

IONIZABLE CATIONIC LIPIDS AND LIPID NANOPARTICLES, AND METHODS OF SYNTHESIS AND USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and the benefit of U.S. Provisional Application No. 63/121,801, filed on December 4, 2020; U.S. Provisional Application No. 63/166,205, filed on March 25, 2021; U.S. Provisional Application No. 63/169,296, filed on April 1, 2021; U.S. Provisional Application No. 63/169,395, filed on April 1, 2021; and U.S. Provisional Application No. 63/172,024, filed on April 7, 2021, the entire disclosures of which are incorporated herein by reference in their entirety.

SUBMISSION OF SEQUENCE LISTING ON ASCII TEXT FILE

[0002] The content of the following submission on ASCII text file is incorporated herein by reference in its entirety: a computer readable form (CRF) of the Sequence Listing (file name: 183952034040SEQLIST.TXT, date recorded: December 3, 2021, size: 205,763 bytes).

FIELD OF THE INVENTION

[0003] The invention provides ionizable cationic lipids and lipid nanoparticles for the delivery of nucleic acids to immune cells, and methods of making and using, such lipids and targeted lipid nanoparticles.

BACKGROUND

[0004] In recent years, a number of therapeutic modalities have been developed that involve the delivery of one or more nucleic acids to a subject. Treatment modalities include, for example, gene therapies where a gene of interest in the form of deoxyribose nucleic acid (DNA) is introduced into a cell, which is then expressed to produce a gene product, for example, protein, for treating a disorder caused by or associated with a deficiency or absence of the gene product. In this approach, the gene is transcribed into a messenger ribonucleic acid (mRNA), whereupon the mRNA is translated to produce the gene product. In another approach, mRNA rather than a gene of interest can be delivered to the cell. The resulting expression product can ameliorate the deficiency or absence of a particular protein in a subject (for example, a protein deficiency occurring in certain forms of cystic fibrosis or lysosomal storage disorders), or can be used to modulate a cellular function, for example, reprogramming immune cells to initiate or otherwise modulate an immune response in the

subject (for example, as a therapeutic agent for treating cancer or as a prophylactic vaccine for preventing or minimizing the risk or severity of a microbial or viral infection).

[0005] However, the delivery of mRNA to a cell for translation within the cell has been challenging for a variety of factors, such as nuclease degradation of the mRNA prior to entry into the cell and then after introduction into the cell but prior to translation.

[0006] RNA may be delivered to a subject using different delivery vehicles, for example, based on cationic polymers or lipids which, together with the RNA, form nanoparticles. The nanoparticles are intended to protect the RNA from degradation, enable delivery of the RNA to the target site and facilitate cellular uptake and processing by the target cells. For delivery efficacy, in addition to the molecular composition, parameters like particle size, charge, or grafting with molecular moieties, such as polyethylene glycol (PEG) or ligands, play a role. Grafting with PEG is believed to reduce serum interactions, increase serum stability and increase time in circulation, which can be helpful for certain targeting approaches.

[0007] Compared with DNA delivery technologies used in certain gene therapies, mRNA-based gene treatment has a number of superior features, for example, ease in manipulation, rapid and transient expression, and adaptive convertibility without mutagenesis.

[0008] However, the delivery of therapeutic RNAs to cells is difficult in view of the relative instability and low cell permeability of RNAs. Thus, there exists a need to develop methods and compositions to facilitate the delivery of RNAs such as mRNA to cells.

SUMMARY

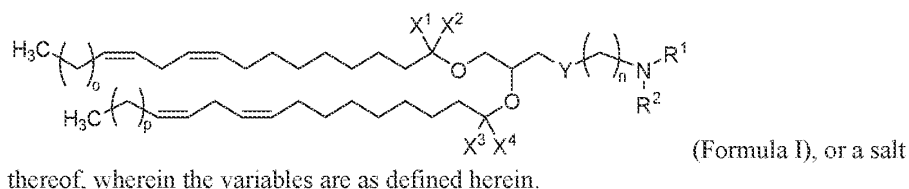
[0009] The invention provides ionizable cationic lipids, lipid-immune cell targeting group conjugates, and lipid nanoparticle compositions comprising such ionizable cationic lipids and/or lipid-immune cell (e.g., T-cell) targeting group conjugates, medical kits containing such lipids and/or conjugates, and methods of making and using, such lipids and conjugates.

[0010] The lipid nanoparticle compositions provided herein may further comprise a nucleic acid, such as an RNA, e.g., a messenger RNA or mRNA. The lipid nanoparticle compositions may be used for mRNA delivery to a cell (e.g., an immune cell, such as T-cell) in a subject. Messenger RNA based gene therapy requires efficient delivery of mRNA to circulating cells (e.g., immune cells, such as T-cells or NK cells) in plasma or to cells in a

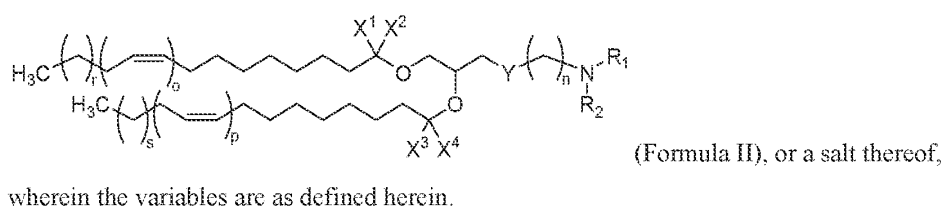
given tissue. The main challenges associated with efficient mRNA delivery to attain robust levels of protein expression include: (a) ability to protect the mRNA payload against prevalent serum nucleases upon administration to a subject; (b) the ability to specifically target mRNA delivery to and thereby maximize protein expression in the target cell (*e.g.*, T-cell) population; and (c) the ability to maximally deliver the mRNA payload to the cytosolic compartment of cells (*e.g.*, T-cells) for translation into proteins within the cytoplasm.

[0011] The invention provides ionizable cationic lipids for producing lipid nanoparticle compositions that facilitate the delivery of a payload (*e.g.*, a nucleic acid, such as a DNA or RNA, such as an mRNA) disposed therein to cells, for example, mammalian cells, for example, immune cells. The lipids are designed to enable intracellular delivery of a nucleic acid, *e.g.*, mRNA, to the cytosolic compartment of a target cell type and rapidly degrade into non-toxic components. These complex functionalities are achieved by the interplay between chemistry and geometry of the ionizable lipid head group, the hydrophobic “acyl-tail” groups and the linker connecting the head group and the acyl tail groups in the ionizable cationic lipids.

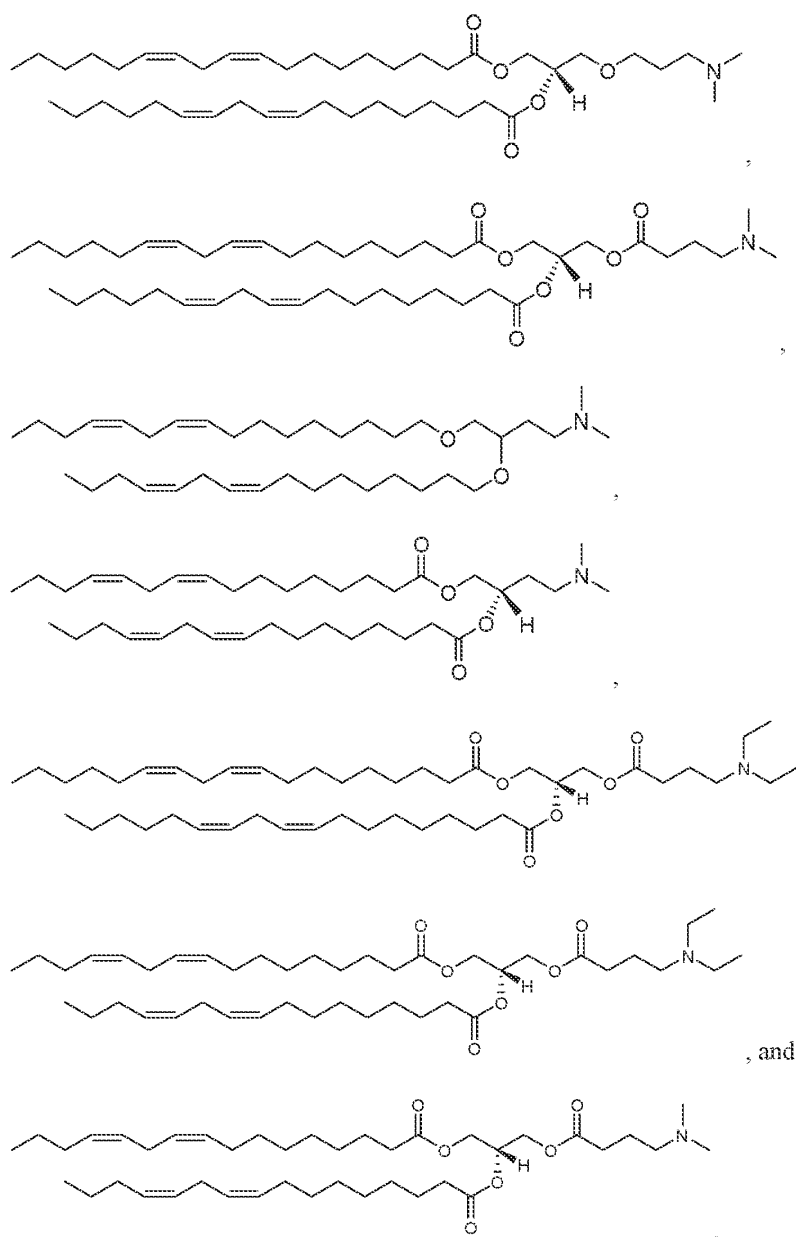
[0012] In one aspect, the present invention provides a compound represented by Formula I:



[0013] In another aspect, the present invention provides a compound represented by Formula II:

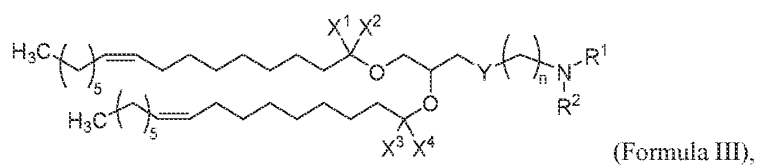


[0014] Provided herein, in part, is a compound selected from the group consisting of:



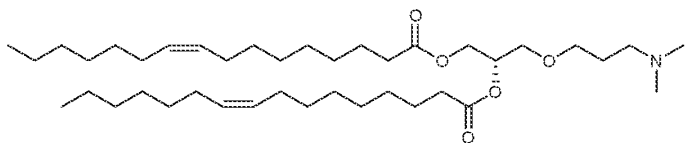
or a salt thereof.

[0015] In certain embodiments, the compound is a compound of Formula III:



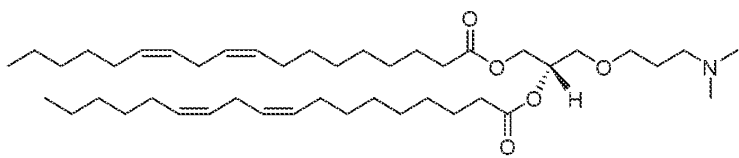
or a salt thereof, wherein the variables are as defined herein.

[0016] Also provided herein is a compound of the formula:



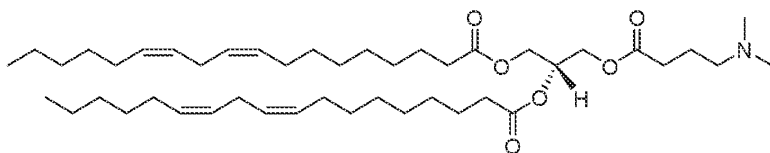
or a salt thereof.

[0017] Also provided herein is a compound of the formula:



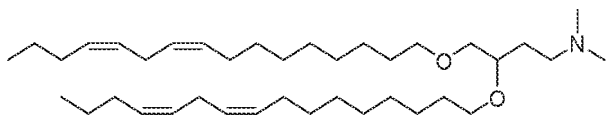
or a salt thereof.

[0018] Also provided herein is a compound of the formula:



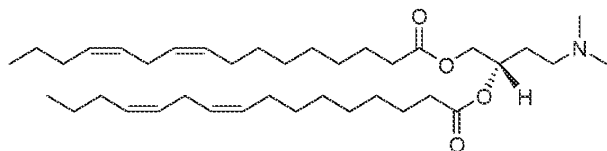
or a salt thereof.

[0019] Also provided herein is a compound of the formula:



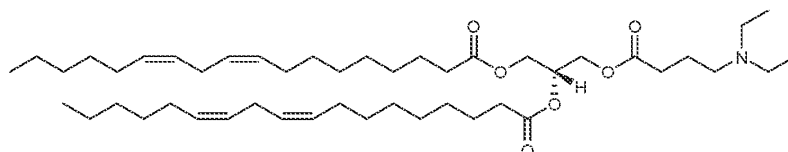
or a salt thereof.

[0020] Also provided herein is a compound of the formula:



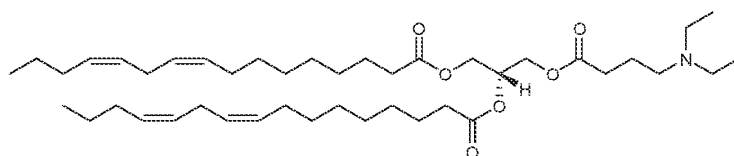
or a salt thereof.

[0021] Also provided herein is a compound of the formula:



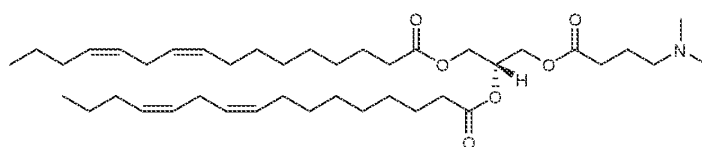
or a salt thereof.

[0022] Also provided herein is a compound of the formula:



or a salt thereof.

[0023] Also provided herein is a compound of the formula:



or a salt thereof.

[0024] Also provided herein is a lipid nanoparticle (LNP) comprising a lipid blend comprising an ionizable cationic lipid and/or lipid-immune cell targeting group conjugate (e.g., a lipid-T-cell targeting group conjugate) provided herein.

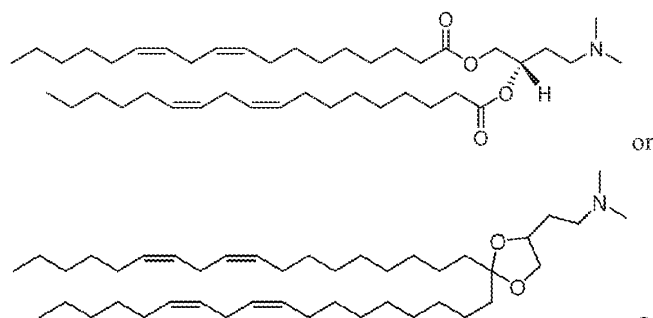
[0025] In another aspect, provided herein is a method of delivering a nucleic acid to an immune cell (e.g., a T-cell), the method comprising exposing the immune cell to an LNP

described herein containing the nucleic acid under conditions that permit the nucleic acid to enter the immune cell.

[0026] In another aspect, provided herein is a method of delivering a nucleic acid to an immune cell (*e.g.*, a T-cell) in a subject in need thereof, the method comprising administering to the subject a composition comprising an LNP described herein containing a nucleic acid thereby to deliver the nucleic acid to the immune cell.

[0027] In another aspect, provided herein is a method of targeting the delivering of a nucleic acid (*e.g.*, mRNA) to an immune cell (*e.g.*, a T-cell) in a subject, the method comprising administering to the subject an LNP described herein containing the nucleic acid so as to facilitate targeted delivery of the nucleic acid to the immune cell.

[0028] In one aspect, provided herein are lipid nanoparticles (LNPs) comprising a lipid blend for targeted delivery of a nucleic acid into an immune cell. In some embodiments, the lipid blend comprises a lipid-immune cell targeting group conjugate comprising the compound of Formula IV: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the lipid blend comprises an ionizable cationic lipid. In some embodiments, the ionizable cationic lipid comprises



. In some embodiments, the LNP comprises a nucleic acid disposed therein.

[0029] In some embodiments, the immune cell targeting group comprises an antibody that binds a T cell antigen. In some embodiments, the T cell antigen is CD3, CD4, CD7, or CD8, or a combination thereof (*e.g.*, both CD3 and CD8, both CD4 and CD8, or both CD7 and CD8). In some embodiments, the immune cell targeting group comprises an antibody that binds a Natural Killer (NK) cell antigen. In some embodiments, the NK cell antigen is

CD7, CD8, or CD56, or a combination thereof (e.g., both CD7 and CD8). In some embodiments, the antibody is a human or humanized antibody.

[0030] In some embodiments, the immune cell targeting group is covalently coupled to a lipid in the lipid blend via a polyethylene glycol (PEG) containing linker. In some embodiments, the lipid covalently coupled to the immune cell targeting group via a PEG containing linker is distearoylglycerol (DSG), distearoyl-phosphatidylethanolamine (DSPE), dimyrstoyl-phosphatidylethanolamine (DMPE), distearoyl-glycero-phosphoglycerol (DSPG), dimyrstoyl-glycerol (DMG), dipalmitoyl-phosphatidylethanolamine (DPPE), dipalmitoyl-glycerol (DPG), or ceramide. In some embodiments, the PEG is PEG 2000.

[0031] In some embodiments, the lipid-immune cell targeting group conjugate is present in the lipid blend in a range of 0.002-0.2 mole percent. In some embodiments, the lipid blend comprises one or more of a structural lipid (e.g., a sterol), a neutral phospholipid, and a free PEG-lipid. In some embodiments, the ionizable cationic lipid is present in the lipid blend in a range of 40-60 mole percent. In some embodiments, the sterol is present in the lipid blend in a range of 30-50 mole percent. In some embodiments, the sterol is present in the lipid blend in a range of 20-70 mole percent. In some embodiments, the sterol is cholesterol.

[0032] In some embodiments, the neutral phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), sphingomyelin (SM). In some embodiments, the neutral phospholipid is present in the lipid blend in a range of 1-10 mole percent.

[0033] In some embodiments, the free PEG-lipid is selected from the group consisting of PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) or PEG-dimyrstoyl-phosphatidylethanolamine (PEG-DMPE), N-(Methylpolyoxyethylene oxycarbonyl)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE-PEG) 1,2-Dimyrstoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DMG), 1,2-Dipalmitoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DPG), 1,2-Dioleoyl-rac-glycerol, methoxypolyethylene Glycol (DOG-PEG) 1,2-Distearoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DSG), N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)]} (PEG-ceramide), and DSPE-PEG-cysteine, or a derivative thereof. In some embodiments, the free PEG-lipid comprises a

diacylphosphatidylethanolamines comprising Dipalmitoyl (C16) chain or Distearoyl (C18) chain. In some embodiments the free PEG-lipid is a mixture of two or more unique free PEG-lipids. In some embodiments, the free PEG-lipid is present in the lipid blend in a range of 1-4 mole percent, such as about 1-2 mole percent, or about 2-4 mole percent, or about 1.5 mole percent. In some embodiments, the free PEG-lipid comprises the same or a different lipid as the lipid in the lipid-immune cell targeting group conjugate.

[0034] In some embodiments, the LNP has a mean diameter in the range of 50-200 nm. In some embodiments, the LNP has a mean diameter of about 100 nm. In some embodiments, the LNP has a polydispersity index in a range from 0.05 to 1. In some embodiments, the LNP has a zeta potential of from about -10 mV to about + 30 mV at pH 5.

[0035] In some embodiments, the nucleic acid is DNA or RNA. In some embodiments, the RNA is an mRNA, tRNA, siRNA, or microRNA. In some embodiments, the mRNA encodes a receptor, a growth factor, a hormone, a cytokine, an antibody, an antigen, an enzyme, or a vaccine. In some embodiments, the mRNA encodes a polypeptide capable of regulating immune response in the immune cell. In some embodiments, the mRNA encodes a polypeptide capable of reprogramming the immune cell. In some embodiments, the mRNA encodes a synthetic T cell receptor (synTCR) or a Chimeric Antigen Receptor (CAR). In some embodiments, the CAR comprises the amino acid sequence of SEQ ID NO: 24. In some embodiments, the mRNA encoding the CAR comprises the polynucleotide sequence of SEQ ID NO: 25.

[0036] In some embodiments, the immune cell targeting group comprises an antibody, and the antibody is a Fab or an immunoglobulin single variable domain, such as a Nanobody. In some embodiments, the immune cell targeting group comprises a Fab, F(ab')₂, Fab'-SH, Fv, or scFv fragment. In some embodiments, the immune cell targeting group comprises a Fab that is engineered to knock out one or more natural interchain disulfide bonds. For example, in some embodiments, the Fab comprises a heavy chain fragment that comprises C233S substitution, numbering according to Kabat, and/or a light chain fragment that comprises C214S substitution, numbering according to Kabat. In some embodiments, the immune cell targeting group comprises a Fab that is engineered to introduce one or more buried interchain disulfide bonds. For example, in some embodiments, the Fab antibody comprises a heavy chain fragment that comprises F174C substitution, numbering according to Kabat, and/or a light chain fragment that comprises S176C substitution, numbering

according to Kabat. In some embodiments, the immune cell targeting group comprises a Fab that is engineered to knock out one or more natural interchain disulfide bonds, and to introduce one or more buried interchain disulfide bonds. In some embodiments, the immune cell targeting group comprises a Fab that comprises a cysteine at the C-terminus of the heavy or light chain fragment. In some embodiments, the Fab further comprises one or more amino acids between the heavy chain fragment of the Fab and the C-terminal cysteine. In some embodiments, the Fab comprises a heavy chain variable domain linked to an antibody CH1 domain and a light chain variable domain linked to an antibody light chain constant domain, wherein the CH1 domain and the light chain constant domain are linked by one or more interchain disulfide bonds, and wherein the immune cell targeting group further comprises a single chain variable fragment (scFv) linked to the C-terminus of the light chain constant domain by an amino acid linker. In some embodiments, the Fab antibody is a DS Fab (Fab with wild type (natural) interchain disulfide bond), a NoDS Fab (Fab with natural disulfide bond knocked out, such as a Fab with C233S substitution on the heavy chain, and/or C214S substitution on the light chain, numbering according to Kabat), a bDS Fab (Fab without natural disulfide bond, and with introduced non-natural interchain buried disulfide bond, such as a Fab with F174C and C233S on the heavy chain, and/or S176C and C214S substitution on the light chain, numbering according to Kabat), or a bDS Fab-ScFv (a bDS Fab linked to a ScFv through a linker, such as (G4S)_x), as demonstrated in FIG 47.

[0037] In some embodiments, the immune cell targeting group comprises an immunoglobulin single variable domain, such as a Nanobody. In some embodiments, the immunoglobulin single variable domain comprises a cysteine at the C-terminus. In some embodiments, the Nanobody further comprises a spacer comprising one or more amino acids between the V_{HH} domain and the C-terminal cysteine. In some embodiments, the immune cell targeting group comprises two or more V_{HH} domains. In some embodiments, the two or more V_{HH} domains are linked by an amino acid linker. In some embodiments, the immune cell targeting group comprises a first V_{HH} domain linked to an antibody CH1 domain and a second V_{HH} domain linked to an antibody light chain constant domain, and wherein the antibody CH1 domain and the antibody light chain constant domain are linked by one or more disulfide bonds. In some embodiments, the immune cell targeting group comprises a V_{HH} domain linked to an antibody CH1 domain, and wherein the antibody CH1 domain is linked to an antibody light chain constant domain by one or more disulfide bonds. In some embodiments, the CH1 domain comprises F174C and C233S substitutions, and/or the light

chain constant domain comprises S176C and C214S substitutions, numbering according to Kabat. In some embodiments, the antibody is a ScFv, a V_{HH} (Nb), a 2xV_{HH}, a V_{HH}-CH1/empty Vk, or a V_{HH}1-CH1/V_{HH}2-Nb bDS, as demonstrated in FIG. 47.

[0038] In some embodiments, the immune cell targeting group comprises a Fab that comprises a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 1 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 2 or 3. In some embodiments, the immune cell targeting group comprises a Fab that comprises a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 6 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 7. In some embodiments, the antibody is an antibody described in the examples.

[0039] In some embodiments, the immune cell targeting group comprises a Fab that comprises:

- (a) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 1 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 2 or 3;
- (b) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 4 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 5;
- (c) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 6 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 7;
- (d) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 8 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 9;
- (e) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 10 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 11;
- (f) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 12 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 13;
- (g) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 14 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 15;
- (h) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 16 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 17;

- (i) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 18 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 19;
- (j) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 20 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 21; or
- (k) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 22 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 23.

[0040] In some embodiments, the immune cell targeting group comprises a Fab, F(ab')₂, Fab'-SH, Fv, or scFv fragment. In some embodiments, the immune cell targeting group comprises a Fab that is engineered to knock out the natural interchain disulfide bond at the C-terminus. In some embodiments, the Fab comprises a heavy chain fragment that comprises C233S substitution, and a light chain fragment that comprises C214S substitution, numbering according to Kabat. In some embodiments, the immune cell targeting group comprises a Fab that has a non-natural interchain disulfide bond (e.g., a engineered, buried interchain disulfide bond). In some embodiments, the Fab comprises F174C substitution in the heavy chain fragment, and S176C substitution in the light chain fragment, numbering according to Kabat. In some embodiments, the immune cell targeting group comprises a Fab that comprises a cysteine at the C-terminus of the heavy or light chain fragment. In some embodiments, the Fab further comprises one or more amino acids between the heavy chain fragment of the Fab and the C-terminal cysteine.

[0041] In some embodiments, the immune cell targeting group comprises an immunoglobulin single variable domain. In some embodiments, the immunoglobulin single variable domain comprises a cysteine at the C-terminus. In some embodiments, the immunoglobulin single variable domain comprises a VHH domain and further comprises a spacer comprising one or more amino acids between the VHH domain and the C-terminal cysteine. In some embodiments, the immune cell targeting group comprises two or more VHH domains. In some embodiments, the two or more VHH domains are linked by an amino acid linker. In some embodiments, the immune cell targeting group comprises a first VHH domain linked to an antibody CH1 domain and a second VHH domain linked to an antibody light chain constant domain. In some embodiments, the antibody CH1 domain and the antibody light chain constant domain are linked by one or more disulfide bonds. In some embodiments, the immune cell targeting group comprises a VHH domain linked to an antibody CH1 domain. In some embodiments, the antibody CH1 domain is linked to an

antibody light chain constant domain by one or more disulfide bonds. In some embodiments, the CH1 domain comprises F174C and C233S substitutions, and the light chain constant domain comprises S176C and C214S substitutions, numbering according to Kabat.

[0042] In some embodiments, the immune cell targeting group comprises a Fab that comprises: (a) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 1 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 2 or 3; or (b) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 6 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 7.

[0043] In another aspect, provided herein are methods of targeting the delivery of a nucleic acid to an immune cell of a subject. In some embodiments, the method comprises contacting the immune cell with a lipid nanoparticle (LNP). In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the compound of the following formula: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid.

[0044] In some embodiments, an aspect of the disclosure relates to an LNP or a pharmaceutical composition containing thereof, as disclosed herein, for use in a method of targeting the delivery of a nucleic acid to an immune cell of a subject. Such a method may be for the treatment of a disease or disorder as disclosed hereafter. In some embodiments, a method as disclosed herein can comprise contacting in vitro or ex vivo the immune cell of a subject with a lipid nanoparticle (LNP). In some embodiments, the LNP is an LNP as described herein in the present disclosure.

[0045] In some embodiments, the LNP provides at least one of the following benefits:

- (i) increased specificity of targeted delivery to the immune cell compared to a reference LNP;
- (ii) increased half-life of the nucleic acid or a polypeptide encoded by the nucleic acid in the immune cell compared to a reference LNP;
- (iii) increased transfection rate compared to a reference LNP; and

(iv) a low level of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies, wherein at least 80% mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation.

[0046] In some aspect, provided are methods of expressing a polypeptide of interest in a targeted immune cell of a subject. In some embodiments, the method comprises contacting the immune cell with a lipid nanoparticle (LNP). In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] -- [optional linker] -- [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises a nucleic acid encoding the polypeptide. In some embodiments, an aspect of the disclosure relates to an LNP or a pharmaceutical composition containing thereof, as disclosed herein, for use in a method of expressing a polypeptide of interest in a targeted immune cell of a subject. Such a method may be for the treatment of a disease or disorder as disclosed hereafter. In some embodiments, a method as disclosed herein can comprise contacting in vitro or ex vivo the immune cell of a subject with a lipid nanoparticle (LNP).

[0047] In some embodiments, the LNP provides at least one of the following benefits:

- (i) increased expression level in the immune cell compared to a reference LNP;
- (ii) increased specificity of expression in the immune cell compared to a reference LNP;
- (iii) increased half-life of the nucleic acid or a polypeptide encoded by the nucleic acid in the immune cell compared to a reference LNP;
- (iv) increased transfection rate compared to a reference LNP; and
- (v) a low level of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies, wherein at least 80% mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation.

[0048] In some aspects, provided are methods of modulating cellular function of a target immune cell of a subject. In some embodiments, the method comprises administering to the subject a lipid nanoparticle (LNP). In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] -- [optional linker] -- [immune cell targeting group]. In some

embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises a nucleic acid encoding a polypeptide for modulating the cellular function of the immune cell. In some embodiments, an aspect of the disclosure relates to an LNP or a pharmaceutical composition containing thereof, as disclosed herein, for use in a method of modulating cellular function of a targeted immune cell of a subject. Such a method may be for the treatment of a disease or disorder as disclosed hereafter. In some embodiments, a method as disclosed herein can comprise contacting in vitro or ex vivo the immune cell of a subject with a lipid nanoparticle (LNP).

[0049] In some embodiments, the LNP provides at least one of the following benefits:

- (i) increased expression level in the immune cell compared to a reference LNP;
- (ii) increased specificity of expression in the immune cell compared to a reference LNP;
- (iii) increased half-life of the nucleic acid or a polypeptide encoded by the nucleic acid in the immune cell compared to a reference LNP;
- (iv) increased transfection rate compared to a reference LNP;
- (v) the LNP can be administered at a lower dose compared to a reference LNP to reach the same biologic effect in the immune cell; and
- (vi) a low level of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies, wherein at least 80% mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation.

[0050] In some embodiments, the modulation of cell function comprises reprogramming the immune cells to initiate an immune response. In some embodiments, the modulation of cell function comprises modulating antigen specificity of the immune cell.

[0051] In some aspect, provided are methods of treating, ameliorating, or preventing a symptom of a disorder or disease in a subject in need thereof. In some embodiments, the method comprises administering to the subject a lipid nanoparticle (LNP) for delivering a nucleic acid into an immune cell of the subject. In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In

some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid.

[0052] In some embodiments, the nucleic acid modulates the immune response of the immune cell, therefore to treat or ameliorate the symptom. In some embodiments, an aspect of the disclosure relates to an LNP or a pharmaceutical composition containing thereof, as disclosed herein, for use in a method of treating, ameliorating, or preventing a symptom of a disorder or disease in a subject in need thereof. A disease or disorder may be as disclosed hereafter. In some embodiments, a method as disclosed herein can comprise contacting in vitro or ex vivo the immune cell of a subject with a lipid nanoparticle (LNP).

[0053] In some embodiments, the LNP provides at least one of the following benefits:

- (i) increased specificity of delivery of the nucleic acid into the immune cell compared to a reference LNP;
- (ii) increased half-life of the nucleic acid or a polypeptide encoded by the nucleic acid in the immune cell compared to a reference LNP;
- (iii) increased transfection rate compared to a reference LNP;
- (iv) the LNP can be administered at a lower dose compared to a reference LNP to reach the same treatment efficacy;
- (v) increased level of gain of function by an immune cell compared to a reference LNP; and
- (vi) a low level of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies, wherein at least 80% mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation.

[0054] In some embodiments, the disorder is an immune disorder, an inflammatory disorder, or cancer. In some embodiments, the nucleic acid encodes an antigen for use in a therapeutic or prophylactic vaccine for treating or preventing an infection by a pathogen. In some embodiments, the Fab antibody comprises a heavy chain fragment that comprises F174C substitution, numbering according to Kabat, and/or a light chain fragment that comprises S176C substitution, numbering according to Kabat

[0055] In some embodiments, no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% of non-immune cells are transfected by the LNP. In some embodiments, no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% of undesired immune cells that are not meant to be the destination of the delivery are transfected by the LNP. In some embodiments, the half-life of the nucleic acid delivered by the LNP to the immune cell or a polypeptide encoded by the nucleic acid delivered by the LNP is at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 1.5 times, 2 times, 3 times, 4 times, 5 times, 10 times, or longer than the half-life of nucleic acid delivered by a reference LNP to the immune cell or a polypeptide encoded by the nucleic acid delivered by the reference LNP.

[0056] In some embodiments, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or more immune cells that are meant to be the destination of the delivery are transfected by the LNP.

[0057] In some embodiments, expression level of the nucleic acid delivered by the LNP is at least 5%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, 1.5 time, 2 times, 3 times, 4 times, 5 times, 10 times, 15 times, 20 times or more higher than expression level of nucleic acid in the same immune cells delivered by a reference LNP.

[0058] In one aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into NK cells of the subject. The LNP comprises (a) An ionizable cationic lipid, (b) A conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]; (c) A sterol or other structural lipid; (d) A neutral phospholipid; (e) A free Polyethylene glycol (PEG) lipid, and (f) the nucleic acid. In some embodiments, the immune cell targeting group comprises an antibody that binds CD56.

[0059] In one aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into immune cells of the subject. The LNP comprises (a) An ionizable cationic lipid, (b) A conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]; (c) A sterol or other structural lipid; (d) A neutral phospholipid; (e) A free Polyethylene glycol (PEG) lipid, and (f) the nucleic acid. In

some embodiments, the immune cell targeting group comprises an antibody that binds CD7 or CD8, and the free PEG lipid is DMG-PEG.

[0060] In one aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into immune cells of the subject. The LNP comprises (a) An ionizable cationic lipid, (b) A conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]; (c) A sterol or other structural lipid; (d) A neutral phospholipid; (e) A free Polyethylene glycol (PEG) lipid, and (f) the nucleic acid. In some embodiments, the immune cell targeting group comprises an antibody, and the antibody is a Fab or an immunoglobulin single variable domain. In some embodiments, the Fab is engineered to knock out the natural interchain disulfide at the C-terminus. In some embodiments, the Fab has a buried interchain disulfide. In some embodiments, the antibody is an immunoglobulin single variable (ISV) domain, and the ISV domain an Nanobody® ISV. In some embodiments, the free PEG lipid comprise a PEG having a molecular weight of at least 2000 daltons. In some embodiments, the PEG has a molecular weight of about 3000 to 5000 daltons. In some embodiments, the Fab is an anti-CD3 antibody, and the free PEG lipid in the LNP comprises a PEG having a molecular weight of about 2000 daltons. In some embodiments, the Fab is an anti-CD4 antibody, and the free PEG lipid in the LNP comprises a PEG having a molecular weight of about 3000 to 3500 daltons.

[0061] In one aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into immune cells of the subject. The LNP comprises (a) An ionizable cationic lipid, (b) A conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]; (c) A sterol or other structural lipid; (d) A neutral phospholipid; (e) A free Polyethylene glycol (PEG) lipid, and (f) the nucleic acid. In some embodiments, the immune cell targeting group comprises an antibody that binds CD3, and an antibody that binds CD11a or CD18.

[0062] In one aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into immune cells of the subject. The LNP comprises (a) An ionizable cationic lipid, (b) A conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]; (c) A sterol or other structural lipid; (d) A neutral phospholipid; (e) A free Polyethylene glycol (PEG) lipid, and (f) the nucleic acid. In some embodiments, the immune cell targeting group comprises an antibody that binds CD7, and an antibody that binds CD8.

[0063] In one aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into two different types of immune cells of the subject. The LNP comprises (a) An ionizable cationic lipid, (b) A conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]; (c) A sterol or other structural lipid; (d) A neutral phospholipid; (e) A free Polyethylene glycol (PEG) lipid, and (f) the nucleic acid.

[0064] In some embodiments, the immune cell targeting group comprise a bispecific targeting moiety. In some embodiments, the bispecific targeting moiety binds to the two different types of immune cells. In some embodiments, the two different types of immune cells are CD4+ T cells and CD8+ T cell. In some embodiments, the bispecific targeting moiety is a bispecific antibody. In some embodiments, the bispecific antibody is a Fab-ScFv.

[0065] In one aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into both CD4+ and CD8+ T cells of a subject. The LNP comprises (a) An ionizable cationic lipid, (b) A conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]; (c) A sterol or other structural lipid; (d) A neutral phospholipid; (e) A free Polyethylene glycol (PEG) lipid, and (f) the nucleic acid. In some embodiments, the immune cell targeting group comprise a single antibody that binds to CD3 or CD7.

[0066] Further provided is a lipid nanoparticle (LNP) for delivering a nucleic acid into an immune cell of a subject, wherein the LNP comprises: (a) an ionizable cationic lipid, (b) a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]; (c) a sterol or other structural lipid; (d) a neutral phospholipid; (e) a free Polyethylene glycol (PEG) lipid, and (f) the nucleic acid, wherein the immune cell targeting group comprises a Fab lacking the native interchain disulfide bond. In some embodiments, the Fab is engineered to replace one or both cysteines on the native constant light chain and the native constant heavy chain that form the native interchain disulfide with a non-cysteine amino acid, therefor to remove the native interchain disulfide bond in the Fab.

[0067] Also provided is an immunoglobulin single variable domain (ISVD) that binds to human CD8. In some embodiments, the ISVD comprises three complementarity determining domains CDR1, CDR2, and CDR3, wherein

(a) the CDR1 comprises GSTFSDYG (SEQ ID NO: 100),

(b) the CDR2 comprises IDWNGEHT (SEQ ID NO: 101), and

(c) the CDR3 comprises AADALPYTVRKYNY (SEQ ID NO: 102).

[0068] In some embodiments, the ISVD is humanized.

[0069] In some embodiments, the ISVD comprises, consists of, or consists essentially of SEQ ID NO: 77.

[0070] Also provided is a polypeptide comprising GSTFSDYG (SEQ ID NO: 100), IDWNGEHT (SEQ ID NO: 101), and AADALPYTVRKYNY (SEQ ID NO: 102).

[0071] In some embodiments, the polypeptide comprises the ISVD as described herein.

[0072] In some embodiments, the polypeptide further comprises a second binding moiety, wherein the second binding moiety binds to CD8 or another different target. In some embodiments, the second binding moiety is also an ISVD.

[0073] In some embodiments, the polypeptide further comprises a detectable marker, or a therapeutic agent.

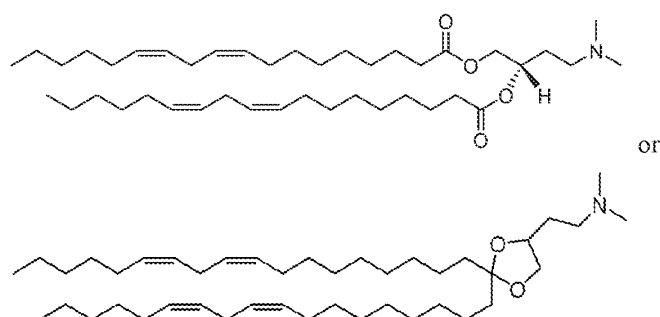
[0074] Also provided is a composition comprising the ISVD or the polypeptide as described herein.

[0075] Further provided is a pharmaceutical composition comprising the ISVD or the polypeptide as described herein, and a pharmaceutically acceptable carrier.

[0076] Further provided is a method of treating a disease or disorder related to CD8 in a subject, comprising administering the pharmaceutical composition as described herein to the subject.

[0077] In some embodiments, the disease is cancer. In some embodiments, the disorder is an immune disorder, an inflammatory disorder, or cancer.

[0078] In some embodiments, the nucleic acid encodes an antigen for use in a therapeutic or prophylactic vaccine for treating or preventing an infection by a pathogen. In some embodiments, the ionizable cationic lipid is



[0079] In some embodiments, the immune cell targeting group comprises an antibody that binds a T cell antigen. In some embodiments, the T cell antigen is CD3, CD8, or both CD3 and CD8.60. In some embodiments, the immune cell targeting group comprises an antibody that binds a Natural Killer (NK) cell antigen. In some embodiments, the NK cell antigen is CD56. In some embodiments, the antibody is a human or humanized antibody.

[0080] In some embodiments, the immune cell targeting group is covalently coupled to a lipid in the lipid blend via a polyethylene glycol (PEG) containing linker. In some embodiments, the lipid covalently coupled to the immune cell targeting group via a PEG containing linker is distearoylglycerol (DSG), distearoyl-phosphatidylethanolamine (DSPE), dimyrstoyl-phosphatidylethanolamine (DMPE), distearoyl-glycero-phosphoglycerol (DSPG), dimyrstoyl-glycerol (DMG), dipalmitoyl-phosphatidylethanolamine (DPPE), dipalmitoyl-glycerol (DPG), or ceramide. In some embodiments, the PEG is PEG 2000.

[0081] In some embodiments, the lipid-immune cell targeting group conjugate is present in the lipid blend in a range of 0.002-0.2 mole percent. In some embodiments, the ionizable cationic lipid is present in the lipid blend in a range of 40-60 mole percent.

[0082] In some embodiments, the sterol is cholesterol. In some embodiments, the sterol is present in the lipid blend in a range of 30-50 mole percent. In some embodiments, the neutral phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), sphingomyelin (SM).

[0083] In some embodiments, the neutral phospholipid is present in the lipid blend in a range of 1-10 mole percent.

[0084] In some embodiments, the free PEG-lipid is selected from the group consisting of PEG-modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides, PEG-modified dialkylamines, PEG-modified diacylglycerols, and PEG-modified dialkylglycerols. For example, a PEG lipid may be PEG- dioleoylglycerol (PEG-DOG), PEG-dimyristoyl-glycerol (PEG-DMG), PEG-dipalmitoyl-glycerol (PEG-DPG), PEG-dilinoleoyl-glycero-phosphatidyl ethanolamine (PEG-DLPE), PEG-dimyristoyl-phosphatidylethanolamine (PEG-DMPE), PEG-dipalmitoyl- phosphatidylethanolamine (PEG-DPPE), PEG-distearoylglycerol (PEG-DSG), PEG-diacylglycerol (PEG-DAG, e.g., PEG-DMG, PEG-DPG, and PEG-DSG), PEG-ceramide, PEG-distearoyl-glycero-phosphoglycerol (PEG-DSPG), PEG-dioleoyl-glycero-phosphoethanolamine (PEG-DOPE), 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide, or a PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) lipid.

[0085] In some embodiments, the free PEG-lipid comprises a diacylphosphatidylethanolamines comprising Dipalmitoyl (C16) chain or Distearoyl (C18) chain. In some embodiments, the free PEG-lipid is present in the lipid blend in a range of 2-4 mole percent. In some embodiments, the free PEG-lipid comprises the same or a different lipid as the lipid in the lipid-immune cell targeting group conjugate.

[0086] In some embodiments, the LNP has a mean diameter in the range of 50-200 nm. In some embodiments, the LNP has a mean diameter of about 100 nm. In some embodiments, the LNP has a polydispersity index in a range from 0.05 to 1. In some embodiments, the LNP has a zeta potential of from about -10 mV to about + 30 mV at pH 5.

[0087] In some embodiments, the nucleic acid is DNA or RNA. In some embodiments, the RNA is an mRNA, tRNA, siRNA, or microRNA. In some embodiments, the mRNA encodes a receptor, a growth factor, a hormone, a cytokine, an antibody, an antigen, an enzyme, or a vaccine. In some embodiments, the mRNA encodes a polypeptide capable of regulating immune response in the immune cell. In some embodiments, the mRNA encodes a polypeptide capable of reprogramming the immune cell. In some embodiments, the mRNA encodes a synthetic T cell receptor (synTCR) or a Chimeric Antigen Receptor (CAR).

[0088] In some embodiments, the immune cell targeting group comprises an antibody, and the antibody is a Fab or an immunoglobulin single variable domain. In some embodiments, the immune cell targeting group comprises an antibody fragment selected from the group consisting of a Fab, F(ab')₂, Fab'-SH, Fv, and scFv fragment. In some embodiments, the immune cell targeting group comprises a Fab that comprises one or more interchain disulfide bonds. In some embodiments, the Fab comprises a heavy chain fragment that comprises F174C and C233S substitutions, and a light chain fragment that comprises S176C and C214S substitutions, numbering according to Kabat. In some embodiments, the immune cell targeting group comprises a Fab that comprises a cysteine at the C-terminus of the heavy or light chain fragment.

[0089] In some embodiments, the Fab further comprises one or more amino acids between the heavy chain fragment of the Fab and the C-terminal cysteine. In some embodiments, the Fab comprises a heavy chain variable domain linked to an antibody CH1 domain and a light chain variable domain linked to an antibody light chain constant domain. In some embodiments, the CH1 domain and the light chain constant domain are linked by one or more interchain disulfide bonds. In some embodiments, the immune cell targeting group further comprises a single chain variable fragment (scFv) linked to the C-terminus of the light chain constant domain by an amino acid linker.

[0090] In some embodiments, the immune cell targeting group comprises an immunoglobulin single variable domain. In some embodiments, the immunoglobulin single variable domain comprises a cysteine at the C-terminus. In some embodiments, the immunoglobulin single variable domain comprises a VHH domain and further comprises a spacer comprising one or more amino acids between the VHH domain and the C-terminal cysteine. In some embodiments, the immune cell targeting group comprises two or more VHH domains. In some embodiments, the two or more VHH domains are linked by an amino acid linker. In some embodiments, the immune cell targeting group comprises a first VHH domain linked to an antibody CH1 domain and a second VHH domain linked to an antibody light chain constant domain. In some embodiments, the antibody CH1 domain and the antibody light chain constant domain are linked by one or more disulfide bonds. In some embodiments, the immune cell targeting group comprises a VHH domain linked to an antibody CH1 domain. In some embodiments, the antibody CH1 domain is linked to an antibody light chain constant domain by one or more disulfide bonds. In some embodiments,

the CH1 domain comprises F174C and C233S substitutions, and the light chain constant domain comprises S176C and C214S substitutions, numbering according to Kabat.

[0091] In some embodiments, the immune cell targeting group comprises a Fab that comprises:

(a) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 1 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 2 or 3;

(b) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 6 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 7.

[0092] In some embodiments, no more than 5% non-immune cells are transfected by the LNP. In some embodiments, half-life of the nucleic acid delivered by the LNP or a polypeptide encoded by the nucleic acid delivered by the LNP is at least 10% longer than half-life of nucleic acid delivered by a reference LNP or a polypeptide encoded by the nucleic acid delivered by the reference LNP. In some embodiments, at least 10% immune cells are transfected by the LNP. In some embodiments, expression level of the nucleic acid delivered by the LNP is at least 10% higher than expression level of nucleic acid delivered by a reference LNP.

[0093] In some aspects, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into an immune cell of the subject. In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid. In some embodiments, the immune cell is an NK cell. In some embodiments, the immune cell targeting group comprises an antibody that binds CD56.

[0094] In some aspect, provided herein are lipid nanoparticles (LNPs) for delivering a nucleic acid into an immune cell of the subject. In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free

Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid. In some embodiments, the immune cell targeting group comprises an antibody that binds CD7 or CD8. In some embodiments, the free PEG lipid is DMG-PEG.

[0095] In some aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into an immune cell of the subject. In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid. In some embodiments, the immune cell targeting group comprises an antibody. In some embodiments, the antibody is a Fab or an immunoglobulin single variable domain.

[0096] In some embodiments, the Fab is engineered to knock out the natural interchain disulfide at the C-terminus. In some embodiments, the Fab comprises a heavy chain fragment that comprises C233S substitutions, and a light chain fragment that comprises C214S substitutions. In some embodiments, the Fab comprises a non-natural interchain disulfide. In some embodiments, the Fab comprises F174C substitution in the heavy chain fragment, and S176C substitution in the light chain fragment. In some embodiments, the antibody is an immunoglobulin single variable (ISV) domain, and the ISV domain is an Nanobody® ISV. In some embodiments, the free PEG lipid comprise a PEG having a molecular weight of at least 2000 daltons. In some embodiments, the PEG has a molecular weight of about 3000 to 5000 daltons. In some embodiments, the antibody is a Fab. In some embodiments, the Fab binds CD3, and the free PEG lipid in the LNP comprises a PEG having a molecular weight of about 2000 daltons. In some embodiments, the Fab is an anti-CD4 antibody, and the free PEG lipid in the LNP comprises a PEG having a molecular weight of about 3000 to 3500 daltons.

[0097] In some embodiments, the immune cell targeting group comprises two or more VHH domains. In some embodiments, the two or more VHH domains are linked by an amino acid linker. In some embodiments, the immune cell targeting group comprises a first VHH domain linked to an antibody CH1 domain and a second VHH domain linked to an antibody light chain constant domain.

[0098] In some aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into an immune cell of the subject. In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid.

[0099] In some embodiments, the LNP binds CD3, and also binds CD11a or CD18. In some embodiments, the LNP comprises two conjugates. In some embodiments, the first conjugate comprises an antibody that binds CD3. In some embodiments, the second conjugate comprises an antibody that binds CD11a or CD18. In some embodiments, the LNP comprises one conjugate. In some embodiments, the conjugate comprises a bispecific antibody that binds both CD3 and CD11a. In some embodiments, the conjugate comprises a bispecific antibody that binds both CD3 and CD18. In some embodiments, the bispecific antibody is an immunoglobulin single variable domain or Fab-ScFv.

[0100] In some aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into an immune cell of the subject. In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid. In some embodiments, the LNP binds CD7 and CD8 of the immune cell.

[0101] In some embodiments, the LNP comprises two conjugates. In some embodiments, the first conjugate comprises an antibody that binds CD7, and a second conjugate that binds CD8. In some embodiments, the LNP comprises one conjugate. In some embodiments, the conjugate comprises a bispecific antibody that binds CD7 and CD8. In some embodiments, the bispecific antibody is an immunoglobulin single variable domain or Fab-ScFv.

[0102] In some aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into two different types of immune cells of the subject. In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate

comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises sterol or other structural lipid. In some embodiments, the LNP comprises neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid. In some embodiments, the LNP binds to a first antigen on the surface of the first type of immune cell, and also binds to a second antigen on the surface of the second type of immune cell. In some embodiments, the two different types of immune cells are CD4+ T cells and CD8+ T cell. In some embodiments, the LNP comprises two conjugates. In some embodiments, the first conjugate comprises a first antibody that binds to the first antigen of the first type of immune cell, and the second conjugate comprises a second antibody that binds to the second antigen of the second type of immune cell. In some embodiments, the LNP comprises one conjugate. In some embodiments, the conjugate comprises a bispecific antibody. In some embodiments, the bispecific antibody binds to both the first antigen on the first type of immune cell, the second antigen on the second type of immune cells. In some embodiments, the bispecific antibody is an immunoglobulin single variable domain or a Fab-ScFv.

[0103] In some aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into both CD4+ and CD8+ T cells of a subject. In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid. In some embodiments, the immune cell targeting group comprises a single antibody that binds to CD3 or CD7.

[0104] In some aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into both T cells and NK cells of a subject. In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid.

In some embodiments, the immune cell targeting group binds to CD7, CD8, or both CD7 and CD8.

[0105] In some aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into both T cells and NK cells of a subject. In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid. In some embodiments, the immune cell targeting group binds to (i) both CD3 and CD56; (ii) both CD8 and CD56; or (iii) both CD7 and CD56.

[0106] In some embodiments, the immune cell targeting group is covalently coupled to a lipid in the lipid blend via a polyethylene glycol (PEG) containing linker. In some embodiments, the lipid covalently coupled to the immune cell targeting group via a PEG containing linker is distearoylglycerol (DSG), distearoyl-phosphatidylethanolamine (DSPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-glycero-phosphoglycerol (DSPG), dimyristoyl-glycerol (DMG), dipalmitoyl-phosphatidylethanolamine (DPPE), dipalmitoyl-glycerol (DPG), or ceramide. In some embodiments, the lipid-immune cell targeting group conjugate is present in the lipid blend in a range of 0.002-0.2 mole percent. In some embodiments, the lipid blend further comprises one or more of a structural lipid (e.g., a sterol), a neutral phospholipid, and a free PEG-lipid.

[0107] In some embodiments, the ionizable cationic lipid is present in the lipid blend in a range of 40-60 mole percent.

[0108] In some embodiments, the sterol is present in the lipid blend in a range of 30-50 mole percent. In some embodiments, the sterol is cholesterol.

[0109] In some embodiments, the neutral phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). In some embodiments, the neutral phospholipid is present in the lipid blend in a range of 1-10 mole percent.

[0110] In some embodiments, the free PEG-lipid is selected from the group consisting of PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) or PEG-dimyristoyl-phosphatidylethanolamine (PEG-DMPE), N-(Methylpolyoxyethylene oxycarbonyl)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE-PEG) 1,2-Dimyristoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DMG), 1,2-Dipalmitoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DPG), 1,2-Dioleoyl-rac-glycerol, methoxypolyethylene Glycol (DOG-PEG) 1,2-Distearoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DSG), N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)]} (PEG-ceramide), and DSPE-PEG-cysteine, or a derivative thereof. In some embodiments, the free PEG-lipid comprises a diacylphosphatidylethanolamines comprising Dipalmitoyl (C16) chain or Distearoyl (C18) chain. In some embodiments, the free PEG-lipid is present in the lipid blend in a range of 1-2 mole percent. In some embodiments, the free PEG-lipid comprises the same or a different lipid as the lipid in the lipid-immune cell targeting group conjugate.

[0111] In some embodiments, the LNP has a mean diameter in the range of 50-200 nm. In some embodiments, the LNP has a mean diameter of about 100 nm. In some embodiments, the LNP has a polydispersity index in a range from 0.05 to 1. In some embodiments, the LNP has a zeta potential of from about -10 mV to about + 30 mV at pH 5.

[0112] In some embodiments, the nucleic acid is DNA or RNA. In some embodiments, the RNA is an mRNA. In some embodiments, the mRNA encodes a receptor, a growth factor, a hormone, a cytokine, an antibody, an antigen, an enzyme, or a vaccine. In some embodiments, the mRNA encodes a polypeptide capable of regulating immune response in the immune cell. In some embodiments, the mRNA encodes a polypeptide capable of reprogramming the immune cell. In some embodiments, the mRNA encodes a synthetic T cell receptor (synTCR) or a Chimeric Antigen Receptor (CAR).

[0113] In some aspect, provided are lipid nanoparticles (LNPs) for delivering a nucleic acid into an immune cell of a subject. In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid.

[0114] In some embodiments, the immune cell targeting group comprises a Fab lacking the native interchain disulfide bond. In some embodiments, the Fab is engineered to replace one or both cysteines on the native constant light chain and the native constant heavy chain that form the native interchain disulfide with a non-cysteine amino acid, therefor to remove the native interchain disulfide bond in the Fab.

[0115] In some aspect, provided are methods of targeting the delivery of a nucleic acid to an immune cell of a subject. In some embodiments, the method comprises contacting the immune cell with a lipid nanoparticle (LNP) provided herein. In some embodiments, the method is for targeting NK cells. In some embodiments, the immune cell targeting group binds to CD56. In some embodiments, the method is for targeting both T cells and NK cells simultaneously. In some embodiments, the immune cell targeting group binds to CD7, CD8, or both CD7 and CD8. In some embodiments, the method is for targeting both CD4+ and CD8+ T cells simultaneously. In some embodiments, the immune cell targeting group comprises a polypeptide that binds to CD3 or CD7.

[0116] In some aspect, provided are methods of expressing a polypeptide of interest in a targeted immune cell of a subject. In some embodiments, the method comprises contacting the immune cell with a lipid nanoparticle (LNP) provided herein.

[0117] In some aspect, provided are method of modulating cellular function of a target immune cell of a subject. In some embodiments, the method comprises administering to the subject a lipid nanoparticle (LNP) provided herein.

[0118] In some aspect, provided are method of treating, ameliorating, or preventing a symptom of a disorder or disease in a subject in need thereof. In some embodiments, the method comprises administering to the subject a lipid nanoparticle (LNP) provided herein.

[0119] In some aspect, provided are immunoglobulin single variable domains (ISVDs) that bind to human CD8. In some embodiments, the ISVD comprises three complementarity determining domains CDR1, CDR2, and CDR3. In some embodiments, the CDR1 comprises GSTFSDYG (SEQ ID NO: 100). In some embodiments, the CDR2 comprises IDWNGEHT (SEQ ID NO: 101). In some embodiments, the CDR3 comprises AADALPYTVRKYNY (SEQ ID NO: 102). In some embodiments, the ISVD is humanized. In some embodiments, the ISVD comprises SEQ ID NO: 77.

[0120] In some aspect, provided are polypeptides comprising GSTFSDYG (SEQ ID NO: 100), IDWNGEHT (SEQ ID NO: 101), and AADALPYTVRKYNY (SEQ ID NO: 102). In another aspect, provided are polypeptides comprising the ISVD provided herein. In some embodiments, the polypeptide comprises a second binding moiety. In some embodiments, the second binding moiety binds to CD8 or another different target. In some embodiments, the second binding moiety is also an ISVD. In some embodiments, the polypeptide comprises a detectable marker. In some embodiments, the polypeptide comprises a therapeutic agent.

[0121] In some aspect, provided are compositions comprising the ISVD provided herein or the polypeptide provided herein.

[0122] In some aspect, provided are pharmaceutical compositions comprising the ISVD provided herein or the polypeptide provided herein, and a pharmaceutically acceptable carrier.

[0123] In some aspect, provided are methods of treating a disease or disorder related to CD8 in a subject. In some embodiments, the method comprises administering a pharmaceutical composition described herein to the subject. In some embodiments, the disease or disorder is cancer.

[0124] Various aspects and embodiments of the invention are described in further detail below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0125] FIG. 1 depicts an NMR spectrum of Lipid 1.

[0126] FIGS. 2A and 2B depict LC-MS spectra of Lipid 1.

[0127] FIG. 3 depicts an NMR spectrum of Lipid 2.

[0128] FIGS. 4A and 4B depict LC-MS spectra of Lipid 2.

[0129] FIG. 5 depicts an NMR spectrum of Lipid 3.

[0130] FIG. 6 depicts an NMR spectrum of Lipid 4.

[0131] FIGS. 7A and 7B depict LC-MS spectra of Lipid 4.

[0132] FIG. 8A depicts Lipid 2 and Lipid 6 LNP pKa (TNS). FIG. 8B depicts Lipid 5 and Lipid 7 LNP pKa (TNS).

[0133] FIGS. 9A depicts hydrodynamic diameter of Lipid 2, and Lipid 6 derived LNPs. FIG. 9B depicts polydispersity (Dynamic Light Scattering) of Lipid 2 and Lipid 6 derived LNPs.

[0134] FIGS. 10A depicts hydrodynamic diameter of Lipid 5, and Lipid 7 derived LNPs. FIG. 10B depicts polydispersity (Dynamic Light Scattering) of Lipid 5 and Lipid 7 derived LNPs.

[0135] FIGS. 11A-D depict in vitro T-cell transfection of GFP mRNA using Lipid 2 and Lipid 6 derived LNPs, % GFP+ cells (FIG. 11A), GFP mean fluorescence intensity (MFI) (FIG. 11B), % Cy5-GFP + cells (FIG. 11C), and Cy5-GFP MFI, E. T-cell viability (FIG. 11D).

[0136] FIGS. 12A-E depict in vitro T-cell transfection of GFP mRNA using Lipid 5 and Lipid 7 derived LNPs, % GFP+ cells (FIG. 12A), GFP mean fluorescence intensity (MFI) (FIG. 12B), % Cy5-GFP + cells (FIG. 12C), Cy5-GFP MFI (FIG. 12D), T-cell viability (FIG. 12E).

[0137] FIG. 13A depicts %GFP+ (translation) human CD8 T cells post 24 hr transfection. FIG. 13B depicts %Cy5+ (binding) human CD8 T cells post 24 hr transfection.

[0138] FIG. 14A depicts %Viable human CD8 T cells post 24 hr transfection. FIG. 14B depicts Human IFN γ measured from cell culture supernatant post 24 hr transfection.

[0139] FIG. 15A depicts %TTR-023+ (anti-CD20 CAR) CD8 T cells post 24 hr transfection with mRNA LNPs. FIG. 15B depicts %TTR-023+ (anti-CD20 CAR) CD4 T cells post 24 hr transfection with mRNA LNPs.

[0140] FIG. 16A depicts %CD69+ CD8 cells post 24 hr transfection with anti-CD20 CAR mRNA LNPs. FIG. 16B depicts %CD69+ CD4 T cells post 24 hr transfection with anti-CD20 CAR mRNA LNPs.

[0141] FIG. 17 depicts Human IFN γ secretion by T cells post 24 hr transfection with anti-CD20 CAR mRNA LNPs.

[0142] FIG. 18A depicts %GFP+ (transfection/translation) of CD8 T cells post 24 hr transfection with Cy5/GFP mRNA at 2.5 ug/mL for 24 hr. FIG. 18B depicts %GFP+ (transfection/translation) Mean Fluorescence Intensity (MFI) of CD8 T cells post 24 hr transfection with Cy5/GFP mRNA at 2.5 ug/mL for 24 hr.

[0143] FIG. 19A depicts %Cy5+ (binding) of CD8 T cells post 24 hr transfection with Cy5/GFP mRNA at 2.5 ug/mL for 24 hr. FIG. 19B depicts Cy5 (binding) Mean Fluorescence Intensity (MFI) of CD8 T cells post 24 hr transfection with Cy5/GFP mRNA at 2.5 ug/mL for 24 hr.

[0144] FIG. 20A depicts %GFP+ (transfection/translation) CD8 cells of human CD3 cells transfected with 2.5 ug/mL Cy5/GFP mRNA LNPs for 24 hr. FIG. 20B depicts %GFP+ (transfection/translation) CD4 cells of human CD3 cells transfected with 2.5 ug/mL Cy5/GFP mRNA LNPs for 24 hr.

[0145] FIG. 21A depicts %Cy5+ (binding) CD8 cells of human CD3 cells transfected with 2.5 ug/mL Cy5/GFP mRNA LNPs for 24 hr. FIG. 21B depicts %Cy5+ (binding) CD4 cells of human CD3 cells transfected with 2.5 ug/mL Cy5/GFP mRNA LNPs for 24 hr.

[0146] FIG. 22A depicts %CD69+ CD8 cells of human CD3 cells transfected with 2.5 ug/mL Cy5/GFP mRNA LNPs for 24 hr. FIG. 22B depicts %CD69+ CD4 cells of human CD3 cells transfected with 2.5 ug/mL Cy5/GFP mRNA LNPs for 24 hr.

[0147] FIG. 23 depicts Human IFN γ secretion from human CD3 cells transfected with 2.5 μ g/mL Cy5/GFP mRNA LNPs for 24 hr.

[0148] FIG. 24A depicts %m Cherry+ CD8 T cells transfected in whole blood at 2.5 μ g/mL mCherry mRNA LNPs for 24 hr. FIG. 24B depicts %m Cherry+ CD4 T cells transfected in whole blood at 2.5 μ g/mL mCherry mRNA LNPs for 24 hr.

[0149] FIG. 25A depicts %m Cherry+ B cells transfected in whole blood at 2.5 μ g/mL mCherry mRNA LNPs for 24 hr. FIG. 25B depicts %m Cherry+ NK cells transfected in whole blood at 2.5 μ g/mL mCherry mRNA LNPs for 24 hr.

[0150] FIG. 26A depicts %m Cherry+ Granulocytes transfected in whole blood at 2.5 μ g/mL mCherry mRNA LNPs for 24 hr. FIG. 26B depicts %CD69+ CD8 T cells transfected

in whole blood at 2.5 ug/mL mCherry mRNA LNPs for 24 hr. **FIG. 26C** depicts %CD69+ CD4 T cells transfected in whole blood at 2.5 ug/mL mCherry mRNA LNPs for 24 hr.

[0151] **FIGS. 27A and 27B** depict time course for *in vivo* reprogramming of CD8+ T cells and CD4+ T cells respectively with CD3 targeted mCherry LNPs in blood. Each symbol represents one mouse. Open symbol represents mice that were buffer control treated and closed symbol represents mcherry LNP treated. Circles represent 24 hr, triangles represent 48 hr and diamonds represents 96 hr. **FIGS. 27C and 27D** depict time course for *in vivo* reprogramming of CD8+ T cells and CD4+ T cells respectively in liver. Each symbol represents one mouse. Open symbol represents mice that were buffer control treated and closed symbol represent mCherry LNP treated. Circles represent 24 hr, triangles represent 48 hr and diamonds represents 96 hr. **FIG. 27E and 27F** depict time course for *in vivo* reprogramming of CD8+ T cells and CD4+ T cells respectively with CD3 targeted mCherry LNPs in spleen. Each symbol represents one mouse. Open symbol represents mice that were buffer control treated and closed symbol represent mCherry LNP treated. Circles represent 24 hr, triangles represent 48 hr and diamonds represents 96hr.

[0152] **FIG. 28** depicts minimal expression of mCherry in liver myeloid and Kupffer cells after 24 hr treated with CD3 targeted mcherry LNP. Each symbol represents one mouse. Open symbols represent mice that were buffer control treated and closed symbol represent mCherry LNP treated.

[0153] **FIG. 29A** depicts *in vivo* reprogramming after 24 hr of 1st dose of mCherry expressing LNPs in blood. Each symbol represents one mouse. Open circles are CD4+ T cells and open square are CD8+ T cells expressing mCherry. **FIG. 29B** depicts *In vivo* reprogramming after 24 hr of 1st dose of TTR-023 expressing LNPs in blood. Each symbol represents one mouse. Open circles are CD4+ T cells and open square are CD8+ T cells expressing anti-CD20 CAR.

[0154] **FIGS. 30A-E** depict *in vivo* reprogramming after 40 hr of 2nd dose of with TTR-023 expressing LNP in blood (**FIG. 30A**), Spleen (**FIG. 30B**), Liver (**FIG. 30C**), Bone Marrow (**FIG. 30D**), and Thymus (**FIG. 30E**). Each symbol represents one mouse. Open circle is CD4+ T cells and open square is CD8+ T cells expressing ant-CD20 CAR.

[0155] **FIGS. 31A-E** depict *in vivo* reprogramming after 40h of 2nd dose of with mCherry expressing LNP in blood in blood (**FIG. 31A**), Spleen (**FIG. 31B**), Liver (**FIG. 31C**), Bone

Marrow (**FIG. 31D**), and Thymus (**FIG. 31E**). Each symbol represents one mouse. Open circle is CD4+ T cells and open square is CD8+ T cells expressing mCherry.

[0156] **FIG. 32** depicts dosing and bleeding schema for the PK study.

[0157] **FIG. 33** depicts calculated mRNA concentration based on converted DiI-C18(3)-DS measurements from mouse serum samples.

[0158] **FIG. 34A** depicts %DiR+ CD4 T-cells after 2 hr incubation with 2.5 µg/mL mRNA LNPs. **FIG. 34B** depicts DiR Mean Fluorescence Intensity (MFI) CD4 T-cells after 2 hr incubation with 2.5 µg/mL mRNA LNPs.

[0159] **FIG. 35A** depicts %DiR+ CD8 T-cells after 2 hr incubation with 2.5 µg/mL mRNA LNPs. **FIG. 35B** depicts DiR Mean Fluorescence Intensity (MFI) CD8 T-cells after 2 hr incubation with 2.5 µg/mL mRNA LNPs.

[0160] **FIG. 36A** depicts %m Cherry+ CD4 T-cells after 24 hr incubation with 2.5 µg/mL mRNA LNPs. **FIG. 36B** depicts %m Cherry+ CD8 T-cells after 24 hr incubation with 2.5 µg/mL mRNA LNPs.

[0161] **FIGS. 37A** depicts hydrodynamic diameter of Lipid 5, Lipid 8 and DLn-MC3-DMA derived LNPs. **FIG. 37B** depicts polydispersity (Dynamic Light Scattering) of Lipid 5, Lipid 8 and DLn-MC3-DMA derived LNPs.

[0162] **FIGS. 38A-E** depict in vitro T-cell transfection of GFP mRNA using Lipid 5, Lipid 8, and DLn-MC3-DMA derived LNPs, % GFP+ cells (**FIG. 38A**), GFP mean fluorescence intensity (MFI) (**FIG. 38B**), % Cy5-GFP + cells (**FIG. 38C**), Cy5-GFP MFI (**FIG. 38D**), and T-cell viability (**FIG. 38E**).

[0163] **FIG. 39** depicts an NMR spectrum of Lipid 5.

[0164] **FIGS. 40A and 40B** depict LC-MS spectra of Lipid 5.

[0165] **FIG. 41** depicts an NMR spectrum of Lipid 6.

[0166] **FIGS. 42A and 42B** depict LC-MS spectra of Lipid 6.

[0167] **FIG. 43** depicts an NMR spectrum of Lipid 7.

[0168] FIGS. 44A and 44B depict LC-MS spectra of Lipid 7.

[0169] FIG. 45A depicts hydrodynamic diameter of Lipid 8, and Lipid 5 derived LNPs. FIG. 45B depicts polydispersity (Dynamic Light Scattering) of Lipid 8 and Lipid 5 derived LNPs.

[0170] FIGS. 46A-E depict in vitro T-cell transfection of GFP mRNA using Lipid 8 and Lipid 5 (O and N) derived LNPs, % GFP+ cells (FIG. 46A), GFP mean fluorescence intensity (MFI) (FIG. 46B), % Cy5-GFP + cells (FIG. 46C), Cy5-GFP MFI (FIG. 46D), T-cell viability (FIG. 46E).

[0171] FIG. 47 depicts structures of various Fab, VHH (Nb), ScFv, Fab-ScFv and Fab-VHH hybrids.

[0172] FIG. 48A depicts and NMR spectrum of Lipid 9. FIG. 48B and FIG. 48C depict the Mass spectrum and LC chromatogram of Lipid 9.

[0173] FIG. 49A depicts and NMR spectrum of Lipid 10. FIG. 49B and FIG. 49C depict the Mass spectrum and LC chromatogram of Lipid 10.

[0174] FIG. 50A depicts and NMR spectrum of Lipid 11. FIG. 50B and FIG. 50C depict the Mass spectrum and LC chromatogram of Lipid 11.

[0175] FIG. 51A depicts and NMR spectrum of Lipid 12. FIG. 51B and FIG. 51C depict the Mass spectrum and LC chromatogram of Lipid 12.

[0176] FIG. 52A depicts and NMR spectrum of Lipid 13. FIG. 52B and FIG. 52C depict the Mass spectrum and LC chromatogram of Lipid 13.

[0177] FIG. 53A depicts hydrodynamic diameter (DLS) of Lipid 5 and Lipid 8 prior to and after antibody conjugate insertion. FIG. 53B depicts polydispersity (DLS) prior to and after antibody conjugate insertion. FIG. 53C and 53D depict LNP surface charge (Zeta Potential, DLS) prior to and after antibody conjugate insertion in pH 5.5 MES and pH 7.4 HEPES buffer.

[0178] FIGS. 54A to 54E depict in vitro T-cell transfection of GFP mRNA using Lipid 5 and Lipid 8 derived LNPs: % GFP+ cells (FIG. 54A), GFP mean fluorescence intensity

(MFI) (FIG. 54B), % DiI + cells (FIG. 54C), and DiI MFI (FIG. 54D), and T-cell viability (FIG. 54E).

[0179] FIG. 55A depicts hydrodynamic diameter (DLS) of Lipid 5, Lipid 8 and DLn-MC3-DMA prior to and after antibody conjugate insertion. FIG. 55B depicts polydispersity (DLS) prior to and after antibody conjugate insertion. FIG. 55C depicts LNP surface charge (Zeta Potential, DLS) prior to antibody conjugate insertion in pH 5.5 MES and pH 7.4 HEPES buffer. FIG. 55D depicts the accessible RNA content and RNA encapsulation efficiency.

[0180] FIGS. 56A to 56E depict in vitro T-cell transfection of GFP mRNA using Lipid 5, Lipid 8 and DLn-MC3-DMA derived LNPs: % GFP+ cells (FIG. 56A), GFP mean fluorescence intensity (MFI) (FIG. 56B), % DiI + cells (FIG. 56C), and DiI MFI (FIG. 56D), and T-cell viability (FIG. 56E).

[0181] FIG. 57A depicts hydrodynamic diameter (DLS) of Lipid 5 formulations stored at 4C or after storage at -80C; Formulations were frozen either by placing in a -80C freezer or flash frozen in Liquid Nitrogen. FIG. 57B depicts formulation polydispersities (DLS) before and after frozen storage.

[0182] FIGS. 58A to 58E depict in vitro T-cell transfection of GFP mRNA and T-cell viability resulting from Lipid 5 LNP formulations that were stored at 4C or after storage at -80C; formulations were frozen either by placing in the -80C freezer or flash frozen in liquid Nitrogen. % GFP+ cells (FIG. 58A), GFP mean fluorescence intensity (MFI) (FIG. 58B), % DiI + cells (FIG. 58C), and DiI MFI (FIG. 58D), and T-cell viability (FIG. 58E).

[0183] FIGS. 59A to 59T depict results of *in vivo* reprogramming of immune cells with CD3-targeted DiI/GFP LNP at the dose of 0.3 mg/kg after 24 or 48h with either DMG, DPG or DSG-PEG 2.5% or after 24h with either DPPE or DSPE 1.5 or 2.5%. Each symbol represents one mouse. Open circle is CD4+ T cells and open square is CD8+ T cells expressing; %GFP (FIG. 59A) in blood, (FIG. 59B) in liver, (FIG. 59C) in lung, (FIG. 59D) in spleen, (FIG. 59E) in bone marrow; GFP MFI (FIG. 59F) in blood, (FIG. 59G) in liver, (FIG. 59H) in lung, (FIG. 59I) in spleen, (FIG. 59J) in bone marrow; % DiI in (FIG. 59K) in blood, (FIG. 59L) in liver, (FIG. 59M) in lung, (FIG. 59N) in spleen, (FIG. 59O) in bone marrow; DiI MFI (FIG. 59P) in blood, (FIG. 59Q) in liver, (FIG. 59R) in lung, (FIG. 59S) in spleen, and (FIG. 59T) in bone marrow.

[0184] FIGS. 60A to 60T depict results of *in vivo* reprogramming with CD3, CD8 antibody/Nanobody targeted DiI/GFP LNP at 0.3 mg/kg of Lipid 5 with either DMG, DPG, 1.5 or 2.5% after 24h. Each symbol represents one mouse. Open circle is CD4+ T cells and open square is CD8+ T cells expressing; %GFP (60A) in blood, (60B) in liver, (60C) in lung, (60D) in spleen, (60E) in bone marrow; GFP MFI (60F) in blood, (60G) in liver, (60H) in lung, (60I) in spleen, (60J) in bone marrow; % DiI in (60K) in blood, (60L) in liver, (60M) in lung, (60N) in spleen, (60O) in bone marrow; DiI MFI (60P) in blood, (60Q) in liver, (60R) in lung, (60S) in spleen, (60T) in bone marrow.

[0185] FIGS. 61A to 61T depict results of *in vivo* reprogramming with either CD8, CD11a, CD4 Nanobody or CD4 antibody targeted DiI/GFP LNP at 0.3 mg/kg of Lipid 5 with either DMG or DPG, 1.5 % after 24h. Each symbol represents one mouse. Open circle is CD4+ T cells and open square is CD8+ T cells expressing; %GFP (61A) in blood, (61B) in liver, (61C) in lung, (61D) in spleen, (61E) in bone marrow; GFP MFI (61F) in blood, (61G) in liver, (61H) in lung, (61I) in spleen, (61J) in bone marrow; % DiI in (61K) in blood, (61L) in liver, (61M) in lung, (61N) in spleen, (61O) in bone marrow; DiI MFI (61P) in blood, (61Q) in liver, (61R) in lung, (61S) in spleen, (61T) in bone marrow.

[0186] FIGS. 62A to 62S depict *in vivo* reprogramming comparing ionizable lipids (DLn-MC3-DMA, Lipid 5 and Lipid 8) with CD3 (hsp34) antibody targeted DiI/GFP LNP at 0.1 mg/kg with DPG-PEG, 1.5 % after 24h. Each symbol represents one mouse. Open circle is CD4+ T cells and open square is CD8+ T cells expressing; %GFP (62A) in blood, (62B) in liver, (62C) in lung, (62D) in spleen, (62E) in bone marrow; GFP MFI (62F) in blood, (62G) in liver, (62H) in lung, (62I) in spleen, (62J) in bone marrow; % DiI in (62K) in blood, (62L) in liver, (62M) in lung, (62N) in spleen, (62O) in bone marrow; DiI MFI (62P) in blood, (62Q) in liver, (62R) in lung, (62S) in spleen, (62T) in bone marrow.

[0187] FIGS. 63A to 63T depict *in vivo* reprogramming with CD7 VHH /Nanobody targeted DiI/GFP LNP at 0.3 mg/kg of Lipid 5 with either DMG, DPG, 1.5 or 2.5% after 24h. Each symbol represents one mouse. Open circle is CD4+ T cells and open square is CD8+ T cells expressing; %GFP (63A) in blood, (63B) in liver, (63C) in lung, (63D) in spleen, (63E) in bone marrow; GFP MFI (63F) in blood, (63G) in liver, (63H) in lung, (63I) in spleen, (63J) in bone marrow; % DiI in (63K) in blood, (63L) in liver, (63M) in lung, (63N) in spleen, (63O) in bone marrow; DiI MFI (63P) in blood, (63Q) in liver, (63R) in lung, (63S) in spleen, (63T) in bone marrow.

[0188] FIG. 64A depicts %GFP Transfection of co-cultured T cells and NK cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 64B depicts GFP Expression levels by mean fluorescence intensity (MFI) co-cultured T cells and NK after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 64C depicts %DiI uptake of co-cultured T cells and NK cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 64D depicts %DiI uptake levels by mean fluorescence intensity (MFI) co-cultured T cells and NK after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated.

[0189] FIG. 65A depicts SDS-PAGE of SP34-hlam DS (contains WT inter-chain disulfide) Fab conjugates produced by reduction at varying TCEP concentrations prior to conjugation. FIG. 65B depicts SDS-PAGE of SP34-hlam NoDS (No inter-chain disulfide, e.g., C to S mutation in HC and LC) Fab conjugates produced by reduction at varying TCEP concentrations prior to conjugation. FIG. 65C depicts R8 RP-HPLC chromatograms of hSP34-hlam DS Fab and Fab conjugate produced with a 0.025 mM TCEP reduction condition prior to conjugation. FIG. 65D depicts R8 RP-HPLC chromatograms of hSP34-hlam NoDS Fab and Fab conjugates produced with various TCEP reduction conditions prior to conjugation. FIG. 65E depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at various densities. FIG. 65F depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at various densities.

[0190] FIG. 66A depicts SDS-PAGE of TS2/18.1 and 9.6 (contain WT inter-chain disulfide) Fab conjugates produced by reduction at varying TCEP concentrations prior to conjugation. Left: TS2/18.1; Right: 9.6 FIG. 66B depicts SDS-PAGE of TS2/18.1, 9.6 and TRX2 NoDS Fab and Fab conjugates produced by reduction at varying TCEP concentrations prior to conjugation. FIG. 66C depicts R8 RP-HPLC chromatograms of TS2/18.1 DS and NoDS Fab and Fab conjugate produced with various TCEP reduction conditions prior to

conjugation. **FIG. 66D** depicts R8 RP-HPLC chromatograms of 9.6 and TRX2 NoDS Fab and Fab conjugate produced with various TCEP reduction conditions prior to conjugation.

[0191] **FIG. 67A** depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 67B** depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 67C** depicts IFN γ secretion into supernatant from T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at densities that gave the highest levels of transfection evaluated.

[0192] **FIG. 68A** depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 68B** depicts GFP Expression levels by mean fluorescence intensity (MFI) of CD8 T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at densities that gave the highest levels of transfection evaluated.

[0193] **FIG. 69A** depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted individually or together at the same densities as the single targeted conditions. **FIG. 69B** depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted individually or together at the same densities as the single targeted conditions. **FIG. 69C** depicts IFN γ secretion into supernatant from T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted individually or together at the same densities as the single targeted conditions.

[0194] **FIG. 70A** depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Fab-ScFv post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 70B** depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Fab-ScFv post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 70C** depicts IFN γ

secretion into supernatant T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Fab-ScFv post inserted at densities that gave the highest levels of transfection evaluated.

[0195] FIG. 71A depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 71B depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 71C depicts IFN γ secretion into supernatant T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at densities that gave the highest levels of transfection evaluated.

[0196] FIG. 72A depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs and Nb post inserted at densities that gave the highest levels of transfection evaluated. FIG. 72B depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs and Nb post inserted at densities that gave the highest levels of transfection evaluated. FIG. 72C depicts IFN γ secretion into supernatant from T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs and Nb post inserted at densities that gave the highest levels of transfection evaluated.

[0197] FIG. 73A depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs and Nb post inserted at densities that gave the highest levels of transfection evaluated. FIG. 73B depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs and Nb post inserted at densities that gave the highest levels of transfection evaluated. FIG. 73C depicts IFN γ secretion into supernatant from T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs and Nb post inserted at densities that gave the highest levels of transfection evaluated.

[0198] FIG. 74A depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at

densities that gave the highest levels of transfection evaluated. **FIG. 74B** depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated.

[0199] **FIG. 75A** depicts %GFP Transfection of CD8 T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 75B** depicts %GFP Transfection of CD4 T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 75C** depicts GFP Expression levels by mean fluorescence intensity (MFI) CD8 T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 75D** depicts GFP Expression levels by mean fluorescence intensity (MFI) CD4 T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated.

[0200] **FIG. 76A** depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs and Nb post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 76B** depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs and Nb post inserted at densities that gave the highest levels of transfection evaluated.

[0201] **FIG. 77A** depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs and Nb post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 77B** depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs and Nb post inserted at densities that gave the highest levels of transfection evaluated.

[0202] **FIG. 78A** depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs and Nb post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 78B** depicts GFP

Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs and Nb post inserted at densities that gave the highest levels of transfection evaluated.

[0203] FIG. 79A depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 79B depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 79C depicts IFN γ secretion into supernatant T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at densities that gave the highest levels of transfection evaluated.

[0204] FIG. 80A depicts %GFP Transfection of CD8 T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 80B depicts %GFP Transfection of CD4 T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 80C depicts GFP Expression levels by mean fluorescence intensity (MFI) CD8 T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 80D depicts GFP Expression levels by mean fluorescence intensity (MFI) CD4 T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 80E depicts IFN γ secretion into supernatant T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs post inserted at densities that gave the highest levels of transfection evaluated.

[0205] FIG. 81A depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 81B depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. FIG. 81C depicts IFN γ secretion from T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for

approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated.

[0206] **FIG. 82A** depicts %GFP Transfection of T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 82B** depicts GFP Expression levels by mean fluorescence intensity (MFI) T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated. **FIG. 82C** depicts IFN γ secretion from T cells after incubation with targeted LNPs at 2.5 ug/mL mRNA for approximately 24 hrs with Fabs or Nbs post inserted at densities that gave the highest levels of transfection evaluated.

[0207] **FIG. 83A** depicts hydrodynamic diameter (DLS) of Lipid 2, Lipid 6, Lipid 12 and Lipid 13 prior to and after antibody conjugate insertion. **FIG. 83B** depicts polydispersity (DLS) prior to and after antibody conjugate insertion. **FIG. 83C** depicts LNP surface charge (Zeta Potential, DLS) prior to antibody conjugate insertion in pH 5.5 MES and pH 7.4 HEPES buffer. **FIG. 83D** depict the percent accessible RNA and total RNA content (ug/mL).

[0208] **FIGS. 84A to 84E** depict in vitro T-cell transfection of GFP mRNA using Lipid 2, Lipid 6, Lipid 12 and Lipid 13 derived LNPs, % GFP+ cells (**FIG. 84A**), GFP mean fluorescence intensity (MFI) (**FIG. 84B**), % DiI + cells (**FIG. 84C**), and DiI MFI (**FIG. 84D**), and T-cell viability (**FIG. 84E**).

[0209] **FIGS. 85A to 85E** depict in vitro T-cell transfection of GFP mRNA using Lipid 2, Lipid 6, Lipid 12 and Lipid 13 derived LNPs, % GFP+ cells (**FIG. 85A**), GFP mean fluorescence intensity (MFI) (**FIG. 85B**), % DiI + cells (**FIG. 85C**), and DiI MFI (**FIG. 85D**), and T-cell viability (**FIG. 85E**).

DETAILED DESCRIPTION

[0210] The invention provides ionizable cationic lipids, lipid-immune cell targeting group conjugates, and lipid nanoparticle compositions comprising such ionizable cationic lipids and/or lipid-immune cell (e.g., T-cell) targeting group conjugates, medical kits containing such lipids and/or conjugates, and methods of making and using, such lipids and conjugates.

[0211] The practice of the present invention employs, unless otherwise indicated, conventional techniques of organic chemistry, pharmacology, cell biology, and biochemistry. Such techniques are explained in the literature, such as in “Comprehensive Organic Synthesis” (B.M. Trost & I. Fleming, eds., 1991-1992); “Current protocols in molecular biology” (F.M. Ausubel *et al.*, eds., 1987, and periodic updates); and “Current protocols in immunology” (J.E. Coligan *et al.*, eds., 1991), each of which is herein incorporated by reference in its entirety. Various aspects of the invention are set forth below in sections; however, aspects of the invention described in one particular section are not to be limited to any particular section.

I. DEFINITIONS

[0212] To facilitate an understanding of the present invention, a number of terms and phrases are defined below.

[0213] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The abbreviations used herein have their conventional meaning within the chemical and biological arts. The chemical structures and formulae set forth herein should be construed according to the standard rules of chemical valency known in the chemical arts. In addition, when a chemical group is a diradical, for example, it is understood that the chemical groups can be bonded to their adjacent atoms in the remainder of the structure in one or both orientations, for example, -OC(O)- is interchangeable with -C(O)O- or -OC(S)- is interchangeable with -C(S)O-.

[0214] The terms “a” and “an” as used herein mean “one or more” and include the plural unless the context is inappropriate.

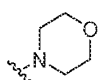
[0215] The term “alkyl” as used herein refers to a saturated straight or branched hydrocarbon, such as a straight or branched group of 1-2, 1-10, or 1-6 carbon atoms, referred to herein as C1-C12alkyl, C1-C10alkyl, and C1-C6alkyl, respectively. Exemplary alkyl groups include, but are not limited to, methyl, ethyl, propyl, isopropyl, 2-methyl-1-propyl, 2-methyl-2-propyl, 2-methyl-1-butyl, 3-methyl-1-butyl, 2-methyl-3-butyl, 2,2-dimethyl-1-propyl, 2-methyl-1-pentyl, 3-methyl-1-pentyl, 4-methyl-1-pentyl, 2-methyl-2-pentyl, 3-methyl-2-pentyl, 4-methyl-2-pentyl, 2,2-dimethyl-1-butyl, 3,3-dimethyl-1-butyl, 2-ethyl-1-butyl, butyl, isobutyl, t-butyl, pentyl, isopentyl, neopentyl, hexyl, heptyl, octyl, etc.

[0216] The term “alkylene” refers to a diradical of an alkyl group. An exemplary alkylene group is $-\text{CH}_2\text{CH}_2-$.

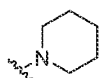
[0217] The term “haloalkyl” refers to an alkyl group that is substituted with at least one halogen. For example, $-\text{CH}_2\text{F}$, $-\text{CHF}_2$, $-\text{CF}_3$, $-\text{CH}_2\text{CF}_3$, $-\text{CF}_2\text{CF}_3$, and the like.

[0218] The term “oxo” is art-recognized and refers to a “ $=\text{O}$ ” substituent. For example, a cyclopentane substituted with an oxo group is cyclopentanone.

[0219] The term “morpholinyl” refers to a substituent having the structure of:



[0220] The term “piperidinyl” refers to a substituent having a structure of:



[0221] In general, the term “substituted”, whether preceded by the term “optionally” or not, means that one or more hydrogens of the designated moiety are replaced with a suitable substituent. Unless otherwise indicated, an “optionally substituted” group may have a suitable substituent at each substitutable position of the group, and when more than one position in any given structure may be substituted with more than one substituent selected from a specified group, the substituent may be either the same or different at each position. Combinations of substituents envisioned under this invention are preferably those that result in the formation of stable or chemically feasible compounds. In some embodiments, an optional substituent may be selected from the group consisting of: C_{1-6} alkyl, cyano, halogen, $-\text{O}-\text{C}_{1-6}$ alkyl, C_{1-6} haloalkyl, C_{3-7} cycloalkyl, 3-7 membered heterocyclyl, 5-6 membered heteroaryl, and phenyl, wherein R^a is hydrogen or C_{1-6} alkyl. In some embodiments, an optional substituent may be selected from the group consisting of: C_{1-6} alkyl, halogen, $-\text{O}-\text{C}_{1-6}$ alkyl, and $-\text{CH}_2\text{N}(\text{R}^a)_2$, wherein R^a is hydrogen or C_{1-6} alkyl.

[0222] The term “haloalkyl” refers to an alkyl group that is substituted with at least one halogen. For example, $-\text{CH}_2\text{F}$, $-\text{CHF}_2$, $-\text{CF}_3$, $-\text{CH}_2\text{CF}_3$, $-\text{CF}_2\text{CF}_3$, and the like.

[0223] The term “cycloalkyl” refers to a monovalent saturated cyclic, bicyclic, bridged cyclic (e.g., adamantyl), or spirocyclic hydrocarbon group of 3-12, 3-8, 4-8, or 4-6 carbons, referred to herein, e.g., as “C₄₋₈cycloalkyl,” derived from a cycloalkane. Exemplary cycloalkyl groups include, but are not limited to, cyclohexanes, cyclopentanes, cyclobutanes and cyclopropanes. Unless specified otherwise, cycloalkyl groups are optionally substituted at one or more ring positions with, for example, alkanoyl, alkoxy, alkyl, haloalkyl, alkenyl, alkynyl, amido, amidino, amino, aryl, arylalkyl, azido, carbamate, carbonate, carboxy, cyano, cycloalkyl, ester, ether, formyl, halogen, haloalkyl, heteroaryl, heterocyclyl, hydroxyl, imino, ketone, nitro, phosphate, phosphonato, phosphinato, sulfate, sulfide, sulfonamido, sulfonyl or thiocarbonyl. In certain embodiments, the cycloalkyl group is not substituted, i.e., it is unsubstituted.

[0224] The terms “heterocyclyl” and “heterocyclic group” are art-recognized and refer to saturated, partially unsaturated, or aromatic 3- to 10-membered ring structures, alternatively 3- to 7-membered rings, whose ring structures include one to four heteroatoms, such as nitrogen, oxygen, and sulfur. The number of ring atoms in the heterocyclyl group can be specified using C_x-C_x nomenclature where x is an integer specifying the number of ring atoms. For example, a C₃-C₇heterocyclyl group refers to a saturated or partially unsaturated 3- to 7-membered ring structure containing one to four heteroatoms, such as nitrogen, oxygen, and sulfur. The designation “C₃-C₇” indicates that the heterocyclic ring contains a total of from 3 to 7 ring atoms, inclusive of any heteroatoms that occupy a ring atom position. One example of a C₃heterocyclyl is aziridinyl. Heterocycles may be, for example, mono-, bi-, or other multi-cyclic ring systems (e.g., fused, spiro, bridged bicyclic). A heterocycle may be fused to one or more aryl, partially unsaturated, or saturated rings. Heterocyclyl groups include, for example, biotinyl, chromenyl, dihydrofuryl, dihydroindolyl, dihydropyranyl, dihydrothienyl, dithiazolyl, homopiperidinyl, imidazolidinyl, isoquinolyl, isothiazolidinyl, isooxazolidinyl, morpholinyl, oxolanyl, oxazolidinyl, phenoxanthenyl, piperazinyl, piperidinyl, pyranyl, pyrazolidinyl, pyrazolyl, pyridyl, pyrimidinyl, pyrrolidinyl, pyrrolidin-2-onyl, pyrrolinyl, tetrahydrofuryl, tetrahydroisoquinolyl, tetrahydropyranyl, tetrahydroquinolyl, thiazolidinyl, thiolanyl, thiomorpholinyl, thiopyranyl, xanthenyl, lactones, lactams such as azetidinones and pyrrolidinones, sultams, sultones, and the like. Unless specified otherwise, the heterocyclic ring is optionally substituted at one or more positions with substituents such as alkanoyl, alkoxy, alkyl, alkenyl, alkynyl, amido, amidino, amino, aryl, arylalkyl, azido, carbamate, carbonate, carboxy, cyano, cycloalkyl, ester, ether,

formyl, halogen, haloalkyl, heteroaryl, heterocyclyl, hydroxyl, imino, ketone, nitro, oxo, phosphate, phosphonato, phosphinato, sulfate, sulfide, sulfonamido, sulfonyl and thiocarbonyl. In certain embodiments, the heterocyclyl group is not substituted, i.e., it is unsubstituted.

[0225] The term “aryl” is art-recognized and refers to a carbocyclic aromatic group. Representative aryl groups include phenyl, naphthyl, anthracenyl, and the like. The term “aryl” includes polycyclic ring systems having two or more carbocyclic rings in which two or more carbons are common to two adjoining rings (the rings are “fused rings”) wherein at least one of the rings is aromatic and, e.g., the other ring(s) may be cycloalkyls, cycloalkenyls, cycloalkynyls, and/or aryls. Unless specified otherwise, the aromatic ring may be substituted at one or more ring positions with, for example, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, alkoxy, amino, nitro, sulfhydryl, imino, amido, carboxylic acid, -C(O)alkyl, CO₂alkyl, carbonyl, carboxyl, alkylthio, sulfonyl, sulfonamido, sulfonamide, ketone, aldehyde, ester, heterocyclyl, aryl or heteroaryl moieties, -CF₃, -CN, or the like. In certain embodiments, the aromatic ring is substituted at one or more ring positions with halogen, alkyl, hydroxyl, or alkoxy. In certain other embodiments, the aromatic ring is not substituted, i.e., it is unsubstituted. In certain embodiments, the aryl group is a 6-10 membered ring structure.

[0226] The term “heteroaryl” is art-recognized and refers to aromatic groups that include at least one ring heteroatom. In certain instances, a heteroaryl group contains 1, 2, 3, or 4 ring heteroatoms. Representative examples of heteroaryl groups include pyrrolyl, furanyl, thiophenyl, imidazolyl, oxazolyl, thiazolyl, triazolyl, pyrazolyl, pyridinyl, pyrazinyl, pyridazinyl and pyrimidinyl, and the like. Unless specified otherwise, the heteroaryl ring may be substituted at one or more ring positions with, for example, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, alkoxy, amino, nitro, sulfhydryl, imino, amido, carboxylic acid, C(O)alkyl, -CO₂alkyl, carbonyl, carboxyl, alkylthio, sulfonyl, sulfonamido, sulfonamide, ketone, aldehyde, ester, heterocyclyl, aryl or heteroaryl moieties, -CF₃, -CN, or the like. The term “heteroaryl” also includes polycyclic ring systems having two or more rings in which two or more carbons are common to two adjoining rings (the rings are “fused rings”) wherein at least one of the rings is heteroaromatic, e.g., the other cyclic rings may be cycloalkyls, cycloalkenyls, cycloalkynyls, and/or aryls. In certain embodiments, the heteroaryl ring is substituted at one or more ring positions with halogen,

alkyl, hydroxyl, or alkoxy. In certain other embodiments, the heteroaryl ring is not substituted, i.e., it is unsubstituted. In certain embodiments, the heteroaryl group is a 5- to 10-membered ring structure, alternatively a 5- to 6-membered ring structure, whose ring structure includes 1, 2, 3, or 4 heteroatoms, such as nitrogen, oxygen, and sulfur.

[0227] The terms “amine” and “amino” are art-recognized and refer to both unsubstituted and substituted amines, e.g., a moiety represented by the general formula $-N(R^{10})(R^{11})$, wherein R^{10} and R^{11} each independently represent hydrogen, alkyl, cycloalkyl, heterocyclyl, alkenyl, aryl, aralkyl, or $(CH_2)_m-R^{12}$; or R^{10} and R^{11} , taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure; R^{12} represents an aryl, a cycloalkyl, a cycloalkenyl, a heterocycle or a polycycle; and m is zero or an integer in the range of 1 to 8. In certain embodiments, R^{10} and R^{11} each independently represent hydrogen, alkyl, alkenyl, or $-(CH_2)_m-R^{12}$.

[0228] The terms “alkoxyl” or “alkoxy” are art-recognized and refer to an alkyl group, as defined above, having an oxygen radical attached thereto. Representative alkoxyl groups include methoxy, ethoxy, propyloxy, tert-butoxy and the like. An “ether” is two hydrocarbons covalently linked by an oxygen. Accordingly, the substituent of an alkyl that renders that alkyl an ether is or resembles an alkoxyl, such as may be represented by one of -O-alkyl, -O-alkenyl, O-alkynyl, $-O-(CH_2)_m-R^{12}$, where m and R^{12} are described above. The term “haloalkoxyl” refers to an alkoxyl group that is substituted with at least one halogen. For example, -O-CH₂F, -O-CHF₂, -O-CF₃, and the like. In certain embodiments, the haloalkoxyl is an alkoxyl group that is substituted with at least one fluoro group. In certain embodiments, the haloalkoxyl is an alkoxyl group that is substituted with from 1-6, 1-5, 1-4, 2-4, or 3 fluoro groups.

[0229] The symbol “~” indicates a point of attachment.

[0230] The compounds of the disclosure may contain one or more chiral centers and/or double bonds and, therefore, exist as stereoisomers, such as geometric isomers, enantiomers or diastereomers. The term “stereoisomers” when used herein consist of all geometric isomers, enantiomers or diastereomers. These compounds may be designated by the symbols “R” or “S,” depending on the configuration of substituents around the stereogenic carbon atom. The present invention encompasses various stereoisomers of these compounds and mixtures thereof. Stereoisomers include enantiomers and diastereomers. Mixtures of

enantiomers or diastereomers may be designated “(±)” in nomenclature, but the skilled artisan will recognize that a structure may denote a chiral center implicitly. It is understood that graphical depictions of chemical structures, e.g., generic chemical structures, encompass all stereoisomeric forms of the specified compounds, unless indicated otherwise.

[0231] Individual stereoisomers of compounds of the present invention can be prepared synthetically from commercially available starting materials that contain asymmetric or stereogenic centers, or by preparation of racemic mixtures followed by resolution methods well known to those of ordinary skill in the art. These methods of resolution are exemplified by (1) attachment of a mixture of enantiomers to a chiral auxiliary, separation of the resulting mixture of diastereomers by recrystallization or chromatography and liberation of the optically pure product from the auxiliary, (2) salt formation employing an optically active resolving agent, or (3) direct separation of the mixture of optical enantiomers on chiral chromatographic columns. Stereoisomeric mixtures can also be resolved into their component stereoisomers by well-known methods, such as chiral-phase gas chromatography, chiral-phase high performance liquid chromatography, crystallizing the compound as a chiral salt complex, or crystallizing the compound in a chiral solvent. Further, enantiomers can be separated using supercritical fluid chromatographic (SFC) techniques described in the literature. Still further, stereoisomers can be obtained from stereomerically-pure intermediates, reagents, and catalysts by well-known asymmetric synthetic methods.

[0232] Geometric isomers can also exist in the compounds of the present invention. The symbol “” denotes a bond that may be a single, double or triple bond as described herein. The present invention encompasses the various geometric isomers and mixtures thereof resulting from the arrangement of substituents around a carbon-carbon double bond or arrangement of substituents around a carbocyclic ring. Substituents around a carbon-carbon double bond are designated as being in the “*Z*” or “*E*” configuration wherein the terms “*Z*” and “*E*” are used in accordance with IUPAC standards. Unless otherwise specified, structures depicting double bonds encompass both the “*E*” and “*Z*” isomers.

[0233] Substituents around a carbon-carbon double bond alternatively can be referred to as “*cis*” or “*trans*,” where “*cis*” represents substituents on the same side of the double bond and “*trans*” represents substituents on opposite sides of the double bond. The arrangement of substituents around a carbocyclic ring are designated as “*cis*” or “*trans*.” The term “*cis*” represents substituents on the same side of the plane of the ring and the term “*trans*”

represents substituents on opposite sides of the plane of the ring. Mixtures of compounds wherein the substituents are disposed on both the same and opposite sides of plane of the ring are designated “cis/trans.”

[0234] The invention also embraces isotopically labeled compounds of the invention which are identical to those recited herein, except that one or more atoms are replaced by an atom having an atomic mass or mass number different from the atomic mass or mass number usually found in nature. Examples of isotopes that can be incorporated into compounds of the invention include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorus, fluorine and chlorine, such as ^2H , ^3H , ^{13}C , ^{14}C , ^{15}N , ^{18}O , ^{17}O , ^{31}P , ^{32}P , ^{35}S , ^{18}F , and ^{36}Cl , respectively.

[0235] Certain isotopically-labeled disclosed compounds (e.g., those labeled with ^3H and ^{14}C) are useful in compound and/or substrate tissue distribution assays. Tritiated (i.e., ^3H) and carbon-14 (i.e., ^{14}C) isotopes are particularly preferred for their ease of preparation and detectability. Further, substitution with heavier isotopes such as deuterium (i.e., ^2H) may afford certain therapeutic advantages resulting from greater metabolic stability (e.g., increased *in vivo* half-life or reduced dosage requirements) and hence may be preferred in some circumstances. Isotopically labeled compounds of the invention can generally be prepared by following procedures analogous to those disclosed in, e.g., the Examples herein by substituting an isotopically labeled reagent for a non-isotopically labeled reagent.

[0236] As used herein, the terms “subject” and “patient” refer to organisms to be treated by the methods of the present invention. Such organisms are preferably mammals (e.g., murines, simians, equines, bovines, porcines, canines, felines, and the like), and more preferably humans.

[0237] As used herein, the term “pharmaceutical composition” refers to the combination of an active agent with a carrier, inert or active, making the composition especially suitable for diagnostic or therapeutic use *in vivo* or *ex vivo*.

[0238] As used herein, the term “pharmaceutically acceptable excipient” refers to any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions (e.g., such as an oil/water or water/oil emulsions), and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Remington's The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro; Lippincott, Williams & Wilkins, Baltimore, MD, 2006.

[0239] As is known to those of skill in the art, “salts” of the compounds of the present invention may be derived from inorganic or organic acids and bases. Examples of acids include, but are not limited to, hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycolic, lactic, salicylic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, naphthalene-2-sulfonic, benzenesulfonic acid, and the like. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts.

[0240] Examples of bases include, but are not limited to, alkali metal (*e.g.*, sodium) hydroxides, alkaline earth metal (*e.g.*, magnesium) hydroxides, ammonia, and compounds of formula NW_4^+ , wherein W is C₁₋₄ alkyl, and the like.

[0241] Examples of salts include, but are not limited to: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, flucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, palmoate, pectinate, persulfate, phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, undecanoate, and the like. Other examples of salts include anions of the compounds of the present invention compounded with a suitable cation such as Na⁺, NH₄⁺, and NW_4^+ (wherein W is a C₁₋₄ alkyl group), and the like.

[0242] Abbreviations as used herein include diisopropylethylamine (DIPEA); 4-dimethylaminopyridine (DMAP); tetrabutylammonium iodide (TBAI); 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC); benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 9-Fluorenylmethoxycarbonyl (Fmoc), tetrabutyltrimethylsilyl chloride (TBDMS-Cl), hydrogen fluoride (HF), phenyl (Ph), bis(trimethylsilyl)amine (HMDS), dimethylformamide (DMF); methylene chloride (DCM); tetrahydrofuran (THF); high-performance liquid chromatography (HPLC); mass spectrometry (MS), evaporative light scattering detector (ELSD), electrospray (ES)); nuclear magnetic resonance spectroscopy (NMR).

[0243] As used herein, the term "effective amount" refers to the amount of a compound (*e.g.*, a nucleic acid, *e.g.*, an mRNA) sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages and is not intended to be limited to a particular formulation or administration route. The term effective amount can be considered to include therapeutically and/or prophylactically effective amounts of a compound.

[0244] The phrase "therapeutically effective amount" as used herein means that amount of a compound (*e.g.*, a nucleic acid, *e.g.*, an mRNA), material, or composition comprising a compound (*e.g.*, a nucleic acid, *e.g.*, an mRNA) which is effective for producing some desired therapeutic effect in at least a sub-population of cells in a mammal, for example, a human, or a subject (*e.g.*, a human subject) at a reasonable benefit/risk ratio applicable to any medical treatment.

[0245] The phrase "prophylactically effective amount" as used herein means that amount of a compound (*e.g.*, a nucleic acid, *e.g.*, an mRNA), material, or composition comprising a compound (*e.g.*, a nucleic acid, *e.g.*, an mRNA) which is effective for producing some desired prophylactic effect in at least a sub-population of cells in a mammal, for example, a human, or a subject (*e.g.*, a human subject) by reducing, minimizing or eliminating the risk of developing a condition or the reducing or minimizing severity of a condition at a reasonable benefit/risk ratio applicable to any medical treatment.

[0246] As used herein, the terms "treat," "treating," and "treatment" include any effect, *e.g.*, lessening, reducing, modulating, ameliorating or eliminating, that results in the improvement of the condition, disease, disorder, and the like, or ameliorating a symptom thereof.

[0247] The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0248] In the application, where an element or component is said to be included in and/or selected from a list of recited elements or components, it should be understood that the element or component can be any one of the recited elements or components, or the element

or component can be selected from a group consisting of two or more of the recited elements or components.

[0249] Further, it should be understood that elements and/or features of a composition or a method described herein can be combined in a variety of ways without departing from the spirit and scope of the present invention, whether explicit or implicit herein. For example, where reference is made to a particular compound, that compound can be used in various embodiments of compositions of the present invention and/or in methods of the present invention, unless otherwise understood from the context. In other words, within this application, embodiments have been described and depicted in a way that enables a clear and concise application to be written and drawn, but it is intended and will be appreciated that embodiments may be variously combined or separated without parting from the present teachings and invention(s). For example, it will be appreciated that all features described and depicted herein can be applicable to all aspects of the invention(s) described and depicted herein.

[0250] It should be understood that the expression “at least one of” includes individually each of the recited objects after the expression and the various combinations of two or more of the recited objects unless otherwise understood from the context and use. The expression “and/or” in connection with three or more recited objects should be understood to have the same meaning unless otherwise understood from the context.

[0251] The use of the term “include,” “includes,” “including,” “have,” “has,” “having,” “contain,” “contains,” or “containing,” including grammatical equivalents thereof, should be understood generally as open-ended and non-limiting, for example, not excluding additional unrecited elements or steps, unless otherwise specifically stated or understood from the context.

[0252] Where the use of the term “about” is before a quantitative value, the present invention also include the specific quantitative value itself, unless specifically stated otherwise. As used herein, the term “about” refers to a $\pm 10\%$ variation from the nominal value unless otherwise indicated or inferred.

[0253] As used herein, unless otherwise indicated, the term “antibody” means any antigen-binding molecule or molecular complex comprising at least one complementarity determining region (CDR) that specifically binds to or interacts with a particular antigen. It is

understood the term encompasses an intact antibody (*e.g.*, an intact monoclonal antibody), or a fragment thereof, such as an Fc fragment of an antibody (*e.g.*, an Fc fragment of a monoclonal antibody), or an antigen-binding fragment of an antibody (*e.g.*, an antigen-binding fragment of a monoclonal antibody), including an intact antibody, antigen-binding fragment, or Fc fragment that has been modified or engineered. Examples of antigen-binding fragments include Fab, Fab', (Fab')₂, Fv, single chain antibodies (*e.g.*, scFv), minibodies, and diabodies. Examples of antibodies that have been modified or engineered include chimeric antibodies, humanized antibodies, and multispecific antibodies (*e.g.*, bispecific antibodies). The term also encompasses an immunoglobulin single variable domain, such as a Nanobody (*e.g.*, a V_{HH}).

[0254] As used here, an “antibody that binds to X” (*i.e.*, X being a particular antigen), or “an anti-X antibody”, is an antibody that specifically recognizes the antigen X.

[0255] As used herein, a “buried interchain disulfide bond” or an “interchain buried disulfide bond” refers to a disulfide bond on a polypeptide which is not readily accessible to water soluble reducing agents, or effectively “buried” in the hydrophobic regions of the polypeptide, such that it is unavailable to both reducing agents and for conjugation to other hydrophilic PEGs. Buried interchain disulfide bonds are further described in WO2017096361A1, which is incorporated by reference in its entirety.

[0256] As used herein, specificity of the targeted delivery by an LNP is defined by the ratio between % of a desired immune cell type that receives the delivered nucleic acid (*e.g.*, on-target delivery), and % of an undesired immune cell type that is not meant to be the destination of the delivery, but receives the delivered nucleic acid (*e.g.*, off-target delivery). For example, the specificity is higher when more desired immune cells receive the delivered nucleic acid, while less undesired immune cells receive the delivered nucleic acid. Specificity of the targeted delivery by an LNP can also be defined the ratio of amount of nucleic acid being delivered to the desired immune cells (*e.g.*, on-target delivery) and amount of nucleic acid being delivered to the undesired immune cells (*e.g.*, off-target delivery). Specificity of the delivery can be determined using any suitable method. As a non-limiting example, expression level of the nucleic acid in the desired immune cell type can be measured and compared to that of a different immune cell type that is not meant to be the destination of the delivery.

[0257] As used herein, in some embodiments, a reference LNP is an LNP that does not have the immune cell targeting group but is otherwise the same as the tested LNP. In some other embodiments, a reference LNP is an LNP that has a different ionizable cationic lipid but is otherwise the same as the tested LNP. In some embodiments, a reference LNP comprises D-Lin-MC3-DMA as the ionizable cationic lipid which is different from the ionizable cationic lipid in a tested LNP, but is otherwise the same as the tested LNP.

[0258] As used herein, a humanized antibody is an antibody which is wholly or partially of non-human origin and whose protein sequence has been modified to replace certain amino acids, for instance that occur at the corresponding position(s) in the framework regions of the VH and VL domains in a sequence of antibody from a human being, to increase its similarity to antibodies produced naturally in humans, in order to avoid or minimize an immune response in humans. For example, using techniques of genetic engineering, the variable domains of a non-human antibodies of interest may be combined with the constant domains of human antibodies. The constant domains of a humanized antibody are most of the time human CH and CL domains.

[0259] As used herein, the term “structural lipid” refers to sterols and also to lipids containing sterol moieties.

[0260] It should be understood that the order of steps or order for performing certain actions is immaterial so long as the present invention remain operable. Moreover, two or more steps or actions may be conducted simultaneously.

[0261] At various places in the present specification, substituents are disclosed in groups or in ranges. It is specifically intended that the description include each and every individual subcombination of the members of such groups and ranges. For example, the term “C₁₋₆ alkyl” is specifically intended to individually disclose C₁, C₂, C₃, C₄, C₅, C₆, C₁-C₆, C₁-C₅, C₁-C₄, C₁-C₃, C₁-C₂, C₂-C₆, C₂-C₅, C₂-C₄, C₂-C₃, C₃-C₆, C₃-C₅, C₃-C₄, C₄-C₆, C₄-C₅, and C₅-C₆ alkyl. By way of other examples, an integer in the range of 0 to 40 is specifically intended to individually disclose 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, and 40, and an integer in the range of 1 to 20 is specifically intended to individually disclose 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20.

[0262] The use of any and all examples, or exemplary language herein, for example, “such as” or “including,” is intended merely to illustrate better the present invention and does not pose a limitation on the scope of the invention unless claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the present invention.

[0263] Throughout the description, where compositions and kits are described as having, including, or comprising specific components, or where processes and methods are described as having, including, or comprising specific steps, it is contemplated that, additionally, there are compositions and kits of the present invention that consist essentially of, or consist of, the recited components, and that there are processes and methods according to the present invention that consist essentially of, or consist of, the recited processing steps.

[0264] As a general matter, compositions specifying a percentage are by weight unless otherwise specified. Further, if a variable is not accompanied by a definition, then the previous definition of the variable controls.

Immunoglobulin single variable domain

[0265] In some embodiments, the immune cell targeting group of the LNPs as described herein comprise an immunoglobulin single variable domain, such as an Nanobody.

[0266] The term “immunoglobulin single variable domain” (ISV), interchangeably used with “single variable domain,” defines immunoglobulin molecules wherein the antigen binding site is present on, and formed by, a single immunoglobulin domain. This sets immunoglobulin single variable domains apart from “conventional” immunoglobulins (e.g., monoclonal antibodies) or their fragments (such as Fab, Fab', F(ab')₂, scFv, di-scFv), wherein two immunoglobulin domains, in particular two variable domains, interact to form an antigen binding site. Typically, in conventional immunoglobulins, a heavy chain variable domain (V_H) and a light chain variable domain (V_L) interact to form an antigen binding site. In this case, the complementarity determining regions (CDRs) of both V_H and V_L will contribute to the antigen binding site, i.e. a total of 6 CDRs will be involved in antigen binding site formation. In view of the above definition, the antigen-binding domain of a conventional 4-chain antibody (such as an IgG, IgM, IgA, IgD or IgE molecule; known in the art) or of a Fab, a F(ab')₂ fragment, an Fv fragment such as a disulfide linked Fv or a scFv fragment, or a diabody (all known in the art) derived from such conventional 4-chain

antibody, would normally not be regarded as an immunoglobulin single variable domain, as, in these cases, binding to the respective epitope of an antigen would normally not occur by one (single) immunoglobulin domain but by a pair of (associating) immunoglobulin domains such as light and heavy chain variable domains, i.e., by a V_H - V_L pair of immunoglobulin domains, which jointly bind to an epitope of the respective antigen.

[0267] In contrast, immunoglobulin single variable domains are capable of specifically binding to an epitope of the antigen without pairing with an additional immunoglobulin variable domain. The binding site of an immunoglobulin single variable domain is formed by a single V_H , a single V_{HH} or single V_L domain. Hence, the antigen binding site of an immunoglobulin single variable domain is formed by no more than three CDRs.

[0268] As such, the single variable domain may be a light chain variable domain sequence (e.g., a V_L -sequence) or a suitable fragment thereof; or a heavy chain variable domain sequence (e.g., a V_H -sequence or V_{HH} sequence) or a suitable fragment thereof; as long as it is capable of forming a single antigen binding unit (i.e., a functional antigen binding unit that essentially consists of the single variable domain, such that the single antigen binding domain does not need to interact with another variable domain to form a functional antigen binding unit).

[0269] An immunoglobulin single variable domain (ISV) can for example be a heavy chain ISV, such as a V_H , V_{HH} , including a camelized V_H or humanized V_{HH} . In one embodiment, it is a V_{HH} , including a camelized V_H or humanized V_{HH} . Heavy chain ISVs can be derived from a conventional four-chain antibody or from a heavy chain antibody.

[0270] For example, the immunoglobulin single variable domain may be a (single) domain antibody (or an amino acid sequence that is suitable for use as a single domain antibody), a "dAb" or dAb (or an amino acid sequence that is suitable for use as a dAb) or a Nanobody® ISV (as defined herein and including but not limited to a V_{HH}); other single variable domains, or any suitable fragment of any one thereof.

[0271] In particular, the immunoglobulin single variable domain may be a Nanobody® ISV (such as a V_{HH} , including a humanized V_{HH} or camelized V_H) or a suitable fragment thereof. [Note: Nanobody® is a registered trademark of Ablynx N.V.].

[0272] “V_{HH} domains”, also known as V_{HHS}, V_{HH} antibody fragments, and V_{HH} antibodies, have originally been described as the antigen binding immunoglobulin variable domain of “heavy chain antibodies” (i.e., of “antibodies devoid of light chains”; Hamers-Casterman et al. 1993 (Nature 363: 446-448). The term “V_{HH} domain” has been chosen in order to distinguish these variable domains from the heavy chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as “V_H domains”) and from the light chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as “V_L domains”). For a further description of V_{HH}'s, reference is made to the review article by Muyldermans 2001 (Reviews in Molecular Biotechnology 74: 277-302).

[0273] For the term “dAb's” and “domain antibody”, reference is for example made to Ward et al. 1989 (Nature 341: 544), to Holt et al. 2003 (Trends Biotechnol. 21: 484); as well as to for example WO 2004/068820, WO 2006/030220, WO 2006/003388 and other published patent applications of Domantis Ltd. It should also be noted that, although less preferred in the context of the present invention because they are not of mammalian origin, single variable domains can be derived from certain species of shark (for example, the so-called “IgNAR domains”, see for example WO 2005/18629).

[0274] Typically, the generation of immunoglobulins involves the immunization of experimental animals, fusion of immunoglobulin producing cells to create hybridomas and screening for the desired specificities. Alternatively, immunoglobulins can be generated by screening of naïve, immune or synthetic libraries e.g. by phage display.

[0275] The generation of immunoglobulin sequences, such as VHHs, has been described extensively in various publications, among which WO 1994/04678, Hamers-Casterman *et al.* 1993 (Nature 363: 446-448) and Muyldermans et al. 2001 (Reviews in Molecular Biotechnology 74: 277-302, 2001). In these methods, camelids are immunized with the target antigen in order to induce an immune response against said target antigen. The repertoire of VHHs obtained from said immunization is further screened for VHHs that bind the target antigen.

[0276] In these instances, the generation of antibodies requires purified antigen for immunization and/or screening. Antigens can be purified from natural sources, or in the

course of recombinant production. Immunization and/or screening for immunoglobulin sequences can be performed using peptide fragments of such antigens.

[0277] Immunoglobulin sequences of different origin, comprising mouse, rat, rabbit, donkey, human and camelid immunoglobulin sequences can be used herein. Also, fully human, humanized or chimeric sequences can be used in the method described herein. For example, camelid immunoglobulin sequences and humanized camelid immunoglobulin sequences, or camelized domain antibodies, e.g. camelized dAb as described by Ward et al. 1989 (Nature 341: 544), WO 1994/04678, and Davis and Riechmann (1994, Febs Lett., 339:285-290; and 1996, Prot. Eng., 9:531-537) can be used herein. Moreover, the ISVs are fused forming a multivalent and/or multispecific construct (for multivalent and multispecific polypeptides containing one or more V_{HH} domains and their preparation, reference is also made to Conrath et al. 2001 (J. Biol. Chem., Vol. 276, 10, 7346-7350) as well as to for example WO 1996/34103 and WO 1999/23221).

[0278] A “humanized V_{HH} ” comprises an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring V_{HH} domain, but that has been “humanized”, i.e. by replacing one or more amino acid residues in the amino acid sequence of said naturally occurring V_{HH} sequence (and in particular in the framework sequences) by one or more of the amino acid residues that occur at the corresponding position(s) in a V_H domain from a conventional 4-chain antibody from a human being (e.g. indicated above). This can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the prior art (e.g. WO 2008/020079). Again, it should be noted that such humanized V_{HH} s can be obtained in any suitable manner known per se and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring V_{HH} domain as a starting material.

[0279] A “camelized V_H ” comprises an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring V_H domain, but that has been “camelized”, i.e. by replacing one or more amino acid residues in the amino acid sequence of a naturally occurring V_H domain from a conventional 4-chain antibody by one or more of the amino acid residues that occur at the corresponding position(s) in a V_{HH} domain of a (camelid) heavy chain antibody. This can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the description in the prior art (e.g. Davies and Riechman 1994, FEBS 339: 285; 1995, Biotechnol. 13: 475; 1996, Prot. Eng. 9: 531; and

Riechman 1999, J. Immunol. Methods 231: 25). Such “camelizing” substitutions are inserted at amino acid positions that form and/or are present at the V_H - V_L interface, and/or at the so-called Camelidae hallmark residues, as defined herein (see for example WO 1994/04678 and Davies and Riechmann (1994 and 1996, *supra*). In one embodiment, the V_H sequence that is used as a starting material or starting point for generating or designing the camelized V_H is a V_H sequence from a mammal, such as the V_H sequence of a human being, such as a V_{H3} sequence. However, it should be noted that such camelized V_H can be obtained in any suitable manner known per se and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring V_H domain as a starting material.

[0280] The structure of an immunoglobulin single variable domain sequence can be considered to be comprised of four framework regions (“FRs”), which are referred to in the art and herein as “Framework region 1” (“FR1”); as “Framework region 2” (“FR2”); as “Framework region 3” (“FR3”); and as “Framework region 4” (“FR4”), respectively; which framework regions are interrupted by three complementary determining regions (“CDRs”), which are referred to in the art and herein as “Complementarity Determining Region 1” (“CDR1”); as “Complementarity Determining Region 2” (“CDR2”); and as “Complementarity Determining Region 3” (“CDR3”), respectively.

[0281] In such an immunoglobulin sequence, the framework sequences may be any suitable framework sequences, and examples of suitable framework sequences will be clear to the skilled person, for example on the basis the standard handbooks and the further disclosure and prior art mentioned herein.

[0282] The framework sequences are (a suitable combination of) immunoglobulin framework sequences or framework sequences that have been derived from immunoglobulin framework sequences (for example, by humanization or camelization). For example, the framework sequences may be framework sequences derived from a light chain variable domain (e.g. a V_L -sequence) and/or from a heavy chain variable domain (e.g. a V_H -sequence or V_{HH} sequence). In one particular aspect, the framework sequences are either framework sequences that have been derived from a V_{HH} -sequence (in which said framework sequences may optionally have been partially or fully humanized) or are conventional V_H sequences that have been camelized (as defined herein).

[0283] In particular, the framework sequences present in the ISV sequence described herein may contain one or more of hallmark residues (as defined herein), such that the ISV sequence is a Nanobody® ISV, such as e.g. a V_{HH}, including a humanized V_{HH} or camelized V_H. Non-limiting examples of (suitable combinations of) such framework sequences will become clear from the further disclosure herein.

[0284] The total number of amino acid residues in a V_H domain and a V_{HH} domain will usually be in the range of from 110 to 120, often between 112 and 115. It should however be noted that smaller and longer sequences may also be suitable for the purposes described herein.

[0285] However, it should be noted that the ISVs described herein is not limited as to the origin of the ISV sequence (or of the nucleotide sequence used to express it), nor as to the way that the ISV sequence or nucleotide sequence is (or has been) generated or obtained. Thus, the ISV sequences may be naturally occurring sequences (from any suitable species) or synthetic or semi-synthetic sequences. In a specific but non-limiting aspect, the ISV sequence is a naturally occurring sequence (from any suitable species) or a synthetic or semi-synthetic sequence, including but not limited to “humanized” (as defined herein) immunoglobulin sequences (such as partially or fully humanized mouse or rabbit immunoglobulin sequences, and in particular partially or fully humanized V_{HH} sequences), “camelized” (as defined herein) immunoglobulin sequences (and in particular camelized V_H sequences), as well as ISVs that have been obtained by techniques such as affinity maturation (for example, starting from synthetic, random or naturally occurring immunoglobulin sequences), CDR grafting, veneering, combining fragments derived from different immunoglobulin sequences, PCR assembly using overlapping primers, and similar techniques for engineering immunoglobulin sequences well known to the skilled person; or any suitable combination of any of the foregoing.

[0286] Similarly, nucleotide sequences may be naturally occurring nucleotide sequences or synthetic or semi-synthetic sequences, and may for example be sequences that are isolated by PCR from a suitable naturally occurring template (e.g. DNA or RNA isolated from a cell), nucleotide sequences that have been isolated from a library (and in particular, an expression library), nucleotide sequences that have been prepared by introducing mutations into a naturally occurring nucleotide sequence (using any suitable technique known per se, such as mismatch PCR), nucleotide sequence that have been prepared by PCR using overlapping

primers, or nucleotide sequences that have been prepared using techniques for DNA synthesis known per se.

[0287] Generally, Nanobody® ISVs (in particular V_{HH} sequences, including (partially) humanized V_{HH} sequences and camelized V_H sequences) can be characterized by the presence of one or more “Hallmark residues” (as described herein) in one or more of the framework sequences (again as further described herein). Thus, generally, a Nanobody® ISV can be defined as an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

[0288] in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which one or more of the Hallmark residues are as further defined herein.

[0289] In particular, a Nanobody® ISV can be an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which the framework sequences are as further defined herein.

[0290] More in particular, a Nanobody® ISV can be an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which: one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table 2A below.

Table 2A: Hallmark Residues in Nanobody® ISVs

Position	Human V _H 3	Hallmark Residues
11	L, V; predominantly L	L, S, V, M, W, F, T, Q, E, A, R, G, K, Y, N, P, I; preferably L
37	V, I, F; usually V	F ⁽¹⁾ , Y, V, L, A, H, S, I, W, C, N, G, D, T, P, preferably F ⁽¹⁾ or Y

44 ⁽⁸⁾	G	E ⁽³⁾ , Q ⁽³⁾ , G ⁽²⁾ , D, A, K, R, L, P, S, V, H, T, N, W, M, I; preferably G ⁽²⁾ , E ⁽³⁾ or Q ⁽³⁾ ; most preferably G ⁽²⁾ or Q ⁽³⁾ .
45 ⁽⁸⁾	L	L ⁽²⁾ , R ⁽³⁾ , P, H, F, G, Q, S, E, T, Y, C, I, D, V; preferably L ⁽²⁾ or R ⁽³⁾ .
47 ⁽⁸⁾	W, Y	F ⁽¹⁾ , L ⁽¹⁾ or W ⁽²⁾ G, I, S, A, V, M, R, Y, E, P, T, C, H, K, Q, N, D; preferably W ⁽²⁾ , L ⁽¹⁾ or F ⁽¹⁾ .
83	R or K; usually R	R, K ⁽⁵⁾ , T, E ⁽⁵⁾ , Q, N, S, I, V, G, M, L, A, D, Y, H; preferably K or R; most preferably K.
84	A, T, D; predominantly A	P ⁽⁵⁾ , S, H, L, A, V, I, T, F, D, R, Y, N, Q, G, E; preferably P.
103	W	W ⁽⁴⁾ , R ⁽⁶⁾ , G, S, K, A, M, Y, L, F, T, N, V, Q, P ⁽⁶⁾ , E, C; preferably W.
104	G	G, A, S, T, D, P, N, E, C, L; preferably G.
108	L, M or T; predominantly L	Q, L ⁽⁷⁾ , R, P, E, K, S, T, M, A, H; preferably Q or L ⁽⁷⁾ .
<p><u>Notes:</u></p> <p>In particular, but not exclusively, in combination with KERE (SEQ ID NO: 103) or KQRE (SEQ ID NO: 104) at positions 43-46.</p> <p>Usually as GLEW (SEQ ID NO: 105) at positions 44-47.</p> <p>Usually as KERE (SEQ ID NO: 103) or KQRE (SEQ ID NO: 104) at positions 43-46, e.g. as KEREL (SEQ ID NO: 106), KERE (SEQ ID NO: 107), KQREL (SEQ ID NO: 108), KQREF (SEQ ID NO: 109), KERE (SEQ ID NO: 110), KQREW (SEQ ID NO: 111) or KQREG (SEQ ID NO: 112) at positions 43-47. Alternatively, also sequences such as TERE (SEQ ID NO: 113) (for example TEREL (SEQ ID NO: 114)), TQRE (SEQ ID NO: 115) (for example TQREL (SEQ ID NO: 116)), KECE (SEQ ID NO: 117) (for example KECER (SEQ ID NO: 118) or KECER (SEQ ID NO: 119)), KQCE (SEQ ID NO: 120) (for example KQCEL (SEQ ID NO: 121)), RERE (SEQ ID NO: 122) (for example REREG (SEQ ID NO: 123)), RQRE (SEQ ID NO: 124) (for example RQREL (SEQ ID NO: 125), RQREF (SEQ ID NO: 126) or RQREW (SEQ ID NO: 127)), QERE (SEQ ID NO: 128) (for example QEREG (SEQ ID NO: 129)), QQRE (SEQ ID NO: 130), (for example QQREW (SEQ ID NO: 131), QQREL (SEQ ID NO: 132) or QQREF (SEQ ID NO: 133)), KGRE (SEQ ID NO: 134) (for example KGREG (SEQ ID NO: 135)), KDRE (SEQ ID NO: 136) (for example KDREV (SEQ ID NO: 137)) are possible. Some other possible, but less preferred sequences include for example DECKL (SEQ ID NO: 138) and NVCEL (SEQ ID NO: 139).</p> <p>With both GLEW (SEQ ID NO: 105) at positions 44-47 and KERE (SEQ ID NO: 103) or KQRE (SEQ ID NO: 104) at positions 43-46.</p> <p>Often as KP or EP at positions 83-84 of naturally occurring V_{HH} domains.</p> <p>In particular, but not exclusively, in combination with GLEW (SEQ ID NO: 105) at positions 44-47.</p> <p>With the proviso that when positions 44-47 are GLEW (SEQ ID NO: 105), position 108 is always Q in (non-humanized) V_{HH} sequences that also contain a W at 103.</p> <p>The GLEW group also contains GLEW-like sequences at positions 44-47, such as for example GVEW (SEQ ID NO: 140), EPEW (SEQ ID NO: 141), GLER (SEQ ID NO: 142), DQEW (SEQ ID NO: 143), DLEW (SEQ ID NO: 144), GIEW (SEQ ID NO: 145), ELEW (SEQ ID NO: 146),</p>		

GPEW (SEQ ID NO: 147), EWLP (SEQ ID NO: 148), GPER (SEQ ID NO: 149), GLER (SEQ ID NO: 142) and ELEW (SEQ ID NO: 146).

[0291] In one embodiment, the immunoglobulin single variable domain has certain amino acid substitutions in the framework regions effective in preventing or reducing binding of so-called “pre-existing antibodies” to the polypeptides. ISVs in which (i) the amino acid residue at position 112 is one of K or Q; and/or (ii) the amino acid residue at position 89 is T; and/or (iii) the amino acid residue at position 89 is L and the amino acid residue at position 110 is one of K or Q; and (iv) in each of cases (i) to (iii), the amino acid at position 11 is preferably V have been described in WO2015/173325.

Polypeptides

[0292] The immunoglobulin single variable domains may form part of a protein or polypeptide, which may comprise or essentially consist of one or more (at least one) immunoglobulin single variable domains and which may optionally further comprise one or more further amino acid sequences (all optionally linked via one or more suitable linkers). The term “immunoglobulin single variable domain” may also encompass such polypeptides. The one or more immunoglobulin single variable domains may be used as a binding unit in such a protein or polypeptide, which may optionally contain one or more further amino acids that can serve as a binding unit, so as to provide a monovalent, multivalent or multispecific polypeptide of the invention, respectively (for multivalent and multispecific polypeptides containing one or more VHH domains and their preparation, reference is also made to Conrath et al. 2001 (J. Biol. Chem. 276: 7346), as well as to for example WO 1996/34103, WO 1999/23221 and WO 2010/115998).

[0293] The polypeptides may comprise or essentially consist of one immunoglobulin single variable domain, as outlined above. Such polypeptides are also referred to herein as monovalent polypeptides.

[0294] The term “multivalent” indicates the presence of multiple ISVs in a polypeptide. In one embodiment, the polypeptide is “bivalent”, i.e., comprises or consists of two ISVs. In one embodiment, the polypeptide is “trivalent”, i.e., comprises or consists of three ISVs. In another embodiment, the polypeptide is “tetravalent”, i.e. comprises or consists of four ISVs. The polypeptide can thus be “bivalent”, “trivalent”, “tetravalent”, “pentavalent”, “hexavalent”, “heptavalent”, “octavalent”, “nonavalent”, etc., i.e., the polypeptide comprises

or consists of two, three, four, five, six, seven, eight, nine, etc., ISVs, respectively. In one embodiment the multivalent ISV polypeptide is trivalent. In another embodiment the multivalent ISV polypeptide is tetravalent. In still another embodiment, the multivalent ISV polypeptide is pentavalent.

[0295] In one embodiment, the multivalent ISV polypeptide can also be multispecific. The term “multispecific” refers to binding to multiple different target molecules (also referred to as antigens). The multivalent ISV polypeptide can thus be “bispecific”, “trispecific”, “tetraspecific”, etc., i.e., can bind to two, three, four, etc., different target molecules, respectively.

[0296] For example, the polypeptide may be bispecific-trivalent, such as a polypeptide comprising or consisting of three ISVs, wherein two ISVs bind to a first target and one ISV binds to a second target different from the first target. In another example, the polypeptide may be trispecific-tetravalent, such as a polypeptide comprising or consisting of four ISVs, wherein one ISV binds to a first target, two ISVs bind to a second target different from the first target and one ISV binds to a third target different from the first and the second target. In still another example, the polypeptide may be trispecific-pentavalent, such as a polypeptide comprising or consisting of five ISVs, wherein two ISVs bind to a first target, two ISVs bind to a second target different from the first target and one ISV binds to a third target different from the first and the second target.

[0297] In one embodiment, the multivalent ISV polypeptide can also be multiparatopic. The term “multiparatopic” refers to binding to multiple different epitopes on the same target molecules (also referred to as antigens). The multivalent ISV polypeptide can thus be “biparatopic”, “triparatopic”, etc., i.e., can bind to two, three, etc., different epitopes on the same target molecules, respectively.

[0298] In another aspect, the polypeptide of the invention that comprises or essentially consists of one or more immunoglobulin single variable domains (or suitable fragments thereof), may further comprise one or more other groups, residues, moieties or binding units. Such further groups, residues, moieties, binding units or amino acid sequences may or may not provide further functionality to the immunoglobulin single variable domain (and/or to the polypeptide in which it is present) and may or may not modify the properties of the immunoglobulin single variable domain.

[0299] For example, such further groups, residues, moieties or binding units may be one or more additional amino acids, such that the compound, construct or polypeptide is a (fusion) protein or (fusion) polypeptide. In a preferred but non-limiting aspect, said one or more other groups, residues, moieties or binding units are immunoglobulins. Even more preferably, said one or more other groups, residues, moieties or binding units are chosen from the group consisting of domain antibodies, amino acids that are suitable for use as a domain antibody, single domain antibodies, amino acids that are suitable for use as a single domain antibody, "dAb"s, amino acids that are suitable for use as a dAb, or Nanobodies.

[0300] Alternatively, such groups, residues, moieties or binding units may for example be chemical groups, residues, moieties, which may or may not by themselves be biologically and/or pharmacologically active. For example, and without limitation, such groups may be linked to the one or more immunoglobulin single variable domain so as to provide a "derivative" of the immunoglobulin single variable domain.

[0301] In another embodiment, said further residues may be effective in preventing or reducing binding of so-called "pre-existing antibodies" to the polypeptides. For this purpose, the polypeptides and constructs may contain a C-terminal extension (X)_n (SEQ ID NO: 150) (in which n is 1 to 10, preferably 1 to 5, such as 1, 2, 3, 4 or 5 (and preferably 1 or 2, such as 1); and each X is an (preferably naturally occurring) amino acid residue that is independently chosen, and preferably independently chosen from the group consisting of alanine (A), glycine (G), valine (V), leucine (L) or isoleucine (I), for which reference is made to WO 2012/175741. Accordingly, the polypeptide may further comprise a C-terminal extension (X)_n (SEQ ID NO: 151), in which n is 1 to 5, such as 1, 2, 3, 4 or 5, and in which X is a naturally occurring amino acid, preferably no cysteine.

[0302] In the polypeptides described above, the one or more immunoglobulin single variable domains and the one or more groups, residues, moieties or binding units may be linked directly to each other and/or via one or more suitable linkers or spacers. For example, when the one or more groups, residues, moieties or binding units are amino acids, the linkers may also be an amino acid, so that the resulting polypeptide is a fusion protein or fusion polypeptide.

[0303] As used herein, the term "linker" denotes a peptide that fuses together two or more ISVs into a single molecule. The use of linkers to connect two or more (poly)peptides is

well known in the art. Further exemplary peptidic linkers are shown in Table 2B. One often used class of peptidic linker are known as the “Gly-Ser” or “GS” linkers. These are linkers that essentially consist of glycine (G) and serine (S) residues, and usually comprise one or more repeats of a peptide motif such as the GGGGS (SEQ ID NO: 154) motif (for example, having the formula (Gly-Gly-Gly-Gly-Ser)_n (SEQ ID NO: 152) in which n may be 1, 2, 3, 4, 5, 6, 7 or more). Some often-used examples of such GS linkers are 9GS linkers (GGGGSGGGGS, SEQ ID NO: 157), 15GS linkers (n=3) and 35GS linkers (n=7). Reference is for example made to Chen et al. 2013 (Adv. Drug Deliv. Rev. 65(10): 1357-1369) and Klein et al. 2014 (Protein Eng. Des. Sel. 27 (10): 325-330).

Table 2B. Linker sequences (“ID” refers to the SEQ ID NO as used herein)

Name	ID	Amino acid sequence
3A linker	153	AAA
5GS linker	154	GGGGS
7GS linker	155	SGGSGGS
8GS linker	156	GGGSGGS
9GS linker	157	GGGSGGGGS
10GS linker	158	GGGSGGGGS
15GS linker	159	GGGSGGGSGGGGS
18GS linker	160	GGGSGGGSGGGSGGGGS
20GS linker	161	GGGSGGGSGGGSGGGSGGGGS
25GS linker	162	GGGSGGGSGGGSGGGSGGGSGGGGS
30GS linker	163	GGGSGGGSGGGSGGGSGGGSGGGSGGGGS
35GS linker	164	GGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGGS
40GS linker	165	GGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGG GGS
G1 hinge	166	EPKSCDKTHTCPPCP
9GS-G1 hinge	167	GGGSGGGSEPKSCDKTHTCPPCP
Llama upper long hinge region	168	EPKTPKPQAAA
G3 hinge	169	ELKTPLGDTTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPP PCPRCPEPKSCDTPPPCPRCP

[0304] In one aspect, the disclosure also relates to such amino acid sequences and/or Nanobodies that can bind to and/or are directed against CD8 and that comprise CDR

sequences that are generally as further defined herein, to suitable fragments thereof, as well as to polypeptides that comprise or essentially consist of one or more of such Nanobodies and/or suitable fragments. In some aspect, the disclosure relates to Nanobodies with SEQ ID NO: 77. In particular, the disclosure in some specific aspects provides:

[0305] I) amino acid sequences that are directed against CD8 and that have at least 80%, preferably at least 85%, such as 90% or 95% or more sequence identity with at least one of the amino acid sequences of SEQ ID NO: 77;

[0306] II) amino acid sequences that cross-block the binding of the amino acid sequence of SEQ ID NO: 77 to CD8 and/or that compete with at least the amino acid sequence of SEQ ID NO: 77 for binding to CD8;

[0307] Such amino acid sequences may be as further described herein (and may for example be Nanobodies); as well as polypeptides of the disclosure that comprise one or more of such amino acid sequences (which may be as further described herein), and particularly bispecific (or multispecific) polypeptides as described herein, and nucleic acid sequences that encode such amino acid sequences and polypeptides. Such amino acid sequences and polypeptides do not include any naturally occurring ligands.

[0308] In some embodiments, the CD8 is derived from a mammalian animal, such as a human being. In one specific, but non-limiting aspect, the disclosure relates to an amino acid sequence directed against CD8, that comprises:

[0309] a) the amino acid sequence of SEQ ID NO: 77;

[0310] b) amino acid sequences that have at least 80% amino acid identity with at least one of the amino acid sequences of SEQ ID NO: 77, or

[0311] c) amino acid sequences that have 3, 2, or 1 amino acid difference with at least one of the amino acid sequences of SEQ ID NO: 77;

[0312] or any suitable combination thereof.

[0313] In some embodiments, disclosed is a Nanobody against CD8, which consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively). In some embodiments, in such a Nanobody:

[0314] (I) CDR1 comprises or essentially consists of an amino acid sequence of GSTFSDYG (SEQ ID NO: 100),

[0315] or amino acid sequences that have at least 80%, at least 90%, at least 95%, at least 99% or more sequence identity with GSTFSDYG (SEQ ID NO: 100), in which (1) any amino acid substitution is a conservative amino acid substitution; and/or (2) said amino acid sequence only contains amino acids substitutions, and no amino acid deletions or insertions, compared to GSTFSDYG (SEQ ID NO: 100);

[0316] and/or from the group consisting of amino acids sequences that have 2 or only 1 amino acid difference(s) with GSTFSDYG (SEQ ID NO: 100), in which

[0317] any amino acid substitution is a conservative amino acid substitution; and/or

[0318] said amino acid sequence only contains amino acid substitutions, and no amino acid deletions or insertions, compared to GSTFSDYG (SEQ ID NO: 100).

[0319] (II) CDR2 comprises or essentially consists of an amino acid sequence of IDWNGEHT (SEQ ID NO: 101),

[0320] or amino acid sequences that have at least 80%, at least 90%, at least 95%, at least 99% or more sequence identity with IDWNGEHT (SEQ ID NO: 101), in which (1) any amino acid substitution is a conservative amino acid substitution; and/or (2) said amino acid sequence only contains amino acids substitutions, and no amino acid deletions or insertions, compared to IDWNGEHT (SEQ ID NO: 101);

[0321] and/or from the group consisting of amino acids sequences that have 2 or only 1 amino acid difference(s) with IDWNGEHT (SEQ ID NO: 101), in which

[0322] any amino acid substitution is a conservative amino acid substitution; and/or

[0323] said amino acid sequence only contains amino acid substitutions, and no amino acid deletions or insertions, compared to IDWNGEHT (SEQ ID NO: 101).

[0324] (III) CDR3 comprises or essentially consists of an amino acid sequence of AADALPYTVRKYNY (SEQ ID NO: 102),

[0325] or amino acid sequences that have at least 80%, at least 90%, at least 95%, at least 99% or more sequence identity with AADALPYTVRKYNY (SEQ ID NO: 102), in which (1) any amino acid substitution is a conservative amino acid substitution; and/or (2) said amino acid sequence only contains amino acids substitutions, and no amino acid deletions or insertions, compared to AADALPYTVRKYNY (SEQ ID NO: 102);

[0326] and/or from the group consisting of amino acids sequences that have 2 or only 1 amino acid difference(s) with AADALPYTVRKYNY (SEQ ID NO: 102), in which

[0327] any amino acid substitution is a conservative amino acid substitution; and/or

[0328] said amino acid sequence only contains amino acid substitutions, and no amino acid deletions or insertions, compared to AADALPYTVRKYNY (SEQ ID NO: 102).

[0329] CD8 Nanobodies as disclosed herein may comprise one, two or all three of the CDRs explicitly listed above. In some embodiments, the CD8 Nanobody comprises:

[0330] CDR1: GSTFSDYG (SEQ ID NO: 100), based on IMGT designation;

[0331] CDR2: IDWNGEHT (SEQ ID NO: 101), based on IMGT designation; and

[0332] CDR3: AADALPYTVRKYNY (SEQ ID NO: 102), based on IMGT designation.

[0333] In the Nanobodies of the disclosure that comprise the combinations of CDR's mentioned above, each CDR can be replaced by a CDR chosen from the group consisting of amino acid sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the mentioned CDR's; in which:

[0334] (1) any amino acid substitution is preferably a conservative amino acid substitution; and/or

[0335] (2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s);

[0336] and/or chosen from the group consisting of amino acid sequences that have 3, 2 or only 1 (as indicated in the preceding paragraph) "amino acid difference(s)" with the mentioned CDR(s) one of the above amino acid sequences, in which:

[0337] (1) any amino acid substitution is preferably a conservative amino acid substitution; and/or

[0338] (2) said amino acid sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the above amino acid sequence(s).

[0339] In one embodiment, the CD8 Nanobody is BDSn:

Anti-CD8 BDSn Nb sequence (CDR1, CDR2, CDR3 underlined based on IMGT designation):

[0340] EVQLVESGGGLVQAGGSLRLSCAASGSTFSDYGVGWFRQAPGKGRFVA
 DIDWNGEHTSYADSVKGRFATSRD**NAKNTAYLQMNSLKPEDTAVYYCAADALPYT**
VRKYNYWGQGTQVTVSSGGCGGHHHHHHH (SEQ ID NO: 77)

[0341] In some embodiments, a CD8 Nanobody of the present disclosure binds to CD8 with an dissociation constant (KD) of 10^{-5} to 10^{-12} moles/liter (M) or less, and preferably 10^{-7} to 10^{-12} moles/liter (M) or less and more preferably 10^{-8} to 10^{-12} moles/liter (M), and/or with an association constant (KA) of at least 10^7 M⁻¹, preferably at least 10^8 M⁻¹, more preferably at least 10^9 M⁻¹, such as at least 10^{12} M⁻¹; and in particular with a KD less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 μ M. The KD and KA values of the Nanobody of the disclosure against vWF can be determined in a manner known per se, for example using the assay described herein. More generally, the Nanobodies described herein preferably have a dissociation constant with respect to vWF that is as described in this paragraph.

[0342] Generally, it should be noted that the term Nanobody as used herein in its broadest sense is not limited to a specific biological source or to a specific method of preparation. For example, as will be discussed in more detail below, the Nanobodies can be obtained (1) by isolating the VHH domain of a naturally occurring heavy chain antibody; (2) by expression of a nucleotide sequence encoding a naturally occurring VHH domain; (3) by "humanization" (as described below) of a naturally occurring VHH domain or by expression of a nucleic acid encoding a such humanized VHH domain; (4) by "camelization" (as described below) of a naturally occurring VH domain from any animal species, in particular a species of mammal, such as from a human being, or by expression of a nucleic acid encoding such a camelized

VH domain; (5) by “camelisation” of a “domain antibody” or “Dab” as described by Ward et al (supra), or by expression of a nucleic acid encoding such a camelized VH domain; (6) using synthetic or semi-synthetic techniques for preparing proteins, polypeptides or other amino acid sequences; (7) by preparing a nucleic acid encoding a Nanobody using techniques for nucleic acid synthesis, followed by expression of the nucleic acid thus obtained; and/or (8) by any combination of the foregoing. Suitable methods and techniques for performing the foregoing will be clear to the skilled person based on the disclosure herein and for example include the methods and techniques described in more detail hereinbelow.

[0343] In some embodiments, the CD8 Nanobodies of the present disclosure do not have an amino acid sequence that is exactly the same as (i.e. as a degree of sequence identity of 100% with) the amino acid sequence of a naturally occurring VH domain, such as the amino acid sequence of a naturally occurring VH domain from a mammal, and in particular from a human being.

[0344] One class of CD8 Nanobodies of the disclosure comprises Nanobodies with an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring VHH domain, but that has been “humanized”, i.e. by replacing one or more amino acid residues in the amino acid sequence of said naturally occurring VHH sequence by one or more of the amino acid residues that occur at the corresponding position(s) in a VH domain from a conventional 4-chain antibody from a human being (e.g. indicated above). It should be noted that such humanized CD8 Nanobodies of the present disclosure can be obtained in any suitable manner known per se (i.e. as indicated under points (1)-(8) above) and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring VHH domain as a starting material.

[0345] Another class of CD8 Nanobodies of the present disclosure comprises Nanobodies with an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring VH domain that has been “camelized”, i.e. by replacing one or more amino acid residues in the amino acid sequence of a naturally occurring VH domain from a conventional 4-chain antibody by one or more of the amino acid residues that occur at the corresponding position(s) in a VHH domain of a heavy chain antibody. This can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the further description below. Reference is also made to WO 94/04678. Such camelization may preferentially occur at amino acid positions which are present at the VH-VL interface and at

the so-called Camelidae hallmark residues (see for example also WO 94/04678), as also mentioned below. In some embodiments, the VH domain or sequence that is used as a starting material or starting point for generating or designing the camelized Nanobody is a VH sequence from a mammal, e.g., VH sequence of a human being. It should be noted that such camelized Nanobodies of the present disclosure can be obtained in any suitable manner known per se and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring VH domain as a starting material.

[0346] For example, both "humanization" and "camelization" can be performed by providing a nucleotide sequence that encodes such a naturally occurring VHH domain or VH domain, respectively, and then changing, in a manner known per se, one or more codons in said nucleotide sequence such that the new nucleotide sequence encodes a humanized or camelized Nanobody of the present disclosure, respectively, and then expressing the nucleotide sequence thus obtained in a manner known per se so as to provide the desired Nanobody. Alternatively, based on the amino acid sequence of a naturally occurring VHH domain or VH domain, respectively, the amino acid sequence of the desired humanized or camelized Nanobody of the present disclosure, respectively, can be designed and then synthesized de novo using techniques for peptide synthesis known per se. Also, based on the amino acid sequence or nucleotide sequence of a naturally occurring VHH domain or VH domain, respectively, a nucleotide sequence encoding the desired humanized or camelized Nanobody can be designed and then synthesized de novo using techniques for nucleic acid synthesis known per se, after which the nucleotide sequence thus obtained can be expressed in a manner known per se so as to provide the desired Nanobody.

[0347] Other suitable ways and techniques for obtaining Nanobodies and/or nucleotide sequences and/or nucleic acids encoding the same, starting from (the amino acid sequence of) naturally occurring VH domains or preferably VHH domains and/or from nucleotide sequences and/or nucleic acid sequences encoding the same will be clear from the skilled person, and may for example comprising combining one or more amino acid sequences and/or nucleotide sequences from naturally occurring VH domains (such as one or more FR's and/or CDR's) with one or more one or more amino acid sequences and/or nucleotide sequences from naturally occurring VHH domains (such an one or more FR's or CDR's), in a suitable manner so as to provide (a nucleotide sequence or nucleic acid encoding) a Nanobody. Also provided are compounds and constructs, and in particular proteins and

polypeptides that comprise or essentially consists of at least one such amino acid sequence and/or Nanobody of the disclosure (or suitable fragments thereof), and optionally further comprises one or more other groups, residues, moieties or binding units. In some embodiments, such further groups, residues, moieties, binding units or amino acid sequences may or may not provide further functionality to the amino acid sequence and/or Nanobody (and/or to the compound or construct in which it is present) and may or may not modify the properties of the amino acid sequence and/or Nanobody.

[0348] The disclosure also encompasses any polypeptide of the present disclosure that has been glycosylated at one or more amino acid positions, usually depending on the host used to express the polypeptide. a polypeptide can comprise an amino acid sequence of a CD8 Nanobody of the present disclosure, which is fused at its amino terminal end, at its carboxy terminal end, or both at its amino terminal end and at its carboxy terminal end with at least one further amino acid sequence. Such further amino acid sequence may comprise at least one further Nanobody, so as to provide a polypeptide that comprises at least two, such as three, four or five, Nanobodies, in which said Nanobodies may optionally be linked via one or more linker sequences (as defined herein). Polypeptides of comprising CD8 Nanobody of the present disclosure and one or more another Nanobodies are multivalent polypeptides. In a multivalent polypeptide, the two or more Nanobodies may be the same or different. For example, the two or more Nanobodies in a multivalent polypeptide:

- may be directed against the same antigen, i.e. against the same parts or epitopes of said antigen or against two or more different parts or epitopes of said antigen; and/or:
- may be directed against the different antigens;
- or a combination thereof.

Thus, a bivalent polypeptide, for example:

- may comprise two identical Nanobodies;
- may comprise a first Nanobody directed against a first part or epitope of an antigen and a second Nanobody directed against the same part or epitope of said antigen or against another part or epitope of said antigen;

or may comprise a first Nanobody directed against a first antigen and a second Nanobody directed against a second antigen different from said first antigen;

whereas a trivalent Polypeptide of the Invention for example:

- may comprises three identical or different Nanobodies directed against the same or different parts or epitopes of the same antigen;
- may comprise two identical or different Nanobodies directed against the same or different parts or epitopes on a first antigen and a third Nanobody directed against a second antigen different from said first antigen; or
- may comprise a first Nanobody directed against a first antigen, a second Nanobody directed against a second antigen different from said first antigen, and a third Nanobody directed against a third antigen different from said first and second antigen,

[0349] The CD8 Nanobodies and polypeptides as disclosed herein can also be introduced and expressed in one or more cells, tissues or organs of a multicellular organism, for example for prophylactic and/or therapeutic purposes (e.g. as a gene therapy). For this purpose, the nucleotide sequences encoding the CD8 Nanobodies or polypeptides as disclosed herein can be introduced into the cells or tissues in any suitable way, for example as such (e.g. using liposomes) or after they have been inserted into a suitable gene therapy vector (for example derived from retroviruses such as adenovirus, or parvoviruses such as adeno-associated virus). As will also be clear to the skilled person, such gene therapy may be performed in vivo and/or in situ in the body of a patient by administering a nucleic acid of the invention or a suitable gene therapy vector encoding the same to the patient or to specific cells or a specific tissue or organ of the patient; or suitable cells (often taken from the body of the patient to be treated, such as explanted lymphocytes, bone marrow aspirates or tissue biopsies) may be treated in vitro with a nucleotide sequence of the invention and then be suitably (re-)introduced into the body of the patient. All this can be performed using gene therapy vectors, techniques and delivery systems which are well known to the skilled person, for Culver, K. W., "Gene Therapy", 1994, p. xii, Mary Ann Liebert, Inc., Publishers, New York, N.Y.). Giordano, Nature F Medicine 2 (1996), 534-539; Schaper, Circ. Res. 79 (1996), 911-919; Anderson, Science 256 (1992), 808-813; Verma, Nature 389 (1994), 239; Isner, Lancet 348 (1996), 370-374; Muhlhauser, Circ. Res. 77 (1995), 1077-1086; Onodera, Blood 91; (1998), 30-36; Verma, Gene Ther. 5 (1998), 692-699; Nabel, Ann. N.Y. Acad. Sci.: 811 (1997), 289-292; Verzeletti, Hum. Gene Ther. 9 (1998), 2243-51; Wang, Nature Medicine 2 (1996), 714-716; WO 94/29469; WO 97/00957, U.S. Pat. No. 5,580,859; 1 U.S. Pat. No. 5,589,5466; or Schaper, Current Opinion in Biotechnology 7 (1996), 635-640. For example, in situ expression of ScFv fragments (Afanasieva et al., Gene Ther., 10, 1850-1859 (2003))

and of diabodies (Blanco et al., J. Immunol, 171, 1070-1077 (2003)) has been described in the art.

[0350] Accordingly, nucleic acid sequences encoding the CD8 Nanobodies as described herein, and expression construct and host cells comprising the nucleic acid sequence are also provided.

[0351] Also disclosed are methods of using CD8 Nanobodies and polypeptides of the present disclosure.

[0352] In some embodiments, a polypeptide comprising a CD8 Nanobody can be used in the lipid nanoparticles of the present disclosure for delivering a nucleic acid into an immune cell, as described herein. In some embodiments, CD8 Nanobodies and polypeptides of the present disclosure can be used to treat a condition or a disease in a subject in need thereof. In some embodiments, such conditions or diseases include, but are not limited to, cancer, infections, immune disorders, autoimmune diseases.

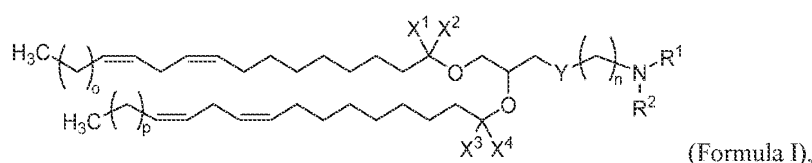
[0353] In some embodiments, a polypeptide comprising a CD8 Nanobody can be used in an imaging agent. In some embodiments, the imaging agent allows for the detection of human CD8 which is a specific biomarker found on the surface of a subset of T-cell for diagnostic imaging of the immune system. Imaging of CD8 allows for the in vivo detection of T-cell localization. Changes in T-cell localization can reflect the progression of an immune response and can occur over time as a result of various therapeutic treatments or even disease states. In some embodiments, it is used for imaging T-cell localization for immunotherapy.

[0354] In addition, CD8 plays a role in activating downstream signaling pathways that are important for the activation of cytolytic T cells that function to clear viral pathogens and provide immunity to tumors. CD8 positive T cells can recognize short peptides presented within the MHC I protein of antigen presenting cells. In some embodiments, a polypeptide comprising a CD8 Nanobody can potentiate signaling through the T cell receptor and enhance the ability of a subject to clear viral pathogens and respond to tumor antigens. Thus, in some embodiments, the antigen binding constructs provided herein can be agonists and can activate the CD8 target.

II. IONIZABLE CATIONIC LIPIDS

[0355] Provided herein are ionizable cationic lipids that can be used to produce lipid nanoparticle compositions to facilitate the delivery of a payload (*e.g.*, a nucleic acid, such as a DNA or RNA, such as an mRNA) disposed therein to cells, *e.g.*, mammalian cells, *e.g.*, immune cells. The ionizable cationic lipids have been designed to enable intracellular delivery of a nucleic acid, *e.g.*, mRNA, to the cytosolic compartment of a target cell type and rapidly degrade into non-toxic components. The complex functionalities of the ionizable cationic lipids are facilitated by the interplay between the chemistry and geometry of the ionizable lipid head group, the hydrophobic “acyl-tail” groups and the linkers connecting the head group and the acyl tail groups. Typically, the pK_a of the ionizable amine head group is designed to be in the range of 6-8, such as between 6.2-7.4, or between 6.5-7.1, such that it remains strongly cationic under acidic formulation conditions (*e.g.*, pH 4 – pH 5.5), neutral in physiological pH (7.4) and cationic in the early and late endosomal compartments (*e.g.*, pH 5.5 – pH 7). The acyl-tail groups play a key role in fusion of the lipid nanoparticle with endosomal membranes and membrane destabilization through structural perturbation. The three-dimensional structure of the acyl-tail (determined by its length, and degree and site of unsaturation) along with the relative sizes of the head group and tail group are thought to play a role in promoting membrane fusion, and hence lipid nanoparticle endosomal escape (a key requirement for cytosolic delivery of a nucleic acid payload). The linker connecting the head group and acyl tail groups is designed to degrade by physiologically prevalent enzymes (*e.g.*, esterases, or proteases) or by acid catalyzed hydrolysis.

[0356] In one aspect, the present invention provides a compound represented by Formula I:



or a salt thereof, wherein:

R^1 and R^2 are independently C_{1-3} alkyl, or R^1 and R^2 are taken together with the nitrogen atom to form an optionally substituted piperidiny1 or morpholiny1;

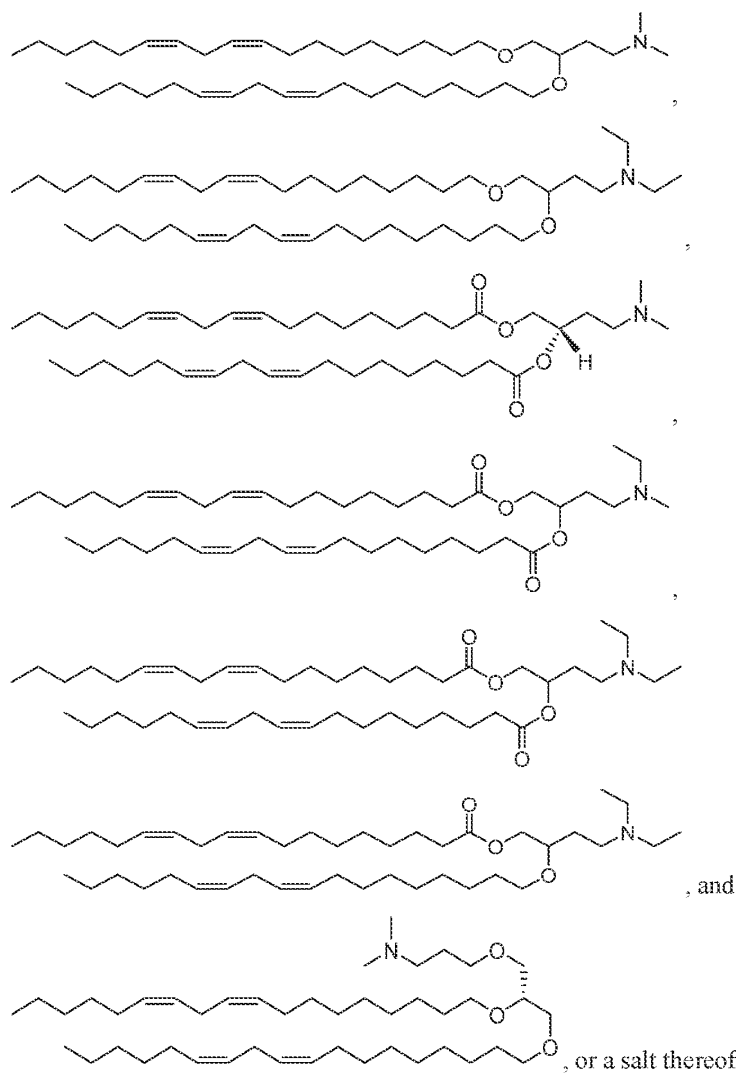
Y is selected from the group consisting of -O-, -OC(O)-, -OC(S)-, and -CH₂-;

X^1 , X^2 , X^3 , and X^4 are hydrogen or X^1 and X^2 or X^3 and X^4 independently are taken together to form an oxo;

n is 0 or 3;

o and p are independently an integer selected from 2-6;

wherein the compound is not a compound selected from the group consisting of



[0357] In certain embodiments, o and p may be 2. In certain embodiments, o and p may be 3. In other embodiments, o and p may be 4. In some embodiments, o and p may be 5. In other embodiments, o and p may be 6.

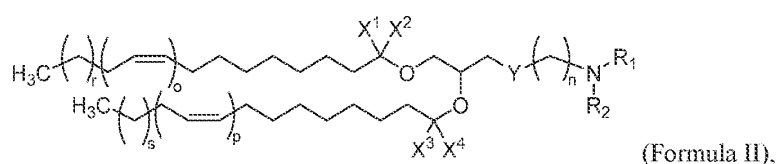
[0358] In certain embodiments, X^1 and X^2 may be taken together to form an oxo and X^3 and X^4 are taken together to form an oxo. In other embodiments, X^1 , X^2 , X^3 , and X^4 may be hydrogen.

[0359] In certain embodiments, Y may be selected from the group consisting of -O-, -OC(O)-, OC(S)- and -CH₂-. For example, in certain embodiments, Y may be -O-. In certain embodiments, Y may be -OC(O)-. In certain embodiments, Y may be -CH₂-. In certain embodiments, Y may be -OC(S)-.

[0360] In certain embodiments, R^1 and R^2 may be independently C₁₋₃alkyl. In other embodiments, R^1 and R^2 may be -CH₃. In certain embodiments, R^1 and R^2 are -CH₂CH₃. In certain embodiments, R^1 and R^2 are C₃ alkyl.

[0361] In certain embodiments, n may be 0. In other embodiments, n may be 3.

[0362] Also provided herein, in part, is a compound represented by Formula II:



or a salt thereof, wherein:

R^1 and R^2 are independently C₁₋₃alkyl, or R^1 and R^2 are taken together with the nitrogen atom to form an optionally substituted piperidinyl or morpholinyl;

Y is selected from the group consisting of -O-, -OC(O)-, -OC(S)-, and -CH₂-;

X^1 , X^2 , X^3 , and X^4 are hydrogen or X^1 and X^2 or X^3 and X^4 are taken together to form an oxo;

n is 0-4;

o is 1 and r is an integer selected from 3-8 or o is 2 and r is an integer selected from 1-8,

p is 1 and s is an integer selected from 3-8 or p is 2 and s is an integer selected from 1-8,

wherein,

when o and p are both 1, r and s are independently 4, 5, 7, or 8, and

when o and p are both 2, r and s are independently 1, 2, 4, or 5.

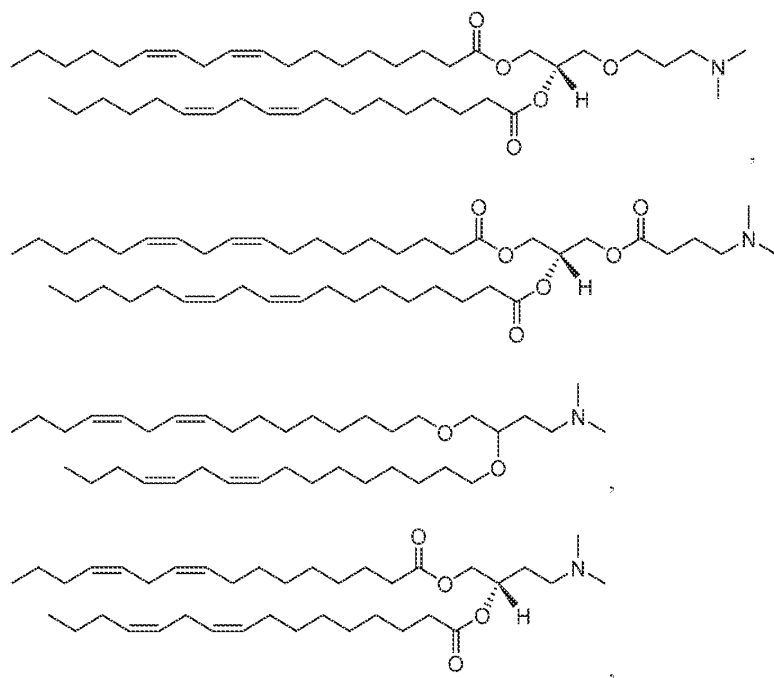
[0363] In certain embodiments, X^1 and X^2 may be taken together to form an oxo and X^3 and X^4 may be taken together to form an oxo. In other embodiments, X^1 , X^2 , X^3 , and X^4 may be hydrogen.

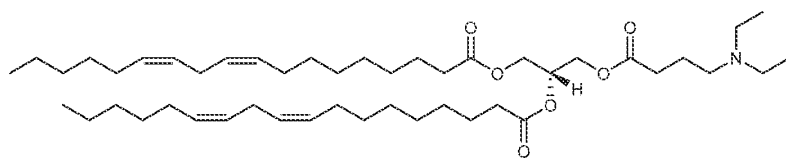
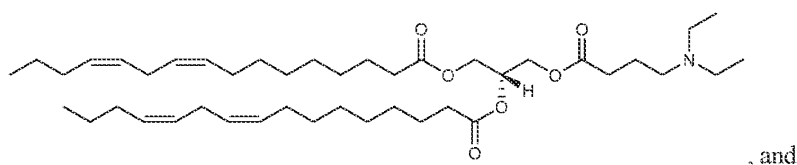
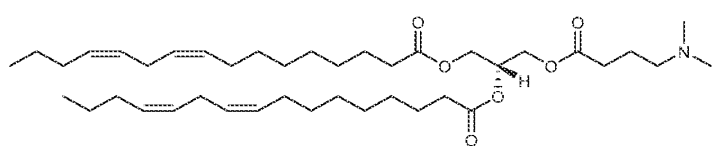
[0364] In certain embodiments, Y may be selected from the group consisting of -O-, -OC(O)-, and -CH₂-. For example, in certain embodiments, Y may be -O-. In certain embodiments, Y may be -OC(O)-. In certain embodiments, Y may be -CH₂-. In certain embodiments, Y may be -OC(S)-.

[0365] In certain embodiments, R^1 and R^2 may be independently C₁₋₃alkyl. In other embodiments, R^1 and R^2 may be -CH₃. In certain embodiments, R^1 and R^2 may be -CH₂CH₃. In some embodiments, R^1 and R^2 may be C₃ alkyl. In certain embodiments, R^1 and R^2 are taken together with the nitrogen atom to form an optionally substituted piperidinyl.

[0366] In certain embodiments, n may be 0. In other embodiments, n may be 3.

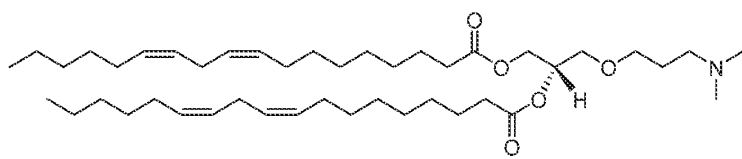
[0367] Provided herein, in part, is a compound selected from the group consisting of:





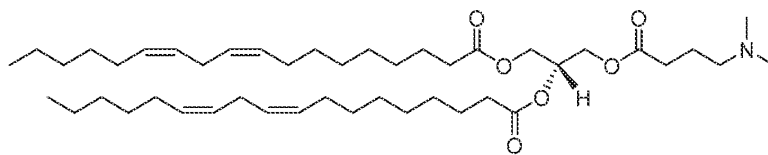
or a salt thereof.

[0368] Provided herein, in part, is a compound of formula:



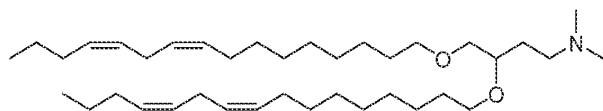
or a salt thereof.

[0369] Provided herein, in part, is a compound of formula:



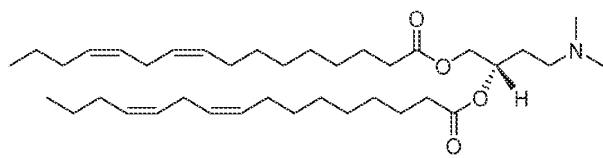
or a salt thereof.

[0370] Provided herein, in part, is a compound of formula:



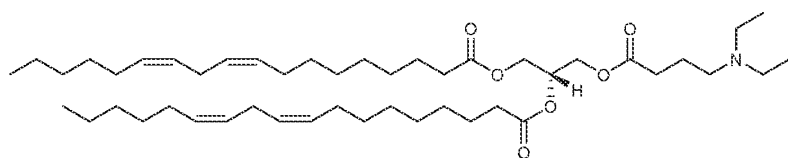
or a salt thereof.

[0371] Provided herein, in part, is a compound of formula:



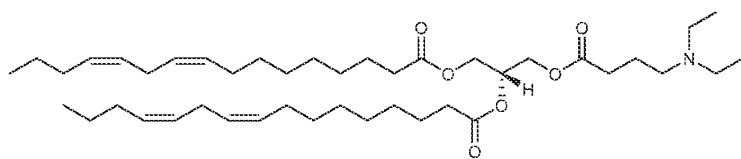
or a salt thereof.

[0372] Provided herein, in part, is a compound of formula:



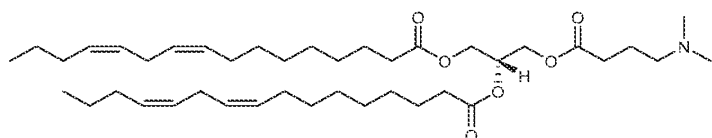
or a salt thereof.

[0373] Provided herein, in part, is a compound of formula:



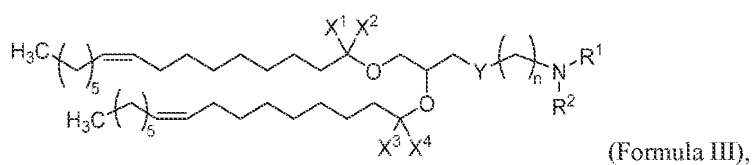
or a salt thereof.

[0374] Provided herein, in part, is a compound of formula:



or a salt thereof.

[0375] In certain embodiments, the compound is a compound of Formula III:



or a salt thereof, wherein:

R^1 and R^2 are independently C_{1-3} alkyl, or R^1 and R^2 are taken together with the nitrogen atom to form an optionally substituted piperidiny1 or morpholiny1;

Y is selected from the group consisting of -O-, -OC(O)-, -OC(S)-, and -CH₂-;

X^1 , X^2 , X^3 , and X^4 are hydrogen or X^1 and X^2 or X^3 and X^4 are taken together to form an oxo; and

n is an integer selected from 0-4.

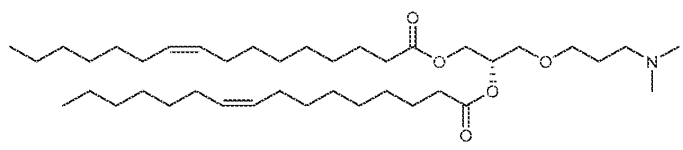
[0376] In certain embodiments, X^1 and X^2 may be taken together to form an oxo and X^3 and X^4 may be taken together to form an oxo. In other embodiments, X^1 , X^2 , X^3 , and X^4 may be hydrogen.

[0377] In certain embodiments, Y may be selected from the group consisting of -O-, -OC(O)-, and -CH₂-. For example, in certain embodiments, Y may be -O-. In certain embodiments, Y may be -OC(O)-. In certain embodiments, Y may be -CH₂-. In certain embodiments, Y may be -OC(S)-.

[0378] In certain embodiments, R^1 and R^2 may be independently C_{1-3} alkyl. In other embodiments, R^1 and R^2 may be -CH₃. In certain embodiments, R^1 and R^2 may be -CH₂CH₃. In some embodiments, R^1 and R^2 may be C_3 alkyl. In certain embodiments, R^1 and R^2 are taken together with the nitrogen atom to form an optionally substituted piperidiny1.

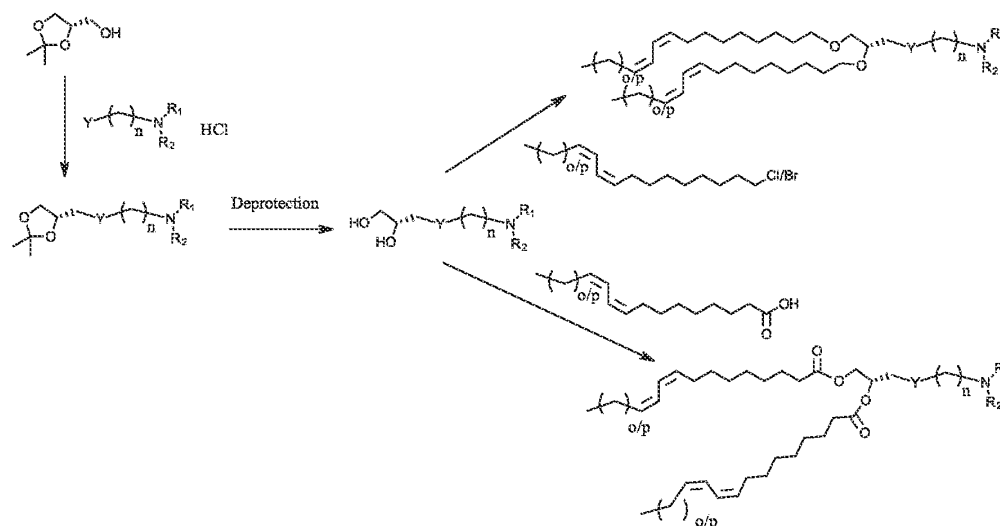
[0379] In certain embodiments, n may be 0. In other embodiments, n may be 3.

[0380] Also provided herein is a compound of the formula:



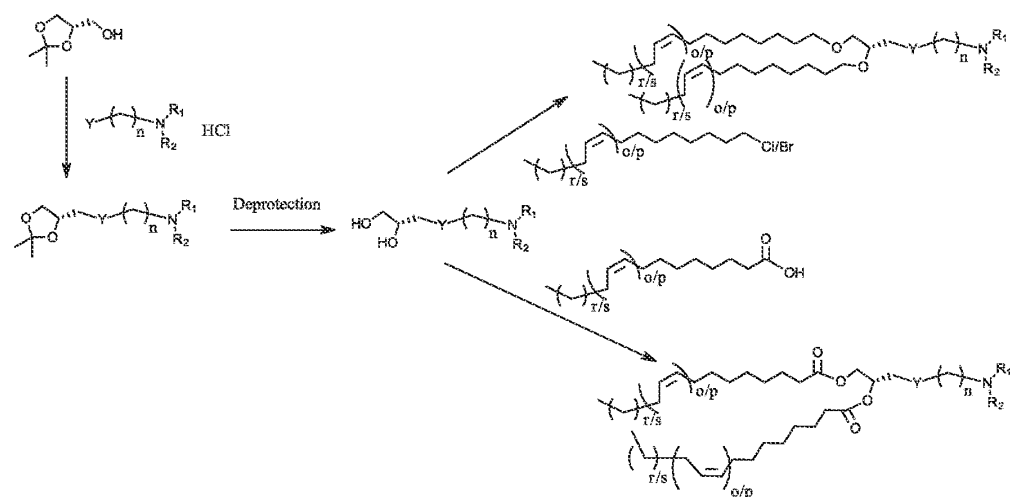
or a salt thereof.

SCHEME 1 - Synthetic scheme for making a lipid of Formula (I)



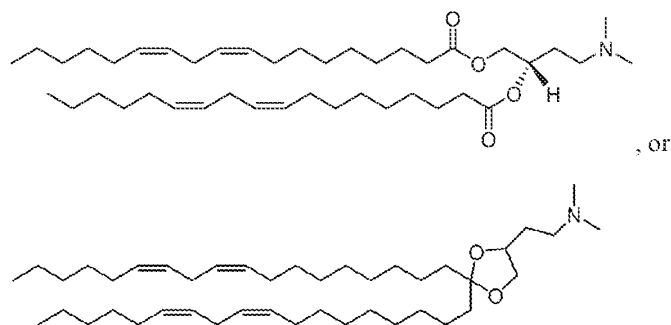
[0381] A compound of Formula I may be prepared, *e.g.*, according to Scheme 1. A hydroxy-functional protected propane diol is converted to the corresponding dimethyl amino-function ether (Y = oxo) or ester (Y = O-C(O)). The ether bond formation results from a reaction of the alkyl halide with alcohol in the presence of tertiary butylammonium iodide / NaOH in THF at 80°C. The ester bond formation utilizes treatment of an acid functional dimethylamine with alcohol under carbodiimide activation (DCM, EDC, DIEPA, DMAP). The diol deprotection yields a vicinal diol intermediate that is subsequently converted to the corresponding ether linked or ester linked diacyl lipids by treatment with TBAI/NaOH and bromo-acyl or by carbodiimide mediated carboxylic acid activation for ester bond formation, respectively.

SCHEME 2 - Synthetic scheme for making a lipid composition of Formula (II)



[0382] A compound of Formula II may be prepared, *e.g.*, according to Scheme 2. The synthetic procedure is as outlined above for Scheme 1; however, in Scheme 2, either bis-unsaturated acyl groups or mono-unsaturated acyl groups may be employed to obtain a lipid of Formula II.

[0383] In some embodiments, ionizable cationic lipid used in the LNPs of the present disclosure is selected from the lipids in Table 1, or a combination thereof. In some embodiments, the ionizable cationic lipid is:



In some embodiments, the ionizable cationic lipid is not Dlin-MC3-DMA.

III. LIPID-IMMUNE CELL TARGETING GROUP CONJUGATES

[0384] As discussed herein, the LNPs may be targeted to a particular cell type, *e.g.*, an immune cell, *e.g.*, a T cell, B cell, or natural killer (NK) cell. This can be accomplished by

using one or more of the lipids described herein. Furthermore, targeting can be enhanced by including a targeting group at a solvent accessible surface of an LNP particle. For example, targeting groups may include a member of a specific binding pair, *e.g.*, an antibody-antigen pair, a ligand-receptor pair, etc. In certain embodiments, the targeting group is an antibody. Targeting can be implemented, for example, by using lipid-immune cell targeting group conjugates described herein.

[0385] Optionally, the targeting moiety is an antibody fragment without an Fc component. Previous attempts to target circulating immune cells with LNPs have employed full antibodies (WO 2016/189532 A1). Liposomes or lipid based particles with conjugated full antibodies clear more quickly from the circulation due to engagement of the Fc, reducing their potential for reaching the target cell of interest (Harding et al. (1997) *Biochim Biophys. Acta* 1327, 181-192; Sapra et al. (2004) *Clin Cancer Res* 10, 1100-1111; Aragnol et al., (1986) *Proc Natl Acad Sci USA* 83, 2699-2703). Liposomes targeted with antibody fragments retain their long circulating properties, like those targeted to EGFR (Mamot et al., (2005) *Cancer Res* 65, 11631-11638), ErbB2 (Park et al. (2002) *Clin Cancer Res* 8, 1172-1181), or EphA2 (Kamoun et al., 2019 *Nat. Biomed. Eng* 3, 264-280). In addition, lipid based carriers can be prepared using a micellar insertion process that allows for the nearly quantitative incorporation of the antibody conjugation following its separate manufacturing (Nellis et al. (2005) *Biotechnol Prog* 21, 221-232), compared to a highly inefficient insertion when conjugating full IgGs (Ishida et al. (1999) *FEBS Lett.* 460, 129-133) or the need to complete conjugation directly on an intact LNP (WO 2016/189532 A1). scFv, Fab, or VHH fragments can also be directly conjugated to activated PEG-lipids to make insertable conjugates.

[0386] In certain embodiments, a targeting group may be a surface-bound antibody or surface bound antigen binding fragment thereof, which can permit tuning of cell targeting specificity. This is especially useful since highly specific antibodies can be raised against an epitope of interest for the desired targeting site. In one embodiment, multiple different antibodies can be incorporated into, and presented at the surface of an LNP, where each antibody binds to different epitopes on the same antigen or different epitopes on different antigens. Such approaches can increase the avidity and specificity of targeting interactions to a particular target cell.

[0387] A targeting group or combination of targeting groups can be selected based on the desired localization, function, or structural features of a given target cell. For example, in order to target a T-cell, T-cell population or T-cell subpopulation, one or more antibodies or antigen binding fragments or antigen binding derivatives thereof may be selected that target a T-cell, such as via a T-cell surface antigen. Exemplary T-cell surface antigens include, but are not limited to, for example, CD2, CD3, CD4, CD5, CD7, CD8, CD28, CD39, CD69, CD103, CD137, CD45, T-cell receptor (TCR) β , TCR- α , TCR- α/β , TCR- γ/δ , PD1, CTLA4, TIM3, LAG3, CD18, IL-2 receptor, CD11a, GL7, TLR2, TLR4, TLR5 and IL-15 receptor. In order to target an NK cell, or NK cell population, one or more antibodies, antigen binding fragments or antigen binding derivatives thereof may be selected that target an NK cell such as via a NK cell surface antigen. Exemplary NK cell surface antigens include, but are not limited to, CD48, CD56, CD85a, CD85c, CD85d, CD85e, CD85f, CD85i, CD85j, CD158b2, CD161, CD244, CD16a, CD16b, IL-2 receptor, CD27, CD28, CD48, CD69, CD70, CD86, CD112, CD122, CD155, CD161, CD244, CD266, CD314 / NKG2D, CD336 / NKP44, CD337 / NKP30. In order to target a B cell or B cell population, one or more antibodies, antigen binding fragments or antigen binding derivatives thereof may be selected that target a B cell such as via a B cell antigen. Exemplary B cell antigens include, but are not limited to, CD19 for all B cells except plasma cells, CD19, CD25, and CD30 for activated B cells, CD27, CD38, CD78, CD138, and CD319 for plasma cells, CD20, CD27, CD40, CD80 and PDL-2 for memory cells, Notch2, CD1, CD21, and CD27 for marginal zone B cells, CD21, CD22, and CD23 for follicular B cells, and CD1, CD5, CD21, CD24, and TLR4 for regulatory B cells.

[0388] In certain embodiments, targeting can be implemented, for example, by using lipid-immune cell targeting group conjugates described herein. Exemplary lipid-immune cell targeting group conjugates can include compounds of Formula IV,

[Lipid] – [optional linker] – [immune cell targeting group, *e.g.*, T-cell targeting molecule,
e.g., an
 anti-CD2 antibody, anti-CD3 antibody, anti-CD7 antibody, or anti-CD8 antibody]

(Formula IV).

[0389] In some embodiments, the immune cell targeting group is a polypeptide, and the lipid is conjugated to the N-terminus, C-terminus, or anywhere in the middle part of the polypeptide.

[0390] In certain embodiments, the targeting group or targeting molecule is a T-cell targeting agent, for example, an antibody, 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. In certain embodiments, the T cell antigen may be CD2, and the targeting group can be, for example, an anti-CD2 antibody. In certain embodiments, the T cell antigen may be CD3, and the targeting group can be, for example, an anti-CD3 antibody. In certain embodiments, the T cell antigen may be CD4, and the targeting group can be, for example, an anti-CD4 antibody. In certain embodiments, the T cell antigen may be CD5, and the targeting group can be, for example, an anti-CD5 antibody. In certain embodiments, the T cell antigen may be CD7, and the targeting group can be, for example, an anti-CD7 antibody. In certain embodiments, the T cell antigen may be CD8, and the targeting group can be, for example, an anti-CD8 antibody. In certain embodiments, the T cell antigen may be TCR β , and the targeting group can be, for example, an anti-TCR β antibody. In some embodiments, the antibody is a human or humanized antibody.

[0391] An exemplary CD2 binding agent can be an antibody selected from the group consisting of 9.6 (<https://academic.oup.com/intimm/article/10/12/1863/744536>), 9-1 (<https://academic.oup.com/intimm/article/10/12/1863/744536>), TS2/18.1.1 (ATCC HB-195), Lo-CD2b (ATCC PTA-802), Lo-CD2a/BTI-322 (US Patent 6849258B1), Sipilzumab/MEDI-507 (US Patent 6849258B1/en), 35.1 (ATCC HB-222), OKT11 (ATCC CRL-8027), RPA-2.1 (PCT Publication WO2020023559A1), AF1856 (R&D Systems), MAB18562 (R&D Systems), MAB18561 (R&D Systems), MAB1856 (R&D Systems), PAB30359 (Abnova Corporation), 10299-1 (Abnova Corporation), and antigen binding fragments thereof. In certain embodiments, the binding agent comprises a heavy chain variable domain (V_H) and a light chain variable domain (V_L) of an antibody selected from the group consisting of AF1856 (R&D Systems), MAB18562 (R&D Systems), MAB18561 (R&D Systems), MAB1856 (R&D Systems), PAB30359 (Abnova Corporation), and 10299-1 (Abnova Corporation). In certain embodiments, the binding agent comprises the heavy chain CDR₁,

CDR₂, and CDR₃ and the light chain CDR₁, CDR₂, and CDR₃, determined under Kabat (see, Kabat *et al.*, (1991) Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242, Bethesda), Chothia (see, *e.g.*, Chothia C & Lesk A M, (1987), J. MOL. BIOL. 196: 901-917), MacCallum (see, MacCallum R M *et al.*, (1996) J. MOL. BIOL. 262: 732-745), or any other CDR determination method known in the art, of the V_H and V_L sequences of an antibody selected from the group consisting of AF1856 (R&D Systems), MAB18562 (R&D Systems), MAB18561 (R&D Systems), MAB1856 (R&D Systems), PAB30359 (Abnova Corporation), and 10299-1 (Abnova Corporation).

[0392] An exemplary CD2 binding agent can also be selected from antibodies or antibody fragments employing CDRs of clones 9.6, 9-1, TS2/18.1.1, Lo-CD2b, Lo-CD2a, BTI-322, sipilzumab, 35.1, OKT11, RPA-2.1, SQB-3.21, LT2, TS1/8, UT329, 4F22, OX-34, UQ2/42, MU3, U7.4, NFN-76, or MOM-181-4-F(E).

[0393] An exemplary CD3 binding agent (CD3 $\gamma/\delta/\epsilon$, CD3 γ , CD3 δ , CD3 γ/ϵ , CD3 δ/ϵ , or CD3 ϵ) can be an antibody selected from the group consisting of MEM-57 (CD3 $\gamma/\delta/\epsilon$, EnzoLife Sciences), MAB100 (CD3 ϵ , R&D Systems), CD3-H5 (CD3 ϵ , Abnova Corporation), CD3-12 (CD3 ϵ , Cell Signaling Technology), LE-CD3 (CD3 ϵ , Santa Cruz Biotechnology, Inc.), NBPI-31250 (CD3 γ , Novus Biologicals), 16669-1-AP (CD3 δ , Invitrogen) and antigen binding fragments thereof. In certain embodiments, the binding agent comprises a V_H domain and a V_L domain of an antibody selected from the group consisting of MEM-57 (CD3 $\gamma/\delta/\epsilon$, EnzoLife Sciences), MAB100 (CD3 ϵ , R&D Systems), CD3-H5 (CD3 ϵ , Abnova Corporation), CD3-12 (CD3 ϵ , Cell Signaling Technology), LE-CD3 (CD3 ϵ , Santa Cruz Biotechnology, Inc.), NBPI-31250 (CD3 γ , Novus Biologicals), and 16669-1-AP (CD3 δ , Invitrogen). In certain embodiments, the binding agent comprises the heavy chain CDR₁, CDR₂, and CDR₃ and the light chain CDR₁, CDR₂, and CDR₃, determined under Kabat (see, Kabat *et al.*, (1991) Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242, Bethesda), Chothia (see, *e.g.*, Chothia C & Lesk A M, (1987), J. MOL. BIOL. 196: 901-917), MacCallum (see, MacCallum R M *et al.*, (1996) J. MOL. BIOL. 262: 732-745), or any other CDR determination method known in the art, of the V_H and V_L sequences of an antibody selected from the group consisting of MEM-57 (CD3 $\gamma/\delta/\epsilon$, EnzoLife Sciences), MAB100 (CD3 ϵ , R&D Systems), CD3-H5 (CD3 ϵ , Abnova Corporation), CD3-12 (CD3 ϵ , Cell Signaling Technology), LE-CD3 (CD3 ϵ , Santa Cruz

Biotechnology, Inc.), NBP1-31250 (CD3 γ , Novus Biologicals), and 16669-1-AP (CD3 δ , Invitrogen).

[0394] An exemplary CD3 binding agent can also be selected from antibodies or antibody fragments employing CDRs of clones hsp34, OKT-3, UCHT1, 38.1, HIT3a, RFT8, SK7, BC3, SP34-2, HU291, TRX4, Catumaxomab, teplizumab, 3-106, 3-114, 3-148, 3-190, 3-271, 3-550, 4-10, 4-48, H2C, F12Q, I2C, SP7, 3F3A1, CD3-12, 301, RIV9, JB38-29, JE17-74, GT0013, 4E2, 7A4, 4D10A6, SPV-T3b, M2AB, ICO-90, 30A1 or Hu38E4.v1 (US Patent Application 20200299409A1), REGN5458 (US Patent Application 20200024356A1), Blinatumomab (https://go.drugbank.com/drugs/DB09052/polypeptide_sequences.fasta). In some embodiments, the conjugate comprises a Fab, wherein the Fab comprises (a) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 1 and a light chain fragment comprising the amino acid sequence of SEQ ID NO:2 or 3.

[0395] An exemplary CD4 binding agent can be an antibody selected from the group consisting of Ibalizumab (https://www.genome.jp/dbget-bin/www_bget?D09575), AF1856 (R&D Systems), MAB554 (R&D Systems), BF0174 (Affinity Biosciences), PAB31115 (Abnova Corporation), CAL4 (Abcam), and antigen binding fragments thereof. In certain embodiments, the binding agent comprises a V_H domain and a V_L domain of an antibody selected from the group consisting of AF1856 (R&D Systems), MAB554 (R&D Systems), BF0174 (Affinity Biosciences), PAB31115 (Abnova Corporation), and CAL4 (Abcam). In certain embodiments, the binding agent comprises the heavy chain CDR₁, CDR₂, and CDR₃ and the light chain CDR₁, CDR₂, and CDR₃, determined under Kabat (see, Kabat *et al.*, (1991) Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242, Bethesda), Chothia (see, *e.g.*, Chothia C & Lesk A M, (1987), J. MOL. BIOL. 196: 901-917), MacCallum (see, MacCallum R M *et al.*, (1996) J. MOL. BIOL. 262: 732-745), or any other CDR determination method known in the art, of the V_H and V_L sequences of an antibody selected from the group consisting of AF1856 (R&D Systems), MAB554 (R&D Systems), BF0174 (Affinity Biosciences), PAB31115 (Abnova Corporation), and CAL4 (Abcam).

[0396] An exemplary CD4 binding agent can also be selected from antibodies or antibody fragments employing CDRs of clones Ibalizumab, OKT4, RPA-T4, S3.5, SK3, NIUG0, RIV6, OTI18E3, MEM-241, B486A1, RFT-4g, 7E14, MDX.2, MEM-115, MEM-16, ICO-86, Edu-2, or ilbalizumab.

[0397] An exemplary CD5 binding agent can be an antibody selected from the group consisting of He3, MAB1636 (R&D Systems), AF1636 (R&D Systems), MAB115 (R&D Systems), C5/473 + CD5/54/F6 (Abcam), CD5/54/F6 (Abcam), 65152 (Proteintech), and antigen binding fragments thereof. In some embodiments, the binding agent comprises a V_H domain and a V_L of an antibody selected from the group consisting of MAB1636 (R&D Systems), AF1636 (R&D Systems), MAB115 (R&D Systems), C5/473 + CD5/54/F6 (Abcam), CD5/54/F6 (Abcam), and 65152 (Proteintech). In certain embodiments, the binding agent comprises the heavy chain CDR₁, CDR₂, and CDR₃ and the light chain CDR₁, CDR₂, and CDR₃, determined under Kabat (see, Kabat *et al.*, (1991) Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242, Bethesda), Chothia (see, *e.g.*, Chothia C & Lesk A M, (1987), J. MOL. BIOL. 196: 901-917), MacCallum (see, MacCallum R M *et al.*, (1996) J. MOL. BIOL. 262: 732-745), or any other CDR determination method known in the art, of the V_H and V_L sequences of an antibody selected from the group consisting of MAB1636 (R&D Systems), AF1636 (R&D Systems), MAB115 (R&D Systems), C5/473 + CD5/54/F6 (Abcam), CD5/54/F6 (Abcam), and 65152 (Proteintech).

[0398] An exemplary CD5 binding agent can also be selected from antibodies or antibody fragments employing CDRs of clones of zolimomab, 5D7, L17F12, and UCHT2, 1D8, 3I21, 4H10, 8J23, 5O4, 4H2, 5G2, 8G8, 6M4, 2E3, 4E24, 4F10, 7J9, 7P9, 8E24, 6L18, 7H7, 1E7, 8J21, 7I11, 8M9, 1P21, 2H11, 3M22, 5M6, 5H8, 7I19, 1A2, 8E15, 8C10, 3P16, 4F3, 5M24, 5O24, 7B16, 1E8, 2H16, BLA1, 1804, DK23, Cris1, MEM-32, H65, 4C7, OX-19, Leu-1, 53-7.3, 4H8E6, T101, EP2952, D-9, H-3, HK231, N-20, Y2/178, H-300, CD5/54/F6, Q-20, CC17, MOM-18539-S(P), or MOM-18885-S(P).

[0399] An exemplary CD7 binding agent can be an antibody selected from the group consisting of MAB7579 (R&D Systems), AF7579 (R&D Systems), EPR22065 (Abcam), 1G10D8 (Proteintech), NBP2-32097 (Novus Biologicals), NBP2-38440 (Novus Biologicals), and antigen binding fragments thereof. In certain embodiments, the binding agent comprises a V_H domain and a V_L of an antibody selected from the group consisting of MAB7579 (R&D Systems), AF7579 (R&D Systems), EPR22065 (Abcam), 1G10D8 (Proteintech), NBP2-32097 (Novus Biologicals), and NBP2-38440 (Novus Biologicals). In certain embodiments, the binding agent comprises the heavy chain CDR₁, CDR₂, and CDR₃ and the light chain CDR₁, CDR₂, and CDR₃, determined under Kabat (see, Kabat *et al.*, (1991) Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242, Bethesda), Chothia (see,

e.g., Chothia C & Lesk A M, (1987), J. MOL. BIOL. 196: 901-917), MacCallum (see, MacCallum R M *et al.*, (1996) J. MOL. BIOL. 262: 732-745), or any other CDR determination method known in the art, of the V_H and V_L sequences of an antibody selected from the group consisting of MAB7579 (R&D Systems), AF7579 (R&D Systems), EPR22065 (Abcam), 1G10D8 (Proteintech), NBP2-32097 (Novus Biologicals), and NBP2-38440 (Novus Biologicals).

[0400] An exemplary CD7 binding agent can also be selected from antibodies or antibody fragments employing CDRs of clones TH-69, 3Af11, T3-3A1, 124-1D1, 3A1f, CD7-6B7, or VHH6.

[0401] An exemplary CD8 (CD8 α , CD8 α/α , CD8 α/β or CD8 β) binding agent can be an antibody selected from the group consisting of 2.43 (Invitrogen), Du CD8-1 (CD8 α , Invitrogen), 9358-CD (CD8 α/β , R&D Systems), MAB116 (CD8 α , R&D Systems), ab4055 (CD8 α , Abcam), C8/144B (CD8 α , Novus Biologicals), YTS105.18 (CD8 α , Novus Biologicals), TRX2 (<https://patents.justia.com/patent/20170198045>), and antigen binding fragments thereof. In certain embodiments, the binding agent comprises a V_H domain and a V_L domain of an antibody selected from the group consisting of 2.43 (Invitrogen), 51.1 (ATCC HB-230), Du CD8-1 (CD8 α , Invitrogen), 9358-CD (CD8 α/β , R&D Systems), MAB116 (CD8 α , R&D Systems), ab4055 (CD8 α , Abcam), C8/144B (CD8 α , Novus Biologicals), and YTS105.18 (CD8 α , Novus Biologicals). In certain embodiments, the binding agent comprises the heavy chain CDR₁, CDR₂, and CDR₃ and the light chain CDR₁, CDR₂, and CDR₃, determined under Kabat (see, Kabat *et al.*, (1991) Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242, Bethesda), Chothia (see, *e.g.*, Chothia C & Lesk A M, (1987), J. MOL. BIOL. 196: 901-917), MacCallum (see, MacCallum R M *et al.*, (1996) J. MOL. BIOL. 262: 732-745), or any other CDR determination method known in the art, of the V_H and V_L sequences of an antibody selected from the group consisting of 2.43 (Invitrogen), Du CD8-1 (CD8 α , Invitrogen), 9358-CD (CD8 α/β , R&D Systems), MAB116 (CD8 α , R&D Systems), ab4055 (CD8 α , Abcam), C8/144B (CD8 α , Novus Biologicals), and YTS105.18 (CD8 α , Novus Biologicals).

[0402] An exemplary CD8 binding agent can also be selected from antibodies or antibody fragments employing CDRs of clones OKT-8, 51.1, S6F1, TRX2, and UCHT4, SP16, 3B5, C8-144B, HIT8a, RAVB3, LT8, 17D8, MEM-31, MEM-87, RIV11, DK-25, YTC141.1HL, or YTC182.20. In some embodiments, the conjugate comprises a Fab, wherein the Fab

comprises a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 6 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 7.

[0403] An exemplary CD137 binding agent can be selected from antibodies or antibody fragments employing CDRs of clones 4B4-1, P566, or Urelumab. An exemplary CD28 binding agent can be selected from antibodies or antibody fragments employing CDRs of clone TAB08. An exemplary CD45 binding agent can be selected from antibodies or antibody fragments employing CDRs of clones BC8, 9.4, 4B2, Tu116, or GAP8.3. An exemplary CD18 binding agent can be selected from antibodies or antibody fragments employing CDRs of clones 1B4, TS1/18, MEM-48, YFC118-3, TA-4, MEM-148, or R3-3, 24. An exemplary CD11a binding agent can be selected from antibodies or antibody fragments employing CDRs of clone MHM24 or Efalizumab. An exemplary IL-2 receptor binding agent can be selected from antibodies or antibody fragments employing CDRs of clones YTH 906.9HL, IL2R.1, BC96, B-B10, 216, MEM-181, ITYV, MEM-140, ICO-105, Daclizumab, or from the group consisting of IL2 or fragments of IL2. An exemplary IL-15R binding agent can be selected from antibodies or antibody fragments employing CDRs of clones JM7A4, or OTI3D5, or from the group consisting of IL15 or fragments of IL15. An exemplary TLR2 binding agent can be selected from antibodies or antibody fragments employing CDRs of clones JM22-41, TL2.1, 11G7, or TLR2.45. An exemplary TLR4 binding agent can be selected from antibodies or antibody fragments employing CDRs of clones HTA125, or 76B357-1. An exemplary TLR5 binding agent can be selected from antibodies or antibody fragments employing CDRs of clones 85B152-5, or 9D759-2. An exemplary GL7 binding agent can be selected from antibodies or antibody fragments employing CDRs of clone GL7.

[0404] An exemplary PD1 binding agent can be selected from antibodies or antibody fragments employing CDRs of clones MIH4, J116, J150, OTIB11, OTI17B10, OTI3A1, or OTI16D4. In addition, exemplary anti-PD-1 antibodies are described, for example, in U.S. Patent Nos. 8,952,136, 8,779,105, 8,008,449, 8,741,295, 9,205,148, 9,181,342, 9,102,728, 9,102,727, 8,952,136, 8,927,697, 8,900,587, 8,735,553, and 7,488,802. Exemplary anti-PD-1 antibodies include, for example, nivolumab (Opdivo®, Bristol-Myers Squibb Co.), pembrolizumab (Keytruda®, Merck Sharp & Dohme Corp.), PDR001 (Novartis Pharmaceuticals), and pidilizumab (CT-011, Cure Tech). Exemplary anti-PD-L1 antibodies are described, for example, in U.S. Patent Nos. 9,273,135, 7,943,743, 9,175,082, 8,741,295,

8,552,154, and 8,217,149. Exemplary anti-PD-L1 antibodies include, for example, atezolizumab (Tecentriq®, Genentech), durvalumab (AstraZeneca), MEDI4736, avelumab, and BMS 936559 (Bristol Myers Squibb Co.).

[0405] An exemplary CTLA-4 binding agent can be selected from antibodies or antibody fragments employing CDRs of clones ER4.7G.11 [7G11], OTI9G4, OTI9F3, OTI3A5, A3.4H2.H12, 14D3, OTI3A12, OTI1A11, OTI1E8, OTI3B11, OTI3D2, OTI10C8, OTI2E9, OTI6F1, OTI7D3, OTI85B, OTI12C6. Exemplary anti-CTLA-4 antibodies are described in U.S. Patent Nos. 6,984,720, 6,682,736, 7,311,910; 7,307,064, 7,109,003, 7,132,281, 6,207,156, 7,807,797, 7,824,679, 8,143,379, 8,263,073, 8,318,916, 8,017,114, 8,784,815, and 8,883,984, International (PCT) Publication Nos. WO98/42752, WO00/37504, and WO01/14424, and European Patent No. EP 1212422 B1. Exemplary CTLA-4 antibodies include ipilimumab or tremelimumab.

[0406] An exemplary TCR β binding agent can be an antibody selected from the group consisting of H57-597 (Invitrogen), 8A3 (Novus Biologicals), R73 (TCR α/β , Abcam), E6Z3S (TRBC1/TCR β , Cell Signaling Technology), and antigen binding fragments thereof. In certain embodiments, the binding agent comprises a V_H domain and a V_L of an antibody selected from the group consisting of H57-597 (Invitrogen), 8A3 (Novus Biologicals), R73 (TCR α/β , Abcam), and E6Z3S (TRBC1/TCR β , Cell Signaling Technology). In certain embodiments, the binding agent comprises the heavy chain CDR₁, CDR₂, and CDR₃ and the light chain CDR₁, CDR₂, and CDR₃, determined under Kabat (see, Kabat *et al.*, (1991) Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242, Bethesda), Chothia (see, *e.g.*, Chothia C & Lesk A M, (1987), J. MOL. BIOL. 196: 901-917), MacCallum (see, MacCallum R M *et al.*, (1996) J. MOL. BIOL. 262: 732-745), or any other CDR determination method known in the art, of the V_H and V_L sequences of an antibody selected from the group consisting of H57-597 (Invitrogen), 8A3 (Novus Biologicals), R73 (TCR α/β , Abcam), and E6Z3S (TRBC1/TCR β , Cell Signaling Technology).

[0407] An exemplary CD137 binding agent can be selected from antibodies or antibody fragments employing CDRs of clones 4B4-1, P566, or Urelumab.

[0408] In some embodiments, the immune cell targeting group comprises an antibody selected from the group consisting of a Fab, F(ab')₂, Fab'-SH, Fv, and scFv fragment. In some embodiments, the antibody is a human or humanized antibody. In some embodiments,

the immune cell targeting group comprises a Fab or an immunoglobulin single variable domain, such as a Nanobody. In some embodiments, the immune cell targeting group comprises a Fab that does not comprise a natural interchain disulfide bond. For example, in some embodiments, the Fab comprises a heavy chain fragment that comprises a C233S substitution, and/or a light chain fragment that comprises a C214S substitution, numbering according to Kabat. In some embodiments, the immune cell targeting group comprises a Fab that comprises one or more non-native interchain disulfide bonds. In some embodiments, the interchain disulfide bonds are between two non-native cysteine residues on the light chain fragment and heavy chain fragment, respectively. For example, in some embodiments, the Fab comprises a heavy chain fragment that comprises F174C substitution, and/or a light chain fragment that comprises S176C substitution, numbering according to Kabat. In some embodiments, the Fab comprises a heavy chain fragment that comprises F174C and C233S substitutions, and/or a light chain fragment that comprises S176C and C214S substitutions, numbering according to Kabat. In some embodiments, the immune cell targeting group comprises a C-terminal cysteine residue. In some embodiments, the immune cell targeting group comprises a Fab that comprises a cysteine at the C-terminus of the heavy or light chain fragment. In some embodiments, the Fab further comprises one or more amino acids between the heavy chain of the Fab and the C-terminal cysteine. For example, in some embodiments, the Fab comprises two or more amino acids derived from an antibody hinge region (e.g., a partial hinge sequence) between the C-terminus of the Fab and the C-terminal cysteine. In some embodiments, the Fab comprises a heavy chain variable domain linked to an antibody CH1 domain and a light chain variable domain linked to an antibody light chain constant domain, wherein the CH1 domain and the light chain constant domain are linked by one or more interchain disulfide bonds, and wherein the immune cell targeting group further comprises a single chain variable fragment (scFv) linked to the C-terminus of the light chain constant domain by an amino acid linker. In some embodiments, the Fab antibody is a DS Fab, a NoDS Fab, a bDS Fab, a bDS Fab-ScFv, as demonstrated in FIG 47.

[0409] In some embodiments, the immune cell targeting group comprises an immunoglobulin single variable domain, such as a Nanobody (e.g., a V_{HH}). In some embodiments, the Nanobody comprises a cysteine at the C-terminus. In some embodiments, the Nanobody further comprises a spacer comprising one or more amino acids between the V_{HH} domain and the C-terminal cysteine. In some embodiments, the spacer comprises one or more glycine residues, e.g., two glycine residues. In some embodiments, the immune cell

targeting group comprises two or more V_{HH} domains. In some embodiments, the two or more V_{HH} domains are linked by an amino acid linker. In some embodiments, the amino acid linker comprises one or more glycine and/or serine residues (*e.g.*, one or more repeats of the sequence GGGGS). In some embodiments, the immune cell targeting group comprises a first V_{HH} domain linked to an antibody CH1 domain and a second V_{HH} domain linked to an antibody light chain constant domain, and wherein the antibody CH1 domain and the antibody light chain constant domain are linked by one or more disulfide bonds (*e.g.*, interchain disulfide bonds). In some embodiments, the immune cell targeting group comprises a V_{HH} domain linked to an antibody CH1 domain, and wherein the antibody CH1 domain is linked to an antibody light chain constant domain by one or more disulfide bonds. In some embodiments, the CH1 domain comprises F174C and C233S substitutions, and the light chain constant domain comprises S176C and C214S substitutions, numbering according to Kabat. In some embodiments, the antibody is a ScFv, a V_{HH} , a $2 \times V_{HH}$, a V_{HH} -CH1/empty Vk, or a V_{HH1} -CH1/ V_{HH2} -Nb bDS, as demonstrated in FIG. 47.

[0410] An exemplary targeting moiety may have an amino sequence as set forth below:

Anti-CD3 hSP34-Fab sequences:

hSP34 heavy chain (HC) sequence (SEQ ID NO: 1):

EVQLVESGGGLVQPGGSLKLSAASGFTFNKYAMNWVRQAPGKGLEWVARIRSKY
NNYATYYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYISY
WAYWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKVEP
KSSDKTHTC

hSP34-mlam light chain (LC) sequence (mouse lambda) (SEQ ID NO: 2):

QTVVTQEPSLTVSPGGTVTLTCGSSTGAVTSGNYPNWVQKPGQAPRGLIGGTFKFLA
PGTPARFSGSLLGGKAALTLGSGVQPEDEAEYYCVLWYSNRWVFGGGTKLTVLGQPK
SSPSVTLFPPSSEELTNKATLVCTITDFYPGVTVTDWKVDGTPVTQGMETTQPSKQS
NNKYMASSYLTLTARAWERHSSYSCQVTHEGHTVEKSLSRADSS

SP34-hlam LC (human lambda) (SEQ ID NO: 3):

QTVVTQEPSLTVSPGGTVTLTCGSSTGAVTSGNYPNWVQKPGQAPRGLIGGTFKFLA
PGTPARFSGSLLGGKAALTLGSGVQPEDEAEYYCVLWYSNRWVFGGGTKLTVLSQPK
AAPSVTLFPPSSEELQANKATLVCLVSDFYPGAVTVAWKADGSPVKVGVETTKPSK
QSNKYAASSYLSLTPEQWKSHRSYSCRVTHEGSTVEKTVAPAESS

Anti-CD3 Hu291-Fab sequences:

Hu291 HC (SEQ ID NO: 4):

QVQLVQSGAEVKKPGASVKVSCKASGYTFISYTMHWVRQAPGQGLEWMGYINPRS
GYTHYNQKLKDKATLTADKSASTAYMELSSLRSEDTAVYYCARSAYYDYDGFAYW
QGGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT

SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDDKKVEPKSSDK
THTC

Hu 291 LC (SEQ ID NO: 5):

MDMRVPAQLLGLLLLWLPQAKCDIQMTQSPSSLSASVGDRVTTTCSASSSVSYMNW
YQKPGKAPKRLIYDTSKLASGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQWSS
NPPTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
DNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSPVTK
SFNRGES

Anti-CD8 TRX2-Fab sequences:

TRX2 HC (SEQ ID NO: 6):

QVQLVESGGGVVQPGRSLRLSCAASGFTFSDFGMNWVRQAPGKGLEWVALIYYDG
SNKFYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAKPHYDGYHFFDS
WGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDDKKVEPKSSD
KTHTC

TRX2 LC (SEQ ID NO: 7):

DIQMTQSPSSLSASVGDRVTTTCKGSQDINNYLAWYQQKPGKAPKLLIYNTDILHTG
VPSRFSGSGSGTDFTFTISLQPEDFATYYCYQYNNGYTFGQGTKVEIKRTVAAPSVFI
FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDY
SLSTLTLSKADYEKHKVYACEVTHQGLSPVTKSFNRGES

Anti-CD8 OKT8-Fab sequences:

OKT8 HC (SEQ ID NO: 8):

QVQLVQSGAEDKKPGASVKVSKASGFNIKDTYIHWVRQAPGQGLEWMGRIDPAN
DNTLYASKFQGRVTITADTSSNTAYMELSSLRSEDVAVYYCGRGYGYVFDHWGQ
GTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
VHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDDKKVEPKSSDKTH
TC

OKT8 LC (SEQ ID NO: 9):

DIVMTQSPSSLSASVGDRVTTTCRTSRISQYLAWYQEKGKAPKLLIYSGSTLQSGVP
SRFSGSGSGTDFTLTISLQPEDFATYYCQQHNNPLTFGQGTKVEIKRTVAAPSVFIF
PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDY
LSSTLTLSKADYEKHKVYACEVTHQGLSPVTKSFNRGES

Anti-CD4 Ibalizumab-Fab sequences:

Ibalizumab HC (SEQ ID NO: 10):

QVQLQQSGPEVVKPGASVKMSCKASGYTFTSYVIHWVRQKPGQGLDWIGYINPYND
GTDYDEKFKGKATLTSDTSTAYMELSSLRSEDVAVYYCAREKDNYATGAWFAY
WGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDDKKVEPKSSD
KTHTC

Ibalizumab LC (SEQ ID NO: 11):

DIVMTQSPDSLAVSLGERVTMNCSSQSLLYSTNQKNYLAWYQQKPGQSPKLLIYW
ASTRESGVPDRFSGSGSGTDFTLTISVQAEDVAVYYCQQYYSYRTFGGGTKLEIKRT

VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

anti-CD5 He3-Fab sequences:

He3 HC (SEQ ID NO: 12):

EIQLVQSGGGLVKPGGVRISCAASGYTFTNYGMNWVRQAPGKGLEWMGWINTHT
GEPTYADSFKGRFTFSLDDSKNTAYLQINSLRAEDTAVYFCTRRGYDWYFDVWGQG
TTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC

He3 LC (SEQ ID NO: 13):

DIQMTQSPSSLSASVGDRVTITCRASQDINSYLSWFQKPGKAPKTLIYRANRLESGVP
SRFSGSGSGTDYTLTISSLQYEDFGIYYCQYDESPWTFGGGTKLEIKRTVAAPSVFIFP
PSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

anti-CD7 TH-69-Fab sequences:

TH-69 HC (SEQ ID NO: 14):

EVQLVESGGGLVKPGGSLKLSCAASGLTFSSYAMSWVRQTPPEKRLEWVASISSGGFT
YYPDSVKGRFTISRDNARNILYLQMSSLRSEDTAMYYCARDEVRGYLDVWGAGTTV
TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP
AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC

TH-69 LC (SEQ ID NO: 15):

DIQMTQTTSSLSASLGDRVTISCSASQGISNYLNWYQKPDGTVKLLIYYTSSLHSGVP
SRFSGSGSGTDYSLTISNLEPEDATYYCQYQSKLPYTFGGGTKLEIKRTVAAPSVFIFP
PSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

anti-CD2 TS2/18.1-Fab sequences:

TS2/18.1 HC (SEQ ID NO: 16):

EVQLVESGGGLVMPGGSLKLSCAASGFAFSSYDMSWVRQTPPEKRLEWVAYISGGGF
TYYPDVTVKGRFTLSRDNAKNTLYLQMSSLSKSEDTAMYYCARQGANWELVYWGQGT
LVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC

TS2/18.1 LC (SEQ ID NO: 17):

DIVMTQSPATLSVTPGDRVFLSCRASQSSDFLHWYQKSHESPRLLIKYASQSIGIPS
RFGSGSGSGDFTLSINSVEPEDVGVYFCQNGHNFPPTFGGGTKLEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

anti-CD2 9.6-Fab sequences:

9.6 HC (SEQ ID NO: 18):

QVQLQQPGAELVRPGSSVKLSCKASGYTFTRYWIHWVKQRPIQGLEWIGNIDPSDSE
THYNQKFKDKATLTVDKSSGTAYMQLSSLTSEDSAVYYCATEDLYYAMEYWGQGT
SVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC

9.6 LC (SEQ ID NO: 19):

NIMMTQSPSSSLAVSAGEKVTMTCKSSQSVLYSSNQKNYLAWYQQKPGQSPKLLIYW
ASTRESGVDPDRFTGSGSGTDFTLTISVQPEDLAVYYCHQYLSSHTFGGGTKLEIKRTV
AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQD
SKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

anti-CD2 9-1-Fab sequences:

9-1 HC (SEQ ID NO: 20):

QVQLQQPGTELVRPGSSVKLSCKASGYTFTSYWVNWVKQRPDQGLEWIGRIDPYDS
ETHYNQKFTDKAISTIDTSSNTAYMQLSTLTSDASAVYYCSRSPRDSSTNLADWGQG
TLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC

9-1 LC (SEQ ID NO: 21):

DIVMTQSPATLSVTPGDRVSLSCRASQISDYLHWYQQKSHESPRLLIKYASQISGIPS
RFSGSGSGDFTLTISNVEPEDVGVYYCQNGHSFPLTFGAGTKLELRRTVAAPSVFIFP
PSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

mutOKT8-Fab sequences:

mutOKT8 HC (SEQ ID NO: 22):

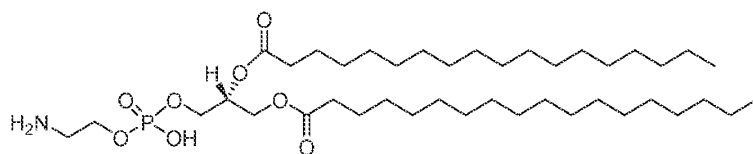
QVQLVQSGAEDKKPGASVKVSCASGFNIKDTYIHWVRQAPGQGLEWMGRIDPAND
NTLYASKFQGRVTITADTSSNTAYMELSSLRSEDTAVYYCGRGAGAYVFDHWGQGT
TVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC

mutOKT8 LC (SEQ ID NO: 23):

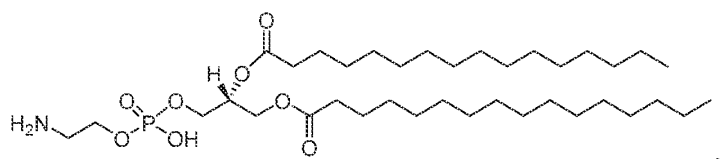
DIVMTQSPSSLSASVGDRVTITCRTSRISAAALAWYQEKPGKAPKLLIYSGSTLQSGVP
SRFSGSGSGDFTLTISLQPEDFATYYCQHNENPLTFGQGTKVEIKRTVAAPSVFIFP
PSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES.

[0411] In certain embodiments, the targeting group or immune cell targeting group (e.g., T cell-targeting agent, B cell-targeting agent, or NK-cell targeting agent) may be covalently coupled to a lipid via a polyethylene glycol (PEG) containing linker.

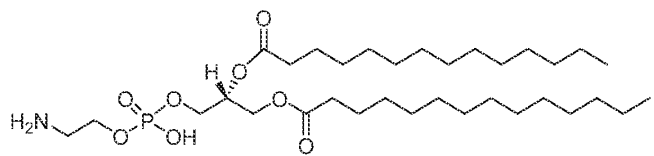
[0412] In other embodiments, the lipid used to create a conjugate may be selected from distearoyl-phosphatidylethanolamine (DSPE):



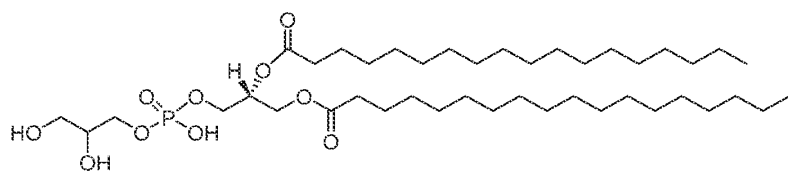
dipalmitoyl-phosphatidylethanolamine (DPPE):



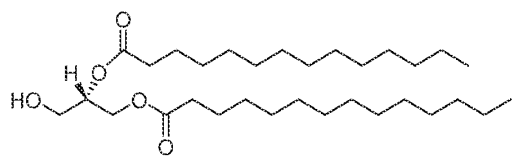
dimyristoyl-phosphatidylethanolamine (DMPE):



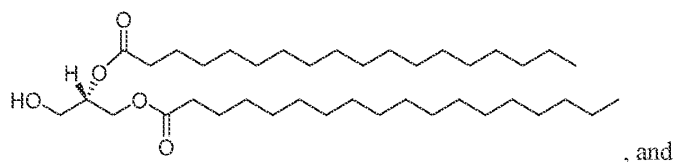
distearoyl-glycero-phosphoglycerol (DSPG):



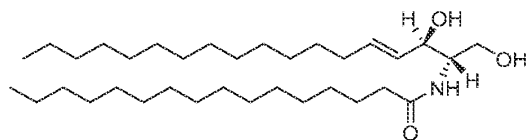
dimyristoyl-glycerol (DMG):



distearoylglycerol (DSG):



N-palmitoyl-sphingosine (C16-ceramide)



[0413] The immune cell targeting group can be covalently coupled to a lipid either directly or via a linker, for example, a polyethylene glycol (PEG) containing linker. In

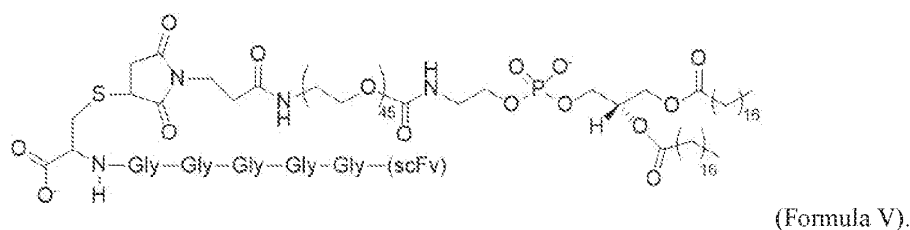
certain embodiments, the PEG is PEG 1000, PEG 2000, PEG 3400, PEG 3000, PEG 3450, PEG 4000, or PEG 5000. In certain, embodiments, the PEG is PEG 2000.

[0414] In some embodiments, the lipid-immune cell targeting group conjugate is present in the lipid blend in a range of 0.001-0.5 mole percent, 0.001-0.3 mole percent, 0.002-0.2 mole percent, 0.01-0.1 mole percent, 0.1-0.3 mole percent, or 0.1-0.2 mole percent.

[0415] In certain embodiments, the lipid immune-cell targeting agent conjugate comprises DSPE, a PEG component and a targeting antibody. In certain embodiments, the antibody is a T-cell targeting agent, for example, an anti-CD2 antibody, an anti-CD3 antibody, an anti-CD4 antibody, an anti-CD5 antibody, an anti-CD7 antibody, an anti CD8 antibody, or an anti-TCR β antibody.

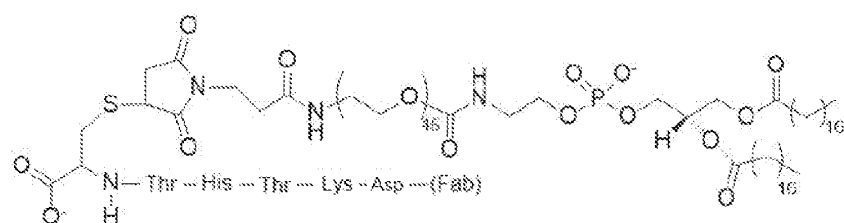
[0416] An exemplary lipid-immune cell targeting group conjugate comprises DSPE and PEG 2000, for example, as described in Nellis *et al.* (2005) BIOTECHNOL. PROG. 21, 205-220. An exemplary conjugate comprises the structure of Formula V, where the scFv represents an engineered antibody binding site that binds to a target of interest. In certain embodiments, the engineered antibody binding site binds to any of the targets described hereinabove. In certain embodiments, the engineered antibody binding site can be, for example, an engineered anti-CD3 antibody or an engineered anti-CD8 antibody. In certain embodiments, the engineered antibody binding site can be, for example, an engineered anti-CD2 antibody or an engineered anti-CD7 antibody.

[0417] An example of a compound of Formula (V) is as shown below:



It is contemplated that the scFv in Formula V may be replaced with an intact antibody or an antigen fragment thereof (*e.g.*, an Fab).

Another example of a compound of Formula (VI) is as shown below:



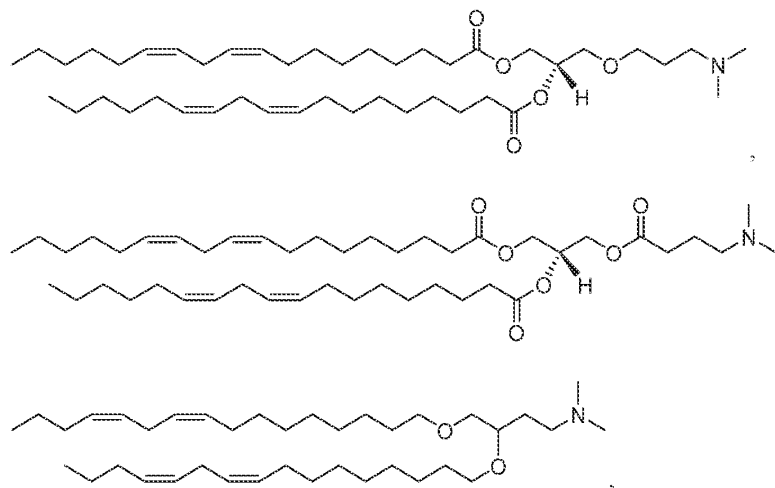
(Formula

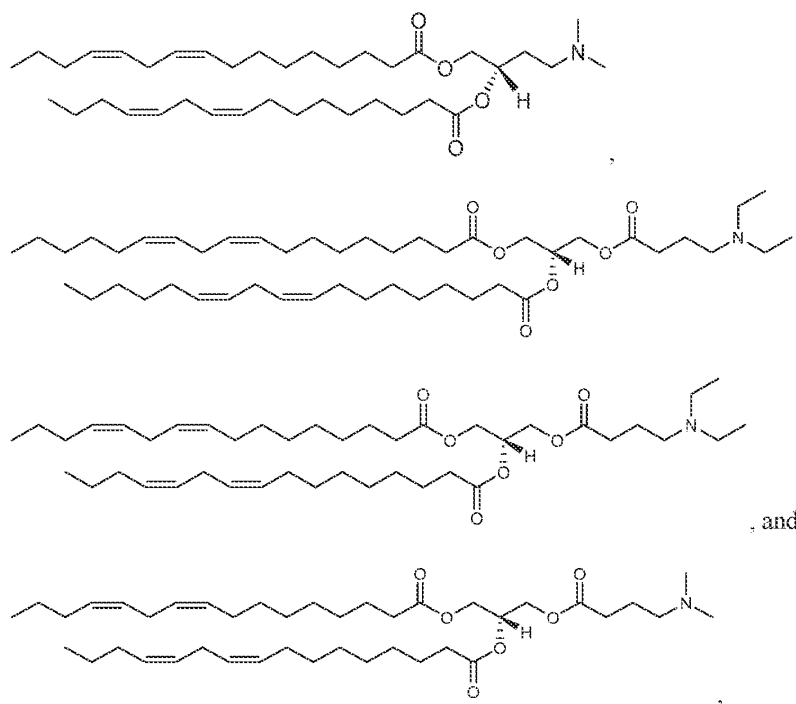
VI),

the production of which is described in Nellis *et al.* (2005) *supra*, or U.S. Patent No. 7,022,336. It is contemplated that the Fab in Formula VI may be replaced with an intact antibody or an antigen fragment thereof (*e.g.*, an (Fab')₂ fragment) or an engineering antibody binding site (*e.g.*, an scFv).

[0418] Other lipid immune cell target group conjugates are described, for example, in U.S. Patent No. 7,022,336, where the targeting group may be replaced with a targeting group of interest, for example, a targeting group that binds an T-cell or NK cell surface antigen as described hereinabove.

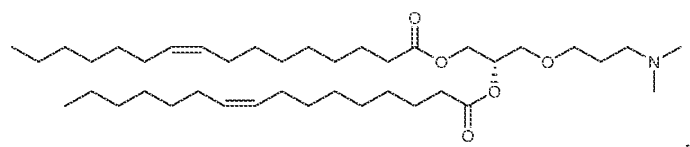
[0419] In certain embodiments, the lipid component of an exemplary conjugate of Formula IV can be based on an ionizable, cationic lipid described herein, for example, an ionizable, cationic lipid of Formula I, Formula II, or Formula III. For example, an exemplary ionizable, cationic lipid can be selected from the group consisting of:





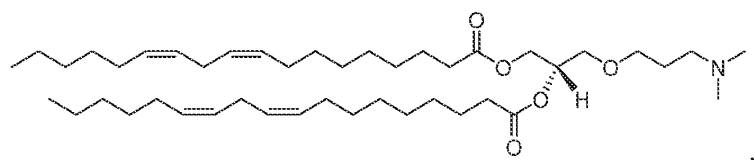
or a salt thereof

[0420] In certain embodiments, an exemplary ionizable, cationic lipid can be a compound of the formula:



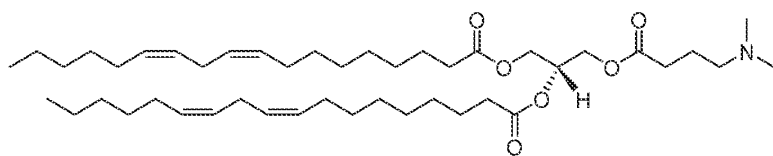
or a salt thereof.

[0421] In some embodiments, an exemplary ionizable, cationic lipid can be a compound of the formula:



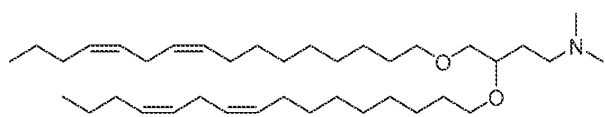
or a salt thereof.

[0422] In other embodiments, an exemplary ionizable, cationic lipid can be a compound of the formula:



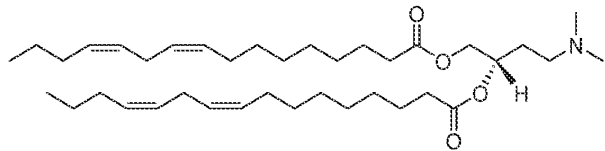
or a salt thereof.

[0423] In certain embodiments, an exemplary ionizable, cationic lipid can be a compound of the formula:



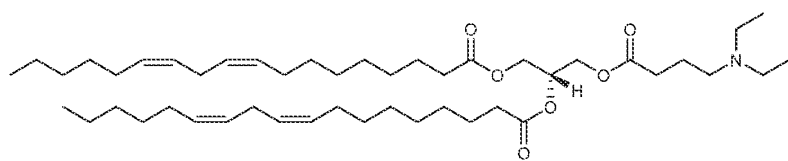
or a salt thereof.

[0424] In some embodiments, an exemplary ionizable, cationic lipid can be a compound of the formula:



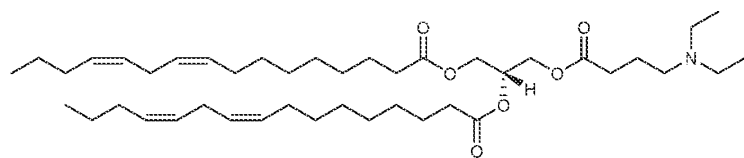
or a salt thereof.

[0425] In other embodiments, an exemplary ionizable, cationic lipid can be a compound of the formula:



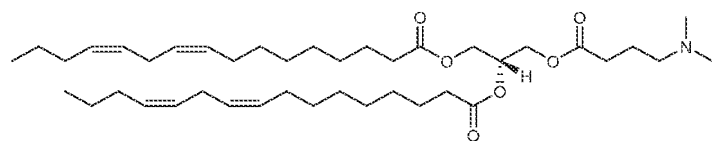
or a salt thereof.

[0426] In certain embodiments, an exemplary ionizable, cationic lipid can be a compound of the formula:



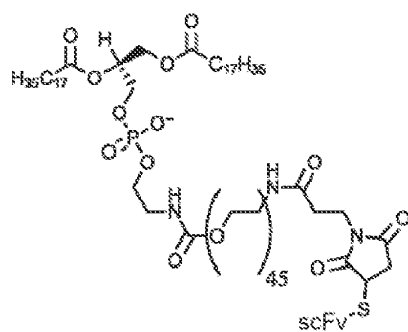
or a salt thereof.

[0427] In some embodiments, an exemplary ionizable, cationic lipid can be a compound of the formula:



or a salt thereof.

[0428] In certain embodiments, the conjugate based on a lipid of Formula III may include:



, where scFv represents an engineered antibody binding site that binds a target described hereinabove, *e.g.*, CD2, CD3, CD7, or CD8.

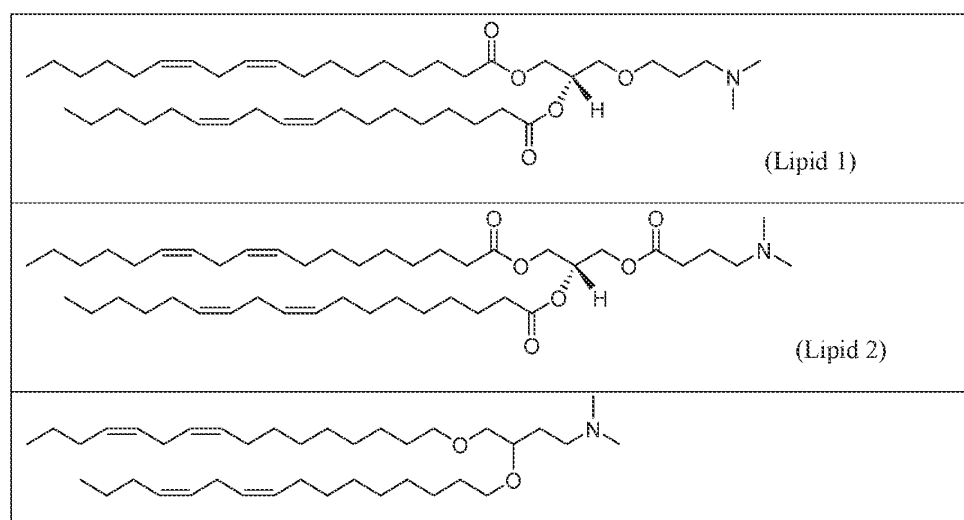
[0429] In certain embodiments, the lipid blend may further comprise free PEG-lipid so as to reduce the amount of non-specific binding via the targeting group. The free PEG-lipid can be the same or different from the PEG-lipid included in the conjugate. In certain embodiments, the free PEG-lipid is selected from the group consisting of PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) or PEG-dimyristoyl-phosphatidylethanolamine (PEG-DMPE), N-(Methylpolyoxyethylene oxycarbonyl)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE-PEG) 1,2-Dimyristoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DMG), 1,2-Dipalmitoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DPG), 1,2-

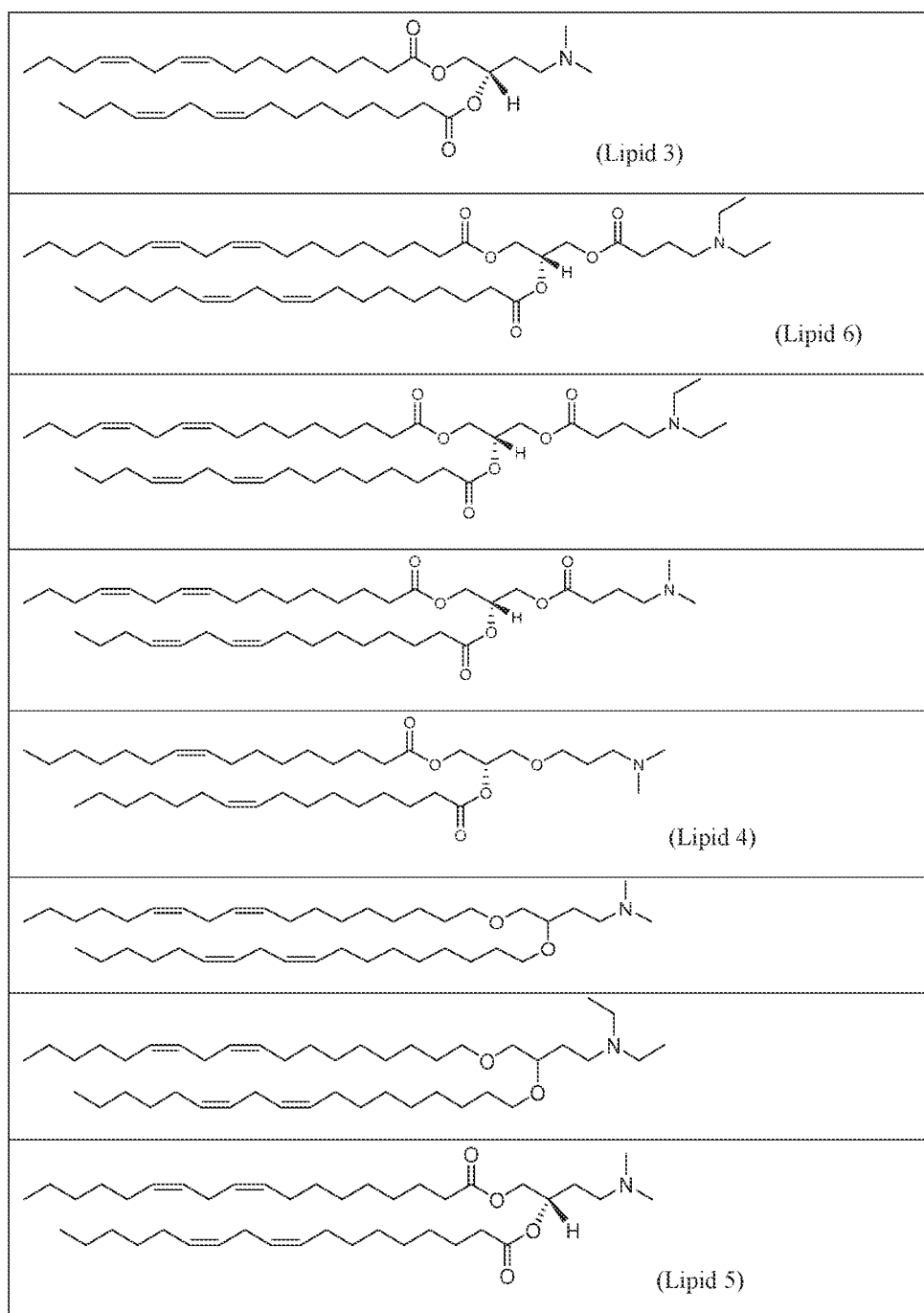
Dioleoyl-rac-glycerol, methoxypolyethylene Glycol (DOG-PEG) 1,2-Distearoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DSG), N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)]} (PEG-ceramide), DSPE-PEG-cysteine, or a derivative thereof, all with average PEG lengths between 2000-5000, with 2000, 3400, or 5000. A final composition may contain a mixture of two or more of these pegylated lipids. In certain embodiments, the LNP composition comprises a mixture of PEG-lipids with myristoyl and stearic acyl chains.

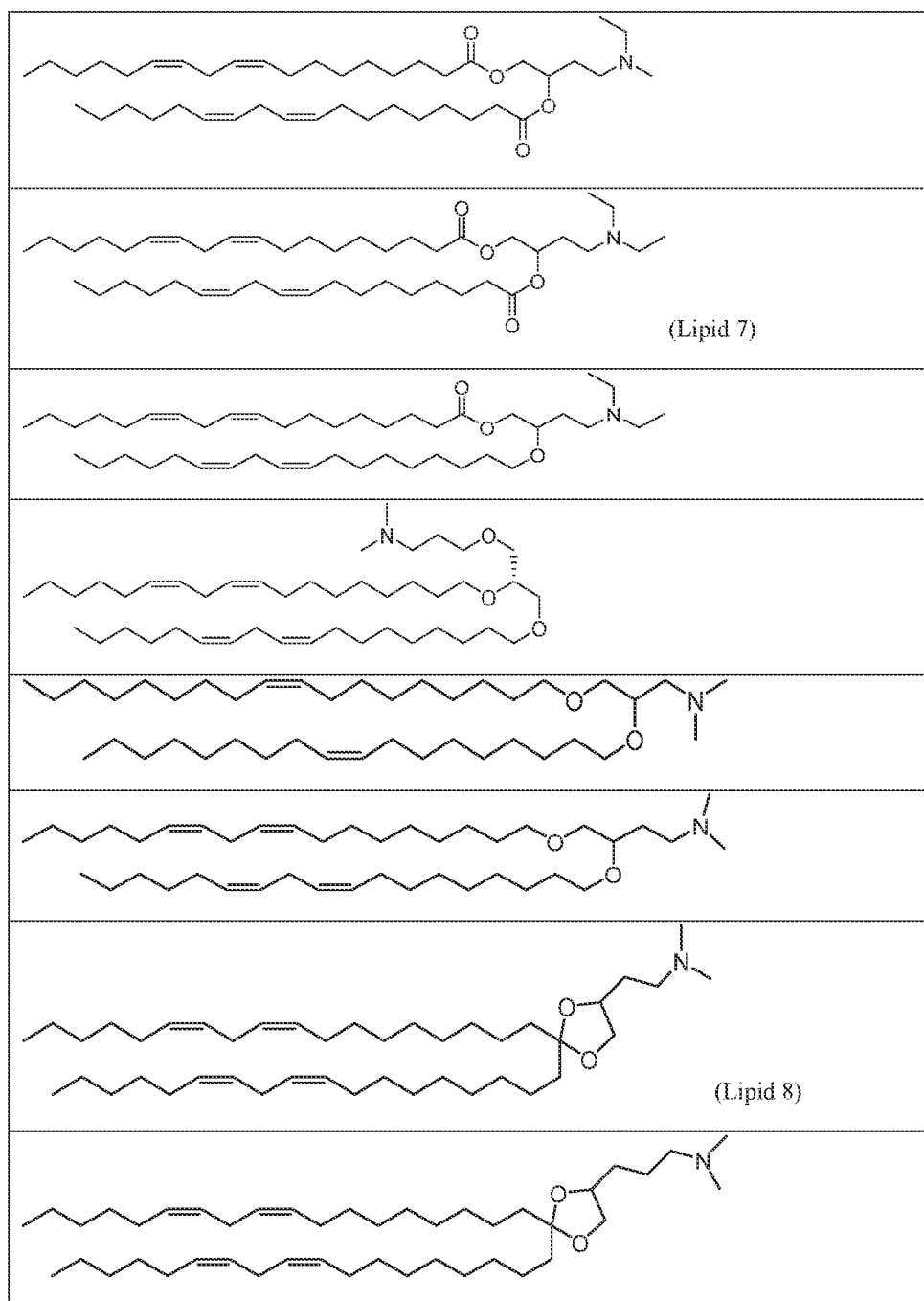
[0430] In certain embodiments, the derivative of the PEG-lipid has a hydroxyl or a carboxylic acid end group at the PEG terminus.

[0431] The lipid-immune cell targeting group conjugate can be incorporated into LNPs as described below, for example, in LNPs containing, for example, an ionizable cationic lipid, a sterol, a neutral phospholipid and a PEG-lipid. It is contemplated that, in certain embodiments, the LNPs containing the lipid-immune cell targeting group can contain an ionizable cationic lipid described herein or a cationic lipid described, for example, in U.S. Patent No. 10,221,127, 10,653,780 or U.S. Published application No. US2018/0085474, US2016/0317676, International Publication No. WO2009/086558, or Miao *et al.* (2019) NATURE BIOTECH 37:1174-1185, or Jayaraman *et al.* (2012) ANGEW CHEM INT. 51: 8529-8533. In other embodiments, the cationic lipid can be selected from an ionizable cationic lipid set forth in the Table 1.

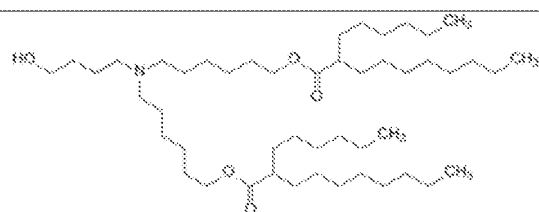
Table 1.





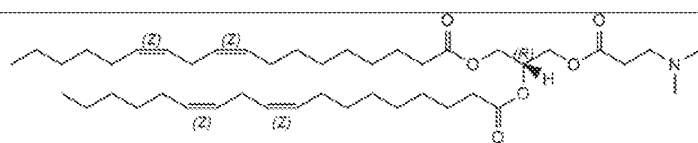


(Lipid 14)

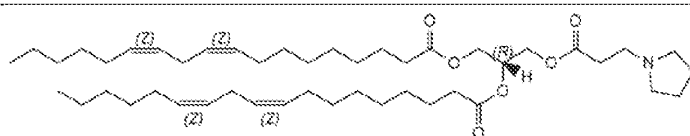


Lipid 15

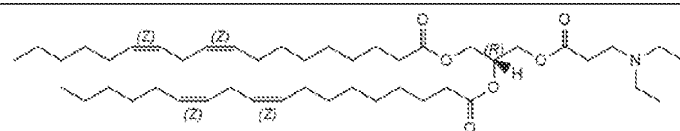
(Lipid 9)



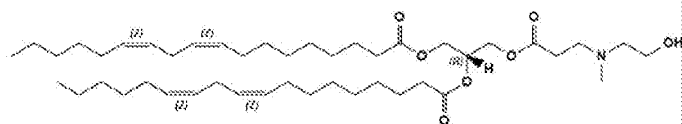
(Lipid 10)

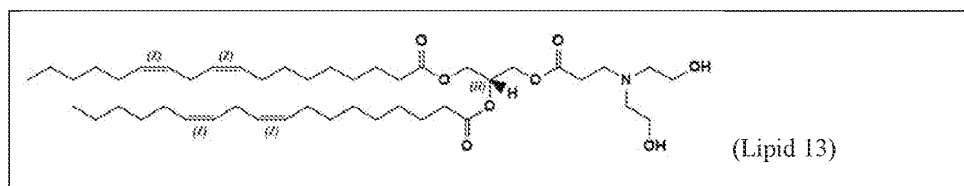


(Lipid 11)



(Lipid 12)





[0432] The LNPs can be formulated using the methods and other components described below in the following sections.

IV. LIPID NANOPARTICLE COMPOSITIONS

[0433] The invention provides a lipid nanoparticle (LNP) composition comprising a lipid blend that contains an ionizable cationic lipid described herein and/or a lipid-immune cell targeting agent conjugate described herein. In certain embodiments, the lipid blend may comprise an ionizable, cationic lipid described herein and one or more of a sterol, a neutral phospholipid, a PEG-lipid, and a lipid-immune cell targeting group conjugate.

[0434] In certain embodiments, the ionizable, cationic lipid described herein may be present in the lipid blend in a range of 30-70 mole percent, 30-60 mole percent, 30-50 mole percent, 40-70 mole percent, 40-60 mole percent, 40-50 mole percent, 50-70 mole percent, 50-60 mole percent, or of about 30 mole percent, about 35 mole percent, about 40 mole percent, about 45 mole percent, about 50 mole percent, about 55 mole percent, about 60 mole percent, about 65 mole percent, or about 70 mole percent.

STEROL

[0435] In certain embodiments, the lipid blend of the lipid nanoparticle may comprise a sterol component, for example, one or more sterols selected from the group consisting of cholesterol, fecosterol, β -sitosterol, ergosterol, campesterol, stigmasterol, stigmastanol, brassicasterol. In certain embodiments, the sterol is cholesterol.

[0436] The sterol (*e.g.*, cholesterol) may be present in the lipid blend in a range of 20-70 mole percent, 20-60 mole percent, 20-50 mole percent, 30-70 mole percent, 30-60 mole percent, 30-50 mole percent, 40-70 mole percent, 40-60 mole percent, 40-50 mole percent, 50-70 mole percent, 50-60 mole percent, or about 20 mole percent, about 25 mole percent, about 30 mole percent, about 35 mole percent, about 40 mole percent, about 45 mole percent,

about 50 mole percent, about 55 mole percent, about 60 mole percent or about 65 mole percent.

NEUTRAL PHOSPHOLIPID

[0437] In certain embodiments, the lipid blend of the lipid nanoparticle may contain one or more neutral phospholipids. The neutral phospholipid can be selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), sphingomyelin (SM).

[0438] Other neutral phospholipids can be selected from the group consisting of distearoyl-phosphatidylethanolamine (DSPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-glycero-phosphocholine (DSPC), dioleoyl-glycero-phosphoethanolamine (DOPE), dilinoleoyl-glycero-phosphocholine (DLPC), dimyristoyl-glycero-phosphocholine (DMPC), dioleoyl-glycero-phosphocholine (DOPC), dipalmitoyl-glycero-phosphocholine (DPPC), diundecanoyl-glycero-phosphocholine (DUPC), palmitoyl-oleoyl-glycero-phosphocholine (POPC), dioctadecenyl-glycero-phosphocholine, oleoyl-cholesterylhemisuccinoyl-glycero-phosphocholine, hexadecyl-glycero-phosphocholine, dilinolenoyl-glycero-phosphocholine, diarachidonoyl-glycero-3-phosphocholine, didocosahexaenoyl-glycero-phosphocholine, or sphingomyelin.

[0439] The neutral phospholipid may be present in the lipid blend in a range of 1-10 mole percent, 1-15 mole percent, 1-12 mole percent, 1-10 mole percent, 3-15 mole percent, 3-12 mole percent, 3-10 mole percent, 4-15 mole percent, 4-12 mole percent, 4-10 mole percent, 4-8 mole percent, 5-15 mole percent, 5-12 mole percent, 5-10 mole percent, 6-15 mole percent, 6-12 mole percent, 6-10 more percent, or about 1 mole percent, about 2 mole percent, about 3 mole percent, about 4 mole percent, about 5 mole percent, about 6 mole percent, about 7 mole percent, about 8 mole percent, about 9 mole percent, about 10 mole percent, about 11 mole percent, about 12 mole percent, about 13 mole percent, about 14 mole percent, or about 15 mole percent.

PEG-LIPID

[0440] The lipid blend of the lipid nanoparticle may include one or more PEG or PEG-modified lipids. Such species may be alternately referred to as PEGylated lipids. A PEG

lipid is a lipid modified with polyethylene glycol. As noted above, free PEG-lipids can be included in the lipid blend to reduce or eliminate non-specific binding via a targeting group when a lipid-immune cell targeting group is included in the lipid blend.

[0441] A PEG lipid may be selected from the non-limiting group consisting of PEG-modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides, PEG-modified dialkylamines, PEG-modified diacylglycerols, and PEG-modified dialkylglycerols. For example, a PEG lipid may be PEG- dioleoylglycerol (PEG-DOG), PEG-dimyristoyl-glycerol (PEG-DMG), PEG-dipalmitoyl-glycerol (PEG-DPG), PEG-dilinoleoyl-glycero-phosphatidyl ethanolamine (PEG-DLPE), PEG-dimyristoyl-phosphatidylethanolamine (PEG-DMPE), PEG-dipalmitoyl- phosphatidylethanolamine (PEG-DPPE), PEG-distearoylglycerol (PEG-DSG), PEG-diacylglycerol (PEG-DAG, e.g., PEG-DMG, PEG-DPG, and PEG-DSG), PEG-ceramide, PEG-distearoyl-glycero-phosphoglycerol (PEG-DSPG), PEG-dioleoyl-glycero-phosphoethanolamine (PEG-DOPE), 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide, or a PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) lipid.

[0442] In certain embodiments, the blend may contain a free PEG-lipid that can be selected from the group consisting of PEG-distearoylglycerol (PEG-DSG), PEG-diacylglycerol (PEG-DAG, e.g., PEG-DMG, PEG-DPG, and PEG-DSG), PEG-dimyristoyl-glycerol (PEG-DMG), PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) and PEG-dimyristoyl-phosphatidylethanolamine (PEG-DMPE). In some embodiments, the free PEG-lipid comprises a diacylphosphatidylcholines comprising Dipalmitoyl (C16) chain or Distearoyl (C18) chain.

[0443] The PEG-lipid may be present in the lipid blend in a range of 1-10 mole percent, 1-8 mole percent, 1-7 mole percent, 1-6 mole percent, 1-5 mole percent, 1-4 mole percent, 1-3 mole percent, 2-8 mole percent, 2-7 mole percent, 2-6 mole percent, 2-5 mole percent, 2-4 mole percent, 2-3 mole percent, or about 1 mole percent, about 2 mole percent, about 3 mole percent, about 4 mole percent, or about 5 mole percent. In some embodiments, the PEG-lipid is a free PEG-lipid.

[0444] In some embodiments, the PEG-lipid may be present in the lipid blend in the range of 0.01-10 mole percent, 0.01-5 mole percent, 0.01-4 mole percent, 0.01-3 mole percent, 0.01-2 mole percent, 0.01-1 mole percent, 0.1-10 mole percent, 0.1-5 mole percent,

0.1-4 mole percent, 0.1-3 mole percent, 0.1-2 mole percent, 0.1-1 mole percent, 0.5-10 mole percent, 0.5-5 mole percent, 0.5-4 mole percent, 0.5-3 mole percent, 0.5-2 mole percent, 0.5-1 mole percent, 1-2 mole percent, 3-4 mole percent, 4-5 mole percent, 5-6 mole percent, or 1.25-1.75 mole percent. In some embodiments, the PET-lipid may be about 0.5 mole percent, about 1 mole percent, about 1.5 mole percent, about 2 mole percent, about 2.5 mole percent, about 3 mole percent, about 3.5 mole percent, about 4 mole percent, about 4.5 mole percent, about 5 mole percent, or about 5.5 mole percent of the lipid blend. In some embodiments, the PEG-lipid is a free PEG-lipid.

[0445] In some embodiments, the lipid anchor length of PEG-lipid is C14 (as in PEG-DMG). In some embodiments, the lipid anchor length of PEG-lipid is C16 (as in DPG). In some embodiments, the lipid anchor length of PEG-lipid is C18 (as in PEG-DSG). In some embodiments, the back bone or head group of PEG-lipid is diacyl glycerol or phosphoethanolamine. In some embodiments, the PEG-lipid is a free PEG-lipid.

[0446] A LNP of the present disclosure may comprise one or more free PEG-lipid that is not conjugated to an immune cell targeting group, and a PEG-lipid that is conjugated to immune cell targeting group. In some embodiments, the free PEG-lipid comprises the same or a different lipid as the lipid in the lipid-immune cell targeting group conjugate.

IMMUNE CELL TARGETING GROUP CONJUGATE

[0447] In certain embodiments, the lipid blend can also include a lipid-immune cell targeting group conjugate as described in Section III above.

[0448] The lipid-immune cell targeting group conjugate may be present in the lipid blend in a range of 0.001-0.5 mol percent, 0.001-0.1 mole percent, 0.01-0.5 mole percent, 0.05-0.5 mole percent, 0.1-0.5 mole percent, 0.1-0.3 mole percent, 0.1-0.2 mole percent, 0.2-0.3 mole percent, of about 0.01 mole percent, about 0.05 mole percent, about 0.1 mole percent, about 0.15 mole percent, about 0.2 mole percent, about 0.25 mole percent, about 0.3 mole percent, about 0.35 mole percent, about 0.4 mole percent, about 0.45 mole percent, or about 0.5 mole percent.

[0449] In addition to the lipids present in the lipid blend, the LNP compositions may further comprise a payload, for example, a payload described hereinbelow. In certain embodiments, the payload is a nucleic acid, for example, DNA or RNA, for example, an mRNA, transfer RNA (tRNA), a microRNA, or small interfering RNA (siRNA).

[0450] In certain embodiments, the number of the nucleotides in the nucleic acid is from about 400 to about 6000.

PRODUCTION OF LIPID NANOPARTICLES

[0451] In general the LNPs are produced by using either rapid mixing by an orbital vortexer or by microfluidic mixing. Orbital vortexer mixing is accomplished by rapid addition of lipids solution in ethanol to the aqueous solution of a nucleic acid of interest followed immediately by vortexing at 2,500 rpm. Microfluidic mixing is achieved mixing the aqueous and organic streams at a controlled flow rates in a microfluidic channel using, *e.g.*, a NanoAssemblr device and microfluidic chips featuring optimized mixing chamber geometry (Precision Nanosystems, Vancouver, BC).

[0452] In certain embodiments, the resulting LNP compositions comprise a lipid blend containing, for example, from about 40 mole percent to about 60 mole percent of one or more ionizable cationic lipids described herein, from about 35 mole percent to about 50 mole percent of one or more sterols, from about 5 mole percent to about 15 mole percent of one or more neutral lipids, and from about 0.5 mole percent to about 5 mole percent of one or more PEG-lipids.

PHYSICAL PROPERTIES OF LIPID NANOPARTICLES

[0453] The characteristics of an LNP composition may depend on the components, their absolute or relative amounts, contained in a lipid nanoparticle (LNP) composition. Characteristics may also vary depending on the method and conditions of preparation of the LNP composition.

[0454] LNP compositions may be characterized by a variety of methods. For example, microscopy (*e.g.*, transmission electron microscopy or scanning electron microscopy) may be used to examine the morphology and size distribution of an LNP composition. Dynamic light scattering or potentiometry (*e.g.*, potentiometric titrations) may be used to measure zeta potentials. Dynamic light scattering may also be utilized to determine particle sizes.

Instruments such as the Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK) may also be used to measure multiple characteristics of an LNP composition, such as particle size, polydispersity index, and zeta potential. RNA encapsulated efficiency is determined by a combination of methods relying on RNA binding dyes (ribogreen, cybergreen to determine dye accessible RNA fraction) and LNP de-formulation followed by HPLC analysis for total RNA content.

[0455] In some embodiments, the LNP may have a mean diameter in the range of 1-250 nm, 1-200 nm, 1-150 nm, 1-100 nm, 50-250 nm, 50-200 nm, 50-150 nm, 50-100 nm, 75-250 nm, 75-200 nm, 75-150 nm, 75-100 nm, 100-250 nm, 100-200 nm, 100-150 nm. In certain embodiments, the LNP compositions may have a mean diameter of about 1 nm, about 10 nm, about 20 nm, about 30 nm, about 40 nm, about 50 nm, about 60 nm, about 70 nm, about 80 nm, about 90 nm, about 100 nm, about 110 nm, about 120 nm, about 130 nm, about 140 nm, about 150 nm, about 160 nm, about 170 nm, about 180 nm, about 190 nm, or about 200 nm. In some embodiments, the LNP has a mean diameter of about 100 nm.

[0456] Alternatively or in addition, the LNP compositions may have a polydispersity index in a range from 0.05-1, 0.05-0.75, 0.05-0.5, 0.05-0.4, 0.05-0.3, 0.05-0.2, 0.08-1, 0.08-0.75, 0.08-0.5, 0.08-0.4, 0.08-0.3, 0.08-0.2, 0.1-1, 0.1-0.75, 0.1-0.5, 0.1-0.4, 0.1-0.3, 0.1-0.2. In certain embodiments, the polydispersity index is in the range of 0.1-0.25, 0.1-0.2, 0.1-0.19, 0.1-0.18, 0.1-0.17, 0.1-0.16, or 0.1-0.15.

[0457] Alternatively or in addition, the LNP compositions may have a zeta potential of about -30 mV to about +30 mV. In certain embodiments, the LNP composition has a zeta potential of about -10 mV to about +20 mV. The zeta potential may vary as a function of pH. As a result, in certain embodiments, the LNP compositions may have a zeta potential of about -10 mV to about +30 mV or about 0 mV to +30 mV or about +5 mV to about +30 mV at pH 5.5 or pH 5, and/or a zeta potential of about -30 mV to about +5 mV or about -20 mV to about +15 mV at pH 7.4.

V. PAYLOADS

[0458] The LNP compositions may comprise an agent, for example, a nucleic acid molecule for delivery to a cell (e.g., an immune cell) or tissue, for example, a cell (e.g., an immune cell) or tissue in a subject.

[0459] The LNP compositions of the present invention may include a nucleic acid, for example, a DNA or RNA, such as an mRNA, tRNA, microRNA, siRNA, or dicer substrate siRNA. It is contemplated that nucleic acids can contain naturally occurring components, such as, naturally occurring bases, sugars or linkage groups (*e.g.*, phosphodiester linkage groups) or may contain non-naturally occurring components or modifications, (*e.g.*, thioester linkage groups). For example, the nucleic acid can be synthesized to contain base, sugar, linker modifications known to those skilled in the art. Furthermore, the nucleic acids can be linear or circular, or have any desired configuration. The LNP compositions can include multiple nucleic acid molecules, for example, multiple RNA molecules, which can be the same or different.

[0460] In certain embodiments, the payload is an mRNA. In certain embodiments, a particular LNP composition may contain a number mRNA molecules that can be the same or different. In certain embodiments, one or more LNP compositions including one or more different mRNAs may be combined, and/or simultaneously contacted, with a cell. It is contemplated that an mRNA may include one or more of a stem loop, a chain terminating nucleoside, a polyA sequence, a polyadenylation signal, and/or a 5' cap structure. The mRNA may encode a receptor, such as a chimeric antigen receptor (CAR), for use in for example, an immune disorder, inflammatory disorder or cancer. In addition, the mRNA may encode an antigen for use in a therapeutic or prophylactic vaccine, for example, for treating or preventing an infection by a pathogen, for example, a microbial or viral pathogen, or for reducing or ameliorating the side effects caused directly or indirectly by such an infection.

[0461] In certain embodiments, the LNP composition may include one or more other components including, but not limited to, one or more pharmaceutically acceptable excipients, small hydrophobic molecules, therapeutic agents, carbohydrates, polymers, permeability enhancing molecules, and surface altering agents.

[0462] In some embodiments, the wt/wt ratio of the lipid component to the payload (*e.g.*, mRNA) in the resulting LNP composition is from about 1:1 to about 50:1. In certain embodiments, the wt/wt ratio of the lipid component to the payload (*e.g.*, mRNA) in the resulting composition is from about 5:1 to about 50:1. In certain embodiments, the wt/wt ratio is from about 5:1 to about 40:1. In certain embodiments, the wt/wt ratio is from about 10:1 to about 40:1. In certain embodiments, the wt/wt ratio is from about 15:1 to about 25:1.

[0463] In certain embodiments, the encapsulation efficiency of the payload (e.g., mRNA) in the lipid nanoparticles is at least 50%. In certain embodiments, the encapsulation efficiency is at least 80%, at least 90% or, or greater than 90%.

RNA PAYLOAD

[0464] In certain embodiments, the RNA payload is an mRNA, tRNA, microRNA, or siRNA payload.

[0465] In certain embodiments, the lipid nanoparticle compositions are optimized for the delivery of RNA, e.g., mRNA, to a target cell for translation within the cell. An mRNA may be a naturally or non-naturally occurring mRNA. An mRNA may include one or more modified nucleobases, nucleosides, or nucleotides.

[0466] The nucleobases may be selected from the non-limiting group consisting of adenine, guanine, uracil, cytosine, 7-methylguanine, 5-methylcytosine, 5-hydroxymethylcytosine, thymine, pseudouracil, dihydrouracil, hypoxanthine, and xanthine.

[0467] A nucleoside of an mRNA is a compound including a sugar molecule (e.g., a 5-carbon or 6-carbon sugar, such as pentose, ribose, arabinose, xylose, glucose, galactose, or a deoxy derivative thereof) in combination with a nucleobase. A nucleoside may be a canonical nucleoside (e.g., adenosine, guanosine, cytidine, uridine, 5-methyluridine, deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine, and thymidine) or an analog thereof and may include one or more substitutions or modifications.

[0468] A nucleotide of an mRNA is a compound containing a nucleoside and a phosphate group or alternative group (e.g., boranophosphate, thiophosphate, selenophosphate, phosphonate, alkyl group, amidate, and glycerol). A nucleotide may be a canonical nucleotide (e.g., adenosine, guanosine, cytidine, uridine, 5-methyluridine, deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine, and thymidine monophosphates) or an analog thereof and may include one or more substitutions or modifications including but not limited to alkyl, aryl, halo, oxo, hydroxyl, alkyloxy, and/or thio substitutions; one or more fused or open rings; oxidation; and/or reduction of the nucleobase, sugar, and/or phosphate or alternative component. A nucleotide may include one or more phosphate or alternative groups. For example, a nucleotide may include a nucleoside and a triphosphate group. A "nucleoside triphosphate" (e.g., guanosine triphosphate, adenosine triphosphate, cytidine

triphosphate, and uridine triphosphate) may refer to the canonical nucleoside triphosphate or an analog or derivative thereof and may include one or more substitutions or modifications as described herein.

[0469] An mRNA may include a 5' untranslated region, a 3' untranslated region, and/or a coding or translating sequence. An mRNA may include any number of base pairs, including tens, hundreds, or thousands of base pairs. Any number (e.g., all, some, or none) of nucleobases, nucleosides, or nucleotides may be an analog of a canonical species, substituted, modified, or otherwise non-naturally occurring. In certain embodiments, all of a particular nucleobase type may be modified. For example, all cytosine in an mRNA may be 5-methylcytosine.

[0470] In certain embodiments, an mRNA may include a 5' cap structure, a chain terminating nucleotide, a stem loop, a polyA sequence, and/or a polyadenylation signal.

[0471] A cap structure or cap species is a compound including two nucleoside moieties joined by a linker and may be selected from a naturally occurring cap, a non-naturally occurring cap or a cap analog. A cap species may include one or more modified nucleosides and/or linker moieties. For example, a natural mRNA cap may include a guanine nucleotide and a guanine (G) nucleotide methylated at the 7 position joined by a triphosphate linkage at their 5' positions, e.g., m7G(5')ppp(5')G, commonly written as m7GpppG. A cap species may also be an anti-reverse cap analog. A non-limiting list of possible cap species includes m7GpppG, m7Gpppm7G, m73'dGpppG, m7Gpppm7G, m73'dGpppG, and m27 02'GppppG.

[0472] Alternatively or in addition, an mRNA may include a chain terminating nucleoside. For example, a chain terminating nucleoside may include those nucleosides deoxygenated at the 2' and/or 3' positions of their sugar group. Such species may include 3'-deoxyadenosine (cordycepin), 3'-deoxyuridine, 3'-deoxycytosine, 3'-deoxyguanosine, 3'-deoxythymine, and 2',3'-dideoxynucleosides, such as 2',3'-dideoxyadenosine, 2',3'-dideoxyuridine, 2',3'-dideoxycytosine, 2',3'-dideoxyguanosine, and 2',3'-dideoxythymine.

[0473] Alternatively or in addition, an mRNA may include a stem loop, such as a histone stem loop. A stem loop may include 1, 2, 3, 4, 5, 6, 7, 8, or more nucleotide base pairs. For example, a stem loop may include 4, 5, 6, 7, or 8 nucleotide base pairs. A stem loop may be located in any region of an mRNA. For example, a stem loop may be located in, before, or

after an untranslated region (a 5' untranslated region or a 3' untranslated region), a coding region, or a polyA sequence or tail.

[0474] Alternatively or in addition, an mRNA may include a polyA sequence and/or polyadenylation signal. A polyA sequence may be comprised entirely or mostly of adenine nucleotides or analogs or derivatives thereof. A polyA sequence may be a tail located adjacent to a 3' untranslated region of an mRNA.

[0475] An mRNA may encode any polypeptide of interest, including any naturally or non-naturally occurring or otherwise modified polypeptide. A polypeptide encoded by an mRNA may be of any size and may have any secondary structure or activity. In some embodiments, a polypeptide encoded by an mRNA may have a therapeutic effect when expressed in a cell. In some embodiments, the mRNA may encode an antibody, enzyme, growth factor, hormone, cytokine, viral protein (*e.g.*, a viral capsid protein), antigen, vaccine, or receptor. In some embodiments, the mRNA may encode an engineered receptor such as a CAR or an antigen for use in a therapeutic vaccine (*e.g.*, a cancer vaccine) or a prophylactic vaccine (*e.g.*, a vaccine for minimizing the risk or severity of an infection by a microbial or viral pathogen). In some embodiments, the mRNA encodes a polypeptide capable of regulating immune response in the immune cell. In some embodiments, the mRNA encodes a polypeptide capable of reprogramming the immune cell. In some embodiments, the mRNA encodes a synthetic T cell receptor (synTCR) or a Chimeric Antigen Receptor (CAR).

[0476] A lipid composition may be designed for one or more specific applications or targets. For example, an LNP composition may be designed to deliver mRNA to a particular cell, tissue, organ, or system or group thereof in a mammal's body, such as the renal system. Physiochemical properties of LNP compositions may be altered in order to increase selectivity for particular target site within a subject. For instance, particle sizes may be adjusted based on the fenestration sizes of different organs. The mRNA included in an LNP composition may also depend on the desired delivery target or targets. For example, an mRNA may be selected for a particular indication, condition, disease, or disorder and/or for delivery to a particular cell, tissue, organ, or system or group thereof (*e.g.*, localized or specific delivery).

[0477] The amount of mRNA in a lipid composition may depend on the size, sequence, and other characteristics of the mRNA. The amount of mRNA in an LNP may also depend

on the size, composition, desired target, and other characteristics of the LNP composition. The relative amounts of mRNA and other elements (e.g., lipids) may also vary. The amount of mRNA in an LNP composition may, for example, be measured using absorption spectroscopy (e.g., ultraviolet-visible spectroscopy).

[0478] In some embodiments, the one or more mRNAs, lipids, and polymers and amounts thereof may be selected to provide a specific N:P ratio (the ratio of positively-chargeable lipid or polymer amine (N = nitrogen) groups to negatively-charged nucleic acid phosphate (P) groups). The N:P ratio of the composition refers to the molar ratio of nitrogen atoms in one or more lipids to the number of phosphate groups in an mRNA. In general, a lower N:P ratio is preferred. A N:P ratio may be dependent on a specific lipid and its pKa. In certain embodiments, the mRNA and LNP composition, and/or their relative amounts may be selected to provide an N:P ratio from about 1:1 to about 30:1, or from about 1:1 to about 20:1. In certain embodiments, the N:P ratio can be, for example, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, or 8:1. In certain embodiments, the N:P ratio may be from about 2:1 to about 5:1. In certain embodiments, the N:P ratio may be about 4:1. In other embodiments, the N:P ratio is from about 4:1 to about 8:1. For example, the N:P ratio may be about 4:1, about 4.5:1, about 4.6:1, about 4.7:1, about 4.8:1, about 4.9:1, about 5.0:1, about 5.1:1, about 5.2:1, about 5.3:1, about 5.4:1, about 5.5:1, about 5.6:1, about 5.7:1, about 6.0:1, about 6.5:1, or about 7.0:1.

[0479] The amount of mRNA in a nanoparticle composition may depend on the size, sequence, and other characteristics of the mRNA. The amount of mRNA in a nanoparticle composition may also depend on the size, composition, desired target, and other characteristics of the nanoparticle composition. The relative amounts of mRNA and other elements (e.g., lipids) may also vary. In some embodiments, the wt/wt ratio of the lipid component to an mRNA in a nanoparticle composition may be from about 5:1 to about 50:1, such as 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 16:1, 17:1, 18:1, 19:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, and 50:1. For example, the wt/wt ratio of the lipid component to an mRNA may be from about 10:1 to about 40:1. The amount of mRNA in a nanoparticle composition may, for example, be measured using absorption spectroscopy (e.g., ultraviolet-visible spectroscopy).

[0480] The efficiency of encapsulation of an mRNA describes the amount of mRNA that is encapsulated or otherwise associated with a lipid composition 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 mRNA in a solution containing the lipid composition before and after breaking up the LNP composition with one or more organic solvents or detergents. Fluorescence may be used to measure the amount of free mRNA in a solution. For the LNP compositions of the invention, the encapsulation efficiency of an mRNA 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 certain embodiments, the encapsulation efficiency may be at least 80%.

VI. FORMULATION AND MODE OF DELIVERY

[0481] LNP compositions of the invention may be formulated in whole or in part as a pharmaceutical composition. The pharmaceutical compositions may further include one or more pharmaceutically acceptable excipients or accessory ingredients such as those described herein. General guidelines for the formulation and manufacture of pharmaceutical compositions and agents are available, for example, in Remington's (2006) *supra*. Conventional excipients and accessory ingredients may be used in any pharmaceutical composition of the invention, except insofar as any conventional excipient or accessory ingredient may be incompatible with one or more components of an LNP composition of the invention. An excipient or accessory ingredient may be incompatible with a component of an LNP composition if its combination with the component may result in any undesirable biological effect or otherwise deleterious effect.

[0482] In some embodiments, one or more excipients or accessory ingredients may make up greater than 50% of the total mass or volume of a pharmaceutical composition including an LNP composition of the invention. For example, the one or more excipients or accessory ingredients may make up 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of a pharmaceutical composition. In certain embodiments, the excipient is approved for use in humans and for veterinary use, for example, by United States Food and Drug Administration. In certain embodiments, the excipient is pharmaceutical grade. In certain embodiments, an excipient meets the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia.

[0483] Relative amounts of the one or more lipids or LNPs, one or more pharmaceutically acceptable excipients, and/or any additional ingredients in a pharmaceutical

composition will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered.

[0484] Lipid compositions and/or pharmaceutical compositions including one or more LNP compositions may be administered to any subject, including a human patient that may benefit from a therapeutic effect provided by the delivery of a nucleic acid, *e.g.*, an RNA (*e.g.*, mRNA, tRNA or siRNA) to one or more particular cells, tissues, organs, or systems or groups thereof, such as the renal system. Although the descriptions provided herein of LNP compositions and pharmaceutical compositions including LNP compositions are principally directed to compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other mammal. Modification of compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is understood.

[0485] A pharmaceutical composition in accordance with the present disclosure may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient (*e.g.*, the payload).

[0486] Pharmaceutical compositions of the invention may be prepared in a variety of forms suitable for a variety of routes and methods of administration. For example, pharmaceutical compositions of the invention may be prepared in liquid dosage forms (*e.g.*, emulsions, microemulsions, nanoemulsions, solutions, suspensions, syrups, and elixirs), injectable forms, solid dosage forms (*e.g.*, capsules, tablets, pills, powders, and granules), dosage forms for topical and/or transdermal administration (*e.g.*, ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants, and patches), suspensions, powders, and other forms.

[0487] Liquid dosage forms for oral and parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, nanoemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-

butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents.

[0488] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

[0489] Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

Other Components

[0490] In addition, it is contemplated that the pharmaceutical compositions may include one or more components in addition to those described hereinabove.

[0491] The pharmaceutical compositions may also include one or more permeability enhancer molecules, carbohydrates, polymers, therapeutic agents, surface altering agents, or other components. A permeability enhancer molecule may be a molecule described, for example, in U.S. patent application publication No. 2005/0222064. Carbohydrates may include simple sugars (e.g., glucose) and polysaccharides (e.g., glycogen and derivatives and analogs thereof).

[0492] The pharmaceutical compositions may also contain a surface altering agent, including for example, anionic proteins (e.g., bovine serum albumin), surfactants (e.g., cationic surfactants such as dimethyldioctadecyl-ammonium bromide), sugars or sugar

derivatives (e.g., cyclodextrin), polymers (e.g., heparin, polyethylene glycol, and poloxamer), mucolytic agents (e.g., acetylcysteine, mugwort, bromelain, papain, clerodendrum, bromhexine, carbocysteine, eprazinone, mesna, ambroxol, sobrerol, domiodol, letosteine, stepronin, tiopronin, gelsolin, thymosin β 4, dornase alfa, neltexine, and erdosteine), and DNases (e.g., rhDNase). A surface altering agent may be disposed within and/or upon the surface of a composition described herein.

[0493] In addition to these components, a pharmaceutical composition containing an LNP composition of the invention may include any substance useful in pharmaceutical compositions. For example, the pharmaceutical composition may include one or more pharmaceutically acceptable excipients or accessory ingredients such as, but not limited to, one or more solvents, dispersion media, diluents, dispersion aids, suspension aids, granulating aids, disintegrants, fillers, glidants, liquid vehicles, binders, surface active agents, isotonic agents, thickening or emulsifying agents, buffering agents, lubricating agents, oils, preservatives, and other species. Excipients such as waxes, butters, coloring agents, coating agents, flavorings, and perfuming agents may also be included. Pharmaceutically acceptable excipients are well known in the art (*see, e.g., Remington's (2006) supra*).

[0494] Dispersing agents may be selected from the non-limiting list consisting of potato starch, corn starch, tapioca starch, sodium starch glycolate, clays, alginic acid, guar gum, citrus pulp, agar, bentonite, cellulose and wood products, natural sponge, cation-exchange resins, calcium carbonate, silicates, sodium carbonate, cross-linked poly(vinyl-pyrrolidone) (crospovidone), sodium carboxymethyl starch (sodium starch glycolate), carboxymethyl cellulose, cross-linked sodium carboxymethyl cellulose (croscarmellose), methylcellulose, pregelatinized starch (starch 1500), microcrystalline starch, water insoluble starch, calcium carboxymethyl cellulose, magnesium aluminum silicate (VEEGUM®), sodium lauryl sulfate, quaternary ammonium compounds, and/or combinations thereof.

[0495] Surface active agents and/or emulsifiers may include, but are not limited to, natural emulsifiers (e.g. acacia, agar, alginic acid, sodium alginate, tragacanth, chondrux, cholesterol, xanthan, pectin, gelatin, egg yolk, casein, wool fat, cholesterol, wax, and lecithin), colloidal clays (e.g. bentonite [aluminum silicate] and VEEGUM® [magnesium aluminum silicate]), long chain amino acid derivatives, high molecular weight alcohols (e.g. stearyl alcohol, cetyl alcohol, oleyl alcohol, triacetin monostearate, ethylene glycol distearate, glyceryl monostearate, and propylene glycol monostearate, polyvinyl alcohol), carbomers

(e.g. carboxy polymethylene, polyacrylic acid, acrylic acid polymer, and carboxyvinyl polymer), carrageenan, cellulosic derivatives (e.g. carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose), sorbitan fatty acid esters (e.g. polyoxyethylene sorbitan monolaurate [TWEEN®20], polyoxyethylene sorbitan [TWEEN® 60], polyoxyethylene sorbitan monooleate [TWEEN®80], sorbitan monopalmitate [SPAN®40], sorbitan monostearate [SPAN®60], sorbitan tristearate [SPAN®65], glyceryl monooleate, sorbitan monooleate [SPAN®80]), polyoxyethylene esters (e.g. polyoxyethylene monostearate [MYRJ® 45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxymethylene stearate, and SOLUTOL®), sucrose fatty acid esters, polyethylene glycol fatty acid esters (e.g. CREMOPHOR®), polyoxyethylene ethers, (e.g. polyoxyethylene lauryl ether [BRIJ® 30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate, PLURONIC®F 68, POLOXAMER® 188, cetrimonium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, and/or combinations thereof.

[0496] Examples of preservatives may include, but are not limited to, antioxidants, chelating agents, antimicrobial preservatives, antifungal preservatives, alcohol preservatives, acidic preservatives, and/or other preservatives. Examples of antioxidants include, but are not limited to, alpha tocopherol, ascorbic acid, acorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, monothioglycerol, potassium metabisulfite, propionic acid, propyl gallate, sodium ascorbate, sodium bisulfite, sodium metabisulfite, and/or sodium sulfite. Examples of chelating agents include ethylenediaminetetraacetic acid (EDTA), citric acid monohydrate, disodium edetate, dipotassium edetate, edetic acid, fumaric acid, malic acid, phosphoric acid, sodium edetate, tartaric acid, and/or trisodium edetate. Examples of antimicrobial preservatives include, but are not limited to, benzalkonium chloride, benzethonium chloride, benzyl alcohol, bronopol, cetrimide, cetylpyridinium chloride, chlorhexidine, chlorobutanol, chlorocresol, chloroxylenol, cresol, ethyl alcohol, glycerin, hexetidine, imidurea, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric nitrate, propylene glycol, and/or thimerosal. Examples of antifungal preservatives include, but are not limited to, butyl paraben, methyl paraben, ethyl paraben, propyl paraben, benzoic acid, hydroxybenzoic acid, potassium benzoate, potassium sorbate, sodium benzoate, sodium propionate, and/or sorbic acid. Examples of alcohol preservatives include, but are not limited to, ethanol, polyethylene glycol, benzyl alcohol, phenol, phenolic compounds, bisphenol,

chlorobutanol, hydroxybenzoate, and/or phenylethyl alcohol. Examples of acidic preservatives include, but are not limited to, vitamin A, vitamin C, vitamin E, beta-carotene, citric acid, acetic acid, dehydroascorbic acid, ascorbic acid, sorbic acid, and/or phytic acid. Other preservatives include, but are not limited to, tocopherol, tocopherol acetate, deteroxime mesylate, cetrimide, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethylenediamine, sodium lauryl sulfate (SLS), sodium lauryl ether sulfate (SLES), sodium bisulfite, sodium metabisulfite, potassium sulfite, potassium metabisulfite.

[0497] Examples of buffering agents include, but are not limited to, citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium gluconate, calcium gluceptate, calcium gluconate, d-gluconic acid, calcium glycerophosphate, calcium lactate, calcium lactobionate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, amino-sulfonate buffers (e.g. HEPES), magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol, and/or combinations thereof.

[0498] In certain embodiments, the lipid nanoparticle compositions and formulations thereof are adapted for administration intravenously, intramuscularly, intradermally, subcutaneously, intra-arterially, intra-tumor, or by inhalation. In certain embodiments, a dose of about 0.001 mg/kg to about 10 mg/kg is administered to a subject. Compositions in accordance with the present disclosure may be formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of a composition of the present disclosure will be decided by an attending physician within the scope of sound medical judgment.

[0499] The specific therapeutically effective, prophylactically effective, or otherwise appropriate dose level (e.g., for imaging) for any particular patient will depend upon a variety of factors including the severity and identify of a disorder being treated, if any; the one or more mRNAs employed; the specific composition employed; the age, body weight, general health, sex, and diet of the patient; the time of administration, route of administration, and

rate of excretion of the specific pharmaceutical composition employed; the duration of the treatment; drugs used in combination or coincidental with the specific pharmaceutical composition employed; and like factors well known in the medical arts.

VII. METHODS

[0500] The present disclosure provides methods of delivering a payload to a target cell or tissue, for example, a target cell or tissue in a subject, and LNPs or pharmaceutical compositions containing the LNPs for use in such methods.

[0501] In certain embodiments, the invention provides a method of producing a polypeptide of interest (e.g., a protein of interest) in a mammalian cell, and LNPs or pharmaceutical compositions containing the LNPs for use in such methods. Methods of producing polypeptides in such a cell involve contacting a cell with an LNP composition comprising an RNA of interest (e.g., an mRNA encoding the polypeptide of interest (e.g., a protein of interest). Upon contacting the cell with the LNP composition, the mRNA may be taken up and translated in the cell to produce the polypeptide of interest.

[0502] In general, the step of contacting a mammalian cell with an LNP composition including an mRNA encoding a polypeptide of interest may be performed *in vivo*, *ex vivo*, or *in vitro*. The amount of an LNP composition contacted with a cell, and/or the amount of mRNA therein, may depend on the type of cell or tissue being contacted, the means of administration, the physiochemical characteristics of the LNP composition and the mRNA (e.g., size, charge, and chemical composition) therein, and other factors. In general, an effective amount of the LNP composition will allow for efficient polypeptide production in the cell. Metrics for efficiency may include polypeptide translation (indicated by polypeptide expression), level of mRNA degradation, and immune response indicators.

[0503] The step of contacting an LNP composition including an mRNA with a cell may involve or cause transfection where the LNP composition may fuse with the membrane of cell to permit the delivery of the mRNA into the cell. Upon introduction into the cytoplasm of the cell, the mRNA is then translated into a protein or peptide via the protein synthesis machinery within the cytoplasm of the cell.

[0504] In certain embodiments, the LNP compositions described herein may be used to deliver therapeutic or prophylactic agents to a subject. For example, an mRNA included in an LNP composition may encode a polypeptide and produce the therapeutic or prophylactic

polypeptide upon contacting and/or entry (e.g., transfection) into a cell. In certain embodiments, an mRNA included in an LNP composition of the invention may encode a polypeptide that may improve or increase the immunity of a subject.

[0505] In certain embodiments, contacting a cell with an LNP composition including an mRNA may reduce the innate immune response of a cell to an exogenous nucleic acid. A cell may be contacted with a first LNP composition including a first amount of a first exogenous mRNA including a translatable region and the level of the innate immune response of the cell to the first exogenous mRNA may be determined. Subsequently, the cell may be contacted with a second composition including a second amount of the first exogenous mRNA, the second amount being a lesser amount of the first exogenous mRNA compared to the first amount. Alternatively, the second composition may include a first amount of a second exogenous mRNA that is different from the first exogenous mRNA. The steps of contacting the cell with the first and second compositions may be repeated one or more times.

[0506] Additionally, efficiency of polypeptide production in the cell may be optionally determined, and the cell may be re-contacted with the first and/or second composition repeatedly until a target protein production efficiency is achieved.

[0507] The present disclosure provides methods of delivering a nucleic acid (e.g., an mRNA) to a mammalian cell or tissue, for example, a mammalian cell or tissue in a subject. Delivery of an mRNA to such a cell or tissue involves administering an LNP composition including the mRNA to a subject, for example, by injection, e.g., via intramuscular injection or intravascular delivery into the subject. After administration, the LNP can target and/or contact a cell, for example, an immune cell, such as a T-cell. Upon contacting the cell with the LNP composition, a translatable mRNA may be translated in the cell to produce a polypeptide of interest.

[0508] In certain embodiments, an LNP composition of the invention may target a particular type or class of cells. This targeting may be facilitated using the lipids described herein to form LNPs, which may also include a targeting group for targeting cells of interest. In certain, embodiments, specific delivery may result in a greater than 2 fold, 5 fold, 10 fold, 15 fold, or 20 fold increase in the amount of mRNA to the targeted destination (e.g., cells that express or express at high levels the receptor of interest which binds to the immune cell

targeting group of the LNPs) as compared to another destinations (e.g., cells that either do not express or only express at low levels the receptor of interest).

[0509] LNP compositions of the invention may be useful for treating a disease, disorder, or condition characterized by missing or aberrant protein or polypeptide activity. Upon delivery of an mRNA encoding the missing or aberrant polypeptide to a cell, translation of the mRNA may produce the polypeptide, thereby reducing or eliminating an issue caused by the absence of or aberrant activity caused by the polypeptide. Because translation may occur rapidly, the methods and compositions of the invention may be useful in the treatment of acute diseases, disorders, or conditions such as sepsis, stroke, and myocardial infarction. An mRNA included in an LNP composition of the invention may also be capable of altering the rate of transcription of a given species, thereby affecting gene expression.

[0510] Diseases, disorders, and/or conditions characterized by dysfunctional or aberrant protein or polypeptide activity for which a composition of the invention may be administered include, but are not limited to, cancer and proliferative diseases, genetic diseases (e.g., cystic fibrosis), autoimmune diseases, diabetes, neurodegenerative diseases, cardio- and reno-vascular diseases, and metabolic diseases. Multiple diseases, disorders, and/or conditions may be characterized by missing (or substantially diminished such that proper protein function does not occur) protein activity. Such proteins may not be present, or they may be essentially non-functional. A specific example of a dysfunctional protein is the missense mutation variants of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which produce a dysfunctional protein variant of CFTR protein, which causes cystic fibrosis. The present disclosure provides a method for treating such diseases, disorders, and/or conditions in a subject by administering an LNP composition including an mRNA and a lipid component including KL10, a phospholipid (optionally unsaturated), a PEG lipid, and a structural lipid, wherein the mRNA encodes a polypeptide that antagonizes or otherwise overcomes an aberrant protein activity present in the cell of the subject.

[0511] The therapeutic and/or prophylactic compositions described herein may be administered to a subject using any reasonable amount and any route of administration effective for preventing, treating, diagnosing, or imaging a disease, disorder, and/or condition and/or any other purpose. The specific amount administered to a given subject may vary depending on the species, age, and general condition of the subject, the purpose of the administration, the particular composition, the mode of administration, and the like.

Compositions in accordance with the present disclosure may be formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of a composition of the present disclosure will be decided by an attending physician within the scope of sound medical judgment.

[0512] A LNP composition including one or more mRNAs may be administered by a variety of routes, for example, orally, intravenously, intramuscularly, intra-arterially, intramedullary, intrathecal, subcutaneously, intraventricularly, trans- or intra-dermally, intradermally, rectally, intravaginally, intraperitoneally, topically, mucosally, nasally, intratumorally. In certain embodiments, an LNP composition may be administered intravenously, intramuscularly, intradermally, intra-arterially, intratumorally, or subcutaneously. However, the present disclosure encompasses the delivery of LNP compositions of the invention by any appropriate route taking into consideration likely advances in the sciences of drug delivery. In general, the most appropriate route of administration will depend upon a variety of factors including the nature of the LNP composition including one or more mRNAs (*e.g.*, its stability in various bodily environments such as the bloodstream and gastrointestinal tract), the condition of the patient (*e.g.*, whether the patient is able to tolerate particular routes of administration), etc.

[0513] In certain embodiments, compositions in accordance with the present disclosure may be administered at dosage levels sufficient to deliver from about 0.0001 mg/kg to about 10 mg/kg, from about 0.001 mg/kg to about 10 mg/kg, from about 0.005 mg/kg to about 10 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.05 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, from about 1 mg/kg to about 10 mg/kg, from about 2 mg/kg to about 10 mg/kg, from about 5 mg/kg to about 10 mg/kg, from about 0.0001 mg/kg to about 5 mg/kg, from about 0.001 mg/kg to about 5 mg/kg, from about 0.005 mg/kg to about 5 mg/kg, from about 0.01 mg/kg to about 5 mg/kg, from about 0.05 mg/kg to about 5 mg/kg, from about 0.1 mg/kg to about 5 mg/kg, from about 1 mg/kg to about 5 mg/kg, from about 2 mg/kg to about 5 mg/kg, from about 0.0001 mg/kg to about 2.5 mg/kg, from about 0.001 mg/kg to about 2.5 mg/kg, from about 0.005 mg/kg to about 2.5 mg/kg, from about 0.01 mg/kg to about 2.5 mg/kg, from about 0.05 mg/kg to about 2.5 mg/kg, from about 0.1 mg/kg to about 2.5 mg/kg, from about 1 mg/kg to about 2.5 mg/kg, from about 2 mg/kg to about 2.5 mg/kg, from about 0.0001 mg/kg to about 1 mg/kg, from about 0.001 mg/kg to about 1 mg/kg, from about 0.005 mg/kg to about 1 mg/kg, from about 0.01 mg/kg

to about 1 mg/kg, from about 0.05 mg/kg to about 1 mg/kg, from about 0.1 mg/kg to about 1 mg/kg, from about 0.0001 mg/kg to about 0.25 mg/kg, from about 0.001 mg/kg to about 0.25 mg/kg, from about 0.005 mg/kg to about 0.25 mg/kg, from about 0.01 mg/kg to about 0.25 mg/kg, from about 0.05 mg/kg to about 0.25 mg/kg, or from about 0.1 mg/kg to about 0.25 mg/kg of a composition in a given dose, where a dose of 1 mg/kg provides 1 mg of a composition per 1 kg of subject body weight.

[0514] In particular embodiments, a dose of about 0.001 mg/kg to about 10 mg/kg of an LNP composition of the invention may be administered. In other embodiments, a dose of about 0.005 mg/kg to about 2.5 mg/kg of an LNP composition may be administered. In certain embodiments, a dose of about 0.1 mg/kg to about 1 mg/kg may be administered. In other embodiments, a dose of about 0.05 mg/kg to about 0.25 mg/kg may be administered. A dose may be administered one or more times per day, in the same or a different amount, to obtain a desired level of mRNA expression and/or therapeutic, diagnostic, prophylactic, or imaging effect. The desired dosage may be delivered, for example, three times a day, two times a day, once a day, every other day, every third day, every week, every two weeks, every three weeks, or every four weeks. In certain embodiments, the desired dosage may be delivered using multiple administrations (e.g., two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more administrations). In some embodiments, a single dose may be administered, for example, prior to or after a surgical procedure or in the instance of an acute disease, disorder, or condition.

[0515] LNP compositions including one or more mRNAs may be used in combination with one or more other therapeutic, prophylactic, diagnostic, or imaging agents. By "in combination with," it is not intended to imply that the agents must be administered at the same time and/or formulated for delivery together, although these methods of delivery are within the scope of the present disclosure. For example, one or more LNP compositions including one or more different mRNAs may be administered in combination. Compositions can be administered concurrently with, prior to, or subsequent to, one or more other desired therapeutics or medical procedures. In general, each agent will be administered at a dose and/or on a time schedule determined for that agent. In some embodiments, the present disclosure encompasses the delivery of compositions of the invention, or imaging, diagnostic, or prophylactic compositions thereof in combination with agents that improve their

bioavailability, reduce and/or modify their metabolism, inhibit their excretion, and/or modify their distribution within the body.

[0516] It will further be appreciated that therapeutically, prophylactically, diagnostically, or imaging active agents utilized in combination may be administered together in a single composition or administered separately in different compositions. In general, it is expected that agents utilized in combination will be utilized at levels that do not exceed the levels at which they are utilized individually. In some embodiments, the levels utilized in combination may be lower than those utilized individually.

[0517] The particular combination of therapies (therapeutics or procedures) to employ in a combination regimen will take into account compatibility of the desired therapeutics and/or procedures and the desired therapeutic effect to be achieved. It will also be appreciated that the therapies employed may achieve a desired effect for the same disorder (for example, a composition useful for treating cancer may be administered concurrently with a chemotherapeutic agent), or they may achieve different effects (e.g., control of any adverse effects).

[0518] In some embodiments, no more than 1%, no more than 2%, no more than 3%, no more than 4%, no more than 5%, no more than 6%, no more than 7%, no more than 8%, no more than 9%, no more than 10%, no more than 15%, no more than 20%, no more than 25%, no more than 30%, no more than 35%, no more than 40%, no more than 45%, or no more than 50% of cells that are not meant to be the destination of the delivery are transfected by the LNP. In some embodiments, the cells that are not meant to be the destination of the delivery are subject's non-immune cells. In some embodiments, the cells that are not meant to be the destination of the delivery are cells not targeted by the method. In some embodiments, the cells that are not meant to be the destination of the delivery are subject's cells not targeted by the method.

[0519] In some embodiments, the half-life of the nucleic acid delivered by the LNP described herein to the immune cell or a polypeptide encoded by the nucleic acid delivered by the LNP and expressed in the immune cell is at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 2 times, at least 3 times, at least 4 times, or at least 5 times longer than the half-life of the nucleic acid delivered by a

reference LNP to the immune cells or a polypeptide encoded by the nucleic acid delivered by the reference LNP and expressed in the immune cell.

[0520] In some embodiments, the composition of the LNP differs from the composition of the reference LNP in the type of ionizable cationic lipid, relative amount of ionizable cationic lipid, length of the lipid anchor in PEG lipid, back bone or head group of the PEG lipid, relative amount of PEG lipid, or type of immune cell targeting group, or any combination thereof. In some embodiments, the composition of the LNP differs from the composition of the reference LNP only in the type of ionizable cationic lipid. In some embodiments, the composition of the LNP differs from the composition of the reference LNP only in the amount of PEG lipid. In some embodiments, the reference LNP comprises cationic Lipid DLin-MC3-DMA or Lipid 7, but otherwise as the same as a tested LNP. In some embodiments, PEG lipid is a free PEG lipid.

[0521] In some embodiments, at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the immune cells are transfected by the LNP. In some embodiments, the immune cells are subject's immune cells. In some embodiments, the immune cells are immune cells targeted by the method. In some embodiments, the immune cells are subject's immune cells targeted by the method.

[0522] In some embodiments, the expression level of the nucleic acid delivered by the LNP is at least at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 2 times, at least 3 times, at least 4 times, at least 5 times, at least 6 times, at least 7 times, at least 8 times, at least 9 times, or at least 10 times higher than the expression level of the nucleic acid delivered by a reference LNP. In some embodiments, the expression level is measured and compared with a method described herein. In some embodiments, the expression level is measured by the ratio of cells expressing the encoded polypeptide. In some embodiments, the expression level is measured with FACS. In some embodiments, the expression level is measured by the average amount of the encoded polypeptide expressed in cells. In some embodiments, the expression level is measured as mean fluorescence intensity. In some embodiments, the expression level is measured by the amount of the encoded polypeptide or other materials secreted by cells.

[0523] In another aspect, provided herein are methods of targeting the delivery of a nucleic acid to an immune cell of a subject. In some embodiments, the method comprises contacting the immune cell with a lipid nanoparticle (LNP). In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the compound of the following formula: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid.

[0524] In some embodiments, an aspect of the disclosure relates to an LNP or a pharmaceutical composition containing thereof, as disclosed herein, for use in a method of targeting the delivery of a nucleic acid to an immune cell of a subject. Such a method may be for the treatment of a disease or disorder as disclosed hereafter. In some embodiments, a method as disclosed herein can comprise contacting in vitro or ex vivo the immune cell of a subject with a lipid nanoparticle (LNP). In some embodiments, the LNP is an LNP as described herein in the present disclosure.

[0525] In some embodiments, the LNP provides at least one of the following benefits:

- (i) increased specificity of targeted delivery to the immune cell compared to a reference LNP;
- (ii) increased half-life of the nucleic acid or a polypeptide encoded by the nucleic acid in the immune cell compared to a reference LNP;
- (iii) increased transfection rate compared to a reference LNP; and
- (iv) a low level of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies, wherein at least 80% mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation.

[0526] In some aspect, provided are methods of expressing a polypeptide of interest in a targeted immune cell of a subject. In some embodiments, the method comprises contacting the immune cell with a lipid nanoparticle (LNP). In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some

embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises a nucleic acid encoding the polypeptide. In some embodiments, an aspect of the disclosure relates to an LNP or a pharmaceutical composition containing thereof, as disclosed herein, for use in a method of expressing a polypeptide of interest in a targeted immune cell of a subject. Such a method may be for the treatment of a disease or disorder as disclosed hereafter. In some embodiments, a method as disclosed herein can comprise contacting in vitro or ex vivo the immune cell of a subject with a lipid nanoparticle (LNP).

[0527] In some embodiments, the LNP provides at least one of the following benefits:

- (i) increased expression level in the immune cell compared to a reference LNP;
- (ii) increased specificity of expression in the immune cell compared to a reference LNP;
- (iii) increased half-life of the nucleic acid or a polypeptide encoded by the nucleic acid in the immune cell compared to a reference LNP;
- (iv) increased transfection rate compared to a reference LNP; and
- (v) a low level of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies, wherein at least 80% mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation.

[0528] In some aspects, provided are methods of modulating cellular function of a target immune cell of a subject. In some embodiments, the method comprises administering to the subject a lipid nanoparticle (LNP). In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises a nucleic acid encoding a polypeptide for modulating the cellular function of the immune cell. In some embodiments, an aspect of the disclosure relates to an LNP or a pharmaceutical composition containing thereof, as disclosed herein, for use in a method of modulating cellular function of a targeted immune cell of a subject. Such a method may be for the treatment of a disease or disorder as disclosed hereafter. In some embodiments, a method as disclosed herein can

comprise contacting in vitro or ex vivo the immune cell of a subject with a lipid nanoparticle (LNP).

[0529] In some embodiments, the LNP provides at least one of the following benefits:

- (i) increased expression level in the immune cell compared to a reference LNP;
- (ii) increased specificity of expression in the immune cell compared to a reference LNP;
- (iii) increased half-life of the nucleic acid or a polypeptide encoded by the nucleic acid in the immune cell compared to a reference LNP;
- (iv) increased transfection rate compared to a reference LNP;
- (v) the LNP can be administered at a lower dose compared to a reference LNP to reach the same biologic effect in the immune cell; and
- (vi) a low level of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies, wherein at least 80% mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation.

[0530] In some embodiments, the modulation of cell function comprises reprogramming the immune cells to initiate an immune response. In some embodiments, the modulation of cell function comprises modulating antigen specificity of the immune cell.

[0531] In some aspect, provided are methods of treating, ameliorating, or preventing a symptom of a disorder or disease in a subject in need thereof. In some embodiments, the method comprises administering to the subject a lipid nanoparticle (LNP) for delivering a nucleic acid into an immune cell of the subject. In some embodiments, the LNP comprises an ionizable cationic lipid. In some embodiments, the LNP comprises a conjugate comprising the following structure: [Lipid] – [optional linker] – [immune cell targeting group]. In some embodiments, the LNP comprises a sterol or other structural lipid. In some embodiments, the LNP comprises a neutral phospholipid. In some embodiments, the LNP comprises a free Polyethylene glycol (PEG) lipid. In some embodiments, the LNP comprises the nucleic acid.

[0532] In some embodiments, the nucleic acid modulates the immune response of the immune cell, therefore to treat or ameliorate the symptom. In some embodiments, an aspect of the disclosure relates to an LNP or a pharmaceutical composition containing thereof, as

disclosed herein, for use in a method of treating, ameliorating, or preventing a symptom of a disorder or disease in a subject in need thereof. A disease or disorder may be as disclosed hereafter. In some embodiments, a method as disclosed herein can comprise contacting in vitro or ex vivo the immune cell of a subject with a lipid nanoparticle (LNP).

[0533] In some embodiments, the LNP provides at least one of the following benefits:

- (i) increased specificity of delivery of the nucleic acid into the immune cell compared to a reference LNP;
- (ii) increased half-life of the nucleic acid or a polypeptide encoded by the nucleic acid in the immune cell compared to a reference LNP;
- (iii) increased transfection rate compared to a reference LNP;
- (iv) the LNP can be administered at a lower dose compared to a reference LNP to reach the same treatment efficacy;
- (v) increased level of gain of function by an immune cell compared to a reference LNP; and
- (vi) a low level of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies, wherein at least 80% mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation.

[0534] In some embodiments, the disorder is an immune disorder, an inflammatory disorder, or cancer. In some embodiments, the nucleic acid encodes an antigen for use in a therapeutic or prophylactic vaccine for treating or preventing an infection by a pathogen.

[0535] In some embodiments, no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% of non-immune cells are transfected by the LNP. In some embodiments, no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% of undesired immune cells that are not meant to be the destination of the delivery are transfected by the LNP. In some embodiments, the half-life of the nucleic acid delivered by the LNP to the immune cell or a polypeptide encoded by the nucleic acid delivered by the LNP is at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 1.5 times, 2 times, 3 times, 4 times, 5 times, 10 times, or longer than the half-life of nucleic acid delivered by a reference LNP to the immune cell or a polypeptide encoded by the nucleic acid delivered by the reference LNP.

[0536] In some embodiments, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or more immune cells that are meant to be the destination of the delivery are transfected by the LNP.

[0537] In some embodiments, expression level of the nucleic acid delivered by the LNP is at least 5%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, at least 10%, 1.5 time, 2 times, 3 times, 4 times, 5 times, 10 times, 15 times, 20 times or more higher than expression level of nucleic acid in the same immune cells delivered by a reference LNP.

[0538] In some aspect, provided are methods of targeting the delivery of a nucleic acid to an immune cell of a subject. In some embodiments, the method comprises contacting the immune cell with a lipid nanoparticle (LNP) provided herein. In some embodiments, the method is for targeting NK cells. In some embodiments, the immune cell targeting group binds to CD56. In some embodiments, the method is for targeting both T cells and NK cells simultaneously. In some embodiments, the immune cell targeting group binds to CD7, CD8, or both CD7 and CD8. In some embodiments, the method is for targeting both CD4+ and CD8+ T cells simultaneously. In some embodiments, the immune cell targeting group comprises a polypeptide that binds to CD3 or CD7.

[0539] In some aspect, provided are methods of expressing a polypeptide of interest in a targeted immune cell of a subject. In some embodiments, the method comprises contacting the immune cell with a lipid nanoparticle (LNP) provided herein.

[0540] In some aspect, provided are method of modulating cellular function of a target immune cell of a subject. In some embodiments, the method comprises administering to the subject a lipid nanoparticle (LNP) provided herein.

[0541] In some aspect, provided are method of treating, ameliorating, or preventing a symptom of a disorder or disease in a subject in need thereof. In some embodiments, the method comprises administering to the subject a lipid nanoparticle (LNP) provided herein.

[0542] In some aspect, provided are methods of treating a disease or disorder related to CD8 in a subject. In some embodiments, the method comprises administering a pharmaceutical composition described herein to the subject. In some embodiments, the disease or disorder is cancer.

[0543] LNPs disclosed in the present disclosure and as claimed are suitable for the methods described above.

VIII. KITS FOR USE IN MEDICAL APPLICATIONS

[0544] Another aspect of the invention provides a kit for treating a disorder. The kit comprises: an ionizable cationic lipid, a lipid-immune cell targeting group conjugate, a lipid nanoparticle composition comprising an ionizable cationic lipid and/or a lipid-immune cell targeting group conjugate with or without an encapsulated payload (*e.g.*, an mRNA), and instructions for treating a medical disorder, such as, cancer or a microbial or viral infection.

EXAMPLES

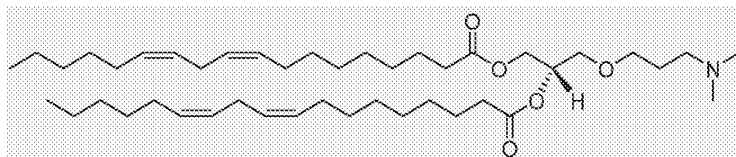
[0545] The invention now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

EXAMPLE 1 -- PREPARATION OF IONIZABLE CATIONIC LIPIDS

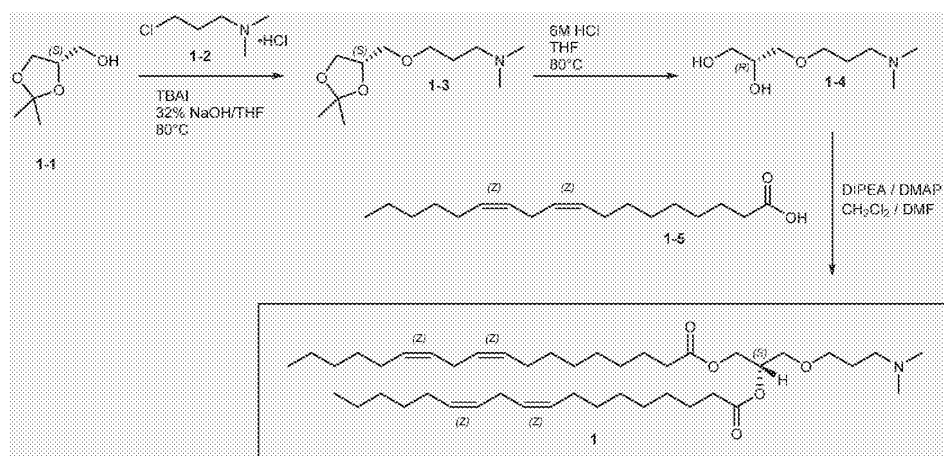
This Example describes the synthesis of various cationic lipids.

Cationic Lipid 1

[0546] The synthesis of Cationic Lipid 1, shown in the following formula,



was prepared as described in the following scheme 1.



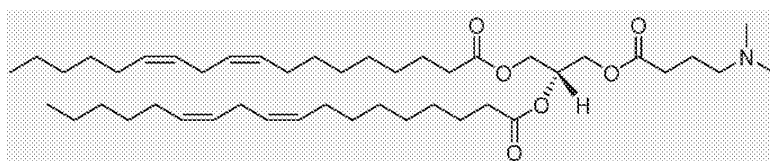
(Scheme 1)

[0547] Ether intermediate **1-3** was prepared by reacting hydroxy-functional, protected 1,2-diol starting material (**1-1**) (0.151 mol, 24 g) with dimethylaminopropylchloride, compound **1-2** (0.051 mol, 24 g, 1 equiv.) and TBAI (0.0015 mol, 554 mg, 0.01 equiv.) in the presence of NaOH (32%)/THF at 80°C overnight to afford ether intermediate compound **1-3** (20.1g, 0.1 mol). Thereafter, compound **1-3** (2.2 g, 0.01 mol) was deprotected (THF, 6 M HCl, 4 hours) to obtain vicinal diol intermediate compound **1-4** (1.6 g, 0.009 mol) in quantitative yield. Compound **1-4** (1.12 g, 0.006 mol) was bis-acylated using fatty acid **1-5** (5.9 mL 0.018 mol, 3 equiv.) using 10 equiv. EQ DIPEA, in DCM using EDC (3.8 g, 0.019 mol, 3.2 equiv.) to afford Lipid Compound **1** (238 mg, 0.0034 mol). Lipid Compound **1** was purified by preparatory HPLC (CombiFlash Nextgen 300+ Teledyne ISCO), and final product purity was 98% (RP-HPLC-ELSD, using Durashell-C18, 4.6 x 50mm, 3 uM, Cat# DC930505-0).

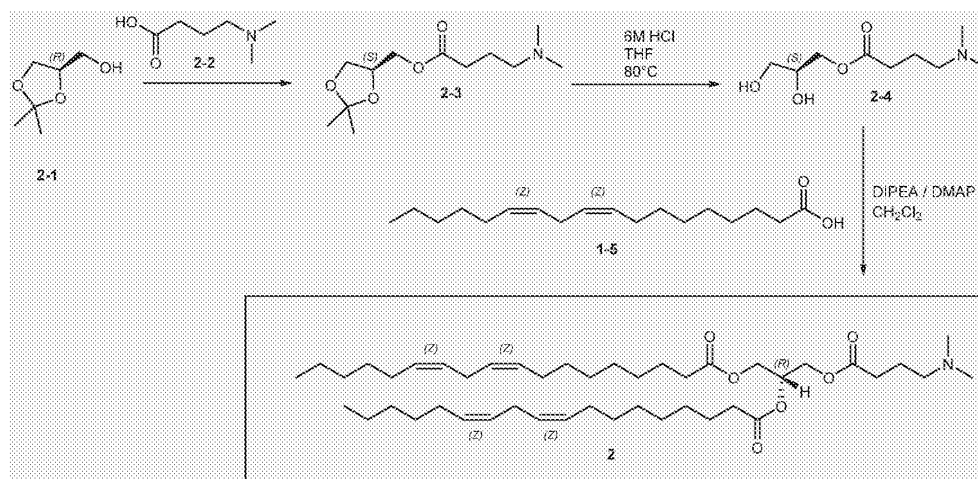
[0548] Purified Lipid **1** free base (C₄₄H₇₉NO₅, molecular weight 702.12 g/mol.) was characterized by proton NMR Spectroscopy (400 MHz) in CDCl₃ as shown in FIG. 1, and by LC-MS to confirm structure (NMR, m/z) and purity (NMR, LC) as shown in FIGS. 2A and 2B.

Cationic Lipid 2

[0549] The synthesis of Cationic Lipid **2**, shown in the following formula,



was prepared as described in the following scheme 2.



(Scheme 2)

[0550] Ester intermediate 2-3 was prepared by acylating protected 1,2-diol (1,2-O-isopropylidene-D-glycerol) starting material (2-1) with dimethylaminobutanoic acid compound 2-2 (0.03 mol, 4.19 g, 0.03 eq.) using EDCI (0.03 mol, 5.73 g, 1 eq.), DIPEA (0.12 mol, 12.9 g, 4.0 eq.) and DMAP (0.006 mol, 733 mg, 0.2 eq.) in DCM (300 mL) solution to obtain 5.44 g (0.02 mol) of 2-3. Intermediate 2-3 (670 mg, 0.0027 mol, 1.0 eq.) was deprotected in a mixture of 10 mL TFA/water (50:50 v/v) and 10 mL 6 M HCl at room temperature to afford 560 mg (0.0027 mol) of compound 2-4. Compound 2-4 (0.003 mol, 641 mg, 1 eq.) was esterified with fatty acid 1-5 (0.009 mol, 2.52 g, 3 eq.) in 50 mL of DCM, using EDC (0.009 mol, 1.72 g, 1 eq.), DIPEA (0.001 mol, 122 mg, 0.33 eq.) to afford cationic lipid 2 (260 mg, 0.0003 mol).

[0551] Lipid Compound 2 was purified by preparatory HPLC. The resulting product was greater than 99% pure (Reverse phase HPLC-ELSD using Durashell-C18, 4.6 x 50mm, 3 μ M, Cat# DC930505-0).

[0552] Purified Lipid 2 free base (C₄₅H₇₉NO₆, molecular weight 730.10 g/mol.) was characterized by proton NMR Spectroscopy (400 MHz) in CDCl₃ as shown in FIG. 3 and by

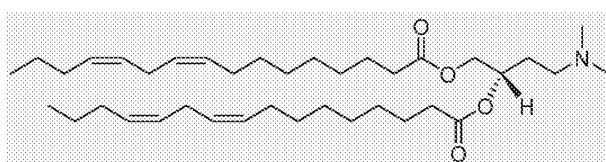
LC-MS to confirm structure (NMR, m/z) and purity (NMR, LC) as shown in FIGS. 4A and 4B.

[0553] Lipid 6 was synthesized using a method analogous to the synthesis of Lipid 2 except that diethylaminobutanoic acid was used to generate the tertiary amine head group instead of dimethylaminobutanoic acid used for Lipid 2.

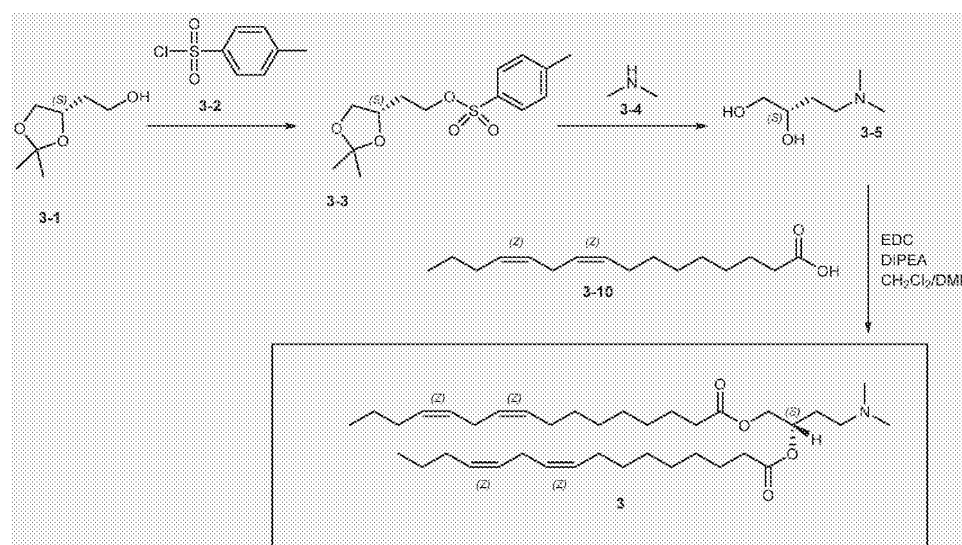
[0554] Purified Lipid 6 was characterized by proton NMR spectroscopy as shown in FIG. 41 and mass spectrometry and reverse phase HPLC as shown in FIGS. 42A and 42B.

Cationic Lipid 3

[0555] The synthesis of Cationic Lipid 3, shown in the following formula,

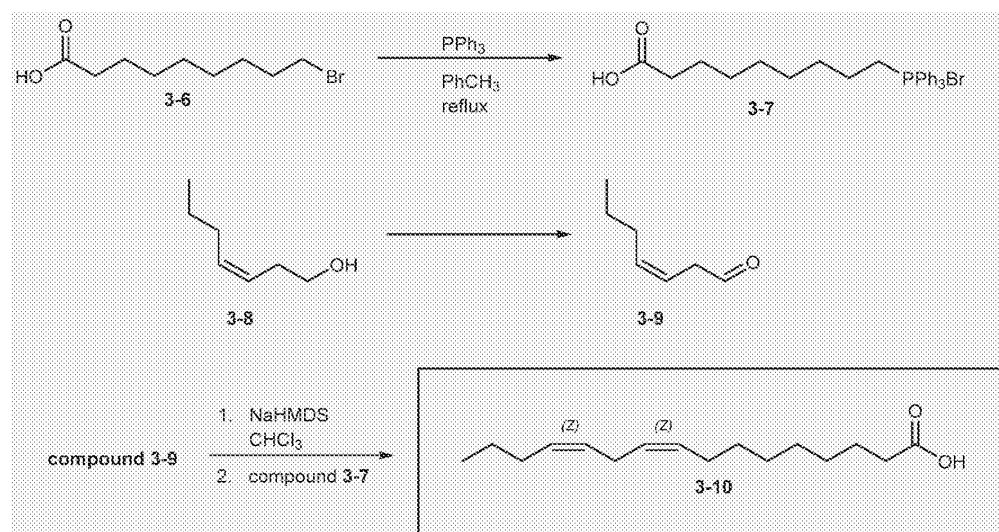


was prepared as described in the following scheme 3.



(Scheme 3)

[0556] Fatty acid 3-10 was prepared using the following scheme 3a:



(Scheme 3a)

[0557] Fatty acid **3-10** (9Z,12Z)-hexadeca-9,12-dienoic acid was synthesized using a Wittig reaction approach as shown in Scheme 3a. In order to produce compound **3-7**, 9-bromononanoic acid (0.0148 mol, 3.50 g) was combined with PPh_3 (0.0148 mol, 3.87g, 1 eq.) in 5 mL toluene and refluxed for 48 hours to afford 7.23 g of phosphonium bromide **3-7**. In order to produce compound **3-9**, compound **3-8** (0.0076 mol, 0.87 g) was oxidized using Dess-Martin periodinane (0.0082 mol, 3.48 g, 1.1 eq.) in 20 mL DCM to afford aldehyde 0.85 g of **3-9**. Compound **3-9** (0.0076 mol, 0.85 g) was reacted with compound **3-7** (0.0076 mol, 3.78 g, 1 eq.) in 7.6 mL of 40% NaHMDS in 60 mL THF to afford 400 mg of fatty acid **3-10**.

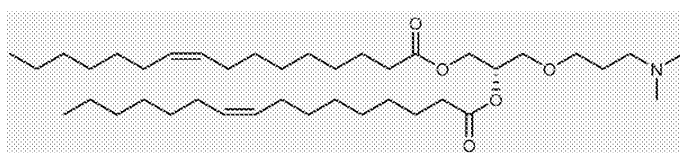
[0558] As shown in Scheme 3, intermediate **3-3** was produced by the tosylation of the free hydroxyl on a protected 1,2,4-butanetriol starting material (**3-1**) (0.0342 mol, 5.0 g, 1 eq.) using tosylchloride (**3-2**) (0.034 mol, 6.5 g, 1 eq.) and triethylamine (0.034 mol, 4.9 mL, 1 eq.), DMAP (0.011 mol, 200 mg, 0.3 eq.) in 250 mL DCM at room temperature. Nucleophilic displacement of compound **3-3** (0.0032 mol, 1.0 g, 1 eq.) with dimethylamine (0.03 mol, 1.5g, 10 eq.) in 16.6 mL of THF to afford 400 mg of tertiary amine **3-4**. Compound **3-4** was deprotected with 2 M HCl in MeOH to afford 410 mg of compound **3-5**. Compound **3-5** (90 mg) was esterified with fatty acid **3-10** (0.0016 mol, 400 mg, 3 eq.) using EDC (0.0018 mol, 306 mg, 3 eq.), DIPEA (0.0024 mol, 8.8 mL, 4.5 eq.) in 5.4 mL of DCM,

2 hours) to afford 12 mg of ionizable lipid **3**. Lipid Compound **3** was purified by preparatory HPLC.

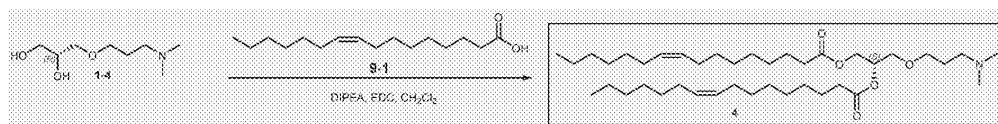
[0559] Lipid Compound **3** was purified by preparatory HPLC (CombiFlash Nextgen 300+ Teledyne ISCO). Product purity of 99% was determined by reverse phase HPLC-ELSD (using Durashell-C18, 4.6 x 50mm, 3 uM, Cat# DC930505-0).

Cationic Lipid 4

[0560] The synthesis of Cationic Lipid **4**, shown in the following formula,



was prepared as described in the following Scheme 4.



(Scheme 4)

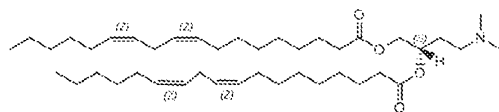
[0561] Fatty acid **9-1** was synthesized using a protocol analogous to that used for synthesis of fatty acid **4-6** above using 9-bromononanoic acid heptanal starting materials.

[0562] The bis-acylation of **1-4** (0.003 mol, 600 mg) by fatty acid **9-1** (0.01 mol, 2.14 g, 3 eq.) proceeded using EDC (0.009 mol, 1.7 g, 3.2 eq.), DIPEA (0.009 mol, 2.45 mL, 3.2 eq.) in 7 mL of DCM provided 203 mg of ionizable lipid **4** (mass, yield). Lipid Compound **4** was purified by preparatory HPLC (CombiFlash Nextgen 300+ Teledyne ISCO) and yield 99% pure product (HPLC-ELSD using Durashell-C18, 4.6 x 50mm, 3 uM, Cat# DC930505-0).

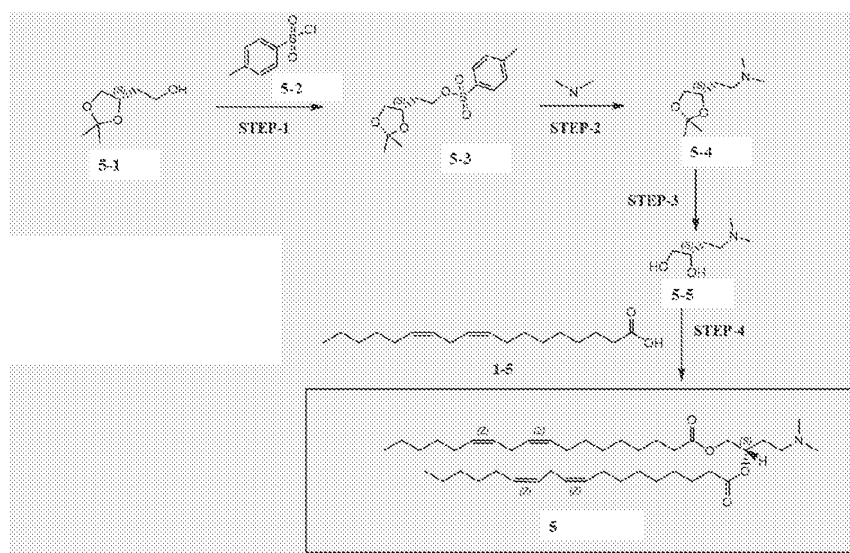
[0563] Purified Lipid **4** was characterized by proton NMR spectroscopy as shown in FIG. 6 and mass spectrometry and reverse phase HPLC as shown in FIGS. 7A and 7B.

Cationic Lipid 5

[0564] The synthesis of Cationic Lipid **5**, shown in the following formula,



was prepared as described in the following scheme 5.



(Scheme 5)

[0565] As shown in Scheme 5, intermediate **5-3** was produced by the tosylation of the free hydroxyl on a protected 1,2,4-butanetriol starting material (**5-1**) (0.0342 mol, 5.0 g, 1 eq.) using tosylchloride (**5-2**) (0.039 mol, 7.5 g, 1 eq.) and triethylamine (0.039 mol, 5.6 mL, 1 eq.), DMAP (0.013 mol, 230 mg, 0.3 eq.) in 250 mL DCM at room temperature. Nucleophilic displacement of compound **5-3** (0.0032 mol, 1.0 g, 1 eq.) with dimethylamine (0.03 mol, 1.5g, 10 eq.) in 16.6 mL of THF overnight to afford 1.1 g of tertiary amine **5-4**. Compound **5-4** (712 mg) was deprotected in 6 M HCl in water (5 mL) to afford 551 mg of compound **5-5**. Compound **5-5** (2 g) was esterified with fatty acid **1-5** (35.5 mmol, 8.88g, 3 eq.) using EDC.HCl (35.5 mol, 6.7 g, 3 eq.), DIPEA (47 mmol, 6.7 mL, 4 eq.) in 55 mL of DCM, 2 hours) to afford 1.09 g of ionizable lipid **5**. Lipid Compound **5** was purified by preparatory HPLC.

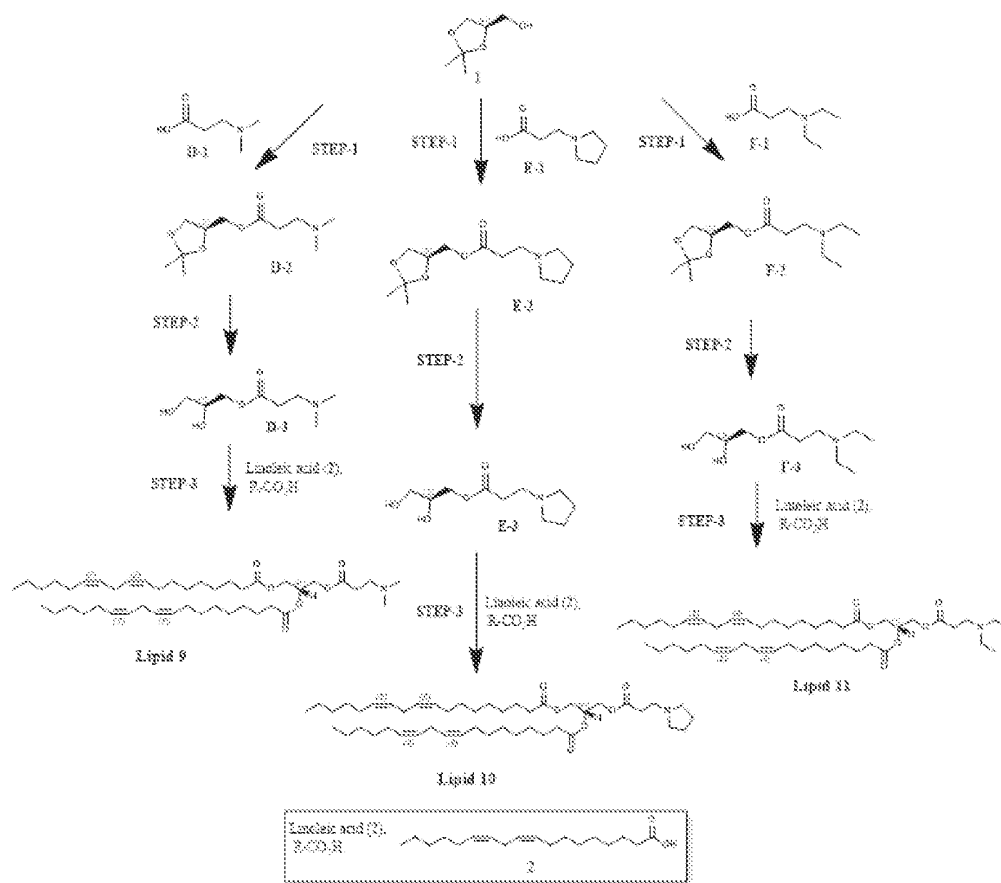
[0566] Lipid Compound **5** was purified by preparatory HPLC (CombiFlash Nextgen 300+ Teledyne ISCO). Product purity of 99% was determined by reverse phase HPLC-ELSD (using Durashell-C18, 4.6 x 50mm, 3 uM, Cat# DC930505-0).

[0567] Purified Lipid 5 free base ($C_{42}H_{74}NO_4$, molecular weight 657.57 g/mol.) was characterized by proton NMR Spectroscopy (400 MHz) in $CDCl_3$ as shown in FIG. 39A and by mass spectrometry to confirm structure (NMR, m/z) and purity (LC-ELSD) as shown in FIGS. 40A and 40B.

[0568] Lipid 7 was synthesized using a method analogous to the synthesis of Lipid 5 except using diethyl amine instead of dimethyl amine to incorporate the tertiary amine head group.

[0569] Purified Lipid 7 was characterized by proton NMR spectroscopy as shown in FIG. 43 and mass spectrometry and reverse phase HPLC as shown in FIGS. 44A and 44B.

[0570] Scheme 6 below depicts the synthesis of Lipid 9, Lipid 10, and Lipid 11:



(Scheme 6)

Cationic Lipid 9

[0571] Ester intermediate D-2 was prepared by acylating 4 g (30.2 mmol) of protected 1,2-diol (1,2-O-isopropylidene-D-glycerol) starting material (1) with dimethylaminopropanoic acid compound D-1 (33.3 mmol, 6.06 g, 1.71 eq), EDCI (33.2 mmol, 6.28 g, 1.1 eq), DIPEA (121.0 mmol, 21.08 mL, 4.0 eq), and DMAP (6 mmol, 740 mg, 0.2 eq) in DCM (100 mL) solution to obtain 2.6 g (0.02 mol) in 37% yield of D-2. Intermediate D-2 (2.5 g, 10.68 mmol, 1.0 eq.) was deprotected in a 1:3 (v/v) mixture of 1M aq. HCl and THF (total volume 20 mL) at room temperature to afford 2.4g (12.6 mmol) of crude compound D-3. Crude compound D-3 (12.6 mmol, 2.4 g, 1 eq.) was esterified with fatty acid 2 (8.9 g, 2.5eq, 31.8 mmol), EDCI (6.1 g, 2.5 eq, 31.8 mmol), DIPEA (5.53 mL, 2.5 eq, 31.8 mmol), DMAP (285 mg, 0.2 eq, 2.6 mmol), DCM (100 mL) to afford 7g of crude ionizable lipid 9. Crude product (3g) was purified by preparatory HPLC to obtain 100 mg of pure Lipid (>99% pure by Reverse phase HPLC-ELSD using Durashell-C18, 4.6 x 50mm, 3 uM, Cat# DC930505-0).

[0572] Purified Lipid 9 free base (C₄₄H₇₇NO₆, molecular weight 716.10 g/mol.) was characterized by proton NMR Spectroscopy (400 MHz) in CDCl₃ and Mass Spectrometry to confirm structure (FIG. 48A and FIG. 48B) and by LC-ELSD to determine purity (FIG. 48C).

Cationic Lipid 10

[0573] Ester intermediate E-2 was prepared by acylating 4 g (30.2 mmol) of protected 1,2-diol (1,2-O-isopropylidene-D-glycerol) starting material (1) with dimethylaminopropanoic acid compound E-1 (4.76 g, 1.1 eq, 33 mmol), EDCI (6.38 g, 1.1 eq, 33 mmol), DIPEA (21.08 mL, 4.0 eq, 120 mmol), and DMAP (680 mg, 0.2 eq, 6 mmol), in DCM (150 mL) solution to obtain 1.9 g (7.38 mmol) in 25% yield of E-2. Intermediate E-2 (1.9 g, 7.38 mmol, 1 eq) was deprotected in a 1:3 (v/v) mixture of 1M aq. HCl and THF (total volume 80 mL) at room temperature to afford 1.58 g (7.27 mmol) of crude compound E-3. Crude compound E-3 (7.27 mmol, 1.58 g, 1 eq.) was esterified with fatty acid 2 (18.2 mmol, 5.1 g, 2.5eq), EDCI (18.2 mmol, 3.48 g, 2.5 eq), DIPEA (18.2 mmol, 3.16 mL, 2.5 eq), and DMAP (1.4 mmol, 160 mg, 0.2 eq) in DCM (80 mL) solution to afford ~7.5 g of crude ionizable lipid 10. Crude product (3g) was purified by preparatory HPLC to obtain 105 mg of pure Lipid (>99% pure by Reverse phase HPLC-ELSD using Durashell-C18, 4.6 x 50mm, 3 uM, Cat# DC930505-0)

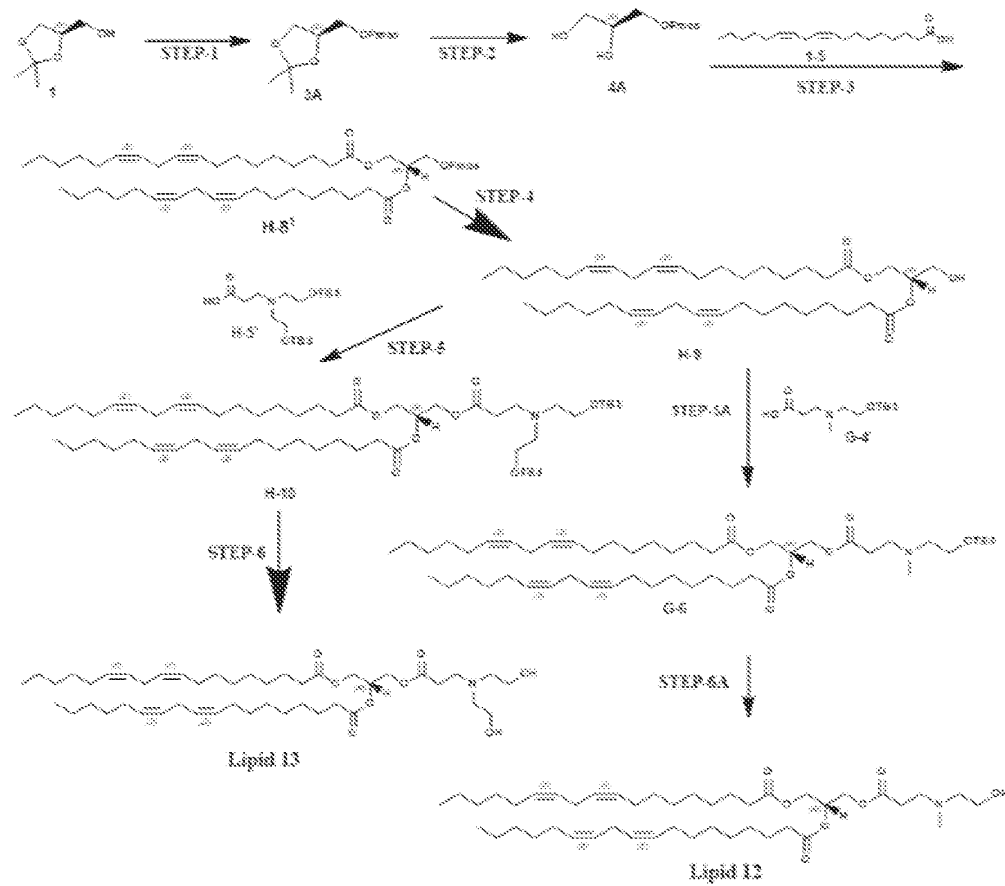
[0574] Purified Lipid 10 free base (C₄₆H₇₉NO₆, molecular weight 742.14 g/mol.) was characterized by proton NMR Spectroscopy (400 MHz) in CDCl₃ and Mass Spectrometry to confirm structure (FIG. 49A and FIG. 49B) and by LC-ELSD to determine purity (FIG. 49C).

Cationic Lipid 11

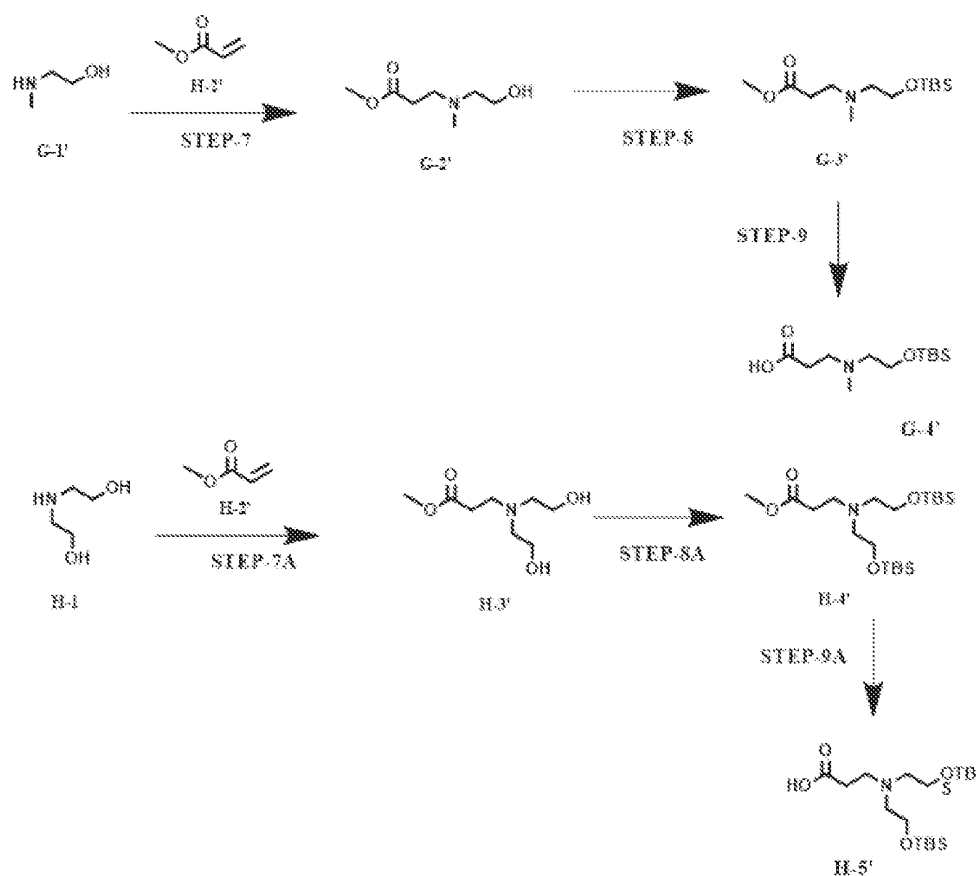
[0575] Ester intermediate F-2 was prepared by acylating 4 g (30.2 mmol) of protected 1,2-diol (1,2-O-isopropylidene-D-glycerol) starting material (1) with dimethylaminopropanoic acid compound F-1 (6.06 g, 1.38 eq, 41.7 mmol), EDCI (6.38 g, 1.1 eq, 33.3 mmol), DIPEA (21.08 mL, 4.0 eq, 121.0 mmol), and DMAP (740 mg, 0.2 eq, 6 mmol), in DCM (100 mL) solution to obtain 3.3 g (12.7 mmol) in 41.7% yield of F-2. Intermediate F-2 (3.2 g, 12.3 mmol, 1 eq) was deprotected in a 1:3 (v/v) mixture of 1M aq. HCl and THF (total volume 80 mL) at room temperature to afford 3.1 g (14.1 mmol) of crude compound F-3. Crude compound F-3 (14.13 mmol, 3.1 g, 1 eq.) was esterified with fatty acid 2 (35.3 mmol, 9.9 g, 2.5eq), EDCI (6.8 g, 2.5 eq, 35.3 mmol), DIPEA (6.2 mL, 2.5 eq, 35.3 mmol), and DMAP (316 mg, 0.2 eq, 2.8 mmol) in DCM (100 mL) solution to afford ~9 g of crude ionizable lipid 11. Crude product (3g) was purified by preparatory HPLC to obtain 55 mg of pure Lipid 11 (>99% pure by Reverse phase HPLC-ELSD using Durashell-C18, 4.6 x 50mm, 3 uM, Cat# DC930505-0).

[0576] Purified Lipid 11 free base (C₄₆H₈₁NO₆, molecular weight 744.16 g/mol.) was characterized by proton NMR Spectroscopy (400 MHz) in CDCl₃ and Mass Spectrometry to confirm structure (FIG. 50A and FIG. 50B) and by LC-ELSD to determine purity (FIG. 50C).

[0577] Scheme 7 and Scheme 8 below depict the synthesis of Lipid 12 and Lipid 13:



(Scheme 7)



(Scheme 8)

Cationic Lipid 12

[0578] Fmoc protected intermediate 3A was produced from (R)-(2,2-dimethyl-1,3-dioxolan-4-yl)methanol (1), 2.0 g (1.0 eq, 15.2 mmol) using Fmoc chloride (30 mmol, 7.9 g, 2.0 eq) in pyridine (20 mL) to afford 3.8 g of 3A in 71% yield (Step 1, Scheme 7). 3A, 2.3 g (1.0 eq, 6.5 mmol) was selectively deprotected in 1M HCl:THF (1:3, 20 mL) and 0.5 mL methanol to afford ~2 g of 4A (Step 2, Scheme 7). O-acylation of 4A, 1.3 g (1.0 eq, 4.2 mmol) with linoleic acid, 1-5 (9.2 mmol, 2.9 mL, 2.2 eq) using PyBOP (9.2 mmol, 4.7 g, 2.2 eq) and DIPEA (9.2 mmol, 1.6 mL, 2.2 eq.) in 3mL DMF. Combined product from two batches afforded a total of 1.6 g (21%) of pure intermediate H-8' (Step 3, Scheme 7). Fmoc removal (Step 4, Scheme 7) from H-8', 2.05 g (1.0 eq, 2.44 mmol) using 1% piperidine in THF (25 mL), 0 °C, 4 hours, yielded 680 mg of purified intermediate H-9 in 45% yield. Key intermediate H-9 was used in subsequent steps for production of Lipid 12 (via O-acylation of

G-4') as well as for production of Lipid 13 (via O-acylation of H-5' as described in relevant section below).

[0579] Protected compound G-4', 3-((2-((tert-butyldimethylsilyl)oxy)ethyl)(methyl)amino)propanoic acid, was prepared using starting materials, methyl acrylate, H-2', and 2-(methylamino)ethan-1-ol, G-1' via Michael addition. Methyl acrylate (H-2'), 1.6 ml (1 eq, 17.8 mmol) was reacted with G-1' (2 g, 1.5 eq, 26.6 mmol) and Aluminum oxide (904 mg, 0.5 eq, 8.9 mmol) under solvent free conditions at room temperature for 3 hours to afford 2.58 g (91%) of G-2' (Step 7, Scheme 8). G-2', 1.33 g (1 eq, 8.26 mmol) was converted to the tertiary butyl dimethylsilyl protected intermediate G-3' (Step 8, Scheme 8) using tert-butyldimethylsilyl chloride, TBDMSCl (1.62 g, 1.3 eq, 10.74 mmol) and TEA (2.3 ml, 2 eq, 16.52 mmol) in 3 ml DCM at room temperature, overnight, resulting in recovery of about 1.13 g (50%) for purified G-3'. Subsequent selective deprotection of G-3', 1.14 g (1 eq, 4 mmol) in THF/MeOH/1 M HCl (3/2/1 (v/v); total volume of 6 ml) at room temperature, overnight, yielded 1.13g of G-4' (Step 9, Scheme 8). G-4' and H-9 were combined to produce intermediate G-6 (Step 5A, Scheme 8). H-9, 400 mg (1.0 eq, 0.65 mmol) was acylated with G-4' (0.98 mmol, 268 mg, 1.5 eq) using EDCI (198 mg, 1.5 eq, 0.98 mmol), DIPEA (167 μ L, 1.5 eq, 0.98 mmol), DMAP (15.9 mg, 0.2 eq, 0.13 mmol) in 2.0 mL DCM to afford 308 mg (55%) of crude G-6. Crude G-6 (308 mg) was deprotected in HF.pyridine (9.0 mmol, 641 μ L, 25 eq) in 6.0 mL of THF (Step 6A, Scheme 7) yielding 308 mg of crude Lipid 12. Crude product was purified twice using preparatory HPLC to isolate 213 mg (79%) of purified Lipid 12. (>99% pure by Reverse phase HPLC-ELSD using Durashell-C18, 4.6 x 50mm, 3 μ M, Cat# DC930505-0).

[0580] Purified Lipid 12 free base (C₄₅H₇₉NO₇, molecular weight 746.13 g/mol.) was characterized by proton NMR Spectroscopy (400 MHz) in CDCl₃ and Mass Spectrometry to confirm structure (FIG. 51A and FIG. 51B) and by LC-ELSD to determine purity (FIG. 51C).

Cationic Lipid 13

[0581] Protected compound 3-(bis(2-((tert-butyldimethylsilyl)oxy)ethyl)amino)propanoic acid (H5') was prepared using starting materials, methyl acrylate, H-2', and 2,2'-azanediyldis(ethan-1-ol), H-1' via Michael addition. Methyl acrylate (H-2'), 1.65 g (1 eq, 19.2 mmol) was reacted with H-1' (28.5 mmol, 3.0 g, 1.5 eq.), Aluminum oxide (960 mg, 0.5 eq, 9.6 mmol) under solvent free conditions at room temperature for 3 hours to afford 3.53 g

(97%) of H-3'. H-3', 830 mg (1 eq, 4.3 mmol) was converted to the tertiary butyl dimethylsilyl protected intermediate H-4' using tert-butyldimethylsilyl chloride, TBDMSCl (1.56 g, 2.5 eq, 10.9 mmol), TEA (1.16 mL, 2.0 eq, 10.9 mmol) in 8 mL DCM at room temperature, overnight, resulting in recovery of about 1.51 g (83%) for purified H-4'. Subsequent selective deprotections of H-4', 600 mg (1 eq, 1.4 mmol) in THF (3 ml)/MeOH (2 ml)/1M LiOH (1 ml) at room temperature, overnight yielded ~500 mg of H-5'. Intermediate H-10 was produced by O-acylation of H-9 using 3-(bis(2-((tert-butyldimethylsilyl)oxy)ethyl)amino)propanoic acid (H5'). H-5' and H-9 were combined to produce intermediate H-10 (Step 5, Scheme 7). H-9, 150 mg (1.0 eq, 0.24 mmol) was acylated with H-5' (0.36 mmol, 150 mg, 1.5 eq) using EDCI (0.36 mmol, 74 mg, 1.5 eq), DIPEA (0.36 mmol, 62 μ L, 1.5 eq), DMAP (0.048 mmol, 6 mg, 0.2 eq) in 1.0 mL DCM to afford 108 mg (44%) of crude H-10. Crude H-10, 108 mg (1.0 eq, 0.11 mmol) was deprotected in HF/pyridine (2.75 mmol, 200 μ L, 25 eq), in 2.0 mL THF yielding 41 mg (48%) of crude Lipid 13. Crude product from two batches was combined and purified twice using preparatory HPLC to isolate 71 mg of purified Lipid 13. (>99% pure by Reverse phase HPLC-ELSD using Durashell-C18, 4.6 x 50mm, 3 μ M, Cat# DC930505-0).

[0582] Purified Lipid 13 free base (C₄₆H₈₁NO₈, molecular weight 776.15 g/mol.) was characterized by proton NMR Spectroscopy (400 MHz) in CDCl₃ and Mass Spectrometry to confirm structure (FIG. 52A and FIG. 52B) and by LC-ELSD to determine purity (FIG. 52C).

EXAMPLE 2 – PREPARATION OF LNPs BY VORTEX MIXING USING EXEMPLARY IONIZABLE LIPIDS

[0583] Exemplary LNPs were created using cationic Lipid 2 and cationic Lipid 5 as synthesized in Example 1 as well as commercially available cationic Lipid 8 and cationic Lipid DLin-MC3-DMA (MedChemExpress, New Jersey, US; Catalog #HY-112758).

[0584] LNPs were created with an encapsulated mRNA payload and lipid blend by vortex mixing an aqueous mRNA solution and an ethanolic lipid solution. The mRNA (a 9:1 w/w mix of mRNA encoding eGFP and eGFP mRNA labeled with Cy5, TriLink Biotechnologies, California, US) was mixed with pH 4 acetate buffer to provide a final aqueous mRNA solution containing 133 μ g/mL mRNA and 21.7 mM acetate buffer. The lipid components were dissolved in anhydrous ethanol at the relative ratios set forth in TABLE 3 below.

TABLE 3

Lipid	Source	Ratio of Lipid to mRNA (nmol lipid/100 μ g mRNA)	Concentration in Lipid Solution (mM)	Theoretical LNP Lipid Composition (mol%)
Ionizable Cationic Lipid	-	1,500	6	48.8
Cholesterol	Dishman Netherlands	1,200	4.8	39.0
DSPC	Avanti Polar Lipids, Alabama, U.S.	300	1.2	9.8
DMG-PEG(2000)	Avanti Polar Lipids, Alabama, U.S.	75	0.3	2.4

[0585] Briefly, the mRNA solution (375 μ L) was transferred into a conical bottom centrifuge tube, and the lipid solution (125 μ L) was rapidly added into the tube containing the mRNA solution (3:1 v/v ratio of mRNA solution to lipid solution). The tube containing the mixture was immediately capped and vortexed for 15 s at 2,500 rpm, followed by incubation at room temperature for not less than 5 min before proceeding to ethanol removal and buffer exchange.

[0586] Following mixing, ethanol removal and buffer exchange was performed on the resulting LNP suspension using a Sephadex G-25 resin packed SEC column (PD MiniTrap G-25, Cytiva, Massachusetts, U.S.), by gravity flow. Briefly, the SEC column was rinsed five times with 2.5 mL of exchange buffer (25 mM pH 7.4 HEPES buffer with 150 mM NaCl) before then loading 425 μ L of LNP suspension. Once the LNP suspension fully moved into the resin bed, a 75 μ L stacker volume of exchange buffer was applied to the column to achieve the specified target load volume of the column and maximize recovery, according to manufacturer specifications. After the stacker fully moved into the resin bed, the SEC column was transferred to a new centrifuge tube, and the LNP suspension was eluted by

adding 1.0 mL of exchange buffer to the column. Eluate containing the LNPs in the exchange buffer was recovered and stored at 4°C until further use.

EXAMPLE 3 – CHARACTERIZATION OF LNPs

[0587] This Example describes the characterization of LNPs produced in Example 2.

[0588] Samples of the LNPs produced in Example 2 were characterized to determine the average hydrodynamic diameter, zeta potential, and mRNA content (total and dye-accessible). The hydrodynamic diameter was determined by dynamic light scattering (DLS) using a Zetasizer model ZEN3600 (Malvern Pananalytical, UK). The zeta potential was measured in 5 mM pH 5.5 MES buffer and 5 mM pH 7.4 HEPES buffer by laser Doppler electrophoresis using the Zetasizer.

[0589] RNA content of the nanoparticles is measured using Thermo Fisher Quant-iT RiboGreen RNA Assay Kit. Dye accessible RNA, which includes both non-incorporated RNA and RNA that is near the surface of the nanoparticle, is measured by diluting the nanoparticles to approximately 1 µg/mL mRNA using HEPES buffered saline, and then adding Quant-iT reagent to the mixture. Total RNA content is measured by diluting the particles to 1 µg/mL mRNA using HEPES buffered saline, disrupting the nanoparticles by heating them to 60 °C for 30 minutes in HEPES buffered saline containing 0.5% Triton, and then adding Quant-It reagent. RNA is quantified by measuring fluorescence at 485/535 nm, and concentration is determined relative to a contemporaneously run RNA standard curve. The results are set forth in TABLE 5.

TABLE 5

Formulation No.	Ionizable Lipid	DLS Z-Avg. Diameter (nm)	DLS PDI	Zeta Potential at pH 5.5 (mV)	Zeta Potential at pH 7.4 (mV)	Dye-Accessible mRNA (%)
1	Cationic Lipid 2	120	0.19	21	5.4	16
2	Cationic Lipid 5	109	0.14	23	0.3	13
3	Cationic Lipid 8	91	0.12	20	0.9	6.3

4	Cationic Lipid DLn-MC3-DMA	96	0.14	16	-0.4	9.4
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Example 4 – Preparation of Fab conjugates to enable T-cell targeting

[0590] This Example describes the production of an exemplary lipid-immune cell targeting group conjugate.

[0591] An anti-CD3 Fab (hSP34 with mouse lambda and human lambda) (see amino acid sequences below) was conjugated to DSPE-PEG(2k)-maleimide via covalent coupling between the maleimide group and a C-terminal cysteine in the heavy chain (HC). Anti-CD3 Fab clone Hu291, anti-CD8 Fab clone TRX2, anti-CD8 Fab clone OKT8, a non-functional mutated OKT8 (mutOKT8), anti-CD4 Fab from Ibalizumab sequence, anti-CD5 Fab clone He3, anti-CD7 Fab clone TH-69, anti-CD2 Fab clone TS2/18.1, anti-CD2 Fab clone 9.6, anti-CD2 Fab clone 9-1 with human kappas were also conjugated using similar methods described herein. The protein (3-4 mg/mL), after buffer exchange into oxygen free, pH 7 phosphate buffer, was reduced in 2 mM TCEP in oxygen free pH 7 phosphate buffer for 1 hour at room temperature. The reduced protein was isolated using a 7 kDa SEC column to remove TCEP and buffer exchanged into fresh oxygen free pH 7 phosphate buffer.

[0592] The conjugation reaction was initiated by addition of a 10 mg/mL micellar suspension of DSPE-PEG-maleimide (Avanti Polar Lipids, Alabama, US) and 30 mg/mL DSPE-PEG-OCH₃ (Avanti Polar Lipids, Alabama, U.S.) (1:1 to 1:3 weight ratio is used depending on protein) in oxygen free pH 5.7 citrate buffer (1 mM Citrate). Protein solution is concentrated to 3 - 4 mg/mL using a 10 kDa Regenerated Cellulose Membrane and subsequently buffer exchanged in oxygen free pH 7 phosphate buffer using a 40 kDa Size Exclusion Column. The conjugation reaction is carried out using 2 – 4 mg/mL protein and a 3.5 molar excess of maleimide at 37°C for 2 hours followed by incubation at room temperature for an additional 12 - 16 hours.

[0593] The production of the resulting conjugate was monitored by HPLC and the reaction quenched in 2 mM cysteine. The resulting conjugate (DSPE-PEG(2k)-anti-hSP34 Fab) is isolated using a 100 kDa Millipore Regenerated Cellulose membrane filtration using pH 7.4 HEPES buffer saline (25 mM HEPES, 150 mM NaCl) buffer and stored at 4°C prior to use. After quenching, the final micelle composition consists of a mixture of DSPE-PEG-

Fab, DSPE-PEG-maleimide(cysteine terminated), and DSPE-PEG-OCH₃. The ratio of the three components is DSPE-PEG-Fab: DSPE-PEG-maleimide(cysteine terminated): DSPE-PEG-OCH₃ = 1: 2.45: 3.45 -10.35 (by mole)).

[0594] The resulting conjugate displayed comparable binding to recombinant Rhesus CD3 epsilon as the unconjugated anti-CD3 Fab by ELISA assay.

Anti-CD3 hSP34-Fab sequence:

hSP34 HC (SEQ ID NO: 1):

EVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAMNWVRQAPGKGLEWVARIRSKY
NNYATYYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYISY
WAYWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP
KSSDKTHTC

hSP34-mlam LC (mouse lambda) (SEQ ID NO: 2):

QTVVTQEPSTLVSPGGTVTLTCGSSTGAVTSGNYPNWVQKPGQAPRGLIGGTKFLA
PGTPARFSGSLGGAALTLGSGVQPEDEAEYYCVLWYSNRWVFGGGTKLTVLGQPK
SSPSVTLFPPSSEELTNKATLVCTITDFYPGVVTVDWKVDGTPVTQGMETTQPSKQS
NNKYMASSYLTLTARAWERHSSYSQVTHEGHTVEKSLSRADSS

SP34-blam LC (human lambda) (SEQ ID NO: 3):

QTVVTQEPSTLVSPGGTVTLTCGSSTGAVTSGNYPNWVQKPGQAPRGLIGGTKFLA
PGTPARFSGSLGGAALTLGSGVQPEDEAEYYCVLWYSNRWVFGGGTKLTVLSQPK
AAPSVTLFPPSSEELQANKATLVCLVSDFYPGA VTAWKADGSPVKVGVETTKPSK
QSNKYAASSYLSLTPEQWKSHRSYSCRVTHEGSTVEKTVAPAESS

Anti-CD3 Hu291-Fab sequence:

Hu291 HC (SEQ ID NO: 4):

QVQLVQSGAEVKKPGASVKVSCKASGYTFISYTMHWVRQAPGQGLEWMGYINPRS
GYTHYNQKLKDKATLTADKSASTAYMELSSLRSEDTAVYYCARSAYYDYDGFAYW
GQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDK
THTC

Hu 291 LC (SEQ ID NO: 5):

MDMRVPAQLLGLLLLWLPGAKCDIQMTQSPSSLSASVGDRVTITCSASSSVSYMNW
YQKPGKAPKRLIYDTSKLASGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQWSS
NPPTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
DNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSPVTK
SFNRGES

Anti-CD8 TRX2-Fab sequence:

TRX2 HC (SEQ ID NO: 6):

QVQLVESGGGVVQPGRSLRLSCAASGFTFSDFGMNWVRQAPGKGLEWVALIYYDG
 SNKFYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPHYDGYHHFFDS
 WGQGT LVT VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGAL
 TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSD
 KTHTC

TRX2 LC (SEQ ID NO: 7):

DIQMTQSPSSLSASVGDRVTITCKGSQDINNYLAWYQQKPGKAPKLLIYNTDILHTG
 VPSRFGSGSGTDFFTISSLPEDIATYYCYQYNNGYTFGQGTKVEIKRTVAAPSVFI
 FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSY
 SLSSLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD8 OKT8-Fab sequence:

OKT8 HC (SEQ ID NO: 8):

QVQLVQSGAEDKKPGASVKVSKASGFNIKDTYIHWVRQAPGQGLEWMGRIDPAN
 DNTLYASKFQGRVTITADTSSNTAYMELSSLRSED TAVYYCGRGYGYVFDHWGQ
 GTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSG
 VHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTH
 TC

OKT8 LC (SEQ ID NO: 9):

DIVMTQSPSSLSASVGDRVTITCRTSRISQYLAWYQEKP G KAPKLLIYSGSTLQSGVP
 SRFGSGSGTDFTLTISSLQPEDFATYYCQQHNNPLTFGQGTKVEIKRTVAAPSVFIF
 PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSY
 LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD4 Ibalizumab-Fab sequence:

Ibalizumab HC (SEQ ID NO: 10):

QVQLQQSGPEVVKPGASVKMSCASGYTFTSYVIHWVRQKPGQGLDWIGYINPYND
 GTDYDEKFKGKATLTSDTSTAYMELSSLRSED TAVYYCAREKDNYATGAWFAY
 WGQGT LVT VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGAL
 TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSD
 KTHTC

Ibalizumab LC (SEQ ID NO: 11):

DIVMTQSPDSLAVSLGERVTMNCSSQSLLYSTNQKNYLAWYQQKPGQSPKLLIYW
 ASTRESGVPDRFGSGSGTDFTLTISSVQAEDVAVYYCQQYYSYRTFGGGTKLEIKRT
 VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
 DSKDSYSLSSLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

anti-CD5 He3-Fab sequence:

He3 HC (SEQ ID NO: 12):

EIQLVQSGGGLVKPGGSRISCAASGYTFTNYGMNWVRQAPGKGLEWMGWINTHT
 GEPTYADSFKGRFTFSLDDSKNTAYLQINSLRAEDTAVYFCTRRGYDWYFDVWGQG
 TTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVH
 TPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC

He3 LC (SEQ ID NO: 13):

DIQMTQSPSSLSASVGDRVITITCRASQDINSYLSWFQQKPGKAPKTLIYRANRLESGVP
SRFSGSGSGTDYTLTISSLQYEDFGIYYCQYDESPWTFGGGKLEIKRTVAAPSVFIFP
PSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSL
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

anti-CD7 TH-69-Fab sequence:

TH-69 HC (SEQ ID NO: 14):

EVQLVESGGGLVPGGSLKLSCAASGLTFSSYAMSWVRQTPEKRLEWVASISSGGFT
YYPDSVKGRFTISRDNARNILYQMSSLRSEDAMYYCARDEVIRGYLDVWGAGTTV
TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP
AVLQSSGLYSLSSVTVPSDDLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC

TH-69 LC (SEQ ID NO: 15):

DIQMTQTTSSLSASLGDRVITISCSASQGISNYLNWYQQKPDGTVKLLIYTSSLHSGVP
SRFSGSGSGTDYSLTISNLEPEDIATYYCQYQSKLPYTFGGGKLEIKRTVAAPSVFIFP
PSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSL
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

anti-CD2 TS2/18.1-Fab sequence:

TS2/18.1 HC (SEQ ID NO: 16):

EVQLVESGGGLVMPGGSLKLSCAASGFAFSSYDMSWVRQTPEKRLEWVAYISGGGF
TYYPDTVKGRFTLSRDNAKNTLYQMSSLKSEDTAMYYCARQGANWELVYWGQGT
LVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVTVPSDDLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC

TS2/18.1 LC (SEQ ID NO: 17):

DIVMTQSPATLSVTPGDRVFLSCRASQSSISDFLHWYQQKSHESPRLLIKYASQSSIGIPS
RFSGSGSGSDFTLINSVEPEDVGVYFCQNGHNFPPTFGGGKLEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSS
TLTSLKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

anti-CD2 9.6-Fab sequence:

9.6 HC (SEQ ID NO: 18):

QVQLQQPGAELVRPGSSVKLSCKASGYTFTRYWIHWVKQRPIQGLEWIGNIDPSDSE
THYNQKFKDKATLTVDKSSGTAYMQLSSLTSEDSAVYYCATEDLYYAMEYWGQGT
SVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVTVPSDDLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC

9.6 LC (SEQ ID NO: 19):

NIMMTQSPSSLAVSAGEKVTMTCKSSQSVLYSSNQKNYLAWYQQKPGQSPKLLIYW
ASTRESGVPDRFTGSGSGTDFTLTISVQPEDLAVYYCHQYLSSHTFGGGKLEIKRTV
AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQD
SKDYSLSSLTSLKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

anti-CD2 9-1-Fab sequence:

9-1 HC (SEQ ID NO: 20):

QVQLQQPGTELVRPGSSVKLSCKASGYTFTSYWVNWVKQRPDQGLEWIGRIDPYDS
ETHYNQKFTDKAISTIDTSSNTAYMQLSTLTSDASAVYYCSRSPRDSSTNLADWGQG
TLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC

9-1 LC (SEQ ID NO: 21):

DIVMTQSPATLSVTPGDRVSLSCRASQSSISDYLHWYQQKSHESPRLLIKYASQSSIGIPS
RFSGSGSGSDFTLINSVEPEDVGYYCQNGHSFPLTFGAGTKLELRRTVAAPSVFIFP
PSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSL
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

mutOKT8-Fab sequence:

mutOKT8 HC (SEQ ID NO: 22):

QVQLVQSGAEDKKPGASVKVSCKASGFNIKDTYIHWVRQAPGQGLEWMGRIDPAND
NTLYASKFQGRVTITADTSSNTAYMELSSLRSEDTAVYYCGRGAGAYVFDHWGQGT
TVTSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC

mutOKT8 LC (SEQ ID NO: 23):

DIVMTQSPSSLSASVGDRVTITCRTSRSISAALAWYQEKGKAPKLLIYSGSTLQSGVP
SRFSGSGSGTDFTLTISSLQPEDFATYYCQQHNENPLTFGQGTKVEIKRTVAAPSVFIFP
PSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSL
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

EXAMPLE 5 – PREPARATION OF LNPs CONTAINING T CELL TARGETING GROUP

[0595] This Example describes the incorporation of an exemplary immune cell targeting conjugate into a preformed LNP.

[0596] LNPs from Example 3 and the conjugate from Example 4 were combined as shown in Table 6 in an Eppendorf tube and vortexed for 10 seconds at 2,500 rpm. The Eppendorf tubes were placed in the ThermoMixer at 37 °C at 300 rpm for 14 hours, and then stored at 4°C until use.

TABLE 6

nmol total lipid / mg RNA	Fab target g/mol lipid	Fab mg/mg RNA	RNA mg/mL LNP	Fab mg/mL Fab	Fab mg/ mL LNP	LNP mL/mL Fab
30,750	17	0.52275	1	1	0.52	1.91

[0597] This Example describes the incorporation of an immune cell targeting conjugate into a preformed LNP.

[0598] LNPs from Example 2 and conjugates (anti-CD3 (hSP34) and anti-CD8 (TRX-2) conjugates) were prepared using methods described in Example 4 were combined as shown

in Table 6A in an Eppendorf tube and vortexed for 10 seconds at 2,500 rpm. The Eppendorf tubes were placed in the ThermoMixer at 37 °C at 300 rpm for 14 hours, and then stored at 4°C until use. Alternatively, the Eppendorf tubes were placed in the ThermoMixer at 60 °C at 300 rpm for 30 minutes to 3 hours, followed by continued mixing at 4 °C and 300 rpm for an additional 12 – 24 hr and then stored at 4°C until use.

TABLE 6A

nmol total lipid / mg RNA	Fab target g/mol lipid	Fab mg/mg RNA	RNA mg/mL LNP	Fab mg/mL Fab	Fab mg/mL LNP	LNP mL/mL Fab	Mol% DSPE-PEG In LNP
30,750	17	0.52275	0.45	1.46	0.235	6.2	0.47

EXAMPLE 6 – Preparation of LNPs By Microfluidic Mixing Using Exemplary Ionizable Lipids

[0599] This example describes the preparation of LNPs using cationic Lipid 5 and cationic Lipid 8 by a microfluidic mixing method.

[0600] LNPs were created with an encapsulated mRNA payload and lipid blend by mixing an aqueous mRNA solution and an ethanolic lipid solution using an in-line microfluidic mixing process. The mRNA (a 9:1 w/w mix of mRNA encoding eGFP and eGFP mRNA labeled with Cy5, TriLink Biotechnologies, California, US) was mixed with pH 4 acetate buffer to provide a final aqueous mRNA solution containing 133 µg/mL mRNA and 21.7 mM acetate buffer. The lipid components were dissolved in anhydrous ethanol at the relative ratios set forth in TABLE 7 below.

TABLE 7

Lipid	Source	Ratio of Lipid to mRNA (nmol lipid/100 µg mRNA)	Concentration in Lipid Solution (mM)	Theoretical LNP Lipid Composition (mol%)
Ionizable Cationic Lipid	-	1,500	6	48.8
Cholesterol	Dishman Netherlands	1,200	4.8	39.0

DSPC	Avanti Polar Lipids, Alabama, US	300	1.2	9.8
DMG-PEG(2000)	Avanti Polar Lipids, Alabama, US	75	0.3	2.4

[0601] The mRNA and lipid solutions were mixed using a NanoAssemblr Ignite microfluidic mixing device (part no. NIN0001) and NxGen mixing cartridge (part no. NIN0002) from Precision Nanosystems Inc. (British Columbia, CA). Briefly, the mRNA and lipid solutions were each loaded into separate polypropylene syringes. A mixing cartridge was inserted into the NanoAssemblr Ignite, and the syringes were then attached to the cartridge. The two solutions were then mixed at a 3:1 v/v ratio of mRNA solution (975 μ L) to lipid solution (325 μ L) at a total flow rate of 9 mL/min using the NanoAssemblr Ignite. The resulting suspension was incubated at room temperature for not less than 5 min before proceeding to ethanol removal and buffer exchange.

[0602] Following mixing, ethanol removal and buffer exchange was performed on the resulting LNP suspension using two Sephadex G-25 resin packed SEC columns (PD MiniTrap G-25, Cytiva, Massachusetts, US), by gravity flow. Briefly, the SEC columns were each rinsed five times with 2.5 mL of exchange buffer (25 mM pH 7.4 HEPES buffer with 150 mM NaCl) before then loading 450 μ L of LNP suspension per column. Once the LNP suspension fully moved into the resin bed, a 50 μ L stacker volume of exchange buffer was applied to each column to achieve the specified target load volume of the column and maximize recovery, according to manufacturer specifications. After the stacker fully moved into the resin bed, the SEC columns were transferred to new centrifuge tubes, and the LNP suspension was eluted by adding 1.0 mL of exchange buffer to each column. Eluate containing the LNPs in the exchange buffer was recovered from each column, combined into a single LNP batch, and stored at 4°C until further use.

[0603] The resulting LNPs were characterized as described in Example 3. The results are summarized in TABLE 8 below. As seen in Table 8, the microfluidic process results in sub-100 nm particles exhibiting narrow polydispersity and good mRNA encapsulation (<20% dye accessible RNA).

TABLE 8

Formulation No.	Ionizable Lipid	DLS Z-Avg. Diameter (nm)	DLS PDI	Zeta Potential at pH 5.5 (mV)	Zeta Potential at pH 7.4 (mV)	Dye-Accessible mRNA (%)
5	Cationic Lipid 5	52	0.11	24	3.8	19
6	Cationic Lipid 8	55	0.15	24	4.0	11

Example 7 – Characterization of LNPs pKa Using Toluidinyl-naphthalene Sulfonate (TNS) fluorescent probe

[0604] This example describes the fluorescent dye based method used for measurement of the apparent pKa of the lipid nanoparticles. Apparent pKa determines the nanoparticle surface charge under physiological pH conditions, typically a pKa value in the endosomal pH range (6 – 7.4) results in LNPs that are neutral or slightly charged at plasma or the extracellular space (pH 7.4) and become strongly positive under acidic endosomal environments. This positive surface charge drives fusion of the LNP surface with negatively charged endosomal membranes resulting in destabilization and rupture of the endosomal compartment and LNP escape into the cytosolic compartment, a critical step in cytosolic delivery of mRNA and protein expression via engagement of the cells ribosomal machinery.

[0605] The apparent pKa of LNPs made using ionizable Lipids 2, 5 (synthesized as described in example 1), 6 and 7 (synthesized using method analogous to Lipid 2, 5, respectively, except using diethyl amine instead of dimethyl amine to incorporate the tertiary amine head group) were determined by 6-(p-Toluidino)-2-naphthalenesulfonic acid (TNS) fluorescence measurement in aqueous buffers covering a range of pH values (pH 4 - pH 10). TNS is non-fluorescent when free in solution, but which fluoresces strongly when associated with a positively charged lipid nanoparticle. At a pH values below the pKa of the nanoparticle, positive LNP surface charge results in dye recruitment at the particle interface resulting in TNS fluorescence. At pH values above the LNP pKa no fluorescence is observed. The apparent pKa of the LNP is reported as the pH at which the fluorescence is at 50% of its maximum, as determined using a four-point logistic curve fit. Lipids 2 exhibited an apparent pKa of 7.5 and chemical modification of tertiary amine head group in Lipid 6 resulted in a pKa shift to lower values (Lipid 6 pKa ~7, FIG. 8A). Similarly, Lipid 5 exhibited an apparent pKa of 6.9 and chemical modification of tertiary amine head group in Lipid 7 resulted in a pKa shift to lower

values (Lipid 7 pKa ~6.3, **FIG. 8B**). As a result, both modifications were found to create LNPs potentially capable of improved ability to fuse with negatively charged endosomal membranes and result in improved cytosolic delivery of the mRNA payload.

Example 8 – In Vitro Transfection Protocol in Primary Human T-cells

[0606] This example describes the protocol used for *in vitro* LNP transfections in primary human T-cells. This method is used to assess LNP *in vitro* efficacy in relevant target cells (CD3+ T cells) by first transfecting the cells using LNPs loaded with mRNA encoding for a reporter gene, such as GFP mRNA, and then assessing transfection by measuring reporter gene expression by fluorescence-actuated cell sorting (FACS). Additionally, particle association with cells may be observed by in the same assay by labeling individual nanoparticle components, such as the mRNA, with a fluorescent dye, such as Cy5, and then observing cell/dye association by FACS.

[0607] CD3+ T cells were isolated from frozen peripheral blood mononuclear cells using an EasySep Human T Cell Isolation Kit on a RoboSep automated cell isolation system from STEMCELL. T cells were plated into a flat bottom 96-well plate in RPMI cell culture media supplemented with glutamax, 10% fetal bovine serum, and 40 ng/mL IL2. 100 μ L of cell suspension was seeded per well at a density of 1M T cells/mL (100K T cells/well). Cells were allowed to rest for two hours in a 37°C incubator, and then were transfected by gently adding 10 μ L of a 22 μ g/mL (by mRNA) nanoparticle suspension, resulting in a final mRNA concentration of 2 μ g/mL. Cells were gently mixed with a pipette and then incubated for 24 hours in a 37 °C incubator. After incubation the cells were analyzed using an ThermoFisher Attune NXT flow cytometer. Cy5 was detected using a 638 nm laser with 670/14 nm filter. eGFP was detected using a 488 nm laser and a 530/30 nm filter. Data were analyzed using FlowJo software from BD biosciences. FACS data were first gated to exclude doublets and dead cells and then gated for GFP and Cy5. Gates for GFP+ and Cy5+ were set such that a control sample (PBS treated T cells) was $\leq$ 0.1% positive.

Example 9 – Lipid 2, lipid 6 LNP properties and in vitro protein expression in primary human T-cells

[0608] This example describes the transfection ability of LNPs derived from Lipid 2 and Lipid 6. Nanoparticles are first produced using a mixing process followed by buffer exchange. Particles thus produced were subsequently tested *in vitro* in human CD3+ T cells to assess LNP association with cells, and expression of a reporter gene.

[0609] Lipid 2 and Lipid 6 LNPs encapsulating a 90-10 (w/w) mixture of GFP-mRNA and Cyanine-5 dye labelled mRNA (TriLink Biotechnologies Inc.) were prepared using the mixing process described in Example 6, the buffer exchange process described below in Example 21. Both formulations resulted in particles exhibiting hydrodynamic diameters in the sub-150 nm range and moderate polydispersities, as well as good mRNA encapsulation and recovery (<25% dye accessible mRNA and > 80% encapsulated mRNA was recovered using the Triton-deformulation procedure described in Example 3).

[0610] As seen in FIGS. 9A and 9B and Table 9, moderate changes in particle size and PDI were observed upon insertion of an anti-CD3 hSP34-PEG2k-DSPE conjugation using the insertion procedure described in Example 4. The resulting targeted LNPs were evaluated in primary human T-cells using the *in vitro* transfection protocol described in example 8. As seen in FIG. 11, both formulations were well tolerated by T-cells below 0.5 µg/mL dose (<40% drop in cell viability relative to PBS control) with Lipid 6 LNPs resulting in moderately higher viability at higher dose of 2 µg/mL. Dose dependent expression of GFP protein was observed with both ionizable lipids (2 and 6) as illustrated by high percentage of GFP+ cells and strong GFP MFI values. As illustrated by the Cy5+ and Cy5 MFI values, both formulations were equally associated with cells suggesting the conjugate insertion process was not dependent of the ionizable lipid chemistry. Both ionizable lipids (2 and 6) resulted in acceptable levels of mRNA encapsulation (<30% dye accessible RNA and >60% total mRNA recovery).

Table 9. Lipid 2 and Lipid 6 LNP mRNA content

Ionizable Lipid	Theoretical total mRNA (µg/mL)	Measured total (Triton method) mRNA (µg/mL)	Ribogreen Dye Accessible mRNA (µg/mL)	% Dye Accessible mRNA
Lipid 2	45	39	8	20%
Lipid 6	45	40	8	15%

These findings demonstrate that lipid nanoparticles made using alternative ionizable lipids 2 and 6 may effectively encapsulate mRNA and transfect T cells *in vitro*.

Example 10 – Lipid 5, lipid 7 LNP properties and *in vitro* protein expression in primary human T-cells

[0611] This example describes the transfection ability of LNPs derived from Lipid 5 and Lipid 7. Nanoparticles are first produced using a mixing process followed by buffer

exchange. Particles thus produced were subsequently tested *in vitro* in human CD3+ T cells to assess LNP association with cells, and expression of a reporter gene.

[0612] Lipid 5 and Lipid 7 LNPs encapsulating a 90-10 (w/w) mixture of GFP-mRNA and Cyanine-5 dye labelled mRNA (TriLink Biotechnologies Inc.) were prepared using the mixing process described in Example 6, the buffer exchange process described below in Example 21. Both formulations resulted in particles exhibiting hydrodynamic diameters in the sub-150 nm range and moderate polydispersities, as well as good mRNA encapsulation and recovery (<25% dye accessible mRNA and > 80% encapsulated mRNA was recovered using the Triton-deformulation procedure described in Example 3). As seen in FIGS. 10A and 10B and Table 10, Lipid 5 LNP exhibited a larger change in hydrodynamic diameter (relative to Lipid 7 LNP) upon insertion of an anti-CD3 hSP34-PEG2k-DSPE conjugate using the insertion procedure described in Example 4. The resulting targeted LNPs were evaluated in primary human T-cells using the *in vitro* transfection protocol described in example 8. As seen in FIGS. 12A-12E, both formulations were well tolerated by T-cells at and below 0.5 µg/mL dose (minimal drop in cell viability was observed relative to the PBS control). As illustrated by the Cy5+ and Cy5 MFI values, both formulations were equally associated with cells suggesting the conjugate insertion process is not dependent on the ionizable lipid chemistry.

[0613] Dose dependent expression of GFP protein was observed with both ionizable lipids (5 and 7) as illustrated by similar % GFP+ and GFP MFI values (FIGS. 12A and B). Both formulations resulted in similar levels of cell association as illustrated by similar %Cy5+ and Cy5 MFI values (FIGS. 12C and D). However, Lipid 7 LNP formulation (apparent pKa ~ 6.4) exhibited significantly lower level of GFP protein expression relative to Lipid 5 LNP formulation (apparent pKa ~ 7) suggesting relatively poor cytosolic access with Lipid 7 LNPs. Both ionizable lipids (5 and 7) resulted in acceptable levels of mRNA encapsulation (<30% dye accessible RNA and >60% total mRNA recovery).

Table 10. Lipid 5 and Lipid 7 LNP mRNA content

Ionizable Lipid	Theoretical total mRNA (µg/mL)	Measured total (Triton method) mRNA (µg/mL)	Ribogreen Dye Accessible mRNA (µg/mL)	% Dye Accessible mRNA
Lipid 5	50	43	19	23%
Lipid 7	50	42	6	14%

Example 11 – Lipid 5, Lipid 8 and DLn-MC3-DMA LNP properties in vitro protein expression in primary human T-cells

[0614] This example compares the GFP protein expression resulting from LNP's derived from Lipid 5 and Lipid 8 to LNPs made using DLn-MC3-DMA. Nanoparticles are first produced using a mixing process followed by buffer exchange. Particles thus produced were subsequently tested *in vitro* in human CD3+ T cells to assess LNP association with cells, and expression of a reporter gene.

[0615] Lipid 5, Lipid 8 and DLn-MC3-DMA LNPs encapsulating a 90-10 (w/w) mixture of GFP-mRNA and Cyanine-5 dye labelled mRNA (TriLink Biotechnologies Inc.) were prepared using the vortexer mixing and buffer exchange process described in Example 4. All three formulations resulted in particles exhibiting hydrodynamic diameters in the sub-150 nm range and moderate polydispersities (FIG. 37A and B). Additionally, all three formulations made using the vortexer method using Lipids 5, 8 and DLn-MC3-DMA exhibited acceptable mRNA encapsulation (<30% dye accessible mRNA) and moderate mRNA recovery (>60% encapsulated mRNA recovered using the Triton-deformation procedure described in Example 3). As seen in FIG. 37, the insertion of an anti-CD3 hSP34-PEG2k-DSPE conjugation using the insertion procedure described in Example 4 resulted in only slight increases in hydrodynamic diameter and PDI. The resulting targeted LNPs were evaluated in primary human T-cells using the *in vitro* transfection protocol described in example 8. As seen in FIG. 38, all three formulations were well tolerated by T-cells below 0.5 µg/mL dose (<40% drop in cell viability relative to PBS control) with Lipid 8 LNPs exhibiting slightly lower viability at higher dose of 2 µg/mL (FIG. 38E). Dose dependent expression of GFP protein was observed with ionizable Lipids 2 and 8 as illustrated by high percentage of GFP+ cells and strong GFP MFI values (FIGS. 38A, B). However, DLn-MC3-DMA (also shown in FIGS. 38A, B) LNPs failed to express GFP protein. Comparison of the Cy5+ and Cy5 MFI values in Lipid 2 or Lipid 8 formulations with the corresponding levels in the DLn-MC3-DMA LNP transfections (FIGS. 38C and D) indicates that all three formulation associated equally with T-cells suggesting that the efficiency of the antibody insertion process is independent of ionizable lipid chemistry. Poor performance of DLn-MC3-DMA LNPs may be attributed to this formulation resulting in poor cytosolic availability of mRNA in primary human T-cells.

TABLE 11A. Lipid 5, Lipid 8 and DLn-MC3-DMA LNP mRNA content

Ionizable Lipid	Theoretical total mRNA ($\mu\text{g/mL}$)	Measured total (Triton method) mRNA ($\mu\text{g/mL}$)	Ribogreen Dye Accessible mRNA ($\mu\text{g/mL}$)
Lipid 8	42.5	30	3
DLn-MC3-DMA	42.5	33	2
Lipid 5	42.5	27	4

EXAMPLE 12 – IN VITRO PROTEIN EXPRESSION - CD3 AND CD8 TARGETED Cy5/GFP LNP WITH VARIOUS DENSITIES

[0616] This Example describes targeting human CD8 T cells with either anti-CD3 or anti-CD8 Fabs post-inserted into Cy5/GFP mRNA LNPs at various Fab densities and their effect on particle binding, transfection, viability, CD69 upregulation and IFN γ secretion.

[0617] LNPs were prepared using the mixing process described in Example 6, the buffer exchange process described in Example 21. hSP34 and TRX2 Fab-lipid conjugates generated from methods described in Example 4 and a non-T cell specific anti-HER2 lipid-conjugate (Nellis DF, Ekstrom DL, Kirpotin DB, Zhu J, Andersson R, Broadt TL, Ouellette TF, Perkins SC, Roach JM, Drummond DC, Hong K, Marks JD, Park JW and Giardina SL (2005) Preclinical manufacture of an anti-HER2 scFv-PEG-DSPE, liposome-inserting conjugate. 1. Gram-scale production and purification. Biotechnol Prog 21:205-220) were post-inserted at various densities (SP34 0.6-17 g/mol; TRX2 3-9 g/mol; anti-HER2 17 g/mol) into LNPs containing Lipid 8, Cy5 labeled GFP mRNA and GFP mRNA (1:9 Cy5:GFP mass ratio) by adding conjugate and LNPs together and heating the solution without mixing in a thermal cycler at 60°C for 60 min and cooled to 4 °C for 3-5 min. Particles were then diluted to 25 $\mu\text{g/mL}$ mRNA with Hepes Buffered Saline pH 7.4 prior to transfection of human CD8 T cells using a method similar to Example 8 where the final concentration was approximately 2.5 $\mu\text{g/mL}$ mRNA for approximately 24 hr (or 10 μL of Hepes Buffered Saline pH 7.4 buffer was added as a mock transfection). After transfection, LNP binding efficiency (Cy5) and transfection efficiency (GFP) was evaluated by flow cytometry. Supernatants were measured for human IFN γ concentration using a commercially available ELISA kit under manufacturers recommended conditions (R&D Systems DuoSet).

[0618] High transfection (FIG. 13A) and binding (FIG. 13B) was observed for SP34 and TRX2 Fab post-inserted LNPs with a broad range of Fab densities mediating transfection while non-specific HER2 targeted LNPs exhibited low binding and transfection. Some loss in cell viability was observed (FIG. 14A) using hSP34 CD3 targeted LNPs while TRX2 CD8

targeted LNPs had similar viabilities to non-specific HER2 targeted LNPs and untransfected (mock buffer added) T cells. Additionally, hSP34 (with mouse or human lambda) CD3-targeted LNPs mediated high IFN γ secretion (**FIG. 14B**) relative to TRX2 CD8-targeted, HER2-targeted LNPs and the mock T cell transfection conditions.

[0619] This study shows that CD8 T cells can be efficiently transfected with CD3 and/or CD8 targeted LNPs using a broad range of Fab densities in all cases. Additionally, using anti-CD8 Fab can mediate efficient LNP transfection while avoiding high CD69 upregulation and IFN γ secretion.

EXAMPLE 13 – IN VITRO PROTEIN EXPRESSION - CD3, CD8 AND CD3/CD8 TARGETED TTR-023 LNP WITH VARIOUS DENSITIES

[0620] This example describes targeting human CD3 T cells with either anti-CD3 or anti-CD8 Fabs post-inserted into anti-CD20 CAR (TTR-023) mRNA LNPs at various Fab densities and their effect on transfection, viability, CD69 upregulation and IFN γ secretion.

[0621] LNPs were prepared using the mixing process described in Example 6, the buffer exchange process described below in Example 21. Using methods similar to Example 12, hSP34 and TRX2 Fab-lipid conjugates and a non-T cell specific anti-HER2 lipid-conjugate (Nellis DF, Ekstrom DL, Kirpotin DB, Zhu J, Andersson R, Broadt TL, Ouellette TF, Perkins SC, Roach JM, Drummond DC, Hong K, Marks JD, Park JW and Giardina SL (2005) Preclinical manufacture of an anti-HER2 scFv-PEG-DSPE, liposome-inserting conjugate. 1. Gram-scale production and purification. *Biotechnol Prog* 21:205-220) were post-inserted at various densities (SP34 0.25-17 g/mol; TRX2 0.25-9 g/mol; SP34 + TRX2 0.25-9 g/mol each conjugate; anti-HER2 17 g/mol) into LNPs containing Lipid 8 and anti-CD20 targeting CART mRNA. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr. For FACS analysis T cells were stained with M1 antibody (Sigma, F3040) that bind a N-terminus FLAG-tag variant sequence on the TTR-023 CAR (sequence provided below) in addition to staining for CD69 (Biolegend, 310930) and CD4 (Biolegend, 344648) to differentiate CD8 from CD4 cells.

[0622] High transfection efficiency (**FIGS. 15A and 15B**) was observed between 2-17 g/mol Fab for hSP34 alone or co-targeted with TRX2 and transfection was detected over background for TRX2 at 6-9 g/mol Fab. Consistent with the transfection results, CD69 was

upregulated in a target specific manner where CD3 targeting by hSP34 Fab induced CD69 expression on both CD8 and CD4 cells while CD8 targeting by TRX2 mediated CD69 expression on CD8 cells only (FIGS. 16A and 16B). CD8-targeted LNPs with TRX2 Fab induced low levels of IFN γ secretion (FIG. 17) relative to CD3 and CD3/CD8 targeted LNPs despite observable CD69 upregulation on CD8 T cells.

[0623] This study shows that both CD4 and CD8 T cells can be efficiently transfected with CD3-targeted and CD3/CD8-targeted LNPs and CD8 cells can be specifically (avoidance of CD4 transfection) transfected with CD8-targeted LNPs using a broad range of Fab densities in all cases. Additionally, using anti-CD8 Fab can mediate efficient transfection with CAR mRNA while avoiding high CD69 upregulation and IFN γ secretion.

[0624] TTR-023 anti-CD20 (Leu-16) CAR sequence (including leader) (SEQ ID NO: 24):

METDTLLLWVLLLWVPGSTGDYKAKEVQLQQSGAELVKPGASVKMSCKASGYTFT
SYNMHWVKQTPGQGLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSS
LTSEDSADYYCARSNYYGSSYWFFDVWGAGTTVTVSSGGSGGGSGGGSSDIVLT
QSPAILSASPGEKVTMTCRASSSVNYMDWYQKKPGSSPKPWYATSNLASGVPARFS
GSGSGTSYSLTISRVEAEDAATYYCQQWSFNPTFGGGTKLEIKGGGGSAAAIEVMY
PPPYLDNEKSNGTIIHVKGKHLCPSPFLPGPSKPFWVLLVVGGVLACYSLLVTVAFIIF
WVRSKRSRLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSRVKFSRSAEPPAY
QQGQNQLYNELNLGRREEYDVLDRRRGRDPEMGGKPRRKNPQEGLYNELQKDKM
AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

Corresponding nucleic acid sequence (SEQ ID NO: 25):

atggagaccgacacccctgtgcttgggtactgttacttgggtgcccgatctaccggtgattacaaggccaaggaggtgcagctg
cagcagagcggagccgagctggtgaagccaggcgctccgtgaagatgtcttgaaggcctccgctacacatccaccagctaca
atatgcactgggtaaagcagactccggggcagggcctggagtgataggtgccatctaccctggcaacggcgacaccagctaca
accagaagttaagggaaggctactctaaccagcgacaagtcgtctctaccgctcatatgcaactcagctccctgacgagcga
ggactecggcggactactactgtgccgctccaactactacggctctagctattgttcttcgacgtgtggcgctggaacgacct
gaccgtgtcttccgtggaggttccggggcggaagcgccgggtggcggcagttccgacatcgtgctgaccagagccctgcc
tcctgtccgcttccccggggagaaaagttacgatgacctgccgagcgagctccaggtgcaactacatggattgtaccagaagaa
gccccgcagcagtcaccaagccgtggatttacgctactagcaacctggcgtccggtgtcccggtctgcttccaggttctgctgg
gtactagtattcatttaaccatttctcgtggaggctgaggacgctgccactactactgccaacagtggcttccaacctccactt
tcggaggcgggcaccagctcgagatcaaggcggggtggctccgcagcagccattgaggtgatgtatcctcctcctatttgg
caacgagaagtcaaatggcaccatcatccagctaaaggcaagcactgtgcccattctccctgttccagggcccctctaagccct
ctgggtctcgtgtgtgtcgccggcgtctcgtgcatgtactctctgctggtgacgtcgcttcatcatcttftgggtccggtccaag
cgagccgctgtctccactccgactacatgaatgactcctctaggtcccggtccaaccgcaagcactaccagccgtacgcgc
cgccagagacttctgtcttaccgatccagagtgaattttctaggtcgccgaacctccgcatafcagcagggccagaaccag
ctgtacaacgaactcaacttgggacggcgaggaatagatgtctggataaacgctgtggcgatcccgagatggcggg
gaagccacgtcgcaaaaacctcaggagggtcttacaacaggttcgagaaggacaaaatggcgaggcctactccgagatcg

gaatgaagggggagcgccggcgcggaaggcatgacggcctctaccagggcctgtccacagccacgaaagacacctatga
cgccctgcatatgcagggcctgccccgcgctgataatga

EXAMPLE 14 – IN VITRO PROTEIN EXPRESSION - CD3 AND CD8 TARGETED WITH OTHER CLONES

[0625] This example describes targeting human CD8 T cells with either anti-CD3 or anti-CD8 Fabs post-inserted into Cy5/GFP mRNA LNPs at various Fab densities and their effect on particle binding, transfection, viability, CD69 upregulation and IFN γ secretion.

[0626] LNPs were prepared using the mixing process described in Example 6, the buffer exchange process described in Example 21. Using methods similar to Example 12, hSP34, Hu291, TRX2, OKT8 Fab-lipid conjugates and a non-T cell specific anti-HER2 lipid-conjugate (Nellis DF, Ekstrom DL, Kirpotin DB, Zhu J, Andersson R, Broadt TL, Ouellette TF, Perkins SC, Roach JM, Drummond DC, Hong K, Marks JD, Park JW and Giardina SL (2005) Preclinical manufacture of an anti-HER2 scFv-PEG-DSPE, liposome-inserting conjugate. 1. Gram-scale production and purification. *Biotechnol Prog* 21:205-220) were post-inserted at various densities (Table 11) into LNPs containing Lipid 8 and Cy5/GFP mRNA. Transfections were performed with human CD8 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr.

[0627] SP34 mediated higher %transfection (FIG. 18A) and substantially higher GFP expression levels (FIG. 18B) quantified by mean fluorescence intensity, MFI) than Hu291 despite both clones showing high levels of %Cy5+ T cells and binding the same target, CD3 (FIGS. 19A and 19B). Similarly for CD8 targeting, TRX2 mediated higher transfection than OKT8 by both metrics of %GFP+ and MFI (FIGS. 18A and 18B) despite both clones mediating high levels of particle binding measured by %Cy5+ (FIG. 19A). Additionally, combining OKT8 and TRX2 increased the amount of particle binding (FIG. 19B) while providing no enhancement of transfection (FIGS. 18A and 18B).

[0628] This data indicates that the epitope that the Fab binds on the target protein may be important in determining its ability to mediate efficient particle uptake, transfection and translation and that efficient binding to the target does not guarantee efficient transfection.

Table 11: Target post-insertion LNP Fab densities

Fab clone	Fab density 1 (g/mol)	Fab density 2 (g/mol)
SP34-mlam	12	17
Hu291	6	12
OKT8	3	6
TRX2	3	6
OKT8 / TRX2	3	6
F5	17	-

EXAMPLE 15 – IN VITRO PROTEIN EXPRESSION – CD3, CD8, CD4 AND CD8/CD4 TARGETED

[0629] This example shows targeting human CD3 T cells with either anti-CD3, anti-CD8 anti-CD4 or anti-CD8 and anti-CD4 Fabs post-inserted into Cy5/GFP mRNA LNPs at various Fab densities and their effect on particle binding, transfection, viability, CD69 upregulation and IFN γ secretion.

[0630] LNPs were prepared using the mixing process described in Example 6, the buffer exchange process described in Example 21. Using methods similar to Example 12, hSP34, TRX2, and Ibalizumab Fab-lipid conjugates and a non-T cell specific anti-HER2 lipid-conjugate were post-inserted at various densities (specified in FIGS. 20A and 20B) into LNPs containing Lipid 8 and Cy5/GFP mRNA. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr and stained for CD69 (Biolegend, 310930) and CD4 (Biolegend, 344648) to differentiate CD8 from CD4 cells by FACS analysis.

[0631] Consistent with previous results, hSP34 and TRX2 mediated specific LNP binding and transfection to CD3 and CD8 cells respectively (FIGS. 20A and 20B and FIGS. 21A and 21B). A CD4 targeting Fab based on the VH and VL sequences of Ibalizumab mediated high binding and transfection of CD4 T cells while displaying minimal off-target binding and transfection of CD8 T cells. When TRX2 and Ibalizumab Fabs were post inserted into the same LNPs, high levels of binding and transfection were observed in both CD4 and CD8 cells using a broad range of Fab densities. While hSP34 drive high levels of CD69 upregulation (FIGS. 22A and 22B), TRX2 alone, Ibalizumab-Fab alone and TRX2 combined

with Ibalizumab-Fab mediated much lower levels of CD69. In addition, SP34 drove higher levels of IFN γ (FIG. 23) than TRX2, Ibalizumab-Fab or the combination thereof.

[0632] This study shows that both CD4 and CD8 T cells can be efficiently transfected with CD3-targeted and CD8/CD4-targeted LNPs, CD8 cells can be specifically (avoidance of CD4 transfection) transfected with CD8-targeted LNPs and CD4 cells can be specifically (avoidance of CD8 transfection) transfected with CD4-targeted LNPs using a broad range of Fab densities in all cases. Additionally, using anti-CD8 Fab, anti-CD4 Fab or both anti-CD8 and anti-CD4 Fab can mediate efficient transfection while avoiding high CD69 upregulation and IFN γ secretion.

EXAMPLE 16 – IN VITRO EXPERIMENTAL PROTOCOL FOR WHOLE BLOOD TRANSFECTION

[0633] This Example describes the method used to transfect immune cells in whole blood using Fab targeted mRNA LNPs.

[0634] Venous blood from healthy volunteers was anti-coagulated in heparin tubes (BD Biosciences #367526) and seeded at 50 μ L in a 96-well round-bottom plate. Transfection of whole blood was carried out simply by adding nanoparticles containing 5 μ g/mL mRNA to the cells and co-culturing at 37°C until the time of analysis. To assess transfection efficiency, cells were analyzed 24-hours post-transfection by flow cytometry. LNPs used (with and without post-inserted targets) at 2.5 μ g/mL:RDM073.23. Cells obtained from human blood were analyzed by flow cytometry. Prior to the analysis of whole blood transfection efficiency, red blood cells were lysed twice with VersaLyse Lysing Solution (Beckman Coulter #A09777) for 10 minutes at room temperature. Primary antibodies applied in the flow cytometry analysis of whole blood included the following: CD4-FITC (1:200) (BD Biosciences #555346), CD19-BUV395 (1:400) (BD Biosciences #563551), CD56-BUV737 (1:400) (BD Biosciences #741842). Fixable Viability Dye eFluor780 (eBiosciences #65-0865-14) was used to assess viability for all samples. For flow analysis, 1×10^5 cells were Fe-blocked (BD Biosciences #564219) for 5 minutes on ice, followed by labeling dead cells with fixable viability dye eFluor780 and surface staining for 30 minutes on ice with specific antibodies.

[0635] Compensation for each fluorochrome was performed in the multicolor flow panels using positive and negative compensation beads. Fluorescence minus one (FMO) samples

and unstained controls were included to determine the level of background fluorescence and to set the gates for the negative cell populations versus the positive cell populations.

[0636] All samples were acquired on a BD LSRFortessa X-20 (BD Biosciences) running FACSDIVA software (Becton Dickinson). All data collected were analyzed using FlowJo 10.7.1 software and GraphPad Prism version 9.0.

Example 17 – *In vitro*-cell specific protein expression (mcherry) in human whole blood

[0637] This example describes specifically targeting human T cells in whole blood with anti-CD3, anti-CD8, anti-CD2, anti-CD5, anti-CD7 Fabs or combinations thereof post-inserted into mCherry mRNA LNPs at various Fab densities and their effect on transfection and CD69 upregulation secretion.

[0638] LNPs were prepared using the vortex mixing process described in Example 2 using the component ratios described in Table 12 below. Conjugate from a process described in Example 4 was post-inserted after particle formation). Particle properties were characterized using methods described in Example 3 and are described below in Table 13.

Table 12

Batch	nmol lipid per 100µg mRNA				
	Lipid 8	Cholesterol	DSPC	DMG-PEG2000	Conjugate
73.23	1500	1200	300	75	0.1-0.6*

*depending on specific conjugate. TRX2 used in 0.6 nmol per 100µg mRNA.

Table 13

Batch	Size (nm)	PDI	EE (%)	Zeta at pH 7.4 (mV)
73.23	123.6	0.217	93.2	N.D.

[0639] Using the methods described in Example 16, the transfection efficiency with the classic ionosphere formulation with Fab clones hSP34 (anti-CD3), TRX2 (anti-CD8), He3

(anti-CD5), anti-CD2 (TS2, 9.6 or 9-1), anti-CD7 (TH-69) or mutOKT8, post-inserted respectively (densities described in Table 14), and combinations of these, directly in human whole blood. LNPs with Lipid 8 were transfected in WB with 2.5 µg/mL mCherry mRNA for 24 hours.

Table 14

Conjugate 1	Conjugate 2	Density (g/mol)	Target 1	Target 2
hSP34	-	9,00	CD3	-
TRX2	-	9,00	CD8	-
TRX2	hSP34	9,00	CD8	CD3
He3	-	3,00	CD5	-
He3	TRX2	3,00	CD5	CD8
TS2/18.1	-	1,50	CD2	-
9.6	-	1,50	CD2	-
TS2/18.1	TRX2	1,50	CD2	CD8
9.6	TRX2	1,50	CD2	CD8
TH-69	-	3,00	CD7	-
TH-69	TRX2	3,00	CD7	CD8
9-1	TS1/18.1	1,50	CD2	CD2
9-1	9.6	1,50	CD2	CD2
He3	TH-69	3,00	CD5	CD7
mutOKT8	-	9,00	NT	-
DSPE-PEG	-	9,00*	NT	-
TH-69	hSP34	3,00	CD7	CD3
TS2/18.1	He3	1,50	CD2	CD5

*DSPE-PEG amount added to match the amount added from 9 g/mol of a ~48kD Fab

[0640] All of the targeting Fabs enable observable transfection relative to background with varying degrees of efficiency depending on the target and clone. The most efficient transfection of both CD8 and CD4 cells was observed using hSP34, He3 and combinations of hSP34/He3, He3/TH-69, hSP34/TRX2, He3/TRX2, hSP34/TH-69, TH-69/TRX2, 9.6/9-1, TS2/9-1 (FIGS. 24A and 24B). TRX2 efficiently transfects CD8 T-cells without transfection observed in CD4 T-cells (FIGS. 24A and 24B). Additive effects were observed in terms of transfection efficiency for combinations with TRX2/He3, 9.6/9-1, TS2/9-1 and He3/TH-69

(FIGS. 24A and 24B) indicating synergistic effects can be mediated by targeting two different targets or two different epitopes on the same target. Transfection was also observed in NK cells with CD3, CD8, CD2 and CD7 targeting in addition to combinations thereof (FIG. 25B). Generally, no off-target transfection is observed in B-cells (FIG. 25A) and Granulocytes (FIG. 26A). High CD69 upregulation was only observed in CD4 and CD8 cells with CD3-targeting or CD3 targeting in combination with other targets such as CD8 or CD7 (FIGS. 26B and 26C). Additionally, non-targeted LNPs post-inserted with similar DPSE-PEG relative to Fab targeted formulations and LNPs post-inserted with mutOKT8 Fab did not exhibit transfection of any of the immune cell types indicating specific transfection is mediated by Fab targeting (FIGS. 24A and 24B).

[0641] This study shows that in whole blood, CD4 and CD8 T cells can be efficiently transfected with CD3-targeted, CD5-targeted, CD7-targeted or CD2-targeted LNPs as well as targeting combinations thereof, CD8 cells can be specifically (avoidance of CD4 transfection) transfected with CD8-targeted LNPs, transfection can be skewed towards CD8 cells versus CD4 cells using CD8-targeting in combination with CD5, CD7 or CD2 targeting.

[0642] Using Fabs targeting different targets or Fab clones that bind the same target but known to target different epitopes (*e.g.*, anti-CD2 clones 9.6 and 9-1) in combination can lead to synergistic increases in transfection efficiency. NK cells were transfected with CD8, CD7 or CD2 targeting Fabs or combinations thereof consistent with known surface expression of these markers on human NK cells or NK cell subsets. While LNPs with anti-CD3, anti-CD8, anti-CD5, anti-CD7 or anti-CD2 Fabs or combinations thereof can mediate efficient transfection of T cells and NK cells (for some Fabs), minimal transfection was observed in B cells or Granulocytes indicating high specific uptake and transfection enabled by Fab targeting given non-targeted Fab (mutOKT8) or nontargeted LNPs did not transfect T cells or NK cells. Additionally, using anti-CD8, anti-CD5, anti-CD7 or anti-CD2 Fabs or combinations thereof can mediate efficient transfection without driving high CD69 expression.

EXAMPLE 18 – IN-VIVO REPROGRAMMING OF IMMUNE CELLS WITH LNP EXPRESSING MCHERRY

[0643] This example describes the time course of reprogramming of immune cells in humanized mice treated with LNP expressing mCherry.

Mice Strains and Humanization

[0644] The NCG mouse (NOD-Prkdc^{em26Cd52} Il2rg^{em26Cd22}/NjuCr1) mouse model was purchased from Charles River Laboratories. 4 weeks old male mice were engrafted with 10 million PBMC of qualified donor (by Charles river) in sterile PBS by tail vein injection and were shipped to Tidal facility. Individual body weight was monitored twice a week and blood samples were collected at appropriate interval to evaluate human immune cells engraftment.

Evaluation of Human T-cell Engraftment in the Immunodeficient Mice

[0645] 50ul blood was collected by tail vein bleed from each mouse. Red blood cells were lysed using Versalyse, RBC lysis solution following protocol as instructed by manufacturer (Beckman Coulter A09777). Cells were stained with hCD45 & hCD3 to determine the engraftment of human T-cells. After 30 days of PBMC injection, mice had anywhere from 30-60% huCD45+. These humanized mice were evaluated for reprogramming of immune cells by LNPs expressing mCherry.

Reprogramming of Immune Cells

[0646] At time zero, 9 mice were injected with mCherry expressing LNPs prepared using cationic Lipid 8 and the mixing process described in Example 6, the buffer exchange process described in Example 21, and targeted with hSP34-lipid using the process described in Example 5 (Lot# 201109APG-NF70-409), by i.v. at 3mg/kg or 6 mice were injected with appropriate buffer. At each time point, 24, 48 and 96 h, 3 mice treated with LNPs or 2 mice treated with buffer were sacrificed. Terminal blood and tissues collection was performed to determine mCherry expression in different organs and immune cells as below.

Tissue and blood sample collection

[0647] At above specified timepoints, mice were anesthetized with CO2 before sample collection. For blood collection, the chest was opened to expose the heart. Up to 300 µl blood was drawn from the left ventricle and dispensed into a K3EDTA mini collect tube (Greiner Bio-One). Then a new syringe was used to draw remaining blood from the heart as much as possible. All the immune organs; spleen, bone marrow, thymus and all the lymph nodes (lingual, axillary, submandibular and mesentery) were isolated along with liver. Immune cells were isolated from spleen, thymus and lymph nodes via smearing and shredding it through syringe and cell suspension was filtered through 70 µm cell strainer and was washed with

PBS. A piece of liver tissue was gently grinded with tissue homogenizer and the homogenized tissue was incubated with digestive solution (10 ml HBSS supplemented with 0.05% of type IV collagenase (Sigma C5138-5G) 0.02% BSA (Sigma A2153-100G), 0.001% DNASE I, Grade II (Sigma 10104159001) and 1 mM calcium chloride (Sigma C7902-500G) for 30 min at 37°C. After 30 mins the digestion was terminated with 10 ml of ice cold solution of HBSS.

Immunophenotyping Analysis

[0648] Immune cells from blood and all the above organs were processed with Versalyse, RBC lysis buffer as per manufacturing instructions. Immune cells were stained with live/dead fixable dye and surface markers with standard flow analysis protocol as shown in below panel. Attune, Thermo flow cytometer was used to determine positive population.

Table 15. Panel 1

Antigen	Fluorophore	Clone	Company	Catalog#
Alive Dead dye	Zombie Aqua	NA	BioLegend	423102
Anti-human CD45	FITC	2D1	BioLegend	368508
Anti-human CD3	PerCP/Cyanine5.5	UCTH1	BioLegend	300430
Anti-human CD8	APC-Fire750	SK1	BioLegend	344746
Anti-human CD4	BV711	SK3	BioLegend	344648
Anti-human CD69	BV421	FN50	BioLegend	310930
Anti-human CD137	BV421	4B4-1	BioLegend	309820
Anti-human 279 PD-1	APC	NAT05	BioLegend	367406
Anti-human CD366	APC	F38-2E2	BioLegend	345012

mCherry	mCherry	NA	NA	NA
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Table 16. Panel 2

Antigen	Fluorophore	Clone	Company	Catalog#
Alive Dead dye	Zombie Aqua	NA	BioLegend	423102
Anti-human	FITC	2D1	BioLegend	368508
Anti-human/mouse CD11b	BV785	M1/70	BioLegend	101243
Anti-mouse F4/80	APC	BM8	BioLegend	123116
Anti-human CD19	BV711	SJ25C1	BioLegend	563036
mCherry	mCherry	NA		NA

huNCG-PBMC mice treated with CD3 targeted LNP expressing mCherry at 3mg/kg showed mcherry in T cells of blood, liver and spleen. CD8+ T cells (FIGS. 27A, 27C and 27E) showed highest mCherry expression, with up to ~30% of CD8+T cells in blood, liver and spleen. CD4+ T cells (FIGS. 27B, 27D and 27F) showed up to ~15% mCherry expression in blood, liver and spleen. No reprogramming was seen in other organs analyzed. The expression of mCherry is restricted to CD3+ cells. Minimal or no mCherry expression was observed in liver myeloid, macrophages or Kupffer cells (FIG. 28).

Overall, CD3 targeted mCherry LNP specifically reprogrammed T cells with minimal or no expression in myeloid population.

EXAMPLE 19 – IN-VIVO REPROGRAMMING OF IMMUNE CELLS WITH LNP EXPRESSING MCHERRY OR CD20 CAR WITH EITHER CD3 AND OR CD8 TARGETING ANTIBODY

[0649] This Example describes *in vivo* reprogramming with LNP expressing either mCherry or CD20 CAR and compares CD3 vs CD8 targeting or combination of both.

Mice Strains and Humanization

[0650] NSG female mice were purchased from Jackson lab. At 8 weeks of age mice were i.v. injected with 20 million PBMC (in house isolated leukopak, donor 555046, Precision for Medicine).

Evaluation of Human T-cell Engraftment in the Immunodeficient Mice

[0651] 50ul blood was collected by tail vein bleed from each mouse. Red blood cells were lysed using Versalyse, RBC lysis solution following protocol as instructed by the manufacturer (Beckman Coulter A09777). Cells were stained with hCD45 & hCD3 to determine the engraftment of human T-cells. After 30 days of PBMC injection, mice had anywhere from 60-80% huCD45+. These humanized mice were evaluated for reprogramming of immune cells as below.

Reprogramming of Immune Cells with mCherry & CD20 CAR with CD3 and/or CD8 targeting antibody

[0652] At time zero, mice (n=5) were i.v. injected at the dose of 3mg/kg with either i) Buffer or LNP expressing; ii) TTR-023 mRNA targeted with 17g/mol of hSP34; iii) TTR-023 mRNA targeted with 9g/mol of hSP34; iv) TTR-023 mRNA targeted with 9g/mol of TRX2; v) TTR-023 mRNA targeted with 9g/mol of TRX2 + 9g/mol of hSP34; vi) mCherry mRNA (n=3 mice) targeted with 17g/mol of hSP34. After 24h, 50ml blood was collected and processed as mentioned in example 18. At 96h, 2nd dose of either LNPs (as above) or buffer was injected in mice at 3mg/kg. After the 2nd dose, terminal blood and organs were collected and processed as described in example 18 at 40h time point.

[0653] LNPs were prepared using cationic Lipid 8 and the mixing process described in Example 6, the buffer exchange process described below in Example 21, and targeted with hSP34-lipid or TRX2-lipid using the process described in Example 5. The table below summarizes the formulations and lot numbers used.

Table 17.

Test Article Description	Lot Number for 1st Dose Material	Lot Number for 2nd Dose Material
TTR-023 LNPs inserted with 17 g/mol hSP34	210128APG-NT317	210201APG-NT317
TTR-023 LNPs inserted with 9 g/mol hSP34	210128APG-NT309	210201APG-NT309
TTR-023 LNPs inserted with 9 g/mol TRX2	210128APG-NT809	210201APG-NT809
TTR-023 LNPs inserted with 9 g/mol hSP34 and 9 g/mol TRX2	210128APG-NT38	210201APG-NT38
mCherry LNPs inserted with 17 g/mol hSP34	210128APG-NM317	210201APG-NM317
5.3 wt% sucrose in HBS	210128APG-S1	210128APG-S1

Immunophenotyping Analysis

[0654] Similar immunophenotyping analysis was done as described in example 18 with panels listed below. CD20 CAR expression was evaluated by detecting M1 tag expressed by CD20 CAR with primary M1 antibody followed by secondary antibody.

Table 18. Panel 1

Antigen	Fluorophore	Clone	Company	Catalog#
Alive Dead dye	Zombie Aqua	NA	BioLegend	423102
Anti-human CD45	FITC	2D1	BioLegend	368508
Anti-human CD3	PerCP/Cyanine5.5	UCTH1	BioLegend	300430
Anti-human CD8	APC-Fire750	SK1	BioLegend	344746
Anti-human CD4	BV711	SK3	BioLegend	344648
Anti-human CD69	BV421	FN50	BioLegend	310930
Anti-human CD137	BV421	4B4-1	BioLegend	309820
M1 tag	NA	NA	Sigma	M1-F3040
Secondary antibody to M1	APC	NA	Southern biotech	1090-11S
mCherry	mCherry	NA	NA	NA

Table 19. Panel 2

Antigen	Fluorophore	Clone	Company	Catalog#
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Alive Dead dye	Zombie Aqua	NA	BioLegend	423102
Anti-human	FITC	2D1	BioLegend	368508
Anti-human/mouse CD11b	BV786	M1/70	BioLegend	101243
Anti-mouse F4/80	BV421	BM8	BioLegend	123132
Anti-human CD19	BV711	SJ25C1	BioLegend	563036
mCherry	mCherry	NA	NA	NA
M1 tag	NA	NA	Sigma	M1-F3040
Secondary antibody to M1	APC	NA	Southern biotech	1090-11S

After 24 hr of first dose at 3mg/kg, >60% T cells are reprogrammed with mCherry mRNA using anti-CD3 targeting (Fig 29A). Both CD3 and CD8 targeting showed >20% of T cells reprogrammed with CD20 CAR mRNA, whereas combination of both showed ~30% T cells reprogrammed with CD20 CAR mRNA (Fig 29B). After 40hr of 2nd dose at 3mg/kg of anti-CD20 CAR expressing LNP >30% of T cells are reprogrammed with anti-CD20 CAR using CD3 targeting; Spleen > Blood > Liver > Bone Marrow > Thymus (Fig 30A-E). No significant increase in reprogramming of T cells is observed with increasing density of anti-CD3 targeting (Fig 30A-E). After 40 hr of 2nd dose at 3mg/kg of anti-CD20 CAR expressing LNP targeted with CD8 showed maximum reprogramming in spleen (>50%) as compared to other tissue (Fig 30A-E). As expected, CD8-targeted selectively reprograms CD8+ T cells over CD4+ cells. Combination of CD3 and CD8 targeting shows the most robust reprogramming in multiple tissues; Blood = Spleen > Bone Marrow > Thymus > Liver (Fig 30A-E). After 40h of 2nd dose >60% of T cells are reprogrammed with mCherry mRNA using anti-CD3 targeting; Spleen > Liver > Bone Marrow > Blood (Fig 31A-E). No

reprogramming is observed in thymus or lymph node at this time point with mCherry expressing LNPs.

Overall, there is difference in distribution of CD3 or CD8 targeted reprogrammed cells that appears to also mRNA cargo dependent. Without wishing to be bound by theory, it could also be due to different kinetics of redistribution of already reprogrammed T cells in periphery and other organs. CD8 targeting shows specificity for reprogramming of CD8 T cells in blood and all the organs tested. No reprogramming was observed in myeloid cells in blood or organs.

Example 20: Pharmacokinetics Study in Mice with LNPs

[0655] This example describes the pharmacokinetics of LNPs in female BALB/c mice.

[0656] LNPs were prepared using the vortex mixing process described in Example 2 using the component ratios described in Table 20 below. Particle properties were characterized using methods described in Example 3 and are described below in Table 21.

Table 20.

Batch	nmol lipid per 100µg mRNA				
	Lipid 8	Cholesterol	DSPC	DMG-PEG2000	DiI-C18(3)-DS
83.1	1500	1200	300	75	9

Table 21.

Batch	Size (nm)	PDI	EE (%)
83.1	157.4	0.165	79.6*

*Encapsulation efficiency possibly affected by DiI-C18(3)-DS fluorescence interfering with mRNA quantification

[0657] 8 female BALB/c mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and acclimated for one week. Food was provided ad libitum.

[0658] Mice were injected intravenously through the tail vein with a single dose of 3 mg/kg LNPs formulated with 1,1'-Dioctadecyl-3,3',3'-Tetramethylindocarbocyanine-5,5'-Disulfonic Acid (DiI-C18(3)-DS), and mCherry mRNA. Blood samples were obtained from the facial vein and sample collection occurred at times ranging from 30 minutes to 24 hours

(n=2 for each time point). Samples were centrifuged at 10,000g x 10 minutes, and serum was stored at 4°C until the time of analysis. The pharmacokinetics of LNPs of the formulation described in Table 20 were determined in Balb/c mice following collection of blood at the timepoints outlined in FIG. 32 and are shown in FIG. 33, where the LNPs are cleared only slowly from the circulation over 24 h.

[0659] The fluorescence quantification was carried out using a fluorescence microplate reader (Spark multimode reader, Tecan). Readings were from the top with excitation/emission wavelengths at 555/570 nm. Quantification of nanoparticles in circulation was performed through interpolation using a standard curve.

[0660] Using an average mouse weight of 25 g and approximate blood volume of 2 mL to calculate a theoretical initial LNP concentration of 37.5 µg/mL in serum, ~80% of the injected dose was detected in plasma after 30 min, ~40% after 1 hour and ~10% after 8 hours (FIG. 33).

[0661] This study shows that the current formulation can maintain mRNA levels in the circulation above a concentration of 0.5 µg/mL for more than 24 hours when dosed at an mRNA dose of 3 mg/kg.

EXAMPLE 21 – PREPARATION OF LNPs BY MICROFLUIDIC IN-LINE MIXING AND TANGENTIAL FLOW FILTRATION USING EXEMPLARY IONIZABLE LIPIDS

[0662] This example describes preparation of LNPs using scalable unit operations, namely in-line microfluidic mixing followed by tangential flow filtration (TFF) for ethanol removal and buffer exchange.

[0663] Using the mixing process in Example 6, multiple LNP batches were pooled together, totaling 60 mL at an RNA concentration of 300 µg/mL. Ethanol removal and buffer exchange was subsequently performed using tangential flow filtration (TFF).

[0664] Following mixing, ethanol removal and buffer exchange were performed on the resulting LNP suspension using a hollow fiber TFF module (Repligen, US P/N D02-E100-05-N). Briefly, the TFF module was rinsed with DI water and pumped dry before use. LNPs were then added to the reservoir, and the exchange buffer (25 mM pH 7.4 HEPES buffer with 150 mM NaCl) was used as the diafiltration buffer. The TFF module was primed, and

diafiltrations (DVs) were then initiated by ramping up the peristaltic pump to target flow rate and adjusting Retentate valve until target transmembrane pressure (TMP) is reached. A flow rate of 212 mL/min and a TMP of 3.5 psi were the target operating parameters for the system once diafiltration was initiated. Throughout the diafiltration process, the TMP was kept constant by adjusting the retentate valve. Permeate flow rate was monitored and did not decrease significantly over time. Six diafiltrations were performed, with samples set aside at the end of each diafiltration to later track the buffer exchange process. Final ethanol content was < 0.1%, as measured by refractive index measurements on DV samples, and pH measurements confirmed the buffer exchange into the exchange buffer. Upon the completion of six diafiltrations, the pump was stopped, and a concentration of the resulting LNP suspension was subsequently performed.

[0665] The concentration of the LNP suspension was performed using the same TFF module that was used during the buffer exchange process. TMP and flow rate, after pump ramp up, from buffer exchange process were maintained and the suspension was allowed to concentrate by stopping the addition of diafiltration buffer. The resulting LNP suspension was collected and filtered with a 0.2 µm syringe filter. The suspension was sampled for analytical purposes and then stored at 4°C until further use.

[0666] Using the LNP characterization process in Example 3, LNP batch was characterized to determine the average hydrodynamic diameter and mRNA content (total and dye-accessible); set forth in Table 22 below. As seen in Table 22, the microfluidic mixing process with ethanol removal and buffer exchange by TFF results in sub-100 nm particles exhibiting narrow polydispersity and good mRNA encapsulation (<20% dye accessible RNA).

TABLE 22

Sample ID/Lot Number	Process Point/Description	DLS Z-Avg. Diameter (nm)	DLS PDI	Total mRNA Content (µg/mL)	Dye-Accessible mRNA Content	Dye-Accessible mRNA (%)
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					($\mu\text{g/mL}$)	
210107MTM-NR20	LNPs after 6 DVs in HBS	64	0.08	282	11	4%
210107MTM-NR30	LNPs after Concentration (pre-filter)	64	0.10	1111	43	4%
210107MTM-NR40	LNPs after Concentration and 0.2 μm filtration	63	0.06	1099	30	3%

Example 22: Effect of PEG in Whole Blood transfection of mRNA LNPs

[0667] This example describes specifically targeting human T cells in whole blood with anti-CD3 Fab post-inserted into mCherry mRNA LNPs with or without DiR labeling and with varying levels of PEG incorporated during particle formation to determine the effect of PEG on LNP binding (DiR signal) and transfection efficiency (mCherry).

[0668] SP34-Fab lipid conjugate from the process described in Example 4 is a mixture of 3 PEG-lipid variants (DSPE-PEG2k-Fab, DSPE-PEG2k-maleimide(quenched), DSPE-PEG2k-OCH₃) therefore the effect of an additional PEG (DMG-PEG200) was explored. LNPs were prepared using the vortex mixing process described in Example 2 using the component ratios described in Table 23 below and conjugate was post-inserted after particle formation. Particle properties were characterized using methods described in Example 3 and are described below in Table 24.

Table 23.

Batch	nmol lipid per 100 μg mCherry mRNA					
	Lipid 8	Cholesterol	DSPC	DMG-PEG2000	DiR	Conjugate

RDM085.8	1500	1200	300	75	4.5	1.0
RDM0138	1575	1200	300	-	4.5	1.0
RDM073.19	1500	1200	300	75	-	1.0
RDM149	1575	1200	300	-	-	1.0

Table 24.

Batch	Size (nm)	PDI	EE (%)
RDM085.8	103.2	0.113	96.3
RDM0138	113.8	0.096	87.4
RDM073.19	119.4	0.129	83.4
RDM149	170.5	0.083	92.3

[0669] Venous blood from healthy volunteers was anti-coagulated in Hirudin tubes (Sarsted #04.1959.001) and seeded at 50 μ L in a 96-well round-bottom plate. Transfection of whole blood was carried out by adding LNPs containing 2.5 μ g/mL mRNA to the cells and co-culturing at 37°C until the time of analysis. To assess binding of DiR labeled LNPs, cells were analyzed 2-hours post-LNP addition and for transfection efficiency, cells were analyzed after 24-hours of incubation by flow cytometry.

[0670] For non-targeted LNPs, only DSPE-PEG2k was post-inserted to match SP34 targeted LNPs, labeled DSPE-PEG in FIGS. 34A-36B. No detectable binding (FIGS. 34A-35B) or transfection (FIGS. 36A and 36B) was observed for non-targeted LNPs indicating SP34 mediated highly specific transfection via CD3 targeting. For SP34-Fab lipid conjugate post-inserted particles, LNPs lacking DMG-PEG200 during particle formation exhibited higher binding by DiR signal for CD4 (FIGS. 34A and 34B) and CD8 (FIGS. 35A and 34B) T cells but lower transfection efficiency (FIGS. 36A and 36B) than LNPs that contained DMG-PEG200 during particle formation.

[0671] This study indicates introduction of a 4th PEG-lipid variant into the particle in addition to the 3 PEG-lipid variants that get added as part of the post-insertion process can have a dramatic effect on particle uptake and transfection efficiency, in this case, an approximate 2-3 fold difference was observed in transfection efficiency.

Example 23 – Lipid 8 and lipid 5 LNP properties, in vitro cell viability and protein expression in primary human T-cells

[0672] This example describes the relative *in vitro* toxicity of CD3 targeted LNPs derived from Lipid 8 and Lipid 5 in primary human T-cell transfection of GFP-mRNA. Nanoparticles are first produced using a mixing process followed by buffer exchange. Particles thus produced were subsequently tested *in vitro* in human CD3+ T cells to assess T-cell viability at three LNP doses, LNP association with cells, and expression of a reporter gene.

[0673] Lipid 8 and Lipid 5 LNPs encapsulating a 90-10 (w/w) mixture of GFP-mRNA and Cyanine-5 dye labelled mRNA (TriLink Biotechnologies Inc.) were prepared using the mixing process described in Example 6, the buffer exchange process described in Example 21. Lipid 5 LNPs were produced using Lipid 5 stock solutions that had been stored frozen at -20C for either 2 weeks or 1 day (Lipid 5 (O) and Lipid 5 (N), respectively). Both formulations resulted in particles exhibiting hydrodynamic diameters in the sub-100 nm range and moderate polydispersities, as well as good mRNA encapsulation and recovery (Table 25, <25% dye accessible mRNA and $\geq 80\%$ encapsulated mRNA was recovered using the Triton-deformulation procedure described in Example 3). As seen in FIGS. 45A and 45B, Lipid 5 LNP exhibited a larger change in hydrodynamic diameter (relative to Lipid 8 LNP) upon insertion of an anti-CD3 hSP34-PEG2k-DSPE conjugate using the insertion procedure described in Example 4. The resulting targeted LNPs were evaluated in primary human T-cells using the *in vitro* transfection protocol described in example 8. As seen in FIGS. 46A-46E, the PBS control arm exhibited about 50% T-cell viability while doses of 0.125, 0.5 and 2 ug mRNA/mL per well of Lipid 8 LNPs exhibited a dose dependent toxicity towards T-cells with T-cell viability dropping from about 45% live at 0.125 ug mRNA/mL per well to about 25% live at 2 ug mRNA/mL per well. In contrast, Lipid 5 LNPs (both sample “O” and “N”) were consistently better tolerated by T-cells with 40 – 45% T-cell viability observed at all three dose levels. Lower toxicities observed with Lipid 5 LNPs may be attributed to more rapid degradation and clearance of Lipid 5 from T-cells driven by hydrolytic and/or enzymatic degradation of labile ester bonds in the Lipid 5 molecule.

[0674] Dose dependent expression of GFP protein was observed with both ionizable lipids (5 and 8), however, as illustrated by both % GFP+ and GFP MFI values (FIGS. 46A and B), Lipid 5 LNPs resulted in greater overall protein expression at all three mRNA dose levels suggesting improved cytosolic availability of the mRNA payload with Lipid 5 LNPs.

Table 25. Lipid 8 and Lipid 5 LNP mRNA content

Ionizable Lipid	Theoretical total mRNA (µg/mL)	Measured total (Triton method) mRNA (µg/mL)	Ribogreen Dye Accessible mRNA (µg/mL)
Lipid 8	45	40	4
Lipid 5 (O)	45	38	7
Lipid 5 (N)	45	36	7

[0675] Overall, these data show that CD3-targeted LNPs formed with Lipid 5 showed both lower cellular toxicity and higher transfection activity in human T-cells compared to LNPs prepared with Lipid 8.

EXAMPLE 24 – Standard Procedure for *In-Vivo* Reprogramming of Immune Cells with DiI LNP expressing GFP

[0676] The following standard procedure for in-vivo reprogramming of immune cells with DiI LNP expressing GFP was used in the experiments in Example 29.

Mice Strains and Humanization

[0677] The NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mouse model was purchased from Jax Laboratories. 6-8 weeks old male mice were engrafted with 10 million PBMC of qualified donor in sterile PBS by tail vein injection. Individual body weight was monitored twice a week and blood samples were collected at appropriate interval to evaluate human immune cells engraftment.

Evaluation of Human T-cell Engraftment in the Immunodeficient Mice

[0678] 50ul blood was collected by tail vein bleed from each mouse. Red blood cells were lysed using Versalyse, RBC lysis solution following protocol as instructed by manufacturer (Beckman Coulter A09777). Cells were stained with hCD45 & hCD3 to determine the engraftment of human T-cells. After 15 days of PBMC injection, mice had anywhere from 30-60% huCD45+. These humanized mice were evaluated for reprogramming of immune cells by LNPs expressing Dil dye and GFP.

Reprogramming of Immune Cells

[0679] At time zero, mice (n=4 per group) were injected with GFP expressing Dil LNPs (by *i.v.* at 0.3mg/kg or 0.1 mg/kg with appropriate buffer. At each time point, 24 or 48h depending on the example mice treated with either LNPs or buffer were sacrificed. Terminal blood and tissues collection was performed to determine Dil and GFP expression in different organs and immune cells as below.

Tissue and blood sample collection.

[0680] At above specified timepoints, mice were anesthetized with CO₂ before sample collection. For blood collection, the chest was opened to expose the heart. Up to 300 µl blood was drawn from the left ventricle and dispensed into a K₃EDTA mini collect tube (Greiner Bio-One). Then a new syringe was used to draw remaining blood from the heart as much as possible. All the immune organs; spleen, bone marrow was isolated along with liver and lung. Immune cells were isolated from spleen, via smearing and shredding it through syringe and cell suspension was filtered through 70 µm cell strainer and was washed with PBS. Bone marrow was flushed with needle to collect all the immune cells. A piece of liver and lung tissue was gently grinded with tissue homogenizer and the homogenized and cells were isolated using miltenyi liver dissociation kit, (Miltenyi Biotec, Catalog# 130-105-807) and lung dissociation kit (Miltenyi Biotec, Catalog# 130-0950927) and instruction were followed according to the manufacturing instruction.

Immunophenotyping Analysis

[0681] Immune cells from blood and all the above organs were processed with Versalyse, RBC lysis buffer as per manufacturing instructions. Immune cells were stained with live/dead fixable dye and surface markers with standard flow analysis protocol as shown in below panel. BD symphony flow cytometer was used to determine positive population.

Panel

Antigen	Fluorophore	Clone	Company	Catalog#
Dil	APC	NA	NA	NA
GFP	mRNA	NA	NA	NA
Anti-CD45	BUV395	HI30	BD Biosciences	563792
Anti-CD3	BUV805	UCHT1	BD Biosciences	612895
Anti-CD4	BV711	SK3	BioLegend	344648
Anti-CD8	BV421	RPA-TB	BD Biosciences	562428
Anti-CD45	BB700	30-F11	BD Biosciences	566439
Anti-CD11b	BV785	MI/70	BioLegend	101243
Anti-F4/80	PE Dazzle	BM8	BioLegend	123146
Anti-CD31	BUV737	MEC 13.3	BD Biosciences	612802
TruStain Monocyte Blocker	NA	NA	BioLegend	462103
Arc Amine Comp beads	NA	NA	NA	01-3333-42
UltraComp eBeads	NA	NA	Invitrogen	01-3333-42
LIVE/DEAD Far Red Stain	NA	NA	Invitrogen	L34974
TruStain Fc X	NA	NA	Invitrogen	422302

Example 25 – ALTERNATIVE ETHANOL REMOVAL AND BUFFER EXCHANGE PROCESS

[0682] In addition to the ethanol removal and buffer exchange process described in Example 6, an alternative process can be used to produce LNPs of the present disclosure. Particularly, following mixing, ethanol removal and buffer exchange was performed on the resulting LNP suspension using a discontinuous diafiltration process. A centrifugal ultrafiltration device with 100,000 kDa MWCO regenerated cellulose membrane (Amicon Ultra-15, MilliporeSigma, Massachusetts, US) was sanitized with 70% ethanol solution and then washed twice with exchange buffer (25 mM pH 7.4 HEPES buffer with 150 mM NaCl). The LNP suspension (1.5 mL) was then loaded into the device and centrifuged at 500 ref until the volume was reduced by half (0.75 mL). The suspension was then diluted with exchange buffer (0.75 mL) to bring the suspension back to the original volume. This process of two-fold concentration and two-fold dilution was repeated five additional times for a total of six discontinuous diafiltration steps. The retentate containing the LNPs in the exchange buffer was recovered from the centrifugal ultrafiltration device and stored at 4°C until further use.

Example 26 – LIPID 8 AND LIPID 5 LNP PROPERTIES, AND IN VITRO CELL VIABILITY AND PROTEIN EXPRESSION IN PRIMARY HUMAN T-CELLS

[0683] This example compares the properties of LNPs prepared using Lipid 5 and Lipid 8 and the GFP protein expression in primary human T-cells. Both LNP formulations (Table 26) were prepared using the microfluidic mixing process as described in Example 6 and using a discontinuous diafiltration process for ethanol removal as described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US) and labeled with 0.01 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US) using the lipid ratios shown in Table 26 below. The LNPs were then inserted with a targeting conjugate using the specified conditions to provide the final targeted LNP formulations. The LNPs were characterized as described in Example 3. The characteristics of the LNPs are shown in Table 27.

Table 26. LNP formulation composition and Antibody conjugate insertion conditions

<u>Ionizable Lipid</u>	<u>Formulation No.</u>	<u>Lipid-PEG</u>	<u>Lipid-PEG Content (mol%)</u>	<u>Targeting Conjugate</u>	<u>Conjugate Insertion Density (g/mol)</u>	<u>Insertion Condition</u>
<u>Lipid 8</u>	<u>EXP210021</u> <u>79-</u> <u>N00H60T</u>	<u>DMG-</u> <u>PEG</u>	<u>2.5</u>	<u>hSP34</u>	<u>9</u>	<u>60 °C for 1 h in</u> <u>pH 7.4 HBS</u>
<u>Lipid 5</u>	<u>EXP210021</u> <u>79-</u> <u>N02H374T</u>	<u>DMG-</u> <u>PEG</u>	<u>2.5</u>	<u>hSP34</u>	<u>9</u>	<u>37 °C for 4 h in</u> <u>pH 7.4 HBS</u>

Table 27. LNP Size, Charge (Dynamic Light Scattering) and mRNA encapsulation (Ribogreen assay)

<u>Ionizable Lipid</u>	<u>Formulation No.</u>	<u>Post-Insertion DLS Z-Avg. Diameter (nm)/ PDI</u>	<u>Pre-Insertion Zeta Potential at pH 5.5 (mV) / pH 7.4 (mV)</u>	<u>Pre-Insertion Dye-Accessible mRNA (%)</u>	<u>Pre-Insertion mRNA content (ug/mL)*%/ encapsulation</u>
<u>Lipid 8</u>	<u>EXP2100217</u> <u>9-N00H60T</u>	<u>75 / 0.24</u>	<u>+21.9 / +3.5</u>	<u>12</u>	
<u>Lipid 5</u>	<u>EXP2100217</u> <u>9-N02H374T</u>	<u>81 / 0.24</u>	<u>+23.3 / +3.0</u>	<u>14</u>	

*mRNA content determined using the Triton-deformulation procedure described in Example 3

[0684] Both Lipid 5 and Lipid 8 formulations resulted in particles exhibiting hydrodynamic diameters in the sub-100 nm range (Table 27 and FIG. 53A) with narrow polydispersity (<0.1) prior to antibody conjugate insertion and moderately higher polydispersities (<0.3) after antibody conjugate insertion. Additionally, low levels of dye accessible mRNA ($<15\%$) and high RNA encapsulation efficiencies ($> 80\%$ mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation) were observed in both formulations. These improvements to LNP size distribution are attributed to the use of discontinuous diafiltration for ethanol removal (described in Example 25) relative to ethanol removal by buffer exchange using a size exclusion column (described in Example 6). As seen in FIGS. 53B and 53C, Lipid 5 and Lipid 8 LNPs exhibited positive zeta potential at pH 5.5, and either near neutral or slightly negative charge at pH 7.4 both prior to and after antibody insertion (FIG. 53D), suggesting a change in LNP ionization state as expected.

[0685] The resulting targeted LNPs were evaluated in primary human T-cells using the in vitro transfection protocol described in Example 8. Dose dependent expression of GFP protein was observed with both ionizable lipids (5 and 8) as illustrated by similar % GFP+ and GFP MFI values (FIGS. 54A and 54B). However, a two-fold higher mean fluorescence intensity (GFP MFI) was observed with the Lipid 5 LNP (at both 0.5 $\mu\text{g/mL}$ and 1.0 $\mu\text{g/mL}$ dose/well) suggesting more efficient cytosolic release of the mRNA payload (and thus greater GFP protein expression) with the Lipid 5 formulation relative to the Lipid 8 formulation. As illustrated by the DiI+ and DiI MFI values, both formulations were equally associated with cells suggesting the conjugate insertion process is not dependent on the ionizable lipid chemistry (FIGS. 54C and 54D). As seen in FIGS. 54E, both formulations were well tolerated by T-cells at and below 1.0 $\mu\text{g/mL}$ dose (minimal drop in cell viability was observed relative to the PBS control).

Example 27 – LIPID 5, LIPID 8 AND DLN-MC3-DMA LNP PROPERTIES AND IN VITRO GFP PROTEIN EXPRESSION IN PRIMARY HUMAN T-CELLS

[0686] This example compares the properties of LNPs prepared using Lipid 5, Lipid 8 and DLn-MC3-DMA LNP properties and in vitro GFP protein expression in primary human T-cells. All LNP formulations (Table 28) were prepared using the microfluidic mixing process (described in Example 6) and using a discontinuous diafiltration process for ethanol removal (described in Example 25). The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US) and labeled with 0.01 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US) using the lipid ratios shown in Table 28 below. The LNPs were then inserted with a targeting conjugate using the specified conditions to provide the final targeted LNP formulations. The LNPs were characterized as described in Example 3.

Table 28. LNP Formulation composition and antibody insertion conditions

Ionizable Lipid	Formulation No.	Lipid-PEG	Lipid-PEG Content (mol%)	Targeting Conjugate / Insertion density (g/mol)	Antibody conjugate insertion condition
DLin-MC3-DMA	EXP210034 71-N1H3	DPG-PEG	1.5	hSP34 / 9	60 °C for 0.5 h in pH 7.4 HBS
Lipid 8	EXP210034 71-N2H3	DPG-PEG	1.5	hSP34 / 9	60 °C for 0.5 h in pH 7.4 HBS
Lipid 5	EXP210034 71-N3H3	DPG-PEG	1.5	hSP34 / 9	37 °C for 4 h in pH 7.4 HBS

Table 29. LNP Size, Charge (Dynamic Light Scattering) and mRNA encapsulation (Ribogreen assay)

Ionizable Lipid	Formulation No.	Post-Insertion DLS Z-Avg. Diameter (nm) / PDI	Pre-Insertion Zeta Potential at pH 5.5 (mV) / pH 7.4 (mV)	Pre-Insertion Dye-Accessible mRNA (%)	Pre-insertion total mRNA content (ug/mL)
DLin-MC3-DMA	EXP210034 71-N1H3	107 / 0.20	16.4 / -0.1	9.1	
Lipid 8	EXP210034 71-N2H3	103 / 0.19	16.2 / 1.6	10	
Lipid 5	EXP210034 71-N3H3	92 / 0.11	20.1 / 4.4	6.4	

[0687] DLn-MC3-DMA, Lipid 5 and Lipid 8 were formulated using 1.5 mole % DPG-PEG. As seen in Table 29 and FIGS. 55A to 55B, all LNPs display sub-100 nm hydrodynamic diameter (DLS) prior to antibody insertion and roughly 100 nm or smaller post antibody conjugate insertion. Lipid 5 LNP polydispersity remains narrow (<0.15) post antibody conjugate insertion, while DLn-MC3-DMA and Lipid 8 exhibit a slightly larger change polydispersity (~0.2 after insertion. Additionally, low levels of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies (> 80% mRNA in parent LNP samples) were observed in all formulations (Table 29 and FIG. 55D). As shown in FIG. 55C, all three formulations exhibited positive zeta potential at pH 5.5, and either near neutral or slightly negative charge at pH 7.4 prior to antibody insertion, suggesting a change in LNP ionization state as expected. The resulting targeted LNPs were evaluated in primary human T-cells using the in vitro transfection protocol described in example 8.

[0688] As seen in FIG. 56E, all formulations were well tolerated by T-cells below 0.125 µg/mL dose (similar to the PBS control). However, the two ester based lipids, DLn-MC3-DMA and Lipid 5 were better tolerated at higher doses, with Lipid 5 being the least toxic at 1 µg/mL dose. As illustrated by the DiI+ and DiI MFI values (FIGS. 56C and 56D), all formulations show similar levels of cell association at most dose levels tested suggesting that the conjugate insertion process is not dependent on the ionizable lipid chemistry. In all cases, dose dependent expression of GFP protein was observed (FIGS. 56A and 56B). However, at all doses tested Lipid 5 outperformed both Lipid 8 and DLn-MC3-DMA, showing >2 fold higher mean fluorescence intensity (GFP MFI) at 0.5 µg/mL and 1.0 µg/mL dose/well relative to Lipid 8 and >5 fold relative to DLn-MC3-DMA, suggesting more efficient cytosolic release of the mRNA payload (and thus greater GFP protein expression) with the Lipid 5 formulation relative to both Lipid 8 and DLn-MC3-DMA formulations.

Example 28 – LIPID 5 LNP FORMULATION STABILITY AFTER FREEZE THAW STRESS

This example illustrates the stability of Lipid 5 LNP formulations after one freeze thaw cycle. Lipid 5 LNP formulation compositions shown in Table 30 were prepared using the microfluidic mixing process (described in Example 6) and using a discontinuous diafiltration process for ethanol removal (described in Example 25). The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US) and labeled with 0.06 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). The LNPs were then inserted with a

targeting conjugate using the specified conditions to provide the final targeted LNP formulation. The LNPs were characterized for size by DLS and mRNA content as described in Example 3.

[0689] Following the preparation of the targeted LNP formulation, the formulation was split into two portions. One portion was exchanged into 40 mM pH 7.4 HEPES buffer, and the other portion was exchanged into 30 mM pH 7.4 Tris buffer. The buffer exchange was performed using a discontinuous diafiltration method where the LNP formulation sample was transferred into a centrifugal ultrafiltration device with 100,000 kDa MWCO regenerated cellulose membrane (Amicon Ultra-4, MilliporeSigma, Massachusetts, US), then diluted 10-fold with the exchange buffer, and concentrated back to the original volume by centrifuging at 500 *rcf*. This dilution and concentration step was repeated one additional time. The exchanged LNP samples were then divided into separate aliquots that were mixed with concentrated sodium chloride and sucrose solutions to provide the final freeze-thaw sample formulations. Samples of each freeze-thaw formulation were then stored at 4 °C, frozen at –80 °C, or flash frozen in liquid nitrogen. The samples were later thawed at room temperature and tested for size by DLS and *in vitro* T cell transfection.

Table 30. LNP Formulation composition and antibody insertion conditions

Formulation No.	Ionizable Lipid	Lipid-PEG	Lipid-PEG Content (mol%)	Targeting Conjugate / Insertion density (g/mol)	Insertion Condition
EXP2100163-9-N14	Lipid 5	DMG-PEG	2.5	hSP34 / 9	37 °C for 14 h in pH 7.4 HBS

Table 31. Lipid 5 LNP Size and Polydispersity (DLS) prior to insertion

Formulation No.	Post-Insertion DLS Z-Avg. Diameter (nm)	Post-Insertion DLS PDI	Pre-Insertion Dye-Accessible mRNA (%)
EXP21001639-N14	106	0.19	<u>24</u>

Table 32. LNP formulation composition and list of storage condition/freezing methods for Lipid 5 LNPs

Lipid 5 LNP, Formulation No.	Buffer Species	Buffer Conc. (mM)	Sucrose Conc. (wt%)	NaCl Conc. (mM)	Storage Condition
EXP21001639-N24	pH 7.4 HEPES	20	9.6	0	4 °C
EXP21001639-N28	pH 7.4 HEPES	20	9.6	0	-80 °C

EXP21001639-N2N	pH 7.4 HEPES	20	9.6	0	LN2*
EXP21001639-N34	pH 7.4 HEPES	20	9.6	25	4 °C
EXP21001639-N38	pH 7.4 HEPES	20	9.6	25	-80 °C
EXP21001639-N3N	pH 7.4 HEPES	20	9.6	25	LN2
EXP21001639-N44	pH 7.4 HEPES	20	9.6	50	4 °C
EXP21001639-N48	pH 7.4 HEPES	20	9.6	50	-80 °C
EXP21001639-N4N	pH 7.4 HEPES	20	9.6	50	LN2
EXP21001639-N54	pH 7.4 HEPES	20	18.6	0	4 °C
EXP21001639-N58	pH 7.4 HEPES	20	18.6	0	-80 °C
EXP21001639-N5N	pH 7.4 HEPES	20	18.6	0	LN2
EXP21001639-N84	pH 7.4 Tris	15	9.6	0	4 °C
EXP21001639-N88	pH 7.4 Tris	15	9.6	0	-80 °C
EXP21001639-N8N	pH 7.4 Tris	15	9.6	0	LN2
EXP21001639-N94	pH 7.4 Tris	15	18.6	0	4 °C
EXP21001639-N98	pH 7.4 Tris	15	18.6	0	-80 °C
EXP21001639-N9N	pH 7.4 Tris	15	18.6	0	LN2

*LN2: Liquid Nitrogen

[0690] As shown in FIGS. 57A and 57B, the freezing method used (flash frozen in Liquid Nitrogen versus storage in -80C freezer) did not impact the post freeze-thaw LNP size distributions with both hydrodynamic radius and polydispersity trending similarly between the two methods. However, the cryoprotectant and buffer composition significantly impacted post freeze-thaw LNP size characteristics. Frozen storage in HEPES buffer with no added salt or with 25 and 50 mM NaCl as well as with either 9.6 wt.% or 18 wt.% sucrose resulted in significant increases in LNP polydispersity. In contrast, storage in TRIS buffer and either 9.6 wt.% or 18 wt.% sucrose preserved the LNP size and polydispersity effectively relative to the 4C stored LNP that were not subjected to freeze-thaw stress. All formulations were evaluated in primary human T-cells using the in vitro transfection protocol described in Example 8.

[0691] As seen in FIGS. 58A and 58B, all LNPs stored in HEPES buffer lost the ability to transfect T-cells after the freeze-thaw cycle, while formulations stored in TRIS buffer retained the ability to transfect T-cells post freeze-thaw activity. As seen in FIGS. 58C and 58D, cell association (as measured by the DiI %+ cells and DiI MFI values) of the HEPES buffer compositions was slightly diminished after the freeze thaw cycle while that of the TRIS buffer formulations was maintained. Lower cell association levels and changes in LNP size properties after freeze-thaw stress in the HEPES buffer formulations is likely responsible for the loss of activity observed. Notably, storage in TRIS buffer maintains both LNP properties and their ability to transfect T-cells after frozen storage and freeze-thaw stress.

Example 29 – IMPACT OF PEG-LIPID ANCHOR AND PEG% ON *IN VIVO* REPROGRAMMING

[0692] The aim of this study was to identify the optimum PEG-lipid and mol % for in vivo reprogramming of immune cells. We had hypothesized that an intermediate anchor length would be optimum for engagement of T-cells/NK cells or other immune cells in the blood since short chained anchors like PEG-DMPE or PEG-DMG (both C14) would be lost too quickly, while PEG-lipids with longer acyl chains, like PEG-DSPE and PEG-DSG (both C18), would be too stable and result in a decrease in transfection efficiency.

Part A. Anti-CD3 Lipid 8 LNPs

[0693] In this study, we used LNPs targeted to CD3 (hsp34 Fab'-PEG-DSPE conjugate) and incorporating the Lipid 8 to test LNPs prepared with DMG-PEG (C14), DPG-PEG (C16), DPPE-PEG (16), or DSG-PEG (C18) at either 1.5 or 2.5 mol % of the total lipid.

[0694] The LNP formulations in the table below were prepared using the microfluidic mixing method described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US) and labeled with 0.06 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). The LNPs were then inserted with a targeting conjugate using the specified conditions to provide the targeted LNP formulations. The final targeted LNPs formulations were prepared by mixing the LNP suspensions with a concentrated sucrose solution to provide the final LNP formulations with 5.3 wt% sucrose. The LNPs were characterized as described in Example 3.

Table 33. Formulation Table

Formulation No.	Ionizable Lipid	Lipid-PEG	Lipid-PEG Content (mol%)	Targeting Conjugate	Conjugate Insertion Density (g/mol)	Insertion Condition
EXP21001532-N1T	Lipid 8	DMG-PEG	2.5	hSP34	9	60 °C for 1 h in pH 7.4 HBS
EXP21001532-N2T	Lipid 8	DPG-PEG	2.5	hSP34	9	60 °C for 1 h in pH 7.4 HBS
EXP21001532-N3T	Lipid 8	DSG-PEG	2.5	hSP34	9	60 °C for 1 h in pH 7.4 HBS
EXP21001532-N4T	Lipid 8	DPPE-PEG	1.5	hSP34	9	60 °C for 1 h in pH 7.4 HBS
EXP21001532-N5T	Lipid 8	DPPE-PEG	2.5	hSP34	9	60 °C for 1 h in pH 7.4 HBS
EXP21001532-N6T	Lipid 8	DSPE-PEG	1.5	hSP34	9	60 °C for 1 h in pH 7.4 HBS
EXP21001532-N7T	Lipid 8	DSPE-PEG	2.5	hSP34	9	60 °C for 1 h in pH 7.4 HBS

Table 34. Formulation Analysis Results

Formulation No.	Post-Insertion DLS Z-Avg. Diameter (nm)	Post-Insertion DLS PDI	Pre-Insertion Zeta Potential at pH 5.5 (mV)	Pre-Insertion Zeta Potential at pH 7.4 (mV)	Pre-Insertion Dye-Accessible mRNA (%)
EXP21001532-N1T	76	0.18	21.3	1.8	13
EXP21001532-N2T	86	0.17	17.8	1.0	8.7
EXP21001532-N3T	96	0.18	17.1	-3.4	10
EXP21001532-N4T	119	0.23	20.1	-5.9	12
EXP21001532-N5T	73	0.15	17.2	-7.0	11
EXP21001532-N6T	108	0.17	17.9	-4.9	10
EXP21001532-N7T	77	0.18	18.6	-6.6	10

[0695] Results of in vivo reprogramming of immune cells with CD3-targeted DiI/GFP LNP at the dose of 0.3 mg/kg after 24 or 48h with either DMG, DPG or DSG-PEG 2.5% or after 24h with either DPPE or DSPE 1.5 or 2.5% are show in FIGS. 59A to 59T.

[0696] All formulations with anti-CD3 hsp34 clone targeted Lipid 8 LNPs showed peak GFP expression at 24h, with maximum expression in blood > lung > spleen > bone marrow > liver. Irrespective of diacyl glycerol or phosphoethanolamine backbone, DPG-PEG or DPPE-PEG, i.e., C16 anchor length shows maximum reprogramming with 1.5% PEG-lipid, compared to PEG-lipids of other acyl chain lengths (C14 or C18). GFP, MFI showed similar trend as GFP% positive T cells. The percent positive DiI or DiI MFI also showed a similar trend as that of GFP positive T cells, indicating that with CD3 targeted Lipid 8 LNPs the binding efficiency of LNPs correlates with their reprogramming ability.

Part B. Anti-CD3 or Anti-CD8 Lipid 5 LNPs

[0697] In this study, we used LNPs targeted to CD3 (hsp34 Fab'-PEG-DSPE conjugate) and LNPs targeted to CD8 (TRX2 Fab'-PEG-DSPE conjugate, or V2 -PEG-DSPE Nb conjugate) incorporating the Lipid 5 to test LNPs prepared with DMG-PEG (C14) or DPG-PEG (C16) at 1.5 mol % of the total lipid.

[0698] The LNP formulations in the table below were prepared using the microfluidic mixing method described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using unmodified eGFP encoding mRNA (TriLink Biotechnologies, California, US; Catalog #L-7601) and labeled with 0.06 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). The LNPs were then inserted with a targeting conjugate using the specified conditions to provide the targeted LNP formulations. The final targeted LNPs formulations were prepared by mixing the LNP suspensions with a concentrated sucrose solution to provide the final LNP formulations with 9.6 wt% sucrose. The LNPs were characterized as described in Example 3.

Table 35. LNP Formulations

Formulation No.	Ionizable Lipid	Lipid-PEG	Lipid-PEG Content (mol%)	Targeting Conjugate	Conjugate Insertion Density (g/mol)	Insertion Condition
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EXP21002551-N6H-TRX2S	Lipid 5	DMG-PEG	1.5	TRX2	9	37 °C for 4 h in pH 7.4 HBS
EXP21002551-N7H-TRX2S	Lipid 5	DMG-PEG	2.5	TRX2	9	37 °C for 4 h in pH 7.4 HBS
EXP21002414-N2H-TRX2S	Lipid 5	DPG-PEG	1.5	TRX2	9	37 °C for 4 h in pH 7.4 HBS
EXP21002551-N5H-TRX2S	Lipid 5	DPG-PEG	2.5	TRX2	9	37 °C for 4 h in pH 7.4 HBS
EXP21002551-N6H-SP34S	Lipid 5	DMG-PEG	1.5	hSP34	9	37 °C for 4 h in pH 7.4 HBS
EXP21002551-N7H-SP34S	Lipid 5	DMG-PEG	2.5	hSP34	9	37 °C for 4 h in pH 7.4 HBS
EXP21002414-N2H-SP34S	Lipid 5	DPG-PEG	1.5	hSP34	9	37 °C for 4 h in pH 7.4 HBS
EXP21002551-N5H-SP34S	Lipid 5	DPG-PEG	2.5	hSP34	9	37 °C for 4 h in pH 7.4 HBS
EXP21002551-N6H-NbV2	Lipid 5	DMG-PEG	1.5	V2	5.86	37 °C for 4 h in pH 7.4 HBS
EXP21002551-N7H-Nb V2	Lipid 5	DMG-PEG	2.5	V2	5.86	37 °C for 4 h in pH 7.4 HBS
EXP21002414-N2H-Nb V2	Lipid 5	DPG-PEG	1.5	V2	5.86	37 °C for 4 h in pH 7.4 HBS
EXP21002551-N5H-Nb V2	Lipid 5	DPG-PEG	2.5	V2	5.86	37 °C for 4 h in pH 7.4 HBS

Table 36. Formulation Analysis Results

Formulation No.	Post-Insertion DLS Z-Avg. Diameter (nm)	Post-Insertion DLS PDI	Pre-Insertion Zeta Potential at pH 5.5 (mV)	Pre-Insertion Zeta Potential at pH 7.4 (mV)	Pre-Insertion Dye-Accessible mRNA (%)
EXP21002551-N6H-TRX2S	83	0.06	23.7	5.2	14
EXP21002551-N7H-TRX2S	70	0.15	21.9	4.0	30

EXP21002414-N2H-TRX2S	90	0.12	27.9	7.3	6.9
EXP21002551-N5H-TRX2S	76	0.19	22.9	5.5	17
EXP21002551-N6H-SP34S	96	0.16	23.7	5.2	14
EXP21002551-N7H-SP34S	138	0.26	21.9	4.0	30
EXP21002414-N2H-SP34S	104	0.14	27.9	7.3	6.9
EXP21002551-N5H-SP34S	79	0.17	22.9	5.5	17
EXP21002551-N6H-Nb V2	85	0.10	23.7	5.2	14
EXP21002551-N7H-Nb V2	69	0.11	21.9	4.0	30
EXP21002414-N2H-Nb V2	90	0.12	27.9	7.3	6.9
EXP21002551-N5H-Nb V2	82	0.21	22.9	5.5	17

[0699] Results of in vivo reprogramming of immune cells with CD3- or CD8- targeted DiI/GFP LNP at 0.3 mg/kg of Lipid 5 with either DMG-PEG or DPG-PEG (1.5 or 2.5%) after 24h are shown in **FIGS. 60A to 60T**.

[0700] Lipid 5 CD3 targeted LNPs showed reprogramming of both CD4 and CD8 T cells, whereas CD8 targeted antibody TRX2 or CD8 Nanobody is specific for reprogramming CD8 T cells as expected. Similarly, CD3 targeted LNPs binds to both CD4 and CD8 T cells, and CD8 targeted LNPs with either antibody or Nanobody only binds to CD8 T cells. CD3 targeted Lipid 5 LNPs showed similar GFP expression with both DMG or DPG (i.e., C14, or C16 lipid anchor length) with 1.5% PEG, whereas in blood CD8 antibody or Nanobody targeted LNPs showed maximum GFP expression with DMG-PEG, which was 2-fold more compared to DPG-PEG at 24h. In other tissues (e.g., lung, spleen and bone marrow), CD8 targeting LNPs with either antibody or Nanobody showed similar GFP expression with both DMG-PEG and DPG-PEG-1.5%. GFP MFI showed similar trend as that of GFP expression. % DiI positive T cells are only observed in blood but not in other compartments, and DiI MFI is also maximum in blood compartment.

Part C. Anti-CD8, Anti-CD11a, and Anti-CD4 Lipid 5 LNPs

[0701] In this study, we used LNPs targeted to CD8 (Nanobody-PEG-DSPE conjugate), LNPs targeted to CD11a (hMHM24 Fab -PEG-DSPE conjugate), and LNPs targeted to CD4 (Nanobody-PEG-DSPE conjugate or Ibalizumab-PEG-DSPE conjugate) incorporating Lipid 5 to test LNPs prepared with DPG-PEG (C16) at 1.5 mol % of the total lipid.

[0702] The LNP formulations in the table below were prepared using the microfluidic mixing method described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US) and labeled with 0.06 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). The LNPs were then inserted with a targeting conjugate using the specified conditions to provide the targeted LNP formulations. The LNPs were characterized as described in Example 3.

Table 37. LNP Formulations

Formulation No.	Ionizable Lipid	Lipid-PEG	Lipid-PEG Content (mol%)	Targeting Conjugate	Conjugate Insertion Density (g/mol)	Insertion Condition
<u>EXP21003027-N201</u>	Lipid 5	DPG-PEG	1.5	hSP34	9	37 °C for 4 h in pH 7.4 HBS
<u>EXP21003027-N105</u>	Lipid 5	DMG-PEG	1.5	BDSn (Nanobody)	5.3	37 °C for 4 h in pH 7.4 HBS
<u>EXP21003027-N206</u>	Lipid 5	DPG-PEG	1.5	BDSn (Nanobody)	5.3	37 °C for 4 h in pH 7.4 HBS
<u>EXP21003027-N107</u>	Lipid 5	DMG-PEG	1.5	hMHM24 Fab	6.12	37 °C for 4 h in pH 7.4 HBS
<u>EXP21003027-N208</u>	Lipid 5	DPG-PEG	1.5	hMHM24 Fab	6.12	37 °C for 4 h in pH 7.4 HBS
<u>EXP21003027-N109</u>	Lipid 5	DMG-PEG	1.5	T023200008 (Nanobody)	1.86	37 °C for 4 h in pH 7.4 HBS
<u>EXP21003027-N210</u>	Lipid 5	DPG-PEG	1.5	T023200008 (Nanobody)	1.86	37 °C for 4 h in pH 7.4 HBS
<u>EXP21003027-N111</u>	Lipid 5	DMG-PEG	1.5	Ibalizumab Fab	6.12	37 °C for 4 h in pH 7.4 HBS

EXP21003027-N212	Lipid 5	DPG-PEG	1.5	Ibalizumab Fab	6.12	37 °C for 4 h in pH 7.4 HBS
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Table 38. Formulation Analysis Results

Formulation No.	Post-Insertion DLS Z-Avg. Diameter (nm)	Post-Insertion DLS PDI	Pre-Insertion Zeta Potential at pH 5.5 (mV)	Pre-Insertion Zeta Potential at pH 7.4 (mV)	Pre-Insertion Dye-Accessible mRNA (%)
EXP21003027-N201	94	0.13	Not measured	Not measured	8.4
EXP21003027-N105	85	0.07	21.5	5.5	11
EXP21003027-N206	89	0.07	Not measured	Not measured	8.4
EXP21003027-N107	81	0.10	21.5	5.5	11
EXP21003027-N208	86	0.08	Not measured	Not measured	8.4
EXP21003027-N109	83	0.10	21.5	5.5	11
EXP21003027-N210	87	0.06	Not measured	Not measured	8.4
EXP21003027-N111	83	0.10	21.5	5.5	11
EXP21003027-N212	88	0.06	Not measured	Not measured	8.4

[0703] Results of in vivo reprogramming of immune cells with above LNPs at 0.3 mg/kg of Lipid 5 with either DMG-PEG or DPG-PEG (1.5 mol%) after 24h are show in **FIGS. 61A to 61T**.

[0704] Lipid 5 LNP with CD8 Nanobody targeted LNPs showed GFP expression specifically in only CD8 T cells and not CD4 T cells. Similarly, CD4 targeting with either Nanobody or antibody specifically showed GFP expression in only CD4 T cells and CD11a targeting showed both CD4 and CD8 T cells expressing GFP. CD8 Nanobody LNP showed maximum GFP expression with DMG-PEG-1.5% as compared to DPG-PEG while CD11a Fab and both CD4 Nanobody and Fab antibody showed similar GFP expression with both DMG-PEG and DPG-PEG (1.5 mol%). GFP MFI showed similar trend as that of GFP % T cells. % DiI positive T cells and MFI were maximum in blood, liver and lung with different CD8, CD11a or CD4 targeted antibody or Nanobody.

Part D. Anti-CD7 Lipid 5 LNPs

[0705] In this study, we used LNP targeted to CD7 (V1-PEG-DSPE conjugate) incorporating the Lipid 5 to test LNPs prepared with DMG-PEG (C14) or DPG-PEG (C16) at either 2.5 or 1.5 mol % of the total lipid.

[0706] The LNP formulations were prepared using the microfluidic mixing method described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US) and labeled with 0.06 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). The LNPs were then inserted with a targeting conjugate using the specified conditions to provide the targeted LNP formulations. The LNPs were characterized as described in Example 3.

[0707] Results of in vivo reprogramming of immune cells with the LNPs at 0.3 mg/kg of Lipid 5 with either DMG-PEG or DPG-PEG (1.5%) after 24h are show in FIGS. 63A to 63T.

[0708] Lipid 5, CD7 Nanobody targeted LNPs showed maximum GFP expressing T cells with DMG-PEG (50%) as compared to DPG-PEG (35%), both with 1.5 mol% PEG-lipid. Other tissues liver and lung showed equal 20% GFP expressing T cells with both DMG-PEG and DPG-PEG-1.5%. GFP MFI showed similar trend. DiI positive T cells and DiI MFI showed maximum binding only in blood where most GFP expression is observed.

Example 30 – LIPID 5, LIPID 8, DLN-MC3-DMA LNPs IN VIVO REPROGRAMMING COMPARISON

[0709] In this example, LNPs utilizing Lipid 5, Lipid 8, or DLn-MC3-DMA (0.1 mg/kg dose) targeted to CD3 (SP34) or CD8 (V2 (Nanobody)) were tested for their ability to reprogram immune cells in vivo.

[0710] The LNP formulations in the table below were prepared using the microfluidic mixing method described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US) and labeled with 0.06 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). The LNPs were then inserted with a targeting conjugate using the

specified conditions to provide the final targeted LNP formulations. The LNPs were characterized as described in Example 3.

Table 39. LNP Formulations

Formulation No.	Ionizable Lipid	Lipid-PEG	Lipid-PEG Content (mol%)	Targeting Conjugate	Conjugate Insertion Density (g/mol)	Insertion Condition
<u>EXP21003471-N101</u>	DLin-MC3-DMA	DPG-PEG	1.5	hSP34	9	60 °C for 0.5 h in pH 7.4 HBS
<u>EXP21003471-N202</u>	Lipid 8	DPG-PEG	1.5	hSP34	9	60 °C for 0.5 h in pH 7.4 HBS
<u>EXP21003471-N303</u>	Lipid 5	DPG-PEG	1.5	hSP34	9	37 °C for 4 h in pH 7.4 HBS
<u>EXP21003471-N111</u>	DLin-MC3-DMA	DPG-PEG	1.5	V2 (Nanobody)	5.86	60 °C for 0.5 h in pH 7.4 HBS

Table 40. Formulation Analysis Results

Formulation No.	Post-Insertion DLS Z-Avg. Diameter (nm)	Post-Insertion DLS PDI	Pre-Insertion Zeta Potential at pH 5.5 (mV)	Pre-Insertion Zeta Potential at pH 7.4 (mV)	Pre-Insertion Dye-Accessible mRNA (%)
<u>EXP21003471-N101</u>	107	0.20	16.4	-0.1	9.1
<u>EXP21003471-N202</u>	103	0.19	16.2	1.6	10
<u>EXP21003471-N303</u>	92	0.11	20.1	4.4	6.4
<u>EXP21003471-N111</u>	92	0.16	16.4	-0.1	9.1

[0711] Results of in vivo reprogramming of immune cells with above LNPs at 0.1 mg/kg with DPG-PEG (1.5%) after 24h are show in FIGS. 62A to 62S.

[0712] Comparison of DLn-MC3-DMA, Lipid 8 and Lipid 5 with DPG-PEG-1.5% and CD3 (hsp34) targeted LNPs at 0.1mg/kg dose, Lipid 5 is superior for reprogramming T cells

and showed maximum reprogramming of T cells with maximum GFP expression in blood and lung about 25% (blood = lung > liver > bone marrow). GFP MFI showed similar trend. All 3 lipids showed similar DiI positive T cells indicating that all lipids may bind equally but Lipid 5 is better at reprogramming T cells.

Example 31 – IN VITRO PROTEIN EXPRESSION – LNP TRANSFECTION OF NK AND T CELLS IN CO-CULTURE

[0713] This example describes targeting co-cultured human NK and T cells with anti-CD3, anti-CD7, anti-CD11a, anti-CD18, anti-CD56 (Lorvotuzumab) anti-CD137 (4B4-1) and anti-CD2 (RPA-2.10v1) Fab or Nanobodies post-inserted into GFP mRNA DiI labeled LNPs and their effect on transfection and translation.

[0714] Primary human T cells were purified using magnetic-based CD3 negative selection. 20 million purified T cells were activated using anti-CD3/anti-CD28 coated beads for 48 hours in media containing 100IU/mL IL-2. Following activation, activation beads were removed, and T cells were expanded for an additional 48 hours in media containing 100IU/mL IL-2. After the expansion period, T cells were concentrated to 1 million cells/mL in preparation for co-transfection with primary human NK cells.

[0715] CD3-depleted PBMCs were purified using magnetic-based CD3 positive selection and retaining of the negative fraction. 20 million CD3 depleted PBMCs were added to 1 well of a 6 well GREX plate in media containing 10ng/mL IL-15 for 7 days. On day 7, each well was split in 2 and cells were cultured further in media containing 10ng/mL IL-15 for an additional 7 days. On day 14, NK cells were concentrated to 1 million cells/mL in preparation for co-transfection with primary human T cells.

[0716] LNPs were prepared using the mixing process described in Example 6, the buffer exchange process described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiI C18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from methods described in Example 4 while generation of Nb-conjugated differed in using 1:1:4 Nb:DSPE-5KPEG-maleimide:DSPE-2KPEG-OCH₃ and a 50 kD UF membrane (Millipore Corp, Billerica, MA USA) for separation of Nb-conjugate from free Nb. Using methods similar to Example 12, conjugated Fabs and conjugated Nb were post-

inserted at various densities (Table 41) into LNPs containing Lipid 8 and GFP mRNA with DiI dye. into the LNPs containing Lipid 8, GFP mRNA, and DiI dye.

Table 41. Fab or Nb density post-inserted onto the surface of the LNP

Targeting moiety	
α CD11a HzMHM24 bDS Fab	3, 5, 9 g/mol
α CD18 h1B4 bDS Fab	3, 5, 9 g/mol
α CD7 V1	3, 5, 9 g/mol
α CD56 A1 Fab bDS	3, 9, 19 g/mol
α CD56 A2 Fab bDS	3, 9, 19 g/mol
α CD56 A3 Fab bDS	3, 9, 19 g/mol
α CD56 Lorvotuzumab Fab bDS	3, 6, 9 g/mol
α CD137 4B4-1 Fab bDS	3, 9, 18 g/mol
α CD2 9.6 Fab bDS	0.75, 1.5, 3 g/mol
α CD2 TS2/18.1 Fab bDS	0.75, 1.5, 3 g/mol
α CD2 RPA-2.10v1 Fab bDS	0.75, 1.5, 3 g/mol
α CD2 Lo-CD2b Fab bDS	0.75, 1.5, 3 g/mol
α CD2 35.1 Fab bDS	0.75, 1.5, 3 g/mol
α CD2 OKT11 Fab bDS	0.75, 1.5, 3 g/mol
Dead Fab mutOKT8	9 g/mol
α CD8 TRX2 NoDS (interchain disulfide knockout)	9 g/mol
HEPES Buffered Saline	No LNP

Anti-CD56 A1 Fab sequence

A1 bDS HC (SEQ ID NO: 26):

QVQLQQSGPGLVKPSQTLTLTCAISGDSVSSNSAAWNWIRQSPSNWIRQSPSGLEWL
GRYYRSKQWYNDYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCARENIAA
WTWAFDIWGQGTMTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT
VSWNSGALTSGVHTCPAVLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVD
KKVEPKSSDKTHTCGGHHHHHH

A1 bDS LC (SEQ ID NO: 27):

EIVMTQSPGTLSPGERATLSCRASQSVSSSYLAWYQQKPGAPRLLIYDTSRATDI
PDRFSGSGSGTAFTLTISRLEPEDFAVYYCQQYGSSPTFGQGKVEIKRTVAAPSVEFIF
PPSDEQLKSGTASVVCLLNNFYPRKAKVQWKVDNALQSGNSQESVTEQDSKDYSTYS
LCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD56 A2 Fab sequence

A2 bDS HC (SEQ ID NO: 28):

EVQLVQSGAEVKKPGSSVKVSCASGGTFTGYMHWRQAPGQGLEWMGWINPN
SGGTNYAQKFQGRVTMTDRDTSISTAYMELSRLRSDDTAVYYCARDLSSGYSGYFDY
WGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTCPAVLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSD
KTHTCGGHHHHHH

A2 bDS LC (SEQ ID NO: 29):

DVVMTQSPSLPVTTPGEPASISCRSSQSLHNSNGYNYLNWYLQKPGQSPQLLIYLGSN
RASGVPDRFSGSGSGTDFTLKISRVEGEDVGDYYCMQALQSPFTFGQGTKLEIKRTV
AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQD
SKDSTYSLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD56 A3 Fab sequence

A3 bDS HC (SEQ ID NO: 30):

EVQLVQSGAEVKKPGSSVKVSCKASGGTFTGYMHVVRQAPGQGLEWMGWINPN
SGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCARDLSSGYSGYFDY
WGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTCPAVLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSD
KTHTCGGHHHHHH

A3 bDS LC (SEQ ID NO: 31):

DVVMTQSPSLPVTTPGEPASISCRSSQSLHNSNGYNFLDWYLQKPGQSPQLLIYLGSN
RASGVPDRFSGSGSGTDFTLKISRVEADDVGYYCMQSLQTPWTFGHGTVKVEIKRTV
AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQD
SKDSTYSLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD56 Lorvotuzumab Fab sequence

Lorvotuzumab bDS HC (SEQ ID NO: 32):

QVQLVESGGG VVQPGRSLRL SCAASGFTFS SFGMHVVRQA
PGKGLEWVAYISSGSFTIYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED
TAVYYCARMR KGYAMDYWGQ
GTLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
VHTCPAVLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKT
HTCHHHHHHH

Lorvotuzumab bDS LC (SEQ ID NO: 33):

DVVMTQSPSLPVTTLGQPASISCRSSQIIHSDGNTYLEWFQQRPGQSPRRLIYKVSNR
FSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQGSHPHTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS
KDSTYSLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD2 RPA-2.10v1 Fab sequence

RPA-2.10v1 bDS HC (SEQ ID NO: 34):

EVKLVESGGGLVKPGGSLKLSAASGFTFSSYDMSWVRQTPEKRLEWVASISGGGFL
YYLDSVKGRFTISRDNARNILYLHMTSLRSEDAMYYCARSSYGEIMDYWGQGTSV
TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTCP
AVLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTCHH
HHHH

RPA-2.10v1 bDS LC (SEQ ID NO: 35):

DILLTQSPAILSVSPGERVSFSCRASQRIGTSIHWYQQRRTTGSPrLLIKYASESISGIPSR
FSGSGSGTDFTLSINSVESEDVADYYCQQSHGWPFTHGGGKLEIERTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLC
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD137 4B4-1 Fab sequence

4B4-1 bDS HC (SEQ ID NO: 36):

QVQLQQPGAELVKPGASVKLSCKASGYTFSSYWMHWVKQRPQGQVLEWIGEINPGN
 GHTNYNEKFKSKATLTVDKSSSTAYMQLSSLTSEDSAVVYCARSFRTARGFAYWGQ
 GTLTVTSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
 HTCPAVLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHT
 CHHHHHH

4B4-1 bDS LC (SEQ ID NO: 37):

DIVMTQSPATQSVTPGDRVSLSCRASQTISDYLHWYQQKSHESPRLLIKYASQSISGIP
 SRFSGSGSGSDFTLINSVEPEDVGVYYCQDGHSPPTFGGGTKLEIKRTVAAPSVFIF
 PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSTYS
 LCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

[0717] On the day of LNP transfection, 50 thousand T cells and 50 thousand NK cells were added to each well of a 96 well culture plate in a total volume of 100µL containing 100IU/mL IL-2. 10µL of each test LNP was added to each well to facilitate simultaneous transfection of primary human T cells and NK cells at 2.5 ug/mL mRNA.

[0718] 24 hours after LNP transfection, cell culture media was aspirated from T cell and NK cell co-cultures after centrifugation. T cells and NK cells were resuspended with anti-CD3 and anti-CD56 fluorescently labeled antibodies to facilitate analysis of LNP transfection independently in each cell type within the co-culture for 20 minutes at room temperature. Following incubation, cells were concentrated by centrifugation and resuspended in 1x PBS for analysis by flow cytometry. Following acquisition by flow cytometry, T cells and NK cells were analyzed independently using FlowJo (flow cytometry analysis software). In either CD3+ cells (T cells) or CD56+ cells (NK cells), the frequency of GFP positive events relative to GFP negative events was calculated (FIG. 64A). Additionally, the overall fluorescence of GFP was quantified by assessment of mean fluorescence intensity (MFI, FIG. 64B). Similar frequency and MFI analysis were performed for the DiI dye (FIG. 64C, FIG. 64D). Together, these metrics enabled quantification of LNP transfection efficiency of all targeted LNPs tested in primary human T cell and NK cell co-cultures.

[0719] In contrast to experiments performed with unstimulated T cells or whole blood, both ex vivo expanded NK and T cells show high %DiI and %GFP of LNPs post-inserted with the non-target specific mutOKT8 Fab. Despite this difference in %frequency, when comparing formulations by MFI, there was clear separation between mutOKT8 non-targeted LNPs and many of the surface antigen targeted LNPs. Consistent with previous studies, T cells were transfected by anti-CD7, anti-CD8, anti-CD2, anti-CD11a and anti-CD18 targeted LNPs while minimal to no transfection of T cells was observed for anti-CD137 or anti-CD56

targeted LNPs. Similarly, NK cells could also be transfected by anti-CD7, anti-CD8, anti-CD2, anti-CD11a and anti-CD18 targeted LNPs. But, in contrast to T cells, the CD56 targeted LNPs with Lorvotuzumab or the A3 clone only show highly specific transfection of NK cells.

[0720] This data indicates that Fabs or nanobodies are capable of enabling transfection/translation for both NK cells and T cells using anti-CD7, anti-CD8, anti-CD2, anti-CD11a or anti-CD18 targeted LNPs, while using anti-CD56 targeted LNPs is capable of translations/translation for NK cells with high specificity over other immune cells.

Example 32 – PEG-LIPID CONJUGATION AND IN VITRO PROTEIN EXPRESSION - CD3 TARGETING FABS WITH AND WITHOUT NATURAL INTER-CHAIN DISULFIDE

[0721] This example describes the conjugation, purity of either anti-CD3 Fabs with and without the natural interchain disulfide as well as their T cell transfection post-inserted into Cy5/GFP mRNA LNPs.

[0722] LNPs were prepared using the mixing process described in Example 6, the buffer exchange process described in Example 21. Conjugates were generated using a method similar to that of Example 4 except 0.025, 0.1, 0.5 mM TCEP was used for hSP34 DS, 0.025, 0.2, 2 mM TCEP was used for hSP34-hlam NoDS (interchain disulfide knockout) for reduction prior to conjugation and the conjugation reactions were performed at 37C for 2 hr. SDS-PAGE (FIGS. 65A, 65B) was performed using the manufacturers recommended conditions with 1 ug of protein (Thermo, 4-12% Bis-Tris MiniGel). RP-HPLC (FIGS. 65C, 65D) was performed using an Agilent 300 SB-C8 at 0.5 mL/min with a column temperature of 60 °C, Mobile Phase A: Water with 0.1% TFA, Mobile Phase B: Acetonitrile with 0.1% TFA, Gradient %B: 0 min 5%, 1 min 5%, 6.5 min 95%, 8 min 95% injecting 10 ul with a target of 1-25 ug of protein. Using methods similar to Example 12, ant-CD3 hSP34 (with and without natural interchain disulfide, DS (with interchain disulfide) vs. NoDS (without interchain disulfide), see sequences below) PEG-lipid conjugated Fabs were post-inserted at various Fab densities (6, 12, 17 g Fab/mol total lipid) into LNPs containing Lipid 8 and Cy5/GFP mRNA. Transfections were performed with human CD3 T cells at approximately 2.5 µg/mL mRNA for approximately 24 hr. Levels of transfection of CD3 T cells was measured by flow cytometry.

anti-HuCD3 hSP34-hlam Fabs NoDS (without interchain disulfide) and DS (with interchain disulfide)

hSP34-hlam NoDS HC (SEQ ID NO: 38):

EVQLVESGGGLVQPGGSLKLSAASGFTFNKYAMNWVRQAPGKGLEWVARIRSKY
NNYATYYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYISY
WAYWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP
KSSDKTHTC

hSP34-hlam NoDS LC (SEQ ID NO: 39):

QTVVTQEPSLTVSPGGTVTLTCGSSTGAVTSGNYPNWVQKPGQAPRGLIGGTKFLA
PGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCVLWYSNRWVFGGGTKLTVLSQPK
AAPSVTLFPPSSEELQANKATLVCLVSDFYPGAFTVAWKADGSPVKVGVETTKPSK
QSNKYAASSYLSLTPEQWKSHRSYSCRVTHEGSTVEKTVAPAESS

hSP34-hlam DS HC (SEQ ID NO: 40):

EVQLVESGGGLVQPGGSLKLSAASGFTFNKYAMNWVRQAPGKGLEWVARIRSKY
NNYATYYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYISY
WAYWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP
KSCDKTHTC

hSP34-hlam DS LC (SEQ ID NO: 41):

QTVVTQEPSLTVSPGGTVTLTCGSSTGAVTSGNYPNWVQKPGQAPRGLIGGTKFLA
PGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCVLWYSNRWVFGGGTKLTVLSQPK
AAPSVTLFPPSSEELQANKATLVCLVSDFYPGAFTVAWKADGSPVKVGVETTKPSK
QSNKYAASSYLSLTPEQWKSHRSYSCRVTHEGSTVEKTVAPAEC

[0723] The SP34 DS Fab runs around 49 kD on a non-reduced gel (**FIG. 65A**) while the SP34 NoDS Fab LC and HC run similar to each other at slightly less than 28 kD on a non-reduced gel (**FIG. 65B**) confirming that the interchain disulfide has been knocked out by mutating the corresponding cysteines in the heavy and light chain to serine. SP34 DS Fab that was reduced at varying levels of TCEP exhibited high levels of LC/HC single and double PEG-lipid conjugation as shown in both the gel and RP-HPLC chromatogram (**FIG. 65C**) where the condition with the highest purity was 0.025 mM TCEP during reduction while 0.1 and 0.5 mM TCEP had intractable amounts of double conjugate. In contrast, the SP34 NoDS Fab shows high purity at the full range of TCEP evaluated up to 2 mM TCEP highlighting that removal of the interchain disulfide has a dramatic effect on the ability to generate highly pure (single lipid) conjugated Fab. For T cell transfection studies, 0.025 mM TCEP generated SP34 DS Fab was selected given it was the purest of the reaction conditions and had similar recoveries to the other conditions after UF purification (**TABLE 42**) and 0.2 mM TCEP

generated SP34 NoDS Fab was selected given it had both high purity and gave the best recovery after UF purification (TABLE 43).

Table 42. Relationship between TCEP concentration during reduction and final recovery of SP34-hlam DS conjugate after UF purification

TCEP mM	%Recovery Post UF
0.5	20.5
0.1	27.6
0.025	24.3

Table 43. Relationship between TCEP concentration during reduction and final recovery of SP34-hlam NoDS conjugate after UF purification

TCEP mM	%Recovery Post UF
2	14.0
0.2	55.5
0.025	25.9

[0724] The SP34 NoDS Fab mediated higher %transfection (FIG. 65E) and higher GFP expression levels (FIG. 65F) than the SP34 DS Fab at the lowest, 6 g/mol, and middle, 12 g/mol, Fab densities indicating that the potency of this conjugate is higher than that of SP34 DS Fab which is consistent with its correspondingly higher purity (1 PEG-Lipid per Fab, no LC-PEG-lipid).

[0725] This data indicates that knocking out the natural interchain disulfide enables highly efficient, site-specific conjugation towards the c-terminal cysteine with a single PEG-lipid. This broadens the range of reducing agent that can be employed to obtain high purity conjugate (1 PEG-Lipid per Fab) with high process recovery and avoids conjugation of 2 or more PEG-Lipids per Fab which can reduce the final transfection efficiency of targeted LNPs for immune cells.

Example 33 – FAB-PEG-LIPID CONJUGATION AND PURITY – CD2 AND CD8 TARGETING FABS WITH AND WITHOUT NATURAL INTER-CHAIN DISULFIDE

[0726] This example describes the conjugation and purity of anti-CD2 and anti-CD8 Fabs with and without their natural interchain disulfide (FIG. 47).

[0727] Conjugates were generated using a method similar to that of Example 4 except 0.025, 0.0375, 0.05, 0.0625 mM TCEP was used for anti-CD2 TS2/18.1 and 9.6 DS Fabs, 0.05, 0.1, 0.2 mM TCEP was used for anti-CD2 TS2/18.1 and 9.6 NoDS Fabs (see sequences

below), 0.025, 0.05, 0.1, 0.2 mM TCEP was used for anti-CD8 TRX2 NoDS Fab for reduction prior to conjugation and the conjugation reactions were performed at 37°C for 2 hr. SDS-PAGE (FIGS. 66A and 66B) was performed using the manufacturers recommended conditions with 1 µg of protein (Thermo, 4-12% Bis-Tris MiniGel). RP-HPLC (FIG. 66C and 66D) was performed using an Agilent 300 SB-C8 at 0.5 mL/min with a column temperature of 60 °C, Mobile Phase A: Water with 0.1% TFA, Mobile Phase B: Acetonitrile with 0.1% TFA, Gradient %B: 0 min 5%, 1 min 5%, 6.5 min 95%, 8 min 95% injecting 10 µl with a target of 1-25 µg of protein.

Anti-CD2 TS2/18.1 DS Fab

TS2/18.1 DS HC (SEQ ID NO: 42):

EVQLVESGGGLVMPGGSLKLSAASGFAFSSYDMSWVRQTPEKRLEWVAYISGGGF
 TYYPDTVKGRFTLSRDNAKNTLYLQMSSLKSEDTAMYYCARQGANWELVYWGQG
 TLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
 HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
 C

TS2/18.1 DS LC (SEQ ID NO: 43):

DIVMTQSPATLSVTPGDRVFLSCRASQSSISDFLHWYQKSHESPRLLIKYASQSSIGIPS
 RFSGSGSGSDFTLINSVEPEDVGIFYFCQNGHNFPPTFGGGTKLEIKRTVAAPSVFIFP
 PSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSTLS
 SSTLTLSKADYEEKHKVYACEVTHQGLSSPVTKSFNRGEC

Anti-CD2 9.6 DS Fab

9.6 DS HC (SEQ ID NO: 44):

QVQLQPGAELVRPGSSVKLSCKASGYTFTRYWIHWVKQRPIQGLEWIGNIDPSDSE
 THYNQKFKDKATLTVDKSSGTAYMQLSSLTSEDSAVYYCATEDLYYAMEYWGQGT
 SVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
 FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC

9.6 DS LC (SEQ ID NO: 45):

NIMMTQSPSSLAVSAGEKVTMTCKSSQSVLYSSNQKNYLAWYQQKPGQSPKLLIYW
 ASTRESGVDPDRFTGSGSGTDFTLTISVQPEDLAVYYCHQYLSSHTFGGGTKLEIKRT
 VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
 DSKDSTYLSSTLTLSKADYEEKHKVYACEVTHQGLSSPVTKSFNRGEC

[0728] The TS2/18.1 and 9.6 DS Fabs run around 49 kD on a non-reduced gel (FIG. 26-1) while the TS2/18.1, 9.6 and TRX2 NoDS Fabs LC and HC run similar to each other at slightly less than 28 kD on a non-reduced gel (FIG. 66B) confirming that the interchain disulfide has been knocked out by mutating the corresponding the cysteines in the heavy and light chain to serine. The TS2/18.1 and 9.6 DS Fabs reduced at varying levels of TCEP prior to conjugation exhibited high levels of LC/HC single and double PEG-lipid conjugation as shown by both the SDS-PAGE (FIG. 66A) and RP-HPLC chromatograms (TS2/18.1 only, FIG. 66C) where the condition with the highest purity was 0.025 mM TCEP during reduction

and higher TCEP levels increased the amount of LC conjugate and double conjugate (2 PEG-lipid per Fab). In contrast, the TS2/18.1, 9.6 and TRX2 NoDS Fabs shows high purity at the full range of TCEP evaluated up to 0.2 mM TCEP highlighting that removal of the interchain disulfide has a dramatic effect on the ability to generate highly pure (1 PEG-lipid per Fab) conjugate.

[0729] This data across a number immune cell targeting Fabs indicates that knocking out the natural interchain disulfide is a generalizable approach to enable highly efficient, site-specific conjugation towards the c-terminal cysteine on the heavy chain while avoiding conjugation to the light chain and conjugating more than one PEG-lipid per Fab.

[0730] **Example 34 – IN VITRO PROTEIN EXPRESSION - CD3 AND TCR TARGETING COMPARISON AND SP34 FAB WITH AND WITHOUT BURIED DISULFIDE** This example describes targeting human CD3 T cells with either anti-CD3 or anti-TCR Fabs and an anti-CD3 Fab with and without a buried interchain disulfide (FIG. 47) post-inserted into Cy5/GFP mRNA LNPs and their effect on transfection and IFN γ secretion.

[0731] LNPs were prepared using the mixing process described in Example 6, the buffer exchange process described in Example 21. Conjugates were generated using a method similar to that of Example 4 except 0.1 mM TCEP was used for reduction prior to conjugation and the conjugation reaction was performed at 37C for 2 hr. Using methods similar to Example 12, anti-CD3 hSP34 (with and without buried disulfide, bDS vs. NoDS), TR66 (Bortoletto et al Optimizing anti-CD3 affinity for effective T cell targeting against tumor cells, Eu. J of Immun. 2002; Frank et al Combining T cell specific activation and in vivo gene delivery through CD3-targeted lentiviral vectors, Blood Adv 2020), anti-CD3 TRX4, anti-CD3 humanized UCHT1 (HzUCHT1), and anti-CD3 Teplizumab PEG-lipid conjugated Fabs were post-inserted into LNPs containing Lipid 8 and Cy5/GFP mRNA. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (clone SK3) to distinguish the two cell types. IFN γ in the supernatants was measured using the manufacturers recommended procedure (R&D Systems, DY285B).

Anti-CD3 hSP34-hlam bDS Fab sequence
hSP34-hlam bDS HC (SEQ ID NO: 46):

EVQLVESGGGLVQPGGSLKLSAASGFTFNKYAMNWVRQAPGKGLEWVARIRSKY
 NNYATYYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYISY
 WAYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
 SGALTSGVHTCPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP
 KSSDKTHTCHHHHHH

hSP34-hlam bDS LC (SEQ ID NO: 47):

QTVVTQEPSTLVSPGGTVTLTCGSSTGAVTSGNYPNWVQKPGQAPRGLIGGTFKFLA
 PGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCVLWYSNRWVFGGGTKLTVLSQPK
 AAPSVTLFPPSSEELQANKATLVCLVSDFYPGAFTVAWKADGSPVKVGVETTKPSK
 QSNKYAACSYLSLTPEQWKSHRSYSCRVTHEGSTVEKTVAPAESS

Anti-CD3 TR66 bDS Fab sequence

TR66 bDS HC (SEQ ID NO: 48):

QVQLQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINPSR
 GYTNYNQKFQDKATLTITDKSSSTAYMQLSSLTSEDSAVYYCARYYDDNYSLDYWG
 QGTTLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
 VHTCPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKT
 HTCHHHHHH

TR66 bDS LC (SEQ ID NO: 49):

QIVLTQSPSSLSASLGEKVTMTCRASSSVSYMNWYQKPGTSPKRWIYDTSKVASGV
 PDRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLELKRTVAAPSV
 FIFPPSDEQLKSGTASVVCCLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
 YSLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD3 TRX4 bDS Fab sequence

TRX4 bDS HC (SEQ ID NO: 50):

EVQLLESGGGLVQPGGSLRLSCAASGFTSSFPMAWVRQAPGKGLEWVSTISTSGGR
 TYRDSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAKFRQYSGGFDYWGGQ
 TLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
 HTCPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHT
 CHHHHHH

TRX4 bDS LC (SEQ ID NO: 51):

DIQLTQPNSTVSTSLGSTVKLSCTLSSGNIENNYVHWYQLYEGRSPTTMIYDDDKRPD
 GVPDRFSGSIDRSSNSAFLTIHNVAIEDEAIYFCHSYVSSFNVFGGGTKLTVLGQPKAN
 PTVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADGSPVKAGVETTKPSKQSN
 NKYAAACSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTESS

Anti-CD3 HzUCHT1 bDS Fab sequence

HzUCHT1(Y59T) bDS HC (SEQ ID NO: 52):

EVQLVESGGGLVQPGGSLRLSCAASGSYFTGYTMNWVRQAPGKGLEWVALINPTK
 GVSTYNQKFQDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCARSGYYGDSWDYFD
 VWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA
 LTSGVHTCPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSS
 DKTHTCHHHHHH

HzUCHT1 bDS LC (SEQ ID NO: 53):

DIQMTQSPSSLSASVGDRVITTCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLESGV
 PSRFSGSGSGTDYTLTISSLPEDFATYYCQQGNTLPWTFGQGTKVEIKRTVAAPSVFI
 FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY
 SLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD3 Teplizumab bDS Fab sequence

Teplizumab bDS HC (SEQ ID NO: 54):

QVQLVQSGGGVVPGRSLRLSCKASGYTFTRYTMHWVRQAPGKGLEWIGYINPSRG
 YTNYNQKVKDRFTISRDN SKNTAFLQMDSL RPEDTG VYFCARYYDDHYCLDYWGQ
 GTPVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
 HTCFAVLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHT
 CHHHHHH

Teplizumab bDS LC (SEQ ID NO: 55):

DIQMTQSPSSLSASVGDRVITTCASSSVSYMNWYQQTPGKAPKRWIYDTSKLASGV
 PSRFSGSGSGTDYTFITISLPEDIAITYYCQQWSSNPFTFGQGTKLQITRTVAAPSVFIF
 PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS
 LCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

[0732] The SP34 NoDS Fab mediated higher %transfection (FIG. 67A) and higher GFP expression levels (FIG. 67B) quantified by mean fluorescence intensity, MFI) than the other Fab constructs. While the SP34 bDS and TR66 bDS Fabs mediated similar levels of %transfection by GFP the expression levels were lower than that of the SP34 NoDS Fab quantified by mean fluorescence intensity. For SP34 the NoDS and bDS Fab formats had similar levels of IFN γ secretion (FIG. 67C). Additionally, while TR66, TRX4, HzUCHT1 and Teplizumab had higher levels of IFN γ secretion than SP34, they exhibited lower levels of GFP expression (FIG. 67B).

[0733] This data indicates that across multiple CD3 targeting Fabs, whether they have kappa or lambda light chains, many are capable of mediating high transfection/translation in either the NoDS or bDS exemplifying CD3 as a robust T cell target for mediating CD8 and CD4 T cell transfection and translation. For the SP34 clone, the NoDS format is preferred over the bDS format with regards to T cell transfection/translation efficiency. Additionally, this data suggests that T cell activation does not guarantee efficient transfection and translation.

Example 35 – IN VITRO PROTEIN EXPRESSION – CD8 TARGETED FAB WITH AND WITHOUT BURIED DISULFIDE AND OTHER CD2 TARGETED FAB CLONES

[0734] This example describes targeting human CD8 T cells with anti-CD8 Fab in a NoDS or bDS format or anti-CD2 Fabs post-inserted into Cy5/GFP mRNA LNPs and their effect on transfection and translation.

[0735] LNPs were prepared using the mixing process described in Example 6, the buffer exchange process described in Example 21. Fab-lipid conjugates generated from methods described in Example 4. Using methods similar to Example 12, anti-CD3 hSP34, anti-CD8 TRX2 and anti-CD2 clones Lo-CD2b (ATCC, PTA-802), 35.1 (ATCC, HB-222) and OKT11 (ATCC, CRL-8027) PEG-lipid conjugated Fabs were post-inserted into LNPs containing Lipid 8 and Cy5/GFP mRNA. Transfections were performed with human CD3 T cells at approximately 2.5 µg/mL mRNA for approximately 24 hr. Levels of transfection of CD8 cells was measured by flow cytometry.

Anti-CD8 TRX2 bDS Fab sequence

TRX2 bDS HC (SEQ ID NO: 56):

QVQLVESGGGVVQPGRSLRLSCAASGFTFSDFGMNWVRQAPGKGLEWVALIYYDG
SNKFYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPHYDGYHHFFDS
WGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTCPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSD
KTHTC

TRX2 bDS LC (SEQ ID NO: 57):

DIQMTQSPSSLSASVGDRVTITCKGSQDINNYLAWYQQKPGKAPKLLIYNTDILHTG
VPSRFSGSGSGTDFTFTISSLQPEDIATYYCYQYNNGYTFGQGTKVEIKRTVAAPSVFI
FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY
SLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD2 Lo-CD2b bDS Fab sequence

Lo-CD2b bDS HC (SEQ ID NO: 58):

EVQLVESGGGLVQPGASLKSLSCVASGFTFSDYWMSWVRQTPGKPMEWIGHIKYDGS
YTNYAPSLKNRFTISRDN AKTTLYLQMSNVRSEDSATYYCAREAPGAASYWGQGTLL
TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
CPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC

Lo-CD2b bDS LC (SEQ ID NO: 59):

DVVLTTQTPVAQPVTLGDAQISCRSSQSLVHSNGNTYLEWFLQKPGQSPQLLIYKVS
NRFSGVPDRFIGSGSGSDFTLKISRVEPEDWGVYYCFQGTHTDPYTFGAGTKLELKRT
VAAPSVFI FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD2 35.1 bDS Fab sequence

35.1 bDS HC (SEQ ID NO: 60):

EVQLQSGAELVKPGASVKLS CRTSGFNIKDTYIHWVKQRPEQGLKWIGRIDPANGN
TKYDPKFQDKATVTADTSSNTAYLQLSSLTSED TAVYYCVTYAYDGNWYFDVWGA
GTAVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
VHTCPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKT
HTC

35.1 bDS LC (SEQ ID NO: 61):

DIKMTQSPSSMYVSLGERVTITCKASQDINSFLSWFQQKPGKSPKTLIYRANRLVDGV
 PSRFSGSGSGQDYSLTSSLEYEDMEIYYCLQYDEFPTYFGGGTKLEMKRTVAAPSVF
 IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDY
 SLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD2 OKT11 bDS Fab sequence

OKT11 bDS HC (SEQ ID NO: 62):

QVQLQQPGAELVRPGTSVKLSCKASGYTFTSYWMHWIKQRPEQGLEWIGRIDPYDS
 ETHYNEKFKDKAILSVDKSSSTAYIQLSSLTSDDSAVYYCSRDAKYDGYALDYWG
 QGTSVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
 VHTCPAVLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKT
 HTC

OKT11 bDS LC (SEQ ID NO: 63):

DIVMTQAAPSVPTPGESVSISCRSSKTLTLLHSNGNTYLYWFLQRPQGSPQVLIYRMSN
 LASGVNRFSGSGSETTFTLRISRVEAEDVGIYYCMQHLEYPYTFGGGTKLEIERTVA
 APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS
 KDSTYSLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

[0736] The interchain disulfide knockout (NoDS) anti-CD8 TRX2 Fab had slightly higher %transfection (FIG. 68A) and GFP expression levels (FIG. 68B) than the buried disulfide (bDS) TRX2 Fab however the difference was small. Similar to Example 17, none of the CD2 targeting Fabs explored herein performed as well as anti-CD3 or anti-CD8 Fab.

[0737] This data indicates that the TRX2 clone in the NoDS format is preferred however Fabs in the bDS format can mediate efficient T cell transfection/translation. Additionally, CD8 and CD3 are preferred targets over CD2 for the clones evaluated.

Example 36 – IN VITRO PROTEIN EXPRESSION – CD8 TARGETED FAB WITH AND WITHOUT BURIED DISULFIDE AND OTHER CD2 TARGETED FAB CLONES

[0738] This example describes targeting human T cells by co-targeting with anti-CD3 and anti-CD11a or anti-CD3 and anti-CD18 Fabs post-inserted into Cy5/GFP mRNA LNPs and their effect on transfection/translation and IFN γ cytokine secretion.

[0739] LNPs were prepared using the mixing process described in Example 6, the buffer exchange process described in Example 21. Fab-lipid conjugates generated from methods described in Example 4. Using methods similar to Example 12, anti-CD3 hSP34, anti-CD11 α HzMHM24, anti-CD18 Erlizumab PEG-lipid conjugated Fabs were post-inserted into LNPs containing Lipid 8 and Cy5/GFP mRNA. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK7)

to distinguish the two cell types. IFN γ in the supernatants was measured using the manufacturers recommended procedure (R&D Systems, DY285B).

[0740] Targeting either CD11a or CD18 alone mediated transfection (FIG. 69A) and GFP expression (FIG. 69B) for both CD8 and CD4 T cells. Importantly, by co-targeting either anti-CD3 clone with any of the anti-CD11a or CD18 clones, the levels of IFN γ secretion was reduced by nearly 50% while the levels of transfection and translation were either similar or higher than CD3 targeting alone (FIG. 69C).

[0741] This data indicates that targeting CD11a and CD18 can mediate efficient immune cell transfection/translation and that co-targeting CD3 with either CD11a or CD18 can substantially reduce the cytokine release from T cells without negatively impacting T cell transfection and protein translation. Another anti-CD3 clone in the NoDS Fab format can approach similar levels of transfection/translation of SP34 in the NoDS Fab format.

Anti-CD11a HzMHM24 bDS Fab sequence

HzMHM24 bDS HC (SEQ ID NO: 64):

EVQLVESGGGLVQPGGSLRLSCAASGYSTFGHWMNWVRQAPGKGLEWVGMIHPSD
SETRYNQKFKDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARGIYFYGTTFDYW
QGGLTLVTSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTCPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSD
KTHTCHHHHHH

HzMHM24 bDS LC (SEQ ID NO: 65):

DIQMTQSPSSLSASVGDRVTITCRASKTISKYLAWYQKPGKAPKLLIYSGSTLQSGV
PSRFGSGSGTDFTLTISSLQPEDFATYYCQQHNEYPLTFGGGTKVEIKRTVAAPSVFI
FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY
SLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD18 h1B4 bDS Fab sequence

h1B4 bDS HC (SEQ ID NO: 66):

EVQLVESGGDLVQPGRSLRLSCAASGFTFSDYYMSWVRQAPGKGLEWVAAIDNDG
GSISYPDTVKGRTISRDNKNSLYLQMNSLRVEDTALYYCARQGRLRRDYFDYWG
QGTLVTVSTASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTCPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDK
THTCHHHHHH

h1B4 bDS LC (SEQ ID NO: 67):

DIQMTQSPSSLSASVGDRVTITCRASESVDSYGNSFMHWYQKPGKAPKLLIYRASN
LESGVPSRFGSGSGTDFTLTISSLQPEDATYYCQQSNEDPLTFGGGTKLEIKRTVAA
PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSK
DSTYSLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD18 Erlizumab bDS Fab sequence

Erlizumab bDS HC (SEQ ID NO: 68):

EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAGINPKN
GGTSHNQRFMDFRTISVDKSTSTAYMQMNSLRAEDTAVYYCARWRGLNYGFDVRY
FDVWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTCPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP
KSSDKTHTCHHHHHH

Erlizumab bDS LC (SEQ ID NO: 69):

DIQMTQSPSSLSASVGDRVTITCRASQDINNYLNWYQQKPGKAPKLLIYYTSTLHSG
VPSRFGSGSGTDYTLTISSLOPEDFATYYCQQGNTLPPTFGQGTKVEIKRTVAAPSVF
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSY
SLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

**Example 37 – IN VITRO PROTEIN EXPRESSION – CO-TARGETED LNPs WITH CD4 AND CD8 FABS
OR CD4 / CD8 Fab-ScFv BISPECIFIC**

[0742] This example describes targeting human T cells with anti-CD4 or anti-CD8 Fabs, anti-CD4 and anti-CD8 Fabs and a CD4 Fab with a CD8 ScFv off the CD4 Fab light chain (Fab-ScFv) post-inserted into Cy5/GFP mRNA LNPs and their effect on transfection and IFN γ secretion.

[0743] LNPs were prepared using the microfluidic mixing process described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiI18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from methods described in Example 4. Using methods similar to Example 12, anti-CD3 hSP34, anti-CD4 Ibalizumab, anti-CD8 TRX2 conjugated Fabs and CD4/CD8 Ibalizumab/TRX2 Fab-ScFv were post-inserted into LNPs containing Lipid 8 and GFP mRNA with DiI dye. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK3) to distinguish the two cell types. IFN γ in the supernatants was measured using the manufacturers recommended procedure (R&D Systems, DY285B).

[0744] Post-inserting both anti-CD8 and anti-CD4 Fab together shows similar CD8 and CD4 T cell transfection and protein expression relative to the Fabs individually post-inserted (FIGS. 70A and 70B). Compared to post-inserting the CD4 and CD8 Fabs together, the CD4/CD8 Fab-ScFv bispecific shows slightly lower CD4 and CD8 T cell transfection. None

of the CD4, CD8 or CD4/CD8 co-targeting conditions mediated substantial IFN γ release in contrast to CD3 targeting with SP34 Fab (FIG. 70C).

[0745] This data indicates that a bispecific targeting moiety can be leveraged to have a single protein construct target 2 different immune cell types with minimal loss in targeting function over post-inserting targeting moieties individually into the same LNP.

Anti-CD4/CD8 Ibalizumab/TRX2 bDS Fab-ScFv sequence

Ibalizumab/TRX2 bDS Fab-ScFv HC (SEQ ID NO: 70):

QVQLQQSGPEVVKPGASVKMSCKASGYTFTSYVIHWVRQKPGQGLDWIGYINPYND
GTDYDEKFKGKATLTSDTSTSTAYMELSSLRSEDVAVYYCAREKDNATGAWFAY
WGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTCPAVLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSD
KTHTCHHHHHH

Ibalizumab/TRX2 bDS Fab-ScFv LC (SEQ ID NO: 71):

DIVMTQSPDSLAVSLGERVTMCKSSQSLLYSTNQKNYLAWYQQKPGQSPKLLIYW
ASTRESGVDPDRFSGSGSGTDFTLTISVQAEDVAVYYCQQYYSYRTFGGGTKLEIKRT
VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKSTYSLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGESGGGGSGGG
GSGGGGSQVQLVESGGGVVQPGRSLRLSCAASGFTFSDFGMNWRQAPGKGLEWV
ALIYYDGSNKFYADSVKGRFTISRDNSTNTLYLQMNSLRAEDTAVYYCAKPHYDGY
YHFFDSWGQGTLLTVSSGGGGSGGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRV
TITCKGSQDINNYLAWYQQKPGKAPKLLIYNNTDILHTGVPSRFSGSGSGTDFTFTISL
QPEDIATYYCYQYNNGYTFGGGTKEIK

Example 38 – IN VITRO PROTEIN EXPRESSION – CD4 TARGETED FABS WITHOUT NATURAL INTERCHAIN DISULFIDE OR WITH BURIED INTERCHAIN DISULFIDE

[0746] This example describes targeting human T cells with anti-CD3 or anti-CD4 Fabs post-inserted into Cy5/GFP mRNA LNPs and their effect on transfection and IFN γ secretion.

[0747] LNPs were prepared using the mixing process described in Example 6, the buffer exchange process described in Example 21. Fab-lipid conjugates generated from methods described in Example 4. Using methods similar to Example 12, anti-CD3 hSP34, anti-CD4 Ibalizumab, anti-CD4 humanized OKT4 PEG-lipid conjugated Fabs and Nb were post-inserted into LNPs containing Lipid 8 and Cy5/GFP mRNA. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK4) to distinguish the two cell types. IFN γ in the supernatants was measured using the manufacturers recommended procedure (R&D Systems, DY285B).

[0748] Amongst the CD4 targeted Fabs, Ibalizumab mediated higher %transfection (FIG. 71A) and GFP expression levels (FIG. 71B) quantified by mean fluorescence intensity, MFI) however it was lower than anti-CD3 SP34 Fab. None of the anti-CD4 Fabs mediated substantial IFN γ secretion levels over non-targeted mutOKT8 Fab while anti-CD3 SP34 Fab exhibited higher levels of IFN γ (FIG. 71C).

[0749] This data indicates that anti-CD4 Fabs without the natural interchain disulfide (Ibalizumab, NoDS) or with a buried interchain disulfide (OKT4, bDS) can mediate highly specific LNP transfection and protein translation of CD4+ T cells versus CD8+ T cells and targeting CD4 can avoid T cell activation and IFN γ release.

Anti-CD4 Ibalizumab NoDS Fab sequence

Ibalizumab NoDS LC (SEQ ID NO: 72):

QVQLQQSGPEVVKPGASVKMSCKASGYTFTSYVIHWVRQKPGQGLDWIGYINPYND
GTDYDEKFKGKATLTSDTSTSTAYMELSSLRSEDTAVYYCAREKDNATGAWFAY
WGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSD
KTHTC

Ibalizumab NoDS HC (SEQ ID NO: 73):

DIVMTQSPDSLAVSLGERVTMNCSSQSLLYSTNQKNYLAWYQQKPGQSPKLLIYW
ASTRESGVPRDFSGSGSDFTLTISVQAEDVAVYYCQYYSYRTFGGGTKLEIKRT
VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKSTYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD4 OKT4 bDS Fab sequence

OKT4 bDS LC (SEQ ID NO: 74):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYAMSWVRQAPGKRLEWVSAISDHST
NTYYPDSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCARKYGGDYDPFDYWG
QGTLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTCPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDK
THTCHHHHHH

OKT4 bDS HC (SEQ ID NO: 75):

DIQMTQSPSSLSASVGDRVTITCQASQDINNYIAWYQHKPGKGPGLLIHYTSTLQPGIP
SRFSGSGSGRDTLTISLQPEDFATYYCLQYDNLLFTFGGGTKVEIKRTVAAPSVFIF
PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYS
LCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Example 39 – IN VITRO PROTEIN EXPRESSION – OTHER CD4 TARGETED FAB CLONES and A CD4 TARGETED NANOBODY

[0750] This example describes targeting human T cells with anti-CD3 or anti-CD4 Fabs post-inserted into GFP mRNA DiI labeled LNPs and their effect on transfection and IFN γ secretion.

[0751] LNPs were prepared using the microfluidic mixing process described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from methods described in Example 4 while generation of Nb-conjugated differed in using 1:1:4 Nb:DSPE-3.4KPEG-maleimide:DSPE-2KPEG-OCH₃ and a 50 kD UF membrane (Millipore Corp, Billerica, MA USA) for separation of Nb-conjugate from free Nb. Using methods similar to Example 12, anti-CD3 hSP34, anti-CD4 Ibalizumab, anti-CD4 hBF5 conjugated Fabs and conjugated Nb (derived from llama immunization) were post-inserted into LNPs containing Lipid 8 and GFP mRNA with DiI dye. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (OKT3) to distinguish the two cell types. IFN γ in the supernatants was measured using the manufacturers recommended procedure (R&D Systems, DY285B).

[0752] Amongst the CD4 targeted conjugates, the anti-CD4 mediated slightly higher %transfection (FIG. 72A) and GFP expression levels (FIG. 72B) quantified by mean fluorescence intensity, MFI) however it was lower than anti-CD3 SP34 Fab. For the CD4 targeted Fabs and Nb the transfection and translation was only observed in the CD4+ T cell population. None of the anti-CD4 Fabs mediated substantial IFN γ secretion levels over non-targeted mutOKT8 Fab while anti-CD3 SP34 Fab exhibited higher levels of IFN γ .

[0753] This data indicates that both Fabs and Nanobodies can mediate highly specific LNP transfection and protein translation by CD4+ T cells versus CD8 T cells and targeting CD4 can avoid T cell activation and IFN γ release.

Anti-CD4 T023200008 Nb sequence (SEQ ID NO: 76)

CDR1, CDR2, CDR3 underlined based on IMGT designation:

EVQLVESGGGSVQPGGSLTLSCGTSGRITFNVMGWFRQAPGKEREFVAAVRVWSSTGI
YYTQYADSVKSRFTISRDNAKNTVYLEMNSLKPEDTAVVYCAADTYSNPARWDG
 YDFRGQGTLVTVSSGGCGGHHHHHH

Example 40- IN VITRO PROTEIN EXPRESSION –CD8 TARGETED NANOBODY CLONE

[0754] This example describes targeting human T cells with anti-CD3, anti-CD8 Fab or anti-CD8 Nanobodies post-inserted into GFP mRNA DiI labeled LNPs and their effect on transfection and IFN γ secretion.

[0755] LNPs were prepared using the microfluidic mixing process described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from methods described in Example 4 while generation of Nb-conjugated differed in using 1:1:4 Nb:DSPE-5KPEG-maleimide:DSPE-2KPEG-OCH₃ and a 50 kD UF membrane (Millipore Corp, Billerica, MA USA) for separation of Nb-conjugate from free Nb. Using methods similar to Example 12 conjugated Fabs and conjugated Nb (derived from llama or alpaca immunization) were post-inserted into LNPs containing Lipid 8 and GFP mRNA with DiI dye. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK3) to distinguish the two cell types. IFN γ in the supernatants was measured using the manufacturers recommended procedure (R&D Systems, DY285B).

[0756] The CD8 targeted Nb conjugate exhibited higher %transfection (**FIG. 73A**) and GFP expression levels (**FIG. 73B**) than anti-CD8 TRX2. For the CD8 targeted Nb, transfection and translation was only observed in the CD8 T cell population relative to the mutOKT8 Fab. The anti-CD8 Nb did not mediate substantial IFN γ secretion levels over non-targeted mutOKT8 Fab while anti-CD3 SP34 Fab exhibited higher levels of IFN γ (**FIG. 73C**).

[0757] This data indicates that both Fabs and Nanobodies can mediate highly specific LNP transfection and protein translation by CD8 T cells versus CD4 T cells and targeting CD8 can avoid T cell activation and IFN γ release.

Anti-CD8 BDSn Nb sequence (SEQ ID NO: 77)

CDR1, CDR2, CDR3 underlined based on IMGT designation:

EVQLVESGGGLVQAGGSLRLSCAASGSTFSDYGVGWFRQAPGKGREFVADIDWNG
 EHTSYADSVKGRFATSRDNAKNTAYLQMNSLKPEDTAVYYCAADALPYTVRKYNY
 WGQGTQVTVSSGGCGGHHHHHH

Example 41 – IN VITRO PROTEIN EXPRESSION –CD3 and CD7 TARGETED NANOBODIES WITH 2K OR 5K PEG

[0758] This example describes targeting human T cells with anti-CD3, anti-CD7 Fab or anti-CD8 Nanobodies post-inserted into GFP mRNA DiI labeled LNPs and their effect on transfection and IFN γ secretion.

[0759] LNPs were prepared using the microfluidic mixing process described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiI18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from methods described in Example 4 while generation of Nb-conjugated differed in using 1:1:4 Nb:DSPE-5KPEG-maleimide or DSPE-3.4KPEG-maleimide:DSPE-2KPEG-OCH₃ and a 50 kD UF membrane (Millipore Corp, Billerica, MA USA) for separation of Nb-conjugate from free Nb. Using methods similar to Example 12 conjugated Fabs and conjugated Nb (E11 and G03), V1 (anti-CD7) were post-inserted into LNPs containing Lipid 8 and GFP mRNA with DiI dye. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK3) to distinguish the two cell types.

[0760] For both anti-CD3 Nb clones and the anti-CD7 Nb the longer, 5K PEG, improved %transfection (FIG. 74A) and GFP expression levels (FIG. 74B) over the 2K PEG. For the anti-CD3 Nbs the difference between the conjugate PEG lengths was more dramatic than for the anti-CD7 Nbs.

[0761] This data indicates that Nanobody conjugates can benefit from a PEG length longer than 2K and that different clones can have varying degrees of improvement.

Anti-CD3 T0170117G03-A Nb sequence (SEQ ID NO: 78)

EVQLVESGGGPVQAGGSLRLSCAASGRTRYGYSMGWFRQAPGKREFVAAIVWSG
 GNTYYEDSVKGRFTISRDNAMIMYLQMTSLKPEDSATYYCAAKIRPYIFKIAGQYD
 YWGQGTTLTVSSAGGSGGHHHHHH

Anti-CD3 T0170060E11 Nb sequence (SEQ ID NO: 79)

EVQLVESGGGLVQPGGSLRLSCAASGDIYKSFDMGWYRQAPGKQRDLVAVIGSRGN
NRGRTNYADSVKGRFTISRDTGTNTVYLLMNKLRPEDTAIYYCNTAPLVAGRPWGR
GTLVTVSSGGGSGGHHHHHC

Anti-CD7 V1 Nb sequence (SEQ ID NO: 80)

DVQLQESGGGLVQAGGSLRLSCAVSGYPYSSYCMGWFRQAPGKEREGVAAIDSDG
RTRYADSVKGRFTISQDNAKNTLYLQMNRMKPEDTAMYYCAARFGPMGCVDLSTL
SFGHWGQGTQVTVSITGGGCHHHHHHHH

Example 42 – IN VITRO PROTEIN EXPRESSION – CD8, CD3, CD28, CD4 and TCR TARGETED NANOBODIES WITH 2K OR 5K PEG

[0762] This example describes targeting human T cells with anti-CD8, anti-CD3, anti-CD4 Fab and anti-CD8, anti-CD3, anti-CD28, anti-CD4 and anti-TCR Nanobodies post-inserted into GFP mRNA DiI labeled LNPs and their effect on transfection and IFN γ secretion.

[0763] LNPs were prepared using the microfluidic mixing process described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from methods described in Example 4 while generation of Nb-conjugated differed in using 1:1:4 Nb:DSPE-2KPEG-maleimide:DSPE-2KPEG-OCH₃ or Nb:DSPE-5KPEG-maleimide:DSPE-2KPEG-OCH₃ and a 50 kD UF membrane (Millipore Corp, Billerica, MA USA) for separation of Nb-conjugate from unconjugated Nb. Using methods similar to Example 12 conjugated Fabs and conjugated Nb (derived from llama or alpaca immunization) were post-inserted into LNPs containing Lipid 8 and GFP mRNA with DiI dye. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK3) to distinguish the two cell types.

[0764] For all of the targets evaluated, nanobodies conjugated with the longer, 5K PEG, generally improved %transfection (FIGS. 75A and 75B) and GFP expression levels (FIGS. 75C and 75D) over the 2K PEG with exception of anti-CD8 clone E05 which showed the reverse relationship. The magnitude of improvement appears to be clone specific.

[0765] This data indicates that regardless of the target, the preferred PEG length for a Nanobody PEG-lipid conjugate is greater than 2K.

Anti-TCR T017000700 Nb sequence (SEQ ID NO: 81)

CDR1, CDR2, CDR3 underlined based on IMGT designation:

EVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVAHISIGDQ
TDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRIWPYDYWGQGT
 LTVVSSGGCGGHHHHHH

Anti-CD28 28CD065G01 Nb sequence (SEQ ID NO: 82)

EVQLVESGGGLVQPGGSLRLSCAASGSIFRLHTMEWYRRTPETQREWVATITSGGTT
 NYPDSVKGRFTISRDDTKKTVYLQMNSLKPEDTAVVYCHAVATEDAGFPSPNYWG
 QGTLLTVVSSGGCGGHHHHHH

Anti-CD3 T0170061C09 Nb sequence (SEQ ID NO: 83)

EVQLVESGGGPVQAGGSLRLSCAASGRTYRGYSMGWFRQAPGREREFVAAIVWSD
 GNTYYEDSVKGRFTISRDNKNTMYLQMTSLKPEDSATYYCAAKIRPYIFKIAGQYD
 YWGQGTLLTVVSSGGCGGHHHHHH

Anti-CD4 T023200008 Nb sequence (SEQ ID NO: 76)

EVQLVESGGGSVQPGGSLTLSCGTSGRTFNVMGWFRQAPGKEREVAAVRRWSSTGI
YYTQYADSVKSRFTISRDNKNTVYLEMNSLKPEDTAVVYCAADTYNSNPARDWG
 YDFRGQGTLLTVVSSGGCGGHHHHHH

EXAMPLE 43 – IN VITRO PROTEIN EXPRESSION –CD8, CD7 AND CD3 TARGETED NANOBOBIES WITH 5K OR 3.4K PEG

[0766] This example describes targeting human T cells with anti-CD8, anti-CD3 Fab and anti-CD8, anti-CD7 and anti-CD3 Nanobodies post-inserted into GFP mRNA DiI labeled LNPs and their effect on transfection.

[0767] LNPs were prepared using the microfluidic mixing process described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from methods described in Example 4 while generation of Nb-conjugated differed in using 1:1:4 Nb:DSPE-3.4KPEG-maleimide:DSPE-2KPEG-OCH₃ or Nb:DSPE-5KPEG-maleimide:DSPE-2KPEG-OCH₃ and a 50 kD UF membrane (Millipore Corp, Billerica, MA USA) for separation of Nb-conjugate from unconjugated Nb. Using methods similar to Example 12 conjugated Fabs and conjugated Nb (derived from

llama or alpaca immunization) were post-inserted into LNPs containing Lipid 5 and GFP mRNA with DiI dye at temperature of 37C for 4 hrs. Transfections were performed with human CD3 T cells at approximately 2.5 µg/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK3) to distinguish the two cell types.

[0768] For all of the targets evaluated, Nb conjugated with the shorter, 3.4K PEG was similar if not slightly better than the longer 5K PEG in %transfection (FIG. 76A) and GFP expression levels (FIG. 76B) or in the case of the anti-CD7 V1 Nb 3.4K PEG mediated higher transfection and expression than the 5K PEG.

[0769] This data indicates that regardless of the target, the generally preferred PEG length for Nanobody-PEG-lipid conjugates is 3.4K PEG versus the shorter 2K PEG as previously described in EXAMPLE 42 or the longer 5K PEG as described herein.

EXAMPLE 44 – IN VITRO PROTEIN EXPRESSION – 2K VERSUS 3.4K PEG SPACER FOR FABS

[0770] This example describes targeting human T cells with anti-CD3, anti-CD4, anti-CD8, anti-CD28, Fabs post-inserted into GFP mRNA DiI labeled LNPs and their effect on transfection.

[0771] LNPs were prepared using the microfluidic mixing process described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from methods described in Example 4. Using methods similar to Example 12 conjugated Fab (12D2) was post-inserted into LNPs containing Lipid 8 and GFP mRNA with DiI dye. Transfections were performed with human CD3 T cells at approximately 2.5 µg/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK3) to distinguish the two cell types.

[0772] While most Fab clones did not differ in %transfection (FIG. 77A) and GFP expression levels (FIG. 77B) between the 2 PEG lengths, anti-CD4 Ibalizumab showed an

increase in transfection efficiency going from 2K PEG to 3.4K PEG while anti-CD3 SP34 transfection efficiency decreased going from 2K PEG to 3.4K PEG.

[0773] This data indicates that generally a 2K PEG spacer is preferred for Fab-PEG-lipid conjugates however some clones can gain benefit from a longer PEG spacer. Additionally, it indicates anti-CD3 clone 12D2 with a buried disulfide with either a 2K or 3.4K PEG can efficiently transfect both CD8 and CD4 T cell subsets.

Anti-CD3 12D2 bDS Fab sequence

12D2 bDS HC (SEQ ID NO: 84):

EVKLVESGGGLVQPGRSLRLSCAASGFNFYAYWMGWVRQAPGKGLEWIGEIKKDG
TTINYTPSLKDRFTISRDNQNTLYLQMTKLGS EDTALYYCAREERDGYFDYWGGQ
VMVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTCPAVLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHT
CGGHHHHHHH

12D2 bDS LC (SEQ ID NO: 85):

QFVLTPQNSVSTNLGSTVKLSCKRSTGNIGSNYVNWYQQHEGRSPTTMIYRDDKRPD
GVPDRFSGSIDRSSNSALLTINNVTQTEADYFCQSYSSGIVFGGGTKLTVLSQPKAA
PSVTLFPPSSEELQANKATLVCLVSDFYPGA VTVAWKADGSPVKVGVETTKPSKQSN
NKYAACSYSLSLTPEQWKSHRSYSCRVTHEGSTVEKTVAPAESS

EXAMPLE 45 – IN VITRO PROTEIN EXPRESSION –CD8 TARGETED NANOBODY MRNA TITRATION

[0774] This example describes targeting human T cells with anti-CD3, anti-CD8 Fab or anti-CD8 Nanobodies post-inserted into GFP mRNA DiI labeled LNPs and their effect on transfection and protein expression.

[0775] LNPs were prepared using the microfluidic mixing process described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from methods described in Example 4 while generation of Nb-conjugated differed in using 1:1:4 Nb:DSPE-3.4KPEG-maleimide:DSPE-2KPEG-OCH3 and a 50 kD UF membrane (Millipore Corp, Billerica, MA USA) for separation of Nb-conjugate from free Nb. Using methods similar to Example 12 conjugated Fabs and conjugated Nb (derived from alpaca immunization) were post-inserted into LNPs containing Lipid 8 and GFP mRNA with DiI dye. Transfections were performed with human CD3 T cells at approximately 2.5, 0.5 and 0.1 µg/mL mRNA for approximately 24 hr. Levels of

transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK3) to distinguish the two cell types.

[0776] The CD8 targeted Nb conjugate exhibited %transfection (FIG. 78A) and GFP expression levels (FIG. 78B) greater than the mutOKT8 negative control down to 0.1 ug/mL mRNA.

[0777] This data indicates that Nanobodies conjugated with 3.4K PEG-lipid can mediate highly potent T cell transfection with low levels of mRNA concentration in solution.

EXAMPLE 46 – IN VITRO PROTEIN EXPRESSION – CD28 TARGETED FAB CLONES

[0778] This example describes targeting human T cells with anti-CD28, anti-CD8, anti-CD4, anti-CD3 Fabs post-inserted into GFP mRNA LNPs (doped with DiI dye) and their effect on transfection and IFN γ secretion.

[0779] LNPs were prepared using the microfluidic mixing process described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from similar methods described in Example 4. Using methods similar to Example 12, anti-CD28 8G8A., anti-CD28 2E12, anti-CD28 CD28.9.3, anti-CD28 HzTN228, anti-CD28 TGN2122.C/H.

[0780] PEG-lipid conjugated Fabs were post-inserted into LNPs containing Lipid 8 and GFP mRNA and doped with DiI dye. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK3) to distinguish the two cell types. IFN γ in the supernatants was measured using the manufacturers recommended procedure (R&D Systems, DY285B).

[0781] While most of the CD28 targeting Fabs show CD8 and CD4 T cell GFP transfection (FIG. 79A) and expression levels (FIG. 79B) greater than that of the mutOKT8 post-inserted particles, none of the clones evaluated surpass single T cell subset targeting with anti-CD4 hBF5, anti-CD8 TRX2 or targeting both subsets with anti-CD3 SP34. Other than SP34, none of the clones evaluated elicited substantial IFN γ secretion over mutOKT8 LNPs (FIG. 79C).

[0782] This data indicates that despite being able to transfect both CD4 and CD8 T cell subsets, there is not an advantage in transfection/translation efficiency by targeting CD28 versus targeting CD4, CD8 or CD3 for the clones evaluated.

Anti-CD28 8G8A Fab sequence

8G8A bDS HC (SEQ ID NO: 86):

EVQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGSINPYND
YTKYNEKFKGKATLTSKSSITAYMEFSLTSEDSALYCARWGDGNYWGRGTLTVSS
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTCPAVL
QSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTCGGHHH
HHH

8G8A bDS LC (SEQ ID NO: 87):

DIEMTQSPAISASLGERVTMTCTASSSVSSSYFHWYQKPGSSPKLCIYSTSNLASGV
PPRFSGSGSTSYSLTISMEAEDAATYFCHQYHRSPFTGGGKLETKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLC
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD28 2E12 Fab sequence

2E12 bDS HC (SEQ ID NO: 88):

QVQLKESGPGLVAPSQSLITCTVSGFSLTGYGVNWVRQPPGKGLEWLGMIWGDGS
TDYNSALKSRLSITKDNSKSQVFLKMNSLQDDTARYYCARDGYSNFHYVMDYW
GQGTSVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTCPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDK
THTCGGHHHHHHH

2E12 bDS LC (SEQ ID NO: 89):

DIVLTQSPASLAVSLGQRATISCRASESVEYYVTSLMQWYQQKPGQPPKLLISAASNV
ESGVPARFSGSGGTDFSLNIHPVEEDDIAMYFCQQSRKVPWTFGGGKLEIKRRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS
KDYSLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD28 CD28.9.3 Fab sequence

CD28.9.3 bDS HC (SEQ ID NO: 90):

QVKLQQSGPGLVTPSQSLITCTVSGFSLSDYGVHWVRQSPGQGLEWLGVIWAGGG
TNYNSALMSRKSISKDNSKSQVFLKMNSLQADDTAVYYCARDKGYSYYYSMYD
GQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTCPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSD
KTHTCGGHHHHHHH

CD28.9.3 bDS LC (SEQ ID NO: 91):

DIVLTQSPAS LAVSLGQRAT ISCRASESVEYYVTSLMQWY QQKPGQPPKL
LIFAASNVES GVPARFSGSG SGTNFSLNHPVDEDDVAMY FCQQSRKVPY
TFGGGKLEIKRRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNA
LQSGNSQESVTEQDSKDYSLCSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFN
RGES

Anti-CD28 HzTN228 Fab sequence

HzTN228 bDS HC (SEQ ID NO: 92):

QVQLQESGPGLVKPSSETLSLTCAVSGFSLTSYGVHWIRQPGKGLEWLGVIWPGTNFN
SALMSRLTISEDTSKNQVSLKLSSVTAADTAVYCARDRAYGNYLYAMDYWGQGT
LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
CPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC
GGHHHHHH

HzTN228 bDS LC (SEQ ID NO: 93):

DIQMTQSPSLASVGDRVITTCRASESVEYVTSMLQWYQKPGKAPKLLIYAASNVD
S
GVPSRFGSGGTDFLTISLQPEDATYCSRKVPFTFGGGTKVEIKRTVAAPSVFIFPP
S
DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSLCS
TLTSLKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD28 TGN2122.C Fab sequence

TGN2122.C bDS HC (SEQ ID NO: 94):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTDYKIHVVRQAPGQGLEWIGYIYPYSG
SSDYNQKFKSRATLTVDNSISTAYMELSRLSDDTAVYYCARGGDAMDYWGQGT
LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
CPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC
GGHHHHHH

TGN2122.C bDS LC (SEQ ID NO: 95):

DIQMTQSPSSLSASVGDRVITTCGASENIYGALNWWYQRKPGKAPKLLIYGATNLADG
VPSRFGSGSGRDTLTISLQPEDFATYFCQNILGTWTFGGGTKEIKRTVAAPSVFI
FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSY
SLCSTLTSLKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

Anti-CD28 TGN2122.H Fab sequence

TGN2122.H bDS HC (SEQ ID NO: 96):

EVQLVESGGGLVQPGGSLRLSCAASGFTFNIYYMSWVRQAPGKGLELVAAINPDGG
NTYYPDTVKGRFTISRDAKNSLYLQMNSLRAEDTAVYYCARYGGPGFDSWGQGT
LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TCPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSSDKTHTC
GGHHHHHH

TGN2122.H bDS LC (SEQ ID NO: 97):

ENVLTQSPATLSLSPGERATLSCASSSVSYMHWYQQKPGQAPRLWIYDTSKLASGIP
ARFSGSGSRNDYLTISLLEPEDFAVYYCFPGSGFPFMYTFGGGTKEIKRTVAAPSV
FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
T
YSLCSTLTSLKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

EXAMPLE 47 – IN VITRO PROTEIN EXPRESSION – CD3, CD4, CD7, CD8, CD11a, CD18, CD28, AND TCR TARGETED FABS AND NANOBODIES

[0783] This example describes targeting human T cells with anti-CD3, anti-CD4, anti-CD7, anti-CD8, anti-CD11a, anti-CD18, anti-CD28 and anti-TCR Fabs and Nbs post-inserted into GFP mRNA Dil labeled LNPs and their effect on transfection and IFN γ secretion.

[0784] LNPs were prepared using the microfluidic mixing process described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiIc18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from methods described in Example 4 while generation of Nb-conjugated differed in using 1:1:4 Nb:DSPE-3.4KPEG-maleimide:DSPE-2KPEG-OCH₃ and a 50 kD UF membrane (Millipore Corp, Billerica, MA USA) for separation of Nb-conjugate from free Nb. Using methods similar to Example 12 conjugated Fabs and conjugated Nbs were post-inserted into LNPs containing Lipid 8 and GFP mRNA with DiI dye. Transfections were performed with human CD3 T cells at approximately 2.5 µg/mL, 0.5, µg/mL and 0.1 µg/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK3) distinguish the two cell types.

[0785] All of the clones evaluated mediated some level of transfection (FIG. 80A, 80B) and GFP expression levels (FIG. 80C, 80D) relative to the mutOKT8 LNP control. For targeting both CD8 and CD4 T cell subset simultaneously anti-CD3 and anti-CD7 stand out as having the highest transfection/translation between both cell subsets at both the highest and second highest mRNA dose. The anti-TCR clone has high transfection/translation efficiency at the highest dose however falls off at the 2nd highest dose. For specific T cell subset targeting, targeting anti-CD8 or anti-CD4 provides the highest specificity for their corresponding subsets regardless of the use of a Fab or Nb. Targeting either CD3 or TCR elicited T cell activation and secretion of IFN γ while the other targeting clones did not elicit levels substantially over the mutOKT8 LNP (FIG. 80E).

[0786] This data indicates that targeting CD3 or CD7 with a Fab or Nb is preferred to enable high transfection of both CD4 and CD8 T cell subsets. For targeting CD4 or CD8 T cell subsets individually, use of subset specific anti-CD4 or anti-CD8 Fab or Nb is preferred to enable high transfection of its corresponding T cell subset. Targeting CD3 or TCR can elicit IFN γ secretion while targeting CD4, CD7, CD8, CD11a, anti-CD18 or anti-CD28 can avoid IFN γ secretion.

EXAMPLE 48 – IN VITRO PROTEIN EXPRESSION –CD7 AND CD8 CO-TARGETED LNPS

[0787] This example describes targeting human T cells with anti-CD7 anti-CD8 Nbs post-inserted alone or together into GFP mRNA DiI labeled LNPs and their effect on transfection and IFN γ secretion.

[0788] LNPs were prepared using the microfluidic mixing process described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiI C18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from methods described in Example 4 while generation of Nb-conjugated differed in using 1:1:4 Nb:DSPE-5KPEG-maleimide:DSPE-2KPEG-OCH₃ and a 50 kD UF membrane (Millipore Corp, Billerica, MA USA) for separation of Nb-conjugate from free Nb. Using methods similar to Example 12 conjugated Fabs and conjugated Nb were post-inserted into LNPs containing Lipid 8 and GFP mRNA with DiI dye. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK3) to distinguish the two cell types. IFN γ in the supernatants was measured using the manufacturers recommended procedure (R&D Systems, DY285B).

[0789] For both anti-CD8 Nb clones (V3 and V4) combined with the anti-CD7 V1 Nb the %transfection (**FIG. 81A**) and GFP expression levels (**FIG. 81B**) in CD8 T cell subset were higher than targeting CD8 alone or CD7 alone with Nb or CD8 targeting with the TRX2 NoDS Fab. The CD7/CD8 targeting combination is approaching similar levels of GFP expression to the anti-CD3 SP34 NoDS Fab for CD8 T cells while maintaining similar if not lower levels of Transfection in CD4 T cells. Despite CD8/CD7 co-targeting achieving similar levels of transfection/translation to that of anti-CD3 Fab in the CD8 T cell population, there was not substantial amounts of IFN γ secreted by the T cells relative to the non-specific mutOKT8 control LNPs (**FIG. 81C**).

[0790] This data indicates that co-targeting CD7 and CD8 can mediate highly efficient transfection in the CD8 T cell population while avoiding substantial amounts of IFN γ secretion.

EXAMPLE 49 -- IN VITRO PROTEIN EXPRESSION --CD7 AND CD8 BISPECIFIC TARGETED LNPs AND CD8 TARGETED SCFV

[0791] This example describes targeting human T cells with anti-CD8 TRX2 Fab NoDS or anti-CD8 TRX2 ScFv, anti-CD7 or anti-CD8 Nbs post-inserted alone or together and bispecific designs described in FIG. 47 including anti-CD7/anti-CD8 2xVHH (V1/V2), anti-CD8/anti-CD7 2xVHH (V2/V1) or anti-CD7/anti-CD8 VHH-CH1/VHH-Vk bDS post-inserted into GFP mRNA DiI labeled LNPs and their effect on transfection/translation and IFN γ secretion.

[0792] LNPs were prepared using the microfluidic mixing process described in Example 6 and discontinuous diafiltration method described in Example 25. The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US), Lipid 8 as the ionizable lipid, and labeled with 0.01 mol% DiIC18(5)-DS (Invitrogen, Massachusetts, US). Fab-lipid conjugates generated from methods described in Example 4 while generation of ScFv or Nb conjugation differed in using 1:1:4 Nb:DSPE-3.4KPEG-maleimide:DSPE-2KPEG-OCH₃ and a 50 kD UF membrane (Millipore Corp, Billerica, MA USA) for separation of ScFv or Nb-conjugate from free protein. Using methods similar to Example 12 conjugated Fabs, conjugated ScFv and conjugated Nb were post-inserted into LNPs containing Lipid 8 and GFP mRNA with DiI dye. Transfections were performed with human CD3 T cells at approximately 2.5 μ g/mL mRNA for approximately 24 hr. Levels of transfection of both CD8 and CD4 cells was measured by flow cytometry using an anti-CD4 antibody (SK3) to distinguish the two cell types. IFN γ in the supernatants was measured using the manufacturers recommended procedure (R&D Systems, DY285B).

[0793] The TRX2 ScFv mediated slightly lower %transfection (FIG. 82A) and GFP expression levels (FIG. 82B) in the CD8 T cell subset relative to the anti-CD8 TRX2 NoDS Fab however the signal was greater than the non-targeted mutOKT8 Fab LNP. The LNPs co-targeting CD8 and CD7 with either anti-CD8 and anti-CD7 Nb post-inserted together or post-inserted bispecifics including anti-CD7/anti-CD8 2xVHH, anti-CD8/anti-CD7 2xVHH and anti-CD7/anti-CD8 VHH-CH1/VHH-Vk bDS all exhibited high levels of CD8 T cell %transfection and higher levels of GFP expression than the anti-CD3 SP34-hlam NoDS Fab indicating a synergistic effect of combining CD8 and CD7 targeting with Nbs similar to the observation in EXAMPLE 17 co-targeting CD8 (clone TRX2) and CD7 (clone TH-69) with Fabs. Despite CD7/CD8 co-targeting achieving similar or better transfection/translation to that of anti-CD3 SP34 NoDS Fab in the CD8 T cell subset there was not substantial amounts

of IFN γ secreted by the T cells relative to the non-specific mutOKT8 control LNPs and in contrast to SP34 (FIG. 82C).

[0794] This data indicates an ScFv alone is capable of mediating similar transfection efficiency to that of Fab. Additionally, it indicates that a synergistic effect on transfection/translation can be achieved when targeting both CD7 and CD8 on the same LNP whether the targeting moieties are post inserted together as individual proteins or post inserted as dual-targeting bi-specifics.

Anti-CD8 TRX2 ScFv sequence (SEQ ID NO: 98):

QVQLVESGGGVVQPGRSLRLSCAASGFTFSDFGMNWVRQAPGKGLEWVALIYYDG
SNKFYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAKPHYDGYHHFDS
WGQGTLLTVSSGGGGSGGGSGGGSGGGSGGGSDIQMTQSPSSLSASVGDRTITCKG
SQDINNYLAWYQQKPKGKAPKLLIYNTDILHTGVPSRFSGSGSGTDFTFTISLQPEDIA
TYYCYQYNNGYTFGQGTKVEIKGGGSGGCGHHHHHH

V1 VHH-CH1 bDS HC (SEQ ID NO: 99):

DVQLQESGGGLVQAGGSLRLSCAASGYPYSSYCMGWFRQAPGKEREGVAAIDSDG
RTRYADSVKGRFTISQDNAKNTLYLQMNRMPEDTAMYYCAARFGPMGCVDLSTL
SFGHWGQGTQVTVSITASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW
NSGALTSGVHTCPAVLQSSGLYSLSVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSSDKTHTCGHHHHHH

EXAMPLE 50 – Lipid 2, Lipid 6, Lipid 12 and Lipid 13 LNP properties and in vitro GFP protein expression in primary human T-cells

[0795] This example compares the properties of LNPs prepared using Lipid 2, Lipid 6, Lipid 12 and Lipid 13 and in vitro GFP protein expression in primary human T-cells. LNP formulations were prepared using the microfluidic mixing process (described in Example 6) and using a discontinuous diafiltration process for ethanol removal (described in Example 25). The LNPs were formulated using eGFP encoding mRNA (TriLink Biotechnologies, California, US) and labeled with 0.01 mol% DiI C18(5)-DS (Invitrogen, Massachusetts, US) using the lipid ratios shown in the Formulation Table 44 below. The LNPs were then inserted with a targeting conjugate using the specified conditions to provide the final targeted LNP formulations. The LNPs were characterized as described in Example 3.

Table 44. LNP Formulation composition and antibody insertion conditions

Ionizable Lipid	Formulation No.	Lipid-PEG	Lipid-PEG Content	Targeting Conjugate / Insertion density (g/mol)	Antibody conjugate insertion condition

			(mol%)		
Lipid 2	EXP21003810-N1M3	DPG-PEG	1.5	hSP34 / 9	37 °C for 4 h in pH 6.5 MBS
Lipid 6	EXP21003810-N2M3	DPG-PEG	1.5	hSP34 / 9	37 °C for 4 h in pH 6.5 MBS
Lipid 12	EXP21003810-N3M3	DPG-PEG	1.5	hSP34 / 9	37 °C for 4 h in pH 6.5 MBS
Lipid 13	EXP21003810-N4M3	DPG-PEG	1.5	hSP34 / 9	37 °C for 4 h in pH 6.5 MBS

Table 45. LNP size, charge (Dynamic Light Scattering) and mRNA encapsulation (Ribogreen assay)

Ionizable Lipid	Formulation No.	Pre-Insertion DLS Z-Avg. Diameter (nm) / PDI	Pre-Insertion Zeta Potential at pH 5.5 (mV) / pH 7.4 (mV)	Pre-Insertion Dye-Accessible mRNA (%)	Pre-insertion total mRNA content (ug/mL)
Lipid 2	EXP21003810-N1H	62.3 / 0.08	26.2 / 11.4	9.1	59.4
Lipid 6	EXP21003810-N2H	82.0 / 0.07	25.5 / 2.27	9.2	54.6
Lipid 12	EXP21003810-N3H	75.9 / 0.02	12.9 / -1.06	10.4	79.2
Lipid 13	EXP21003810-N4H	76.3 / 0.09	12.2 / -5.20	16.5	77.4

[0796] Lipid 2, Lipid 6, Lipid 12 and Lipid 13 were formulated using 1.5 mole % DPG-PEG, as seen in Table 44 and Table 45, all LNPs display sub-100 nm hydrodynamic diameter (DLS) in pH 7.4 HEPES buffer. Buffer exchange into pH 6.5 MES and antibody insertion resulted in size and polydispersity increase in all four lipid compositions. However, Lipid 2 and Lipid 6 LNPs showed significantly greater size distribution changes compared to Lipid 12 and Lipid 13 LNPs (FIG 83A and FIG. 83B). As seen in FIG 83C, under both physiological and acidic pH conditions (pH 7.4 and pH 5.5), Lipid 2 and Lipid 6 showed a greater positive surface charge relative to Lipid 12 and 13 indicating a significant shift in the LNP apparent pK_a (in Lipid 12 and 13 LNPs) to lower values resulting from the mono- and di-hydroxyethyl substitution of the ionizable amine head groups, respectively. Additionally, in all four LNP compositions, low levels of dye accessible mRNA (<20%) and good RNA encapsulation efficiencies (> 80% mRNA in parent LNP samples) were observed (Table 45 and FIG. 83D). The resulting targeted LNPs were evaluated in primary human T-cells using the in vitro transfection protocol described in example 8. As seen in FIG. 84E, all formulations were well tolerated by T-cells at all LNP doses tested (T-cell viability remained

similar to the PBS control). As illustrated by the DiI⁺ and DiI MFI values (FIGS. 84C and 84D), all formulations show similar levels of cell association at most dose levels tested suggesting that the conjugate insertion process is not dependent on the ionizable lipid chemistry. Lipids 2 and Lipid 6 LNPs exhibited dose dependent expression of GFP protein (FIGS. 84A and 84B). However, at all doses tested Lipid 12 and Lipid 13 LNPs performed poorly potentially due to non-optimal LNP surface charge properties and diminished cytosolic access in T-cells. Additionally, Lipid 2 and Lipid 6 LNPs retained function after being subjected to freeze-thaw stress as illustrated in FIG. 85. Both compositions showed minor changes in particle size distributions after frozen storage at -80C relative to particles stored at 4C as seen in FIG. 83A and FIG. 83B. Furthermore, both compositions retained the ability to bind and transfect primary human T-cells post freeze-thaw with similar levels of %DiI⁺ and DiI MFI values as well as similar levels protein expression (% GFP⁺ cells and GFP MFI values) observed after refrigerated (4C) and frozen (-80C) storage conditions.

EXAMPLE 51 – DiI T Cell Transfection Experiments:

[0797] CD3⁺ T cells were isolated from frozen peripheral blood mononuclear cells using an EasySep Human T Cell Isolation Kit on a RoboSep automated cell isolation system from STEMCELL. T cells were plated into a round bottom 96-well plate in RPMI cell culture media supplemented with glutamax, 10% fetal bovine serum, pen-strep, and 40 ng/mL IL-2. 100 μ L of cell suspension was seeded per well at a density of 1M T cells/mL (100K T cells/well). Cells were allowed to rest for two hours in a 37°C incubator, and then were transfected by gently adding 10 μ L of a 22 μ g/mL (by mRNA) nanoparticle suspension, resulting in a final mRNA concentration of 2 μ g/mL (unless otherwise noted). Cells were gently mixed with a pipette and then incubated for 24 hours in a 37 °C incubator. After incubation the cells were diluted with FACS buffer (BD 554657) and analyzed using a BD Fortessa flow cytometer. Data were analyzed using FlowJo software from BD biosciences.

EXAMPLE 52 – CD4 and CD69 Staining

[0798] After 24 hours, cells were transferred to a 96-well conical bottom polypropylene plate and centrifuged at $350 \times g$ for 5 minutes. Supernatants were removed and transferred to a fresh conical bottom polypropylene plate for further analysis. Cells were washed by adding 200 μ L FACS buffer (BD 554657), centrifuging at $350 \times g$ for 5 min, and then aspirating the supernatant from each well. BV421 anti-human CD69 (BioLegend 310930 clone FN50) and BV711 anti-human CD4 (BioLegend 344648 clone SK3) antibodies were diluted 100 $\times$ by adding 100 μ L of each antibody to 10 mL FACS buffer. 100 μ L of the diluted antibody solution was added to each well and the plate was incubated at room temperature for 20 minutes. The plates were then washed by centrifuging at $350 \times g$ for 5 min, removing the supernatant, re-suspending in 200 μ L FACS buffer, centrifuging at $350 \times g$ for 5 min and aspirating the supernatant from each well. Following the wash, cells were resuspended in 100 μ L of 1.6% formaldehyde and stored at 4°C until FACS analysis. FACS analysis was performed using a BD Fortessa equipped with a High Throughput Sampler.

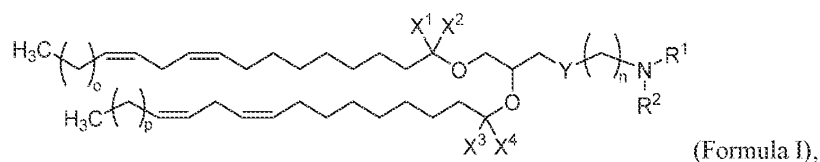
EXAMPLE 53 – Human IFN- γ ELISA:

[0799] IFN- γ was assayed using an R&D Duoset IL-2 ELISA kit, PN DY285B. Briefly: an Immulon 2HB 96-well plate (Thermo X1506319) was coated by adding 100 μ L of a 2 μ g/mL solution of the R&D IL-2 capture antibody to each well and then incubating the plate overnight at 4 °C. The plate was washed three times with wash buffer (0.05 TWEEN-20 in pH 7.4 TRIS buffered saline, Thermo 28360), blocked with reagent diluent (0.1% BSA in wash buffer) for one hour at room temperature, and then washed an additional three times with wash buffer. Supernatants were diluted three-fold in reagent diluent and then 100 μ L of diluted supernatant was added to each well. IFN- γ standards were prepared on the same plate by serial dilution. Plates were incubated for two hours at room temperature, washed three times with wash buffer. 100 μ L of detection antibody diluted in reagent diluent was added, incubated for 2 hours at room temperature, and then the plate was washed three times with wash buffer. 100 μ L of Streptavidin-HRP was added, incubated for 20 minutes at room temperature, and then the plate was washed three times with wash buffer. 100 μ L of substrate solution (Thermo N301) was added, incubated for 20 minutes at room temperature and then the reaction was quenched by adding 100 μ L of stop solution (Invitrogen SS04). Optical density at 450 nm was read on a SpectraMax M5 plate reader. IFN- γ concentration was

quantified relative to a standard curve based on contemporaneously analyzed IFN- γ standards.

ENUMERATED EMBODIMENTS

1. A compound represented by Formula I:



or a salt thereof, wherein:

R¹ and R² are independently C₁₋₃alkyl, or R¹ and R² are taken together with the nitrogen atom to form an optionally substituted piperidiny1 or morpholinyl;

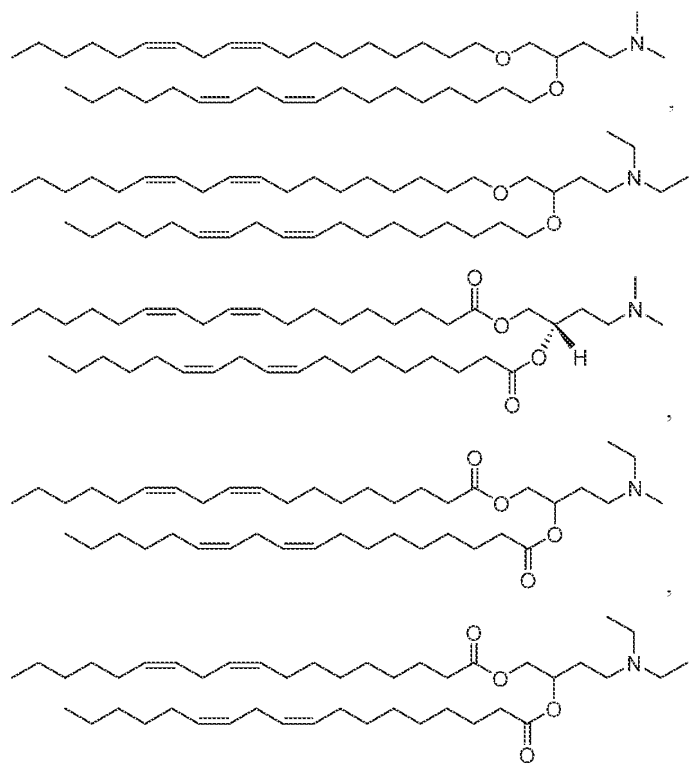
Y is selected from the group consisting of -O-, -OC(O)-, -OC(S)-, and -CH₂-;

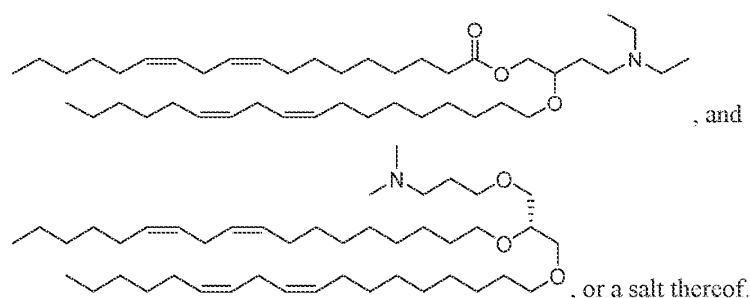
X¹, X², X³, and X⁴ are hydrogen or X¹ and X² or X³ and X⁴ are taken together to form an oxo;

n is 0 or 3;

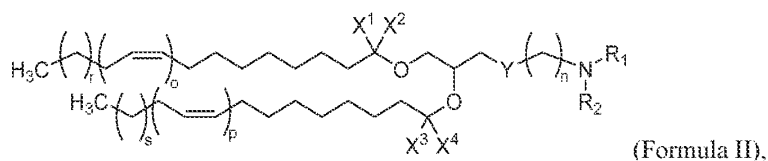
o and p are independently an integer selected from 2-6;

wherein the compound is not a compound selected from the group consisting of





2. The compound of embodiment 1, wherein o and p are 2.
3. The compound of embodiment 1, wherein o and p are 4.
4. The compound of embodiment 1, wherein o and p are 6.
5. The compound of any one of embodiments 1-4, wherein X1 and X2 are taken together to form an oxo and X3 and X4 are taken together to form an oxo.
6. The compound of any one of embodiments 1-4, wherein X1, X2, X3, and X4 are hydrogen.
7. The compound of any one of embodiments 1-6, wherein Y is selected from the group consisting of -O-, -OC(O)-, and -CH2-.
8. The compound of embodiment 7, wherein Y is -O-.
9. The compound of embodiment 7, wherein Y is -OC(O)-.
10. The compound of embodiment 7, wherein Y is -CH2-.
11. The compound of any one of embodiments 1-10, wherein R1 and R2 are independently C1-3alkyl.
12. The compound of embodiment 11, wherein R1 and R2 are -CH3.
13. The compound of embodiment 11, wherein R1 and R2 are -CH2CH3.
14. The compound of any one of embodiments 1-13, wherein n is 0.
15. The compound of any one of embodiments 1-13, wherein n is 3.
16. A compound represented by Formula II:



or a salt thereof, wherein:

R^1 and R^2 are independently C_{1-3} alkyl, or R^1 and R^2 are taken together with the nitrogen atom to form an optionally substituted piperidinyl or morpholinyl;

Y is selected from the group consisting of -O-, -OC(O)-, -OC(S)-, and -CH₂-;

X^1 , X^2 , X^3 , and X^4 are hydrogen or X^1 and X^2 or X^3 and X^4 are taken together to form an oxo;

n is 0-4;

o is 1 and r is an integer selected from 3-8 or o is 2 and r is an integer selected from 1-8,

p is 1 and s is an integer selected from 3-8 or p is 2 and s is an integer selected from 1-8,

wherein,

when o and p are both 1, r and s are independently 4, 5, 7, or 8, and

when o and p are both 2, r and s are independently 1, 2, 4, or 5.

17. The compound of embodiment 16, wherein X^1 and X^2 are taken together to form an oxo and X^3 and X^4 are taken together to form an oxo.

18. The compound of embodiment 16 or 17, wherein X^1 , X^2 , X^3 , and X^4 are hydrogen.

19. The compound of any one of embodiments 16-18, wherein Y is selected from the group consisting of -O-, -OC(O)-, and -CH₂-.

20. The compound of embodiment 19, wherein Y is -O-.

21. The compound of embodiment 19, wherein Y is -OC(O)-.

22. The compound of embodiment 19, wherein Y is -CH₂-.

23. The compound of any one of embodiments 16-22, wherein R^1 and R^2 are independently C_{1-3} alkyl.

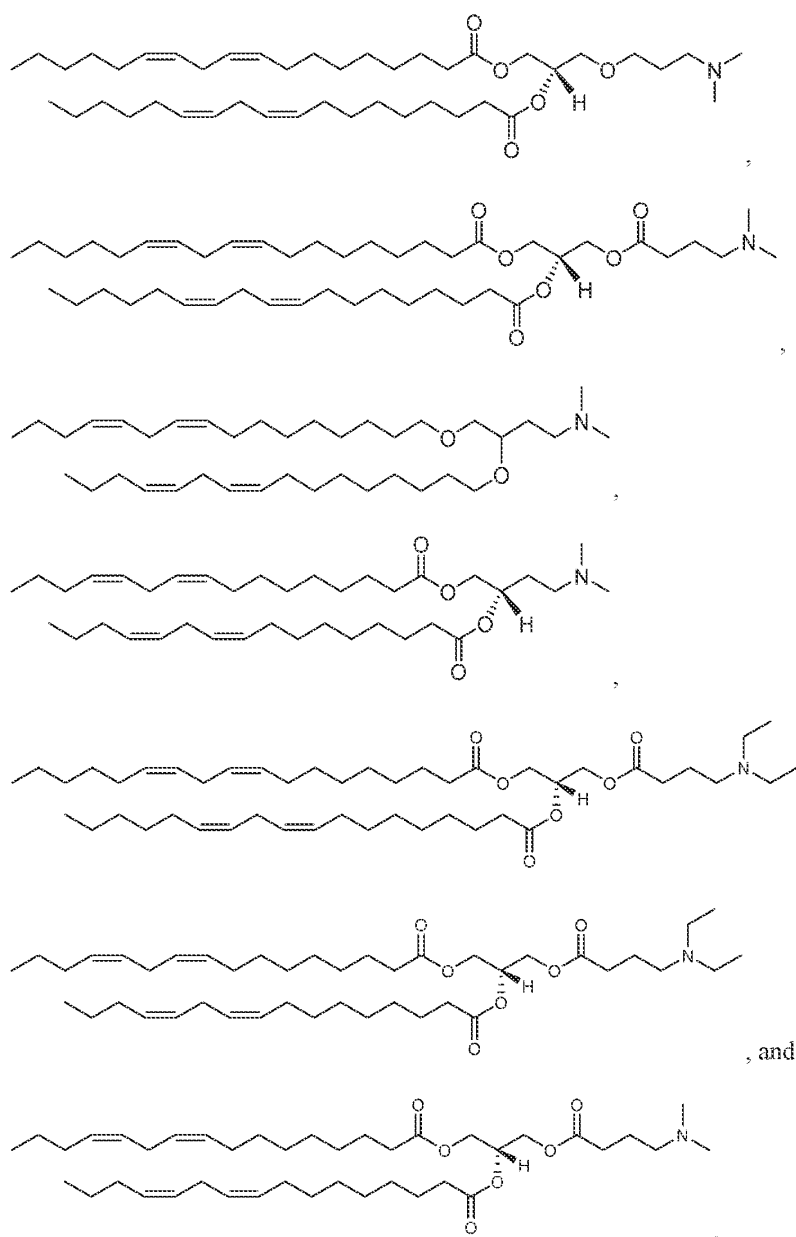
24. The compound of embodiment 23, wherein R^1 and R^2 are -CH₃.

25. The compound of embodiment 23, wherein R^1 and R^2 are -CH₂CH₃.

26. The compound of any one of embodiments 16-25, wherein n is 0.

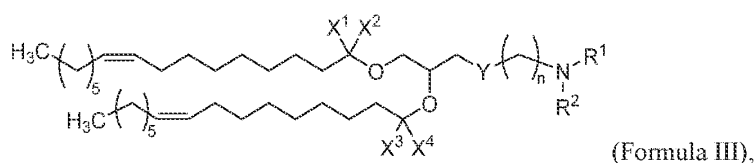
27. The compound of any one of embodiments 16-25, wherein n is 3.

28. A compound selected from the group consisting of:



or a salt thereof.

29. The compound of embodiment 16, wherein the compound is a compound of Formula III:



or a salt thereof, wherein:

R^1 and R^2 are independently C_{1-3} alkyl, or R^1 and R^2 are taken together with the nitrogen atom to form an optionally substituted piperidinyl;

Y is selected from the group consisting of -O-, -OC(O)-, -OC(S)-, and -CH₂-;

X^1 , X^2 , X^3 , and X^4 are hydrogen or X^1 and X^2 or X^3 and X^4 are taken together to form an oxo;
and

n is an integer selected from 0-4.

30. The compound of embodiment 29, wherein R^1 and R^2 are independently C_{1-3} alkyl.

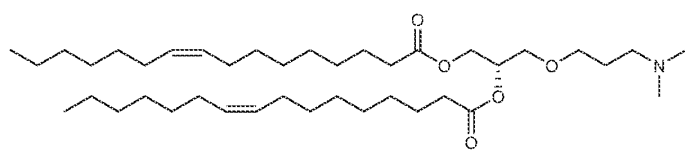
31. The compound of embodiments 29 or 30, wherein R^1 and R^2 are -CH₃.

32. The compound of any one of embodiments 29-31, wherein Y is -O-.

33. The compound of any one of embodiments 29-32, wherein X^1 and X^2 are taken together to form an oxo and X^3 and X^4 are taken together to form an oxo.

34. The compound of any one of embodiments 29-33, wherein n is 3.

35. A compound of formula:



or a salt thereof.

36. A lipid nanoparticle (LNP) comprising a lipid blend comprising the compound of any one of embodiments 1-35 or a lipid of Table 1.

37. The LNP of embodiment 36, wherein the lipid blend further comprises one or more of a sterol, a neutral phospholipid, a PEG-lipid, and a lipid-immune cell targeting group conjugate.

38. The LNP of embodiment 35 or 36, wherein the compound is present in the lipid blend in a range of 30-70-60 mole percent.

39. The LNP of any one of embodiments 36-38, wherein the sterol (e.g., cholesterol) is present in the lipid blend in a range of 20-70 mole percent.
40. The LNP of any one of embodiments 36-39, wherein the neutral phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).
41. The LNP of any one of embodiments 36-40, wherein the neutral phospholipid is present in the lipid blend in a range of 1-10 mole percent.
42. The LNP of any one of embodiments 36-41, wherein the PEG-lipid is selected from the group consisting of PEG-distearoylglycerol (PEG-DSG), PEG-dipalmitoylglycerol (PEG-DAG, e.g., PEG-DMG, PEG-DPG, and PEG-DSG), PEG-dimyristoyl-glycerol (PEG-DMG), PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE), PEG-dipalmitoyl-phosphatidylethanolamine (PEG-DPPE) and PEG-dimyristoyl-phosphatidylethanolamine (PEG-DMPE).
43. The LNP of any one of embodiments 36-42, wherein the PEG-lipid is present in the lipid blend in a range of 1-10 mole percent.
44. The LNP of any one of embodiments 36-43, wherein the lipid-immune cell targeting group conjugate is present in the lipid blend in a range of 0.1-0.3 mole percent or 0.002-0.2 mole percent.
45. The LNP of embodiment 44, wherein the targeting group is a T cell targeting group.
46. The LNP of embodiment 45, wherein the T cell targeting group is an antibody or antigen binding fragment thereof that binds a T cell antigen.
47. The LNP of embodiment 46, wherein the T cell antigen is selected from the group consisting of CD2, CD3, CD4, CD5, CD7, CD8, CD28, CD137, and T-cell receptor (TCR) β (e.g., CD3 or CD8).
48. The LNP of any one of embodiments 36-47, wherein the T cell-targeting group is covalently coupled to a lipid via a polyethylene glycol (PEG) containing linker.
49. The LNP of embodiment 48, wherein the lipid is distearoyl-phosphatidylethanolamine (DSPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-

phosphatidylethanolamine (DMPE), distearoyl-glycero-phosphoglycerol (DSPG), distearoyl-glycerol (DSG), dimyristoyl-glycerol (DMG), or ceramide.

50. The LNP of embodiment 48 or 49, wherein the PEG is selected from the group consisting of PEG 2000, PEG 1000, PEG 3000, PEG 3450, PEG 4000, or PEG 5000.

51. The LNP of any one of embodiments 36-50, wherein the lipid blend further comprises free PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE), PEG-dimyristoyl-phosphatidylethanolamine (PEG-DMPE), N-(Methylpolyoxyethylene oxycarbonyl)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE-PEG) 1,2-Dimyristoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DMG), 1,2-Dipalmitoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DPG), 1,2-Dioleoyl-rac-glycerol, methoxypolyethylene Glycol (DOG-PEG) 1,2-Distearoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DSG), N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)]} (PEG-ceramide), and DSPE-PEG-cysteine, or a derivative thereof.

52. The LNP of embodiment 51, wherein the derivative of the PEG-lipid has a hydroxyl or a carboxylic acid end group at the PEG terminus.

53. The LNP of any one of embodiments 36-52, wherein the LNP has a mean diameter in the range of 50-200 nm.

54. The LNP of embodiment 53, wherein the LNP has a mean diameter of about 100 nm.

55. The LNP of any one of embodiments 36-54, wherein the LNP has a polydispersity index in a range from 0.05 to 1.

56. The LNP of any one of embodiments 36-55, wherein the LNP has a zeta potential of from about -10 mV to about + 30 mV at pH 5.

57. The LNP of any one of embodiments 36-55, wherein the LNP has a zeta potential of from about -30 mV to about + 5 mV at pH 7.4.

58. The LNP of any one of embodiments 36-57, further comprising a nucleic acid disposed therein.

59. The LNP of embodiment 58, wherein the nucleic acid is DNA or RNA.

60. A lipid nanoparticle (LNP) comprising a lipid blend comprising a lipid-T-cell-targeting group conjugate and optionally a lipid set forth in Table 1.

61. The LNP of embodiment 60, wherein the T-cell targeting group is an antibody that binds a T cell antigen.
62. The LNP of embodiment 61, wherein the T cell antigen is selected from the group consisting of CD2, CD3, CD4, CD5, CD7, CD8, CD28, CD137, and T-cell receptor (TCR) β .
63. The LNP of embodiment 62, wherein the T cell antigen is CD2, CD3, CD7, or CD8.
64. The LNP of any one of embodiments 60-63, wherein the T-cell-targeting group is covalently coupled to the lipid via a polyethylene glycol (PEG) containing linker.
65. The LNP of embodiment 64, wherein the lipid is distearoyl-phosphatidylethanolamine (DSPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-glycero-phosphoglycerol (DSPG), dimyristoyl-glycerol (DMG), dipalmitoyl-phosphatidylethanolamine (DPPE), dipalmitoyl-glycerol (DPG), or ceramide.
66. The LNP of embodiment 64 or 65, wherein the PEG is PEG 2000, PEG 1000, PEG 3000, PEG 3450, PEG 4000, or PEG 5000.
67. The LNP of any one of embodiments 60-66, wherein the lipid-T-cell targeting group conjugate is present in the lipid blend in a range of 0.002-0.2 mole percent.
68. The LNP of any one of embodiments 60-67, wherein the lipid blend further comprises one or more of a cationic lipid, sterol, a neutral phospholipid, and a PEG-lipid.
69. The LNP of embodiment 68, wherein the ionizable cationic lipid is a compound of any one of embodiments 1-35, or a lipid set forth in Table 1.
70. The LNP of embodiment 68 or 69, wherein the ionizable cationic lipid is present in the lipid blend in a range of 40-60 mole percent.
71. The LNP of any one of embodiments 68-70, wherein the sterol (e.g., cholesterol) is present in the lipid blend in a range of 30-50 mole percent.
72. The LNP of any one of embodiments 68-71, wherein the neutral phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), sphingomyelin.
73. The LNP of any one of embodiments 68-72, wherein the neutral phospholipid is present in the lipid blend in a range of 1-10 mole percent.

74. The LNP of any one of embodiments 68-73, wherein the PEG-lipid is selected from the group consisting of PEG-distearoylglycerol (PEG-DSG), PEG-diacylglycerol (PEG-DAG, e.g., PEG-DMG, PEG-DPG, and PEG-DSG), PEG-dimyristoyl-glycerol (PEG-DMG), PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) and PEG-dimyristoyl-phosphatidylethanolamine (PEG-DMPE), N-(Methylpolyoxyethylene oxycarbonyl)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE-PEG), 1,2-Dipalmitoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DPG), 1,2-Dioleoyl-rac-glycerol, methoxypolyethylene Glycol (DOG-PEG) and N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)]} (PEG-ceramide).
75. The LNP of any one of embodiments 68-74, wherein the PEG-lipid is present in the lipid blend in a range of 2-4 mole percent.
76. The LNP of any one of embodiments 68-75, wherein the lipid blend further comprises free PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) or PEG-dimyristoyl-phosphatidylethanolamine (PEG-DMPE), N-(Methylpolyoxyethylene oxycarbonyl)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE-PEG) 1,2-Dimyristoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DMG), 1,2-Dipalmitoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DPG), 1,2-Dioleoyl-rac-glycerol, methoxypolyethylene Glycol (DOG-PEG) 1,2-Distearoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DSG), N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)]} (PEG-ceramide), and DSPE-PEG-cysteine, or a derivative thereof.
77. The LNP of any one of embodiments 60-75, wherein the LNP has a mean diameter in the range of 50-200 nm.
78. The LNP of embodiment 77, where the LNP has a mean diameter of about 100 nm.
79. The LNP of any one of embodiments 60-78, wherein the LNP has a polydispersity index in a range from 0.05 to 1.
80. The LNP of any one of embodiments 60-79, wherein the LNP has a zeta potential of from about -10 mV to about +30 mV at pH 5.
81. The LNP of any one of embodiments 60-80, further comprising a nucleic acid disposed therein.
82. The LNP of embodiment 81, wherein the nucleic acid is DNA or RNA (e.g., an mRNA, tRNA, or siRNA).

83. The LNP of embodiment 81 or 82, wherein the number of the nucleotides in the nucleic acid is from about 400 to about 6000.

84. A method of delivering a nucleic acid to an immune cell (*e.g.*, a T-cell), the method comprising exposing the immune cell to an LNP of any one of embodiments 36-83 containing a nucleic acid under conditions that permit the nucleic acid to enter the immune cell.

85. A method of delivering a nucleic acid to an immune cell (*e.g.*, a T-cell) in a subject in need thereof, the method comprising administering to the subject a composition comprising the LNP of any one of embodiments 36-83 containing a nucleic acid thereby to deliver the nucleic acid to the immune cell.

86. A method of targeting the delivering of a nucleic acid (*e.g.*, mRNA) to an immune cell (*e.g.*, a T-cell) in a subject, the method comprising administering to the subject an LNP of any one of embodiments 36-83 containing the nucleic acid so as to facilitate targeted delivery of the nucleic acid to the immune cell.

INCORPORATION BY REFERENCE

[0800] Unless defined otherwise, all technical and scientific terms herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials, similar or equivalent to those described herein, can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications, scientific articles, patents, and patent publications cited are incorporated by reference herein in their entirety for all purposes.

[0801] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

EQUIVALENTS

[0802] The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are intended to be embraced therein. While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

CLAIMS

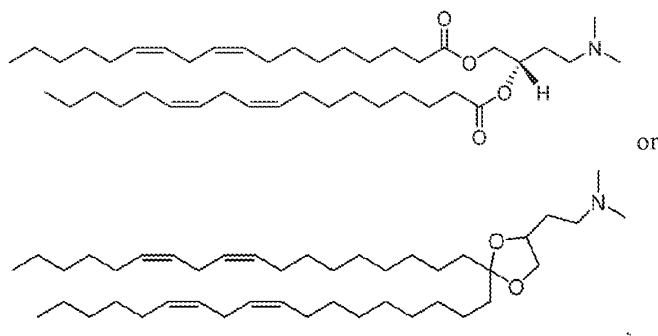
What is claimed is:

1. A lipid nanoparticle (LNP) comprising a lipid blend for targeted delivery of a nucleic acid into an immune cell, the lipid blend comprising:

(a) a lipid-immune cell targeting group conjugate comprising the compound of Formula IV:

[Lipid] – [optional linker] – [immune cell targeting group], and

(b) an ionizable cationic lipid comprising



wherein the LNP further comprises a nucleic acid disposed therein.

2. The LNP of claim 1, wherein the immune cell targeting group comprises an antibody that binds a T cell antigen.

3. The LNP of claim 2, wherein the T cell antigen is CD3, CD4, CD7, CD8, or a combination thereof (e.g., both CD3 and CD8, both CD4 and CD8, or both CD7 and CD8).

4. The LNP of claim 2, wherein the immune cell targeting group comprises an antibody that binds a Natural Killer (NK) cell antigen.

5. The LNP of claim 4, wherein the NK cell antigen is CD7, CD8, CD56, or a combination thereof (e.g., both CD7 and CD8).

6. The LNP of any one of claims 1 to 5, wherein the immune cell targeting group is covalently coupled to a lipid in the lipid blend via a polyethylene glycol (PEG) containing linker.

7. The LNP of claim 6, wherein the lipid covalently coupled to the immune cell targeting group via a PEG containing linker is distearoylglycerol (DSG), distearoyl-phosphatidylethanolamine (DSPE), dimyrstoyl-phosphatidylethanolamine (DMPE),

distearoyl-glycero-phosphoglycerol (DSPG), dimyristoyl-glycerol (DMG), dipalmitoyl-phosphatidylethanolamine (DPPE), dipalmitoyl-glycerol (DPG), or ceramide.

8. The LNP of claim 6 or 7, wherein the PEG is PEG 2000 or PEG 3400.

9. The LNP of any one of claims 1-8, wherein the lipid-immune cell targeting group conjugate is present in the lipid blend in a range of 0.001 -0.5 mole percent (e.g., 0.002-0.2 mole percent).

10. The LNP of any one of claims 1 to 9, wherein the lipid blend further comprises one or more of a structural lipid (e.g., a sterol), a neutral phospholipid, and a free PEG-lipid.

11. The LNP of any one of claims 1 to 10, wherein the ionizable cationic lipid is present in the lipid blend in a range of 30-70 (e.g., 40-60) mole percent.

12. The LNP of claim 10, wherein the sterol is present in the lipid blend in a range of 20-70 (e.g., 30-50) mole percent.

13. The LNP of claim 10 or 12, wherein the sterol is cholesterol.

14. The LNP of any one of claims 10 to 13, wherein the neutral phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), sphingomyelin.

15. The LNP of any one of claims 10 to 14, wherein the neutral phospholipid is present in the lipid blend in a range of 1-10 mole percent.

16. The LNP of any one of claims 10 to 15, wherein the free PEG-lipid is selected from the group consisting of PEG-modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides, PEG-modified dialkylamines, PEG-modified diacylglycerols, and PEG-modified dialkylglycerols. For example, a PEG lipid may be PEG-dioleoylglycerol (PEG-DOG), PEG-dimyristoyl-glycerol (PEG-DMG), PEG-dipalmitoyl-glycerol (PEG-DPG), PEG-dilinoleoyl-glycero-phosphatidyl ethanolamine (PEG-DLPE), PEG-dimyristoyl-phosphatidylethanolamine (PEG-DMPE), PEG-dipalmitoyl-phosphatidylethanolamine (PEG-DPPE), PEG-distearoylglycerol (PEG-DSG), PEG-diacylglycerol (PEG-DAG, e.g., PEG-DMG, PEG-DPG, and PEG-DSG), PEG-ceramide, PEG-distearoyl-glycero-phosphoglycerol (PEG-DSPG), PEG-dioleoyl-glycero-

phosphoethanolamine (PEG-DOPE), 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide, or a PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) lipid.

17. The LNP of any one of claims 10 to 15, wherein the free PEG-lipid comprises a diacylphosphatidylethanolamine comprising Dipalmitoyl (C16) chain or Distearoyl (C18) chain.

18. The LNP of any one of claims 10 to 17, wherein the free PEG-lipid is present in the lipid blend in a range of 1-4 mole percent.

19. The LNP of any one of claims 10 to 18, wherein the free PEG-lipid comprises the same or a different lipid as the lipid in the lipid-immune cell targeting group conjugate.

20. The LNP of any one of claims 1 to 19, wherein the LNP has a mean diameter in the range of 50-200 nm.

21. The LNP of claim 20, where the LNP has a mean diameter of about 100 nm.

22. The LNP of any one of claims 1 to 21, wherein the LNP has a polydispersity index in a range from 0.05 to 1.

23. The LNP of any one of claims 1 to 22, wherein the LNP has a zeta potential of from about -10 mV to about + 30 mV at pH 5.

24. The LNP of any one of claims 1 to 23, wherein the nucleic acid is DNA or RNA.

25. The LNP of claim 24, wherein the RNA is an mRNA.

26. The LNP of claim 25, wherein the mRNA encodes a receptor, a growth factor, a hormone, a cytokine, an antibody, an antigen, an enzyme, or a vaccine.

27. The LNP of claim 25, wherein the mRNA encodes a polypeptide capable of regulating immune response in the immune cell.

28. The LNP of claim 25, wherein the mRNA encodes a polypeptide capable of reprogramming the immune cell.

29. The LNP of claim 27, wherein the mRNA encodes a synthetic T cell receptor (synTCR) or a Chimeric Antigen Receptor (CAR).

30. The LNP of any one of claims 1 to 29, wherein the immune cell targeting group comprises an antibody, and the antibody is a Fab or an immunoglobulin single variable domain (e.g., a Nanobody).

31. The LNP of any one of claims 1 to 29, wherein the immune cell targeting group comprises a Fab, F(ab')₂, Fab'-SH, Fv, or scFv fragment.
32. The LNP of claim 30 or claim 31, wherein the immune cell targeting group comprises a Fab that is engineered to knock out the natural interchain disulfide bond at the C-terminus.
33. The LNP of claim 32, wherein the Fab comprises a heavy chain fragment that comprises C233S substitution, and a light chain fragment that comprises C214S substitution, numbering according to Kabat.
34. The LNP of any one of claims 31-33, wherein the immune cell targeting group comprises a Fab that has a non-natural interchain disulfide bond (e.g., a engineered, buried interchain disulfide bond).
35. The LNP of claim 34, wherein the Fab comprises F174C substitution in the heavy chain fragment, and S176C substitution in the light chain fragment, numbering according to Kabat.
36. The LNP of claims 31 to 35, wherein the immune cell targeting group comprises a Fab that comprises a cysteine at the C-terminus of the heavy or light chain fragment.
37. The LNP of claim 36, wherein the Fab further comprises one or more amino acids between the heavy chain fragment of the Fab and the C-terminal cysteine.
38. The LNP of claim 30, wherein the immune cell targeting group comprises an immunoglobulin single variable domain.
39. The LNP of claim 30 or claim 38, wherein the immunoglobulin single variable domain comprises a cysteine at the C-terminus.
40. The LNP of claim 39, wherein the immunoglobulin single variable domain comprises a VHH domain and further comprises a spacer comprising one or more amino acids between the VHH domain and the C-terminal cysteine.
41. The LNP of any one of claims 31 and 38-40, wherein the immune cell targeting group comprises two or more VHH domains.
42. The LNP of claim 41, wherein the two or more VHH domains are linked by an amino acid linker.
43. The LNP of claim 41, wherein the immune cell targeting group comprises a first VHH domain linked to an antibody CH1 domain and a second VHH domain linked to an antibody

light chain constant domain, and wherein the antibody CH1 domain and the antibody light chain constant domain are linked by one or more disulfide bonds.

44. The LNP of any one of claims 30 and 38-40, wherein the immune cell targeting group comprises a VHH domain linked to an antibody CH1 domain, and wherein the antibody CH1 domain is linked to an antibody light chain constant domain by one or more disulfide bonds.

45. The LNP of claim 43 or 44, wherein the CH1 domain comprises F174C and C233S substitutions, and the light chain constant domain comprises S176C and C214S substitutions, numbering according to Kabat.

46. The LNP of any one of claims 1 to 27, wherein the immune cell targeting group comprises a Fab that comprises:

(a) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 1 and a light chain fragment comprising the amino acid sequence of SEQ ID NO:2 or 3; or

(b) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 6 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 7.

47. A method of targeting the delivery of a nucleic acid to an immune cell of a subject, comprising contacting the immune cell with a lipid nanoparticle (LNP), wherein the LNP comprises:

(a) An ionizable cationic lipid,

(b) A conjugate comprising the compound of the following formula:

[Lipid] – [optional linker] – [immune cell targeting group];

(c) A sterol or other structural lipid;

(d) A neutral phospholipid

(e) A free Polyethylene glycol (PEG) lipid, and

(f) the nucleic acid,

wherein the LNP provides at least one of the following benefits:

(i) increased specificity of targeted delivery to the immune cell compared to a reference LNP;

(ii) increased half-life of the nucleic acid or a polypeptide encoded by the nucleic acid in the immune cell compared to a reference LNP;

- (iii) increased transfection rate compared to a reference LNP; and
- (iv) a low level of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies, wherein at least 80% mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation.

48. A method of expressing a polypeptide of interest in a targeted immune cell of a subject, comprising contacting the immune cell with a lipid nanoparticle (LNP), wherein the LNP comprises:

- (a) An ionizable cationic lipid;
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];
- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid
- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) a nucleic acid encoding the polypeptide.

49. The method of claim 48, wherein the LNP provides at least one of the following benefits:

- (i) increased expression level in the immune cell compared to a reference LNP;
- (ii) increased specificity of expression in the immune cell compared to a reference LNP;
- (iii) increased half-life of the nucleic acid or a polypeptide encoded by the nucleic acid in the immune cell compared to a reference LNP;
- (iv) increased transfection rate compared to a reference LNP; and
- (v) a low level of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies, wherein at least 80% mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation.

50. A method of modulating cellular function of a target immune cell of a subject, comprising administering to the subject a lipid nanoparticle (LNP), wherein the LNP comprises:

- (a) An ionizable cationic lipid,
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];

- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid;
- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) a nucleic acid encoding a polypeptide for modulating the cellular function of the immune cell.

51. The method of claim 50, wherein the LNP provides at least one of the following benefits:

- (i) increased expression level in the immune cell compared to a reference LNP;
- (ii) increased specificity of expression in the immune cell compared to a reference LNP;
- (iii) increased half-life of the nucleic acid or a polypeptide encoded by the nucleic acid in the immune cell compared to a reference LNP;
- (iv) increased transfection rate compared to a reference LNP; and
- (v) the LNP can be administered at a lower dose compared to a reference LNP to reach the same biologic effect in the immune cell; and
- (vi) a low level of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies, wherein at least 80% mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation.

52. The method of claim 50 or 51, wherein the modulation of cell function comprises reprogramming the immune cells to initiate an immune response.

53. The method of claim 50 or 51, wherein the modulation of cell function comprises modulating antigen specificity of the immune cell.

54. A method of treating, ameliorating, or preventing a symptom of a disorder or disease in a subject in need thereof, comprising administering to the subject a lipid nanoparticle (LNP) for delivering a nucleic acid into an immune cell of the subject, wherein the LNP comprises:

- (a) An ionizable cationic lipid,
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];
- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid;

- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) the nucleic acid,

Wherein the nucleic acid modulates the immune response of the immune cell, therefore to treat or ameliorate the symptom.

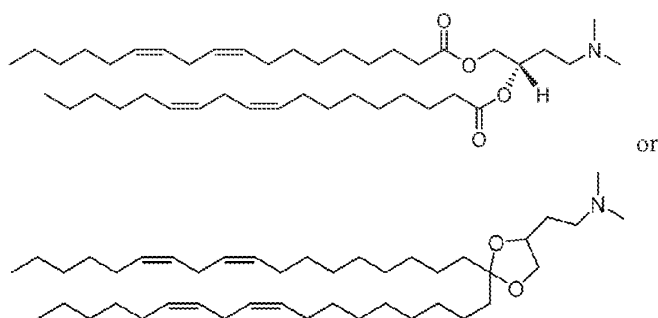
55. The method of claim 50, wherein the LNP provides at least one of the following benefits:

- (i) increased specificity of delivery of the nucleic acid into the immune cell compared to a reference LNP;
- (ii) increased half-life of the nucleic acid or a polypeptide encoded by the nucleic acid in the immune cell compared to a reference LNP;
- (iii) increased transfection rate compared to a reference LNP;
- (v) the LNP can be administered at a lower dose compared to a reference LNP to reach the same treatment efficacy; and
- (vi) a low level of dye accessible mRNA (<15%) and high RNA encapsulation efficiencies, wherein at least 80% mRNA was recovered in final formulation relative to the total RNA used in LNP batch preparation.

56. The method of claim 54 or 55, wherein the disorder is an immune disorder, an inflammatory disorder, or cancer.

57. The method of claim 54 or 55, wherein the nucleic acid encodes an antigen for use in a therapeutic or prophylactic vaccine for treating or preventing an infection by a pathogen.

58. The method of any one of claims 44 to 57, wherein the ionizable cationic lipid is



59. The method of any one of claims 44 to 57, wherein the immune cell targeting group comprises an antibody that binds a T cell antigen.

60. The method of claim 57, wherein the T cell antigen is CD3, CD8, or both CD3 and CD8.
60. The method of any one of claims 44 to 56, wherein the immune cell targeting group comprises an antibody that binds a Natural Killer (NK) cell antigen.
61. The method of claim 60, wherein the NK cell antigen is CD7, CD8, or CD56.
62. The method of any one of claims 58-61, wherein the antibody is a human or humanized antibody.
63. The method of any one of claims 44 to 62, wherein the immune cell targeting group is covalently coupled to a lipid in the lipid blend via a polyethylene glycol (PEG) containing linker.
64. The method of claim 63, wherein the lipid covalently coupled to the immune cell targeting group via a PEG containing linker is distearoylglycerol (DSG), distearoyl-phosphatidylethanolamine (DSPE), dimyrstoyl-phosphatidylethanolamine (DMPE), distearoyl-glycero-phosphoglycerol (DSPG), dimyrstoyl-glycerol (DMG), dipalmitoyl-phosphatidylethanolamine (DPPE), dipalmitoyl-glycerol (DPG), or ceramide.
65. The method of claim 63 or 64, wherein the PEG is PEG 2000.
66. The method of any one of claims 46 to 65, wherein the lipid-immune cell targeting group conjugate is present in the lipid blend in a range of 0.002-0.2 mole percent.
67. The method of any one of claims 46 to 66, wherein the ionizable cationic lipid is present in the lipid blend in a range of 40-60 mole percent.
68. The method of claim 44 to 67, wherein the sterol is cholesterol.
69. The method of any one of claims 44 to 68, wherein the sterol is present in the lipid blend in a range of 30-50 mole percent.
70. The method of claim 44 to 69, wherein the neutral phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), sphingomyelin (SM).
71. The method of claim 44 to 70, wherein the neutral phospholipid is present in the lipid blend in a range of 1-10 mole percent.

72. The method of any one of claims 44 to 71, wherein the free PEG-lipid is selected from the group consisting of PEG-modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides, PEG-modified dialkylamines, PEG-modified diacylglycerols, and PEG-modified dialkylglycerols. For example, a PEG lipid may be PEG-dioleoylglycerol (PEG-DOG), PEG-dimyristoyl-glycerol (PEG-DMG), PEG-dipalmitoyl-glycerol (PEG-DPG), PEG-dilinoyleoyl-glycero-phosphatidyl ethanolamine (PEG-DLPE), PEG-dimyristoyl-phosphatidylethanolamine (PEG-DMPE), PEG-dipalmitoyl-phosphatidylethanolamine (PEG-DPPE), PEG-distearoylglycerol (PEG-DSG), PEG-diacylglycerol (PEG-DAG, e.g., PEG-DMG, PEG-DPG, and PEG-DSG), PEG-ceramide, PEG-distearoyl-glycero-phosphoglycerol (PEG-DSPG), PEG-dioleoyl-glycero-phosphoethanolamine (PEG-DOPE), 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide, or a PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) lipid.
73. The method of claims 44 to 71, wherein the free PEG-lipid comprises a diacylphosphatidylethanolamines comprising Dipalmitoyl (C16) chain or Distearoyl (C18) chain.
74. The method of any one of claims 44 to 73, wherein the free PEG-lipid is present in the lipid blend in a range of 2-4 mole percent.
75. The method of any one of claims 65 to 74, wherein the free PEG-lipid comprises the same or a different lipid as the lipid in the lipid-immune cell targeting group conjugate.
76. The method of claims 44 to 73, wherein the LNP has a mean diameter in the range of 50-200 nm.
77. The method of claim 74, where the LNP has a mean diameter of about 100 nm.
78. The method of claims 44 to 77, wherein the LNP has a polydispersity index in a range from 0.05 to 1.
79. The method of claims 44 to 78, wherein the LNP has a zeta potential of from about -10 mV to about +30 mV at pH 5.
80. The method of claims 44 to 79, wherein the nucleic acid is DNA or RNA.
81. The method of claim 80, wherein the RNA is an mRNA, tRNA, siRNA, or microRNA.
82. The method of claim 81, wherein the mRNA encodes a receptor, a growth factor, a hormone, a cytokine, an antibody, an antigen, an enzyme, or a vaccine.

83. The method of claim 81, wherein the mRNA encodes a polypeptide capable of regulating immune response in the immune cell.
84. The method of claim 81, wherein the mRNA encodes a polypeptide capable of reprogramming the immune cell.
85. The method of claim 81, wherein the mRNA encodes a synthetic T cell receptor (synTCR) or a Chimeric Antigen Receptor (CAR).
86. The method of any one of claims 44 to 85, wherein the immune cell targeting group comprises an antibody, and the antibody is a Fab or an immunoglobulin single variable domain.
87. The method of any one of claims 44 to 85, wherein the immune cell targeting group comprises an antibody fragment selected from the group consisting of a Fab, F(ab')₂, Fab'-SH, Fv, and scFv fragment.
88. The method of claim 86 or 87, wherein the immune cell targeting group comprises a Fab that comprises one or more interchain disulfide bonds.
89. The method of claim 88, wherein the Fab comprises a heavy chain fragment that comprises F174C and C233S substitutions, and a light chain fragment that comprises S176C and C214S substitutions, numbering according to Kabat.
90. The method of any one of claims 86 to 89, wherein the immune cell targeting group comprises a Fab that comprises a cysteine at the C-terminus of the heavy or light chain fragment.
91. The method of claim 86, wherein the Fab further comprises one or more amino acids between the heavy chain fragment of the Fab and the C-terminal cysteine.
92. The method of any one of claims 87-91, wherein the Fab comprises a heavy chain variable domain linked to an antibody CH1 domain and a light chain variable domain linked to an antibody light chain constant domain, wherein the CH1 domain and the light chain constant domain are linked by one or more interchain disulfide bonds, and wherein the immune cell targeting group further comprises a single chain variable fragment (scFv) linked to the C-terminus of the light chain constant domain by an amino acid linker.
93. The method of claim 86, wherein the immune cell targeting group comprises an immunoglobulin single variable domain.

94. The method of claim 86 or 93, wherein the immunoglobulin single variable domain comprises a cysteine at the C-terminus.
95. The method of claim 94, wherein the immunoglobulin single variable domain comprises a VHH domain and further comprises a spacer comprising one or more amino acids between the VHH domain and the C-terminal cysteine.
96. The method of any one of claims 86, and 93 to 95, wherein the immune cell targeting group comprises two or more VHH domains.
97. The method of claim 96, wherein the two or more VHH domains are linked by an amino acid linker.
98. The method of claim 96, wherein the immune cell targeting group comprises a first VHH domain linked to an antibody CH1 domain and a second VHH domain linked to an antibody light chain constant domain, and wherein the antibody CH1 domain and the antibody light chain constant domain are linked by one or more disulfide bonds.
99. The method of any one of claims 86, and 93 to 95, wherein the immune cell targeting group comprises a VHH domain linked to an antibody CH1 domain, and wherein the antibody CH1 domain is linked to an antibody light chain constant domain by one or more disulfide bonds.
100. The method of claim 96 or 97, wherein the CH1 domain comprises F174C and C233S substitutions, and the light chain constant domain comprises S176C and C214S substitutions, numbering according to Kabat.
101. The method of any one of claims 44 to 85, wherein the immune cell targeting group comprises a Fab that comprises:
- (a) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 1 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 2 or 3;
 - (b) a heavy chain fragment comprising the amino acid sequence of SEQ ID NO: 6 and a light chain fragment comprising the amino acid sequence of SEQ ID NO: 7.
102. The method of any one of claims 44 to 101, wherein no more than 5% non-immune cells are transfected by the LNP.
103. The method of any one of claims 44 to 102, wherein half-life of the nucleic acid delivered by the LNP or a polypeptide encoded by the nucleic acid delivered by the LNP is at

least 10% longer than half-life of nucleic acid delivered by a reference LNP or a polypeptide encoded by the nucleic acid delivered by the reference LNP.

104. The method of any one of claims 44 to 103, wherein at least 10% immune cells are transfected by the LNP.

105. The method of any one of claims 44 to 104, wherein expression level of the nucleic acid delivered by the LNP is at least 10% higher than expression level of nucleic acid delivered by a reference LNP.

106. A lipid nanoparticle (LNP) for delivering a nucleic acid into an immune cell of the subject, wherein the LNP comprises:

- (a) An ionizable cationic lipid,
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];
- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid;
- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) the nucleic acid,

wherein the immune cell is an NK cell, and the immune cell targeting group comprises an antibody that binds CD56.

107. A lipid nanoparticle (LNP) for delivering a nucleic acid into an immune cell of the subject, wherein the LNP comprises:

- (a) An ionizable cationic lipid,
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];
- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid;
- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) the nucleic acid,

wherein the immune cell targeting group comprises an antibody that binds CD7 or CD8, and the free PEG lipid is DMG-PEG.

108. A lipid nanoparticle (LNP) for delivering a nucleic acid into an immune cell of the subject, wherein the LNP comprises:

- (a) An ionizable cationic lipid,
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];
- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid;
- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) the nucleic acid,

wherein the immune cell targeting group comprises an antibody, and the antibody is a Fab or an immunoglobulin single variable domain.

109. The LNP of claim 108, wherein the Fab is engineered to knock out the natural interchain disulfide at the C-terminus.

110. The LNP of claim 109, wherein the Fab comprises a heavy chain fragment that comprises C233S substitutions, and a light chain fragment that comprises C214S substitutions.

111. The LNP of claim 110, wherein the Fab comprises a non-natural interchain disulfide.

112. The LNP of claim 110, wherein the Fab comprises F174C substitution in the heavy chain fragment, and S176C substitution in the light chain fragment.

113. The LNP of claim 108, wherein the antibody is an immunoglobulin single variable (ISV) domain, and the ISV domain is an Nanobody® ISV.

114. The LNP of claim 113, wherein the free PEG lipid comprise a PEG having a molecular weight of at least 2000 daltons.

115. The LNP of claim 114, wherein the PEG has a molecular weight of about 3000 to 5000 daltons.

116. The LNP of claim 108, wherein the antibody is a Fab.

117. The LNP of claim 116, wherein the Fab binds CD3, and the free PEG lipid in the LNP comprises a PEG having a molecular weight of about 2000 daltons.

118. The LNP of claim 116, wherein the Fab is an anti-CD4 antibody, and the free PEG lipid in the LNP comprises a PEG having a molecular weight of about 3000 to 3500 daltons.

119. The LNP of claim 108, wherein the immune cell targeting group comprises two or more VHH domains.

120. The LNP of claim 119, wherein the two or more VHH domains are linked by an amino acid linker.

121. The LNP of claim 120, wherein the immune cell targeting group comprises a first VHH domain linked to an antibody CH1 domain and a second VHH domain linked to an antibody light chain constant domain.

122. A lipid nanoparticle (LNP) for delivering a nucleic acid into an immune cell of the subject, wherein the LNP comprises:

- (a) An ionizable cationic lipid,
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];
- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid;
- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) the nucleic acid,

wherein the LNP binds CD3, and also binds CD11a or CD18.

123. The LNP of claim 122, wherein the LNP comprises two conjugates, wherein the first conjugate comprises an antibody that binds CD3, and the second conjugate comprises an antibody that binds CD11a or CD18.

124. The LNP of claim 122, wherein the LNP comprises one conjugate, and the conjugate comprises a bispecific antibody that binds both CD3 and CD11a.

125. The LNP of claim 122, wherein the LNP comprises one conjugate, and the conjugate comprises a bispecific antibody that binds both CD3 and CD18.

126. The LNP of claim 124 or 125, wherein the bispecific antibody is an immunoglobulin single variable domain or Fab-ScFv.

127. A lipid nanoparticle (LNP) for delivering a nucleic acid into an immune cell of the subject, wherein the LNP comprises:

- (a) An ionizable cationic lipid,
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];
- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid;
- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) the nucleic acid,

wherein the LNP binds CD7 and CD8 of the immune cell.

128. The LNP of claim 127, wherein the LNP comprises two conjugates, wherein the first conjugate comprises an antibody that binds CD7, and a second conjugate that binds CD8.

129. The LNP of claim 127, wherein the LNP comprises one conjugate, wherein the conjugate comprises a bispecific antibody that binds CD7 and CD8.

130. The LNP of claim 129, wherein the bispecific antibody is an immunoglobulin single variable domain or Fab-ScFv.

131. A lipid nanoparticle (LNP) for delivering a nucleic acid into two different types of immune cells of the subject, wherein the LNP comprises:

- (a) An ionizable cationic lipid,
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];
- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid;
- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) the nucleic acid,

wherein the LNP binds to a first antigen on the surface of the first type of immune cell, and also binds to a second antigen on the surface of the second type of immune cell.

132. The LNP of claim 131, wherein the two different types of immune cells are CD4+ T cells and CD8+ T cell.

133. The LNP of claim 131, wherein the LNP comprises two conjugates, and the first conjugate comprises a first antibody that binds to the first antigen of the first type of immune cell, and the second conjugate comprises a second antibody that binds to the second antigen of the second type of immune cell.

134. The LNP of claim 131, wherein the LNP comprises one conjugate, and the conjugate comprises a bispecific antibody, and the bispecific antibody binds to both the first antigen on the first type of immune cell, and the second antigen on the second type of immune cells.

135. The LNP of claim 134, wherein the bispecific antibody is an immunoglobulin single variable domain or a Fab-ScFv.

136. A lipid nanoparticle (LNP) for delivering a nucleic acid into both CD4+ and CD8+ T cells of a subject, wherein the LNP comprises:

- (a) An ionizable cationic lipid,
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];
- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid;
- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) the nucleic acid,

wherein the immune cell targeting group comprises a single antibody that binds to CD3 or CD7.

137. A lipid nanoparticle (LNP) for delivering a nucleic acid into both T cells and NK cells of a subject, wherein the LNP comprises:

- (a) An ionizable cationic lipid,
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];

- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid;
- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) the nucleic acid,

wherein the immune cell targeting group binds to CD7, CD8, or both CD7 and CD8.

138. A lipid nanoparticle (LNP) for delivering a nucleic acid into both T cells and NK cells of a subject, wherein the LNP comprises:

- (a) An ionizable cationic lipid,
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];
- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid;
- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) the nucleic acid,

wherein the immune cell targeting group binds to

- (i) both CD3 and CD56;
- (ii) both CD8 and CD56; or
- (iii) both CD7 and CD56.

139. The LNP of any one of claims 106 to 138, wherein the immune cell targeting group is covalently coupled to a lipid in the lipid blend via a polyethylene glycol (PEG) containing linker.

140. The LNP of claim 139, wherein the lipid covalently coupled to the immune cell targeting group via a PEG containing linker is distearoylglycerol (DSG), distearoyl-phosphatidylethanolamine (DSPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-glycero-phosphoglycerol (DSPG), dimyristoyl-glycerol (DMG), dipalmitoyl-phosphatidylethanolamine (DPPE), dipalmitoyl-glycerol (DPG), or ceramide.

141. The LNP of any one of claims 106 to 130, wherein the lipid-immune cell targeting group conjugate is present in the lipid blend in a range of 0.002-0.2 mole percent.

142. The LNP of any one of claims 106 to 141, wherein the lipid blend further comprises one or more of a structural lipid (e.g., a sterol), a neutral phospholipid, and a free PEG-lipid.
143. The LNP of any one of claims 106 to 142, wherein the ionizable cationic lipid is present in the lipid blend in a range of 40-60 mole percent.
144. The LNP of claim 142, wherein the sterol is present in the lipid blend in a range of 30-50 mole percent.
145. The LNP of claim 142 or 144, wherein the sterol is cholesterol.
146. The LNP of any one of claims 138 to 145, wherein the neutral phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).
147. The LNP of any one of claims 106 to 146, wherein the neutral phospholipid is present in the lipid blend in a range of 1-10 mole percent.
148. The LNP of any one of claims 106 to 146, wherein the free PEG-lipid is selected from the group consisting of PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) or PEG-dimyristoyl-phosphatidylethanolamine (PEG-DMPE), N-(Methylpolyoxyethylene oxycarbonyl)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE-PEG) 1,2-Dimyristoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DMG), 1,2-Dipalmitoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DPG), 1,2-Dioleoyl-rac-glycerol, methoxypolyethylene Glycol (DOG-PEG) 1,2-Distearoyl-rac-glycero-3-methylpolyoxyethylene (PEG-DSG), N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)]} (PEG-ceramide), and DSPE-PEG-cysteine, or a derivative thereof.
149. The LNP of any one of claims 106 to 148, wherein the free PEG-lipid comprises a diacylphosphatidylethanolamines comprising Dipalmitoyl (C16) chain or Distearoyl (C18) chain.
150. The LNP of any one of claims 106 to 149, wherein the free PEG-lipid is present in the lipid blend in a range of 1-2 mole percent.
151. The LNP of any one of claims 106 to 150, wherein the free PEG-lipid comprises the same or a different lipid as the lipid in the lipid-immune cell targeting group conjugate.

152. The LNP of any one of claims 106 to 151, wherein the LNP has a mean diameter in the range of 50-200 nm.

153. The LNP of claim 152, where the LNP has a mean diameter of about 100 nm.

154. The LNP of any one of claims 106 to 153, wherein the LNP has a polydispersity index in a range from 0.05 to 1.

155. The LNP of any one of claims 106 to 154, wherein the LNP has a zeta potential of from about -10 mV to about + 30 mV at pH 5.

156. The LNP of any one of claims 106 to 155, wherein the nucleic acid is DNA or RNA.

157. The LNP of claim 156, wherein the RNA is an mRNA.

158. The LNP of claim 157, wherein the mRNA encodes a receptor, a growth factor, a hormone, a cytokine, an antibody, an antigen, an enzyme, or a vaccine.

159. The LNP of claim 157, wherein the mRNA encodes a polypeptide capable of regulating immune response in the immune cell.

160. The LNP of claim 159, wherein the mRNA encodes a polypeptide capable of reprogramming the immune cell.

161. The LNP of claim 160, wherein the mRNA encodes a synthetic T cell receptor (synTCR) or a Chimeric Antigen Receptor (CAR).

162. A lipid nanoparticle (LNP) for delivering a nucleic acid into an immune cell of a subject, wherein the LNP comprises:

- (a) An ionizable cationic lipid,
- (b) A conjugate comprising the following structure:
[Lipid] – [optional linker] – [immune cell targeting group];
- (c) A sterol or other structural lipid;
- (d) A neutral phospholipid;
- (e) A free Polyethylene glycol (PEG) lipid, and
- (f) the nucleic acid,

wherein the immune cell targeting group comprises a Fab lacking the native interchain disulfide bond.

163. The LNP of claim 162, wherein the Fab is engineered to replace one or both cysteines on the native constant light chain and the native constant heavy chain that form the native interchain disulfide with a non-cysteine amino acid, therefor to remove the native interchain disulfide bond in the Fab.

164. A method of targeting the delivery of a nucleic acid to an immune cell of a subject, comprising contacting the immune cell with a lipid nanoparticle (LNP) of any one of claims to 106 to 163.

165. The method of claim 164, wherein the method is for targeting NK cells, wherein the immune cell targeting group binds to CD56.

166. The method of claim 164, wherein the method is for targeting both T cells and NK cells simultaneously, wherein the immune cell targeting group binds to CD7, CD8, or both CD7 and CD8.

167. The method of claim 164, wherein the method is for targeting both CD4+ and CD8+ T cells simultaneously, wherein the immune cell targeting group comprises a polypeptide that binds to CD3 or CD7.

168. A method of expressing a polypeptide of interest in a targeted immune cell of a subject, comprising contacting the immune cell with a lipid nanoparticle (LNP) of any one of claims to 106 to 163.

169. A method of modulating cellular function of a target immune cell of a subject, comprising administering to the subject a lipid nanoparticle (LNP) of any one of claims to 106 to 159.

170. A method of treating, ameliorating, or preventing a symptom of a disorder or disease in a subject in need thereof, comprising administering to the subject a lipid nanoparticle (LNP) of any one of claims to 106 to 163.

171. An immunoglobulin single variable domain (ISVD) that binds to human CD8, wherein the ISVD comprises three complementarity determining domains CDR1, CDR2, and CDR3, wherein

(a) the CDR1 comprises GSTFSDYG (SEQ ID NO: 100),

(b) the CDR2 comprises IDWNGEHT (SEQ ID NO: 101), and

(c) the CDR3 comprises AADALPYTVRKYNY (SEQ ID NO: 102).

172. The ISVD of claim 171, wherein the ISVD is humanized.
173. The ISVD of claim 172, wherein the ISVD comprises SEQ ID NO: 77.
174. A polypeptide comprising GSTFSDYG (SEQ ID NO: 100), IDWNGEHT (SEQ ID NO: 101), and AADALPYTVRKYNY (SEQ ID NO: 102).
175. A polypeptide comprising the ISVD of claim 171.
176. The polypeptide of claim 175, further comprising a second binding moiety, wherein the second binding moiety binds to CD8 or another different target.
177. The polypeptide of claim 176, wherein the second binding moiety is also an ISVD.
178. The polypeptide of claim 175, further comprising a detectable marker.
179. The polypeptide of claim 175, further comprising a therapeutic agent.
180. A composition comprising the ISVD of any one of claims 171 to 173, or the polypeptide of any one of claims 174 to 179.
181. A pharmaceutical composition comprising the ISVD of any one of claims 171 to 173, or the polypeptide of any one of claims 174 to 179, and a pharmaceutically acceptable carrier.
182. A method of treating a disease or disorder related to CD8 in a subject, comprising administering the pharmaceutical composition of claim 181 to the subject.
183. The method of claim 182, wherein the disease or disorder is cancer.