

AGENTS AND METHODS FOR ACTIVATION AND TARGETING OF IMMUNE EFFECTOR CELLS

The invention relates to agents and methods for activation of immune effector cells and targeted delivery of the activated immune effector cells to target cells. In one embodiment, the invention involves providing to a subject immune effector cells genetically modified to express a chimeric antigen receptor (CAR). In one embodiment, the invention involves administering RNA encoding a peptide or polypeptide (activation compound) comprising a binding moiety for the CAR and administering RNA encoding a peptide or polypeptide (docking compound) comprising a binding moiety binding to target cells (primary targeting moiety) and a further binding moiety for the CAR (secondary target). In one embodiment, the RNA encoding an activation compound is expressed by antigen presenting cells on their cell surface. Following expression of the activation compound, the binding moiety for the CAR may be available for binding by the immune effector cells, wherein said binding may result in expansion of the immune effector cells. In one embodiment, the RNA encoding a docking compound is expressed and secreted by cells of the subject such as liver cells. Following expression of the docking compound, the primary targeting moiety may bind to a target antigen such as a cancer antigen on cancer cells and then the immune effector cells may target the secondary target to thereby precisely deliver the immune effector cells to the target cells such as cancer cells. The binding moiety for the CAR comprised in the activation compound and the binding moiety for the CAR comprised in the docking compound may be a peptide tag and/or may be identical or different.

In many areas of medical therapy and diagnosis, it is desired to selectively deliver an agent, such as a therapeutic agent (a drug) or a diagnostic (e.g. imaging) agent, to a specific site, or a confined region, in the body of a subject such as a patient.

Active targeting of an organ or a tissue may be achieved by the direct or indirect conjugation of the desired active moieties (e.g. cytotoxic compound) to a targeting construct, which binds to cell surfaces at the target site of interest. The targeting moieties used to target such agents

are typically constructs that have affinity for cell surface targets, e.g., membrane proteins, and include antibodies or antibody fragments.

The present invention relates to an approach wherein an RNA-encoded docketing compound that labels target cells, e.g., by binding to a primary target (e.g. a cell surface antigen), is used. The docketing compound comprises a secondary target, which will eventually be targeted by immune effector cells, equipped with an antigen receptor targeting the secondary target. Thus, according to the invention, RNA encoding a docketing compound is administered. After the RNA has been expressed, the docketing compound may bind to target cells, e.g., by binding to a primary target. Immune effector cells bind to the secondary target on the docketing compound via their antigen receptor. Further, according to the invention, RNA encoding an activation compound is administered. After the RNA has been expressed, the activation compound may be present at the cell surface of antigen presenting cells and may present a moiety on the cell surface that can be bound by immune effector cells. Immune effector cells bind to the binding moiety via their antigen receptor, said binding resulting in expansion of the immune effector cells. The concept described herein allows to use a single type of immune effector cells for targeting a wide range of target cells, i.e., by using a single type of immune effector cells and optionally a single type of activation compound in combination with different docketing compounds targeting different primary targets and comprising an identical secondary target.

Summary

In one aspect, the invention relates to a method for treating a subject having a disease, disorder or condition characterized by cells expressing a target antigen, comprising:

- (i) providing to the subject immune effector cells genetically modified to express a chimeric antigen receptor (CAR);
- (ii) administering to the subject first RNA encoding a first peptide or polypeptide, wherein the first peptide or polypeptide comprises a binding moiety for the CAR;
- (iii) allowing expression of the first peptide or polypeptide by antigen presenting cells in the subject such that the binding moiety for the CAR is available for binding by the immune effector cells said binding resulting in expansion of the immune effector cells;
- (iv) administering to the subject second RNA encoding a second peptide or polypeptide, wherein the second peptide or polypeptide comprises a binding moiety binding to the target antigen and a binding moiety for the CAR; and
- (v) allowing expression of the second peptide or polypeptide by cells in the subject such that the second peptide or polypeptide becomes associated with cells expressing the target antigen and the binding moiety for the CAR is available for binding by the immune effector cells.

In some embodiments, the antigen presenting cells are transfected with the first RNA.

In some embodiments, the first RNA is administered as particulate formulation such as formulated as lipoplex particles.

In some embodiments, the cells expressing the second peptide or polypeptide are transfected with the second RNA.

In some embodiments, the second RNA is administered as particulate formulation such as formulated as lipid nanoparticles.

In some embodiments, the antigen presenting cells express the first peptide or polypeptide such that it remains associated with the antigen presenting cells.

In some embodiments, the first peptide or polypeptide is a membrane peptide or polypeptide.

In some embodiments, the first peptide or polypeptide is a fusion protein of the binding moiety for the CAR and a membrane peptide or polypeptide.

In some embodiments, binding of the immune effector cells to the second peptide or polypeptide associated with cells expressing the target antigen results in killing of cells expressing the target antigen.

In some embodiments, the cells expressing the second peptide or polypeptide secrete the second peptide or polypeptide.

In some embodiments, the cells expressing the second peptide or polypeptide express the second peptide or polypeptide such that it is released into the bloodstream.

In some embodiments, the target antigen is a cell surface antigen.

In some embodiments, the second peptide or polypeptide is a fusion peptide or polypeptide of the binding moiety binding to the target antigen and the binding moiety for the CAR.

In some embodiments, the binding moiety binding to the target antigen comprises an antibody or an antibody derivative.

In some embodiments, the binding moiety for the CAR comprises a peptide tag.

In some embodiments, the CAR comprises an antibody or an antibody derivative.

In some embodiments, the antibody derivative is an antibody fragment.

In some embodiments, the method comprises administering the immune effector cells genetically modified to express a CAR to the subject.

In some embodiments, the method comprises generating the immune effector cells genetically modified to express a CAR in the subject.

In some embodiments, the disease, disorder or condition is cancer.

In some embodiments, the cells expressing a target antigen are diseased cells.

In some embodiments, the cells expressing a target antigen are cancer cells.

In some embodiments, the target antigen is a tumor antigen.

In a further aspect, the invention relates to a method for treating a subject having a disease, disorder or condition characterized by cells expressing a target antigen, comprising:

- (i) providing to the subject immune effector cells genetically modified to express a chimeric antigen receptor (CAR) binding to a peptide tag;

- (ii) administering to the subject first RNA encoding a first peptide or polypeptide such that the first peptide or polypeptide is expressed in antigen presenting cells of the subject, wherein the first peptide or polypeptide is a membrane protein which comprises in an extracellular domain a peptide tag to which the CAR binds; and
- (iii) administering to the subject second RNA encoding a second peptide or polypeptide such that the second peptide or polypeptide is expressed in and secreted by cells of the subject, wherein the second peptide or polypeptide comprises a binding moiety binding to the target antigen and a peptide tag to which the CAR binds.

In some embodiments, binding of the immune effector cells to the first peptide or polypeptide results in expansion of the immune effector cells.

In some embodiments, binding of the immune effector cells to the second peptide or polypeptide bound to target antigen results in killing of cells expressing the target antigen.

In some embodiments, the antigen presenting cells are transfected with the first RNA.

In some embodiments, the first RNA is administered as particulate formulation such as formulated as lipoplex particles.

In some embodiments, the cells expressing the second peptide or polypeptide are transfected with the second RNA.

In some embodiments, the second RNA is administered as particulate formulation such as formulated as lipid nanoparticles.

In some embodiments, the antigen presenting cells express the first peptide or polypeptide such that it remains associated with the antigen presenting cells.

In some embodiments, the first peptide or polypeptide is a fusion protein of a peptide tag to which the CAR binds and a membrane peptide or polypeptide.

In some embodiments, the second peptide or polypeptide is secreted into the bloodstream.

In some embodiments, the target antigen is a cell surface antigen.

In some embodiments, the second peptide or polypeptide is a fusion peptide or polypeptide of the binding moiety binding to the target antigen and a peptide tag to which the CAR binds.

In some embodiments, the binding moiety binding to the target antigen comprises an antibody or an antibody derivative.

In some embodiments, the CAR comprises an antibody or an antibody derivative.

In some embodiments, the antibody derivative is an antibody fragment.

In some embodiments, the method comprises administering the immune effector cells genetically modified to express a CAR to the subject.

In some embodiments, the method comprises generating the immune effector cells genetically modified to express a CAR in the subject.

In some embodiments, the disease, disorder or condition is cancer.

In some embodiments, the cells expressing a target antigen are diseased cells.

In some embodiments, the cells expressing a target antigen are cancer cells.

In some embodiments, the target antigen is a tumor antigen.

In a further aspect, the invention relates to a kit, comprising:

- (i) nucleic acid for genetically modifying immune effector cells to express a chimeric antigen receptor (CAR) binding to a peptide tag, or immune effector cells genetically modified to express a chimeric antigen receptor (CAR) binding to a peptide tag;
- (ii) first RNA encoding a first peptide or polypeptide which is a membrane protein which comprises in an extracellular domain a peptide tag to which the CAR binds, or nucleic acid for obtaining said first RNA; and optionally
- (iii) second RNA encoding a second peptide or polypeptide which comprises a binding moiety binding to a target antigen and a peptide tag to which the CAR binds, or nucleic acid for obtaining said second RNA.

Embodiments of the nucleic acid for genetically modifying immune effector cells to express a chimeric antigen receptor (CAR) binding to a peptide tag, the immune effector cells genetically modified to express a chimeric antigen receptor (CAR) binding to a peptide tag, the first RNA, the first peptide or polypeptide, the second RNA and/or the second peptide or polypeptide are described herein, e.g., in the context of the method of the invention.

In one aspect, the invention relates to an agent or composition described herein (e.g., nucleic acid for genetically modifying immune effector cells, immune effector cells genetically

modified to express a chimeric antigen receptor (CAR), first RNA, second RNA and/or kit) for use in a method described herein.

Brief description of the drawings**Figure 1: Modular CAR-T cell approach**

The figure contains a schematic illustration of a universal CAR-T approach based on a modular interaction pair on the example of ALFA-tag/NbALFA. Herein a generic and off-the shelf producible CART cell is generated (1) that bears a tag-binding moiety on its surface (e.g. the NbALFA VHH). These CAR-T cells are administered to the patient and can be specifically expanded *in vivo* via RNA lipoplexes encoding the tag fused to a membrane-anchoring protein domain (e.g. truncated CLDN6) (2). A second binding moiety, the so-called targeting ligand (TL) consisting of the ALFA-tag fused to a tumor-antigen specific ligand (e.g. a scFv, VHH or Fab fragment) is administered as a lipid nanoparticle formulated RNA to the patient (3). The RNA is translated *in vivo* into a bispecific protein that is released into the bloodstream. After the targeting ligand is accumulated in the tumor based on its specific binding to a certain tumor antigen, it will be bound by NbALFA-CAR T cells that will be activated as a consequence leading to specific lysis of the tumor cell (4). By using different targeting ligands, different tumor antigens can be addressed either sequentially or in parallel using the same CART cell product of the patient.

Figure 2: Adaptors bind to CAR+ effector cells

Jurkat T cells were transfected with mRNA encoding for a modular CAR. Modular CAR expressing cells were armed with a soluble adaptor and stained with an adaptor-specific antibody. Figures represent the frequency and intensity of adaptor detection at the surface of the modular CAR expressing cells.

Figure 3: Primary human T cells can express the modular CAR

Primary human T cells were activated and subsequently engineered to express the modular CAR using mRNA. CAR molecules were stained and expression was detected using flow cytometric methodology.

Figure 4: Primary human T cells expressing the modular CAR can be armed with soluble adaptors

Modular CAR T cells were armed with a soluble adaptor (100 nM) generated by nucleic acid transfected cells. Adaptor was stained with a specific antibody and expression was detected using flow cytometric methodology. The figure represents the frequency and intensity of adaptor detection at the surface of the modular CAR expressing T cells.

Figure 5: Modular CAR-T Platform mediates tumor cell lysis

Adaptor armed modular CAR T cells were brought into co-culture with antigen positive (Ag+) tumor cells. CAR-T cell responses were assessed using impedance-based cytotoxicity methodology. Cytotoxicity was normalized to tumor cells cultured with non-mRNA transfected T cells.

Figure 6: Modular CAR mediated proliferation stimulated by universal antigen transfected into iDCs

Human immature dendritic cells were transfected with mRNA coding for membrane anchored binding moiety. Concomittantly, T cells were transfected with modular CAR encoding mRNA and stained with a proliferation dye. Modular CAR-T cells and CARVac expressing iDCs were co-cultured. In Figure 6 A and B the membrane anchored binding moiety comprises the ALFA peptide, while the CAR comprises the VHH(aALFA). Figure6A depicts the proliferation of the engineered CAR-T cells armored with adaptors in response to seeing their target on the iDCs. Similarly, to Figure 6A, proliferation of modular CAR expressing human T cells is measured. Here the modular CAR used the VHH(aALFA) SEQ ID NO: 30 which has a higher affinity to it's target antigen expressed at the surface of iDCs than the VHH(aALFA)PE CAR used in figure 6A.

In Figure 6C and D, the membrane anchored binding moiety comprises the VHH(aALFA), while the CAR comprises the ALFA peptide.

Figure 7: Modular ALFA CAR-Ts loaded with CD19 specific adaptors mediate proliferation against CD19 transfected iDCs or primary human B cells

Human primary T cells were transfected with modular CAR encoding mRNA and stained with a proliferation dye. Modular CAR T cells were co-cultured with B cells (CD19+) or CD19 mRNA transfected iDCs. Proliferation was assessed by flow cytometry.

Description of the sequences

The following table provides a listing of certain sequences referenced herein.

DESCRIPTION OF SEQUENCES		
SEQ ID NO:	Description	SEQUENCE
5'-UTR (hAg-Kozak)		
1	5'-UTR	AACUAGUAUUUCUUCUGGUCCCCACAGACUCAGAGAGAACCCGCCACC
3'-UTR (2hBg)		
2	3'-UTR	CUCGAGAGCUCGCUUUUCUUGCUGUCCAAUUUCUAUUAAAGGUUCCUUUGUCCUAAGUCCAACUACUAAACUGGGGGAUUAUUGAAGGCCCUUGAGCAUCUGGUAUUCUGCCUAA AAGGCCUUGAGCAUCUGGAUUCUGCCUAAUAAAAACAUAUUUAUUCUUGCUGGAGAGCUCGCUUUCUGUCCAAUU UCUAUUAAAGGUUCCUUUGUCCUAAAGUCCAACUAAACUGGGGGAUUAUUGAAGGCCCUUGAGCAUCUGGUAUUCUGCCUAA UAAAAACAUAUUUAUUCUUGCUGGUCGAGACCUUGUCCAGAGUCGCUAGC
3'-UTR (element)		
3	UTR	CUGGUACUGCAUGCACGCAAUAGCUGGCCCCUUUCCCGUCCUUGGGUACCCCGAGUCUCCCGACCUCCGGGUCCCAGGUUAUGCUCCC ACCUCCACCUGCCCCACUCACACCUAGCUAGUCCAGACACCUCCCAAGCACGAGCAUUGCAGCUCUAAACGCUUAGCCUAGCCACA CCCCCACGGGAACAGCAGUGAUUAACCUUUAAGCAAUAACGAAAGUUUAACUAAAGCUUAUACUAAACCCCGGGUUGGUAUUUUCGU GCCAGCCACACC
A30		
4	A30L70	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Detailed description

Although the present disclosure is described in detail below, it is to be understood that this disclosure is not limited to the particular methodologies, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present disclosure which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art.

Preferably, the terms used herein are defined as described in "A multilingual glossary of biotechnological terms: (IUPAC Recommendations)", H.G.W. Leuenberger, B. Nagel, and H. Kölbl, Eds., Helvetica Chimica Acta, CH-4010 Basel, Switzerland, (1995).

The practice of the present disclosure will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, cell biology, immunology, and recombinant DNA techniques which are explained in the literature in the field (cf., e.g., Molecular Cloning: A Laboratory Manual, 2nd Edition, J. Sambrook et al. eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989).

In the following, the elements of the present disclosure will be described. These elements are listed with specific embodiments, however, it should be understood that they may be combined in any manner and in any number to create additional embodiments. The variously described examples and embodiments should not be construed to limit the present disclosure to only the explicitly described embodiments. This description should be understood to disclose and encompass embodiments which combine the explicitly described embodiments with any number of the disclosed elements. Furthermore, any permutations and combinations of all described elements should be considered disclosed by this description unless the context indicates otherwise.

The term "about" means approximately or nearly, and in the context of a numerical value or range set forth herein in one embodiment means $\pm 20\%$, $\pm 10\%$, $\pm 5\%$, or $\pm 3\%$ of the numerical value or range recited or claimed.

The terms "a" and "an" and "the" and similar reference used in the context of describing the disclosure (especially in the context of the claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it was individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as"), provided herein is intended merely to better illustrate the disclosure and does not pose a limitation on the scope of the claims. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the disclosure.

Unless expressly specified otherwise, the term "comprising" is used in the context of the present document to indicate that further members may optionally be present in addition to the members of the list introduced by "comprising". It is, however, contemplated as a specific embodiment of the present disclosure that the term "comprising" encompasses the possibility of no further members being present, i.e., for the purpose of this embodiment "comprising" is to be understood as having the meaning of "consisting of" or "consisting essentially of".

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, etc.), whether supra or infra, are hereby incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the present disclosure was not entitled to antedate such disclosure.

Definitions

In the following, definitions will be provided which apply to all aspects of the present disclosure. The following terms have the following meanings unless otherwise indicated. Any undefined terms have their art recognized meanings.

Terms such as "reduce", "decrease", "inhibit" or "impair" as used herein relate to an overall reduction or the ability to cause an overall reduction, preferably of at least 5%, at least 10%, at least 20%, at least 50%, at least 75% or even more, in the level. These terms include a complete or essentially complete inhibition, i.e., a reduction to zero or essentially to zero.

Terms such as "increase", "enhance" or "exceed" preferably relate to an increase or enhancement by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 80%, at least 100%, at least 200%, at least 500%, or even more.

According to the disclosure, the term "peptide" comprises oligo- and polypeptides and refers to substances which comprise about two or more, about 3 or more, about 4 or more, about 6 or more, about 8 or more, about 10 or more, about 13 or more, about 16 or more, about 20 or more, and up to about 50, about 100 or about 150, consecutive amino acids linked to one another via peptide bonds. The term "protein" or "polypeptide" refers to large peptides, in particular peptides having at least about 150 amino acids, but the terms "peptide", "protein" and "polypeptide" are used herein usually as synonyms.

"Fragment", with reference to an amino acid sequence (peptide or protein), relates to a part of an amino acid sequence, i.e. a sequence which represents the amino acid sequence shortened at the N-terminus and/or C-terminus. A fragment shortened at the C-terminus (N-terminal fragment) is obtainable e.g. by translation of a truncated open reading frame that lacks the 3'-end of the open reading frame. A fragment shortened at the N-terminus (C-terminal fragment) is obtainable e.g. by translation of a truncated open reading frame that lacks the 5'-end of the open reading frame, as long as the truncated open reading frame comprises a start codon that serves to initiate translation. A fragment of an amino acid sequence comprises e.g. at least 50 %, at least 60 %, at least 70 %, at least 80%, at least 90%

of the amino acid residues from an amino acid sequence. A fragment of an amino acid sequence preferably comprises at least 6, in particular at least 8, at least 12, at least 15, at least 20, at least 30, at least 50, or at least 100 consecutive amino acids from an amino acid sequence.

By "variant" herein is meant an amino acid sequence that differs from a parent amino acid sequence by virtue of at least one amino acid modification. The parent amino acid sequence may be a naturally occurring or wild type (WT) amino acid sequence, or may be a modified version of a wild type amino acid sequence. Preferably, the variant amino acid sequence has at least one amino acid modification compared to the parent amino acid sequence, e.g., from 1 to about 20 amino acid modifications, and preferably from 1 to about 10 or from 1 to about 5 amino acid modifications compared to the parent.

By "wild type" or "WT" or "native" herein is meant an amino acid sequence that is found in nature, including allelic variations. A wild type amino acid sequence, peptide or protein has an amino acid sequence that has not been intentionally modified.

For the purposes of the present disclosure, "variants" of an amino acid sequence (peptide, protein or polypeptide) comprise amino acid insertion variants, amino acid addition variants, amino acid deletion variants and/or amino acid substitution variants. The term "variant" includes all mutants, splice variants, posttranslationally modified variants, conformations, isoforms, allelic variants, species variants, and species homologs, in particular those which are naturally occurring. The term "variant" includes, in particular, fragments of an amino acid sequence.

Amino acid insertion variants comprise insertions of single or two or more amino acids in a particular amino acid sequence. In the case of amino acid sequence variants having an insertion, one or more amino acid residues are inserted into a particular site in an amino acid sequence, although random insertion with appropriate screening of the resulting product is also possible. Amino acid addition variants comprise amino- and/or carboxy-terminal fusions of one or more amino acids, such as 1, 2, 3, 5, 10, 20, 30, 50, or more amino acids. Amino acid

deletion variants are characterized by the removal of one or more amino acids from the sequence, such as by removal of 1, 2, 3, 5, 10, 20, 30, 50, or more amino acids. The deletions may be in any position of the protein. Amino acid deletion variants that comprise the deletion at the N-terminal and/or C-terminal end of the protein are also called N-terminal and/or C-terminal truncation variants. Amino acid substitution variants are characterized by at least one residue in the sequence being removed and another residue being inserted in its place. Preference is given to the modifications being in positions in the amino acid sequence which are not conserved between homologous proteins or peptides and/or to replacing amino acids with other ones having similar properties. Preferably, amino acid changes in peptide and protein variants are conservative amino acid changes, i.e., substitutions of similarly charged or uncharged amino acids. A conservative amino acid change involves substitution of one of a family of amino acids which are related in their side chains. Naturally occurring amino acids are generally divided into four families: acidic (aspartate, glutamate), basic (lysine, arginine, histidine), non-polar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), and uncharged polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine) amino acids. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In one embodiment, conservative amino acid substitutions include substitutions within the following groups:

glycine, alanine;

valine, isoleucine, leucine;

aspartic acid, glutamic acid;

asparagine, glutamine;

serine, threonine;

lysine, arginine; and

phenylalanine, tyrosine.

Preferably the degree of similarity, preferably identity between a given amino acid sequence and an amino acid sequence which is a variant of said given amino acid sequence will be at

least about 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%. The degree of similarity or identity is given preferably for an amino acid region which is at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or about 100% of the entire length of the reference amino acid sequence. For example, if the reference amino acid sequence consists of 200 amino acids, the degree of similarity or identity is given preferably for at least about 20, at least about 40, at least about 60, at least about 80, at least about 100, at least about 120, at least about 140, at least about 160, at least about 180, or about 200 amino acids, in some embodiments continuous amino acids. In some embodiments, the degree of similarity or identity is given for the entire length of the reference amino acid sequence. The alignment for determining sequence similarity, preferably sequence identity can be done with art known tools, preferably using the best sequence alignment, for example, using Align, using standard settings, preferably EMBOSS::needle, Matrix: Blosum62, Gap Open 10.0, Gap Extend 0.5.

"Sequence similarity" indicates the percentage of amino acids that either are identical or that represent conservative amino acid substitutions. "Sequence identity" between two amino acid sequences indicates the percentage of amino acids that are identical between the sequences. "Sequence identity" between two nucleic acid sequences indicates the percentage of nucleotides that are identical between the sequences.

The terms "% identical", "% identity" or similar terms are intended to refer, in particular, to the percentage of nucleotides or amino acids which are identical in an optimal alignment between the sequences to be compared. Said percentage is purely statistical, and the differences between the two sequences may be but are not necessarily randomly distributed over the entire length of the sequences to be compared. Comparisons of two sequences are usually carried out by comparing the sequences, after optimal alignment, with respect to a segment or "window of comparison", in order to identify local regions of corresponding sequences. The optimal alignment for a comparison may be carried out manually or with the

aid of the local homology algorithm by Smith and Waterman, 1981, *Adv. App. Math.* 2, 482, with the aid of the local homology algorithm by Needleman and Wunsch, 1970, *J. Mol. Biol.* 48, 443, with the aid of the similarity search algorithm by Pearson and Lipman, 1988, *Proc. Natl Acad. Sci. USA* 88, 2444, or with the aid of computer programs using said algorithms (GAP, BESTFIT, FASTA, BLAST P, BLAST N and TFASTA in Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.). In some embodiments, percent identity of two sequences is determined using the BLASTN or BLASTP algorithm, as available on the United States National Center for Biotechnology Information (NCBI) website (e.g., at blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq). In some embodiments, the algorithm parameters used for BLASTN algorithm on the NCBI website include: (i) Expect Threshold set to 10; (ii) Word Size set to 28; (iii) Max matches in a query range set to 0; (iv) Match/Mismatch Scores set to 1, -2; (v) Gap Costs set to Linear; and (vi) the filter for low complexity regions being used. In some embodiments, the algorithm parameters used for BLASTP algorithm on the NCBI website include: (i) Expect Threshold set to 10; (ii) Word Size set to 3; (iii) Max matches in a query range set to 0; (iv) Matrix set to BLOSUM62; (v) Gap Costs set to Existence: 11 Extension: 1; and (vi) conditional compositional score matrix adjustment.

Percentage identity is obtained by determining the number of identical positions at which the sequences to be compared correspond, dividing this number by the number of positions compared (e.g., the number of positions in the reference sequence) and multiplying this result by 100.

In some embodiments, the degree of similarity or identity is given for a region which is at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or about 100% of the entire length of the reference sequence. For example, if the reference nucleic acid sequence consists of 200 nucleotides, the degree of identity is given for at least about 100, at least about 120, at least about 140, at least about 160, at least about 180, or

about 200 nucleotides, in some embodiments continuous nucleotides. In some embodiments, the degree of similarity or identity is given for the entire length of the reference sequence.

Homologous amino acid sequences exhibit according to the disclosure at least 40%, in particular at least 50%, at least 60%, at least 70%, at least 80%, at least 90% and preferably at least 95%, at least 98 or at least 99% identity of the amino acid residues.

The amino acid sequence variants described herein may readily be prepared by the skilled person, for example, by recombinant DNA manipulation. The manipulation of DNA sequences for preparing peptides or proteins having substitutions, additions, insertions or deletions, is described in detail in Sambrook et al. (1989), for example. Furthermore, the peptides and amino acid variants described herein may be readily prepared with the aid of known peptide synthesis techniques such as, for example, by solid phase synthesis and similar methods.

In one embodiment, a fragment or variant of an amino acid sequence (peptide or protein) is preferably a "functional fragment" or "functional variant". The term "functional fragment" or "functional variant" of an amino acid sequence relates to any fragment or variant exhibiting one or more functional properties identical or similar to those of the amino acid sequence from which it is derived, i.e., it is functionally equivalent. With respect to sequences of binding agents such as antibodies, one particular function is one or more binding activities displayed by the amino acid sequence from which the fragment or variant is derived. The term "functional fragment" or "functional variant", as used herein, in particular refers to a variant molecule or sequence that comprises an amino acid sequence that is altered by one or more amino acids compared to the amino acid sequence of the parent molecule or sequence and that is still capable of fulfilling one or more of the functions of the parent molecule or sequence, e.g., binding to a target molecule. In one embodiment, the modifications in the amino acid sequence of the parent molecule or sequence do not significantly affect or alter the characteristics of the molecule or sequence. In different embodiments, the function of the functional fragment or functional variant may be reduced but still significantly present, e.g., binding of the functional variant may be at least 50%, at least 60%, at least 70%, at least 80%,

or at least 90% of the parent molecule or sequence. However, in other embodiments, binding of the functional fragment or functional variant may be enhanced compared to the parent molecule or sequence.

An amino acid sequence (peptide, protein or polypeptide) "derived from" a designated amino acid sequence (peptide, protein or polypeptide) refers to the origin of the first amino acid sequence. Preferably, the amino acid sequence which is derived from a particular amino acid sequence has an amino acid sequence that is identical, essentially identical or homologous to that particular sequence or a fragment thereof. Amino acid sequences derived from a particular amino acid sequence may be variants of that particular sequence or a fragment thereof. For example, it will be understood by one of ordinary skill in the art that the sequences suitable for use herein may be altered such that they vary in sequence from the naturally occurring or native sequences from which they were derived, while retaining the desirable activity of the native sequences.

As used herein, an "instructional material" or "instructions" includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the compositions and methods of the invention. The instructional material of the kit of the invention may, for example, be affixed to a container which contains the compositions of the invention or be shipped together with a container which contains the compositions. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the compositions be used cooperatively by the recipient.

"Isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated", but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is "isolated". An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

The term "recombinant" in the context of the present invention means "made through genetic

engineering". Preferably, a "recombinant object" such as a recombinant nucleic acid in the context of the present invention is not occurring naturally.

The term "naturally occurring" as used herein refers to the fact that an object can be found in nature. For example, a peptide or nucleic acid that is present in an organism (including viruses) and can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally occurring.

The term "bind" or "binding" relates to the non-covalent interaction with a target. In one embodiment, the term "bind" or "binding" relates to a specific binding. By the term "specific binding" or "specifically binds", as used herein, is meant a molecule such as an antibody or antigen receptor which recognizes a specific target molecule, but does not substantially recognize or bind other molecules in a sample or in a subject. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more other species. But, such cross-species reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific.

In some instances, the terms "specific binding" or "specifically binds", can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A", the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody.

"Physiological pH" as used herein refers to a pH of about 7.5.

The term "genetic modification" or simply "modification" includes the transfection of cells with nucleic acid. The term "transfection" relates to the introduction of nucleic acids, in

particular RNA, into a cell. For purposes of the present invention, the term "transfection" also includes the introduction of a nucleic acid into a cell or the uptake of a nucleic acid by such cell, wherein the cell may be present in a subject, e.g., a patient. Thus, according to the present invention, a cell for transfection of a nucleic acid described herein can be present *in vitro* or *in vivo*, e.g. the cell can form part of an organ, a tissue and/or an organism of a patient. According to the invention, transfection can be transient or stable. For some applications of transfection, it is sufficient if the transfected genetic material is only transiently expressed. RNA can be transfected into cells to transiently express its coded protein. Since the nucleic acid introduced in the transfection process is usually not integrated into the nuclear genome, the foreign nucleic acid will be diluted through mitosis or degraded. Cells allowing episomal amplification of nucleic acids greatly reduce the rate of dilution. If it is desired that the transfected nucleic acid actually remains in the genome of the cell and its daughter cells, a stable transfection must occur. Such stable transfection can be achieved by using virus-based systems or transposon-based systems for transfection. Generally, cells that are genetically modified to express an antigen receptor are stably transfected with nucleic acid encoding the antigen receptor. Generally, nucleic acid encoding an activation compound and/or nucleic acid encoding a docking compound is transiently transfected into cells. RNA can be transfected into cells to transiently express its coded protein.

Primary target

A "primary target" as used in the present invention relates to a target to be bound, or otherwise addressed, e.g., in a therapeutic method.

The primary target can be selected from any suitable targets within the human or animal body and can be a cell, pathogen or parasite or can be present on a cell, pathogen or parasite.

According to a particular embodiment, the primary target is a structure such as a protein present on the surface of a target cell such as a cell surface antigen or cell surface receptor. The primary target may be upregulated during a disease, e.g. infection or cancer. In diseased

tissues, markers can differ from healthy tissue and offer unique possibilities for therapy, especially targeted therapy.

In some embodiments the primary target or simply "target" is a disease-associated antigen, such as a tumor antigen, a viral antigen, or a bacterial antigen.

The term "disease-associated antigen" is used in its broadest sense to refer to any antigen associated with a disease. Disease-associated antigens may be associated with infection by microbes, typically microbial antigens, or associated with cancer, typically tumors.

In some embodiments the primary target or simply "target" is a tumor antigen. In the context of the present invention, the term "tumor antigen" or "tumor-associated antigen" relates to proteins that are under normal conditions specifically expressed in a limited number of tissues and/or organs or in specific developmental stages, for example, the tumor antigen may be under normal conditions specifically expressed in stomach tissue, preferably in the gastric mucosa, in reproductive organs, e.g., in testis, in trophoblastic tissue, e.g., in placenta, or in germ line cells, and are expressed or aberrantly expressed in one or more tumor or cancer tissues. In this context, "a limited number" preferably means not more than 3, more preferably not more than 2. The tumor antigens in the context of the present invention include, for example, differentiation antigens, preferably cell type specific differentiation antigens, i.e., proteins that are under normal conditions specifically expressed in a certain cell type at a certain differentiation stage, cancer/testis antigens, i.e., proteins that are under normal conditions specifically expressed in testis and sometimes in placenta, and germ line specific antigens. In the context of the present invention, the tumor antigen is preferably associated with the cell surface of a cancer cell and is preferably not or only rarely expressed in normal tissues. Preferably, the tumor antigen or the aberrant expression of the tumor antigen identifies cancer cells. In the context of the present invention, the tumor antigen that is expressed by a cancer cell in a subject, e.g., a patient suffering from a cancer disease, is preferably a self-protein in said subject. In preferred embodiments, the tumor antigen in the context of the present invention is expressed under normal conditions specifically in a tissue

or organ that is non-essential, i.e., tissues or organs which when damaged by the immune system do not lead to death of the subject, or in organs or structures of the body which are not or only hardly accessible by the immune system. Preferably, the amino acid sequence of the tumor antigen is identical between the tumor antigen which is expressed in normal tissues and the tumor antigen which is expressed in cancer tissues.

Examples for tumor antigens include p53, ART-4, BAGE, beta-catenin/m, Bcr-abL CAMEL, CAP-1, CASP-8, CDC27/m, CDK4/m, CEA, the cell surface proteins of the claudin family, such as CLAUDIN-6, CLAUDIN-18.2 and CLAUDIN-12, c-MYC, CT, Cyp-B, DAM, ELF2M, ETV6-AML1, G250, GAGE, GnT-V, Gap100, HAGE, HER-2/neu, HPV-E7, HPV-E6, HAST-2, hTERT (or hTRT), LAGE, LDLR/FUT, MAGE-A, preferably MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, or MAGE-A12, MAGE-B, MAGE-C, MART-1/Melan-A, MC1R, Myosin/m, MUC1, MUM-1, -2, -3, NA88-A, NF1, NY-ESO-1, NY-BR-1, p190 minor BCR-abL, Pm1/RARa, PRAME, proteinase 3, PSA, PSM, RAGE, RU1 or RU2, SAGE, SART-1 or SART-3, SCGB3A2, SCP1, SCP2, SCP3, SSX, SURVIVIN, TEL/AML1, TPI/m, TRP-1, TRP-2, TRP-2/INT2, TPTE and WT. Particularly preferred tumor antigens include CLAUDIN-18.2 (CLDN18.2) and CLAUDIN-6 (CLDN6).

According to the invention, immune effector cells may be delivered specifically to a target such as a target cell by providing the docketing compound with a moiety that binds to a target, e.g., an antigen on target cells. The docketing compound further comprises a binding moiety for immune effector cells for bringing a target and immune effector cells together.

Docketing compound

According to the invention, an RNA-encoded “docketing compound” is used to form a connection, such as a non-covalent connection, between a primary target, e.g., a target cell or an antigen on target cells, and the docketing compound. The docketing compound may form a connection, such as a non-covalent connection, to immune effector cells. The RNA-encoded docketing compound is also called “RiboDocker” herein.

In one embodiment, a docketing compound comprises a "primary targeting moiety", also referred to as "binding moiety binding to a target", in particular "binding moiety binding to a target on target cells", or "binding moiety binding to a target antigen" that is capable of binding to the primary target of interest. A "primary targeting moiety" as used in the present invention relates to the part of the docketing compound which binds to a primary target. Such targeting moieties are typically moieties that have affinity for cell surface targets (e.g., membrane receptors), or structural proteins (e.g., amyloid plaques). These moieties can be any peptide or protein (e.g. antibodies or antibody fragments) binding to the primary target. Particular embodiments of suitable primary targeting moieties for use herein include cell surface antigen binding peptides and antibodies. Other examples of primary targeting moieties are peptides or proteins which bind to a receptor.

A primary targeting moiety preferably binds with high specificity and/or high affinity and the bond with the primary target is preferably stable within the body.

In order to allow specific targeting of the above-listed primary targets, the primary targeting moiety of the docketing compound can comprise compounds including but not limited to antibodies, antibody fragments, e.g. Fab2, Fab, scFV, VHH domains, and other proteins or peptides.

According to a particular embodiment of the present invention, the primary target is a receptor and suitable primary targeting moieties include but are not limited to, the ligand of such a receptor or a part thereof which still binds to the receptor, e.g. a receptor binding peptide in the case of receptor binding protein ligands.

Other examples of primary targeting moieties of protein nature include interferons, e.g. alpha, beta, and gamma interferon, interleukins, and protein growth factors, such as transforming growth factor (TGF), or platelet-derived growth factor (PDGF).

According to a further particular embodiment of the invention, the primary target and primary targeting moiety are selected so as to result in the specific or increased targeting of a tissue or disease, such as cancer or an infection. This can be achieved by selecting primary targets

with tissue-, cell- or disease-specific expression. For example, tumor antigens may be overexpressed in various tumor cell types while they are not expressed or expressed in a lower amount in normal cells.

The docking compound further comprises a group which serves as a "secondary target", i.e. as the part of the docking compound that provides the binding partner for the immune effector cells. The binding moiety comprised in the docking compound binding to the immune effector cells ("secondary target") and the binding moiety (i.e., antigen receptor) comprised in the immune effector cells binding to the docking compound ("secondary targeting moiety") bind to each other.

In one embodiment, the docking compound comprises a fusion protein which comprises a binding domain binding to a primary target and a binding domain binding to a secondary targeting moiety on immune effector cells.

The term "fusion protein" as used herein refers to a polypeptide or protein comprising two or more subunits. Preferably, the fusion protein is a translational fusion between the two or more subunits. The translational fusion may be generated by genetically engineering the coding nucleotide sequence for one subunit in a reading frame with the coding nucleotide sequence of a further subunit. Subunits may be interspersed by a linker.

In one embodiment, the docking compound comprises a single peptide chain. In one embodiment, the single peptide chain comprises an antibody fragment binding to a primary target and a peptide moiety binding to a secondary targeting moiety (i.e., antigen receptor) on immune effector cells. In one embodiment, the antibody fragments are VHH, scFv, or a mixture thereof.

In one embodiment, the secondary target comprised in the docking compound comprises a peptide or protein, e.g., a peptide tag, and the secondary targeting moiety, i.e., antigen receptor, comprised in the immune effector cells comprises a binder, e.g., an antibody fragment, binding to the peptide or protein.

In one embodiment, the secondary target/secondary targeting moiety system used herein comprises an epitope tag/binder system.

In one embodiment, the epitope tag/binder system comprises an epitope tag comprising the sequence SRLEEELRRRLTE and the binder comprises a camelid VHH domain comprising the CDR1 sequence GVTISALNAMAMG, the CDR2 sequence AVSERGNAM, and the CDR3 sequence LEDRVDSFHDY. In one embodiment, the epitope tag/binder system comprises an epitope tag comprising the sequence SRLEEELRRRLTE and the binder comprises a camelid VHH domain comprising the amino acid sequence EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRVMVAASVSESGNAMYSRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVYYCHVLEDRVDSFHDYWGQGTQTVSS, an amino acid sequence having at least 99%, 98%, 97%, 96%, 95%, 90%, 85%, or 80% identity to said amino acid sequence, or a fragment of said amino acid sequence or the amino acid sequence having at least 99%, 98%, 97%, 96%, 95%, 90%, 85%, or 80% identity to said amino acid sequence.

As used herein, an “epitope tag” refers to a stretch of amino acids to which an antibody or proteinaceous molecule with antibody-like function can bind.

In one embodiment, the docketing compound comprises a signal peptide, e.g., an N-terminal signal peptide, which allows secretion of the docketing compound from the cell expressing the RNA.

Activation compound

The present disclosure further involves an activation compound for enhancing the efficiency of the immune effector cells such as T cells.

As described herein, antigen receptor-engineered immune effector cells may be provided to a subject by administering the antigen receptor-engineered immune effector cells or by generating the antigen receptor-engineered immune effector cells in the subject. In one embodiment, the antigen receptor-engineered immune effector cells are generated in the subject treated. Such *in vivo* generation generally will only provide small amounts of antigen

receptor-engineered immune effector cells in the subject. However, it is expected that these small amounts of antigen receptor-engineered immune effector cells will be therapeutically effective due to the strong stimulatory effect achieved by provision of an activation compound for the antigen receptor. Generally, antigen receptor-engineered immune effector cells may be provided to a subject in sub-therapeutic amounts, because the methods described herein further provide for the administration of an activation compound comprising a moiety to which the immune effector cells described herein bind to, wherein said binding results in activation and/or expansion of the immune effector cells. In one embodiment, the immune effector cells by means of an antigen receptor bind to the activation compound when present on cells of secondary lymphoid organs such as antigen presenting cells, in particular dendritic cells.

In one embodiment of all aspects of the invention, the RNA encoding the activation compound is expressed in cells of a subject to provide a moiety for binding by the antigen receptor expressed by immune effector cells, said binding resulting in stimulation, priming and/or expansion of the immune effector cells.

The activation compound comprises a moiety to which the immune effector cells can bind to. In certain embodiments, the activation compound comprises a moiety which corresponds to the secondary target of the docking compound or a fragment or variant thereof to which the immune effector cells are able to bind, e.g., a peptide tag as described herein.

The activation compound may further comprise a moiety which presents the moiety to which the immune effector cells can bind to on a cell surface, e.g., on the cell surface of antigen presenting cells. Such moiety may comprise a membrane protein, e.g., a transmembrane protein or receptor. Accordingly, the activation compound may comprise a fusion protein comprising a moiety to which the immune effector cells can bind to (e.g., a peptide tag) and a moiety which presents the moiety to which the immune effector cells can bind to on a cell surface (e.g., a membrane protein). The moiety to which the immune effector cells can bind to is generally present on the cell surface, i.e., on the cell exterior.

In one embodiment, RNA encoding an activation compound is administered to provide (following expression of the RNA by appropriate target cells) activation compound for stimulation, priming and/or expansion of immune effector cells. Immune effector cells such as T cells stimulated, primed and/or expanded in the patient are able to recognize via a docking compound a target cell population or target tissue expressing an antigen resulting in the eradication of diseased cells. In one embodiment, RNA encoding an activation compound is targeted to secondary lymphoid organs.

"Activation" or "stimulation", as used herein, refers to the state of an immune effector cell such as T cell that has been sufficiently stimulated to induce detectable cellular proliferation. Activation can also be associated with initiation of signaling pathways, induced cytokine production, and detectable effector functions. The term "activated immune effector cells" refers to, among other things, immune effector cells that are undergoing cell division.

The term "priming" refers to a process wherein an immune effector cell such as a T cell has its first contact with its specific antigen and causes differentiation into effector cells such as effector T cells.

The term "clonal expansion" or "expansion" refers to a process wherein a specific entity is multiplied. In the context of the present disclosure, the term is preferably used in the context of an immunological response in which lymphocytes are stimulated by an antigen, proliferate, and the specific lymphocyte recognizing said antigen is amplified. Preferably, clonal expansion leads to differentiation of the lymphocytes.

In general, the activation compound is expressed on the cell surface such that the antigen receptor binding moiety thereof is available for binding by the antigen receptor expressed by immune effector cells.

The term "expressed on the cell surface" or "associated with the cell surface" means that a molecule such as a receptor or antigen is associated with and located at the plasma membrane of a cell, wherein at least a part of the molecule faces the extracellular space of said cell and is accessible from the outside of said cell, e.g., by an antigen receptor located outside the cell.

In this context, a part is preferably at least 4, preferably at least 8, preferably at least 12, more preferably at least 20 amino acids. The association may be direct or indirect. For example, the association may be by one or more transmembrane domains, one or more lipid anchors, or by the interaction with any other protein, lipid, saccharide, or other structure that can be found on the outer leaflet of the plasma membrane of a cell. For example, a molecule associated with the surface of a cell may be a transmembrane protein having an extracellular portion or may be a protein associated with the surface of a cell by interacting with another protein that is a transmembrane protein.

"Cell surface" or "surface of a cell" is used in accordance with its normal meaning in the art, and thus includes the outside of the cell which is accessible to binding by proteins and other molecules. An antigen is expressed on the surface of cells if it is located at the surface of said cells and is accessible to binding by e.g. antigen-specific antibodies added to the cells. In one embodiment, an antigen expressed on the surface of cells is an integral membrane protein having an extracellular portion recognized by an antigen receptor.

The term "extracellular portion" or "exodomain" in the context of the present invention refers to a part of a molecule such as a protein that is facing the extracellular space of a cell and preferably is accessible from the outside of said cell, e.g., by binding molecules such as antibodies located outside the cell.

Immune effector cells

The immune effector cells used herein comprise a secondary targeting moiety. This secondary targeting moiety is also referred to herein as chimeric antigen receptor (CAR) or simply antigen receptor. The secondary targeting moiety relates to the part of the immune effector cells that forms the binding partner for the binding moiety for immune effector cells comprised in the activation compound and/or for the secondary target comprised in the docking compound. Immune effector cells are preferably capable of providing or bringing about the desired effect, e.g., therapeutic effect.

The immune effector cells used in connection with the present invention and into which nucleic acids (DNA or RNA) encoding antigen receptors may be introduced include, in particular, immune effector cells such as cells with lytic potential, in particular lymphoid cells, and are preferably T cells, in particular cytotoxic lymphocytes, preferably selected from cytotoxic T cells, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells. Upon activation, each of these cytotoxic lymphocytes triggers the destruction of target cells. For example, cytotoxic T cells trigger the destruction of target cells by either or both of the following means. First, upon activation T cells release cytotoxins such as perforin, granzymes, and granulysin. Perforin and granulysin create pores in the target cell, and granzymes enter the cell and trigger a caspase cascade in the cytoplasm that induces apoptosis (programmed cell death) of the cell. Second, apoptosis can be induced via Fas-Fas ligand interaction between the T cells and target cells. The cells used in connection with the present invention will preferably be autologous cells, although heterologous cells or allogenic cells can be used.

The term "effector functions" in the context of the present invention includes any functions mediated by components of the immune system that result, for example, in the killing of diseased cells such as tumor cells, or in the inhibition of tumor growth and/or inhibition of tumor development, including inhibition of tumor dissemination and metastasis. Preferably, the effector functions in the context of the present invention are T cell mediated effector functions. Such functions comprise in the case of a helper T cell (CD4⁺ T cell) the release of cytokines and/or the activation of CD8⁺ lymphocytes (CTLs) and/or B cells, and in the case of CTL the elimination of cells, i.e., cells characterized by expression of an antigen, for example, via apoptosis or perforin-mediated cell lysis, production of cytokines such as IFN- γ and TNF- α , and specific cytolytic killing of antigen expressing target cells.

The term "immune effector cell" or "immunoreactive cell" in the context of the present invention relates to a cell which exerts effector functions during an immune reaction. An "immune effector cell" in one embodiment is capable of binding an antigen (e.g., a peptide antigen) such as an antigen presented by an activation compound and a docking compound

as secondary target. For example, immune effector cells comprise T cells (cytotoxic T cells, helper T cells, tumor infiltrating T cells), B cells, natural killer cells, neutrophils, macrophages, and dendritic cells. Preferably, in the context of the present invention, "immune effector cells" are T cells, preferably CD4⁺ and/or CD8⁺ T cells, most preferably CD8⁺ T cells. According to the invention, the term "immune effector cell" also includes a cell which can mature into an immune cell (such as T cell, in particular T helper cell, or cytolytic T cell) with suitable stimulation. Immune effector cells comprise CD34⁺ hematopoietic stem cells, immature and mature T cells and immature and mature B cells. The differentiation of T cell precursors into a cytolytic T cell, when exposed to an antigen, is similar to clonal selection of the immune system.

The immune effector cells to be used according to the invention may express an endogenous antigen receptor such as T cell receptor or B cell receptor or may lack expression of an endogenous antigen receptor.

A "lymphoid cell" is a cell which, optionally after suitable modification, e.g. after transfer of an antigen receptor such as a TCR or a CAR, is capable of producing an immune response such as a cellular immune response, or a precursor cell of such cell, and includes lymphocytes, preferably T lymphocytes, lymphoblasts, and plasma cells. A lymphoid cell may be an immune effector cell as described herein. A preferred lymphoid cell is a T cell which can be modified to express an antigen receptor on the cell surface. In one embodiment, the lymphoid cell lacks endogenous expression of a T cell receptor.

The terms "T cell" and "T lymphocyte" are used interchangeably herein and include T helper cells (CD4⁺ T cells) and cytotoxic T cells (CTLs, CD8⁺ T cells) which comprise cytolytic T cells.

T cells belong to a group of white blood cells known as lymphocytes, and play a central role in cell-mediated immunity. They can be distinguished from other lymphocyte types, such as B cells and natural killer cells by the presence of a special receptor on their cell surface called T cell receptors (TCR). The thymus is the principal organ responsible for the maturation of T cells. Several different subsets of T cells have been discovered, each with a distinct function.

T helper cells assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and activation of cytotoxic T cells and macrophages, among other functions. These cells are also known as CD4⁺ T cells because they express the CD4 glycoprotein on their surface. Helper T cells become activated when they are presented with peptide antigens by MHC class II molecules that are expressed on the surface of antigen presenting cells (APCs). Once activated, they divide rapidly and secrete small proteins called cytokines that regulate or assist in the active immune response.

Cytotoxic T cells destroy virally infected cells and tumor cells, and are also implicated in transplant rejection. These cells are also known as CD8⁺ T cells since they express the CD8 glycoprotein on their surface. These cells recognize their targets by binding to antigen associated with MHC class I, which is present on the surface of nearly every cell of the body.

"Regulatory T cells" or "Tregs" are a subpopulation of T cells that modulate the immune system, maintain tolerance to self-antigens, and prevent autoimmune disease. Tregs are immunosuppressive and generally suppress or downregulate induction and proliferation of effector T cells. Tregs express the biomarkers CD4, FoxP3, and CD25.

As used herein, the term "naïve T cell" refers to mature T cells that, unlike activated or memory T cells, have not encountered their cognate antigen within the periphery. Naïve T cells are commonly characterized by the surface expression of L-selectin (CD62L), the absence of the activation markers CD25, CD44 or CD69 and the absence of the memory CD45RO isoform.

As used herein, the term "memory T cells" refers to a subgroup or subpopulation of T cells that have previously encountered and responded to their cognate antigen. At a second encounter with the antigen, memory T cells can reproduce to mount a faster and stronger immune response than the first time the immune system responded to the antigen. Memory T cells may be either CD4⁺ or CD8⁺ and usually express CD45RO.

According to the invention, the term "T cell" also includes a cell which can mature into a T cell with suitable stimulation.

A majority of T cells have a T cell receptor (TCR) existing as a complex of several proteins. The actual T cell receptor is composed of two separate peptide chains, which are produced from the independent T cell receptor alpha and beta (TCR α and TCR β) genes and are called α - and β -TCR chains. $\gamma\delta$ T cells (gamma delta T cells) represent a small subset of T cells that possess a distinct T cell receptor (TCR) on their surface. However, in $\gamma\delta$ T cells, the TCR is made up of one γ -chain and one δ -chain. This group of T cells is much less common (2% of total T cells) than the $\alpha\beta$ T cells.

All T cells originate from hematopoietic stem cells in the bone marrow. Hematopoietic progenitors derived from hematopoietic stem cells populate the thymus and expand by cell division to generate a large population of immature thymocytes. The earliest thymocytes express neither CD4 nor CD8, and are therefore classed as double-negative (CD4 $^-$ CD8 $^-$) cells. As they progress through their development they become double-positive thymocytes (CD4 $^+$ CD8 $^+$), and finally mature to single-positive (CD4 $^+$ CD8 $^-$ or CD4 $^-$ CD8 $^+$) thymocytes that are then released from the thymus to peripheral tissues.

T cells may generally be prepared *in vitro* or *ex vivo*, using standard procedures. For example, T cells may be isolated from bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood of a mammal, such as a patient, using a commercially available cell separation system. Alternatively, T cells may be derived from related or unrelated humans, non-human animals, cell lines or cultures. A sample comprising T cells may, for example, be peripheral blood mononuclear cells (PBMC).

As used herein, the term "NK cell" or "Natural Killer cell" refers to a subset of peripheral blood lymphocytes defined by the expression of CD56 or CD16 and the absence of the T cell receptor. As provided herein, the NK cell can also be differentiated from a stem cell or progenitor cell.

Antigen receptor

Immune effector cells described herein may be genetically modified *ex vivo/in vitro* or *in vivo* in a subject being treated to express an antigen receptor such as a chimeric antigen receptor

(CAR) binding antigen on an activation compound and on a docking compound, respectively. In one embodiment, modification to express an antigen receptor takes place *ex vivo/in vitro*. Subsequently, modified cells may be administered to a patient.

Adoptive cell transfer therapy with CAR-engineered T cells expressing chimeric antigen receptors is a promising anti-cancer therapeutic as CAR-modified T cells can be engineered to target virtually any tumor antigen, preferably in an MHC-independent manner. For example, patient's T cells may be genetically engineered (genetically modified) to express CARs specifically directed towards antigens on the patient's tumor cells, then infused back into the patient.

The disclosure described herein is not limited to the use of autologous immune effector cells. According to this disclosure, immune effector cells may be genetically modified to express an antigen receptor. Immune effector cells may be genetically modified *ex vivo* or *in vivo* to express an antigen receptor. Such genetic modification may be effected *ex vivo* or *in vitro* and subsequently the immune effector cells may be administered to a subject in need of treatment, or may be effected *in vivo* in a subject in need of treatment.

In various embodiments, immune effector cells either from a subject to be treated or from a different subject are administered to the subject to be treated. The administered immune effector cells may be genetically modified *ex vivo* prior to administration or genetically modified *in vivo* in the subject following administration to express an antigen receptor described herein. In one embodiment, the immune effector cells are endogenous in a subject to be treated (thus, are not administered to the subject to be treated) and are genetically modified *in vivo* in the subject to express an antigen receptor described herein.

According to the invention, the term "CAR" (or "chimeric antigen receptor") is synonymous with the terms "chimeric T cell receptor" and "artificial T cell receptor" and relates to an artificial receptor comprising a single molecule or a complex of molecules which recognizes, i.e. binds to, a target structure (e.g. an antigen) (e.g. by binding of an antigen binding domain to an antigen) and may confer specificity onto an immune effector cell such as a T cell

expressing said CAR on the cell surface. Such cells do not necessarily require processing and presentation of an antigen for recognition but rather may recognize preferably with specificity any antigen. Preferably, recognition of the target structure by a CAR results in activation of an immune effector cell expressing said CAR. A CAR may comprise one or more protein units said protein units comprising one or more domains as described herein. The term "CAR" does not include T cell receptors.

A CAR comprises a target-specific binding element otherwise referred to as an antigen binding moiety or antigen binding domain that is generally part of the extracellular domain of the CAR. Specifically, the CAR of the invention targets the antigen on an activation compound and on a docketing compound.

In one embodiment of the invention, an antigen binding domain comprises a variable region of a heavy chain of an immunoglobulin (VH) with a specificity for the antigen and a variable region of a light chain of an immunoglobulin (VL) with a specificity for the antigen. In one embodiment, an immunoglobulin is an antibody. In one embodiment, said heavy chain variable region (VH) and the corresponding light chain variable region (VL) are connected via a peptide linker. Preferably, the antigen binding moiety portion in the CAR is a scFv. In one embodiment of the invention, an antigen binding domain comprises a VHH domain.

The CAR is designed to comprise a transmembrane domain that is fused to the extracellular domain of the CAR. In one embodiment, the transmembrane domain is not naturally associated with one of the domains in the CAR. In one embodiment, the transmembrane domain is naturally associated with one of the domains in the CAR. In one embodiment, the transmembrane domain is modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex. The transmembrane domain may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. Transmembrane regions of particular use in this invention may be derived from (i.e. comprise

at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154. Alternatively the transmembrane domain may be synthetic, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. Preferably a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain.

In some instances, the CAR of the invention comprises a hinge domain which forms the linkage between the transmembrane domain and the extracellular domain.

The cytoplasmic domain or otherwise the intracellular signaling domain of the CAR is responsible for activation of at least one of the normal effector functions of the immune cell in which the CAR has been placed in. The term "effector function" refers to a specialized function of a cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines. Thus the term "intracellular signaling domain" refers to the portion of a protein which transduces the effector function signal and directs the cell to perform a specialized function. While usually the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the intracellular signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The term intracellular signaling domain is thus meant to include any truncated portion of the intracellular signaling domain sufficient to transduce the effector function signal.

It is known that signals generated through the TCR alone are insufficient for full activation of the T cell and that a secondary or co-stimulatory signal is also required. Thus, T cell activation can be said to be mediated by two distinct classes of cytoplasmic signaling sequence: those that initiate antigen-dependent primary activation through the TCR (primary cytoplasmic signaling sequences) and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal (secondary cytoplasmic signaling sequences).

In one embodiment, the CAR comprises a primary cytoplasmic signaling sequence derived from CD3-zeta. Further, the cytoplasmic domain of the CAR may comprise the CD3-zeta signaling domain combined with a costimulatory signaling region.

The identity of the co-stimulation domain is limited only in that it has the ability to enhance cellular proliferation and survival upon binding of the targeted moiety by the CAR. Suitable co-stimulation domains include CD28, CD137 (4-1BB), a member of the tumor necrosis factor receptor (TNFR) superfamily, CD134 (OX40), a member of the TNFR-superfamily of receptors, and CD278 (ICOS), a CD28-superfamily co-stimulatory molecule expressed on activated T cells. The skilled person will understand that sequence variants of these noted co-stimulation domains can be used without adversely impacting the invention, where the variants have the same or similar activity as the domain on which they are modeled. Such variants will have at least about 80% sequence identity to the amino acid sequence of the domain from which they are derived. In some embodiments of the invention, the CAR constructs comprise two co-stimulation domains. While the particular combinations include all possible variations of the four noted domains, specific examples include CD28+CD137 (4-1BB) and CD28+CD134 (OX40). The cytoplasmic signaling sequences within the cytoplasmic signaling portion of the CAR may be linked to each other in a random or specified order. Optionally, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage. A glycine-serine doublet provides a particularly suitable linker.

In one embodiment, the CAR comprises a signal peptide which directs the nascent protein into the endoplasmic reticulum. In one embodiment, the signal peptide precedes the antigen binding domain. In one embodiment, the signal peptide is derived from an immunoglobulin such as IgG.

A CAR may comprise the above domains, together in the form of a fusion protein. Such fusion proteins will generally comprise an antigen binding domain, one or more co-stimulation domains, and a signaling sequence, linked in a N-terminal to C-terminal direction. However, the CARs of the present invention are not limited to this arrangement and other arrangements

are acceptable and include a binding domain, a signaling domain, and one or more co-stimulation domains. It will be understood that because the binding domain must be free to bind antigen, the placement of the binding domain in the fusion protein will generally be such that display of the region on the exterior of the cell is achieved. In the same manner, because the co-stimulation and signaling domains serve to induce activity and proliferation of the cytotoxic lymphocytes, the fusion protein will generally display these two domains in the interior of the cell.

In one embodiment, a CAR molecule comprises:

- i) a target antigen (e.g., epitope tag) binding domain;
- ii) a transmembrane domain; and
- iii) an intracellular domain that comprises a 4-1BB costimulatory domain, and a CD3-zeta signaling domain.

In one embodiment, the antigen binding domain comprises an scFv. In one embodiment, the transmembrane domain comprises a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD154, KIRDS2, OX40, CD2, CD27, LFA-1 (CD11a, CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD40, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), CD160, CD19, IL2R beta, IL2R gamma, IL7Ra, ITGA1, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, DNAMI (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRT AM, Ly9 (CD229), CD160 (BY55), PSGLI, CD100 (SEMA4D), SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, PAG/Cbp, NKp44, NKp30, NKp46, NKG2D, and NKG2C, or a functional variant thereof. In one embodiment, the transmembrane domain comprises a CD8 α transmembrane domain. In one embodiment, the antigen binding domain is connected to the transmembrane domain by a hinge domain. In one embodiment, the hinge domain is a CD8 α hinge domain.

In one embodiment, the CAR molecule of the invention comprises:

- i) a target antigen binding domain;
- ii) a CD8 α hinge domain;
- iii) a CD8 α transmembrane domain; and
- iv) an intracellular domain that comprises a 4-1BB costimulatory domain, and a CD3-zeta signaling domain.

A variety of methods may be used to introduce antigen receptors such as CAR constructs into cells such as T cells to produce cells genetically modified to express the antigen receptors. Such methods including non-viral-based DNA transfection, non-viral-based RNA transfection, e.g., mRNA transfection, transposon-based systems, and viral-based systems. Non-viral-based DNA transfection has low risk of insertional mutagenesis. Transposon-based systems can integrate transgenes more efficiently than plasmids that do not contain an integrating element. Viral-based systems include the use of γ -retroviruses and lentiviral vectors. γ -Retroviruses are relatively easy to produce, efficiently and permanently transduce T cells, and have preliminarily proven safe from an integration standpoint in primary human T cells. Lentiviral vectors also efficiently and permanently transduce T cells but are more expensive to manufacture. They are also potentially safer than retrovirus based systems.

A "lentivirus" as used herein refers to a genus of the Retroviridae family. Lentiviruses are unique among the retroviruses in being able to infect non-dividing cells; they can deliver a significant amount of genetic information into the DNA of the host cell, so they are one of the most efficient methods of a gene delivery vector. HIV, SIV, and FIV are all examples of lentiviruses. Vectors derived from lentiviruses offer the means to achieve significant levels of gene transfer *in vivo*.

In one embodiment, T cells or T cell progenitors are transfected either *ex vivo* or *in vivo* with nucleic acid encoding the antigen receptor. In one embodiment, a combination of *ex vivo* and *in vivo* transfection may be used. In one embodiment, the T cells or T cell progenitors are from

the subject to be treated. In one embodiment of all aspects of the invention, the T cells or T cell progenitors are from a subject which is different to the subject to be treated.

CAR T cells may be produced *in vivo*, and therefore nearly instantaneously, using nanoparticles targeted to T cells. For example, poly(β -amino ester)-based nanoparticles may be coupled to anti-CD3e F(ab) fragments for binding to CD3 on T cells. Upon binding to T cells, these nanoparticles are endocytosed. Their contents, for example plasmid DNA encoding an anti-tumor antigen CAR, may be directed to the T cell nucleus due to the inclusion of peptides containing microtubule-associated sequences (MTAS) and nuclear localization signals (NLSs). The inclusion of transposons flanking the CAR gene expression cassette and a separate plasmid encoding a hyperactive transposase, may allow for the efficient integration of the CAR vector into chromosomes. Such system that allows for the *in vivo* production of CAR T cells following nanoparticle infusion is described in Smith et al. (2017) Nat. Nanotechnol. 12:813-820.

Furthermore, CD19-CAR T cells can be generated directly *in vivo* using the lentiviral vector CD8-LV specifically targeting human CD8⁺ cells (Pfeiffer A. et al., EMBO Mol. Med. Nov;10(11), 2018, 9158).

Another possibility is to use the CRISPR/Cas9 method to deliberately place a CAR coding sequence at a specific locus. For example, existing T cell receptors (TCR) may be knocked out, while knocking in the CAR and placing it under the dynamic regulatory control of the endogenous promoter that would otherwise moderate TCR expression; c.f., e.g., Eyquem et al. (2017) Nature 543:113-117.

In one embodiment, the cells genetically modified to express an antigen receptor are stably or transiently transfected with nucleic acid encoding the antigen receptor. Thus, the nucleic acid encoding the antigen receptor is integrated or not integrated into the genome of the cells. In one embodiment, the cells genetically modified to express an antigen receptor are inactivated for expression of an endogenous T cell receptor and/or endogenous HLA.

In one embodiment, the cells described herein may be autologous, allogeneic or syngeneic to the subject to be treated. In one embodiment, the present disclosure envisions the removal of cells from a patient and the subsequent re-delivery of the cells to the patient. In one embodiment, the present disclosure does not envision the removal of cells from a patient. In the latter case all steps of genetic modification of cells are performed *in vivo*.

The term "autologous" is used to describe anything that is derived from the same subject. For example, "autologous transplant" refers to a transplant of tissue or organs derived from the same subject. Such procedures are advantageous because they overcome the immunological barrier which otherwise results in rejection.

The term "allogeneic" is used to describe anything that is derived from different individuals of the same species. Two or more individuals are said to be allogeneic to one another when the genes at one or more loci are not identical.

The term "syngeneic" is used to describe anything that is derived from individuals or tissues having identical genotypes, i.e., identical twins or animals of the same inbred strain, or their tissues.

The term "heterologous" is used to describe something consisting of multiple different elements. As an example, the transfer of one individual's bone marrow into a different individual constitutes a heterologous transplant. A heterologous gene is a gene derived from a source other than the subject.

Binding moieties and agents

The present disclosure describes binding moieties or agents such as antibodies or antibody derivatives.

The term "epitope" refers to a part or fragment of a molecule or antigen that is recognized by a binding agent. For example, the epitope may be recognized by an antibody or any other binding protein. An epitope may include a continuous or discontinuous portion of the antigen and may be between about 5 and about 100, such as between about 5 and about 50, more

preferably between about 8 and about 30, most preferably between about 8 and about 25 amino acids in length, for example, the epitope may be preferably 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acids in length. In one embodiment, an epitope is between about 10 and about 25 amino acids in length. The term "epitope" includes structural epitopes.

The term "immunoglobulin" refers to a class of structurally related glycoproteins consisting of two pairs of polypeptide chains, one pair of light (L) low molecular weight chains and one pair of heavy (H) chains, all four inter-connected by disulfide bonds. The structure of immunoglobulins has been well characterized. See for instance Fundamental Immunology Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)). Briefly, each heavy chain typically is comprised of a heavy chain variable region (abbreviated herein as V_H or VH) and a heavy chain constant region (abbreviated herein as C_H or CH). The heavy chain constant region typically is comprised of three domains, $CH1$, $CH2$, and $CH3$. The hinge region is the region between the $CH1$ and $CH2$ domains of the heavy chain and is highly flexible. Disulphide bonds in the hinge region are part of the interactions between two heavy chains in an IgG molecule. Each light chain typically is comprised of a light chain variable region (abbreviated herein as V_L or VL) and a light chain constant region (abbreviated herein as C_L or CL). The light chain constant region typically is comprised of one domain, CL . The VH and VL regions may be further subdivided into regions of hypervariability (or hypervariable regions which may be hypervariable in sequence and/or form of structurally defined loops), also termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FRs). Each VH and VL is typically composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 (see also Chothia and Lesk J. Mol. Biol. 196, 901-917 (1987)). Unless otherwise stated or contradicted by context, reference to amino acid positions in the constant regions in the present invention is according to the EU-numbering (Edelman et al., Proc Natl Acad Sci U S A. 1969 May;63(1):78-85; Kabat et al., Sequences of Proteins of Immunological

Interest, Fifth Edition. 1991 NIH Publication No. 91-3242). In general, CDRs described herein are Kabat defined.

The term "amino acid corresponding to position..." as used herein refers to an amino acid position number in a human IgG1 heavy chain. Corresponding amino acid positions in other immunoglobulins may be found by alignment with human IgG1. Thus, an amino acid or segment in one sequence that "corresponds to" an amino acid or segment in another sequence is one that aligns with the other amino acid or segment using a standard sequence alignment program such as ALIGN, ClustalW or similar, typically at default settings and has at least 50%, at least 80%, at least 90%, or at least 95% identity to a human IgG1 heavy chain. It is considered well-known in the art how to align a sequence or segment in a sequence and thereby determine the corresponding position in a sequence to an amino acid position according to the present invention.

The term "antibody" (Ab) in the context of the present invention refers to an immunoglobulin molecule, a fragment of an immunoglobulin molecule, or a derivative of either thereof, which has the ability to bind, preferably specifically bind to an antigen. In one embodiment, binding takes place under typical physiological conditions with a half-life of significant periods of time, such as at least about 30 minutes, at least about 45 minutes, at least about one hour, at least about two hours, at least about four hours, at least about 8 hours, at least about 12 hours, about 24 hours or more, about 48 hours or more, about 3, 4, 5, 6, 7 or more days, etc., or any other relevant functionally-defined period (such as a time sufficient to induce, promote, enhance, and/or modulate a physiological response associated with antibody binding to the antigen). The variable regions of the heavy and light chains of the immunoglobulin molecule contain a binding domain that interacts with an antigen. The term "antigen-binding region", "binding region" or "binding domain", as used herein, refers to the region or domain which interacts with the antigen and typically comprises both a VH region and a VL region. The term antibody when used herein comprises not only monospecific antibodies, but also multispecific antibodies which comprise multiple, such as two or more, e.g. three or more, different

antigen-binding regions. The constant regions of the antibodies (Abs) may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (such as effector cells) and components of the complement system such as C1q, the first component in the classical pathway of complement activation. As indicated above, the term antibody as used herein, unless otherwise stated or clearly contradicted by context, includes fragments of an antibody that are antigen-binding fragments, *i.e.*, retain the ability to specifically bind to the antigen, and antibody derivatives, *i.e.*, constructs that are derived from an antibody. It has been shown that the antigen-binding function of an antibody may be performed by fragments of a full-length antibody. Examples of antigen-binding fragments encompassed within the term "antibody" include (i) a Fab' or Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains, or a monovalent antibody as described in WO2007059782 (Genmab); (ii) F(ab')₂ fragments, bivalent fragments comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting essentially of the VH and CH1 domains; (iv) a Fv fragment consisting essentially of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., *Nature* 341, 544-546 (1989)), which consists essentially of a VH domain and also called domain antibodies (Holt et al; *Trends Biotechnol.* 2003 Nov;21(11):484-90); (vi) camelid or Nanobody molecules (Revets et al; *Expert Opin Biol Ther.* 2005 Jan;5(1):111-24) and (vii) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they may be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain antibodies or single chain Fv (scFv), see for instance Bird et al., *Science* 242, 423-426 (1988) and Huston et al., *PNAS USA* 85, 5879-5883 (1988)). Such single chain antibodies are encompassed within the term antibody unless otherwise noted or clearly indicated by context. Although such fragments are generally included within the meaning of antibody, they collectively and each independently are unique features of the present invention, exhibiting

different biological properties and utility. These and other useful antibody fragments in the context of the present invention, as well as bispecific formats of such fragments, are discussed further herein. It also should be understood that the term antibody, unless specified otherwise, also includes polyclonal antibodies, monoclonal antibodies (mAbs), antibody-like polypeptides, such as chimeric antibodies and humanized antibodies, and antibody fragments retaining the ability to specifically bind to the antigen (antigen-binding fragments) provided by any known technique, such as enzymatic cleavage, peptide synthesis, and recombinant techniques.

The phrase "single chain Fv" or "scFv" refers to an antibody in which the variable domains of the heavy chain and of the light chain (V_H and V_L) of a traditional two chain antibody have been joined to form one chain. Optionally, a linker (usually a peptide) is inserted between the two chains to allow for proper folding and creation of an active binding site.

A single-domain antibody, also known as a nanobody, is an antibody fragment consisting of a single monomeric variable antibody domain. In one embodiment, a single-domain antibody is a variable domain (V_H) of a heavy-chain antibody. These are called VHH fragments. Like a whole antibody, a single-domain antibody is able to bind selectively to a specific antigen. The first single-domain antibodies were engineered from heavy-chain antibodies found in camelids. Cartilaginous fishes also have heavy-chain antibodies (IgNAR, 'immunoglobulin new antigen receptor'), from which single-domain antibodies called VNAR fragments can be obtained. An alternative approach is to split the dimeric variable domains from common immunoglobulin G (IgG) from humans or mice into monomers. Although most research into single-domain antibodies is currently based on heavy chain variable domains, nanobodies derived from light chains have also been shown to bind specifically to target epitopes.

An antibody can possess any isotype. As used herein, the term "isotype" refers to the immunoglobulin class (for instance IgG1, IgG2, IgG3, IgG4, IgD, IgA, IgE, or IgM) that is encoded by heavy chain constant region genes. When a particular isotype, e.g. IgG1, is mentioned herein, the term is not limited to a specific isotype sequence, e.g. a particular IgG1 sequence,

but is used to indicate that the antibody is closer in sequence to that isotype, e.g. IgG1, than to other isotypes. Thus, e.g. an IgG1 antibody of the invention may be a sequence variant of a naturally-occurring IgG1 antibody, including variations in the constant regions.

In various embodiments, an antibody is an IgG1 antibody, more particularly an IgG1, kappa or IgG1, lambda isotype (i.e. IgG1, κ , λ), an IgG2a antibody (e.g. IgG2a, κ , λ), an IgG2b antibody (e.g. IgG2b, κ , λ), an IgG3 antibody (e.g. IgG3, κ , λ) or an IgG4 antibody (e.g. IgG4, κ , λ).

The term "monoclonal antibody" as used herein refers to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. Accordingly, the term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions derived from human germline immunoglobulin sequences. The human monoclonal antibodies may be generated by a hybridoma which includes a B cell obtained from a transgenic or transchromosomal non-human animal, such as a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene, fused to an immortalized cell.

The term "chimeric antibody" as used herein, refers to an antibody wherein the variable region is derived from a non-human species (e.g. derived from rodents) and the constant region is derived from a different species, such as human. Chimeric monoclonal antibodies for therapeutic applications are developed to reduce antibody immunogenicity. The terms "variable region" or "variable domain" as used in the context of chimeric antibodies, refer to a region which comprises the CDRs and framework regions of both the heavy and light chains of the immunoglobulin. Chimeric antibodies may be generated by using standard DNA techniques as described in Sambrook et al., 1989, *Molecular Cloning: A laboratory Manual*, New York: Cold Spring Harbor Laboratory Press, Ch. 15. The chimeric antibody may be a genetically or an enzymatically engineered recombinant antibody. It is within the knowledge of the skilled person to generate a chimeric antibody, and thus, generation of the chimeric

antibody according to the present invention may be performed by other methods than described herein.

The term "humanized antibody" as used herein, refers to a genetically engineered non-human antibody, which contains human antibody constant domains and non-human variable domains modified to contain a high level of sequence homology to human variable domains. This can be achieved by grafting of the six non-human antibody complementarity-determining regions (CDRs), which together form the antigen binding site, onto a homologous human acceptor framework region (FR) (see WO92/22653 and EP0629240). In order to fully reconstitute the binding affinity and specificity of the parental antibody, the substitution of framework residues from the parental antibody (i.e. the non-human antibody) into the human framework regions (back-mutations) may be required. Structural homology modeling may help to identify the amino acid residues in the framework regions that are important for the binding properties of the antibody. Thus, a humanized antibody may comprise non-human CDR sequences, primarily human framework regions optionally comprising one or more amino acid back-mutations to the non-human amino acid sequence, and fully human constant regions. Optionally, additional amino acid modifications, which are not necessarily back-mutations, may be applied to obtain a humanized antibody with preferred characteristics, such as affinity and biochemical properties.

The term "human antibody" as used herein, refers to antibodies having variable and constant regions derived from human germline immunoglobulin sequences. Human antibodies may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse or rat, have been grafted onto human framework sequences. Human monoclonal antibodies can be produced by a variety of techniques, including conventional monoclonal antibody methodology, e.g., the standard somatic cell hybridization technique of

Kohler and Milstein, *Nature* 256: 495 (1975). Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can be employed, *e.g.*, viral or oncogenic transformation of B-lymphocytes or phage display techniques using libraries of human antibody genes. A suitable animal system for preparing hybridomas that secrete human monoclonal antibodies is the murine system. Hybridoma production in the mouse is a very well established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (*e.g.*, murine myeloma cells) and fusion procedures are also known. Human monoclonal antibodies can thus *e.g.* be generated using transgenic or transchromosomal mice or rats carrying parts of the human immune system rather than the mouse or rat system. Accordingly, in one embodiment, a human antibody is obtained from a transgenic animal, such as a mouse or a rat, carrying human germline immunoglobulin sequences instead of animal immunoglobulin sequences. In such embodiments, the antibody originates from human germline immunoglobulin sequences introduced in the animal, but the final antibody sequence is the result of said human germline immunoglobulin sequences being further modified by somatic hypermutations and affinity maturation by the endogenous animal antibody machinery, see *e.g.* Mendez et al. 1997 *Nat Genet.* 15(2):146-56.

When used herein, unless contradicted by context, the term “Fab-arm”, “binding arm” or “arm” includes one heavy chain-light chain pair and is used interchangeably with “half-molecule” herein.

The term “full-length” when used in the context of an antibody indicates that the antibody is not a fragment, but contains all of the domains of the particular isotype normally found for that isotype in nature, *e.g.* the VH, CH1, CH2, CH3, hinge, VL and CL domains for an IgG1 antibody.

When used herein, unless contradicted by context, the term “Fc region” refers to an antibody region consisting of the two Fc sequences of the heavy chains of an immunoglobulin, wherein said Fc sequences comprise at least a hinge region, a CH2 domain, and a CH3 domain.

As used herein, the terms "binding" or "capable of binding" in the context of the binding of an antibody to a predetermined antigen or epitope typically is a binding with an affinity corresponding to a K_D of about 10^{-7} M or less, such as about 10^{-8} M or less, such as about 10^{-9} M or less, about 10^{-10} M or less, or about 10^{-11} M or even less, when determined using Bio-Layer Interferometry (BLI), or, for instance, when determined using surface plasmon resonance (SPR) technology in a BIAcore 3000 instrument using the antigen as the ligand and the antibody as the analyte. The antibody binds to the predetermined antigen with an affinity corresponding to a K_D that is at least ten-fold lower, such as at least 100-fold lower, for instance at least 1,000-fold lower, such as at least 10,000-fold lower, for instance at least 100,000-fold lower than its affinity for binding to a non-specific antigen (e.g., BSA, casein) other than the predetermined antigen or a closely-related antigen. The amount with which the affinity is lower is dependent on the K_D of the antibody, so that when the K_D of the antibody is very low (that is, the antibody is highly specific), then the degree to which the affinity for the antigen is lower than the affinity for a non-specific antigen may be at least 10,000-fold. The term " k_d " (sec^{-1}), as used herein, refers to the dissociation rate constant of a particular antibody-antigen interaction. Said value is also referred to as the k_{off} value.

The term " K_D " (M), as used herein, refers to the dissociation equilibrium constant of a particular antibody-antigen interaction.

The present invention also envisions antibodies comprising functional variants of the VL regions, VH regions, or one or more CDRs of the antibodies described herein. A functional variant of a VL, VH, or CDR used in the context of an antibody still allows the antibody to retain at least a substantial proportion (at least about 50%, 60%, 70%, 80%, 90%, 95% or more) of the affinity and/or the specificity/selectivity of the "reference" or "parent" antibody and in some cases, such an antibody may be associated with greater affinity, selectivity and/or specificity than the parent antibody.

Such functional variants typically retain significant sequence identity to the parent antibody.

Exemplary variants include those which differ from VH and/or VL and/or CDR regions of the parent antibody sequences mainly by conservative substitutions; for instance, up to 10, such as 9, 8, 7, 6, 5, 4, 3, 2 or 1 of the substitutions in the variant are conservative amino acid residue replacements.

Functional variants of antibody sequences described herein such as VL regions, or VH regions, or antibody sequences having a certain degree of homology or identity to antibody sequences described herein such as VL regions, or VH regions preferably comprise modifications or variations in the non-CDR sequences, while the CDR sequences preferably remain unchanged. The term "specificity" as used herein is intended to have the following meaning unless contradicted by context. Two antibodies have the "same specificity" if they bind to the same antigen and the same epitope.

The term "competes" and "competition" may refer to the competition between a first antibody and a second antibody to the same antigen. Alternatively "competes" and "competition" may also refer to the competition between an antibody and an endogenous ligand for binding to the corresponding receptor of the endogenous ligand. If an antibody prevents the binding of the endogenous ligand to its receptor, such an antibody is said to block the endogenous interaction of the ligand with its receptor and therefore is competing with the endogenous ligand. It is well known to a person skilled in the art how to test for competition of antibodies for binding to a target antigen. An example of such a method is a so-called cross-competition assay, which may e.g. be performed as an ELISA or by flow-cytometry. Alternatively, competition may be determined using biolayer interferometry.

Antibodies which compete for binding to a target antigen may bind different epitopes on the antigen, wherein the epitopes are so close to each other that a first antibody binding to one epitope prevents binding of a second antibody to the other epitope. In other situations, however, two different antibodies may bind the same epitope on the antigen and would compete for binding in a competition binding assay. Such antibodies binding to the same epitope are considered to have the same specificity herein. Thus, in one embodiment,

antibodies binding to the same epitope are considered to bind to the same amino acids on the target molecule. That antibodies bind to the same epitope on a target antigen may be determined by standard alanine scanning experiments or antibody-antigen crystallization experiments known to a person skilled in the art. Preferably, antibodies or binding domains binding to different epitopes are not competing with each other for binding to their respective epitopes.

As described above, various formats of antibodies have been described in the art. The binding agent of the invention can in principle comprise an antibody of any isotype. The choice of isotype typically will be guided by the desired Fc-mediated effector functions, such as ADCC induction, or the requirement for an antibody devoid of Fc-mediated effector function ("inert" antibody). Exemplary isotypes are IgG1, IgG2, IgG3, and IgG4. Either of the human light chain constant regions, kappa or lambda, may be used. The effector function of the antibodies of the present invention may be changed by isotype switching to, e.g., an IgG1, IgG2, IgG3, IgG4, IgD, IgA, IgE, or IgM antibody for various therapeutic uses. In one embodiment, both heavy chains of an antibody of the present invention are of the IgG1 isotype, for instance an IgG1, κ . Optionally, the heavy chain may be modified in the hinge and/or CH3 region as described elsewhere herein.

Preferably, each of the antigen-binding regions or domains comprises a heavy chain variable region (VH) and a light chain variable region (VL), and wherein said variable regions each comprise three CDR sequences, CDR1, CDR2 and CDR3, respectively, and four framework sequences, FR1, FR2, FR3 and FR4, respectively. Furthermore, preferably, the antibody comprises two heavy chain constant regions (CH), and two light chain constant regions (CL). In one embodiment, the binding agent comprises a full-length antibody, such as a full-length IgG1 antibody.

In other embodiment, the binding agent comprises an antibody fragment, such as a Fab' or Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains, a monovalent antibody as described in WO2007059782 (Genmab), a F(ab')₂ fragment, a Fd

fragment, a Fv fragment, a dAb fragment, camelid or nanobodies, or an isolated complementarity determining region (CDR).

The term "binding agent" in the context of the present invention refers to any agent capable of binding to desired antigens. In certain embodiments of the invention, the binding agent is or comprises an antibody, antibody fragment, or any other binding protein, or any combination thereof.

The term "binding moiety" in the context of the present invention refers to any moiety, group or domain capable of binding to desired antigens. In certain embodiments of the invention, the binding moiety is or comprises an antibody, antibody fragment, or any other binding protein, or any combination thereof.

Nucleic acids

The term "polynucleotide" or "nucleic acid", as used herein, is intended to include DNA and RNA such as genomic DNA, cDNA, mRNA, recombinantly produced and chemically synthesized molecules. A nucleic acid may be single-stranded or double-stranded. RNA includes *in vitro* transcribed RNA (IVT RNA) or synthetic RNA. According to the invention, a polynucleotide is preferably isolated.

Nucleic acids may be comprised in a vector. The term "vector" as used herein includes any vectors known to the skilled person including plasmid vectors, cosmid vectors, phage vectors such as lambda phage, viral vectors such as retroviral, adenoviral or baculoviral vectors, or artificial chromosome vectors such as bacterial artificial chromosomes (BAC), yeast artificial chromosomes (YAC), or P1 artificial chromosomes (PAC). Said vectors include expression as well as cloning vectors. Expression vectors comprise plasmids as well as viral vectors and generally contain a desired coding sequence and appropriate DNA sequences necessary for the expression of the operably linked coding sequence in a particular host organism (e.g., bacteria, yeast, plant, insect, or mammal) or in *in vitro* expression systems. Cloning vectors are generally used to engineer and amplify a certain desired DNA fragment and may lack

functional sequences needed for expression of the desired DNA fragments.

In one embodiment of all aspects of the invention, the RNA encoding the activation compound, described herein is expressed in cells of the subject treated to provide the activation compound. If an activation compound comprises more than one polypeptide chain the different polypeptide chains may be encoded by the same or different RNA molecules.

In one embodiment of all aspects of the invention, the RNA encoding the docketing compound, described herein is expressed in cells of the subject treated to provide the docketing compound. If a docketing compound comprises more than one polypeptide chain the different polypeptide chains may be encoded by the same or different RNA molecules.

The nucleic acids described herein may be recombinant and/or isolated molecules.

In the present disclosure, the term "RNA" relates to a nucleic acid molecule which includes ribonucleotide residues. In preferred embodiments, the RNA contains all or a majority of ribonucleotide residues. As used herein, "ribonucleotide" refers to a nucleotide with a hydroxyl group at the 2'-position of a β -D-ribofuranosyl group. RNA encompasses without limitation, double stranded RNA, single stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as modified RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations may refer to addition of non-nucleotide material to internal RNA nucleotides or to the end(s) of RNA. It is also contemplated herein that nucleotides in RNA may be non-standard nucleotides, such as chemically synthesized nucleotides or deoxynucleotides. For the present disclosure, these altered RNAs are considered analogs of naturally-occurring RNA.

In certain embodiments of the present disclosure, the RNA is messenger RNA (mRNA) that relates to a RNA transcript which encodes a peptide or protein. As established in the art, mRNA generally contains a 5' untranslated region (5'-UTR), a peptide coding region and a 3' untranslated region (3'-UTR). In some embodiments, the RNA is produced by *in vitro* transcription or chemical synthesis. In one embodiment, the mRNA is produced by *in vitro*

transcription using a DNA template where DNA refers to a nucleic acid that contains deoxyribonucleotides.

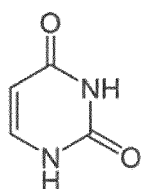
In one embodiment, RNA is *in vitro* transcribed RNA (IVT-RNA) and may be obtained by *in vitro* transcription of an appropriate DNA template. The promoter for controlling transcription can be any promoter for any RNA polymerase. A DNA template for *in vitro* transcription may be obtained by cloning of a nucleic acid, in particular cDNA, and introducing it into an appropriate vector for *in vitro* transcription. The cDNA may be obtained by reverse transcription of RNA.

In certain embodiments of the present disclosure, the RNA is "replicon RNA" or simply a "replicon", in particular "self-replicating RNA" or "self-amplifying RNA". In one particularly preferred embodiment, the replicon or self-replicating RNA is derived from or comprises elements derived from a ssRNA virus, in particular a positive-stranded ssRNA virus such as an alphavirus. Alphaviruses are typical representatives of positive-stranded RNA viruses. Alphaviruses replicate in the cytoplasm of infected cells (for review of the alphaviral life cycle see José et al., *Future Microbiol.*, 2009, vol. 4, pp. 837–856). The total genome length of many alphaviruses typically ranges between 11,000 and 12,000 nucleotides, and the genomic RNA typically has a 5'-cap, and a 3' poly(A) tail. The genome of alphaviruses encodes non-structural proteins (involved in transcription, modification and replication of viral RNA and in protein modification) and structural proteins (forming the virus particle). There are typically two open reading frames (ORFs) in the genome. The four non-structural proteins (nsP1–nsP4) are typically encoded together by a first ORF beginning near the 5' terminus of the genome, while alphavirus structural proteins are encoded together by a second ORF which is found downstream of the first ORF and extends near the 3' terminus of the genome. Typically, the first ORF is larger than the second ORF, the ratio being roughly 2:1. In cells infected by an alphavirus, only the nucleic acid sequence encoding non-structural proteins is translated from the genomic RNA, while the genetic information encoding structural proteins is translatable from a subgenomic transcript, which is an RNA molecule that resembles eukaryotic messenger RNA (mRNA; Gould et al., 2010, *Antiviral Res.*, vol. 87 pp. 111–124). Following infection, i.e. at

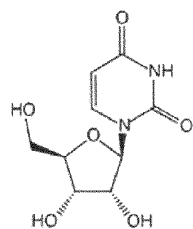
early stages of the viral life cycle, the (+) stranded genomic RNA directly acts like a messenger RNA for the translation of the open reading frame encoding the non-structural poly-protein (nsP1234). Alphavirus-derived vectors have been proposed for delivery of foreign genetic information into target cells or target organisms. In simple approaches, the open reading frame encoding alphaviral structural proteins is replaced by an open reading frame encoding a protein of interest. Alphavirus-based trans-replication systems rely on alphavirus nucleotide sequence elements on two separate nucleic acid molecules: one nucleic acid molecule encodes a viral replicase, and the other nucleic acid molecule is capable of being replicated by said replicase in trans (hence the designation trans-replication system). Trans-replication requires the presence of both these nucleic acid molecules in a given host cell. The nucleic acid molecule capable of being replicated by the replicase in trans must comprise certain alphaviral sequence elements to allow recognition and RNA synthesis by the alphaviral replicase.

In one embodiment, the RNA described herein may have modified nucleosides. In some embodiments, the RNA comprises a modified nucleoside in place of at least one (e.g. every) uridine.

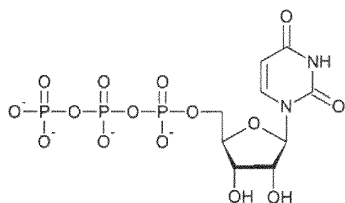
The term "uracil," as used herein, describes one of the nucleobases that can occur in the nucleic acid of RNA. The structure of uracil is:



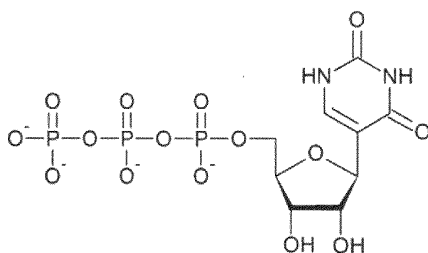
The term "uridine," as used herein, describes one of the nucleosides that can occur in RNA. The structure of uridine is:



UTP (uridine 5'-triphosphate) has the following structure:

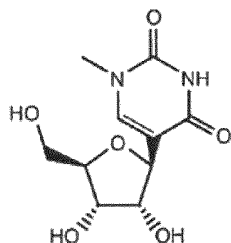


Pseudo-UTP (pseudouridine 5'-triphosphate) has the following structure:

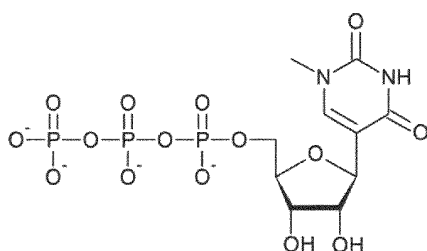


"Pseudouridine" is one example of a modified nucleoside that is an isomer of uridine, where the uracil is attached to the pentose ring via a carbon-carbon bond instead of a nitrogen-carbon glycosidic bond.

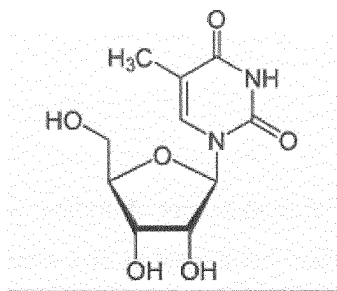
Another exemplary modified nucleoside is N1-methyl-pseudouridine (m1Ψ), which has the structure:



N1-methyl-pseudo-UTP has the following structure:



Another exemplary modified nucleoside is 5-methyl-uridine (m5U), which has the structure:



In some embodiments, one or more uridine in the RNA described herein is replaced by a modified nucleoside. In some embodiments, the modified nucleoside is a modified uridine. In some embodiments, RNA comprises a modified nucleoside in place of at least one uridine. In some embodiments, RNA comprises a modified nucleoside in place of each uridine. In some embodiments, the modified nucleoside is independently selected from pseudouridine (ψ), N1-methyl-pseudouridine (m1 ψ), and 5-methyl-uridine (m5U). In some embodiments, the modified nucleoside comprises pseudouridine (ψ). In some embodiments, the modified nucleoside comprises N1-methyl-pseudouridine (m1 ψ). In some embodiments, the modified

nucleoside comprises 5-methyl-uridine (m5U). In some embodiments, RNA may comprise more than one type of modified nucleoside, and the modified nucleosides are independently selected from pseudouridine (ψ), N1-methyl-pseudouridine (m1 ψ), and 5-methyl-uridine (m5U). In some embodiments, the modified nucleosides comprise pseudouridine (ψ) and N1-methyl-pseudouridine (m1 ψ). In some embodiments, the modified nucleosides comprise pseudouridine (ψ) and 5-methyl-uridine (m5U). In some embodiments, the modified nucleosides comprise N1-methyl-pseudouridine (m1 ψ) and 5-methyl-uridine (m5U). In some embodiments, the modified nucleosides comprise pseudouridine (ψ), N1-methyl-pseudouridine (m1 ψ), and 5-methyl-uridine (m5U).

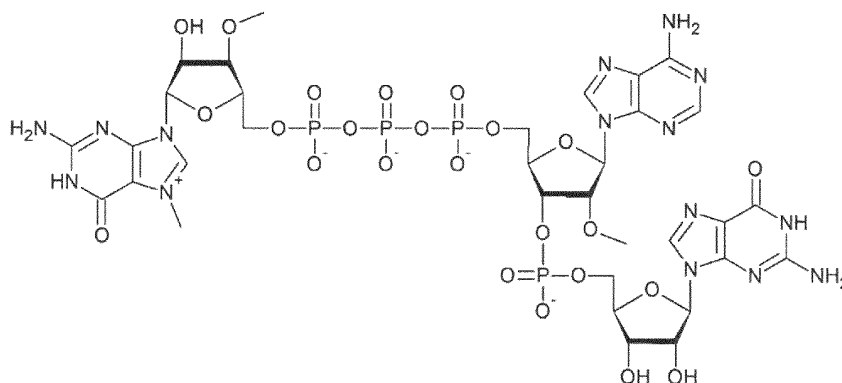
In some embodiments, the modified nucleoside replacing one or more, e.g., all, uridine in the RNA may be any one or more of 3-methyl-uridine (m³U), 5-methoxy-uridine (mo⁵U), 5-aza-uridine, 6-aza-uridine, 2-thio-5-aza-uridine, 2-thio-uridine (s²U), 4-thio-uridine (s⁴U), 4-thio-pseudouridine, 2-thio-pseudouridine, 5-hydroxy-uridine (ho⁵U), 5-aminoallyl-uridine, 5-halo-uridine (e.g., 5-iodo-uridine or 5-bromo-uridine), uridine 5-oxyacetic acid (cmo⁵U), uridine 5-oxyacetic acid methyl ester (mcmo⁵U), 5-carboxymethyl-uridine (cm⁵U), 1-carboxymethyl-pseudouridine, 5-carboxyhydroxymethyl-uridine (chm⁵U), 5-carboxyhydroxymethyl-uridine methyl ester (mchm⁵U), 5-methoxycarbonylmethyl-uridine (mcm⁵U), 5-methoxycarbonylmethyl-2-thio-uridine (mcm⁵s²U), 5-aminomethyl-2-thio-uridine (nm⁵s²U), 5-methylaminomethyl-uridine (mnm⁵U), 1-ethyl-pseudouridine, 5-methylaminomethyl-2-thio-uridine (mnm⁵s²U), 5-methylaminomethyl-2-seleno-uridine (mnm⁵se²U), 5-carbamoylmethyl-uridine (ncm⁵U), 5-carboxymethylaminomethyl-uridine (cmnm⁵U), 5-carboxymethylaminomethyl-2-thio-uridine (cmnm⁵s²U), 5-propynyl-uridine, 1-propynyl-pseudouridine, 5-aurinomethyl-uridine (τ m⁵U), 1-aurinomethyl-pseudouridine, 5-aurinomethyl-2-thio-uridine (τ m⁵s²U), 1-aurinomethyl-4-thio-pseudouridine, 5-methyl-2-thio-uridine (m⁵s²U), 1-methyl-4-thio-pseudouridine (m¹s⁴ ψ), 4-thio-1-methyl-pseudouridine, 3-methyl-pseudouridine (m³ ψ), 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydrouridine (D),

dihydropseudouridine, 5,6-dihydrouridine, 5-methyl-dihydrouridine (m^5D), 2-thio-dihydrouridine, 2-thio-dihydropseudouridine, 2-methoxy-uridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, N1-methyl-pseudouridine, 3-(3-amino-3-carboxypropyl)uridine (acp^3U), 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine ($acp^3\psi$), 5-(isopentenylaminomethyl)uridine (inm^5U), 5-(isopentenylaminomethyl)-2-thio-uridine (inm^5s^2U), α -thio-uridine, 2'-O-methyl-uridine (Um), 5,2'-O-dimethyl-uridine (m^5Um), 2'-O-methyl-pseudouridine (ψm), 2-thio-2'-O-methyl-uridine (s^2Um), 5-methoxycarbonylmethyl-2'-O-methyl-uridine (mcm^5Um), 5-carbamoylmethyl-2'-O-methyl-uridine (ncm^5Um), 5-carboxymethylaminomethyl-2'-O-methyl-uridine ($cmnm^5Um$), 3,2'-O-dimethyl-uridine (m^3Um), 5-(isopentenylaminomethyl)-2'-O-methyl-uridine (inm^5Um), 1-thio-uridine, deoxythymidine, 2'-F-ara-uridine, 2'-F-uridine, 2'-OH-ara-uridine, 5-(2-carbomethoxyvinyl) uridine, 5-[3-(1-E-propenylamino)uridine, or any other modified uridine known in the art.

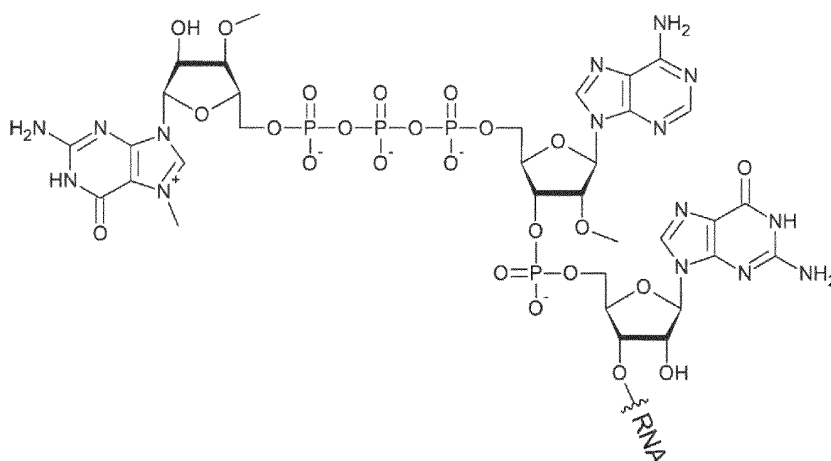
In one embodiment, the RNA comprises other modified nucleosides or comprises further modified nucleosides, e.g., modified cytidine. For example, in one embodiment, in the RNA 5-methylcytidine is substituted partially or completely, preferably completely, for cytidine. In one embodiment, the RNA comprises 5-methylcytidine and one or more selected from pseudouridine (ψ), N1-methyl-pseudouridine ($m1\psi$), and 5-methyl-uridine ($m5U$). In one embodiment, the RNA comprises 5-methylcytidine and N1-methyl-pseudouridine ($m1\psi$). In some embodiments, the RNA comprises 5-methylcytidine in place of each cytidine and N1-methyl-pseudouridine ($m1\psi$) in place of each uridine.

In some embodiments, the RNA according to the present disclosure comprises a 5'-cap. In one embodiment, the RNA of the present disclosure does not have uncapped 5'-triphosphates. In one embodiment, the RNA may be modified by a 5'-cap analog. The term "5'-cap" refers to a structure found on the 5'-end of an mRNA molecule and generally consists of a guanosine nucleotide connected to the mRNA via a 5'- to 5'-triphosphate linkage. In one embodiment, this guanosine is methylated at the 7-position. Providing an RNA with a 5'-cap or 5'-cap analog

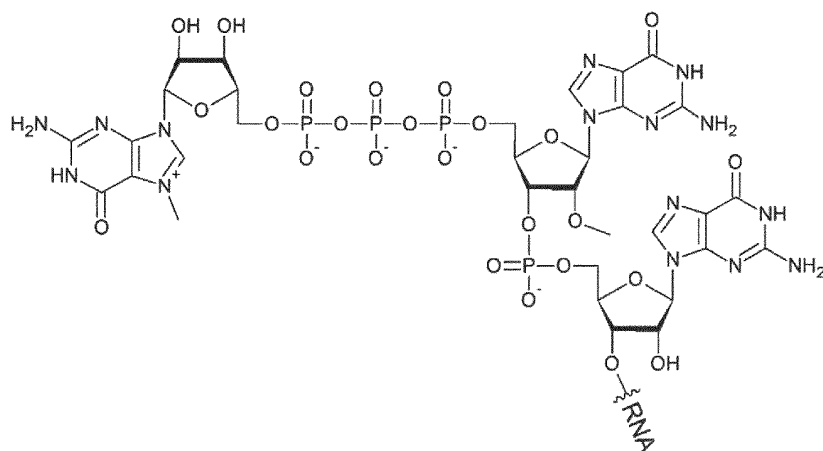
may be achieved by *in vitro* transcription, in which the 5'-cap is co-transcriptionally expressed into the RNA strand, or may be attached to RNA post-transcriptionally using capping enzymes. In some embodiments, the building block cap for RNA is $m_2^{7,3'-O}Gppp(m_1^{2'-O})ApG$ (also sometimes referred to as $m_2^{7,3'-O}G(5')ppp(5')m^{2'-O}ApG$), which has the following structure:



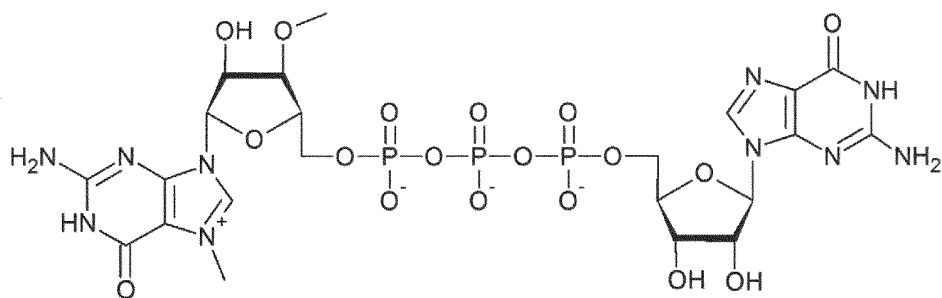
Below is an exemplary Cap1 RNA, which comprises RNA and $m_2^{7,3'-O}G(5')ppp(5')m^{2'-O}ApG$:



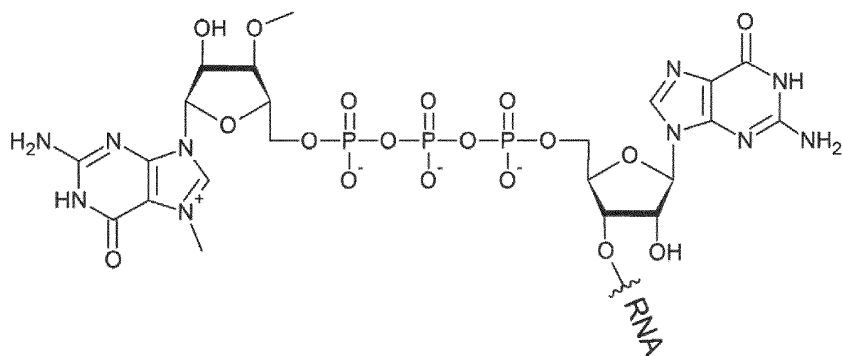
Below is another exemplary Cap1 RNA (no cap analog):



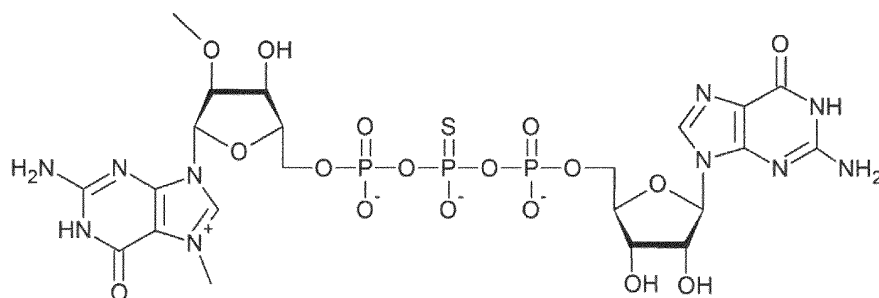
In some embodiments, the RNA is modified with "Cap0" structures using, in one embodiment, the cap analog anti-reverse cap (ARCA Cap ($m_2^{7,3'}G(5')ppp(5')G$)) with the structure:



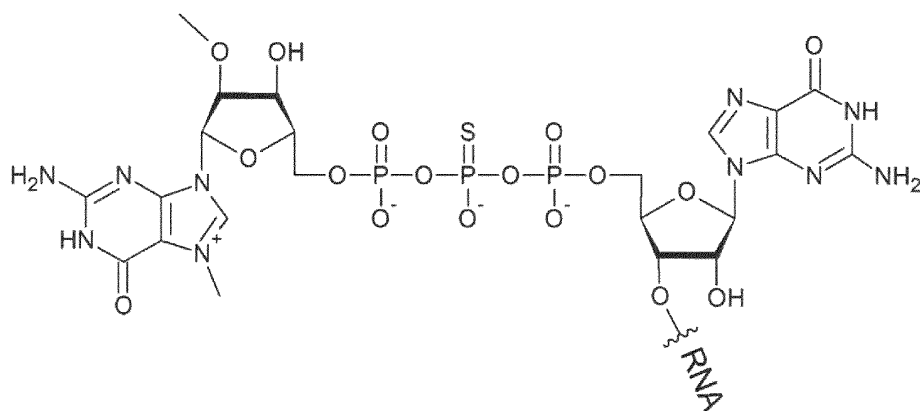
Below is an exemplary Cap0 RNA comprising RNA and $m_2^{7,3'}G(5')ppp(5')G$:



In some embodiments, the "Cap0" structures are generated using the cap analog Beta-S-ARCA ($m_2^{7,2'}G(5')ppSp(5')G$) with the structure:



Below is an exemplary Cap0 RNA comprising Beta-S-ARCA ($m_2^{7,2'}G(5')ppSp(5')G$) and RNA:



The "D1" diastereomer of beta-S-ARCA or "beta-S-ARCA(D1)" is the diastereomer of beta-S-ARCA which elutes first on an HPLC column compared to the D2 diastereomer of beta-S-ARCA (beta-S-ARCA(D2)) and thus exhibits a shorter retention time (cf., WO 2011/015347, herein incorporated by reference).

A particularly preferred cap is beta-S-ARCA(D1) ($m_2^{7,2'-O}GppSpG$) or $m_2^{7,3'-O}Gppp(m_1^{2'-O})ApG$.

In some embodiments, RNA according to the present disclosure comprises a 5'-UTR and/or a 3'-UTR. The term "untranslated region" or "UTR" relates to a region in a DNA molecule which is transcribed but is not translated into an amino acid sequence, or to the corresponding region in an RNA molecule, such as an mRNA molecule. An untranslated region (UTR) can be present 5' (upstream) of an open reading frame (5'-UTR) and/or 3' (downstream) of an open

reading frame (3'-UTR). A 5'-UTR, if present, is located at the 5' end, upstream of the start codon of a protein-encoding region. A 5'-UTR is downstream of the 5'-cap (if present), e.g. directly adjacent to the 5'-cap. A 3'-UTR, if present, is located at the 3' end, downstream of the termination codon of a protein-encoding region, but the term "3'-UTR" does preferably not include the poly(A) sequence. Thus, the 3'-UTR is upstream of the poly(A) sequence (if present), e.g. directly adjacent to the poly(A) sequence.

In some embodiments, RNA comprises a 5'-UTR comprising the nucleotide sequence of SEQ ID NO: 1, or a nucleotide sequence having at least 99%, 98%, 97%, 96%, 95%, 90%, 85%, or 80% identity to the nucleotide sequence of SEQ ID NO: 1.

In some embodiments, RNA comprises a 3'-UTR comprising the nucleotide sequence of SEQ ID NO: 2 or 3, or a nucleotide sequence having at least 99%, 98%, 97%, 96%, 95%, 90%, 85%, or 80% identity to the nucleotide sequence of SEQ ID NO: 2 or 3.

A particularly preferred 5'-UTR comprises the nucleotide sequence of SEQ ID NO: 1. A particularly preferred 3'-UTR comprises the nucleotide sequence of SEQ ID NO: 2 or 3.

In some embodiments, the RNA according to the present disclosure comprises a 3'-poly(A) sequence.

As used herein, the term "poly(A) sequence" or "poly-A tail" refers to an uninterrupted or interrupted sequence of adenylate residues which is typically located at the 3'-end of an RNA molecule. Poly(A) sequences are known to those of skill in the art and may follow the 3'-UTR in the RNAs described herein. An uninterrupted poly(A) sequence is characterized by consecutive adenylate residues. In nature, an uninterrupted poly(A) sequence is typical. RNAs disclosed herein can have a poly(A) sequence attached to the free 3'-end of the RNA by a template-independent RNA polymerase after transcription or a poly(A) sequence encoded by DNA and transcribed by a template-dependent RNA polymerase.

It has been demonstrated that a poly(A) sequence of about 120 A nucleotides has a beneficial influence on the levels of RNA in transfected eukaryotic cells, as well as on the levels of protein

that is translated from an open reading frame that is present upstream (5') of the poly(A) sequence (*Holtkamp et al.*, 2006, *Blood*, vol. 108, pp. 4009-4017).

The poly(A) sequence may be of any length. In some embodiments, a poly(A) sequence comprises, essentially consists of, or consists of at least 20, at least 30, at least 40, at least 80, or at least 100 and up to 500, up to 400, up to 300, up to 200, or up to 150 A nucleotides, and, in particular, about 120 A nucleotides. In this context, "essentially consists of" means that most nucleotides in the poly(A) sequence, typically at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% by number of nucleotides in the poly(A) sequence are A nucleotides, but permits that remaining nucleotides are nucleotides other than A nucleotides, such as U nucleotides (uridylate), G nucleotides (guanylate), or C nucleotides (cytidylate). In this context, "consists of" means that all nucleotides in the poly(A) sequence, i.e., 100% by number of nucleotides in the poly(A) sequence, are A nucleotides. The term "A nucleotide" or "A" refers to adenylate.

In some embodiments, a poly(A) sequence is attached during RNA transcription, e.g., during preparation of *in vitro* transcribed RNA, based on a DNA template comprising repeated dT nucleotides (deoxythymidylate) in the strand complementary to the coding strand. The DNA sequence encoding a poly(A) sequence (coding strand) is referred to as poly(A) cassette.

In some embodiments, the poly(A) cassette present in the coding strand of DNA essentially consists of dA nucleotides, but is interrupted by a random sequence of the four nucleotides (dA, dC, dG, and dT). Such random sequence may be 5 to 50, 10 to 30, or 10 to 20 nucleotides in length. Such a cassette is disclosed in WO 2016/005324 A1, hereby incorporated by reference. Any poly(A) cassette disclosed in WO 2016/005324 A1 may be used in the present invention. A poly(A) cassette that essentially consists of dA nucleotides, but is interrupted by a random sequence having an equal distribution of the four nucleotides (dA, dC, dG, dT) and having a length of e.g., 5 to 50 nucleotides shows, on DNA level, constant propagation of plasmid DNA in *E. coli* and is still associated, on RNA level, with the beneficial properties with respect to supporting RNA stability and translational efficiency is encompassed. Consequently,

in some embodiments, the poly(A) sequence contained in an RNA molecule described herein essentially consists of A nucleotides, but is interrupted by a random sequence of the four nucleotides (A, C, G, U). Such random sequence may be 5 to 50, 10 to 30, or 10 to 20 nucleotides in length.

In some embodiments, no nucleotides other than A nucleotides flank a poly(A) sequence at its 3'-end, i.e., the poly(A) sequence is not masked or followed at its 3'-end by a nucleotide other than A.

In some embodiments, the poly(A) sequence may comprise at least 20, at least 30, at least 40, at least 80, or at least 100 and up to 500, up to 400, up to 300, up to 200, or up to 150 nucleotides. In some embodiments, the poly(A) sequence may essentially consist of at least 20, at least 30, at least 40, at least 80, or at least 100 and up to 500, up to 400, up to 300, up to 200, or up to 150 nucleotides. In some embodiments, the poly(A) sequence may consist of at least 20, at least 30, at least 40, at least 80, or at least 100 and up to 500, up to 400, up to 300, up to 200, or up to 150 nucleotides. In some embodiments, the poly(A) sequence comprises at least 100 nucleotides. In some embodiments, the poly(A) sequence comprises about 150 nucleotides. In some embodiments, the poly(A) sequence comprises about 120 nucleotides.

In some embodiments, RNA comprises a poly(A) sequence comprising the nucleotide sequence of SEQ ID NO: 4, or a nucleotide sequence having at least 99%, 98%, 97%, 96%, 95%, 90%, 85%, or 80% identity to the nucleotide sequence of SEQ ID NO: 4.

A particularly preferred poly(A) sequence comprises comprises the nucleotide sequence of SEQ ID NO: 4.

According to the disclosure, an activation compound is preferably administered as single-stranded, 5'-capped mRNA that is translated into the respective protein upon entering cells of a subject being administered the RNA. Preferably, the RNA contains structural elements optimized for maximal efficacy of the RNA with respect to stability and translational efficiency (5'-cap, 5'-UTR, 3'-UTR, poly(A) sequence).

According to the disclosure, a docking compound is preferably administered as single-stranded, 5'-capped mRNA that is translated into the respective protein upon entering cells of a subject being administered the RNA. Preferably, the RNA contains structural elements optimized for maximal efficacy of the RNA with respect to stability and translational efficiency (5'-cap, 5'-UTR, 3'-UTR, poly(A) sequence).

In one embodiment, beta-S-ARCA(D1) is utilized as specific capping structure at the 5'-end of the RNA. In one embodiment, $m_2^{7,3'-O}Gppp(m_1^{2'-O})ApG$ is utilized as specific capping structure at the 5'-end of the RNA. In one embodiment, the 5'-UTR sequence is derived from the human alpha-globin mRNA and optionally has an optimized 'Kozak sequence' to increase translational efficiency. In one embodiment, a combination of two sequence elements (FI element) derived from the "amino terminal enhancer of split" (AES) mRNA (called F) and the mitochondrial encoded 12S ribosomal RNA (called I) are placed between the coding sequence and the poly(A) sequence to assure higher maximum protein levels and prolonged persistence of the mRNA. These were identified by an *ex vivo* selection process for sequences that confer RNA stability and augment total protein expression (see WO 2017/060314, herein incorporated by reference). In one embodiment, two re-iterated 3'-UTRs derived from the human beta-globin mRNA are placed between the coding sequence and the poly(A) sequence to assure higher maximum protein levels and prolonged persistence of the mRNA. In one embodiment, a poly(A) sequence measuring 110 nucleotides in length, consisting of a stretch of 30 adenosine residues, followed by a 10 nucleotide linker sequence and another 70 adenosine residues is used. This poly(A) sequence was designed to enhance RNA stability and translational efficiency.

In one embodiment of all aspects of the invention, RNA encoding an activation compound is expressed in cells of the subject treated to provide the activation compound. In one embodiment of all aspects of the invention, the RNA is transiently expressed in cells of the subject. In one embodiment of all aspects of the invention, the RNA is *in vitro* transcribed RNA.

In one embodiment of all aspects of the invention, expression of the activation compound is at the cell surface, i.e., the activation compound remains associated with the cell.

In one embodiment of all aspects of the invention, RNA encoding a docketing compound is expressed in cells of the subject treated to provide the docketing compound. In one embodiment of all aspects of the invention, the RNA is transiently expressed in cells of the subject. In one embodiment of all aspects of the invention, the RNA is *in vitro* transcribed RNA. In one embodiment of all aspects of the invention, expression of the docketing compound is into the extracellular space, i.e., the docketing compound is secreted.

In the context of the present disclosure, the term "transcription" relates to a process, wherein the genetic code in a DNA sequence is transcribed into RNA. Subsequently, the RNA may be translated into peptide or protein.

According to the present invention, the term "transcription" comprises "*in vitro* transcription", wherein the term "*in vitro* transcription" relates to a process wherein RNA, in particular mRNA, is *in vitro* synthesized in a cell-free system, preferably using appropriate cell extracts. Preferably, cloning vectors are applied for the generation of transcripts. These cloning vectors are generally designated as transcription vectors and are according to the present invention encompassed by the term "vector". According to the present invention, the RNA used in the present invention preferably is *in vitro* transcribed RNA (IVT-RNA) and may be obtained by *in vitro* transcription of an appropriate DNA template. The promoter for controlling transcription can be any promoter for any RNA polymerase. Particular examples of RNA polymerases are the T7, T3, and SP6 RNA polymerases. Preferably, the *in vitro* transcription according to the invention is controlled by a T7 or SP6 promoter. A DNA template for *in vitro* transcription may be obtained by cloning of a nucleic acid, in particular cDNA, and introducing it into an appropriate vector for *in vitro* transcription. The cDNA may be obtained by reverse transcription of RNA.

With respect to RNA, the term "expression" or "translation" relates to the process in the ribosomes of a cell by which a strand of mRNA directs the assembly of a sequence of amino

acids to make a peptide or protein.

In one embodiment, after administration of the RNA described herein, e.g., formulated as RNA lipid particles, at least a portion of the RNA is delivered to a cell for expression. In one embodiment, at least a portion of the RNA is delivered to the cytosol of the target cell. In one embodiment, the RNA is translated by the target cell to produce the peptide or protein it encodes.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

In one embodiment, the RNA encoding activation compound to be administered according to the invention is non-immunogenic.

In one embodiment, the RNA encoding docketing compound to be administered according to the invention is non-immunogenic.

The term "non-immunogenic RNA" as used herein refers to RNA that does not induce a response by the immune system upon administration, e.g., to a mammal, or induces a weaker response than would have been induced by the same RNA that differs only in that it has not been subjected to the modifications and treatments that render the non-immunogenic RNA non-immunogenic, i.e., than would have been induced by standard RNA (stdRNA). In one preferred embodiment, non-immunogenic RNA, which is also termed modified RNA (modRNA) herein, is rendered non-immunogenic by incorporating modified nucleosides

suppressing RNA-mediated activation of innate immune receptors into the RNA and removing double-stranded RNA (dsRNA).

For rendering the non-immunogenic RNA non-immunogenic by the incorporation of modified nucleosides, any modified nucleoside may be used as long as it lowers or suppresses immunogenicity of the RNA. Particularly preferred are modified nucleosides that suppress RNA-mediated activation of innate immune receptors. In one embodiment, the modified nucleosides comprises a replacement of one or more uridines with a nucleoside comprising a modified nucleobase. In one embodiment, the modified nucleobase is a modified uracil. In one embodiment, the nucleoside comprising a modified nucleobase is selected from the group consisting of 3-methyl-uridine (m^3U), 5-methoxy-uridine (mo^5U), 5-aza-uridine, 6-aza-uridine, 2-thio-5-aza-uridine, 2-thio-uridine (s^2U), 4-thio-uridine (s^4U), 4-thio-pseudouridine, 2-thio-pseudouridine, 5-hydroxy-uridine (ho^5U), 5-aminoallyl-uridine, 5-halo-uridine (e.g., 5-iodo-uridine or 5-bromo-uridine), uridine 5-oxyacetic acid (cmo^5U), uridine 5-oxyacetic acid methyl ester ($mcmo^5U$), 5-carboxymethyl-uridine (cm^5U), 1-carboxymethyl-pseudouridine, 5-carboxyhydroxymethyl-uridine (chm^5U), 5-carboxyhydroxymethyl-uridine methyl ester ($mchm^5U$), 5-methoxycarbonylmethyl-uridine (mcm^5U), 5-methoxycarbonylmethyl-2-thio-uridine (mcm^5s^2U), 5-aminomethyl-2-thio-uridine (nm^5s^2U), 5-methylaminomethyl-uridine (mnm^5U), 1-ethyl-pseudouridine, 5-methylaminomethyl-2-thio-uridine (mnm^5s^2U), 5-methylaminomethyl-2-seleno-uridine (mnm^5se^2U), 5-carbamoylmethyl-uridine (ncm^5U), 5-carboxymethylaminomethyl-uridine ($cmnm^5U$), 5-carboxymethylaminomethyl-2-thio-uridine ($cmnm^5s^2U$), 5-propynyl-uridine, 1-propynyl-pseudouridine, 5-aurinomethyl-uridine (τm^5U), 1-aurinomethyl-pseudouridine, 5-aurinomethyl-2-thio-uridine (τm^5s^2U), 1-aurinomethyl-4-thio-pseudouridine, 5-methyl-2-thio-uridine (m^5s^2U), 1-methyl-4-thio-pseudouridine ($m^1s^4\psi$), 4-thio-1-methyl-pseudouridine, 3-methyl-pseudouridine ($m^3\psi$), 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydrouridine (D), dihydropseudouridine, 5,6-dihydrouridine, 5-methyl-dihydrouridine (m^5D), 2-thio-dihydrouridine, 2-thio-dihydropseudouridine, 2-methoxy-uridine, 2-methoxy-4-

thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, N1-methyl-pseudouridine, 3-(3-amino-3-carboxypropyl)uridine (acp^3U), 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine ($\text{acp}^3\psi$), 5-(isopentenylaminomethyl)uridine (inm^5U), 5-(isopentenylaminomethyl)-2-thio-uridine ($\text{inm}^5\text{s}^2\text{U}$), α -thio-uridine, 2'-O-methyl-uridine (Um), 5,2'-O-dimethyl-uridine (m^5Um), 2'-O-methyl-pseudouridine (ψm), 2-thio-2'-O-methyl-uridine (s^2Um), 5-methoxycarbonylmethyl-2'-O-methyl-uridine (mcm^5Um), 5-carbamoylmethyl-2'-O-methyl-uridine (ncm^5Um), 5-carboxymethylaminomethyl-2'-O-methyl-uridine (cmnm^5Um), 3,2'-O-dimethyl-uridine (m^3Um), 5-(isopentenylaminomethyl)-2'-O-methyl-uridine (inm^5Um), 1-thio-uridine, deoxythymidine, 2'-F-ara-uridine, 2'-F-uridine, 2'-OH-ara-uridine, 5-(2-carbomethoxyvinyl) uridine, and 5-[3-(1-E-propenylamino)uridine. In one particularly preferred embodiment, the nucleoside comprising a modified nucleobase is pseudouridine (ψ), N1-methyl-pseudouridine ($\text{m}1\psi$) or 5-methyl-uridine ($\text{m}5\text{U}$), in particular N1-methyl-pseudouridine.

In one embodiment, the replacement of one or more uridines with a nucleoside comprising a modified nucleobase comprises a replacement of at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 10%, at least 25%, at least 50%, at least 75%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% of the uridines.

During synthesis of mRNA by *in vitro* transcription (IVT) using T7 RNA polymerase significant amounts of aberrant products, including double-stranded RNA (dsRNA) are produced due to unconventional activity of the enzyme. dsRNA induces inflammatory cytokines and activates effector enzymes leading to protein synthesis inhibition. dsRNA can be removed from RNA such as IVT RNA, for example, by ion-pair reversed phase HPLC using a non-porous or porous C-18 polystyrene-divinylbenzene (PS-DVB) matrix. Alternatively, an enzymatic based method using *E. coli* RNaseIII that specifically hydrolyzes dsRNA but not ssRNA, thereby eliminating dsRNA contaminants from IVT RNA preparations can be used. Furthermore, dsRNA can be separated from ssRNA by using a cellulose material. In one embodiment, an RNA preparation is contacted with a cellulose material and the ssRNA is separated from the cellulose material

under conditions which allow binding of dsRNA to the cellulose material and do not allow binding of ssRNA to the cellulose material.

As the term is used herein, "remove" or "removal" refers to the characteristic of a population of first substances, such as non-immunogenic RNA, being separated from the proximity of a population of second substances, such as dsRNA, wherein the population of first substances is not necessarily devoid of the second substance, and the population of second substances is not necessarily devoid of the first substance. However, a population of first substances characterized by the removal of a population of second substances has a measurably lower content of second substances as compared to the non-separated mixture of first and second substances.

In one embodiment, the removal of dsRNA from non-immunogenic RNA comprises a removal of dsRNA such that less than 10%, less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.5%, less than 0.3%, or less than 0.1% of the RNA in the non-immunogenic RNA composition is dsRNA. In one embodiment, the non-immunogenic RNA is free or essentially free of dsRNA. In some embodiments, the non-immunogenic RNA composition comprises a purified preparation of single-stranded nucleoside modified RNA. For example, in some embodiments, the purified preparation of single-stranded nucleoside modified RNA is substantially free of double stranded RNA (dsRNA). In some embodiments, the purified preparation is at least 90%, at least 91%, at least 92%, at least 93 %, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% single stranded nucleoside modified RNA, relative to all other nucleic acid molecules (DNA, dsRNA, etc.).

In one embodiment, the non-immunogenic RNA is translated in a cell more efficiently than standard RNA with the same sequence. In one embodiment, translation is enhanced by a factor of 2-fold relative to its unmodified counterpart. In one embodiment, translation is enhanced by a 3-fold factor. In one embodiment, translation is enhanced by a 4-fold factor. In one embodiment, translation is enhanced by a 5-fold factor. In one embodiment, translation

is enhanced by a 6-fold factor. In one embodiment, translation is enhanced by a 7-fold factor. In one embodiment, translation is enhanced by an 8-fold factor. In one embodiment, translation is enhanced by a 9-fold factor. In one embodiment, translation is enhanced by a 10-fold factor. In one embodiment, translation is enhanced by a 15-fold factor. In one embodiment, translation is enhanced by a 20-fold factor. In one embodiment, translation is enhanced by a 50-fold factor. In one embodiment, translation is enhanced by a 100-fold factor. In one embodiment, translation is enhanced by a 200-fold factor. In one embodiment, translation is enhanced by a 500-fold factor. In one embodiment, translation is enhanced by a 1000-fold factor. In one embodiment, translation is enhanced by a 2000-fold factor. In one embodiment, the factor is 10-1000-fold. In one embodiment, the factor is 10-100-fold. In one embodiment, the factor is 10-200-fold. In one embodiment, the factor is 10-300-fold. In one embodiment, the factor is 10-500-fold. In one embodiment, the factor is 20-1000-fold. In one embodiment, the factor is 30-1000-fold. In one embodiment, the factor is 50-1000-fold. In one embodiment, the factor is 100-1000-fold. In one embodiment, the factor is 200-1000-fold. In one embodiment, translation is enhanced by any other significant amount or range of amounts.

In one embodiment, the non-immunogenic RNA exhibits significantly less innate immunogenicity than standard RNA with the same sequence. In one embodiment, the non-immunogenic RNA exhibits an innate immune response that is 2-fold less than its unmodified counterpart. In one embodiment, innate immunogenicity is reduced by a 3-fold factor. In one embodiment, innate immunogenicity is reduced by a 4-fold factor. In one embodiment, innate immunogenicity is reduced by a 5-fold factor. In one embodiment, innate immunogenicity is reduced by a 6-fold factor. In one embodiment, innate immunogenicity is reduced by a 7-fold factor. In one embodiment, innate immunogenicity is reduced by a 8-fold factor. In one embodiment, innate immunogenicity is reduced by a 9-fold factor. In one embodiment, innate immunogenicity is reduced by a 10-fold factor. In one embodiment, innate immunogenicity is reduced by a 15-fold factor. In one embodiment, innate immunogenicity is reduced by a 20-

fold factor. In one embodiment, innate immunogenicity is reduced by a 50-fold factor. In one embodiment, innate immunogenicity is reduced by a 100-fold factor. In one embodiment, innate immunogenicity is reduced by a 200-fold factor. In one embodiment, innate immunogenicity is reduced by a 500-fold factor. In one embodiment, innate immunogenicity is reduced by a 1000-fold factor. In one embodiment, innate immunogenicity is reduced by a 2000-fold factor.

The term "exhibits significantly less innate immunogenicity" refers to a detectable decrease in innate immunogenicity. In one embodiment, the term refers to a decrease such that an effective amount of the non-immunogenic RNA can be administered without triggering a detectable innate immune response. In one embodiment, the term refers to a decrease such that the non-immunogenic RNA can be repeatedly administered without eliciting an innate immune response sufficient to detectably reduce production of the protein encoded by the non-immunogenic RNA. In one embodiment, the decrease is such that the non-immunogenic RNA can be repeatedly administered without eliciting an innate immune response sufficient to eliminate detectable production of the protein encoded by the non-immunogenic RNA.

"Immunogenicity" is the ability of a foreign substance, such as RNA, to provoke an immune response in the body of a human or other animal. The innate immune system is the component of the immune system that is relatively unspecific and immediate. It is one of two main components of the vertebrate immune system, along with the adaptive immune system. As used herein "endogenous" refers to any material from or produced inside an organism, cell, tissue or system.

As used herein, the term "exogenous" refers to any material introduced from or produced outside an organism, cell, tissue or system.

The term "expression" as used herein is defined as the transcription and/or translation of a particular nucleotide sequence.

As used herein, the terms "linked," "fused", or "fusion" are used interchangeably. These terms refer to the joining together of two or more elements or components or domains.

Codon-optimization / Increase in G/C content

In some embodiment, the amino acid sequence of an activation compound described herein and/or the amino acid sequence of a docking compound described herein is encoded by a coding sequence which is codon-optimized and/or the G/C content of which is increased compared to wild type coding sequence. This also includes embodiments, wherein one or more sequence regions of the coding sequence are codon-optimized and/or increased in the G/C content compared to the corresponding sequence regions of the wild type coding sequence. In one embodiment, the codon-optimization and/or the increase in the G/C content preferably does not change the sequence of the encoded amino acid sequence.

The term "codon-optimized" refers to the alteration of codons in the coding region of a nucleic acid molecule to reflect the typical codon usage of a host organism without preferably altering the amino acid sequence encoded by the nucleic acid molecule. Within the context of the present invention, coding regions are preferably codon-optimized for optimal expression in a subject to be treated using the RNA molecules described herein. Codon-optimization is based on the finding that the translation efficiency is also determined by a different frequency in the occurrence of tRNAs in cells. Thus, the sequence of RNA may be modified such that codons for which frequently occurring tRNAs are available are inserted in place of "rare codons".

In some embodiments of the invention, the guanosine/cytosine (G/C) content of the coding region of the RNA described herein is increased compared to the G/C content of the corresponding coding sequence of the wild type RNA, wherein the amino acid sequence encoded by the RNA is preferably not modified compared to the amino acid sequence encoded by the wild type RNA. This modification of the RNA sequence is based on the fact that the sequence of any RNA region to be translated is important for efficient translation of that mRNA. Sequences having an increased G (guanosine)/C (cytosine) content are more stable than sequences having an increased A (adenosine)/U (uracil) content. In respect to the fact that several codons code for one and the same amino acid (so-called degeneration of the

genetic code), the most favourable codons for the stability can be determined (so-called alternative codon usage). Depending on the amino acid to be encoded by the RNA, there are various possibilities for modification of the RNA sequence, compared to its wild type sequence. In particular, codons which contain A and/or U nucleotides can be modified by substituting these codons by other codons, which code for the same amino acids but contain no A and/or U or contain a lower content of A and/or U nucleotides.

In various embodiments, the G/C content of the coding region of the RNA described herein is increased by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 55%, or even more compared to the G/C content of the coding region of the wild type RNA.

Nucleic acid containing particles

Nucleic acids described herein such as RNA encoding an activation compound and/or RNA encoding a docking compound may be administered formulated as particles.

In the context of the present disclosure, the term "particle" relates to a structured entity formed by molecules or molecule complexes. In one embodiment, the term "particle" relates to a micro- or nano-sized structure, such as a micro- or nano-sized compact structure dispersed in a medium. In one embodiment, a particle is a nucleic acid containing particle such as a particle comprising DNA, RNA or a mixture thereof.

Electrostatic interactions between positively charged molecules such as polymers and lipids and negatively charged nucleic acid are involved in particle formation. This results in complexation and spontaneous formation of nucleic acid particles. In one embodiment, a nucleic acid particle is a nanoparticle.

As used in the present disclosure, "nanoparticle" refers to a particle having an average diameter suitable for parenteral administration.

A "nucleic acid particle" can be used to deliver nucleic acid to a target site of interest (e.g., cell, tissue, organ, and the like). A nucleic acid particle may be formed from at least one cationic or cationically ionizable lipid or lipid-like material, at least one cationic polymer such

as protamine, or a mixture thereof and nucleic acid. Nucleic acid particles include lipid nanoparticle (LNP)-based and lipoplex (LPX)-based formulations.

Without intending to be bound by any theory, it is believed that the cationic or cationically ionizable lipid or lipid-like material and/or the cationic polymer combine together with the nucleic acid to form aggregates, and this aggregation results in colloiddally stable particles.

In one embodiment, particles described herein further comprise at least one lipid or lipid-like material other than a cationic or cationically ionizable lipid or lipid-like material, at least one polymer other than a cationic polymer, or a mixture thereof

In some embodiments, nucleic acid particles comprise more than one type of nucleic acid molecules, where the molecular parameters of the nucleic acid molecules may be similar or different from each other, like with respect to molar mass or fundamental structural elements such as molecular architecture, capping, coding regions or other features,

Nucleic acid particles described herein may have an average diameter that in one embodiment ranges from about 30 nm to about 1000 nm, from about 50 nm to about 800 nm, from about 70 nm to about 600 nm, from about 90 nm to about 400 nm, or from about 100 nm to about 300 nm.

Nucleic acid particles described herein may exhibit a polydispersity index less than about 0.5, less than about 0.4, less than about 0.3, or about 0.2 or less. By way of example, the nucleic acid particles can exhibit a polydispersity index in a range of about 0.1 to about 0.3 or about 0.2 to about 0.3.

With respect to RNA lipid particles, the N/P ratio gives the ratio of the nitrogen groups in the lipid to the number of phosphate groups in the RNA. It is correlated to the charge ratio, as the nitrogen atoms (depending on the pH) are usually positively charged and the phosphate groups are negatively charged. The N/P ratio, where a charge equilibrium exists, depends on the pH. Lipid formulations are frequently formed at N/P ratios larger than four up to twelve, because positively charged nanoparticles are considered favorable for transfection. In that case, RNA is considered to be completely bound to nanoparticles.

Nucleic acid particles described herein can be prepared using a wide range of methods that may involve obtaining a colloid from at least one cationic or cationically ionizable lipid or lipid-like material and/or at least one cationic polymer and mixing the colloid with nucleic acid to obtain nucleic acid particles.

The term "colloid" as used herein relates to a type of homogeneous mixture in which dispersed particles do not settle out. The insoluble particles in the mixture are microscopic, with particle sizes between 1 and 1000 nanometers. The mixture may be termed a colloid or a colloidal suspension. Sometimes the term "colloid" only refers to the particles in the mixture and not the entire suspension.

For the preparation of colloids comprising at least one cationic or cationically ionizable lipid or lipid-like material and/or at least one cationic polymer methods are applicable herein that are conventionally used for preparing liposomal vesicles and are appropriately adapted. The most commonly used methods for preparing liposomal vesicles share the following fundamental stages: (i) lipids dissolution in organic solvents, (ii) drying of the resultant solution, and (iii) hydration of dried lipid (using various aqueous media).

In the film hydration method, lipids are firstly dissolved in a suitable organic solvent, and dried down to yield a thin film at the bottom of the flask. The obtained lipid film is hydrated using an appropriate aqueous medium to produce a liposomal dispersion. Furthermore, an additional downsizing step may be included.

Reverse phase evaporation is an alternative method to the film hydration for preparing liposomal vesicles that involves formation of a water-in-oil emulsion between an aqueous phase and an organic phase containing lipids. A brief sonication of this mixture is required for system homogenization. The removal of the organic phase under reduced pressure yields a milky gel that turns subsequently into a liposomal suspension.

The term "ethanol injection technique" refers to a process, in which an ethanol solution comprising lipids is rapidly injected into an aqueous solution through a needle. This action disperses the lipids throughout the solution and promotes lipid structure formation, for

example lipid vesicle formation such as liposome formation. Generally, the RNA lipoplex particles described herein are obtainable by adding RNA to a colloidal liposome dispersion. Using the ethanol injection technique, such colloidal liposome dispersion is, in one embodiment, formed as follows: an ethanol solution comprising lipids, such as cationic lipids and additional lipids, is injected into an aqueous solution under stirring. In one embodiment, the RNA lipoplex particles described herein are obtainable without a step of extrusion.

The term "extruding" or "extrusion" refers to the creation of particles having a fixed, cross-sectional profile. In particular, it refers to the downsizing of a particle, whereby the particle is forced through filters with defined pores.

Other methods having organic solvent free characteristics may also be used according to the present disclosure for preparing a colloid.

LNPs typically comprise four components: ionizable cationic lipids, neutral lipids such as phospholipids, a steroid such as cholesterol, and a polymer conjugated lipid such as polyethylene glycol (PEG)-lipids. Each component is responsible for payload protection, and enables effective intracellular delivery. LNPs may be prepared by mixing lipids dissolved in ethanol rapidly with nucleic acid in an aqueous buffer.

The term "average diameter" refers to the mean hydrodynamic diameter of particles as measured by dynamic laser light scattering (DLS) with data analysis using the so-called cumulant algorithm, which provides as results the so-called Z_{average} with the dimension of a length, and the polydispersity index (PI), which is dimensionless (Koppel, D., J. Chem. Phys. 57, 1972, pp 4814-4820, ISO 13321). Here "average diameter", "diameter" or "size" for particles is used synonymously with this value of the Z_{average} .

The "polydispersity index" is preferably calculated based on dynamic light scattering measurements by the so-called cumulant analysis as mentioned in the definition of the "average diameter". Under certain prerequisites, it can be taken as a measure of the size distribution of an ensemble of nanoparticles.

Different types of nucleic acid containing particles have been described previously to be suitable for delivery of nucleic acid in particulate form (e.g. Kaczmarek, J. C. et al., 2017, *Genome Medicine* 9, 60). For non-viral nucleic acid delivery vehicles, nanoparticle encapsulation of nucleic acid physically protects nucleic acid from degradation and, depending on the specific chemistry, can aid in cellular uptake and endosomal escape.

The present disclosure describes particles comprising nucleic acid, at least one cationic or cationically ionizable lipid or lipid-like material, and/or at least one cationic polymer which associate with nucleic acid to form nucleic acid particles and compositions comprising such particles. The nucleic acid particles may comprise nucleic acid which is complexed in different forms by non-covalent interactions to the particle. The particles described herein are not viral particles, in particular infectious viral particles, i.e., they are not able to virally infect cells.

Suitable cationic or cationically ionizable lipids or lipid-like materials and cationic polymers are those that form nucleic acid particles and are included by the term "particle forming components" or "particle forming agents". The term "particle forming components" or "particle forming agents" relates to any components which associate with nucleic acid to form nucleic acid particles. Such components include any component which can be part of nucleic acid particles.

Cationic polymer

Given their high degree of chemical flexibility, polymers are commonly used materials for nanoparticle-based delivery. Typically, cationic polymers are used to electrostatically condense the negatively charged nucleic acid into nanoparticles. These positively charged groups often consist of amines that change their state of protonation in the pH range between 5.5 and 7.5, thought to lead to an ion imbalance that results in endosomal rupture. Polymers such as poly-L-lysine, polyamidoamine, protamine and polyethyleneimine, as well as naturally occurring polymers such as chitosan have all been applied to nucleic acid delivery and are suitable as cationic polymers herein. In addition, some investigators have synthesized

polymers specifically for nucleic acid delivery. Poly(β -amino esters), in particular, have gained widespread use in nucleic acid delivery owing to their ease of synthesis and biodegradability. Such synthetic polymers are also suitable as cationic polymers herein.

A "polymer," as used herein, is given its ordinary meaning, i.e., a molecular structure comprising one or more repeat units (monomers), connected by covalent bonds. The repeat units can all be identical, or in some cases, there can be more than one type of repeat unit present within the polymer. In some cases, the polymer is biologically derived, i.e., a biopolymer such as a protein. In some cases, additional moieties can also be present in the polymer, for example targeting moieties such as those described herein.

If more than one type of repeat unit is present within the polymer, then the polymer is said to be a "copolymer." It is to be understood that the polymer being employed herein can be a copolymer. The repeat units forming the copolymer can be arranged in any fashion. For example, the repeat units can be arranged in a random order, in an alternating order, or as a "block" copolymer, i.e., comprising one or more regions each comprising a first repeat unit (e.g., a first block), and one or more regions each comprising a second repeat unit (e.g., a second block), etc. Block copolymers can have two (a diblock copolymer), three (a triblock copolymer), or more numbers of distinct blocks.

In certain embodiments, the polymer is biocompatible. Biocompatible polymers are polymers that typically do not result in significant cell death at moderate concentrations. In certain embodiments, the biocompatible polymer is biodegradable, i.e., the polymer is able to degrade, chemically and/or biologically, within a physiological environment, such as within the body.

In certain embodiments, polymer may be protamine or polyalkyleneimine, in particular protamine.

The term "protamine" refers to any of various strongly basic proteins of relatively low molecular weight that are rich in arginine and are found associated especially with DNA in place of somatic histones in the sperm cells of various animals (as fish). In particular, the term

"protamine" refers to proteins found in fish sperm that are strongly basic, are soluble in water, are not coagulated by heat, and yield chiefly arginine upon hydrolysis. In purified form, they are used in a long-acting formulation of insulin and to neutralize the anticoagulant effects of heparin.

According to the disclosure, the term "protamine" as used herein is meant to comprise any protamine amino acid sequence obtained or derived from natural or biological sources including fragments thereof and multimeric forms of said amino acid sequence or fragment thereof as well as (synthesized) polypeptides which are artificial and specifically designed for specific purposes and cannot be isolated from native or biological sources.

In one embodiment, the polyalkyleneimine comprises polyethylenimine and/or polypropylenimine, preferably polyethyleneimine. A preferred polyalkyleneimine is polyethyleneimine (PEI). The average molecular weight of PEI is preferably $0.75 \cdot 10^2$ to 10^7 Da, preferably 1000 to 10^5 Da, more preferably 10000 to 40000 Da, more preferably 15000 to 30000 Da, even more preferably 20000 to 25000 Da.

Preferred according to the disclosure is linear polyalkyleneimine such as linear polyethyleneimine (PEI).

Cationic polymers (including polycationic polymers) contemplated for use herein include any cationic polymers which are able to electrostatically bind nucleic acid. In one embodiment, cationic polymers contemplated for use herein include any cationic polymers with which nucleic acid can be associated, e.g. by forming complexes with the nucleic acid or forming vesicles in which the nucleic acid is enclosed or encapsulated.

Particles described herein may also comprise polymers other than cationic polymers, i.e., non-cationic polymers and/or anionic polymers. Collectively, anionic and neutral polymers are referred to herein as non-cationic polymers.

Lipid and lipid-like material

The terms "lipid" and "lipid-like material" are broadly defined herein as molecules which comprise one or more hydrophobic moieties or groups and optionally also one or more hydrophilic moieties or groups. Molecules comprising hydrophobic moieties and hydrophilic moieties are also frequently denoted as amphiphiles. Lipids are usually poorly soluble in water. In an aqueous environment, the amphiphilic nature allows the molecules to self-assemble into organized structures and different phases. One of those phases consists of lipid bilayers, as they are present in vesicles, multilamellar/unilamellar liposomes, or membranes in an aqueous environment. Hydrophobicity can be conferred by the inclusion of apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). The hydrophilic groups may comprise polar and/or charged groups and include carbohydrates, phosphate, carboxylic, sulfate, amino, sulfhydryl, nitro, hydroxyl, and other like groups.

As used herein, the term "amphiphilic" refers to a molecule having both a polar portion and a non-polar portion. Often, an amphiphilic compound has a polar head attached to a long hydrophobic tail. In some embodiments, the polar portion is soluble in water, while the non-polar portion is insoluble in water. In addition, the polar portion may have either a formal positive charge, or a formal negative charge. Alternatively, the polar portion may have both a formal positive and a negative charge, and be a zwitterion or inner salt. For purposes of the disclosure, the amphiphilic compound can be, but is not limited to, one or a plurality of natural or non-natural lipids and lipid-like compounds.

The term "lipid-like material", "lipid-like compound" or "lipid-like molecule" relates to substances that structurally and/or functionally relate to lipids but may not be considered as lipids in a strict sense. For example, the term includes compounds that are able to form amphiphilic layers as they are present in vesicles, multilamellar/unilamellar liposomes, or membranes in an aqueous environment and includes surfactants, or synthesized compounds

with both hydrophilic and hydrophobic moieties. Generally speaking, the term refers to molecules, which comprise hydrophilic and hydrophobic moieties with different structural organization, which may or may not be similar to that of lipids. As used herein, the term "lipid" is to be construed to cover both lipids and lipid-like materials unless otherwise indicated herein or clearly contradicted by context.

Specific examples of amphiphilic compounds that may be included in an amphiphilic layer include, but are not limited to, phospholipids, aminolipids and sphingolipids.

In certain embodiments, the amphiphilic compound is a lipid. The term "lipid" refers to a group of organic compounds that are characterized by being insoluble in water, but soluble in many organic solvents. Generally, lipids may be divided into eight categories: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides (derived from condensation of ketoacyl subunits), sterol lipids and prenol lipids (derived from condensation of isoprene subunits). Although the term "lipid" is sometimes used as a synonym for fats, fats are a subgroup of lipids called triglycerides. Lipids also encompass molecules such as fatty acids and their derivatives (including tri-, di-, monoglycerides, and phospholipids), as well as sterol-containing metabolites such as cholesterol.

Fatty acids, or fatty acid residues are a diverse group of molecules made of a hydrocarbon chain that terminates with a carboxylic acid group; this arrangement confers the molecule with a polar, hydrophilic end, and a nonpolar, hydrophobic end that is insoluble in water. The carbon chain, typically between four and 24 carbons long, may be saturated or unsaturated, and may be attached to functional groups containing oxygen, halogens, nitrogen, and sulfur. If a fatty acid contains a double bond, there is the possibility of either a cis or trans geometric isomerism, which significantly affects the molecule's configuration. Cis-double bonds cause the fatty acid chain to bend, an effect that is compounded with more double bonds in the chain. Other major lipid classes in the fatty acid category are the fatty esters and fatty amides. Glycerolipids are composed of mono-, di-, and tri-substituted glycerols, the best-known being the fatty acid triesters of glycerol, called triglycerides. The word "triacylglycerol" is sometimes

used synonymously with "triglyceride". In these compounds, the three hydroxyl groups of glycerol are each esterified, typically by different fatty acids. Additional subclasses of glycerolipids are represented by glycosylglycerols, which are characterized by the presence of one or more sugar residues attached to glycerol via a glycosidic linkage.

The glycerophospholipids are amphipathic molecules (containing both hydrophobic and hydrophilic regions) that contain a glycerol core linked to two fatty acid-derived "tails" by ester linkages and to one "head" group by a phosphate ester linkage. Examples of glycerophospholipids, usually referred to as phospholipids (though sphingomyelins are also classified as phospholipids) are phosphatidylcholine (also known as PC, GPCho or lecithin), phosphatidylethanolamine (PE or GPEtn) and phosphatidylserine (PS or GPSer).

Sphingolipids are a complex family of compounds that share a common structural feature, a sphingoid base backbone. The major sphingoid base in mammals is commonly referred to as sphingosine. Ceramides (N-acyl-sphingoid bases) are a major subclass of sphingoid base derivatives with an amide-linked fatty acid. The fatty acids are typically saturated or mono-unsaturated with chain lengths from 16 to 26 carbon atoms. The major phosphosphingolipids of mammals are sphingomyelins (ceramide phosphocholines), whereas insects contain mainly ceramide phosphoethanolamines and fungi have phytoceramide phosphoinositols and mannose-containing headgroups. The glycosphingolipids are a diverse family of molecules composed of one or more sugar residues linked via a glycosidic bond to the sphingoid base. Examples of these are the simple and complex glycosphingolipids such as cerebroside and gangliosides.

Sterol lipids, such as cholesterol and its derivatives, or tocopherol and its derivatives, are an important component of membrane lipids, along with the glycerophospholipids and sphingomyelins.

Saccharolipids describe compounds in which fatty acids are linked directly to a sugar backbone, forming structures that are compatible with membrane bilayers. In the saccharolipids, a monosaccharide substitutes for the glycerol backbone present in

glycerolipids and glycerophospholipids. The most familiar saccharolipids are the acylated glucosamine precursors of the Lipid A component of the lipopolysaccharides in Gram-negative bacteria. Typical lipid A molecules are disaccharides of glucosamine, which are derivatized with as many as seven fatty-acyl chains. The minimal lipopolysaccharide required for growth in *E. coli* is Kdo2-Lipid A, a hexa-acylated disaccharide of glucosamine that is glycosylated with two 3-deoxy-D-manno-octulosonic acid (Kdo) residues.

Polyketides are synthesized by polymerization of acetyl and propionyl subunits by classic enzymes as well as iterative and multimodular enzymes that share mechanistic features with the fatty acid synthases. They comprise a large number of secondary metabolites and natural products from animal, plant, bacterial, fungal and marine sources, and have great structural diversity. Many polyketides are cyclic molecules whose backbones are often further modified by glycosylation, methylation, hydroxylation, oxidation, or other processes.

According to the disclosure, lipids and lipid-like materials may be cationic, anionic or neutral. Neutral lipids or lipid-like materials exist in an uncharged or neutral zwitterionic form at a selected pH.

Cationic or cationically ionizable lipids or lipid-like materials

The nucleic acid particles described herein may comprise at least one cationic or cationically ionizable lipid or lipid-like material as particle forming agent. Cationic or cationically ionizable lipids or lipid-like materials contemplated for use herein include any cationic or cationically ionizable lipids or lipid-like materials which are able to electrostatically bind nucleic acid. In one embodiment, cationic or cationically ionizable lipids or lipid-like materials contemplated for use herein can be associated with nucleic acid, e.g. by forming complexes with the nucleic acid or forming vesicles in which the nucleic acid is enclosed or encapsulated.

As used herein, a "cationic lipid" or "cationic lipid-like material" refers to a lipid or lipid-like material having a net positive charge. Cationic lipids or lipid-like materials bind negatively charged nucleic acid by electrostatic interaction. Generally, cationic lipids possess a lipophilic

moiety, such as a sterol, an acyl chain, a diacyl or more acyl chains, and the head group of the lipid typically carries the positive charge.

In certain embodiments, a cationic lipid or lipid-like material has a net positive charge only at certain pH, in particular acidic pH, while it has preferably no net positive charge, preferably has no charge, i.e., it is neutral, at a different, preferably higher pH such as physiological pH. This ionizable behavior is thought to enhance efficacy through helping with endosomal escape and reducing toxicity as compared with particles that remain cationic at physiological pH. For purposes of the present disclosure, such "cationically ionizable" lipids or lipid-like materials are comprised by the term "cationic lipid or lipid-like material" unless contradicted by the circumstances.

In one embodiment, the cationic or cationically ionizable lipid or lipid-like material comprises a head group which includes at least one nitrogen atom (N) which is positive charged or capable of being protonated.

Examples of cationic lipids include, but are not limited to 1,2-dioleoyl-3-trimethylammonium propane (DOTAP); N,N-dimethyl-2,3-dioleyloxypropylamine (DODMA), 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), 3-(N—(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), dimethyldioctadecylammonium (DDAB); 1,2-dioleoyl-3-dimethylammonium-propane (DODAP); 1,2-diacyloxy-3-dimethylammonium propanes; 1,2-dialkyloxy-3-dimethylammonium propanes; dioctadecyldimethyl ammonium chloride (DODAC), 1,2-distearoyloxy-N,N-dimethyl-3-aminopropane (DSDMA), 2,3-di(tetradecoxy)propyl-(2-hydroxyethyl)-dimethylazanium (DMRIE), 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine (DMEPC), 1,2-dimyristoyl-3-trimethylammonium propane (DMTAP), 1,2-dioleyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DORIE), and 2,3-dioleyloxy-N-[2(spermine carboxamide)ethyl]-N,N-dimethyl-1-propanamium trifluoroacetate (DOSPA), 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-oc-

tadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxo]-3-dimethyl-1-(cis,cis-9',12'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleoyloxybenzylamine (DMOBA), 1,2-N,N'-dioleoylcarbonyl-3-dimethylaminopropane (DOcarbDAP), 2,3-Dilinoleoyloxy-N,N-dimethylpropylamine (DLinDAP), 1,2-N,N'-Dilinoleoylcarbonyl-3-dimethylaminopropane (DLincarbDAP), 1,2-Dilinoleoylcarbonyl-3-dimethylaminopropane (DLinCDAP), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-K-XTC2-DMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate (DLin-MC3-DMA), N-(2-Hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (DMRIE), ($\pm$)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(cis-9-tetradecenyl)-1-propanaminium bromide (GAP-DMORIE), ($\pm$)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide (GAP-DLRIE), ($\pm$)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (GAP-DMRIE), N-(2-Aminoethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (β AE-DMRIE), N-(4-carboxybenzyl)-N,N-dimethyl-2,3-bis(oleoyloxy)propan-1-aminium (DOBAQ), 2-({8-[(3 β)-cholest-5-en-3-yloxy]octyl}oxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Octyl-CLinDMA), 1,2-dimyristoyl-3-dimethylammonium-propane (DMDAP), 1,2-dipalmitoyl-3-dimethylammonium-propane (DPDAP), N1-[2-((1S)-1-[(3-aminopropyl)amino]-4-[di(3-amino-propyl)amino]butylcarboxamido)ethyl]-3,4-di[oleoyloxy]-benzamide (MVL5), 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (DOEPC), 2,3-bis(dodecyloxy)-N-(2-hydroxyethyl)-N,N-dimethylpropan-1-aminium bromide (DLRIE), N-(2-aminoethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)propan-1-aminium bromide (DMORIE), di((Z)-non-2-en-1-yl) 8,8'-((((2(dimethylamino)ethyl)thio)carbonyl)azanediyl)dioctanoate (ATX), N,N-dimethyl-2,3-bis(dodecyloxy)propan-1-amine (DLDMA), N,N-dimethyl-2,3-bis(tetradecyloxy)propan-1-amine (DMDMA), Di((Z)-non-2-en-1-yl)-9-((4-(dimethylaminobutanoyl)oxy)heptadecanedioate (L319), N-Dodecyl-3-((2-dodecylcarbonyl-

ethyl)-{2-[(2-dodecylcarbamoylethyl)-2-[(2-dodecylcarbamoylethyl)-[2-(2-dodecylcarbamoylethylamino)ethyl]amino]ethylamino}propionamide (lipidoid 98N₁₂-5), 1-[2-[bis(2-hydroxydodecyl)amino]ethyl]-[2-[4-[2-[bis(2-hydroxydodecyl)amino]ethyl]piperazin-1-yl]ethyl]amino]dodecan-2-ol (lipidoid C12-200).

In some embodiments, the cationic lipid may comprise from about 10 mol % to about 100 mol %, about 20 mol % to about 100 mol %, about 30 mol % to about 100 mol %, about 40 mol % to about 100 mol %, or about 50 mol % to about 100 mol % of the total lipid present in the particle.

Additional lipids or lipid-like materials

Particles described herein may also comprise lipids or lipid-like materials other than cationic or cationically ionizable lipids or lipid-like materials, i.e., non-cationic lipids or lipid-like materials (including non-cationically ionizable lipids or lipid-like materials). Collectively, anionic and neutral lipids or lipid-like materials are referred to herein as non-cationic lipids or lipid-like materials. Optimizing the formulation of nucleic acid particles by addition of other hydrophobic moieties, such as cholesterol and lipids, in addition to an ionizable/cationic lipid or lipid-like material may enhance particle stability and efficacy of nucleic acid delivery.

An additional lipid or lipid-like material may be incorporated which may or may not affect the overall charge of the nucleic acid particles. In certain embodiments, the additional lipid or lipid-like material is a non-cationic lipid or lipid-like material. The non-cationic lipid may comprise, e.g., one or more anionic lipids and/or neutral lipids. As used herein, an "anionic lipid" refers to any lipid that is negatively charged at a selected pH. As used herein, a "neutral lipid" refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. In preferred embodiments, the additional lipid comprises one of the following neutral lipid components: (1) a phospholipid, (2) cholesterol or a derivative thereof; or (3) a mixture of a phospholipid and cholesterol or a derivative thereof. Examples of cholesterol derivatives include, but are not limited to, cholestanol, cholestanone,

cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, tocopherol and derivatives thereof, and mixtures thereof.

Specific phospholipids that can be used include, but are not limited to, phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidic acids, phosphatidylserines or sphingomyelin. Such phospholipids include in particular diacylphosphatidylcholines, such as distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dimyristoylphosphatidylcholine (DMPC), dipentadecanoylphosphatidylcholine, dilauroylphosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC), diarachidoylphosphatidylcholine (DAPC), dibehenoylphosphatidylcholine (DBPC), ditricosanoylphosphatidylcholine (DTPC), dilignoceroylphatidylcholine (DLPC), palmitoyloleoyl-phosphatidylcholine (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC) and phosphatidylethanolamines, in particular diacylphosphatidylethanolamines, such as dioleoylphosphatidylethanolamine (DOPE), distearoyl-phosphatidylethanolamine (DSPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), dilauroyl-phosphatidylethanolamine (DLPE), diphytanoyl-phosphatidylethanolamine (DPyPE), and further phosphatidylethanolamine lipids with different hydrophobic chains.

In certain preferred embodiments, the additional lipid is DSPC or DSPC and cholesterol.

In certain embodiments, the nucleic acid particles include both a cationic lipid and an additional lipid.

In one embodiment, particles described herein include a polymer conjugated lipid such as a pegylated lipid. The term "pegylated lipid" refers to a molecule comprising both a lipid portion and a polyethylene glycol portion. Pegylated lipids are known in the art.

Without wishing to be bound by theory, the amount of the at least one cationic lipid compared to the amount of the at least one additional lipid may affect important nucleic acid particle characteristics, such as charge, particle size, stability, tissue selectivity, and bioactivity of the

nucleic acid. Accordingly, in some embodiments, the molar ratio of the at least one cationic lipid to the at least one additional lipid is from about 10:0 to about 1:9, about 4:1 to about 1:2, or about 3:1 to about 1:1.

In some embodiments, the non-cationic lipid, in particular neutral lipid, (e.g., one or more phospholipids and/or cholesterol) may comprise from about 0 mol % to about 90 mol %, from about 0 mol % to about 80 mol %, from about 0 mol % to about 70 mol %, from about 0 mol % to about 60 mol %, or from about 0 mol % to about 50 mol %, of the total lipid present in the particle.

Lipoplex Particles

In certain embodiments of the present disclosure, the RNA described herein may be present in RNA lipoplex particles.

In the context of the present disclosure, the term "RNA lipoplex particle" relates to a particle that contains lipid, in particular cationic lipid, and RNA. Electrostatic interactions between positively charged liposomes and negatively charged RNA results in complexation and spontaneous formation of RNA lipoplex particles. Positively charged liposomes may be generally synthesized using a cationic lipid, such as DOTMA, and additional lipids, such as DOPE. In one embodiment, a RNA lipoplex particle is a nanoparticle.

In certain embodiments, the RNA lipoplex particles include both a cationic lipid and an additional lipid. In an exemplary embodiment, the cationic lipid is DOTMA and the additional lipid is DOPE.

In some embodiments, the molar ratio of the at least one cationic lipid to the at least one additional lipid is from about 10:0 to about 1:9, about 4:1 to about 1:2, or about 3:1 to about 1:1. In specific embodiments, the molar ratio may be about 3:1, about 2.75:1, about 2.5:1, about 2.25:1, about 2:1, about 1.75:1, about 1.5:1, about 1.25:1, or about 1:1. In an exemplary embodiment, the molar ratio of the at least one cationic lipid to the at least one additional lipid is about 2:1.

RNA lipoplex particles described herein have an average diameter that in one embodiment ranges from about 200 nm to about 1000 nm, from about 200 nm to about 800 nm, from about 250 to about 700 nm, from about 400 to about 600 nm, from about 300 nm to about 500 nm, or from about 350 nm to about 400 nm. In specific embodiments, the RNA lipoplex particles have an average diameter of about 200 nm, about 225 nm, about 250 nm, about 275 nm, about 300 nm, about 325 nm, about 350 nm, about 375 nm, about 400 nm, about 425 nm, about 450 nm, about 475 nm, about 500 nm, about 525 nm, about 550 nm, about 575 nm, about 600 nm, about 625 nm, about 650 nm, about 700 nm, about 725 nm, about 750 nm, about 775 nm, about 800 nm, about 825 nm, about 850 nm, about 875 nm, about 900 nm, about 925 nm, about 950 nm, about 975 nm, or about 1000 nm. In an embodiment, the RNA lipoplex particles have an average diameter that ranges from about 250 nm to about 700 nm. In another embodiment, the RNA lipoplex particles have an average diameter that ranges from about 300 nm to about 500 nm. In an exemplary embodiment, the RNA lipoplex particles have an average diameter of about 400 nm.

The RNA lipoplex particles and compositions comprising RNA lipoplex particles described herein are useful for delivery of RNA to a target tissue after parenteral administration, in particular after intravenous administration. The RNA lipoplex particles may be prepared using liposomes that may be obtained by injecting a solution of the lipids in ethanol into water or a suitable aqueous phase. In one embodiment, the aqueous phase has an acidic pH. In one embodiment, the aqueous phase comprises acetic acid, e.g., in an amount of about 5 mM. Liposomes may be used for preparing RNA lipoplex particles by mixing the liposomes with RNA. In one embodiment, the liposomes and RNA lipoplex particles comprise at least one cationic lipid and at least one additional lipid. In one embodiment, the at least one cationic lipid comprises 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) and/or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). In one embodiment, the at least one additional lipid comprises 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE), cholesterol (Chol) and/or 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). In one

embodiment, the at least one cationic lipid comprises 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) and the at least one additional lipid comprises 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE). In one embodiment, the liposomes and RNA lipoplex particles comprise 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) and 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE).

Lipid nanoparticles (LNPs)

In one embodiment, nucleic acid such as RNA described herein is administered in the form of lipid nanoparticles (LNPs). The LNP may comprise any lipid capable of forming a particle to which the one or more nucleic acid molecules are attached, or in which the one or more nucleic acid molecules are encapsulated.

In one embodiment, the LNP comprises one or more cationic lipids, and one or more stabilizing lipids. Stabilizing lipids include neutral lipids and pegylated lipids.

In one embodiment, the LNP comprises a cationic lipid, a neutral lipid, a steroid, a polymer conjugated lipid; and the RNA, encapsulated within or associated with the lipid nanoparticle.

In one embodiment, the LNP comprises from 40 to 55 mol percent, from 40 to 50 mol percent, from 41 to 49 mol percent, from 41 to 48 mol percent, from 42 to 48 mol percent, from 43 to 48 mol percent, from 44 to 48 mol percent, from 45 to 48 mol percent, from 46 to 48 mol percent, from 47 to 48 mol percent, or from 47.2 to 47.8 mol percent of the cationic lipid. In one embodiment, the LNP comprises about 47.0, 47.1, 47.2, 47.3, 47.4, 47.5, 47.6, 47.7, 47.8, 47.9 or 48.0 mol percent of the cationic lipid.

In one embodiment, the neutral lipid is present in a concentration ranging from 5 to 15 mol percent, from 7 to 13 mol percent, or from 9 to 11 mol percent. In one embodiment, the neutral lipid is present in a concentration of about 9.5, 10 or 10.5 mol percent.

In one embodiment, the steroid is present in a concentration ranging from 30 to 50 mol percent, from 35 to 45 mol percent or from 38 to 43 mol percent. In one embodiment, the steroid is present in a concentration of about 40, 41, 42, 43, 44, 45 or 46 mol percent.

In one embodiment, the LNP comprises from 1 to 10 mol percent, from 1 to 5 mol percent, or from 1 to 2.5 mol percent of the polymer conjugated lipid.

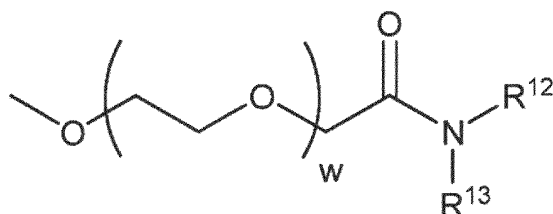
In one embodiment, the LNP comprises from 40 to 50 mol percent a cationic lipid; from 5 to 15 mol percent of a neutral lipid; from 35 to 45 mol percent of a steroid; from 1 to 10 mol percent of a polymer conjugated lipid; and the RNA, encapsulated within or associated with the lipid nanoparticle.

In one embodiment, the mol percent is determined based on total mol of lipid present in the lipid nanoparticle.

In one embodiment, the neutral lipid is selected from the group consisting of DSPC, DPPC, DMPC, DOPC, POPC, DOPE, DOPG, DPPG, POPE, DPPE, DMPE, DSPE, and SM. In one embodiment, the neutral lipid is selected from the group consisting of DSPC, DPPC, DMPC, DOPC, POPC, DOPE and SM. In one embodiment, the neutral lipid is DSPC.

In one embodiment, the steroid is cholesterol.

In one embodiment, the polymer conjugated lipid is a pegylated lipid. In one embodiment, the pegylated lipid has the following structure:

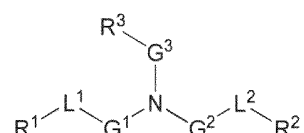


or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein:

R¹² and R¹³ are each independently a straight or branched, saturated or unsaturated alkyl chain containing from 10 to 30 carbon atoms, wherein the alkyl chain is optionally interrupted by one or more ester bonds; and w has a mean value ranging from 30 to 60. In one embodiment, R¹² and R¹³ are each independently straight, saturated alkyl chains containing from 12 to 16 carbon atoms. In one embodiment, w has a mean value ranging from 40 to 55. In one embodiment, the average w is about 45. In one embodiment, R¹² and R¹³ are each

independently a straight, saturated alkyl chain containing about 14 carbon atoms, and w has a mean value of about 45.

In some embodiments, the cationic lipid component of the LNPs has the structure of Formula (III):



(III)

or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein: one of L^1 or L^2 is $-\text{O}(\text{C}=\text{O})-$, $-(\text{C}=\text{O})\text{O}-$, $-\text{C}(\text{O})-$, $-\text{O}-$, $-\text{S}(\text{O})_x-$, $-\text{S}-\text{S}-$, $-\text{C}(\text{O})\text{S}-$, $\text{SC}(\text{O})-$, $-\text{NR}^a\text{C}(\text{O})-$, $-\text{C}(\text{O})\text{NR}^a-$, $\text{NR}^a\text{C}(\text{O})\text{NR}^a-$, $-\text{OC}(\text{O})\text{NR}^a-$ or $-\text{NR}^a\text{C}(\text{O})\text{O}-$, and the other of L^1 or L^2 is $-\text{O}(\text{C}=\text{O})-$, $-(\text{C}=\text{O})\text{O}-$, $-\text{C}(\text{O})-$, $-\text{O}-$, $-\text{S}(\text{O})_x-$, $-\text{S}-\text{S}-$, $-\text{C}(\text{O})\text{S}-$, $\text{SC}(\text{O})-$, $-\text{NR}^a\text{C}(\text{O})-$, $-\text{C}(\text{O})\text{NR}^a-$, $\text{NR}^a\text{C}(\text{O})\text{NR}^a-$, $-\text{OC}(\text{O})\text{NR}^a-$ or $-\text{NR}^a\text{C}(\text{O})\text{O}-$ or a direct bond;

G^1 and G^2 are each independently unsubstituted C_1 - C_{12} alkylene or C_1 - C_{12} alkenylene;

G^3 is C_1 - C_{24} alkylene, C_1 - C_{24} alkenylene, C_3 - C_8 cycloalkylene, C_3 - C_8 cycloalkenylene;

R^a is H or C_1 - C_{12} alkyl;

R^1 and R^2 are each independently C_6 - C_{24} alkyl or C_6 - C_{24} alkenyl;

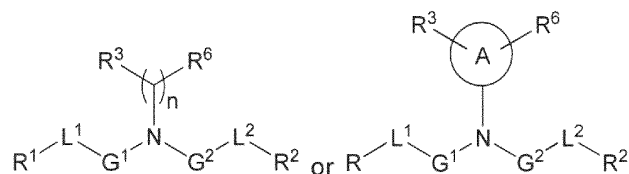
R^3 is H, OR^5 , CN, $-\text{C}(\text{O})\text{OR}^4$, $-\text{OC}(\text{O})\text{R}^4$ or $-\text{NR}^5\text{C}(\text{O})\text{R}^4$;

R^4 is C_1 - C_{12} alkyl;

R^5 is H or C_1 - C_6 alkyl; and

x is 0, 1 or 2.

In some of the foregoing embodiments of Formula (III), the lipid has one of the following structures (IIIA) or (IIIB):



(IIIA)

(IIIB)

wherein:

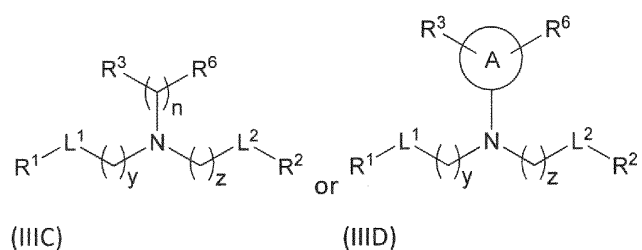
A is a 3 to 8-membered cycloalkyl or cycloalkylene ring;

R⁶ is, at each occurrence, independently H, OH or C₁-C₂₄ alkyl;

n is an integer ranging from 1 to 15.

In some of the foregoing embodiments of Formula (III), the lipid has structure (IIIA), and in other embodiments, the lipid has structure (IIIB).

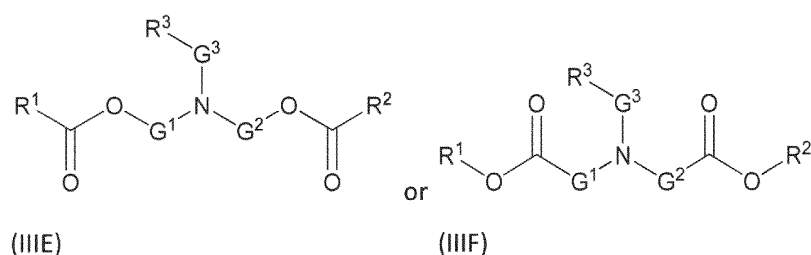
In other embodiments of Formula (III), the lipid has one of the following structures (IIIC) or (IIID):



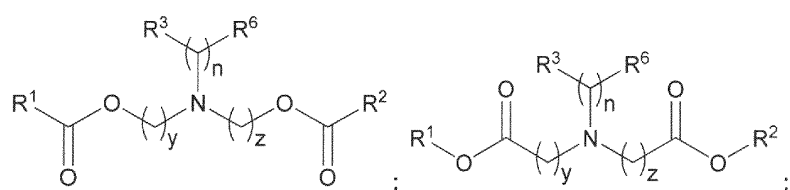
wherein y and z are each independently integers ranging from 1 to 12.

In any of the foregoing embodiments of Formula (III), one of L¹ or L² is -O(C=O)-. For example, in some embodiments each of L¹ and L² are -O(C=O)-. In some different embodiments of any of the foregoing, L¹ and L² are each independently -(C=O)O- or -O(C=O)-. For example, in some embodiments each of L¹ and L² is -(C=O)O-.

In some different embodiments of Formula (III), the lipid has one of the following structures (IIIE) or (IIIF):

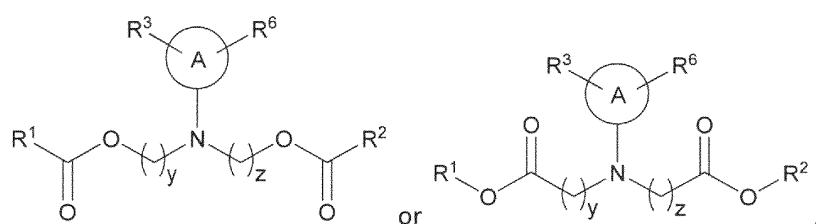


In some of the foregoing embodiments of Formula (III), the lipid has one of the following structures (IIIG), (IIIH), (IIII), or (IIIJ):



(IIIG)

(IIIH)



(IIII)

(IIIJ)

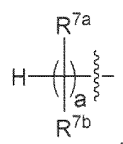
In some of the foregoing embodiments of Formula (III), n is an integer ranging from 2 to 12, for example from 2 to 8 or from 2 to 4. For example, in some embodiments, n is 3, 4, 5 or 6. In some embodiments, n is 3. In some embodiments, n is 4. In some embodiments, n is 5. In some embodiments, n is 6.

In some other of the foregoing embodiments of Formula (III), y and z are each independently an integer ranging from 2 to 10. For example, in some embodiments, y and z are each independently an integer ranging from 4 to 9 or from 4 to 6.

In some of the foregoing embodiments of Formula (III), R^6 is H. In other of the foregoing embodiments, R^6 is C_1 - C_{24} alkyl. In other embodiments, R^6 is OH.

In some embodiments of Formula (III), G^3 is unsubstituted. In other embodiments, G^3 is substituted. In various different embodiments, G^3 is linear C_1 - C_{24} alkylene or linear C_1 - C_{24} alkenylene.

In some other foregoing embodiments of Formula (III), R^1 or R^2 , or both, is C_6 - C_{24} alkenyl. For example, in some embodiments, R^1 and R^2 each, independently have the following structure:



wherein:

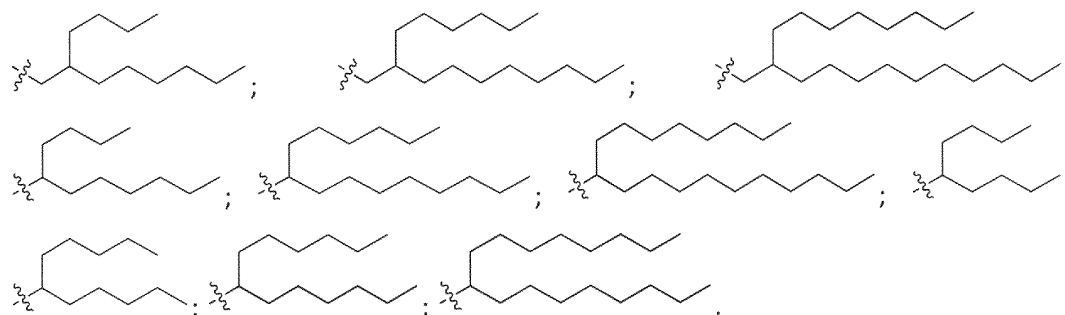
R^{7a} and R^{7b} are, at each occurrence, independently H or C_1 - C_{12} alkyl; and

a is an integer from 2 to 12,

wherein R^{7a} , R^{7b} and a are each selected such that R^1 and R^2 each independently comprise from 6 to 20 carbon atoms. For example, in some embodiments a is an integer ranging from 5 to 9 or from 8 to 12.

In some of the foregoing embodiments of Formula (III), at least one occurrence of R^{7a} is H. For example, in some embodiments, R^{7a} is H at each occurrence. In other different embodiments of the foregoing, at least one occurrence of R^{7b} is C_1 - C_8 alkyl. For example, in some embodiments, C_1 - C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

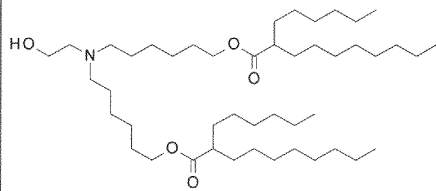
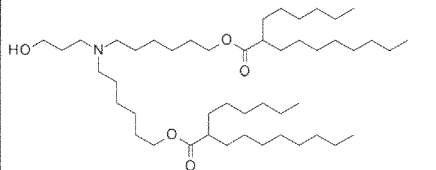
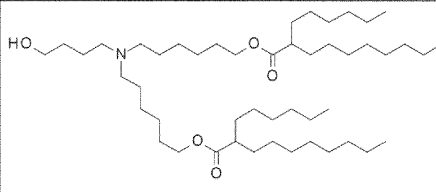
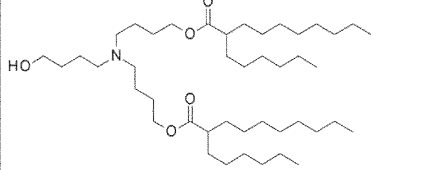
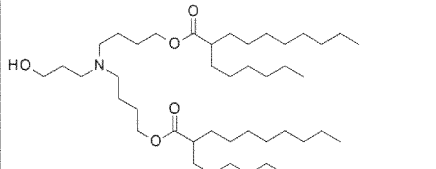
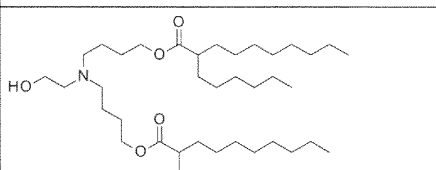
In different embodiments of Formula (III), R^1 or R^2 , or both, has one of the following structures:

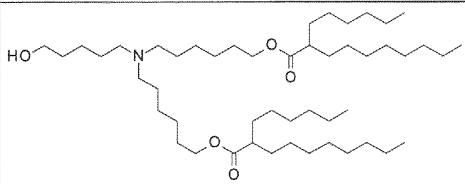
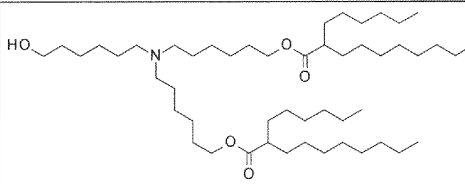
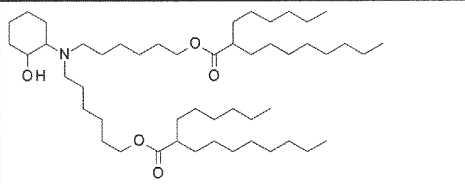
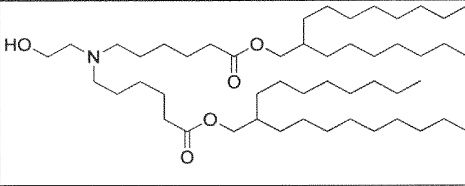
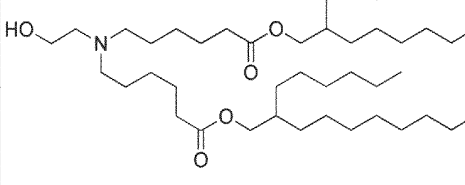
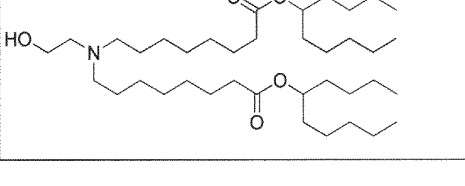


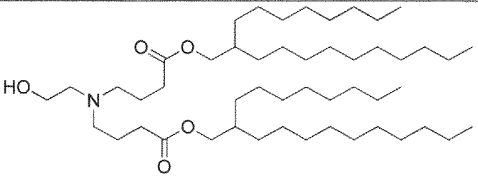
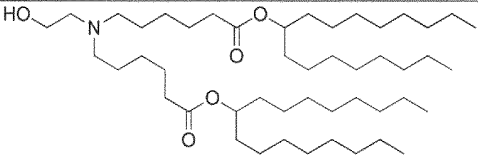
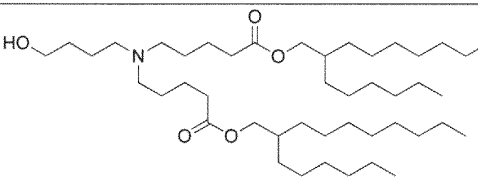
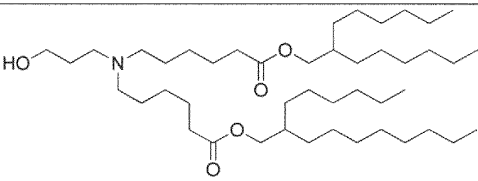
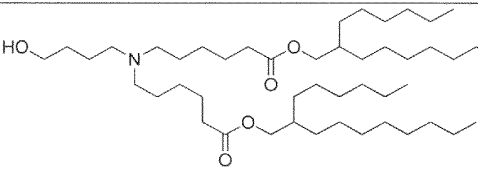
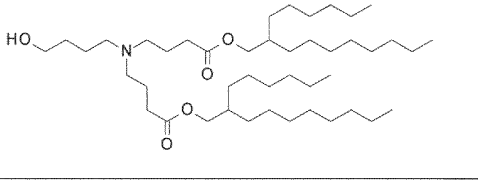
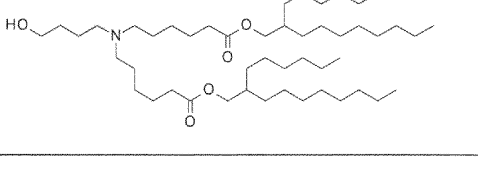
In some of the foregoing embodiments of Formula (III), R^3 is OH, CN, $-C(=O)OR^4$, $-OC(=O)R^4$ or $-NHC(=O)R^4$. In some embodiments, R^4 is methyl or ethyl.

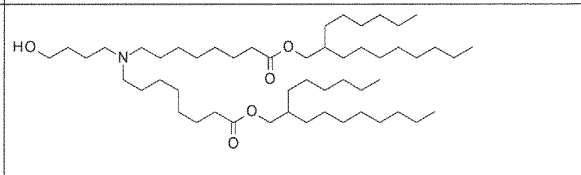
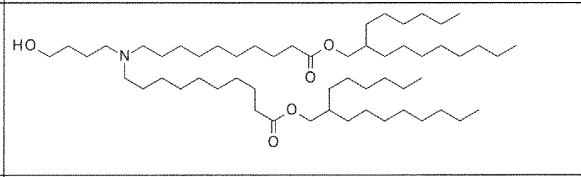
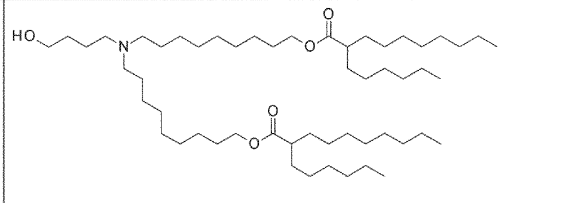
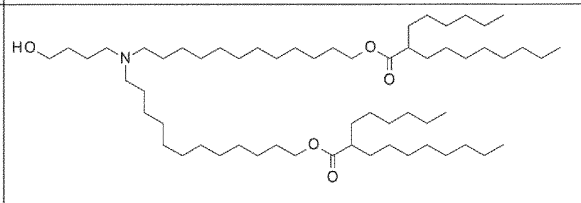
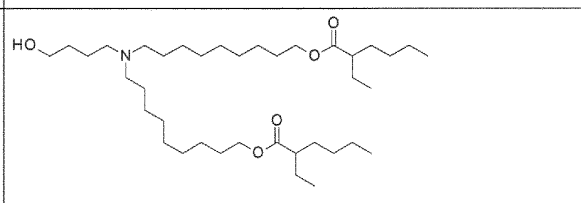
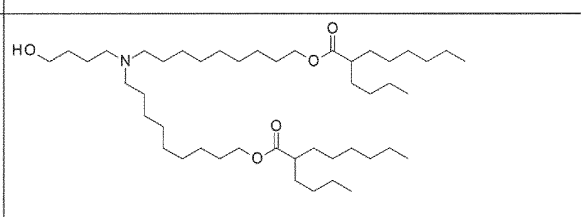
In various different embodiments, the cationic lipid of Formula (III) has one of the structures set forth in the table below.

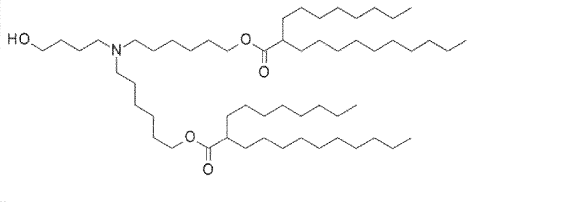
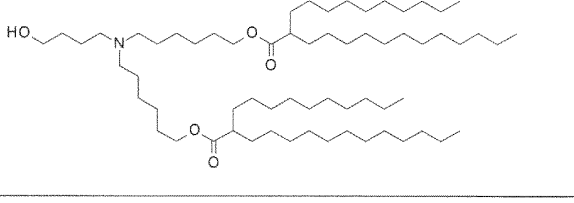
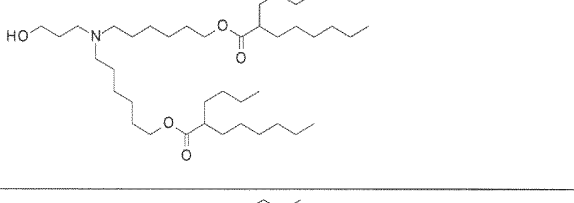
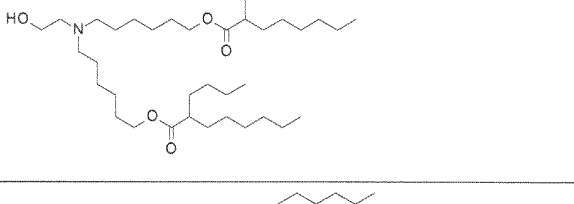
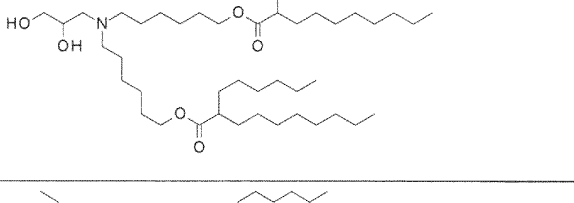
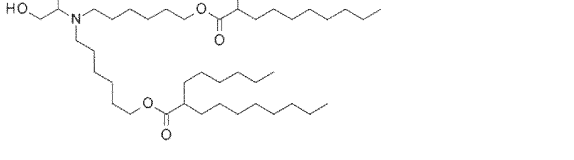
Representative Compounds of Formula (III).

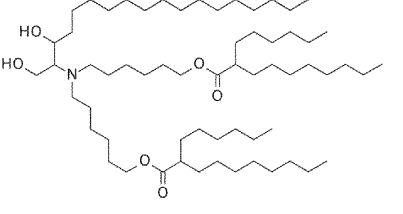
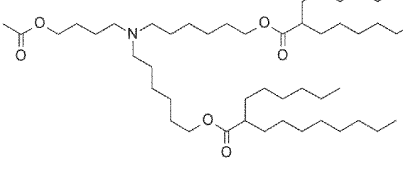
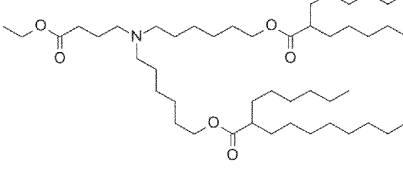
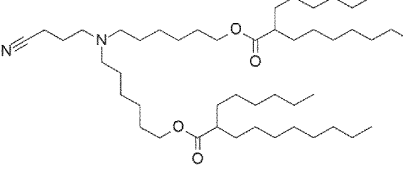
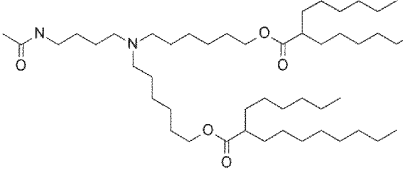
No.	Structure
III-1	
III-2	
III-3	
III-4	
III-5	
III-6	

No.	Structure
III-7	
III-8	
III-9	
III-10	
III-11	
III-12	

No.	Structure
III-13	
III-14	
III-15	
III-16	
III-17	
III-18	
III-19	

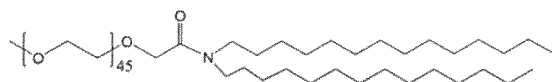
No.	Structure
III-20	
III-21	
III-22	
III-23	
III-24	
III-25	

No.	Structure
III-26	
III-27	
III-28	
III-29	
III-30	
III-31	

No.	Structure
III-32	
III-33	
III-34	
III-35	
III-36	

In some embodiments, the LNP comprises a lipid of Formula (III), RNA, a neutral lipid, a steroid and a pegylated lipid. In some embodiments, the lipid of Formula (III) is compound III-3. In some embodiments, the neutral lipid is DSPC. In some embodiments, the steroid is cholesterol. In some embodiments, the pegylated lipid is ALC-0159.

ALC-0159:



In some embodiments, the cationic lipid is present in the LNP in an amount from about 40 to about 50 mole percent. In one embodiment, the neutral lipid is present in the LNP in an amount from about 5 to about 15 mole percent. In one embodiment, the steroid is present in the LNP in an amount from about 35 to about 45 mole percent. In one embodiment, the pegylated lipid is present in the LNP in an amount from about 1 to about 10 mole percent.

In some embodiments, the LNP comprises compound III-3 in an amount from about 40 to about 50 mole percent, DSPC in an amount from about 5 to about 15 mole percent, cholesterol in an amount from about 35 to about 45 mole percent, and ALC-0159 in an amount from about 1 to about 10 mole percent.

In some embodiments, the LNP comprises compound III-3 in an amount of about 47.5 mole percent, DSPC in an amount of about 10 mole percent, cholesterol in an amount of about 40.7 mole percent, and ALC-0159 in an amount of about 1.8 mole percent.

The N/P value is preferably at least about 4. In some embodiments, the N/P value ranges from 4 to 20, 4 to 12, 4 to 10, 4 to 8, or 5 to 7. In one embodiment, the N/P value is about 6.

LNP described herein may have an average diameter that in one embodiment ranges from about 30 nm to about 200 nm, or from about 60 nm to about 120 nm.

RNA Targeting

Some aspects of the disclosure involve the targeted delivery of the RNA disclosed herein (e.g., RNA encoding an activation compound and/or RNA encoding a docketing compound).

In one embodiment, the disclosure involves targeting the lymphatic system, in particular secondary lymphoid organs, more specifically spleen. Targeting the lymphatic system, in particular secondary lymphoid organs, more specifically spleen is in particular preferred if the RNA administered is RNA encoding an activation compound.

In one embodiment, the target cell is a spleen cell. In one embodiment, the target cell is an antigen presenting cell such as a professional antigen presenting cell in the spleen. In one embodiment, the target cell is a dendritic cell in the spleen.

The "lymphatic system" is part of the circulatory system and an important part of the immune system, comprising a network of lymphatic vessels that carry lymph. The lymphatic system consists of lymphatic organs, a conducting network of lymphatic vessels, and the circulating lymph. The primary or central lymphoid organs generate lymphocytes from immature progenitor cells. The thymus and the bone marrow constitute the primary lymphoid organs. Secondary or peripheral lymphoid organs, which include lymph nodes and the spleen, maintain mature naïve lymphocytes and initiate an adaptive immune response.

RNA may be delivered to spleen by so-called lipoplex formulations, in which the RNA is bound to liposomes comprising a cationic lipid and optionally an additional or helper lipid to form injectable nanoparticle formulations. The liposomes may be obtained by injecting a solution of the lipids in ethanol into water or a suitable aqueous phase. RNA lipoplex particles may be prepared by mixing the liposomes with RNA. Spleen targeting RNA lipoplex particles are described in WO 2013/143683, herein incorporated by reference. It has been found that RNA lipoplex particles having a net negative charge may be used to preferentially target spleen tissue or spleen cells such as antigen-presenting cells, in particular dendritic cells. Accordingly, following administration of the RNA lipoplex particles, RNA accumulation and/or RNA expression in the spleen occurs. Thus, RNA lipoplex particles of the disclosure may be used for expressing RNA in the spleen. In an embodiment, after administration of the RNA lipoplex particles, no or essentially no RNA accumulation and/or RNA expression in the lung and/or liver occurs. In one embodiment, after administration of the RNA lipoplex particles, RNA accumulation and/or RNA expression in antigen presenting cells, such as professional antigen presenting cells in the spleen occurs. Thus, RNA lipoplex particles of the disclosure may be used for expressing RNA in such antigen presenting cells. In one embodiment, the antigen presenting cells are dendritic cells and/or macrophages.

The electric charge of the RNA lipoplex particles of the present disclosure is the sum of the electric charges present in the at least one cationic lipid and the electric charges present in the RNA. The charge ratio is the ratio of the positive charges present in the at least one cationic lipid to the negative charges present in the RNA. The charge ratio of the positive charges present in the at least one cationic lipid to the negative charges present in the RNA is calculated by the following equation: charge ratio=[(cationic lipid concentration (mol)) * (the total number of positive charges in the cationic lipid)] / [(RNA concentration (mol)) * (the total number of negative charges in RNA)].

The spleen targeting RNA lipoplex particles described herein at physiological pH preferably have a net negative charge such as a charge ratio of positive charges to negative charges from about 1.9:2 to about 1:2, or about 1.6:2 to about 1:2, or about 1.6:2 to about 1.1:2. In specific embodiments, the charge ratio of positive charges to negative charges in the RNA lipoplex particles at physiological pH is about 1.9:2.0, about 1.8:2.0, about 1.7:2.0, about 1.6:2.0, about 1.5:2.0, about 1.4:2.0, about 1.3:2.0, about 1.2:2.0, about 1.1:2.0, or about 1:2.0.

Docking compounds may be provided to a subject by administering to the subject RNA encoding a docking compound in a formulation for preferential delivery of RNA to liver or liver tissue. The delivery of RNA to such target organ or tissue is preferred, in particular, if it is desired to express large amounts of the encoded peptide/polypeptide and/or if systemic presence of the the encoded peptide/polypeptide, in particular in significant amounts, is desired or required.

RNA delivery systems have an inherent preference to the liver. This pertains to lipid-based particles, cationic and neutral nanoparticles, in particular lipid nanoparticles such as liposomes, nanomicelles and lipophilic ligands in bioconjugates. Liver accumulation is caused by the discontinuous nature of the hepatic vasculature or the lipid metabolism (liposomes and lipid or cholesterol conjugates).

For *in vivo* delivery of RNA to the liver, a drug delivery system may be used to transport the RNA into the liver by preventing its degradation. For example, polyplex nanomicelles

consisting of a poly(ethylene glycol) (PEG)-coated surface and an mRNA-containing core is a useful system because the nanomicelles provide excellent *in vivo* stability of the RNA, under physiological conditions. Furthermore, the stealth property provided by the polyplex nanomicelle surface, composed of dense PEG palisades, effectively evades host immune defenses.

Examples of suitable immunostimulants for targeting liver are cytokines involved in T cell proliferation and/or maintenance. Examples of suitable cytokines include IL2 or IL7, fragments and variants thereof, and fusion proteins of these cytokines, fragments and variants.

In another embodiment, RNA encoding an immunostimulant may be administered in a formulation for preferential delivery of RNA to the lymphatic system, in particular secondary lymphoid organs, more specifically spleen. The delivery of an immunostimulant to such target tissue is preferred, in particular, if presence of the immunostimulant in this organ or tissue is desired (e.g., for inducing an immune response, in particular in case immunostimulants such as cytokines are required during T-cell priming or for activation of resident immune cells), while it is not desired that the immunostimulant is present systemically, in particular in significant amounts (e.g., because the immunostimulant has systemic toxicity).

Examples of suitable immunostimulants are cytokines involved in T cell priming. Examples of suitable cytokines include IL12, IL15, IFN- α , or IFN- β , fragments and variants thereof, and fusion proteins of these cytokines, fragments and variants.

Pharmaceutical compositions

The agents described herein such as RNA encoding an activation compound, RNA encoding a docking compound and/or immune effector cells may be administered in pharmaceutical compositions or medicaments and may be administered in the form of any suitable pharmaceutical composition.

In one embodiment of all aspects of the invention, the components described herein may be administered in a pharmaceutical composition which may comprise a pharmaceutically

acceptable carrier and may optionally comprise one or more adjuvants, stabilizers etc. In one embodiment, the pharmaceutical composition is for therapeutic or prophylactic treatments, e.g., for use in treating or preventing a disease.

The term "pharmaceutical composition" relates to a formulation comprising a therapeutically effective agent, preferably together with pharmaceutically acceptable carriers, diluents and/or excipients. Said pharmaceutical composition is useful for treating, preventing, or reducing the severity of a disease or disorder by administration of said pharmaceutical composition to a subject. A pharmaceutical composition is also known in the art as a pharmaceutical formulation.

The pharmaceutical compositions according to the present disclosure are generally applied in a "pharmaceutically effective amount" and in "a pharmaceutically acceptable preparation".

The term "pharmaceutically acceptable" refers to the non-toxicity of a material which does not interact with the action of the active component of the pharmaceutical composition.

The term "pharmaceutically effective amount" or "therapeutically effective amount" refers to the amount which achieves a desired reaction or a desired effect alone or together with further doses and/or agents. In the case of the treatment of a particular disease, the desired reaction preferably relates to inhibition of the course of the disease. This comprises slowing down the progress of the disease and, in particular, interrupting or reversing the progress of the disease. The desired reaction in a treatment of a disease may also be delay of the onset or a prevention of the onset of said disease or said condition. An effective amount of the compositions described herein will depend on the condition to be treated, the severeness of the disease, the individual parameters of the patient, including age, physiological condition, size and weight, the duration of treatment, the type of an accompanying therapy (if present), the specific route of administration and similar factors. Accordingly, the doses administered of the compositions described herein may depend on various of such parameters. In the case that a reaction in a patient is insufficient with an initial dose, higher doses (or effectively higher doses achieved by a different, more localized route of administration) may be used.

The term "sub-therapeutic amount" typically refers to a less than standard therapeutic amount of a pharmaceutical agent, meaning that the amount required for the desired effect is lower than when the pharmaceutical agent is used alone. As used herein, the term "sub-therapeutic amount" means that the dosage or amount of a particular pharmaceutical agent is insufficient to achieve the desired pharmacological action in the absence of other compounds, drugs or pharmaceutical agents, e.g., in the absence of activation compound. Such desired pharmacological action may include the complete or essentially complete rejection of solid tumors. Subtherapeutic amounts and doses will usually not be less than about 5%, typically not less than about 10%, and typically not greater than about 75%, more typically not greater than about 60%, of the therapeutic dosage or amount. Normally, the number of immune effector cells administered (including *in vivo* generation in a subject) for CAR T cell therapy of human beings is about 10^9 per dose (equivalent to 1.33×10^7 per kg) or higher (equivalent to 1.3×10^7 per kg). Furthermore, some therapeutic approaches comprise repetitive administration of CAR T cells in a short time period (e.g. less than 4 weeks) to improve safety by dose escalation and/or to maintain the number of effective T cells in the patient. This leads to even higher "accumulated doses" within such time periods. Thus, a "sub-therapeutic amount" of immune effector cells is an amount of such cells per initial dose and/or accumulated dose over a time period of at least 2 days, at least 3 days, at least 4 days, at least 5 days, at least 6 days, at least 7 days, at least 14 days, at least 21 days, at least 28 days or even longer of 10^8 or less, 10^7 or less, 10^6 or less, 10^5 or less, 10^4 or less, 10^3 or less or even lower. In one embodiment, a "sub-therapeutic amount" of immune effector cells genetically modified to express an antigen receptor relates to a single dose of such cells in an amount of 10^8 or less, 10^7 or less, 10^6 or less, 10^5 or less, 10^4 or less, 10^3 or less or even lower. The term "single dose" means that one dose of a therapeutic substance is administered for a prolonged time. The term "prolonged time" comprises a period of at least 14 days, at least 21 days, at least 28 days, at least 3 months, at least 6 months or even longer.

The pharmaceutical compositions of the present disclosure may contain salts, buffers, preservatives, and optionally other therapeutic agents. In one embodiment, the pharmaceutical compositions of the present disclosure comprise one or more pharmaceutically acceptable carriers, diluents and/or excipients.

Suitable preservatives for use in the pharmaceutical compositions of the present disclosure include, without limitation, benzalkonium chloride, chlorobutanol, paraben and thimerosal.

The term "excipient" as used herein refers to a substance which may be present in a pharmaceutical composition of the present disclosure but is not an active ingredient. Examples of excipients, include without limitation, carriers, binders, diluents, lubricants, thickeners, surface active agents, preservatives, stabilizers, emulsifiers, buffers, flavoring agents, or colorants.

The term "diluent" relates a diluting and/or thinning agent. Moreover, the term "diluent" includes any one or more of fluid, liquid or solid suspension and/or mixing media. Examples of suitable diluents include ethanol, glycerol and water.

The term "carrier" refers to a component which may be natural, synthetic, organic, inorganic in which the active component is combined in order to facilitate, enhance or enable administration of the pharmaceutical composition. A carrier as used herein may be one or more compatible solid or liquid fillers, diluents or encapsulating substances, which are suitable for administration to subject. Suitable carriers include, without limitation, sterile water, Ringer, Ringer lactate, sterile sodium chloride solution, isotonic saline, polyalkylene glycols, hydrogenated naphthalenes and, in particular, biocompatible lactide polymers, lactide/glycolide copolymers or polyoxyethylene/polyoxy-propylene copolymers. In one embodiment, the pharmaceutical composition of the present disclosure includes isotonic saline.

Pharmaceutically acceptable carriers, excipients or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R Gennaro edit. 1985).

Pharmaceutical carriers, excipients or diluents can be selected with regard to the intended route of administration and standard pharmaceutical practice.

In one embodiment, pharmaceutical compositions described herein may be administered intravenously, intraarterially, subcutaneously, intradermally or intramuscularly. In certain embodiments, the pharmaceutical composition is formulated for local administration or systemic administration. Systemic administration may include enteral administration, which involves absorption through the gastrointestinal tract, or parenteral administration. As used herein, "parenteral administration" refers to the administration in any manner other than through the gastrointestinal tract, such as by intravenous injection. In a preferred embodiment, the pharmaceutical composition is formulated for intramuscular administration. In another embodiment, the pharmaceutical composition is formulated for systemic administration, e.g., for intravenous administration.

The term "co-administering" as used herein means a process whereby different compounds or compositions such as RNA encoding an activation compound, RNA encoding a docketing compound and optionally immune effector cells are administered to the same patient. The different compounds or compositions may be administered simultaneously, at essentially the same time, or sequentially.

Treatments

The agents, compositions and methods described herein can be used to treat a subject with a disease, e.g., a disease characterized by the presence of diseased cells expressing an antigen (which may serve as primary target). Particularly preferred diseases are cancer diseases. For example, if the antigen is derived from a virus, the agents, compositions and methods may be useful in the treatment of a viral disease caused by said virus. If the antigen is a tumor antigen, the agents, compositions and methods may be useful in the treatment of a cancer disease wherein cancer cells express said tumor antigen.

The agents, compositions and methods described herein may be used in the therapeutic or prophylactic treatment of various diseases. In one embodiment, the agents, compositions and methods described herein are useful in a prophylactic and/or therapeutic treatment of a disease involving an antigen.

The methods and agents described herein are, in particular, useful for the treatment of diseases characterized by diseased cells expressing an antigen the docking compound is directed to, e.g., a tumor antigen. The cells may express the antigen on the cell surface for recognition by a docking compound. The methods may provide for the selective eradication of such cells expressing an antigen, thereby minimizing adverse effects to normal cells not expressing the antigen. Immune effector cells genetically modified to express a chimeric antigen receptor (CAR) targeting the cells through binding to the docking compound are provided to a subject such as by administration of genetically modified immune effector cells to the subject or generation of genetically modified immune effector cells in the subject.

In one embodiment, the immune effector cells described herein are T cells. In one embodiment, the immune effector cells are directed against a tumor or cancer. In one embodiment, the target cell population or target tissue is tumor cells or tumor tissue, in particular of a solid tumor. In one embodiment, the target antigen (primary target) is a tumor antigen.

In one aspect, the present invention generally embraces the treatment of diseases by targeting cells expressing an antigen such as diseased cells, in particular cancer cells expressing a tumor antigen. In one embodiment, the cancer cells or cancer tissue is solid cancer. The target cells may express the antigen on the cell surface. In one embodiment, the antigen is a tumor-associated antigen and the disease is cancer. Such treatment provides for the selective eradication of cells that express an antigen, thereby minimizing adverse effects to normal cells not expressing the antigen.

In one embodiment, the immune effector cells genetically modified to express a CAR are provided to the subject in sub-therapeutic amounts.

The term "disease" refers to an abnormal condition that affects the body of an individual. A disease is often construed as a medical condition associated with specific symptoms and signs. A disease may be caused by factors originally from an external source, such as infectious disease, or it may be caused by internal dysfunctions, such as autoimmune diseases. In humans, "disease" is often used more broadly to refer to any condition that causes pain, dysfunction, distress, social problems, or death to the individual afflicted, or similar problems for those in contact with the individual. In this broader sense, it sometimes includes injuries, disabilities, disorders, syndromes, infections, isolated symptoms, deviant behaviors, and atypical variations of structure and function, while in other contexts and for other purposes these may be considered distinguishable categories. Diseases usually affect individuals not only physically, but also emotionally, as contracting and living with many diseases can alter one's perspective on life, and one's personality.

In the present context, the term "treatment", "treating" or "therapeutic intervention" relates to the management and care of a subject for the purpose of combating a condition such as a disease or disorder. The term is intended to include the full spectrum of treatments for a given condition from which the subject is suffering, such as administration of the therapeutically effective compound to alleviate the symptoms or complications, to delay the progression of the disease, disorder or condition, to alleviate or relief the symptoms and complications, and/or to cure or eliminate the disease, disorder or condition as well as to prevent the condition, wherein prevention is to be understood as the management and care of an individual for the purpose of combating the disease, condition or disorder and includes the administration of the active compounds to prevent the onset of the symptoms or complications.

The term "therapeutic treatment" relates to any treatment which improves the health status and/or prolongs (increases) the lifespan of an individual. Said treatment may eliminate the disease in an individual, arrest or slow the development of a disease in an individual, inhibit or slow the development of a disease in an individual, decrease the frequency or severity of

symptoms in an individual, and/or decrease the recurrence in an individual who currently has or who previously has had a disease.

The terms "prophylactic treatment" or "preventive treatment" relate to any treatment that is intended to prevent a disease from occurring in an individual. The terms "prophylactic treatment" or "preventive treatment" are used herein interchangeably.

The terms "individual" and "subject" are used herein interchangeably. They refer to a human or another mammal (e.g. mouse, rat, rabbit, dog, cat, cattle, swine, sheep, horse or primate) that can be afflicted with or is susceptible to a disease or disorder (e.g., cancer) but may or may not have the disease or disorder. In many embodiments, the individual is a human being. Unless otherwise stated, the terms "individual" and "subject" do not denote a particular age, and thus encompass adults, elderlies, children, and newborns. In embodiments of the present disclosure, the "individual" or "subject" is a "patient".

The term "patient" means an individual or subject for treatment, in particular a diseased individual or subject.

In one embodiment of the disclosure, the aim is to deliver a pharmaceutically active agent (including compounds and cells) to diseased cells expressing an antigen such as cancer cells expressing a tumor antigen, and to treat a disease such as a cancer disease involving cells expressing an antigen such as a tumor antigen.

The term "disease involving an antigen", "disease involving cells expressing an antigen" or similar terms refer to any disease which implicates an antigen, e.g. a disease which is characterized by the presence of an antigen. The disease involving an antigen can be an infectious disease, or a cancer disease or simply cancer. As mentioned above, the antigen may be a disease-associated antigen, such as a tumor-associated antigen, a viral antigen, or a bacterial antigen. In one embodiment, a disease involving an antigen is a disease involving cells expressing an antigen, preferably on the cell surface.

The term "infectious disease" refers to any disease which can be transmitted from individual to individual or from organism to organism, and is caused by a microbial agent (e.g. common

cold). Infectious diseases are known in the art and include, for example, a viral disease, a bacterial disease, or a parasitic disease, which diseases are caused by a virus, a bacterium, and a parasite, respectively. In this regard, the infectious disease can be, for example, hepatitis, sexually transmitted diseases (e.g. chlamydia or gonorrhea), tuberculosis, HIV/acquired immune deficiency syndrome (AIDS), diphtheria, hepatitis B, hepatitis C, cholera, severe acute respiratory syndrome (SARS), the bird flu, and influenza.

The terms "cancer disease" or "cancer" refer to or describe the physiological condition in an individual that is typically characterized by unregulated cell growth. Examples of cancers include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particularly, examples of such cancers include bone cancer, blood cancer, lung cancer, liver cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, colon cancer, breast cancer, prostate cancer, uterine cancer, carcinoma of the sexual and reproductive organs, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the bladder, cancer of the kidney, renal cell carcinoma, carcinoma of the renal pelvis, neoplasms of the central nervous system (CNS), neuroectodermal cancer, spinal axis tumors, glioma, meningioma, and pituitary adenoma. The term "cancer" according to the disclosure also comprises cancer metastases.

The term "solid tumor" or "solid cancer" as used herein refers to the manifestation of a cancerous mass, as is well known in the art for example in Harrison's Principles of Internal Medicine, 14th edition. Preferably, the term refers to a cancer or carcinoma of body tissues other than blood, preferably other than blood, bone marrow, and lymphoid system. For example, but not by way of limitation, solid tumors include cancers of the prostate, lung cancer, colorectal tissue, bladder, oropharyngeal/laryngeal tissue, kidney, breast, endometrium, ovary, cervix, stomach, pancreas, brain, and central nervous system.

The methods and agents described herein are, in particular, useful for the treatment of cancers, e.g., solid cancers, characterized by diseased cells expressing an antigen the docketing compound is directed to.

In one embodiment of the disclosure, the aim is to provide an immune response against diseased cells expressing an antigen such as cancer cells expressing a tumor antigen, and to treat a disease such as a cancer disease involving cells expressing an antigen such as a tumor antigen.

An immune response against an antigen may be elicited which may be therapeutic or partially or fully protective. Pharmaceutical compositions described herein are applicable for inducing or enhancing an immune response. Pharmaceutical compositions described herein are thus useful in a prophylactic and/or therapeutic treatment of a disease involving an antigen.

As used herein, "immune response" refers to an integrated bodily response to an antigen or a cell expressing an antigen and refers to a cellular immune response and/or a humoral immune response.

"Cell-mediated immunity", "cellular immunity", "cellular immune response", or similar terms are meant to include a cellular response directed to cells characterized by expression of an antigen, in particular characterized by presentation of an antigen with class I or class II MHC. The cellular response relates to cells called T cells or T lymphocytes which act as either "helpers" or "killers". The helper T cells (also termed CD4+ T cells) play a central role by regulating the immune response and the killer cells (also termed cytotoxic T cells, cytolytic T cells, CD8+ T cells or CTLs) kill diseased cells such as cancer cells, preventing the production of more diseased cells.

The present disclosure contemplates an immune response that may be protective, preventive, prophylactic and/or therapeutic. As used herein, "induces [or inducing] an immune response" may indicate that no immune response against a particular antigen was present before induction or it may indicate that there was a basal level of immune response against a

particular antigen before induction, which was enhanced after induction. Therefore, "induces [or inducing] an immune response" includes "enhances [or enhancing] an immune response". The term "macrophage" refers to a subgroup of phagocytic cells produced by the differentiation of monocytes. Macrophages which are activated by inflammation, immune cytokines or microbial products nonspecifically engulf and kill foreign pathogens within the macrophage by hydrolytic and oxidative attack resulting in degradation of the pathogen. Peptides from degraded proteins are displayed on the macrophage cell surface where they can be recognized by T cells, and they can directly interact with antibodies on the B cell surface, resulting in T and B cell activation and further stimulation of the immune response. Macrophages belong to the class of antigen presenting cells. In one embodiment, the macrophages are splenic macrophages.

The term "dendritic cell" (DC) refers to another subtype of phagocytic cells belonging to the class of antigen presenting cells. In one embodiment, dendritic cells are derived from hematopoietic bone marrow progenitor cells. These progenitor cells initially transform into immature dendritic cells. These immature cells are characterized by high phagocytic activity and low T cell activation potential. Immature dendritic cells constantly sample the surrounding environment for pathogens such as viruses and bacteria. Once they have come into contact with a presentable antigen, they become activated into mature dendritic cells and begin to migrate to the spleen or to the lymph node. Immature dendritic cells phagocytose pathogens and degrade their proteins into small pieces and upon maturation present those fragments at their cell surface using MHC molecules. Simultaneously, they upregulate cell-surface receptors that act as co-receptors in T cell activation such as CD80, CD86, and CD40 greatly enhancing their ability to activate T cells. They also upregulate CCR7, a chemotactic receptor that induces the dendritic cell to travel through the blood stream to the spleen or through the lymphatic system to a lymph node. Here they act as antigen-presenting cells and activate helper T cells and killer T cells as well as B cells by presenting them antigens, alongside non-antigen specific

co-stimulatory signals. Thus, dendritic cells can actively induce a T cell- or B cell-related immune response. In one embodiment, the dendritic cells are splenic dendritic cells.

The term "antigen presenting cell" (APC) is a cell of a variety of cells capable of displaying, acquiring, and/or presenting at least one antigen or antigenic fragment on (or at) its cell surface. Antigen-presenting cells can be distinguished in professional antigen presenting cells and non-professional antigen presenting cells.

The term "professional antigen presenting cells" relates to antigen presenting cells which constitutively express the Major Histocompatibility Complex class II (MHC class II) molecules required for interaction with naive T cells. If a T cell interacts with the MHC class II molecule complex on the membrane of the antigen presenting cell, the antigen presenting cell produces a co-stimulatory molecule inducing activation of the T cell. Professional antigen presenting cells comprise dendritic cells and macrophages.

The term "non-professional antigen presenting cells" relates to antigen presenting cells which do not constitutively express MHC class II molecules, but upon stimulation by certain cytokines such as interferon-gamma. Exemplary, non-professional antigen presenting cells include fibroblasts, thymic epithelial cells, thyroid epithelial cells, glial cells, pancreatic beta cells or vascular endothelial cells.

"Antigen processing" refers to the degradation of an antigen into procession products, which are fragments of said antigen (e.g., the degradation of a protein into peptides) and the association of one or more of these fragments (e.g., via binding) with MHC molecules for presentation by cells, such as antigen presenting cells to specific T cells.

Citation of documents and studies referenced herein is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the contents of these documents are based on the information available to the applicants and do not constitute any admission as to the correctness of the contents of these documents.

The following description is presented to enable a person of ordinary skill in the art to make and use the various embodiments. Descriptions of specific devices, techniques, and applications are provided only as examples. Various modifications to the examples described herein will be readily apparent to those of ordinary skill in the art, and the general principles defined herein may be applied to other examples and applications without departing from the spirit and scope of the various embodiments. Thus, the various embodiments are not intended to be limited to the examples described herein and shown, but are to be accorded the scope consistent with the claims.

Examples

mRNA constructs

All constructs were generated by gene synthesis comprising or encoding the sequence elements described below:

Sequence elements

Region	nt-sequence	SEQ ID NO:	aa-Sequence	SEQ ID NO:
IgG- Leader	ATGGACTGGATCTGGCGAATACTGTTTCTGG TGGGAGCCGCCACTGGGGCCATTCT	35	MDWIWRILFLV GAATGAHS	10
ALFA	CCTAGCAGGCTGGAGGAGGAAGTCCGCAGA CGGCTGACCGAACCC	36	PSRLEELRRRLT EP	11
Human CD8 hinge	ACTACTACCCAGCACCTAGACCGCCTACAC CCGCACCCACTATCGCGTCTCAGCCCTTGAG TCTGCGGCCCGAGGCTTGTCGGCCCGCAGCT GGCGGGGCTGTGCATACCCGAGGACTCGAC TTTGATGCGACATCTACATTTGGGCCCCTCT GGCCGGCACTTGCGGCGTCCTTCTTGAGT CTGGTCATAACGTTGTATTGC	37	TTTPAPRPPTPAP TIASQPLSLRPEA CRPAAGGAVHT RGLDFACDIYW APLAGTCGVLLLS LVITLYC	12
Human 4-1BB domain	AAACGGGGCAGGAAGAAAGTCTGTATATC TTCAAGCAGCCTTTCATGCGCCAGTTCAGA CCTCAGGAGGAGGATGGGTGTTCTGTC GTTTCCCTGAGGAAGAAGAAGGCGGGTGCG AATTG	38	KRGRKKLLYIFKQ PFMRPVQTTQEE DGCSCRFPEEEE GGCEL	13

Human CD3 zeta domain	AGGGTCAAGTTTAGCCGATCAGCTGACGCC CCTGCTTACAAACAGGGGCAAAATCAGCTTT ACAATGAGCTGAACCTCGGGCGTAGAGAGG AGTACGACGTGTTGGACAAGCGCAGAGGGA GAGATCCCAGATGGGCGGCAAACCAAGAC GCAAGAATCCCCAAGAAGGCCTCTACAACG AGCTGCAGAAGGATAAAATGGCCGAAGCCT ATAGCGAGATTGGCATGAAAGGAGAACGG AGGAGAGGGAAAGGTCACGATGGACTCTAC CAAGGCCTGAGCACAGCTACCAAAGACACG TATGATGCCTTGACATGCAAGCACTGCCAC CCAGGTAG	39	RVKFSRSADAPA YKQGQNQLYNEL NLGRREEYDVLD KRRGRDPEMGG KPRRKNPQEGLY NELQDKMAEA YSEIGMKGERRR GKGHDGLYQGLS TATKDTYDALHM QALPPR*	14
G4S- Linker	GGAGGAGGCGGGAGC	40	GGGGS	15
TNFR1 (full sequence)	ATGGAGTATGCTAGCGATGCCTCTCTGGACC CGGAAGCTCCATGGCCACCAGCTCCTAGGG CTCGCGCTTGCCGTGTGCTTCCTTGGGCCCT GGTGGCTGGCCTCCTGTTGCTGCTGCTGCTG GCTGCCGATGCGCTGTGTTCTCGCCTGTC CATGGGCGGTAAGTGGCGCGAGAGCCTCTC CTGGTTCAGCCGATCACCAGGCTGAGGG AAGGGCCAGAGCTTAGCCCCGATGACCCTG CTGGGTTGCTCGACCTGAGACAGGGGATGT TTGCCAGTTGGTAGCGCAGAACGTGCTGCT GATCGACGGTCCCTTGAGCTGGTATTCCGAT CCCGGTCTTGCCGAGTCAGCCTCACTGGCG GCCTGAGTTACAAGGAGGACACCAAGGAAC	41	MEYASDASLDPE APWPPAPRARA CRVLPWALVAGL LLLLLLAAACAVF LACPWAVSGAR ASPGSAASPRLRE GPELSPDDPAGL LDLRQGMFAQL VAQNVLLIDGPLS WYSDPGLAGVSL TGGLSYKEDTKEL VVAKAGVYVFF QLELRRV VAGEG	16

	TGGTGGTTGCCAAAGCCGGTGTCTACTACGT GTTCTTCCAGCTCGAACTCAGGCGCGTGGTT GCAGGAGAGGGGTCTAGGCTCTGTGAGTCTT GCCCTTCATCTCCAGCCCCTGAGAAGCGCAG CCGGAGCCGCTGCACTGGCGCTGACCGTGG ATCTCCACCCGCCTCCTCCGAAGCCCGCAA TAGCGCTTTTGGCTTCCAAGGGCGACTGTTG CACCTGTCTGCAGGCCAACGGCTGGGAGTC CATCTGCACACGGAGGCCAGAGCACGACAC GCATGGCAGCTGACACAGGGAGCCACTGTC CTGGGACTGTTTCGGGTTACCCCGAGATTC CTGCAGGCCTCCCTCCCCTCGGTCCGAG		SGSVSLALHLQPL RSAAGAAALALT VDLPPASSEARN SAFGFQGRLLHLS AGQRLGVHLHTE ARARHAWQLTQ GATVLGLFRVTP EIPAGLPSRSE	
Human sec signal	ATGAGAGTGATGGCCCCAGAACCCTGATCCTG CTGCTGTCTGGCGCCCTGGCCCTGACA GAGACATGGGCCGGAAGC	42	MRVMAPRTLILL LSGALALTETWA GS	17
(EA ₃ K) ₃ Linker	GAGGCGGCAGCTAAGGAAGCTGCTGCTAAAGA GGCTGCGGCTAAG	43	EAAAKEAAAKEA AAK	18
GPI Anchor (human CD55)	CCAAATAAAGGAAGTGGAACCACTTCAGGTACT ACCCGTCTTCTATCTGGGCACACGTGTTTCACGT TGACAGGTTTGCTTGGGACGCTAGTAACCATGG GCTTGCTGACTTGA	44	PNKSGTTS GTT RLLSGHTCFTLTG LLGTLVTMGLLT	19
IgG- Leader2	ATGGATTGGA CTGGCGGGTCTTTTGCCTGTTAG CAGTGGCCCCCGGCGCTCATAGC	45	MDWTWRVFCLL AVAPGAHS	20
GS- Linker2	GGAGGAGGCGGGAGCGGCGGGGGTAGT	46	GGGSGGGGS	21
aCLDN6 VH	GAAGTTCAGCTGCAGCAGTCAGGGCCGGAAGT GTGAAACCCGGCGCTAGCATGAAGATCTCCTGT	47	EVQLQQSGPELV KPGASMKISCKA	22

	AAGGCGAGCGGGTATTCCTTTACCGGCTACACC ATGAACTGGGTAAAGCAATCTCACGGCAAGAAC CTTGAGTGGATAGGACTCATTAATCCTTACAATG GCGGCACAATCTACAACCAGAAGTTCAAAGGCA AAGCAACCCTTACCGTGGACAAGAGCAGCTCTA CAGCCTATATGGAGCTGCTCTCACTGACGTCCGA GGATAGCGCAGTGTACTATTGTGCGCGGGATTA CGGGTTTGTGCTGGATTATTGGGGACAGGGCAC CACACTGACCGTAAGCAGT		SGYSFTGYTMN WVKQSHGKNLE WIGLINPYNGGTI YNQKFKGKATLT VDKSSSTAYMEL LSLTSEDSAVYYC ARDYGFVLDYW GQGTTLTVSS	
aCLDN6 VL	GACATTGTCCTGACACAGAGTCCATCCATTATGA GCGTGAGTCCTGGTGAAAAGGTTACAATCACCT GCAGTGCAAGCTCATCAGTGTCTTACATGCATTG GTTTCAGCAGAAGCCGGGAACCTCTCCTAAGCT GAGCATCTACTCTACGAGCAATCTCGCCTCTGGT GTCCAGCGAGGTTCTCAGGACGCGGGTCCGGG ACTTCCTATTCCTCACAATTTCCAGGGTAGCCG CTGAAGATGCTGCCACCTATTATTGCCAACAGCG CAGCAACTACCCACCCTGGACATTCGGTGGTGG AACAAACTGGAGATTAAGCGGTCCGACCCAGC C	48	DIVLTQSPSIMSV SPGEKVTITCSAS SSVSYMHWFQQ KPGTSPKLSIYSTS NLASGVPARFSG RSGTSYSLTISR VAAEDAATYYCQ QRSNYPPWTFG GGTKLEIKRSDPA	23
GS- Linker3	GGAGGCGGTGGAAGTGGCGGTGGAGGGTCAG GAGGTGGTGGATCT	49	GGGSGGGGSG GGGS	24
GS- Linker4	GGCGGCTCT	50	GGG	25
His-Tag	CACCACCACCACCATCAT	51	HHHHHH	26

The proteins encoded by the constructs to be used for the examples below are as follows:

Name	Sequence	consist of SEQ ID NO:	SEQ ID NO:
Leader- ALFA- CD8h- BBz-CAR	MDWIWRILFLVGAATGAHS PSRLEEELRRRLTEP TTTPAPRPPTPAPTASQPLSLRPEACRPAAGGAVHTRGLDFACDIYWAPL AGTCGVLLLSLVITLYC KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCEL RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATK DTYDALHMQALPPR	10, 11, 12, 13, 14	27
Leader- ALFA- G4S- CD8h- BBz-CAR	MDWIWRILFLVGAATGAHS PSRLEEELRRRLTEP GGGGS TTTPAPRPPTPAPTASQPLSLRPEACRPAAGGAVHTRGLDFACDIYWAPL AGTCGVLLLSLVITLYC KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCEL RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATK DTYDALHMQALPPR	10, 11, 15, 12, 13, 14	28
Leader- VHH(aAL FA)- CD8h- BBz-CAR	MDWIWRILFLVGAATGAHS EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERR VMVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGGTQVTVSSTTPAPRPPTPAPTASQPLSLRPE ACRPAAGGAVHTRGLDFACDIYWAPLAGTCGVLLLSLVITLYC KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCEL RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATK DTYDALHMQALPPR	10, 9, 12, 13, 14	29

Leader- VHH(aAL FA)-G4S- CD8h- BBz-CAR	MDWIWRILFLVGAATGAHS EVQLQESGGGLVQPGGSLRLSCTASGVVISALNAMAMGWYRQAPGERR VMVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSS GGGGG TTTPAPRPPTPAPTASQPLSLRPEACRPAAGGAVHTRGLDFACDIYWAPL AGTCGVLLLSLVITLYC KRGRKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL RVKFSRSADAPAYKQGQNQLYNELNLRREEYDVLDRRGRDPEMGGKP RRKNPQEGLYNELQDKMAEAYSEIGMKGERRRGKGDGLYQGLSTATK DTYDALHMQALPPR	10, 9, 15, 12, 13, 14	30
TNFL9- ALFA	MEYASDASLDPEAPWPPAPRARACRVLPWALVAGLLLLLLAAACAVFLA CPWAVSGARASPGSAASPRREGPELSPDDPAGLLDLRQGMFAQLVAQN VLLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVAKAGVYVFFQLELRR VVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQG RLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGLPSP RSE PSRLEELRRRLTE	16, 11	31
hu-sec- ALFA- (EA3K)3- GPI	MRVMAPRTLILLSGALALTETWAGS SRLEELRRRLTEP EAAAKEAAAKEAAK PNKSGTTS GTTRLLSGHTCFTLTGLGLVTMGLLT	17, 5, Pro, 18, 19	32
hu-sec- VHH(aAL FA)- (EA3K)3- GPI	MRVMAPRTLILLSGALALTETWAGS EVQLQESGGGLVQPGGSLRLSCTASGVVISALNAMAMGWYRQAPGERR VMVAAVSERGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVY YCHVLEDRVDSFHDYWGQGTQVTVSSEAAAKEAAAKEAAK PNKSGTTS GTTRLLSGHTCFTLTGLGLVTMGLLT	17, 9, 18, 19	33
Leader2- ALFA-	MDWTWRVFCLLAVAPGAHS <u>PSRLEELRRRLTEP</u>	20, 11, 21, 22,	34

VH/VL(a) CLDN6)- His	GGGSGGGG EVQLQQSGPELVKPGASMKISCKASGYSFTGYTMNWVKQSHGKNLEWIG LINPYNGGTIYNQKFKGKATLTVDKSSSTAYMELLSLTSEDSAVYYCARDYG FVLDYWGGQTTLTVSS GGGSGGGSGGGG <u>DIVLTQSPSIMSVSPGEKVTITCSASSSVSYMHWFQQKPGTSPKLSIYSTSN</u> <u>LASGVPARFSGRSGTSYSLTISRVAEDAATYYCQQRSNYPPWTFGGGT</u> <u>KLEIKRSDPA</u> GGG <u>HHHHHH</u>	24, 23, 25, 26	
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Example 1: Universal CART approach.

In this example, illustrated in figure 1, CAR-T cells are generated in which the catcher is fused as a recognition domain to the hinge, transmembrane and intracellular signaling domain of the chimeric antigen receptor. To reduce costs and allow for fast patient supply, allogenic CAR-T cells are preferred for this purpose, which can be generated via established methods like gene engineering of $\alpha\beta$ T-cell depletion. The generic CAR-T cells are administered to the patient where they can be amplified via liposome formulated mRNA encoding the CAR-specific tag fused to a membrane-anchoring protein domain based on our recently published CARVAC approach (Reinhard et al., 2020). As a second component, the tumor-specific targeting ligand, e.g. an anti-CLDN6 scFv, is fused to the tag sequence and administered to the patient as RNA encapsulated in lipid nanoparticles. The lipid nanoparticles are taken up by liver cells, which then express the bispecific fusion protein and release it into the bloodstream. The targeting ligand accumulates at the tumor site where finally the generic CAR-T cells can bind and mediate tumor-cell killing. The described procedure is in theory universally applicable for different tumor antigens and would in principle enhance efficacy and also enable for simultaneous targeting of several antigens via providing a mixture of RNAs encoding for different targeting ligands. Another major advantage of this approach is the safe persistence of CAR-T cells in the absence of the targeting ligand. This enables to pause the therapy without depletion of the CAR-T cells, if all tumor cells are eliminated and in parallel providing the option to continue therapy using the same or another targeting ligand in case of a tumor relapse.

Example 2: Modular CAR-T in Jurkat cells

Jurkat T cells were engineered with mRNA encoding the modular CAR and analyzed 24h later by flow cytometry. Soluble modular CAR adaptors were produced by nucleic acid transfected cells. Modular CAR Jurkat cells were armed with 1 or 10 nM of soluble adaptor and counter stained with an adaptor-specific antibody (anti idotype antibody against aCLDN6 antibody) in

order to detect binding of the adaptor on the modular CAR. Adaptor-bound CARs were detected using flow cytometric methodology. Mock Jurkat cells (non-mRNA transfected) were used as controls where the adaptor moiety at the surface of the cells is not detected. Adaptors are detected at the surface of the T cell line modified to express the modular CAR (VHH(aALFA)-G4S-CAR, SEQ ID NO: 30). The dose range used shows that the modular CAR-T cells can effectively bind the adaptors at low doses (1nM, % of adaptor binding cells) and the intensity of binding (MFI) can be increased with higher doses of adaptor. In this experiment an ALFA-VH-VL adaptor format was used (SEQ ID NO: 34). Figure 2 shows the assembly of the CAR and adaptor.

Example 3: Modular CAR-T in primary cells

Primary human T cells were activated over 5 days using CD3 agonistic antibodies and subsequently transfected with modular CAR encoding mRNA (VHH(aALFA)-G4S-CAR, SEQ ID NO: 30). CAR molecules were stained 24 hours later with a labeled ALFA peptide. Expression was detected using flow cytometric methodology. Figure 3 shows that the CAR is well expressed in primary human T cells (39.5 %).

In parallel, primary cells activated and transfected as described above were armed with 100 nM soluble adaptor generated by nucleic acid transfected cells and stained with an adaptor-specific antibody 24 hours later. Percent CAR-bound adaptor was assessed using flow cytometric methodology. The adaptor binds the modular CAR expressed at the surface of engineered primary human T cells (12.4 % for 100 nM adaptor). In this experiment an ALFA-VH-VL-His-Tag adaptor format (SEQ ID NO: 34) was used. Figure 4 shows that the modular CAR has assembled. The lower detection of the adaptor compared to Figure 2 is based on the accessibility of the VH-VL domain, which is enclosed by 2 tags here.

Example 4: Modular CAR-T cells mediates tumor cell lysis

Antigen positive tumor cells were seeded on E-plates (ACEA) and incubated for 24 hours prior to co-culture. Activated primary human T cells, engineered to express the modular CAR (see

figure 3), were armed 24 hours after transfection with 10 nM or 100 nM soluble adaptor generated by nucleic acid transfected cells. Armed modular CAR-T cells were co-cultured with tumor cells at an effector to target ratio of 10:1. Unarmed modular CAR-T cells were used as a negative control. Tumor cell lysis was assessed over 27 hours using impedance-based methodology on an xCELLigence system. Cytotoxicity was normalized to non-mRNA transfected T cells and calculated in relation to maximum lysis achieved by Triton-X.

Unarmed modular CAR-T cells did not induce cytotoxic lysis of antigen expressing tumor cells. Modular CAR-T cells armed with adaptor promoted strong cytolytic activity. Figure 5 shows the killing of tumor cells in an adaptor dose dependent manner.

Example 5: Proliferation of Modular CAR-T cells induced by universal ALFA-peptide containing mRNA transfected iDCs

Human immature dendritic cells (iDC) were differentiated from CD14 positive cells via GM-CSF and IL-4. iDCs were transfected with mRNA coding for membrane anchored binding moiety (CARVac). Autologous T cells were transfected with modular CAR encoding mRNA and stained with the proliferation dye brilliant violet. Modular CAR-T cells and CARVac expressing iDCs were co-cultured for 4 days at an effector to target ratio of 10:1. Modular CAR-T cell proliferation was assessed by a reduction of proliferation dye over daughter generations using flow cytometric methodology. Mock mRNA transfected iDCs served as a negative control (control). In Figure 6 A and B the membrane anchored binding moiety expressed on iDCs comprises the ALFA peptide, while the CAR comprises the anti-ALFA VHH. In Figure 6C and D, the membrane anchored binding moiety expressed on iDCs comprises the anti-ALFA VHH, while the CAR comprises the ALFA peptide.

While modular CAR-T cells did not proliferate against control iDCs, VHH(aALFA) CAR (SEQ ID NO: 30) T cells as well as the similar VHH(aALFA)PE-CAR showed prominent proliferation against ALFA-peptide containing CARVac constructs (Figure 6 A & B). Here the target of the

modular CAR was expressed at the surface of the antigen presenting cells using 2 different scaffolds for transmembrane anchoring. The TNFL9 anchor (SEQ ID NO: 31) or the (EA3K)GPI anchor (SEQ ID NO: 32). In Figure 6 C & D, the modular CARs (SEQ ID NO: 27 and 28) facilitate prominent proliferation against the VHH-containing iDC presented construct (SEQ ID NO: 33).

Example 6: Modular ALFA CAR-Ts loaded with CD19 specific adaptors mediate proliferation against CD19 transfected iDCs or primary human B cells

Human primary T cells were engineered to express the ALFA CAR and labelled with the proliferation dye brilliant violet. They were then co-incubated with either primary human B cells (left) or autologous iDCs transfected with CD19 encoding mRNA for 4 days in presence of 0, 1 or 10 nM adaptor (nbALFA/VHH(aALFA)-antiCD19(FMC63)) generated from nucleic acid engineered producing cells. In the case of B cells the effector to target ratio was varied from 10:1, 1:1 to 1:10. In the case of iDCs as target cells, the E:T ratio used was 10:1. The frequency of proliferating CAR-T cells is depicted after flow cytometer acquisition and analysis.

As observed in Figure 7, the proliferation of engineered ALFA-CAR T cells required presence of the adaptor as well as target cells expressing the CD19 target.

Note that in this example, the adaptor is made of a VHH(aALFA) antigen binding domain linked to an scFv antigen binding domain.

Claims

1. A method for treating a subject having a disease, disorder or condition characterized by cells expressing a target antigen, comprising:
 - (i) providing to the subject immune effector cells genetically modified to express a chimeric antigen receptor (CAR);
 - (ii) administering to the subject first RNA encoding a first peptide or polypeptide, wherein the first peptide or polypeptide comprises a binding moiety for the CAR;
 - (iii) allowing expression of the first peptide or polypeptide by antigen presenting cells in the subject such that the binding moiety for the CAR is available for binding by the immune effector cells said binding resulting in expansion of the immune effector cells;
 - (iv) administering to the subject second RNA encoding a second peptide or polypeptide, wherein the second peptide or polypeptide comprises a binding moiety binding to the target antigen and a binding moiety for the CAR; and
 - (v) allowing expression of the second peptide or polypeptide by cells in the subject such that the second peptide or polypeptide becomes associated with cells expressing the target antigen and the binding moiety for the CAR is available for binding by the immune effector cells.
2. The method of claim 1, wherein the antigen presenting cells are transfected with the first RNA.
3. The method of claim 1 or 2, wherein the first RNA is administered as particulate formulation such as formulated as lipoplex particles.
4. The method of any one of claims 1 to 3, wherein the cells expressing the second peptide or polypeptide are transfected with the second RNA.
5. The method of any one of claims 1 to 4, wherein the second RNA is administered as particulate formulation such as formulated as lipid nanoparticles.

6. The method of any one of claims 1 to 5, wherein the antigen presenting cells express the first peptide or polypeptide such that it remains associated with the antigen presenting cells.
7. The method of any one of claims 1 to 6, wherein the first peptide or polypeptide is a membrane peptide or polypeptide.
8. The method of any one of claims 1 to 7, wherein the first peptide or polypeptide is a fusion protein of the binding moiety for the CAR and a membrane peptide or polypeptide.
9. The method of any one of claims 1 to 8, wherein binding of the immune effector cells to the second peptide or polypeptide associated with cells expressing the target antigen results in killing of cells expressing the target antigen.
10. The method of any one of claims 1 to 9, wherein the cells expressing the second peptide or polypeptide secrete the second peptide or polypeptide.
11. The method of any one of claims 1 to 10, wherein the cells expressing the second peptide or polypeptide express the second peptide or polypeptide such that it is released into the bloodstream.
12. The method of any one of claims 1 to 11, wherein the target antigen is a cell surface antigen.
13. The method of any one of claims 1 to 12, wherein the second peptide or polypeptide is a fusion peptide or polypeptide of the binding moiety binding to the target antigen and the binding moiety for the CAR.
14. The method of any one of claims 1 to 13, wherein the binding moiety binding to the target antigen comprises an antibody or an antibody derivative.

15. The method of any one of claims 1 to 14, wherein the binding moiety for the CAR comprises a peptide tag.
16. The method of any one of claims 1 to 16, wherein the CAR comprises an antibody or an antibody derivative.
17. The method of any one of claims 14 to 16, wherein the antibody derivative is an antibody fragment.
18. The method of any one of claims 1 to 17, wherein the method comprises administering the immune effector cells genetically modified to express a CAR to the subject.
19. The method of any one of claims 1 to 17, wherein the method comprises generating the immune effector cells genetically modified to express a CAR in the subject.
20. The method of any one of claims 1 to 19, wherein the disease, disorder or condition is cancer.
21. The method of any one of claims 1 to 20, wherein the cells expressing a target antigen are diseased cells.
22. The method of any one of claims 1 to 21, wherein the cells expressing a target antigen are cancer cells.
23. The method of any one of claims 1 to 22, wherein the target antigen is a tumor antigen.
24. A method for treating a subject having a disease, disorder or condition characterized by cells expressing a target antigen, comprising:
 - (i) providing to the subject immune effector cells genetically modified to express a chimeric antigen receptor (CAR) binding to a peptide tag;

- (ii) administering to the subject first RNA encoding a first peptide or polypeptide such that the first peptide or polypeptide is expressed in antigen presenting cells of the subject, wherein the first peptide or polypeptide is a membrane protein which comprises in an extracellular domain a peptide tag to which the CAR binds; and
- (iii) administering to the subject second RNA encoding a second peptide or polypeptide such that the second peptide or polypeptide is expressed in and secreted by cells of the subject, wherein the second peptide or polypeptide comprises a binding moiety binding to the target antigen and a peptide tag to which the CAR binds.

25. The method of claim 24, wherein binding of the immune effector cells to the first peptide or polypeptide results in expansion of the immune effector cells.

26. The method of claim 24 or 25, wherein binding of the immune effector cells to the second peptide or polypeptide bound to target antigen results in killing of cells expressing the target antigen.

27. The method of any one of claims 24 to 26, wherein the antigen presenting cells are transfected with the first RNA.

28. The method of any one of claims 24 to 27, wherein the first RNA is administered as particulate formulation such as formulated as lipoplex particles.

29. The method of any one of claims 24 to 28, wherein the cells expressing the second peptide or polypeptide are transfected with the second RNA.

30. The method of any one of claims 24 to 29, wherein the second RNA is administered as particulate formulation such as formulated as lipid nanoparticles.

31. The method of any one of claims 24 to 30, wherein the antigen presenting cells express the first peptide or polypeptide such that it remains associated with the antigen presenting cells.

32. The method of any one of claims 24 to 31, wherein the first peptide or polypeptide is a fusion protein of a peptide tag to which the CAR binds and a membrane peptide or polypeptide.
33. The method of any one of claims 24 to 32, wherein the second peptide or polypeptide is secreted into the bloodstream.
34. The method of any one of claims 24 to 33, wherein the target antigen is a cell surface antigen.
35. The method of any one of claims 24 to 34, wherein the second peptide or polypeptide is a fusion peptide or polypeptide of the binding moiety binding to the target antigen and a peptide tag to which the CAR binds.
36. The method of any one of claims 24 to 35, wherein the binding moiety binding to the target antigen comprises an antibody or an antibody derivative.
37. The method of any one of claims 24 to 36, wherein the CAR comprises an antibody or an antibody derivative.
38. The method of claim 36 or 37, wherein the antibody derivative is an antibody fragment.
39. The method of any one of claims 24 to 38, wherein the method comprises administering the immune effector cells genetically modified to express a CAR to the subject.
40. The method of any one of claims 24 to 38, wherein the method comprises generating the immune effector cells genetically modified to express a CAR in the subject.
41. The method of any one of claims 24 to 40, wherein the disease, disorder or condition is cancer.

42. The method of any one of claims 24 to 41, wherein the cells expressing a target antigen are diseased cells.
43. The method of any one of claims 24 to 42, wherein the cells expressing a target antigen are cancer cells.
44. The method of any one of claims 24 to 43, wherein the target antigen is a tumor antigen.
45. A kit, comprising:
- (i) nucleic acid for genetically modifying immune effector cells to express a chimeric antigen receptor (CAR) binding to a peptide tag, or immune effector cells genetically modified to express a chimeric antigen receptor (CAR) binding to a peptide tag;
 - (ii) first RNA encoding a first peptide or polypeptide which is a membrane protein which comprises in an extracellular domain a peptide tag to which the CAR binds, or nucleic acid for obtaining said first RNA; and optionally
 - (iii) second RNA encoding a second peptide or polypeptide which comprises a binding moiety binding to a target antigen and a peptide tag to which the CAR binds, or nucleic acid for obtaining said second RNA.