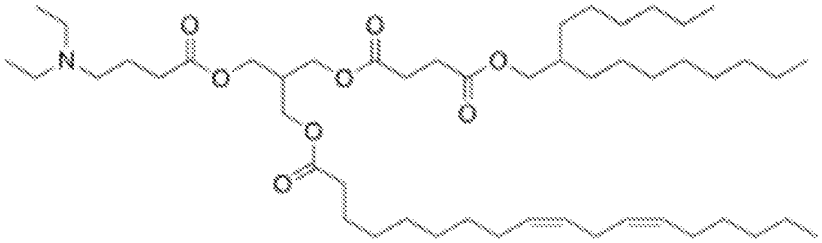
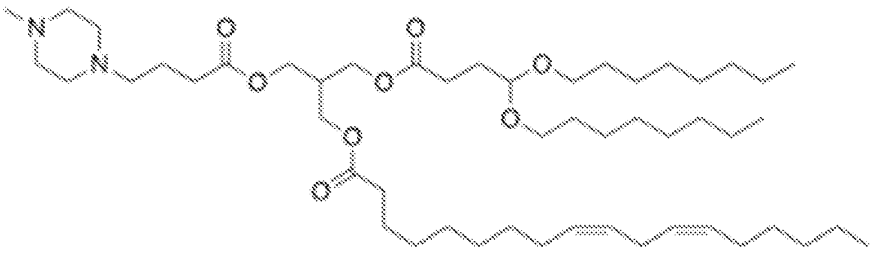
[0301] In some embodiments, the LNP (e.g., targeted LNP) comprises the ionizable lipid shown in **Table 1**. In some cases, an LNP containing an ionizable lipid of **Table 1** exhibits higher levels of transduction in immune cells (e.g., T cells) and/or higher expression of a payload protein in immune cells (e.g., T cells) relative to an LNP that contains V003 as the ionizable lipid. In other embodiments, Lipid 092 is used as an ionizable lipid to generate LNPs for delivery to immune cells (e.g., T cells). In other embodiments, Lipid 093 is used as an ionizable lipid to generate LNPs for delivery to immune cells (e.g., T cells). In other embodiments, Lipid 153 is used as an ionizable lipid to generate LNPs for delivery to immune cells (e.g., T cells). In other embodiments, Lipid154 is used as an ionizable lipid to generate LNPs for delivery to immune cells (e.g., T cells). In other embodiments, Lipid 155 is used as an ionizable lipid to generate LNPs for delivery to immune cells (e.g., T cells). In other embodiments, Lipid 162 is

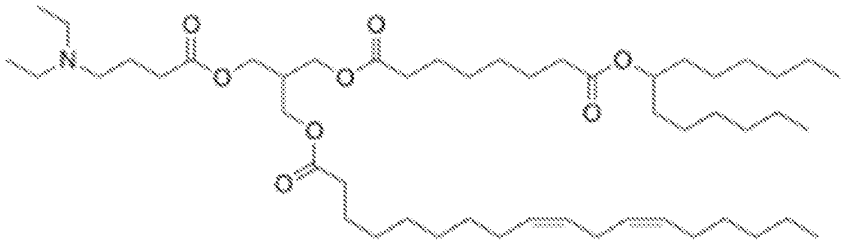
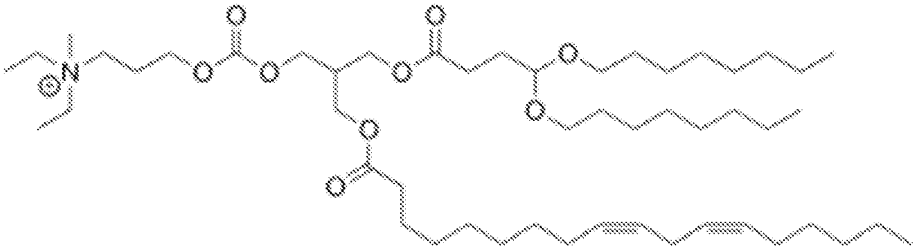
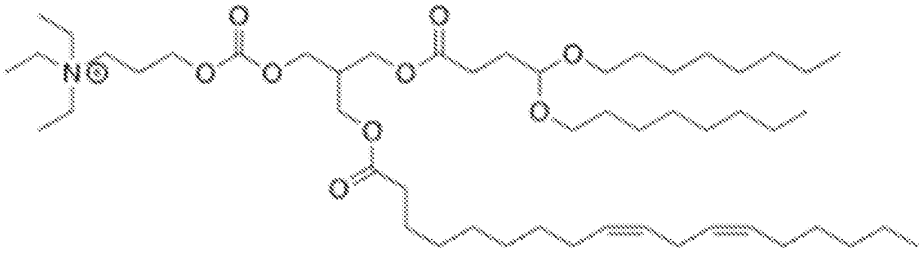
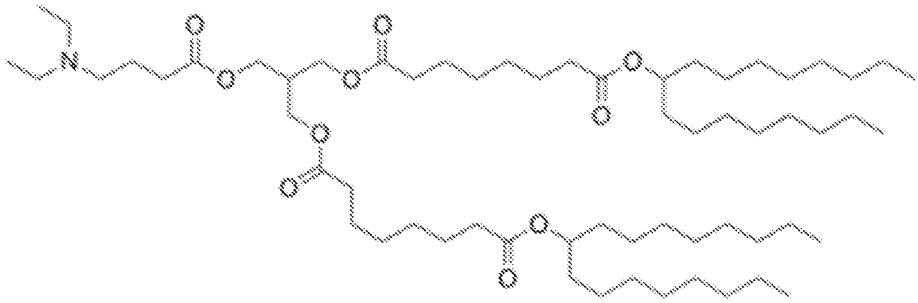
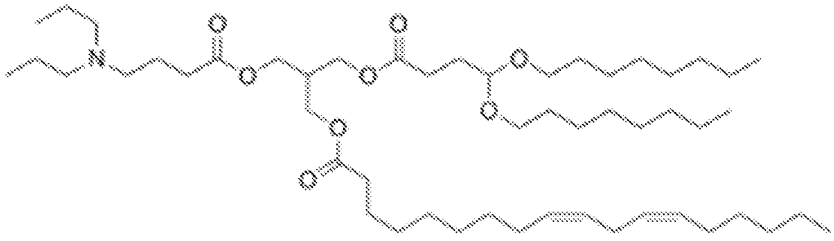
used as an ionizable lipid to generate LNPs for delivery to immune cells (e.g., T cells). In other embodiments, Lipid 163 is used as an ionizable lipid to generate LNPs for delivery to immune cells (e.g., T cells). In other embodiments, Lipid 169 is used as an ionizable lipid to generate LNPs for delivery to immune cells (e.g., T cells). In other embodiments, Lipid 176 is used as an ionizable lipid to generate LNPs for delivery to immune cells (e.g., T cells). In other embodiments, Lipid 178 is used as an ionizable lipid to generate LNPs for delivery to immune cells (e.g., T cells). In other embodiments, Lipid 183 is used as an ionizable lipid to generate LNPs for delivery to immune cells (e.g., T cells).

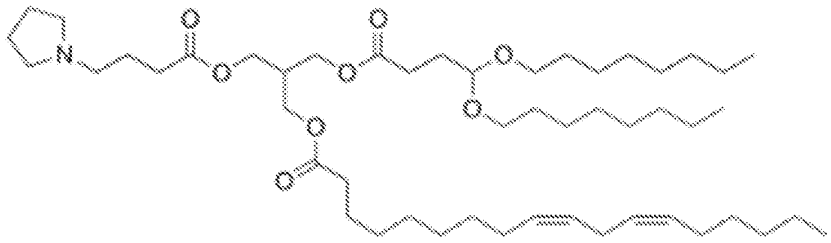
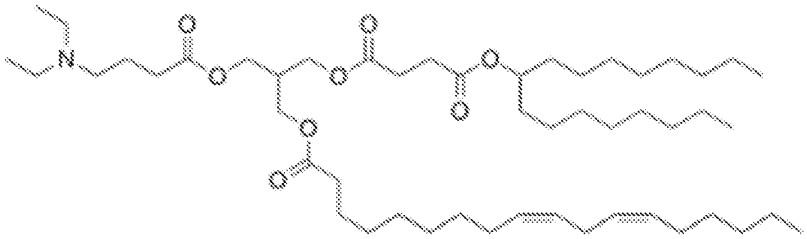
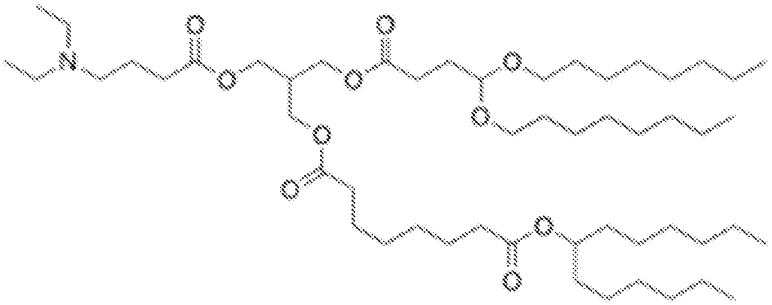
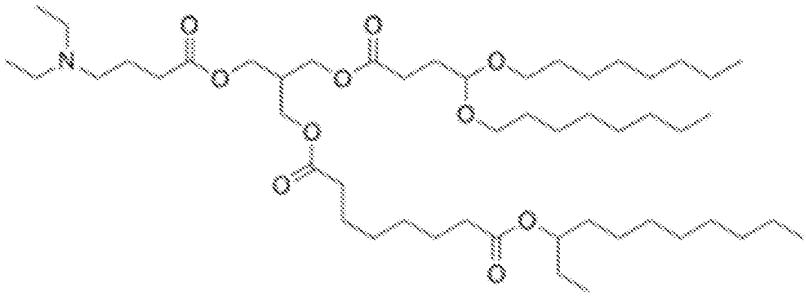
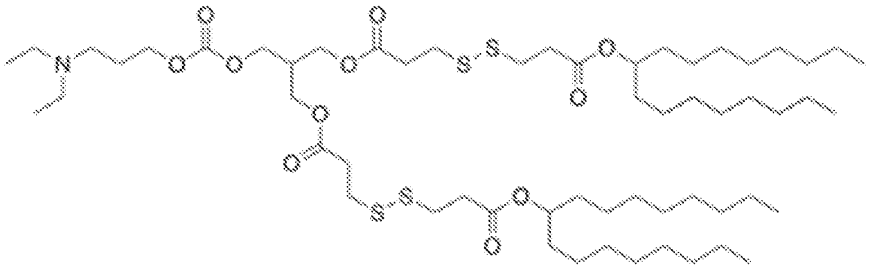
Table 1: Exemplary Ionizable Lipids

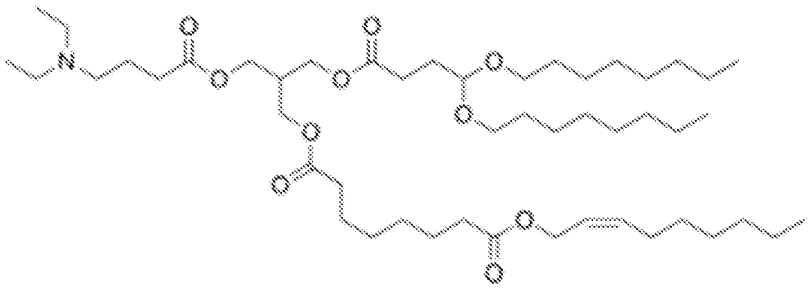
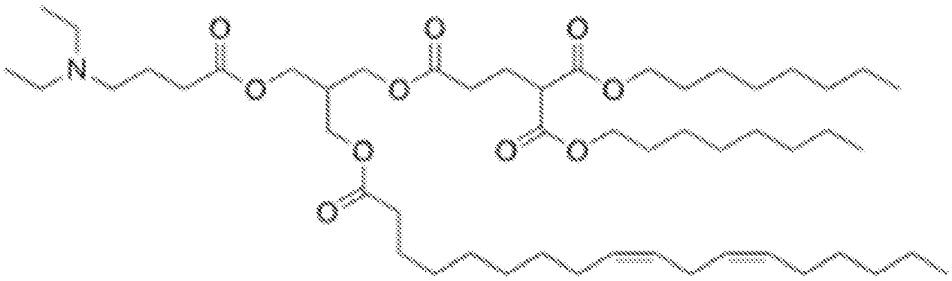
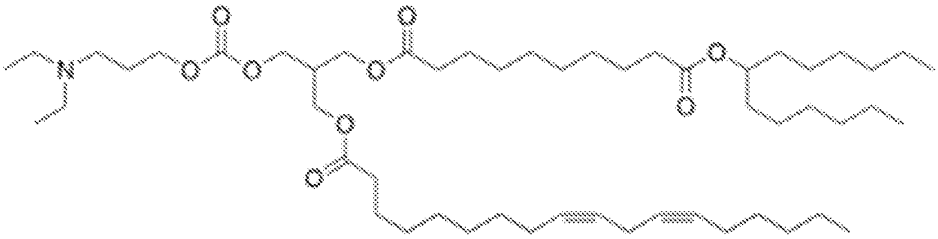
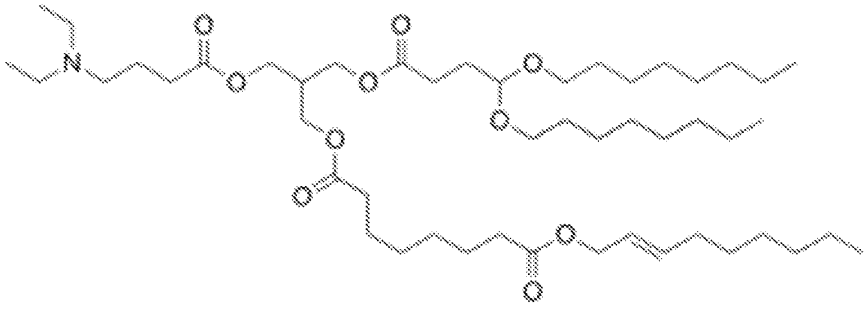
Lipid ID	Structure
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LIPID093	
LIPID095	
LIPID096	

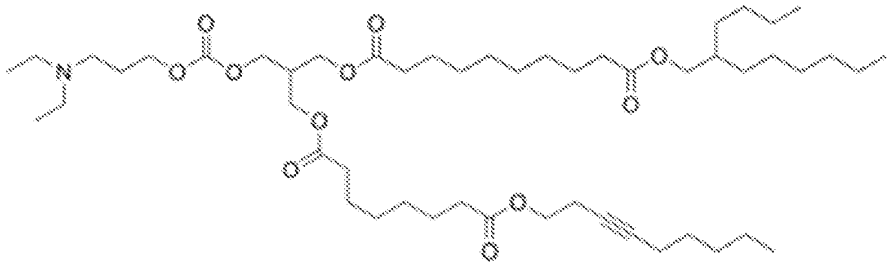
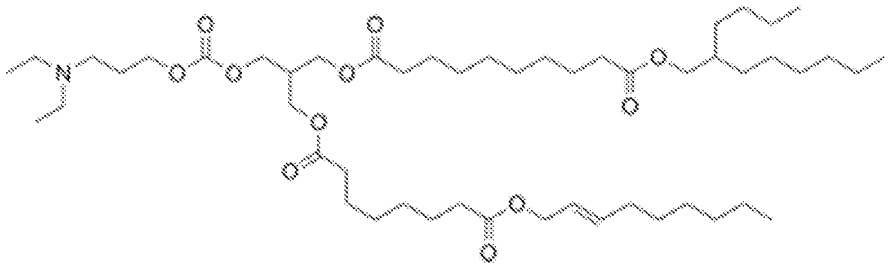
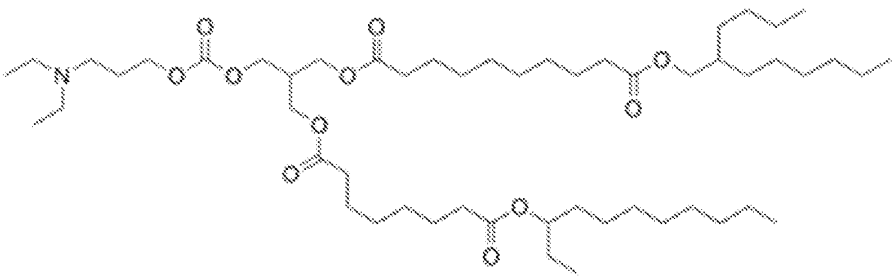
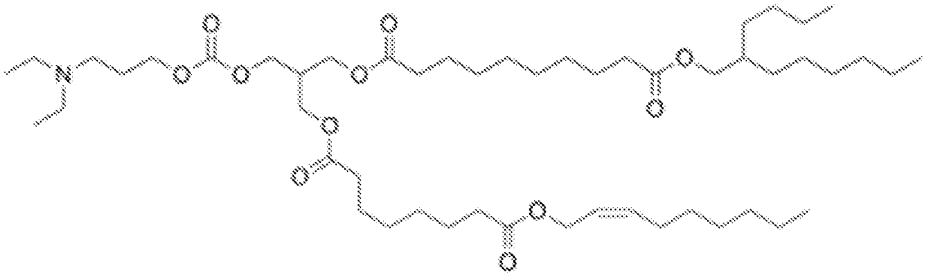
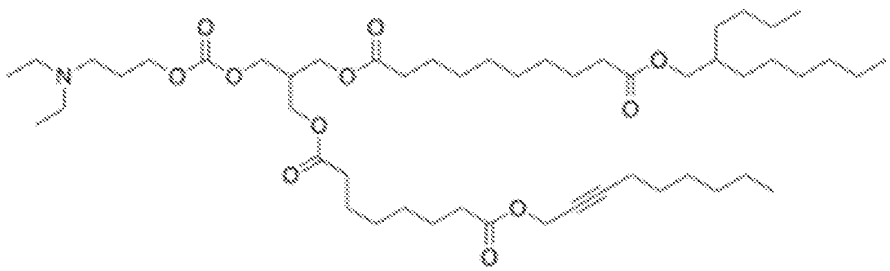
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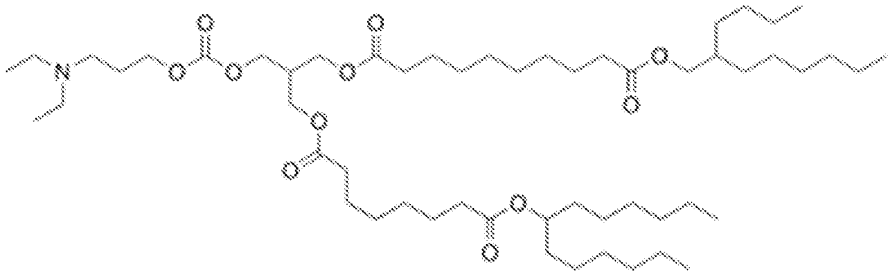
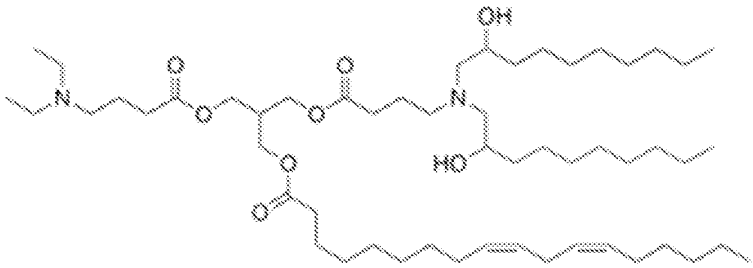
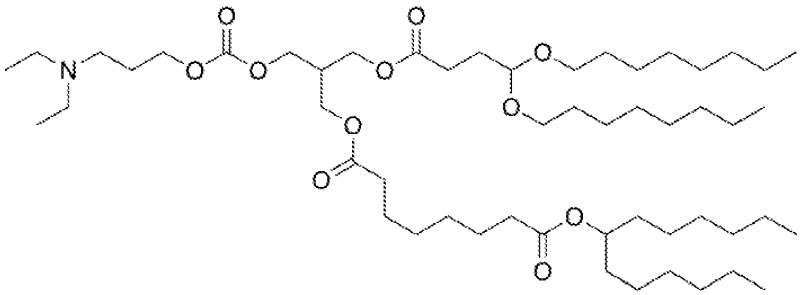
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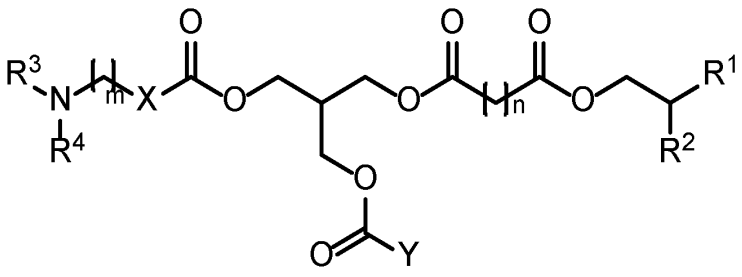
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LIPID163	
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LIPID174	
LIPID175	
LIPID176	
LIPID178	
LIPID179	

LIPID183	
LIPID 184	
Lipid 232	

[0302] In some embodiments, the LNP, e.g., targeted LNP, comprises an ionizable lipid having a structure of formula (I):



formula (I),

or a pharmaceutically acceptable salt thereof, wherein:

X is O or CH_2;

m is 1, 2, or 3;

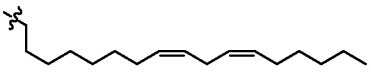
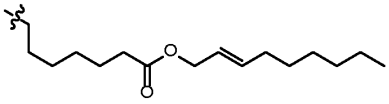
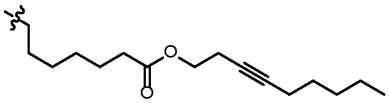
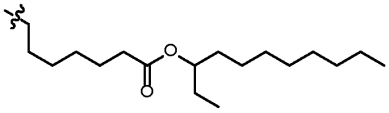
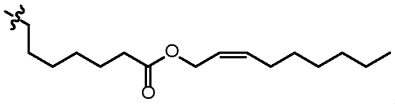
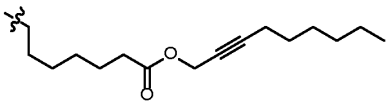
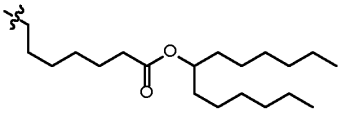
n is 2, 3, 4, 5, 6, 7, 8, 9, or 10;

R^1 is $C_{4\text{-}10}$alkyl;

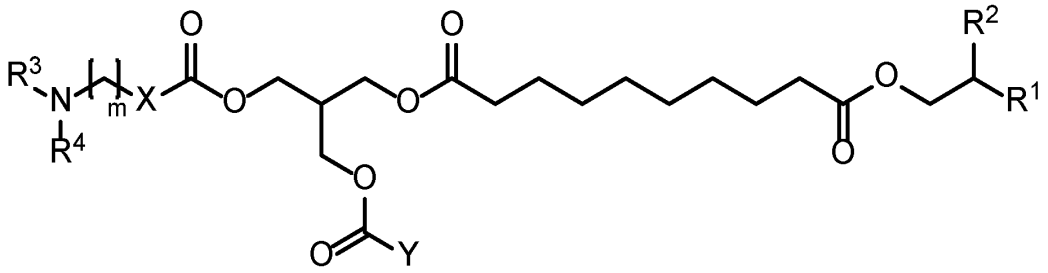
R^2 is $C_{1\text{-}4}$alkyl;

R^3 and R^4 are independently $C_{1\text{-}4}$ alkyl; and

Y is a lipophilic tail, branched or unbranched.

[0303] In some embodiments of the formula (I), R^1 is $C_{6\text{-}8}$alkyl. In some embodiments, R^1 is C_6alkyl. In some embodiments, R^2 is C_3alkyl. In some embodiments, R^2 is C_4alkyl. In some embodiments, n is 8. In some embodiments, n is 6. In some embodiments, n is 4. In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, X is O. In some embodiments, X is methylene. In some embodiments, m is 2. In some embodiments, m is 3. In some embodiments, m is 4. In some embodiments, R^3 is *n*-propyl. In some embodiments, R^3 is ethyl. In some embodiments, R^3 is methyl. In some embodiments, R^4 is *n*-propyl. In some embodiments, R^4 is ethyl. In some embodiments, R^4 is methyl. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a pyrrolidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperazine.

[0304] In some embodiments, compounds of formula (I) are compounds of formula (I-A):



formula (I-A),

or a pharmaceutically acceptable salt thereof, wherein:

X is O or CH_2;

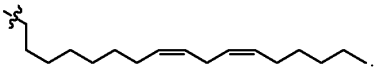
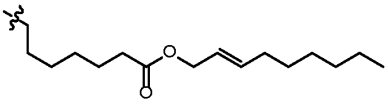
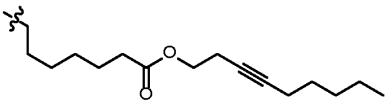
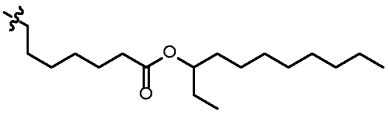
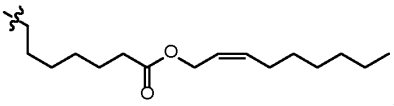
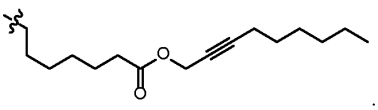
m is 1, 2, or 3;

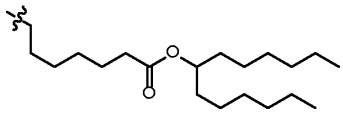
R^1 is $C_{4\text{-}10}$alkyl;

R^2 is $C_{1\text{-}4}$alkyl;

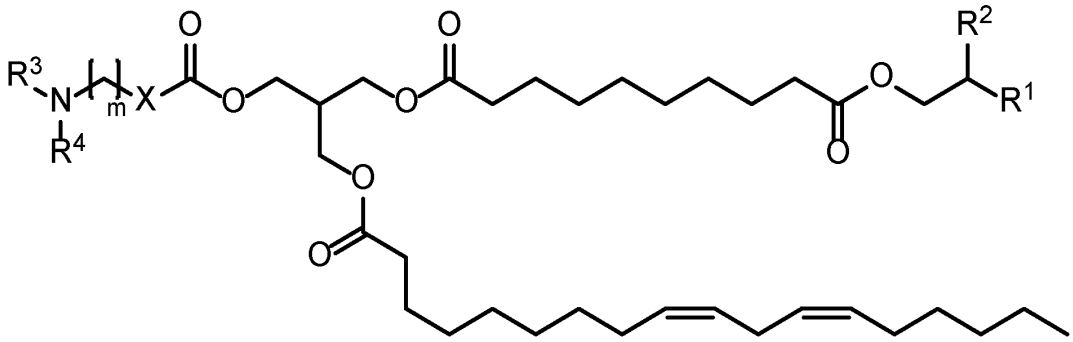
R^3 and R^4 are independently $C_{1\text{-}4}$ alkyl, or R^3 and R^4 are taken together with the nitrogen to which they are attached to form a heterocycle; and

Y is a lipophilic tail, branched or unbranched.

[0305] In some embodiments of the formula (I-A), R^1 is $C_{6\text{-}8}$alkyl. In some embodiments, R^1 is C_6alkyl. In some embodiments, R^2 is C_3alkyl. In some embodiments, R^2 is C_4alkyl. In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  .

In some embodiments, Y is  . In some embodiments, X is O. In some embodiments, X is methylene. In some embodiments, m is 2. In some embodiments, m is 3. In some embodiments, m is 4. In some embodiments, R^3 is *n*-propyl. In some embodiments, R^3 is ethyl. In some embodiments, R^3 is methyl. In some embodiments, R^4 is *n*-propyl. In some embodiments, R^4 is ethyl. In some embodiments, R^4 is methyl. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a pyrrolidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperazine.

[0306] In some embodiments, compounds of formula (I) are compounds of formula (I-B):



formula (I-B),

or a pharmaceutically acceptable salt thereof, wherein:

X is O or CH_2;FTab

m is 1, 2, or 3;

R^1 is $C_{4\text{-}10}$alkyl;

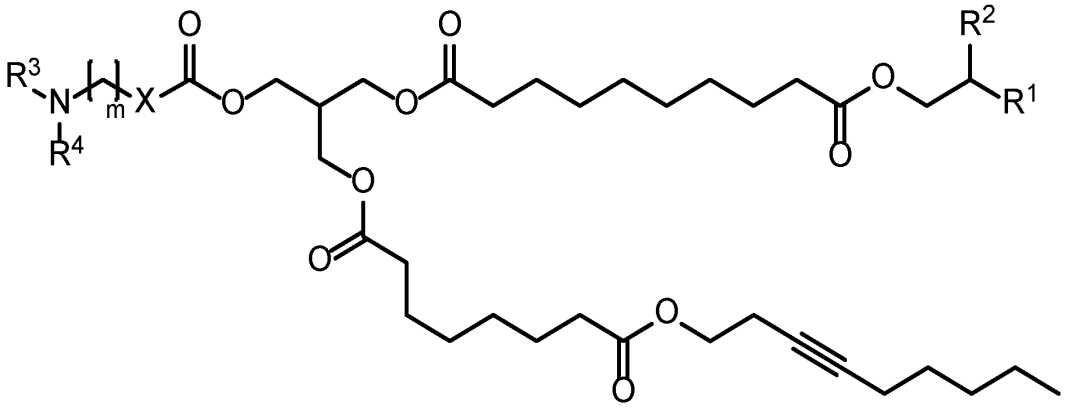
R^2 is $C_{1\text{-}4}$alkyl; and

R^3 and R^4 are independently $C_{1\text{-}4}$ alkyl, or R^3 and R^4 are taken together with the nitrogen to which they are attached to form a heterocycle.

[0307] In some embodiments of the formula (I-B), R^1 is $C_{6\text{-}8}$alkyl. In some embodiments, R^1 is C_6alkyl. In some embodiments, R^2 is C_3alkyl. In some embodiments, R^2 is C_4alkyl. In some embodiments, X is O. In some embodiments, X is methylene. In some embodiments, m is 2. In some embodiments, m is 3. In some embodiments, m is 4. In some embodiments, R^3 is *n*-propyl.

In some embodiments, R^3 is ethyl. In some embodiments, R^3 is methyl. In some embodiments, R^4 is *n*-propyl. In some embodiments, R^4 is ethyl. In some embodiments, R^4 is methyl. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a pyrrolidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperazine.

[0308] In some embodiments, compounds of formula (I) are compounds of formula (I-C):



formula (I-C),

or a pharmaceutically acceptable salt thereof, wherein:

X is O or CH_2;

m is 1, 2, or 3;

R^1 is $C_{4\text{-}10}$alkyl;

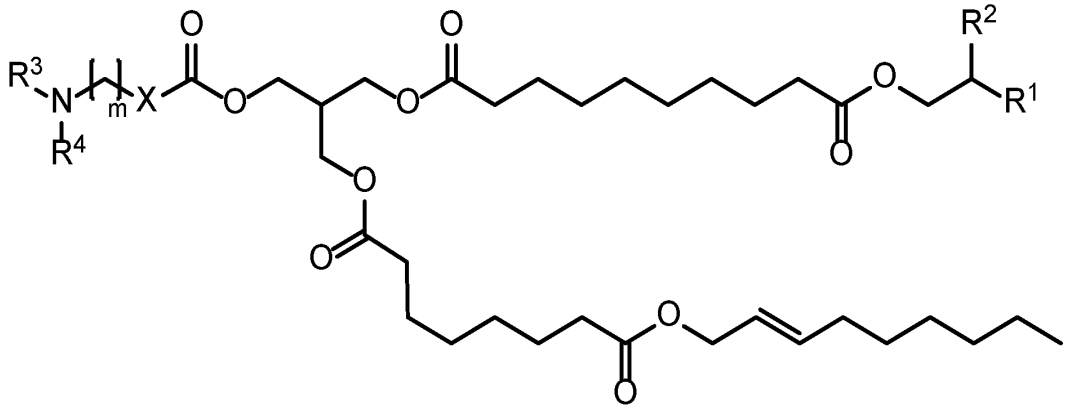
R^2 is $C_{1\text{-}4}$alkyl; and

R^3 and R^4 are independently $C_{1\text{-}4}$ alkyl, or R^3 and R^4 are taken together with the nitrogen to which they are attached to form a heterocycle.

[0309] In some embodiments of the formula (I-C), R^1 is $C_{6\text{-}8}$alkyl. In some embodiments, R^1 is C_6alkyl. In some embodiments, R^2 is C_3alkyl. In some embodiments, R^2 is C_4alkyl. In some embodiments, X is O. In some embodiments, X is methylene. In some embodiments, m is 2. In some embodiments, m is 3. In some embodiments, m is 4. In some embodiments, R^3 is *n*-propyl. In some embodiments, R^3 is ethyl. In some embodiments, R^3 is methyl. In some embodiments, R^4 is *n*-propyl. In some embodiments, R^4 is ethyl. In some embodiments, R^4 is methyl. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a pyrrolidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are

attached to form a piperidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperazine.

[0310] In some embodiments, compounds of formula (I) are compounds of formula (I-D):



formula (I-D),

or a pharmaceutically acceptable salt thereof, wherein:

X is O or CH_2;

m is 1, 2, or 3;

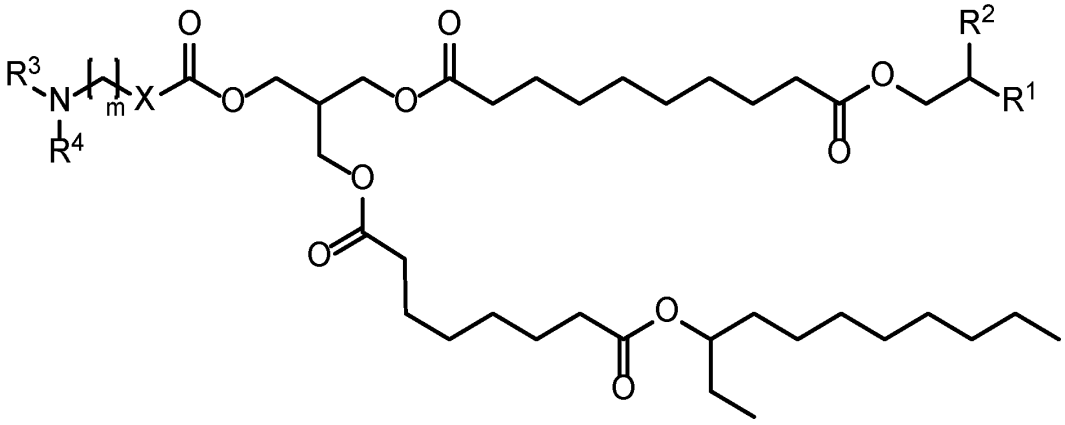
R^1 is $C_{4\text{-}10}$alkyl;

R^2 is $C_{1\text{-}4}$alkyl; and

R^3 and R^4 are independently $C_{1\text{-}4}$ alkyl, or R^3 and R^4 are taken together with the nitrogen to which they are attached to form a heterocycle.

[0311] In some embodiments of the formula (I-D), R^1 is $C_{6\text{-}8}$alkyl. In some embodiments, R^1 is C_6alkyl. In some embodiments, R^2 is C_3alkyl. In some embodiments, R^2 is C_4alkyl. In some embodiments, X is O. In some embodiments, X is methylene. In some embodiments, m is 2. In some embodiments, m is 3. In some embodiments, m is 4. In some embodiments, R^3 is *n*-propyl. In some embodiments, R^3 is ethyl. In some embodiments, R^3 is methyl. In some embodiments, R^4 is *n*-propyl. In some embodiments, R^4 is ethyl. In some embodiments, R^4 is methyl. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a pyrrolidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperazine.

[0312] In some embodiments, compounds of formula (I) are compounds of formula (I-E):



formula (I-E),

or a pharmaceutically acceptable salt thereof, wherein:

X is O or CH_2;

m is 1, 2, or 3;

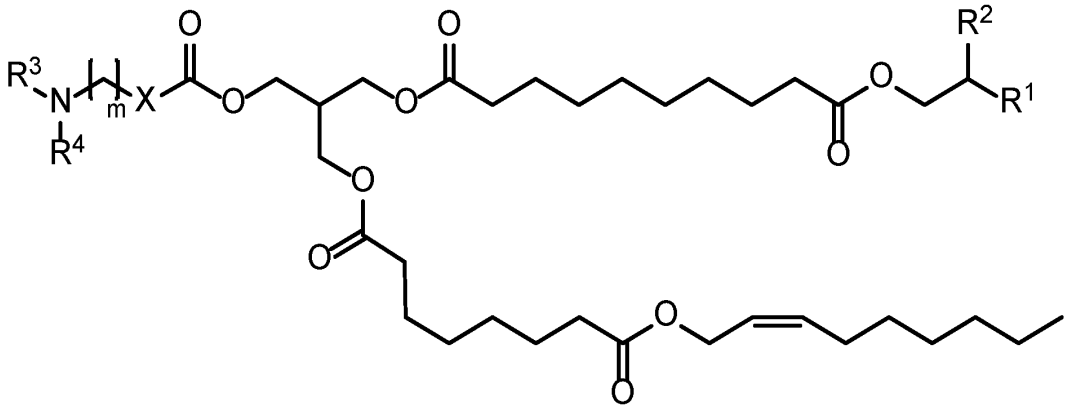
R^1 is C_{4-10}alkyl;

R^2 is C_{1-4}alkyl; and

R^3 and R^4 are independently C_{1-4} alkyl, or R^3 and R^4 are taken together with the nitrogen to which they are attached to form a heterocycle.

[0313] In some embodiments of the formula (I-E), R^1 is C_{6-8}alkyl. In some embodiments, R^1 is C_6alkyl. In some embodiments, R^2 is C_3alkyl. In some embodiments, R^2 is C_4alkyl. In some embodiments, X is O. In some embodiments, X is methylene. In some embodiments, m is 2. In some embodiments, m is 3. In some embodiments, m is 4. In some embodiments, R^3 is *n*-propyl. In some embodiments, R^3 is ethyl. In some embodiments, R^3 is methyl. In some embodiments, R^4 is *n*-propyl. In some embodiments, R^4 is ethyl. In some embodiments, R^4 is methyl. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a pyrrolidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperazine.

[0314] In some embodiments, compounds of formula (I) are compounds of formula (I-F):



formula (I-F),

or a pharmaceutically acceptable salt thereof, wherein:

X is O or CH_2;

m is 1, 2, or 3;

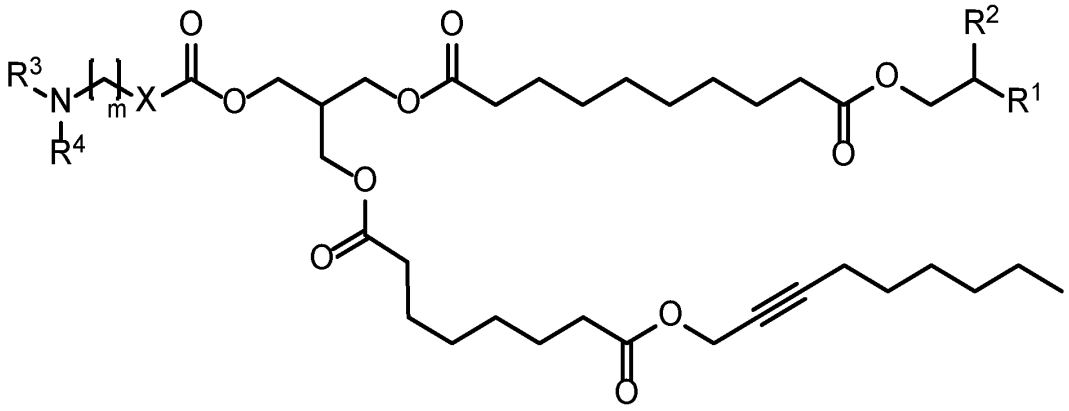
R^1 is $C_{4\text{-}10}$alkyl;

R^2 is $C_{1\text{-}4}$alkyl; and

R^3 and R^4 are independently $C_{1\text{-}4}$ alkyl, or R^3 and R^4 are taken together with the nitrogen to which they are attached to form a heterocycle.

[0315] In some embodiments of the formula (I-F), R^1 is $C_{6\text{-}8}$alkyl. In some embodiments, R^1 is C_6alkyl. In some embodiments, R^2 is C_3alkyl. In some embodiments, R^2 is C_4alkyl. In some embodiments, X is O. In some embodiments, X is methylene. In some embodiments, m is 2. In some embodiments, m is 3. In some embodiments, m is 4. In some embodiments, R^3 is *n*-propyl. In some embodiments, R^3 is ethyl. In some embodiments, R^3 is methyl. In some embodiments, R^4 is *n*-propyl. In some embodiments, R^4 is ethyl. In some embodiments, R^4 is methyl. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a pyrrolidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperazine.

[0316] In some embodiments, compounds of formula (I) are compounds of formula (I-G):



formula (I-G),

or a pharmaceutically acceptable salt thereof, wherein:

X is O or CH_2;

m is 1, 2, or 3;

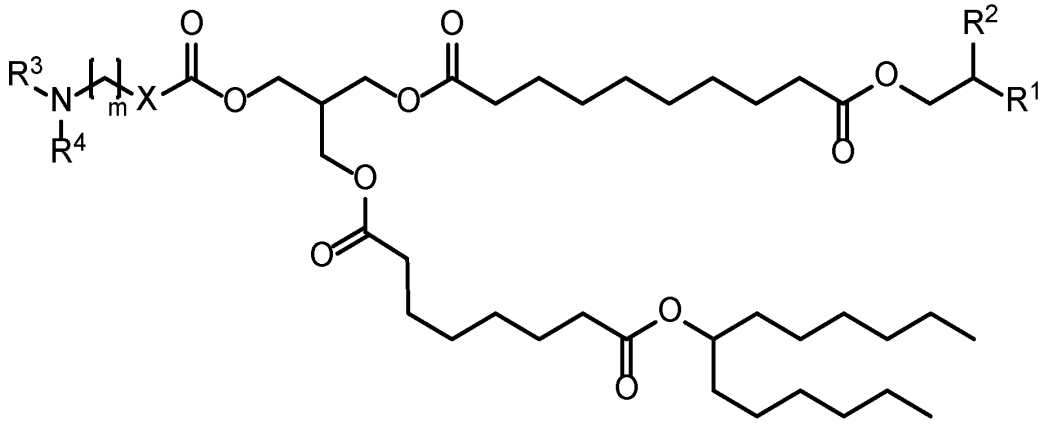
R^1 is $C_{4\text{-}10}$alkyl;

R^2 is $C_{1\text{-}4}$alkyl; and

R^3 and R^4 are independently $C_{1\text{-}4}$ alkyl, or R^3 and R^4 are taken together with the nitrogen to which they are attached to form a heterocycle.

[0317] In some embodiments of the formula (I-G), R^1 is $C_{6\text{-}8}$alkyl. In some embodiments, R^1 is C_6alkyl. In some embodiments, R^2 is C_3alkyl. In some embodiments, R^2 is C_4alkyl. In some embodiments, X is O. In some embodiments, X is methylene. In some embodiments, m is 2. In some embodiments, m is 3. In some embodiments, m is 4. In some embodiments, R^3 is *n*-propyl. In some embodiments, R^3 is ethyl. In some embodiments, R^3 is methyl. In some embodiments, R^4 is *n*-propyl. In some embodiments, R^4 is ethyl. In some embodiments, R^4 is methyl. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a pyrrolidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperazine.

[0318] In some embodiments, compounds of formula (I) are compounds of formula (I-H):



formula (I-H),

or a pharmaceutically acceptable salt thereof, wherein:

X is O or CH_2;

m is 1, 2, or 3;

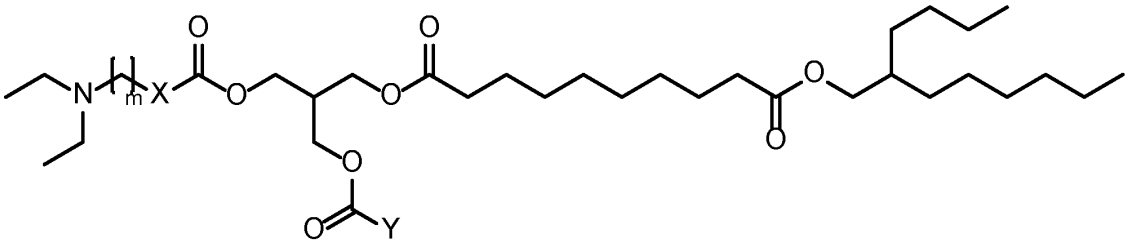
R^1 is C_{4-10}alkyl;

R^2 is C_{1-4}alkyl; and

R^3 and R^4 are independently C_{1-4} alkyl, or R^3 and R^4 are taken together with the nitrogen to which they are attached to form a heterocycle.

[0319] In some embodiments of the formula (I-H), R^1 is C_{6-8}alkyl. In some embodiments, R^1 is C_6alkyl. In some embodiments, R^2 is C_3alkyl. In some embodiments, R^2 is C_4alkyl. In some embodiments, X is O. In some embodiments, X is methylene. In some embodiments, m is 2. In some embodiments, m is 3. In some embodiments, m is 4. In some embodiments, R^3 is *n*-propyl. In some embodiments, R^3 is ethyl. In some embodiments, R^3 is methyl. In some embodiments, R^4 is *n*-propyl. In some embodiments, R^4 is ethyl. In some embodiments, R^4 is methyl. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a pyrrolidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperazine.

[0320] In some embodiments, the LNP, e.g., targeted LNP, comprises an ionizable lipid having a structure of formula (II):



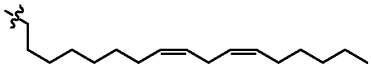
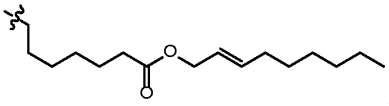
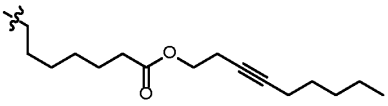
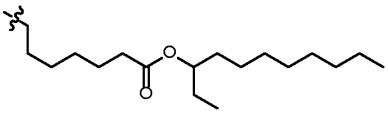
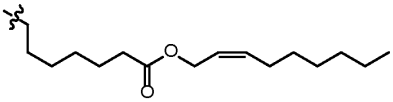
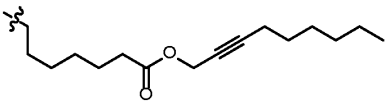
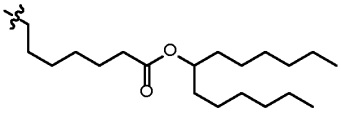
formula (II),

or a pharmaceutically acceptable salt thereof, wherein:

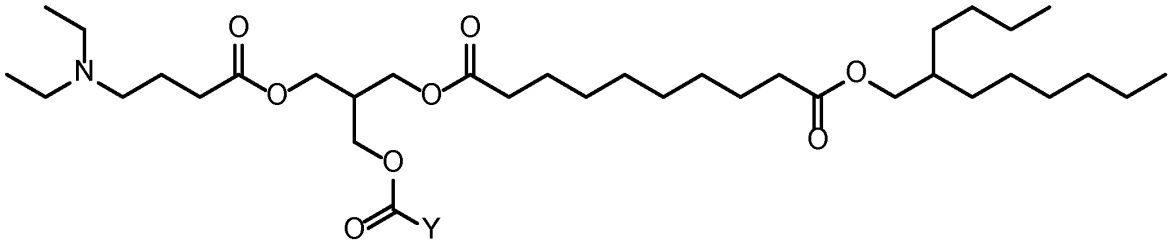
X is O or CH_2;

m is 1, 2, or 3; and

Y is a lipophilic tail, branched or unbranched.

[0321] In some embodiments of the formula (II), Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, X is O. In some embodiments, X is methylene. In some embodiments, m is 2. In some embodiments, m is 3. In some embodiments, m is 4.

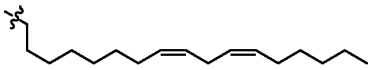
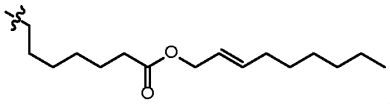
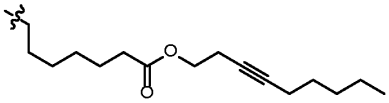
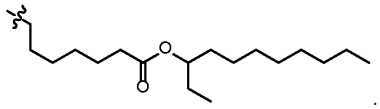
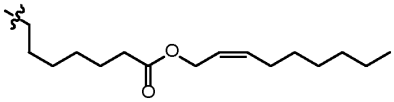
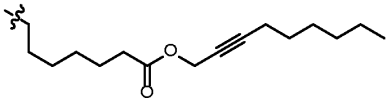
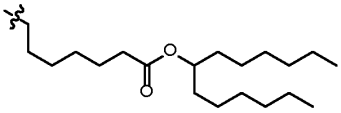
[0322] In some embodiments, compounds of formula (II) are compounds of formula (II-A):



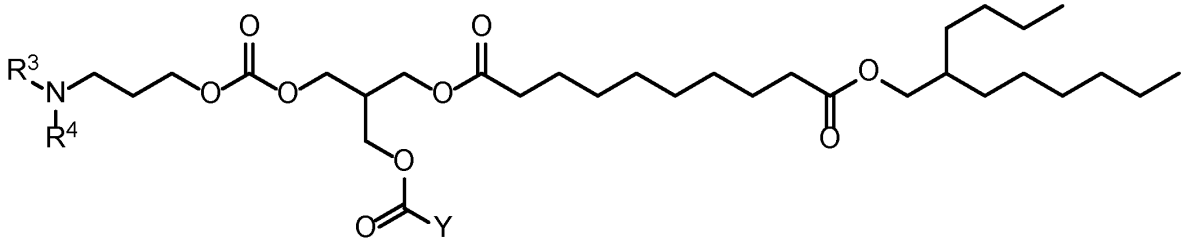
formula (II-A),

or a pharmaceutically acceptable salt thereof, wherein:

Y is a lipophilic tail, branched or unbranched.

[0323] In some embodiments of the formula (II-A), Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  .

[0324] In some embodiments, the LNP, e.g., targeted LNP, comprises an ionizable lipid having a structure of formula (III):

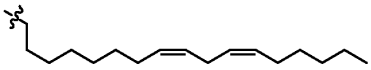
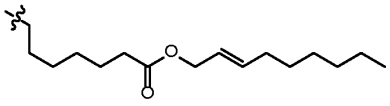
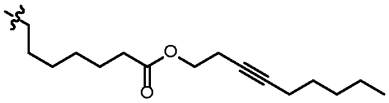
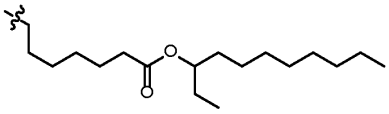
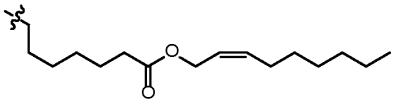
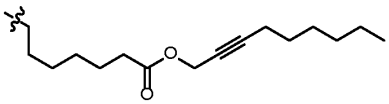
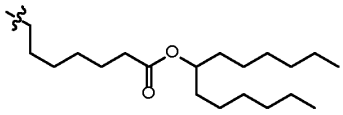


formula (III),

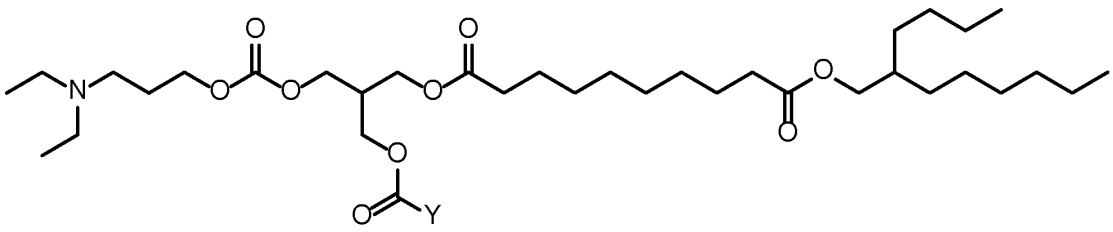
or a pharmaceutically acceptable salt thereof, wherein:

R^3 and R^4 are independently C_{1-4} alkyl, or R^3 and R^4 are taken together with the nitrogen to which they are attached to form a heterocycle; and

Y is a lipophilic tail, branched or unbranched.

[0325] In some embodiments of the formula (III), Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, R^3 is *n*-propyl. In some embodiments, R^3 is ethyl. In some embodiments, R^3 is methyl. In some embodiments, R^4 is *n*-propyl. In some embodiments, R^4 is ethyl. In some embodiments, R^4 is methyl. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a pyrrolidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperidine. In some embodiments, R^3 and R^4 are taken to together with the N to which they are attached to form a piperazine.

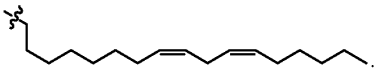
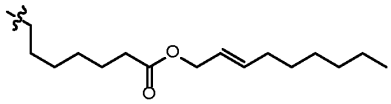
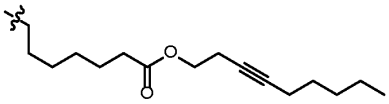
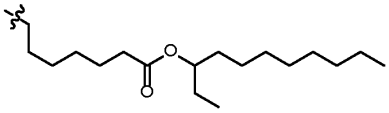
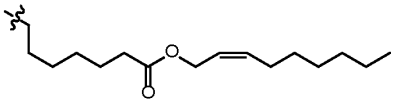
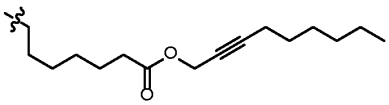
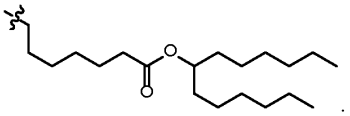
[0326] In some embodiments, compounds of formula (III) are compounds of formula (III-A):



formula (III-A),

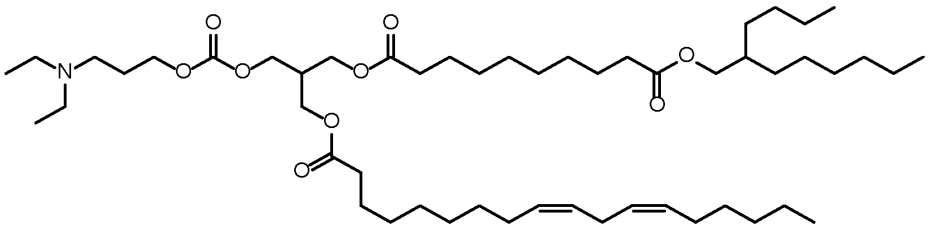
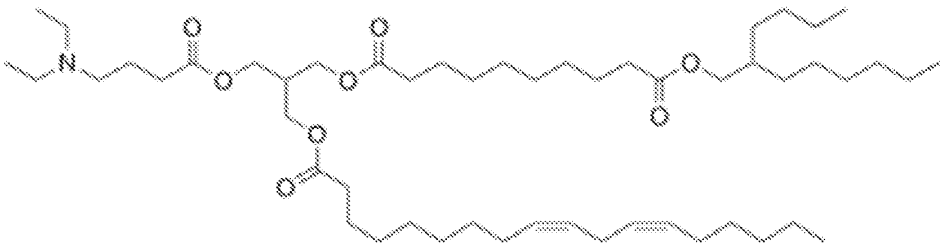
or a pharmaceutically acceptable salt thereof, wherein:

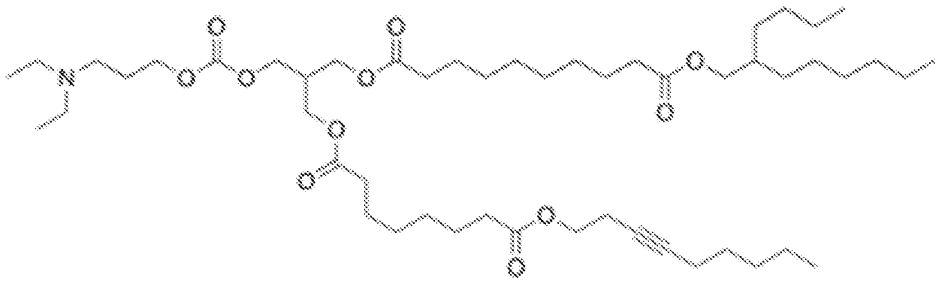
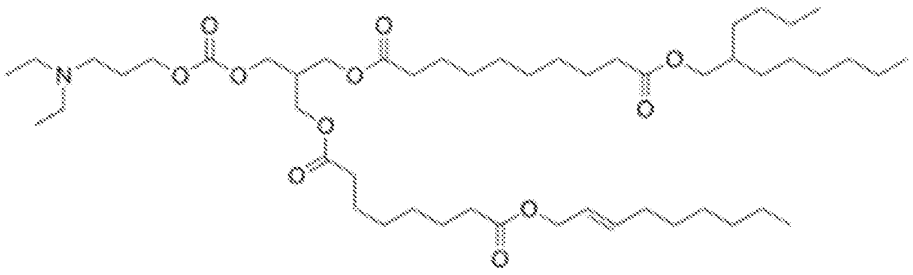
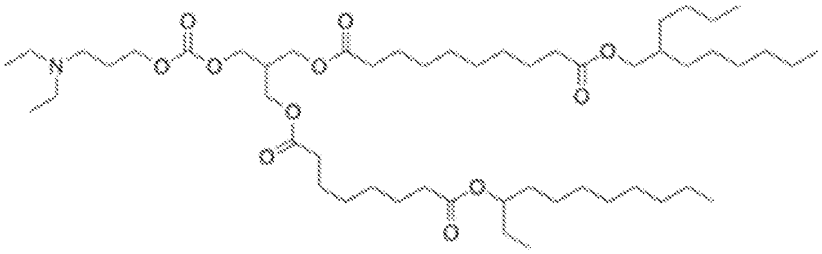
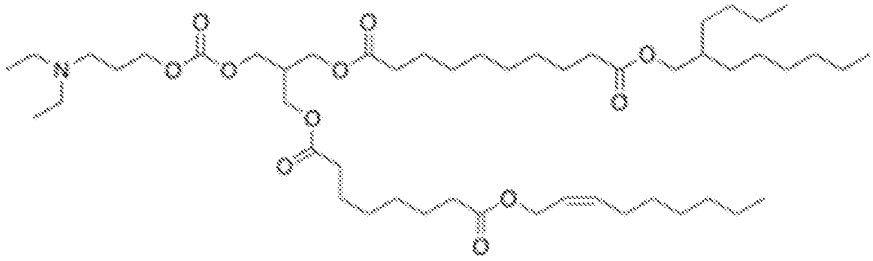
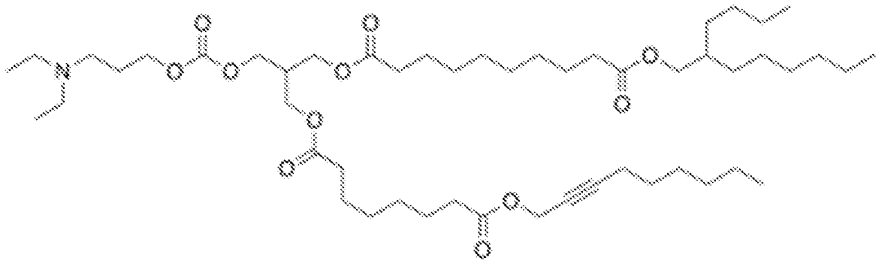
Y is a lipophilic tail, branched or unbranched.

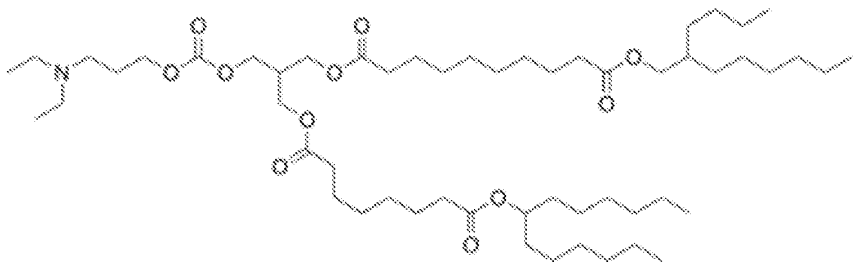
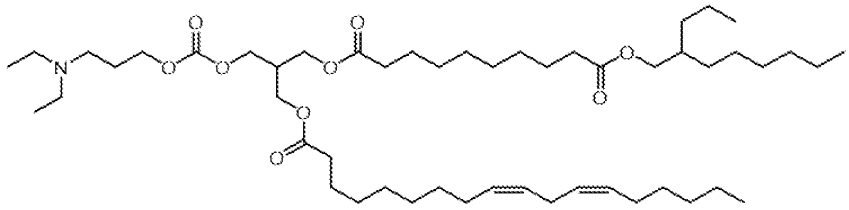
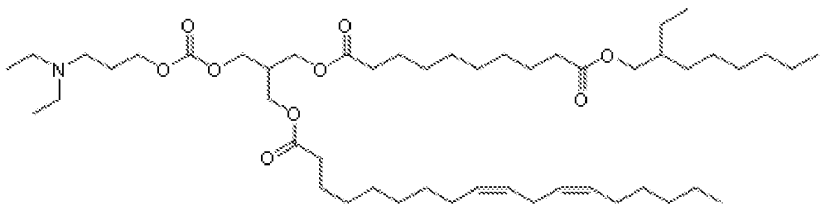
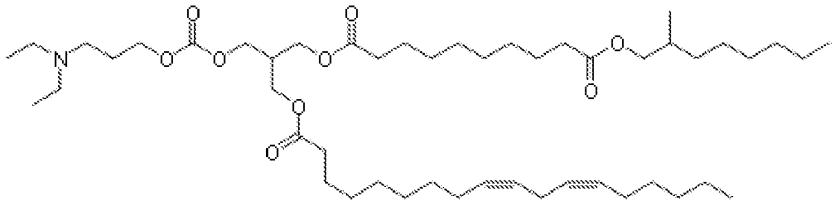
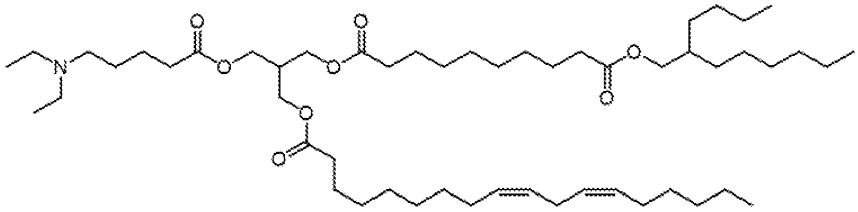
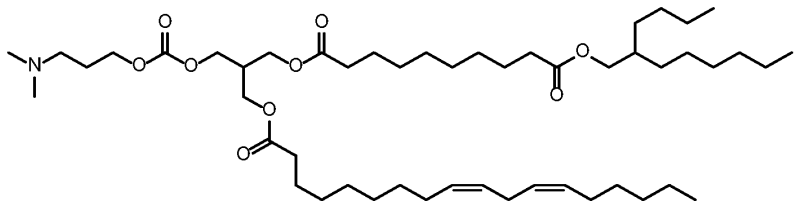
[0327] In some embodiments of the formula (III-A), Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  . In some embodiments, Y is  .

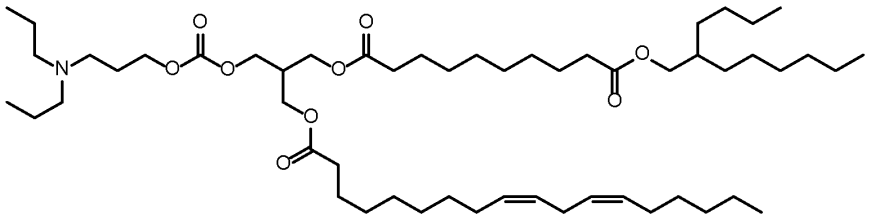
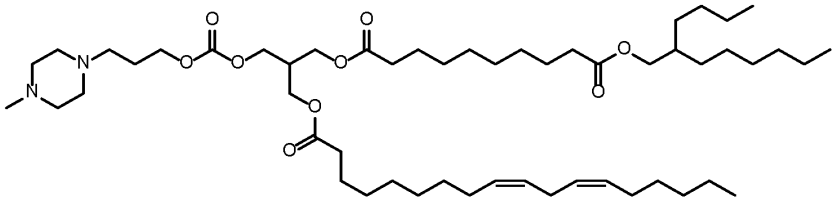
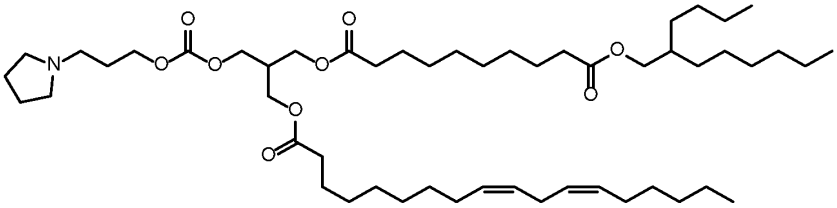
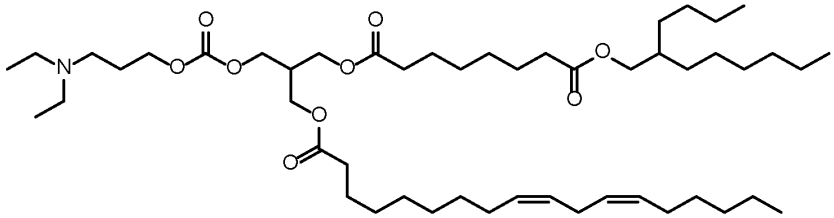
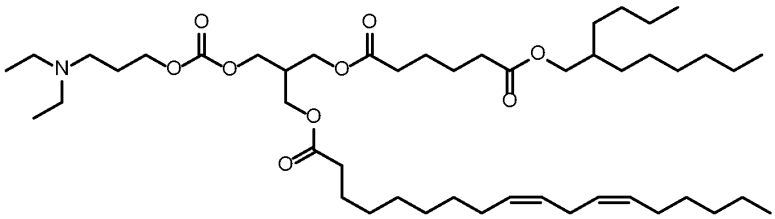
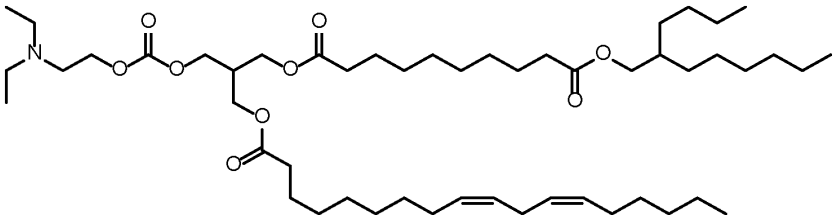
[0328] In some embodiments, the compound of formula (I) is a compound selected from the exemplary compounds of **Table 2**.

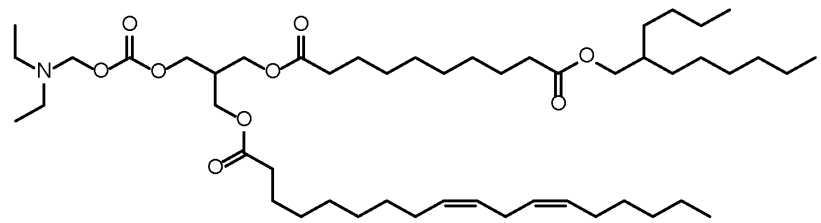
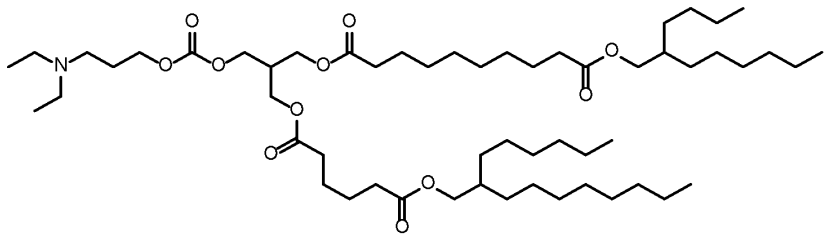
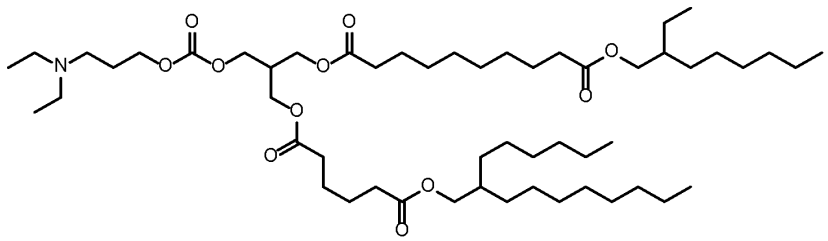
Table 2: Exemplary Ionizable Lipids

Compound No.	Structure
Lipid 093	
Lipid 141	

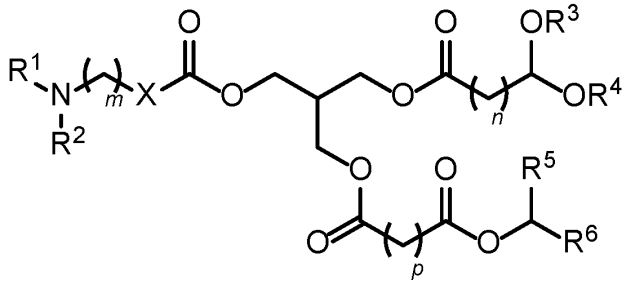
Lipid 174	
Lipid 175	
Lipid 176	
Lipid 178	
Lipid 179	

Lipid 183	
Lipid 710	
Lipid 711	
Lipid 712	
Lipid 713	
Lipid 714	

Lipid 715	
Lipid 716	
Lipid 717	
Lipid 718	
Lipid 719	
Lipid 720	

Lipid 721	
Lipid 722	
Lipid 723	

[0329] In some embodiments, the LNP, e.g., targeted LNP, comprises an ionizable lipid having a structure of formula (IV):



(IV),

or a pharmaceutically acceptable salt thereof, wherein:

X is -O- or -CH_2-;

m is 0, 1, 2, or 3;

R^1 is C_{1-4}alkyl;

R^2 is C_{1-4}alkyl;

n is 1, 2, 3, or 4;

R^3 is C_{4-10}alkyl;

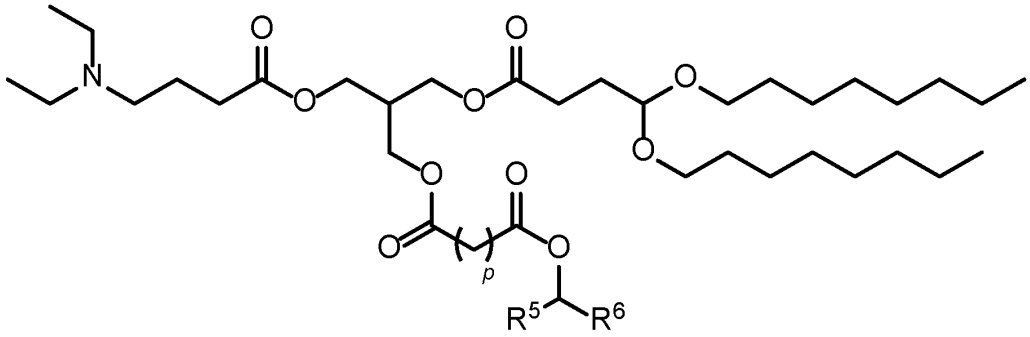
R^4 is C_{4-10}alkyl;

p is 2, 3, 4, 5, or 6;

R^5 is C_{4-10}alkyl; and

R^6 is C_{4-10}alkyl.

[0330] In some embodiments, compounds of formula (IV) are compounds of formula (IV-A):



(IV-A),

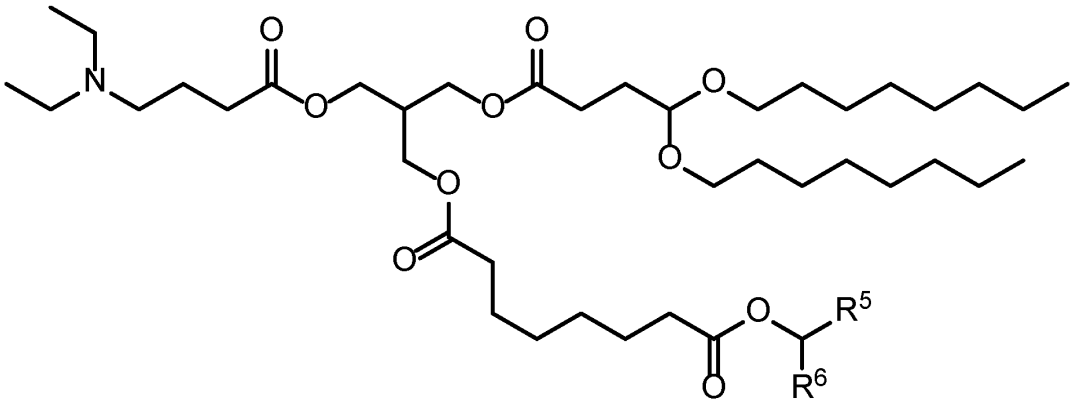
or a pharmaceutically acceptable salt thereof, wherein:

p is 2, 3, 4, 5, or 6;

R^5 is C_{4-10}alkyl; and

R^6 is C_{4-10}alkyl.

[0331] In some embodiments, compounds of formula (IV) are compounds of formula (IV-B):



(IV-B),

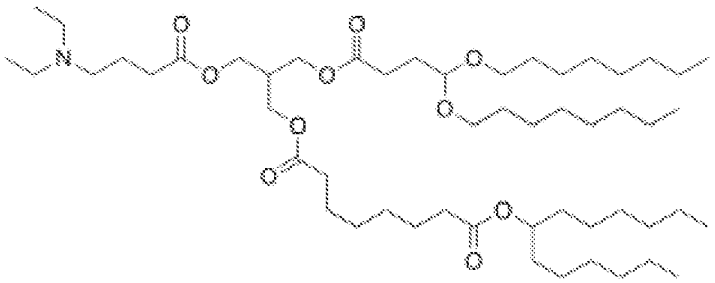
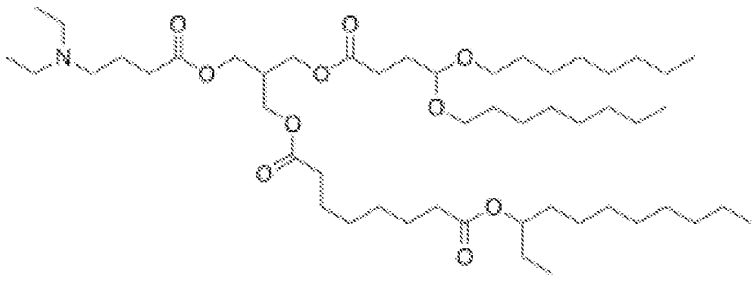
or a pharmaceutically acceptable salt thereof, wherein:

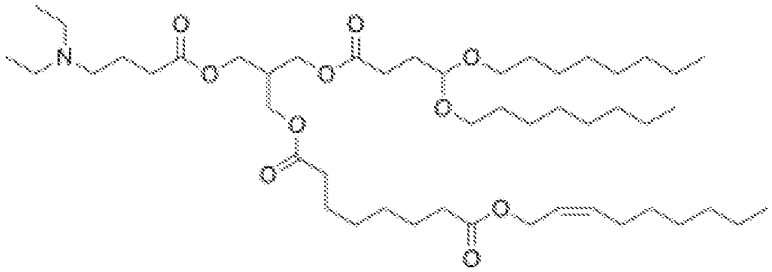
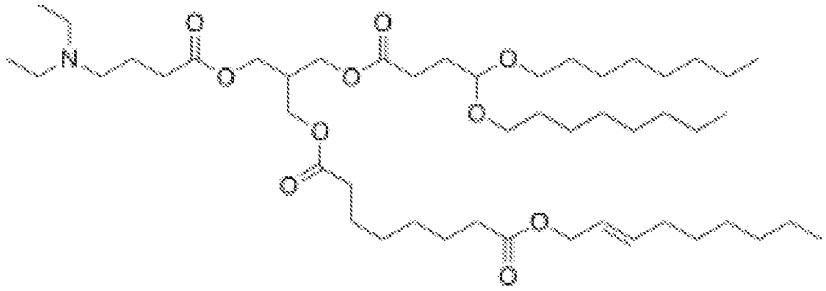
R^5 is C_{4-6}alkyl; and

R^6 is C_{4-6}alkyl.

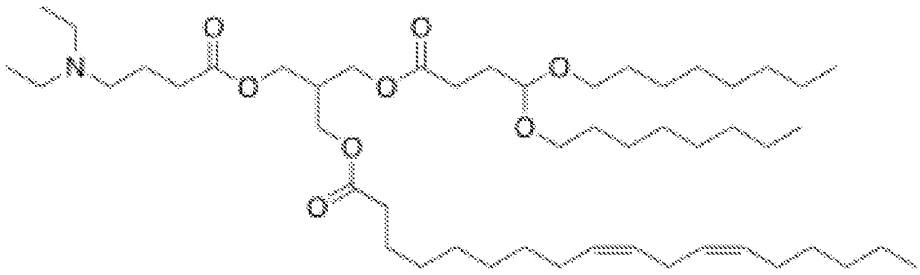
[0332] In some embodiments, the compound of formula (I) is a compound selected from the exemplary compounds of **Table 3**.

Table 3: Exemplary Ionizable Lipids

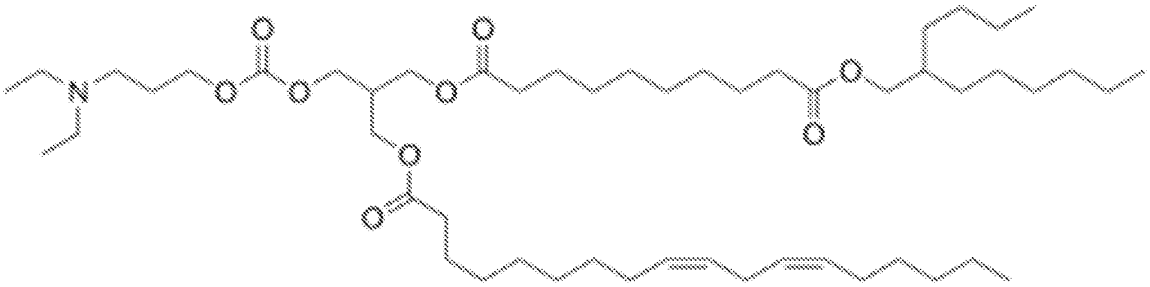
Compound	Structure
LIPID 154	
LIPID 155	

LIPID 163	
LIPID 173	

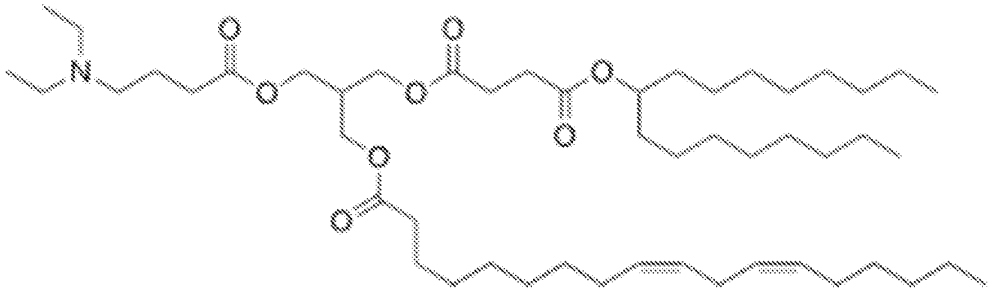
[0333] In some embodiments, the ionizable lipid has one of the structures depicted below:



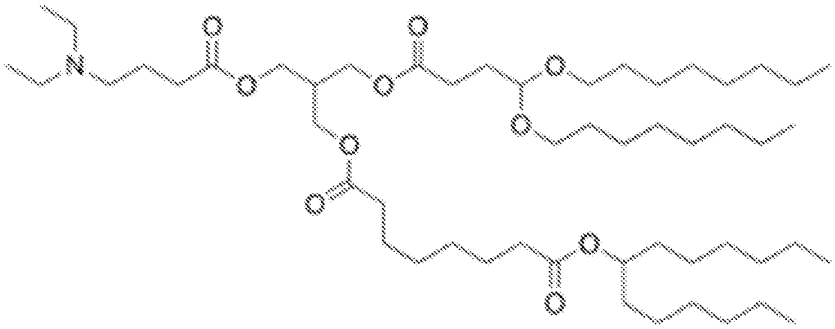
Lipid 092



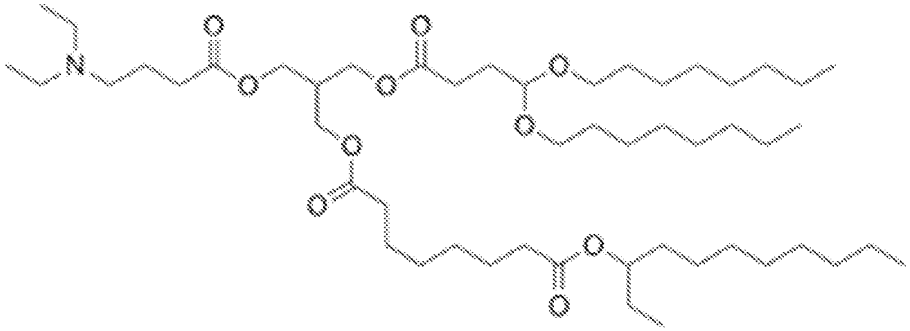
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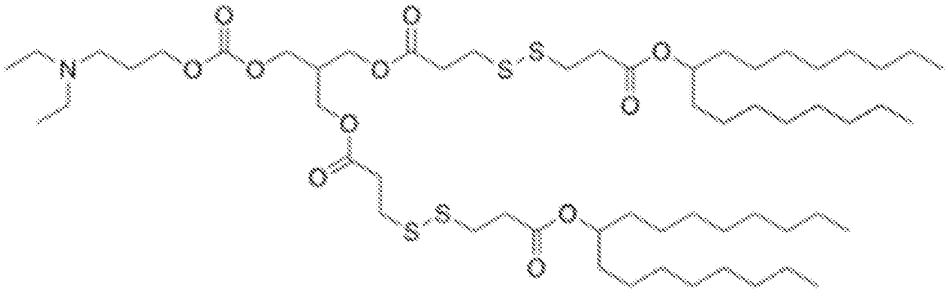
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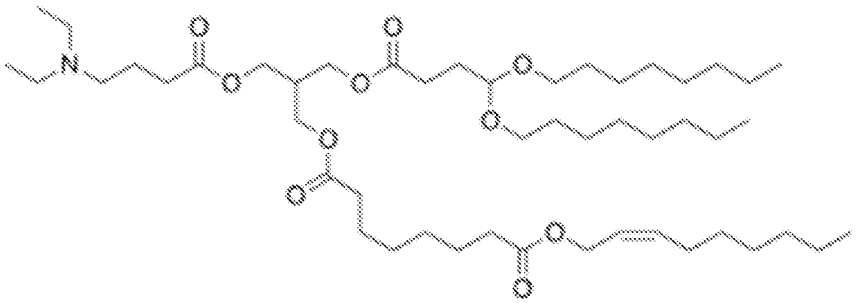
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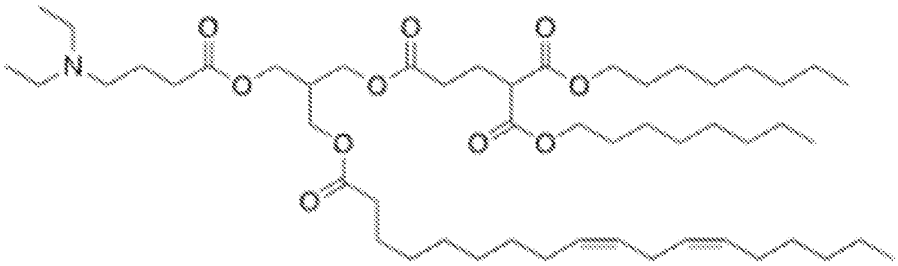
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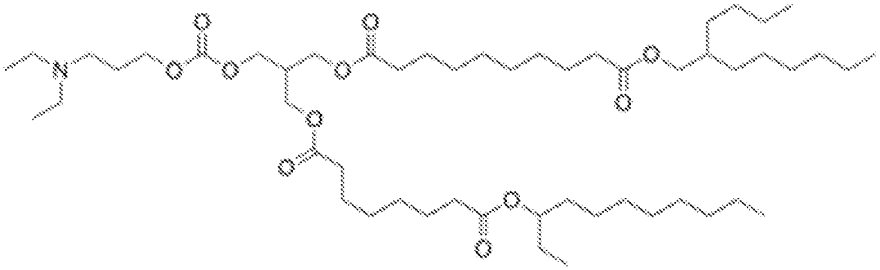
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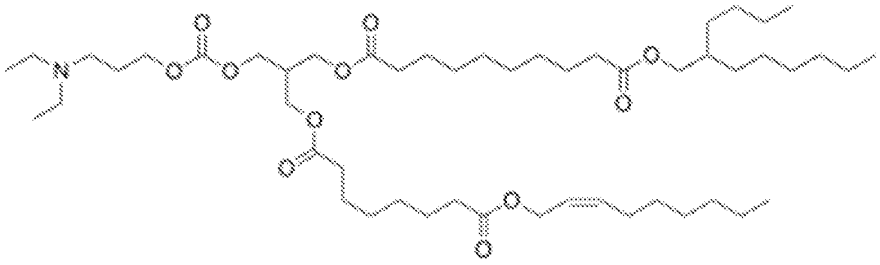
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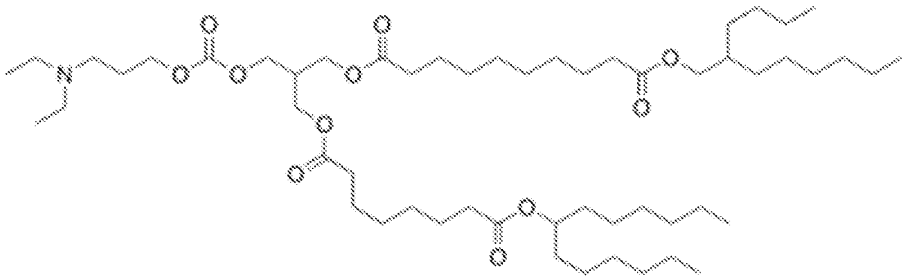
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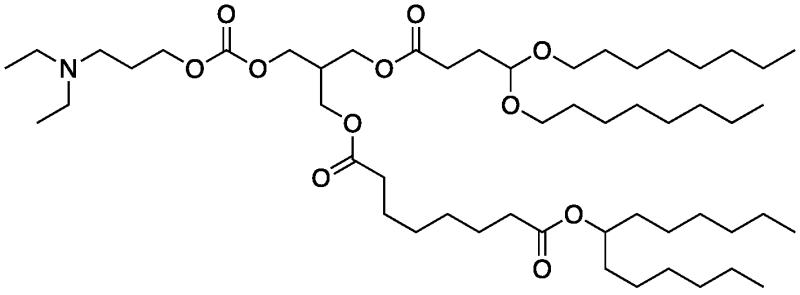
Lipid 176



Lipid 178



Lipid 183



Lipid 232

[0334] Other exemplary ionizable lipids that can be used in lipid nanoparticle formulations include, without limitation, those listed in Table 1 of WO2019051289, incorporated herein by reference. Additional exemplary lipids include, without limitation, one or more of the following formulae: X of US2016/0311759; I of US20150376115 or in US2016/0376224; I, II or III of US20160151284; I, IA, II, or IIA of US20170210967; I-c of US20150140070; A of US2013/0178541; I of US2013/0303587 or US2013/0123338; I of US2015/0141678; II, III, IV, or V of US2015/0239926; I of US2017/0119904; I or II of WO2017/117528; A of US2012/0149894; A of US2015/0057373; A of WO2013/116126; A of US2013/0090372; A of US2013/0274523; A of US2013/0274504; A of US2013/0053572; A of W02013/016058; A of W02012/162210; I of US2008/042973; I, II, III, or IV of US2012/01287670; I or II of US2014/0200257; I, II, or III of US2015/0203446; I or III of US2015/0005363; I, IA, IB, IC, ID, II, IIA, IIB, IIC, IID, or III-XXIV of US2014/0308304; of US2013/0338210; I, II, III, or IV of W02009/132131; A of US2012/01011478; I or XXXV of US2012/0027796; XIV or XVII of US2012/0058144; of US2013/0323269; I of US2011/0117125; I, II, or III of US2011/0256175; I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII of US2012/0202871; I, II, III, IV, V, VI, VII, VIII, X, XII, XIII, XIV, XV, or XVI of US2011/0076335; I or II of US2006/008378; I of US2013/0123338; I or X-A-Y-Z of US2015/0064242; XVI, XVII, or XVIII of US2013/0022649; I, II, or III of US2013/0116307; I, II, or III of US2013/0116307; I or II of US2010/0062967; I-X of US2013/0189351; I of US2014/0039032; V of US2018/0028664; I of

US2016/0317458; I of US2013/0195920; 5, 6, or 10 of US10,221,127; III-3 of WO2018/081480; I-5 or I-8 of WO2020/081938; 18 or 25 of US9,867,888; A of US2019/0136231; II of WO2020/219876; 1 of US2012/0027803; OF-02 of US2019/0240349; 23 of US10,086,013; cKK-E12/A6 of Miao et al (2020); C12-200 of WO2010/053572; 7C1 of Dahlman et al (2017); 304-O13 or 503-O13 of Whitehead et al; TS-P4C2 of US9,708,628; I of WO2020/106946; I of WO2020/106946.

[0335] In some embodiments, the ionizable lipid is MC3 (6Z,9Z,28Z,3 lZ)-heptatriaconta-6,9,28,3 l-tetraen-l9-yl-4-(dimethylamino) butanoate (DLin-MC3-DMA or MC3), e.g., as described in Example 9 of WO2019051289A9 (incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is the lipid ATX-002, e.g., as described in Example 10 of WO2019051289A9 (incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is (l3Z,l6Z)-A,A-dimethyl-3- nonyldocosa-l3, l6-dien-l-amine (Compound 32), e.g., as described in Example 11 of WO2019051289A9 (incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is Compound 6 or Compound 22, e.g., as described in Example 12 of WO2019051289A9 (incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is heptadecan-9-yl 8-((2-hydroxyethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (SM-102); e.g., as described in Example 1 of US9,867,888(incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is 9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate (LP01) e.g., as synthesized in Example 13 of WO2015/095340(incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is Di((Z)-non-2-en-1-yl) 9-((4-dimethylamino)butanoyl)oxy)heptadecanedioate (L319), , e.g. as synthesized in Example 7, 8, or 9 of US2012/0027803(incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is 1,1'-((2-(4-(2-((2-(Bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl) amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200), e.g., as synthesized in Examples 14 and 16 of WO2010/053572(incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is; Imidazole cholesterol ester (ICE) lipid (3S, 10R, 13R, 17R)-10, 13-dimethyl-17- ((R)-6-methylheptan-2-yl)-2, 3, 4, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17-tetradecahydro-lH- cyclopenta[a]phenanthren-3-yl 3-(1H-imidazol-4-yl)propanoate, e.g., Structure (I) from WO2020/106946 (incorporated by reference herein in its entirety).

[0336] In some embodiments, the mol% of the ionizable lipid in the LNP, e.g., targeted LNP, is from about 25% to about 65%. In some embodiments, the mol% of the ionizable lipid in the LNP, e.g., targeted LNP, is from about 35% to about 60%. In some embodiments, the mol% of

the ionizable lipid in the LNP, e.g., targeted LNP, is from about 40% to about 50%. In some embodiments, the mol% of the ionizable lipid in the LNP, e.g., targeted LNP, is from about 45% to about 50%. In some embodiments, the mol% of the ionizable lipid in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the ionizable lipid in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the ionizable lipid in the LNP, e.g., targeted LNP, is from about 25% to about 40%.

[0337] %. In some embodiments, the mol% of the ionizable lipid in the LNP, e.g., targeted LNP, is from about 15% to about 30%.

[0338] The compounds disclosed herein (e.g., lipids in **Table 1, Table 2**, and **Table 3**), or their pharmaceutically acceptable salts, may include an asymmetric center and may thus give rise to enantiomers, diastereomers, and other stereoisomeric forms. The present disclosure contemplates various stereoisomers and mixtures thereof and includes "enantiomers," which refers to two stereoisomers whose molecules are nonsuperimposeable mirror images of one another and "diastereomers," which refers to stereoisomers that have at least two asymmetric atoms, but which are not mirror-images of each other. The present disclosure is meant to include all such possible isomers, as well as their racemic and optically pure forms. Optically active (+) and (-), or (*R*)- and (*S*)- isomers may be prepared using chiral synthons or chiral reagents, or resolved using conventional techniques, for example, chromatography and fractional crystallization. Conventional techniques for the preparation/isolation of individual enantiomers include chiral synthesis from a suitable optically pure precursor or resolution of the racemate using, for example, chiral high pressure liquid chromatography (HPLC). When the compounds described herein contain olefinic double bonds or other centers of geometric asymmetry, and unless specified otherwise, it is intended that the compounds include both E and Z geometric isomers.

Helper Lipids

[0339] The LNPs, e.g., targeted LNPs, of the disclosure comprise one or more ionizable lipids. Exemplary helper lipids include, but are not limited to, distearoyl-sn-glycero-phosphoethanolamine, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane- 1 - carboxylate (DOPE-mal), dipalmitoyl phosphatidyl

ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), monomethyl-phosphatidylethanolamine (such as 16-O-monomethyl PE), dimethyl- phosphatidylethanolamine (such as 16-O-dimethyl PE), l8-l-trans PE, l-stearoyl-2-oleoyl- phosphatidyethanolamine (SOPE), hydrogenated soy phosphatidylcholine, egg phosphatidylcholine (EPC), dioleoylphosphatidylserine (DOPS), sphingomyelin (SM), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), distearoylphosphatidylglycerol (DSPG), dierucoylphosphatidylcholine (DEPC), palmitoyloleyolphosphatidylglycerol (POPG), dielaidoyl-phosphatidylethanolamine (DEPE), lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidicacid,cerebrosides, dicetylphosphate, lysophosphatidylcholine, dilinoleoylphosphatidylcholine, or mixtures thereof. It is understood that other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C10-C24 carbon chains, e.g., lauroyl, myristoyl, paimitoyl, stearoyl, or oleoyl. Additional exemplary lipids, in certain embodiments, include, without limitation, those described in Kim et al. (2020) dx.doi.org/10.1021/acs.nanolett.0c01386, incorporated herein by reference.

[0340] Other examples of non-cationic lipids suitable for use in the lipid nanopartieles include, without limitation, nonphosphorous lipids such as, e.g., stearylamine, dodeeylamine, hexadecylamine, acetyl palmitate, glycerol ricinoleate, hexadecyl stereate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyl dimethyl ammonium bromide, ceramide, sphingomyelin, and the like. Other non-cationic lipids are described in WO2017/099823 or US patent publication US2018/0028664, the contents of which is incorporated herein by reference in their entirety. In some embodiments, the non-cationic lipid is oleic acid or a compound of Formula I, II, or IV of US2018/0028664, incorporated herein by reference in its entirety.

[0341] In some embodiments, the helper lipid is a sphingolipid. In some such embodiments, the non-pegylated lipid is a sphingomyelin. In some embodiments, the sphingomyelin has a head group selected from, phosphocholine, phosphoethanolamine or ceramide. In some embodiments, the sphingomyelin is egg sphingomyelin.

[0342] In some embodiments, the helper lipid comprises 5-40% (mol), 8%-30%, 10%-28%, 20%-40%, 20%-36%, 24%-38%, 22%-32%, or 10-15% (mol) of the total lipid present in the

lipid nanoparticle. In embodiments, the molar ratio of ionizable lipid to the neutral lipid ranges from about 2:1 to about 8:1 (e.g., about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, or 8:1).

[0343] In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 18% to about 32%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 25% to about 30%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 22% to about 32%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP is from about 21% to about 23%. In some embodiments, the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, or about 32%. As set forth in the examples below, *in vivo* delivery of certain payloads following administration of the disclosed LNPs (e.g., targeted LNPs) with these percentages of helper lipids provides enhanced transduction and expression of the payloads relative to targeted LNPs with smaller or larger quantities of helper lipid.

[0344] In some embodiments, the disclosure provides a lipid nanoparticle (LNP) comprising: (i) a lipid component comprising an ionizable lipid and a helper lipid; (ii) one or two targeting moieties conjugated to the LNP, wherein the one or two targeting moieties are antibodies or fragments thereof configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; (iii) an mRNA encoding a gene modifying polypeptide; and (iv) a template RNA comprising (a) a sequence that binds to the polypeptide and (b) a heterologous object sequence. In some embodiments, the LNP comprises a CD3 targeting moiety, optionally wherein the CD3 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from

Table 19 or Table 32 (or CDR sequences (bolded) selected from Table 19 or Table 32). In some embodiments, the LNP comprises a CD2 targeting moiety, optionally wherein the CD2 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD5 targeting moiety, optionally wherein the CD5 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD7 targeting moiety, optionally wherein the CD7 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD28 targeting moiety. In some embodiments, the LNP comprises a CD4 targeting moiety. In some embodiments, the LNP comprises a CD8 targeting moiety. In some embodiments, the LNP comprises two targeting moieties configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28. In some embodiments, the two targeting moieties are configured to bind to CD3 and CD2, CD3 and CD4, CD3 and CD5, CD3 and CD7, CD3 and CD8 or CD3 and CD28. In some embodiments, the CD3 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 19 or Table 32, and the CD2, CD5, or CD7 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 20. In some embodiments, the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, about 20 mol% to about 40 mol% of the lipid component, about 20 mol% to about 30 mol% of the lipid component or about 25% to about 40% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component. In some embodiments, the template RNA comprises a sequence present in Table 5B or a sequence selected from SEQ ID NOs: 395-404.

[0345] In some embodiments, the disclosure provides a lipid nanoparticle (LNP), comprising: (i) a lipid component comprising an ionizable lipid and a helper lipid, wherein the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, about 20 mol% to about 40 mol% of the lipid component, about 20 mol% to about 30 mol% of the lipid component or about 25% to about 40% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component; (ii) one or two targeting moieties conjugated to the LNP, wherein the one or two targeting moieties are antibodies or fragments thereof configured to bind one or more receptors on the surface of T cells selected from the

group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; and (iii) a therapeutic agent encapsulated within the LNP, wherein the therapeutic agent comprises one or more nucleic acid molecules (e.g., one or more RNA molecules). In some embodiments, the LNP comprises a CD3 targeting moiety, optionally wherein the CD3 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 19 or Table 32 (or CDR sequences (bolded) selected from Table 19 or Table 32). In some embodiments, the LNP comprises a CD2 targeting moiety, optionally wherein the CD2 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD5 targeting moiety, optionally wherein the CD5 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD7 targeting moiety, optionally wherein the CD7 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD28 targeting moiety. In some embodiments, the LNP comprises a CD4 targeting moiety. In some embodiments, the LNP comprises a CD8 targeting moiety. In some embodiments, the LNP comprises two targeting moieties configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28. In some embodiments, the two targeting moieties are configured to bind to CD3 and CD2, CD3 and CD4, CD3 and CD5, CD3 and CD7, CD3 and CD8 or CD3 and CD28. In some embodiments, the CD3 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 19 or Table 32, and the CD2, CD5, or CD7 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 20. In some embodiments, the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, about 20 mol% to about 40 mol% of the lipid component, about 20 mol% to about 30 mol% of the lipid component or about 25% to about 40% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component. In some embodiments, the therapeutic agent comprises a DNA molecule. In some embodiments, the therapeutic agent comprises an mRNA molecule. In some embodiments, the therapeutic agent comprises two nucleic acid molecules, wherein one nucleic acid is an mRNA and the other nucleic acid is a template RNA. In some embodiments, the therapeutic agent comprises a gene modifying system. In some embodiments, the therapeutic agent comprises a heterologous gene modifying system. In some

embodiments, the therapeutic agent comprises a gene modifying system, and the template RNA of the system comprises a sequence present in Table 5B or a sequence selected from SEQ ID NOs: 395-404.

[0346] In some embodiments, the disclosure provides a lipid nanoparticle (LNP), comprising: (i) a lipid component comprising an ionizable lipid and a helper lipid, wherein the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, about 20 mol% to about 40 mol% of the lipid component, about 20 mol% to about 30 mol% of the lipid component or about 25% to about 40% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component; (ii) means for binding a cell surface receptor selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; and (iii) a therapeutic agent encapsulated within the LNP, wherein the therapeutic agent comprises one or more nucleic acid molecules (e.g., one or more RNA molecules). In some embodiments, the means for binding the cell surface receptor is present on the surface of the LNP. In some embodiments, the means for binding a cell surface receptor comprises a targeting moiety, such as an antibody or fragment thereof. In some embodiments, the LNP comprises a CD3 targeting moiety, optionally wherein the CD3 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 19 or Table 32 (or CDR sequences (bolded) selected from Table 19 or Table 32). In some embodiments, the LNP comprises a CD2 targeting moiety, optionally wherein the CD2 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD5 targeting moiety, optionally wherein the CD5 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD7 targeting moiety, optionally wherein the CD7 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD28 targeting moiety. In some embodiments, the LNP comprises a CD4 targeting moiety. In some embodiments, the LNP comprises a CD8 targeting moiety. In some embodiments, the LNP comprises two targeting moieties configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28. In some embodiments, the two targeting moieties are configured to bind to CD3 and CD2, CD3 and CD4, CD3 and CD5, CD3 and CD7, CD3 and CD8 or CD3 and CD28. In some embodiments, the CD3 targeting moiety comprises variable

heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 19 or Table 32, and the CD2, CD5, or CD7 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 20. In some embodiments, the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, about 20 mol% to about 40 mol% of the lipid component, about 20 mol% to about 30 mol% of the lipid component or about 25% to about 40% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component. In some embodiments, the therapeutic agent comprises a DNA molecule. In some embodiments, the therapeutic agent comprises an mRNA molecule. In some embodiments, the therapeutic agent comprises two nucleic acid molecules, wherein one nucleic acid is an mRNA and the other nucleic acid is a template RNA. In some embodiments, the therapeutic agent comprises a gene modifying system. In some embodiments, the therapeutic agent comprises a heterologous gene modifying system. In some embodiments, the therapeutic agent comprises a gene modifying system, and the template RNA of the system comprises a sequence present in Table 5B or a sequence selected from SEQ ID NOs: 395-404.

[0347] In some embodiments, the disclosure provides a lipid nanoparticle (LNP), comprising: (i) a lipid component comprising an ionizable lipid and a helper lipid, wherein the ionizable lipid is present at about 35 mol% to about 65 mol% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component; (ii) one or two targeting moieties conjugated to the LNP, wherein the one or two targeting moieties are antibodies or fragments thereof configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; and (iii) a therapeutic agent encapsulated within the LNP, wherein the therapeutic agent comprises one or more nucleic acid molecules (e.g., one or more RNA molecules). In some embodiments, the LNP comprises a CD3 targeting moiety, optionally wherein the CD3 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 19 or Table 32 (or CDR sequences (bolded) selected from Table 19 or Table 32). In some embodiments, the LNP comprises a CD2 targeting moiety, optionally wherein the CD2 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD5 targeting moiety, optionally wherein the CD5 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD7 targeting moiety, optionally

wherein the CD7 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD28 targeting moiety. In some embodiments, the LNP comprises a CD4 targeting moiety. In some embodiments, the LNP comprises a CD8 targeting moiety. In some embodiments, the LNP comprises two targeting moieties configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28. In some embodiments, the two targeting moieties are configured to bind to CD3 and CD2, CD3 and CD4, CD3 and CD5, CD3 and CD7, CD3 and CD8 or CD3 and CD28. In some embodiments, the CD3 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 19 or Table 32, and the CD2, CD5, or CD7 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 20. In some embodiments, the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component or is present at about 40 mol% to about 50 mol% of the lipid component or is present at about 20 mol% to about 40 mol% of the lipid component, or is present at about 20 mol% to about 30 mol% of the lipid component or is present at about 40 mol% to about 50 mol% of the lipid component and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component. In some embodiments, the therapeutic agent comprises a DNA molecule. In some embodiments, the therapeutic agent comprises an mRNA molecule. In some embodiments, the therapeutic agent comprises two nucleic acid molecules, wherein one nucleic acid is an mRNA and the other nucleic acid is a template RNA. In some embodiments, the therapeutic agent comprises a gene modifying system. In some embodiments, the therapeutic agent comprises a heterologous gene modifying system. In some embodiments, the therapeutic agent comprises a gene modifying system, and the template RNA of the system comprises a sequence present in Table 5B or a sequence selected from SEQ ID NOs: 395-404. In some embodiments, the ionizable lipid is present at about 47% of the lipid component and the helper lipid is present at about 8% of the lipid component. In some embodiments, the ionizable lipid is present at about 47% of the lipid component, the helper lipid is present at about 8% of the lipid component, and the cholesterol is present at between about 42% and 44% of the lipid component..

[0348] In some embodiments, the disclosure provides a lipid nanoparticle (LNP), comprising: (i) a lipid component comprising an ionizable lipid and a helper lipid, wherein the ionizable lipid is present at about 35 mol% to about 65 mol% of the lipid component, and the helper lipid is present at about 5 mol% to about 15 mol% of the lipid component; (ii) means for

binding a cell surface receptor selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; and (iii) a therapeutic agent encapsulated within the LNP, wherein the therapeutic agent comprises one or more nucleic acid molecules (e.g., one or more RNA molecules). In some embodiments, the means for binding the cell surface receptor is present on the surface of the LNP. In some embodiments, the means for binding a cell surface receptor comprises a targeting moiety, such as an antibody or fragment thereof. In some embodiments, the LNP comprises a CD3 targeting moiety, optionally wherein the CD3 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 19 or Table 32 (or CDR sequences (bolded) selected from Table 19 or Table 32). In some embodiments, the LNP comprises a CD2 targeting moiety, optionally wherein the CD2 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD5 targeting moiety, optionally wherein the CD5 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD7 targeting moiety, optionally wherein the CD7 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences selected from Table 20 (or CDR sequences (bolded) selected from Table 20). In some embodiments, the LNP comprises a CD28 targeting moiety. In some embodiments, the LNP comprises a CD4 targeting moiety. In some embodiments, the LNP comprises a CD8 targeting moiety. In some embodiments, the LNP comprises two targeting moieties configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28. In some embodiments, the two targeting moieties are configured to bind to CD3 and CD2, CD3 and CD4, CD3 and CD5, CD3 and CD7, CD3 and CD8 or CD3 and CD28. In some embodiments, the CD3 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 19 or Table 32, and the CD2, CD5, or CD7 targeting moiety comprises variable heavy and light chain sequences (or CDR sequences (bolded)) selected from Table 20. In some embodiments, the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component or is present at about 40 mol% to about 50 mol% of the lipid component, and the helper lipid is present at about 5 mol% to about 10 mol% of the lipid component (e.g., 5 mol%, 6 mol%, 7 mol%, 8 mol%, 9 mol% or 10 mol% of the lipid component). In some embodiments, the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, and the helper lipid is present at about 10 mol% to about 15 mol%

of the lipid component (e.g., 10 mol%, 11 mol%, 12 mol%, 13 mol%, 14 mol% or 15 mol% of the lipid component). In some embodiments, the therapeutic agent comprises a DNA molecule. In some embodiments, the therapeutic agent comprises an mRNA molecule. In some embodiments, the therapeutic agent comprises two nucleic acid molecules, wherein one nucleic acid is an mRNA and the other nucleic acid is a template RNA. In some embodiments, the therapeutic agent comprises a gene modifying system. In some embodiments, the therapeutic agent comprises a heterologous gene modifying system. In some embodiments, the therapeutic agent comprises a gene modifying system, and the template RNA of the system comprises a sequence present in Table 5B or a sequence selected from SEQ ID NOs: 395-404. In some embodiments, the ionizable lipid is present at about 47% of the lipid component and the helper lipid is present at about 8% of the lipid component. In some embodiments, the ionizable lipid is present at about 47% of the lipid component, the helper lipid is present at about 8% of the lipid component, and the cholesterol is present at between about 42% and 44% of the lipid component..

[0349] In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) in the LNP, e.g., targeted LNP, is from about 1:1 to about 7:1. In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 1:1 to about 4:1. In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 1:1 to about 3:1. In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC) is from about 1:1 to about 2.5:1. In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 1:1 to about 2:1. In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC) is from about 1.5:1 to about 2.5:1. In some embodiments, the molar ratio between the ionizable lipid and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 2:1 to about 2.5:1.

[0350] In some embodiments, the LNP, e.g., targeted LNP, comprises an ionizable lipid in **Table 1**, **Table 2** or **Table 3** and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises an ionizable lipid in **Table 1**, **Table 2** or **Table 3** and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In

some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0351] In some embodiments, the LNP, e.g., targeted LNP, comprises an ionizable lipid of Formula I and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises an ionizable lipid of Formula I and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0352] In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 20% to about 40% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP is from about 20% to about 40%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 20% to about 30% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 25% to about 40% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 28% to about 32% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 20% to about 40%. In some of the foregoing

embodiments, the mol% of cholesterol in the targeted LNP is from about 30%-40% or from about 32% to about 37% (e.g., about 33%, about 34%, about 35% or about 36%).

[0353] In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 20% to about 40% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 25% to about 35%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 20% to about 30% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 25% to about 35%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 25% to about 40% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 25% to about 35%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 28% to about 32% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 25% to about 35%. In some of the foregoing embodiments, the mol% of cholesterol in the targeted LNP is from about 30%-40% or from about 32% to about 37% (e.g., about 33%, about 34%, about 35% or about 36%).

[0354] In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 20% to about 40% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 30% to about 35% (e.g., about 31%, about 32%, about 33%, about 34% or about 35%). In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 20% to about 30% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 30% to about 35% (e.g., about 31%, about 32%, about 33%m about 34% or about 35%). In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 25% to about 40% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 30% to about 35% (e.g., about 31%, about 32%, about 33%, about 34% or about 35%). In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 28% to about 32% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 30% to about 35% (e.g., about 31%, about 32%, about 33%, about 34% or about 35%). In some of the foregoing embodiments, the mol% of cholesterol in the targeted LNP is from about 30%-40% or from about 32% to about 37% (e.g., about 33%, about 34%, about 35% or about 36%).

[0355] In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 35% to about 60% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 35% to about 60% and the mol% of the helper

lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 30% to about 40%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 35% to about 50% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the ionizable lipid in the targeted LNP is from about 35% to about 50% and the mol% of the helper lipid (e.g., DSPC or sphingomyelin) in the targeted LNP, is from about 30% to about 40%. In some of the foregoing embodiments, the mol% of cholesterol in the targeted LNP is from about 25%-40%,

[0356] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 093 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 093 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 20%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0357] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 092 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 092 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about

25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0358] In some embodiments, the LNP, e.g., targeted LNP comprises Lipid 153 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 153 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0359] In some embodiments, the LNP, e.g., targeted LNP comprises Lipid 154 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 154 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%,

about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0360] In some embodiments, the LNP, e.g., targeted LNP comprises Lipid 155 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 155 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0361] In some embodiments, the LNP, e.g., targeted LNP comprises Lipid 162 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 163 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0362] In some embodiments, the LNP, e.g., targeted LNP comprises Lipid 169 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 169 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0363] In some embodiments, the LNP, e.g., targeted LNP comprises Lipid 176 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 176 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0364] In some embodiments, the LNP, e.g., targeted LNP comprises Lipid 178 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 178 and a sphingomyelin.

In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0365] In some embodiments, the LNP, e.g., targeted LNP comprises Lipid 183 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 183 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0366] In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid V003 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid V003 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the

DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

[0367] In some embodiments, the LNP, e.g., targeted LNP comprises Lipid 232 and DSPC. In some embodiments, the LNP, e.g., targeted LNP, comprises Lipid 232 and a sphingomyelin. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 40%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 25%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 20% to about 30%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 28%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 22% to about 36%. In some embodiments, the mol% of the DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is from about 24% to about 38%. In some embodiments, the mol% of DSPC or sphingomyelin in the LNP, e.g., targeted LNP, is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about33%, about 34%, about 35%, about 36%, about 27%, about 38%, about 39%, or about 40%.

Sterols

[0368] In some embodiments, the LNPs, e.g., targeted LNPs, of the disclosure can comprise a component, such as a sterol, to provide membrane integrity. One exemplary sterol that can be used in the lipid nanoparticle is cholesterol and derivatives thereof. Non-limiting examples of cholesterol derivatives include polar analogues such as 5a-choiestanol, 53-coprostanol, choiesteryl-(2'-hydroxy)-ethyl ether, choiesteryl-(4'- hydroxy)-butyl ether, and 6-

ketocholestanol; non-polar analogues such as 5a-cholestane, cholestenone, 5a-cholestanone, 5p-cholestanone, and cholesteryl decanoate; and mixtures thereof. In some embodiments, the cholesterol derivative is a polar analogue, e.g., choiesteryl-(4 '-hydroxy)-buty1 ether. Exemplary cholesterol derivatives are described in PCT publication W02009/127060 and US patent publication US2010/0130588, each of which is incorporated herein by reference in its entirety.

[0369] In some embodiments, the component providing membrane integrity, such as a sterol, can comprise 0-50% (mol) (e.g., 0-10%, 10-20%, 20-30%, 30-40%, or 40-50%) of the total lipid present in the lipid nanoparticle. In some embodiments, such a component is 20-50% (mol) 30-40% (mol) of the total lipid content of the lipid nanoparticle.

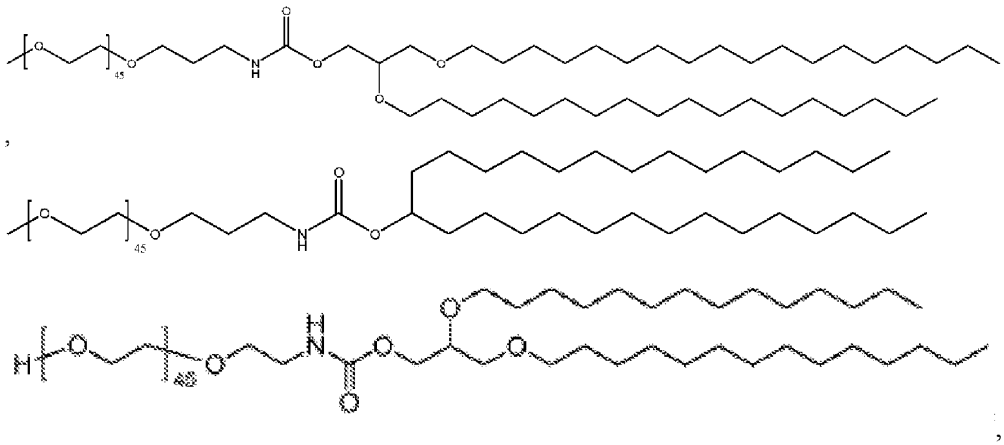
[0370] In some embodiments, the molar ratio between the cholesterol molecule and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 6:1 to about 0.5:1. In some embodiments, the ratio between the cholesterol molecule and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 3:1 to about 0.5:1. In some embodiments, the ratio between the cholesterol molecule and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 2:1 to about 0.5:1. In some embodiments, the ratio between the cholesterol molecule and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 1.5:1 to about 0.5:1. In some embodiments, the ratio between the cholesterol molecule and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 1:1 to about 0.5:1. In some embodiments, the ratio between the cholesterol molecule and the non-pegylated helper lipid (e.g., DSPC or sphingomyelin) is from about 1:2 to about 0.8:1.

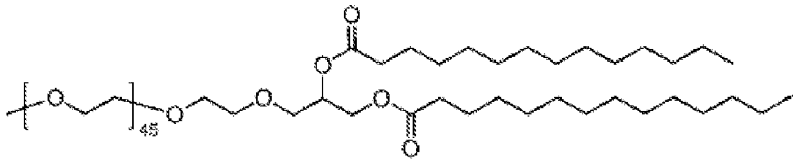
Pegylated Lipids

[0371] In some embodiments, the LNPs, e.g., targeted LNPs, of the disclosure can comprise a polyethylene glycol (PEG) or a conjugated lipid molecule. Generally, these are used to inhibit aggregation of lipid nanoparticles and/or provide steric stabilization. Exemplary conjugated lipids include, but are not limited to, PEG-lipid conjugates, polyoxazoline (POZ)-lipid conjugates, polyamide-lipid conjugates (such as ATTA-lipid conjugates), cationic-polymer lipid (CPL) conjugates, and mixtures thereof. In some embodiments, the conjugated lipid molecule is a PEG-lipid conjugate, for example, a (methoxy polyethylene glycol)-conjugated lipid.

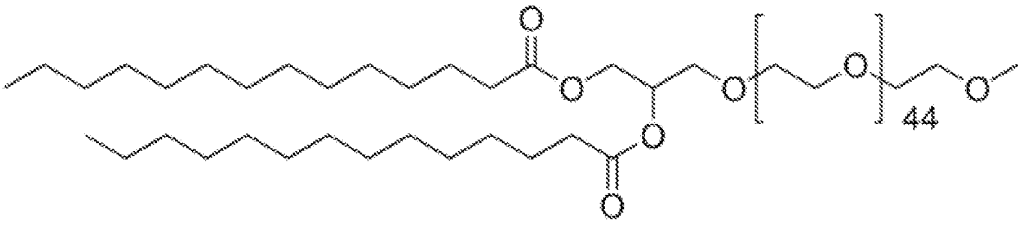
[0372] Exemplary PEG-lipid conjugates include, but are not limited to, PEG-diacylglycerol (DAG) (such as l-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG)), PEG-dialkyloxypropyl (DAA), PEG-phospholipid, PEG-ceramide (Cer), a pegylated phosphatidylethanoloamine (PEG-PE), 1,2-dimyristoyl-sn-glycerol, methoxypoly ethylene glycol (DMG-PEG-2K), PEG succinate diacylglycerol (PEGS-DAG) (such as 4-0-(2',3'-

di(tetradecanoyloxy)propyl-l-0-(w-methoxy(polyethoxy)ethyl) butanedioate (PEG-S-DMG)), PEG dialkoxypropylcarbam, N-(carbonyl-methoxypolyethylene glycol 2000)-l,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt, or a mixture thereof. Additional exemplary PEG-lipid conjugates are described, for example, in US5,885,6l3, US6,287,59l, US2003/0077829, US2003/0077829, US2005/0175682, US2008/0020058, US2011/0117125, US2010/0130588, US2016/0376224, US2017/0119904, and US/099823, the contents of all of which are incorporated herein by reference in their entirety. In some embodiments, a PEG-lipid is a compound of Formula III, III-a-I, III-a-2, III-b-1, III-b-2, or V of US2018/0028664, the content of which is incorporated herein by reference in its entirety. In some embodiments, a PEG-lipid is of Formula II of US20150376115 or US2016/0376224, the content of both of which is incorporated herein by reference in its entirety. In some embodiments, the PEG-DAA conjugate can be, for example, PEG-dilauryloxypropyl, PEG- dimyristyloxypropyl, PEG-dipalmityloxypropyl, or PEG-distearyloxypropyl. The PEG-lipid can be one or more of PEG-DMG, PEG-dilaurylglycerol, PEG-dipalmitoylglycerol, PEG- disterylglycerol, PEG-dilaurylglycamide, PEG-dimyristylglycamide, PEG- dipalmitoylglycamide, PEG-disterylglycamide, PEG-cholesterol (l-[8'-(Cholest-5-en-3[beta]- oxy)carboxamido-3',6'-dioxaoctanyl] carbamoyl-[omega]-methyl-poly(ethylene glycol), PEG- DMB (3,4-Ditetradecoxylbenzyl- [omega]-methyl-poly(ethylene glycol) ether), and 1,2- dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]. In some embodiments, the PEG-lipid comprises PEG-DMG, 1,2- dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]. In some embodiments, the PEG-lipid comprises a structure selected from:





, and



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[0373] In some embodiments, lipids conjugated with a molecule other than a PEG can also be used in place of PEG-lipid. For example, polyoxazoline (POZ)-lipid conjugates, polyamide-lipid conjugates (such as ATTA-lipid conjugates), and cationic-polymer lipid (GPL) conjugates can be used in place of or in addition to the PEG-lipid.

[0374] Exemplary conjugated lipids, i.e., PEG-lipids, (POZ)-lipid conjugates, ATTA-lipid conjugates and cationic polymer-lipids are described in the PCT and LIS patent applications listed in Table 2 of WO2019051289A9 and in WO2020106946A1, the contents of all of which are incorporated herein by reference in their entirety.

[0375] In some embodiments, the pegylated lipid has at least one C16 (palmitoyl) PEG lipid anchor. In some embodiments, the pegylated lipid has two C16 PEG lipid anchors (i.e., dialkyl chains of 16 carbons long). In some embodiments, the pegylated lipid is 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE-PEG2000.). In some embodiments, the pegylated lipid is 1,2-Dipalmitoyl-rac-glycero-3-methylpolyoxyethylene (DPG-PEG2000). In some embodiments, the pegylated lipid is C16 PEG ceramide. In some embodiments, the targeted LNPs comprising the C16 pegylated lipids show reduced liver uptake than otherwise identical LNPs comprising C14 pegylated lipids.

[0376] In some embodiments, the LNP further comprises a pegylated lipid comprising at least one C14 alkyl chain (e.g., two C14 alkyl chains). In some such embodiments, the pegylated lipid is DMG-PEG2000.

[0377] In some embodiments, the pegylated lipid has at least one C18 PEG lipid anchor. In some embodiments, the pegylated lipid has two C18 PEG lipid anchors (i.e., dialkyl chains of 18 carbons long). In some embodiments, the C18 pegylated lipid is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000). In some embodiments, the C18 pegylated lipid is distearoyl-rac-glycerol-PEG2000 (DSG-PEG2000).

[0378] In some embodiments, the PEG or the conjugated lipid can comprise 0-20% (mol) of the total lipid present in the lipid nanoparticle. In some embodiments, PEG or the conjugated lipid content is 0.5- 10% or 2-5% (mol) of the total lipid present in the lipid nanoparticle.

Additional components

[0379] In some embodiments, one or more additional compounds can also be included in the LNPs, e.g., targeted LNPs, of the disclosure. Those compounds can be administered separately, or the additional compounds can be included in the lipid nanoparticles of the invention. In other words, the lipid nanoparticles can contain other compounds, such as a payload, e.g., a therapeutic agent, as described herein. In some embodiments, the lipid nanoparticles can contain one or more nucleic acids. In some embodiments, the lipid nanoparticles can contain at least a first nucleic acid and a second nucleic acid, wherein the second nucleic acid is different than the first nucleic acid. Without limitations, other additional compounds can be selected from the group consisting of small or large organic or inorganic molecules, monosaccharides, disaccharides, trisaccharides, oligosaccharides, polysaccharides, peptides, proteins, peptide analogs and derivatives thereof, peptidomimetics, nucleic acids, nucleic acid analogs and derivatives, an extract made from biological materials, or any combinations thereof.

[0380] In some embodiments, a lipid nanoparticle (or a formulation comprising lipid nanoparticles) lacks reactive impurities (e.g., aldehydes or ketones), or comprises less than a preselected level of reactive impurities (e.g., aldehydes or ketones). While not wishing to be bound by theory, in some embodiments, a lipid reagent is used to make a lipid nanoparticle formulation, and the lipid reagent may comprise a contaminating reactive impurity (e.g., an aldehyde or ketone). A lipid regent may be selected for manufacturing based on having less than a preselected level of reactive impurities (e.g., aldehydes or ketones). Without wishing to be bound by theory, in some embodiments, aldehydes can cause modification and damage of RNA, e.g., cross-linking between bases and/or covalently conjugating lipid to RNA (e.g., forming lipid-RNA adducts). This may, in some instances, lead to failure of a reverse transcriptase reaction and/or incorporation of inappropriate bases, e.g., at the site(s) of lesion(s), e.g., a mutation in a newly synthesized target DNA.

[0381] In some embodiments, a lipid nanoparticle formulation is produced using a lipid reagent comprising less than 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% total reactive impurity (e.g., aldehyde) content. In some embodiments, a lipid nanoparticle formulation is produced using a lipid reagent comprising less than 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% of any single reactive

impurity (e.g., aldehyde) species. In some embodiments, a lipid nanoparticle formulation is produced using a lipid reagent comprising: (i) less than 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% total reactive impurity (e.g., aldehyde) content; and (ii) less than 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% of any single reactive impurity (e.g., aldehyde) species. In some embodiments, the lipid nanoparticle formulation is produced using a plurality of lipid reagents, and each lipid reagent of the plurality independently meets one or more criterion described in this paragraph. In some embodiments, each lipid reagent of the plurality meets the same criterion, e.g., a criterion of this paragraph.

[0382] In some embodiments, the lipid nanoparticle formulation comprises less than 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% total reactive impurity (e.g., aldehyde) content. In some embodiments, the lipid nanoparticle formulation comprises less than 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% of any single reactive impurity (e.g., aldehyde) species. In some embodiments, the lipid nanoparticle formulation comprises: (i) less than 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% total reactive impurity (e.g., aldehyde) content; and (ii) less than 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% of any single reactive impurity (e.g., aldehyde) species.

[0383] In some embodiments, one or more, or optionally all, of the lipid reagents used for a lipid nanoparticle as described herein or a formulation thereof comprise less than 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% total reactive impurity (e.g., aldehyde) content. In some embodiments, one or more, or optionally all, of the lipid reagents used for a lipid nanoparticle as described herein or a formulation thereof comprise less than 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% of any single reactive impurity (e.g., aldehyde) species. In some embodiments, one or more, or optionally all, of the lipid reagents used for a lipid nanoparticle as described herein or a formulation thereof comprise: (i) less than 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% total reactive impurity (e.g., aldehyde) content; and (ii) less than 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% of any single reactive impurity (e.g., aldehyde) species.

[0384] In some embodiments, total aldehyde content and/or quantity of any single reactive impurity (e.g., aldehyde) species is determined by liquid chromatography (LC), e.g., coupled with tandem mass spectrometry (MS/MS), e.g., according to the method described in Example

34. In some embodiments, reactive impurity (e.g., aldehyde) content and/or quantity of reactive impurity (e.g., aldehyde) species is determined by detecting one or more chemical modifications of a nucleic acid molecule (e.g., an RNA molecule, e.g., as described herein) associated with the presence of reactive impurities (e.g., aldehydes), e.g., in the lipid reagents. In some embodiments, reactive impurity (e.g., aldehyde) content and/or quantity of reactive impurity (e.g., aldehyde) species is determined by detecting one or more chemical modifications of a nucleotide or nucleoside (e.g., a ribonucleotide or ribonucleoside, e.g., comprised in or isolated from a template nucleic acid, e.g., as described herein) associated with the presence of reactive impurities (e.g., aldehydes), e.g., in the lipid reagents. In embodiments, chemical modifications of a nucleic acid molecule, nucleotide, or nucleoside are detected by determining the presence of one or more modified nucleotides or nucleosides, e.g., using LC-MS/MS analysis.

IV. PAYLOADS

[0385] The LNPs, e.g., targeted LNPs, of the disclosure can be used to deliver payloads to cells, e.g., immune cells (e.g., T cells). In some embodiments, the LNPs can be used to deliver a payload into T cells. In some embodiments, a targeted LNP as described herein can be used to deliver a payload to cells expressing cell-surface receptors targeted by the targeting moiety, e.g., antibody, Fab fragment, scFv or nanobody component, of the targeted LNP (conjugate).

[0386] The conjugates disclosed herein can be used to deliver a therapeutic of interest to a cell. For instance, in some embodiments, the conjugates disclosed herein can be used to deliver a nucleic acid (e.g., mRNA) encoding a vaccine. In other embodiments, the conjugates disclosed herein can be used to deliver a nucleic acid (e.g., mRNA) encoding an enzyme. In other embodiments, the conjugates disclosed herein can be used to deliver a nucleic acid, e.g., a DNA or RNA molecule, encoding a chimeric antigen receptor (CAR) to T cells.

[0387] In some embodiments, the payload is one or more nucleic acids. In some embodiments, the payload is one or more RNA molecules. In some embodiments, the payload is an mRNA (e.g., an mRNA encoding an enzyme). In some embodiments, the RNA molecule is a non-coding RNA (ncRNA). In some embodiments, the payload is an RNA template (for example, an RNA template for reverse transcription, e.g., for Target Primed Reverse Transcription (TPRT)). In other embodiments the payload is a siRNA or a microRNA (miRNA). In other embodiments, the payload is a guide RNA. In other embodiments, the payload is a tRNA. In other embodiments, the payload is an antisense oligonucleotide (ASO). In other embodiments, the payload is a DNA molecule, for example, a DNA plasmid, closed-ended DNA (ceDNA), or a small circular DNA (e.g., a minicircle or nanoplasmid). Nucleic acid payloads

can be linear, circular, covalently closed, single-stranded, double-stranded, or hybrid RNA/DNA molecules. In other embodiments, the payload is a small molecule. In some embodiments, the payload is a peptide or protein. In some embodiments, the conjugates disclosed herein can include two or more payloads, for example, selected from mRNA, ncRNA, guide RNA, siRNA, miRNA, ASO, DNA vector, small molecule, peptide, or protein.

[0388] The LNPs described herein, e.g., targeted LNPs, can be used to deliver a therapeutic of interest to a cell, e.g., an immune cell (e.g., T cell). In some embodiments, the LNP, e.g., targeted LNP, contains a payload that is a therapeutic agent. In some embodiments, the therapeutic agent can be a therapeutic peptide or protein, a nucleic acid comprising a therapeutic agent, or a nucleic acid encoding a therapeutic agent. In some embodiments, the therapeutic agent can be a genetic medicine (e.g., for gene therapy or gene editing), wherein the therapeutic agent is capable of modifying, altering or effecting a change in the genomic DNA of a cell (e.g., an immune cell (e.g., T cell)). In some embodiments, the therapeutic agent is a gene therapy agent or gene editing agent. In some embodiments, the therapeutic agent is a gene modifying polypeptide, as described herein. In some embodiments, the therapeutic agent is a gene modifying system, as described herein.

[0389] In some embodiments, the therapeutic agent can be a peptide or protein, such as an enzyme, or a nucleic acid (e.g., mRNA or DNA) encoding the peptide or protein (e.g., an enzyme). In some embodiments, the enzyme can be a nuclease, recombinase, integrase, transposase, retrotransposase, helicase, transcriptase, polymerase, reverse transcriptase, deaminase, methylase, demethylase, or ligase, or can have a combination of enzymatic activities thereof. In some embodiments, the therapeutic agent can be a peptide or protein, or a nucleic acid encoding the peptide or protein, for use as a replacement gene therapy. In some embodiments, the therapeutic agent can be a peptide or protein, or a nucleic acid encoding the peptide or protein, for use in modifying or altering the genome or epigenome of a cell, e.g., an immune cell of a subject. In some embodiments, the therapeutic agent can comprise one or more components of a system for modifying or altering the genome or epigenome of a cell, e.g., an immune cell (e.g., T cell) of a subject. In some embodiments, the system for modifying or altering the genome or epigenome of a cell of a subject comprises one or more proteins, one or more nucleic acids (e.g., RNA and/or DNA), or combinations thereof.

[0390] In some embodiments, the therapeutic agent can be one or more components of a ribonucleoprotein (RNP) complex for modifying or altering the genome or epigenome of a cell, e.g., immune cell such as a T cell. For example, in some cases the therapeutic agent can be a

protein, or a nucleic acid (e.g., mRNA) encoding the protein, and/or an RNA molecule (e.g., a gRNA) for guiding the protein to a particular location in the genome or epigenome, wherein the protein is capable of modifying or altering the genome or epigenome as a nuclease, recombinase, integrase, transposase, helicase, reverse transcriptase, deaminase, methylase, demethylase, or ligase, or combinations thereof. In certain embodiments, the therapeutic agent is a nuclease. In some embodiments, the nuclease cleaves DNA (e.g., both strands of the DNA), thereby introducing insertion and/or deletion (indel) mutations in DNA, e.g., genomic DNA. In certain embodiments, the nuclease is a nickase (i.e., it cleaves a single stand of DNA). In certain embodiments, the nuclease is mutated such that it is inactive or comprises reduced nuclease activity. In certain embodiments, the therapeutic agent can be used to introduce a substitution in DNA, e.g., genomic DNA, via base editing. In certain embodiments, the therapeutic agent can be used for epigenome editing. In certain embodiments, the therapeutic agent can be used to introduce an indel or a substitution in DNA, e.g., genomic DNA, by inducing target-primed reverse transcription (TPRT). In certain embodiments, the therapeutic agent (i.e., payload) delivered by the LNP, e.g., targeted LNP, can be a gene modifying protein, a nucleic acid encoding a gene modifying protein, or a gene modifying system, as described herein. In certain embodiments, the therapeutic agent can be a small molecule. In certain embodiments, the therapeutic agent can be an siRNA or miRNA, In some embodiments, the LNPs, e.g., targeted LNPs, of the disclosure can be used to deliver gene editing components into immune cells (e.g., T cells). In some embodiments, the LNPs, e.g., targeted LNPs, can be used to deliver a CRISPR-Cas system into immune cells (e.g., T cells). In some embodiments, the LNPs, e.g., targeted LNPs, can be used to deliver a Class 1 (type I, type III, or type IV) CRISPR system into immune cells (e.g., T cells). In some embodiments, the LNPs, e.g., targeted LNPs, can be used to deliver a Class 2 (type II, type V, or type VI) CRISPR system into immune cells (e.g., T cells). In some embodiments, the LNPs, e.g., targeted LNPs, can be used to deliver a CRISPR-Cas9 system, or a nucleic acid encoding one or more components of the CRISPR-Cas9 system, into immune cells (e.g., T cells). In some embodiments, the LNPs, e.g., targeted LNPs, can be used to deliver a CRISPR-Cas12 system (e.g., a Cas12a system), or a nucleic acid encoding one or more components of the CRISPR-Cas12 system, into immune cells (e.g., T cells). In some such embodiments, the LNPs, e.g., targeted LNPs, can comprise two RNA molecules, such as an RNA comprising a guide RNA (gRNA) and an mRNA encoding the Cas protein . In some embodiments, the Cas is Cas9 or Cas12a. In some embodiments, the Cas is Cas9. In some embodiments, the Cas is Cas12a. In some embodiments, the gRNA is a single guide RNA (sgRNA). In some embodiments, the LNPs, e.g., targeted LNPs, comprise a payload consisting

of or comprising a Cas9 or an mRNA encoding a Cas9. In some embodiments, the LNPs, e.g., targeted LNPs, comprise a payload consisting of or comprising a Cas12 (e.g., Cas12a) or an mRNA encoding a Cas12 (e.g., Cas12a). In some embodiments, the payload further consists of or comprises a gRNA.

[0391] In some embodiments, the mRNA component of a gene modifying system comprises a recombinant nuclease, or a nucleic acid encoding the nuclease, for example a restriction endonuclease, meganuclease, homing endonuclease, zinc finger nuclease (ZFN), or a transcription activator-like effector nuclease (TALEN).

[0392] In certain embodiments, the therapeutic agent (i.e., payload) delivered by the LNP, e.g., targeted LNP, can be a gene modifying system, or one or more nucleic acids (e.g., RNA molecules) comprising the gene modifying system (e.g., an mRNA encoding a gene modifying polypeptide and a template RNA), as described herein. In some embodiments, the gene modifying system is a retrotransposon gene modifying system, as described herein. In some embodiments, the gene modifying system is a heterologous gene modifying system, as described herein.

Retrotransposon Gene Modifying Systems

[0393] In some embodiments, provided herein are systems used to insert a heterologous object sequence (e.g., a CAR) into the genome of a cell. In some embodiments, the system comprises: (A) a gene modifying polypeptide or a nucleic acid encoding the gene modifying polypeptide, wherein the gene modifying polypeptide comprises: (i) an endonuclease and/or DNA binding domain; and (ii) a reverse transcriptase (RT) domain, where (i) and (ii) are both derived from a retrotransposon (e.g., from the same retrotransposon or different retrotransposons); and (B) a template RNA (or DNA encoding the template RNA) comprising (i) a sequence that binds the polypeptide and (ii) a heterologous object sequence. A gene modifying polypeptide, in some embodiments, acts as a substantially autonomous protein machine capable of integrating a template nucleic acid sequence into a target DNA molecule (e.g., in a mammalian host cell, such as a genomic DNA molecule in the host cell), substantially without relying on host machinery. The heterologous object sequence may include, e.g., a coding sequence, a regulatory sequence, or a gene expression unit.

Gene modifying polypeptides

[0394] Non-long terminal repeat (LTR) retrotransposons are a type of mobile genetic elements that are widespread in eukaryotic genomes. They include, for example, the

apurinic/apyrimidinic endonuclease (APE)-type, the restriction enzyme-like endonuclease (RLE)-type, and the Penelope-like element (PLE)-type.

[0395] The APE class retrotransposons are comprised of two functional domains: an endonuclease/DNA binding domain, and a reverse transcriptase domain. Examples of APE-class retrotransposons can be found, for example, in Table 1 of PCT Application No. PCT/US2019/048607, incorporated herein by reference in its entirety, including the sequence listing and sequences referred to in Table 1 therein.

[0396] The RLE class are comprised of three functional domains: a DNA binding domain, a reverse transcription domain, and an endonuclease domain. Examples of RLE-class retrotransposons can be found, for example, in Table 2 of PCT Application No. PCT/US2019/048607, incorporated herein by reference in its entirety, including the sequence listing and sequences referred to in Table 2 therein.

[0397] Penelope-like elements (PLEs) are distinct from both LTR and non-LTR retrotransposons. PLEs generally comprise a reverse transcriptase domain distinct from that of APE and RLE elements, but similar to that of telomerases and Group II introns, and an optional GIY-YIG endonuclease domain.

[0398] Other exemplary classes of retrotransposon include, without limitation, RTE (e.g., RTE-1_MD, RTE-3_BF, and RTE-25_LMi), CR1 (e.g., CR1-1_PH), Crack (e.g., Crack-28_RF), L2 (e.g., L2-2_Dre and L2-5_GA), and Vingi (e.g., Vingi-1_Acar) retrotransposons.

[0399] In some embodiments, an amino acid sequence encoded by an element of Table 4 is an amino acid sequence encoded by the full length sequence of an element listed in Table 4, or a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the full-length sequence of an element listed in Table 4 may comprise one or more (e.g., all of) of a 5' UTR, polypeptide-encoding sequence, or 3' UTR of a retrotransposon as described herein. In some embodiments, an amino acid sequence of Table 4 is an amino acid sequence encoded by the full length sequence of an element listed in Table 4, or a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, a 5' UTR of an element of Table 4 comprises a 5' UTR of the full length sequence of an element listed in Table 4, or a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, a 3' UTR of an element of Table 4 comprises a 3' UTR of the full length sequence of an element listed in Table 4, or a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto.

[0400] Also indicated in Table 4 are the host organisms from which the nucleic acid sequences were obtained and a listing of domains present within the polypeptide encoded by the open reading frame of the nucleic acid sequence.

[0401] Tables 4 and 5A provide gene modifying polypeptides comprising retrotransposon elements. In some embodiments, a template RNA described herein comprises one or both of a first homology domain comprising a sequence of a 5' Human Homology Arm of Table 4 (or a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto) and a second homology domain comprising a sequence of a 3' Human Homology Arm of Table 4 (or a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto).

Table 4: Retrotransposase systems

Element, Organism, Domains	Target Motif	Consensus Optimized Protein Sequence	5' Human Homology Arm	3' Human Homology Arm	5' UTR	3' UTR
L2-2_DRe (1), Danio rerio, RT and EN	TGAGCAC GGTAGCA TTAGTCGa (SEQ ID NO:26)	(SEQ ID NO: 30) MCFLIPVVTNTRKTREVRCRRNPHNLRSIH VSTISQLSLSVGLWNCQSAVNKADFITSIAT YSDYNLMALTETWLRPEDTATHATLSANF SFSHTPRQTGRGGGTGLLISKEWKFTLIPSL PTISSFEFHAVTIIHPFYINVVVIYRPPGKLG HFLDELDVLLSSFSNFATPLLVLGDFNIYV DKPQAADFQTLLASFDLKRAPTSATHKSG NQLDLIYTRHCFTDQTIVTPLQISDHFLLSL NIHITPEPPHTPTLVTFRRNLRSLSPNRLSTI VSDSLPPSRKLTALDSNSATNTLCSTLASCL DRLCPLASRPARASPPAPWLSDALREHRSK LRAAERIWRKTKNPAHLLTYQTLLSSFSAE VTSAKQTYYRLKINNATNPRLLFKTFSSLL YPPPPPASSTLTTIDDFATFFCTKTAKISAQF AAPTTNTQDTTPTPHTLTSFSQLSESEVSKL VLSSHATTCPLDPIPSHLLQAISPAVIPTLTH IINTSLDSGLFPTTFKQARVTPLLKKPNLDH TLLENYRPVSLLPFMAKILEKVVFNQVLDF LTQNNLMDNKQSGFKKGHSTETALLSVVE DLRLAKADSKSSVLLLLDLSAAFDTVNHQI LLSTLESLGVAGTVIQWFRSYLSDRSFRVS WRGEVSNLQHLNTGVPQGSVLGPLLFSIYT SSLGPVIQRHGFSYHCYADDTQLYLSFHPD DPSVPARISACLLDISHWMKDHHLQLNLA KTEMLVVSANPTLHHNFSIQMDGATITASK MVKSLGVTIDDQLNFSDHISRTARSCRFAL YNIRKIRPFLSEHAAQLLVQALVLSKLDYC NSLLAGLPANSIKPLQLLQNAAARVVFNEP KRAHVTPLLVRLHWLPVAARIKFKTLMFA YKVTSGLAPSYLHSLLQIYVPSRNLRSVNE RRLVVPSQRGKKSLSRTLTLNLPSWWNEL PNCIRTAESLAIFKKRLKTQLFSLHFTS	(SEQ ID NO: 41) GTTTCGCGT CGTCCGCCT CAGTTTCGG CCCTTGTTT TGACTCGG GAGGCGTG TCCAAACG CCAGGTAA TTAGTCG	a	(SEQ ID NO: 42) GCAGGAGAAGCACTTT AGCAGCATCTAGAACA GCAGCCTGTAAGTACA TTTAAGATTTGTTTCAG TTGTTGTGTATGGTTTG AGGACTTGTTTCCAGCT GTTTGTGTAAAGTTGTA GGACATTTAAACTTGCT TTCAGTTGTGTATAATA CTAGAAGTTGTTTTAGCC ACTGCGTCGTCCGCCTC AGTTTCGGCCCTTGTTT TGACTCGGGAGGCGTG TCCAAACGCCAGGTAA CCACTATATAGTGAGC ACGGTAGCATTAGTCG GCAGGAGAAGCACTTT AGCAGCATCTAGAACA GCAGCCTGTAAGTACA TTTAAGATTTGTTTCAG TTGTTGTGTATGGTTTG AGGACTTGTTTCCAGCT GTTTGTGTAAAGTTGTA GGACATTTAAACTTGCT TTCAGTTGTGTATAATA CTAGAAGTTGTTTTAGCC ACTGTTTCCTTGGTTAC TATAAGAGGCTTGTGTA GCGAACGGCAGACGCGG	(SEQ ID NO: 53) TAATCTGCAATTGCC TCTCTGAATATCACA CTAACTGTACCCAA AAAAAAAAAAAAAA AAAAAAAAAATAAA AAWACTACTAATAC TTCCCTTCTTAGACT TTACAGACCTGAAA CTTGCCTATAGCACT TATTCATTGTTGCTC TTAGTTGTGTAAATT GCTTCCTTGTCCTCA TTTGTAAGTCGCTTT GGATAAAAGCGTCT GCTAAATGACTAAA TGTAAATGTAAATGT

Element, Organism, Domains	Target Motif	Consensus Optimized Protein Sequence	5' Human Homology Arm	3' Human Homology Arm	5' UTR	3' UTR
					TTCGCGTCGTCCGCCTC AGTTTCGGCCCTTGTTT TGACTCGGGAGGCGTG TCCAAACGCCAGGTAA CCACTATATAGTGAGC ACGGTAGCATTAGTCG GCAGGAGAAGCACTTT AGCAGCATCTAGAACA GCAGCCTGTAAGTACA TTTAAGATTTGTTTCAG TTGTTGTGTATGGTTTG AGGACTTGTTTCCAGCT GTTTGTGTAAAGTTGTA GGACATTTAAACTTGCT TTCAGTTGTGTATAATA CTAGAAGTTGTTTAGCC ACTGTTTCCTTGGTTAC TATAAGAGCTTGTGTA GCGAACGCAGACGCGG TTCGCGTCGTCCGCCTC AGTTTCGGCCCTTGTTT TGACTCTCGAGGCGTGT CCAGCTGAATTCAATC AGCTAGTGCTTTGGGGT TATATAAACAACTAGTT CACCGCGGCAGCGGTC GCGGCAGCCTCGTGTG AAGACCGACGAGGGTA AAGACCATCGACTCTA CCTGCGCGACTCCACC GAGCAAAGACACCGAC AAAGCACTTGAGTACT TTACTGTATTGTTTTTAC TTTACACTTATTTTTTG TTGTCAGTGCACTTTTA TT	

Element, Organism, Domains	Target Motif	Consensus Optimized Protein Sequence	5' Human Homology Arm	3' Human Homology Arm	5' UTR	3' UTR
L2-2_Dre (2), Danio rerio, RT and EN	TGAGCAC GGTAGCA TTAGTCGa (SEQ ID NO:26)	(SEQ ID NO: 31) MCFLIPVVTNTRKTREVRCRRNPHNLRSIH VSTISQLSLSVGLWNCQSAVNKADFITSIAT YSDYNLMALTETWLRPEDTATHATLSANF SFSHTPRQTGRGGGTGLLISKEWKFTLIPSL PTISSFEFHAVTIIHPFYINVVVIYRPPGKLG HFLDELDVLLSSFSNFATPLLVLGDFNIYV DKPQAADFQTILLASFDLKRAPTSATHKSG NQLDLIYTRHCFTDQTIVTPLQISDHFLLSL NHHITPEPPHTPTLVTFRRNLRSLSPNRLSTI VSDSLPPSRKLTALDSNSATNTLCSTLASCL DRLCPLASRPARASPPAPWLSDALREHRSK LRAAERIWRKTKNPAHLLTYQTLLSSFSAE VTSAKQTYYRLKINNATNPRLLFKTFSSLL YPPPPPASSTLTTDDFATFFCTKTAKISAQF AAPTTNTQDTTPTPHTLTSFSQLSESEVSKL VLSSHATTCPLDPIPSHLLQAISPAVIPTLTH IINTSLDSGLFPTTFKQARVTPLLKKPNLDH TLLENYRPVSLLPFMAKILEKVVFNQVLDF LTQNNLMDNKQSGFKKGHSTETALLSVVE DLRLAKADSKSSVLILLDLSAAFDTVNHQI LLSTLESLGVAGTVIQWFRSYLSDRSFRVS WRGEVSNLQHLNTGVPQGSVLGPLLFSIYT SSLGPVIQRHGFSYHCYADDTQLYLSFHPD DPSVPARISACLLDISHWMKDHHLQLNLA KTEMLVVSANPTLHHNFSIQMDGATITASK MVKSLGVTIDDQLNFSDHISRTARSCRFAL YNIRKIRPFLSEHAAQLLVQALVLSKLDYC NSLLAGLPANSIKPLQLLQNAAARVVFNEP KRAHVTPLLVRLHWLPVAARIKFKALMFA YKVTSGLAPSYLLSLLQIYVPSRNLRSVNE RRLVVPSQRGKKSLSRTLTLNLPSWWNEL PNCIRTAESLAIFKKRLKTQLFSLHFTS	a	(SEQ ID NO: 42) GCAGGAGAAGCACTTT AGCAGCATCTAGAACA GCAGCCTGTAAGTACA TTTAAGATTTGTTTCAG TTGTTGTGTATGGTTG AGGACTTGTTTCCAGCT GTTTGTGTAAAGTTGTA GGACATTTAAACTTGCT TTCAGTTGTGTATAATA CTAGAAGTTGTTTAGCC ACTGTTTCCTTGGTTAC TATAAGAGCTTGTGTAC GCGAACGCAGACGCGG TTCGTCGGTCCTTGTTT TGACTCGGGAGGCGTG	(SEQ ID NO: 53) TAATCTGCAATTGCC TCTCTGAATATCACA CTAACTGTACCCAA AAAAAAAAAAAAAA AAAAAAAAAATAAA AAWACTACTAATAC TTCCCTTCTTAGACT TTACAGACCTGAAA CTTGCCTATAGCACT TATTCATTGTTGCTC TTAGTTGTGTAAATT GCTTCCTTGTCCTCA TTTGTAAGTCGCTTT GGATAAAAGCGTCT GCTAAATGACTAAA TGTAAATGTAAATGT

Element, Organism, Domains	Target Motif	Consensus Optimized Protein Sequence	5' Human Homology Arm	3' Human Homology Arm	5' UTR	3' UTR
					TCCAAACGCCAGGTAA CCACTATATAGTGAGC ACGGTAGCATTAGTCG GCAGGAGAAGCACTTT AGCAGCATCTAGAACA GCAGCCTGTAAGTACA TTTAAGATTTGTTTCAG TTGTTGTGTATGGTTTG AGGACTTGTTTCCAGCT GTTTGTGTAAAGTTGTA GGACATTTAAACTTGCT TTCAGTTGTGTATAATA CTAGAAGTTGTTTAGCC ACTGTTTCCTTGGTTAC TATAAGAGCTTGTGTA GCGAACGCAGACGCGG TTCGCGTCGTCCGCCTC AGTTTCGGCCCTTGTTT TGACTCTCGAGGCGTGT CCAGCTGAATTCAATC AGCTAGTGCTTTGGGGT TATATAAACAACTAGTT CACCGCGGCAGCGGTC GCGGCAGCCTCGTGTG AAGACCGACGAGGGTA AAGACCATCGACTCTA CCTGCGCGACTCCACC GAGCAAAGACACCGAC AAAGCACTTGAGTACT TTACTGTATTGTTTTAC TTTACACTTATTTTTTG TTGTCAGTGCACTTTTA TT	
RTE-1_MD (1), Monodelph	NA	(SEQ ID NO: 32) MDSTAHPNQGRGLEKVSQTLPALQTPGQH TAAGGSSPLSGRNQRKNTKKLLLGAWNIR			(SEQ ID NO: 44) gggtgtatgggggtgctcagggaggg gtagtatctctggtatggagggcttgtc	(SEQ ID NO: 55) tgaaactgcacaaagacaatagt

Element, Organism, Domains	Target Motif	Consensus Optimized Protein Sequence	5' Human Homology Arm	3' Human Homology Arm	5' UTR	3' UTR
is domestica, L1-EN, RT, ZF		TLLDRENTPRPERRTALIGKELARYNIDIAA LSETRLPEEGSLSEPTTGYTFFWKGRASNE DRIHGVGLAIKTSLLKQLPDLPVGISERLM KIRLPLSKDRYATIISAYAPTLTSTEETIEQF YSDLSAVLHSVPTNDKLILLGDFNARVGQ DHERWKGVLGKHGVGKMNNNGLLLLSK CSEFELTITNTVFRMANKYKTTWMHPRSK QWHLIDYIIVRRRDIQDVKITRAMRGAEC WTDHRLVRATLQMRIAPRHPKRAQTVRAF YNVSRLRDPSYLQTFQSCLDDKLSAKGPLT GSSTEKWNQFRDAVKETSKAVLGPKQRN HQDWFDENNTAIEDLLSKKNKAFMEWQN NPNSAPKKDRFKSLQATAQREIRKMQDRW WEKKAEEIQRFADMKNYKQFFSALKTVY GPLKPTTTPLLSSDGDTLIKDKKGISNRWK EHFSQLLNRPSSVDQSALDQIPQNRTIEQLD VPPSIEEVQKAIKQMSAGKAPGKDGIPTEV YKALNGKALQAFHIVLTSIWEEEDMPPELR DASIVALYKNKGSRAACDNYRGISLLSTAG KILARVILNRLLSSVSEQNLPESQCGFRPDR STIDMVFTVRQMQEKCLEQNLSLYIVFIDL TKAFDTVNRDALWVILSKLGCPAKFVKLI QLFHVDMTGEVLSGGETSDRFNISNGVKQ GCVLAPVLFNLFFTQVLRHAVMDLDLGVY IKYRLDGSLFDLRRLTAKTKTTERLILEALF ADDCALMAHQENHLQTIVDRFSTATKLFG LTISLSKTEVLFQPAPGRPTNQPCITIDGTQL SNVNTFKYLGSTIANDGSLDHEINARIQKA SQALGRLRCKVLQHRGVSTATKLKVYNA VVLSSLLYGCETWTLYRKHMKQLEQFHQ RSLRSIMRIRWQDRITNQEVLDRANSTSIEV MVLKTQLRWSGHVIRMDPQRIPRQVFYGE LSAGLRKQGRPKKRFKDQLKSNLKWAGIT PKQLELAASDRSSWRTHINHAATTFEDERR RRLAAARERRHQATTAPPVTTGVPCPMCH KLCASAFGLQSHMRVHRR			gtgccctcctaggcagctctccagc ctctgaccccacctgacacccagctc tcacttgtggctcccagtagctgctagc atgtggcagcggccacacccccgggc aacggcttcgacaggccggctaaacc ttgtgagggtagccatcgggtcatcga ccctggtgaaccaggggcttgctcac ccagcatgtgaagactgcttcggctga acagacggaagaaaccaataagaag gttcaacggctgagaggggcgacgca gcaaagcactgtggagtgcttagggc gtgttggagcacaaaggacaacacg gccatccaatgcagctgaggaagtct ccagatgtaacaatttttcgtgccactg gacccaggcttccaacgccgagaga gtgggactgtctctgtgcatcggctttt ccacttaaatctctttcacgcacaagtat ctttgtgcacactcatctatcctaacccc gtccacccttcttcaagacctgcggcga tgggggagtggcgacgcaacaggtg gaggtgaccactggcagttgtagtcac gatcctgcacgtaggcggcccacgg accagtggtcgctcggccctgtgggc agcagggacgttcggcagcatcctgg gcgactgagcagccctctctag	cattctcgatcaccgagagactac cact

Element, Organism, Domains	Target Motif	Consensus Optimized Protein Sequence	5' Human Homology Arm	3' Human Homology Arm	5' UTR	3' UTR
RTE-1_MD (2), Monodelphis domestica, L1-EN, RT, ZF	NA	(SEQ ID NO: 33) MDSTAHPNQGRGLEKVSQTLPALQTPGQH TAAGGSSPLSGRNQRKNTKKLLLGAWNIR TLLDRENTPRPERRTALIGKELARYNIDIAA LSETRLPEEGSLSEPTTGYTFFWKGRASNE DRIHGVGLAIKTSLLKQLPDLPVGISERLM KIRLPLSKDRYATIISAYAPTLTSTEETIEQF YSDLSAVLHSVPTNDKLILLGDFNARVGQ DHERWKGVLGKHGVGKMNNNGLLLLSK CSEFELTITNTVFRMANKYKTTWMHPRSK QWHLIDYIIVRRRDIQDVKITRAMRGAEC WTDHRLVRATLQMRIAPRHPKRAQTVRAF YNVSRLRDPSYLQTFQSCLDNKLSAKGPLT GSSTEKWNQFRDAVKETSKAVLGPKQRN HQDWFDENNTAIEDLLSKKNKAFMEWQN NPNSAPKKDRFKSLQATAQREIRKMQDRW WEKKAEEIQRFADMKNYKQFFSALKTVY GPLKPTTTPLLSSDGDTLIKDKKGISNRWK EHFSQLLNRPSSVDQSALDQIPQNRSIEQLD VPPSIEEVQKAIKQMSAGKAPGKDGIPTEV YKALNGKALQAFHIVLTSIWEEEDMPPELR DASIVALYKNKGSRAACDNYRGISLLSTAG KILARVILNRLLSSVSEQNLPESQCGFRPDR STIDMVFTVRQMQEKCLEQNLSLYIVFIDL TKAFDTVNRDALWVILSKLGCPAKFVKLI QLFHVDMTGEVLSGGETSDRFNISNGVKQ GCVLAPVLFNLFFTQVLRHAVMDLDLGVY IKYRLDGSLFDLRRLTAKTKTTERLILEALF ADDCALMAHQENHLQTIVDRFSTATKLFG LTISLSKTEVLFQPAPGRPTNQPCITIDGTQL SNVNTFKYLGSTIANDGSLDHEINARIQKA SQALGRLRCKVLQHRGVSTATKLKVYNA VVLSSLLYGCETWTLYRKHMKQLEQFHQ RSLRSIMRIRWQDRITNQEVLDRANSTSIEV MVLKTQLRWSGHVIRMDPQRIPRQVFYGE			(SEQ ID NO: 44) gggtgtatgggtgctcagggaggg gtagtatctctgatatgagaggcttgtc gtgccctcctagggcagctctccagc ctctgacccccacctgacacccagctc tcacttgtggctcccagtagctgctagc atgtggcagcggccacacccgggc aacggcttcgacaggccggctaaacc ttgtgagggtagccatcgggtcatcga ccccctggtgaaccagggcttlgctcac ccagcatgtgaagactgcttcggctga acagacggaagaaaccaataagaag gttcaacggctgagagggcgacgca gcaaagcactgtggagtgcttaggge gtgttggagcacaaaggacaacacg gccatccaatgcagctgaggaagtct ccagatgtaacaattttcgtgccactg gacccaggcttccaacgccgagaga gtgggactgtctctgtgcatcggctttt ccacttaaatctctttcacgcacaagtat ctttgtgcacactcatctatcctaacccc gtccacctcttcaagacctgcggcga tgggggagtggcgacgcaacaggtg gaggtgaccactggcagttgtagtcac gatcctgcacgtaggcggcccacgg accagtggtcgctcggccctgtgggc agcagggacgttcggcagcatcctgg gcgactgagcagccctctctag	(SEQ ID NO: 55) tgaaactgcacaaagacaatagt cattctcgatcaccgagagactac cact

Element, Organism, Domains	Target Motif	Consensus Optimized Protein Sequence	5' Human Homology Arm	3' Human Homology Arm	5' UTR	3' UTR
		LSAGLRKQGRPKKRFKDQLKSNLKWAGIT PKQLELAASDRSSWRTHINHAATTFEDERR RRLAAARERRHQATTAPPVTTGVPCPMCH KLCASAFGLQSHMRVHRR				
Vingi-1_Acar (1), Anolis carolinensis, DNAse-like, RT	NA	(SEQ ID NO: 34) MDEYQRSLSRPLLTIMSINIEGLSLAKEELL AKMSEDISCDILCIQETHRDITMRRPKILGM QLAVERPHRQYGSAIFVRSGVAISATSLTE VNNIEILSVELDSCTVSSLYKPPGADFYFTP PTSCHNHEAHFVVGDFNSHSCVWGYDED DRNGEAVLTWADNSRMSLLHDSKLPPSFN SGRWKRGYNPDLIFVKESISHQCTKRVLNP IPNTQHRPICCVAYAAVRPKSVPFRRRYNF NKANWTKFTETLEAAISDIEPSIENYDLFVE AVKRSSRLSIPRGCRTSYLPGLNEESLNQL QEYLRLFQENPYSDGTIAAGQKLSTALAN AKKDRWIELLENLDMSKSSRKAWQLLRRL DSDPLVNPGHANVTPDQIAHQLIQNGKTN CSRIKMKINRVPELETHQLSSPLNLKELREA IKRCKTGKAPGLDDLMMEQIKHLGPKAEN WLLKFYNQCLAHKQIPRAWRKTKIIAILKP GKDASNARNYRPISLLCHLYKVYERMLLN RLGPVIEPKLIAQQAGFRPGKNCTGQILHLT EHIEEGYEKGCITGTVFVDLTAAYDTVQHR KMLHKVYHITRDFDFTKTVQTLLENRSFY VEFQGQKSRWRRQKNGLPQGSVLAPTLFN IFTNDQPQPPLTKSFIYADDLGLTTQAKDFE TVEKQLTNALKDLSSYYKENHLKPNPAKT QVCAFHLRNREANRKLKVTWEGQELEHC FHPKYLGVTLDRTLTYRKHCMNTKHKVA ARNNILRKLTGSAWGADPQVIRTSALALSF STAEYACPVWHKSAHAKQVDIALNETCRII TGCLKPTPVDKLYKLAGIAPPDVRREVAA NGERKKVEHCESHPLHDYHPPPTRLKSRK GFMRTTTPLDVPPATARVSLWAAKPGNSN WMAPQEGLPPGANQEWATWKSLNRLRSG			(SEQ ID NO: 46) GGGGGACACCGAAAGA GCCTCCCCGAAGATTG AGTGAATTCAGTCGGG CGTCCCCTGGGCAACG TTTCTTGTAAGCGGCCG ATCTTTCCACCCCAAAA GCATTGGATGA	(SEQ ID NO: 57) TAGTTGCTTGTGATT TCTTTTCTTTTTTATT TTATTTCCATTATTT GAAATGTATTTGCTG TACCAATGCTTTTGA CACGAAATAAATAA A

Element, Organism, Domains	Target Motif	Consensus Optimized Protein Sequence	5' Human Homology Arm	3' Human Homology Arm	5' UTR	3' UTR
		VGRSKDNLARWHYLEESSTLCDCGAEQTT QHMYACPQCPASCTEEELFKATDNAVAVA RFWSKTI				
Vingi-1_Acar (2), Anolis carolinensis, DNAse-like, RT	NA	(SEQ ID NO: 35) MDEYQRSLSRPLLTIMSINIEGLSLAKEELL AKMSEDISCDILCIQETHRDITMRRPKILGM QLAVERPHRQYGSAIFVRSGVAISATSLTE VNNIELSVELDSCTVSSLYKPPGADFYFTP PTSCHNHEAHFVVGDFNSHSCVWGYDED DRNGEAVLTWADNSRMSLLHDSKLPPSFN SGRWKRGYNPDLIFVKESISHQCTKRVLNP IPNTQHRPICCVAYAAVRPKSVPFRRRYNF NKANWTKFTETLEAAISDIEPSIENYDLFVE AVKRSSRLSIPRGCRTSYLPGLNEESLNQL QEYLRLFQENPYSDGTIAAGQKLSTALAAN AKKDRWIELLENLDMSKSSRKAWQLLRRL DSDPLVNPGHANVTPDQIAHQLIQNGKTN CSRIKMKINRVPELETHQLSSPLNLKELREA IKRCKTGKAPGLDDLMMEQIKHLGAKAEN WLLKFYNQCLAHKQIPRAWRKTKIIAILKP GKDASNARNYRPISLLCHLYKVYERMLLN RLGPVIEPKLIAQQAGFRPGKNCTGQILHLT EHIEEGYEKGCITGTVFVDLTAAYDTVQHR KMLHKVYHITRDFDFTKTVQTLLLENRSFY VEFQGQKSRWRRQKNGLPQGSVLAPTLFN IFTNDQPQPPLTKSFIYADDLGLTTQAKDFE TVEKQLTNALKDLSSYYKENHLKPNPAKT QVCAFHLRNREANRKLKVTWEGQELEHC FHPKYLGVTLDRTLTYRKHCMNTKHKVA ARNNILRKLTGSAWGADPQVIRTSALALSF STAEYACPVWHKSAHAKQVDIALNETCRII TGCLKPTPVDKLYKLAGIAPPDVRREVAA NGERKKVEHCESHPLHGYHPPPTRLKSRK GFMRTTTPLDVPPAAARVSLWAAKPGNSN WMAPQEGLPPPGANQEWATWKSLNRLRSG VGRSKDNLARWHYLEESSTLCDCGAEQTT			(SEQ ID NO: 46) GGGGGACACCGAAAGA GCCTCCCCCGAAGATTG AGTGAATTCAGTCGGG CGTCCCCTGGGCAACG TTTCTTGTAAGCGGCCG ATCTTTCCACCCCAAAA GCATTGGATGA	(SEQ ID NO: 57) TAGTTGCTTGTGATT TCTTTTCTTTTTTATT TTATTTCCATTATTT GAAATGTATTTGCTG TACCAATGCTTTTGA CACGAAATAAATAA A

Element, Organism, Domains	Target Motif	Consensus Optimized Protein Sequence	5' Human Homology Arm	3' Human Homology Arm	5' UTR	3' UTR
		QHMYACPQCPASCTEEELFKATDNAVAVA RFWSKTI				
CR1-1_PH, Parhyale hawaiensis, DNAse I-like, RT	NA	(SEQ ID NO: 36) MLYTIFLVYGILCFFFVYFCIYLYITVYLCF LFFLFLLLCGDVESNPGPGRARGCRLLYCN IRGLHANLAELDFVSRGVDVVCCSETLVA GRRHDAELALAGFQSPFRRLCGSGPGFRG MAVYVRSGFCAYRQSVHECACHEIIVVRV CGRLNNYYLFSLYRSPATDDSLYDCLLTA MASIQSTDPKAAFVFVGDVNAHHRDWLG SASPTDCHGVAALDFCTLSSCVQLVRGSTH IAGNCLDLVMTDVPDLMTVTVGSPIGSSD HSHLVVSLDLNQVVPVVDTRRVVFVKSRA NWVAITRAVRTLPWRQIIHSEDPVSELNDL TVSILERFVPKRTILVRSRDKPWFDDQCRL AFEAKQAAYRAWRRSRDRTLWQTYVDRR AEAKRVYEEAQRRLRQRSRESLLSIDHPHR WWSELKGSVFGAEPSLPPLVGPGGGIITDP LARAELLSAHFDGKQSRDVIALPHGCHPEP RLTSLAFRSGAIKVLLEGLDPYGGVDPVG MFPLFYKQLADVLAPKLAVIFRRLIRLGNF PRCWRTGNITPIPKGPVSPYVANYRPTLTP ILSKVFEKLIAGKLGRFAEVTGLLPAGQFA YRIGLGCCDALLSVSHHLQSALDCRSEARL VQLDFSAAFDRVNHRGLLYKLESFGVGGR VLSIIRDFVSERTQSVSVDGVLSASVGVVS GVPQGSVLGPLLFVLYTSDMFSSLENTLIN YADDSTLMAVIPAPRLRDAVAQSLNRDLS RISAWCSAWSMKLNASKTKSMIISRSRTLV PQHPQLEIDDTLLQESSSLEILGVVFDEKLT FEPHIRRLVSRASTKIGLLRKVNSVFGDSQ VARRCFYAFLLPVLEYCSPVWASAADTHL RLLDRLVSSASRLCADNDLVNLSHRRRVA ELCMFYKVYNNERHWLYSSLPALKVFGRE TRAACGAHSFTLEAVRCRTNQFCRCFVPW			(SEQ ID NO: 48) gcgtggcctgcgcgttatcaggccgc ccccattgtccggccaccggacgcct ctttgttgtatgagctggcttgggttg gggctctggtagccccctgagttgga ggaaatgccactctaaaggcggatga gacctcttttggtccaacggcatcctg agaggagatgaacaggctggtgccat agctgggtactcagtcttccgcagtct gctcctctctctccatcaggtagtac cacctgatgctcttttccttccttctctt cttcttctttttcggccaaagcctgtactg gtgatggcaggggtctgggggcgcgtag atagctggccgctttcgtagctatcgtt actgtcgatttgttttgctttatt	(SEQ ID NO: 59) tagcagcgttttgggcttcccggt gttgcaagatgcttcatttgggttgt attacctgtccgtgggagctcaag cgtgaagtttaataataataataa

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		SAKVWNLLPASAFGRIGLQAFKSAVNGFL LDFL				
Crack-28_BF, Branchiostoma floridae, RT and EN	NNNNNNN NNNNNNN NNNNNTN NNNNCNN GTNNNNA NNNNNNN NNNNNNN CNNNNNN NNNNNNN NNNTNNN NNNNNNN NNNNNNN NNNTNNN NNNNNNN NNnntnnnn nnnnnnnnn nnnnnncnnn nnnnnnnnn nnnnngcnnn nnnnntnnnn nnnnnnnnn nnnnnnnnn ntnnnnnnn nnnannnnnn nnn (SEQ ID NO:27)	(SEQ ID NO: 37) MWEKATSNVHLQSGTWITQNAYSVKINPG LSGVRLIGRSTTCTERTERTLNLLVCATLLL AGDVSPNPGPDTGGLPVWRKGIVYAFYNV VSLPRHLDEIQQLLLRNTRIHVLGLNETRLS DSIPDSSVDINGYTLYRTDRDRQGGVGV YVKQTIASQRRCELEQEDLEVCCVEIKPEK ARKTLLTCVYRPPTSGPDWRNSAESLVHK LNQTAEKENADVAIMGDFNSDLLTSTQAM SSVEFLMGLYQLVPVIREPTRITEKTESCID NIFVSNPDRYKSSASVAWGPSDHNLILTCA KAGSEAGAAHRCEYRSYKLYTQQSFIDSL KSVRWDTVFDCTDVSEAWNAFKDIFLNVA DEHAPLRTKTARENNRPAPWMTDTVKNM MGRRDAARRKAIRTKDVQDWDTYRSLRN QTTSIIRKEKKSHFATAVSEAKGDQSLMW KIINSFTGKSKSTKRVQKLLRADNTSMSDP GEMAQEFNDYFTSCASRLTDGMPDSEEDP LRHIPDSTTKFSFDCVEETEVLNELQKLKT KKATGLDKIPAKLLKDSAPVVAKPLAHIFN LSLASGEVPSDWKEAQITPVHKSGSCADV GNYRPVSVLSVTSKVMEKLVCNQVTRYLT RCKLLTTHQSGFRRHHSTATAVQKVVEDI TSGYNCSKVTVALFLDLRKAFDSVNHEIM LSKLKKFGFDSDAMKWFTSYLSERLQCTC LQGQYSSKTRVSCGVPQGSVLGPLLFCLY VNDLPNVIQKCSIHMYADDTVLYYSAVSV KVCEETVSMDMKRVVKWLSENRLLLHPD KTKSMLFGLPQKLKHAGTTVNITDGVNVY EQVDSFTYLGITLDPALRWAAHVQKITKK LLSGLGAMGRARAFVTNEVLKTMYQTLLL AHLEYCATAWLPSLAQGNKTLMLQLDRL VNRAAARLITGHKLRDHVTVDNLRAEAGID SVRKRTEITTLVTVFKTTRGKAPAYLASLFK				

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		WEAPPTMSVRPTRSEVKRLRDYDPHLLWC PPARVIAFRNSLQSYGPFLWNSLPLKQRQL LSLRTFKKFIEN				
L2-5_GA, Gasterosteus aculeatus, RT and EN, signal peptide	NNNNNNN NNNNNNN NNNNNNN NNNNNNN NNNNNNN NNNNNNN NNNNNNN NNNNNNN NNNNNNN NNNNNNN NNnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnnnnnnnn nnn	(SEQ ID NO: 38) MRRLLLLFLIMCLTPSPVPVRISSRRYYRPR ARSALYRNLSSLSYPTRSTHVQHLVTGGL WNCQSATRKADFISGFAIQQSLDFLALTET WITPENTSTPAALSSAFSFSHTPRPTGRGGG TGLLISPKWSFSLYPLPPSTPLSFEFHAVTIT HPVQLTIIVLYRPPGSLGHFLEELDILLSNFP ENGPPLILLGDFNIQTEKSSDLLHLLSSFALS LSPSPPTHKAGNHLDYIFTRNCSTTNLSVTP LHVSDHFFISYSLPLSITNKPPSLTNSIPARR NIRSLSPSSLASSVLSALPSTDSFSLLHPNAA AETLLSTLSSSLDSLCPLTTRRTGKSPPAPW LSQPVRAMRATMRASERRWRKYKRPDDL LEFQSLLSSFSASISAAKSSFYQSKIESSFSN PKKLFSIFSNLLEPPTPPPPSTLLPGDFVNYF TKKIADIRSSFSNPPPTSRVPPTSPLSPSLSSF TALSPNQILTLVTSARPTTCPLDPIPSHLLQS IAPDLLPFLTCLINNALSSGCFPNSLKEARV NPLLKKPTLNPSEENNYRPVSLLPFLSKTLE RAIFNQLSSYLHCNNLLDPHQSGFKAGHST ETALLAVSEQLHTARAASLSSVLILLDLSA AFDTVNHQILISSLQELGVTGSALSLLSSYL DGRTYRVTWRGSVSEPCPLTTGVPQGSVL GPLLFSLYTNSLGAVIRSHGFSYHSYADDT QLILSFPHSDTQVAARISACLTDISQWMSA HHLKINPDKTELLLLFPGKDSLTQDLTVNFG NSVLTPTSTAKNLGVTLDSQLSLTPNITATT RSCRYTLYNIRRIRPLLTQKAAQVLIQALVI SRLDYCNSLLAGLPATAIRPLQLIQNAAAR LVFNLPKFSHITTPLLRSLHWLPVAARIQFK TLVLTYHAVNGSGPAYIQDMVKPYIPTRTL RSASAKLLVPPSLRAKHSTRSRLFAVLAPK			(SEQ ID NO: 50) CAGTGTGCATCTCTTCT ACAAGGCCACCAGACA TCCAGGCGACCTGAAA TCGGACTAAGTATCTCT TTCTTTAAACCAAGCTA GTCTGCTAAATTTGCTG TTGATTCTAGTGTCTTA AGTTAATTTGATTGCTG ATTTGCTTTAGTTTTTG TCGTCTCACGGCCAGTT CTCTTTGTTTTGAAGTTA TCTTGGTTGCTAGTTTG CTCAAGCTCTTTCAGTC TTTTAGTGCCAGTTTGT GTTCTAGATTCTACTAT TAAGTTAACTGCCAGT GTGCCCTCCTAACTCTG CTAGGTTTTGACCTGAT CTAAGTCTGTTTTTGCCT TGTTTTCTGAAGCAAAT AAGACTTGTATCCTTAA ACTCTCATTTTGTCAAA ACACCACATGGTGTTTG TTCACTGATTAGGGTGT CAAGCTATTGGTGCTTT GCATTTTGAGGAGTTTC TGTCGAGGCCAATCGA CCTTTTGTCTCCTAAAC GACGGGGGAGCGGCCA GCGCAGCCGCAGCCGA CTTCCACTAACGAGGG AGTATTTAACTAGAAA	(SEQ ID NO: 61) TAAAGACTAACAAA TTGTAGCACTTAAAT TGTACTTGTAACGTC ACTCATCTATAGCAA ATTGTAAATTGGCTT ATTTGAGGAAATTG CACTTTCTTGTTTCT TGTTCTCCTGAGTTT GTACCCTATGGTTGA ATGCACTTATTGTAC GTCGCTTTGGATAAA AGCGTCAGCTAAAT GACATGTAATGTAA TGTAATGTAATG

Element, Organism, Domains	Target Motif	Consensus Optimized Protein Sequence	5' Human Homology Arm	3' Human Homology Arm	5' UTR	3' UTR
		WWNELSEDTRTAESLHIFRRKLKTHLFRLY LD			TCGTGGGAAGCGTTAG CTTATCCTCGCAGCACG AAGACGAGCAGAACAA AGACCAGGGAGTCTTT CGTGGAGTCAAGACGA GAGACAAAGACCAGGG AGTCTTTCGTGGAGTCA AGACGAGCAGAACAAA GACAAGGGAGTCTTTC GTGGCAGTCTTCGGGG CGGCTGA	
RTE-3_BF, Branchiostoma floridae, RT and EN	NTNNNNN NNNNNNT NNNNNNN NNNNNNN NNNNNNN GNNNNNN NNNNNNN NNNNNNN NNNNNNN NNNNNNN NNTNNNT NNNNNNN NNNNNAN NNNNNNN NNnnnnnnn nnnnntnnnn nnnnnnnnnn gngnnnnnnt nnnnnennnn nnnntnnnnn nnnnnnnnnt nnnnnnnnnn nnnannnnnn nnnnnnnnnn	(SEQ ID NO: 39) MSGTPRVAFDSGKDLRNPIGQSPPALSRAA PGQLGTDPSRSACFIGCLELRVLLDKWIVC RAPDKQESKEKRRQKTQPIRIGSWNVRTM RTGLSDDLTVIEDIRKTAAIDRELYRLNIDI VALQETRLPDSGSLKEDSYTFFWQGKGME ETREHGVGFAVRNTLLHMIEPPTGGTERIIT LRLSTHEGPVNLLCVYAPTLQATSEVKDQF YGQLDSAIKKIPVSEHIFILGDFNARVGTDQ ESWQTVLGHHGIGKMNENGQRLLELCCY HNLCVTNTFFQNKAIHKASWRHPRSQRWH QLDLVITRRTSLNSVCNTRAYHSADCDTD HSLIAARIKLRPKKLHHMKKKGQPKIDVSK TMLPDRNQKFLECLEGTLNNIQPQDAEHR WETLSKTIYSAAAQSYGKKERKNTDWFEA YISELEPVMDTKRKALVSYKQNPSSQNLQ ALKAARQEAQRASRRCANNYWLLLSERIQ LASATGDIRRMYEGIKQATGKPIKKSAPLK AKSGEIITDKDKQMARWVEHYLDIYSTEN SVSQDALDNIEDFSVLAELDADPTIEELSKA IDSMSNGKAPGEDNIPAEIIKSGKSVLLEPL HELLRLCWKEGKVPQSMRNSKIVTLYKNK GDRTIDCNSYRGISLLSIVGKVFAKVVLTRL QVLADRVYPESQCGFRAERSTTDMIFSVRQ LQEKCREQQRPLYIAFIDLTKAFDLVSRRG	-		(SEQ ID NO: 51) TCTGTAAATGGCTGTGT GATGCGTCTAGTGTGTA GGCGTAGCGGTAGTGT GCTTGCCACTTCTCGCT TTCAGCCCTCACCGCTG GCTAGCGGAGCTGCAT GCAGCACTGCGAAAAG AGAGCCGAGTGCGTAA GTCTCTCCTGCCAGTAC ATAGCCTCTCTCCACAGC AAGTCCCCCATTGCAGT GCCTCCTCGTGGCTACA GACGGAAACTGGGCAC CGTAAGGCCCCCAGGCT AAACTGCAGGGAGCTC GGAGGAGGTCTGGCCC CCAGACGCACGGCATG CATGGCCCACCGGCGT GTGGACACGCCCTGAT GCCTGCGAACCAGACC CCCAGCTATGGGCAAA TAGCACGGGTAGACGG AGCTCGTCAGCCTTGG ATGGCAGTTCGTCTAG	(SEQ ID NO: 62) TGACCTGATACAGA GCGCTACCATCATCT GGAAAGATGGAAGG ATGCCTACTAC

Element, Organism, Domains	Target Motif	Consensus Optimized Protein Sequence	5' Human Homology Arm	3' Human Homology Arm	5' UTR	3' UTR
	nnn (SEQ ID NO:28)	LFQLLRKIGCPPQLLDIIISFHEDMKGVVSF DGETSEPFAIRSGVKQGCVLAPTLFGIFFSL LLKSAFGHSTQGVHLHTRSDGKLFNLARL RAKTKVRSVLIRDMLFADDAALVAHVEDE LQQLLNQFAHACSEFALTISIKKTVVMGQD VPQPPVVTIGSEVLEVTDHFTYLGSTVTSN LSLDKEIDRRIARAAGVMTKLGTRVWNNS HLTLNTKLEVYRSCVLSTLLYGSETWTTY AKQENRLESFHLRCLRRILGISWRDRVPNT TVLERSCSLSIHLLLCQRRLRWLGHVSRMK DGRIPKDILFGELATGKRPVGRPALRFRDV CKRDLKLTDIDPASWEQIAADRNRWRHTV KDGLAKGQERRTEHLESRRRKRKEKPQQG NPSAFICPNCGRDCHARIGLQSHSRRCQPP			GGGAAGGAAAACCCTG ATTCAAAAACCTCCGCT GCCTTGCGGCTATACCC AGTCCTGGGAAAGGCT ACGGGAGTTAACCCAG AGAGAAAATCCGGAGT GGAGTACGTGAGGCGG TTGGCTGTCAAACTCTG TCATCCTTCCGGCAACT CCTGCAGCCAAACCAA CGCCAAGTGTCACGCC TCGCGTTCCCTTGGACC ACGTCGGTGAGGTCGA GAGGGGGGTCCTGTTG TGTTTTTGGGCAGCGCA GGTCCTCCATAAACCTG CCCAGGCTAGCGCTCT GGAGAGGCCACTCCAG TCGCCCCCATCACTGGG GGTGAGAAACAAACCG GGAGACAGCAGTTTAC GGGTTATAAGTCCTTGC TAAATTGACGTAA	
RTE-25_LMi, Locusta migratoria, RT and EN	NNNNNNNN NNNNNNNN NNNNNNNN NANNNNNN NNNNNNNN NNNNANNN NNNCNNNN ANNNNNNN NNNNNNNN NNNNNNNN NNNNNNNN NNNNNNNN NNNNNNNN	(SEQ ID NO: 40) MPRKNWNCGRRTGDEKRKMMFGCWNVQ GISTKIDLLPAELDMFNIDVVVLSETKRKG KGEEELDNYVHIWSGVSKAVRAKAGVSIM IQKKWKKRITNWTFINERITVVEMTLFARE VVIIGVYAPTNDTKDKEKDAFWDTLRETIE KIPRRKELIIMGDMNGRVGIRESCKIVGKH GEAEYNDNGERLIDICAQFDLKITNTFFKH KDIHKYTWQQNTKELRSIIDYIIIRQTSSFK AADVRSYRGAQCGSDHYLVKMKSFWPW KNATNDTSNINKMNCSEKVQNVHFNIDSL QDESIRTFFKARMERTLDESFEGSTEEIYEY IKTKVKNVASEVLGIKENNPKRAAEWWSE			(SEQ ID NO: 52) CCCGTGTGGAGTTTGCT GGTCTTCCATCGGGCAC CTCCCCAGGTGGCGGA TAGGGGAATGCTCACC AGATATGGTGGGTACC GGGGAAATAAAATACC CGGGGTGGACCAAAAC CAGCAACTGCTGCCTTG TAGTATGATATTGGCTT ATCAAAGGCTAAAGGA AGAAAACCTTGAATAC AAATTACCTGGTCCTCC	(SEQ ID NO: 63) TAGTGTAAAACCTTA TGTACTAGGTGTATT CATTTCTGGGCGTAT TAGTATGTTGGAGGT AAACCTCTGTAATG AGGACAATCTCAAT AATAAAATAAAATA AA

Element, Organism, Domains	Target Motif	Consensus Optimized Protein Sequence	5' Human Homology Arm	3' Human Homology Arm	5' UTR	3' UTR
	NNNNNCNNNnnnnnnnnnnannnnnnnnnnnnantnntnnnnnnnnnnnnnnnannnnnnnnnnnnngnnnnnnannnnntnnnnnnannnnnnnnnnnannnnnn (SEQ ID NO:29)	EIETSVKEKRNAFVQWLNDKSEGTRSKYKEKKNEVEKKIRLAKNEAWERTCANVNSKLGFGRAKEAWSVLKALRQDTKGKSNLQLVTQKEWEEYFKKLLNEDRDEYLEEGTVEENEHHDDEILISESEVLQVLRTGKNGKSPGPGNINMEFLKYGGDKIVKLILQLFNKMLHGDSVPKEMKLGYISTIFKKGDRKICSNYRGICVTNTLMRIFGKIIKNKLEKNFRTQQEQCGFTAGRSCVDHIFTLRQILEKHREKSKNVGLIFIDLEKAYDTVPRKLLWRALHRANINTSLIKIIEQMYKDNICQVKIGNTLSQKFRTSKGLLQGCPMSPTLFKIYIDICLRTWSQKCNSMGLEIRDGVYLHHLLFADDQVVIAQDGEDANYMCNQLAIAYKNWGLKINYQKTEYLTNDPHELRIEGKKIKKVNTFCYLGSILETEGKSDSEINKRISSGRKVIGMLNSVLWSRNVMNRTKKIIYKSIFESAVLYGAETWTINQKHTKKLQALEMDFWRRSARISRKEKRRNTEVIKRMEIIERIDEVMDRKKLRWYGHVRRMEDTRIPKLVLEWQPEGRRRRGRPVTTWIKNVQLTMNRLGAEEEDTQDRHTWRNIVNN			AGGTTGGGGGTTGTGCAGTGGGCCAGCTCCTCACTCACATAAAAATATAAAATGCTAAAAAACCTAATAAT	

Table 5A. Additional retrotransposon amino acid sequences

Name	Amino Acid Sequence
RTE-1_MD RNAIVT 1901	MPKKKRKVGGSDSTAHPNQGRGLEKVSQTLPALQTPGQHTAAGGSSPLSGRNQRKNTKKLLLGAWNIRTLLDRENTPRPERRTALIGKELARYNIDIAALSETRLPEEGSLSEPTTGYTFFWKGRASNEDRIHGVGLAIKTSLLKQLPDLPVGISERLMKIRLPLSKDRYATIISAYAPTLTSTEETIEQFYSDLSAVLHSVPTNDKLILLGDFNARVGQDHERWKGVLGKHGVGKMNNNGLLLLSKCSEFELTITNTVFRMANKYKTTWMHPRSKQWHLIDYIIVRRRDIQDVKITRAMRGAECWTDHRLVRATLQMRIAPRHPKRAQTVRAFYNVSRLRDPSYLQTFQSCLDDKLSAKGPLTGSSTEKWNQFRDAVKETSKAVLGPKQRNHQDWFDENNTAIEDLLSKKNKAFMEWQNNPNSAPKKDRFKSLQATAQREIRKMQDRWWEKKAEEIQRFADMKNYKQFFSALKTVYGPLKPTTTPLLSSDGDTLIKDKKGISNRWKEHFSQLLNRPSSVDQSALDQIPQNRTIEQLDVPPSIEEVQKAIKQMSAGKAPGKDGIPTEVYKALNGKALQAFHIVLTSIWEEEDMPPELRDASIVALYKNKGSRAACDNYRGISLLSTAGKILARVILNRLLSSVSEQNLPESQCGFRPDRSTIDMVFTVRQMQEKCLEQNLSLYIVFIDLTKAFDTVNRDALWVILSKLGCPAKFVKLIQLFHVDMTGEVLSGGETSDRFNISNGVKQGCVLAPVLFNLFFTQVLRHAVMDLDLGVYIKYRLDGSLFDLRRLTAKTKTTERLILEALFADDCALMAHQENHLQTIVDRFSTATKLFGLTISLSKTEVLFQPAPGRPTNQPCITIDGTQLSNVNTFKYLGSTIANDGSLDHEINARIQKASQALGRLRCKVLQHRGVSTATKLKVYNAVVLSSLLYGC

	ETWTLYRKHMKQLEQFHQRSLRSIMRIRWQDRITNQEVLDRANSTSIEVMVLKTQLRWSGHVIRMDPQRIPRQVFYGELSAGLRKQGRPKKRF KDQLKSNLKWAGITPKQLELAASDRSSWRTHINHAATTFEDERRRRLAAARERRHQATTAPPVTTGVPCPMCHKLCASAFGLQSHMRVHRRS GSETPGTSESATPESVSGWRLFKKIS (SEQ ID NO:64)
Vingi-1_Acar RNAIVT 696	MDEYQRSLSRPLLTIMSINIEGLSLAKEELLAKMSEDISCDILCIQETHRDITMRRPKILGMQLAVERPHRQYGSAIFVRSGVAISATSLTEVNNIEI LSVELDSCTVSSLYKPPGADFYFTPPTSCHNHEAHFVVGDFNSHSCVWGYDEDDRNGEAVLTWADNSRMSLLHDSKLPPSFNSGRWKRGYNP DLIFVKESISHQCTKRVLNPIPNTQHRPICCVAYAAVRPKSVPFRRRYNFNKANWTKFTETLEAAISDIEPSIENYDLFVEAVKRSSRLSIPRGCRT SYLPGLNEESLNQLQEYLRLFQENPYSDGTIAAGQKLSTALANAKKDRWIELLENLDMSKSSRKAWQLLRRLDSDPLVNPGHANVTPDQIAHQ LIQNGKTNCSRIKMKINRVPELETHQLSSPLNLKELREAIKRCKTGKAPGLDDLMMEQIKHLGAKAENWLLKFYNQCLAHKQIPRAWRKTKIIA ILKPGKDASNARNYRPISLLCHLYKVYERMLLNRLGPVIEPKLIAQQAGFRPGKNCTGQILHLTEHIEEGYEKGCTTGTVFVDLTAAYDTVQHR KMLHKVYHITRDFDFTKTVQTLLENRSFYVEFQGQKSRWRRQKNGLPQGSVLAPTLFNIFTNDQPQPPLTKSFIYADDLGLTTQAKDFETVEKQ LTNALKDLSSYYKENHLKPNPAKTQVCAFHLRNREANRKLKVTWEGQELEHCFHPKYLGVTLDRTLTYRKHCMNTKHKVAARNNILRKLTG SAWGADPQVIRTSALALSFSTAEYACPVWHKSAHAKQVDIALNETCRIITGCLKPTPVDKLYKLAGIAPPDVRREVAANGERKKVEHCESHPL HGYHPPPTRLKSRKGFMRTTTPLDVPPAAARVSLWAAKPGNSNWMAPQEGLPPGANQEWATWKSLNRLRSGVGRSKDNLARWHYLEESSTL CDCGAEQTTQHMYACPQCPASCTEEELFKATDNAVAVARFWSKTIGGGSPKKKRKVSGSETPGTSESATPESVSGWRLFKKIS (SEQ ID NO:65)
CR1-1_PH RNAIVT 636	MLYTIFLVYGILCFFFVYFCIYLYTTVYLCFLFFLFLLLCGDVESNPGPGRARGCRLLYCNIRGLHANLAELDFVSRGVDVVCCSETLVAGRRHD AELALAGFQSPFRRLCGSGPGFRGMAVYVRSGFCAYRQSVHECACHEIIVVRVCGRLNNYYLFSLYRSPATDDSLYDCLLTAMASIQSTDPKA AFVFVGDVNAHHRDWLGSASPTDCHGVAALDFCTLSSCVQLVRGSTHIAGNCLDLVMTDVPDLMTVTVGSPIGSSDHSHLVVSLDLNQVVPV VDTRRVVFVKSRANWVAITRAVRTLPWRQIIHSEDPVSELNDLTVSILERFVPKRTILVRSRDKPWFDDQCRLAFEAKQAAYRAWRRSRDRTL WQTYVDRRAEAKRVYEEAQRRLRQRSRESLLSIDHPHRWWSELKGSVFGAEPSLPPLVGPGGGIITDPLARAELLSAHFDGKQSRDVIALPHGC HPEPRLTSLAFRSGAIKVLLEGLDPYGGVDPVGMFPLFYKQLADVLAPKLAVIFRRLIRLGNFPRCWRTGNITPIPKGPVSPYVVANYRPITLTPILS KVFEKLIAGKLGRFAEVTGLLPAGQFAYRIGLGCCDALLSVSHHLQSALDCRSEARLVQLDFSAAFDRVNHRGLLYKLESFGVGGRVLSIIRDF VSERTQSVSVDGVLSASVGVVSGVPQGSVLGPLLFVLYTSDMFSSLENTLINYADDSTLMAVIPAPRLRDAVAQSLNRDLSRISAWCSAWSMK LNASKTKSMIISRSRTLVPQHPQLEIDDTLLQESSSLEILGVVFDEKLTFEPHIRRLVSRASTKIGLLRKVNSVFGDSQVARRCFYAFLLPVLEYCS PVWASAADTHLRLLLDRLVSSASRLCADNDLVNLSHRRRVAELCMFYKVYNNERHWLYSSLPALKVFGRETRAACGAHSFTLEAVRCRTNQF CRCFVPWSAKVWNLLPASAFGRIGLQAFKSAVNGFLLDFLGGGSPKKKRKVSGSETPGTSESATPESVSGWRLFKKIS (SEQ ID NO:66)
RTE-3_BF RNAIVT 1770	MPKKKRKVGGSSGTPRVAFDSGKDLRNPIGQSPPALSRAAPGQLGTDPSRSACFIGCLELRVLLLDKWIVCRAPDKQESKEKRRQKTQPIRIGSW NVRTMRTGLSDDLTVIEDIRKTAAIDRELYRLNIDIVALQETRLPDSGSLKEDSYTFFWQGKGMEETREHGVGFAVRNTLLLHMIEPPTGGTERII TLRLSTHEGPVNLLCVYAPTLQATSEVKDQFYGQLDSAIKKIPVSEHIFILGDFNARVGTDQESWQTVLGHHGIGKMNENGQRLLELCCYHNLC VTNTFFQNKAIHKASWRHPRSQRWHQLDLVITRRTSLNSVCNTRAYHSADCDTDHSLIAARIKLRPKKLHHHMKKKGQPKIDVSKTMLPDRNQ KFLECLEGTLNNIQPDAEHRWETLSKTIYSAAAQSYGKKERKNTIDWFEAYISELEPVMDTKRKALVSYKQNPSSQNLQALKAARQEAQRAS RRCANNYWLLLSERIQLASATGDIRRMYEGIKQATGKPIKKSAPLKAKSGEIITDKDKQMARWVVEHYLDIYSTENSVSQDALDNIEDFSVLAEL DADPTIEELSKAIDSMSNGKAPGEDNIPAEIIKSGKSVLLEPLHELLRLCWKEGKVPQSMRNSKIVTLYKNKGDRTDCNSYRGISLLSIVGKVFA KVVLTRLQVLADRVYPESQCGFRAERSTTDMIFSVRQLQEKCREQQRPLYIAFIDLTKAFDLVSRRGLFQLLRKIGCPPQLLDIIISFHEDMKGVV SFDGETSEPFAIRSGVKQGCVLAPTLFGIFFSLLLKSAFGHSTQGVHLHTRSDGKLFNLARLRAKTKVRSVLIRDMLFADDAALVAHVEDELQQ LLNQFAHACSEFALTISIKKTVVMGQDVPQPPVVTIGSEVLEVTDHFTYLGSTVTSNLSLDKEIDRRIARAAGVMTKLGTRVWNNSHLTLNTKL EVYRSCVLSTLLYGSETWTTYAKQENRLESFHLRCLRRILGISWRDRVPNTTVLERSCSLSIHLLLCQRRLRWLGHVSRMKDGRIPKDILFGELA TGKRPVGRPALRFRDVCKRDLKLTDIDPASWFQIAADRNRWRHTVKDGLAKGQERRTFEHLESRRRKREKPQQGNPSAFICPNCGRDCHARI GLQSHSRRCQPPSGSETPGTSESATPESVSGWRLFKKIS (SEQ ID NO:67)

RTE-25_LMi RNAIVT 1772	MPRKNWNCGRRTGDEKRKMMFGCWNVQGISTKIDLLPAELDMFNIDVVVLSETKRKGKGEEELDNYVHIWSGVSKAVRAKAGVSIMIQKK WKKRITNWTFINERIITVEMTLFAREVVIIGVYAPTNDTKDKEKDAFWDTLRETIEKIPRRKELIIMGDMNGRVGIRESCKIVGKHGEAEYNDNG ERLIDICAQFDLKITNTFFKHKDIHKYTWQQNTKELRSIIDYIIIRQTSSFKAADVRSYRGAQCGSDHYLVKMKSFWPWKNATNDTSNINKMNC SEKVQNVHFNIDSLQDESIRTFFKARMERTLDESFEGSTEEIYEYIKTKVKNVASEVLGIKENNPKRAAEWWSEEIETSVKEKRNAFVQWLNDK SEGTRSKYKEKKNEVEKKIRLAKNEAWERTCANVNSKLGFGRAKEAWSVLKALRQDTKGKSNLQLVTQKEWEEYFKKLLNEDRDFEYLEEGT VEENEHHDDEILISESEVLQVLRTGKNGKSPGPGNINMEFLKYGGDKIVKLILQLFNKMLHGDSVPKEMKLGYISTIFKKGDRKICSNYRGICVT NTLMRIFGKIIKNKLEKNFRTQQEQCGFTAGRSCVDHIFTLRQILEKHREKSKNVGLIFIDLEKAYDTVPRKLLWRALHRANINTSLIKIIEQMYK DNICQVKIGNTLSQKFRTSKGLLQGCPMSPTLFKIYIDICLRTWSQKCNSMGLEIRDGVYLHHLLFADDQVVIAQDGEDANYMCNQLAIAYKN WGLKINYQKTEYLTNDPHELRIEGKKIKKVNIFCYLGSILETEGKSDSEINKRISSGRKVIGMLNSVLWSRNVMNRTKKIYKSIFESAVLYGAET WTINQKHTKKLQALEMDFWRRSARISRKEKRRNTEVIKRMEIIERIDEVMDRKKLRWYGHVRRMEDTRIPKLVLEWQPEGRRRRGRPVTTWI KNVQLTMNRLGAEEEDTQDRHTWRNIVNNGGGSPKKKRKVSGSETPGTSESATPESVSGWRLFKKIS (SEQ ID NO:68)
RTE-1_MD RNAIVT 1123	MDSTAHPNQGRGLEKVSQTLPALQTPGQHTAAGGSSPLSGRNQRKNTKKLLLGAWNIRTLLDRENTPRERTALIGKELARYNIDIAALSETR LPEEGSLSEPTTGYTFFWKGRASNEDRIHGVGLAIKTSLLKQLPDLPVGISERLMKIRLPLSKDRYATIISAYAPTLTSTEETIEQFYSDLSAVLHS VPTNDKLILLGDFNARVGQDHERWKGVLGKHGVGKMNNNGLLLLSKCSEFELTITNTVFRMANKYKTTWMHPRSKQWHLIDYIIVRRRDIQD VKITRAMRGAECWTDHRLVRATLQMRIAPRHPKRAQTVRAFYNVSRLRDPSYLQTFQSCLDDKLSAKGPLTGSSTEKWNQFRDAVKETSKAV LGPKQRNHQDWFDENNTAIEDLLSKKNKAFMEWQNNPNSAPKKDRFKSLQATAQREIRKMQDRWWEKKAEEIQRFADMKNYKQFFSALKT VYGPLKPTTTPLLSSDGDTLIKDKKGISNRWKEHFSQLLNRPSSVDQSALDQIPQNRTIEQLDVPPSIEEVQKAIKQMSAGKAPGKDGIPTEVYK ALNGKALQAFHIVLTSIWEEEDMPPELRDASIVALYKNKGSRAACDNYRGISLLSTAGKILARVILNRLLSSVSEQNLPESQCGFRPDRSTIDMV FTVRQMQEKCLEQNLSLYIVFIDLTKAFDTVNRDALWVILSKLGCPAKFVKLIQLFHVDMTGEVLSGGETSDRFNISNGVKQGCVLAPVLFNLF FTQVLRHAVMDLDLGVYIKYRLDGSLFDLRRLTAKTKTTERLILEALFADDCALMAHQENHLQTIVDRFSTATKLFGLTISLSKTEVLFQPAPG RPTNQPCTITIDGTQLSNVNTFKYLGSTIANDGSLDHEINARIQKASQALGRLRCKVLQHRGVSTATKLKVYNAVVLSSLLYGCETWTLYRKHM KQLEQFHQRSLRSIMRIRWQDRITNQEVLDRANSTSIEVMVLKTQLRWSGHVIRMDPQRIPRQVFYGELSAGLRKQGRPKKRFKDQLKSNLKW AGITPKQLELAASDRSSWRTHINHAATTFEDERRRRLAAARERRHQATTAPPVTTGVPCPMCHKLCASAFGLQSHMRVHRRSGSETPGTSESA TPESVSGWRLFKKIS (SEQ ID NO:69)
Vingi-1_Acar RNAIVT 696	MDEYQRSLSRPLLTIMSINIEGLSLAKEELLAKMSEDISCDILCIQETHRDITMRRPKILGMQLAVERPHRQYGSAIFVRSGVAISATSLTEVNNIEI LSVELDSCTVSSLYKPPGADFYFTPPTSCHNHEAHFVVGDFNSHSCVWGYDEDDRNGEAVLTWADNSRMSLLHDSKLPPSFNSGRWKRGYNP DLIFVKESISHQCTKRVLNPIPNTQHRPICCVAYAAVRPKSVPFRRRYNFNKANWTKFTETLEAAISDIEPSIENYDLFVEAVKRSSSRLSIPRGCRT SYLPGLNEESLNQLQEYLRLFQENPYSDGTIAAGQKLSTALANAKKDRWIELLENLDMSKSSRKAWQLLRRLDSDPLVNPGHANVTPDQIAHQ LIQNGKTNCSRIKMKINRVPELETHQLSSPLNLKELREAIKRCKTGKAPGLDDLMMEQIKHLGAKAENWLLKFYNQCLAHKQIPRAWRKTKIIA ILKPGKDASNARNYRPISLLCHLYKVVERMLLNRLGPVIEPKLIAQQAGFRPGKNCTGQILHLTEHIEEGYEYEKGGCITGTVFVDLTAAYDTVQHR KMLHKVYHITRDFDFTKTVQTLLENRSFYVEFQGQKSRWRRQKNGLPQGSVLAPTLFNIFTNDQPQPPLTKSFIYADDLGLTTQAKDFETVEKQ LTNALKDLSSYYKENHLKPNPAKTQVCAFHLRNREANRKLKVTWEGQELEHCFHPKYLGVTLDRTLTYRKHCMNTKHKVAARNNILRKLTG SAWGADPQVIRTSALALSFSTAEYACPVWHKSAHAKQVDIALNETCRIITGCLKPTPVDKLYKLAGIAPPDVRREVAAANGERKKVEHCESHPL HGYHPPPTRLKSRKGFMRTTTPLDVPPAAARVSLWAAKPGNSNWMAPQEGLPPGANQEWATWKSLNRLRSGVGRSKDNLARWHYLEESSTL CDCGAEQTTQHMYACPQCPASCTEEELFKATDNAVAVARFWSKTIGGGSPKKKRKVSGSETPGTSESATPESVSGWRLFKKIS (SEQ ID NO:65)
CR1-1_PH	MLYTIFLVYGILCFFFVYFCIYLYITVYLCFLFFLFLLLCGDVESNPGPGRARGCRLLYCNIRGLHANLAELDFVSRGVDVVCCSETLVAGRRHD AELALAGFQSPFRRLCGSGPGFRGMAVYVRSGFCAYRQSVHECACHEIIVVRVCGRLNNYYLFSLYRSPATDDSLYDCLLTAMASIQSTDPKA AFVFVGDVNAHHRDWLGSASPTDCHGVAAALDFCTLSSCVQLVRGSTHIAGNCLDLVMTDVPDLMTVTVGSPIGSSDHSHLVVSLDLNQVVPV

RNAIVT 636	VDTRRVVFVKSRANWVAITRAVRTLPWRQIIHSEDPVSELNDLTVSILERFVPKRTILVRSRDKPWFDDQCRLAFEAKQAAYRAWRRSRDRTL WQTYVDRRAEAKRVYEEAQRRLRQRSRESLLSIDHPHRWWSELKGSVFGAEPSLPPLVGPGGGIITDPLARAELLSAHFDGKQSRDVIALPHGC HPEPRLTSLAFRSGAIKVLLEGLDPYGGVDPVGMFPLFYKQLADVLAPKLAVIFRRLIRLGNFPRCWRTGNITPIPKGPVSPYVVANYRPITLTPILS KVFEKLIAGKLGRFAEVTGLLPAGQFAYRIGLGCCDALLSVSHHLQSALDCRSEARLVQLDFSAAFDRVNHRGLLYKLESFGVGGRVLSIIRDF VSERTQSVSVDGVLSASVGVVSGVPQGSVLGPLLFVLYTSDMFSSLENTLINYADDSTLMAVIPAPRLRDAVAQSLNRDLSRISAWCSAWSMK LNASKTKSMIISRSRTLVPQHPQLEIDDTLLQESSSLEILGVVFDEKLTFEPHIRRLVSRASTKIGLLRKVNSVFGDSQVARRCFYAFLLPVLEYCS PVWASAADTHLRLLDRLVSSASRLCADNDLVNLSHRRRVAELCMFYKVYNNERHWLYSSLPALKVFGRETRAACGAHSFTLEAVRCRTNQF CRCFVPWSAKVWNLLPASAFGRIGLQAFKSAVNGFLLDFLGGGSPKKKRKVSGSETPGTSESATPESVSGWRLFKKIS (SEQ ID NO:66)
RTE-3_BF RNAIVT 635	MSGTPRVAFDSGKDLRNPIGQSPPALSRAAPGQLGTDPSRSACFIGCLELRVLLDKWIVCRAPDKQESKEKRRQKTQPIRIGSWNVRTMRTGLS DDLTVIEDIRKTAAIDRELYRLNIDIVALQETRLPDSGSLKEDSYTFFWQGKGMEETREHGVGFAVRNTLLHMIEPPTGGTERIITLRLSTHEGPV NLLCVYAPTLQATSEVKDQFYGQLDSAIKKIPVSEHIFILGDFNARVGTDQESWQTVLGHHGIGKMNENGQRLLELCCYHNLCVTNTFFQNKAI HKASWRHPRSQRWHQLDLVITRRTSLNSVCNTRAYHSADCDTDHSLIAARIKLRPKKLHHMKKKGQPKIDVSKTMLPDRNQKFLECLEGTLN NIQPQDAEHRWETLSKTIYSAAAQSYGKKERKNTDWFEAYISELEPVMDTKRKALVSYKQNPSSQNLQALKAARQEAQRASRRCANNYWLL LSERIQLASATGDIRRMYEGIKQATGKPIKKSAPLKAKSGEIITDKDKQMARWVEHYLDIYSTENSVSQDALDNIEDFSVLAELDADPTIEELSK AIDSMSNGKAPGEDNIPAEIIKSGKSVLLEPLHELLRLCWKEGKVPQSMRNSKIVTLYKNKGDRTDCNSYRGISLLSIVGKVFAKVVLTRLQVL ADRVYPESQCGFRAERSTTDMIFSVRQLQEKCREQQRPLYIAFIDLTKAFDLVSRRGLFQLLRKIGCPPQLLDIIISFHEDMKGVVSFDGETSEPFA IRSGVKQGCVLAPTLFGIFFSLLLKSAFGHSTQGVHLHTRSDGKLFNLARLRAKTKVRSVLIRDMLFADDAALVAHVEDELQQLLNQFAHACS EFALTISIKKTVVMGQDVPQPPVVTIGSEVLEVTDHFTYLGSTVTSNLSLDKEIDRRIARAAGVMTKLGTRVWNNSHLTLNTKLFVYRSCVLST LLYGSETWTTYAKQENRLESFHLRCLRRILGISWRDRVPNTTVLERSCSLSIHLLLCQRRLRWLGHVSRMKDGRIPKDILFGELATGKRPVGRP ALRFRDVCKRDLKLTDIDPASWEQIAADRNRWRHTVKDGLAKGQERRTEHLESRRRKRKFEKPQQGNPSAFICPNCGRDCHARIGLQSHSRRC QPPSGSETPGTSESATPESVSGWRLFKKIS (SEQ ID NO:70)
RTE-25_LMi RNAIVT 927	MPRKNWNCGRRTGDEKRKMMFGCWNVQGISTKIDLLPAELDMFNIDVVVLSETKRKGKGEEELDNYVHIWSGVSKAVRAKAGVSIMIQKK WKKRITNWTFINERIITVEMTLFARFVVIIGVYAPTNDTKDKEKDAFWDTLRETIEKIPRRKFLIIMGDMNGRVGIRESCKIVGKHGEAEYNDNG ERLIDICAQFDLKITNTFFKHKDIHKYTWQQNTKELRSIIDYIIIRQTSSFKAADVRSYRGAQCGSDHYLVKMKSFWPWKNATNDTSNINKMNC SEKVQNVHFNIDSLQDESIRTFFKARMERTLDESFEGSTEEIYEYIKTKVKNVASEVLGIKENNPKRAAEWWSEEIETSVKEKRNAFVQWLNDK SEGTRSKYKEKKNEVEKKIRLAKNEAWERTCANVNSKLGFGRAKEAWSVLKALRQDTKGKSNLQLVTQKEWEEYFKKLLNEDRDEYLEEGT VEENEHHDDEILISESEVLQVLRTGKNGKSPGPGNINMEFLKYGGDKIVKLILQLFNKMLHGDSVPKEMKLGYISTIFKKGDRKICSNYRGICVT NTLMRIFGKIIKNKLEKNFRTQQEQCGFTAGRSCVDHIFTLRQILEKHREKSKNVGLIFIDLEKAYDTVPRKLLWRALHRANINTSLIKIIEQMYK DNICQVKIGNTLSQKFRTSKGLLQGCPMSPTLFKIYIDICLRTWSQKCNSMGLEIRDGVYLHHLLFADDQVVIAQDGEDANYMCNQLAIAYKN WGLKINYQKTEYLTNDPHELRIEGKKIKKVNTFCYLGSILETEGKSDSEINKRISSGRKVIGMLNSVLWSRNVMNRTKKIIYKSIFESAVLYGAET WTINQKHTKKLQALEMDFWRRSARISRKEKRRNTEVIKRMEIIERIDEVMDRKKLRWYGHVRRMEDTRIPKLVLEWQPEGRRRRGRPVTTWI KNVQLTMNRLGAEEEDTQDRHTWRNIVNNSGSETPGTSESATPESVSGWRLFKKIS (SEQ ID NO:71)

[0402] In some embodiments, a gene modifying system is capable of producing an insertion into the target site of at least 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 nucleotides (and optionally no more than 500, 400, 300, 200, or 100 nucleotides). In some embodiments, a gene modifying system is capable of producing an insertion into the target site of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 nucleotides (and optionally no more than 500, 400, 300, 200, or 100 nucleotides). In some embodiments, a gene modifying system is capable of producing an insertion into the target site of at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 or 10 kilobases (and optionally no more than 1, 5, 10, or 20 kilobases).

Template RNA component of retrotransposon gene modifying systems

[0403] The gene modifying polypeptides described herein can be used together with a template RNA, to install a mutation into a target RNA. In some embodiments, the template RNA comprises a 5' UTR, a heterologous object sequence, and a 3' UTR.

[0404] In some embodiments, the template RNA has a 3' untranslated region derived from a retrotransposon, e.g., a retrotransposon described herein. In some embodiments the template RNA has a 3' region of at least 10, 15, 20, 25, 30, 40, 50, 60, 80, 100, 120, 140, 160, 180, 200 or more bases of at least 50%, 60%, 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% homology to the 3' sequence of a retrotransposon, e.g., a retrotransposon described herein, e.g. a retrotransposon in Table 4. In some embodiments, the template RNA has a 5' untranslated region derived from a retrotransposon, e.g., a retrotransposon described herein. In some embodiments the template RNA has a 5' region of at least 10, 15, 20, 25, 30, 40, 50, 60, 80, 100, 120, 140, 160, 180, or 200 or more bases of at least 40%, 50%, 60%, 70%, 80%, 90%, 95% or greater homology to the 5' sequence of a retrotransposon, e.g., a retrotransposon described herein, e.g., a retrotransposon described in **Table 4**.

[0405] The template RNA may have some homology to the target DNA. In some embodiments the template RNA has at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 175, 200 or more bases of exact homology to the target DNA at the 3' end of the RNA. In some embodiments the template RNA has at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 175, 180, or 200 or more bases of at least 50%, 60%, 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% homology to the target DNA, e.g., at the 5' end of the template RNA.

[0406] In some embodiments, the template RNA has a poly-A tail at the 3' end. In some embodiments, the template RNA does not have a poly-A tail at the 3' end.

[0407] In some embodiments, a system or method described herein comprises a single template RNA. In some embodiments, a system or method described herein comprises a plurality of template RNAs.

[0408] In some embodiments, the template nucleic acid may comprise one or more UTRs (e.g., a 5' UTR or a 3' UTR, e.g., from an R2-type retrotransposon). In some embodiments, the UTR facilitates interaction of the template with the reverse transcriptase domain of the polypeptide. In some embodiments, the template possesses one or more sequences aiding in association of the template with the gene modifying polypeptide. In some embodiments, these sequences may be derived from retrotransposon UTRs. In some embodiments, the UTRs may be located flanking the desired insertion sequence. In some embodiments, a sequence with target site homology may be located outside of one or both UTRs. In some embodiments, the sequence with target site homology can anneal to the target sequence to prime reverse transcription. In some embodiments, the 5' and/or 3' UTR may be located terminal to the target site homology sequence. In some embodiments, the gene modifying system may result in the insertion of a desired payload without any additional sequence (e.g., a gene expression unit without UTRs used to bind the gene modifying protein).

[0409] In some embodiments, the template RNA comprises one or more chemically modified nucleotides. In some embodiments, the template RNA comprises a backbone modification, e.g., a modification to a sugar or phosphate group in the backbone. In some embodiments, the template RNA comprises a nucleobase modification.

[0410] In some embodiments, the heterologous object sequence encodes a membrane protein, e.g., a CAR or a membrane protein other than a CAR, and/or an endogenous human membrane protein. In some embodiments, the heterologous object sequence encodes an extracellular protein. In some embodiments, the heterologous object sequence encodes an enzyme, a structural protein, a signaling protein, a regulatory protein, a transport protein, a sensory protein, a motor protein, a defense protein, or a storage protein. Other exemplary proteins that may be encoded by a heterologous object sequence include, without limitation, an immune receptor protein, e.g. a synthetic immune receptor protein such as a chimeric antigen receptor protein (CAR), a T cell receptor, a B cell receptor, or an antibody.

[0411] In some embodiments, an LNP described herein comprises a retrotransposon gene modifying system, as described herein. In some embodiments, the template RNA of the

retrotransposon gene modifying system comprises a template RNA sequence present in **Table 5B** or a template RNA sequence comprising 80% to 99% sequence identity (e.g., 80%, 85%, 90%, 95%, or 99% sequence identity) to a template RNA sequence present in **Table 5B**. In some embodiments, the template RNA of the retrotransposon gene modifying system comprises a heterologous object sequence comprising a CAR coding sequence present in **Table 5B** or a heterologous object sequence comprising 80% to 99% sequence identity (e.g., 80%, 85%, 90%, 95%, or 99% sequence identity) to a CAR coding sequence present in **Table 5B**.

Table 5B. Template RNA sequences encoding a CAR

Construct	Template RNA sequence	CAR coding sequence (RNA)
RNAIVT7266	AGGGUGUAUGGGGUGC	UUAUCUGGGGGGCAGG
	UCAGGGAGGGGUAGUA	GCCUGCAUGUGCAGGGC
	UCUCUGGUAUGGAGGG	GUCGUAGGUGUCCUUG
	CUUGUCGUGCCCUCCUA	GUGGCGGUGCUCAGGCC
	GGGCAGCUCUCCAGCCU	CUGGUACAGGCCAUCGU
	CUGACCCCCACCUGACA	GGCCCUUGCCGCGCCUC
	CCCAGCUCUCACUUGUG	CGCUCGCCCUUCAUGCC
	GCUCCCAGUAGCUGCUA	GAUCUCGCUGUAGGCCU
	GCAUGUGGCAGCGGCCA	CGGCCAUCUUGUCUUUC
	CACCCCGGGCAACGGCU	UGCAGUUCGUUAUACA
	UCGACAGGCCGGCUAAA	GGCCUUCCUGGGGGUUC
	CCUUGUGAGGGUAGCCA	UUCCGUCUGGGCUUUCC
	UCGGGUCAUCGACCCCU	GCCCAUCUCGGGGUCCC
	GGUGAACCAGGGCUUU	GGCCUCUCCGCUUGUCC
	GCUCACCCAGCAUGUGA	AGCACGUCGUACUCUUC
	AGACUGCUUCGGCUGAA	CCGUCUGCCCAGGUUCA
	CAGACGGAAGAAACCAA	GCUCGUUGUACAGCUGG
	UAAGAAGGUUCAACGG	UUCUGUCCCUGCUGGUA
	CUGAGAGGGCGACGCAG	GGCAGGGGCGUCGGCGG
	CAAAGCACUGUGGAGU	AUCUGCUGAACUUCACU
	GCUUAGGGCGUGUUGG	CUCAGUUCACAUCCUCC
	AGCACAAAGGACAACAC	UUCUUCUUCUUCUGGAA
	GGCCAUCCAAUGCAGCU	AUCGGCAGCUACAGCCA
	GAGGAAGUCUCCAGAU	UCUUCCUCUUGAGUAGU
	GUAACAAUUUUUCGUG	UUGUACUGGUCUCAUA
	CCACUGGACCCAGGCUU	AAUGGUUGUUUGAAUA
	CCAACGCCGAGAGAGUG	UAUACAGGAGUUUCUU
	GGACUGUCUCUGUGCAU	UCUGCCCCGUUUGUUCC
	CGGCUUUUCCACUUAAA	GGUGGUUGCAGUACAG
	UCUCUUUCACGCACAAG	GGUGAUCACCAGGCUCA
	UAUCUUUGUGCACACUC	GCAGCAGCACGCCACAG
	AUCUAUCCUAACCCCGU	GUGCCGGCCAGAGGGGC
	CCACCCUCUUCAAGACC	CCAGAUGUAGAUGUCGC
	UGCGGCGAUGGGGGAG	AGGCGAAGUCCAGGCCU
	UGGCGACGCAACAGGUG	CUGGUGUGCACGGCUCC
	GAGGUGACCACUGGCAG	GCCGGCAGCAGGUCUAC

	UUGUAGUCACGAUCCUG	AGGCUUCGGGCCGCAGA
	CACGUAGGCGGCCCACG	GACAGAGGCUGGCUGGC
	GACCAGUGGUCGCUCGG	GAUUGUUGGGGCUGGG
	CCCUGUGGGCAGCAGGG	GUGGGAGGUCUAGGGG
	ACGUUCGGCAGCAUCCU	CAGGGGUGGUGGUAGG
	GGGCGACUGAGCAGCCC	UUUGGCGGGCAGGAAC
	UCUCUAGGUUCUUUCCG	ACGGGCACGAAUGAGCU
	CCUCAGAAGCCAUAGAG	GACGGUGACCAUUGUAC
	CCCACCGCAUCCCCAGC	CCUGACCCCAUACGUCU
	AUGCCUGCUAUUGUCUU	AGUAUGGUGUCUCCACG
	CCCAAUCCUCCCCCUUG	AUCUCUGGCGCAGUAGU
	CUGUCCUGCCCCACCCC	ACACCGCCGUGUCUGCG
	ACCCCCCAGAAUAGAAU	GCGGUCACAGAACUCAG
	GACACCUACUCAGACAA	CUUCAGGGAGAACUGG
	UGCGAUGCAAUUUCCUC	UUCUUGGACGUGUCUAC
	AUUUUAUUAGGAAAGG	GGAUAUGGUGACUCGA
	ACAGUGGGAGUGGCACC	CUCUUGAGGGACGGGU
	UUCCAGGGUCAAGGAA	UGUAGUAGGUGCUCCCA
	GGCACGGGGGAGGGGC	CUAUAGGAGAUACUCCC
	AAACAACAGAUGGCUG	AAUCCACUCCAGCCCCU
	GCAACUAGAAGGCACAG	UCCCUGGGGGCUGGCGG
	UCGAGGCUGAUCAGCGA	AUCCAGCCCCAGUAGUA
	UUAUCUGGGGGGCAGG	ACUACUACUGCUGAUGG
	GCCUGCAUGUGCAGGGC	AGCCACCAGAGACAGUA
	GUCGUAGGUGUCCUUG	CAGGUGAGGGACAGGG
	GUGGCGGUGCUCAGGCC	UCUCCGAAGGCUUCACC
	CUGGUACAGGCCAUCGU	AGUCCUGGGCCCGACUC
	GGCCCUUGCCGCGCCUC	CUGCAGCUGCAGCUGUC
	CGCUCGCCCUUCAUGCC	CCUUUGUGCUGCCCUCG
	GAUCUCGCUGUAGGCCU	CCAGAGCCAGGCUUGCC
	CGGCCAUCUUGUCUUUC	GGAGCCGCUGGUGCUGC
	UGCAGUUCGUUAUACA	CUUUGAUCUCAACCUUG
	GGCCUUCCUGGGGGUUC	GUCCCUCCGCCAAAAGU
	UUCCGUCUGGGCUUUCC	GAGGAGGUCGUAUUUU
	GCCCAUCUCGGGGUCCC	UGCUGACAGUAGUAAG
	GGCCUCUCCGCUUGUCC	UUGCAAAAUCUUCAGG
	AGCACGUCGUACUCUUC	UUGCAGACUGCUGAUG
	CCGUCUGCCCAGGUUCA	GUGAGAGUGAAAUCUG
	GCUCGUUGUACAGCUGG	UCCCAGAUCCACUGCCA
	UUCUGUCCCUGCUGGUA	CUGAACCUUGAUGGGAC
	GGCAGGGGCGUCGGCGG	CCCACUUUGCAAACUGG
	AUCUGCUGAACUUCACU	AUGCAGCAUAGAUCAG
	CUCAGUUCACAUCCUCC	GAGCUUAGGGGCUUUCC
	UUCUUCUUCUUCUGGAA	CUGGUUUCUGCUGAUAC
	AUCGGCAGCUACAGCCA	CAAUUUAAAUAGCUGC
	UCUUCCUCUUGAGUAGU	UAAUGCUCUGACUUGCC
	UUGUACUGGUCUCAUA	CGGCAAGUGAUGGUGA
	AAUGGUUGUUUGAAUA	CUCUGUCUCCUACAGAU
	UAUACAGGAGUUUCUU	GCAGACAGGGAGGAUG
	UCUGCCCCGUUUGUUCC	GAGACUGGGUCAUCUG
	GGUGGUUGCAGUACAG	GAUGUCCGGGCGAGCGG

	GGUGAUCACCAGGCUCA GCAGCAGCACGCCACAG GUGCCGGCCAGAGGGGC CCAGAUGUAGAUGUCGC AGGCGAAGUCCAGGCCU CUGGUGUGCACGGCUCC GCCGGCAGCAGGUCUAC AGGCUUCGGGCCGCAGA GACAGAGGCUGGCUGGC GAUUGUUGGGGCUGGG GUGGGAGGUCUAGGGG CAGGGGUGGUGGUAGG UUUGGCGGGCAGGAAC ACGGGCACGAAUGAGCU GACGGUGACCAUUGUAC CCUGACCCCAUACGUCU AGUAUGGUGUCUCCACG AUCUCUGGCGCAGUAGU ACACCGCCGUGUCUGCG GCGGUCACAGAACUCAG CUUCAGGGAGAACUGG UUCUUGGACGUGUCUAC GGAUAUGGUGACUCGA CUCUUGAGGGACGGGU UGUAGUAGGUGCUCCCA CUAUAGGAGAUACUCCC AAUCCACUCCAGCCCCU UCCCUGGGGGCUGGCGG AUCCAGCCCCAGUAGUA ACUACUACUGCUGAUGG AGCCACCAGAGACAGUA CAGGUGAGGGACAGGG UCUCCGAAGGCUUCACC AGUCCUGGGCCCGACUC CUGCAGCUGCAGCUGUC CCUUUGUGCUGCCCUCG CCAGAGCCAGGCUUGCC GGAGCCGCUGGUGCUGC CUUUGAUCUCAACCUUG GUCCCUCCGCCAAAAGU GAGGAGGUCGUAUUUU UGCUGACAGUAGUAAG UUGCAAAAUCUUCAGG UUGCAGACUGCUGAUG GUGAGAGUGAAAUCUG UCCCAGAUCCACUGCCA CUGAACCUUGAUGGGAC CCCACUUUGCAAACUGG AUGCAGCAUAGAUCAG GAGCUUAGGGGCUUUCC CUGGUUUCUGCUGAUAC	CAUGCAAGAGCAGGGCA AGAGGCAGAAGCAGGG CGGUCACCGGCAGAGCC AU (SEQ ID NO:396)

	CAAUUUAAAUAGCUGC UAAUGCUCUGACUUGCC CGGCAAGUGAUGGUGA CUCUGUCUCCUACAGAU GCAGACAGGGAGGAUG GAGACUGGGUCAUCUG GAUGUCCGGGCGAGCGG CAUGCAAGAGCAGGGCA AGAGGCAGAAGCAGGG CGGUCACCGGCAGAGCC AUGGUGGCUUUACCAAC AGUACCGGAAUGCCAAG CUUGGGUCUGGGCUCUU UUAUUGAGCUCGGGGA GCAGAAGCGCGCGAACA GAAGCGAGAAGCGAAC UGAUUGGUUAGUUCAA AUAAGGCACAGGGUCA UUUCAGGUCCUUGGGGC ACCCUGGAAACAUCUGA UGGUUCUCUAGAAACU GCUGAGGGCGGGACCGC AUCUGGGGACCAUAUG UUCUUGGCCCUGAGCCG GGGCAGGAACUGCUUAC CACAGAUAUCCUGUUUG GCCCAUAUUCUGCUGUU CCAACUGUUCUUGGCCC UGAGCCGGGGCAGGAAC UGCUUACCACAGAUAUC CUGUUUGGCCCAUAUUC UGCUGUCUCUCUGUUCC UAACCUUGAUCCUAGCU UGCCAAACCUACAGGUG GGGUCUUUCAUUCCCCC CUUUUUCUGGAGACUA AAUAAAUGAAACUGCA CAAAGACAAUAGUCAU UCUCGAUCACCGAGAGA CUACCACUAAAAAAAAA AAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAA AAAAAAA (SEQ ID NO:395)	
RNAIVT7260	AGGGUGUAUGGGGUGC UCAGGGAGGGGUAGUA UCUCUGGUAUGGAGGG CUUGUCGUGCCCUCCUA GGGCAGCUCUCCAGCCU	UCACUAUUACCGGGGGG GAAGGGCCUGCAUGUGC AGGGCGUCGUAGGUGU CCUUGGUGGCGGUGCUC AGGCCUUGGUACAGGCC

	CUGACCCCCACCUGACA	AUCGUGUCCCUUGCCCC
	CCCAGCUCUCACUUGUG	GCCUCCGCUCGCCCUUC
	GCUCCCAGUAGCUGCUA	AUGCCGAUCUCGCUGUA
	GCAUGUGGCAGCGGCCA	AGCCUCGGCCAUCUUGU
	CACCCCGGGCAACGGCU	CCUUCUGCAGCUCGUUG
	UCGACAGGCCGGCUAAA	UACAGGCCCUCCUGGGG
	CCUUGUGAGGGUAGCCA	GUUCUUCCGUCUGGGCU
	UCGGGUCAUCGACCCCU	UUCCGCCCAUCUCGGGG
	GGUGAACCAGGGCUUU	UCCCGGCCUCUCCGCUU
	GCUCACCCAGCAUGUGA	GUCCAGCACGUCGUACU
	AGACUGCUUCGGCUGAA	CCUCCCGUCUGCCCAGG
	CAGACGGAAGAAACCAA	UUCAGCUCGUUGUACAG
	UAAGAAGGUUCAACGG	CUGGUUCUGGCCCUGCU
	CUGAGAGGGCGACGCAG	GGUAGGCAGGGGCGUC
	CAAAGCACUGUGGAGU	GGCGCUUCUGCUGAACU
	GCUUAGGGCGUGUUGG	UCACCCGCAGCUCACAG
	AGCACAAAGGACAACAC	CCGCCCUCCUCCUCCUC
	GGCCAUCCAAUGCAGCU	GGGAAACCUGCAGCUGC
	GAGGAAGUCUCCAGAU	AGCCGUCCUCCUCCUGG
	GUAACAAUUUUUCGUG	GUGGUCUGCACGGGCCG
	CCACUGGACCCAGGCUU	CAUGAAGGGCUGCUUG
	CCAACGCCGAGAGAGUG	AAGAUGUACAGCAGCU
	GGACUGUCUCUGUGCAU	UCUUCCGGCCCCGCUUG
	CGGCUUUUCCACUUAAA	UUCCGGUGGUUGCAGU
	UCUCUUUCACGCACAAG	ACAGGGUGAUCACCAGG
	UAUCUUUGUGCACACUC	CUCAGCAGCAGCACGCC
	AUCUAUCCUAACCCCGU	ACAUGUGCCGGCCAGAG
	CCACCCUCUUCAAGACC	GGGCCCAGAUGUAGAU
	UGCGGCGAUGGGGGAG	GUCGCAGGCGAAGUCCA
	UGGCGACGCAACAGGUG	GGCCUCUGGUGUGCACA
	GAGGUGACCACUGGCAG	GCUCCGCCGGCAGCAGG
	UUGUAGUCACGAUCCUG	UCUACAGGCUUCGGGCC
	CACGUAGGCGGCCCACG	GCAGAGACAGAGGCUG
	GACCAGUGGUCGCUCGG	GCUGGCGAUUGUAGGA
	CCCUGUGGGCAGCAGGG	GCAGGAGUAGGAGGUC
	ACGUUCGGCAGCAUCCU	UAGGAGCAGGAGUUGU
	GGGCGACUGAGCAGCCC	GGUAGGCUUGGCGGGC
	UCUCUAGGUUCUUUCCG	AGGAACACGGGCACGAA
	CCUCAGAAGCCAUAGAG	GCUGCUCACGGUCACCA
	CCCACCGCAUCCCCAGC	UGGUGCCCUGGCCCCAC
	AUGCCUGCUAUUGUCUU	ACGUCCAGGAUGGUGUC
	CCCAAUCCUCCCCCUUG	UCCCCGAUCUCUGGCGC
	CUGUCCUGCCCCACCCC	AGUAGUACACGGCGGU
	ACCCCCCAGAAUAGAAU	GUCGGCGGCGGUCACGC
	GACACCUACUCAGACAA	UGCUCAGCUUCAGGCUG
	UGCGAUGCAAUUUCCUC	AACUGGUUCUUGCUGG
	AUUUUAUUAGGAAAGG	UGUCCACGCUGAUGGUC
	ACAGUGGGAGUGGCACC	ACCCGGGACUUCAGGCU
	UUCCAGGGUCAAGGAA	GGGGUUGUAGUAGGUG
	GGCACGGGGGAGGGGC	CUGCCGCUGUAGCUGAU
	AAACAACAGAUGGCUG	AGAGCCAAUCCACUCCA

	GCAACUAGAAGGCACAG UCGAGGCUGAUCAGCGA UCACUAUUACCGGGGGG GAAGGGCCUGCAUGUGC AGGGCGUCGUAGGUGU CCUUGGUGGCGGUGCUC AGGCCUUGGUACAGGCC AUCGUGUCCCUUGCCCC GCCUCCGCUCGCCCUUC AUGCCGAUCUCGCUGUA AGCCUCGGCCAUCUUGU CCUUCUGCAGCUCGUUG UACAGGCCCUCCUGGGG GUUCUUCCGUCUGGGCU UUCCGCCCAUCUCGGGG UCCCGGCCUCUCCGCUU GUCCAGCACGUCGUACU CCUCCCGUCUGCCCAGG UUCAGCUCGUUGUACAG CUGGUUCUGGCCCUGCU GGUAGGCAGGGGCGUC GGCGCUUCUGCUGAACU UCACCCGCAGCUCACAG CCGCCCUCCUCCUCCUC GGGAAACCUGCAGCUGC AGCCGUCCUCCUCCUGG GUGGUCUGCACGGGCCG CAUGAAGGGCUGCUUG AAGAUGUACAGCAGCU UCUUCCGGCCCCGCUUG UUCCGGUGGUUGCAGU ACAGGGUGAUCACCAGG CUCAGCAGCAGCACGCC ACAUGUGCCGGCCAGAG GGGCCCAGAUGUAGAU GUCGCAGGCGAAGUCCA GGCCUCUGGUGUGCACA GCUCCGCCGGCAGCAGG UCUACAGGCUUCGGGCC GCAGAGACAGAGGCUG GCUGGCGAUUGUAGGA GCAGGAGUAGGAGGUC UAGGAGCAGGAGUUGU GGUAGGCUUGGCGGGC AGGAACACGGGCACGAA GCUGCUCACGGUCACCA UGGUGCCCUGGCCCCAC ACGUCCAGGAUGGUGUC UCCCCGAUCUCUGGCGC AGUAGUACACGGCGGU GUCGGCGGCGGUCACGC	GGCCCUUGCCAGGGGGC UGCCGGAUCCAGCCCCA GUAGUAGCUGCUGCUGC UGAUGCUGCCGCCGCUC ACUGUGCAGGUCAGGCU CAGUGUCUCGCUAGGCU UCACCAGGCCAGGGCCG CUCUCCUGCAGCUGCAG CUGUCCCUUUGUGCUGC CCUCGCCAGAGCCAGGC UUGCCGCUGCCGCUGGU GCUGCCUUUGAUCUCCA CCUUGGUGCCGCCGCCG AAGGUCAGCAGGUCGU ACUUCUGCUGGCAGUAG UAGGUGGCGAAGUCCUC GGGCUGCAGGCUGCUGA UGGUCAGGGUGAAGUC GGUGCCGCUGCCAGAGC CAGAGAACCUGCUGGGC ACGCCAGACUGCAGGCU GCUGGCGGCGUAGAUCA GCAGCUUGGGGGCCUUG CCGGGCUUCUGCUGGUA CCAGUUCAGGUAGCUGC UGAUGCUCUGGCUGGCC CGGCAUGUGAUGGUCAC UCUGUCGCCCACGCUGG CAGACAGGCUGCUGGGG CUCUGGGUCAUCUGGAU GUCGGGCCGAGCGGCAU GCAACAGCAGGGCAAGA GGCAGAAGCAGGGCGG UCACGGGCAGGGCCAU (SEQ ID NO:398)

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	GUUCUCUAGAAACUGCU GAGGGCGGGACCGCAUC UGGGGACCAUAUGUUC UUGGCCCUGAGCCGGGG CAGGAACUGCUUACCAC AGAUAUCCUGUUUGGCC CAUAUUCUGCUGUUCCA ACUGUUCUUGGCCCUGA GCCGGGGCAGGAACUGC UUACCACAGAUAUCCUG UUUGGCCCAUAUUCUGC UGUCUCUCUGUUCCUAA CCUUGAUCCUAGCUUGC CAAACCUACAGGUGGGG UCUUUCAUUCCCCCCUU UUUCUGGAGACUAAAU AAAUGAAACUGCACAA AGACAAUAGUCAUUCUC GAUCACCGAGAGACUAC CACUAAAAAAAAAAAAA AAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAA AAAA (SEQ ID NO:397)	
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	CCACUGGACCCAGGCUU	GCAUGAAGGGCUGCUU
	CCAACGCCGAGAGAGUG	GAAGAUGUACAGCAGC
	GGACUGUCUCUGUGCAU	UUCUUCCGGCCCCGCUU
	CGGCUUUUCCACUUAAA	GUUCCGGUGGUUGCAG
	UCUCUUUCACGCACAAG	UACAGGGUGAUCACCAG
	UAUCUUUGUGCACACUC	GCUAAGCAGAAGCACUC
	AUCUAUCCUAACCCCGU	CACAGGUGCCGGCCAAG
	CCACCCUCUUCAAGACC	GGAGCCCAGAUGUAGA
	UGCGGCGAUGGGGGAG	UAUCGCAAGCGAAGUCC
	UGGCGACGCAACAGGUG	AGGCCCCUUGUAUGCAC
	GAGGUGACCACUGGCAG	AGCUCCUCCAGCAGCAG
	UUGUAGUCACGAUCCUG	GUCUGCAAGCUUCGGGC
	CACGUAGGCGGCCCACG	CGAAGAGACAGGGGUU
	GACCAGUGGUCGCUCGG	GAGAGGCGAUUGUAGG
	CCCUGUGGGCAGCAGGG	GGCGGGUGUAGGAGGU
	ACGUUCGGCAGCAUCCU	CUAGGGGCAGGAGUGG
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	CCUCAGAAGCCAUAGAG	AGCUGCUCACGGUCACC
	CCCACCGCAUCCCCAGC	AUUGUUCCCUGGCCCCA
	AUGCCUGCUAUUGUCUU	CACGUCCAGGAUGGUAU
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	CUGUCCUGCCCCACCCC	CAGUAGUACACGGCGGU
	ACCCCCCAGAAUAGAAU	GUCAGCGGCUGUCACAG
	GACACCUACUCAGACAA	AGCUCAGCUUCAGGCUG
	UGCGAUGCAAUUUCCUC	AACUGGUUCUUGCUGG
	AUUUUAUUAGGAAAGG	UGUCCACGCUGAUGGUC
	ACAGUGGGAGUGGCACC	ACCCGGGACUUCAGGCU
	UUCCAGGGUCAAGGAA	GGGGUUGUAGUAGGUG
	GGCACGGGGGAGGGGC	CUGCCGCUGUAGCUGAU
	AAACAACAGAUGGCUG	AGAUCCAAUCCACUCCA
	GCAACUAGAAGGCACAG	GUCCCUUGCCAGGAGGC
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	AAGGCCCUGAUACAGGC	UUCACUAAGCCAGGGCC
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	UCCUUCUGCAGCUCGUU	UGCUUCCUUUAAUCUCA
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	UGUCAAGCACGUCGUAU	UCGGGCUGCAGGCUGCU
	UCUUCCCGUCUGCCCAG	GAUGGUCAGGGUGAAG
	GUUCAGCUCGUUGUACA	UCGGUGCCAGAUCCAGA
	GCUGGUUCUGUCCCUGU	GCCAGAGAACCUGCUGG

	UGGUAGGCAGGGGCGU CGGCGCUUCUGCUGAAC UUCACUCUCAGUUCACA GCCGCCCUCCUCCUCCU CGGGAAACCUGCAGCUA CAGCCAUCUUCCUCCUG GGUGGUCUGCACGGGCC GCAUGAAGGGCUGCUU GAAGAUGUACAGCAGC UUCUUCCGGCCCCGCUU GUUCCGGUGGUUGCAG UACAGGGUGAUCACCAG GCUAAGCAGAAGCACUC CACAGGUGCCGGCCAAG GGAGCCCAGAUGUAGA UAUCGCAAGCGAAGUCC AGGCCCCUUGUAUGCAC AGCUCCUCCAGCAGCAG GUCUGCAAGCUUCGGGC CGAAGAGACAGGGGUU GAGAGGCGAUUGUAGG GGCGGGUGUAGGAGGU CUAGGGGCAGGAGUGG UGGUAGGUUUAGCAGG CAGGAACACGGGCACAA AGCUGCUCACGGUCACC AUUGUUCCCUGGCCCCA CACGUCCAGGAUGGUAU CUCCCCGAUCUCUGGCG CAGUAGUACACGGCGGU GUCAGCGGCUGUCACAG AGCUCAGCUUCAGGCUG AACUGGUUCUUGCUGG UGUCCACGCUGAUGGUC ACCCGGGACUUCAGGCU GGGGUUGUAGUAGGUG CUGCCGCUGUAGCUGAU AGAUCCAAUCCACUCCA GUCCCUUGCCAGGAGGC UGCCGGAUCCAGCCCCA GUAGUAAGAGCUAGAG CUGAUGCUGCCGCCGCU CACUGUGCAGGUCAGGC UCAGUGUCUCGCUAGGC UUCACUAAGCCAGGGCC GCUCUCCUGCAGUUGCA GCUGUCCCUUUGUGCUU CCCUCUCCAGAGCCAGG UUUGCCGCUGCCGCUGG UGCUUCCUUUAAUCUCA ACCUUGGUGCCGCCGCC	GCACGCCAGACUGCAAG CUGCUAGCAGCGUAGAU CAGCAGCUUGGGGGCCU UGCCGGGCUUCUGCUGG UACCAGUUCAGGUAGCU GCUGAUGCUCUGAGAA GCCCGGCAUGUGAUGGU CACUCUGUCGCCCACGC UGGCAGACAGGCUAGA AGGAGACUGGGUCAUC UGGAUGUCGGGCCGAGC GGCAUGCAAUAACAGG GCAAGAGGCAGAAGCA GGGCUGUCACGGGCAGG GCCAU (SEQ ID NO:400)

	GAAGGUCAGCAGGUCG UACUUCUGCUGGCAGUA GUAGGUGGCAAAGUCC UCGGGCUGCAGGCUGCU GAUGGUCAGGGUGAAG UCGGUGCCAGAUCCAGA GCCAGAGAACCUGCUGG GCACGCCAGACUGCAAG CUGCUAGCAGCGUAGAU CAGCAGCUUGGGGGCCU UGCCGGGCUUCUGCUGG UACCAGUUCAGGUAGCU GCUGAUGCUCUGAGAA GCCCGGCAUGUGAUGGU CACUCUGUCGCCCACGC UGGCAGACAGGCUAGA AGGAGACUGGGUCAUC UGGAUGUCGGGCCGAGC GGCAUGCAAUAACAGG GCAAGAGGCAGAAGCA GGGCUGUCACGGGCAGG GCCAUGGUGGCUUUACC AACAGUACCGGAAUGCC AAGCUUGGGUCUGGGC UCUUUUAUUGAGCUCG GGGAGCAGAAGCGCGCG AACAGAAGCGAGAAGC GAACUGAUUGGUUAGU UCAAAUAAGGCACAGG GUCAUUUCAGGUCCUUG GGGCACCCUGGAAACAU CUGAUGGUUCUCUAGA AACUGCUGAGGGCGGG ACCGCAUCUGGGGACCA UAUGUUCUUGGCCCUGA GCCGGGGCAGGAACUGC UUACCACAGAUAUCCUG UUUGGCCCAUAUUCUGC UGUUCCAACUGUUCUUG GCCCUGAGCCGGGGCAG GAACUGCUUACCACAGA UAUCCUGUUUGGCCCAU AUUCUGCUGUCUCUCUG UUCCUAACCUUGAUCCU AGCUUGCCAAACCUACA GGUGGGGUCUUUCAUU CCCCCCUUUUUCUGGAG ACUAAAUAAAUGAAAC UGCACAAAGACAAUAG UCAUUCUCGAUCACCGA GAGACUACCACUAAAAA	

	AAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAA AAAAAAAAAA (SEQ ID NO:399)	
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	AUGCCUGCUAUUGUCUU CCCAAUCCUCCCCCUUG CUGUCCUGCCCCACCCC ACCCCCCAGAAUAGAAU GACACCUACUCAGACAA UGCGAUGCAAUUUCCUC AUUUUAUUAGGAAAGG ACAGUGGGAGUGGCACC UUCCAGGGUCAAGGAA GGCACGGGGGAGGGGC AAACAACAGAUGGCUG GCAACUAGAAGGCACAG UCGAGGCUGAUCAGCGA UCACUAUUACCGGGGGG GAAGGGCCUGCAUAUGC AGGGCAUCAUAGGUGU CCUUGGUGGCGGUAGAC AGGCCCUGAUAAAGGCC GUCAUGUCCCUUUCCCC GCCUCCUUUCGCCCUUC AUGCCAAUCUCGCUGUA GGCCUCGGCCAUCUUGU CCUUCUGCAGCUCGUUG UACAGGCCCUCCUGGGG GUUUUUCCGUCUGGGCU UUCCGCCCAUUUCGGGA UCCCGGCCUCUCCUCUU GUCCAACACGUCGUACU CCUCCCGUCUACCCAGG UUCAGCUCGUUGUACAG CUGGUUCUGUCCUUGCU GGUAGGCAGGGGCGUC GGCGCUUCUGCUGAACU UCACUCUCAGUUCACAG CCGCCCUCCUCCUCCUC GGGAAACCUGCAGCUAC AGCCAUCUUCCUCCUGG GUGGUCUGCACGGGCCG CAUGAAGGGCUGCUUG AAGAUGUACAGCAGCU UCUUCCGGCCCCGCUUG UUCCGGUGGUUGCAGU ACAGGGUGAUCACCAGA GACAGCAGCAGCACGCC ACAGGUUCCGGCAAGAG GAGCCCAGAUGUAGAU GUCGCAGGCGAAGUCCA GGCCUCUUGUGUGAACA GCUCCGCCAGCAGCAGG UCUACAGGCUUCGGGCC GCAAAGAAAGAGGUUG	ACGUCCAGGAUGGUGUC UCCCCUAUCUCUGGCGC AGUAAUACACGGCGGU GUCAGCGGCGGUCACAG AGCUCAGCUUCAGGCUG AACUGGUUCUUGCUGG UGUCCACGCUGAUGGUC ACCCGGGACUUCAGGCU GGGGUUGUAGUAGGUG CUGCCGCUGUAGCUGAU AGAGCCAAUCCACUCCA GUCCUUUGCCAGGGGGC UGCCGAAUCCAGCCCCA GUAGUAGCUGCUAGAG CUGAUGCUGCCGCCGCU CACUGUGCAGGUCAGGC UCAGUGUCUCGCUAGGC UUCACCAGUCCAGGGCC AGACUCCUGCAACUGCA GCUGUCCCUUUGUGCUG CCUUCUCCAGAGCCAGG CUUUCCGCUGCCGCUGG UGCUGCCUUUGAUCUCA ACCUUGGUGCCUCCGCC GAAGGUCAGCAGGUCG UACUUCUGCUGGCAGUA GUAGGUGGCAAAGUCC UCGGGCUGCAGGCUGCU GAUGGUCAGGGUGAAG UCGGUGCCAGAUCCAGA GCCAGAGAACCUGCUGG GCACGCCAGACUGCAAG CUGCUAGCAGCGUAGAU CAGCAGCUUGGGGGCCU UGCCGGGCUUCUGCUGG UACCAGUUCAGGUAGCU GCUAAUGCUCUGGCUAG CCCGGCAUGUGAUGGUC ACUCUGUCGCCAACGCU GGCAGACAGGCUGCUAG GGCUCUGGGUCAUCUGG AUGUCGGGCCUAGCAGC AUGCAACAGCAGGGCAA GAGGCAGAAGCAGAGC GGUCACGGGCAGAGCCA U (SEQ ID NO:402)

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	CAAAGCACUGUGGAGU	GCGGACCGGGAGAACUU
	GCUUAGGGCGUGUUGG	CACUCGGAGCUCGCAUC
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	GGCCAUCCAAUGCAGCU	GGGAAUCGGCAGGAGC
	GAGGAAGUCUCCAGAU	AGCCAUCCUCCUCCUGC
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	CCAACGCCGAGAGAGUG	AAGAUAUACAGGAGCU
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	CGGCUUUUCCACUUAAA	UUCCGGUGGUUGCAGU
	UCUCUUUCACGCACAAG	ACAGGGUGAUCACCAGG
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	CCACCCUCUUCAAGACC	GGGCCCAGAUGUAGAU
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	ACGUUCGGCAGCAUCCU	UAGGCGCUGGGGUGGU
	GGGCGACUGAGCAGCCC	GGUAGGUUUGGCGGGC
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	CCCACCGCAUCCCCAGC	UCGUACCCUGACCCCAC
	AUGCCUGCUAUUGUCUU	ACGUCGAGGAUGGUGU
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	CUGUCCUGCCCCACCCC	CAGUAGUACACAGCCGU
	ACCCCCCAGAAUAGAAU	GUCUGCGGCGGUGACCG
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	ACAGUGGGAGUGGCACC	UGACUCGGGACUUGAG
	UUCCAGGGUCAAGGAA	GGACGGGUUGUAGUAG
	GGCACGGGGGAGGGGC	GUGGACCCGGAGUAGG
	AAACAACAGAUGGCUG	AGAUACUCCCAAUCCAC
	GCAACUAGAAGGCACAG	UCCAGCCCCUUCCCUGG
	UCGAGGCUGAUCAGCGA	GGGCUGCCGGAUCCACC
	UCACUAUUACCGGGGGG	CCCAGUAGUAGGAGGA
	GCAGGGCCUGCAUGUGC	GGAGGAGAUGGACCCAC
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	CUUGGUUGCCGUGGACA	GAGGGACAGUGUCUCCG
	GACCCUGGUAUAAGCCG	ACGGCUUCACCAGACCU
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	CCUUCUCUCCCCCUUCA	CUGGAGCUGUCCCUUUG

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	CCCAGUAGUAGGAGGA GGAGGAGAUGGACCCAC CGGAGACAGUACAGGU GAGGGACAGUGUCUCCG ACGGCUUCACCAGACCU GGGCCCGACUCCUGCAG CUGGAGCUGUCCCUUUG UGCUCCCCUCGCCAGAC CCAGGCUUCCCGGAGCC GGAGGUGGAGCCUUUG AUCUCGACCUUCGUCCC CCCGCCGAACGUGAGGA GGUCGUACUUCUGCUGG CAGUAGUACGUAGCGA AGUCCUCCGGCUGCAGG GAGGAGAUGGUGAGCG UGAAGUCCGUCCCAGAC CCGGACCCGGAGAACCU GGAGGGGACCCCACUCU GCAAGGAGGAUGCAGC GUAGAUCAGGAGCUUC GGGGCCUUCCCGGGCUU CUGCUGAUACCAGUUGA GGUAGGAGGAGAUGGA CUGGGAUGCCCGGCACG UGAUCGUGACUCUGUCC CCGACGGAGGCGGACAG GGAGGACGGAGACUGG GUCAUCUGGAUGUCGG GCCGAGCGGCAUGCAAG AGCAGGGCAAGAGGCA GAAGCAGGGCCGUCACC GGCAGGGCCAUGGUGGC UUUACCAACAGUACCGG AAUGCCAAGCUUGGGUC UGGGCUCUUUUAUUGA GCUCGGGGAGCAGAAGC GCGCGAACAGAAGCGAG AAGCGAACUGAUUGGU UAGUUCAAAUAAGGCA CAGGGUCAUUUCAGGUC CUUGGGGCACCCUGGAA ACAUCUGAUGGUUCUCU AGAAACUGCUGAGGGC GGGACCGCAUCUGGGGA CCAUAUGUUCUUGGCCC UGAGCCGGGGCAGGAAC UGCUUACCACAGAUAUC CUGUUUGGCCCAUAUUC UGCUGUUCCAACUGUUC UUGGCCCUGAGCCGGGG	

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Chimeric Antigen Receptors

[0412] In some embodiments, the heterologous object sequence encodes a chimeric antigen receptor (CAR) comprising an antigen binding domain. In some embodiments, the CAR is or comprises a first generation CAR comprising an antigen binding domain, a transmembrane domain, and a single intracellular signaling domain. In some embodiments, the CAR is or comprises a second generation CAR comprising an antigen binding domain, a transmembrane domain, and two intracellular signaling domains (e.g., a first intracellular signaling domain and a second intracellular signaling domain). In some embodiments, the CAR comprises a third generation CAR comprising an antigen binding domain, a transmembrane domain, and at least three intracellular signaling domains. In some embodiments, a fourth generation CAR comprising an antigen binding domain, a transmembrane domain, three or four intracellular signaling domains, and a domain which upon successful signaling of the CAR induces expression of a cytokine gene. In some embodiments, the antigen binding domain is or comprises an scFv, Fab, a diabody, a D domain binder, centyrins (e.g., antibody-like scaffolds, e.g., a CARTyrin), one or more single domain antibodies such as VHH domains (e.g., comprises two VHH binding domains).

Antigen Binding Domains

[0413] In some embodiments, a CAR antigen binding domain is or comprises an antibody or antigen-binding portion thereof. In some embodiments, a CAR antigen binding domain is or comprises an scFv or Fab. In some embodiments, an antigen binding domain binds to a cell surface antigen of a cell. In some embodiments, a cell surface antigen is characteristic of one

type of cell. In some embodiments, a cell surface antigen is characteristic of more than one type of cell. In some embodiments, the CAR antigen binding domain binds to two epitopes of the target antigen (e.g., is a biepitopic binding domain). In some embodiments, the CAR comprises two antigen binding domains, such that each antigen binding domain binds to a different target antigen on a cell, e.g., a neoplastic cell.

[0414] In some embodiments, the antigen binding domain targets an antigen characteristic of a neoplastic cell. In some embodiments, the antigen characteristic of a neoplastic cell is selected from a receptor listed in **Table 6**, or an antigenic fragment or antigenic portion thereof. In some embodiments, the antigen binding domain binds one or more antigens of a blood cancer (e.g., a leukemia or a lymphoma, or a multiple myeloma). In some embodiments, the blood cancer antigen is a B cell antigen. In some embodiments, the antigen binding domain binds an antigen of a solid tumor.

Table 6: Exemplary Neoplastic Cell Antigens

cell surface receptor	NAV1.6	Carbonic anhydrase IX	prostate specific membrane antigen (PSMA)	p53 mutant
an ion channel-linked receptor	NAV1.7	PSMA	ROR1	prostein
an enzyme-linked receptor	NAV1.8	Folate binding protein	FLT3	survivin
a G protein-coupled receptor	NAV1.9	Gangliosides	FAP	telomerase
receptor tyrosine kinase	sphingosin-1-phosphate receptor (S1P1R)	CD2	TAG72	PCTA-1/Galectin 8
tyrosine kinase associated receptor	NMDA channel	CD3	CD38	MelanA/MART1
receptor-like tyrosine phosphatase	transmembrane protein	GM2)	CD44v6	Ras mutant
receptor serine/ threonine kinase	multispan transmembrane protein	Lewis-γ^2	CEA	hTERT
receptor guanylyl cyclase	T-cell receptor motifs	VEGF	EPCAM	sarcoma translocation breakpoints
histidine kinase associated receptor	T-cell alpha chains	VEGFR 1/2/3	B7H3	ML-IAP
ErbB1/EGFR	T-cell β chains	αVβ3	KIT	ERG (TMPRSS2 ETS fusion gene)

ErbB2/HER2	T-cell γ chains	α5β1	interleukin-11 receptor a (IL-11Ra)	NA17
ErbB3/HER3	T-cell δ chains	ErbB1/EGFR	PSCA	PAX3
and ErbB4/HER4)	CCR7	ErbB1/HER2	PRSS21	Androgen receptor
FGF1	CD3	ErB3	VEGFR2	Cyclin B1
FGF2	CD4	c-MET	LewisY	MYCN
FGF3	CD5	IGF1R	CD24	RhoC
FGF4	CD7	EphA3	platelet-derived growth factor receptor-beta (PDGFR-beta)	TRP-2
FGF5	CD8	TRAIL-R1	SSEA-4	CYPIB I
FGF6	CD11b	TRAIL-R2	CD20	BORIS
FGF7	CD11c	RANKL	MUC1	SART3
FGF18	CD16	FAP	NCAM	PAX5
FGF21	CD19	Tenascin	Prostase	OY-TES1
VEGF-A	CD20	PDL-1	PAP	LCK
VEGF-B	CD21	BAFF	ELF2M	AKAP-4
VEGF-C	CD22	HDAC	Ephrin B2	SSX2
VEGF-D	CD25	ABL	IGF-1 receptor	RAGE-1
PIGF	CD28	FLT3	CAIX	human telomerase reverse transcriptase
RET Receptor and the Eph Receptor Family including EphA1	CD34	KIT	LMP2	RU1
EphA2	CD35	MET	gplOO	RU2
EphA3	CD40	RET	bcr-abl	intestinal carboxyl esterase
EphA4	CD45RA	IL-1β	tyrosinase	mut hsp70-2
EphA5	CD45RO	ALK	Fucosyl GM1	CD79a
EphA6	CD52	RANKL	sLe	CD79b
EphA7	CD56	mTOR	GM3	CD72
EphA8	CD62L	CTLA-4	TGS5	LAIR1
EphA9	CD68	IL-6	HMWMAA	FCAR
EphA10	CD80	IL-6R	o-acetyl-GD2	LILRA2
EphB1	CD95	JAK3	Folate receptor beta	CD300LF
EphB2. EphB3	CD117	BRAF	TEM1/CD248	CLEC12A
EphB4	CD127	PTCH	TEM7R	BST2
EphB6	CD133	PIGF	CLDN6	EMR2
CXCR1	CD137 (4-1 BB)	ANPEP	GPRC5D	LY75
CXCR2	CD163	TIMP1	CXORF61	GPC3

CXCR3	F4/80	PLAUR	CD97	FCRL5
CXCR4	IL-4Ra	PTPRJ	CD179a	IGLL1
CXCR6	Sca-1	LTBR	ALK	a neoantigen
CCR1	CTLA-4	ANTXR1	Polysialic acid	CD133
CCR2	GITR	Folate receptor alpha (FRa)	PLACl	CD15
CCR3	GARP	ERBB2 (Her2/neu)	GloboH	CD184
CCR4	LAP	EphA2	NY-BR-1	CD24
CCR5	granzyme B	IL-13Ra2	UPK2	CD56
CCR6	LFA-1	epidermal growth factor receptor (EGFR)	HAVCR1	CD26
CCR8	transferrin receptor	Mesothelin	ADRB3	CD29
CFTR	NKp46	TSHR	PANX3	CD44
CIC-1	perforin	CD19	GPR20	HLA-A
CIC-2	CD4+	CD123	LY6K	HLA-B
CIC-4	Th1	CD22	OR51E2	HLA-C
CIC-5	Th2	CD30	TARP	(HLA-A
CIC-7	Th17	CD171	WT1	B
CIC-Ka	Th40	CS-1	NY-ESO-1	CD49f
CIC-Kb	Th22	CLL-1	LAGE-la	CD151 CD340
Bestrophins	Th9	CD33	MAGE-A1	CD200
TMEM16A	Tfh	EGFRvIII	legumain	tkrA
GABA receptor	Canonical Treg. FoxP3+	GD2	HPV E6	trkB
glycin receptor	Tr1	GD3	E7	or trkC
ABC transporters	Th3	BCMA	ETV6-AML	TAG-72
NAV1.1	Treg17	MUC16 (CA125)	sperm protein 17	Tn Ag
NAV1.2	$T_{RE}G$	L1CAM	XAGE1	p53
NAV1.3	CDCP1	LeY	Tie 2	Mucins
NAV1.4	NT5E	MSLN	MAD-CT-1	Claudin 18.2 (CLDN18.2)
NAV1.5	EpCAM	IL13Ra1	MAD-CT-2	
CEA	gpA33	Fos-related antigen 1	L1-CAM	

[0415] In some embodiments, the antigen binding domain targets an antigen characteristic of a T-cell. In some embodiments, the antigen characteristic of a T-cell is selected from an exemplary T cell antigen listed in **Table 7**, or an antigenic fragment thereof.

Table 7: Exemplary T-cell Antigens

a cell surface receptor	CALM1	HLA-DRB1	MAP2K6 (MKK6)	NFKBIA	RAF1
a membrane transport protein	CD3D (CD3δ)	HLA-DRB3	MAP2K7 (MKK7)	NRAS	RELA
an active or passive transport protein	CD3E (CD3ε)	HLA-DRB4	MAP3K1 (MEKK1)	PAK1	SDF1
an ion channel protein	CD3G (CD3γ)	HLA-DRB5	MAP3K3	PAK2	SHP2
a pore-forming protein	CD4	HRAS	MAP3K4	PAK3	SLP76
a transmembrane receptor	CD8	IKBKA (CHUK)	MAP3K5	PAK4	SOS
a membrane enzyme	CD28	IKBKB	MAP3K8	PIK3C2B	SRC
a cell adhesion protein characteristic of a T-cell	CD45	IKBKE	MAP3K14 (NIK)	PIK3C3 (VPS34)	TBK1
a G protein-coupled receptor	CD80 (B7-1)	IKBKG (NEMO)	MAPK8 (JNK1)	PIK3CA	TCRA
receptor tyrosine kinase	CD86 (B7-2)	IL2	MAPK9 (JNK2)	PIK3CB	TEC
tyrosine kinase associated receptor	CD247 (CD3ζ)	ITPR1	MAPK10 (JNK3)	PIK3CD	TRAF6
receptor-like tyrosine phosphatase	CTLA4 (CD152)	ITK	MAPK11 (p38β)	PIK3R1	VAV1
receptor serine/threonine kinase	ELK1	JUN	MAPK12 (p38γ)	PKCA	VAV2
receptor guanylyl cyclase	ERK1 (MAPK3)	KRAS2	MAPK13 (p38δ)	PKCB	ZAP70
histidine kinase associated receptor	ERK2	LAT	MAPK14 (p38α)	PKCM	
AKT1	FOS	LCK	NCK	PKCQ	
AKT2	FYN	MAP2K1 (MEK1)	NFAT1	PLCY1	
AKT3	GRAP2 (GADS)	MAP2K2 (MEK2)	NFAT2	PRF1 (Perforin)	
ATF2	GRB2	MAP2K3 (MKK3)	NFKB1	PTEN	
BCL10	HLA-DRA	MAP2K4 (MKK4)	NFKB2	RAC1	

[0416] In some embodiments, the antigen binding domain targets an antigen characteristic of an autoimmune or inflammatory disorder. In some embodiments, the autoimmune or inflammatory disorder is selected from chronic graft-vs-host disease (GVHD), lupus, arthritis, immune complex glomerulonephritis, goodpasture, uveitis, hepatitis, systemic sclerosis or scleroderma, type I diabetes, multiple sclerosis, cold agglutinin disease, Pemphigus vulgaris, Grave's disease, autoimmune hemolytic anemia, Hemophilia A, Primary Sjogren's Syndrome, thrombotic thrombocytopenia purrpura, neuromyelits optica, Evan's syndrome, IgM mediated

neuropathy, cyroglobulinemia, dermatomyositis, idiopathic thrombocytopenia, ankylosing spondylitis, bullous pemphigoid, acquired angioedema, chronic urticarial, antiphospholipid demyelinating polyneuropathy, and autoimmune thrombocytopenia or neutropenia or pure red cell aplasias, while exemplary non-limiting examples of alloimmune diseases include allosensitization (see, for example, Blazar et al., 2015, Am. J. Transplant, 15(4):931-41) or xenosensitization from hematopoietic or solid organ transplantation, blood transfusions, pregnancy with fetal allosensitization, neonatal alloimmune thrombocytopenia, hemolytic disease of the newborn, sensitization to foreign antigens such as can occur with replacement of inherited or acquired deficiency disorders treated with enzyme or protein replacement therapy, blood products, and gene therapy. In some embodiments, the antigen characteristic of an autoimmune or inflammatory disorder is selected from an exemplary antigen listed in **Table 8**, or an antigenic fragment thereof.

Table 8: Exemplary Autoimmune or Inflammatory Disorder Antigens

a cell surface receptor	a G protein-coupled receptor	receptor-like tyrosine phosphatase	histidine kinase associated receptor
an ion channel-linked receptor	receptor tyrosine kinase	receptor serine/ threonine kinase	citrullinated vimentin
an enzyme-linked receptor	tyrosine kinase associated receptor	receptor guanylyl cyclase	Human leukocyte antigen (HLA)

[0417] In some embodiments, a CAR antigen binding domain binds to a ligand expressed on B cells, plasma cells, plasmablasts. In some embodiments, the antigen expressed on B cells, plasma cells, or plasmablasts is selected from an exemplary antigen listed in **Table 9**, or an antigenic fragment thereof.

Table 9: Exemplary B cell, Plasma Cell, and Plasmablast Antigens

CD10	CD24	CD138	TNF	LFA-1
CD19	CD27	CD319	interferon receptors	CD3 gamma
CD20	CD38	BCMA	GM-CSF	CD5
CD22	CD45R	CD28	ZAP-70	CD2
GPRC5D				

[0418] In some embodiments, the antigen binding domain targets an antigen characteristic of an infectious disease. In some embodiments, wherein the infectious disease is selected from HIV, hepatitis B virus, hepatitis C virus, Human herpes virus, Human herpes virus 8 (HHV-8, Kaposi sarcoma-associated herpes virus (KSHV)), Human T-lymphotrophic virus-1 (HTLV-1), Merkel cell polyomavirus (MCV), Simian virus 40 (SV40), Eptstein-Barr virus, CMV, human

papillomavirus. In some embodiments, the antigen characteristic of an infectious disease is selected from an exemplary antigen listed in **Table 10**, or an antigenic fragment thereof.

Table 10: Exemplary Infectious Disease Antigens

a cell surface receptor	receptor serine/threonine kinase
an ion channel-linked receptor	receptor guanylyl cyclase
an enzyme-linked receptor	histidine kinase associated receptor
a G protein-coupled receptor	HIV Env
receptor tyrosine kinase	gpl20
tyrosine kinase associated receptor	CD4-induced epitope on HIV-1 Env
receptor-like tyrosine phosphatase	

Transmembrane Domain

[0419] In some embodiments, the CAR transmembrane domain comprises at least a transmembrane region of an exemplary transmembrane domain listed in **Table 11**, or a functional fragment thereof.

Table 11: Exemplary Transmembrane Domains

alpha chain of the T cell receptor	CD22	4-1BB/CD137	TCRα	CD80
beta chain of the T cell receptor	CD33	CD28	TCRβ	CD86
zeta chain of the T-cell receptor	CD37	CD34	TCRζ	CD40
CD28	CD64	CD4	CD32	CD40L/CD154
CD3 epsilon	CD80	FcεRIγ	CD64	VEGFR2
CD45	CD86	CD16	CD64	FAS
CD4	CD134	OX40/CD134	CD45	FGFR2B
CD5	CD137	CD3ζ	CD5	
CD8	CD154	CD3ε	CD9	
CD9	CD8α	CD3γ	CD22	
CD16	CD8β	CD3δ	CD37	

[0420] In some embodiments, the transmembrane domain is a CD8 transmembrane domain. In some embodiments, the CD8 transmembrane domain has an amino acid sequence of a CD8 transmembrane domain listed in Table C6A, or sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto. In some embodiments, the CD8 transmembrane domain is encoded by a nucleic acid sequence of a CD8 transmembrane domain listed in **Table 12**, or sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

Table 12: Sequences of Exemplary Transmembrane Domains

Name	Sequence	SEQ ID NO:
CD8 transmembrane (amino acid)	IYIWAPLAGTCGVLLLSLVITLYC	72
CD8 transmembrane (amino acid)	IYIWAPLAGTCGVLLLSLVITLYCNHRN	73
CD8α transmembrane domain (amino acid)	IYIWAPLAGTCGVLLLSLVITYC	74

Intracellular Signaling Domains

[0421] In some embodiments, the CAR comprises at least one signaling domain selected from one or more intracellular signaling domains listed in **Table 13**, or a functional fragment thereof. In some embodiments, the CAR comprises a first intracellular signaling domain and a second intracellular signaling domain. In some embodiments, the first intracellular signaling domain mediates downstream signaling during T-cell activation. In some embodiments, the second intracellular signaling domain is a costimulatory domain.

Table 13: Exemplary Intracellular Signaling Domains

B7-1/CD80	4-1BB Ligand/TNFSF9	RELT/TNFRSF19L	CD7
B7-2/CD86	BAFF/BLyS/TNFSF13B	TACI/TNFRSF13B	CD53
B7-H1/PD-L1	BAFF R/TNFRSF13C	TL1A/TNFSF15	CD82/Kai-1
B7-H2	CD27/TNFRSF7	TNF-alpha	CD90/Thy1
B7-H3	CD27 Ligand/TNFSF7	TNF RII/TNFRSF1B	CD96
B7-H4	CD30/TNFRSF8	2B4/CD244/SLAMF4	CD160
B7-H6	CD30 Ligand/TNFSF8	BLAME/SLAMF8	CD200
B7-H7	CD40/TNFRSF5	CD2	CD300a/LMIR1
BTLA/CD272	CD40/TNFSF5	CD2F-10/SLAMF9	HLA Class I
CD28	CD40 Ligand/TNFSF5	CD48/SLAMF2	HLA-DR
CTLA-4	DR3/TNFRSF25	CD58/LFA-3	Ikaros
Gi24/VISTA/B7-H5	GITR/TNFRSF18	CD84/SLAMF5	Integrin alpha 4/CD49d
ICOS/CD278	GITR Ligand/TNFSF18	CD229/SLAMF3	Integrin alpha 4 beta 1
PD-1	HVEM/TNFRSF14	CRACC/SLAMF7	Integrin alpha 4 beta 7/LPAM-1
PD-L2/B7-DC	LIGHT/TNFSF14	NTB-A/SLAMF6	LAG-3
PDCD6	Lymphotoxin-alpha/TNF-beta	SLAM/CD150	TCL1A
4-1BB/TNFSF9/CD137	OX40/TNFRSF4	CD2	TCL1B
CRTAM	CD30	TIM-1/KIM-1/HAVCR	CD2
DAP12	CD40	TIM-4	CD7
Dectin-1/CLEC7A	PD-1	TSLP	LIGHT
DPPIV/CD26	ICOS	TSLP R	NKG2C

EphB6	lymphocyte function-associated antigen-1 (LFA-1)	lymphocyte function associated antigen-1 (LFA-1)	B7-H3
NKG2C	a ligand that specifically binds with CD83	a CD3 zeta domain	an immunoreceptor tyrosine-based activation motif (ITAM)
CD27	CD28	4-1BB	CD134/OX40

[0422] In some embodiments, the CAR comprises a (i) a CD3 zeta domain, or an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof; (ii) a CD28 domain or functional variant thereof; and (iii) a 4-1BB domain, or a CD134 domain, or functional variant thereof. In some embodiments, the CAR comprises a CD3 zeta domain or an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof. In some embodiments, the CAR comprises (i) a CD3 zeta domain, or an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof; (ii) a CD28 domain, or a 4-1BB domain, or functional variant thereof, and/or (iii) a 4-1BB domain, or a CD134 domain, or functional variant thereof. In some embodiments, the CAR comprises a (i) a CD3 zeta domain, or an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof; (ii) a CD28 domain or functional variant thereof; (iii) a 4-1BB domain, or a CD134 domain, or functional variant thereof; and (iv) a cytokine or costimulatory ligand transgene.

[0423] In some embodiments, the CAR comprises a CD28z co-stimulatory domain.

[0424] In some embodiments, the CAR comprises a CD3z signaling domain.

[0425] In some embodiments, intracellular signaling domain comprises an intracellular signaling domain listed in **Table 14**, or sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

Table 14: Sequences of Exemplary Intracellular Signaling Domains

Name	**Sequence**
CD28 signaling domain	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS (SEQ ID NO:75)
CD3z signaling domain	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:76)
4-1BB signaling domain	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO:77)

[0426] In some embodiments, the CAR further comprises one or more spacers, e.g., wherein the spacer is a first spacer between the antigen binding domain and the transmembrane domain. In some embodiments, the first spacer includes at least a portion of an immunoglobulin constant region or variant or modified version thereof. In some embodiments, the spacer is a second spacer between the transmembrane domain and a signaling domain. In some embodiments, the second spacer is an oligopeptide, e.g., wherein the oligopeptide comprises glycine-serine doublets. In some embodiments, the CAR further comprises a hinge domain. In some embodiments, the hinge domain is a CD8 hinge domain. In some embodiments, the CD8 hinge domain has an amino acid sequence of a CD8 hinge domain in **Table 15**, or sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

Table 15: Sequences of Exemplary Hinge Domains

Name	Sequence
CD8 Hinge A	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD FACD (SEQ ID NO:78)
CD8 Hinge B	FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAG GAVHTRGLDFACD (SEQ ID NO:79)

[0427] In some embodiments, the CAR comprises a sequence of a CAR listed in **Table 16**, or sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

Table 16: Sequences of Exemplary CAR Molecules

BCMA CAR 1		
Description	Sequence	SEQ ID NO
BCMA CAR1 VL (amino acid)	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGK APKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATY YCQQKYDLLTFGGGTKVEIK	80
BCMA CAR1 VH (amino acid)	QLQLQESGPGLVKPSETLSLTCTVSGGSISSSSYYWGWIRQPP GKGLEWIGSISYSGSTYYNPSLKSRVTISVDTSKNQFSLKLSS VTAADTAVYYCARDRGDTILDVWGQGTMVTVSS	81
BCMA CAR1 Full CAR (no signal peptide) (amino acid)	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGK APKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATY YCQQKYDLLTFGGGTKVEIKGSTSGSGKPGSGEGSTKGQLQ LQESGPGLVKPSETLSLTCTVSGGSISSSSYYWGWIRQPPGKG LEWIGSISYSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTA ADTAVYYCARDRGDTILDVWGQGTMVTVSSFVPVFLPAKPT TTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFAC DIYIWAPLAGTCGVLLLSLVITLYCNHRNKRGRKKLLYIFKQ PFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAY QQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGL STATKDTYDALHMQALPPR	82

BCMA CAR1 Full CAR (with signal peptide) (amino acid)	MALPVTALLLPLALLLHAARPDIQMTQSPSSLSASVGDRVTIT CRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGS GSGTDFTLTISSLQPEDFATYYCQQKYDLLTFGGGTKVEIKGS TSGSGKPGSGEGSTKGQLQLQESGPGLVKPSETLSLTCTVSG GSISSSSYYWGWIRQPPGKGLEWIGSISYSGSTYYNPSLKSRV TISVDTSKNQFSLKLSSVTAADTAVYYCARDRGDTILDVWG QGTMVTVSSFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPE ACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITL YCNHRNKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEE EGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVL DKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	83
BCMA CAR2		
BCMA CAR2 VHH1 (amino acid)	QVKLEESGGGLVQAGRSLRLSCAASEHTFSSHVMGWFRQAP GKERESVAVIGWRDISTSYADSVKGRFTISRDNAKKTLYLQM NSLKPEDTAVYYCAARRIDAADFDSWGQGTQVTVSS	83
BCMA CAR2 VHH2 (amino acid)	EVQLVESGGGLVQAGGSLRLSCAASGRTFTMGWFRQAPGK EREFVAAISLSPTLAYYAESVKGRFTISRDNAKNTVVLQMNS LKPEDTALYYCAADRKSVMSIRPDYWGQGTQVTVSS	84
BCMA CAR2 Full CAR (no signal peptide) (amino acid)	QVKLEESGGGLVQAGRSLRLSCAASEHTFSSHVMGWFRQAP GKERESVAVIGWRDISTSYADSVKGRFTISRDNAKKTLYLQM NSLKPEDTAVYYCAARRIDAADFDSWGQGTQVTVSSGGGGS EVQLVESGGGLVQAGGSLRLSCAASGRTFTMGWFRQAPGK EREFVAAISLSPTLAYYAESVKGRFTISRDNAKNTVVLQMNS LKPEDTALYYCAADRKSVMSIRPDYWGQGTQVTVSSTTTPA PRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQT TQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQL YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYN ELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDT YDALHMQALPPR	85
BCMA CAR2 Full CAR (with signal peptide) (amino acid)	MALPVTALLLPLALLLHAARPQVKLEESGGGLVQAGRSLRL SCAASEHTFSSHVMGWFRQAPGKERESVAVIGWRDISTSYA DSVKGRFTISRDNAKKTLYLQMNSLKPEDTAVYYCAARRID AADFDSWGQGTQVTVSSGGGGSEVQLVESGGGLVQAGGSL RLSCAASGRTFTMGWFRQAPGKEREFVAAISLSPTLAYYAES VKGRFTISRDNAKNTVVLQMNSLKPEDTALYYCAADRKSV MSIRPDYWGQGTQVTVSSTTTPAPRPPTPAPTIASQPLSLRPE ACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITL YCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGC ELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKR RGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKG ERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	86
BCMA CAR3		
BCMA CAR3 scFv (amino acid)	MALPVTALLLPLALLLHAARPDIVLTQSPPSLAMSLGKRATIS CRASESVTILGSHLIHWYQQKPGQPPTLLIQLASNVQTGVPA RFSGSGSRTDFTLTIDPVEEDDVAVYYCLQSRTIPRTFGGGTK LEIKGSTSGSGKPGSGEGSTKGQIQLVQSGPELKKPGETVKIS CKASGYTFTDYSINWVKRAPGKGLKWMGWINTETREPAYA YDFRGRFAFSLETSASTAYLQINNLKYEDTATYFCALDYSYA MDYWGQGTSVTVSS	87

BCMA CAR4		
BCMA CAR4 Full CAR (amino acid)	MALPVTALLLPLALLLHAARPDIVLTQSPPSLAMSLGKRATIS CRASESVTILGSHLIHWYQQKPGQPPTLLIQLASNVQTGVPA RFSGSGSRTDFTLTIDPVEEDDVAVYYCLQSRTIPRTFGGGTK LEIKGSTSGSGKPGSGEGSTKGQIQLVQSGPELKKPGETVKIS CKASGYTFTDYSINWVKRAPGKGLKWMGWINTETREPAYA YDFRGRFAFSLETSASTAYLQINNLKYEDTATYFCALDYSYA MDYWGQGTSVTVSSFVPVFLPAKPTTTPAPRPPTPAPTIASQP LSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLL SLVITLYCNHRNKRGRKKLLYIFKQPFMRPVQTTQEEDGCSC RFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRR EEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAE AYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQAL PPR	88
BCMA CAR5		
BCMA CAR5 Full CAR (amino acid)	MALPVTALLLPLALLLHAARPEVQLLESGGGLVQPGGSLRLS CAASGFTFSSYAMSWVRQAPGKGLEWVSSISGSGDYIYYAD SVKGRFTISRDISKNTLYLQMNSLRAEDTAVYYCAKEGTGA NSSLADYRGQGTLVTVSSFVPVFLPAKPTTTPAPRPPTPAPTI ASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCG VLLLSLVITLYCNHRNRSKRSRLLHSDYMNMTPRRPGPTRKH YQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNL GRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDK MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPR	89
BCMA CAR6		
BCMA CAR6 Full CAR (amino acid)	MALPVTALLLPLALLLHAARPEVQLLESGGGLVQPGGSLRLS CAASGFTFSSYAMSWVRQAPGKGLEWVSSISGSGDYIYYAD SVKGRFTISRDISKNTLYLQMNSLRAEDTAVYYCAKEGTGA NSSLADYRGQGTLVTVSSFVPVFLPAKPTTTPAPRPPTPAPTI ASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCG VLLLSLVITLYCNHRNKRGRKKLLYIFKQPFMRPVQTTQEED GCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELN LGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKD KMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALH MQALPPR	90
BCMA CAR7		
BCMA CAR7 VH (amino acid)	QVQLVQSGAEVKKPGASVKVSCKASGYSFPDYYINWVRQA PGQGLEWMGWIYFASGNSEYNQKFTGRVTMTRDTSSSTAY MELSSLRSEDTAVYFCASLYDYDWYFDVWGQGTMVTVSS	91
BCMA CAR 7 VL (amino acid)	DIVMTQTPLSLSVTPGEPASISCKSSQSLVHSNGNTYLHWYL QKPGQSPQLLIYKVSNRFSGVPDRFSGSGSGADFTLKISRVEA EDVGVYYCAETSHVPWTFGQGTKLEIK	92
BCMA CAR-8		
BCMA CAR-8 CD8α signal peptide (amino acid)	MALPVTALLLPLALLLHAARP	93
BCMA CAR-8 VH (amino acid)	EVQLLESGGGLVQPGGSLRLSCAAS**GFTFSSY**AMNWVRQAP GKGLEWVS**AISDSGGSTYYADSVKG**RFTISRDNSKNTLYLQ MNSLRAEDTAVYYCAR**YWPMDI**WGQGTLVTVSS	**94**

BCMA CAR-8 GS linker (amino acid)	GGGGSGGGGSGGGGS	**95**
BCMA CAR-8 VL (amino acid)	EIVLTQSPGTLSLSPGERATLSC**RASQSVSSSYLA**WYQQKPG QAPRLLMY**DASIRAT**GIPDRFSGSGSGTDFTLTISRLEPEDFA VYYC**QQYGSWPLT**FGQGTKVEIK	**96**
BCMA CAR-8 CD20 epitope (amino acid)	GSGGGGSCPYSNPSLCSGGGGSCPYSNPSLCSGGGGS	**97**
BCMA CAR-8 CD8α hinge (amino acid)	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFA CD	**78**
BCMA CAR-8 CD8α transmembrane domain (amino acid)	IYIWAPLAGTCGVLLLSLVITYC	**74**
BCMA CAR-8 4-1BB intracellular signaling domain (amino acid)	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	**77**
BCMA CAR-8 CD3z instracellular signaling domain (amino acid)	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGER RRGKGHDGLYQGLSTATKDTYDALHMQALPPR	**76**
BCMA CAR-8 Full CAR (amino acid)	MALPVTALLLPLALLLHAARPEVQLLESGGGLVQPGGSLRLS CAASGFTFSSYAMNWVRQAPGKGLEWVSAISDSGGSTYYA DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARYWP MDIWGQGTLVTVSSGGGGSGGGGSGGGGSEIVLTQSPGTLS LSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLMYDASI RATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSWPL TFGQGTKVEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG AVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKK LLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHD GLYQGLSTATKDTYDALH MQALPPR	**102**
BCMA CAR-9		
BCMA CAR-9 VH (amino acid)	EVQLLESGGGLVQPGGSLRLSCAAS**GFTFSSY**PMSWVRQAP GKGLEWVS**AIGGSGGSLPYADSVKG**RFTISRDNSKNTLYLQ MNSLRAEDTAVYYCAR**YWPMDI**WGQGTLVTVSS	**103**
BCMA CAR-9 VL (amino acid)	EIVLTQSPGTLSLSPGERATLSC**RASQSVSSSYLA**WYQQKPG QAPRLLMY**DASIRAT**GIPDRFSGSGSGTDFTLTISRLEPEDFA VYYC**QQYQSWPLT**FGQGTKVEIK	**104**
BCMA CAR-10		

BCMA CAR-10 D domain (amino acid)	MGSWVEFWDRLGAIRERLEALGGSEAELAAFEKEIAAFESEL QAYKGKGNPEVEKLRYVAAVIRHRLQAYRHN	**105**
BCMA CAR-11		
BCMA CAR-11 D domain (amino acid)	MGSWSEFWVRLGAIRERLDALGGSEAELAAFEKEIAAFESEL QAYKGKGNPEVEKLRYTAATIRRFLQAYRHN	**106**
BCMA CAR-12		
BCMA CAR-12 D domain (amino acid)	MGSWSEFWARLGAIRERLDALGGSEAELAAFEKEIAAFESEL QAYKGKGNPEVEKLRYTAGTIKRFLQAYRHN	**107**
CD19 CAR1		
CD19 CAR1 VL (amino acid)	DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDG TVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATY FCQQGNTLPYTFGGGTKLEIT	108
CD19 CAR1 VH (amino acid)	EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPR KGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNS LQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSS	109
CD19 CAR1 scFv (amino acid)	DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDG TVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATY FCQQGNTLPYTFGGGTKLEITGSTSGSGKPGSGEGSTKGEVK LQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGL EWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQT DDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSS	110
CD19 CAR1 Full CAR (amino acid)	DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDG TVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATY FCQQGNTLPYTFGGGTKLEITGSTSGSGKPGSGEGSTKGEVK LQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGL EWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQT DDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSSTTTPAPR PPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWA PLAGTCGVLLLSLVITLYCRSKRSRLLHSDYMNMTPRRPGPT RKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYN ELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNEL QKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD ALHMQALPPRKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCR FPEEEEGGCEL	111
GPRC5D CAR1		
GPRC5D CAR1 VH (amino acid)	QMQLVQSGAEVKKPGASVKVSCKASGYTFNRYAITWVRQA PGQGLEWMGWISAYNGNSHYAQKLQGRVTMTTDTSTGTAY MELRRLRSDDTAVYYCARMAYDSWGQGTLVTVSS	112
GPRC5D CAR1 VL (amino acid)	QSVLTQPASVSGSPGQSLTISCTGTSNDVGAYKYVSWYQQY PGKAPKLILYDVFKRPSGVSNRFSGSKSDNTASLTISGLQAED EADYYCFSLTSSNTYVFGTGTKVTVLG	113
GPRC5D CAR1 scFv (amino acid)	QSVLTQPASVSGSPGQSLTISCTGTSNDVGAYKYVSWYQQY PGKAPKLILYDVFKRPSGVSNRFSGSKSDNTASLTISGLQAED EADYYCFSLTSSNTYVFGTGTKVTVLGSRGGGGSGGGGSGG GGSLEMAQMQLVQSGAEVKKPGASVKVSCKASGYTFNRYA ITWVRQAPGQGLEWMGWISAYNGNSHYAQKLQGRVTMTT DTSTGTAYMELRRLRSDDTAVYYCARMAYDSWGQGTLVTV SS	114

GPRC5D CAR2		
GPRC5D CAR2 VH (amino acid)	EVQLVESGGGLVKPGGSLRLSCAASGFTFSDYYMSWIRQAP GKGLEWVSYISSSGSTIYYADSVKGRFTISRDNAKNSLYLQM NSLRAEDTAVYYCARGYGKAYDQWGQGTLVTVSS	115
GPRC5D CAR2 VL (amino acid)	QSVLTQPPSASGTPGQRVTISCSGSRSNVGGNYVFWYQQVPG ATPKLLIYRSNQRPSGVPDRFAGSKSGSSASLAISGLRSEDEA DYYCATWDDSLSGFVFGTGTKVTVLG	116
GPRC5D CAR2 scFv (amino acid)	QSVLTQPPSASGTPGQRVTISCSGSRSNVGGNYVFWYQQVPG ATPKLLIYRSNQRPSGVPDRFAGSKSGSSASLAISGLRSEDEA DYYCATWDDSLSGFVFGTGTKVTVLGSRGGGGSGGGGSGG GGSLEMAEVQLVESGGGLVKPGGSLRLSCAASGFTFSDYYM SWIRQAPGKGLEWVSYISSSGSTIYYADSVKGRFTISRDNAK NSLYLQMNSLRAEDTAVYYCARGYGKAYDQWGQGTLVTV SS	117
GPRC5D CAR3		
GPRC5D CAR3 VH (amino acid)	QVQLVESGGGLVHPGGSLRLSCAASGFTFRSHSMNWVRQAP GKGLEWVSSISSDSTYTYYADSVKGRFTISRDNAKNSLYLQM NSLRAEDTAVYYCARSGGQWKYYDYWGQGTLVTVSS	118
GPRC5D CAR3 VL (amino acid)	SSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQ APVLVIYGKNNRPSGIPDRFSGSSSGNTASLTITGAQAEDEAD YYCNSRDSSGNPPVVFGGGTKLTVLG	119
GPRC5D CAR3 scFv (amino acid)	SSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQ APVLVIYGKNNRPSGIPDRFSGSSSGNTASLTITGAQAEDEAD YYCNSRDSSGNPPVVFGGGTKLTVLGSRGGGGSGGGGSGGG GSLEMAQVQLVESGGGLVHPGGSLRLSCAASGFTFRSHSMN WVRQAPGKGLEWVSSISSDSTYTYYADSVKGRFTISRDNAK NSLYLQMNSLRAEDTAVYYCARSGGQWKYYDYWGQGTLV TVSS	120
GPRC5D CAR4		
GPRC5D CAR4 VH (amino acid)	EVQLVESGGGLIQPGGSLRLSCAASGFTFSNYAMNWVRQAP GKGLEWVSTINGRGSSTIYADSVKGRFTISRDNSKNTLYLQM NSLRAEDTATYYCARYISRGLGDSWGQGTLVTV	121
GPRC5D CAR4 VL (amino acid)	QSVVTQPPSMSAAPGQQVTISCSGGNSNIERNYVSWYLQLPG TAPKLVIFDNDRRPSGIPDRFSGSKSGTSATLGITGLQTGDEA DYYCGTWDSSLRGWVFGGGTKLTVLG	122
GPRC5D CAR4 scFv (amino acid)	QSVVTQPPSMSAAPGQQVTISCSGGNSNIERNYVSWYLQLPG TAPKLVIFDNDRRPSGIPDRFSGSKSGTSATLGITGLQTGDEA DYYCGTWDSSLRGWVFGGGTKLTVLGSRGGGGSGGGGSGG GGSLEMAEVQLVESGGGLIQPGGSLRLSCAASGFTFSNYAM NWVRQAPGKGLEWVSTINGRGSSTIYADSVKGRFTISRDNSK NTLYLQMNSLRAEDTATYYCARYISRGLGDSWGQGTLVTV	123
CD20 CAR 1 scFv (amino acid)	IVLTQSPAILSASPGEKVTMTCRASSSVNYMDWYQKKPGSSP KPWIYATSNLASGVPARFSGSGSGTSYSLTISRVEAEDAATY YCQQWSFNPPTFGGGTKLEIKGSTSGGGSGGGSGGGGSSEV QLQQSGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPG QGLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQL SSLTSEDSADYYCARSNYYGSSYWFFDVWGAGTTVTVSS	124
CD20 CAR 1 Full CAR (amino acid)	METDTLLLWVLLLWVPGSTGDIVLTQSPAILSASPGEKVTMT CRASSSVNYMDWYQKKPGSSPKPWIYATSNLASGVPARFSG SGSGTSYSLTISRVEAEDAATYYCQQWSFNPPTFGGGTKLEI	125

	KGSTSGGGSGGGSGGGGSSEVQLQQSGAELVKPGASVKMSC KASGYTFTSYNMHWVKQTPGQGLEWIGAIYPGNGDTSYNQ KFKGKATLTADKSSSTAYMQLSSLTSEDSADYYCARSNYYG SSYWFFDVWGAGTTVTVSSESKYGPPCPPCPMFWVLVVVG GVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPT RKHYQPYAPPRDFAAYRSFSRSADAPAYQQGQNQLYNELNL GRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDK MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPR	

CAR Compositions, Methods of Manufacture, and Uses

[0428] A system described herein may comprise:

(a) a gene modifying polypeptide or a nucleic acid (e.g., DNA or mRNA) encoding the gene modifying polypeptide, as disclosed herein, and

(b) a template RNA (or DNA encoding the template RNA) comprising (i) a sequence that binds the polypeptide and (ii) a heterologous object sequence encoding a chimeric antigen receptor (CAR), wherein the CAR comprises an antigen-binding domain, a transmembrane domain, a first intracellular signaling domain, and a second intracellular signaling domain, as disclosed herein.

[0429] In some embodiments, one or more of:

(i) the CAR comprises an antigen binding domain that binds one or more antigens of a blood cancer (e.g., a leukemia or lymphoma), wherein optionally the antigen is a B cell antigen;

(ii) the CAR comprises an antigen binding domain that binds one or more antigens of a solid tumor;

(iii) the CAR comprise an antigen binding domain of any one of **Tables 5-10** or **16**;

(iv) the CAR comprises a transmembrane domain of **Table 11** or **12**;

(v) the CAR comprises a hinge domain (e.g., a hinge domain of **Table 15**);

(vi) the CAR comprises an intracellular signaling domain of **Table 13** or **14**;

(vii) the CAR comprises a costimulatory domain of **Table 13** or **14**;

(viii) the CAR comprises an antigen binding domain which comprises an scFv; or

(ix) the CAR comprises an amino acid sequence according to of **Table 16**;

(x) wherein the CAR comprises a first intracellular signaling domain and a second intracellular signaling domain.

[0430] Additionally provided herein are methods of modifying a mammalian cell to express a CAR. In some embodiments, the method involves contacting the cell (e.g., an immune effector cell) with a composition disclosed herein. In some embodiments, the immune effector cell is a cell that expresses one or more Fc receptors and mediates one or more effector functions. In some embodiments, the immune effector cell may include, but may not be limited to, one or more of a monocyte, macrophage, neutrophil, dendritic cell, eosinophil, mast cell, platelet, large granular lymphocyte, Langerhans' cell, natural killer (NK) cell, T-lymphocyte (e.g., T-cell), a Gamma delta T cell, B-lymphocyte (e.g., B-cell) and may be from any organism including but not limited to humans, mice, rats, rabbits, and monkeys. In some embodiments, the mammalian cell is a T cell, e.g., a primary T cell.

[0431] In some embodiments, the method is performed *ex vivo* or *in vitro*.

[0432] In some embodiments, a retrotransposon gene modifying system described herein may be used in a multiplex format. For instance, the retrotransposon gene modifying system may be used to install a large sequence, such as a CAR sequence, and at the same time a heterologous gene modifying system may be used to make a smaller edit.

Heterologous Gene Modifying Systems

[0433] A heterologous gene modifying system may comprise a heterologous gene modifying polypeptide and a template RNA. The heterologous gene modifying polypeptide may comprise a Cas domain, a linker, and a reverse transcriptase domain derived from a retrovirus. The template RNA compatible with the heterologous gene modifying polypeptide may comprise (e.g., from 5′ to 3′) (i) a gRNA spacer that binds a target site, (ii) a gRNA scaffold that binds the heterologous gene modifying polypeptide, e.g., the Cas domain of the heterologous gene modifying polypeptide, (iii) a heterologous object sequence, and (iv) a primer binding site (PBS) sequence. These components are now described in more detail.

[0434] In some aspects, a heterologous gene modifying polypeptide described herein comprises (e.g., a system described herein comprises a gene modifying polypeptide that comprises): 1) a Cas domain (e.g., a Cas nickase domain, e.g., a Cas9 nickase domain); 2) a reverse transcriptase (RT) domain, wherein the RT domain is C-terminal of the Cas domain; and a linker disposed between the RT domain and the Cas domain.

[0435] In some embodiments, the heterologous gene modifying polypeptide comprises a sequence of SEQ ID NO:126 which comprises the first NLS and the Cas domain, or a sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% identity thereto. In some embodiments, the heterologous gene modifying polypeptide comprises a sequence of SEQ ID NO:127 which comprises the second NLS, or a sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% identity thereto.

Exemplary N-terminal NLS-Cas9 domain

MPAAKRVKLDGGDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLI
GALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVE
EDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGH
FLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQ
LPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYA
DLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKY
KEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDN
GSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRK
SEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKV
KYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDR
FNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKV
MKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKE
DIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMAR
ENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMY
VDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKARGKSDNVPSEEVVKKMKN
YWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMN
TKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIK
KYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRK
RPLIETNGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKL
IARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNP
IDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLY
LASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKH
RDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETR
IDLSQLGGDGG (SEQ ID NO:126)

Exemplary C-terminal sequence comprising an NLS

AGKRTADGSEFEKRTADGSEFESPKKKAKVE (SEQ ID NO:127)

[0436] In some embodiments, a heterologous gene modifying polypeptide described herein comprises an RT domain having an amino acid sequence according to Table 6 of International Application WO/2023/039440 (which Table is incorporated herein by reference in its entirety), or a sequence having at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity thereto.

[0437] In some embodiments, a heterologous gene modifying polypeptide comprises: (i) a linker comprising a linker sequence as listed in a row of **Table 17**, or an amino acid sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity thereto; and (ii) an RT domain comprising an RT domain sequence as listed in the same row of **Table 17,** or an amino acid sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity thereto. In some embodiments, a heterologous gene modifying polypeptide comprises an amino acid sequence according to **Table 18**, or an amino acid sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity thereto.

Table 17. Selection of exemplary gene modifying polypeptides

SEQ ID NO: for Full Polypeptide Sequence	Linker Sequence	RT name and sequence
129	AEAAAKEAAAKEAAAKEAAAKALEAEAAAKEAAAKEAAAKEAAAKA (SEQ ID NO: 128)	AVIRE_P03360_3mutA TAPLEEEYRLFLEAPIQNVTLLEQWKREIPKVVWAEINPPG LASTQAPIHVQLLSTALPVRVRQYPITLEAKRSLRETIRKF RAAGILRPVHSPWNTPLLPVRKSGTSEYRMVQDLREVN KRVETIHPTVPNPYTLLSLLPPDRIWYSVLDLKDAFFCIPL APESQLIFAFEWADAEEGESGQLTWTRLPQGFKNSPTLF NEALNRDLQGFRLDHPSVSLLQYVDDLLIAADTQAACLS ATRDLLMTLAELGYRVSGKKAQLCQEEVTYLGFKIHKG SRSLSNSRTQAILQIPVPKTKRQVREFLGKIGYCRLFIPGF AELAQPLYAATRPGNDPLVWGEKEEEAFQSLKLALTQP PALALPSLDKPFQLFVEETSGAAKGVLTQALGPWKRPV AYLSKRLDPVAAGWPRCLRAIAAAALLTREASKLTFGQ DIEITSSHNLESLLRSPPDKWLTNARITQYQVLLLDPPRV RFKQTAALNPATLLPETDDTLPIHHCLDTLDSLTSTRPDL TDQPLAQAEATLFTDGSSYIRDGKRYAGAAVVTLDSVI WAEPLPIGTSAQKAELIALTKALEWSKDKSVNIYTDSRY AFATLHVHGMIYRERGWLTAGGKAIKNAPEILALLTAV WLPKRVAVMHCKGHQKDDAPTSTGNRRADEVAREVAI RPLSTQATIS (SEQ ID NO:129)
130	AEAAAKEAAAKEAAAK	FLV_P10273_3mutA

	EAAAKALE AEAAAKEA AAKEAAAK EAAAKA (SEQ ID NO: 128)	TLQLEEEYRLFEPESTQKQEMDIWLKNFPQAWAETGGM GTAHCQAPVLIQLKATATPISIRQYPMPHEAYQGIKPHIR RMLDQGILKPCQSPWNTPLLPVKKPGTEDYRPVQDLRE VNKRVEDIHPTVPNPYNLLSTLPPSHPWYTVLDLKDAFF CLRLHSESQLLFAFEWRDPEIGLSGQLTWTRLPQGFKNS PTLFNEALHSDLADFRVRYPALVLLQYVDDLLLAAATR TECLEGTKALLETLGNKGYRASAKKAQICLQEVTYLGY SLKDGQRWLTKARKEAILSIPVPKNSRQVREFLGKAGYC RLFIPGFAELAAPLYPLTRPGTLFQWGTEQQLAFEDIKKA LLSSPALGLPDITKPFELFIDENSGFAKGVLVQKLGPWKR PVAYLSKKLDTVASGWPPCLRMVAAIAILVKDAGKLTL GQPLTILTSHPVEALVRQPPNKWLSNARMTHYQAMLLD AERVHFGPTVSLNPATLLPLPSGGNHHDCLQILAETHGT RPDLTDQPLPDADLTWYTDGSSFIRNGEREAGAAVTTES EVIWAAPLPPGTSAQRAELIALTQALKMAEGKKLTVYT DSRYAFATTHVHGEIYRRRGWLTSEGKEIKNKNEILALL EALFLPKRLSIIHCPGHQKGDSPQAKGNRLADDTAKKAA TETHSSLTVLP (SEQ ID NO:130)
131	AEAAAKEA AAKEAAAK EAAAKALE AEAAAKEA AAKEAAAK EAAAKA (SEQ ID NO: 128)	MLVMS_P03355_3mutA_WS TLNIEDEHRLHETSKEPDVSLGSTWLSDFPQAWAETGG MGLAVRQAPLIIPLKATSTPVSIKQYPMSQEARLGIKPHI QRLLDQGILVPCQSPWNTPLLPVKKPGTNDYRPVQDLRE VNKRVEDIHPTVPNPYNLLSGLPPSHQWYTVLDLKDAFF CLRLHPTSQPLFAFEWRDPEMGISGQLTWTRLPQGFKNS PTLFNEALHRDLADFRIQHPDLILLQYVDDLLLAATSELD CQQGTRALLQTLGNLGYRASAKKAQICQKQVKYLGYLL KEGQRWLTEARKETVMGQPTPKTPRQLREFLGKAGFCR LFIPGFAEMAAPLYPLTKPGTLFNWGPDQQKAYQEIKQA LLTAPALGLPDLTKPFELFVDEKQGYAKGVLTQKLGPW RRPVAYLSKKLDPVAAGWPPCLRMVAAIAVLTKDAGK LTMGQPLVILAPHAVEALVKQPPDRWLSNARMTHYQAL LLDTDRVQFGPVVALNPATLLPLPEEGLQHNCLDILAEA HGTRPDLTDQPLPDADHTWYTDGSSLLQEGQRKAGAA VTTETEVIWAKALPAGTSAQRAELIALTQALKMAEGKK LNVYTDSRYAFATAHIHGEIYRRRGWLTSEGKEIKNKDE ILALLKALFLPKRLSIIHCPGHQKGHSAEARGNRMADQA ARKAAITETPDTSTLL (SEQ ID NO:131)
132	AEAAAKEA AAKEAAAK EAAAKALE AEAAAKEA AAKEAAAK EAAAKA (SEQ ID NO: 128)	SFV3L_P27401_2mutA MDPLQLLQPLEAEIKGTKLKAHWNSGATITCVPQAFLEE EVPIKNIWIKTIHGEKEQPVYYLTFKIQGRKVEAEVISSPY DYILVSPSDIPWLMKKPLQLTTLVPLQEYEERLLKQTML TGSYKEKLQSLFLKYDALWQHWENQVGHRRIKPHHIAT GTVNPRPQKQYPINPKAKASIQTVINDLLKQGVLIQQNSI MNTPVYPVPKPDGKWRMVLDYREVNKTIPLIAAQNQHS AGILSSIFRGKYKTTLDLSNGFWAHSITPESYWLTAFTWL GQQYCWTRLPQGFLNSPALFNADVVDLLKEVPNVQVY VDDIYISHDDPREHLEQLEKVFSLLLNAGYVVSLKKSEIA QHEVEFLGFNITKEGRGLTETFKQKLLNITPPRDLKQLQS ILGKLNFARNFIPNFSELVKPLYNIIATAPGKYITWTTDNS QQLQNIISMLNSAENLEERNPEVRLIMKVNTSPSAGYIRF YNEFAKRPIMYLNYVYTKAEVKFTNTEKLLTTIHKGLIK

		ALDLGMGQEILVYSPIVSMTKIQKTPLPERKALPIRWITW MSYLEDPRIQFHYDKTLPELQQVPTVTDDIIAKIKHPSEFS MVFYTDGSAIKHPNVNKSHNAGMGIAQVQFKPEFTVIN TWSIPLGDHTAQLAEVAAVEFACKKALKIDGPVLIVTDS FYVAESVNKELPYWQSNGFFNNKKKPLKHVSKWKSIAD CIQLKPDIIIIHEKGHQPTASTFHTEGNNLADKLATQGSY VVN (SEQ ID NO:132)

Table 18. Full length amino acid sequence corresponding to Table T1

SEQ ID NO:	Amino acid sequence
133	MPAAKRVKLDGGDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNL IGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESF LVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIK FRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRR LENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLL AQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKA LVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNR EDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVG PLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKH SLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYF KKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFE DREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKS DGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKV VDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPV ENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTR SDKARGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFI KRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKV REINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKAT AKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSMPQ VNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKV EKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELE NGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHY LDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAA FKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDGGAEAAAKEAAAK EAAAKEAAAKALEAEAAAKEAAAKEAAAKEAAAKAGGTAPLEEEYRLFLEAPIQNVTL LEQWKREIPKVWAEINPPGLASTQAPIHVQLLSTALPVRVRQYPITLEAKRSLRETIR KFRAAGILRPVHSPWNTPLLPVRKSGTSEYRMVQDLREVNKRVETIHPTVPNPYTLLS LLPPDRIWYSVLDLKDAFFCIPLAPESQLIFAFEWADAEEGESGQLTWTRLPQGFKNS PTLFNEALNRDLQGFRLDHPSVSLLQYVDDLLIAADTQAACLSATRDLLMTLAELGYR VSGKKAQLCQEEVTYLGFKIHKGSRSLSNSRTQAILQIPVPKTKRQVREFLGKIGYCR LFIPGFAELAQPLYAATRPGNDPLVWGEKEEEAFQSLKLALTQPPALALPSLDKPFQL FVEETSGAAKGVLTQALGPWKRPVAYLSKRLDPVAAGWPRCLRAIAAAALLTREASKL TFGQDIEITSSHNLESLLRSPPDKWLTNARITQYQVLLLDPPRVRFKQTAALNPATLL PETDDTLPIHHCLDTLDSLTSTRPDLTDQPLAQAEATLFTDGSSYIRDGKRYAGAAVV TLDSVIWAEPLPIGTSAQKAELIALTKALEWSKDKSVNIYTDSRYAFATLHVHGMIYR ERGWLTAGGKAIKNAPEILALLTAVWLPKRVAVMHCKGHQKDDAPTSTGNRRADEVAR EVAIRPLSTQATISAGKRTADGSEFEKRTADGSEFESPKKKAKVE
134	MPAAKRVKLDGGDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNL IGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESF

	LVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIK FRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRR LENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLL AQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKA LVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNR EDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVG PLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKH SLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYF KKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFE DREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKS DGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKV VDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPV ENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTR SDKARGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFI KRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKV REINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKAT AKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSMPQ VNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKV EKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELE NGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHY LDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAA FKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDGGAEAAAKEAAAK EAAAKEAAAKALEAEAAAKEAAAKEAAAKEAAAKAGGTLQLEEEYRLFEPESTQKQEM DIWLKNFPQAWAETGGMGTAHCQAPVLIQLKATATPISIRQYPMPHEAYQGIKPHIRR MLDQGILKPCQSPWNTPLLPVKKPGTEDYRPVQDLREVNKRVEDIHPTVPNPYNLLST LPPSHPWYTVLDLKDAFFCLRLHSESQLLFAFEWRDPEIGLSGQLTWTRLPQGFKNSP TLFNEALHSDLADFRVRYPALVLLQYVDDLLLAAATRTECLEGTKALLETLGNKGYRA SAKKAQICLQEVTYLGYSLKDGQRWLTKARKEAILSIPVPKNSRQVREFLGKAGYCRL FIPGFAELAAPLYPLTRPGTLFQWGTEQQLAFEDIKKALLSSPALGLPDITKPFELFI DENSGFAKGVLVQKLGPWKRPVAYLSKKLDTVASGWPPCLRMVAAIAILVKDAGKLTL GQPLTILTSHPVEALVRQPPNKWLSNARMTHYQAMLLDAERVHFGPTVSLNPATLLPL PSGGNHHDCLQILAETHGTRPDLTDQPLPDADLTWYTDGSSFIRNGEREAGAAVTTES EVIWAAPLPPGTSAQRAELIALTQALKMAEGKKLTVYTDSRYAFATTHVHGEIYRRRG WLTSEGKEIKNKNEILALLEALFLPKRLSIIHCPGHQKGDSPQAKGNRLADDTAKKAA TETHSSLTVLPAGKRTADGSEFEKRTADGSEFESPKKKAKVE
135	MPAAKRVKLDGGDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNL IGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESF LVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIK FRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRR LENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLL AQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKA LVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNR EDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVG PLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKH SLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYF KKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFE DREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKS DGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKV VDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPV ENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTR SDKARGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFI KRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKV

	REINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKAT AKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSMPQ VNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKV EKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELE NGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHY LDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAA FKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDGGAEAAAKEAAAK EAAAKEAAAKALEAEAAAKEAAAKEAAAKEAAAKAGGTLNIEDEHRLHETSKEPDVSL GSTWLSDFPQAWAETGGMGLAVRQAPLIIPLKATSTPVSIKQYPMSQEARLGIKPHIQ RLLDQGILVPCQSPWNTPLLPVKKPGTNDYRPVQDLREVNKRVEDIHPTVPNPYNLLS GLPPSHQWYTVLDLKDAFFCLRLHPTSQPLFAFEWRDPEMGISGQLTWTRLPQGFKNS PTLFNEALHRDLADFRIQHPDLILLQYVDDLLLAATSELDCQQGTRALLQTLGNLGYR ASAKKAQICQKQVKYLGYLLKEGQRWLTEARKETVMGQPTPKTPRQLREFLGKAGFCR LFIPGFAEMAAPLYPLTKPGTLFNWGPDQQKAYQEIKQALLTAPALGLPDLTKPFELF VDEKQGYAKGVLTQKLGPWRRPVAYLSKKLDPVAAGWPPCLRMVAAIAVLTKDAGKLT MGQPLVILAPHAVEALVKQPPDRWLSNARMTHYQALLLDTDRVQFGPVVALNPATLLP LPEEGLQHNCLDILAEAHGTRPDLTDQPLPDADHTWYTDGSSLLQEGQRKAGAAVTTE TEVIWAKALPAGTSAQRAELIALTQALKMAEGKKLNVYTDSRYAFATAHIHGEIYRRR GWLTSEGKEIKNKDEILALLKALFLPKRLSIIHCPGHQKGHSAEARGNRMADQAARKA AITETPDTSTLLAGKRTADGSEFEKRTADGSEFESPKKKAKVE
136	MPAAKRVKLDGGDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNL IGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESF LVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIK FRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRR LENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLL AQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKA LVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNR EDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVG PLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKH SLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYF KKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFE DREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKS DGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKV VDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPV ENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTR SDKARGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFI KRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKV REINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKAT AKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSMPQ VNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKV EKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELE NGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHY LDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAA FKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDGGAEAAAKEAAAK EAAAKEAAAKALEAEAAAKEAAAKEAAAKEAAAKAGGMDPLQLLQPLEAEIKGTKLKA HWNSGATITCVPQAFLEEEVPIKNIWIKTIHGEKEQPVYYLTFKIQGRKVEAEVISSP YDYILVSPSDIPWLMKKPLQLTTLVPLQEYEERLLKQTMLTGSYKEKLQSLFLKYDAL WQHWENQVGHRRIKPHHIATGTVNPRPQKQYPINPKAKASIQTVINDLLKQGVLIQQN SIMNTPVYPVPKPDGKWRMVLDYREVNKTIPLIAAQNQHSAGILSSIFRGKYKTTLDL SNGFWAHSITPESYWLTAFTWLGQQYCWTRLPQGFLNSPALFNADVVDLLKEVPNVQV YVDDIYISHDDPREHLEQLEKVFSLLLNAGYVVSLKKSEIAQHEVEFLGFNITKEGRG LTETFKQKLLNITPPRDLKQLQSILGKLNFARNFIPNFSELVKPLYNIIATAPGKYIT

	WTTDNSQQLQNIISMLNSAENLEERNPEVRLIMKVNTSPSAGYIRFYNEFAKRPIMYL NYVYTKAEVKFTNTEKLLTTIHKGLIKALDLGMGQEILVYSPIVSMTKIQKTPLPERK ALPIRWITWMSYLEDPRIQFHYDKTLPELQQVPTVTDDIIAKIKHPSEFSMVFYTDGS AIKHPNVNKSHNAGMGIAQVQFKPEFTVINTWSIPLGDHTAQLAEVAAVEFACKKALK IDGPVLIVTDSFYVAESVNKELPYWQSNGFFNNKKKPLKHVSKWKSIADCIQLKPDII IIHEKGHQPTASTFHTEGNNLADKLATQGSYVVNAGKRTADGSEFEKRTADGSEFESP KKKAKVE

[0438] In some embodiments, a heterologous gene modifying polypeptide may comprise a linker, e.g., a peptide linker, e.g., a linker as described in Table 10 of International Application WO/2023/039440 (which Table is incorporated herein by reference in its entirety).

[0439] In some embodiments, a template RNA molecule for use in the system comprises, from 5′ to 3′ (1) a gRNA spacer; (2) a gRNA scaffold; (3) heterologous object sequence (4) a primer binding site (PBS) sequence. In some embodiments:

(1) Is a gRNA spacer of ~18-22 nt, e.g., is 20 nt

(2) Is a gRNA scaffold comprising one or more hairpin loops, e.g., 1, 2, of 3 loops for associating the template with a Cas domain, e.g., a nickase Cas9 domain. In some embodiments, the gRNA scaffold comprises the sequence, from 5′ to 3′, GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA ACTTGAAAAAGTGGGACCGAGTCGGTCC (SEQ ID NO:137).

(3) In some embodiments, the heterologous object sequence is, e.g., 7-74, e.g., 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, or 70-80 nt or, 80-90 nt in length.

(4) In some embodiments, the PBS sequence that binds the target priming sequence after nicking occurs is e.g., 3-20 nt, e.g., 7-15 nt, e.g., 12-14 nt. In some embodiments, the PBS sequence has 40-60% GC content.

[0440] In some embodiments, the template RNA is configured to alter a genomic sequence at nucleotide TRAC and/or B2M genetic locus to disrupt the TRAC gene and/or B2M gene. In some embodiments, the gRNA spacer of the template RNA is complementary to a portion of the human TRAC gene, the heterologous object sequence installs a mutation in the TRAC gene, and the PBS is complementary to another portion of the human TRAC gene. In some embodiments, the template RNA directs a mutation that leads to a loss of TRAC mRNA. In some embodiments, the mutation leads to an inactive TRAC protein. In some embodiments, the gRNA spacer of the template RNA is complementary to a portion of the human B2M gene, the heterologous object sequence installs a mutation in the B2M gene, and the PBS is complementary to another portion of the human B2M gene. In some embodiments, the template

RNA directs a mutation that leads to a loss of B2M mRNA. In some embodiments, the mutation leads to an inactive B2M protein.

Combined Systems for Installing a Gene and Editing a Gene within a Cell

[0441] In some embodiments, a retrotransposase gene modifying system, used to insert a heterologous object sequence (e.g., a CAR) into the genome of a cell, may be combined with an additional system for modifying DNA to install a mutation (e.g., resulting in knock down or knock out of a target gene, or a correction or deletion of an aberrant gene) into a human gene. The additional system may be a heterologous gene modifying system.

[0442] In some embodiments, two systems may be utilized to introduce multiple edits to the same cell. In some embodiments, the two systems may be introduced into cells simultaneously or separately. In some embodiments, editing efficiency when inserting whole genes using the retrotransposase gene modifying system is not affected by the co-delivery of another exemplary system containing a heterologous gene modifying polypeptide (e.g., directing a short edit, e.g., an indel or substitution in the DNA). In some embodiments, editing efficiency when making short edits (e.g., editing of 5 nucleotides or fewer) is not affected by the co-delivery of a retrotransposase gene modifying system (e.g., directing the insertion of a gene). In some embodiments, treating T cells with both the first and second systems results in high level of co-occurrence of the two editing events in the same cell. In some embodiments, no skewing in the distribution of the subpopulations or phenotypes of T cells is observed when comparing mock treated T cells, T cells edited by just the first gene modifying system, and T cells edited by both the first and second gene modifying systems.

Targeted LNPs for delivering gene modifying systems to immune cells

[0443] In some embodiments, multiple components of a gene modifying system may be prepared as a single LNP formulation, e.g., an LNP formulation comprises mRNA encoding for the gene modifying polypeptide and an RNA template. Ratios of nucleic acid components may be varied in order to maximize the properties of a therapeutic. In some embodiments, the ratio of RNA template to mRNA encoding a gene modifying polypeptide is about 1:1 to 100:1, e.g., about 1:1 to 20:1, about 20:1 to 40:1, about 40:1 to 60:1, about 60:1 to 80:1, or about 80:1 to 100:1, by molar ratio. In other embodiments, a system of multiple nucleic acids may be prepared by separate formulations, e.g., one LNP formulation comprising a template RNA and a second LNP formulation comprising an mRNA encoding a gene modifying polypeptide. In some embodiments, the system may comprise more than two nucleic acid components formulated into

LNPs. In some embodiments, the system may comprise a protein, e.g., a gene modifying polypeptide, and a template RNA formulated into at least one LNP formulation.

[0444] In some embodiments, the targeted LNPs (conjugates) of the disclosure may be used to simultaneously deliver gene modifying components to T cells that are capable of inserting CAR into the genome of the T cells and altering the T cell receptor alpha constant (TRAC) locus and/or the B2M genetic locus in T cells, hence disrupting the TRAC gene and/or B2M gene. In some embodiments, simultaneous insertion of a CAR sequence and knock out of a TRAC and/or B2M gene can be accomplished via LNP delivery of a retrotransposase gene modifying system. In some such embodiments, a single targeted LNP (conjugate) can deliver all of the components of the gene modifying system including (i) a gene modifying polypeptide; (ii) a template RNA (or DNA encoding the template RNA) that binds to the gene modifying polypeptide and a heterologous CAR sequence; and (iii) an additional system for modifying DNA to install a mutation (e.g., resulting in knock down or knock out of the TRAC gene and/or B2M gene. In alternative embodiments, multiple targeted LNPs (conjugates) can be used to deliver the components of the gene modifying system. For instance, one conjugate can be used to deliver including (i) a gene modifying polypeptide; and (ii) a template RNA (or DNA encoding the template RNA) that binds to the gene modifying polypeptide and a heterologous CAR sequence and a second conjugate can be used to deliver an additional system for modifying DNA to install a mutation (e.g., resulting in knock down or knock out of the TRAC gene and/or B2M gene.

[0445] In some aspects, the disclosure provides a targeted lipid nanoparticle (LNP) for *ex vivo* or *in vivo* delivery of a therapeutic agent to immune cells (e.g., T cells), wherein the targeted LNP comprises:

i. an ionizable lipid;

ii. a helper lipid;

iii. two different targeting moieties conjugated to the LNP, wherein each of the two different targeting moieties binds to a target on the surface of the immune cells (e.g., T cells); and

iv. one or more nucleic acids (e.g., two or more nucleic acids) encoding components of a system for modifying or altering genomic DNA (e.g., a gene modifying system), wherein the one or more nucleic acids (e.g., two or more nucleic acids) are RNA molecules.

[0446] In some embodiments, the dual binders can be added to the surface of the LNP by methods described in Section II of the Detailed Description. For instance, in some embodiments, each of the targeting moieties is conjugated to the lipid nanoparticle through a linker, wherein each linker comprises a Click product formed from a Click reaction between a first Click handle on the targeting moiety and a second Click handle on the LNP. In some such embodiments, the targeting moieties are antibodies or antigen binding fragments thereof. In other such embodiments, the targeting moieties are scFvs. In some embodiments, the targeting moieties are Fab fragments.

[0447] In some embodiments, one of the RNA molecules encoding a component of a system for modifying or altering genomic DNA (e.g., a gene modifying system) is an mRNA encoding a gene modifying polypeptide, as described herein. In some embodiments, one of the components of a system for modifying or altering genomic DNA (e.g., a gene modifying system) is an mRNA encoding a Cas9 nickase fused to a reverse transcriptase (RT) domain. In some embodiments, one of the components of a system for modifying or altering genomic DNA is an mRNA encoding a Cas9-RT fusion protein.

[0448] In some embodiments, one of the RNA molecules encoding a component of a system for modifying or altering genomic DNA (e.g., a gene modifying system) is a guide RNA (gRNA). In some embodiments, one of the RNA molecules encoding a component of a system for modifying or altering genomic DNA (e.g., a gene modifying system) is a template RNA encoding a heterologous nucleic acid for use with a gene modifying polypeptide to insert the heterologous nucleic acid sequence into a DNA sequence, e.g., the genomic DNA of a cell. In some embodiments, the components of a system for modifying or altering genomic DNA (e.g., a gene modifying system) have nuclease activity, e.g., nickase activity. In some embodiments, the components of a system for modifying or altering genomic DNA (e.g., a gene modifying system) do not have nuclease activity. In some embodiments, the components of a system for modifying or altering genomic DNA (e.g., a gene modifying system) do not elicit a double-stranded break in the genomic DNA. In such embodiments, the system for modifying or altering genomic DNA elicits a single-stranded break in the genomic DNA. In some embodiments, a system for modifying or altering genomic DNA induces target-primed reverse transcription (TPRT) to insert a heterologous sequence into the genomic DNA.

[0449] In some embodiments, the ionizable lipid is V003 or a lipid from **Table 1**, **Table 2**, or **Table 3**. In some embodiments, the ionizable lipid is Lipid 093. In some embodiments, the ionizable lipid is Lipid 092. In some embodiments, the ionizable lipid is Lipid 154.

[0450] In some embodiments, the helper lipid of the targeted LNP is DSPC or sphingomyelin. In some embodiments, the mo1% of the DSPC or sphingomyelin in the targeted LNP is from about 20% to about 30%. In some embodiments, the mol% of DSPC or sphingomyelin in the targeted LNP is about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 25%, about 27%, about 28%, about 29%, or about 30%.

[0451] In some embodiments, the targeted LNP further comprises a pegylated lipid. In some embodiments, the pegylated lipid comprises at least one C16 alkyl chain (e.g., two C16 alkyl chains). In some such embodiments, the pegylated is DPPE-PEG2000 or DPG-PEG2000.

[0452] In some embodiments, the targeted delivery (*ex vivo* or *in vivo*) of a system for modifying or altering genomic DNA (e.g., a gene modifying system) with an LNP comprising two or more T cells specific binders conjugated to the LNP results in synergistic enhancement of gene editing or modification of the target T cells. In some embodiments, the LNPs comprising dual binders are capable of modifying at least 5% of the genomes of the T cells. In some embodiments, the LNPs comprising dual binders are capable of modifying at least 10% of the genomes of the T cells. In some embodiments, the LNPs comprising dual binders are capable of modifying at least 15% of the genomes of the T cells. In some embodiments, the LNPs comprising dual binders are capable of modifying at least 20% of the genomes of the T cells. In some embodiments, the LNPs comprising dual binders are capable of modifying at least 25% of the genomes of the T cells. In some embodiments, the LNPs comprising dual binders are capable of modifying at least 30% of the genomes of the T cells. In some embodiments, the LNPs comprising dual binders are capable of modifying at least 30% of the genomes of the T cells. In some embodiments, the LNPs comprising dual binders are capable of modifying from about 10% to about 30% of the genomes of the T cells. In some embodiments, the LNPs comprising dual binders are capable of modifying from about 20% to about 30% of the genomes of the T cells. In some embodiments, the LNPs comprising dual binders are capable of modifying from about 10% to about 20% of the genomes of the T cells. In any of the foregoing embodiments, the system for modifying or altering genomic DNA elicits a single-stranded break in the genomic DNA. In some such embodiments, a system for modifying or altering genomic DNA includes a gene modifying polypeptide and a template RNA for a nucleic acid sequence to be inserted at a specific location of the genomic DNA, hence resulting in modification of the genomic DNA. In some embodiments, the gene modifying system comprises a Cas9 nickase fused to a reverse transcriptase (RT) domain. In some embodiments, a system for modifying or altering genomic DNA induces target-primed reverse transcription (TPRT) to insert a heterologous nucleic acid sequence into a DNA sequence, e.g., genomic DNA.

V. METHODS OF DELIVERY

[0453] In some embodiments, an LNP, e.g., targeted LNP, disclosed herein is delivered *ex vivo* to isolated immune cells (e.g., T cells) of a subject (e.g., human patient) in need thereof. In some such embodiments, the immune cells (e.g., T cells) are collected from the blood or bone marrow of a subject. Following isolation of the immune cells (e.g., T cells) the LNPs of the disclosure can be mixed with the immune cells (e.g., T cells), thereby resulting in effective transduction of the payload (e.g., gene modifying system) into the immune cells (e.g., T cells). The modified immune cells (e.g., T cells) can be administered to the patient. In some embodiments, the immune cells (e.g., T cells) are activated, e.g., with an activating agent (such as TransAct (Miltenyi Biotec)), prior to incubation with the LNPs. In some embodiments, the immune cells (e.g., T cells) are not activated prior to incubation with the LNPs.

[0454] In other embodiments, an LNP, e.g., targeted LNP, as disclosed herein can be introduced into cells, tissues and/or multicellular organisms. In other embodiments, the LNPs, e.g., targeted LNPs, are delivered to the cells via mechanical means or physical means.

[0455] Formulation of protein therapeutics is described in Meyer (Ed.), Therapeutic Protein Drug Products: Practical Approaches to formulation in the Laboratory, Manufacturing, and the Clinic, Woodhead Publishing Series (2012).

[0456] In some embodiments, the LNP described herein, e.g., targeted LNPs, are administered *in vivo* to a patient in need thereof. The high level of transduction and protein expression following *in vivo* administration allows for sufficient levels of transduction and expression of proteins (e.g., gene modifying proteins), and hence can be used to treat various diseases.

[0457] In some embodiments, an LNP, e.g., targeted LNP, described herein is delivered to a tissue or cell from or in the blood or bone marrow. In some such embodiments, the LNP, e.g., targeted LNP, is administered by parenteral administration (e.g., intravenous, intramuscular, subcutaneous, intradermal, epidural, intracerebral, intracerebroventricular, epicutaneous, nasal, intra-arterial, intra-articular, intracavernous, intraocular, intraosseous infusion, intraperitoneal, intrathecal, intrauterine, intravaginal, intravesical, perivascular, or transmucosal administration).

[0458] In some embodiments, an LNP, e.g., targeted LNP, of the disclosure delivers a therapeutic agent, as described above, to immune cells (e.g., T cells) of a patient following *in vivo* administration. In some embodiments, an LNP, e.g., targeted LNP, of the disclosure delivers a gene modifying polypeptide, or a nucleic acid encoding the gene modifying polypeptide, to immune cells (e.g., T cells) of a patient following *in vivo* administration. In some

embodiments, an LNP, e.g., targeted LNP, of the disclosure delivers a gene modifying system to immune cells (e.g., T cells) of a patient following *in vivo* administration. In some embodiments, an LNP, e.g., targeted LNP, of the disclosure delivers gene editing components to immune cells (e.g., T cells) of a patient following *in vivo* administration. Exemplary gene editing systems are described above. Following *in vivo* administration, the nucleic acids encoding components of a system for modifying or altering a genome can be expressed, resulting in *in vivo* gene editing of the immune cells (e.g., T cells)

[0459] It has surprisingly been discovered that particular combinations of targeting moieties (e.g., antibodies, Fab fragments or scFvs), and ionizable lipids in particular LNP formulations (e.g., comprising particular amounts of helper lipid) are capable of more effectively delivering therapeutic agents to immune cells (e.g., T cells) using an *ex vivo* procedure and/or delivering greater amounts of therapeutic agents to immune cells (e.g., T cells) following *in vivo* administration to a subject. In some cases, the LNPs described herein result in enhanced levels of modification or alteration of the genomic DNA of immune cells (e.g., T cells), e.g., enhanced levels of gene editing in the immune cells (e.g., T cells). In some embodiments, the percentage of immune cells (e.g., T cells) exhibiting modified or altered (e.g., edited) genomic DNA in the subject following *ex vivo* incubation or after administration of the LNPs, e.g., targeted LNPs, is at least 5%. In some embodiments, the percentage of immune cells (e.g., T cells) exhibiting modified or altered (e.g., edited) genomic DNA in the subject following *ex vivo* incubation or after administration of the LNPs, e.g., targeted LNPs, is at least 10%. In some embodiments, the percentage of immune cells (e.g., T cells) exhibiting modified or altered (e.g., edited) genomic DNA in the subject following *ex vivo* incubation or after administration of the LNPs, e.g., targeted LNPs, is at least 15%. In some embodiments, the percentage of immune cells (e.g., T cells) exhibiting modified or altered (e.g., edited) genomic DNA in the subject following *ex vivo* incubation or after administration of the LNPs, e.g., targeted LNPs, is at least 20%. In some cases, the LNPs described herein result in enhanced levels of modification or alteration of the genomic DNA of immune cells (e.g., T cells) e.g., enhanced levels of gene editing in the immune cells (e.g., T cells). In some embodiments, the percentage of immune cells (e.g., T cells) having modified or altered (e.g., edited) genomic DNA in the subject after administration of the LNPs, e.g., targeted LNPs, is at least 30%. In some embodiments, the percentage of immune cells (e.g., T cells) having modified or altered (e.g., edited) genomic DNA in the subject after administration of the LNPs, e.g., targeted LNPs, is at least 40%. In some embodiments, the percentage of immune cells (e.g., T cells) having modified or altered (e.g., edited) genomic DNA in the subject after administration of the LNPs, e.g., targeted LNPs, is at least 50%. In some

embodiments, the percentage of immune cells (e.g., T cells) having modified or altered (e.g., edited) genomic DNA in the subject after administration of the LNPs, e.g., targeted LNPs, is at least 60%. In some embodiments, the percentage of immune cells (e.g., T cells) having modified or altered (e.g., edited) genomic DNA in the subject after administration of the LNPs, e.g., targeted LNPs, is at least 70%. In some embodiments, the percentage of immune cells (e.g., T cells) having modified or altered (e.g., edited) genomic DNA in the subject after administration of the LNPs, e.g., targeted LNPs, is from about 20% to about 50%, from about 20% to about 40%, or from about 20% to about 30%.

[0460] In some embodiments, 10% more immune cells (e.g., T cells) have modified or altered (e.g., edited) genomic DNA in the subject after administration of an LNP as described herein, e.g., a targeted LNP, comprising a therapeutic agent (e.g., a nucleic acid encoding a gene modifying polypeptide or one or more nucleic acids encoding a gene modifying system) relative to a baseline LNP (e.g., a LNP comprising V003) comprising the same therapeutic agent. In some embodiments, 15% more immune cells (e.g., T cells) have modified or altered (e.g., edited) genomic DNA in the subject after administration of an LNP as described herein, e.g., a targeted LNP, comprising a therapeutic agent (e.g., a nucleic acid encoding a gene modifying polypeptide or one or more nucleic acids encoding a gene modifying system) relative to a baseline LNP (e.g., a LNP comprising V003) comprising the same therapeutic agent. In some embodiments, 20% more immune cells (e.g., T cells) have modified or altered (e.g., edited) genomic DNA in the subject after administration of an LNP as described herein, e.g., a targeted LNP, comprising a therapeutic agent (e.g., a nucleic acid encoding a gene modifying polypeptide or one or more nucleic acids encoding a gene modifying system) relative to a baseline LNP (e.g., a LNP comprising V003) comprising the same therapeutic agent. In some embodiments, 25% more immune cells (e.g., T cells) have modified or altered (e.g., edited) genomic DNA in the subject after administration of an LNP as described herein, e.g., a targeted LNP, comprising a therapeutic agent (e.g., a nucleic acid encoding a gene modifying polypeptide or one or more nucleic acids encoding a gene modifying system) relative to a baseline LNP (e.g., a LNP comprising V003) comprising the same therapeutic agent. In some embodiments, 30% more immune cells (e.g., T cells) have modified or altered (e.g., edited) genomic DNA in the subject after administration of an LNP as described herein, e.g., a targeted LNP, comprising a therapeutic agent (e.g., a nucleic acid encoding a gene modifying polypeptide or one or more nucleic acids encoding a gene modifying system) relative to a baseline LNP (e.g., a LNP comprising V003) comprising the same therapeutic agent. In some embodiments, 35% more immune cells (e.g., T cells) have modified or altered (e.g., edited) genomic DNA in the subject

after administration of an LNP as described herein, e.g., a targeted LNP, comprising a therapeutic agent (e.g., a nucleic acid encoding a gene modifying polypeptide or one or more nucleic acids encoding a gene modifying system) relative to a baseline LNP (e.g., a LNP comprising V003) comprising the same therapeutic agent. In some embodiments, 40% more immune cells (e.g., T cells) have modified or altered (e.g., edited) genomic DNA in the subject after administration of an LNP as described herein, e.g., a targeted LNP, comprising a therapeutic agent (e.g., a nucleic acid encoding a gene modifying polypeptide or one or more nucleic acids encoding a gene modifying system) relative to a baseline LNP (e.g., a LNP comprising V003) comprising the same therapeutic agent. In some embodiments, 45% more immune cells (e.g., T cells) have modified or altered (e.g., edited) genomic DNA in the subject after administration of an LNP as described herein, e.g., a targeted LNP, comprising a therapeutic agent (e.g., a nucleic acid encoding a gene modifying polypeptide or one or more nucleic acids encoding a gene modifying system) relative to a baseline LNP (e.g., a LNP comprising V003) comprising the same therapeutic agent. In some embodiments, 50% more immune cells (e.g., T cells) have modified or altered (e.g., edited) genomic DNA in the subject after administration of an LNP as described herein, e.g., a targeted LNP, comprising a therapeutic agent (e.g., a nucleic acid encoding a gene modifying polypeptide or one or more nucleic acids encoding a gene modifying system) relative to a baseline LNP (e.g., a LNP comprising V003) comprising the same therapeutic agent. In some embodiments, the number of immune cells (e.g., T cells) comprising modified or altered (e.g., edited) genomic DNA can be determined by DNA sequencing.

[0461] In some embodiments, the LNPs described herein can deliver greater amounts of a payload, e.g., a therapeutic agent or a reporter, to a immune cells (e.g., T cells) when administered *ex vivo* or *in vivo* to a subject compared to a baseline LNP (e.g., V003). In some embodiments, the targeted LNPs described herein can deliver greater amounts of a payload, e.g., a therapeutic agent or a reporter, to an immune cell (e.g., T cell) when administered *in vivo* to a subject compared to a baseline LNP (e.g., an LNP comprising V003) comprising the same targeting moieties as the targeted LNP. In some embodiments, the LNPs described herein, e.g., targeted LNPs, can deliver greater amounts of a nucleic acid, e.g., a nucleic acid (e.g., mRNA) encoding a gene modifying polypeptide or one or more nucleic acids comprising a gene modifying system), to an immune cell (e.g., T cell) when administered *in vivo* to a subject compared to a baseline LNP (e.g., an LNP comprising V003). In some cases, a nucleic acid payload (e.g., a therapeutic agent such as a nucleic acid (e.g., mRNA) encoding a gene modifying polypeptide or one or more nucleic acids encoding a gene modifying system) that is

delivered to immune cells (e.g., T cells) *in vivo* using an LNP as described herein (e.g., a targeted LNP) is expressed at higher levels relative to the same payload delivered using a baseline LNP (e.g., an LNP comprising V003). In some cases, a nucleic acid payload (e.g., mRNA) that is delivered to immune cells (e.g., T cells) *in vivo* using an LNP as described herein (e.g., a targeted LNP) exhibits 10% higher expression relative to the same payload delivered using a baseline LNP (e.g., an LNP comprising V003). In some cases, a nucleic acid payload (e.g., mRNA) that is delivered to immune cells (e.g., T cells) *in vivo* using an LNP as described herein (e.g., a targeted LNP) exhibits 15% higher expression relative to the same payload delivered using a baseline LNP (e.g., an LNP comprising V003). In some cases, a nucleic acid payload (e.g., mRNA) that is delivered to immune cells (e.g., T cells) *in vivo* using an LNP as described herein (e.g., a targeted LNP) exhibits 20% higher expression relative to the same payload delivered using a baseline LNP (e.g., an LNP comprising V003). In some cases, a nucleic acid payload (e.g., mRNA) that is delivered to immune cells (e.g., T cells) *in vivo* using an LNP as described herein (e.g., a targeted LNP) exhibits 25% higher expression relative to the same payload delivered using a baseline LNP (e.g., an LNP comprising V003). In some cases, a nucleic acid payload (e.g., mRNA) that is delivered to immune cells (e.g., T cells) *in vivo* using an LNP as described herein (e.g., a targeted LNP) exhibits 30% higher expression relative to the same payload delivered using a baseline LNP (e.g., an LNP comprising V003). In some cases, a nucleic acid payload (e.g., mRNA) that is delivered to immune cells (e.g., T cells) *in vivo* using an LNP as described herein (e.g., a targeted LNP) exhibits 35% higher expression relative to the same payload delivered using a baseline LNP (e.g., an LNP comprising V003). In some cases, a nucleic acid payload (e.g., mRNA) that is delivered to immune cells (e.g., T cells) *in vivo* using an LNP as described herein (e.g., a targeted LNP) exhibits 40% higher expression relative to the same payload delivered using a baseline LNP (e.g., an LNP comprising V003). In some cases, a nucleic acid payload (e.g., mRNA) that is delivered to immune cells (e.g., T cells) *in vivo* using an LNP as described herein (e.g., a targeted LNP) exhibits 45% higher expression relative to the same payload delivered using a baseline LNP (e.g., an LNP comprising V003). In some cases, a nucleic acid payload (e.g., mRNA) that is delivered to immune cells (e.g., T cells) *in vivo* using an LNP as described herein (e.g., a targeted LNP) exhibits 50% higher expression relative to the same payload delivered using a baseline LNP (e.g., an LNP comprising V003). In some embodiments, a nucleic acid payload (e.g., mRNA) that is delivered to immune cells (e.g., T cells) *in vivo* using an LNP as described herein (e.g., a targeted LNP) exhibits between 10% to 50%, between 20% to 40%, between 10% to 30%, or between 30% to 50% higher expression relative to the same payload delivered using a baseline LNP (e.g., an LNP comprising V003). In

some cases, the level of expression of the payload can be determined by measuring protein levels, e.g., by flow cytometry. In some cases, for example, when the payload comprises a gene modifying polypeptide or gene modifying system, the expression level of the payload can be measured by determining the level of modification or alteration of the genomic DNA of the immune cells (e.g., T cells) e.g., by DNA sequencing.

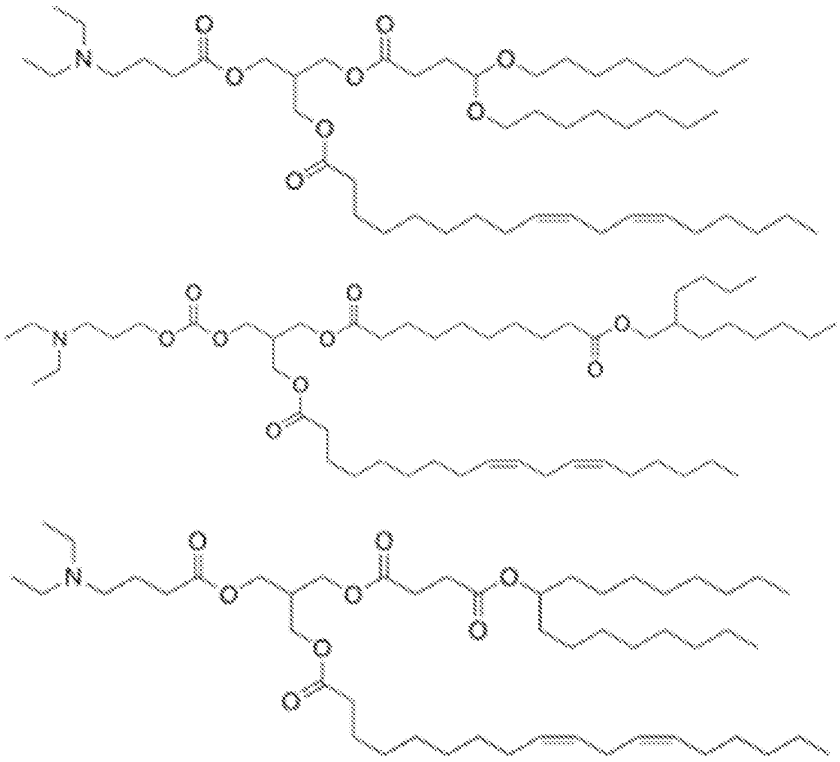
EXEMPLARY EMBODIMENTS

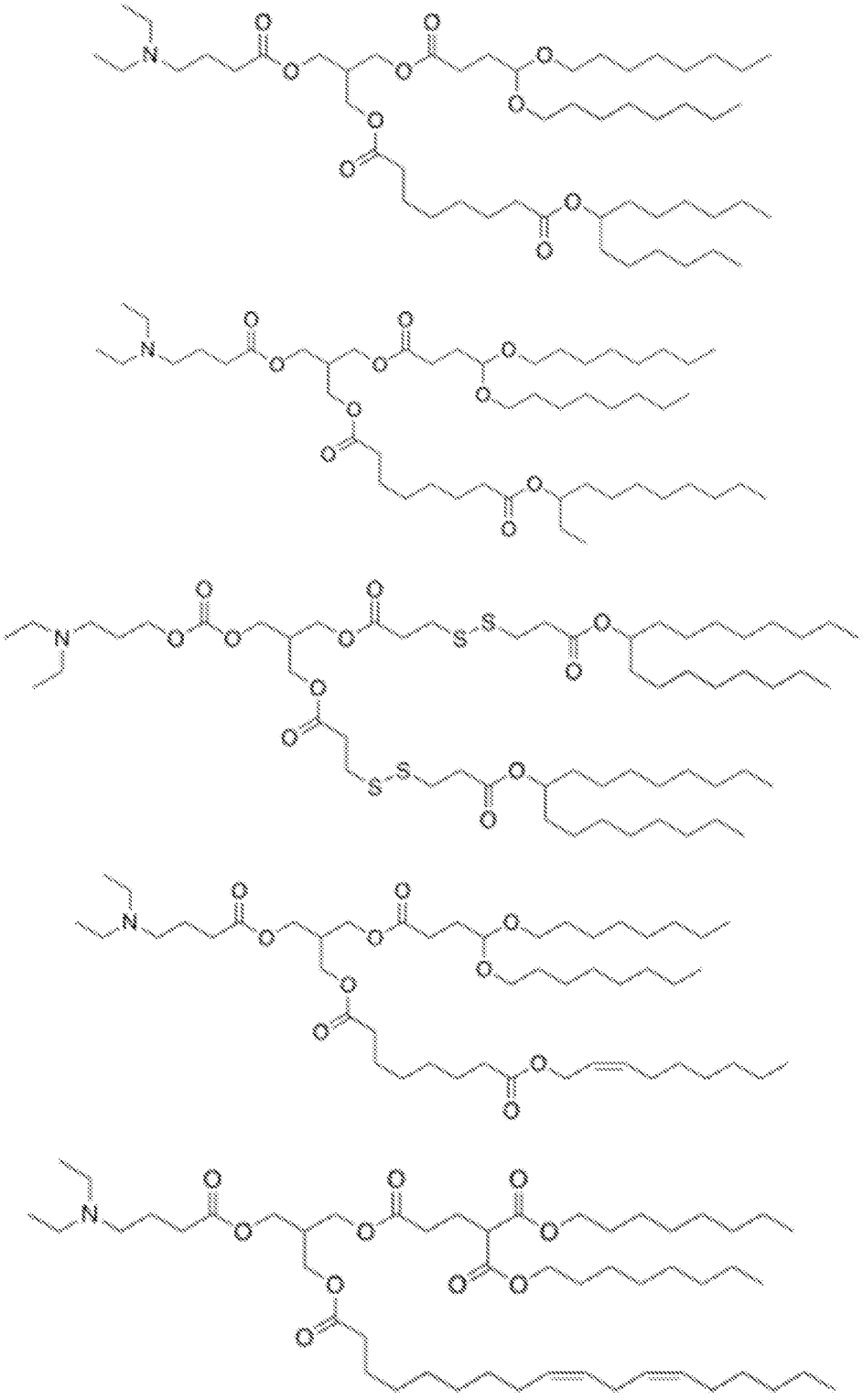
[0462] A lipid nanoparticle (LNP) for delivery of a therapeutic agent to immune cells *ex vivo* or *in vivo*, the LNP comprising:

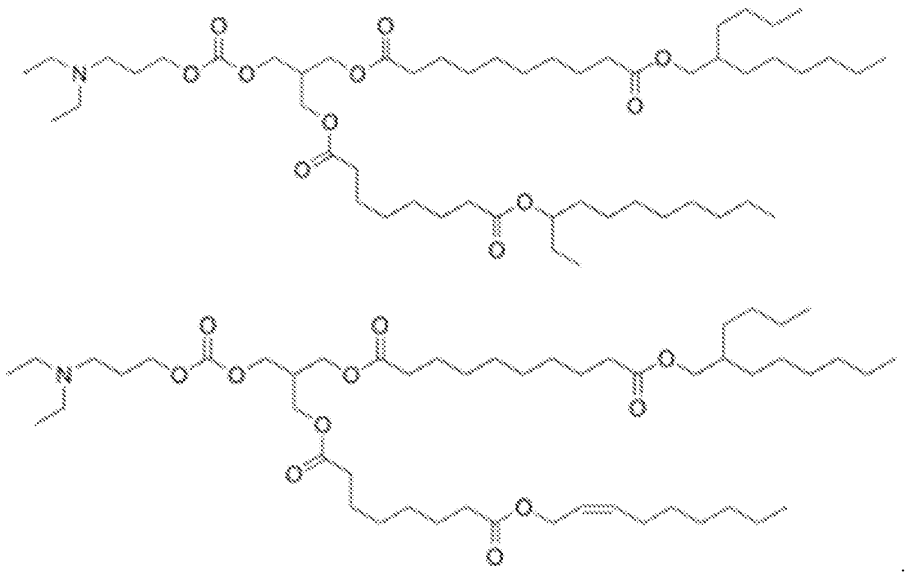
an ionizable lipid and a helper lipid, wherein the ionizable lipid is selected from the lipids in **Table 1**, **Table 2** and **Table 3**; and

a therapeutic agent encapsulated within the LNP.

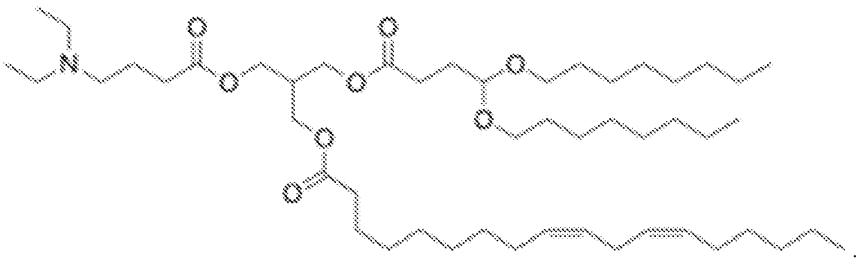
[0463] The LNP of embodiment 1, wherein the ionizable lipid has one of the following structures:



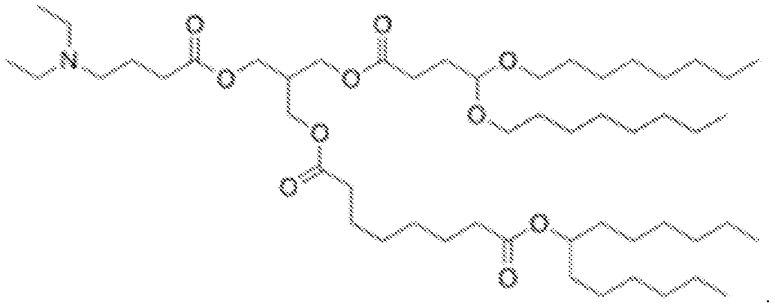




[0464] The LNP of embodiment 1, wherein the ionizable lipid has the structure:



[0465] LNP of embodiment 1, wherein the ionizable lipid has the structure:



[0466] The LNP of any one of embodiments 2-4, wherein the therapeutic agent comprises at least one nucleic acid molecule.

[0467] The LNP of embodiment 5, wherein the nucleic acid molecule is an mRNA molecule or a DNA molecule.

[0468] The LNP of embodiment 5, wherein the nucleic acid molecule is an mRNA molecule.

[0469] The LNP of embodiment 5, wherein the nucleic acid molecule is a siRNA or miRNA molecule.

[0470] The LNP of embodiment 5, wherein the therapeutic agent comprises two nucleic acid molecules.

[0471] The LNP of embodiment 9, wherein the two nucleic acid molecules are RNA molecules.

[0472] The LNP of embodiment 10, wherein one nucleic acid molecule comprises an mRNA molecule and one nucleic acid molecule comprises an RNA molecule.

[0473] The LNP of embodiment 11, wherein one nucleic acid molecule comprises an mRNA molecule and one nucleic acid molecule comprises a guide RNA.

[0474] The LNP of embodiment 9, wherein one nucleic acid molecule is an mRNA molecule and one nucleic acid is a DNA molecule.

[0475] The LNP of any one of embodiments 9-13, wherein at least one of the nucleic acid molecules encodes at least one component for altering a genome.

[0476] The LNP of embodiment 14, wherein the at least one component comprises an mRNA encoding a CRISPR-associated nuclease (Cas) and a guide RNA.

[0477] The LNP of embodiment 15, wherein the Cas is a Cas9 (e.g., spCas9) or a Cas12 (e.g., Cas12a).

[0478] The LNP of any one of embodiments 1-4, wherein the therapeutic agent comprises a gene modifying system, e.g., a retrotransposon gene modifying system or a heterologous gene modifying system.

[0479] The LNP of embodiment 7, wherein the mRNA encodes a gene modifying polypeptide.

[0480] The LNP of embodiment 11, wherein the mRNA encodes a gene modifying polypeptide and the RNA encodes a template RNA, e.g., a template RNA encoding a CAR.

[0481] The LNP any one of embodiments 1-19, wherein the mol% of ionizable lipids in the LNP ranges from about 35% to about 60%.

[0482] The LNP of embodiment 20, wherein the mol% of ionizable lipids in the LNP ranges from about 40% to about 50%.

[0483] The LNP of embodiment 20, wherein the mol% of ionizable lipids in the LNP ranges from about 45% to about 50%.

[0484] The LNP of any one of embodiments 1-22, wherein the helper lipid is selected from the group consisting of distearoyl-sn-glycero- phosphoethanolamine, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane- 1 - carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), monomethyl-phosphatidylethanolamine (such as 16-O-monomethyl PE), dimethyl- phosphatidylethanolamine (such as 16-O-dimethyl PE), l8-l-trans PE, l-stearoyl-2-oleoyl- phosphatidyethanolamine (SOPE), hydrogenated soy phosphatidylcholine, egg phosphatidylcholine (EPC), dioleoylphosphatidylserine (DOPS), sphingomyelin (SM), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), distearoylphosphatidylglycerol (DSPG), dierucoylphosphatidylcholine (DEPC), palmitoyloleyolphosphatidylglycerol (POPG), dielaidoyl-phosphatidylethanolamine (DEPE), lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidicacid,cerebrosides, dicetylphosphate, lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof.

[0485] The LNP of embodiment 23, wherein the helper lipid is DSPC.

[0486] The LNP of embodiment 23, wherein the helper lipid is sphingomeylin.

[0487] The LNP of embodiment 25, wherein the sphingomeylin has a head group selected from phosphocholine, phosphoethanolamine and ceramide.

[0488] The LNP of embodiment 26, wherein the sphingomeylin is egg sphingomeylin.

[0489] The LNP of any one of embodiments 23-27, wherein the mol% of helper lipid is from about 18% to about 32%.

[0490] The LNP of embodiment 28, wherein the mol% of helper lipid is from about 20% to about 25%.

[0491] The LNP of embodiment 28, wherein the mol% of helper lipid is from about 22% to about 28%.

[0492] A conjugate for targeted delivery of a therapeutic agent to T cells *ex vivo* or *in vivo*, the conjugate comprising:

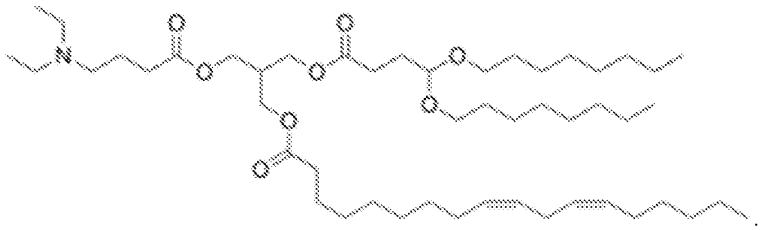
a lipid nanoparticle (LNP) comprising an ionizable lipid and a helper lipid, wherein the ionizable lipid is selected from the lipids in **Table 1**, **Table 2** and **Table 3**;

a plurality of targeting moieties conjugated to the LNP, wherein the plurality of targeting moieties is configured to target T cells.; and

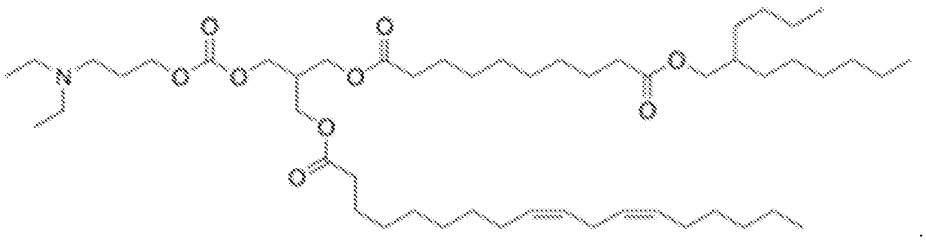
a therapeutic agent encapsulated within the LNP.

[0493] The conjugate of embodiment 31, wherein the ionizable lipid has one of the following structures depicted in embodiment 2.

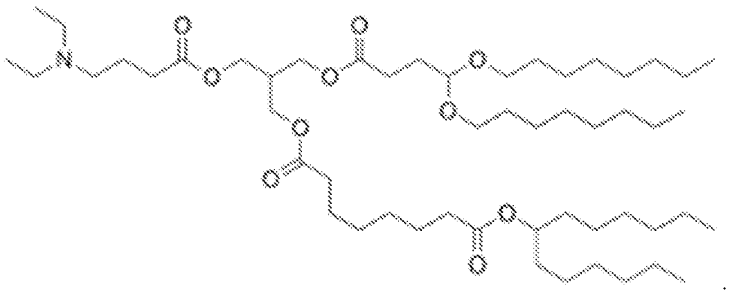
[0494] The conjugate of embodiment 31, wherein the ionizable lipid has the structure:



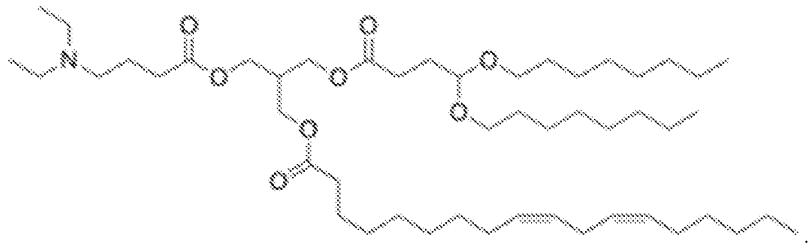
[0495] The conjugate of embodiment 31, wherein the ionizable lipid has the structure:



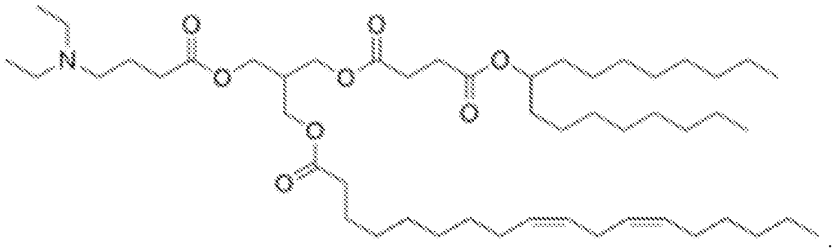
[0496] The conjugate of embodiment 31, wherein the ionizable lipid has the structure:



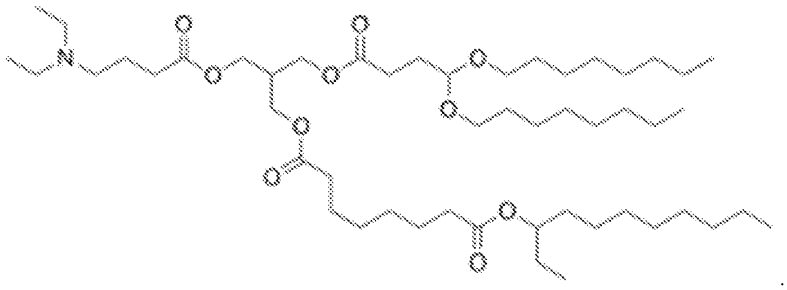
[0497] The conjugate of embodiment 31, wherein the ionizable lipid has the structure:



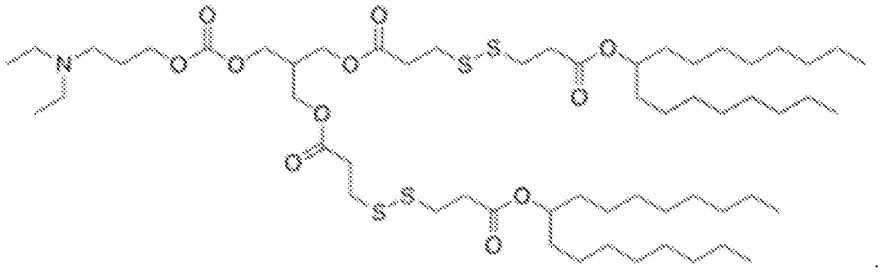
[0498] The conjugate of embodiment 31 wherein the ionizable lipid has the structure:



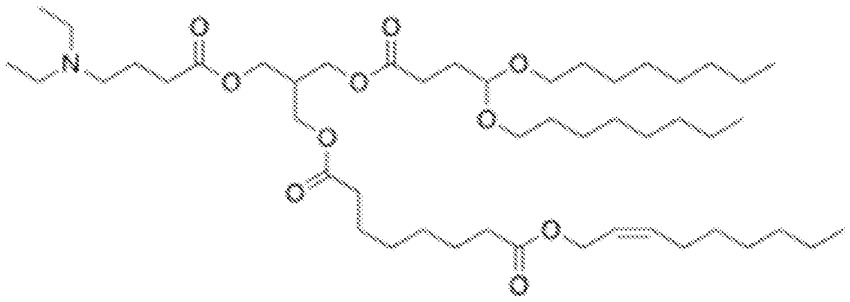
[0499] The conjugate of embodiment 31, wherein the ionizable lipid has the structure:



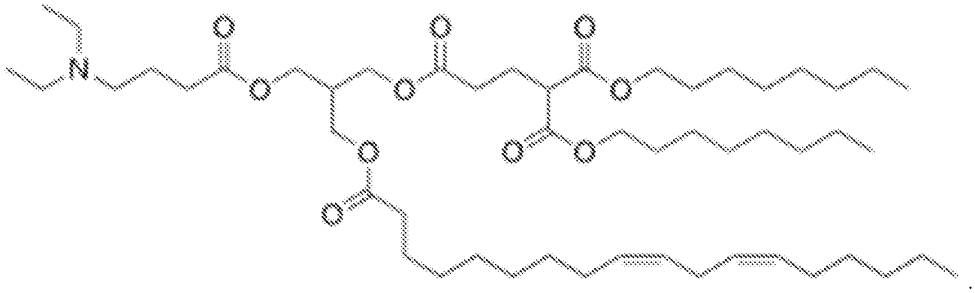
[0500] The conjugate of embodiment 31 wherein the ionizable lipid has the structure:



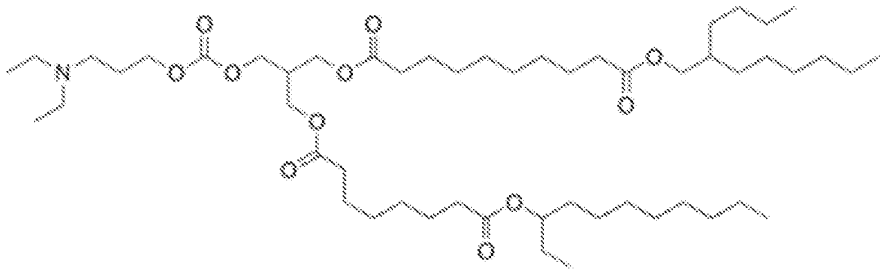
[0501] The conjugate of embodiment 31, wherein the ionizable lipid has the structure:



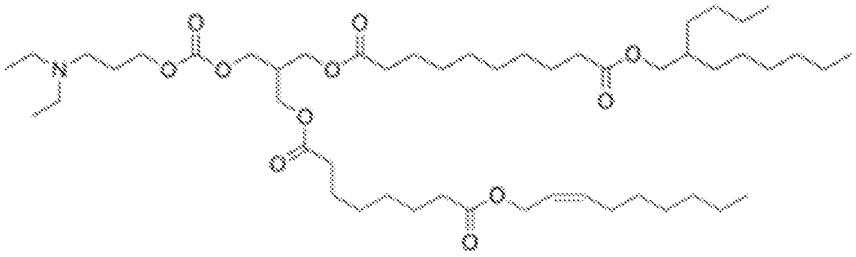
[0502] The conjugate of embodiment 31, wherein the ionizable lipid has the structure:



[0503] The conjugate of embodiment 31, wherein the ionizable lipid has the structure:



[0504] The conjugate of embodiment 31, wherein the ionizable lipid has the structure:



[0505] The conjugate of any one of embodiment 31-43, wherein the plurality of targeting moieties is configured to target one or more receptors on the surface of T cells selected from CD2, CD3, CD4, CD5, CD6, CD7, CD8, and CD28.

[0506] The conjugate of embodiment 44, wherein the plurality of targeting moieties is configured to target CD3.

[0507] The conjugate of embodiment 44, wherein the plurality of targeting moieties is configured to target CD7.

[0508] The conjugate of embodiment 44, wherein the plurality of targeting moieties is configured to target CD5.

[0509] The conjugate of any one of embodiments 31-47, wherein the plurality of targeting moieties comprises antibodies or fragments thereof.

[0510] The conjugate of any one of embodiments 31-47, wherein the plurality of targeting moieties comprises Fab fragments.

[0511] The conjugate of any one of embodiments 31-47, wherein the plurality of targeting moieties comprises single chain variable fragments (scFv).

[0512] The conjugate of any one of embodiments 31-50, wherein the therapeutic agent comprises at least one nucleic acid molecule.

[0513] The conjugate of embodiment 51, wherein the nucleic acid molecule is an mRNA molecule or a DNA molecule.

[0514] The conjugate of embodiment 51, wherein the nucleic acid molecule is an mRNA molecule.

[0515] The conjugate of embodiment 51, wherein the nucleic acid molecule is a siRNA or miRNA molecule.

[0516] The conjugate of embodiment 51, wherein the therapeutic agent comprises two nucleic acid molecules.

[0517] The conjugate of embodiment 55, wherein the two nucleic acid molecules are RNA molecules.

[0518] The conjugate of embodiment 55, wherein one nucleic acid molecule comprises an mRNA molecule and one nucleic acid molecule comprises an RNA molecule.

[0519] The conjugate of embodiment 55, wherein one nucleic acid molecule comprises an mRNA molecule and one nucleic acid comprises a guide RNA.

[0520] The conjugate of embodiment 55, wherein one nucleic acid molecule is an mRNA molecule and one nucleic acid molecule is a DNA molecule.

[0521] The conjugate of any one of embodiments 55-59, wherein at least one of the nucleic acid molecules encodes at least one component for altering a genome.

[0522] The conjugate of embodiment 60, wherein the at least one component comprises an mRNA encoding a CRISPR-associated nuclease (Cas) and a guide RNA.

[0523] The conjugate of embodiment 61, wherein the Cas is a Cas9 (e.g., spCas9) or a Cas12 (e.g., Cas12a).

[0524] The LNP of any one of embodiments 31-50, wherein the therapeutic agent comprises a gene modifying system, e.g., a retrotransposon gene modifying system or a heterologous gene modifying system.

[0525] The LNP of embodiment 53, wherein the mRNA encodes a gene modifying polypeptide.

[0526] The LNP of embodiment 58, wherein the mRNA encodes a gene modifying polypeptide and the RNA encodes a template RNA, e.g., a template RNA encoding a CAR.

[0527] The conjugate of embodiment 58, wherein the guide RNA is a single guide RNA.

[0528] The conjugate of any one of embodiments 31-66, wherein the mol% of ionizable lipids in the LNP ranges from about 35% to about 60%.

[0529] The conjugate of embodiment 67, wherein the mol% of ionizable lipids in the LNP ranges from about 40% to about 50%.

[0530] The conjugate of embodiment 67, wherein the mol% of ionizable lipids in the LNP ranges from about 45% to about 50%.

[0531] The conjugate of any one of embodiments 28-69, wherein the helper lipid is selected from the group consisting of distearoyl-sn-glycero- phosphoethanolamine, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane- 1 - carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), monomethyl-phosphatidylethanolamine (such as 16-O-monomethyl PE), dimethyl- phosphatidylethanolamine (such as 16-O-dimethyl PE), l8-l-trans PE, l-stearoyl-2-

oleoyl- phosphatidyethanolamine (SOPE), hydrogenated soy phosphatidylcholine, egg phosphatidylcholine (EPC), dioleoylphosphatidylserine (DOPS), sphingomyelin (SM), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), distearoylphosphatidylglycerol (DSPG), dierucoylphosphatidylcholine (DEPC), palmitoyloleyolphosphatidylglycerol (POPG), dielaidoyl-phosphatidylethanolamine (DEPE), lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidicacid,cerebrosides, dicetylphosphate, lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof.

[0532] The conjugate of embodiment 70, wherein the helper lipid is DSPC.

[0533] The conjugate of embodiment 670 wherein the helper lipids is sphingomeylin.

[0534] The conjugate of embodiment 72, wherein the sphingomeylin has a head group selected from phosphocholine, phosphoethanolamine and ceramide.

[0535] The conjugate of embodiment 72, wherein the sphingomeylin is egg sphingomeylin.

[0536] The conjugate of any one of embodiments 28-74, wherein the mol% of helper lipid in the conjugate is from about 18% to about 32%.

[0537] The conjugate of embodiment 75, wherein the mol% of helper lipid in the conjugate is from about 20% to about 25%.

[0538] The conjugate of embodiment 75, wherein the mol% of helper lipid in the conjugate is from about 21% to about 23%.

[0539] The conjugate of embodiment 75, wherein the mol% of helper lipid in the conjugate is from about 22% to about 28%.

[0540] The conjugate of any one of embodiments 28-78, wherein the LNP further comprises one or more PEGylated lipids.

[0541] The conjugate of embodiment 79, wherein the targeting moieties are conjugated to the LNP through the PEGylated lipids.

[0542] The conjugate of embodiment 79 or embodiment 80, wherein the PEGylated lipids each comprise a lipid component, wherein the lipid component of the PEGylated lipids is selected from the group consisting of DMG, DPG, DSG, DTA, DOPE, DPPE, DMPE, DSPE, sphingosine, sphingomyelin, stearic acid, and any combination thereof.

[0543] The conjugate of embodiment 81, wherein the lipid component of the PEGylated lipids is DSPE.

[0544] The conjugate of any one of embodiments 79-82, wherein the molar ratio of PEGylated lipids in the LNP ranges from about 0.05% to about 2%.

[0545] The conjugate of any one of embodiments 28-83, wherein the plurality of lipid molecules comprises one or more cholesterol molecules.

[0546] The conjugate of embodiment 84, wherein the cholesterol molecules are selected from beta-sitoesterol, hydroxycholesterol, stigmastanol, and any combination thereof.

[0547] The conjugate of embodiment 84 or embodiment 85, wherein the molar ratio of cholesterol molecules in the LNP ranges from about 35% to about 55%.

[0548] The conjugate of any one of embodiments 28-86, wherein the plurality of targeting moieties comprises more than 10 targeting moieties.

[0549] The conjugate of embodiment 87, wherein the plurality of targeting moieties comprises more than 20 targeting moieties.

[0550] The conjugate of embodiment 87, wherein the plurality of targeting moieties comprises more than 50 targeting moieties.

[0551] The conjugate of embodiment 87, wherein the plurality of targeting moieties comprises more than 100 targeting moieties.

[0552] The conjugate of any one of embodiments 28-90, wherein the conjugate is formed via a via a Click reaction between a first Click handle on the targeting moiety and a second Click handle on the LNP, thereby generating a Click product.

[0553] The conjugate of embodiment 91, wherein the second Click handle is covalently bonded to a pegylated lipid on the LNP.

[0554] The conjugate of embodiment 91 or embodiment 92, wherein the LNP is conjugated to the targeting moiety site specifically.

[0555] The conjugate of any one of embodiments 91-93, wherein the Click reaction is an inverse electron demand Diels-Alder reaction between a trans-cyclooctene (TCO) moiety on the first or second Click handle and a tetrazine ring on the first or second Click handle.

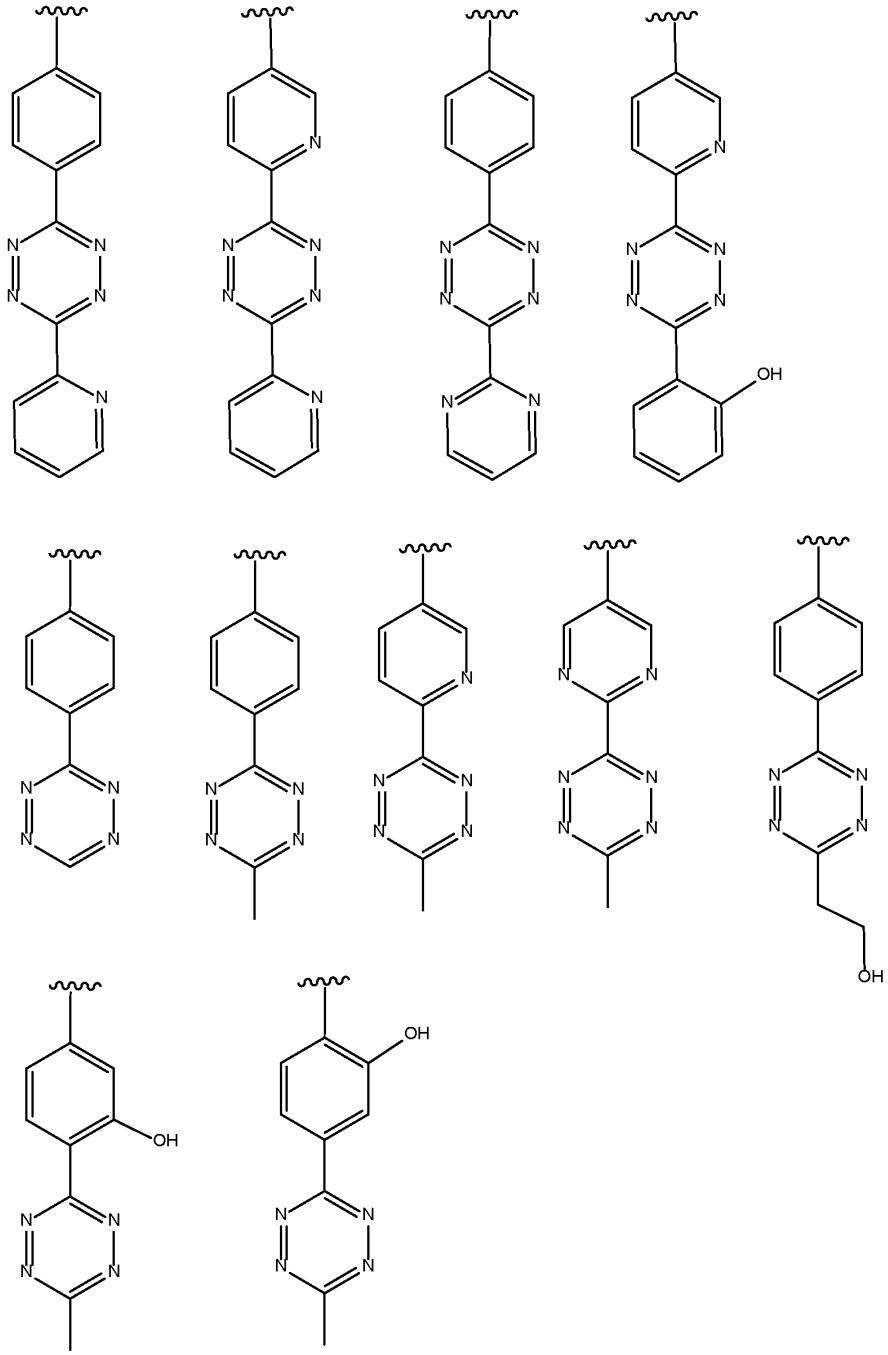
[0556] The conjugate of embodiment 94, wherein the first Click handle comprises a tetrazine ring and the second Click handle comprises a TCO moiety.

[0557] The conjugate of embodiment 94 or embodiment 95, wherein the tetrazine ring is unsubstituted.

[0558] The conjugate of any one of embodiment 95 or embodiment 96, wherein the tetrazine ring is methyltetrazine.

[0559] The conjugate of embodiment 97, wherein the tetrazine ring is 6-methyltetrazine.

[0560] The conjugate of embodiment 95, wherein the tetrazine ring has one of the following structures:



[0561] The conjugate of any one of embodiments 91-99, wherein the first Click handle is conjugated to the targeting moiety through a sortase mediated ligation of a sortase recognition motif of the targeting moiety and a plurality of glycine residues conjugated to the first Click handle.

[0562] The conjugate of embodiment 100, wherein the sortase recognition motif comprises an LPXT motif, wherein X is any amino acid.

[0563] The conjugate of embodiment 101, wherein the sortase recognition motif is LPET (SEQ ID NO:10).

[0564] The conjugate of any one of embodiments 100-102, wherein the plurality of glycine residues comprises 3-10 glycine.

[0565] The conjugate of embodiment 103, wherein the plurality of glycine residues comprises 3-6 glycine residues.

[0566] The conjugate of embodiment 104, wherein the plurality of glycine residues comprises 3 glycine residues.

[0567] The conjugate of any one of embodiments 100-105, wherein the targeting moiety comprises a C-terminus, wherein the sortase recognition motif is covalently bonded to the C-terminus.

[0568] The conjugate of any one of embodiments 91-99, wherein the first Click handle is conjugated to the targeting moiety through a lipoic acid ligase (LplA) mediated ligation of an LplA acceptor peptide of the targeting moiety and a carboxylic acid group conjugated to the first Click handle.

[0569] The conjugate of embodiment 107, wherein the LplA acceptor peptide has the sequence GFEDKVWYDLDA (SEQ ID NO:23).

[0570] The conjugate of embodiment 107 or embodiment 108, wherein the targeting moiety comprises a C-terminus, wherein the LplA acceptor peptide is covalently bonded to the C-terminus.

[0571] The conjugate of any one of embodiments 91-99, wherein the first Click handle is conjugated to the targeting moiety through a transglutaminase enzyme mediated ligation of an LLQG enzyme recognition sequence (SEQ ID NO:25) of the targeting moiety and an alkyl-primary amine group conjugated to the first Click handle.

[0572] The conjugate of embodiment 110, wherein the targeting moiety comprises a C-terminus, wherein the LLQG enzyme recognition sequence (SEQ ID NO:25) is covalently bonded to the C-terminus.

[0573] The conjugate of any one of embodiments 91-99, wherein the first Click handle is conjugated to the targeting moiety through a formylglycine generating enzyme mediated ligation of a CXPXR enzyme recognition sequence of the targeting moiety and an aminooxy group or a hydrazine group conjugated to the first Click handle, wherein X is any amino acid.

[0574] The conjugate of embodiment 112, wherein the targeting moiety comprises a C-terminus, wherein the CXPXR enzyme recognition sequence is covalently bonded to the C-terminus.

[0575] The conjugate of any one of embodiments 91-99, wherein the first Click handle is conjugated to the targeting moiety through a reaction of a free cysteine residue of the targeting moiety and a thiol-reactive moiety conjugated to the first Click handle.

[0576] The conjugate of embodiment 114, wherein the thiol-reactive moiety is maleimide.

[0577] The conjugate of embodiment 114 or embodiment 115, wherein the targeting moiety comprises a C-terminus, wherein a hinge region is covalently bonded to the C-terminus, and wherein the hinge region comprises the free cysteine residue.

[0578] A method of treating and/or delaying the progression of a disease in a subject, the method comprising administering to the subject a therapeutically effective amount of a conjugate of any one of embodiments 28-116.

EXAMPLES

[0579] The invention is further illustrated by the following examples. The examples are provided for illustrative purposes only and are not to be construed as limiting the scope or content of the invention in any way.

Example 1: *In vitro* screen for anti-CD3 targeting moieties

[0580] A screen was conducted to identify anti-CD3 targeting moieties that can be conjugated to the surface of an LNP to create targeted LNPs (tLNPs) capable of enhanced delivery of a payload (e.g., a therapeutic agent) to T cells. A base LNP was conjugated to select anti-CD3 targeting moieties to generate tLNPs. The capability of these tLNPs to deliver an mRNA encoding a GFP reporter to activated or resting T cells, either in the presence or absence of serum, was assayed to determine the potential suitability of each targeting moiety for *ex vivo* and/or *in vivo* T cell delivery applications.

T Cell Activation and Culture

[0581] Cryopreserved primary T cells from a single human donor (HemaCare, donor D328798) were thawed and transferred into PBS with 2% BSA by volume. Cells were counted on a Cellometer K2 Cell Counter (Nexcelom), then pelleted by centrifugation at 400xg for 5 minutes and resuspended in complete T cell media at a density of $1x10^6$ cells/mL. The complete T cell media was comprised of CTS AIM V SFM basal media (Gibco #0870112DK) with 5%

human serum AB (Gemini Bio #100-512) and the following cytokines: human IL-2 at 20ng/mL (Miltenyi Biotec #130-097-746), human IL-7 at 10ng/mL (Miltenyi Biotec #130-095-362), and human IL-15 at 5ng/mL (Miltenyi Biotec #130-095-764). TransAct Research Grade T cell activation reagent (Miltenyi Biotec #130-111-160) was added to cell suspension at a volume of 10uL per $1x10^6$ cells. Activated cells were transferred to flasks and incubated at 37°C, 5% CO_2 for 72 hours ("Activated" condition).

[0582] At 48 hours post-activation, additional T cells from the same donor were thawed and resuspended in complete T cell media as above, without TransAct stimulation. These cells were transferred to flasks and incubated at 37°C, 5% CO_2 for 24 hours ("Rested" condition).

Preparation of T Cells for Transfection

[0583] Following the incubation periods above, the activated and rested T cells were collected separately and counted. Two aliquots of $1.5x10^7$ cells each were taken from each activation condition, then pelleted by centrifugation, and the supernatant aspirated. The cell pellets were resuspended in CTS AIM V SFM at a concentration of $1.33x10^6$ cells/mL, supplemented with human IL-2, human IL-7, and human IL-15, at concentrations of 26.6ng/mL, 13.3ng/mL and 6.6ng/mL, respectively.

[0584] One aliquot per condition (activated and rested) was resuspended in basal media without serum, while the second aliquot was resuspended in basal media with 5% fetal bovine serum (FBS, Gibco #A31604-01). Previous data (not shown) demonstrated that transfection of activated T cells with non-targeted base LNPs was enhanced in the presence of FBS, while select targeted LNPs (notably LNPs with the anti-CD3-8 clone (see **Table 19**) demonstrated higher levels of transfection of activated T cells in the absence of serum (see **FIG. 1**). All candidate anti-CD3 targeting moieties were screened both with and without FBS present in the media during transfection.

[0585] The resuspended T cells were plated in non-treated 96-well U-bottom polystyrene plates (Falcon # 08-772-54) at a density of $2x10^5$ cells (150uL) per well, and returned to incubation at 37°C, 5% CO_2 while the LNPs were prepared.

Preparation of Targeted LNPs for Transfection

[0586] Targeted LNPs were formulated as follows. An ethanol phase was prepared with five lipids, containing 47% ionizable lipid (V003), 8% DSPC, 42.5% cholesterol, 2% PEG-DMG and 0.5% DSPE-PEG2K-TCO. An aqueous phase was composed of mRNA (encoding eGFP) dissolved in 25 mM acetate buffer. The two phases were mixed using a microfluidic device to

formulate LNP-TCO at a total flow rate of 18 ml/min (aqueous phase: ethanol phase=3:1). 3x volume of CBS was added to LNP solution and the resulting solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes. Following this, the anti-CD3 targeting moiety was added to the LNP solution at 1:1 ratio of mRNA:targeting moiety. The mixture was incubated at 25 C for 2 hours, followed by overnight incubation at 4 C. The targeted LNPs were then buffer exchanged to the desired concentration either via Tangential Flow Filtration (TFF) and/or centrifugation (100k MWCO).

[0587] **Table 19** provides the anti-CD3 targeting moieties that were tested. Each anti-CD3 targeting moiety was produced with an additional sortase tag (LPETG, SEQ ID NO:11) on the C terminus of the heavy chain (i.e., GGGGSLPETGHHHHHH, SEQ ID NO:17). 10uM anti-CD3 Fab-LPETG and 1mM Triglycine methyltetrazine (GGG-meTz) were prepared in sortase buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl2, pH 7.4). 2uM Sortase A5 was then added to initiate the reaction. The above solution was incubated at 30°C for 3 hours with shaking at 800 rpm. Excess reagents were removed, and buffer was exchanged to PBS containing 10mM EDTA by a 10K Amicon centrifugal filter. To confirm a DOL (degree of labeling) of close to 1meTz per antibody, sortase modified anti-CD3 targeting moiety was allowed to react with 5eq AF594 containing TCO group in PBS for 2 hours at room temperature. After the reaction, excess dye was removed by two runs with Zeba desalting column. DOL was measured by absorbances at A280 and A590 using DOL calculator. The resulting meTz-modified anti-CD3 targeting moiety was added to the TCO modified-LNP solution (as prepared above) for antibody-LNP conjugation, with a mass ratio between antibody and mRNA (encoding eGFP) of 1:1, except where otherwise noted (**Table 1**). The solution was incubated at room temperature for 2 hours and then at 4°C overnight. Any unreacted anti-CD3 targeting moiety was removed by a 300K MPES TFF membrane. The resulting antibody-LNP product was further concentrated using an Amicon column.

Table 19. Anti-CD3 targeting moieties tested with targeted LNPs

Targeting moiety	**Targeting moiety ratio (Ab (mg):RNA (mg)**	**HC sequence**	**HC sequence (without sortase tag)**	**LC sequence**	**VH sequence**	**VL sequence**
Anti-CD3-1 (Mosunetuzumab)	1:1	EVQLVQSGAEVKKPGASVKVSCKASGYTFT**NYYIH**W	EVQLVQSGAEVKKPGASVKVSCKASGYTFT**NYYIH**WVRQAPG	DIVMTQSPDSLAVSLGERATINC**KSSQSLL****NSRTRKN****YLA**WYQ	EVQLVQSGAEVKKPGASVKVSCKASGYTFT**NYYIH**W	DIVMTQSPDSLAVSLGERATINC**KSSQSL****LNSRT**

		VRQAPG QGLEWI G**WIYPG** **DGNTKY** **NEKFKG** RATLTA DTSTSTA YLELSSL RSEDTA VYYCAR **DSYSNY** **YFDY**W GQGTLV TVSSAST KGPSVFP LAPSSKS TSGGTA ALGCLV KDYFPE PVTVSW NSGALT SGVHTF PAVLQS SGLYSLS SVVTVP SSSLGTQ TYICNV NHKPSN TKVDKK VEPKSC *GGGGSL PETGHH HHHH* (SEQ ID NO:138)	QGLEWIG **WIYPGDG** **NTKYNEK** **FKG**RATL TADTSTST AYLELSSL RSEDTAV YYCAR**DS** **YSNYYFD** **Y**WGQGTL VTVSSAST KGPSVFPL APSSKSTS GGTAALG CLVKDYF PEPVTVS WNSGALT SGVHTFP AVLQSSG LYSLSSVV TVPSSSLG TQTYICN VNHKPSN TKVDKKV EPKSC (SEQ ID NO:139)	QKPGQPP KLLIY**WA** **STRES**GV PDRFSGSG SGTDFTLT ISSLQAED VAVYYC**T** **QSFILRT**F GQGTKVE IKRTVAAP SVFIFPPS DEQLKSG TASVVCL LNNFYPR EAKVQW KVDNALQ SGNSQES VTEQDSK DSTYSLSS TLTLSKA DYEKHKV YACEVTH QGLSSPVT KSFNRGE C (SEQ ID NO:140)	VRQAPG QGLEWI G**WIYPG** **DGNTK** **YNEKFK** **G**RATLT ADTSTS TAYLEL SSLRSED TAVYYC ARDSYS **NYYFDY** WGQGTL VTVSS (SEQ ID NO:141)	**RKNYL** **AWY**QQ KPGQPP KLLIY **WASTR** **ES**GVPD RFSGSG SGTDFT LTISSL QAEDV AVYYC **TQSFIL** **RT**FGQ GTKVEI K (SEQ ID NO:142)
Anti-CD3-2 (Odronextamab)	1:1	EVQLVE SGGGLV QPGRSL RLSCAA SGFTFD **DYTMH** WVRQAP GKGLEW VS**GISW** **NSGSIG** **YADSVK** **G**RFTISR DNAKKS LYLQMN SLRAED TALYYC	EVQLVES GGGLVQP GRSLRLSC AASGFTF D**DYTMH** WVRQAPG KGLEWVS **GISWNSG** **SIGYADS** **VKG**RFTIS RDNAKKS LYLQMNS LRAEDTA LYYCAK**D** **NSGYGHY** **YYGMDV**	EIVMTQSP ATLSVSPG ERATLSC **RASQSVS** **SNLA**WYQ QKPGQAP RLLIY**GAS** **TRAT**GIP ARFSGSGS GTEFTLTI SSLQSEDF AVYYC**Q** **HYINWPL** **T**FGGGTK VEIKRTV AAPSVFIF	EVQLVE SGGGLV QPGRSL RLSCAA SGFTFD **DYTMH** WVRQAP GKGLEW VS**GISW** **NSGSIG** **YADSVK** **G**RFTISR DNAKKS LYLQMN SLRAED TALYYC	EIVMTQ SPATLS VSPGER ATLSC**R** **ASQSVS** **SNLA**W YQQKP GQAPR LLIY**GA** **STRAT** GIPARF SGSGSG TEFTLTI SSLQSE DFAVY YC**QHY**

		AK**DNSG** **YGHYY** **YGMDV** WGQGTT VTVASA STKGPS VFPLAPS SKSTSG GTAALG CLVKDY FPEPVTV SWNSGA LTSGVH TFPAVL QSSGLY SLSSVVT VPSSSLG TQTYICN VNHKPS NTKVDK KVEPKS C*GGGGS LPETGH HHHHH* (SEQ ID NO:149)	WGQGTTV TVASAST KGPSVFPL APSSKSTS GGTAALG CLVKDYF PEPVTVS WNSGALT SGVHTFP AVLQSSG LYSLSSVV TVPSSSLG TQTYICN VNHKPSN TKVDKKV EPKSC (SEQ ID NO:150)	PPSDEQLK SGTASVV CLLNNFY PREAKVQ WKVDNA LQSGNSQ ESVTEQD SKDSTYSL SSTLTLSK ADYEKHK VYACEVT HQGLSSP VTKSFNR GEC (SEQ ID NO:151)	AK**DNSG** **YGHYY** **YGMDV** WGQGTT VTVAS (SEQ ID NO:152)	**INWPL** **T**FGGG TKVEIK (SEQ ID NO:153)
Anti-CD3-3 (Tebentafusp)	1:1	EVQLVE SGGGLV QPGGSL RLSCAA SGYSFT **GYTMN** WVRQAP GKGLEW VA**LINP** **YKGVST** **YNQKFK** **D**RFTISV DKSKNT AYLQMN SLRAED TAVYYC AR**SGYY** **GDSDW** **YFDV**W GQGTLV TVSSAST KGPSVFP LAPSSKS TSGGTA ALGCLV	EVQLVES GGGLVQP GGSLRLS CAASGYS FT**GYTMN** WVRQAPG KGLEWVA **LINPYKG** **VSTYNQK** **FKD**RFTIS VDKSKNT AYLQMNS LRAEDTA VYYCAR**S** **GYYGDSD** **WYFDV**W GQGTLVT VSSASTK GPSVFPLA PSSKSTSG GTAALGC LVKDYFP EPVTVSW NSGALTS GVHTFPA	AIQMTQS PSSLSASV GDRVTITC **RASQDIR** **NYLN**WY QQKPGKA PKLLIY**YT** **SRLES**GV PSRFSGSG SGTDYTL TISSLQPE DFATYYC **QQGNTLP** **WT**FGQGT KVEIKRT VAAPSVFI FPPSDEQL KSGTASV VCLLNNF YPREAKV QWKVDN ALQSGNS QESVTEQ DSKDSTY SLSSTLTL	EVQLVE SGGGLV QPGGSL RLSCAA SGYSFT **GYTMN** WVRQAP GKGLEW VA**LINP** **YKGVST** **YNQKF** **KD**RFTIS VDKSKN TAYLQM NSLRAE DTAVYY CAR**SGY** **YGDSD** **WYFDV** WGQGTL VTVSS (SEQ ID NO:163)	AIQMT QSPSSL SASVG DRVTIT C**RASQ** **DIRNYL** **N**WYQQ KPGKA PKLLIY **YTSRL** **ES**GVPS RFSGSG SGTDYT LTISSL QPEDFA TYYC**Q** **QGNTL** **PWT**FG QGTKV EIK (SEQ ID NO:164)

		KDYFPE PVTVSW NSGALT SGVHTF PAVLQS SGLYSLS SVVTVP SSSLGTQ TYICNV NHKPSN TKVDKK VEPKSC *GGGGSL PETGHH HHHH* (SEQ ID NO:160)	VLQSSGL YSLSSVVT VPSSSLGT QTYICNV NHKPSNT KVDKKVE PKSC (SEQ ID NO:161)	SKADYEK HKVYACE VTHQGLS SPVTKSFN RGEC (SEQ ID NO:162)		
Anti-CD3-4 (Teplizumab)	1:1	QVQLVQ SGGGVV QPGRSL RLSCKA SGYTF**T** **RYTMH** WVRQAP GKGLEW IG**YINPS** **RGYTNY** **NQKVK** **D**RFTISR DNSKNT AFLQMD SLRPEDT GVYFCA R**YYDDH** **YCLDY** WGQGTP VTVSSA STKGPS VFPLAPS SKSTSG GTAALG CLVKDY FPEPVTV SWNSGA LTSGVH TFPAVL QSSGLY SLSSVVT VPSSSLG TQTYICN VNHKPS	QVQLVQS GGGVVQP GRSLRLSC KASGYTF **TRYTMH** WVRQAPG KGLEWIG **YINPSRG** **YTNYNQ** **KVKD**RFT ISRDNSKN TAFLQMD SLRPEDTG VYFCAR**Y** **YDDHYCL** **DY**WGQG TPVTVSSA STKGPSVF PLAPSSKS TSGGTAA LGCLVKD YFPEPVTV SWNSGAL TSGVHTFP AVLQSSG LYSLSSVV TVPSSSLG TQTYICN VNHKPSN TKVDKKV EPKSC (SEQ ID NO:172)	DIQMTQS PSSLSASV GDRVTITC **SASSSVSY** **MN**WYQQ TPGKAPK RWIY**DTS** **KLAS**GVP SRFSGSGS GTDYTFTI SSLQPEDI ATYYC**QQ** **WSSNPFT** FGQGTKL QITRTVA APSVFIFP PSDEQLKS GTASVVC LLNNFYP REAKVQ WKVDNA LQSGNSQ ESVTEQD SKDSTYSL SSTLTLSK ADYEKHK VYACEVT HQGLSSP VTKSFNR GEC (SEQ ID NO:173)	QVQLVQ SGGGVV QPGRSL RLSCKA SGYTFT **RYTMH** WVRQAP GKGLEW IG**YINPS** **RGYTNY** **NQKVK** **D**RFTISR DNSKNT AFLQMD SLRPEDT GVYFCA R**YYDDH** **YCLDY** WGQGTP VTVSS (SEQ ID NO:174)	DIQMT QSPSSL SASVG DRVTIT C**SASSS** **VSYMN** WYQQT PGKAP KRWIY **DTSKL** **AS**GVPS RFSGSG SGTDYT FTISSLQ PEDIAT YYC**QQ** **WSSNP** **FT**FGQ GTKLQI T (SEQ ID NO:175)

		NTKVDK KVEPKS C*GGGGS LPETGH HHHHH* (SEQ ID NO:171)				
Anti-CD3-5 (Teclistamab)	1:1	EVQLVE SGGGLV QPGGSL RLSCAA SGFTF**NT YAMN**W VRQAPG KGLEWV A**RIRSK YNNYAT YYAASV KG**RFTIS RDDSKN SLYLQM NSLKTE DTAVYY CAR**HGN FGNSYV SWFAY** WGQGTL VTVSSA STKGPS VFPLAPS SKSTSG GTAALG CLVKDY FPEPVTV SWNSGA LTSGVH TFPAVL QSSGLY SLSSVVT VPSSSLG TQTYICN VNHKPS NTKVDK KVEPKS C*GGGGS LPETGH HHHHH* (SEQ ID NO:182)	EVQLVES GGGLVQP GGSLRLS CAASGFT **FNTYAMN** WVRQAPG KGLEWVA **RIRSKYN NYATYYA ASVKG**RF TISRDDSK NSLYLQM NSLKTED TAVYYCA R**HGNFGN SYVSWFA Y**WGQGTL VTVSSAST KGPSVFPL APSSKSTS GGTAALG CLVKDYF PEPVTVS WNSGALT SGVHTFP AVLQSSG LYSLSSVV TVPSSSLG TQTYICN VNHKPSN TKVDKKV EPKSC (SEQ ID NO:183)	QTVVTQE PSLTVSPG GTVTLTC **RSSTGAV TTSNYAN** WVQQKP GQAPRGLI G**GTNKR AP**GTPAR FSGSLLGG KAALTLS GVQPEDE AEYYC**AL WYSNLW V**FGGGTK LTVLGQP KANPTVT LFPPSSEE LQANKAT LVCLISDF YPGAVTV AWKADG SPVKAGV ETTKPSK QSNNKYA ASSYLSLT PEQWKSH RSYSCQV THEGSTV EKTVAPT ECS (SEQ ID NO:184)	EVQLVE SGGGLV QPGGSL RLSCAA SGFTF**N TYAMN** WVRQAP GKGLEW VA**RIRS KYNNY ATYYAA SVKG**RF TISRDDS KNSLYL QMNSLK TEDTAV YYCAR**H GNFGNS YVSWFA Y**WGQG TLVTVS S (SEQ ID NO:185)	QTVVT QEPSLT VSPGGT VTLTC**R SSTGA VTTSN YAN**WV QQKPG QAPRG LIG**GTN KRAP**G TPARFS GSLLGG KAALT LSGVQP EDEAE YYC**AL WYSNL WV**FGG GTKLT VL (SEQ ID NO:186)
Anti-CD3-6	1:1	QVQLVQ SGAEVK	QVQLVQS GAEVKKP	DIQMTQS PSSLSASV	QVQLVQ SGAEVK	DIQMT QSPSSL

(Visilizumab)		KPGASV KVSCKA SGYTFI**S** **YTMH**W VRQAPG QGLEW MG**YINP** **RSGYTH** **YNQKL** **KD**KATL TADKSA STAYME LSSLRSE DTAVYY CAR**SAY** **YDYDGF** **AY**WGQ GTLVTV SSASTK GPSVFPL APSSKST SGGTAA LGCLVK DYFPEP VTVSWN SGALTS GVHTFP AVLQSS GLYSLSS VVTVPS SSLGTQT YICNVN HKPSNT KVDKKV EPKSC*G GGGSLP ETGHHH HHH* (SEQ ID NO:193)	GASVKVS CKASGYT F**ISYTMH** WVRQAPG QGLEWM G**YINPRS** **GYTHYN** **QKLKD**K ATLTADK SASTAYM ELSSLRSE DTAVYYC AR**SAYYD** **YDGFAY** WGQGTLV TVSSASTK GPSVFPLA PSSKSTSG GTAALGC LVKDYFP EPVTVSW NSGALTS GVHTFPA VLQSSGL YSLSSVVT VPSSSLGT QTYICNV NHKPSNT KVDKKVE PKSC (SEQ ID NO:194)	GDRVTITC **SASSSVSY** **MN**WYQQ KPGKAPK RLIY**DTSK** **LAS**GVPS RFSGSGSG TDFTLTIS SLQPEDFA TYYC**QQ** **WSSNPPT** FGGGTKV EIKRTVA APSVFIFP PSDEQLKS GTASVVC LLNNFYP REAKVQ WKVDNA LQSGNSQ ESVTEQD SKDSTYSL SSTLTLSK ADYEKHK VYACEVT HQGLSSP VTKSFNR GEC (SEQ ID NO:195)	KPGASV KVSCKA SGYTFI**S** **YTMH**W VRQAPG QGLEW MG**YINP** **RSGYTH** **YNQKL** **KD**KATL TADKSA STAYME LSSLRSE DTAVYY CAR**SAY** **YDYDGF** **AY**WGQ GTLVTV SS (SEQ ID NO:196)	SASVG DRVTIT **CSASSS** **VSYMN** WYQQK PGKAP KRLIY**D** **TSKLAS** GVPSRF SGSGSG TDFTLT ISSLQPE DFATY YC**QQW** **SSNPPT** FGGGT KVEIK (SEQ ID NO:197)
Anti-CD3-7 (Muromonab)	1:1	QVQLQQ SGAELA RPGASV KMSCKA SGYTFT **RYTMH** WVKQRP GQGLEW IG**YINPS** **RGYTNY** **NQKFKD**	QVQLQQS GAELARP GASVKMS CKASGYT FT**RYTMH** WVKQRPG QGLEWIG **YINPSRG** **YTNYNQ** **KFKD**KAT LTTDKSSS	QIVLTQSP AIMSASPG EKVTMTC **SASSSVSY** **MN**WYQQ KSGTSPK RWIY**DTS** **KLAS**GVP AHFRGSG SGTSYSLT ISGMEAE	QVQLQQ SGAELA RPGASV KMSCKA SGYTFT **RYTMH** WVKQRP GQGLEW IG**YINPS** **RGYTNY** **NQKFK**	QIVLTQ SPAIMS ASPGEK VTMTC **SASSSV** **SYMN**W YQQKS GTSPKR WIY**DTS** **KLAS**G VPAHFR

		KATLTT DKSSST AYMQLS SLTSEDS AVYYCA R**YYDDH YCLDY** WGQGTT LTVSSA KTTAPS VYPLAP VCGGTT GSSVTL GCLVKG YFPEPVT LTWNSG SLSSGV HTFPAV LQSDLY TLSSSVT VTSSTW PSQSITC NVAHPA SSTKVD KKIEPRP KSCDKT H*GGGGS LPETGH HHHHH* (SEQ ID NO:204)	TAYMQLS SLTSEDSA VYYCAR**Y YDDHYCL DY**WGQG TTLTVSSA KTTAPSV YPLAPVC GGTTGSS VTLGCLV KGYFPEP VTLTWNS GSLSSGV HTFPAVL QSDLYTL SSSVTVTS STWPSQSI TCNVAHP ASSTKVD KKIEPRPK SCDKTH (SEQ ID NO:205)	DAATYYC **QQWSSNP FT**FGSGT KLEINRA DTAPTVSI FPPSSEQL TSGGASV VCFLNNF YPKDINV KWKIDGS ERQNGVL NSWTDQD SKDSTYS MSSTLTLT KDEYERH NSYTCEA THKTSTSP IVKSFNRN EC* (SEQ ID NO:206)	**D**KATLT TDKSSST AYMQLS SLTSEDS AVYYCA R**YYDDH YCLDY** WGQGTT LTVSS (SEQ ID NO:207)	GSGSGT SYSLTIS GMEAE DAATY YC**QQW SSNPFT** FGSGTK LEIN (SEQ ID NO:208)
Anti-CD3-8 (SP34)	1:1	EVQLVE SGGGLV QPGGSL KLSCAA SGFTFN **KYAMN** WVRQAP GKGLEW VA**RIRS KYNNYA TYYADS VKD**RFTI SRDDSK NTAYLQ MNNLKT EDTAVY YCVR**HG NFGNSY ISYWAY** WGQGTL	EVQLVES GGGLVQP GGSLKLS CAASGFT FN**KYAM N**WVRQAP GKGLEWV A**RIRSKY NNYATYY ADSVKD**R FTISRDDS KNTAYLQ MNNLKTE DTAVYYC VR**HGNFG NSYISYW AY**WGQG TLVTVSS ASTKGPS VFPLAPSS	QTVVTQE PSLTVSPG GTVTLTC **GSSTGAV TSGNYPN** WVQQKP GQAPRGLI G**GTKFLA P**GTPARFS GSLLGGK AALTLSG VQPEDEA EYYC**VL WYSNRW V**FGGGTK LTVLGQP KANPTVT LFPPSSEE LQANKAT LVCLISDF	EVQLVE SGGGLV QPGGSL KLSCAA SGFTFN **KYAMN** WVRQAP GKGLEW VA**RIRS KYNNY ATYYAD SVKD**RF TISRDDS KNTAYL QMNNL KTEDTA VYYCVR **HGNFG NSYISY WAY**WG	QTVVT QEPSLT VSPGGT VTLTC **GSSTG AVTSG** **NYPN**W VQQKP GQAPR GLIG**GT KFLAP** GTPARF SGSLLG GKAAL TLSGVQ PEDEAE YYC**VL WYSNR WV**FGG GTKLT

		VTVSSA STKGPS VFPLAPS SKSTSG GTAALG CLVKDY FPEPVTV SWNSGA LTSGVH TFPAVL QSSGLY SLSSVVT VPSSSLG TQTYICN VNHKPS NTKVDK KVEPKS SDKTHT C*GGGGS LPETGH HHHHH* (SEQ ID NO:215)	KSTSGGT AALGCLV KDYFPEP VTVSWNS GALTSGV HTFPAVL QSSGLYSL SSVVTVPS SSLGTQT YICNVNH KPSNTKV DKKVEPK SSDKTHT C (SEQ ID NO:216)	YPGAVTV AWKADG SPVKAGV ETTKPSK QSNNKYA ASSYLSLT PEQWKSH RSYSCQV THEGSTV EKTVAPT ECS (SEQ ID NO:217)	QGTLVT VSS (SEQ ID NO:218)	VL (SEQ ID NO:219)
The sortase tag sequence in the heavy chain (HC) sequences are underlined and italicized. The CDR sequences for the anti-CD3-9 targeting moiety are in bolded text.						

[0588] Targeted LNPs were screened with a dose titration ranging from 0.125ug/1x10^6 cells up to 4ug/1x10^6 cells. tLNPs were diluted to a top concentration of 16ug/mL in basal CTS AIM V SFM media (with no serum or cytokines), then serially diluted 1:1 in additional basal CTS AIM V SFM media to the lowest concentration of 0.5ug/mL. tLNPs were further diluted 1 in 4 upon addition to the plated cells, for the final concentration range of 0.125ug/mL to 4ug/mL.

LNP Transfection

[0589] 50uL of diluted tLNPs were added to plated activated and rested T cells, in singlicate, and mixed gently by pipetting. After addition of the tLNPs, all plated cells were at a final density of 1x10^6 cells/mL in 200uL, with a final concentration of 5% FBS (for serum-containing conditions) and cytokines at the following concentrations: human IL-2 at 20ng/mL, human IL-7 at 10ng/mL, and human IL-15 at 5ng/mL. Transfected cells were returned to incubation at 37°C, 5% CO_2 overnight.

Flow Cytometry

[0590] Following overnight incubation, cells in plates were pelleted by centrifugation at 800xg for 2 minutes, washed once with 100uL of 1X PBS (Gibco #), then resuspended in 50uL PBS containing LIVE/DEAD™ Fixable Near-IR Dead Cell Stain (Invitrogen # L34976) at

1:1,000 dilution, plus the following antibodies: mouse anti-human CD3 APC (clone OKT3, Biolegend #317318), mouse anti-human CD25 Alexa Fluor 700 (clone BC96, Biolegend # 302622), and mouse anti-human LDLR PE (clone C7, BD Biosciences # 565653). All antibodies were used at a 1:200 final dilution. Cells were stained on ice for 20 minutes, then centrifuged, washed as above and resuspended in 100uL of 1X PBS containing 2% FBS.

[0591] Flow cytometry data was acquired on a NovoCyte 3000RYB (Agilent # 2010044), then analyzed for the proportion of live cells expressing GFP as well as the median fluorescence intensity (MFI) of GFP signal in live cells.

Cytokine Quantification

[0592] Supernatants from transfected T cells were collected and assayed using the MSD Multi-Spot Assay for human pro-inflammatory panel 1 (MesoScale Discovery #K15049D). Supernatants were diluted 1:50 in Diluent 2 and assayed in duplicate according to the manufacturer's directions.

Results

[0593] **FIGs. 2B** and **2C** show that in the absence of serum, all anti-CD3 targeting moieties screened improved transfection of activated T cells when conjugated to an LNP relative to the base LNP (non-conjugated to an anti-CD3 targeting moiety). The tLNPs with the anti-CD3-8 targeting moiety boosted GFP expression by 75-fold relative to non-targeted LNPs (**FIG. 2C**) and increased the proportion of transfected cells from 53.6% to 99.4% of cells at the highest doses tested (**FIG. 2B**). The tLNPs conjugated to the other tested anti-CD3 targeting moieties also increased both GFP expression and the proportion of GFP+ cells (to GFP- cells) relative to the base LNP, with the tLNPs having the anti-CD3-5 targeting moiety exhibiting the second highest GFP expression levels after the anti-CD3-8 tLNPs (**FIGs. 2B** and **2C**).

[0594] As shown by **FIGs. 2A** and **2D**, when FBS was present, the transfection efficiency in activated T cells appeared more normalized across the anti-CD3 targeting moieties. The increase in MFI between anti-CD3-8 tLNPs and non-targeted base LNPs was less than 4-fold higher. Additionally, **FIG. 2D** shows that the overall levels of expression were enhanced in the presence of serum for most anti-CD3 targeting moieties screened, except for the anti-CD3-8 and anti-CD3-5 targeting moieties.

[0595] **FIGs. 3A-3D** show that in rested T cells, all tLNPs conjugated to the anti-CD3 targeting moieties that were screened enhanced transfection efficiency above that of non-targeted base LNPs, both in the presence and absence of serum. As in activated cells, rested cells

transfected with tLNPs conjugated to the anti-CD3-8 or the anti-CD3-5 targeting moieties had higher GFP expression in serum-free conditions compared to cells transfected in the presence of FBS. Serum had less of a normalization effect in rested cells (compared to activated cells), as rested cells transfected with anti-CD3-8 tLNPs showed over a 120-fold increase in MFI relative to cells transfected with non-targeted base LNPs at the highest dose tested (**FIG. 3D**). In the absence of serum, GFP expression is over 370-fold higher in cells transfected by anti-CD8-8 tLNPs compared to non-targeted base LNPs (**FIG. 3C**). This trend was similar for the tLNPs conjugated to the anti-CD3-5 targeting moiety.

[0596] Targeted LNPs comprising the anti-CD3 targeting moieties induced variable expression of the T cell activation marker CD25 (**FIG. 4A**). Anti-CD3-2 was minimally activating, even at the highest dose tested, while anti-CD3-4 and anti-CD3-6 induced high levels of CD25 expression. Targeted LNPs comprising the anti-CD3-1, anti-CD3-5, anti-CD3-7, or anti-CD3-8 targeting moieties induced moderately high levels of CD25 expression.

Example 2: *In vitro* screen for anti-CD2, anti-CD5 and anti-CD7 targeting moieties

[0597] A screen was conducted to identify anti-CD2, anti-CD5, and anti-CD7 targeting moieties that can be conjugated to the surface of an LNP to create targeted LNPs (tLNPs) capable of enhanced delivery of a payload (e.g., a therapeutic agent) to T cells. A base LNP was conjugated to select anti-CD2, anti-CD5, or anti-CD7 Fab fragments to generate tLNPs. The capability of these tLNPs to deliver an mRNA encoding a GFP reporter to activated or resting T cells, either in the presence or absence of serum, was assayed to determine the potential suitability of each targeting moiety for *ex vivo* and/or *in vivo* T cell delivery applications.

[0598] T cells were cultured and activated as described in Example 1. Targeted LNPs were prepared as described in Example 1 except that the base LNP was conjugated to one of the targeting moieties present in **Table 20** (an anti-CD2, anti-CD7, anti-CD5, or anti-CD3 targeting moiety or a CD28 targeting protein derived from the CD80 extracellular domain ("CD80 ECD") at a 1:1 antibody weight: mRNA weight ratio. In some cases, both the anti-CD3 targeting moiety and CD80 ECD were both conjugated to the surface of the base LNP at a 1:1 molar ratio (anti-CD3 targeting moiety:CD80 ECD:mRNA) to create dual targeted LNPs. **Table 20** provides the targeting moieties that were tested.

Table 20. Anti-CD2, anti-CD5, anti-CD7, and-CD3 targeting moieties and CD80 ECD tested with targeted LNPs

Targeting moiety	Targeting moiety Ratio Ab (mg):RNA (mg)	HC sequence	HC sequence (without sortase tag)	LC sequence	VH sequence	VL sequence
Anti-CD2-1	1:1	QVQLQQP GTELVRPG SSVKLSCK ASGYTFT**S** **YWVN**WV KQRPDQGL EWIG**RIDP** **YDSETHY** **NQKFTD**K AISTIDTSS NTAYMQL STLTSDAS AVYYCSR**S** **PRDSSTNL** **AD**WGQGT LVTVSSAS TKGPSVFP LAPSSKSTS GGTAALG CLVKDYFP EPVTVSWN SGALTSGV HTFPAVLQ SSGLYSLSS VVTVPSSS LGTQTYIC NVNHKPSN TKVDKKV EPKSC*GGG GSLPETGH HHHHH* (SEQ ID NO:266)	QVQLQQPGT ELVRPGSSV KLSCKASGY TFT**SYWVN** WVKQRPDQ GLEWIG**RID** **PYDSETHYN** **QKFTD**KAIS TIDTSSNTAY MQLSTLTSD ASAVYYCSR **SPRDSSTNL** **AD**WGQGTL VTVSSASTK GPSVFPLAPS SKSTSGGTA ALGCLVKDY FPEPVTVSW NSGALTSGV HTFPAVLQS SGLYSLSSV VTVPSSSLGT QTYICNVNH KPSNTKVDK KVEPKSC (SEQ ID NO:267)	DIVMTQSP ATLSVTPG DRVSLSC**R** **ASQSISDY** **LH**WYQQK SHESPRLLI K**YASQSIS** GIPSRFSGS GSGSDFTL SINSVEPED VGVYYC**Q** **NGHSFPLT** FGAGTKLE LRRTVAAP SVFIFPPSD EQLKSGTA SVVCLLNN FYPREAKV QWKVDNA LQSGNSQE SVTEQDSK DSTYSLSS TLTLSKAD YEKHKVY ACEVTHQ GLSSPVTK SFNRGEC (SEQ ID NO:268)	QVQLQ QPGTEL VRPGSS VKLSC KASGY TFT**SY** **WVN**W VKQRP DQGLE WIG**RID** **PYDSET** **HYNQK** **FTD**KAI STIDTSS NTAYM QLSTLT SDASA VYYCS R**SPRDS** **STNLA** **D**WGQG TLVTVS S (SEQ ID NO:269)	DIVMTQS PATLSVT PGDRVSL SC**RASQS** **ISDYLH** WYQQKS HESPRLLI K**YASQSI** **S**GIPSRFS GSGSGSD FTLSINSV EPEDVGV YYC**QNG** **HSFPLT**F GAGTKLE LR (SEQ ID NO:270)
Anti-CD2-2	1:1	QVQLQQP GAELVRPG SSVKLSCK ASGYTFT**R** **YWIH**WVK QRPIQGLE WIG**NIDPS** **DSETHYN** **QKFKD**KA TLTVDKSS GTAYMQL SSLTSEDS AVYYCAT	QVQLQQPGA ELVRPGSSV KLSCKASGY TFT**RYWIH** WVKQRPIQG LEWIG**NIDPS** **DSETHYNQ** **KFKD**KATLT VDKSSGTAY MQLSSLTSE DSAVYYCAT **EDLYYAME** **Y**WGQGTSV	NIMMTQSP SSLAVSAG EKVTMTC **KSSQSVLY** **SSNQKNY** **LA**WYQQK PGQSPKLLI Y**WASTRE** **S**GVPDRFT GSGSGTDF TLTISSVQP EDLAVYY **CHQYLSS**	QVQLQ QPGAEL VRPGSS VKLSC KASGY TFT**RY** **WIH**WV KQRPIQ GLEWIG **NIDPSD** **SETHY** **NQKFK** **D**KATL	NIMMTQS PSSLAVS AGEKVT MTC**KSS** **QSVLYSS** **NQKNYL** **A**WYQQK PGQSPKL LIY**WAST** **RES**GVPD RFTGSGS GTDFTLT ISSVQPE

		EDLYYAM EYWGQGT SVTVSSAS TKGPSVFP LAPSSKSTS GGTAALG CLVKDYFP EPVTVSWN SGALTSGV HTFPAVLQ SSGLYSLSS VVTVPSSS LGTQTYIC NVNHKPSN TKVDKKV EPKSC*GGG GSLPETGH HHHHH* (SEQ ID NO:277)	TVSSASTKG PSVFPLAPSS KSTSGGTAA LGCLVKDYF PEPVTVSWN SGALTSGVH TFPAVLQSS GLYSLSSVV TVPSSSLGTQ TYICNVNHK PSNTKVDKK VEPKSC (SEQ ID NO:278)	**HT**FGGGT KLEIKRTV AAPSVFIFP PSDEQLKS GTASVVCL LNNFYPRE AKVQWKV DNALQSG NSQESVTE QDSKDSTY SLSSTLTLS KADYEKH KVYACEV THQGLSSP VTKSFNRG EC (SEQ ID NO:279)	TVDKSS GTAYM QLSSLT SEDSAV YYCAT **EDLYY AMEY** WGQGT SVTVSS (SEQ ID NO:280)	DLAVYY C**HQYLSS HT**FGGG TKLEIK (SEQ ID NO:281)
Anti-CD2-3	1:1	QVQLVQS GAEVKKP GASVKVSC KASGYTFT **EYYMY**WV RQAPGQGL ELMG**RIDP EDGSIDYV EKFKK**KV TLTADTSS STAYMELS SLTSDDTA VYYCAR**G KFNYRFA Y**WGQGTL VTVSSAST KGPSVFPL APSSKSTS GGTAALG CLVKDYFP EPVTVSWN SGALTSGV HTFPAVLQ SSGLYSLSS VVTVPSSS LGTQTYIC NVNHKPSN TKVDKKV EPKSC*GGG GSLPETGH HHHHH*	QVQLVQSGA EVKKPGASV KVSCKASGY TFT**EYYMY** WVRQAPGQ GLELMG**RID PEDGSIDYV EKFKK**KVT LTADTSSST AYMELSSLT SDDTAVYYC AR**GKFNYR FAY**WGQGT LVTVSSAST KGPSVFPLA PSSKSTSGGT AALGCLVKD YFPEPVTVS WNSGALTSG VHTFPAVLQ SSGLYSLSSV VTVPSSSLGT QTYICNVNH KPSNTKVDK KVEPKSC (SEQ ID NO:289)	DVVMTQS PPSLLVTL GQPASISC **RSSQSLLH SSGNTYLN** WLLQRPG QSPQPLIY**L VSKLES**GV PDRFSGSG SGTDFTLKI SGVEAEDV GVYYC**MQ FTHYPYT**F GQGTKLEI KRTVAAPS VFIFPPSDE QLKSGTAS VVCLLNNF YPREAKVQ WKVDNAL QSGNSQES VTEQDSKD STYSLSSTL TLSKADYE KHKVYAC EVTHQGLS SPVTKSFN RGEC (SEQ ID NO:290)	QVQLV QSGAE VKKPG ASVKV SCKASG YTFT**EY YMY**W VRQAP GQGLE LMG**RI DPEDG SIDYVE KFKK**K VTLTA DTSSST AYMEL SSLTSD DTAVY YCAR**G KFNYR FAY**WG QGTLV TVSS (SEQ ID NO:291)	DVVMTQ SPPSLLV TLGQPAS ISC**RSSQS LLHSSGN TYLN**WL LQRPGQS PQPLIY**L VSKLES**G VPDRFSG SGSGTDF TLKISGV EAEDVG VYYC**MQ FTHYPY T**FGQGTK LEIK (SEQ ID NO:292)

		(SEQ ID NO:288)				
Anti-CD2-4	1:1	QVQLVQS GAEVQRPG ASVKVSCK ASGYIFT**E** **YYMY**WVR QAPGQGLE LVG**RIDPE** **DGSIDYVE** **KFKK**KVT LTADTSSS TAYMELSS LTSDDTAV YYCAR**GK** **FNYRFAY** WGQGTLV TVSSASTK GPSVFPLA PSSKSTSG GTAALGCL VKDYFPEP VTVSWNS GALTSGVH TFPAVLQS SGLYSLSS VVTVPSSS LGTQTYIC NVNHKPSN TKVDKKV EPKSC*GGG GSLPETGH HHHHH* (SEQ ID NO:299)	QVQLVQSGA EVQRPGASV KVSCKASGY IFT**EYYMY**W VRQAPGQGL ELVG**RIDPE** **DGSIDYVEK** **FKK**KVTLTA DTSSSTAYM ELSSLTSDDT AVYYCAR**G** **KFNYRFAY** WGQGTLVT VSSASTKGP SVFPLAPSSK STSGGTAAL GCLVKDYFP EPVTVSWNS GALTSGVHT FPAVLQSSG LYSLSSVVT VPSSSLGTQT YICNVNHKP SNTKVDKKV EPKSC (SEQ ID NO:300)	DVVMTQS PPSLLVTL GQPASISC **RSSQSLLH** **SSGNTYLN** WLLQRPG QSPQPLIY**L** **VSKLES**GV PDRFSGSG SGTDFTLKI SGVEAEDV GVYYC**MQ** **FTHYPYT**F GQGTKLEI KRTVAAPS VFIFPPSDE QLKSGTAS VVCLLNNF YPREAKVQ WKVDNAL QSGNSQES VTEQDSKD STYSLSSTL TLSKADYE KHKVYAC EVTHQGLS SPVTKSFN RGEC (SEQ ID NO:290)	QVQLV QSGAE VQRPG YIFT**EY** **YMY**W VRQAP GQGLE LVG**RID** **PEDGSI** **DYVEK** **FKK**KV TLTADT SSSTAY MELSSL TSDDTA VYYCA R**GKFN** **YRFAY** WGQGT LVTVSS (SEQ ID NO:302)	DVVMTQ SPPSLLV TLGQPAS ISC**RSSQS** **LLHSSGN** **TYLN**WL LQRPGQS PQPLIY**L** **VSKLES**G VPDRFSG SGSGTDF TLKISGV EAEDVG VYYC**MQ** **FTHYPY** **T**FGQGTK LEIK (SEQ ID NO:292)
Anti-CD7-1 (Grisnilimab)	1:1	QIQLVQSG PELKKPGE TVKISCKA SGYTFT**NY** **GMN**WVK QAPGKGL MWLG**WIN** **TYTGEPT** **YADDFKG** RFAFSLETS ASTAYLQI NNLKNEDT ATYFCAR **WAYFYGS** **SPYFFDY** WGQGTTL	QIQLVQSGP ELKKPGETV KISCKASGY TFT**NYGMN** WVKQAPGK GLMWLG**WI** **NTYTGEPTY** **ADDFKG**RF AFSLETSAST AYLQINNLK NEDTATYFC AR**WAYFYG** **SSPYFFDY**W GQGTTLTVS SASTKGPSV FPLAPSSKST	QAVVTQES ALTTSPGE TVTLTC**RS** **STGAVTTS** **NYAN**WVQ EKPDHLFT GLIG**GTNN** **RAP**GVPAR FSGSLIGD KAALTITG AQTEDEAI YFC**ALWC** **SNHLV**FGG GTKLTVLG QPKANPTV QPKANPTV TLFPPSSEE	QIQLVQ SGPELK KPGETV KISCKA SGYTFT **NYGMN** WVKQA PGKGL MWLG **WINTY** **TGEPT** **YADDF** **KG**RFA FSLETS ASTAYL QINNLK	QAVVTQ ESALTTS PGETVTL TC**RSSTG** **AVTTSN** **YAN**WVQ EKPDHLF TGLIG**GT** **NNRAP**G VPARFSG SLIGDKA ALTITGA QTEDEAI YFC**ALW** **CSNHLV**F GGGTKLT

		TVSSASTK GPSVFPLA PSSKSTSG GTAALGCL VKDYFPEP VTVSWNS GALTSGVH TFPAVLQS SGLYSLSS VVTVPSSS LGTQTYIC NVNHKPSN TKVDKKV EPKSC*GGG GSLPETGH HHHHH* (SEQ ID NO:310)	SGGTAALGC LVKDYFPEP VTVSWNSG ALTSGVHTF PAVLQSSGL YSLSSVVTV PSSSLGTQTY ICNVNHKPS NTKVDKKV EPKSC (SEQ ID NO:311)	LQANKATL VCLISDFYP GAVTVAW KADGSPVK AGVETTKP SKQSNNKY AASSYLSL TPEQWKSH RSYSCQVT HEGSTVEK TVAPTECS (SEQ ID NO:312)	NEDTA TYFCAR **WAYFY GSSPYF FDY**WG QGTTLT VSS (SEQ ID NO:313)	VL (SEQ ID NO:314)
Anti-CD7-2	1:1	EVQLVESG GGLVQLG GSLRLSCA ASGLTFN**N YAMS**WVR QAPGKGLE WVS**TISGS GGSTYYA DSAKG**RFT ISRDNSKN TLYLQMNS LRAEDTAV YYCAK**GG LLYFGEF HFDY**WGQ GTLVTVSS ASTKGPSV FPLAPSSKS TSGGTAAL GCLVKDYF PEPVTVSW NSGALTSG VHTFPAVL QSSGLYSL SSVVTVPS SSLGTQTYI CNVNHKPS NTKVDKK VEPKSC*GG GGSLPETG HHHHHH* (SEQ ID NO:321)	EVQLVESGG GLVQLGGSL RLSCAASGL TFN**NYAMS** WVRQAPGK GLEWVS**TIS GSGGSTYY ADSAKG**RFT ISRDNSKNTL YLQMNSLRA EDTAVYYCA K**GGLLYFG EFHFDY**WG QGTLVTVSS ASTKGPSVF PLAPSSKSTS GGTAALGCL VKDYFPEPV TVSWNSGAL TSGVHTFPA VLQSSGLYS LSSVVTVPSS SLGTQTYIC NVNHKPSNT KVDKKVEPK SC (SEQ ID NO:322)	DIQMTQSP SSLSASVG DRVTIIC**R ASQGISNY LA**WFQQK PGKAPKSL IY**AASSLQ S**GVPSKFS GSGSGTDF TLTISSLQP EDFATYYC **QHYNSYP LT**FGGGTK VEIKRTVA APSVFIFPP SDEQLKSG TASVVCLL NNFYPREA KVQWKVD NALQSGNS QESVTEQD SKDSTYSL SSTLTLSK ADYEKHK VYACEVT HQGLSSPV TKSFNRGE C (SEQ ID NO:323)	EVQLV ESGGGL VQLGG SLRLSC AASGLT FN**NYA MS**WVR QAPGK GLEWV S**TISGS GGSTY YADSA KG**RFTI SRDNSK NTLYL QMNSL RAEDT AVYYC AK**GGL LYFGE FHFDY** WGQGT LVTVSS (SEQ ID NO:324)	DIQMTQS PSSLSAS VGDRVTI IC**RASQG ISNYLA**W FQQKPGK APKSLIY **AASSLQS** GVPSKFS GSGSGTD FTLTISSL QPEDFAT YYC**QHY NSYPLT**F GGGTKV EIK (SEQ ID NO:325)

Anti-CD7-3	1:1	EVQLVESG GGLVKPG GSPRLSCA AYGVTFS**N** **AWMS**WV RQAPGKGL EWVG**RIKS** **KTDGGTT** **DYAAPVK** **G**RFTISRD DSKNTLYL QMNSLKTE DTAVYYC **TIEAVAGH** **FDY**WGQG TLVTVSSA STKGPSVF PLAPSSKST SGGTAALG CLVKDYFP EPVTVSWN SGALTSGV HTFPAVLQ SSGLYSLSS VVTVPSSS LGTQTYIC NVNHKPSN TKVDKKV EPKSC*GGG GSLPETGH HHHHH* (SEQ ID NO:332)	EVQLVESGG GLVKPGGSP RLSCAAYGV TFS**NAWMS** WVRQAPGK GLEWVG**RIK** **SKTDGGTT** **DYAAPVKG** RFTISRDDSK NTLYLQMNS LKTEDTAVY YC**TIEAVAG** **HFDY**WGQG TLVTVSSAS TKGPSVFPL APSSKSTSG GTAALGCLV KDYFPEPVT VSWNSGALT SGVHTFPAV LQSSGLYSLS SVVTVPSSSL GTQTYICNV NHKPSNTKV DKKVEPKSC (SEQ ID NO:333)	DIQMTQSP STLSASVG DRVTITC**R** **ASQSISSW** **LA**WYQQK PGKAPKLL IY**KASSLE** **S**GVPSRFS GSGSGTEF TLTISSLQP DDFATYYC **QQYNNYS** **PT**FGQGTK VEIKRTVA APSVFIFPP SDEQLKSG TASVVCLL NNFYPREA KVQWKVD NALQSGNS QESVTEQD SKDSTYSL SSTLTLSK ADYEKHK VYACEVT HQGLSSPV TKSFNRGE C (SEQ ID NO:334)	EVQLV ESGGGL VKPGG SPRLSC AAYGV TFS**NA** WYQQKP GKAPKLL IY**KASSL** **ES**GVPSR FSGSGSG TEFTLTIS SLQPDDF ATYYC**Q** **QYNNYS** **PT**FGQGT KVEIK (SEQ ID NO:335)	DIQMTQS PSTLSAS VGDRVTI TC**RASQS** **ISSWLA** WYQQKP GKAPKLL IY**KASSL** **ES**GVPSR FSGSGSG TEFTLTIS SLQPDDF ATYYC**Q** **QYNNYS** **PT**FGQGT KVEIK (SEQ ID NO:336)
Anti-CD7-4	1:1	EVQLVESG GGLVQPG GSLRLSCA ASGFTFS**R** **YAMS**WVR QAPGKGLE WVS**SISAS** **GATTFYA** **DPVKG**RFT ISRDNSKK TLYLQMNS LRAEDTAV YYCAK**DQ** **DFDILTGY** **LNWFDP**W GQGTLVTV SSASTKGP SVFPLAPSS	EVQLVESGG GLVQPGGSL RLSCAASGF TFS**RYAMS** WVRQAPGK GLEWVS**SIS** **ASGATTFYA** **DPVKG**RFTI SRDNSKKTL YLQMNSLRA EDTAVYYCA K**DQDFDILT** **GYLNWFDP** WGQGTLVT VSSASTKGP SVFPLAPSSK STSGGTAAL GCLVKDYFP	EIVLTQSPA TLSLSPGE RVILSC**RV** **SQSVSSYL** **A**WYQQKP GQAPRLLI Y**DTSNRA** **T**GIPARFS GSGSGTDF TLTISSLEP EDFAVYYC **QQRRNWP** **LT**FGGGTK VEIKRTVA APSVFIFPP SDEQLKSG TASVVCLL NNFYPREA	EVQLV ESGGGL VQPGG SLRLSC AASGFT FS**RYA** YQQKPG QAPRLLI Y**DTSNR** **AT**GIPAR FSGSGSG TDFTLTIS SLEPEDF AVYYC**Q** **QRRNWP** **LT**FGGGT KVEIK (SEQ ID NO:347)	EIVLTQSP ATLSLSP GERVILS C**RVSQSV** **SSYLA**W YQQKPG QAPRLLI Y**DTSNR** **AT**GIPAR FSGSGSG TDFTLTIS SLEPEDF AVYYC**Q** **QRRNWP** **LT**FGGGT KVEIK (SEQ ID NO:347)

		KSTSGGTA ALGCLVK DYFPEPVT VSWNSGA LTSGVHTF PAVLQSSG LYSLSSVV TVPSSSLG TQTYICNV NHKPSNTK VDKKVEP KSC*GGGGS LPETGHHH HHH* (SEQ ID NO:343)	EPVTVSWNS GALTSGVHT FPAVLQSSG LYSLSSVVT VPSSSLGTQT YICNVNHKP SNTKVDKKV EPKSC (SEQ ID NO:344)	KVQWKVD NALQSGNS QESVTEQD SKDSTYSL SSTLTLSK ADYEKHK VYACEVT HQGLSSPV TKSFNRGE C (SEQ ID NO:345)	AK**DQD FDILTG YLNWF DP**WGQ GTLVT VSS (SEQ ID NO:346)	
Anti-CD3-8 (SP34) + anti-CD3-6 (Visilizumab) (1:1)	1:1	See Example 1	See Example 1	See Example 1	See Example 1	See Example 1
Anti-CD3-8 (SP34)	1:1	See Example 1	See Example 1	See Example 1	See Example 1	See Example 1
Anti-CD5-1	1:1	QVTLKESG PVLVKPTE TLTLTCTFS GFSLS**TSG MGVG**WIR QAPGKGLE WVA**HIW WDDDVYY NPSLKS**RL TITKDASK DQVSLKLS SVTAADTA VYYCVR**R RATGTGF DY**WGQGT LVTVSSAS TKGPSVFP LAPSSKSTS GGTAALG CLVKDYFP EPVTVSWN SGALTSGV HTFPAVLQ SSGLYSLSS VVTVPSSS LGTQTYIC NVNHKPSN	QVTLKESGP VLVKPTETL TLTCTFSGFS LS**TSGMGV G**WIRQAPGK GLEWVA**HI WWDDDVY YNPSLKS**RL TITKDASKD QVSLKLSSV TAADTAVY YCVR**RRAT GTGFDY**WG QGTLVTVSS ASTKGPSVF PLAPSSKSTS GGTAALGCL VKDYFPEPV TVSWNSGAL TSGVHTFPA VLQSSGLYS LSSVVTVPSS SLGTQTYIC NVNHKPSNT KVDKKVEPK SCDKTH (SEQ ID NO:355)	NIVMTQSP SSLSASVG DRVTITC**Q ASQDVGT AVA**WYQQ KPDQSPKL LIY**WTSTR HT**GVPDRF TGSGSGTD FTLTISSLQ PEDIATYF C**HQYNSY NT**FGSGTK LEIKRTVA APSVFIFPP SDEQLKSG TASVVCLL NNFYPREA KVQWKVD NALQSGNS QESVTEQD SKDSTYSL SSTLTLSK ADYEKHK VYACEVT HQGLSSPV TKSFNRGE	QVTLK ESGPVL VKPTET LTLTCT FSGFSL S**TSGM GVG**WI RQAPG KGLEW VA**HIW WDDDV YYNPS LKS**RLT ITKDAS KDQVS LKLSSV TAADT AVYYC VR**RRA TGTGF DY**WGQ GTLVT VSS (SEQ ID NO:357)	NIVMTQS PSSLSAS VGDRVTI TC**QASQ DVGTAV A**WYQQK PDQSPKL LIY**WTST RHT**GVP DRFTGSG SGTDFTL TISSLQPE DIATYFC **HQYNSY NT**FGSGT KLEIK (SEQ ID NO:358)

		TKVDKKV EPKSCDKT H*GGGGSLP ETGHHHH HH* (SEQ ID NO:354)		C (SEQ ID NO:356)		
The sortase tag sequence in the heavy chain (HC) sequences are underlined and italicized. The CDR sequences for the anti-CD3-9 targeting moiety are in bolded text.						
Targeting moiety	**Ratio**		**Sequence**			
CD80 ECD	1:1		VIHVTKEVKEVATLSCGHNVSVEELAQTRIYWQK EKKMVLTMMSGDMNIWPEYKNRTIFDITNNLSIVI LALRPSDEGTYECVVLKYEKDAFKREHLAEVTLS VKADFPTPSISDFEIPTSNIRRIICSTSGGFPEPHLSW LENGEELNAINTTVSQDPETELYAVSSKLDFNMTT NHSFMCLIKYGHLRVNQTFNWNTTKQEHFPDN*G GGGSLPETGHHHHHH* (SEQ ID NO:365)			
Anti-CD3-8 (SP34) + CD80 ECD	1:1		See above			

LNP Transfection and Flow Cytometry

[0599] T cells were transfected with LNPs as described in Example 1. Following overnight incubation, cells in plates were pelleted by centrifugation at 800xg for 2 minutes, washed once with 100uL of 1X PBS (Gibco #), then resuspended in 50uL PBS containing LIVE/DEAD™ Fixable Near-IR Dead Cell Stain (Invitrogen # L34976) at 1:1,000 dilution, plus the following antibodies: mouse anti-human CD3 PE (clone OKT3, Biolegend # 317308), mouse anti-human CD25 Alexa Fluor 700 (clone BC96, Biolegend # 302622), mouse anti-human CD69 APC (clone FN50, Biolegend # 310910)and mouse anti-human LDLR PE (clone C7, BD Biosciences # 565653). All antibodies were used at a 1:200 final dilution. Cells were stained on ice for 20 minutes, then centrifuged, washed as above and resuspended in 100uL of 1X PBS containing 2% FBS.

[0600] Flow cytometry data was acquired on a NovoCyte 3000RYB (Agilent # 2010044), then analyzed for the proportion of live cells expressing GFP as well as the median fluorescence intensity (MFI) of GFP signal in live cells.

[0601] Cytokines were quantified as described in Example 1.

Results

[0602] **FIGs. 5A-5D** show that all of the anti-CD2, anti-CD7, anti-CD3, and anti-CD5 targeted tLNPs outperform non-targeted base LNPs in activated T cells, either when the cells were cultured with serum (FBS) or without serum. All of the tLNPs conjugated to anti-CD7

targeting moieties drove higher GFP expression when transfected in activated T cells in the presence or absence of serum compared to tLNPs conjugated with anti-CD2 targeting moieties, especially at higher LNP concentrations (**FIGs. 5C-5D**). There was an average fold increase in GFP expression of 20-fold (with serum, **FIG. 5D**) and 140-fold (without serum, **FIG. 5C**) across all of the tLNPs with anti-CD7 targeting moieties. In the presence of FBS, all four anti-CD7-targeted tLNPs also outperformed the anti-CD3 tLNP tested. Transfection by anti-CD7 tLNPs was somewhat lower in the absence of serum (FBS) (**FIG. 5C**).

[0603] In rested T cells, transduction by tLNPs was generally weaker than in activated T cells. **FIGs. 6A-6D** show that targeted LNPs conjugated to anti-CD7, anti-CD5, and anti-CD3 targeting moieties outperformed non-targeted base LNPs, both with and without serum. However, **FIGs. 6C** and **6D** show that the tLNPs with the anti-CD3 targeting moiety resulted in an average four-fold higher GFP expression than the tLNPs conjugated to the anti-CD7 targeting moieties.

[0604] Dual targeted LNPs comprising both the anti-CD3 targeting moiety and the CD80 extracellular domain (ECD) on their surface boosted transfection capacity in both activated and resting T cells relative to the other tLNPs tested (**FIGs. 5A-5D** and **FIGs. 6A-6D**). While tLNPs with the CD80 ECD alone provided a minimal increase to transfection efficiency compared to non-targeted LNPs, the tLNPs with both the anti-CD3 targeting moiety and the CD80 ECD increased GFP expression at least two-fold compared to tLNPs with the anti-CD3 targeting moiety alone in all conditions tested. Indeed, the dual targeted anti-CD3/CD80 ECD tLNPs generated higher GFP expression levels in activated T cells without serum and in the rested T cells with or without serum (**FIGs. 5C, 6C**, and **6D**).

[0605] In addition to increasing transfection efficiency, **FIGs. 7A** and **7B** show that dual targeted LNPs conjugated to the anti-CD3 targeting moiety and the CD80 ECD were also capable of activating rested T cells. These dual tLNPs drove increased expression of surface activation markers CD25 and CD69 relative to non-targeted base LNPs, and increased expression of the activation markers by at least two-fold relative to tLNPs with the anti-CD3 targeting moiety alone. A dual targeted LNP comprising two different anti-CD3 targeting moieties (anti-CD3-6 and anti-CD3-8) similarly increased expression of the CD25 and CD69 activation markers relative to the base LNP and tLNPs with a single anti-CD3 targeting moiety (**FIGs. 7A-7B**). None of the tLNPs conjugated to the anti-CD2, anti-CD7, or anti-CD5 targeting moieties increased the activation markers.

Example 3: Screening for LNPs comprising novel ionizable lipids that enhance delivery to activated and resting T cells

[0606] A screen was conducted to identify novel ionizable lipids that can be formulated in LNPs to enhance delivery of a payload to human T cells. Base LNPs (lacking targeting moieties) were formulated with Lipid092, Lipid110, Lipid133, Lipid134, or Lipid154, and an mRNA encoding GFP, and then the transduction efficiency of each LNP was tested in activated and resting T cells. LNPs containing the V003, SM102, or Lipid177 ionizable lipids were also generated to establish baseline transduction controls for the purpose of comparing transduction efficiency.

T Cell Activation and Culture

[0607] Cryopreserved primary T cells from a single human donor (HemaCare, donor D328798) were thawed and transferred into PBS with 2% BSA by volume. Cells were counted on a Cellometer K2 Cell Counter (Nexcelom), then pelleted by centrifugation at 400xg for 5 minutes and resuspended in complete T cell media at a density of $1x10^6$ cells/mL. The complete T cell media was comprised of CTS AIM V SFM basal media (Gibco #0870112DK) with 5% human serum AB (Gemini Bio #100-512) and the following cytokines: human IL-2 at 20ng/mL (Miltenyi Biotec #130-097-746), human IL-7 at 10ng/mL (Miltenyi Biotec #130-095-362), and human IL-15 at 5ng/mL (Miltenyi Biotec #130-095-764). TransAct Research Grade T cell activation reagent (Miltenyi Biotec #130-111-160) was added to cell suspension at a volume of 10uL per $1x10^6$ cells. Activated cells were transferred to flasks and incubated at 37°C, 5% CO_2 for 72 hours ("Activated" condition).

[0608] At 48 hours post-activation, additional T cells from the same donor were thawed and resuspended in complete T cell media as above, without TransAct stimulation. These cells were transferred to flasks and incubated at 37°C, 5% CO_2 for 24 hours ("Rested" condition).

Preparation of T Cells for Transfection

[0609] Following the incubation periods above, the activated and rested T cells were collected separately and counted. $2.0x10^7$ cells were taken from each activation condition, then pelleted by centrifugation, and the supernatant aspirated. The cell pellets were resuspended in CTS AIM V SFM at a concentration of $1.33x10^6$ cells/mL, supplemented with 6.67% fetal bovine serum (FBS, Gibco #A31604-01), and human IL-2, human IL7, and human IL-15, at concentrations of 26.6ng/mL, 13.3ng/mL and 6.6ng/mL, respectively.

[0610] The resuspended T cells were plated in non-treated 96-well U-bottom polystyrene plates (Falcon # 08-772-54) at a density of $2x10^5$ cells (150uL) per well, and returned to incubation at 37°C, 5% CO_2 while the LNPs were prepared.

Preparation of Base LNPs for Transfection

[0611] LNPs were formulated as follows. An ethanol phase was prepared with five lipids, containing 47% ionizable lipid (Lipid092, Lipid110, Lipid133, Lipid134, Lipid154, Lipid177, V003, or SM102), 8% DSPC, 42.5% cholesterol, 2% DMG-PEG2K and 0.5% DSPE-PEG2K-TCO. An aqueous phase was composed of mRNA (encoding eGFP) dissolved in 25 mM acetate buffer. The two phases were mixed using a microfluidic device to formulate LNP-TCO at a total flow rate of 18 ml/min (aqueous phase: ethanol phase=3:1). 3x volume of CBS was added to LNP solution and the resulting solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes. The LNPs were then subjected to overnight dialysis in Tris/sucrose buffer at 4 C followed by centrifugation (100k MWCO) and/or via Tangential Flow Filtration (TFF) to the desired concentration.

LNP Transfection

[0612] 50uL of diluted LNPs were added to plated activated and rested T cells at 100ng, 200ng, 400ng, 600ng and 800ng of LNP per 2 $x10^5$ cells, and mixed gently by pipetting. After addition of the LNPs, all plated cells were at a final density of $1x10^6$ cells/mL in 200uL, with a final concentration of 5% FBS (for serum-containing conditions) and cytokines at the following concentrations: human IL-2 at 20ng/mL, human IL-7 at 10ng/mL, and human IL-15 at 5ng/mL. Transfected cells were returned to incubation at 37°C, 5% CO_2 overnight.

Flow Cytometry

[0613] Following overnight incubation, flow cytometry was conducted on the cells to detect GFP as described in Example 1.

Results

[0614] The base LNPs formulated with Lipid092 and Lipid154 exhibited the highest transfection levels in activated T cells out of all the LNPs tested. **FIG. 8A** shows that close to 100% of activated T cells transfected with LNPs comprising Lipid092 or Lipid154 expressed GFP, starting at the lowest dose. Fewer T cells were transfected with the other LNPs tested, including the baseline control LNPs, across all dose levels. **FIG. 8B** shows that transfection with Lipid154 LNPs resulted in the highest GFP expression levels (MFI) in the cells, followed by LNPs comprising Lipid092. The MFI levels increased in a dose-dependent fashion for these

LNPs. Activated T cells transfected with the other LNPs exhibited much lower GFP expression levels at all doses tested.

[0615] The base LNPs formulated with Lipid092 and Lipid154 also exhibited the highest transfection levels in rested T cells out of all the LNPs tested. **FIG. 8C** shows that Lipid092 and Lipid154 LNPs transfected the largest numbers of cells at the 100ng to 400 ng doses (per $2x10^5$ cells), but then the percentage of GFP+ cells fell at higher doses of the Lipid154 LNP. The LNPs formulated with the V003 ionizable lipid transfected smaller numbers of rested T cells at all doses tested. **FIG. 8D** shows that transfection of LNPs with Lipid092 GFP expression resulted in the highest levels of GFP expression at most doses, followed by LNPs with Lipid154. By comparison, LNPs formulated with V003 induced the lowest GFP expression levels out of all LNPs tested.

Example 4: Testing of ionizable lipids for LNP delivery of an exemplary gene modifying system to activated T cells *in vitro*

[0616] The Lipid092 and Lipid154 ionizable lipids that exhibited enhanced delivery to T cells in the lipid screen described in Example 3 were next formulated in base LNPs with a gene modifying system comprised of mRNA encoding an exemplary gene modifying polypeptide and a template RNA encoding GFP for use with the gene modifying polypeptide. Activated T cells were then transfected to determine whether these base LNPs could deliver the gene modifying system to activated T cells to enable insertion of the heterologous GFP gene into the genomic DNA of the cells. The gene writing results were compared to baseline writing results using a base LNP formulated with the V003 ionizable lipid.

T Cell Activation and Culture

[0617] Cryopreserved primary T cells from a single human donor (HemaCare, donor D328798) were thawed and transferred into PBS with 2% BSA by volume. Cells were counted on a Cellometer K2 Cell Counter (Nexcelom), then pelleted by centrifugation at 400xg for 5 minutes and resuspended in complete T cell media at a density of $1x10^6$ cells/mL. The complete T cell media was comprised of CTS AIM V SFM basal media (Gibco #0870112DK) with 5% human serum AB (Gemini Bio #100-512) and the following cytokines: human IL-2 at 20ng/mL (Miltenyi Biotec #130-097-746), human IL-7 at 10ng/mL (Miltenyi Biotec #130-095-362), and human IL-15 at 5ng/mL (Miltenyi Biotec #130-095-764). TransAct Research Grade T cell activation reagent (Miltenyi Biotec #130-111-160) was added to cell suspension at a volume of 10uL per $1x10^6$ cells. Activated cells were transferred to flasks and incubated at 37°C, 5% CO_2 for 72 hours ("Activated" condition).

Preparation of T Cells for Transfection

[0618] Following the incubation periods above, the activated T cells were collected separately and counted. $2.0x10^7$ cells were taken from each activation condition, then pelleted by centrifugation, and the supernatant aspirated. The cell pellets were resuspended in CTS AIM V SFM at a concentration of $1.33x10^6$ cells/mL, supplemented with 6.67% fetal bovine serum (FBS, Gibco #A31604-01), and human IL-2, human IL-7, and human IL-15, at concentrations of 26.6ng/mL, 13.3ng/mL and 6.6ng/mL, respectively.

[0619] The resuspended T cells were plated in non-treated 96-well U-bottom polystyrene plates (Falcon # 08-772-54) at a density of $2x10^5$ cells (150uL) per well, and returned to incubation at 37°C, 5% CO_2 while the LNPs were prepared.

Preparation of Base LNPs for Transfection

[0620] LNPs were formulated as follows. An ethanol phase was prepared with five lipids, containing 47% ionizable lipid (Lipid092, Lipid154, or V003), 8% DSPC, 42.5% cholesterol, 2% PEG-DMG and 0.5% DSPE-PEG2K-TCO. An aqueous phase was composed of mRNA encoding the exemplary gene modifying polypeptide and a template RNA encoding GFP dissolved in 25 mM acetate buffer. The two phases were mixed using a microfluidic device to formulate LNP-TCO at a total flow rate of 18 ml/min (aqueous phase: ethanol phase=3:1). 3x volume of CBS was added to LNP solution and the resulting solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes. The LNPs were then buffer exchanged to the desired concentration either via Tangential Flow Filtration (TFF) and/or centrifugation (100k MWCO).

[0621] The gene modifying system formulated in the LNPs comprised an mRNA encoding an exemplary gene modifying polypeptide comprised the following components.

[0622] The gene modifying polypeptide comprised the amino acid sequence of:

MDSTAHPNQGRGLEKVSQTLPALQTPGQHTAAGGSSPLSGRNQRKNTKKLLLGAWNIR
TLLDRENTPRPERRTALIGKELARYNIDIAALSETRLPEEGSLSEPTTGYTFFWKGRASNE
DRIHGVGLAIKTSLLKQLPDLPVGISERLMKIRLPLSKDRYATIISAYAPTLTSTEETIEQFY
SDLSAVLHSVPTNDKLILLGDFNARVGQDHERWKGVLGKHGVGKMNNNGLLLLSKCS
EFELTITNTVFRMANKYKTTWMHPRSKQWHLIDYIIVRRRDIQDVKITRAMRGAECWT
DHRLVRATLQMRIAPRHPKRAQTVRAFYNVSRLRDPSYLQTFQSCLDDKLSAKGPLTGS
STEKWNQFRDAVKETSKAVLGPKQRNHQDWFDENNTAIEDLLSKKNKAFMEWQNNPN
SAPKKDRFKSLQATAQREIRKMQDRWWEKKAEEIQRFADMKNYKQFFSALKTVYGPL
KPTTTPLLSSDGDTLIKDKKGISNRWKEHFSQLLNRPSSVDQSALDQIPQNRTIEQLDVPP

SIEEVQKAIKQMSAGKAPGKDGIPTEVYKALNGKALQAFHIVLTSIWEEEDMPPELRDAS
IVALYKNKGSRAACDNYRGISLLSTAGKILARVILNRLLSSVSEQNLPESQCGFRPDRSTI
DMVFTVRQMQEKCLEQNLSLYIVFIDLTKAFDTVNRDALWVILSKLGCPAKFVKLIQLF
HVDMTGEVLSGGETSDRFNISNGVKQGCVLAPVLFNLFFTQVLRHAVMDLDLGVYIKY
RLDGSLFDLRRLTAKTKTTERLILEALFADDCALMAHQENHLQTIVDRFSTATKLFGLTI
SLSKTEVLFQPAPGRPTNQPCITIDGTQLSNVNTFKYLGSTIANDGSLDHEINARIQKASQ
ALGRLRCKVLQHRGVSTATKLKVYNAVVLSSLLYGCETWTLYRKHMKQLEQFHQRSL
RSIMRIRWQDRITNQEVLDRANSTSIEVMVLKTQLRWSGHVIRMDPQRIPRQVFYGELS
AGLRKQGRPKKRFKDQLKSNLKWAGITPKQLELAASDRSSWRTHINHAATTFEDERRR
RLAAARERRHQATTAPPVTTGVPCPMCHKLCASAFGLQSHMRVHRR (SEQ ID NO:32)

[0623] The gene modifying polypeptide comprised a GGS Linker having an amino acid sequence of:

GGS

[0624] The gene modifying polypeptide comprised an SV40 NLS having an amino acid sequence of:

PKKKRKV (SEQ ID NO:367)

[0625] The gene modifying polypeptide comprised an XTEN linker having an amino acid sequence of:

SGSETPGTSESATPES (SEQ ID NO:368)

[0626] The gene modifying polypeptide comprised an HiBit tag having an amino acid sequence of:

VSGWRLFKKIS (SEQ ID NO:369)

[0627] The full-length gene modifying polypeptide with all components was structured as follows: NLS - GGS linker - gene modifying polypeptide - XTEN linker - HiBit tag. The full-length sequences are provided in **Table 21**.

[0628] Table 21: Nucleic Acid and Amino Acid Sequences of the Gene Modifying Polypeptide

Name	Nucleic Acid Sequence	Amino Acid Sequence
Gene modifying polypeptide	AGGAAAUAAGAGAGAAAAGAAGAGUAAGA AGAAAUAUAAGAGCCACCAUGCCAAAGAAG AAAAGAAAAGUAGGAGGUUCCGACUCCACA GCUCAUCCCAACCAGGGACGUGGCCUGGAG AAGGUGUCCCAGACUCUGCCUGCGCUGCAG ACCCCAGGGCAGCACACUGCCGCUGGGGGA	MPKKKRKVGGSDSTAHPNQGRGLE KVSQTLPALQTPGQHTAAGGSSPLS GRNQRKNTKKLLLGAWNIRTLLDR ENTPRPERRTALIGKELARYNIDIAA LSETRLPEEGSLSEPTTGYTFFWKGR ASNEDRIHGVGLAIKTSLLKQLPDLP

RNAI VT1901	UCCUCUCCCCUGUCCGGCAGGAAUCAGCGG AAGAACACCAAAAAGCUGUUGCUGGGCGCC UGGAACAUCCGCACCCUGCUCGACCGCGAA AAUACUCCCCGUCCUGAAAGACGCACGGCA CUGAUUGGGAAAGAGCUCGCCAGAUAUAAC AUCGACAUCGCUGCCCUGUCCGAAACCCGC CUGCCAGAGGAAGGAUCUCUGAGCGAGCCU ACCACUGGCUACACGUUCUUUUGGAAGGGC AGAGCAAGCAACGAGGACCGUAUCCAUGGU GUAGGCCUGGCUAUCAAGACCUCUCUCCUG AAACAACUCCCAGAUCUGCCAGUCGGCAUC UCAGAGAGACUGAUGAAGAUCAGGCUUCCU CUCUCAAAAGACCGCUACGCAACCAUCAUU UCCGCAUACGCGCCGACCCUGACUAGCACC GAGGAAACCAUUGAGCAGUUCUACUCUGAU CUUUCAGCUGUUUUGCACUCCGUGCCUACC AACGACAAGCUGAUCCUCCUGGGCGACUUC AACGCUCGCGUGGGUCAGGACCAUGAGCGC UGGAAAGGCGUGCUGGGUAAGCAUGGCGU GGGUAAGAUGAACAAUAACGGCCUCCUGCU CCUGUCUAAGUGUAGCGAAUUCGAGCUGAC UAUUACCAACACUGUUUUUCGUAUGGCUAA CAAGUACAAAACUACAUGGAUGCACCCCAG GAGCAAGCAGUGGCACCUGAUUGACUACAU UAUCGUGCGUAGGCGGGAUAUCCAGGACGU GAAGAUUACCCGUGCAAUGCGCGGCGCUGA GUGCUGGACAGACCAUCGCCUGGUGAGAGC UACUCUCCAGAUGAGGAUCGCACCGCGUCA CCCUAAGCGCGCCCAGACCGUGCGGGCCUU UUACAACGUAUCACGGCUUCGGGAUCCUUC CUACCUUCAGACAUUUCAAUCUUGCCUCGA UGACAAGCUCUCUGCUAAGGGUCCCCUCAC CGGAUCUUCAACAGAGAAGUGGAACCAAUU CCGCGAUGCGGUGAAGGAAACCAGCAAAGC AGUCCUGGGACCGAAGCAGCGCAACCACCA AGACUGGUUUGACGAAAAUAACACUGCCAU CGAAGACCUCCUGUCUAAAAAGAACAAAGC AUUUAUGGAGUGGCAGAAUAACCCAAACA GUGCACCCAAGAAAGACCGUUUCAAAAGCC UGCAGGCCACCGCGCAGCGCGAGAUCCGGA AGAUGCAGGACCGCUGGUGGGAAAAAAAG GCCGAAGAGAUCCAGAGGUUCGCGGAUAUG AAAAACUACAAGCAGUUCUUUAGCGCACUG AAGACCGUGUACGGACCUCUCAAACCCACC ACGACUCCCCUGCUCUCUUCCGAUGGUGAC ACUCUCAUCAAGGACAAAAAGGGAAUCAGC AACCGCUGGAAAGAACACUUUUCUCAACUU CUCAACCGCCCAAGCUCCGUGGACCAGAGC GCUCUGGACCAGAUCCCGCAGAACCGUACC AUCGAGCAGCUGGACGUGCCUCCCUCCAUC GAAGAGGUCCAGAAGGCUAUCAAGCAGAU GUCUGCUGGGAAGGCUCCUGGCAAGGAUGG GAUCCCAACAGAGGUCUAUAAAGCUCUCAA CGGAAAGGCGCUGCAGGCCUUUCACAUUGU CCUGACCUCCAUCUGGGAGGAAGAGGACAU GCCACCUGAACUUAGGGACGCCUCUAUUGU GGCCCUGUACAAGAAUAAGGGCAGCCGCGC AGCUUGUGAUAACUAUCGUGGCAUCUCACU CCUGAGUACAGCUGGCAAGAUUCUGGCUCG UGUGAUCCUGAACCGGUUGCUGAGUUCCGU UUCCGAGCAGAACCUGCCCGAAUCACAGUG	VGISERLMKIRLPLSKDRYATIISAY APTLTSTEETIEQFYSDLSAVLHSVP TNDKLILLGDFNARVGQDHERWKG VLGKHGVGKMNNNGLLLLSKCSEF ELTITNTVFRMANKYKTTWMHPRS KQWHLIDYIIVRRRDIQDVKITRAM RGAECWTDHRLVRATLQMRIAPRH PKRAQTVRAFYNVSRLRDPSYLQTF QSCLDDKLSAKGPLTGSSTEKWNQF RDAVKETSKAVLGPKQRNHQDWFD ENNTAIEDLLSKKNKAFMEWQNNP NSAPKKDRFKSLQATAQREIRKMQ DRWWEKKAEEIQRFADMKNYKQFF SALKTVYGPLKPTTTPLLSSDGDTLI KDKKGISNRWKEHFSQLLNRPSSVD QSALDQIPQNRTIEQLDVPPSIEEVQ KAIKQMSAGKAPGKDGIPTEVYKAL NGKALQAFHIVLTSIWEEEDMPPEL RDASIVALYKNKGSRAACDNYRGIS LLSTAGKILARVILNRLLSSVSEQNL PESQCGFRPDRSTIDMVFTVRQMQE KCLEQNLSLYIVFIDLTKAFDTVNRD ALWVILSKLGCPAKFVKLIQLFHVD MTGEVLSGGETSDRFNISNGVKQGC VLAPVLFNLFFTQVLRHAVMDLDL GVYIKYRLDGSLFDLRRLTAKTKTT ERLILEALFADDCALMAHQENHLQT IVDRFSTATKLFGLTISLSKTEVLFQP APGRPTNQPCITIDGTQLSNVNTFKY LGSTIANDGSLDHEINARIQKASQAL GRLRCKVLQHRGVSTATKLKVYNA VVLSSLLYGCETWTLYRKHMKQLE QFHQRSLRSIMRIRWQDRITNQEVL DRANSTSIEVMVLKTQLRWSGHVIR MDPQRIPRQVFYGELSAGLRKQGRP KKRFKDQLKSNLKWAGITPKQLEL AASDRSSWRTHINHAATTFEDERRR RLAAARERRHQATTAPPVTTGVPCP MCHKLCASAFGLQSHMRVHRRSGS ETPGTSESATPESVSGWRLFKKIS (SEQ ID NO:64)

	CGGCUUCCGCCCCGAUCGGUCCACCAUCGA UAUGGUGUUCACCGUGCGUCAGAUGCAGGA GAAGUGCCUGGAACAGAAUCUGAGCCUGUA CAUCGUUUUCAUCGAUCUGACUAAGGCCUU CGACACGGUGAACCGCGAUGCCCUGUGGGU GAUCCUGAGCAAGCUGGGGUGCCCCGCUAA GUUCGUAAAGCUCAUCCAGUUGUUCCACGU UGAUAUGACGGGAGAGGUCCUGUCCGGUG GAGAAACCUCCGACCGGUUUAACAUCUCCA AUGGCGUGAAACAGGGCUGCGUCCUGGCCC CAGUGCUGUUCAACCUGUUUUUCACACAGG UGCUGAGACAUGCGGUUAUGGAUCUUGACC UGGGUGUGUAUAUCAAGUAUCGCUUGGAC GGCAGUCUGUUCGAUCUCAGACGCCUGACA GCUAAGACUAAGACUACCGAGCGCCUCAUU CUCGAAGCCCUGUUUGCCGACGAUUGUGCC CUUAUGGCCCACCAGGAGAACCACCUGCAG ACGAUUGUCGACCGCUUCUCUACAGCGACC AAGCUGUUCGGCCUCACCAUCUCCCUCUCA AAGACCGAAGUCCUGUUUCAGCCCGCACCC GGGAGACCCACGAAUCAGCCGUGCAUUACC AUUGACGGGACCCAACUUUCCAACGUCAAC ACCUUCAAGUAUCUGGGAUCUACCAUCGCC AAUGAUGGCAGCCUGGAUCAUGAAAUCAAC GCAAGGAUCCAAAAGGCUAGCCAGGCACUG GGCCGCCUCCGGUGCAAGGUCCUGCAGCAC CGGGGCGUUUCCACUGCCACUAAACUGAAG GUGUACAACGCUGUCGUGCUCUCUUCCCUG CUUUAUGGCUGCGAAACAUGGACUCUGUAC CGGAAGCAUAUGAAACAGCUUGAGCAGUUC CACCAGAGGAGCUUGCGUAGCAUCAUGAGG AUCCGCUGGCAGGACCGCAUCACCAACCAG GAGGUGCUGGACAGGGCUAAUUCCACCAGC AUCGAAGUUAUGGUGCUGAAGACCCAGCUC CGUUGGUCUGGCCAUGUCAUUCGGAUGGAC CCUCAGCGUAUCCCCCGCCAGGUAUUCUAC GGGGAACUGUCUGCGGGGCUCAGAAAGCAG GGCCGCCCAAAGAAACGCUUUAAGGAUCAG CUGAAAAGCAAUCUGAAGUGGGCCGGCAUC ACCCCCAAGCAGCUGGAGCUGGCGGCAUCU GACCGCUCCAGCUGGCGCACCCACAUUAAC CACGCAGCUACUACCUUUGAGGACGAACGC CGGCGCAGACUGGCCGCUGCACGCGAGAGG CGCCACCAAGCCACUACCGCCCCUCCCGUG ACCACUGGGGUCCCUUGCCCUAUGUGCCAC AAGCUGUGUGCGUCUGCCUUCGGUUUGCAG UCCCACAUGCGUGUGCAUCGCCGCAGCGGC AGCGAGACUCCCGGGACCUCAGAGUCCGCC ACACCCGAAAGUGUGAGCGGCUGGCGGCUG UUCAAGAAGAUUAGCUGAGCUGGAGCCUCG GUGGCCAUGCUUCUUGCCCCUUGGGCCUCC CCCCAGCCCCUCCUCCCCUUCCUGCACCCGU ACCCCCGUGGUCUUUGAAUAAAGUCUGAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAA (SEQ ID NO:370)	

[0629] The gene modifying system further comprised an exemplary template RNA comprising the following sequences from 5' to 3': 5'UTR_{retro}, GFP reporter, MND promoter, 3'UTR_{retro}.

[0630] The template RNA comprised a 5'UTR having the sequence of:

GGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGUCG
UGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUCAC
UUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACGGC
UUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUGGU
GAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACGGA
AGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUGUG
GAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAGCU
GAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAACG
CCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUCAC
GCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCAAG
ACCUGCGGCGAUGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCAGU
UGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCCUG
UGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAG
(SEQ ID NO:372)

[0631] The template RNA comprised a 3'UTR having the sequence of:

UGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCGAGAGACUACCACU (SEQ ID NO:373)

[0632] The exemplary template RNA comprised a nucleic acid sequence encoding a GFP protein having a protein sequence of:

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWP
TLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEG
DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGS
VQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGM
DELYK (SEQ ID NO:374)

LNP Transfection and Flow Cytometry

[0633] 50uL of diluted LNPs were added to plated activated T cells at 0.25, 0.5, 1, 2.5, 5, or 10 µg of LNP per 1 ^{x106} cells, and mixed gently by pipetting. After addition of the LNPs, all plated cells were at a final density of $1x10^6$ cells/mL in 200uL, with a final concentration of 5%

FBS (for serum-containing conditions) and cytokines at the following concentrations: human IL-2 at 20ng/mL, human IL-7 at 10ng/mL, and human IL-15 at 5ng/mL. Transfected cells were returned to incubation at 37°C, 5% CO_2 for 4 hours. After the 4 hour tLNP incubation, cells were centrifuged at 350xg for 7min, supernatant aspirated, and cells resuspended in complete T cell media as described above at 1e6 cells/mL in flat bottom plates. Flow cytometry was conducted on the cells, as described in Example 1, at 4 days, 7 days, and 10 days following tLNP transfection.

Results

[0634] Base LNPs formulated with Lipid1092 and Lipid154 both delivered the exemplary gene modifying system more effectively to activated T cells than the baseline LNP formulated with the V003 lipid. **FIG. 9A** shows that at 4 days following transfection, substantially more activated T cells expressed GFP at all doses when Lipid092 LNPs or Lipid154 LNPs delivered the gene modifying system compared to activated T cells that were contacted with the V003 LNPs, with the Lipid154 LNPs showing the highest levels of delivery. The peak dose for the LNPs was at 2.5 μg per 1 x10^6 cells. The GFP expression was reliant on the presence of the gene modifying system since base LNPs carrying the template RNA only (without the gene modifying polypeptide mRNA) did not create GFP positive cells when administered to the activated T cells. **FIG. 9B** shows that activated T cells transduced with the Lipid092 or Lipid154 expressed GFP at higher levels (higher MFI) relative to activated T cells transduced with LNPs comprising the V003 ionizable lipid.

[0635] The results show that LNPs formulated with the Lipid092, Lipid154 or V003 ionizable lipids were all capable of delivering an exemplary all-RNA gene modifying system to activated T cells to insert a heterologous gene encoded by template RNA. However, LNPs formulated with Lipid092 or Lipid154 can deliver a gene modifying system more effectively to activated T cells to enable higher insertion rates of a heterologous gene into genomic DNA.

Example 5: Testing of ionizable lipids for targeted LNP delivery of an exemplary gene modifying system to activated T cells *in vitro*

[0636] Base LNPs formulated with the Lipid092 and Lipid154 ionizable lipids were conjugated to the anti-CD3-8 targeting moiety, as described in Example 1, to generate targeted LNPs. The tLNPs were then tested to determine whether they could more effectively deliver a gene modifying system (mRNA encoding an exemplary gene modifying polypeptide and a template RNA encoding GFP) to activated T cells relative to the base LNPs. The efficiency of LNP delivery and insertion of the heterologous GFP sequence encoded by the template RNA

using these tLNPs was compared to the efficiency of delivery and GFP insertion using a tLNP formulated with the V003 ionizable lipid. Dual targeted LNPs were also generated that comprised the Lipid092 ionizable lipid conjugated to both the anti-CD3-8 targeting moiety and a CD80 ECD and compared to the tLNPs conjugated to a single targeting moiety.

T Cell Activation and Culture

[0637] Cryopreserved primary T cells from a single human donor (HemaCare, donor D328798) were thawed and transferred into PBS with 2% BSA by volume. Cells were counted on a Cellometer K2 Cell Counter (Nexcelom), then pelleted by centrifugation at 400xg for 5 minutes and resuspended in complete T cell media at a density of $1x10^6$ cells/mL. The complete T cell media was comprised of CTS AIM V SFM basal media (Gibco #0870112DK) with 5% human serum AB (Gemini Bio #100-512) and the following cytokines: human IL-2 at 20ng/mL (Miltenyi Biotec #130-097-746), human IL-7 at 10ng/mL (Miltenyi Biotec #130-095-362), and human IL-15 at 5ng/mL (Miltenyi Biotec #130-095-764). TransAct Research Grade T cell activation reagent (Miltenyi Biotec #130-111-160) was added to cell suspension at a volume of 10uL per $1x10^6$ cells. Activated cells were transferred to flasks and incubated at 37°C, 5% CO_2 for 72 hours ("Activated" condition).

Preparation of T Cells for Transfection

[0638] Following the incubation periods above, the activated T cells were collected separately and counted. $2.0x10^7$ cells were taken from each activation condition, then pelleted by centrifugation, and the supernatant aspirated. The cell pellets were resuspended in CTS AIM V SFM at a concentration of $1.33x10^6$ cells/mL, supplemented with 6.67% fetal bovine serum (FBS, Gibco #A31604-01), and human IL-2, human IL-7, and human IL-15, at concentrations of 26.6ng/mL, 13.3ng/mL and 6.6ng/mL, respectively.

[0639] The resuspended T cells were plated in non-treated 96-well U-bottom polystyrene plates (Falcon # 08-772-54) at a density of $2x10^5$ cells (150uL) per well, and returned to incubation at 37°C, 5% CO_2 while the LNPs were prepared.

Preparation of Targeted LNPs for Transfection

[0640] LNPs were formulated as follows. An ethanol phase was prepared with five lipids, containing 47% ionizable lipid (Lipid092, Lipid154, or V003), 8% DSPC, 42.5% cholesterol, 2% PEG-DMG and 0.5% DSPE-PEG2K-TCO. An aqueous phase was composed of mRNA encoding the exemplary gene modifying polypeptide and a template RNA encoding GFP dissolved in 25 mM acetate buffer. The two phases were mixed using a microfluidic device to

formulate LNP-TCO at a total flow rate of 18 ml/min (aqueous phase: ethanol phase=3:1). 3x volume of CBS was added to LNP solution and the resulting solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes. The LNPs were then buffer exchanged to the desired concentration either via Tangential Flow Filtration (TFF) and/or centrifugation (100k MWCO).

[0641] The anti-CD3-8 targeting moiety was produced with an additional sortase tag (LPETG, SEQ ID NO:11) on the C terminus of the heavy chain. 10uM anti-CD3 Fab-LPETG and 1mM Triglycine methyltetrazine (GGG-meTz) were prepared in sortase buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl2, pH 7.4). 2uM Sortase A5 was then added to initiate the reaction. The above solution was incubated at 30°C for 3 hours with shaking at 800 rpm. Excess reagents were removed, and buffer was exchanged to PBS containing 10mM EDTA by a 10K Amicon centrifugal filter. To confirm a DOL (degree of labeling) of close to 1meTz per antibody, sortase modified anti-CD3 targeting moiety was allowed to react with 5eq AF594 containing TCO group in PBS for 2 hours at room temperature. After the reaction, excess dye was removed by two runs with Zeba desalting column. DOL was measured by absorbances at A280 and A590 using DOL calculator. The resulting meTz-modified anti-CD3 targeting moiety was added to the TCO modified-LNP solution (as prepared above) for antibody-LNP conjugation, with a mass ratio between antibody and mRNA (encoding eGFP) of 1:1, except where otherwise noted (**Table 19**). The solution was incubated at room temperature for 2 hours and then at 4°C overnight. Any unreacted anti-CD3 targeting moiety was removed by a 300K MPES TFF membrane. The resulting antibody-LNP product was further concentrated using an Amicon column.

[0642] For dual targeted LNPs, the anti-CD3-8 Fab and the CD80 ECD were produced with sortase tags and conjugated to the surface of the base LNP as described above, but at a 1:1:1 molar ratio (anti-CD3:CD80 ECD:mRNA) to create the dual targeted LNPs.

[0643] The gene modifying system formulated in the LNPs comprised an mRNA encoding an exemplary gene modifying polypeptide comprised the following components.

[0644] The gene modifying polypeptide comprised the amino acid sequence of:

MDSTAHPNQGRGLEKVSQTLPALQTPGQHTAAGGSSPLSGRNQRKNTKKLLLGAWNIR
TLLDRENTPRPERRTALIGKELARYNIDIAALSETRLPEEGSLSEPTTGYTFFWKGRASNE
DRIHGVGLAIKTSLLKQLPDLPVGISERLMKIRLPLSKDRYATIISAYAPTLTSTEETIEQFY
SDLSAVLHSVPTNDKLILLGDFNARVGQDHERWKGVLGKHGVGKMNNNGLLLLSKCS
EFELTITNTVFRMANKYKTTWMHPRSKQWHLIDYIIVRRRDIQDVKITRAMRGAECWT
DHRLVRATLQMRIAPRHPKRAQTVRAFYNVSRLRDPSYLQTFQSCLDDKLSAKGPLTGS
STEKWNQFRDAVKETSKAVLGPKQRNHQDWFDENNTAIEDLLSKKNKAFMEWQNNPN

SAPKKDRFKSLQATAQREIRKMQDRWWEKKAEEIQRFADMKNYKQFFSALKTVYGPL
KPTTTPLLSSDGDTLIKDKKGISNRWKEHFSQLLNRPSSVDQSALDQIPQNRTIEQLDVPP
SIEEVQKAIKQMSAGKAPGKDGIPTEVYKALNGKALQAFHIVLTSIWEEEDMPPELRDAS
IVALYKNKGSRAACDNYRGISLLSTAGKILARVILNRLLSSVSEQNLPESQCGFRPDRSTI
DMVFTVRQMQEKCLEQNLSLYIVFIDLTKAFDTVNRDALWVILSKLGCPAKFVKLIQLF
HVDMTGEVLSGGETSDRFNISNGVKQGCVLAPVLFNLFFTQVLRHAVMDLDLGVYIKY
RLDGSLFDLRRLTAKTKTTERLILEALFADDCALMAHQENHLQTIVDRFSTATKLFGLTI
SLSKTEVLFQPAPGRPTNQPCITIDGTQLSNVNTFKYLGSTIANDGSLDHEINARIQKASQ
ALGRLRCKVLQHRGVSTATKLKVYNAVVLSSLLYGCETWTLYRKHMKQLEQFHQRSL
RSIMRIRWQDRITNQEVLDRANSTSIEVMVLKTQLRWSGHVIRMDPQRIPRQVFYGELS
AGLRKQGRPKKRFKDQLKSNLKWAGITPKQLELAASDRSSWRTHINHAATTFEDERRR
RLAAARERRHQATTAPPVTTGVPCPMCHKLCASAFGLQSHMRVHRR (SEQ ID NO:32)

[0645] The gene modifying polypeptide comprised a GGS Linker having an amino acid sequence of:

GGS

[0646] The gene modifying polypeptide comprised an SV40 NLS having an amino acid sequence of:

PKKKRKV (SEQ ID NO:367)

[0647] The gene modifying polypeptide comprised an XTEN linker having an amino acid sequence of:

SGSETPGTSESATPES (SEQ ID NO:368)

[0648] The gene modifying polypeptide comprised an HiBit tag having an amino acid sequence of:

VSGWRLFKKIS (SEQ ID NO:369)

[0649] The full-length gene modifying polypeptide with all components was structured as follows: NLS - GGS linker – gene modifying polypeptide - XTEN linker - HiBit tag. The full-length sequences are provided in **Table 21** (Example 4).

[0650] The gene modifying system further comprised a template RNA comprising the following sequences from 5’ to 3’: 5’UTR$_{retro}$, GFP reporter, MND promoter, 3’UTR$_{retro}$.

[0651] The template RNA comprised a 5’UTR having the sequence of:

GGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGUCG
UGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUCAC
UUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACGGC
UUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUGGU
GAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACGGA
AGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUGUG
GAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAGCU
GAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAACG
CCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUCAC
GCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCAAG
ACCUGCGGCGAUGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCAGU
UGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCCUG
UGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAG
(SEQ ID NO:372)

[0652] The template RNA comprised a 3'UTR having the sequence of:

UGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCGAGAGACUACCACU (SEQ ID NO373)

[0653] The template RNA comprised a nucleic acid sequence encoding a GFP protein having a protein sequence of:

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWP
TLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEG
DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGS
VQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGM
DELYK (SEQ ID NO:374)

LNP Transfection and Flow Cytometry

[0654] 50uL of diluted tLNPs were added to plated activated T cells at 0.25, 0.5, 1, 2.5, 5, or 10 μg of LNP per 1 $x10^6$ cells, and mixed gently by pipetting. After addition of the tLNPs, all plated cells were at a final density of $1x10^6$ cells/mL in 200uL, with a final concentration of 5% FBS (for serum-containing conditions) and cytokines at the following concentrations: human IL-2 at 20ng/mL, human IL-7 at 10ng/mL, and human IL-15 at 5ng/mL. Transfected cells were returned to incubation at 37°C, 5% CO_2 for 4 hours. After the 4 hour tLNP incubation, cells were centrifuged at 350xg for 7min, supernatant aspirated, and cells resuspended in complete T cell media as described above at 1e6 cells/mL in flat bottom plates. Flow cytometry was

conducted on the cells, as described in Example 1, at 4 days, 7 days, and 10 days following tLNP transfection.

Results

[0655] **FIG. 10A** shows that targeted LNPs formulated with Lipid092 and Lipid154 both delivered the exemplary gene modifying system more effectively to activated T cells than the baseline tLNP formulated with the V003 lipid. **FIG. 10A** shows that at 4 days following transfection, substantially more activated T cells expressed GFP at all doses when Lipid092 tLNPs or Lipid154 tLNPs delivered the gene modifying system relative to activated T cells that were contacted with the V003 tLNPs. More activated T cells expressed GFP at the lower doses (0.25, 0.5, or 1.0 µg of LNP) of Lipid154 tLNPs compared to the Lipid092 tLNPs. Similarly, Lipid092 dual tLNPs that had an anti-CD3 targeting moiety in combination with the CD80 ECD exhibited higher delivery levels. **FIG. 10B** shows that activated T cells transduced with the Lipid092 or Lipid154 tLNPs expressed GFP at higher levels (higher MFI) relative to activated T cells transduced with tLNPs comprising the V003 ionizable lipid.

[0656] All tLNPs tested (comprising the Lipid092, Lipid154, or V003 ionizable lipids) delivered the gene modifying system to substantially more activated T cells, resulting in increases in GFP insertion into the genomic DNA of T cells and GFP expression, relative to the base LNPs formulated without the CD3 targeting moiety (compare **FIGs. 9A-9B** to **FIGs. 10A-10B**). Thus, the addition of the anti-CD3 targeting moiety to the surface of the LNPs increases delivery of a gene modifying system payload to activated T cells.

Example 6: Targeted LNP delivery of an exemplary gene modifying system to human T cells in a humanized mouse model

[0657] Targeted LNPs formulated with the Lipid092 ionizable lipid were tested to determine whether they could deliver a gene modifying system comprising mRNA encoding an exemplary gene modifying polypeptide and a template RNA encoding GFP to human T cells in a humanized mouse model (naïve human T cells engrafted 2-3 weeks prior to study) effectively enough to insert the exogenous GFP gene into the genomic DNA of the T cells. The tLNPs were conjugated to the anti-CD3-8 targeting moiety, as described in Example 1. A negative control tLNP was also formulated to carry the GFP template RNA only but was otherwise identical.

Preparation of Targeted LNPs for In Vivo Studies

[0658] LNPs were formulated as follows. An ethanol phase was prepared with five lipids, containing 47% ionizable lipid (Lipid092), 8% DSPC, 42.5% cholesterol, 2% PEG-DMG and

0.5% DSPE-PEG2K-TCO. An aqueous phase was composed of mRNA encoding the gene modifying polypeptide and a template RNA encoding GFP dissolved in 25 mM acetate buffer. The two phases were mixed using a microfluidic device to formulate LNP-TCO at a total flow rate of 18 ml/min (aqueous phase: ethanol phase=3:1). 3x volume of CBS was added to LNP solution and the resulting solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes. The LNPs were then buffer exchanged to the desired concentration either via Tangential Flow Filtration (TFF) and/or centrifugation (100k MWCO).

[0659] The anti-CD3-8 targeting moiety was produced with an additional sortase tag (LPETG, SEQ ID NO:11) on the C terminus of the heavy chain. 10uM anti-CD3 Fab-LPETG and 1mM Triglycine methyltetrazine (GGG-meTz) were prepared in sortase buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl2, pH 7.4). 2uM Sortase A5 was then added to initiate the reaction. The above solution was incubated at 30°C for 3 hours with shaking at 800 rpm. Excess reagents were removed, and buffer was exchanged to PBS containing 10mM EDTA by a 10K Amicon centrifugal filter. To confirm a DOL (degree of labeling) of close to 1meTz per antibody, sortase modified anti-CD3 targeting moiety was allowed to react with 5eq AF594 containing TCO group in PBS for 2 hours at room temperature. After the reaction, excess dye was removed by two runs with Zeba desalting column. DOL was measured by absorbances at A280 and A590 using DOL calculator. The resulting meTz-modified anti-CD3 targeting moiety was added to the TCO modified-LNP solution (as prepared above) for targeting moiety-LNP conjugation, with a mass ratio between targeting moiety and mRNA (encoding eGFP) of 1:1, except where otherwise noted (**Table 1**). The solution was incubated at room temperature for 2 hours and then at 4°C overnight. Any unreacted anti-CD3 targeting moiety was removed by a 300K MPES TFF membrane. The resulting targeting moiety-LNP product was further concentrated using an Amicon column.

[0660] The gene modifying system formulated in the LNPs comprised an mRNA encoding an exemplary gene modifying polypeptide comprised the following components.

[0661] The gene modifying polypeptide comprised the amino acid sequence of:

MDSTAHPNQGRGLEKVSQTLPALQTPGQHTAAGGSSPLSGRNQRKNTKKLLLGAWNIR
TLLDRENTPRPERRTALIGKELARYNIDIAALSETRLPEEGSLSEPTTGYTFFWKGRASNE
DRIHGVGLAIKTSLLKQLPDLPVGISERLMKIRLPLSKDRYATIISAYAPTLTSTEETIEQFY
SDLSAVLHSVPTNDKLILLGDFNARVGQDHERWKGVLGKHGVGKMNNNGLLLLSKCS
EFELTITNTVFRMANKYKTTWMHPRSKQWHLIDYIIVRRRDIQDVKITRAMRGAECWT
DHRLVRATLQMRIAPRHPKRAQTVRAFYNVSRLRDPSYLQTFQSCLDDKLSAKGPLTGS

STEKWNQFRDAVKETSKAVLGPKQRNHQDWFDENNTAIEDLLSKKNKAFMEWQNNPN
SAPKKDRFKSLQATAQREIRKMQDRWWEKKAEEIQRFADMKNYKQFFSALKTVYGPL
KPTTTPLLSSDGDTLIKDKKGISNRWKEHFSQLLNRPSSVDQSALDQIPQNRTIEQLDVPP
SIEEVQKAIKQMSAGKAPGKDGIPTEVYKALNGKALQAFHIVLTSIWEEEDMPPELRDAS
IVALYKNKGSRAACDNYRGISLLSTAGKILARVILNRLLSSVSEQNLPESQCGFRPDRSTI
DMVFTVRQMQEKCLEQNLSLYIVFIDLTKAFDTVNRDALWVILSKLGCPAKFVKLIQLF
HVDMTGEVLSGGETSDRFNISNGVKQGCVLAPVLFNLFFTQVLRHAVMDLDLGVYIKY
RLDGSLFDLRRLTAKTKTTERLILEALFADDCALMAHQENHLQTIVDRFSTATKLFGLTI
SLSKTEVLFQPAPGRPTNQPCITIDGTQLSNVNTFKYLGSTIANDGSLDHEINARIQKASQ
ALGRLRCKVLQHRGVSTATKLKVYNAVVLSSLLYGCETWTLYRKHMKQLEQFHQRSL
RSIMRIRWQDRITNQEVLDRANSTSIEVMVLKTQLRWSGHVIRMDPQRIPRQVFYGELS
AGLRKQGRPKKRFKDQLKSNLKWAGITPKQLELAASDRSSWRTHINHAATTFEDERRR
RLAAARERRHQATTAPPVTTGVPCPMCHKLCASAFGLQSHMRVHRR (SEQ ID NO:32)

[0662] The gene modifying polypeptide comprised a GGS Linker having an amino acid sequence of:

GGS

[0663] The gene modifying polypeptide comprised an SV40 NLS having an amino acid sequence of:

PKKKRKV (SEQ ID NO:367)

[0664] The gene modifying polypeptide comprised an XTEN linker having an amino acid sequence of:

SGSETPGTSESATPES (SEQ ID NO:368)

[0665] The gene modifying polypeptide comprised an HiBit tag having an amino acid sequence of:

VSGWRLFKKIS (SEQ ID NO:369)

[0666] The full-length gene modifying polypeptide with all components was structured as follows: NLS - 3GS linker – gene modifying polypeptide - XTEN linker - HiBit tag. The full-length sequences are provided in Table 21 (Example 4).

[0667] The gene modifying system further comprised a template RNA comprising the following sequences from 5’ to 3’: 5’UTR_{retro}, GFP reporter, MND promoter, 3’UTR_{retro}.

[0668] The template RNA comprised a 5’UTR having the sequence of:

GGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGUCG
UGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUCAC
UUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACGGC
UUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUGGU
GAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACGGA
AGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUGUG
GAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAGCU
GAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAACG
CCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUCAC
GCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCAAG
ACCUGCGGCGAUGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCAGU
UGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCCUG
UGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAG
(SEQ ID NO:372)

[0669] The template RNA comprised a 3'UTR having the sequence of:

UGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCGAGAGACUACCACU (SEQ ID NO:373)

[0670] The template RNA comprised a nucleic acid sequence encoding a GFP protein having a protein sequence of:

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWP
TLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEG
DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGS
VQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGM
DELYK (SEQ ID NO:374)

Preparation and Dosing of a Humanized Mouse Model

[0671] Briefly, female NSG mice 6-8 weeks of age (Catalog #005557) were purchased from Jackson Labs (Bar Harbor, ME). Following acclimation to the vivarium for 3-5 days, animals received a tail vein injection of 5 X 10^6 human CD3+ T cells (Source) and waited for 2-3 weeks for T cell engraftment. For each *in vivo* study, "humanized" NSG mice were randomized into groups of 3-4 animals and baseline weights were obtained. Animals were then treated via intravenous tail vein injection with 100 ul of candidate tLNPs formulated with gene modifying system at a dose of 4 mg/kg. Following a 16-hour incubation, animals were euthanized via CO2 asphyxiation, weighed and their spleens were collected and the splenic cells were isolated.

Flow Cytometry

[0672] Splenic cells (1 X 10^6 in 0.1 ml PBS) from each test mouse were subsequently aliquoted to individual wells in 96 well plates and antibody fluorochrome conjugate mixtures (**Table 22**) were added to the cells in a total of 0.1 ml. Following a 30-minute incubation at room temperature, the plates were centrifuged for 5 minutes at 500G and supernatants were removed. The cells were then resuspended in 0.2 ml PBS and evaluated on an Aligent NovoCyte Quanteon (2010098) or Cytek Northern Lights (N7-00008) flow cytometer using FlowJo software (Ashland OR). Cells stained with relevant isotype control fluorochrome conjugated antibodies were used to set the negative control gates. Total T cells were defined as CD45+CD3+CD5+. CD4+ and CD8+ T cells were defined as CD45+CD3+CD5+CD4+ and CD45+CD3+CD5+CD8+, respectively.

Table 22. Flow cytometry antibodies

Marker	Fluorophore	Clone	Vendor	Catalog
Live/Dead	Fixable Yellow		ThermoFisher	L34959
hCD45	PE-Cy7	HI30	Biolegend	304016
CD3	BV421	OKT3	Biolegend	317344
CD4	BV605	OKT4	Biolegend	317438
CD5	BV510	L17F12	Biolegend	364018
GFP				
CD8	PE	SK1	Biolegend	344706
CCR7	APC	G043H7	Biolegend	353214
mCD45	PE Cy5	30-F11	Biolegend	103110
CD45RA	BV785	HI100	Biolegend	304140

Results

[0673] **FIG. 11** shows that the tLNPs carrying an all-RNA gene modifying system generated human T cells expressing GFP in the engrafted mice, as determined by flow cytometry. **FIG. 11A** shows that an average of at least 5 percent of human cells expressed GFP, including about 5 percent of human CD4+ T cells and about 5 percent of CD8+ T cells, when the gene modifying polypeptide mRNA and the GFP template RNA were formulated within the same tLNP. The percentages of GFP positive cells were slightly lower, on average, when the gene modifying polypeptide mRNA and the GFP template RNA were formulated in separate tLNPs. By contrast, no engrafted human T cells expressed GFP in mice that were administered a negative control tLNP comprising a GFP template but lacking the gene modifying polypeptide mRNA. **FIG. 11B** shows that engrafted human cells, including CD4+ and CD8+ T cells, expressed GFP at higher levels when tLNPs comprising the gene modifying system were administered to the humanized

mice compared to engrafted cells in mice that were administered the negative control tLNP. Together, the results show that the tLNPs carrying a gene modifying system can insert an exogenous nucleic acid sequence into the genomic DNA of human T cells *in vivo*, leading to the expression of that exogenous nucleic acid sequence.

Example 7: Improved LNP formulation for delivery of an exemplary gene modifying system to activated T cells

[0674] Alternative formulations of targeted LNPs were tested to determine whether they could improve delivery of a gene modifying system payload to activated T cells, leading to increased insertion of an exogenous gene in the cells. LNPs were formulated with the Lipid154 ionizable lipid and a higher amount of helper lipid (22% DSCP or sphingomyelin) relative to the LNPs in prior examples (formulated with 8% helper lipid) and then conjugated to the anti-CD3-8 targeting moiety, as described in Example 1. The LNPs were formulated with an all-RNA gene modifying system composed of an mRNA encoding an exemplary gene modifying polypeptide and a template RNA encoding GFP. A baseline control anti-CD3 tLNP comprising Lipid154 and 8% helper lipid (8% DSPC) was also prepared as described in prior examples. Negative control tLNPs were formulated to carry either the gene modifying polypeptide mRNA payload only or a GFP template RNA payload only.

T Cell Activation and Culture

[0675] Cryopreserved primary T cells from two human donors (HemaCare, donor D328798 and donor DXXX405) were thawed and transferred into PBS with 2% BSA by volume. Cells were counted on a Cellometer K2 Cell Counter (Nexcelom), then pelleted by centrifugation at 400xg for 5 minutes and resuspended in complete T cell media at a density of $1x10^6$ cells/mL. The complete T cell media was comprised of CTS AIM V SFM basal media (Gibco #0870112DK) with 5% human serum AB (Gemini Bio #100-512) and the following cytokines: human IL-2 at 20ng/mL (Miltenyi Biotec #130-097-746), human IL-7 at 10ng/mL (Miltenyi Biotec #130-095-362), and human IL-15 at 5ng/mL (Miltenyi Biotec #130-095-764). TransAct Research Grade T cell activation reagent (Miltenyi Biotec #130-111-160) was added to cell suspension at a volume of 10uL per $1x10^6$ cells. Activated cells were transferred to flasks and incubated at 37°C, 5% CO_2 for 72 hours.

Preparation of T Cells for Transfection

[0676] Following the incubation periods above, the activated T cells were collected separately and counted. $2.0x10^7$ cells were taken from each activation condition, then pelleted by centrifugation, and the supernatant aspirated. The cell pellets were resuspended in CTS AIM V

SFM at a concentration of $2x10^6$ cells/mL, supplemented with 10% fetal bovine serum (FBS, Gibco #A31604-01), and human IL-2, human IL-7, and human IL-15, at concentrations of 40ng/mL, 20ng/mL and 10ng/mL, respectively.

[0677] The resuspended T cells were plated in non-treated 96-well U-bottom polystyrene plates (Falcon # 08-772-54) at a density of $2x10^5$ cells (100uL) per well, and returned to incubation at 37°C, 5% CO_2 while the LNPs were prepared.

Preparation of Targeted LNPs for Transfection

[0678] LNPs were formulated as follows. An ethanol phase was prepared with five lipids, containing 47% ionizable lipid (Lipid154), 22% helper lipid (DSPC or egg sphingomyelin), 28.5% cholesterol, 2% DMG-PEG-2K and 0.5% DSPE-PEG2K-TCO. An aqueous phase was composed of mRNA encoding the gene modifying polypeptide and a template RNA encoding GFP dissolved in 25 mM acetate buffer. The two phases were mixed using a microfluidic device to formulate LNP-TCO at a total flow rate of 18 ml/min (aqueous phase: ethanol phase=3:1). 3x volume of CBS was added to LNP solution and the resulting solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes. The LNPs were then buffer exchanged to the desired concentration either via Tangential Flow Filtration (TFF) and/or centrifugation (100k MWCO). The control tLNP was prepared using the same approach, except the ethanol phase was prepared with five lipids, containing 47% ionizable lipid (Lipid154), 8% DSPC, 42.5% cholesterol, 2% DMG-PEG2K and 0.5% DSPE-PEG2K-TCO. For the negative control tLNPs, an aqueous phase containing either the gene modifying polypeptide mRNA only or the GFP template RNA only was mixed with the ethanol phase followed by the process described earlier.

[0679] The anti-CD3-8 targeting moiety was produced with an additional sortase tag (LPETG, SEQ ID NO:11) on the C terminus of the heavy chain. 10uM anti-CD3 Fab-LPETG and 1mM Triglycine methyltetrazine (GGG-meTz) were prepared in sortase buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl2, pH 7.4). 2uM Sortase A5 was then added to initiate the reaction. The above solution was incubated at 30°C for 3 hours with shaking at 800 rpm. Excess reagents were removed, and buffer was exchanged to PBS containing 10mM EDTA by a 10K Amicon centrifugal filter. To confirm a DOL (degree of labeling) of close to 1meTz per antibody, sortase modified anti-CD3 targeting moiety was allowed to react with 5eq AF594 containing TCO group in PBS for 2 hours at room temperature. After the reaction, excess dye was removed by two runs with Zeba desalting column. DOL was measured by absorbances at A280 and A590 using DOL calculator. The resulting meTz-modified anti-CD3 targeting moiety was added to the TCO modified-LNP solution (as prepared above) for antibody-LNP conjugation, with a mass ratio

between antibody and mRNA (encoding eGFP) of 1:1, except where otherwise noted (**Table 19**). The solution was incubated at room temperature for 2 hours and then at 4°C overnight. Any unreacted anti-CD3 targeting moiety was removed by a 300K MPES TFF membrane. The resulting antibody-LNP product was further concentrated using an Amicon column.

[0680] The gene modifying system formulated in the LNPs comprised an mRNA encoding an exemplary gene modifying polypeptide comprised the following components.

[0681] The gene modifying polypeptide comprised the amino acid sequence of:

MDSTAHPNQGRGLEKVSQTLPALQTPGQHTAAGGSSPLSGRNQRKNTKKLLLGAWNIR
TLLDRENTPRPERRTALIGKELARYNIDIAALSETRLPEEGSLSEPTTGYTFFWKGRASNE
DRIHGVGLAIKTSLLKQLPDLPVGISERLMKIRLPLSKDRYATIISAYAPTLTSTEETIEQFY
SDLSAVLHSVPTNDKLILLGDFNARVGQDHERWKGVLGKHGVGKMNNNGLLLLSKCS
EFELTITNTVFRMANKYKTTWMHPRSKQWHLIDYIIVRRRDIQDVKITRAMRGAECWT
DHRLVRATLQMRIAPRHPKRAQTVRAFYNVSRLRDPSYLQTFQSCLDDKLSAKGPLTGS
STEKWNQFRDAVKETSKAVLGPKQRNHQDWFDENNTAIEDLLSKKNKAFMEWQNNPN
SAPKKDRFKSLQATAQREIRKMQDRWWEKKAEEIQRFADMKNYKQFFSALKTVYGPL
KPTTTPLLSSDGDTLIKDKKGISNRWKEHFSQLLNRPSSVDQSALDQIPQNRTIEQLDVPP
SIEEVQKAIKQMSAGKAPGKDGIPTEVYKALNGKALQAFHIVLTSIWEEEDMPPELRDAS
IVALYKNKGSRAACDNYRGISLLSTAGKILARVILNRLLSSVSEQNLPESQCGFRPDRSTI
DMVFTVRQMQEKCLEQNLSLYIVFIDLTKAFDTVNRDALWVILSKLGCPAKFVKLIQLF
HVDMTGEVLSGGETSDRFNISNGVKQGCVLAPVLFNLFFTQVLRHAVMDLDLGVYIKY
RLDGSLFDLRRLTAKTKTTERLILEALFADDCALMAHQENHLQTIVDRFSTATKLFGLTI
SLSKTEVLFQPAPGRPTNQPCITIDGTQLSNVNTFKYLGSTIANDGSLDHEINARIQKASQ
ALGRLRCKVLQHRGVSTATKLKVYNAVVLSSLLYGCETWTLYRKHMKQLEQFHQRSL
RSIMRIRWQDRITNQEVLDRANSTSIEVMVLKTQLRWSGHVIRMDPQRIPRQVFYGELS
AGLRKQGRPKKRFKDQLKSNLKWAGITPKQLELAASDRSSWRTHINHAATTFEDERRR
RLAAARERRHQATTAPPVTTGVPCPMCHKLCASAFGLQSHMRVHRR (SEQ ID NO:32)

[0682] The gene modifying polypeptide comprised a GGS Linker having an amino acid sequence of:

GGS

[0683] The gene modifying polypeptide comprised an SV40 NLS having an amino acid sequence of:

PKKKRKV (SEQ ID NO:367)

[0684] The gene modifying polypeptide comprised an XTEN linker having an amino acid sequence of:

SGSETPGTSESATPES (SEQ ID NO:368)

[0685] The gene modifying polypeptide comprised an HiBit tag having an amino acid sequence of:

VSGWRLFKKIS (SEQ ID NO:369)

[0686] The full-length gene modifying polypeptide with all components was structured as follows: NLS - 3GS linker – gene modifying polypeptide - XTEN linker - HiBit tag. The full-length sequences are provided in **Table 21** (Example 4).

[0687] The gene modifying system further comprised a template RNA comprising the following sequences from 5' to 3': 5'UTR_{retro}, GFP reporter, MND promoter, 3'UTR_{retro}.

[0688] The template RNA comprised a 5'UTR having the sequence of:

GGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGUCG
UGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUCAC
UUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACGGC
UUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUGGU
GAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACGGA
AGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUGUG
GAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAGCU
GAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAACG
CCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUCAC
GCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCAAG
ACCUGCGGCGAUGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCAGU
UGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCCUG
UGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAG
(SEQ ID NO:372)

[0689] The template RNA comprised a 3'UTR having the sequence of:

UGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCGAGAGACUACCACU (SEQ ID NO:373)

[0690] The template RNA comprised a nucleic acid sequence encoding a GFP protein having a protein sequence of:

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWP TLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEG DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGS VQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGM DELYK (SEQ ID NO:374)

LNP Transfection and Flow Cytometry

[0691] 50uL of diluted tLNPs were added to plated activated T cells at 0.25, 0.5, 1, 2.5, 5, or 10 µg of LNP per 1 x10^6 cells, and mixed gently by pipetting. Negative control tLNPs were dosed at 2.5 µg of LNP per 1 x10^6 cells. After addition of the tLNPs, all plated cells were at a final density of 1x10^6 cells/mL in 200uL, with a final concentration of 5% FBS (for serum-containing conditions) and cytokines at the following concentrations: human IL-2 at 20ng/mL, human IL-7 at 10ng/mL, and human IL-15 at 5ng/mL. Transfected cells were returned to incubation at 37°C, 5% CO_2 for 4 hours. After the 4 hour tLNP incubation, cells were centrifuged at 350xg for 7min, supernatant aspirated, and cells resuspended in complete T cell media as described above at 1e6 cells/mL in flat bottom plates. Flow cytometry was conducted on the cells, as described in Example 1, at 4 days, 7 days, and 10 days following tLNP transfection.

Results

[0692] **FIG. 12A-D** show that delivery of a gene modifying system payload to activated cells using targeted LNPs formulated with Lipid154 and 22% DSPC generated more cells that expressed GFP (%GFP+) and at higher levels (MFI) relative to the baseline control tLNP that was the identical except that it was formulated with 8% DSPC. At four days (**FIGs. 12A** and **B**) and at 7 days (**FIGs. 12C** and **D**) following transfection of tLNPs comprising 22% DSPC at all doses tested, more activated T cells expressed GFP at higher levels relative to the cells transfected with tLNPs comprising 8% DSPC. The differences in percent GFP+ cells and MFI were especially large at lower doses. The tLNPs formulated with 22% egg sphingomyelin also generated more cells expressing GFP relative to the tLNPs formulated with 8% DSPC at 4 days and 7 days post-transfection, but the expression differences were not as large when cells were transfected with tLNPs comprising 22% DSPC. The negative control tLNPs comprising the gene modifying polypeptide mRNA alone or the GFP template RNA alone did not produce any GFP-expressing cells. Thus, delivery of a gene modifying system to activated T cells using tLNPs comprising higher helper lipid levels can increase the number of cells that are engineered

by the gene modifying system to comprise genomic integration of an exogenous nucleic acid sequence.

Example 8: Improved LNP formulation for delivery of an exemplary gene writer system to resting T cells

[0693] The tLNP formulations comprising higher helper lipid of Example 7 were tested to determine whether they could improve delivery of a gene modifying system payload to rested T cells, leading to increased levels of exogenous gene insertion in the cells. LNPs were formulated with the Lipid154 ionizable lipid and a higher amount of helper lipid (22% DSPC or sphingomyelin) relative to the LNPs in prior examples (formulated with 8% helper lipid) and then conjugated to the anti-CD3-8 targeting moiety, as described in Example 1. The LNPs were formulated with an all-RNA gene modifying system composed of an mRNA encoding an exemplary gene modifying polypeptide and a template RNA encoding GFP. A baseline control tLNP comprising Lipid154 and 8% helper lipid (8% DSPC) and conjugated to the anti-CD3-8 targeting moiety, and a base LNP comprising Lipid 154 and 8% helper lipid (8% DSPC) but no targeting moieties, as described in Examples 3 and 4, was also generated. Negative control tLNPs were also formulated with either the gene modifying polypeptide mRNA payload alone or a GFP template RNA payload alone, as described in Example 7.

T Cell Culture

[0694] Cryopreserved primary T cells from a single human donor (HemaCare, donor D328798) were thawed and transferred into PBS with 2% BSA by volume. Cells were counted on a Cellometer K2 Cell Counter (Nexcelom), then pelleted by centrifugation at 400xg for 5 minutes and resuspended in complete T cell media at a density of $1x10^6$ cells/mL. The T cells were resuspended and cultured overnight in CTS AIM V SFM basal media (Gibco #0870112DK) with 5% human serum AB (Gemini Bio #100-512) and the following cytokines: human IL-7 at 10ng/mL (Miltenyi Biotec #130-095-362) and human IL-15 at 5ng/mL (Miltenyi Biotec #130-095-764).

Preparation of T Cells for Transfection

[0695] All cells were pelleted by centrifugation and the supernatant aspirated. The cell pellets were resuspended in CTS AIM V SFM at a concentration of $2x10^6$ cells/mL. The resuspended T cells were plated in non-treated 96-well U-bottom polystyrene plates (Falcon # 08-772-54) at a density of $2x10^5$ cells (100uL) per well, and returned to incubation at 37°C, 5% CO_2 while the LNPs were prepared.

Preparation of Targeted LNPs for Transfection

[0696] LNPs were formulated as follows. An ethanol phase was prepared with five lipids, containing 47% ionizable lipid (Lipid154), 22% helper lipid (DSPC or egg sphingomyelin), 28.5% cholesterol, 2% PEG-DMG and 0.5% DSPE-PEG2K-TCO. An aqueous phase was composed of mRNA encoding the gene modifying polypeptide and a template RNA encoding GFP dissolved in 25 mM acetate buffer. The two phases were mixed using a microfluidic device to formulate LNP-TCO at a total flow rate of 18 ml/min (aqueous phase: ethanol phase=3:1). 3x volume of CBS was added to LNP solution and the resulting solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes. The LNPs were then buffer exchanged to the desired concentration either via Tangential Flow Filtration (TFF) and/or centrifugation (100k MWCO). The base and baseline control tLNPs were prepared using the same approach, except the ethanol phase was prepared with five lipids, containing 47% ionizable lipid (Lipid154), 8% DSPC, 42.5% cholesterol, 2% PEG-DMG and 0.5% DSPE-PEG2K-TCO. For the negative control tLNPs, an aqueous phase containing either the gene modifying polypeptide mRNA only or the GFP template RNA only was mixed with the ethanol phase.

[0697] The anti-CD3-8 targeting moiety was produced with an additional sortase tag (LPETG, SEQ ID NO:11) on the C terminus of the heavy chain. 10uM anti-CD3 Fab-LPETG and 1mM Triglycine methyltetrazine (GGG-meTz) were prepared in sortase buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl2, pH 7.4). 2uM Sortase A5 was then added to initiate the reaction. The above solution was incubated at 30°C for 3 hours with shaking at 800 rpm. Excess reagents were removed, and buffer was exchanged to PBS containing 10mM EDTA by a 10K Amicon centrifugal filter. To confirm a DOL (degree of labeling) of close to 1meTz per antibody, sortase modified anti-CD3 targeting moiety was allowed to react with 5eq AF594 containing TCO group in PBS for 2 hours at room temperature. After the reaction, excess dye was removed by two runs with Zeba desalting column. DOL was measured by absorbances at A280 and A590 using DOL calculator. The resulting meTz-modified anti-CD3 targeting moiety was added to the TCO modified-LNP solution (as prepared above) for antibody-LNP conjugation, with a mass ratio between antibody and mRNA (encoding eGFP) of 1:1, except where otherwise noted (Table 1). The solution was incubated at room temperature for 2 hours and then at 4°C overnight. Any unreacted anti-CD3 targeting moiety was removed by a 300K MPES TFF membrane. The resulting antibody-LNP product was further concentrated using an Amicon column.

[0698] The gene modifying system formulated in the LNPs comprised an mRNA encoding an exemplary gene modifying polypeptide comprised the following components.

[0699] The gene modifying polypeptide comprised the amino acid sequence of:

MDSTAHPNQGRGLEKVSQTLPALQTPGQHTAAGGSSPLSGRNQRKNTKKLLLGAWNIR
TLLDRENTPRPERRTALIGKELARYNIDIAALSETRLPEEGSLSEPTTGYTFFWKGRASNE
DRIHGVGLAIKTSLLKQLPDLPVGISERLMKIRLPLSKDRYATIISAYAPTLTSTEETIEQFY
SDLSAVLHSVPTNDKLILLGDFNARVGQDHERWKGVLGKHGVGKMNNNGLLLLSKCS
EFELTITNTVFRMANKYKTTWMHPRSKQWHLIDYIIVRRRDIQDVKITRAMRGAECWT
DHRLVRATLQMRIAPRHPKRAQTVRAFYNVSRLRDPSYLQTFQSCLDDKLSAKGPLTGS
STEKWNQFRDAVKETSKAVLGPKQRNHQDWFDENNTAIEDLLSKKNKAFMEWQNNPN
SAPKKDRFKSLQATAQREIRKMQDRWWEKKAEEIQRFADMKNYKQFFSALKTVYGPL
KPTTTPLLSSDGDTLIKDKKGISNRWKEHFSQLLNRPSSVDQSALDQIPQNRTIEQLDVPP
SIEEVQKAIKQMSAGKAPGKDGIPTEVYKALNGKALQAFHIVLTSIWEEEDMPPELRDAS
IVALYKNKGSRAACDNYRGISLLSTAGKILARVILNRLLSSVSEQNLPESQCGFRPDRSTI
DMVFTVRQMQEKCLEQNLSLYIVFIDLTKAFDTVNRDALWVILSKLGCPAKFVKLIQLF
HVDMTGEVLSGGETSDRFNISNGVKQGCVLAPVLFNLFFTQVLRHAVMDLDLGVYIKY
RLDGSLFDLRRLTAKTKTTERLILEALFADDCALMAHQENHLQTIVDRFSTATKLFGLTI
SLSKTEVLFQPAPGRPTNQPCITIDGTQLSNVNTFKYLGSTIANDGSLDHEINARIQKASQ
ALGRLRCKVLQHRGVSTATKLKVYNAVVLSSLLYGCETWTLYRKHMKQLEQFHQRSL
RSIMRIRWQDRITNQEVLDRANSTSIEVMVLKTQLRWSGHVIRMDPQRIPRQVFYGELS
AGLRKQGRPKKRFKDQLKSNLKWAGITPKQLELAASDRSSWRTHINHAATTFEDERRR
RLAAARERRHQATTAPPVTTGVPCPMCHKLCASAFGLQSHMRVHRR (SEQ ID NO:32)

[0700] The gene modifying polypeptide comprised a GGS Linker having an amino acid sequence of:

GGS

[0701] The gene modifying polypeptide comprised an SV40 NLS having an amino acid sequence of:

PKKKRKV (SEQ ID NO:367)

[0702] The gene modifying polypeptide comprised an XTEN linker having an amino acid sequence of:

SGSETPGTSESATPES (SEQ ID NO:368)

[0703] The gene modifying polypeptide comprised an HiBit tag having an amino acid sequence of:

VSGWRLFKKIS (SEQ ID NO:369)

[0704] The full-length gene modifying polypeptide with all components was structured as follows: NLS - 3GS linker – gene modifying polypeptide - XTEN linker - HiBit tag. The full-length sequences are provided in **Table 21** (Example 4).

[0705] The gene modifying system further comprised a template RNA comprising the following sequences from 5' to 3': 5'UTR_{retro}, GFP reporter, MND promoter, 3'UTR_{retro}.

[0706] The template RNA comprised a 5'UTR having the sequence of:

GGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGUCG
UGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUCAC
UUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACGGC
UUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUGGU
GAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACGGA
AGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUGUG
GAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAGCU
GAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAACG
CCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUCAC
GCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCAAG
ACCUGCGGCGAUGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCAGU
UGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCCUG
UGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAG
(SEQ ID NO:372)

[0707] The template RNA comprised a 3'UTR having the sequence of:

UGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCGAGAGACUACCACU (SEQ ID NO:373)

[0708] The template RNA comprised a nucleic acid sequence encoding a GFP protein having a protein sequence of:

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWP
TLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEG
DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGS
VQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGM
DELYK (SEQ ID NO:374)

LNP Transfection and Flow Cytometry

[0709] 50uL of diluted tLNPs were added to plated activated T cells at 0.25, 0.5, 1, 2.5, 5, or 10 µg of LNP per 1 x10^6 cells, and mixed gently by pipetting. Negative control tLNPs were dosed at 2.5 µg of LNP per 1 x10^6 cells. In some conditions, TransAct Research Grade T cell activation reagent (Miltenyi Biotec #130-111-160) was also added to the rested T cell suspension with the tLNPs at a volume of 10uL per 1x10^6 cells. After addition of the tLNPs, all plated cells were at a final density of 1x10^6 cells/mL in 200uL, with a final concentration of 5% FBS (for serum-containing conditions) and cytokines at the following concentrations: human IL-2 at 20ng/mL, human IL-7 at 10ng/mL, and human IL-15 at 5ng/mL. Transfected cells were returned to incubation at 37°C, 5% CO_2 for four hours. Four hours post tLNP transfection, cells were centrifuged at 350xg for 7min and resuspended in CTS AIM V SFM basal media (Gibco #0870112DK) with 5% human serum AB (Gemini Bio #100-512) and the following cytokines: human IL-7 at 10ng/mL (Miltenyi Biotec #130-095-362) and human IL-15 at 5ng/mL (Miltenyi Biotec #130-095-764) at 1e6 cells/mL and returned to 37°C, 5% CO_2 incubation. Flow cytometry was conducted on the cells, as described in Example 1, at 4 days, 7 days, and 10 days following tLNP transfection.

Results

[0710] **FIG. 13A -C** show that delivery of a gene modifying system payload to rested T cells using targeted LNPs formulated with Lipid154 and a higher percentage of helper lipid (22% DSPC) generated many more cells that expressed GFP (%GFP+) and at higher levels (MFI) relative to the baseline control tLNP that was the identical except that it was formulated with 8% DSPC. At four days (**FIG. 13A**) and at 7 days (**FIG. 13B**) following transfection of tLNPs comprising higher helper lipid (22% DSPC) at all doses tested, more rested T cells expressed GFP at higher levels relative to the cells transfected with Lipid154 tLNPs comprising 8% DSPC. The tLNPs formulated with 22% egg sphingomyelin also generated more cells expressing GFP relative to the tLNPs formulated with 8% DSPC at 4 days and 7 days post-transfection, but the effects were not as pronounced compared to the effects generated using tLNPs formulated with 22% DSPC. Overall, a greater number of cells expressed GFP at Day 7 relative to Day 4 for all LNPs tested. When the tLNP comprising Lipid 154 and 22% DSPC was co-administered with TransAct, a T cell activating agent, the number of GFP positive cells increased at Days 4 and 7 following dosing. A base LNP formulated with Lipid154 and 8% DSPC, but no targeting moieties ("Base L154" in **FIG. 13**), did not generate an appreciable number of cells expressing GFP, showing that the anti-CD3 targeting moiety increases the number of cells expressing

exogenous GFP. **FIG. 13C** shows that the GFP+ cells generated using tLNPs comprising 22% DSPC expressed the GFP at higher levels than cells transfected with the baseline tLNPs. The negative control tLNPs comprising a gene modifying polypeptide mRNA alone or the GFP template RNA alone did not produce any GFP-expressing cells.

[0711] In summary, tLNPs comprising a combination of Lipid154 as the ionizable lipid and a higher percentage of helper lipid (such as DSPC or sphingomyelin), and an anti-CD3 targeting moiety with a gene modifying system enables effective insertion of an exogenous nucleic acid sequence into the genomes of rested T cells. At Day 4 post-transfection, up to approximately 30% of the T cells expressed the exogenous GFP nucleic acid sequence, while up to over 40% of the T cells expressed GFP at Day 7 post-transfection. The addition of TransAct can further increase the percentage of cells expressing the exogenous nucleic acid.

Example 9: Improved LNP formulation for delivery of an exemplary gene writer system to resting T cells to produce CAR-T cells

[0712] The targeted LNPs of Example 8 comprising Lipid154 and a higher helper lipid percentage (22% DSPC) and conjugated to the anti-CD3-8 targeting moiety were tested to determine whether they could deliver a gene modifying system payload into rested T cells to integrate an exogenous chimeric antigen receptor (CAR) into the genomic DNA of the rested T cells. The tLNPs were formulated with an all-RNA gene modifying system composed of an mRNA encoding an exemplary gene modifying polypeptide and a template RNA encoding a CAR. A control tLNP comprising Lipid154 and 8% DSPC and conjugated to the anti-CD3-8 targeting moieties was also generated to establish a baseline CAR insertion level for purposes of comparison.

T Cell Culture

[0713] Cryopreserved primary T cells from a single human donor (HemaCare, donor D328798) were thawed and transferred into PBS with 2% BSA by volume. Cells were counted on a Cellometer K2 Cell Counter (Nexcelom), then pelleted by centrifugation at 400xg for 5 minutes and resuspended in complete T cell media at a density of $1x10^6$ cells/mL. The complete T cell media was comprised of CTS AIM V SFM basal media (Gibco #0870112DK) with 5% human serum AB (Gemini Bio #100-512) and the following cytokines: human IL-7 at 10ng/mL (Miltenyi Biotec #130-095-362), and human IL-15 at 5ng/mL (Miltenyi Biotec #130-095-764).

Preparation of T Cells for Transfection

[0714] $2.0x10^7$ cells were pelleted by centrifugation and the supernatant aspirated. The cell pellets were resuspended in CTS AIM V SFM at a concentration of $2x10^6$ cells/mL. The resuspended T cells were plated in non-treated 96-well U-bottom polystyrene plates (Falcon # 08-772-54) at a density of $2x10^5$ cells (100uL) per well, and returned to incubation at 37°C, 5% CO_2 while the LNPs were prepared.

Preparation of Targeted LNPs for Transfection

[0715] LNPs were formulated as follows. An ethanol phase was prepared with five lipids, containing 47% ionizable lipid (Lipid154), 22% DSPC, 28.5% cholesterol, 2% PEG-DMG and 0.5% DSPE-PEG2K-TCO. An aqueous phase was composed of mRNA encoding the gene modifying polypeptide and a template RNA encoding the CAR dissolved in 25 mM acetate buffer. The two phases were mixed using a microfluidic device to formulate LNP-TCO at a total flow rate of 18 ml/min (aqueous phase: ethanol phase=3:1). 3x volume of CBS was added to LNP solution and the resulting solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes. The LNPs were then buffer exchanged to the desired concentration either via Tangential Flow Filtration (TFF) and/or centrifugation (100k MWCO). The baseline control tLNP was prepared using the same approach, except the ethanol phase was prepared with five lipids, containing 47% ionizable lipid (Lipid154), 8% DSPC, 42.5% cholesterol, 2% PEG-DMG and 0.5% DSPE-PEG2K-TCO. For the negative control tLNPs, an aqueous phase containing either the gene modifying polypeptide mRNA only or the GFP template RNA only was mixed with the ethanol phase.

[0716] The anti-CD3-8 targeting moiety was produced with an additional sortase tag (LPETG, SEQ ID NO:11) on the C terminus of the heavy chain. 10uM anti-CD3 Fab-LPETG and 1mM Triglycine methyltetrazine (GGG-meTz) were prepared in sortase buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl2, pH 7.4). 2uM Sortase A5 was then added to initiate the reaction. The above solution was incubated at 30°C for 3 hours with shaking at 800 rpm. Excess reagents were removed, and buffer was exchanged to PBS containing 10mM EDTA by a 10K Amicon centrifugal filter. To confirm a DOL (degree of labeling) of close to 1meTz per targeting moiety, sortase modified anti-CD3 targeting moiety was allowed to react with 5eq AF594 containing TCO group in PBS for 2 hours at room temperature. After the reaction, excess dye was removed by two runs with Zeba desalting column. DOL was measured by absorbances at A280 and A590 using DOL calculator. The resulting meTz-modified anti-CD3 targeting moiety was added to the TCO modified-LNP solution (as prepared above) for antibody-LNP conjugation, with a mass

ratio between antibody and mRNA (encoding eGFP) of 1:1, except where otherwise noted (**Table 1**). The solution was incubated at room temperature for 2 hours and then at 4°C overnight. Any unreacted anti-CD3 targeting moiety was removed by a 300K MPES TFF membrane. The resulting targeting moiety-LNP product was further concentrated using an Amicon column.

[0717] The gene modifying system formulated in the tLNPs comprised an mRNA encoding an exemplary gene modifying polypeptide comprised the following components.

[0718] The gene modifying polypeptide comprising the amino acid sequence of:

MDSTAHPNQGRGLEKVSQTLPALQTPGQHTAAGGSSPLSGRNQRKNTKKLLLGAWNIR
TLLDRENTPRPERRTALIGKELARYNIDIAALSETRLPEEGSLSEPTTGYTFFWKGRASNE
DRIHGVGLAIKTSLLKQLPDLPVGISERLMKIRLPLSKDRYATIISAYAPTLTSTEETIEQFY
SDLSAVLHSVPTNDKLILLGDFNARVGQDHERWKGVLGKHGVGKMNNNGLLLLSKCS
EFELTITNTVFRMANKYKTTWMHPRSKQWHLIDYIIVRRRDIQDVKITRAMRGAECWT
DHRLVRATLQMRIAPRHPKRAQTVRAFYNVSRLRDPSYLQTFQSCLDDKLSAKGPLTGS
STEKWNQFRDAVKETSKAVLGPKQRNHQDWFDENNTAIEDLLSKKNKAFMEWQNNPN
SAPKKDRFKSLQATAQREIRKMQDRWWEKKAEEIQRFADMKNYKQFFSALKTVYGPL
KPTTTPLLSSDGDTLIKDKKGISNRWKEHFSQLLNRPSSVDQSALDQIPQNRTIEQLDVPP
SIEEVQKAIKQMSAGKAPGKDGIPTEVYKALNGKALQAFHIVLTSIWEEEDMPPELRDAS
IVALYKNKGSRAACDNYRGISLLSTAGKILARVILNRLLSSVSEQNLPESQCGFRPDRSTI
DMVFTVRQMQEKCLEQNLSLYIVFIDLTKAFDTVNRDALWVILSKLGCPAKFVKLIQLF
HVDMTGEVLSGGETSDRFNISNGVKQGCVLAPVLFNLFFTQVLRHAVMDLDLGVYIKY
RLDGSLFDLRRLTAKTKTTERLILEALFADDCALMAHQENHLQTIVDRFSTATKLFGLTI
SLSKTEVLFQPAPGRPTNQPCITIDGTQLSNVNTFKYLGSTIANDGSLDHEINARIQKASQ
ALGRLRCKVLQHRGVSTATKLKVYNAVVLSSLLYGCETWTLYRKHMKQLEQFHQRSL
RSIMRIRWQDRITNQEVLDRANSTSIEVMVLKTQLRWSGHVIRMDPQRIPRQVFYGELS
AGLRKQGRPKKRFKDQLKSNLKWAGITPKQLELAASDRSSWRTHINHAATTFEDERRR
RLAAARERRHQATTAPPVTTGVPCPMCHKLCASAFGLQSHMRVHRR (SEQ ID NO:32)

[0719] The gene modifying polypeptide comprised a GGS Linker having an amino acid sequence of:

GGS

[0720] The gene modifying polypeptide comprised an SV40 NLS having an amino acid sequence of:

PKKKRKV (SSEQ ID NO:367)

[0721] The gene modifying polypeptide comprised an XTEN linker having an amino acid sequence of:

SGSETPGTSESATPES (SEQ ID NO:368)

[0722] The gene modifying polypeptide comprised an HiBit tag having an amino acid sequence of:

VSGWRLFKKIS (SEQ ID NO:369)

[0723] The full-length gene modifying polypeptide with all components was structured as follows: NLS - 3GS linker – gene modifying polypeptide - XTEN linker - HiBit tag. The full-length sequences are provided in Table N (Example 21).

[0724] The gene modifying system further comprised a template RNA with the following sequences from 5' to 3': 5'UTR_{retro}, BCMA CAR, MND promoter, 3'UTR_{retro}.

[0725] The template RNA comprised a 5'UTR having the sequence of:

GGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGUCG
UGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUCAC
UUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACGGC
UUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUGGU
GAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACGGA
AGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUGUG
GAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAGCU
GAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAACG
CCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUCAC
GCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCAAG
ACCUGCGGCGAUGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCAGU
UGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCCUG
UGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAG
(SEQ ID NO:372)

[0726] The template RNA comprised a 3'UTR having the sequence of:

UGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCGAGAGACUACCACU (SEQ ID NO:373)

[0727] The template RNA comprised a nucleic acid sequence encoding a BCMA-CAR fusion polypeptide, wherein the nucleic acid sequence was:

AGGGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGU
CGUGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUC
ACUUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACG
GCUUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUG
GUGAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACG
GAAGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUG
UGGAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAG
CUGAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAA
CGCCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUC
ACGCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCA
AGACCUGCGGCGAUGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCA
GUUGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCC
UGUGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAG
GUUUACGGAUCCGAAUUCUCGACGGAUCGAUCCGUUCUUUCCGCCUCAGAAGCCA
UAGAGCCCACCGCAUCCCCAGCAUGCCUGCUAUUGUCUUCCCAAUCCUCCCCCUU
GCUGUCCUGCCCCACCCCACCCCCCAGAAUAGAAUGACACCUACUCAGACAAUGC
GAUGCAAUUUCCUCAUUUUAUUAGGAAAGGACAGUGGGAGUGGCACCUUCCAGG
GUCAAGGAAGGCACGGGGGAGGGGCAAACAACAGAUGGCUGGCAACUAGAAGGC
ACAGUCGAGGCUGAUCAGCGAAUCCCCCGGCCGCUUUAUCUGGGGGGCAGGGCCU
GCAUGUGCAGGGCGUCGUAGGUGUCCUUGGUGGCGGUGCUCAGGCCCUGGUACA
GGCCAUCGUGGCCCUUGCCGCGCCUCCGCUCGCCCUUCAUGCCGAUCUCGCUGUA
GGCCUCGGCCAUCUUGUCUUUCUGCAGUUCGUUAUACAGGCCUUCCUGGGGGUUC
UUCCGUCUGGGCUUUCCGCCCAUCUCGGGGUCCCGGCCUCUCCGCUUGUCCAGCA
CGUCGUACUCUUCCCGUCUGCCCAGGUUCAGCUCGUUGUACAGCUGGUUCUGUCC
CUGCUGGUAGGCAGGGGCGUCGGCGGAUCUGCUGAACUUCACUCUCAGUUCACAU
CCUCCUUCUUCUUCUUCUGGAAAUCGGCAGCUACAGCCAUCUUCCUCUUGAGUAG
UUUGUACUGGUCUCAUAAAUGGUUGUUUGAAUAUAUACAGGAGUUUCUUUCUGC
CCCGUUUGUUCCGGUGGUUGCAGUACAGGGUGAUCACCAGGCUCAGCAGCAGCAC
GCCACAGGUGCCGGCCAGAGGGGCCCAGAUGUAGAUGUCGCAGGCGAAGUCCAGG
CCUCUGGUGUGCACGGCUCCGCCGGCAGCAGGUCUACAGGCUUCGGGCCGCAGAG
ACAGAGGCUGGCUGGCGAUUGUUGGGGCUGGGGUGGGAGGUCUAGGGGCAGGGG
UGGUGGUAGGUUUGGCGGGCAGGAACACGGGCACGAAUGAGCUGACGGUGACCA

UUGUACCCUGACCCCAUACGUCUAGUAUGGUGUCUCCACGAUCUCUGGCGCAGUA
GUACACCGCCGUGUCUGCGGCGGUCACAGAACUCAGCUUCAGGGAGAACUGGUUC
UUGGACGUGUCUACGGAUAUGGUGACUCGACUCUUGAGGGACGGGUUGUAGUAG
GUGCUCCCACUAUAGGAGAUACUCCCAAUCCACUCCAGCCCCUUCCCUGGGGGCU
GGCGGAUCCAGCCCCAGUAGUAACUACUACUGCUGAUGGAGCCACCAGAGACAGU
ACAGGUGAGGGACAGGGUCUCCGAAGGCUUCACCAGUCCUGGGCCCGACUCCUGC
AGCUGCAGCUGUCCCUUUGUGCUGCCCUCGCCAGAGCCAGGCUUGCCGGAGCCGC
UGGUGCUGCCUUUGAUCUCAACCUUGGUCCCUCCGCCAAAAGUGAGGAGGUCGUA
UUUUUGCUGACAGUAGUAAGUUGCAAAAUCUUCAGGUUGCAGACUGCUGAUGGU
GAGAGUGAAAUCUGUCCCAGAUCCACUGCCACUGAACCUUGAUGGGACCCCACUU
UGCAAACUGGAUGCAGCAUAGAUCAGGAGCUUAGGGGCUUUCCCUGGUUUCUGC
UGAUACCAAUUUAAAUAGCUGCUAAUGCUCUGACUUGCCCGGCAAGUGAUGGUG
ACUCUGUCUCCUACAGAUGCAGACAGGGAGGAUGGAGACUGGGUCAUCUGGAUG
UCCGGGCGAGCGGCAUGCAAGAGCAGGGCAAGAGGCAGAAGCAGGGCGGUCACCG
GCAGAGCCAUGGUGGCUUUACCAACAGUACCGGAAUGCCAAGCUUGGGUCUGGGC
UCUUUUAUUGAGCUCGGGGAGCAGAAGCGCGCGAACAGAAGCGAGAAGCGAACU
GAUUGGUUAGUUCAAAUAAGGCACAGGGUCAUUUCAGGUCCUUGGGGCACCCUG
GAAACAUCUGAUGGUUCUCUAGAAACUGCUGAGGGCGGGACCGCAUCUGGGGAC
CAUCUGUUCUUGGCCCUGAGCCGGGGCAGGAACUGCUUACCACAGAUAUCCUGUU
UGGCCCAUAUUCUGCUGUUCCAACUGUUCUUGGCCCUGAGCCGGGGCAGGAACUG
CUUACCACAGAUAUCCUGUUUGGCCCAUAUUCUGCUGUCUCUCUGUUCCUAACCU
UGAUCCUAGCUUGCCAAACCUACAGGUGGGGUCUUUCAUUCCCCCCUUUUUCUGG
AGACUAAAUAAAUGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCGAGAG
ACUACCACUAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA (SEQ ID NO:375)

[0728] The BCMA-CAR fusion polypeptide comprised an amino acid sequence of:

DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSR
FSGSGSGTDFTLTISSLQPEDFATYYCQQKYDLLTFGGGTKVEIKGSTSGSGKPGSGEGST
KGQLQLQESGPGLVKPSETLSLTCTVSGGSISSSSYYWGWIRQPPGKGLEWIGSISYSGST
YYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDRGDTILDVWGQGTMVTV
SSFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW
APLAGTCGVLLLSLVITLYCNHRNKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEE
EGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR

KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQ ALPPR (SEQ ID NO:82)

[0729] The BCMA-CAR fusion polypeptide comprised a CD8 hinge domain having an amino acid sequence of:

FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO:79)

[0730] The BCMA-CAR fusion polypeptide comprised a CD8 transmembrane domain having an amino acid sequence of:

IYIWAPLAGTCGVLLLSLVITLYCNHRN (SEQ ID NO:73)

[0731] The BCMA-CAR fusion polypeptide comprised a 4-1BB signaling domain having an amino acid sequence of:

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO:77)

[0732] The BCMA-CAR fusion polypeptide comprised a CD3z signaling domain having an amino acid sequence of:

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEG LYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:76)

[0733] The BCMA-CAR fusion polypeptide comprised a GSG Linker having an amino acid sequence of:

GSG

[0734] The BCMA-CAR fusion polypeptide comprised a T2A peptide having an amino acid sequence of:

EGRGSLLTCGDVEENPGP (SEQ ID NO:376)

LNP Transfection and Flow Cytometry

[0735] 50uL of diluted tLNPs were added to plated activated T cells at 0.25, 0.5, 1, 2.5, 5, or 10 µg of LNP per 1 x10^6 cells, and mixed gently by pipetting. Negative conrtol tLNPs were dosed at 2.5 µg of LNP per 1 x10^6 cells. In some conditions, TransAct Research Grade T cell activation reagent (Miltenyi Biotec #130-111-160) was also added to the rested T cell suspension at the same time as the tLNPs at a volume of 10uL per 1x10^6 cells. After addition of the tLNPs, all plated cells were at a final density of 1x10^6 cells/mL in 200uL, with a final concentration of

5% FBS (for serum-containing conditions) and cytokines at the following concentrations: human IL-2 at 20ng/mL, human IL-7 at 10ng/mL, and human IL-15 at 5ng/mL. Transfected cells were returned to incubation at 37°C, 5% CO_2 for four hours. After the 4-hour tLNP incubation, cells were centrifuged at 350xg for 7min, supernatant aspirated, and cells resuspended in complete T cell media as previously described at 1e6 cells/mL in flat bottom plates. Flow cytometry was conducted on the cells, as described in Example 1, at 4 days, 7 days, and 10 days following tLNP transfection.

Results

[0736] **FIG. 14A** shows that delivery of an exemplary gene modifying system payload to rested T cells using targeted LNPs formulated with Lipid154 and higher helper lipid (22% DSPC) generated many more CAR-T cells (%CAR+) at all dose levels at 3 and 7 days post transfection relative to the baseline control tLNP that was the identical except that it was formulated with 8% DSPC. **FIGs. 14B** and **14C** show that the rested T cells transfected with the tLNPs comprising higher helper lipid exhibited higher CAR expression levels (MFI) relative to the cells transfected with the baseline control tLNPs at most doses at 3 and 7 days, respectively, following LNP transfection. The coadministration of TransAct ("TA") with the tLNPs increased the number of CAR expressing cells and CAR expression levels for all tLNPs tested.

[0737] In summary, tLNPs comprising a combination of Lipid154 as the ionizable lipid and a higher percentage of helper lipid (such as DSPC), and an anti-CD3 targeting moiety with a gene modifying system enables effective insertion of an exogenous nucleic acid sequence encoding a CAR into the genomes of rested T cells. At Day 4 and Day 7 post-transfection, over 5% of the T cells expressed the exogenous CAR nucleic acid sequence at the 1 ug dose (or higher) without TransAct and over 10% expressed the CAR at doses of 2.5 ug or higher. The addition of TransAct further increased CAR expression levels in the cells. Furthermore, the CAR-T cells generated using these tLNPs were functional, as they were capable of killing target tumor cells in an *in vitro* tumor cell killing assay (data not shown).

Example 10: Testing a dual targeted-LNP conjugated to anti-CD3 and anti-CD7 targeting moieties for delivery of an exemplary gene writer system to resting T cells to produce CAR-T cells

[0738] Dual targeted LNPs conjugated to anti-CD3 and anti-CD7 targeting moieties were administered to resting T cells to determine whether they could effectively deliver an exemplary gene modifying system into the cells to enable TPRT-mediated insertion of a CAR sequence into the genomic DNA of the cells. The tLNPs were formulated with an all-RNA gene modifying

system composed of an mRNA encoding an exemplary gene modifying polypeptide and a template RNA encoding a CAR. Targeted LNPs were also generated that were identical except that they were conjugated to either an anti-CD3 targeting moiety or an anti-CD7 targeting moiety alone, as described herein, for the purposes of comparing delivery efficiency of the gene modifying system in the rested T cells.

T Cell Culture

[0739] Cryopreserved primary peripheral blood monocyte cells (PBMCs) from two human donors (HemaCare, donor D354 and D448) were thawed and transferred into PBS with 2% BSA by volume. Cells were counted on a Cellometer K2 Cell Counter (Nexcelom), then pelleted by centrifugation at 400xg for 5 minutes and resuspended in T cell media at a density of $20x10^6$ cells/mL in 50ul of media. The T cell media was comprised of Tex MACS (Miltenyi Biotec # 130-097-196) with 2% Human serum albumin (AkronBio: AK8228-0100). The resuspended T cells were plated in non-treated 96-well U-bottom polystyrene plates (Falcon # 08-772-54) and returned to incubation at 37°C, 5% CO_2 while the LNPs were prepared.

Preparation of Targeted LNPs for Transfection

[0740] The inter-chain disulfide bonds of an anti-CD3 Fab fragment and an anti-CD7 Fab fragment were specifically reduced before conjugation to the LNPs. A solution containing each of the Fab fragments was buffer exchanged with PBS containing 10mM EDTA. Ten equivalents of TCEP were added to each solution and the reduction was carried out for 1 hour at room temperature. Complete reduction of the Fab fragments was confirmed by SDS PAGE gels.

[0741] Base LNPs were prepared at a molar ratio of 47% ionizable lipid (Lipid154): 28.5% cholesterol: 22% DSPC: 2.0% DMG-PEG2000: 0.5% DSPE-PEG2000-maleimide at an N/P of 6 in the ethanol phase. The aqueous phase was composed of mRNA encoding the exemplary gene modifying polypeptide and the exemplary template RNA at a mass ratio of 1:4 in 25 mM acetate buffer. The two phases were mixed using a microfluidic device to formulate base LNP at a total flow rate of 18 mL/min (aqueous phase: ethanol phase=3:1). 3x volume of citrate-buffered saline was added to LNP solution and resulted solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes.

[0742] The neutralized base LNPs were treated with the reduced anti-CD3 Fab fragments added at a mass ratio of 0.4:1 (mg of Fab to mg of total RNA), and the reduced anti-CD7 Fab fragments added at a mass ratio of 0.4:1 (mg of Fab to mg of total RNA). The solution was incubated at room temperature for 2 hours for conjugation and then stored at 4°C overnight. The unreacted maleimide was quenched with cysteine (3 equivalents) for 1 hour at room

temperature. Unbound Fab fragments were removed and the LNPs were purified and concentrated either via buffer exchange using a centrifugal filter (100K MWCO) or via TFF (300kD mPES flat sheet) using Tris buffer containing 9% sucrose. The resulting dual tLNP was characterized by Zetasizer for size and PDI and by Ribogreen assays for mRNA concentration. For the control tLNPs, either only the reduced anti-CD3 Fab fragments or the reduced anti-CD7 Fab fragments were added to the LNP-maleimide solution at a mass ratio of 0.4:1 (mg of Fab to mg of total RNA) and characterized as described above.

LNP Transfection and Flow Cytometry

[0743] The tLNPs were diluted in TexMACs media alone that was sterile filtered, and 50uL of this diluted tLNPs (with dual or single targeting moieties) were added to the plated cells at 0.5, 1, and 5 µg of LNP per 10 $x10^6$ cells, and mixed gently by pipetting. After addition of the tLNPs, all plated cells were at a final density of $10x10^6$ cells/mL in 100uL, with a final concentration of 1% HSA in TexMACs media. Transfected cells were returned to incubation at 37°C, 5% CO_2 for four hours. After the 4-hour tLNP incubation, cells were centrifuged at 350xg for 7min, supernatant aspirated, and cells resuspended in complete T cell media which comprised of TexMACs media at 5% Human Ab Serum (Gemini Bio 100-152) with IL-2 (100 IU/ml) (Miltenyi Biotec #130-097-746), human IL-7 at 10ng/mL (Miltenyi Biotec #130-095-362), and human IL-15 at 5ng/mL (Miltenyi Biotec #130-095-764) at 1e6 cells/mL in flat bottom plates. Flow cytometry was conducted on the cells, as described in Example 1, at days 1, 5, 7 days, following tLNP transfection.

Results

[0744] Delivery of an exemplary gene modifying system payload (dose of 1 µg of LNP per 10 $x10^6$ cells) to rested PBMCs cells using the dual targeted LNPs conjugated to the anti-CD3 and anti-CD7 targeting moieties resulted in an average of about 15.7% of CAR-T cells (%CAR+), as assayed by FACs analysis at 7 days following LNP administration. For comparison, administration of the targeted LNPs conjugated to the anti-CD3 targeting moiety generated about an average of 21% of CAR-T cells, while the anti-CD7 tLNPs generated about 1% of CAR-T cells, at the same dose and same day post-administration.

Example 11: Targeted LNP delivery of gene modifying systems for multiplex editing in T cells

[0745] Lipid154 tLNPs were used to deliver two gene modifying systems to effect multiplex editing of the genomic DNA of activated primary human T cells. LNPs were formulated to contain 47% ionizable lipid (Lipid154), 22% DSPC, 28.5% cholesterol, 2% PEG-DMG and

0.5% DSPE-PEG2K-TCO, and then conjugated to an anti-CD3 targeting moiety (anti-CD3-8), as described in Example 9. Targeted LNPs were formulated with a first exemplary heterologous gene modifying system containing an mRNA encoding a first heterologous gene modifying polypeptide and 2 template RNAs to produce an insertion of 5 nucleotides (TAGTG) in the first exon of the human TRAC gene and an insertion of 5 nucleotides (TAGTG) in the first exon of the Beta 2 Microglobulin (B2M) gene in human T cells. Each insertion into the TRAC and B2M locus generated a frameshift mutation that resulted in a non-functional gene (also referred to as a knock-out). Targeted LNPs were also formulated with a second exemplary gene modifying system (a retrotransposon gene modifying system) containing an mRNA encoding a retrotransposon gene modifying polypeptide and a template RNA to insert either an expression cassette for a chimeric antigen receptor (CAR) or an expression cassette for GFP into the genome of the human T cells.

[0746] Each template RNA of the first heterologous gene modifying system contained:

1. a gRNA spacer;
2. a gRNA scaffold;
3. a heterologous object sequence; and
4. a primer binding site (PBS) sequence.

[0747] The exemplary template RNAs generated and used in the first heterologous gene modifying system are given in **Table 23**. Nucleotide modifications are noted as follows: phosphorothioate linkages denoted by an asterisk, 2'-O-methyl groups denoted by an 'm' preceding a nucleotide.

Table 23 – Exemplary Template RNAs and Sequences

Name	Descriptor	Sequence
RNACS5630	TRAC4_Ins 5 Nick P9R18	mG*mA*mG*rArArUrCrArArArArUrCrGrGrUrGrArArUr GrUrUrUrUrArGrGrAmGmCmUmAmGmAmAmAmUmAmG mCrArArGrUrUrArArArArUrArArGrGrCrUrArGrUrCrCrG rUrUrArUrCrAmAmCmUmUmGmAmAmAmAmAmGmU mGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCrC rUrGrUrCrUrGrCrCrUrArUrUrUrArGrUrGrCrArCrCrGrA* mU*mU*mU (SEQ ID NO:377)
RNACS7755	B2m1_P11R 18_ins5_nic k_v1	mA*mC*mU*rCrArCrGrCrUrGrGrArUrArGrCrCrUrCrCrG rUrUrUrUrArGrGrAmGmCmUmAmGmAmAmAmUmAmGm CrArArGrUrUrArArArArUrArArGrGrCrUrArGrUrCrCrGrU

		rUrArUrCrAmAmCmUmUmGmAmAmAmAmAmGmUmG mGmCmAmCmCmGmAmGmUmCmGmGmUmGmCrUrUr UrCrUrGrGrCrCrUrGrGrArUrGrArUrGrGrGrCrUrArUrCrC *mA*mG*mC (SEQ ID NO:378)

[0748] **Table 24** shows the sequences of **Table 24** without chemical modifications. In some embodiments, the sequences of **Table 24** may be used without chemical modifications, or with one or more chemical modifications.

Table 24: Table E7 Sequences without Chemical Modifications

RNACS	Name	Sequence	SEQ ID NO
RNACS5630	TRAC 4_Ins 5 Nick P9R18	GAGAAUCAAAAUCGGUGAAUGUUUUAGAGCUAG AAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUC AACUUGAAAAAGUGGCACCGAGUCGGUGCCUGUC UGCCUAUUUAGUGCACCGAUUU	379
RNACS7755	B2m1_ P11R1 8_ins5 _nick_ v1	ACUCACGCUGGAUAGCCUCCGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCA ACUUGAAAAAGUGGCACCGAGUCGGUGCUUUCUG GCCUGGAUGAUGGGCUAUCCAGC	380

[0749] The exemplary heterologous gene modifying polypeptide of the first heterologous gene modifying system contained:

1. an endonuclease and/or DNA binding domain;
2. a peptide linker; and
3. a reverse transcriptase (RT) domain,

where (1), (2), and (3) are heterologous to each other. Accordingly, the first heterologous gene modifying polypeptide is an example of heterologous gene modifying polypeptides.

[0750] The exemplary heterologous gene modifying polypeptide of the first gene modifying system was RNAIVT1564, and comprised the amino acid sequence indicated in **Table 25**.

Table 25 – Exemplary heterologous gene modifying polypeptide and Sequence

Name	AA sequence	SEQ ID NO
RNAI VT15 64	MPAAKRVKLDGGDKKYSIGLDIGTNSVGWAVITDEYKVPSK KFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRY TRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHER HPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLAL AHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPI NASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIAL	381

	SLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQY ADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHH QDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEE FYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIH LGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGN SRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKN LPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGE QKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDR FNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDRE MIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIR DKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVS GQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKP ENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEH PVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDH IVPQSFLKDDSIDNKVLTRSDKARGKSDNVPSEEVVKKMKNY WRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVET RQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFR KDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFV YGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEIT LANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSMPQVNI VKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDS PTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPI DFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQ KGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQH KHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQ AENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIH QSITGLYETRIDLSQLGGDSGGSSGGSSGSETPGTSESATPESS GGSSGGSSTLNIEDEYRLHETSKEPDVSLGSTWLSDFPQAWAE TGGMGLAVRQAPLIIPLKATSTPVSIKQYPMSQEARLGIKPHIQ RLLDQGILVPCQSPWNTPLLPVKKPGTNDYRPVQDLREVNKR VEDIHPTVPNPYNLLSGLPPSHQWYTVLDLKDAFFCLRLHPTS QPLFAFEWRDPEMGISGQLTWTRLPQGFKNSPTLFNEALHRD LADFRIQHPDLILLQYVDDLLLAATSELDCQQGTRALLQTLG NLGYRASAKKAQICQKQVKYLGYLLKEGQRWLTEARKETV MGQPTPKTPRQLREFLGKAGFCRLFIPGFAEMAAPLYPLTKPG TLFNWGPDQQKAYQEIKQALLTAPALGLPDLTKPFELFVDEK QGYAKGVLTQKLGPWRRPVAYLSKKLDPVAAGWPPCLRMV AAIAVLTKDAGKLTMGQPLVILAPHAVEALVKQPPDRWLSN ARMTHYQALLLDTDRVQFGPVVALNPATLLPLPEEGLQHNC LDILAEAHGTRPDLTDQPLPDADHTWYTDGSSLLQEGQRKAG AAVTTETEVIWAKALPAGTSAQRAELIALTQALKMAEGKKL NVYTDSRYAFATAHIHGEIYRRRGWLTSEGKEIKNKDEILALL KALFLPKRLSIIHCPGHQKGHSAEARGNRMADQAARKAAITE TPDTSTLLIENSSPSGGSKRTADGSEFEKRTADGSEFESPKKKA KVE	

[0751] The exemplary gene modifying polypeptide of the second retrotransposon gene modifying system contained:

1. an endonuclease and/or DNA binding domain; and

2. a reverse transcriptase (RT) domain,

where (1) and (2) are both derived from a retrotransposon (from the same retrotransposons). Accordingly, the second gene modifying polypeptide was an example of retrotransposon gene modifying polypeptides.

[0752] The second retrotransposon gene modifying system used comprised the amino acid sequence in **Table 26**. The mRNA sequence encoding the second retrotransposon gene modifying system is also given in **Table 26**.

Table 26 – Exemplary gene modifying polypeptides and sequences

Name	Nucleic Acid Sequence	Amino Acid Sequence
RNAI VT19 01	AGGAAAUAAGAGAGAAAAGAAGAGUAAG AAGAAAUAUAAGAGCCACCAUGCCAAAG AAGAAAAGAAAAGUAGGAGGUUCCGACU CCACAGCUCAUCCCAACCAGGGACGUGGC CUGGAGAAGGUGUCCCAGACUCUGCCUG CGCUGCAGACCCCAGGGCAGCACACUGCC GCUGGGGGAUCCUCUCCCCUGUCCGGCAG GAAUCAGCGGAAGAACACCAAAAAGCUG UUGCUGGGCGCCUGGAACAUCCGCACCCU GCUCGACCGCGAAAAUACUCCCCGUCCUG AAAGACGCACGGCACUGAUUGGGAAAGA GCUCGCCAGAUAUAACAUCGACAUCGCU GCCCUGUCCGAAACCCGCCUGCCAGAGGA AGGAUCUCUGAGCGAGCCUACCACUGGC UACACGUUCUUUUGGAAGGGCAGAGCAA GCAACGAGGACCGUAUCCAUGGUGUAGG CCUGGCUAUCAAGACCUCUCUCCUGAAAC AACUCCCAGAUCUGCCAGUCGGCAUCUCA GAGAGACUGAUGAAGAUCAGGCUUCCUC UCUCAAAAGACCGCUACGCAACCAUCAU UUCCGCAUACGCGCCGACCCUGACUAGCA CCGAGGAAACCAUUGAGCAGUUCUACUC UGAUCUUUCAGCUGUUUUGCACUCCGUG CCUACCAACGACAAGCUGAUCCUCCUGGG CGACUUCAACGCUCGCGUGGGUCAGGAC CAUGAGCGCUGGAAAGGCGUGCUGGGUA AGCAUGGCGUGGGUAAGAUGAACAAUAA CGGCCUCCUGCUCCUGUCUAAGUGUAGC GAAUUCGAGCUGACUAUUACCAACACUG UUUUUCGUAUGGCUAACAAGUACAAAAC UACAUGGAUGCACCCCAGGAGCAAGCAG UGGCACCUGAUUGACUACAUUAUCGUGC GUAGGCGGGAUAUCCAGGACGUGAAGAU UACCCGUGCAAUGCGCGGCGCUGAGUGC UGGACAGACCAUCGCCUGGUGAGAGCUA CUCUCCAGAUGAGGAUCGCACCGCGUCAC CCUAAGCGCGCCCAGACCGUGCGGGCCUU UUACAACGUAUCACGGCUUCGGGAUCCU UCCUACCUUCAGACAUUUCAAUCUUGCC UCGAUGACAAGCUCUCUGCUAAGGGUCC CCUCACCGGAUCUUCAACAGAGAAGUGG AACCAAUUCCGCGAUGCGGUGAAGGAAA CCAGCAAAGCAGUCCUGGGACCGAAGCA GCGCAACCACCAAGACUGGUUUGACGAA	MPKKKRKVGGSDSTAHPNQGRGLEKVSQTL PALQTPGQHTAAGGSSPLSGRNQRKNTKKL LLGAWNIRTLLDRENTPRPERRTALIGKELA RYNIDIAALSETRLPEEGSLSEPTTGYTFFWK GRASNEDRIHGVGLAIKTSLLKQLPDLPVGIS ERLMKIRLPLSKDRYATIISAYAPTLTSTEETI EQFYSDLSAVLHSVPTNDKLILLGDFNARVG QDHERWKGVLGKHGVGKMNNNGLLLLSK CSEFELTITNTVFRMANKYKTTWMHPRSKQ WHLIDYIIVRRRDIQDVKITRAMRGAECWTD HRLVRATLQMRIAPRHPKRAQTVRAFYNVS RLRDPSYLQTFQSCLDDKLSAKGPLTGSSTE KWNQFRDAVKETSKAVLGPKQRNHQDWFD ENNTAIEDLLSKKNKAFMEWQNNPNSAPKK DRFKSLQATAQREIRKMQDRWWEKKAEEIQ RFADMKNYKQFFSALKTVYGPLKPTTTPLLS SDGDTLIKDKKGISNRWKEHFSQLLNRPSSV DQSALDQIPQNRTIEQLDVPPSIEEVQKAIKQ MSAGKAPGKDGIPTEVYKALNGKALQAFHI VLTSIWEEEDMPPELRDASIVALYKNKGSRA ACDNYRGISLLSTAGKILARVILNRLLSSVSE QNLPESQCGFRPDRSTIDMVFTVRQMQEKC LEQNLSLYIVFIDLTKAFDTVNRDALWVILS KLGCPAKFVKLIQLFHVDMTGEVLSGGETS DRFNISNGVKQGCVLAPVLFNLFFTQVLRHA VMDLDLGVYIKYRLDGSLFDLRRLTAKTKT TERLILEALFADDCALMAHQENHLQTIVDRF STATKLFGLTISLSKTEVLFQPAPGRPTNQPCI TIDGTQLSNVNTFKYLGSTIANDGSLDHEIN ARIQKASQALGRLRCKVLQHRGVSTATKLK VYNAVVLSSLLYGCETWTLYRKHMKQLEQ FHQRSLRSIMRIRWQDRITNQEVLDRANSTSI EVMVLKTQLRWSGHVIRMDPQRIPRQVFYG ELSAGLRKQGRPKKRFKDQLKSNLKWAGIT PKQLELAASDRSSWRTHINHAATTFEDERRR RLAAARERRHQATTAPPVTTGVPCPMCHKL CASAFGLQSHMRVHRRSGSETPGTSESATPE SVSGWRLFKKIS (SEQ ID NO:64)

	AAUAACACUGCCAUCGAAGACCUCCUGU CUAAAAAGAACAAAGCAUUUAUGGAGUG GCAGAAUAACCCAAACAGUGCACCCAAG AAAGACCGUUUCAAAAGCCUGCAGGCCA CCGCGCAGCGCGAGAUCCGGAAGAUGCA GGACCGCUGGUGGGAAAAAAAGGCCGAA GAGAUCCAGAGGUUCGCGGAUAUGAAAA ACUACAAGCAGUUCUUUAGCGCACUGAA GACCGUGUACGGACCUCUCAAACCCACCA CGACUCCCCUGCUCUCUUCCGAUGGUGAC ACUCUCAUCAAGGACAAAAAGGGAAUCA GCAACCGCUGGAAAGAACACUUUUCUCA ACUUCUCAACCGCCCAAGCUCCGUGGACC AGAGCGCUCUGGACCAGAUCCCGCAGAA CCGUACCAUCGAGCAGCUGGACGUGCCUC CCUCCAUCGAAGAGGUCCAGAAGGCUAU CAAGCAGAUGUCUGCUGGGAAGGCUCCU GGCAAGGAUGGGAUCCCAACAGAGGUCU AUAAAGCUCUCAACGGAAAGGCGCUGCA GGCCUUUCACAUUGUCCUGACCUCCAUCU GGGAGGAAGAGGACAUGCCACCUGAACU UAGGGACGCCUCUAUUGUGGCCCUGUAC AAGAAUAAGGGCAGCCGCGCAGCUUGUG AUAACUAUCGUGGCAUCUCACUCCUGAG UACAGCUGGCAAGAUUCUGGCUCGUGUG AUCCUGAACCGGUUGCUGAGUUCCGUUU CCGAGCAGAACCUGCCCGAAUCACAGUGC GGCUUCCGCCCCGAUCGGUCCACCAUCGA UAUGGUGUUCACCGUGCGUCAGAUGCAG GAGAAGUGCCUGGAACAGAAUCUGAGCC UGUACAUCGUUUUCAUCGAUCUGACUAA GGCCUUCGACACGGUGAACCGCGAUGCCC UGUGGGUGAUCCUGAGCAAGCUGGGGUG CCCCGCUAAGUUCGUAAAGCUCAUCCAG UUGUUCCACGUUGAUAUGACGGGAGAGG UCCUGUCCGGUGGAGAAACCUCCGACCG GUUUAACAUCUCCAAUGGCGUGAAACAG GGCUGCGUCCUGGCCCCAGUGCUGUUCA ACCUGUUUUUCACACAGGUGCUGAGACA UGCGGUUAUGGAUCUUGACCUGGGUGUG UAUAUCAAGUAUCGCUUGGACGGCAGUC UGUUCGAUCUCAGACGCCUGACAGCUAA GACUAAGACUACCGAGCGCCUCAUUCUC GAAGCCCUGUUUGCCGACGAUUGUGCCC UUAUGGCCCACCAGGAGAACCACCUGCA GACGAUUGUCGACCGCUUCUCUACAGCG ACCAAGCUGUUCGGCCUCACCAUCUCCCU CUCAAAGACCGAAGUCCUGUUUCAGCCC GCACCCGGGAGACCCACGAAUCAGCCGUG CAUUACCAUUGACGGGACCCAACUUUCC AACGUCAACACCUUCAAGUAUCUGGGAU CUACCAUCGCCAAUGAUGGCAGCCUGGA UCAUGAAAUCAACGCAAGGAUCCAAAAG GCUAGCCAGGCACUGGGCCGCCUCCGGUG CAAGGUCCUGCAGCACCGGGGCGUUUCC ACUGCCACUAAACUGAAGGUGUACAACG CUGUCGUGCUCUCUUCCCUGCUUUAUGG CUGCGAAACAUGGACUCUGUACCGGAAG CAUAUGAAACAGCUUGAGCAGUUCCACC AGAGGAGCUUGCGUAGCAUCAUGAGGAU CCGCUGGCAGGACCGCAUCACCAACCAGG	

	AGGUGCUGGACAGGGCUAAUUCCACCAG CAUCGAAGUUAUGGUGCUGAAGACCCAG CUCCGUUGGUCUGGCCAUGUCAUUCGGA UGGACCCUCAGCGUAUCCCCCGCCAGGUA UUCUACGGGGAACUGUCUGCGGGGCUCA GAAAGCAGGGCCGCCCAAAGAAACGCUU UAAGGAUCAGCUGAAAAGCAAUCUGAAG UGGGCCGGCAUCACCCCCAAGCAGCUGGA GCUGGCGGCAUCUGACCGCUCCAGCUGGC GCACCCACAUUAACCACGCAGCUACUACC UUUGAGGACGAACGCCGGCGCAGACUGG CCGCUGCACGCGAGAGGCGCCACCAAGCC ACUACCGCCCCUCCCGUGACCACUGGGGU CCCUUGCCCUAUGUGCCACAAGCUGUGU GCGUCUGCCUUCGGUUUGCAGUCCCACA UGCGUGUGCAUCGCCGCAGCGGCAGCGA GACUCCCGGGACCUCAGAGUCCGCCACAC CCGAAAGUGUGAGCGGCUGGCGGCUGUU CAAGAAGAUUAGCUGAGCUGGAGCCUCG GUGGCCAUGCUUCUUGCCCCUUGGGCCUC CCCCCAGCCCCUCCUCCCCUUCCUGCACC CGUACCCCCGUGGUCUUUGAAUAAAGUC UGAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAA (SEQ ID NO:370)	

[0753] The template RNA for use with the retrotransposon gene modifying polypeptide comprised the following sequences from 5' to 3': either 5'UTR_{retro}, BCMA CAR, MND promoter, 3'UTR_{retro}or 5'UTR_{retro}, GFP, MND promoter, 3'UTR_{retro}

[0754] The template RNAs comprised a 5'UTR having the sequence of:

GGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGUCG
UGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUCAC
UUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACGGC
UUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUGGU
GAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACGGA
AGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUGUG
GAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAGCU
GAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAACG
CCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUCAC
GCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCAAG
ACCUGCGGCGAUGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCAGU
UGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCCUG
UGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAG
(SEQ ID NO:372)

[0755] The template RNAs comprised a 3'UTR having the sequence of:

UGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCGAGAGACUACCACU (SEQ ID NO:373)

[0756] The CAR template RNA comprised a nucleic acid sequence encoding a BCMA-CAR fusion polypeptide, wherein the nucleic acid sequence was:

AGGGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGU
CGUGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUC
ACUUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACG
GCUUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUG
GUGAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACG
GAAGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUG
UGGAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAG
CUGAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAA
CGCCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUC
ACGCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCA
AGACCUGCGGCGAUGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCA
GUUGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCC
UGUGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAG
GUUUACGGAUCCGAAUUCUCGACGGAUCGAUCCGUUCUUUCCGCCUCAGAAGCCA
UAGAGCCCACCGCAUCCCCAGCAUGCCUGCUAUUGUCUUCCCAAUCCUCCCCCUU
GCUGUCCUGCCCCACCCCACCCCCCAGAAUAGAAUGACACCUACUCAGACAAUGC
GAUGCAAUUUCCUCAUUUUAUUAGGAAAGGACAGUGGGAGUGGCACCUUCCAGG
GUCAAGGAAGGCACGGGGGAGGGGCAAACAACAGAUGGCUGGCAACUAGAAGGC
ACAGUCGAGGCUGAUCAGCGAAUCCCCCGGCCGCUUUAUCUGGGGGGCAGGGCCU
GCAUGUGCAGGGCGUCGUAGGUGUCCUUGGUGGCGGUGCUCAGGCCCUGGUACA
GGCCAUCGUGGCCCUUGCCGCGCCUCCGCUCGCCCUUCAUGCCGAUCUCGCUGUA
GGCCUCGGCCAUCUUGUCUUUCUGCAGUUCGUUAUACAGGCCUUCCUGGGGGUUC
UUCCGUCUGGGCUUUCCGCCCAUCUCGGGGUCCCGGCCUCUCCGCUUGUCCAGCA
CGUCGUACUCUUCCCGUCUGCCCAGGUUCAGCUCGUUGUACAGCUGGUUCUGUCC
CUGCUGGUAGGCAGGGGCGUCGGCGGAUCUGCUGAACUUCACUCUCAGUUCACAU
CCUCCUUCUUCUUCUUCUGGAAAUCGGCAGCUACAGCCAUCUUCCUCUUGAGUAG
UUUGUACUGGUCUCAUAAAUGGUUGUUUGAAUAUAUACAGGAGUUUCUUUCUGC
CCCGUUUGUUCCGGUGGUUGCAGUACAGGGUGAUCACCAGGCUCAGCAGCAGCAC

GCCACAGGUGCCGGCCAGAGGGGCCCAGAUGUAGAUGUCGCAGGCGAAGUCCAGG
CCUCUGGUGUGCACGGCUCCGCCGGCAGCAGGUCUACAGGCUUCGGGCCGCAGAG
ACAGAGGCUGGCUGGCGAUUGUUGGGGCUGGGGUGGGAGGUCUAGGGGCAGGGG
UGGUGGUAGGUUUGGCGGGCAGGAACACGGGCACGAAUGAGCUGACGGUGACCA
UUGUACCCUGACCCCAUACGUCUAGUAUGGUGUCUCCACGAUCUCUGGCGCAGUA
GUACACCGCCGUGUCUGCGGCGGUCACAGAACUCAGCUUCAGGGAGAACUGGUUC
UUGGACGUGUCUACGGAUAUGGUGACUCGACUCUUGAGGGACGGGUUGUAGUAG
GUGCUCCCACUAUAGGAGAUACUCCCAAUCCACUCCAGCCCCUUCCCUGGGGGCU
GGCGGAUCCAGCCCCAGUAGUAACUACUACUGCUGAUGGAGCCACCAGAGACAGU
ACAGGUGAGGGACAGGGUCUCCGAAGGCUUCACCAGUCCUGGGCCCGACUCCUGC
AGCUGCAGCUGUCCCUUUGUGCUGCCCUCGCCAGAGCCAGGCUUGCCGGAGCCGC
UGGUGCUGCCUUUGAUCUCAACCUUGGUCCCUCCGCCAAAAGUGAGGAGGUCGUA
UUUUUGCUGACAGUAGUAAGUUGCAAAAUCUUCAGGUUGCAGACUGCUGAUGGU
GAGAGUGAAAUCUGUCCCAGAUCCACUGCCACUGAACCUUGAUGGGACCCCACUU
UGCAAACUGGAUGCAGCAUAGAUCAGGAGCUUAGGGGCUUUCCCUGGUUUCUGC
UGAUACCAAUUUAAAUAGCUGCUAAUGCUCUGACUUGCCCGGCAAGUGAUGGUG
ACUCUGUCUCCUACAGAUGCAGACAGGGAGGAUGGAGACUGGGUCAUCUGGAUG
UCCGGGCGAGCGGCAUGCAAGAGCAGGGCAAGAGGCAGAAGCAGGGCGGUCACCG
GCAGAGCCAUGGUGGCUUUACCAACAGUACCGGAAUGCCAAGCUUGGGUCUGGGC
UCUUUUAUUGAGCUCGGGGAGCAGAAGCGCGCGAACAGAAGCGAGAAGCGAACU
GAUUGGUUAGUUCAAAUAAGGCACAGGGUCAUUUCAGGUCCUUGGGGCACCCUG
GAAACAUCUGAUGGUUCUCUAGAAACUGCUGAGGGCGGGACCGCAUCUGGGGAC
CAUCUGUUCUUGGCCCUGAGCCGGGGCAGGAACUGCUUACCACAGAUAUCCUGUU
UGGCCCAUAUUCUGCUGUUCCAACUGUUCUUGGCCCUGAGCCGGGGCAGGAACUG
CUUACCACAGAUAUCCUGUUUGGCCCAUAUUCUGCUGUCUCUCUGUUCCUAACCU
UGAUCCUAGCUUGCCAAACCUACAGGUGGGGUCUUUCAUUCCCCCCUUUUUCUGG
AGACUAAAUAAAUGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCGAGAG
ACUACCACUAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA (SEQ ID NO:375)

[0757] The BCMA-CAR fusion polypeptide comprised an amino acid sequence of:

DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSR
FSGSGSGTDFTLTISSLQPEDFATYYCQQKYDLLTFGGGTKVEIKGSTSGSGKPGSGEGST
KGQLQLQESGPGLVKPSETLSLTCTVSGGSISSSSYYWGWIRQPPGKGLEWIGSISYSGST
YYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDRGDTILDVWGQGTMVTV

SSFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW APLAGTCGVLLLSLVITLYCNHRNKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEE EGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQ ALPPR (SEQ ID NO:82)

[0758] The BCMA-CAR fusion polypeptide comprised a CD8 hinge having an amino acid sequence of:

FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO:79)

[0759] The BCMA-CAR fusion polypeptide comprised a CD8 transmembrane domain having an amino acid sequence of:

IYIWAPLAGTCGVLLLSLVITLYCNHRN (SEQ ID NO:73)

[0760] The BCMA-CAR fusion polypeptide comprised a 4-1BB signaling domain having an amino acid sequence of:

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO:77)

[0761] The BCMA-CAR fusion polypeptide comprised a CD3z signaling domain having an amino acid sequence of:

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEG LYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:76)

[0762] The BCMA-CAR fusion polypeptide comprised a GSG Linker having an amino acid sequence of:

GSG

[0763] The BCMA-CAR fusion polypeptide comprised a T2A peptide having an amino acid sequence of:

EGRGSLLTCGDVEENPGP (SEQ ID NO:376)

[0764] The GFP template RNA comprised a nucleic acid sequence encoding a GFP protein having a protein sequence of:

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWP TLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEG

DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGS VQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGM DELYK (SEQ ID NO:374)

[0765] Cryopreserved primary T cells from a single human donor (HemaCare, donor D328798) were thawed and transferred into PBS with 2% BSA by volume. Cells were counted on a Cellometer K2 Cell Counter (Nexcelom), then pelleted by centrifugation at 400xg for 5 minutes and resuspended in complete T cell media at a density of $1x10^6$ cells/mL. The complete T cell media was comprised of TexMACS basal media (Miltenyi #130-097-196) with 5% human serum AB (Gemini Bio #100-512) and the following cytokines: human IL-2 at 20ng/mL (Miltenyi Biotec #130-097-746), human IL-7 at 10ng/mL (Miltenyi Biotec #130-095-362), and human IL-15 at 5ng/mL (Miltenyi Biotec #130-095-764). TransAct Research Grade T cell activation reagent (Miltenyi Biotec #130-111-160) was added to cell suspension at a volume of 10uL per $1x10^6$ cells. Activated cells were transferred to flasks and incubated at 37°C, 5% CO_2 for 48 hours. After washing in PBS, T cells were plated in 96-well flat bottom plates at a density of $2.5x10^5$ cells (100uL) per well in Xvivo15 medium (Lonza) supplemented with ApoE3 10mg/ml. Targeted LNPs comprising the first heterologous gene modifying system and tLNPs comprising the second retrotransposon gene modifying system (comprising mRNA encoding gene modifying polypeptide and a template RNA encoding GFP or the CAR) were co-administered to the plated human T cells and mixed. The tLNPs containing the first heterologous gene modifying system were formulated at a mass ratio of 1:0.5:1.25 of gene modifying polypeptide mRNA:RNACS7755:RNACS5630. LNPs containing the first heterologous gene modifying system were used at a dose of 8 µg of LNP per 1 $x10^6$ cells. The tLNPs containing the second retrotransposon gene modifying system were co-mixed such that there was a mass ratio of 1:4 of gene modifying polypeptide mRNA:template for the gene modifying system comprising GFP template and there was a mass ratio of 1:4-8 of gene modifying polypeptide mRNA:template for the gene modifying system comprising the CAR template. LNP containing the second retrotransposon gene modifying system were used at a total dose (template and polypeptide) of 2.5-1.25 µg of LNP per 1 $x10^6$ cells for GFP and 5 µg of LNP per 1 $x10^6$ cells for CAR.

[0766] Transfected cells were returned to incubation at 37°C, 5% CO_2 for four hours, washed in PBS and then cultured for additional 4 days in TexMACS basal media supplemented with 5% human serum AB and the following cytokines: human IL-2 at 20ng/mL, human IL-7 at 10ng/mL, and human IL-15 at 5ng/mL.

Flow Cytometry

[0767] To determine whether insertion by the second retrotransposon gene modifying system was successful, flow cytometry was used to determine the fraction of cells expressing GFP or BCMA CAR as detected by staining cells with fluorescently labeled Recombinant Human BCMA Protein. To determine whether editing of the TRAC locus by the first heterologous gene modifying system was successful, CD3 levels were measured by flow cytometry as a surrogate readout. Functional loss of TRAC is known to be associated with loss of surface expression of the CD3 complex. To determine whether editing of the B2M locus by the first heterologous gene modifying system was successful, expression of B2M was measured by flow cytometry.

Results

[0768] **FIG. 15A** shows the levels of editing by the first heterologous gene modifying system and the second retrotransposon gene modifying system in the bulk T cell population when the cells were co-transfected with tLNPs formulated with a payload comprising the first heterologous gene modifying system and tLNPs formulated with a payload comprising the second retrotransposon gene modifying system designed to integrate a GFP sequence into the genome of cells. The results showed that an average of 51.9% of the bulk T cells lacked B2M and TRAC by flow cytometry, showing that those loci were edited to reduce function, and also expressed GFP, showing that the exogenous GFP-encoding nucleic acid was inserted into the genomes of the cells. Overall, 87.2% of cells were edited such that they lacked B2M; 84% were edited such that they lacked TRAC; and 63.5% of cells were modified such that they expressed GFP.

[0769] **FIG. 15B** shows the levels of editing by the first heterologous gene modifying system and the second retrotransposon gene modifying system in the bulk T cell population when the cells were co-transfected with tLNPs formulated with a payload comprising the first heterologous gene modifying system and tLNPs formulated a payload comprising the second retrotransposon gene modifying system designed to integrate a CAR sequence into the genome of cells. The results showed that an average of 19.5% of the bulk T cells were edited such that they lacked B2M and TRAC while also expressing the CAR. Overall, 76.5% of cells were edited such that they lacked B2M; 81% were edited such that they lacked TRAC; and 28.8% of cells were modified such that they expressed the CAR.

Example 12: Synthesis of ionizable lipids

[0770] A number of ionizable lipids were synthesized for formulation in LNPs that were tested for delivery to HSPCs.

[0771] **General:** All reactions were run using anhydrous grade solvents under an atmosphere of nitrogen in flasks or vials with magnetic stirring, unless otherwise noted. Anhydrous solvents were from Wuxi EHS and used as received. Column silica gel chromatography using silica gel 100-200 plates, and compounds were visualized using phosphomolybdic acid. Nuclear magnetic resonance (NMR) spectroscopy was performed either using 400 MHz spectrometer; chemical shifts are reported in & parts per million (ppm) referenced to tetramethylsilane at $\delta = 0.00$ ppm for $CDCl_3$ samples, and residual solvent peak ($\delta = 7.27$ ppm) for $CDCl_3$ samples. Liquid Chromatography-Mass Spectrum (LC-MS) was performed using a detector (ESI*) using one of the following general methods.

[0772] **Method A** (20 min run): 60-100AB-20MIN_Phenyl-Hexyl-ELSD-3 : LC/MS (The gradient was 60% B in 0-0.4 min, 60-100% B in 0.4-17 min, 100% B in 17-20 min, 60% B in 0.01, the flow rate was 0.8 ml/min. Mobile phase A was 0.05% Trifluoroacetic Acid in water, mobile phase B was 0.05% Trifluoroacetic Acid in acetonitrile. The column used for chromatography was a Waters XSelect CSH Phenyl-Hexyl 150*4.6mm, 3.5 um. Detection methods are diode array (DAD) and evaporative light scattering (ELSD) detection as well as positive electrospray ionization. MS range was 100-2000.

[0773] **Method B** (22 min run): 50-100AB-22MIN-CSH_Phenyl-Hexyl-ELSD-3M : LC/MS (The gradient was 50% B in 0-0.4 min, 50-100% B in 0.4-17 min, 100% B in 17-20 min, 50% B in 0.01, the flow rate was 0.8 ml/min. Mobile phase A was 0.05% Trifluoroacetic Acid in water, mobile phase B was 0.05% Trifluoroacetic Acid in acetonitrile. The column used for chromatography was a Waters XSelect CSH Phenyl-Hexyl 150*4.6mm, 3.5 um. Detection methods are diode array (DAD) and evaporative light scattering (ELSD) detection as well as positive electrospray ionization. MS range was 100-2000.

[0774] **Method C** (10 min run): WUXIAB60-Phenyl-Hexyl-10MIN-1: LCMS (The gradient was 60% B in 0.4 min and 60-100% B at 0.4-8.0 min, hold at 100% B for 1 min, 100-0% B in 0.01 min, and then held at 60% B for 2min. Mobile phase A was 0.05% Trifluoroacetic Acid in water, mobile phase B was 0.05% Trifluoroacetic Acid in acetonitrile. The column used for chromatography was a Waters XSelect CSH Phenyl-Hexyl 50*2.1mm, 3.5 um. Detection methods are ELSD. The ESI MS range was 100-2000.

[0775] **Method D** (10 min run): WUXIAB40-Phenyl -Hexyl_10min : LC/MS (The gradient was 40% B in 0-0.4 min, 40-100% B in 0.4-8 min, 100% B in 8-9 min, 40% B in 9.01-10 min, the flow rate was 0.8 ml/min. Mobile phase A was 0.05% Trifluoroacetic Acid in water, mobile phase B was 0.05% Trifluoroacetic Acid in acetonitrile. The column used for chromatography

was a Waters XSelect CSH Phenyl-Hexyl 50*2.1mm, 3.5 um. Detection methods are diode array (DAD) and evaporative light scattering (ELSD) detection as well as positive electrospray ionization. MS range was 100-2000.

List of abbreviations

DCM: dichloromethane

DIEA: N,N-diisopropylethylamine.

DMAP: 4-(dimethylamino)pyridine

EDCI: 3-(ethyliminomethylideneamino)propyl-dimethylazanium;chloride

Eq or eqv: equivalents

Py: pyridine

Lipid 092

General procedure for preparation of compound 3

[0776] Compound 3 was prepared according to the synthetic scheme shown in **FIG. 24A**. In a 250 mL round-bottom flask equipped with a stir bar, (9Z,12Z)-octadeca-9,12-dienoic acid (20 g, 71.32 mmol, 20.00 mL, 1.0 eq) was dissolved in DCM (200 mL). DIEA (13.83 g, 107.0 mmol, 18.63 mL, 1.5 eq), EDCI (20.51 g, 107.0 mmol, 1.5 eq) and DMAP (1.74 g, 14.26 mmol, 0.2 eq) was added at 20°C, followed by (2,2-dimethyl-1,3-dioxan-5-yl)methanol (12.51 g, 85.58 mmol, 1.2 eq). The mixture was degassed and purged with N_2 for 3 times and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 5:1, the starting material R_f = 0.3, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with H_2O (50 mL) and extracted with DCM (100 mL * 2). The combined organic layers were washed with brine (50 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue (38 g). DCM (100 mL) and 60 g of silica was added to the residue. Solvent was concentrated under reduced pressure again. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 100:1 (800 mL, impurity eluted) to 80:1 to 10:1 (product eluted)) to give (2,2-dimethyl-1,3-dioxan-5-yl)methyl (9Z,12Z)-octadeca-9,12-dienoate (compound 3) (20 g, 48.95 mmol, 68.6% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 5.43 - 5.30 (m, 4H), 4.17 (d, *J* = 7.0 Hz, 2H), 3.99 (dd, *J* = 4.2, 12.0 Hz, 2H), 3.74 (dd, *J* = 6.0, 12.0 Hz, 2H), 2.78 (t, *J* = 6.4 Hz, 2H), 2.31 (t, *J* = 7.6 Hz, 2H), 2.10 - 1.98 (m, 5H), 1.67 - 1.60 (m, 2H), 1.46 - 1.26 (m, 21H), 0.90 (t, *J* = 6.8 Hz, 3H).

General procedure for preparation of compound 4

[0777] Compound 4 was prepared according to the synthetic scheme shown in **FIG. 24B**. In a 250 mL round-bottom flask equipped with a stir bar, compound 3 (13.0 g, 31.81 mmol, 1.0 eq) was dissolved in isopropanol (65 mL). HCl (1 M, 65 mL) was added to the mixture by dropwise. The mixture was stirred at 15°C for 0.5 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 1:1, the starting material R_f = 0.7, product R_f = 0.5) showed the reaction was completed. Then saturated $NaHCO_3$ solution (100 mL) was added to the mixture by dropwise (pH = 7) and extracted with DCM (100 mL * 2). The combined organic layers were washed with brine (100 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue (11 g). DCM (60 mL) and 20 g of silica was added to the residue. Solvent was concentrated under reduced pressure again. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 50:1 (800 mL), 30:1 (800 mL, impurity eluted), 10:1, product 5:1 to 1:1 (product eluted)) to give [3-hydroxy-2-(hydroxymethyl)propyl] (9Z,12Z)-octadeca-9,12-dienoate (compound 4) (9.0 g, 24.42 mmol, 76.8% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 5.42 - 5.30 (m, 4H), 4.26 (d, *J* = 6.4 Hz, 2H), 3.77 (dq, *J* = 5.2, 10.9 Hz, 4H), 2.78 (t, *J* = 6.4 Hz, 2H), 2.34 (t, *J* = 7.6 Hz, 4H), 2.18 (s, 1H), 2.06 (q, *J* = 7.0 Hz, 5H), 1.63 (br t, *J* = 7.2 Hz, 2H), 1.40 - 1.25 (m, 15H), 0.90 (t, *J* = 6.8 Hz, 3H).

General procedure for preparation of compound 6

[0778] Compound 6 was prepared according to the synthetic scheme shown in **FIG. 24C**. In a 100 mL round-bottom flask equipped with a stir bar, 4,4-dioctoxybutanoic acid (4.49 g, 13.02 mmol, 1.2 eq) was dissolved in DCM (40 mL). Then DIEA (2.81 g, 21.71 mmol, 3.78 mL, 2.0 eq), DMAP (265.2 mg, 2.17 mmol, 0.2 eq) and EDCI (4.16 g, 21.71 mmol, 2.0 eq) was added to the mixture in order at 20°C. Then compound 4 (4.0 g, 10.85 mmol, 1.0 eq) was added by one portion at 20°C. The mixture was degassed and purged with N_2 for 3 times, and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 3:1, the starting material R_f = 0.3, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with H_2O (20 mL) and extracted with DCM (30 mL * 2). The combined organic layers were washed with brine (30 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 30:1 (400 mL) to 20:1 (400 mL, impurity eluted) to 10:1 ~ 4:1 (product eluted)) to give [2-(4,4-dioctoxybutanoyloxymethyl)-3-hydroxy-propyl] (9Z,12Z)-octadeca-9,12-dienoate (compound 6) (2.5 g, 3.60 mmol, 33.1% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 5.43 - 5.30 (m, 4H), 4.50 (s, 1H), 4.25 - 4.12 (m, 4H), 3.67 - 3.54 (m, 4H), 3.46 - 3.36 (m, 2H), 2.78 (t, *J* = 6.6 Hz, 2H), 2.45 - 2.37 (m, 2H), 2.33 (t, *J* = 7.6 Hz, 2H), 2.20 (s, 1H), 2.06 (q, *J* = 6.8 Hz, 4H), 1.99 - 1.89 (m, 2H), 1.67 - 1.46 (m, 7H), 1.39 - 1.24 (m, 35H), 0.94 - 0.84 (m, 9H).

General procedure for preparation of compound 9

[0779] Compound 9 was prepared according to the synthetic scheme shown in **FIG. 24D**. In a 500 mL round-bottom flask equipped with a stir bar, benzyl 4-bromobutanoate (20 g, 77.78 mmol, 1.0 eq) was dissolved in DCM (200 mL). Then N-ethylethanamine (19.91 g, 272.2 mmol, 28.04 mL, 3.5 eq) was added at 20°C. The mixture was stirred at 45°C for 16 hr. TLC (Petroleum ether: Ethyl acetate = 1:1, the starting material R_f = 0.7, product R_f = 0.3) showed the reaction was completed. The reaction mixture was diluted with H_2O 20 mL and extracted with DCM (30mL * 2). The combined organic layers were washed with brine (30 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue (40 g). DCM (100 mL) and 80 g of silica was added to the residue. Solvent was concentrated under reduced pressure again. The residue was purified by column chromatography (SiO_2, 0.25% TEA alkalized silica gel column) petroleum ether: ethyl acetate = from 80:1 to 30:1 to remove impurities, from 30:1 to 0:1 and DCM:MeOH = 100:1 to 10:1 to get product.) to give benzyl 4-(diethylamino)butanoate (compound 9) (18.0 g, 72.19 mmol, 92.8% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 7.39 - 7.32 (m, 5H), 5.12 (s, 2H), 2.54 - 2.37 (m, 8H), 1.84 - 1.75 (m, 2H), 1.00 (t, *J* = 7.2 Hz, 6H).

General procedure for preparation of compound 10

[0780] Compound 10 was prepared according to the synthetic scheme shown in **FIG. 24E**. In a 250 mL round-bottom flask equipped with a stir bar, compound 9 (11 g, 44.12 mmol, 1.0 eq) was dissolved in EtOH (110 mL). Pd/C (11.0 g, 10% purity) was added under N_2 atmosphere. The suspension was degassed under vacuum and purged with H_2 for 3 times. The mixture was stirred under H_2 (15 psi) at 15°C for 2 hr. TLC (Dichloromethane: Methanol = 10:1, the starting material R_f = 0.8, product R_f = 0.3) showed the reaction was completed. The mixture was filtered through a pad of Celite and the filter cake was washed with EtOH (10 mL * 3). The filtrate was combined and concentrated under reduced pressure to give 4-(diethylamino)butanoic acid (compound 10) (7 g, crude) as colorless oil. There was no further purification, the product was used directly in next step. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 2.90 - 2.76 (m, 6H), 2.62 - 2.52 (m, 2H), 1.89 - 1.80 (m, 2H), 1.22 (t, *J* = 7.4 Hz, 6H)

General procedure for preparation of Lipid 092

[0781] Lipid 092 was prepared according to the synthetic scheme shown in **FIG. 24F**. In a 100 mL round-bottom flask equipped with a stir bar, compound 10 (3.21 g, 20.14 mmol, 2.0 eq) was dissolved in DCM (70 mL). DIEA (2.60 g, 20.14 mmol, 3.51 mL, 2.0 eq), and DMAP (615.2 mg, 5.04 mmol, 0.5 eq) and EDCI (3.86 g, 20.14 mmol, 2.0 eq) was added in order at

20°C. Then compound 6 (7.0 g, 10.07 mmol, 1 eq) was added to the mixture by one portion at 20°C. The mixture was degassed and purged with N_2 for 3 times, and then the mixture was stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Dichloromethane: Methanol = 10:1, the starting material R_f = 0.7, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with H_2O 50 mL and extracted with DCM (50 mL * 2). The combined organic layers were washed with brine (50 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = from 100:1 to 5:1 to remove impurities, from 5:1 to 3:1 to get product) for 3 times to give Lipid 092 (15.4 g, 18.42 mmol, 81.1% yield) as light-yellow oil. LCMS (Method A): Rt 12.11 min, m/z calculated [M+H]: 836.7, found: 836.7. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 5.42 - 5.30 (m, 4H), 4.49 (t, *J* = 5.4 Hz, 1H), 4.13 (d, *J* = 5.8 Hz, 6H), 3.60 - 3.53 (m, 2H), 3.44 - 3.37 (m, 2H), 2.78 (t, *J* = 6.6 Hz, 2H), 2.54 - 2.29 (m, 13H), 2.06 (q, *J* = 6.8 Hz, 4H), 1.96 - 1.89 (m, 2H), 1.81 - 1.71 (m, 2H), 1.66 - 1.50 (m, 7H), 1.39 - 1.23 (m, 34H), 1.01 (t, *J* = 7.1 Hz, 6H), 0.95 - 0.83 (m, 9H).

Lipid 093

General procedure for preparation of compound 13

[0782] Compound 13 was prepared according to the synthetic scheme shown in **FIG. 25A**. To a mixture of decanedioic acid (40 g, 197.78 mmol, 1.0 eq) in DCM (400 mL) was added DIEA (30.67 g, 237.3 mmol, 41.34 mL, 1.2 eq), DMAP (4.83 g, 39.56 mmol, 0.2 eq) and EDCI (37.91 g, 197.78 mmol, 1.0 eq) at 20°C. Then 2-butyloctan-1-ol (29.48 g, 158.2 mmol, 0.8 eq) was added by one portion at 20°C. The resulting mixture was degassed and purged with N_2 for 3 times and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 5:1, the starting material R_f = 0.8, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with H_2O (200 mL) and extracted with DCM (200 mL * 2). The combined organic layers were washed with brine (200 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 80:1 to 10:1 remove impurities, 10:1 to 1:1 to get product) to give 10-(2-butyloctoxy)-10-oxo-decanoic acid (compound 13) (50 g, 134.9 mmol, 34.1% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 3.98 (d, *J* = 5.8 Hz, 2H), 2.33 (td, *J* = 7.6, 19.2 Hz, 4H), 1.68 - 1.47 (m, 6H), 1.37 - 1.23 (m, 24H), 0.96 - 0.84 (m, 6H).

General procedure for preparation of compound 14

[0783] Compound 14 was prepared according to the synthetic scheme shown in **FIG. 25B**. Compound 4 was prepared according to the synthetic scheme shown in **FIG. 24B**. In a 500 mL

round-bottom flask equipped with a stir bar, compound 13 (22.0 g, 59.37 mmol, 1 eq) was dissolved in DCM (220 mL). Then DIEA (11.51 g, 89.05 mmol,15.51 mL, 1.5 eq), DMAP (2.18 g, 17.81 mmol, 0.3 eq) and EDCI (17.07 g, 89.05 mmol, 1.5 eq) was added in order at 20°C. Then compound 4 (21.88 g, 59.37 mmol, 1 eq) was added at 20°C. The mixture was degassed and purged with N_2 for 3 times, and then stirred at 20°C for 16 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 3:1, the starting material R_f = 0.65, product R_f = 0.55) showed the reaction was completed. The reaction mixture was diluted with H_2O (100 mL) and extracted with DCM (100 mL * 3). The combined organic layers were washed with brine (50 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = from 80:1 to 10:1 to remove impurities; from 10:1 to 5:1 to get product) to give O1-(2-butyloctyl) O10-[2-(hydroxymethyl)-3-[(9Z,12Z)-octadeca-9,12-dienoyl]oxy-propyl] decanedioate (compound 14) (40 g, 55.47 mmol, 93.4% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 5.42 - 5.30 (m, 4H), 4.23 - 4.14 (m, 4H), 3.97 (d, *J* = 6.0 Hz, 2H), 3.62 (d, *J* = 5.4 Hz, 2H), 2.78 (t, *J* = 6.4 Hz, 2H), 2.36 - 2.27 (m, 6H), 2.25 - 2.14 (m, 1H), 2.06 (q, *J* = 6.6 Hz, 4H), 1.62 (br d, J = 4.8 Hz, 8H), 1.49 - 1.36 (m, 3H), 1.34 - 1.25 (m, 35H), 0.94 - 0.86 (m, 9H).

General procedure for preparation of Lipid 093

[0784] Lipid 093 was prepared according to the synthetic scheme shown in **FIG. 25C**. In a 500 mL round-bottom flask equipped with a stir bar, compound 14 (10 g, 13.87 mmol, 1.0 eq) was dissolved in DCM (100 mL). Then Py (1.76 g, 22.19 mmol, 1.79 mL, 1.6 eq), DMAP (508.26 mg, 4.16 mmol, 0.3 eq) were added, followed by (4-nitrophenyl) carbonochloridate (4.47 g, 22.19 mmol, 1.6 eq) at 20°C. The mixture was degassed and purged with N_2 for 3 times and stirred at 20°C for 1 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate= 3:1, the starting material R_f = 0.5, product R_f = 0.7) showed the reaction was completed. 3-(diethylamino)propan-1-ol (5.42 g, 41.30 mmol, 6.16 mL, 3.0 eq) was added to the reaction mixture. The mixture was stirred at 20°C for another 16 hr. TLC (Dichloromethane: Methanol = 10:1, the starting material Cpd.12: R_f = 0.7, product Lipid 093: R_f = 0.5) showed the reaction was completed. The mixture was diluted with H_2O (150 mL) and extracted with DCM (100 mL * 2). The organic phase was separated, washed with Na_2CO_3 solution (100 mL * 5), brine (100 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = from 100:1 to 5:1 to remove impurities, from 5:1 to 3:1 to get product) for two times to give Lipid 093 (15.6 g, 17.76 mmol, 64.5% yield, 100% purity) as colorless oil. LCMS (Method A): Rt 11.80 min, m/z calculated $[M+H]^+$: 878.7, found: 878.7. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 5.42

- 5.30 (m, 4H), 4.23 - 4.12 (m, 8H), 3.97 (d, J = 5.8 Hz, 2H), 2.78 (t, J = 6.4 Hz, 2H), 2.62 - 2.49 (m, 6H), 2.49 - 2.38 (m, 1H), 2.35 - 2.27 (m, 6H), 2.06 (q, J = 6.8 Hz, 4H), 1.91 - 1.69 (m, 2H), 1.62 (br s, 8H), 1.38 - 1.24 (m, 40H), 1.04 (br t, J = 6.8 Hz, 6H), 0.94 - 0.86 (m, 9H).

Lipid 162

General procedure for preparation of compound 19

[0785] Compound 19 was prepared according to the synthetic scheme shown in **FIG. 26A**. To a solution of 3-(2-carboxyethyldisulfanyl) propanoic acid (2 g, 9.50 mmol, 1.0 eq) in DCM (20.0 mL) was added DIEA (1.48 g, 11.41 mmol, 1.99 mL, 1.2 eq), DMAP (581.0 mg, 4.76 mmol, 0.5 eq) and EDCI (1.82 g, 9.51 mmol, 1.0 eq). Then heptadecan-9-ol (2.44 g, 9.51 mmol, 1.0 eq) was added at 20°C. The mixture was stirred at 45°C for 16 hr. TLC (Petroleum ether: Ethyl acetate = 2:1, product R_f = 0.3) showed heptadecan-9-ol was consumed completely. The reaction mixture was quenched by addition H_2O (20 mL), and then extracted with DCM (20 mL * 3). The organic layers were dried over Na_2SO_4, the filtrate was filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, Petroleum ether: Ethyl acetate = 1:0 to 0:1) to give 3-[[3-(1-octylnonoxy)-3-oxo-propyl]disulfanyl]propanoic acid (compound 19) (5.30 g, 11.81 mmol, 41.4% yield) as yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.87 (t, J = 6.2 Hz, 1H), 3.66 (m, J = 8.0, 13.4 Hz, 3H), 3.09 (m, J = 8.0 Hz, 3H), 2.91 (m, J = 4.0, 7.2 Hz, 4H), 2.76 - 2.65 (m, 4H), 1.68 - 1.38 (m, 26H), 1.35 - 1.16 (m, 26H), 0.86 (t, J = 8.0 Hz, 6H).

General procedure for preparation of compound 21

[0786] Compound 21 was prepared according to the synthetic scheme shown in **FIG. 26B**. To a solution of compound 19 (4.20 g, 9.36 mmol, 1.0 eq) in DCM (40 mL) was added DMAP (228.7 mg, 1.87 mmol, 0.2 eq), DIEA (3.63 g, 28.08 mmol, 4.89 mL, 3.0 eq) and EDCI (4.49 g, 23.40 mmol, 2.5 eq) at 20°C. Then 2-(hydroxymethyl) propane-1,3-diol (496.6 mg, 4.68 mmol, 0.5 eq) was added by one portion at 20°C. The mixture was stirred at 40°C for 16 hr. TLC (Petroleum ether: Ethyl acetate = 3:1, product R_f = 0.30) showed compound 19 was consumed completely. The reaction mixture was quenched by addition of H_2O (40 mL) and extracted with DCM (50 mL * 3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, Petroleum ether: Ethyl acetate = 1:0 to 0:1) to give compound 21 (0.745 g, 462.0 μmol, 10% yield, 60% purity) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.90 (t, J = 6.2 Hz, 2H), 4.28 - 4.19 (m, 4H), 3.67 (t, J = 5.8 Hz, 2H), 2.94 (t, J = 8.0 Hz, 8H), 2.82 -

2.69 (m, 8H), 2.26 - 2.16 (m, 2H), 1.59 - 1.49 (m, 10H), 1.34 - 1.23 (m, 50H), 0.93 - 0.85 (m, 12H).

General procedure for preparation of Lipid162

[0787] Lipid 162 was prepared according to the synthetic scheme shown in **FIG. 26C**. To a solution of compound 21 (0.5 g, 516.8 μmol, 1.0 eq) in DCM (5 mL) was added Py (65.40 mg, 826.9 μmol, 66.74 μL, 1.6 eq) and DMAP (18.94 mg, 155.0 μmol, 0.3 eq). Then (4-nitrophenyl) carbonochloridate (166.7 mg, 826.9 μmol, 1.6 eq) was added to the mixture at 20°C. The resulting mixture was stirred at 20°C for 2 hr. TLC (Petroleum ether: Ethyl acetate = 2:1, product R_f = 0.5) showed the reaction was completed. 3-(diethylamino)propan-1-ol (208.5 mg, 1.59 mmol, 237.0 μL, 3.0 eq) was added to the reaction mixture at 20°C by one portion. The mixture was stirred at 20°C for another 16 hr. TLC (Dichloromethane: Methanol = 10:1, product R_f = 0.4) showed the reaction was completed. The reaction mixture was quenched by addition H_2O (10 mL) and extracted with DCM (10 mL * 3). The combined organic layers were dried over Na_2SO_4, filtered and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, Petroleum ether: Ethyl acetate = 1:0 to 0:1) for 3 times to give Lipid162 (0.245 g, 215.7 μmol, 40.7% yield, 99% purity) as colorless oil. ^{1}H NMR (400 MHz, CDCl3-*d*) δ 4.89 (m, *J* = 4.0 Hz, 2H), 4.26 - 4.15 (m, 8H), 3.00 - 2.85 (m, 8H), 2.81 - 2.67 (m, 8H), 2.56 (m, *J* = 4.0 Hz, 6H), 2.51 - 2.40 (m, 1H), 1.91 - 1.78 (m, 2H), 1.52 (m, *J* = 5.5 Hz, 8H), 1.26 (br s, 48H), 1.08 - 0.99 (m, 6H), 0.88 (t, *J* = 8.0 Hz, 12H). LCMS (Method A): Rt 14.42 min, m/z calculated $[M+H]^+$: 1124.7, found: 1124.7.

Lipid 153

General procedure for preparation of compound 24

[0788] Compound 24 was prepared according to the synthetic scheme shown in **FIG. 27A**. A mixture of tetrahydrofuran-2,5-dione (2.0 g, 19.99 mmol, 1.0 eq), heptadecan-9-ol (5.13 g, 19.99 mmol, 1.0 eq) in Tol. (20 mL) was added Py (316.2 mg, 4.00 mmol, 322.62 μL, 0.2 eq). The mixture was degassed and purged with N_2 for 3 times, and then the mixture was refluxed for 16 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 5:1, product R_f = 0.5) showed the reaction was completed. The reaction mixture was quenched by addition of HCl (1M, 3 mL) at 20°C, and then diluted with H_2O (15 mL) and extracted with EtOAc (15 mL * 3). The combined organic layers were washed with brine (20 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, Petroleum ether: Ethyl acetate = 80:1 to 5:1) to give 4-(1-octylnonoxy)-4-oxo-butanoic acid (compound 24) (3.6 g, 10.10 mmol, 50.5% yield, 100% purity) as colorless

oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.90 (t, *J* = 6.2 Hz, 1H), 2.71 - 2.60 (m, 4H), 1.52 (br d, *J* = 5.8 Hz, 4H), 1.33 - 1.21 (m, 24H), 0.93 - 0.85 (m, 6H).

General procedure for preparation of compound 25

[0789] Compound 25 was prepared according to the synthetic scheme shown in **FIG. 27B**. Compound 4 was prepared according to the synthetic scheme shown in **FIG. 24B**. To a solution of compound 24 (1.5 g, 4.21 mmol, 1.0 eq) in DCM (15 mL) was added DIEA (815.6 mg, 6.31 mmol, 1.10 mL, 1.5 eq), DMAP (102.8 mg, 841.4 μmol, 0.2 eq) and EDCI (1.21 g, 6.31 mmol, 1.5 eq). Then compound 4 (1.55 g, 4.21 mmol, 1.0 eq) was added by one portion. The mixture was stirred at 25°C for 16 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 5:1, the starting material R_f = 0.52, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with H_2O (20 mL) and extracted with DCM (20 mL * 2). The combined organic layers were washed with brine (20 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 100:1 to 3:1) to give O1-[2-(hydroxymethyl)-3-[(9Z,12Z)-octadeca-9,12-dienoyl]oxy-propyl] O4-(1-octylnonyl) butanedioate (compound 25) (1.48 g, 2.09 mmol, 49.8% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 5.43 - 5.30 (m, 4H), 4.88 (t, *J* = 6.2 Hz, 1H), 4.27 - 4.14 (m, 4H), 3.63 (br d, *J* = 5.0 Hz, 2H), 2.78 (t, *J* = 6.4 Hz, 2H), 2.69 - 2.58 (m, 4H), 2.33 (t, *J* = 7.6 Hz, 2H), 2.26 - 2.14 (m, 2H), 2.06 (q, *J* = 6.8 Hz, 4H), 1.69 - 1.56 (m, 3H), 1.56 - 1.52 (m, 2H), 1.40 - 1.22 (m, 40H), 0.95 - 0.83 (m, 9H).

General procedure for preparation of Lipid153

[0790] Lipid153 was prepared according to the synthetic scheme shown in **FIG. 27C**. Compound 10 was prepared according to the synthetic scheme shown in **FIG. 24E**. To the mixture of compound 10 (225.2 mg, 1.41 mmol, 2.0 eq) in DCM (5 mL) was added DIEA (182.8 mg, 1.41 mmol, 246.3 μL, 2.0 eq), DMAP (43.19 mg, 353.6 μmol, 0.5 eq) and EDCI (271.1 mg, 1.41 mmol, 2.0 eq) in order. Then compound 25 (500 mg, 707.1 μmol, 1.0 eq) was added by one portion. The mixture was stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Dichloromethane: Methanol = 10:1, the starting material R_f = 0.7, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with H_2O (15 mL) and extracted with DCM (20 mL * 2). The combined organic layers were washed with brine (20 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = from 80:1 to 10:1 to remove impurities, from 10:1 to 3:1 to get product) for 3 times to give Lipid153 (221.8 mg, 261.5 μmol,

37.0% yield) as colorless oil. LCMS (Method A): Rt 12.05 min, m/z calculated $[M+H]^+$: 848.7, found: 848.7. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 5.42 - 5.30 (m, 4H), 4.88 (t, *J* = 6.2 Hz, 1H), 4.18 - 4.11 (m, 6H), 2.78 (t, *J* = 6.4 Hz, 2H), 2.67 - 2.59 (m, 4H), 2.54 - 2.29 (m, 11H), 2.06 (q, *J* = 6.8 Hz, 4H), 1.81 - 1.72 (m, 2H), 1.66 - 1.56 (m, 3H), 1.56 - 1.52 (m, 2H), 1.40 - 1.23 (m, 39H), 1.01 (t, *J* = 7.2 Hz, 6H), 0.93 - 0.85 (m, 9H).

Lipid 154

General procedure for preparation of compound 5

[0791] Compound 5 was prepared according to the synthetic scheme shown in **FIG. 28A**. KOH (25.85 g, 460.8 mmol, 3.0 eq) was dissolved in H_2O (150 mL), the resulting solution was added to a solution of 4,4-dioctoxybutanenitrile (50 g, 153.6 mmol, 1.0 eq) in EtOH (150 mL). The mixture was stirred at 110°C for 20 hr. TLC (Petroleum ether: Ethyl acetate = 10:1, the starting material R_f = 0.7, product R_f = 0.5) showed the reaction was completed. The reaction mixture was concentrated under reduced pressure to remove EtOH. The residue was diluted with water (10 mL). The aqueous layer was acidified to pH=5 with (HCl, 1 M, 50 mL), and extracted with EtOAc (50 mL * 3). The combined organic layers were washed with brine (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give compound 5 (50 g, crude) as colorless oil. There was no further purification, the product was used directly in next step. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 5.28 (br s, 2H), 4.63 (br s, 3H), 4.45 (br s, 1H), 3.57 - 3.37 (m, 4H), 2.13 (br t, J = 7.6 Hz, 2H), 1.87 - 1.65 (m, 2H), 1.55 (br d, J = 6.0 Hz, 4H), 1.36 - 1.25 (m, 19H), 0.96 - 0.82 (m, 6H).

General procedure for preparation of compound 29

[0792] Compound 29 was prepared according to the synthetic scheme shown in **FIG. 28B**. To a solution of octanedioic acid (10 g, 57.41 mmol, 1.0 eq) in DCM (100 mL) was added DIEA (11.13 g, 86.11 mmol, 15.0 mL, 1.5 eq), DMAP (1.40 g, 11.48 mmol, 0.2 eq) and EDCI (16.51 g, 86.11 mmol, 1.5 eq) in order. Then tridecan-7-ol (11.50 g, 57.41 mmol, 1.0 eq) was added by one portion at 20°C. The mixture was degassed and purged with N_2 for 3 times and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 5:1, product R_f = 0.5) showed the reaction was completed. The residue was extracted with DCM (50 mL * 3) and H_2O (50 mL). The combined organic layers were washed with brine (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, Petroleum ether: Ethyl acetate = 30:1 to 1:1) to give 8-(1-hexylheptoxy)-8-oxo-octanoic acid (compound 29) (10 g, 28.05 mmol, 48.9%

yield) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.87 (t, *J* = 6.2 Hz, 1H), 2.39 - 2.25 (m, 4H), 1.70 - 1.57 (m, 4H), 1.56 - 1.51 (m, 2H), 1.41 - 1.22 (m, 22H), 0.88 (t, *J* = 6.8 Hz, 6H).

General procedure for preparation of compound 30

[0793] Compound 30 was prepared according to the synthetic scheme shown in **FIG. 28C**. To a mixture of compound 29 (8 g, 22.44 mmol, 1.0 eq) in DCM (80 mL) was added DIEA (4.35 g, 33.66 mmol, 5.86 mL,1.5 eq), DMAP (548.2 mg, 4.49 mmol, 0.2 eq) and EDCI (6.45 g, 33.66 mmol, 1.5 eq). Then (2,2-dimethyl-1,3-dioxan-5-yl)methanol (3.94 g, 26.93 mmol, 1.2 eq) was added. The mixture was degassed and purged with N_2 for 3 times and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 5:1, the starting material R_f = 0.3, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with H_2O (50 mL) and extracted with DCM (100 mL * 2). The combined organic layers were washed with brine (50 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 100:1 to 3:1) to give O1-[(2,2-dimethyl-1,3-dioxan-5-yl)methyl] O8-(1-hexylheptyl) octanedioate (compound 30) (9.7 g, 20.01 mmol, 89.2% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.87 (t, *J* = 6.2 Hz, 1H), 4.17 (d, *J* = 7.0 Hz, 2H), 4.00 (dd, *J* = 4.0, 12.0 Hz, 2H), 3.74 (dd, *J* = 5.8, 12.0 Hz, 2H), 2.30 (td, *J* = 7.6, 12.6 Hz, 4H), 2.01 (br s, 1H), 1.69 - 1.64 (m, 1H), 1.62 - 1.48 (m, 6H), 1.43 (d, *J* = 13.2 Hz, 6H), 1.37 - 1.22 (m, 20H), 0.93 - 0.84 (m, 6H).

General procedure for preparation of compound 31

[0794] Compound 31 was prepared according to the synthetic scheme shown in **FIG. 28D**. To a solution of O1-[(2,2-dimethyl-1,3-dioxan-5-yl)methyl] O8-(1-hexylheptyl) octanedioate (7.7 g, 15.89 mmol, 1.0 eq) in isopropanol (39 mL) was added HCl (1 M, 39 mL). The mixture was stirred at 15°C for 1 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 1:1, the starting material R_f = 0.7, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with saturated $NaHCO_3$ solution (60 mL) and extracted with DCM (50 mL * 2). The combined organic layers were washed with brine (50 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = from 50:1 to 10:1 to remove impurities, from 5:1 to 1:1 to get product) to give O8-(1-hexylheptyl) O1-[3-hydroxy-2-(hydroxymethyl)propyl] octanedioate (compound 31) (9.7 g, crude) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.87 (t, *J* = 6.4 Hz, 1H), 4.27 (d, *J* = 6.4 Hz, 2H), 3.78 (dq, *J* = 5.2, 11.1

Hz, 4H), 2.40 - 2.24 (m, 6H), 2.09 - 2.00 (m, 1H), 1.69 - 1.64 (m, 2H), 1.62 - 1.57 (m, 1H), 1.51 (br d, J = 6.2 Hz, 4H), 1.38 - 1.22 (m, 21H), 0.93 - 0.84 (m, 6H).

General procedure for preparation of compound 32

[0795] Compound 32 was prepared according to the synthetic scheme shown in **FIG. 28E**. To a solution of 4,4-dioctoxybutanoic acid (7.25 g, 21.05 mmol, 1.2 eq) in DCM (80 mL) was added DIEA (4.53 g, 35.08 mmol, 6.11 mL, 2.0 eq), DMAP (428.6 mg, 3.51 mmol, 0.2 eq) and EDCI (6.73 g, 35.08 mmol, 2.0 eq). Then compound 31 (7.8 g, 17.54 mmol, 1.0 eq) was added. The resulting mixture was degassed and purged with N_2 for 3 times and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 3:1, the starting material R_f = 0.3, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with H_2O 50 mL and extracted with DCM (50 mL * 2). The combined organic layers were washed with brine (50 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 100:1 to 3:1) to give compound 32 (8.1 g, 10.50 mmol, 59.9% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.87 (t, J = 6.4 Hz, 1H), 4.50 (t, J = 5.6 Hz, 1H), 4.23 - 4.14 (m, 4H), 3.65 - 3.54 (m, 4H), 3.41 (td, J = 6.8, 9.3 Hz, 2H), 2.42 (t, J = 7.6 Hz, 2H), 2.35 - 2.17 (m, 6H), 1.98 - 1.91 (m, 2H), 1.69 - 1.65 (m, 1H), 1.62 - 1.47 (m, 10H), 1.38 - 1.22 (m, 42H), 0.93 - 0.85 (m, 12H).

General procedure for preparation of Lipid 154

[0796] Lipid 154 was prepared according to the synthetic scheme shown in **FIG. 28F**. To a mixture of 4-(diethylamino)butanoic acid (2.48 g, 15.56 mmol, 3.0 eq) in DCM (40 mL) was added DIEA (2.01 g, 15.56 mmol, 2.71 mL, 3.0 eq), DMAP (316.8 mg, 2.59 mmol, 0.5 eq) and EDCI (2.98 g, 15.56 mmol, 3.0 eq). Then compound 32 (4 g, 5.19 mmol, 1.0 eq) was added to the mixture. The resulting mixture was degassed and purged with N_2 for 3 times, and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Dichloromethane: Methanol = 10:1, the starting material R_f = 0.7, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with H_2O (50 mL) and extracted with DCM (50 mL * 2). The combined organic layers were washed with brine (50 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = from 80:1 to 10:1 to remove impurity, from 10:1 to 3:1 to get product) for 2 times to give Lipid 154 (3.52 g, 3.86 mmol, 74.4% yield) as colorless oil. LCMS (Method C): Rt 3.47 min, m/z calculated $[M+H]^+$: 912.7, found: 912.8. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.87 (t, J = 6.4 Hz, 1H), 4.49 (t, J = 5.6 Hz, 1H), 4.13 (d, J = 5.8 Hz, 6H), 3.57 (td, J

= 6.8, 9.2 Hz, 2H), 3.41 (td, *J* = 6.8, 9.2 Hz, 2H), 2.54 - 2.26 (m, 15H), 1.97 - 1.90 (m, 2H), 1.81 - 1.70 (m, 2H), 1.68 - 1.47 (m, 14H), 1.38 - 1.22 (m, 42H), 1.01 (t, *J* = 7.2 Hz, 6H), 0.92 - 0.85 (m, 12H).

Lipid 155

General procedure for preparation of compound 34

[0797] Compound 34 was prepared according to the synthetic scheme shown in **FIG. 29A**. In a 100 mL round-bottom flask equipped with a stir bar, octanedioic acid (5.0 g, 28.70 mmol, 1.0 eq) was dissolved in DCM (50 mL). DIEA (5.56 g, 43.06 mmol, 7.50 mL, 1.5 eq), DMAP (701.3 mg, 5.74 mmol, 0.2 eq) and EDCI (8.25 g, 43.06 mmol, 1.5 eq) was added in order. Then undecan-3-ol (4.95 g, 28.70 mmol, 1.0 eq) was added to the mixture, the mixture was degassed and purged with N_2 for 3 times. The mixture was stirred at 20°C for 16 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 5:1, the starting material R_f = 0.8, product R_f = 0.5) showed undecan-3-ol was consumed completely and one major new spot was detected. The reaction mixture was diluted with H_2O (50 mL) and extracted with DCM (50 mL * 2). The combined organic layers were washed with brine (50 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 30:1 to 1:1) to give 8-(1-ethylnonoxy)-8-oxo-octanoic acid(compound 34) (3.0 g, 9.13 mmol, 31.8% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.82 (t, *J* = 6.2 Hz, 1H), 2.31 (td, *J* = 7.6, 12.1 Hz, 4H), 1.69 - 1.65 (m, 1H), 1.73 - 1.46 (m, 7H), 1.43 - 1.23 (m, 17H), 0.93 - 0.83 (m, 6H).

General procedure for preparation of compound 35

[0798] Compound 35 was prepared according to the synthetic scheme shown in **FIG. 29B**. In a 100 mL round-bottom flask equipped with a stir bar, compound 34 (2.5 g, 7.61 mmol, 1.0 eq) was dissolved in DCM (25 mL). DIEA (1.48 g, 11.42 mmol, 1.99 mL, 1.5 eq), DMAP (186.0 mg, 1.52 mmol, 0.2 eq) and EDCI (2.19 g, 11.42 mmol, 1.5 eq) was added in order. Then (2,2-dimethyl-1,3-dioxan-5-yl)methanol (1.34 g, 9.13 mmol, 1.2 eq) was added to the mixture by one portion. The mixture was degassed and purged with N_2 for 3 times and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 5:1, the starting material R_f = 0.3, product R_f = 0.5) showed starting material was consumed completely and one major new spot was detected. The reaction mixture was diluted with H_2O (25 mL) and extracted with DCM (25 mL * 2). The combined organic layers were washed with brine (25 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 100:1 to 14:1) to give O1-[(2,2-

dimethyl-1,3-dioxan-5-yl)methyl] O8-(1-ethylnonyl) octanedioate (compound 35) (3.1 g, 6.31 mmol, 83.0% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.82 (quin, *J* = 6.2 Hz, 1H), 4.17 (d, *J* = 7.0 Hz, 2H), 4.00 (dd, *J* = 4.0, 12.0 Hz, 2H), 3.74 (dd, *J* = 5.8, 12.0 Hz, 2H), 2.35 - 2.26 (m, 4H), 2.05 - 1.96 (m, 1H), 1.68 - 1.47 (m, 9H), 1.43 (d, *J* = 13.4 Hz, 6H), 1.37 - 1.21 (m, 16H), 0.93 - 0.83 (m, 6H).

General procedure for preparation of compound 36

[0799] Compound 36 was prepared according to the synthetic scheme shown in **FIG. 29C**. In a 50 mL round-bottom flask equipped with a stir bar, compound 35 (1.9 g, 4.16 mmol, 1.0 eq) was dissolved in IPA (10 mL) and HCl (1 M, 10 mL). The mixture was degassed and purged with N_2 for 3 times, and then the mixture was stirred at 20°C for 2 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 1:1, the starting material R_f = 0.8, product R_f = 0.5) showed starting material was consumed completely and one major new spot was detected. Adjust the pH to basic (pH=8~9) with $NaHCO_3$ (10 mL). The reaction mixture was diluted with H_2O (20mL) and extracted with DCM (20 mL * 2). The combined organic layers were washed with brine (20 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 100:1 to 13:1) to give O8-(1-ethylnonyl) O1-[3-hydroxy-2-(hydroxymethyl)propyl] octanedioate (compound 36) (1.6 g, 3.84 mmol, 92.3% yield, 100% purity) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.82 (quin, *J* = 6.2 Hz, 1H), 4.27 (d, *J* = 6.4 Hz, 2H), 3.78 (dq, J = 5.2, 11.0 Hz, 4H), 2.32 (td, *J* = 7.4, 17.8 Hz, 6H), 2.05 (td, *J* = 5.6, 10.9 Hz, 1H), 1.69 - 1.47 (m, 9H), 1.39 - 1.22 (m, 17H), 0.92 - 0.84 (m, 6H).

General procedure for preparation of compound 37

[0800] Compound 37 was prepared according to the synthetic scheme shown in **FIG. 29D**. In a 50 mL round-bottom flask equipped with a stir bar, compound 36 (900 mg, 2.16 mmol, 1.0 eq) was dissolved in DCM (10 mL). DIEA (418.8 mg, 3.24 mmol, 564.5 μL, 1.5 eq), DMAP (52.79 mg, 432.1 μmol, 0.2 eq) and EDCI (621.2 mg, 3.24 mmol, 1.5 eq) was added in order. Then 4,4-dioctoxybutanoic acid (893.2 mg, 2.59 mmol, 1.2 eq) was added to the mixture. The mixture was degassed and purged with N_2 for 3 times, and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 5:1, the starting material R_f = 0.5, product R_f = 0.4) showed the reaction was completed. The reaction mixture was diluted with H_2O (10 mL) and extracted with DCM (10 mL * 2). The combined organic layers were washed with brine (10 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 100:

1 to 13: 1) to give compound 37 (1.4 g, 1.86 mmol, 86.3% yield, 98.9% purity) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.82 (quin, *J* = 6.2 Hz, 1H), 4.50 (t, *J* = 5.4 Hz, 1H), 4.23 - 4.13 (m, 4H), 3.66 - 3.53 (m, 4H), 3.41 (td, *J* = 6.8, 9.2 Hz, 2H), 2.42 (t, *J* = 7.4 Hz, 2H), 2.36 - 2.16 (m, 6H), 1.98 - 1.90 (m, 2H), 1.68 - 1.48 (m, 14H), 1.40 - 1.21 (m, 37H), 0.94 - 0.85 (m, 12H).

General procedure for preparation of Lipid 155

[0801] Lipid155 was prepared according to the synthetic scheme shown in **FIG. 29E**. In a 50 mL round-bottom flask equipped with a stir bar, compound 37 (600 mg, 807.4 μmol, 1.0 eq) was dissolved in DCM (10 mL). DIEA (156.5 mg, 1.21 mmol, 211.0 μL, 1.5 eq), DMAP (19.73 mg, 161.5 μmol, 0.2 eq) and EDCI (232.2 mg, 1.21 mmol, 1.5 eq) was added in order. Then 4-(diethylamino)butanoic acid (385.7 mg, 2.42 mmol, 3.0 eq) was added to the mixture. The mixture was degassed and purged with N_2 for 3 times and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Dichloromethane: Methanol = 10:1, the starting material R_f = 0.8, product R_f = 0.5) showed the reaction was completed. The mixture was washed by H_2O (10 mL *3) and extracted with DCM (10 mL * 3). The organic phase was separated, washed with Na_2CO_3 solution (10 mL * 5), brine (10 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = from 100:1 to 13:1 to remove impurities, from 13:1 to 1:1 to get product) for 3 times to give Lipid 155 (217 mg, 245.4 μmol, 30.4% yield, 100% purity) as colorless oil. LCMS (Method A): Rt 11.04 min, m/z calculated $[M+H]^+$: 884.7, found: 884.7. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.82 (quin, *J* = 6.2 Hz, 1H), 4.49 (t, *J* = 5.4 Hz, 1H), 4.13 (d, *J* = 5.8 Hz, 6H), 3.57 (td, *J* = 6.8, 9.2 Hz, 2H), 3.41 (td, *J* = 6.8, 9.2 Hz, 2H), 2.56 - 2.26 (m, 15H), 1.97 - 1.89 (m, 2H), 1.81 - 1.72 (m, 2H), 1.67 - 1.47 (m, 13H), 1.39 - 1.21 (m, 37H), 1.01 (br t, *J* = 6.8 Hz, 6H), 0.93 - 0.84 (m, 12H).

Lipid 176

General procedure for preparation of compound 13

[0802] Compound 13 was prepared according to the synthetic scheme shown in **FIG. 30A**. To a mixture of decanedioic acid (20 g, 98.89 mmol, 1 eq) in DCM (200 mL) was added DIEA (15.34 g, 118.7 mmol, 20.67 mL, 1.2 eq), DMAP (2.42 g, 19.78 mmol, 0.2 eq) and EDCI (18.96 g, 98.89 mmol, 1 eq) in order. Then 2-butyloctan-1-ol (14.74 g, 79.11 mmol, 0.8 eq) was added to the mixture by one portion. The resulting mixture was degassed and purged with N_2 for 3 times and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 5:1, product R_f = 0.5) showed the reaction was completed. The residue was extracted with DCM (100 mL * 3) and H_2O (100 mL). The combined organic layers were washed with brine (100

mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, Petroleum ether: Ethyl acetate = from 80: 1 to 10:1 to remove impurities; from 10:1 to 0:1 and Dichloromethane: Methanol = 50:1 to 5:1 to get product) to give 10-(2-butyloctoxy)-10-oxo-decanoic acid (compound 13) (29 g, 78.26 mmol, 79.1% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 3.98 (d, *J* = 5.8 Hz, 2H), 2.32 (td, *J* = 7.4, 14.4 Hz, 4H), 1.68 - 1.63 (m, 2H), 1.40 (br d, *J* = 6.6 Hz, 2H), 1.35 - 1.24 (m, 24H), 0.95 - 0.83 (m, 6H).

General procedure for preparation of compound 38

[0803] Compound 38 was prepared according to the synthetic scheme shown in **FIG. 30B**. To a mixture of compound 13 (22 g, 59.37 mmol, 1.0 eq) in DCM (250 mL) was added, DIEA (11.51 g, 89.05 mmol, 15.51 mL, 1.5 eq), DMAP (1.45 g, 11.87 mmol, 0.2 eq) and EDCI (17.07 g, 89.05 mmol, 1.5 eq) in order. Then (2,2-dimethyl-1,3-dioxan-5-yl)methanol (10.41 g, 71.24 mmol, 1.2 eq) was added to the mixture. The mixture was degassed and purged with N_2 for 3 times and stirred at 20°C for 16 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 5:1, the starting material R_f = 0.3, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with H_2O 200 mL and extracted with DCM (100 mL * 2). The combined organic layers were washed with brine (100 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 100:1 to 10:1) to give O10-(2-butyloctyl) O1-[(2,2-dimethyl-1,3-dioxan-5-yl)methyl] decanedioate (compound 38) (19 g, 38.10 mmol, 64.2% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.17 (d, *J* = 7.0 Hz, 2H), 4.02 - 3.95 (m, 4H), 3.74 (dd, *J* = 6.0, 12.0 Hz, 2H), 2.31 (dt, *J* = 3.6, 7.5 Hz, 4H), 2.01 (br s, 1H), 1.62 (br s, 5H), 1.43 (d, J = 12.9 Hz, 6H), 1.35 - 1.24 (m, 25H), 0.94 - 0.86 (m, 6H).

General procedure for preparation of compound 39

[0804] Compound 39 was prepared according to the synthetic scheme shown in **FIG. 30C**. To a solution of compound 38 (19 g, 38.10 mmol, 1 eq) in IPA (100 mL) was added HCl (1 M, 100 mL). The mixture was stirred at 15°C for 30 min under N2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 1:1, the starting material R_f = 0.7, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with saturated $NaHCO_3$ solution (100 mL) and extracted with DCM (100 mL * 2). The combined organic layers were washed with brine (100 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = from 50:1 to 10:1 to remove impurities; from 5:1 to 1:1 to get product) to give compound 39 (16

g, 34.88 mmol, 91.6% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.27 (d, *J* = 6.4 Hz, 2H), 3.98 (d, *J* = 5.8 Hz, 2H), 3.78 (dq, *J* = 5.2, 10.8 Hz, 4H), 2.32 (td, *J* = 7.6, 13.6 Hz, 6H), 2.16 - 2.00 (m, 1H), 1.62 (br s, 6H), 1.37 - 1.22 (m, 25H), 0.96 - 0.84 (m, 6H).

General procedure for preparation of compound 34

[0805] Compound 34 was prepared according to the synthetic scheme shown in **FIG. 30D**. To a mixture of octanedioic acid (5.0 g, 28.7 mmol, 1.0 eq) in DCM (50 mL) was added DIEA (5.5 g, 43.06 mmol, 7.5 mL, 1.5 eq), DMAP (701.3 mg, 5.7 mmol, 0.2 eq) and EDCI (5.5 g, 28.7 mmol, 1.0 eq) in order. Then the undecan-3-ol (3.9 g, 22.9 mmol, 0.8 eq) was added to the mixture. The mixture was degassed and purged with N_2 for 3 times and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 2:1, product R_f = 0.3) showed the starting material was consumed completely. The reaction mixture was diluted with H_2O (20 mL) and extracted with DCM (10 mL * 2). The combined organic layers were washed with brine (10 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, Petroleum ether: Ethyl acetate = 20:1, 10:1 to 3:1 and Dichloromethane: Methanol = 30:1, 10:1 to 0:1) to give 8-(1-ethylnonoxy)-8-oxo-octanoic acid (compound 34) (5.6 g, 17.1 mmol, 59.7% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.87 - 4.78 (m, 1H), 2.40 - 2.27 (m, 4H), 1.70 - 1.60 (m, 4H), 1.59 - 1.47 (m, 4H), 1.41 - 1.34 (m, 4H), 1.33 - 1.20 (m, 13H), 0.93 - 0.83 (m, 6H).

General procedure for preparation of compound 40

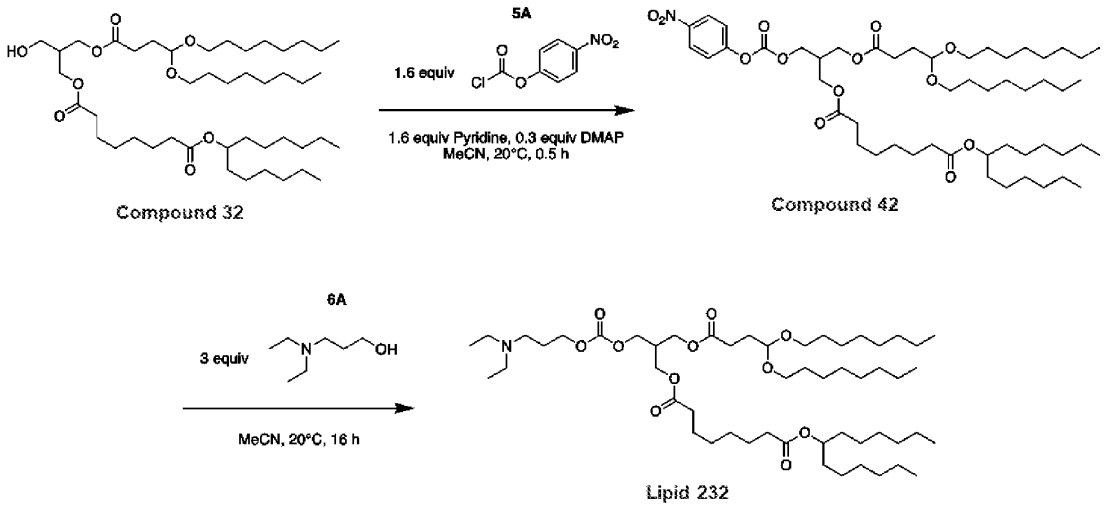
[0806] Compound 40 was prepared according to the synthetic scheme shown in **FIG. 30E**. To a mixture of compound 39 (1.4 g, 3.0 mmol, 1.0 eq) in DCM (10 mL) was added DIEA (786.9 mg, 6.0 mmol, 1.0 mL, 2.0 eq), DMAP (74.3 mg, 608.8 μmol, 0.2 eq) and EDCI (875.3 mg, 4.5 mmol, 1.5 eq) in order. Then compound 34 (1.0 g, 3.0 mmol, 1.0 eq) was added to the mixture. The mixture was degassed and purged with N_2 for 3 times and stirred at 20°C for 12 hr under N_2 atmosphere. TLC (Petroleum ether: Ethyl acetate = 3:1, the starting material R_f = 0.5, product R_f = 0.4) showed the starting material was consumed completely and the reaction was completed. The reaction mixture was diluted with H_2O (20 mL) and extracted with DCM (10 mL * 2). The combined organic layers were washed with brine (10 mL * 2), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, Petroleum ether: Ethyl acetate = from 20:1 to 3:1 to remove impurities; from 3: 1 to 0:1 and Dichloromethane: Methanol = 50:1 to 3:1 to get product) to give compound 40 (2.3 g, 2.9 mmol, 98.2% yield) as colorless oil. ^{1}H NMR (400 MHz, CDCl3-d) δ 4.87 - 4.76 (m, 1H), 4.28 - 4.12 (m, 4H), 4.02 - 3.94 m, 2H), 3.67 - 3.57 m, 2H), 2.39 - 2.14 (m,

10H), 1.67 - 1.58 (m, 10H), 1.57 - 1.49 (m, 4H), 1.38 - 1.35 (m, 3H), 1.33 - 1.23 (m, 36H), 0.93 - 0.85 (m, 12H).

General procedure for preparation of Lipid 176

[0807] Lipid 176 was prepared according to the synthetic scheme shown in **FIG. 30F**. To a solution of compound 40 (1.0 g, 1.3 mmol, 1.0 eq) in DCM (10 mL) was added Py (164.5 mg, 2.0 mmol, 167.9 μL, 1.6 eq), DMAP (47.6 mg, 390.0 μmol, 0.3 eq) and (4-nitrophenyl)carbonochloridate (419.3 mg, 2.0 mmol, 1.6 eq) in order. The mixture was stirred at 20°C for 16 hr. TLC (Petroleum ether: Ethyl acetate = 3:1, the starting material R_f = 0.3, product (Cpd.8) R_f = 0.5) showed the starting material was consumed completely and the reaction was completed. Then 3-(diethylamino)propan-1-ol (509.8 mg, 3.8 mmol, 579.3 μL, 3.0 eq) was added to the mixture by one portion and the mixture was stirred at 20°C for 16 hr. TLC (Dichloromethane: Methanol = 10:1, product R_f = 0.5) showed the reaction was completed. The reaction mixture was quenched by addition H_2O (20 mL) at 20°C, and then diluted with Na_2CO_3 (50 mL) and extracted with DCM (10 mL * 3) and H_2O (10 mL). The combined organic layers were washed with brine (20 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, Petroleum ether: Ethyl acetate = from 20:1 to 10:1 to remove impurities; from to 5:1 to 0:1 and Dichloromethane: Methanol = 50:1 to 3:1 to get product) for 2 times to give Lipid 176 (316.8 mg, 342.0 μmol, 26.4% yield) as colorless oil. LCMS (Method D): Rt 5.44 min, m/z calculated $[M+H]^+$: 926.7, found: 926.8. ^1H NMR (400 MHz, CDCl3-*d*) δ 4.87 - 4.73 (m, 1H), 4.23 - 4.18 (m, 4H), 4.16 - 4.13 (m, 3H), 4.02 - 3.91 (m, 2H), 2.61 - 2.50 (m, 5H), 2.48 - 2.39 (m, 1H), 2.35 - 2.25 (m, 8H), 1.90 - 1.79 (m, 2H), 1.69 - 1.47 (m, 14H), 1.38 - 1.21 (m, 41H), 1.08 - 1.00 (m, 6H), 0.95 - 0.82 (m, 12H).

[0808] General procedure for preparation of Lipid 232



[0809] To a solution of compound **32** (20 g, 25.94 mmol, 1 eq) in MeCN (200 mL) was added pyridine (3.28 g, 41.50 mmol, 3.35 mL, 1.6 eq), DMAP (950.5 mg, 7.78 mmol, 0.3 eq) and chloroformate **5A** (8.36 g, 41.50 mmol, 1.6 eq) at 20°C. The mixture was stirred at 20°C for 0.5 hr. LCMS and TLC (Petroleum ether: Ethyl acetate = 3: 1, R_f = 0.7) showed reactant **32** was consumed completely and desired MS of **42** was detected. The reaction mixture was used in situ to next step without any workup. LCMS: m/z calculated $[M+Na]^+$: 958.6, found: 958.8.

[0810] To the resulting mixture of intermediate **42** in MeCN (total ~250 mL) was added alcohol **6A** (10.09 g, 76.90 mmol, 11.47 mL, 3 eq) at 20°C. Then the mixture was stirred at 20°C for 16 hr. LCMS and TLC (Dichloromethane: Methanol = 10:1, the starting material R_f = 0.7, product R_f = 0.5) showed the reaction was completed. The reaction mixture was diluted with H_2O (200 mL) and extracted with CH_2Cl_2 (100 mL * 2). The combined organic layers were washed with Na_2CO_3(aq) (200 mL x 2) and brine (200 mL x 2), dried over Na_2SO_4, filtrated, and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether: ethyl acetate = 20:1, 5:1 to 3:1) for 2 times to give **Lipid 232** (14.48 g, 15.60 mmol, 60.85% yield, 100% purity ELSD, 96.0% purity CAD) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 4.87 (t, *J* = 6.2 Hz, 1H), 4.49 (t, *J* = 5.6 Hz, 1H), 4.22 - 4.13 (m, 8H), 3.60 - 3.53 (m, 2H), 3.44 - 3.37 (m, 2H), 2.51 (q, *J* = 6.8 Hz, 6H), 2.45 - 2.37 (m, 3H), 2.30 (td, *J* = 7.6, 11.4 Hz, 4H), 1.97 - 1.88 (m, 2H), 1.85 - 1.77 (m, 2H), 1.65 - 1.49 (m, 12H), 1.36 - 1.23 (m, 40H), 1.01 (t, *J* = 7.2 Hz, 6H), 0.92 - 0.85 (m, 12H). LCMS: m/z calculated $[M+H]^+$: 928.7, found: 928.6.

Example 13: Conjugating multiple targeting moieties to the surface of an LNP

[0811] A methodology was developed to conjugate multiple targeting moieties to the surface of an LNP using a site-specific conjugation process described herein. This process can be used to efficiently conjugate two or more targeting moieties to the surface of an LNP at specific ratios.

Conjugating two targeting moieties on LNPs

[0812] The ethanol phase was prepared with five lipids, containing ionizable lipid (in this case V003), DSPC, cholesterol, DMG-PEG and DSPE-PEG2K-TCO with a molar ratio of 47%: 8%: 42.5%: 2%: 0.5%, respectively. The aqueous phase was composed of mRNA in 25 mM acetate buffer. Two phases were mixed using a microfluidic device to formulate base LNP at a total flow rate of 18 ml/min (aqueous phase: ethanol phase=3:1). 3x volume of citrate-buffered saline was added to LNP solution and resulted solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes.

[0813] Two methyltetrazine modified antibody fragments (anti-CD117 Fab-MeTz and anti-CD45 Fab-MeTz) were labeled by Alexa Fluor 647 and TAMRA NHS esters separately and then mixed at mass ratios of 1:3, 1:1, or 3:1 and then added to the LNP-TCO solution for targeting moiety-LNP conjugation. While targeting moiety ratios of 1:3, 1:1, and 3:1 were tested in this experiment, other ratios of two targeting moieties could also be used efficiently. The mass ratio between the total targeting moieties and mRNA was 1:1. The solution was incubated at room temperature for 2 hours and then at 4°C overnight.

Conjugation efficiency analysis for dual targeting moiety conjugation on LNPs

[0814] To quantify the conjugation efficiency for the dual targeting moieties on the LNP, the above crude solution was submitted to SEC (size exclusion chromatography) using Sepharose CL-4B resin. Elution was collected as 250ul fractions. Fractions containing tLNP were confirmed by Ribogreen assay and the fluorescent intensity was measured by plate reader. The conjugation efficiency was calculated by the fluorescence ratio between factions containing tLNP and overall fractions.

[0815] **FIG. 31** shows that this site-specific conjugation process can effectively and accurately conjugate two targeting moieties (anti-CD117 Fab and anti-CD45 Fab, in this case) to the surface of LNPs at specific ratios. The molar ratio of the targeting moieties that are added to the base LNPs are highly predictive of the molar ratio of targeting moieties that become conjugated to the surface of the dual tLNPs, suggesting that the conjugation process described herein is highly efficient.

Example 14: Targeted LNPs can deliver RNA payloads comprising an exemplary heterologous gene modifying system to T cells *in vivo*

[0816] Targeted LNPs conjugated to an anti-CD5 targeting moiety were tested for their ability to deliver gene modifying systems to T cells *in vivo*. The tLNP was formulated with either an exemplary gene editing system comprised of an mRNA encoding a wild-type Cas9-reverse transcriptase (RT) fusion protein and a sgRNA specific for the first exon of the human Beta 2 Microglobulin (B2M) gene or an exemplary heterologous gene modifying system comprised of an mRNA encoding Cas9 nickase fused to an RT and a template RNA designed to insert five nucleotides (TAGTG) into the first exon of B2M via a target-primed reverse transcription process. The introduction of the wtCas9-RT and sgRNA leads to the introduction and repair of a DNA double-stranded break induced at this site, which results in the formation of small insertions and deletions (indels), largely resulting in frameshifts and thus loss of B2M expression from the cell surface. The introduction of the nCas9-RT and B2M template RNA leads to a single-stranded DNA break and the insertion of the 5 nucleotides thereby generating a frameshift mutation, which resulted in loss of expression of B2M from the cell surface. The tLNPs were administered to humanized mice and evaluated for their effectiveness in delivering the payloads *in vivo* as measured by the percentage of either B2M indels in T cells or the percentage of reads that included the desired insertion of 5 nucleotides (TAGTG) in the first exon of B2M (assayed by Amp-seq).

[0817] As described above, tLNPs containing the gene editing system were formulated to encapsulate an mRNA encoding a wtCas9 molecule fused to a reverse transcriptase domain ("wtCas9-RT", SEQ ID NO:383) and the sgRNA ("B2M sgRNA1", SEQ ID NO:384) at a mass ratio of 1:1. The tLNPs containing the heterologous gene modifying system were formulated to encapsulate an mRNA encoding Cas9 nickase fused to an RT ("nCas9-RT", SEQ ID NO:133) and the template RNA ("B2M template RNA", SEQ ID NO: 103) at a mass ratio of 1:1.

[0818] The wtCas9-RT and nCas9-RT gene modifying polypeptides comprised the amino acid sequences provided in **Table 27**.

Table 27: Gene modifying polypeptide sequences

Name	AA sequence	SEQ ID NO
wtCas 9 RT	MPAAKRVKLDGGDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFK VLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRI CYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDE VAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIE GDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLS	383

	KSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAK LQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRV NTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQ SKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLR KQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRI PYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIE RMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKP AFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGV EDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDRE MIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQ SGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSL HEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMAR ENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKL YLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKV LTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNL TKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDE NDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLN AVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAK YFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFAT VRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWD PKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERS SFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAG ELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHK HYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENII HLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYE TRIDLSQLGGDGGAEAAAKEAAAKEAAAKEAAAKALEAEAAAK EAAAKEAAAKEAAAKAGGTAPLEEEYRLFLEAPIQNVTLLEQWK REIPKVWAEINPPGLASTQAPIHVQLLSTALPVRVRQYPITLEAKRS LRETIRKFRAAGILRPVHSPWNTPLLPVRKSGTSEYRMVQDLREVN KRVETIHPTVPNPYTLLSLLPPDRIWYSVLDLKDAFFCIPLAPESQLI FAFEWADAEEGESGQLTWTRLPQGFKNSPTLFNEALNRDLQGFRL DHPSVSLLQYVDDLLIAADTQAACLSATRDLLMTLAELGYRVSGK KAQLCQEEVTYLGFKIHKGSRSLSNSRTQAILQIPVPKTKRQVREF LGKIGYCRLFIPGFAELAQPLYAATRPGNDPLVWGEKEEEAFQSLK LALTQPPALALPSLDKPFQLFVEETSGAAKGVLTQALGPWKRPVA YLSKRLDPVAAGWPRCLRAIAAAALLTREASKLTFGQDIEITSSHN LESLLRSPPDKWLTNARITQYQVLLLDPPRVRFKQTAALNPATLLP ETDDTLPIHHCLDTLDSLTSTRPDLTDQPLAQAEATLFTDGSSYIRD GKRYAGAAVVTLDSVIWAEPLPIGTSAQKAELIALTKALEWSKDK SVNIYTDSRYAFATLHVHGMIYRERGWLTAGGKAIKNAPEILALL TAVWLPKRVAVMHCKGHQKDDAPTSTGNRRADEVAREVAIRPLS TQATISAGKRTADGSEFEKRTADGSEFESPKKKAKVE	
nCas9 -RT	MPAAKRVKLDGGDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFK VLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRI CYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDE VAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIE GDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLS KSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAK LQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRV NTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQ SKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLR	133

	KQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRI PYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIE RMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKP AFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGV EDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDRE MIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQ SGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSL HEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMAR ENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKL YLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKV LTRSDKARGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNL TKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDE NDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLN AVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAK YFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFAT VRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWD PKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERS SFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAG ELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHK HYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENII HLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYE TRIDLSQLGGDGGAEAAAKEAAAKEAAAKEAAAKALEAEAAAK EAAAKEAAAKEAAAKAGGTAPLEEEYRLFLEAPIQNVTLLEQWK REIPKVWAEINPPGLASTQAPIHVQLLSTALPVRVRQYPITLEAKRS LRETIRKFRAAGILRPVHSPWNTPLLPVRKSGTSEYRMVQDLREVN KRVETIHPTVPNPYTLLSLLPPDRIWYSVLDLKDAFFCIPLAPESQLI FAFEWADAEEGESGQLTWTRLPQGFKNSPTLFNEALNRDLQGFRL DHPSVSLLQYVDDLLIAADTQAACLSATRDLLMTLAELGYRVSGK KAQLCQEEVTYLGFKIHKGSRSLSNSRTQAILQIPVPKTKRQVREF LGKIGYCRLFIPGFAELAQPLYAATRPGNDPLVWGEKEEEAFQSLK LALTQPPALALPSLDKPFQLFVEETSGAAKGVLTQALGPWKRPVA YLSKRLDPVAAGWPRCLRAIAAAALLTREASKLTFGQDIEITSSHN LESLLRSPPDKWLTNARITQYQVLLLDPPRVRFKQTAALNPATLLP ETDDTLPIHHCLDTLDSLTSTRPDLTDQPLAQAEATLFTDGSSYIRD GKRYAGAAVVTLDSVIWAEPLPIGTSAQKAELIALTKALEWSKDK SVNIYTDSRYAFATLHVHGMIYRERGWLTAGGKAIKNAPEILALL TAVWLPKRVAVMHCKGHQKDDAPTSTGNRRADEVAREVAIRPLS TQATISAGKRTADGSEFEKRTADGSEFESPKKKAKVE	

[0819] The B2M sgRNA and template RNA sequences are provided in **Table 28**. Nucleotide modifications are noted as follows: phosphorothioate linkages denoted by an asterisk, 2'-O-methyl groups denoted by an 'm' preceding a nucleotide. SEQ ID NO:380 shows the sequence of the B2M template RNA without the phosphorothioate linkages.

Table 28 – B2M gRNA and template sequences

Name	Sequence	SEQ ID NO
B2M sgRNA1	mA*mC*mU*rCrArCrGrCrUrGrGrArUrArGrCrCrUrCrCrGrUr UrUrUrArGrAmGmCmUmAmGmAmAmAmUmAmGmCrArA	384

	rGrUrUrArArArArUrArArGrGrCrUrArGrUrCrCrGrUrUrArUrC rAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAm CmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU	
B2M template RNA (with modifications)	mA*mC*mU*rCrArCrGrCrUrGrGrArUrArGrCrCrUrCrCrGrUr UrUrUrArGrAmGmCmUmAmGmAmAmAmUmAmGmCrArA rGrUrUrArArArArUrArArGrGrCrUrArGrUrCrCrGrUrUrArUrC rAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAm CmCmGmAmGmUmCmGmGmUmGmCrUrUrUrCrUrGrGrCr CrUrGrGrArUrGrArUrGrGrGrCrUrArUrCrC*mA*mG*mC	386
B2M template RNA (without modifications)	ACUCACGCUGGAUAGCCUCCGUUUUAGAGCUAGAAAU AGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUG AAAAAGUGGCACCGAGUCGGUGCUUUCUGGCCUGGA UGAUGGGCUAUCCAGC	380

[0820] The B2M sgRNA1 contained a spacer (ACUCACGCUGGAUAGCCUCC) (SEQ ID NO:388) and a sgRNA scaffold (GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGA AAAAGUGGCACCGAGUCGGUGCUUUU) (SEQ ID NO:389).

[0821] The B2M template RNA contained:

1. a gRNA spacer;
2. a gRNA scaffold;
3. a heterologous object sequence; and
4. a primer binding site (PBS) sequence.

[0822] The heterologous object sequences and PBS sequences were designed to introduce an insertion of 5 nucleotides (TAGTG) in the first exon of B2M, which generates a frameshift mutation.

Targeted LNP (tLNP) production

[0823] Briefly, base LNPs were prepared at a molar ratio of 47% ionizable lipid (V003): 42.5% cholesterol: 8% DSPC: 2.0% PEG-DMG: 0.5% DSPE-PEG2000-TCO at an N/P of 6 in the ethanol phase. The aqueous phase was composed of either mRNA encoding wtCas9-RT and the B2M sgRNA or mRNA encoding nCas9-RT and the B2M template RNA at a mass ratio of 1:1 in 25 mM acetate buffer. The two phases were mixed using a microfluidic device to formulate base LNP at a total flow rate of 18 mL/min (aqueous phase: ethanol phase=3:1). 3x volume of citrate-buffered saline was added to LNP solution and resulted solution was

neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes. The LNPs were further concentrated using 100kDa Amicon Filters (Millipore) at 4°C.

[0824] The anti-CD5 targeting moiety (Fab) was produced with a sortase tag (LPETG, SEQ ID NO:11) on the C terminus of the heavy chain. 10uM Fab-LPETG and 1mM Triglycine methyltetrazine (GGG-meTz) were prepared in sortase buffer (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.4). 2uM Sortase A5 was then added to initiate the reaction. The above solution was incubated at 30°C for 3 hours with gentle mixing. Excess reagents were removed, and buffer was exchanged to PBS containing 10mM EDTA by a 10K MWCO Amicon centrifugal UF device. To confirm a DOL (degree of labeling) of close to 1 meTz per targeting moiety, sortase modified Fab was allowed to react with 5eq AF594 containing TCO group in PBS for 2 hours at room temperature. After the reaction, excess dye was removed via Zeba desalting columns. DOL was determined by first measuring absorbances at A280 and A590 using a Nanodrop, and then calculating the DOL using extinction coefficients and correction factors. The resulting meTz-modified Fabs were added to the TCO modified-LNP solution described above for Fab-LNP conjugation, with a 1:1 mass ratio between Fab and total RNA payload. The solution was incubated at room temperature for 2 hours and then at 4°C overnight. Any unreacted Fabs were removed by a 300K mPES TFF membrane. The resulting tLNP products were further concentrated using a 100k MWCO Amicon centrifugal UF device. The tLNPs were characterized using a ribogreen assay to quantify RNA content and encapsulation efficiency and by DLS.

Preparation and Dosing of a Humanized Mouse Model

[0825] Briefly, female NSG mice 6-8 weeks of age (Catalog #005557) were purchased from Jackson Labs (Bar Harbor, ME). Following acclimation to the vivarium for 3-5 days, animals received a tail vein injection of 5 X 10^6 human CD3+ T cells and kept for 2-3 weeks for T cell engraftment. For each *in vivo* study, "humanized" NSG mice were randomized into groups of 4 animals and baseline weights were obtained. A group of four animals was then treated via intravenous tail vein injection with 100 ul of either the tLNPs comprising the gene editing system or the tLNPs comprising the heterologous gene modifying system at a dose of 4 mg/kg. Following a 16-hour incubation, animals were euthanized via CO2 asphyxiation, weighed and their spleens were collected and the splenic cells were isolated. Gene editing of the B2M locus by the gene editing system was quantified by Amp-Seq.

Results

[0826] **FIG. 32** shows that the administered tLNPs can deliver the exemplary gene modifying systems to human T cells in an animal model effectively, leading to *in vivo* gene editing in T cells. Targeted LNPs formulated with the exemplary gene editing system payload (wtCas9-RT mRNA and B2M sgRNA) resulted in a mean of about 64% indels in B2M in T cells, as measured by Amp-seq. Control animals that did not receive tLNPs did not exhibit any B2M indels. The tLNPs formulated with the exemplary heterologous gene modifying system payload (nCas9-RT and B2M template RNA) ("Rewriting" in **FIG. 32**) resulted in a mean of about 31% of the human T cells comprising the intended edit, as measured by Amp-seq. The control animals that did not receive tLNPs did not exhibit any reduction in B2M.

Example 15: CAR expression visible in T cells in as little as 1 hour

[0827] This Example demonstrates that an anti-CD3 targeted LNP comprising an exemplary all-RNA gene modifying system can transduce T cells in human peripheral blood mononuclear cells (PBMCs), a complex mixture of cells comprising lymphocytes, to generate functional CAR-T cells, such as in conditions and in a timeframe suitable for same-day manufacturing, e.g., in an extracorporeal device (ECD). In this instance, BCMA-targeted CAR-T cells were generated and shown to be capable of killing human cancer cells (multiple myeloma tumor cells) *in vitro*.

Preparation of Targeted LNPs for Transfection

[0828] LNPs were formulated as follows. The inter-chain disulfide bond of the anti-CD3-9 Fab fragment (sequences provided in **Table 30**) was specifically reduced before conjugation to an LNP.

Table 30: Anti-CD3-9 Fab fragment

	Heavy Chain Sequence	Light Chain Sequence	VH Sequence	VL Sequence
Anti-CD3-9 (Plamotamab)	EVQLVESGGGLV QPGGSLRLSCAA SGFTFS**TYAMN** WVRQAPGKGLE WVG**RIRSKYNN YATYYADSVKG** RFTISRDDSKNTL YLQMNSLRAED TAVYYCVR**HGN FGDSYVSWFAY** WGQGTLVTVSS ASTKGPSVFPLA	ETVVTQEPSLTV SPGGTVTLTC**GS STGAVTTSNYA** **N**WVQQKPGKSP RGLIG**GTNKRA** **P**GVPARFSGSLL GGKAALTISGA QPEDEADYYC**A** **LWYSNHWV**FG GGTKLTVL GQPKANPTVTL FPPSSEELQANK	EVQLVESGGGL VQPGGSLRLSC AASGFTFS**TYA** **MN**WVRQAPG KGLEWVG**RIR** **SKYNNYATYY** **ADSVKG**RFTIS RDDSKNTLYL QMNSLRAEDT AVYYCVR**HGN** **FGDSYVSWFA** **Y**WGQGTLVTV	ETVVTQEPSLT VSPGGTVTLTC **GSSTGAVTTS** **NYAN**WVQQKP GKSPRGLIG**GT** **NKRAP**GVPAR FSGSLLGGKAA LTISGAQPEDE ADYYC**ALWYS** **NHWV**FGGGTK LTVL

	PSSKSTSGGTAA LGCLVKDYFPEP VTVSWNSGALTS GVHTFPAVLQSS GLYSLSSVVTVP SSSLGTQTYICN VNHKPSNTKVD KKVEPKSCDKT (SEQ ID NO:226)	ATLVCLISDFYP GAVTVAWKAD GSPVKAGVETT KPSKQSNNKYA ASSYLSLTPEQ WKSHRSYSCQV THEGSTVEKTV APTECS (SEQ ID NO:227)	SS (SEQ ID NO:228)	(SEQ ID NO:229)
Note: The CDR sequences for the anti-CD3-9 targeting moiety are in **bolded** text.				

[0829] A solution containing the Fab fragment was buffer exchanged with PBS containing 10mM EDTA. Ten equivalents of TCEP were added to each solution and the reduction was carried out for 1 hour at room temperature. Complete reduction of the Fab fragment was confirmed by SDS PAGE gels.

[0830] Base LNPs were prepared at a molar ratio of 47% ionizable lipid (Lipid154): 28.5% cholesterol: 22% DSPC: 2.0% PEG-DMG: 0.5% DSPE-PEG2000-maleimide at an N/P of 6 in the ethanol phase. The aqueous phase was composed of mRNA encoding the exemplary gene modifying polypeptide and the exemplary template RNA encoding the CAR at a mass ratio of 1:25 in 25 mM acetate buffer. The two phases were mixed using a T-mixer or microfluidic device to formulate base LNP (aqueous phase: ethanol phase=3:1). 3x volume of citrate-buffered saline was added to LNP solution and resulted solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes.

[0831] After the base LNP was formulated, the reduced anti-CD3-9 Fab fragment was added to the LNP-maleimide solution at specified mass ratio to the total RNA mass (example 0.4:1). The solution was incubated at room temperature for 2 hours for conjugation and then stored at 4°C overnight. The unreacted maleimide was quenched by addition of excess cysteine (3 equivalent) for 1 hour at room temperature. The LNPs were purified and concentrated by a centrifugal filter (100K MWCO) or via TFF. The resulting tLNP were characterized by Zetasizer for size and PDI and by Ribogreen assays for mRNA concentration and encapsulation efficiency.

[0832] The gene modifying system formulated in the tLNPs comprised an mRNA encoding an exemplary gene modifying polypeptide comprising the following components.

[0833] The gene modifying polypeptide comprised an SV40 NLS having an amino acid sequence of:

PKKKRKV (SEQ ID NO:367)

[0834] The gene modifying polypeptide comprised a GGS Linker having an amino acid sequence of:

GGS

[0835] The gene modifying polypeptide comprised the amino acid sequence of:

MDSTAHPNQGRGLEKVSQTLPALQTPGQHTAAGGSSPLSGRNQRKNTKKLLLGAWNIR
TLLDRENTPRPERRTALIGKELARYNIDIAALSETRLPEEGSLSEPTTGYTFFWKGRASNE
DRIHGVGLAIKTSLLKQLPDLPVGISERLMKIRLPLSKDRYATIISAYAPTLTSTEETIEQFY
SDLSAVLHSVPTNDKLILLGDFNARVGQDHERWKGVLGKHGVGKMNNNGLLLLSKCS
EFELTITNTVFRMANKYKTTWMHPRSKQWHLIDYIIVRRRDIQDVKITRAMRGAECWT
DHRLVRATLQMRIAPRHPKRAQTVRAFYNVSRLRDPSYLQTFQSCLDDKLSAKGPLTGS
STEKWNQFRDAVKETSKAVLGPKQRNHQDWFDENNTAIEDLLSKKNKAFMEWQNNPN
SAPKKDRFKSLQATAQREIRKMQDRWWEKKAEEIQRFADMKNYKQFFSALKTVYGPL
KPTTTPLLSSDGDTLIKDKKGISNRWKEHFSQLLNRPSSVDQSALDQIPQNRTIEQLDVPP
SIEEVQKAIKQMSAGKAPGKDGIPTEVYKALNGKALQAFHIVLTSIWEEEDMPPELRDAS
IVALYKNKGSRAACDNYRGISLLSTAGKILARVILNRLLSSVSEQNLPESQCGFRPDRSTI
DMVFTVRQMQEKCLEQNLSLYIVFIDLTKAFDTVNRDALWVILSKLGCPAKFVKLIQLF
HVDMTGEVLSGGETSDRFNISNGVKQGCVLAPVLFNLFFTQVLRHAVMDLDLGVYIKY
RLDGSLFDLRRLTAKTKTTERLILEALFADDCALMAHQENHLQTIVDRFSTATKLFGLTI
SLSKTEVLFQPAPGRPTNQPCITIDGTQLSNVNTFKYLGSTIANDGSLDHEINARIQKASQ
ALGRLRCKVLQHRGVSTATKLKVYNAVVLSSLLYGCETWTLYRKHMKQLEQFHQRSL
RSIMRIRWQDRITNQEVLDRANSTSIEVMVLKTQLRWSGHVIRMDPQRIPRQVFYGELS
AGLRKQGRPKKRFKDQLKSNLKWAGITPKQLELAASDRSSWRTHINHAATTFEDERRR
RLAAARERRHQATTAPPVTTGVPCPMCHKLCASAFGLQSHMRVHRR (SEQ ID NO:32)

Table 31: Nucleic Acid and Amino Acid Sequences of the Gene Modifying Polypeptide

Name	Nucleic Acid Sequence	Amino Acid Sequence
RNAIV T1901	AGGACAACAAUAACUCUAAUAAACAA ACGAAUUCUAUUAUAUCACCACAUUA UAUCUCUAAUCUGCCACCAUGCCAAA GAAGAAAAGAAAAGUAGGAGGUUCCG ACUCCACAGCUCAUCCCAACCAGGGAC GUGGCCUGGAGAAGGUGUCCCAGACU CUGCCUGCGCUGCAGACCCCAGGGCAG CACACUGCCGCUGGGGGAUCCUCUCCC CUGUCCGGCAGGAAUCAGCGGAAGAA CACCAAAAAGCUGUUGCUGGGCGCCU GGAACAUCCGCACCCUGCUCGACCGCG AAAAUACUCCCCGUCCUGAAAGACGC ACGGCACUGAUUGGGAAAGAGCUCGC CAGAUAUAACAUCGACAUCGCUGCCC UGUCCGAAACCCGCCUGCCAGAGGAA	MPKKKRKVGGSDSTAHPNQGRGLEKVS QTLPALQTPGQHTAAGGSSPLSGRNQRK NTKKLLLGAWNIRTLLDRENTPRPERRTA LIGKELARYNIDIAALSETRLPEEGSLSEPT TGYTFFWKGRASNEDRIHGVGLAIKTSLL KQLPDLPVGISERLMKIRLPLSKDRYATII SAYAPTLTSTEETIEQFYSDLSAVLHSVPT NDKLILLGDFNARVGQDHERWKGVLGK HGVGKMNNNGLLLLSKCSEFELTITNTVF RMANKYKTTWMHPRSKQWHLIDYIIVRR RDIQDVKITRAMRGAECWTDHRLVRATL QMRIAPRHPKRAQTVRAFYNVSRLRDPS YLQTFQSCLDDKLSAKGPLTGSSTEKWN QFRDAVKETSKAVLGPKQRNHQDWFDE NNTAIEDLLSKKNKAFMEWQNNPNSAPK

	GGAUCUCUGAGCGAGCCUACCACUGG CUACACGUUCUUUUGGAAGGGCAGAG CAAGCAACGAGGACCGUAUCCAUGGU GUAGGCCUGGCUAUCAAGACCUCUCU CCUGAAACAACUCCCAGAUCUGCCAGU CGGCAUCUCAGAGAGACUGAUGAAGA UCAGGCUUCCUCUCUCAAAAGACCGCU ACGCAACCAUCAUUUCCGCAUACGCGC CGACCCUGACUAGCACCGAGGAAACCA UUGAGCAGUUCUACUCUGAUCUUUCA GCUGUUUUGCACUCCGUGCCUACCAAC GACAAGCUGAUCCUCCUGGGCGACUU CAACGCUCGCGUGGGUCAGGACCAUG AGCGCUGGAAAGGCGUGCUGGGUAAG CAUGGCGUGGGUAAGAUGAACAAUAA CGGCCUCCUGCUCCUGUCUAAGUGUA GCGAAUUCGAGCUGACUAUUACCAAC ACUGUUUUUCGUAUGGCUAACAAGUA CAAAACUACAUGGAUGCACCCCAGGA GCAAGCAGUGGCACCUGAUUGACUAC AUUAUCGUGCGUAGGCGGGAUAUCCA GGACGUGAAGAUUACCCGUGCAAUGC GCGGCGCUGAGUGCUGGACAGACCAU CGCCUGGUGAGAGCUACUCUCCAGAU GAGGAUCGCACCGCGUCACCCUAAGCG CGCCCAGACCGUGCGGGCCUUUUACAA CGUAUCACGGCUUCGGGAUCCUUCCU ACCUUCAGACAUUUCAAUCUUGCCUC GAUGACAAGCUCUCUGCUAAGGGUCC CCUCACCGGAUCUUCAACAGAGAAGU GGAACCAAUUCCGCGAUGCGGUGAAG GAAACCAGCAAAGCAGUCCUGGGACC GAAGCAGCGCAACCACCAAGACUGGU UUGACGAAAAUAACACUGCCAUCGAA GACCUCCUGUCUAAAAAGAACAAAGC AUUUAUGGAGUGGCAGAAUAACCCAA ACAGUGCACCCAAGAAAGACCGUUUC AAAAGCCUGCAGGCCACCGCGCAGCGC GAGAUCCGGAAGAUGCAGGACCGCUG GUGGGAAAAAAAGGCCGAAGAGAUCC AGAGGUUCGCGGAUAUGAAAAACUAC AAGCAGUUCUUUAGCGCACUGAAGAC CGUGUACGGACCUCUCAAACCCACCAC GACUCCCCUGCUCUCUUCCGAUGGUGA CACUCUCAUCAAGGACAAAAAGGGAA UCAGCAACCGCUGGAAAGAACACUUU UCUCAACUUCUCAACCGCCCAAGCUCC GUGGACCAGAGCGCUCUGGACCAGAU CCCGCAGAACCGUACCAUCGAGCAGCU GGACGUGCCUCCCUCCAUCGAAGAGG UCCAGAAGGCUAUCAAGCAGAUGUCU GCUGGGAAGGCUCCUGGCAAGGAUGG GAUCCCAACAGAGGUCUAUAAAGCUC UCAACGGAAAGGCGCUGCAGGCCUUU CACAUUGUCCUGACCUCCAUCUGGGA GGAAGAGGACAUGCCACCUGAACUUA GGGACGCCUCUAUUGUGGCCCUGUAC AAGAAUAAGGGCAGCCGCGCAGCUUG UGAUAACUAUCGUGGCAUCUCACUCC UGAGUACAGCUGGCAAGAUUCUGGCU CGUGUGAUCCUGAACCGGUUGCUGAG	KDRFKSLQATAQREIRKMQDRWWEKKA EEIQRFADMKNYKQFFSALKTVYGPLKP TTTPLLSSDGDTLIKDKKGISNRWKEHFS QLLNRPSSVDQSALDQIPQNRTIEQLDVPP SIEEVQKAIKQMSAGKAPGKDGIPTEVYK ALNGKALQAFHIVLTSIWEEEDMPPELRD ASIVALYKNKGSRAACDNYRGISLLSTAG KILARVILNRLLSSVSEQNLPESQCGFRPD RSTIDMVFTVRQMQEKCLEQNLSLYIVFI DLTKAFDTVNRDALWVILSKLGCPAKFV KLIQLFHVDMTGEVLSGGETSDRFNISNG VKQGCVLAPVLFNLFFTQVLRHAVMDLD LGVYIKYRLDGSLFDLRRLTAKTKTTERL ILEALFADDCALMAHQENHLQTIVDRFST ATKLFGLTISLSKTEVLFQPAPGRPTNQPC ITIDGTQLSNVNTFKYLGSTIANDGSLDHE INARIQKASQALGRLRCKVLQHRGVSTA TKLKVYNAVVLSSLLYGCETWTLYRKH MKQLEQFHQRSLRSIMRIRWQDRITNQE VLDRANSTSIEVMVLKTQLRWSGHVIRM DPQRIPRQVFYGELSAGLRKQGRPKKRFK DQLKSNLKWAGITPKQLELAASDRSSWR THINHAATTFEDERRRRLAAARERRHQA TTAPPVTTGVPCPMCHKLCASAFGLQSH MRVHRR (SEQ ID NO:64)

	UUCCGUUUCCGAGCAGAACCUGCCCGA AUCACAGUGCGGCUUCCGCCCCGAUCG GUCCACCAUCGAUAUGGUGUUCACCG UGCGUCAGAUGCAGGAGAAGUGCCUG GAACAGAAUCUGAGCCUGUACAUCGU UUUCAUCGAUCUGACUAAGGCCUUCG ACACGGUGAACCGCGAUGCCCUGUGG GUGAUCCUGAGCAAGCUGGGGUGCCC CGCUAAGUUCGUAAAGCUCAUCCAGU UGUUCCACGUUGAUAUGACGGGAGAG GUCCUGUCCGGUGGAGAAACCUCCGA CCGGUUUAACAUCUCCAAUGGCGUGA AACAGGGCUGCGUCCUGGCCCCAGUGC UGUUCAACCUGUUUUUCACACAGGUG CUGAGACAUGCGGUUAUGGAUCUUGA CCUGGGUGUGUAUAUCAAGUAUCGCU UGGACGGCAGUCUGUUCGAUCUCAGA CGCCUGACAGCUAAGACUAAGACUAC CGAGCGCCUCAUUCUCGAAGCCCUGUU UGCCGACGAUUGUGCCCUUAUGGCCC ACCAGGAGAACCACCUGCAGACGAUU GUCGACCGCUUCUCUACAGCGACCAAG CUGUUCGGCCUCACCAUCUCCCUCUCA AAGACCGAAGUCCUGUUUCAGCCCGC ACCCGGGAGACCCACGAAUCAGCCGUG CAUUACCAUUGACGGGACCCAACUUU CCAACGUCAACACCUUCAAGUAUCUG GGAUCUACCAUCGCCAAUGAUGGCAG CCUGGAUCAUGAAAUCAACGCAAGGA UCCAAAAGGCUAGCCAGGCACUGGGC CGCCUCCGGUGCAAGGUCCUGCAGCAC CGGGGCGUUUCCACUGCCACUAAACU GAAGGUGUACAACGCUGUCGUGCUCU CUUCCCUGCUUUAUGGCUGCGAAACA UGGACUCUGUACCGGAAGCAUAUGAA ACAGCUUGAGCAGUUCCACCAGAGGA GCUUGCGUAGCAUCAUGAGGAUCCGC UGGCAGGACCGCAUCACCAACCAGGA GGUGCUGGACAGGGCUAAUUCCACCA GCAUCGAAGUUAUGGUGCUGAAGACC CAGCUCCGUUGGUCUGGCCAUGUCAU UCGGAUGGACCCUCAGCGUAUCCCCCG CCAGGUAUUCUACGGGGAACUGUCUG CGGGGCUCAGAAAGCAGGGCCGCCCA AAGAAACGCUUUAAGGAUCAGCUGAA AAGCAAUCUGAAGUGGGCCGGCAUCA CCCCCAAGCAGCUGGAGCUGGCGGCAU CUGACCGCUCCAGCUGGCGCACCCACA UUAACCACGCAGCUACUACCUUUGAG GACGAACGCCGGCGCAGACUGGCCGCU GCACGCGAGAGGCGCCACCAAGCCACU ACCGCCCCUCCCGUGACCACUGGGGUC CCUUGCCCUAUGUGCCACAAGCUGUG UGCGUCUGCCUUCGGUUUGCAGUCCC ACAUGCGUGUGCAUCGCCGCUAAUAG UGAUUGCCAUGUGUAUGUGGGUUUUU UUUUUCCCACAUACUCUGAUGAUCCU UUUUUUUUUGGAUCAUUCAUGGCAAA AAAAAAAAAAAAAAAUUAAAAAAAAAA AAAAAAAAAAAAAAAAUUUAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAA	

	UUUUUAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAA (SEQ ID NO:406)	

[0836] The gene modifying system further comprised a template RNA with the following sequences from 5' to 3': 5'UTR_{retro}, BCMA CAR, MND promoter, 3'UTR_{retro}.

[0837] The template RNA comprised a 5'UTR having the sequence of:

GGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGUCG
UGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUCAC
UUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACGGC
UUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUGGU
GAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACGGA
AGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUGUG
GAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAGCU
GAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAACG
CCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUCAC
GCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCAAG
ACCUGCGGCGAUGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCAGU
UGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCCUG
UGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAG
(SEQ ID NO:372)

[0838] The template RNA comprised a 3'UTR having the sequence of:

UGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCGAGAGACUACCACU (SEQ ID NO:373)

[0839] The template RNA comprised a nucleic acid sequence encoding a BCMA-CAR fusion polypeptide, wherein the nucleic acid sequence was:

AGGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGUC
GUGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUCA
CUUGUGGCUCCCAGUAGCUGCUAGCAUGCGGCAGCGGCCACACCCCGGGCAACGG
CUUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCGUCGACCCCUGG
UGAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCGGAACAGGCGG
AAGAAACCAAUAAGAAGGUUCAACGGCUGAGAUGGCGACGCAGCAAAGCACUGU
GGAGUGCUUAGGGCGUGUUGGAGCACAAAAGACAACACGGCCAUCCAAUGCAGC
UGAGGAAGUCUCCAGACGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAAC

GCCGAGAGAGUGGGACUGUCUCUGUGCAUCGACUUUUCCACUUAAAUCUCCUUCA
CGCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCAA
GACCUGCGGCGAUGGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCAG
UUGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCCU
GUGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAGG
UUCUUUCCGCCUCAGAAGCCAUAGAGCCCACCGCAUCCCCAGCAUGCCUGCUAUU
GUCUUCCCAAUCCUCCCCCUUGCUGUCCUGCCCCACCCCACCCCCCAGAAUAGAAU
GACACCUACUCAGACAAUGCGAUGCAAUUUCCUCAUUUUAUUAGGAAAGGACAG
UGGGAGUGGCACCUUCCAGGGUCAAGGAAGGCACGGGGGAGGGGCAAACAACAG
AUGGCUGGCAACUAGAAGGCACAGUCGAGGCUGAUCAGCGAUCACUAUUACCGG
GGGGGAAGGGCCUGCAUGUGCAGGGCGUCGUAGGUGUCCUUGGUGGCGGUGCUC
AGGCCUUGGUACAGGCCAUCGUGUCCCUUGCCCCGCCUCCGCUCGCCCUUCAUGC
CGAUCUCGCUGUAAGCCUCGGCCAUCUUGUCCUUCUGCAGCUCGUUGUACAGGCC
CUCCUGGGGGUUCUUCCGUCUGGGCUUUCCGCCCAUCUCGGGGUCCCGGCCUCUC
CGCUUGUCCAGCACGUCGUACUCCUCCCGUCUGCCCAGGUUCAGCUCGUUGUACA
GCUGGUUCUGGCCCUGCUGGUAGGCAGGGGCGUCGGCGCUUCUGCUGAACUUCAC
CCGCAGCUCACAGCCGCCCUCCUCCUCCUCGGGAAACCUGCAGCUGCAGCCGUCCU
CCUCCUGGGUGGUCUGCACGGGCCGCAUGAAGGGCUGCUUGAAGAUGUACAGCAG
CUUCUUCCGGCCCCGCUUGUUCCGGUGGUUGCAGUACAGGGUGAUCACCAGGCUC
AGCAGCAGCACGCCACAUGUGCCGGCCAGAGGGGCCCAGAUGUAGAUGUCGCAGG
CGAAGUCCAGGCCUCUGGUGUGCACAGCUCCGCCGGCAGCAGGUCUACAGGCUUC
GGGCCGCAGAGACAGAGGCUGGCUGGCGAUUGUAGGAGCAGGAGUAGGAGGUCU
AGGAGCAGGAGUUGUGGUAGGCUUGGCGGGCAGGAACACGGGCACGAAGCUGCU
CACGGUCACCAUGGUGCCCUGGCCCCACACGUCCAGGAUGGUGUCUCCCCGAUCU
CUGGCGCAGUAGUACACGGCGGUGUCGGCGGCGGUCACGCUGCUCAGCUUCAGGC
UGAACUGGUUCUUGCUGGUGUCCACGCUGAUGGUCACCCGGGACUUCAGGCUGGG
GUUGUAGUAGGUGCUGCCGCUGUAGCUGAUAGAGCCAAUCCACUCCAGGCCCUUG
CCAGGGGGCUGCCGGAUCCAGCCCCAGUAGUAGCUGCUGCUGCUGAUGCUGCCGC
CGCUCACUGUGCAGGUCAGGCUCAGUGUCUCGCUAGGCUUCACCAGGCCAGGGCC
GCUCUCCUGCAGCUGCAGCUGUCCCUUUGUGCUGCCCUCGCCAGAGCCAGGCUUG
CCGCUGCCGCUGGUGCUGCCUUUGAUCUCCACCUUGGUGCCGCCGCCGAAGGUCA
GCAGGUCGUACUUCUGCUGGCAGUAGUAGGUGGCGAAGUCCUCGGGCUGCAGGC
UGCUGAUGGUCAGGGUGAAGUCGGUGCCGCUGCCAGAGCCAGAGAACCUGCUGG
GCACGCCAGACUGCAGGCUGCUGGCGGCGUAGAUCAGCAGCUUGGGGGCCUUGCC

GGGCUUCUGCUGGUACCAGUUCAGGUAGCUGCUGAUGCUCUGGCUGGCCCGGCAU
GUGAUGGUCACUCUGUCGCCCACGCUGGCAGACAGGCUGCUGGGGCUCUGGGUCA
UCUGGAUGUCGGGCCGAGCGGCAUGCAACAGCAGGGCAAGAGGCAGAAGCAGGG
CGGUCACGGGCAGGGCCAUGGUGGCUUUACCAACAGUACCGGAAUGCCAAGCUUG
GGUCUGGGCUCUUUUAUUGAGCUCGGGGAGCAGAAGCGCGCGAACAGAAGCGAG
AAGCGAACUGAUUGGUUAGUUCAAAUAAGGCACAGGGUCAUUUCAGGUCCUUGG
GGCACCCUGGAAACAUCUGAUGGUUCUCUAGAAACUGCUGAGGGCGGGACCGCAU
CUGGGGACCAUAUGUUCUUGGCCCUGAGCCGGGGCAGGAACUGCUUACCACAGAU
AUCCUGUUUGGCCCAUAUUCUGCUGUUCCAACUGUUCUUGGCCCUGAGCCGGGGC
AGGAACUGCUUACCACAGAUAUCCUGUUUGGCCCAUAUUCUGCUGUCUCUCUGUU
CCUAACCUUGAUCCUAGCUUGCCAAACCUACAGGUGGGGUCUUUCAUUCCCCCCU
UUUUCUGGAGACUAAAUAAAUGAAACUGCACAAAGACAAUAGUCAUUCUCGUCA
CCGAGAGACUACCACUAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA (SEQ ID NO:405)

[0840] The BCMA-CAR fusion polypeptide comprised an amino acid sequence of:MALPVTALLLPLALLLHAARPDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQ QKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQKYDLLTFG GGTKVEIKGSTSGSGKPGSGEGSTKGQLQLQESGPGLVKPSETLSLTCTVSGGSISSSSYY WGWIRQPPGKGLEWIGSISYSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVY YCARDRGDTILDVWGQGTMVTVSSFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEA CRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNKRGRKKLLYIFK QPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGR REEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKG HDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:390)

[0841] The BCMA-CAR fusion polypeptide comprised a CD8 signal peptide domain having an amino acid sequence of:

MALPVTALLLPLALLLHAARP (SEQ ID NO:93)

[0842] The BCMA-CAR fusion polypeptide comprised a CD8 hinge domain having an amino acid sequence of:

FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO:79)

[0843] The BCMA-CAR fusion polypeptide comprised a CD8 transmembrane domain having an amino acid sequence of:

IYIWAPLAGTCGVLLLSLVITLYCNHRN (SEQ ID NO:73)

[0844] The BCMA-CAR fusion polypeptide comprised a 4-1BB signaling domain having an amino acid sequence of:

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO:77)

[0845] The BCMA-CAR fusion polypeptide comprised a CD3z signaling domain having an amino acid sequence of:

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEG LYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:76)

Peripheral Blood Monocyte Cells (PBMCs) Thaw and Overnight Rest

[0846] Cryopreserved PBMCs from two donors (Hemacare, donor 448 and 354) were thawed at 37°C and washed in T cell medium comprised of TexMACS™ cell culture medium (Miltenyi #170-076-307) with 5% human AB serum (Gemini Bio 100-152). Cells were counted after pelleting by centrifugation at 300xg for 5 minutes and resuspended in cytokine-free medium at a target concentration of 1 x10^6 cells/mL. Cells were transferred to cell culture flasks and maintained at a target cell concentration of 1 x10^6 cells/mL in TexMACs + 5% human AB serum + IL-7(Miltenyi-130-095-362, 10ng/mL) and IL-15(Miltenyi-130-095-764, 5ng/mL) and placed inside of a cell culture incubator at 37°C and 5% CO_2 for overnight rest.

LNP Transfection

[0847] After overnight rest, cells were harvested and combined into a 50 mL centrifuge tube. The flask was washed with PBS and transferred to a conical tube. 15 mL of versene (FisherSci:15-040-066) was added to the flask to detach adherent cells. The cells were then incubated at 37°C until they detached, and then transferred to a conical tube with PBMCs and spun cells for 7 minutes at 350xg. The cells were then resuspended in Plasma-Lyte A (Baxter #2B2544) plus 2% Human Serum Albumin (AkronBio: AK8228-0100) for cell counting. The cells were counted and plated for final resuspension at 40e6 cells/mL in a 96- well cell repellant U- bottom plate (FisherSci:07-000-612).

[0848] TransAct™ and the tLNPs were mixed together in Plasma-Lyte A and added to the cell suspension such that the final cell concentration was 20e6 cells/mL in Plasma-Lyte A 1% Human Serum Albumin. MACS® GMP T Cell TransAct™, a polymeric nanomatrix comprising human CD3 and CD28, was added to the transfection medium at a final volume dilution of 1:17.5. For the transfected condition, LNPs comprising an mRNA encoding the gene modifying

polypeptide and the template RNA were dosed at a total concentration of 5 ug tLNPs per million cells, and a ratio of one part gene modifying polypeptide tLNPs to 25 parts template tLNPs. For the untransfected condition, no tLNPs were added. Incubation occurred for four hours at room temperature. After the 4-hour incubation, cells were washed by spinning at 350xg for 7 minutes at room temperature. The supernatant was pipetted out, and the cells were then resuspended in complete T cell medium comprised of TexMACS cell culture medium, 5% human AB serum by volume, 100 IU/mL IL-2, 10 ng/mL IL-7, and 5 ng/mL IL-15 at a cell concentration of $1x10^6$ cells/mL in a 96 well flat bottom plate (Greiner Bio-One: 655970).

Culture ex vivo

[0849] Complete T cell medium was added as needed to maintain cultures at $1x10^6$ cells/mL. Samples were obtained for flow cytometry to measure CAR frequency on days 5 and 7.

[0850] For the staining procedure, $0.5x10^6$ cells were first quenched with stain buffer (BD #554657), pelleted by centrifugation at 500xg for 5 minutes, resuspended in 100 µL of stain solution containing 0.125 µL of rh-BCMA Fc Chimera AF647, and incubated for 30 minutes in the dark at 2-8°C. Following incubation, cells were quenched with stain buffer, pelleted by centrifugation at 500xg for 5 minutes, then resuspended in 200 µL of stain buffer 7-AAD (Invitrogen #A1310) live/dead stain at 1:1000 ratio. Flow cytometry data was acquired on a NovoCyte flow cytometer (Agilent), then analyzed for the proportion of live cells expressing anti-BCMA CAR.

In vitro tumor killing assay

[0851] On day 10, CAR-T cells were harvested, and function was assessed via a BCMA target cell killing assay. Briefly, BCMA-CAR-T cells were co-cultured for 16 hours with BCMA-positive tumor cell lines (MM1.S), stably transduced with ffLuc, at serially decreasing CAR+T cell (effector) to tumor cell ratios. The CellTiter-Glo® ((Promega #G7572) Luminescent Cell Viability Assay, homogeneous method of determining the number of viable cells based on quantitation of the ATP present, an indicator of metabolically active cells, where % cytotoxicity = $(BLI_{Mock} - BLI_{Sample})/BLI_{Mock}$, BLI_{Mock} = mean target cell alone value of that experiment. Additionally, after 16 hours of co-culture, supernatants were assessed for their IFN-γ cytokine levels relative to control T cells via ELISA(MSD). The experiment was also conducted using a non-codon optimized version of the template RNA (see sequence in example 9 below), the results were not as strong (data not shown).

[0852] **FIG. 33** shows that the anti-CD3 tLNPs described in this Example delivered an exemplary gene modifying system to human donor PBMCs to generate human CAR-T cells

effectively. An average of about 42% of the T cells expressed the CAR. None of the control cells ("Mock") expressed the CAR. **FIG. 34** shows that the CAR-T cells generated using the methods described herein killed BCMA tumor cells in a cytotoxicity assay, showing that they were functionally capable of killing cancer cells. **FIG. 35** shows that the CAR-T cells generated using the tLNPs formulated with the gene modifying system also expressed much higher levels of the IFN-γ cytokine relative to control T cells that were not administered the tLNPs, demonstrating functional potency of the CAR-T cells.

Example 16: Testing Different Anti-CD3 Targeting Moieties in ECD Conditions

[0853] This Example provides the results of a screen to identify anti-CD3 targeting moieties useful for targeting an LNP to T cells. In particular, the LNP can be used to deliver a gene modifying system to human T cells to generate functional CAR-T cells, such as in conditions and in a timeframe suitable for same-day manufacturing, e.g., in an extracorporeal device (ECD). In this instance, BCMA-targeted CAR-T cells were generated and shown to be capable of killing human cancer cells (multiple myeloma tumor cells) *in vitro*.

Preparation of Targeted LNPs for Transfection

[0854] A solution containing the anti-CD3 Fab fragment to be tested was buffer exchanged with PBS containing 10mM EDTA. Ten equivalents of TCEP were added to each solution and the reduction was carried out for 1 hour at room temperature. Complete reduction of the Fab fragment was confirmed by SDS PAGE gels.

[0855] Base LNPs were prepared at a molar ratio of 47% ionizable lipid (Lipid154): 28.5% cholesterol: 22% DSPC: 2.0% PEG-DMG: 0.5% DSPE-PEG2000-maleimide at an N/P of 6 in the ethanol phase. The aqueous phase was composed of mRNA encoding the exemplary gene modifying polypeptide and the exemplary template RNA encoding the CAR at a mass ratio of 1:1 in 25 mM acetate buffer. The two phases were mixed using a T-mixer or microfluidic device to formulate base LNP (aqueous phase: ethanol phase=3:1). 3x volume of citrate-buffered saline was added to LNP solution and resulted solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes.

[0856] After the base LNP was formulated, the reduced anti-CD3 Fab fragment was added to the LNP-maleimide solution at specified mass ratio to the total RNA mass. The solution was incubated at room temperature for 2 hours for conjugation and then stored at 4°C overnight. The unreacted maleimide was quenched by the addition of excess cysteine (3 equivalent) for 1 hour at room temperature. The LNPs were purified and concentrated by a centrifugal filter (100K

MWCO) or via TFF. The resulting tLNPs were characterized by Zetasizer for size and PDI and by Ribogreen assays for mRNA concentration and encapsulation efficiency.

[0857] The sequences for each tested anti-CD3 targeting moiety (each Fab fragment) are presented in **Table 32**.

Table 32: Exemplary anti-CD3 targeting moiety sequences

	Heavy Chain Sequence	**Light Chain Sequence**	**VH Sequence**	**VL Sequence**
Anti-CD3-9 (Plamotamab)	EVQLVESGGGLVQPGGSLRLSCAASGFTFS**TYAMN**WVRQAPGKGLEWVG**RIRSKYNNYATYYADSVKG**RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVR**HGNFGDSYVSWFAY**WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT (SEQ ID NO:226)	ETVVTQEPSLTVSPGGTVTLTC**GSSTGAVTTSNYAN**WVQQKPGKSPRGLIG**GTNKRAP**GVPARFSGSLLGGKAALTISGAQPEDEADYYC**ALWYSNHWV**FGGGTKLTVLGQPKANPTVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADGSPVKAGVETTKPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS (SEQ ID NO:227)	EVQLVESGGGLVQPGGSLRLSCAASGFTFS**TYAMN**WVRQAPGKGLEWVG**RIRSKYNNYATYYADSVKG**RFTISRDDSKNTLYLQMNSLRAEDTAVYYCVR**HGNFGDSYVSWFAY**WGQGTLVTVSS (SEQ ID NO:228)	ETVVTQEPSLTVSPGGTVTLTC**GSSTGAVTTSNYAN**WVQQKPGKSPRGLIG**GTNKRAP**GVPARFSGSLLGGKAALTISGAQPEDEADYYC**ALWYSNHWV**FGGGTKLTVL(SEQ ID NO:229)
Anti-CD3-10 (HPN536)	EVQLVESGGGLVQPGGSLKLSCAASGFTFN**KYAIN**WVRQAPGKGLEWVA**RIRSKYNNYATYYADQVKD**RFTISRDDSKNTAYLQMNNLKTEDTAVYYCVR**HANFGNSYISYWAY**WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT (SEQ ID NO:230)	ETVVTQEPSLTVSPGGTVTLTC**ASSTGAVTSGNYPN**WVQQKPGQAPRGLIG**GTKFLVP**GTPARFSGSLLGGKAALTLSGVQPEDEAEYYC**TLWYSNRWV**FGGGTKLTVLGQPKANPTVTLFPPSSEELQANKATLV	EVQLVESGGGLVQPGGSLKLSCAASGFTFN**KYAIN**WVRQAPGKGLEWVA**RIRSKYNNYATYYADQVKD**RFTISRDDSKNTAYLQMNNLKTEDTAVYYCVR**HANFGNSYISYWAY**WGQGTLV	ETVVTQEPSLTVSPGGTVTLTC**ASSTGAVTSGNYPN**WVQQKPGQAPRGLIG**GTKFLVP**GTPARFSGSLLGGKAALTLSGVQPEDEAEYYC**TLWYSNRWV**FGGGTKLTVL(SEQ ID NO:239)

	WGQGTLVTVS SASTKGPSVFP LAPSSKSTSGG TAALGCLVKD YFPEPVTVSWN SGALTSGVHTF PAVLQSSGLYS LSSVVTVPSSSL GTQTYICNVNH KPSNTKVDKK VEPKSCDKT (SEQ ID NO:236)	CLISDFYPGAVTV AWKADGSPVKAG VETTKPSKQSNNK YAASSYLSLTPEQ WKSHRSYSCQVT HEGSTVEKTVAPT ECS (SEQ ID NO:237)	TVSS (SEQ ID NO:238)	
Anti-CD3-11 (Pasotuxizumab)	EVQLVESGGGL VQPGGSLKLSC AASGFTFN**KYA MN**WVRQAPG KGLEWVA**RIR SKYNNYATYY ADSVKD**RFTIS RDDSKNTAYL QMNNLKTEDT AVYYCVR**HGN FGNSYISYWA Y**WGQGTLVTV SSASTKGPSVFP LAPSSKSTSGG TAALGCLVKD YFPEPVTVSWN SGALTSGVHTF PAVLQSSGLYS LSSVVTVPSSSL GTQTYICNVNH KPSNTKVDKK VEPKSCDKT (SEQ ID NO:246)	ETVVTQEPSLTVS PGGTVTLTC**GSST GAVTSGNYPN**W VQQKPGQAPRGLI G**GTKFLAP**GTPA RFSGSLLGGKAAL TLSGVQPEDEAEY YC**VLWYSNRWV** FGGGTKLTVLGQ PKANPTVTLFPPS SEELQANKATLV CLISDFYPGAVTV AWKADGSPVKAG VETTKPSKQSNNK YAASSYLSLTPEQ WKSHRSYSCQVT HEGSTVEKTVAPT ECS (SEQ ID NO:247)	EVQLVESGGGLV QPGGSLKLSCAAS GFTFN**KYAMN**W VRQAPGKGLEWV A**RIRSKYNNYAT YYADSVKD**RFTIS RDDSKNTAYLQM NNLKTEDTAVYY CVR**HGNFGNSYI SYWAY**WGQGTL VTVSS (SEQ ID NO:248)	ETVVTQEPSLTVS PGGTVTLTC**GSST GAVTSGNYPN**W VQQKPGQAPRGLI G**GTKFLAP**GTPA RFSGSLLGGKAAL TLSGVQPEDEAEY YC**VLWYSNRWV** FGGGTKLTVL(SEQ ID NO:249)
Anti-CD3-12 (Flotetuzumab)	EVQLVESGGGL VQPGGSLRLSC AASGFTFS**TYA MN**WVRQAPG KGLEWVG**RIR SKYNNYATYY ADSVKD**RFTIS RDDSKNSLYLQ MNSLKTEDTA VYYCVR**HGNF GNSYVSWFAY** WGQGTLVTVS SASTKGPSVFP LAPSSKSTSGG TAALGCLVKD YFPEPVTVSWN	ETVVTQEPSLTVS PGGTVTLTC**RSST GAVTTSNYAN**W VQQKPGQAPRGLI G**GTNKRAP**WTPA RFSGSLLGGKAAL TITGAQAEDEADY YC**ALWYSNLWV** FGGGTKLTVLGQ PKANPTVTLFPPS SEELQANKATLV CLISDFYPGAVTV AWKADGSPVKAG VETTKPSKQSNNK YAASSYLSLTPEQ WKSHRSYSCQVT	EVQLVESGGGLV QPGGSLRLSCAAS GFTFS**TYAMN**WV RQAPGKGLEWVG **RIRSKYNNYATY YADSVKD**RFTISR DDSKNSLYLQMN SLKTEDTAVYYC VR**HGNFGNSYVS WFAY**WGQGTLV TVSS (SEQ ID NO:258)	ETVVTQEPSLTVS PGGTVTLTC**RSST GAVTTSNYAN**W VQQKPGQAPRGLI G**GTNKRAP**WTPA RFSGSLLGGKAAL TITGAQAEDEADY YC**ALWYSNLWV** FGGGTKLTVL(SEQ ID NO:259)

	SGALTSGVHTF PAVLQSSGLYS LSSVVTVPSSSL GTQTYICNVNH KPSNTKVDKK VEPKSCDKT (SEQ ID NO:256)	HEGSTVEKTVAPT ECS (SEQ ID NO:257)		
Note: The CDR sequences for each targeting moiety are in **bolded** text.				

[0858] The gene modifying system formulated in the tLNPs comprised an mRNA encoding an exemplary gene modifying polypeptide comprising the following components.

[0859] The gene modifying polypeptide comprised an SV40 NLS having an amino acid sequence of:

PKKKRKV (SEQ ID NO:267)

[0860] The gene modifying polypeptide comprised a GGS Linker having an amino acid sequence of:

GGS

[0861] The gene modifying polypeptide comprised the amino acid sequence of:

MDSTAHPNQGRGLEKVSQTLPALQTPGQHTAAGGSSPLSGRNQRKNTKKLLLGAWNIR
TLLDRENTPRPERRTALIGKELARYNIDIAALSETRLPEEGSLSEPTTGYTFFWKGRASNE
DRIHGVGLAIKTSLLKQLPDLPVGISERLMKIRLPLSKDRYATIISAYAPTLTSTEETIEQFY
SDLSAVLHSVPTNDKLILLGDFNARVGQDHERWKGVLGKHGVGKMNNNGLLLLSKCS
EFELTITNTVFRMANKYKTTWMHPRSKQWHLIDYIIVRRRDIQDVKITRAMRGAECWT
DHRLVRATLQMRIAPRHPKRAQTVRAFYNVSRLRDPSYLQTFQSCLDDKLSAKGPLTGS
STEKWNQFRDAVKETSKAVLGPKQRNHQDWFDENNTAIEDLLSKKNKAFMEWQNNPN
SAPKKDRFKSLQATAQREIRKMQDRWWEKKAEEIQRFADMKNYKQFFSALKTVYGPL
KPTTTPLLSSDGDTLIKDKKGISNRWKEHFSQLLNRPSSVDQSALDQIPQNRTIEQLDVPP
SIEEVQKAIKQMSAGKAPGKDGIPTEVYKALNGKALQAFHIVLTSIWEEEDMPPELRDAS
IVALYKNKGSRAACDNYRGISLLSTAGKILARVILNRLLSSVSEQNLPESQCGFRPDRSTI
DMVFTVRQMQEKCLEQNLSLYIVFIDLTKAFDTVNRDALWVILSKLGCPAKFVKLIQLF
HVDMTGEVLSGGETSDRFNISNGVKQGCVLAPVLFNLFFTQVLRHAVMDLDLGVYIKY
RLDGSLFDLRRLTAKTKTTERLILEALFADDCALMAHQENHLQTIVDRFSTATKLFGLTI
SLSKTEVLFQPAPGRPTNQPCITIDGTQLSNVNTFKYLGSTIANDGSLDHEINARIQKASQ
ALGRLRCKVLQHRGVSTATKLKVYNAVVLSSLLYGCETWTLYRKHMKQLEQFHQRSL
RSIMRIRWQDRITNQEVLDRANSTSIEVMVLKTQLRWSGHVIRMDPQRIPRQVFYGELS

AGLRKQGRPKKRFKDQLKSNLKWAGITPKQLELAASDRSSWRTHINHAATTFEDERRR
RLAAARERRHQATTAPPVTTGVPCPMCHKLCASAFGLQSHMRVHRR (SEQ ID NO:32)

[0862] The full-length gene modifying polypeptide with all components was structured as follows: NLS - 3GS linker – gene modifying polypeptide - XTEN linker - HiBit tag. The full-length sequences are provided in Table E6.

[0863] The gene modifying system further comprised a template RNA with the following sequences from 5' to 3': 5'UTR_{retro}, BCMA CAR, MND promoter, 3'UTR_{retro}.

[0864] The template RNA comprised a 5'UTR having the sequence of:

GGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGUCG
UGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUCAC
UUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACGGC
UUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUGGU
GAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACGGA
AGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUGUG
GAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAGCU
GAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAACG
CCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUCAC
GCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCAAG
ACCUGCGGCGAUGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCAGU
UGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCCUG
UGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAG
(SEQ ID NO:372)

[0865] The template RNA comprised a 3'UTR having the sequence of:

UGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCGAGAGACUACCACU (SEQ ID NO:373)

[0866] The template RNA comprised a nucleic acid sequence encoding a BCMA-CAR fusion polypeptide, wherein the nucleic acid sequence was SEQ ID NO:7.

SEQ ID NO:7:
AGGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGUC
GUGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUCA
CUUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACGG
CUUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUGG

UGAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACGG
AAGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUGU
GGAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAGC
UGAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAAC
GCCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUCA
CGCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCAA
GACCUGCGGCGAUGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCAG
UUGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCCU
GUGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAGU
UUAUUUAGUCUCCAGAAAAAGGGGGGAAUGAAAGACCCCACCUGUAGGUUUGGC
AAGCUAGGAUCAAGGUUAGGAACAGAGAGACAGCAGAAUAUGGGCCAAACAGGA
UAUCUGUGGUAAGCAGUUCCUGCCCCGGCUCAGGGCCAAGAACAGUUGGAACAGC
AGAAUAUGGGCCAAACAGGAUAUCUGUGGUAAGCAGUUCCUGCCCCGGCUCAGG
GCCAAGAACAGAUGGUCCCCAGAUGCGGUCCCGCCCUCAGCAGUUUCUAGAGAAC
CAUCAGAUGUUUCCAGGGUGCCCCAAGGACCUGAAAUGACCCUGUGCCUUAUUUG
AACUAACCAAUCAGUUCGCUUCUCGCUUCUGUUCGCGCGCUUCUGCUCCCCGAGC
UCAAUAAAAGAGCCCAGACCCAAGCUUGGCAUUCCGGUACUGUUGGUAAAGCCAC
CAUGGCCCUGCCCGUGACCGCCCUGCUUCUGCCUCUUGCCCUGCUGUUGCAUGCC
GCUCGGCCCGACAUCCAGAUGACCCAGAGCCCCAGCAGCCUGUCUGCCAGCGUGG
GCGACAGAGUGACCAUCACAUGCCGGGCCAGCCAGAGCAUCAGCAGCUACCUGAA
CUGGUACCAGCAGAAGCCCGGCAAGGCCCCCAAGCUGCUGAUCUACGCCGCCAGC
AGCCUGCAGUCUGGCGUGCCCAGCAGGUUCUCUGGCUCUGGCAGCGGCACCGACU
UCACCCUGACCAUCAGCAGCCUGCAGCCCGAGGACUUCGCCACCUACUACUGCCA
GCAGAAGUACGACCUGCUGACCUUCGGCGGCGGCACCAAGGUGGAGAUCAAAGGC
AGCACCAGCGGCAGCGGCAAGCCUGGCUCUGGCGAGGGCAGCACAAAGGGACAGC
UGCAGCUGCAGGAGAGCGGCCCUGGCCUGGUGAAGCCUAGCGAGACACUGAGCCU
GACCUGCACAGUGAGCGGCGGCAGCAUCAGCAGCAGCAGCUACUACUGGGGCUGG
AUCCGGCAGCCCCCUGGCAAGGGCCUGGAGUGGAUUGGCUCUAUCAGCUACAGCG
GCAGCACCUACUACAACCCCAGCCUGAAGUCCCGGGUGACCAUCAGCGUGGACAC
CAGCAAGAACCAGUUCAGCCUGAAGCUGAGCAGCGUGACCGCCGCCGACACCGCC
GUGUACUACUGCGCCAGAGAUCGGGGAGACACCAUCCUGGACGUGUGGGGCCAGG
GCACCAUGGUGACCGUGAGCAGCUUCGUGCCCGUGUUCCUGCCCGCCAAGCCUAC
CACAACUCCUGCUCCUAGACCUCCUACUCCUGCUCCUACAAUCGCCAGCCAGCCUC
UGUCUCUGCGGCCCGAAGCCUGUAGACCUGCUGCCGGCGGAGCUGUGCACACCAG

AGGCCUGGACUUCGCCUGCGACAUCUACAUCUGGGCCCCUCUGGCCGGCACAUGU
GGCGUGCUGCUGCUGAGCCUGGUGAUCACCCUGUACUGCAACCACCGGAACAAGC
GGGGCCGGAAGAAGCUGCUGUACAUCUUCAAGCAGCCCUUCAUGCGGCCCGUGCA
GACCACCCAGGAGGAGGACGGCUGCAGCUGCAGGUUUCCCGAGGAGGAGGAGGGC
GGCUGUGAGCUGCGGGUGAAGUUCAGCAGAAGCGCCGACGCCCCUGCCUACCAGC
AGGGCCAGAACCAGCUGUACAACGAGCUGAACCUGGGCAGACGGGAGGAGUACG
ACGUGCUGGACAAGCGGAGAGGCCGGGACCCCGAGAUGGGCGGAAAGCCCAGACG
GAAGAACCCCCAGGAGGGCCUGUACAACGAGCUGCAGAAGGACAAGAUGGCCGAG
GCUUACAGCGAGAUCGGCAUGAAGGGCGAGCGGAGGCGGGGCAAGGGACACGAU
GGCCUGUACCAAGGCCUGAGCACCGCCACCAAGGACACCUACGACGCCCUGCACA
UGCAGGCCCUUCCCCCCCGGUAAUAGUGAUCGCUGAUCAGCCUCGACUGUGCCUU
CUAGUUGCCAGCCCAACUGGUUUGCCCCUCCCCCGUGCCUUCCUUGACCCUGGAA
GGUGCCACUCCCACUGUCCUUUCCUAAUAAAAUGAGGAAAUUGCAUCGCAUUGUC
UGAGUAGGUGUCAUUCUAUUCUGGGGGGUGGGGUGGGGCAGGACAGCAAGGGGG
AGGAUUGGGAAGACAAUAGCAGGCAUGCUGGGGAUGCGGUGGGCUCUAUGGCUU
CUGAGGCGGAAAGAACUGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCG
AGAGACUACCACUAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

[0867] The BCMA-CAR fusion polypeptide comprised an amino acid sequence of:

MALPVTALLLPLALLLHAARPDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQK
PGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQKYDLLTFGGG
TKVEIKGSTSGSGKPGSGEGSTKGQLQLQESGPGLVKPSETLSLTCTVSGGSISSSSYYWG
WIRQPPGKGLEWIGSISYSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYC
ARDRGDTILDVWGQGTMVTVSSFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRP
AAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNKRGRKKLLYIFKQPF
MRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREE
YDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHD
GLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:390)

[0868] The BCMA-CAR fusion polypeptide comprised a CD8 signal peptide domain having an amino acid sequence of:

MALPVTALLLPLALLLHAARP (SEQ ID NO:93)

[0869] The BCMA-CAR fusion polypeptide comprised a CD8 hinge domain having an amino acid sequence of:

FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO:79)

[0870] The BCMA-CAR fusion polypeptide comprised a CD8 transmembrane domain having an amino acid sequence of:

IYIWAPLAGTCGVLLLSLVITLYCNHRN (SEQ ID NO:73)

[0871] The BCMA-CAR fusion polypeptide comprised a 4-1BB signaling domain having an amino acid sequence of:

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO:77)

[0872] The BCMA-CAR fusion polypeptide comprised a CD3z signaling domain having an amino acid sequence of:

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEG LYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:76)

LNP Transfection

[0873] Fresh leukopaks from two different donors were processed using a CUE instrument (Fresnius). The platelet-washed and concentrated leukapheresis material was then pelleted by centrifugation at 350xg for 7 minutes and resuspended in transfection medium comprised of Plasma-Lyte A solution (Baxter #2B2544) with 2% Human Serum Albumin by volume at a target cell stock concentration of 40 x10^6 cells/mL. Cells were plated in a 96-well U-bottom plate at 2e6 cells/well. For the transfected condition, LNPs comprising an mRNA encoding the gene modifying polypeptide and the template RNA were dosed at a total concentration of 5ug and 2.5 ug LNPs per million cells, diluted in Plasma-Lyte A solution, and a ratio of one part gene modifying polypeptide LNPs to four parts template LNPs. For the untransfected condition, no LNPs were added. MACS® GMP T Cell TransAct™, a polymeric nanomatrix comprising human CD3 and CD28, was added to the LNP transfection medium at a final volume dilution of 1:8.75. The final target cell concentration for transfection was 20x10^6 cells/mL with 1:17.5 dilution of MACS® GMP T Cell TransAct™ in 1% Human Serum Albumin by volume. Incubation occurred for four hours incubated at 25°C with 5% CO_2. After the 4-hour incubation, cells were washed with equal volume PBS and resuspended in complete T cell medium at a target cell concentration of 1x10^6 cells/mL in a 24 well G-rex plate, then placed inside of a cell culture incubator at 37°C and 5% CO_2. Complete T cell medium was comprised of TexMACS

cell culture medium (Miltenyi #170-076-307), 5% human AB serum by volume, 100 IU/mL IL-2, 10 ng/mL IL-7, and 5 ng/mL IL-15.

Culture ex vivo

[0874] Complete T cell medium was added as needed to maintain cultures at $1x10^6$ cells/mL. Samples were obtained for flow cytometry to measure CAR frequency on day 7.

[0875] For the staining procedure, $0.5x10^6$ cells were first quenched with stain buffer (BD #554657), pelleted by centrifugation at 500xg for 5 minutes, resuspended in 100 µL of stain solution containing 0.125 µL of rh-BCMA Fc Chimera AF647, and incubated for 30 minutes in the dark at 2-8°C. Following incubation, cells were quenched with stain buffer, pelleted by centrifugation at 500xg for 5 minutes, then resuspended in 100 µL of stain buffer containing and 5 µL of 7-AAD at a dilution of 1:1000. stain. Flow cytometry data was acquired on a Novocyte Quanteon flow cytometer (Agilent), then analyzed for the proportion of live cells expressing anti-BCMA CAR.

In vitro tumor killing assay

[0876] On day 8, CART cells were harvested, and function was assessed via a BCMA target cell killing assay using live cell imaging. Briefly, BCMA-CART cells were co-cultured for 24 hours with BCMA-positive tumor cell lines (MM.1S-GFP-fluc) at serially decreasing T cell (effector) to tumor cell ratios and tracked over 48 hours, these data represent the 24-hour read where optimal killing window was determined. Tumor targets were tracked via GFP expression and cell death was tracked via expression of Annexin V. Tumor cell death was quantified by co-expression of both markers and normalized to a positive tumor cell only control treated with Camptothecin to induce apoptosis.

[0877] The experiment was also conducted using a non-codon optimized version of the template RNA (see sequence in example 9 below), the results were not as strong (data not shown).

[0878] **FIG. 36** shows that tLNPs conjugated to anti-CD3-9, anti-CD3-10, anti-CD3-11, or anti-CD3-12 targeting moieties (fab fragments) were all capable of delivering an exemplary gene modifying system to human leukocytes resulting in the generation of human CAR-T cells under conditions suitable for same day manufacturing of CAR-T cells, e.g., in an extracorporeal device. For all four anti-CD3 tLNPs tested, delivery of an exemplary gene modifying system resulted in an average of over 10% of CD4+ CD8+ T cells expressing CAR at both dose levels (2.5 ug and 5 ug). **FIG. 37** shows the CAR expression levels (MFI) in the CAR-T cells generated

by each of the anti-CD3 tLNPs. **FIG. 38** shows that CAR-T cells generated using each of the anti-CD3 tLNPs comprising an exemplary gene modifying system were capable of killing BCMA-expressing tumor cells in a cytotoxicity assay.

Example 17: Targeted LNP delivery of an exemplary gene modifying system results in writing of a CAR sequence into the genomic DNA of human T cells *in vivo*

[0879] Example 6 showed that tLNPs formulated with an exemplary gene modifying system were capable of writing an exogenous nucleic acid sequence encoding GFP into the genomic DNA of human T cells engrafted in a mouse model. Next, targeted LNPs were conjugated to different targeting moieties (an anti-CD2 targeting moiety, an anti-CD3 targeting moiety, an anti-CD5 targeting moiety, or an anti-CD7 targeting moiety), formulated with an exemplary gene modifying system designed to write a CAR sequence into the genomic DNA of human cells, and then administered to a mouse model engrafted with human T cells. The engrafted human T cells were then assayed by FACs analysis to determine whether the tLNPs could effectively deliver the gene modifying system, leading to appreciable levels of CAR writing and expression *in vivo*. The human T cells in untreated humanized mice were used as a negative control.

[0880] The exemplary gene modifying system comprised an mRNA encoding a gene modifying polypeptide and a template RNA encoding a CAR.

Preparation of Targeted LNPs for In Vivo Studies

[0881] Base LNPs were prepared at a molar ratio of 47% ionizable lipid (Lipid154): 42.5% cholesterol: 8% DSPC: 2.0% PEG-DMG: 0.5% DSPE-PEG2000-maleimide at an N/P of 6 in the ethanol phase. The aqueous phase was composed of mRNA encoding the gene modifying polypeptide and the template RNA encoding the CAR at a mass ratio of 1:4 in 25 mM acetate buffer. The two phases were mixed using a microfluidic device to formulate base LNP at a total flow rate of 18 mL/min (aqueous phase: ethanol phase=3:1). 3x volume of citrate-buffered saline was added to LNP solution and resulted solution was neutralized by 1 vol% of 1M Tris-HCl, pH 8 after 15 minutes.

[0882] The interchain disulfide bonds of the anti-CD2, anti-CD3, anti-CD5, or anti-CD7 Fab fragments were selectively reduced prior to conjugation to the base LNPs. A solution containing each of the Fab fragments was buffer exchanged with PBS containing 10mM EDTA. Ten equivalents of TCEP were added to the solution and the reduction was carried out for 1 hour at room temperature. Complete reduction of each of the Fab fragments was confirmed by SDS PAGE gels.

[0883] The reduced Fab fragments were added to the LNP-maleimide solution to create each tLNP. The solution was incubated at room temperature for 3 hours for conjugation and then stored at 4°C overnight. The unreacted maleimide was quenched by cysteine for 1 hour at room temperature. The LNPs were concentrated, and unbound Fab fragments were removed by a centrifugal filter (100K MWCO) or TFF. The solution was exchanged with Tris buffer containing 9% sucrose during the process. The resulting tLNPs were characterized by Zetasizer for size and PDI and by Ribogreen assays for RNA concentration.

[0884] The gene modifying system formulated in the LNPs comprised an mRNA encoding an exemplary gene modifying polypeptide comprising the following components.

[0885] The gene modifying polypeptide comprised an SV40 NLS having an amino acid sequence of:

PKKKRKV (SEQ ID NO:367)

[0886] The gene modifying polypeptide comprised a GGS Linker having an amino acid sequence of:

GGS

[0887] The gene modifying polypeptide comprised a polypeptide having the amino acid sequence of:

MPKKKRKVGGSDSTAHPNQGRGLEKVSQTLPALQTPGQHTAAGGSSPLSGRNQRKNTK
KLLLGAWNIRTLLDRENTPRPERRTALIGKELARYNIDIAALSETRLPEEGSLSEPTTGYT
FFWKGRASNEDRIHGVGLAIKTSLLKQLPDLPVGISERLMKIRLPLSKDRYATIISAYAPT
LTSTEETIEQFYSDLSAVLHSVPTNDKLILLGDFNARVGQDHERWKGVLGKHGVGKMN
NNGLLLLSKCSEFELTITNTVFRMANKYKTTWMHPRSKQWHLIDYIIVRRRDIQDVKITR
AMRGAECWTDHRLVRATLQMRIAPRHPKRAQTVRAFYNVSRLRDPSYLQTFQSCLDD
KLSAKGPLTGSSTEKWNQFRDAVKETSKAVLGPKQRNHQDWFDENNTAIEDLLSKKNK
AFMEWQNNPNSAPKKDRFKSLQATAQREIRKMQDRWWEKKAEEIQRFADMKNYKQF
FSALKTVYGPLKPTTTPLLSSDGDTLIKDKKGISNRWKEHFSQLLNRPSSVDQSALDQIPQ
NRTIEQLDVPPSIEEVQKAIKQMSAGKAPGKDGIPTEVYKALNGKALQAFHIVLTSIWEE
EDMPPELRDASIVALYKNKGSRAACDNYRGISLLSTAGKILARVILNRLLSSVSEQNLPES
QCGFRPDRSTIDMVFTVRQMQEKCLEQNLSLYIVFIDLTKAFDTVNRDALWVILSKLGC
PAKFVKLIQLFHVDMTGEVLSGGETSDRFNISNGVKQGCVLAPVLFNLFFTQVLRHAVM
DLDLGVYIKYRLDGSLFDLRRLTAKTKTTERLILEALFADDCALMAHQENHLQTIVDRF
STATKLFGLTISLSKTEVLFQPAPGRPTNQPCITIDGTQLSNVNTFKYLGSTIANDGSLDHE

INARIQKASQALGRLRCKVLQHRGVSTATKLKVYNAVVLSSLLYGCETWTLYRKHMK QLEQFHQRSLRSIMRIRWQDRITNQEVLDRANSTSIEVMVLKTQLRWSGHVIRMDPQRIP RQVFYGELSAGLRKQGRPKKRFKDQLKSNLKWAGITPKQLELAASDRSSWRTHINHAA TTFEDERRRRLAAARERRHQATTAPPVTTGVPCPMCHKLCASAFGLQSHMRVHRR (SEQ ID NO:64)

[0888] The full-length gene modifying polypeptide with all components was structured as follows: NLS - 3GS linker – gene modifying polypeptide. The full-length sequences are provided in **Table 33**.

Table 33: Nucleic Acid and Amino Acid Sequences of the Gene Modifying Polypeptide

Name	Nucleic Acid Sequence	Amino Acid Sequence
RNAIV	AGGAAAUAAGAGAGAAAAGAAGAGUAAGA	MPKKKRKVGGSDSTAHPNQGRGLE
T2113	AGAAAUAUAAGAGCCACCAUGCCCAAGAAG	KVSQTLPALQTPGQHTAAGGSSPLS
	AAGAGAAAAGUAGGAGGUUCCGAUUCCAC	GRNQRKNTKKLLLGAWNIRTLLDR
	AGCUCAUCCCAACCAGGGACGGGGACUGGA	ENTPRPERRTALIGKELARYNIDIAA
	AAAGGUUUCCCAGACUCUGCCUGCACUGCA	LSETRLPEEGSLSEPTTGYTFFWKGR
	AACCCCAGGUCAGCAUACUGCAGCUGGUGG	ASNEDRIHGVGLAIKTSLLKQLPDLP
	AUCCUCUCCUCUGAGCGGAAGAAACCAGCG	VGISERLMKIRLPLSKDRYATIISAY
	GAAGAACACCAAGAAGCUGCUGCUGGGAGC	APTLTSTEETIEQFYSDLSAVLHSVP
	CUGGAACAUACGCACACUGCUGGAUCGCGA	TNDKLILLGDFNARVGQDHERWKG
	AAAUACUCCCAGGCCUGAAAGACGCACCGC	VLGKHGVGKMNNNGLLLLSKCSEF
	ACUGAUUGGGAAAGAGCUGGCCAGAUACA	ELTITNTVFRMANKYKTTWMHPRS
	ACAUCGACAUAGCCGCCCUGUCCGAAACCA	KQWHLIDYIIVRRRDIQDVKITRAM
	GACUGCCUGAAGAAGGAUCCUUAUCCGAGC	RGAECWTDHRLVRATLQMRIAPRH
	CUACCACCGGAUACACCUUCUUUUGGAAGG	PKRAQTVRAFYNVSRLRDPSYLQTF
	GGAGAGCAAGCAACGAGGACAGGAUCCAUG	QSCLDDKLSAKGPLTGSSTEKWNQF
	GAGUGGGACUGGCUAUCAAGACCUCCCUGC	RDAVKETSKAVLGPKQRNHQDWFD
	UGAAGCAGCUGCCAGAUCUGCCAGUGGGAA	ENNTAIEDLLSKKNKAFMEWQNNP
	UCUCCGAGAGACUGAUGAAGAUCAGGCUGC	NSAPKKDRFKSLQATAQREIRKMQ
	CUCUGUCCAAGGACCGCUACGCAACAAUCA	DRWWEKKAEEIQRFADMKNYKQFF
	UUUCCGCAUACGCCCCCACCCUGACUUCCA	SALKTVYGPLKPTTTPLLSSDGDTLI
	CCGAGGAAACCAUUGAGCAGUUCUACUCCG	KDKKGISNRWKEHFSQLLNRPSSVD
	AUCUGUCCGCCGUGUUGCACUCCGUGCCUA	QSALDQIPQNRTIEQLDVPPSIEEVQ
	CCAACGACAAGCUGAUCCUGCUUGGAGACU	KAIKQMSAGKAPGKDGIPTEVYKAL
	UCAACGCUCGCGUGGGACAAGACCAUGAGC	NGKALQAFHIVLTSIWEEEDMPPEL
	GCUGGAAAGGAGUGCUGGGUAAACACGGG	RDASIVALYKNKGSRAACDNYRGIS
	GUGGGGAAGAUGAACAACAACGGGCUGCU	LLSTAGKILARVILNRLLSSVSEQNL
	GCUGCUGUCCAAGUGCAGCGAGUUCGAGCU	PESQCGFRPDRSTIDMVFTVRQMQE
	GACCAUUACCAACACCGUGUUUAGGAUGGC	KCLEQNLSLYIVFIDLTKAFDTVNRD
	CAACAAGUACAAGACCACCUGGAUGCACCC	ALWVILSKLGCPAKFVKLIQLFHVD
	CAGGAGCAAGCAGUGGCACCUGAUCGAUUA	MTGEVLSGGETSDRFNISNGVKQGC
	CAUCAUCGUGCGGAGGAGGGAUAUCCAGGA	VLAPVLFNLFFTQVLRHAVMDLDL
	CGUGAAAAUCACCCGUGCAAUGCGCGGAGC	GVYIKYRLDGSLFDLRRLTAKTKTT
	UGAAUGCUGGACAGACCAUAGGCUGGUGA	ERLILEALFADDCALMAHQENHLQT
	GAGCAACACUGCAGAUGAGGAUUGCACCUA	IVDRFSTATKLFGLTISLSKTEVLFQP
	GGCACCCUAAGAGGGCUCAAACCGUGCGGG	APGRPTNQPCITIDGTQLSNVNTFKY
	CCUUUUACAAUGUGUCCCGGCUGAGAGAUC	LGSTIANDGSLDHEINARIQKASQAL
	CCUCCUACCUGCAGACCUUUCAGUCUUGCC	GRLRCKVLQHRGVSTATKLKVYNA
	UGGAUGACAAGCUGUCUGCCAAGGGACCCC	VVLSSLLYGCETWTLYRKHMKQLE
	UGACCGGAUCUUCCACAGAGAAGUGGAACC	QFHQRSLRSIMRIRWQDRITNQEVL
	AGUUUCGCGACGCAGUGAAGGAAACCAGCA	DRANSTSIEVMVLKTQLRWSGHVIR

	AAGCAGUGCUGGGACCCAAACAGCGCAACC ACCAGGACUGGUUCGACGAGAACAACACCG CUAUCGAGGACCUGCUGUCCAAGAAGAACA AGGCCUUUAUGGAGUGGCAGAACAACCCCA ACUCCGCCCCCAAGAAAGACAGGUUCAAAA GCCUGCAAGCCACCGCCCAGAGAGAGAUCC GGAAAAUGCAGGACAGAUGGUGGGAAAAG AAGGCCGAGGAGAUCCAGAGGUUCGCCGAU AUGAAGAACUACAAGCAGUUCUUUAGCGCA CUGAAAACCGUGUACGGACCUCUGAAACCC ACCACCACUCCCCUGCUCUCUUCCGAUGGA GACACCCUGAUCAAGGACAAGAAGGGAAUC AGCAACCGCUGGAAAGAGCACUUUUCCCAG CUGCUGAACAGGCCAUCCUCCGUGGACCAA AGCGCUUUAGACCAGAUCCCCCAGAACCGU ACUAUCGAGCAGCUGGAUGUGCCUCCCUCC AUCGAAGAGGUGCAGAAGGCCAUCAAGCAG AUGUCUGCUGGGAAGGCUCCUGGGAAGGA UGGGAUCCCAACAGAGGUGUACAAAGCCCU GAACGGAAAGGCCCUGCAGGCCUUUCACAU UGUGCUGACCUCCAUCUGGGAGGAAGAGGA CAUGCCUCCUGAGCUUAGGGACGCCUCUAU UGUGGCCCUGUACAAGAAUAAGGGAAGCCG CGCCGCUUGUGAUAACUACCGGGGAAUCUC ACUGCUGAGCACAGCUGGGAAGAUUCUGGC CCGUGUGAUCCUGAACCGGUUGCUGAGUUC CGUGUCCGAGCAGAACUUACCAGAAUCACA GUGCGGGUUUCGCCCCGAUCGGUCCACCAU CGAUAUGGUGUUCACAGUGAGGCAGAUGC AGGAGAAGUGCCUGGAGCAGAACCUGAGCC UGUACAUCGUGUUUAUCGACCUGACCAAGG CCUUUGAUACCGUGAACCGCGACGCACUGU GGGUUAUCCUUAGCAAGCUGGGAUGUCCCG CCAAGUUCGUGAAGCUGAUCCAGCUGUUCC ACGUUGAUAUGACCGGAGAGGUGCUGUCCG GAGGAGAAACCUCCGACCGGUUUAACAUCU CCAAUGGGGUGAAACAGGGGUGUGUGCUG GCCCCAGUGCUGUUCAACCUGUUUUUCACC CAGGUGCUGAGACACGCCGUGAUGGAUCUG GACCUGGGUGUGUACAUCAAGUACCGCCUG GACGGAAGUCUGUUCGAUCUCAGACGCCUG ACCGCCAAGACUAAGACUACCGAGCGCCUG AUUCUGGAAGCCCUGUUUGCCGACGAUUGU GCCCUUAUGGCCCACCAGGAGAACCACCUC CAGACCAUUGUGGACAGAUUCUCCACCGCC ACCAAGCUGUUCGGACUGACCAUCUCCCUG UCAAAGACCGAAGUGCUGUUUCAACCCGCA CCUGGAAGGCCCACCAAUCAGCCUUGCAUC ACUAUUGACGGGACCCAGCUGUCCAACGUG AACACCUUCAAGUACCUGGGAUCCACCAUA GCCAACGAUGGAAGCCUGGAUCACGAGAUC AACGCCAGGAUCCAGAAGGCCAGCCAAGCA CUUGGGAGGCUAAGAUGUAAGGUGCUGCA GCACAGAGGGGUGAGCACUGCCACCAAGCU GAAGGUGUACAACGCUGUGGUGCUGUCCUC CCUGCUGUAUGGAUGCGAAACCUGGACCCU GUACCGGAAGCACAUGAAGCAGCUGGAGCA GUUCCACCAGAGGAGCUUGAGGAGCAUCAU GAGGAUCCGCUGGCAGGAUAGAAUCACCAA CCAGGAGGUGCUGGACAGGGCUAACUCCAC CAGCAUCGAGGUUAUGGUGCUGAAAACCCA	MDPQRIPRQVFYGELSAGLRKQGRP KKRFKDQLKSNLKWAGITPKQLEL AASDRSSWRTHINHAATTFEDERRR RLAAARERRHQATTAPPVTTGVPCP MCHKLCASAFGLQSHMRVHRR (SEQ ID NO:392)

	GCUGCGUUGGUCUGGACACGUGAUUCGGAU GGAUCCUCAGCGUAUUCCCCGCCAGGUAUU CUAUGGGGAGCUGUCUGCCGGACUGAGAAA ACAGGGCAGACCCAAGAAGCGCUUUAAGGA UCAGCUGAAGUCCAACCUGAAGUGGGCUGG GAUCACCCCUAAACAGCUGGAGUUGGCAGC AUCUGAUCGCUCCAGCUGGCGCACACAUAU CAACCACGCAGCCACCACCUUUGAGGAUGA GAGGAGGCGCAGAUUAGCUGCAGCAAGAG AGCGUCGCCAUCAAGCAACUACAGCCCCCC CAGUUACCACAGGAGUACCUUGCCCUAUGU GCCACAAGCUAUGUGCCUCCGCCUUCGGGU UGCAAUCCCACAUGAGAGUGCAUAGGCGAA GUGGGAGCGAGACUCCUGGAACAUCAGAAU CCGCUACACCUGAAUCCGUGAGCGGGUGGC GGCUGUUCAAGAAGAUCAGCUGAGCUGGA GCCUCGGUGGCCAUGCUUCUUGCCCCUUGG GCCUCCCCCCAGCCCCUCCUCCCCUUCCUGC ACCCGUACCCCCGUGGUCUUUGAAUAAAGU CUGAAAAAAAAAAAAAAAAAUUAAAAAAAA AAAAAAAAAAAAAAAAAAUUUAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAUUUU UAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAA (SEQ ID NO:391)	

[0889] The gene modifying system further comprised a template RNA comprising the following sequences from 5' to 3': 5'UTR_{retro}, CAR sequence, MND promoter, 3'UTR_{retro}.

[0890] The template RNA comprised a 5'UTR having the sequence of:

GGGUGUAUGGGGUGCUCAGGGAGGGGUAGUAUCUCUGGUAUGGAGGGCUUGUCG
UGCCCUCCUAGGGCAGCUCUCCAGCCUCUGACCCCCACCUGACACCCAGCUCUCAC
UUGUGGCUCCCAGUAGCUGCUAGCAUGUGGCAGCGGCCACACCCCGGGCAACGGC
UUCGACAGGCCGGCUAAACCUUGUGAGGGUAGCCAUCGGGUCAUCGACCCCUGGU
GAACCAGGGCUUUGCUCACCCAGCAUGUGAAGACUGCUUCGGCUGAACAGACGGA
AGAAACCAAUAAGAAGGUUCAACGGCUGAGAGGGCGACGCAGCAAAGCACUGUG
GAGUGCUUAGGGCGUGUUGGAGCACAAAGGACAACACGGCCAUCCAAUGCAGCU
GAGGAAGUCUCCAGAUGUAACAAUUUUUCGUGCCACUGGACCCAGGCUUCCAACG
CCGAGAGAGUGGGACUGUCUCUGUGCAUCGGCUUUUCCACUUAAAUCUCUUUCAC
GCACAAGUAUCUUUGUGCACACUCAUCUAUCCUAACCCCGUCCACCCUCUUCAAG
ACCUGCGGCGAUGGGGGGAGUGGCGACGCAACAGGUGGAGGUGACCACUGGCAGU
UGUAGUCACGAUCCUGCACGUAGGCGGCCCACGGACCAGUGGUCGCUCGGCCCUG
UGGGCAGCAGGGACGUUCGGCAGCAUCCUGGGCGACUGAGCAGCCCUCUCUAG
(SEQ ID NO:372)

[0891] The template RNA comprised a 3'UTR having the sequence of:

UGAAACUGCACAAAGACAAUAGUCAUUCUCGAUCACCGAGAGACUACCACU (SEQ ID NO:373)

[0892] The template RNA comprised a nucleic acid sequence encoding the CAR polypeptide, wherein the nucleic acid sequence was:

UUUAUUUAGUCUCCAGAAAAAGGGGGGAAUGAAAGACCCCACCUGUAGGUUUGG
CAAGCUAGGAUCAAGGUUAGGAACAGAGAGACAGCAGAAUAUGGGCCAAACAGG
AUAUCUGUGGUAAGCAGUUCCUGCCCCGGCUCAGGGCCAAGAACAGUUGGAACAG
CAGAAUAUGGGCCAAACAGGAUAUCUGUGGUAAGCAGUUCCUGCCCCGGCUCAGG
GCCAAGAACAGAUGGUCCCCAGAUGCGGUCCCGCCCUCAGCAGUUUCUAGAGAAC
CAUCAGAUGUUUCCAGGGUGCCCCAAGGACCUGAAAUGACCCUGUGCCUUAUUUG
AACUAACCAAUCAGUUCGCUUCUCGCUUCUGUUCGCGCGCUUCUGCUCCCCGAGC
UCAAUAAAAGAGCCCAGACCCAAGCUUGGCAUUCCGGUACUGUUGGUAAAGCCAC
CAUGCUGCUGCUGGUGACCAGCCUGCUGCUGUGCGAACUGCCCCACCCCGCCUUC
CUGCUGAUCCCCGACAUCCAGAUGACCCAGACCACCAGCAGCCUGAGCGCCAGCC
UGGGCGACAGAGUGACCAUCAGCUGCAGAGCCAGCCAGGACAUCAGCAAGUACCU
GAACUGGUACCAGCAGAAGCCCGACGGCACCGUGAAGCUGCUGAUCUACCACACC
AGCAGGUUACACAGCGGCGUGCCCAGCAGAUUCAGCGGCAGCGGCAGCGGCACAG
ACUACAGCCUGACCAUCAGCAACCUGGAGCAGGAGGACAUUGCCACCUACUUCUG
CCAGCAGGGCAACACCCUGCCCUACACCUUCGGCGGCGGCACCAAGCUGGAGAUC
ACCGGCUCUACAAGCGGCAGCGGCAAGCCCGGCAGCGGAGAGGGAUCUACCAAGG
GCGAAGUGAAGCUGCAGGAGAGCGGACCCGGACUUGUGGCCCCCAGCCAAAGCCU
GAGCGUGACAUGCACCGUGAGCGGCGUGAGCCUGCCCGAUUACGGCGUGAGCUGG
AUCAGACAGCCCCCCAGAAAGGGCCUGGAGUGGCUGGGCGUGAUCUGGGGCAGCG
AGACAACCUACUACAACAGCGCCCUGAAGUCCCGGCUGACCAUCAUCAAGGACAA
CAGCAAGAGCCAGGUGUUCCUGAAGAUGAACAGCCUGCAGACCGACGACACCGCC
AUCUACUACUGCGCCAAGCACUACUACUACGGCGGCAGCUACGCCAUGGACUAUU
GGGGCCAGGGCACCAGCGUGACCGUGAGCAGCUUCGUGCCCGUGUUCCUGCCCGC
CAAACCUACCACCACCCCUGCCCCUAGACCUCCCACCCCAGCCCCAACAAUCGCCA
GCCAGCCUCUGUCUCUGCGGCCCGAAGCCUGUAGACCUGCUGCCGGCGGAGCCGU
GCACACCAGAGGCCUGGACUUCGCCUGCGACAUCUACAUCUGGGCCCCUCUGGCC
GGCACCUGUGGCGUGCUGCUGCUGAGCCUGGUGAUCACCCUGUACUGCAACCACC
GGAACAAACGGGGCAGAAAGAAACUCCUGUAUAUAUUCAAACAACCAUUUAUGA
GGCCAGUACAAACUACUCAAGAGGAAGAUGGCUGUAGCUGCCGAUUUCCAGAAG
AAGAAGAAGGAGGAUGUGAACUGAGAGUGAAGUUCAGCAGAUCCGCCGACGCCC

CUGCCUACCAGCAGGGACAGAACCAGCUGUACAACGAGCUGAACCUGGGCAGACG
GGAAGAGUACGACGUGCUGGACAAGCGGAGAGGCCGGGACCCCGAGAUGGGCGG
AAAGCCCAGACGGAAGAACCCCCAGGAAGGCCUGUAUAACGAACUGCAGAAAGAC
AAGAUGGCCGAGGCCUACAGCGAGAUCGGCAUGAAGGGCGAGCGGAGGCGCGGC
AAGGGCCACGAUGGCCUGUACCAGGGCCUGAGCACCGCCACCAAGGACACCUACG
ACGCCCUGCACAUGCAGGCCCUGCCCCCCAGAUAAUAAAGCGGCCGGGGGAUUCG
CUGAUCAGCCUCGACUGUGCCUUCUAGUUGCCAGCCAUAUGUUGUUUGCCCCUCC
CCCGUGCCUUCCUUGACCCUGGAAGGUGCCACUCCCACUGUCCUUUCCUAAUAAA
AUGAGGAAAUUGCAUCGCAUUGUCUGAGUAGGUGUCAUUCUAUUCUGGGGGGUG
GGGUGGGGCAGGACAGCAAGGGGGAGGAUUGGGAAGACAAUAGCAGGCAUGCUG
GGGAUGCGGUGGGCUCUAUGGCUUCUGAGGCGGAAAGAAC (SEQ ID NO:393)

[0893] The template RNA comprised a nucleic acid sequence encoding a CAR polypeptide having an amino acid sequence of:

MLLLVTSLLLCELPHPAFLLIPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQK
PDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGG
GTKLEITGSTSGSGKPGSGEGSTKGEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVS
WIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYY
CAKHYYYGGSYAMDYWGQGTSVTVSSFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRP
EACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNKRGRKKLLYI
FKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNL
GRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRG
KGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:394)

Preparation and Dosing of a Humanized Mouse Model

[0894] Briefly, female NSG mice 6-8 weeks of age (Catalog #005557) were purchased from Jackson Labs (Bar Harbor, ME). Following acclimation to the vivarium for 3-5 days, animals received a tail vein injection of 5 X 10^6 human CD3+ T cells (Source) and kept for 2-3 weeks for T cell engraftment. For each *in vivo* study, "humanized" NSG mice were randomized into groups of 3-4 animals and baseline weights were obtained. Animals were then treated via intravenous tail vein injection with 100 ul of the test tLNPs formulated with the gene modifying system at a dose of 1 mg/kg. Following a 16-hour incubation, animals were euthanized via CO_2 asphyxiation, weighed and their spleens were collected and the splenic cells were isolated.

Flow Cytometry

[0895] Splenic cells (1 X 10^6 in 0.1 ml PBS) from each test mouse were subsequently aliquoted to individual wells in 96 well plates and antibody fluorochrome conjugate mixtures (**Table 34**) were added to the cells in a total of 0.1 ml. Following a 30-minute incubation at room temperature, the plates were centrifuged for 5 minutes at 500G and supernatants were removed. The cells were then resuspended in 0.2 ml PBS and evaluated on an Aligent NovoCyte Quanteon (2010098) or Cytek Northern Lights (N7-00008) flow cytometer using FlowJo software (Ashland OR). Cells stained with relevant isotype control fluorochrome conjugated antibodies were used to set the negative control gates. Total T cells were defined as CD45+CD3+CD5+. CD4+ and CD8+ T cells were defined as CD45+CD3+CD5+CD4+ and CD45+CD3+CD5+CD8+, respectively.

Table 34. Flow cytometry antibodies

Marker	Fluorophore	Clone	Vendor	Catalog
Live/Dead	Fixable Yellow		ThermoFisher	L34959
hCD45	PE-Cy7	HI30	Biolegend	304016
CD3	BV421	OKT3	Biolegend	317344
CD4	BV605	OKT4	Biolegend	317438
CD5	BV510	L17F12	Biolegend	364018
GFP				
CD8	PE	SK1	Biolegend	344706
CCR7	APC	G043H7	Biolegend	353214
mCD45	PE Cy5	30-F11	Biolegend	103110
CD45RA	BV785	HI100	Biolegend	304140

Results

[0896] **FIG. 50** shows that administration of each of the tLNPs carrying an exemplary all-RNA gene modifying system to the engrafted mice leads to the generation of human CD3+ T cells expressing CAR *in vivo*, as determined by flow cytometry. The animals that were administered the tLNPs conjugated to the anti-CD3 targeting moiety exhibited an average of about 5% of the CD3+ cells expressing CAR *in vivo*. Administration of the other non-CD3 tLNPs led to smaller numbers of T cells expressing the CAR (an average of about 1.2%, 2.2% and 0.8% of cells for the anti-CD2 tLNPs, the anti-CD5 tLNPs and the anti-CD7 tLNPs, respectively) although the numbers of CAR positive cells were significantly higher than in negative control animals.

[0897] To try to increase the amount of CAR expression in T cells, tLNPs conjugated to the anti-CD2, anti-CD5, and anti-CD7 targeting moieties were reformulated to try to increase LNP

stability and circulation time *in vivo*. More specifically, the amount of helper lipid in the tLNPs formulations was increased. The tLNPs were prepared at a molar ratio of 47% ionizable lipid (Lipid154): 28.5% cholesterol: 22% DSPC: 2.0% DPG-PEG2000: 0.5% DSPE-PEG2000-maleimide at an N/P of 6. They comprised the same exemplary gene modifying system designed to introduce the nucleic acid encoding the CAR into human cells. Humanized mice were dosed with the tLNPs as described above except at 2 mg/kg per animal, and FACs analysis was done on the human T cells as described above to detect CAR expression.

[0898] **FIG. 51** shows that a larger number of human CD3+ T cells expressed the CAR following administration of the reformulated anti-CD2 and anti-CD7 tLNPs at the 2 mg/kg dose.

CLAIMS

1. A lipid nanoparticle (LNP), comprising:

   (i) a lipid component comprising an ionizable lipid and a helper lipid, wherein the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, about 20 mol% to about 40 mol% of the lipid component, about 20 mol% to about 30 mol% of the lipid component, or about 25 mol% to about 40 mol% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component;

   (ii) one or two targeting moieties conjugated to the LNP, wherein the one or two targeting moieties are antibodies or fragments thereof configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; and

   (iii) a therapeutic agent encapsulated within the LNP, wherein the therapeutic agent comprises one or more nucleic acid molecules.

2. A lipid nanoparticle (LNP), comprising:

   (i) a lipid component comprising an ionizable lipid and a helper lipid, wherein the ionizable lipid is present at about 35 mol% to about 60 mol% of the lipid component, about 20 mol% to about 40 mol% of the lipid component, about 20 mol% to about 30 mol% of the lipid component, or about 25 mol% to about 40 mol% of the lipid component, and the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component;

   (ii) means for binding a cell surface receptor selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; and

   (iii) a therapeutic agent encapsulated within the LNP, wherein the therapeutic agent comprises one or more nucleic acid molecules.

3. The LNP of claim 2, wherein the means for binding the cell surface receptor is present on the surface of the LNP.

4. The LNP of claim 2 or claim 3, wherein the LNP comprises means for binding a first cell surface receptor and means for binding a second cell surface receptor, wherein the first cell surface receptor and the second cell surface receptor are different and wherein the

first cell surface receptor and the second cell surface receptor are each independently selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28.

5. The LNP of any one of claims 1-4, wherein the helper lipid is present at about 22 mol% to about 36 mol% of the lipid component.

6. The LNP of any one of claims 1-4, wherein the helper lipid is present at about 20 mol% to about 30 mol% of the lipid component.

7. The LNP of any one of claims 1-6, wherein the helper lipid is present at about 25 mol% to about 30 mol% of the lipid component.

8. The LNP of any one of claims 1-6, wherein the helper lipid is present at about 20 mol% to about 25 mol% of the lipid component.

9. The LNP of any one of claims 1-8, wherein ionizable lipid is present at about 40 mol% to about 50 mol% of the lipid component.

10. The LNP of any one of claims 1-9, wherein ionizable lipid is present at about 20 mol% to about 30 mol% of the lipid component.

11. The LNP of any one of claims 1-10, wherein the helper lipid is selected from the group consisting of distearoyl-sn-glycero-phosphoethanolamine, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane- 1 - carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), monomethyl-phosphatidylethanolamine (such as 16-O-monomethyl PE), dimethyl- phosphatidylethanolamine (such as 16-O-dimethyl PE), l8-l-trans PE, l-stearoyl-2-oleoyl- phosphatidyethanolamine (SOPE), hydrogenated soy phosphatidylcholine, egg phosphatidylcholine (EPC), dioleoylphosphatidylserine (DOPS), sphingomyelin (SM), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), distearoylphosphatidylglycerol (DSPG), dierucoylphosphatidylcholine (DEPC), palmitoyloleyolphosphatidylglycerol (POPG), dielaidoyl-phosphatidylethanolamine (DEPE), lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine,

phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidicacid,cerebrosides, dicetylphosphate, lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof.

12. The LNP of claim 11, wherein the helper lipid is DSPC.

13. The LNP of claim 11, wherein the helper lipid is sphingomeylin.

14. The LNP of any one of claims 1-13, further comprising a cholesterol molecule.

15. The LNP of claim 14, wherein the cholesterol molecule is selected from the group consisting of beta-sitoesterol, hydroxycholesterol, stigmastanol, and any combination thereof.

16. The LNP of claim 14 or claim 15, wherein the cholesterol molecule is present at about 20 mol% to about 55 mol% of the lipid component.

17. The LNP of claim 14 or claim 15 wherein the cholesterol molecule is present at about 20 mol% to about 35 mol% of the lipid component.

18. The LNP of any one of claims 1 and 5-17, wherein the lipid component further comprises a PEGylated lipid.

19. The LNP of claim 18, wherein the one or two targeting moieties are conjugated to the LNP through the PEGylated lipid.

20. The LNP of claim 3 or claim 4, wherein the lipid component further comprises a PEGylated lipid.

21. The LNP of claim 20, wherein the means for binding the cell surface receptor is conjugated to the surface of the LNP through a PEGylated lipid.

22. The LNP of any one of claims 18-21, wherein the PEGylated lipid comprises a lipid portion selected from the group consisting of DMG, DPG, DSG, DTA, DOPE, DPPE, DMPE, DSPE, sphingosine, sphingomyelin, stearic acid, and any combination thereof.

23. The LNP of claim 22, wherein the lipid portion of the PEGylated lipid is DSPE.

24. The LNP of any one of claims 18-23, wherein the PEGylated lipid is present at about 0.05 mol% to about 2.5 mol% of the lipid component.

25. The LNP of any one of claims 1, 5-19 and 22-24, wherein the LNP comprises one targeting moiety conjugated to the surface of the LNP.

26. The LNP of claim 25, wherein the one targeting moiety binds CD2.

27. The LNP of claim 25, wherein the one targeting moiety binds CD3.

28. The LNP of claim 25, wherein the one targeting moiety binds CD5.

29. The LNP of claim 25, wherein the one targeting moiety binds CD6.

30. The LNP of claim 25, wherein the one targeting moiety binds CD7.

31. The LNP of claim 25, wherein the one targeting moiety binds CD8.

32. The LNP of claim 25, wherein the one targeting moiety binds CD28.

33. The LNP of claim 25, wherein the one targeting moiety binds CD4.

34. The LNP of any one of claims 1, 5-19 and 22-24, wherein the LNP comprises two targeting moieties conjugated to the surface of the LNP.

35. The LNP of claim 34, wherein the two targeting moieties bind CD3 and CD2.

36. The LNP of claim 34, wherein the two targeting moieties bind CD3 and CD4.

37. The LNP of claim 34, wherein the two targeting moieties bind CD3 and CD5.

38. The LNP of claim 34, wherein the two targeting moieties bind CD3 and CD6.

39. The LNP of claim 34, wherein the two targeting moieties bind CD3 and CD7.

40. The LNP of claim 34, wherein the two targeting moieties bind CD3 and CD8.

41. The LNP of claim 34, wherein the two targeting moieties bind CD3 and CD28.

42. The LNP of any one of claims 35-40, further comprising a third targeting moiety, wherein the third targeting moiety binds CD28.

43. The LNP of any one of claims 1, 5-19 and 22-24, wherein the LNP comprises two different targeting moieties conjugated to the surface of the LNP, wherein the two different targeting moieties bind the same cell surface receptor.

44. The LNP of claim 43, wherein the cell surface receptor is CD3.

45. The LNP of claim 27 or any one of claims 35-42 or 44, wherein the CD3 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences of Table 19 or Table 32, or variable heavy and light chain sequences having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

46. The LNP of claim 30 or claim 39, wherein the CD7 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences of Table

20, or variable heavy and light chain sequences having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

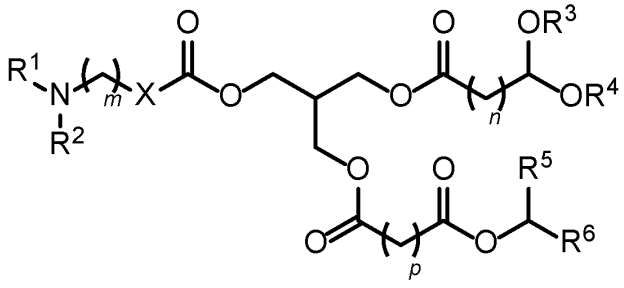
47. The LNP of claim 26 or claim 35, wherein the CD2 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences of Table 20, or variable heavy and light chain sequences having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

48. The LNP of claim 33 or claim 36, wherein the CD4 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences of Table 20.

49. The LNP of claim 28 or claim 37, wherein the CD5 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences of Table 20, or variable heavy and light chain sequences having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

50. The LNP of claim 31 or claim 40, wherein the CD8 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences of Table 20.

51. The LNP of any one of claims 1-50, wherein the ionizable lipid is a compound of formula (I):



(I),

or a pharmaceutically acceptable salt thereof, wherein:

X is -O- or -CH_2-;

m is 0, 1, 2, or 3;

R^1 is C_{1-4}alkyl;

R^2 is C_{1-4}alkyl;

n is 1, 2, 3, or 4;

R^3 is C_{4-10}alkyl;

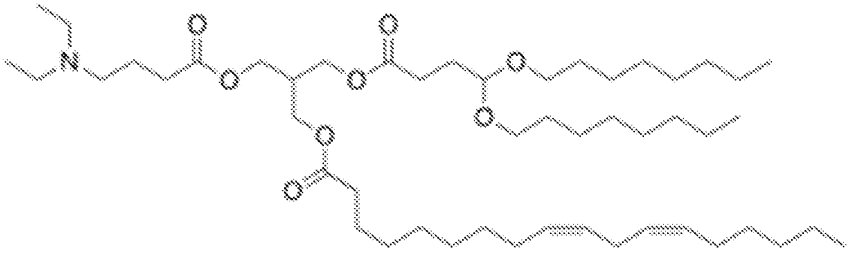
R^4 is C_{4-10}alkyl;

p is 2, 3, 4, 5, or 6;

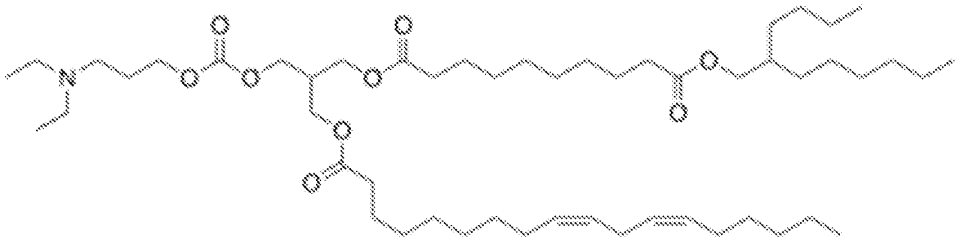
R^5 is C_{4-10}alkyl; and

R^6 is C_{4-10}alkyl.

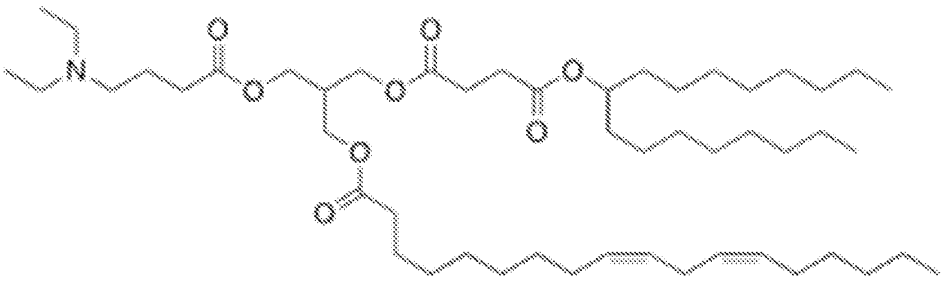
52. The LNP of any one of claims 1-50, wherein the ionizable lipid is a compound of one of the structures depicted below:



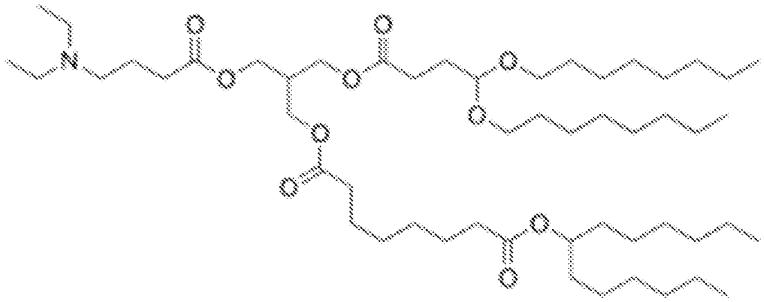
Lipid 092



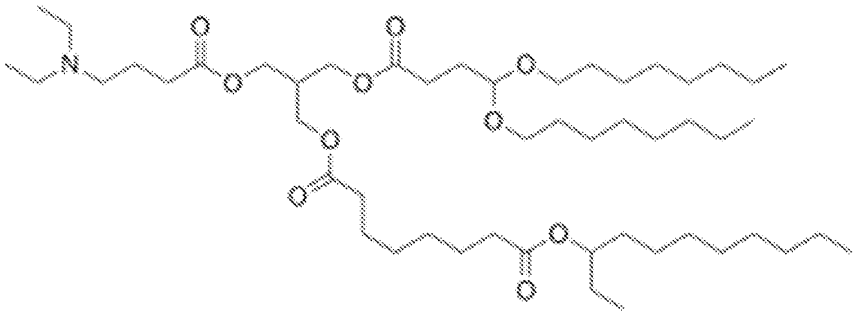
Lipid 093



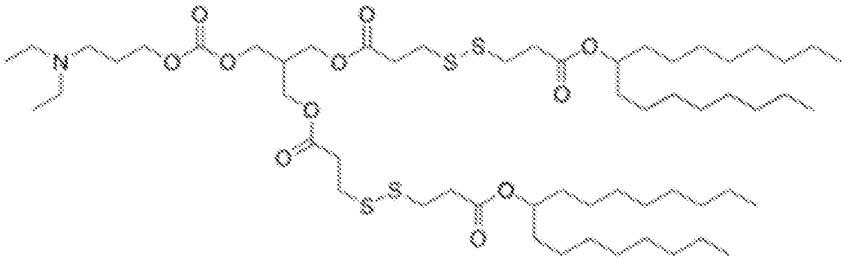
Lipid 153



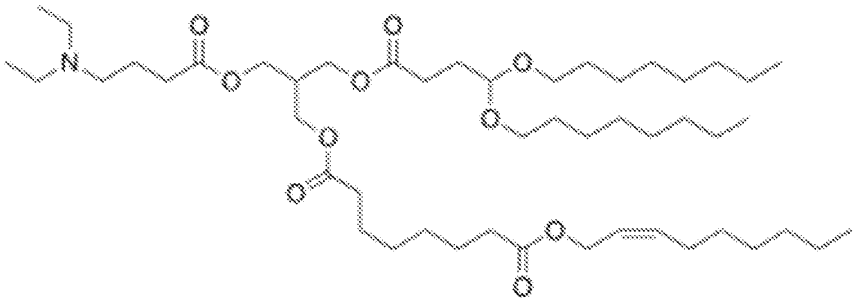
Lipid 154



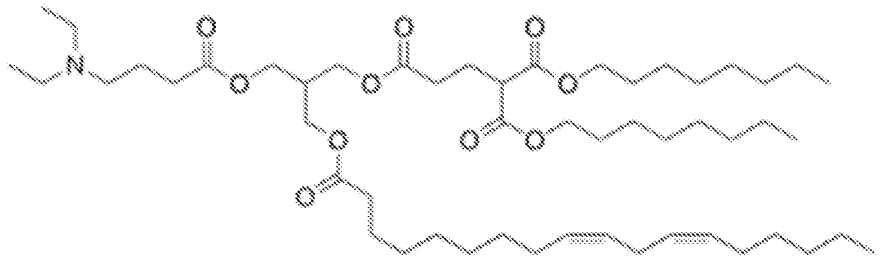
Lipid 155



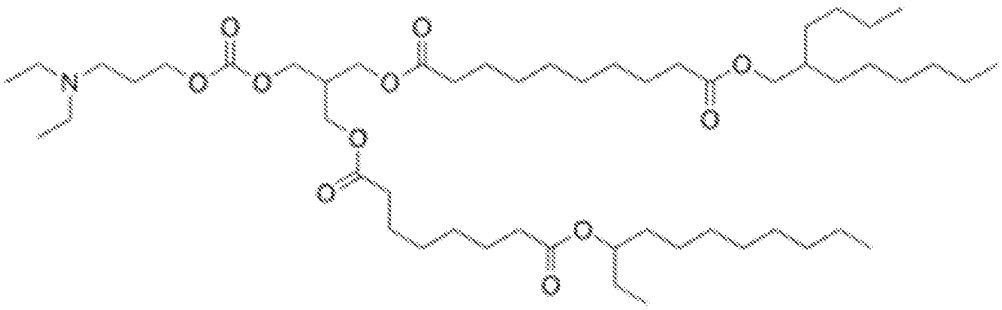
Lipid 162



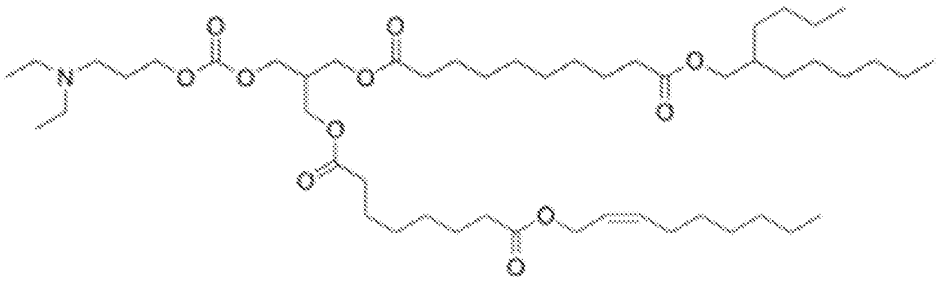
Lipid 163



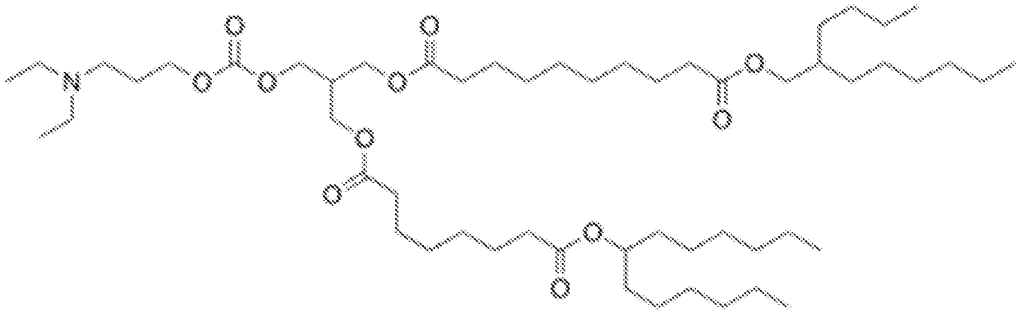
Lipid 169



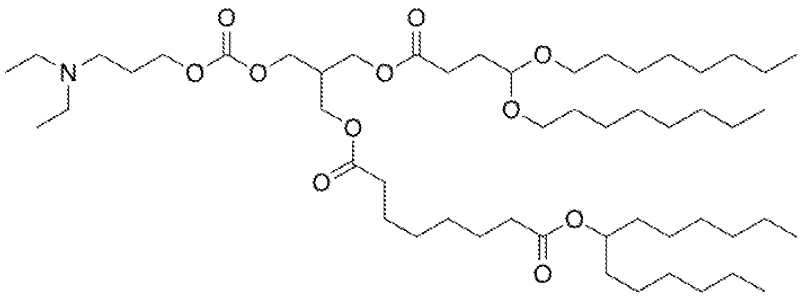
Lipid 176



Lipid 178



Lipid 183



Lipid 232

53. The LNP of any one of claims 1-52, wherein the therapeutic agent comprises one nucleic acid molecules, wherein the one nucleic acid molecule is an RNA molecule.

54. The LNP of claim 53, wherein the therapeutic agent comprises two nucleic acid molecules, wherein the two nucleic acid molecules are RNA molecules.

55. The LNP of claim 54, wherein one RNA molecule is an mRNA and the other RNA molecule is a template RNA.

56. The LNP of claim 55, wherein the mRNA encodes a gene modifying polypeptide polypeptide or a heterologous gene modifying polypeptide.

57. The LNP of claim 56, wherein the mRNA encodes a gene modifying polypeptide.

58. The LNP of any one of claims 55-57, wherein the template RNA is configured to insert a heterologous sequence encoding a protein into the genome of an immune cell.

59. The LNP of any one of claims 55-58, wherein the template RNA encodes a chimeric antigen receptor (CAR).

60. The LNP of any one of claims 1-59, wherein the therapeutic agent comprises three nucleic acid molecules.

61. The LNP of any one of claims 1-52, wherein the therapeutic agent comprises an mRNA encoding a CAR.

62. The LNP of any one of claims 1-52, wherein the therapeutic agent comprises a DNA molecule encoding a CAR

63. The LNP of claim 61 or claim 62, wherein the CAR comprises a sequence provided in Table 16.

64. A lipid nanoparticle (LNP) comprising:

(i) a lipid component comprising an ionizable lipid and a helper lipid;

(ii) one or two targeting moieties conjugated to the LNP, wherein the one or two targeting moieties are antibodies or fragments thereof configured to bind to one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; and

(iii) an mRNA encoding a gene modifying polypeptide; and

(iv) a template RNA comprising (a) sequence that binds to the polypeptide and (b) a heterologous object sequence.

65. The LNP of claim 64, wherein the heterologous object sequence encodes a chimeric antigen receptor (CAR).

66. The LNP of claim 65, wherein

(i) the CAR comprises an antigen binding domain that binds one or more antigens of a blood cancer (e.g., a leukemia or lymphoma), wherein optionally the antigen is a B cell antigen;

(ii) the CAR comprises an antigen binding domain that binds one or more antigens of a solid tumor;

(iii) the CAR comprises an antigen binding domain of any one of Tables 6-10 or 16;

(iv) the CAR comprises a transmembrane domain of Table 11 or 12;

(v) the CAR comprises a hinge domain (e.g., a hinge domain of Table 15);

(vi) the CAR comprises an intracellular signaling domain of Table 13 or 14;

(vii) the CAR comprises a costimulatory domain of Table 13 or 14;

(viii) the CAR comprises an antigen binding domain which comprises an scFv, a Fab, a diabody, a D domain binder, a centryin, or one or more single domain antibodies (e.g., VHH domains); or

(ix) the CAR comprises an amino acid sequence of Table 16 or an amino acid sequence according to any one of SEQ ID NOs: 74, 76-78, 80-87 or 102-125; and/or

(x) wherein the CAR comprises a first intracellular signaling domain and a second intracellular signaling domain.

67. The LNP of claim 65 or claim 66, wherein the template RNA sequence comprises an RNA sequence of Table 5B or an RNA sequence of any one of SEQ ID NOs: 395-404.

68. The LNP of any one of claims 64-67, wherein the gene modifying polypeptide comprises an amino acid sequence of Table 4 or Table 5A or a sequence having no more than 10,

20, 30, 40, 50, 60, 70, 80, 90, or 100 amino acid differences thereto, or a nucleic acid (e.g., DNA or mRNA) encoding the gene modifying polypeptide.

69. The LNP of any one of claims 64-68, wherein the gene modifying polypeptide comprises an amino acid sequence listed in any of Examples 4-11 or 15-17 or a sequence having no more than 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 amino acid differences thereto, or a nucleic acid (e.g., DNA or mRNA) encoding the gene modifying polypeptide.

70. The LNP of any one of claims 69, wherein the gene modifying polypeptide comprises an amino acid sequence of any one of SEQ ID NOs: 32, 33, 64 or 65, or a sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

71. The LNP of claim 64, wherein the sequence that binds the polypeptide comprises a 5'UTR_{retro} or a 3' UTR_{retro}.

72. The LNP of claim 64, wherein the template RNA comprises both of a 5'UTR_{retro} and a 3' UTR_{retro}.

73. The LNP of claim 71 or 72, wherein the 5'UTR_{retro} and the 3' UTR_{retro} comprise 5' or 3' sequences of Table 4 (e.g., SEQ ID NO: 44 and/or SEQ ID NO: 55) or any of Examples 4-11 or 15-17.

74. The LNP of any one of claims 64-73, wherein the helper lipid is present at about 20 mol% to about 40 mol% of the lipid component.

75. The LNP of any one of claims 64-73, wherein the helper lipid is present at about 22 mol% to about 36 mol% of the lipid component.

76. The LNP of any one of claims 64-73, wherein the helper lipid is present at about 20 mol% to about 30 mol% of the lipid component.

77. The LNP of any one of claims 64-73, wherein the ionizable lipid is present at about 40 mol% to about 50 mol% of the lipid component or about 20 mol% to about 30 mol% of the lipid component.

78. The LNP of any one of claims 64-77, wherein the LNP comprises one targeting moiety conjugated to the surface of the LNP.

79. The LNP of claim 78, wherein the one targeting moiety binds CD2.

80. The LNP of claim 78, wherein the one targeting moiety binds CD3.

81. The LNP of claim 78, wherein the one targeting moiety binds CD4.

82. The LNP of claim 78, wherein the one targeting moiety binds CD5.

83. The LNP of claim 78, wherein the one targeting moiety binds CD6.

84. The LNP of claim 78, wherein the one targeting moiety binds CD7.

85. The LNP of claim 78, wherein the one targeting moiety binds CD8.

86. The LNP of claim 78, wherein the one targeting moiety binds CD28.

87. The LNP of any one of claims 64-77. wherein the LNP comprises two targeting moieties conjugated to the surface of the LNP.

88. The LNP of claim 87, wherein the two targeting moieties bind CD3 and CD2.

89. The LNP of claim 87, wherein the two targeting moieties bind CD3 and CD4.

90. The LNP of claim 87, wherein the two targeting moieties bind CD3 and CD5.

91. The LNP of claim 87, wherein the two targeting moieties bind CD3 and CD6.

92. The LNP of claim 87, wherein the two targeting moieties bind CD3 and CD7.

93. The LNP of claim 87, wherein the two targeting moieties bind CD3 and CD8.

94. The LNP of claim 87, wherein the two targeting moieties bind CD3 and CD28.

95. The LNP of any one of claims 78-93, further comprising a third targeting moiety, wherein the third targeting moiety binds CD28.

96. The LNP of any one of claims 64-73, wherein the LNP comprises two different targeting moieties conjugated to the surface of the LNP, wherein the two different targeting moieties bind the same cell surface receptor.

97. The LNP of claim 96, wherein the cell surface receptor is CD3.

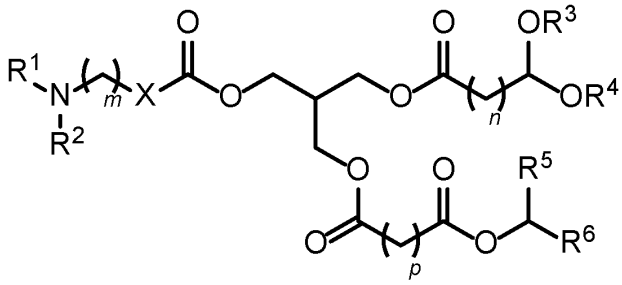
98. The LNP of claim 80 or any one of claims 88-94, wherein the CD3 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences of Table 19 or Table 32, or variable heavy and light chain sequences having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

99. The LNP of claim 79 or claim 88, wherein the CD2 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences of Table 20, or variable heavy and light chain sequences having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

100. The LNP of claim 82 or claim 90, wherein the CD2 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences of Table 20.

101. The LNP of claim 84 or claim 92, wherein the CD7 targeting moiety is an antibody or fragment thereof comprising variable heavy and variable light chain sequences of Table 20, or variable heavy and light chain sequences having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

102. The LNP of any one of claims 64-101, wherein the ionizable lipid is a compound of formula (I):



(I),

or a pharmaceutically acceptable salt thereof, wherein:

X is -O- or -CH_2-;

m is 0, 1, 2, or 3;

R^1 is C_{1-4}alkyl;

R^2 is C_{1-4}alkyl;

n is 1, 2, 3, or 4;

R^3 is C_{4-10}alkyl;

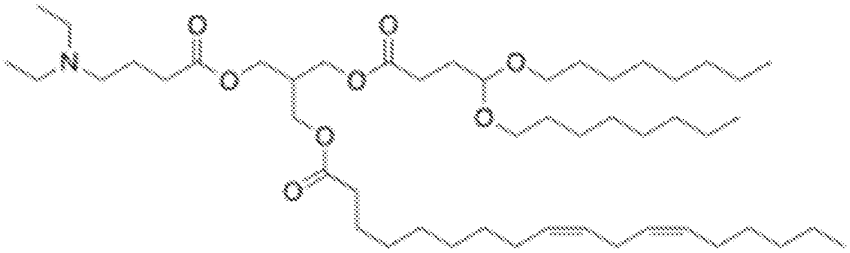
R^4 is C_{4-10}alkyl;

p is 2, 3, 4, 5, or 6;

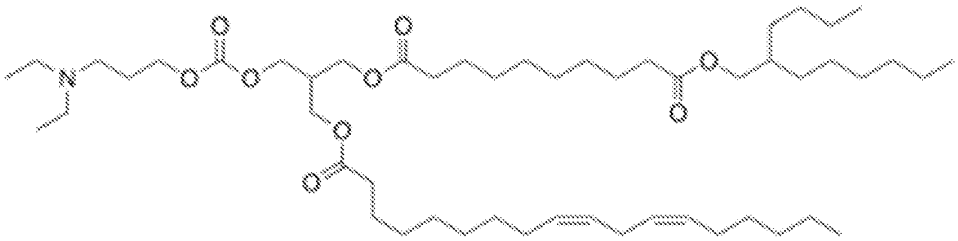
R^5 is C_{4-10}alkyl; and

R^6 is C_{4-10}alkyl.

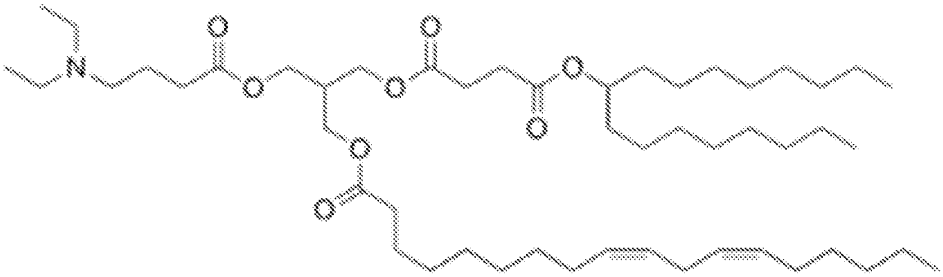
103. The LNP of any one of claims 64-101, wherein the ionizable lipid is a compound of one of the structures depicted below:



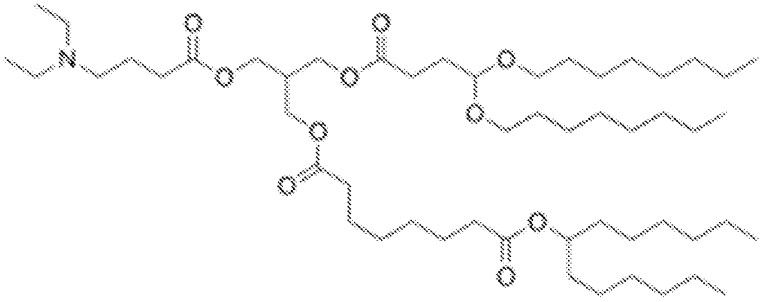
Lipid 092



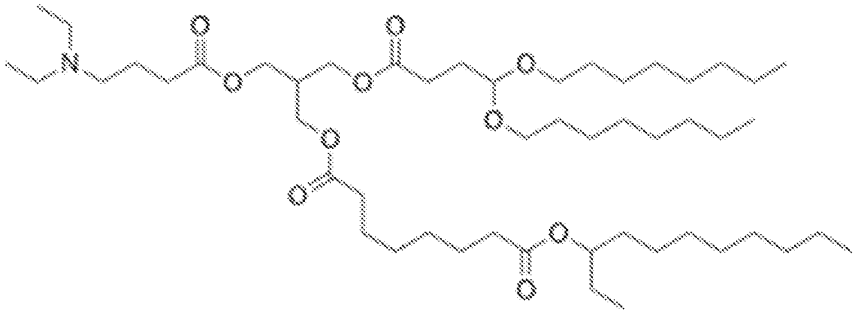
Lipid 093



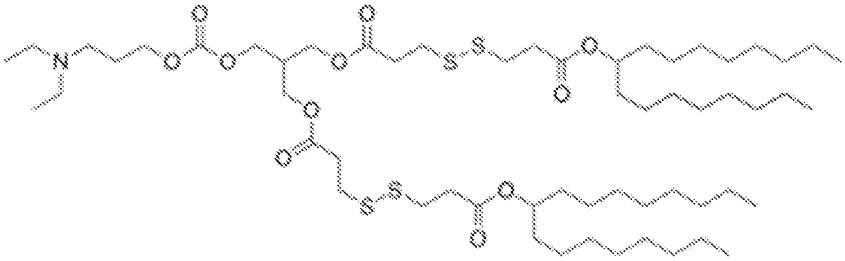
Lipid 153



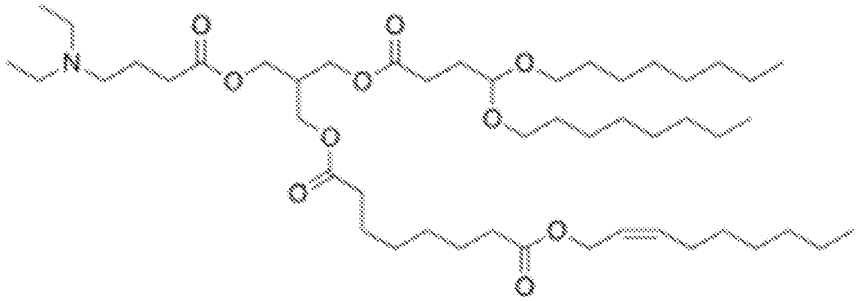
Lipid 154



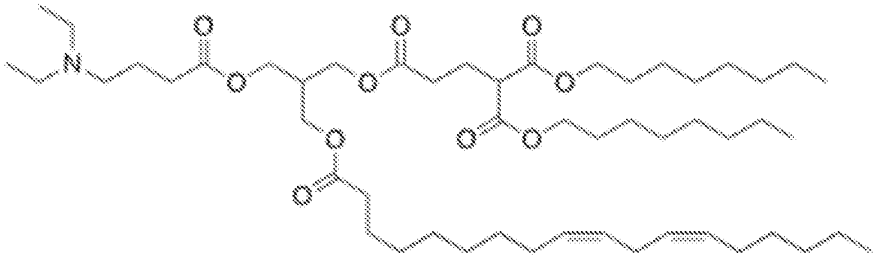
Lipid 155



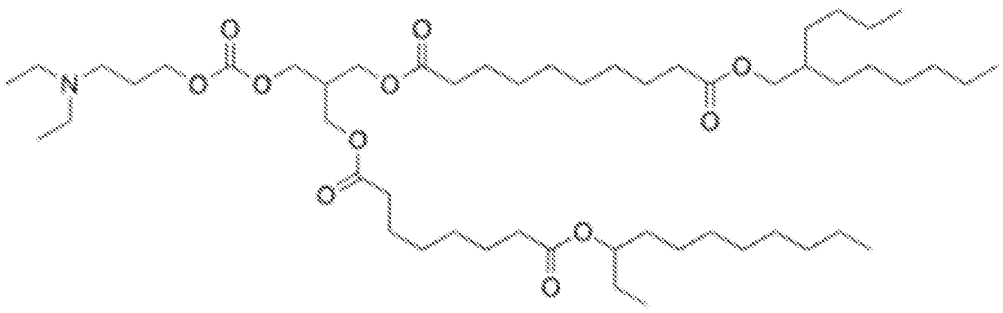
Lipid 162



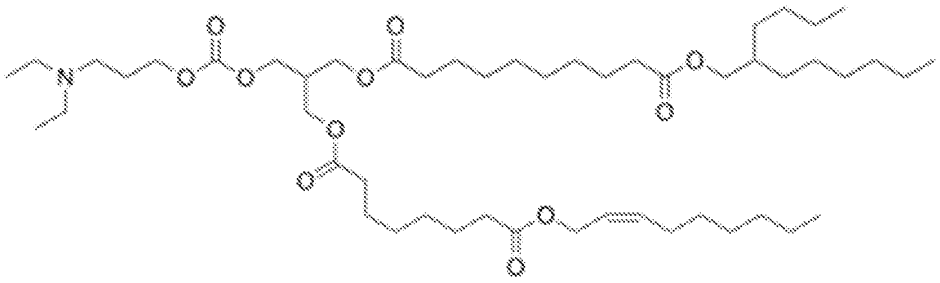
Lipid 163



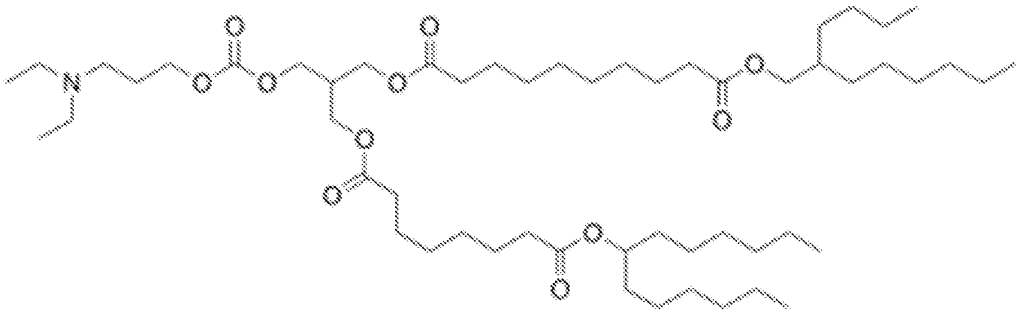
Lipid 169



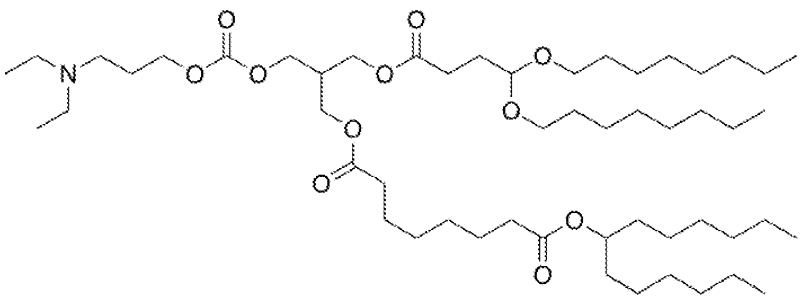
Lipid 176



Lipid 178



Lipid 183



Lipid 232

104. The LNP of any one of claims 1-103, wherein the one or two targeting moieties are Fab fragments.

105. The LNP of claim 104, wherein the conjugation is performed by a method comprising:

(i) contacting a Fab fragment with a reducing reagent, wherein the Fab fragment comprises a heavy chain and a light chain and an interchain disulfide bond linking the constant light chain domain (C_L) and the constant heavy chain domain 1 (C_H1), whereby the reducing reagent reduces the interchain disulfide bond of the Fab fragment to generate two free cysteine residues; and

(ii) contacting the product of step (i) with a precursor LNP comprising a plurality of thiol-reactive groups covalently bonded to one or more lipids of the precursor LNP, thereby forming a conjugate.

106. The LNP of claim 105, wherein the reducing agent is selected from the group consisting of 2-mercaptoethanol, 2-mercaptoethylamine, dithiothreitol (DTT), dithioerythritol (DTE), and tris(carboxyethyl)phosphine (TCEP).

107. The LNP of claim 105 or claim 106, wherein the thiol-reactive group is maleimide.

108. The LNP of claim 105 or claim 106, wherein the thiol-reactive group is 2,3-dibromomaleimide (DBM).

109. The LNP of any one of claims 105-108, wherein the thiol-reactive group is covalently bonded to one or more of the lipids comprising the precursor LNP.

110. A pharmaceutical composition comprising an LNP of any one of claims 1-109.

111. A method of delivering a therapeutic agent to the T cells of a subject, said method comprising *in vivo* administration to the subject of a pharmaceutical composition of claim 110.

112. The method of claim 111, wherein the subject is a human patient.

113. The method of claim 111 or claim 112, wherein the therapeutic agent is an anti-cancer agent or an immunosuppressant targeting an antigen associated with an autoimmune disease.

114. A method of treating cancer in a human patient in need thereof, said method comprising *in vivo* administration of a pharmaceutical composition of claim 110 to the human patient.

115. The method of claim 114, wherein the cancer is an advanced leukemia or lymphoma.

116. The method of claim 114, wherein cancer is acute lymphoblastic leukemia (ALL), adult ALL, chronic lymphoblastic leukemia (CLL), non-Hodgkin lymphoma (NHL), or Diffuse Large B-Cell Lymphoma (DLBCL).

117. The method of any one of claims 111-116, wherein the pharmaceutical composition is administered parenterally.

118. The method of claim 117, wherein the pharmaceutical composition is administered intravenously, intramuscularly, subcutaneously.

119. A method of delivering a therapeutic agent to the T cells of a subject, said method comprising:

a. isolating T cells from the subject,

b. mixing a pharmaceutical composition of claim 110 with the T cells for a sufficient time to allow for encapsulation of the therapeutic agent in the T cells; and

c. administering the T cells encapsulating the therapeutic agent to the subject.

120. The method of claim 119, wherein the subject is a human patient.

121. The method of claim 119 or 120, wherein the therapeutic agent is an anti-cancer agent or an immunosuppressant targeting an antigen associated with an autoimmune disease.

122. A method of treating cancer in a human patient in need thereof, said method comprising

a. isolating T cells from the subject,

b. mixing a pharmaceutical composition of claim 110 with the T cells for a sufficient time to allow for encapsulation of the therapeutic agent in the T cells; and

c. administering the T cells encapsulating the therapeutic agent to the subject.

123. The method of claim 122, wherein the cancer is an advanced leukemia or lymphoma.

124. The method of claim 122 wherein cancer is acute lymphoblastic leukemia (ALL), adult ALL, chronic lymphoblastic leukemia (CLL), non-Hodgkin lymphoma (NHL), or Diffuse Large B-Cell Lymphoma (DLBCL).

125. The method of any one of claims 119-124, wherein the T cells are not activated prior to mixing with the pharmaceutical composition of claim 104.

126. The method of claim 125, wherein the T cells become activated after mixing with the pharmaceutical composition of claim 104.

127. The method of claim 125 or claim 126, wherein at least one or two targeting moieties conjugated to the LNP binds CD3.

128. The method of claim 125 or claim 126, wherein the LNP comprises two targeting moieties, wherein one targeting moiety binds CD3 and the other targeting moiety binds CD28.

129. The method of claim 125 or claim 126, wherein the LNP comprises two targeting moieties, wherein one targeting moiety binds CD3 and the other targeting moiety binds CD2, one targeting moiety binds CD3 and the other targeting moiety binds CD4, one targeting moiety binds CD3 and the other targeting moiety binds CD5, one targeting moiety binds CD3 and the other targeting moiety binds CD6, one targeting moiety binds CD3 and the other targeting moiety binds CD7, or one targeting moiety binds CD3 and the other targeting moiety binds CD8.

130. The method of claim 125 or claim 126, wherein the LNP comprises a first targeting moiety and a second targeting moiety, wherein the first targeting moiety and the second targeting moiety bind CD3.

131. A method for administering a therapeutic composition to a patient, comprising:

a. collecting a blood fraction comprising lymphocytes from the patient;

b. isolating T cells from the blood fraction;

c. contacting the isolated T cells with lipid nanoparticles (LNPs) according to any one of claims 1-109, thereby producing T cells encapsulating a therapeutic payload; and

d. reinfusing the T cells of step (c) into the patient.

132. A method for treating cancer in a patient, comprising:

a. collecting a blood fraction comprising lymphocytes from the patient;

b. isolating T cells from the blood fraction;

c. contacting the isolated T cells with lipid nanoparticles (LNPs) according to any one of claims 1-109, thereby producing T cells encapsulating a therapeutic payload; and

d. reinfusing the T cells of step (c) into the patient.

133. The method of claim 131 or claim 132, wherein the T cells are not activated prior to step (c).

134. The method of any one of claims 131-133, wherein the two targeting moieties bind CD3 and CD28.

135. The method of any one of claims 131-133, wherein the two targeting moieties bind CD3 and CD2.

136. The method of any one of claims 131-133, wherein the two targeting moieties bind CD3 and CD4.

137. The method of any one of claims 131-133, wherein the two targeting moieties bind CD3 and CD5.

138. The method of any one of claims 131-133, wherein the two targeting moieties bind CD3 and CD6.

139. The method of any one of claims 131-133, wherein the two targeting moieties bind CD3 and CD7.

140. The method of any one of claims 131-133, wherein the two targeting moieties bind CD3 and CD8.

141. A lipid nanoparticle (LNP) comprising:

a. a lipid component comprising an ionizable lipid and a helper lipid,;

b. one or two targeting moieties conjugated to the LNP, wherein the one or two targeting moieties are configured to bind one or more receptors on the surface of T cells selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8 and CD28; and

c. an mRNA encoding a heterologous gene modifying polypeptide; and a template RNA (or DNA encoding the template RNA) comprising (1) a gRNA spacer, (2) a gRNA scaffold, (3) a heterologous object sequence, and (4) a primer binding site (PBS) sequence.

142. The LNP of claim 141, wherein the LNP further comprises a second template RNA (or DNA encoding the template RNA) comprising (1) a gRNA spacer, (2) a gRNA scaffold, (3) a heterologous object sequence, and (4) a primer binding site (PBS) sequence.

143. The LNP of any of claim 141 or claim 142, wherein the heterologous gene modifying polypeptide comprises:

a reverse transcriptase (RT) domain (e.g., an RT domain from a retrovirus, or a polypeptide domain having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% amino acids sequence identity thereto); and

a Cas domain that binds to the bind DNA molecule and is heterologous to the RT domain (e.g., a Cas9 domain); and optionally, a linker disposed between the RT domain and the Cas domain.

144. The LNP of claim 143, wherein the RT domain comprises an amino acid sequence of Table 17, or a sequence having at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity thereto.

145. The LNP of claim 143 or claim 144, wherein the Cas domain comprises a Cas9.

146. The LNP of any one of claims 143-145, wherein the Cas domain comprises a Cas domain of SEQ ID NO: 126 and/or SEQ ID NO: 127, or a sequence having at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity thereto.

147. The LNP of any of any of claims 143-146, wherein the heterologous gene modifying polypeptide comprises a linker, optionally wherein the linker comprises an amino acid sequence of Table 17, or a sequence having at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity thereto.

148. The LNP of claim 143, wherein the template RNA is configured to alter a nucleotide sequence at the human TRAC or B2M locus to disrupt the TRAC or B2M gene.

149. The LNP of claim 144, wherein the second template is configured to alter a nucleotide sequence at the human TRAC or B2M locus to disrupt the TRAC or B2M gene.

150. The LNP of claim 148, wherein the template RNA comprises a sequence of Table 23 or Table 24.

151. The LNP of claim 149, wherein the second template RNA comprises a sequence of Table 23 or 24.