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(54) **Title:** EXPANDED T CELL ASSAY

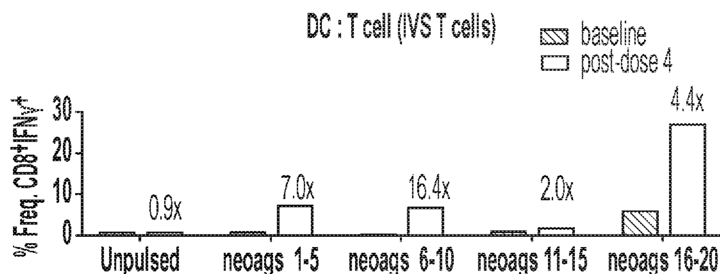

FIG. 2A

(57) **Abstract:** Assays for assessing the therapeutic efficacy of vaccines, including personalized cancer vaccines are provided. Improved mRNA vaccines are also provided.

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EXPANDED T CELL ASSAY

RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application, U.S.S.N. 62/855,718, filed May 31, 2019, the entire contents of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Nucleic acid vaccines based on plasmid DNA, viral vectors or messenger RNA (mRNA) have been evaluated for several clinical applications including cancer, allergy and gene replacement therapies, and have proven to be effective as vaccines against infectious diseases. There has been considerable focus on modified mRNA vaccines during the last decade, as they are safe, scalable and offer precision in antigen design. They circumvent the problem of pre-existing immunity associated with viral vectors and appear to be more potent than DNA vaccines. Personalized mRNA vaccines may be especially valuable for treating cancer.

SUMMARY OF THE INVENTION

In some aspects the invention is a method for detecting antigen specific T cell activation in a population of T cells, comprising: in vitro stimulation (IVS) of a population of T cells, wherein the IVS involves culturing the T cells in an enriched media, stimulation of the cultured T cells with neoantigen matured autologous dendritic cells (DCs), and expanding the stimulated T cells to produce a population of expanded T cells; restimulating the expanded T cells with neoantigen matured autologous DCs; and analyzing the restimulated T cells to detect antigen specific T cells.

In some embodiments the enriched media includes IL-2, IL-7, or IL-2 and IL-7. In other embodiments the T cells are cultured in the enriched media for about 24 hours before stimulation with neoantigen matured autologous DCs. The stimulated T cells are expanded for 12-16 days or 14 days in some embodiments. In other embodiments the stimulated T cells are expanded while cultured in a media comprising IL-2 and IL-7 for 2 days and then in a media comprising IL-2 for 12 days.

In some embodiments the restimulated T cells are analyzed using flow cytometry.

In some embodiments the population of T cells is a sample of pan T cells purified from a patient's PBMCs. In some embodiments the patient's PBMCs are obtained from patient apheresis at baseline of a putative therapeutic treatment. In other embodiments the patient's PBMCs are obtained from patient apheresis at 7 days post-dose of a putative therapeutic treatment. In some embodiments the putative therapeutic treatment is a personalized cancer

vaccine. The personalized cancer vaccine may be an mRNA having one or more open reading frames encoding 3-50 peptide epitopes, wherein each of the peptide epitopes are personalized cancer antigens, formulated in a lipid nanoparticle formulation.

In some embodiments the antigen specific T cell activation is measured as a percent frequency (% freq) of CD8+IFNγ+ cells. In some embodiments a % freq of CD8+IFNγ+ cells greater than or equal to 3x over baseline indicates that a T cell population exceeds a threshold level of T cell activation.

In some embodiments the analysis of T cell activation is performed on a patient receiving a personalized cancer vaccine and wherein the personalized cancer vaccine is reformulated based on the analysis and the patient is administered the reformulated personalized cancer vaccine. In some embodiments the reformulated personalized cancer vaccine includes at least one neoantigen that is not in the personalized cancer vaccine initially administered to the patient.

In some embodiments the analysis of T cell activation is performed on a patient receiving a therapeutic treatment with a cancer vaccine and wherein the therapeutic treatment is modified based on the analysis. In some embodiments the therapeutic treatment is modified. In some embodiments the administration schedule of the therapeutic treatment is modified. In some embodiments a co-therapy is administered to the patient.

A personalized cancer vaccine is provided in other aspects of the invention. The vaccine is an mRNA having one or more open reading frames encoding 8-50 peptide epitopes, wherein each of the peptide epitopes are neoantigens, formulated in a lipid nanoparticle formulation, wherein at least 8 of the neoantigens demonstrated an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells as compared to baseline greater than 3x in an in vitro stimulation (IVS) assay.

In some embodiments the IVS assay is an assay as described herein. In some embodiments at least 80% of the neoantigens demonstrated an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells as compared to baseline greater than 3x in an in vitro stimulation (IVS) assay. In some embodiments at least 90% of the neoantigens demonstrated an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells as compared to baseline greater than 3x in an in vitro stimulation (IVS) assay. In other embodiments all of the neoantigens demonstrated an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells as compared to baseline greater than 3x in an in vitro stimulation (IVS) assay.

A method for vaccinating a patient by administering to a mammalian patient a vaccine composition described herein in an effective amount to vaccinate the patient is provided in other aspects of the invention.

Each of the limitations of the disclosure can encompass various embodiments of the disclosure. It is, therefore, anticipated that each of the limitations of the disclosure involving any one element or combinations of elements can be included in each aspect of the disclosure. This disclosure is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The disclosure is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF DRAWINGS

The accompanying drawings are not intended to be drawn to scale. In the drawings, each identical or nearly identical component that is illustrated in various figures is represented by a like numeral. For purposes of clarity, not every component may be labeled in every drawing. In the drawings:

FIG. 1 shows a schematic of immune monitoring assays performed on an exemplary human patient.

FIGs. 2A-2B show results of neoantigen peptide pool pulsed DC restimulation of in vitro stimulated (IVS) T cells. **FIG. 2A** is a bar graph showing % freq CD8+IFNγ+ T cells based on antigen pool used for stimulation. **FIG. 2B** shows dot blot results of flow cytometry data for CD8 and IFNγ.

FIGs. 3A-3B show results of individual neoantigen peptide pulsed DC restimulation of in vitro stimulated (IVS) T cells. **FIG. 3A** is a bar graph showing % freq CD8+IFNγ+ T cells based on individual antigens used for stimulation. **FIG. 3B** shows dot blot results of flow cytometry data for CD8 and IFNγ.

FIGs. 4A-4C show differences in assay sensitivity using two prior art assays (4A and 4B) and the assay of the invention (4C).

DETAILED DESCRIPTION OF THE INVENTION

A new immune based assay for assessing the efficacy of a therapeutic such as a vaccine is provided. The new assay provides a several fold improvement in sensitivity over existing assays, providing several improvements in therapeutic treatment. The highly sensitive assays disclosed herein are useful for assessing the efficacy of an antigen based immunotherapy earlier than traditional assessments of antigen specific immune activation. For instance, in a cancer vaccine therapy, a patient’s immune response to the vaccine may be assessed within a week, or even less, of receiving a vaccine dose. The sensitivity of the assay allows a practitioner to assess whether a vaccine antigen or antigens are producing a sufficient antigen specific T cell based immune response in the patient to determine whether to continue the therapy, modify the therapy, add to the therapy or discontinue the therapy. The data produced by the assay also enables the

production of a modified vaccine with different antigens, based on the functionality of the antigens used in the initial vaccine.

The assay disclosed herein is a peptide pulsed dendritic cell (DC) : T cell assay in which T cells are initially stimulated with peptide pool pulsed DCs, followed by an extended expansion period, i.e., 14 days, before restimulation with peptide pulsed DCs at the neoantigen pool and/or individual neoantigen level. The stimulation/expansion aspects of the assay are referred to herein as an *in vitro* stimulated (IVS) T cells assay. This assay differs from the assays previously run on patient samples, both in the stimulation/expansion of the T cells prior to measuring antigen specific responses and in the analytical techniques (i.e. use of flow cytometry instead of ELISpot to measure antigen specific responses). A schematic depicting the assay described herein (bottom panel) in comparison with an ELISpot based assays (top 2 panels) shown in Fig. 1.

As shown in Fig. 1, bottom panel, pan T cells are purified from a patient's PBMCs obtained from apheresis at baseline and 7 days after dosing an mRNA vaccine encoding multiple neoantigens. The T cells are then cultured in IL-2 supplemented media for 24 hours before restimulation in IL-2 containing media with autologous monocyte-derived DCs previously matured and exposed to pools of peptides. T cells are then expanded for 2d in IL-2 and IL-7, and an additional 12d in IL-2, this process of expansion of T cells in the presence of neoantigens (IVS). After IVS, cells are restimulated with newly thawed and matured autologous DCs exposed to peptide pools or individual neoantigens. In the exemplified case a 25mer and minimal epitope for each neoantigen are used to pulse DCs.

A human patient received a mRNA encoding a personalized concatemeric cancer vaccine having several neoantigens. Blood was collected from the patient at a baseline (day zero) and 7 days after administration of the vaccine construct. Data on the antigen specific activation of T cells was generated using each of the three assays summarized in Fig. 1. Significantly increased responses were observed with the DC : T cell co-culture method when T cells have undergone IVS as compared to previously reported data using ex vivo T cells. The IVS T cell population has been in vitro stimulated for instance, for 14 days, allowing for the expansion of neoantigen specific T cell clones. This method amplified the neoantigen specific T cells present in the collected samples, thus delivering significantly increased sensitivity to the assay.

Peptide pulsed DC restimulation of IVS T cell, as measured by % freq. of CD8+IFNγ+ cells are shown in Figs. 2A-4C and described in the Examples. The results of the RNA-seq analysis and antigen-specific T cell responses as measured by IFNγ ELISpot in both direct peptide restimulation ELISpot assay were also performed.

The assessment of % frequency in the assay is useful for establishing a baseline and a level or activation over a threshold level. When antigen specific responses of % freq of

$CD8^{+}IFN\gamma^{+}$ of at least 3x over baseline an antigen is considered to have produced a significant antigen specific immune response.

When expanded T cells were restimulated with DCs pulsed with peptide (neoantigen) pools, fold changes over baseline at 7 days after 4th dose of vaccine ranged from 2x to neoantigen pool #11-16, to 16.4x to neoantigen pool #6-10. Three out of four peptide pools had greater than 3x increase at 7 days after 4th dose as compared to baseline in % freq. of CD8+IFNγ+ cells. Interestingly, restimulation with peptide pool 16-20 produced the highest magnitude response in all assay formats tested for this patient at this time point (Figures 4A-C), which, when responses were deconvoluted to the individual neoantigen level, seems to be driven by a single response to neoantigen 16 (Figures 3A-B). Understanding how results obtained with different assay formats compare to one another may inform development of more sensitive assays to measure neoantigen specific responses ex vivo with whole blood collections.

These results evidence the ability to interrogate responses at the individual neoantigen level. 18 out of the 20 neoantigens included in the patient's vaccine were predicted to elicit a class I (CD8) T cell response. 10 out of the 18 predicted class I neoantigens had an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells at C4D8 as compared to baseline greater than 3x.

The data provides the most in depth insight into the ability to predict and incorporate immunogenic neoepitopes into the vaccines (55% of predicted class I epitopes elicited a $\geq$ 3x increase in CD8+IFNγ+ cells post-dose 4 as compared to baseline) and demonstrates the ability of the platform to elicit neoantigen specific CD8 T cell responses in humans.

Therapeutic Agents

In some embodiments a subject or patient is treated with a therapeutic agent. The assay of the invention may be used to assess the effectiveness of the therapeutic agent in the subject or patient at a particular time, dose, combination etc. The information obtained from the assay may be used to alter the therapy. For instance, if it is demonstrated that an effective antigen specific immune response is not generated, the therapy may be halted or altered, for instance, by changing one or more antigens, doses, routes of administration, length of therapy, combinations etc. In some instances a new vaccine is designed based on the information generated using the assay. Such vaccines are included within the scope of the invention.

Thus, in some embodiments the therapeutic treatment is a vaccine such as a cancer vaccine. Vaccines include peptide based vaccines, nucleic acid vaccines (RNA, DNA) and whole vaccines, such as heat killed organisms.

Embodiments of the present disclosure provide RNA (*e.g.*, mRNA) vaccines that include a polynucleotide encoding one or more antigens formulated in a carrier. mRNA vaccines, as

provided herein may be used to induce a balanced immune response, comprising cellular and/or humoral immunity, without many of the risks associated with DNA vaccination. In some embodiments, a vaccine comprises at least one RNA (*e.g.*, mRNA) polynucleotide having an open reading frame encoding an antigen. The mRNA vaccine of the present disclosure comprises a carrier. The term “carrier” denotes an organic or inorganic ingredient, natural or synthetic, with which the mRNA is combined to facilitate administration.

Thus, the invention relates to mRNA vaccines. The mRNA vaccines provide unique therapeutic alternatives to peptide based or DNA vaccines. When the mRNA vaccine is delivered to a cell, the mRNA will be processed into a polypeptide by the intracellular machinery which can then process the polypeptide into immunosensitive fragments capable of stimulating an immune response against the infectious disease or tumor.

The vaccines described herein include at least one ribonucleic acid (RNA) polynucleotide having an open reading frame encoding at least one antigenic polypeptide or an immunogenic fragment thereof (e.g., an immunogenic fragment capable of inducing an immune response to cancer or infectious disease). As used herein, the term “open reading frame”, abbreviated as “ORF”, refers to a segment or region of an mRNA molecule that encodes a polypeptide. The ORF comprises a continuous stretch of non-overlapping, in-frame codons, beginning with the initiation codon and ending with a stop codon, and is translated by the ribosome.

The vaccines may be traditional or personalized cancer or infectious disease vaccines. A traditional cancer vaccine, for instance, is a vaccine including a cancer antigen that is known to be found in cancers or tumors generally or in a specific type of cancer or tumor. Antigens that are expressed in or by tumor cells are referred to as “tumor associated antigens”. A particular tumor associated antigen may or may not also be expressed in non-cancerous cells. Many tumor mutations are known in the art. Personalized vaccines, for instance, may include RNA encoding for one or more known cancer antigens specific for the tumor or cancer antigens specific for each subject, referred to as neoepitopes or patient specific epitopes or antigens. A “patient specific cancer antigen” is an antigen that has been identified as being expressed in a tumor of a particular patient. The patient specific cancer antigen may or may not be typically present in tumor samples generally. Tumor associated antigens that are not expressed or rarely expressed in non-cancerous cells, or whose expression in non-cancerous cells is sufficiently reduced in comparison to that in cancerous cells and that induce an immune response induced upon vaccination, are referred to as neoepitopes.

The mRNA vaccines of the invention may include one or more antigens. In some embodiments the mRNA vaccine is composed of 3 or more, 4 or more, 5 or more 6 or more 7 or more, 8 or more, 9 or more antigens. In other embodiments the mRNA vaccine is composed of

1000 or less, 900 or less, 500 or less, 100 or less, 75 or less, 50 or less, 40 or less, 30 or less, 20 or less or 100 or less cancer antigens. In yet other embodiments the mRNA vaccine has 3-100, 5-100, 10-100, 15-100, 20-100, 25-100, 30-100, 35-100, 40-100, 45-100, 50-100, 55-100, 60-100, 65-100, 70-100, 75-100, 80-100, 90-100, 5-50, 10-50, 15-50, 20-50, 25-50, 30-50, 35-50, 40-50, 45-50, 100-150, 100-200, 100-300, 100-400, 100-500, 50-500, 50-800, 50-1,000, or 100-1,000 antigens.

In some embodiments the mRNA vaccines and vaccination methods include epitopes or antigens based on specific mutations (neoepitopes) and those expressed by cancer-germline genes (antigens common to tumors found in multiple patients) or infectious agents.

An epitope, also known as an antigenic determinant, as used herein is a portion of an antigen that is recognized by the immune system in the appropriate context, specifically by antibodies, B cells, or T cells. Epitopes include B cell epitopes and T cell epitopes. B-cell epitopes are peptide sequences which are required for recognition by specific antibody producing B-cells. B cell epitopes refer to a specific region of the antigen that is recognized by an antibody. The portion of an antibody that binds to the epitope is called a paratope. An epitope may be a conformational epitope or a linear epitope, based on the structure and interaction with the paratope. A linear, or continuous, epitope is defined by the primary amino acid sequence of a particular region of a protein. The sequences that interact with the antibody are situated next to each other sequentially on the protein, and the epitope can usually be mimicked by a single peptide. Conformational epitopes are epitopes that are defined by the conformational structure of the native protein. These epitopes may be continuous or discontinuous, i.e. components of the epitope can be situated on disparate parts of the protein, which are brought close to each other in the folded native protein structure.

T-cell epitopes are peptide sequences which, in association with proteins on APC, are required for recognition by specific T-cells. T cell epitopes are processed intracellularly and presented on the surface of APCs, where they are bound to MHC molecules including MHC class II and MHC class I. The peptide epitope may be any length that is reasonable for an epitope. In some embodiments the peptide epitope is 9-30 amino acids. In other embodiments the length is 9- 22, 9-29, 9-28, 9-27, 9-26, 9-25, 9-24, 9-23, 9-21, 9-20, 9-19, 9-18, 10-22, 10-21, 10-20, 11-22, 22-21, 11-20, 12-22, 12-21, 12-20,13-22, 13-21, 13-20, 14–19, 15-18, or 16-17 amino acids.

In some embodiments, the peptide epitopes comprise at least one MHC class I epitope and at least one MHC class II epitope. In some embodiments, at least 10% of the epitopes are MHC class I epitopes. In some embodiments, at least 20% of the epitopes are MHC class I epitopes. In some embodiments, at least 30% of the epitopes are MHC class I epitopes. In some

embodiments, at least 40% of the epitopes are MHC class I epitopes. In some embodiments, at least 50%, 60%, 70%, 80%, 90% or 100% of the epitopes are MHC class I epitopes. In some embodiments, at least 10% of the epitopes are MHC class II epitopes. In some embodiments, at least 20% of the epitopes are MHC class II epitopes. In some embodiments, at least 30% of the epitopes are MHC class II epitopes. In some embodiments, at least 40% of the epitopes are MHC class II epitopes. In some embodiments, at least 50%, 60%, 70%, 80%, 90% or 100% of the epitopes are MHC class II epitopes. In some embodiments, the ratio of MHC class I epitopes to MHC class II epitopes is a ratio selected from about 10%:about 90%; about 20%:about 80%; about 30%:about 70%; about 40%:about 60%; about 50%:about 50%; about 60%:about 40%; about 70%:about 30%; about 80%: about 20%; about90%: about 10% MHC class 1: MHC class II epitopes. In some embodiments, the ratio of MHC class II epitopes to MHC class I epitopes is a ratio selected from about 10%:about 90%; about 20%:about 80%; about 30%:about 70%; about 40%: about 60%; about 50%: about 50%; about 60%:about 40%; about 70%:about 30%; about 80%: about 20%; about 90%: about 10% MHC class II: MHC class I epitopes. In some embodiments, at least one of the peptide epitopes of the cancer vaccine is a B cell epitope. In some embodiments, the T cell epitope of the cancer vaccine comprises between 8-11 amino acids. In some embodiments, the B cell epitope of the cancer vaccine comprises between 13-17 amino acids. In some embodiments the methods of the invention are particularly useful with MHC class I epitopes.

A. mRNAs

Exemplary aspects of the invention feature mRNA vaccines. Described herein are mRNA vaccines designed to achieve particular biologic effects. Exemplary vaccines of the invention feature mRNAs encoding a particular antigen of interest (or and mRNA or mRNAs encoding antigens of interest), optionally formulated with additional components designed to facilitate efficacious delivery of mRNAs *in vivo*. In exemplary aspects, the vaccines of the invention feature and mRNA or mRNAs encoding antigen(s) of interest, complexed with polymeric or lipid components, or in certain aspects, encapsulated in liposomes, or alternatively, in lipid nanoparticles (LNPs). Chemical modification of mRNAs can facilitate certain desirable properties of vaccines on the invention, for example, influencing the type of immune response to the vaccine. For example, appropriate chemical modification of mRNAs can reduce unwanted innate immune responses against mRNA components and/or can facilitate desirable levels of protein expression of the antigen or antigens of interest. Further description of such features of the invention is provided *infra*.

1. Chemically-modified mRNAs

In some embodiments, the polynucleotide (e.g., a RNA, e.g., an mRNA) of the invention comprises a chemically modified nucleobase. The invention includes modified polynucleotides comprising a polynucleotide described herein (e.g., a polynucleotide comprising a nucleotide sequence encoding an antigen polypeptide). The modified polynucleotides can be chemically modified and/or structurally modified. When the polynucleotides of the present invention are chemically and/or structurally modified the polynucleotides can be referred to as "modified polynucleotides."

The present disclosure provides for modified nucleosides and nucleotides of a polynucleotide (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) encoding an antigen polypeptide. As used herein, the term “nucleic acid” is used in its broadest sense and encompasses any compound and/or substance that includes a polymer of nucleotides, or derivatives or analogs thereof. These polymers are often referred to as “polynucleotides”. Accordingly, as used herein the terms “nucleic acid” and “polynucleotide” are equivalent and are used interchangeably. Exemplary nucleic acids or polynucleotides of the disclosure include, but are not limited to, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), DNA-RNA hybrids, RNAi-inducing agents, RNAi agents, siRNAs, shRNAs, mRNAs, modified mRNAs, miRNAs, antisense RNAs, ribozymes, catalytic DNA, RNAs that induce triple helix formation, threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β-D-ribo configuration, α-LNA having an α-L-ribo configuration (a diastereomer of LNA), 2'-amino-LNA having a 2'-amino functionalization, and 2'-amino-α-LNA having a 2'-amino functionalization) or hybrids thereof. As used herein, the term “nucleobase” (alternatively “nucleotide base” or “nitrogenous base”) refers to a purine or pyrimidine heterocyclic compound found in nucleic acids, including any derivatives or analogs of the naturally occurring purines and pyrimidines that confer improved properties (e.g., binding affinity, nuclease resistance, chemical stability) to a nucleic acid or a portion or segment thereof. Adenine, cytosine, guanine, thymine, and uracil are the nucleobases predominately found in natural nucleic acids. Other natural, non-natural, and/or synthetic nucleobases, as known in the art and/or described herein, can be incorporated into nucleic acids. Nucleoside/Nucleotide: As used herein, the term “nucleoside” refers to a compound containing a sugar molecule (e.g., a ribose in RNA or a deoxyribose in DNA), or derivative or analog thereof, covalently linked to a nucleobase (e.g., a purine or pyrimidine), or a derivative or analog thereof (also referred to herein as “nucleobase”), but lacking an internucleoside linking group (e.g., a phosphate group). As used herein, the term “nucleotide” refers to a nucleoside covalently bonded to an internucleoside linking group (e.g., a phosphate group), or any derivative, analog, or modification thereof that

confers improved chemical and/or functional properties (e.g., binding affinity, nuclease resistance, chemical stability) to a nucleic acid or a portion or segment thereof. Modified nucleotides can by synthesized by any useful method, such as, for example, chemically, enzymatically, or recombinantly, to include one or more modified or non-natural nucleosides. Polynucleotides can comprise a region or regions of linked nucleosides. Such regions can have variable backbone linkages. The linkages can be standard phosphodiester linkages, in which case the polynucleotides would comprise regions of nucleotides.

The modified polynucleotides disclosed herein can comprise various distinct modifications. In some embodiments, the modified polynucleotides contain one, two, or more (optionally different) nucleoside or nucleotide modifications. In some embodiments, a modified polynucleotide, introduced to a cell can exhibit one or more desirable properties, e.g., improved protein expression, reduced immunogenicity, or reduced degradation in the cell, as compared to an unmodified polynucleotide.

In some embodiments, a polynucleotide of the present invention (e.g., a polynucleotide comprising a nucleotide sequence encoding an antigen polypeptide) is structurally modified, *i.e.,* comprises one or more nucleic acid structure modifications. As used herein, a "structural" modification is one in which two or more linked nucleosides are inserted, deleted, duplicated, inverted or randomized in a polynucleotide without significant chemical modification to the nucleotides themselves. Further, the term “nucleic acid structure” (used interchangeably with “polynucleotide structure”) refers to the arrangement or organization of atoms, chemical constituents, elements, motifs, and/or sequence of linked nucleotides, or derivatives or analogs thereof, that comprise a nucleic acid (e.g., an mRNA). The term also refers to the two-dimensional or three-dimensional state of a nucleic acid. Accordingly, the term “RNA structure” refers to the arrangement or organization of atoms, chemical constituents, elements, motifs, and/or sequence of linked nucleotides, or derivatives or analogs thereof, comprising an RNA molecule (e.g., an mRNA) and/or refers to a two-dimensional and/or three dimensional state of an RNA molecule. Nucleic acid structure can be further demarcated into four organizational categories referred to herein as “molecular structure”, “primary structure”, “secondary structure”, and “tertiary structure” based on increasing organizational complexity. Because chemical bonds will necessarily be broken and reformed to effect a structural modification, structural modifications are of a chemical nature and hence are chemical modifications. However, structural modifications will result in a different sequence of nucleotides. For example, the polynucleotide "ATCG" can be chemically modified to "AT-5meC-G". The same polynucleotide can be structurally modified from "ATCG" to "ATCCCG". Here, the dinucleotide "CC" has been inserted, resulting in a structural modification to the polynucleotide.

In some embodiments, the polynucleotides of the present invention are chemically modified. As used herein in reference to a polynucleotide, the terms "chemical modification" or, as appropriate, "chemically modified" refer to modification with respect to adenosine (A), guanosine (G), uridine (U), or cytidine (C) ribo- or deoxyribonucleosides in one or more of their position, pattern, percent or population. Generally, herein, these terms are not intended to refer to the ribonucleotide modifications in naturally occurring 5′-terminal mRNA cap moieties.

In some embodiments, the polynucleotides of the present invention can have a uniform chemical modification of all or any of the same nucleoside type or a population of modifications produced by mere downward titration of the same starting modification in all or any of the same nucleoside type, or a measured percent of a chemical modification of all any of the same nucleoside type but with random incorporation, such as where all uridines are replaced by a uridine analog, *e.g.,* pseudouridine or 5-methoxyuridine. In another embodiment, the polynucleotides can have a uniform chemical modification of two, three, or four of the same nucleoside type throughout the entire polynucleotide (such as all uridines and all cytosines, etc. are modified in the same way).

Modified nucleotide base pairing encompasses not only the standard adenosine-thymine, adenosine-uracil, or guanosine-cytosine base pairs, but also base pairs formed between nucleotides and/or modified nucleotides comprising non-standard or modified bases, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard base structures. One example of such non-standard base pairing is the base pairing between the modified nucleotide inosine and adenine, cytosine or uracil. Any combination of base/sugar or linker can be incorporated into polynucleotides of the present disclosure.

The skilled artisan will appreciate that, except where otherwise noted, polynucleotide sequences set forth in the instant application will recite "T"s in a representative DNA sequence but where the sequence represents RNA, the "T"s would be substituted for "U"s.

In some embodiments, the polynucleotide (*e.g.*, RNA polynucleotide, such as mRNA polynucleotide) includes a combination of at least two (*e.g.*, 2, 3, 4 or more) of the modified nucleobases.

In some embodiments, the mRNA comprises at least one chemically modified nucleoside. In some embodiments, the chemical modification is selected from the group consisting of pseudouridine, N1-methylpseudouridine, N1-ethylpseudouridine, 2-thiouridine, 4′-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-

thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine, 5-methyluridine, 5-methoxyuridine, and 2′-O-methyl uridine. In some embodiments, the one or more mRNA is fully modified.

In some embodiments, the at least one chemically modified nucleoside is selected from the group consisting of pseudouridine (ψ), 2-thiouridine (s2U), 4'-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine, 5-methoxyuridine, 2'-O-methyl uridine, 1-methyl-pseudouridine (m1ψ), 1-ethyl-pseudouridine (e1ψ), 5-methoxy-uridine (mo5U), 5-methyl-cytidine (m5C), α-thio-guanosine, α-thio-adenosine, 5-cyano uridine, 4'-thio uridine 7-deaza-adenine, 1-methyl-adenosine (m1A), 2-methyl-adenine (m2A), N6-methyl-adenosine (m6A), and 2,6-Diaminopurine, (I), 1-methyl-inosine (m1I), wyosine (imG), methylwyosine (mimG), 7-deaza-guanosine, 7-cyano-7-deaza-guanosine (preQ0), 7-aminomethyl-7-deaza-guanosine (preQ1), 7-methyl-guanosine (m7G), 1-methyl-guanosine (m1G), 8-oxo-guanosine, 7-methyl-8-oxo-guanosine, 2,8-dimethyladenosine, 2-geranylthiouridine, 2-lysidine, 2-selenouridine, 3-(3-amino-3-carboxypropyl)-5,6-dihydrouridine, 3-(3-amino-3-carboxypropyl)pseudouridine, 3-methylpseudouridine, 5-(carboxyhydroxymethyl)-2′-O-methyluridine methyl ester, 5-aminomethyl-2-geranylthiouridine, 5-aminomethyl-2-selenouridine, 5-aminomethyluridine, 5-carbamoylhydroxymethyluridine, 5-carbamoylmethyl-2-thiouridine, 5-carboxymethyl-2-thiouridine, 5-carboxymethylaminomethyl-2-geranylthiouridine, 5-carboxymethylaminomethyl-2-selenouridine, 5-cyanomethyluridine, 5-hydroxycytidine, 5-methylaminomethyl-2-geranylthiouridine, 7-aminocarboxypropyl-demethylwyosine, 7-aminocarboxypropylwyosine, 7-aminocarboxypropylwyosine methyl ester, 8-methyladenosine, N4,N4-dimethylcytidine, N6-formyladenosine, N6-hydroxymethyladenosine, agmatidine, cyclic N6-threonylcarbamoyladenosine, glutamyl-queuosine, methylated undermodified hydroxywybutosine, N4,N4,2′-O-trimethylcytidine, geranylated 5-methylaminomethyl-2-thiouridine, geranylated 5-carboxymethylaminomethyl-2-thiouridine, Qbase , preQ0base, preQ1base, and two or more combinations thereof. In some embodiments, the at least one chemically modified nucleoside is selected from the group consisting of pseudouridine, 1-methyl-pseudouridine, 1-ethyl-pseudouridine, 5-methylcytosine, 5-methoxyuridine, and a combination thereof. In some embodiments, the polynucleotide (*e.g.*, RNA polynucleotide, such as mRNA polynucleotide) includes a combination of at least two (*e.g.*, 2, 3, 4 or more) of the aforementioned modified nucleobases.

2. mRNA Flanking Regions

In certain aspects, the present disclosure provides nucleic acid molecules, specifically polynucleotides that encode one or more antigens, or functional fragments thereof. Features, which can be considered beneficial in some embodiments of the present disclosure, can be encoded by regions of the polynucleotide and such regions can be upstream (5') or downstream (3') to, or within, a region that encodes a polypeptide. These regions can be incorporated into the polynucleotide before and/or after sequence optimization of the protein encoding region or open reading frame (ORF). It is not required that a polynucleotide contain both a 5′ and 3′ flanking region. Examples of such features include, but are not limited to, untranslated regions (UTRs), Kozak sequences, an oligo(dT) sequence, and detectable tags and can include multiple cloning sites that can have XbaI recognition.

In some embodiments, a 5′ UTR and/or a 3′ UTR region can be provided as flanking regions. Multiple 5′ or 3′ UTRs can be included in the flanking regions and can be the same or of different sequences. Any portion of the flanking regions, including none, can be sequence-optimized and any can independently contain one or more different structural or chemical modifications, before and/or after sequence optimization.

Untranslated regions (UTRs) are nucleic acid sections of a polynucleotide before a start codon (5'UTR) and after a stop codon (3'UTR) that are not translated. In some embodiments, a polynucleotide (e.g., a ribonucleic acid (RNA), e.g., a messenger RNA (mRNA)) of the invention comprising an open reading frame (ORF) encoding an antigen polypeptide further comprises UTR (e.g., a 5′UTR or functional fragment thereof, a 3′UTR or functional fragment thereof, or a combination thereof).

A UTR can be homologous or heterologous to the coding region in a polynucleotide. In some embodiments, the UTR is homologous to the ORF encoding the antigen polypeptide. In some embodiments, the UTR is heterologous to the ORF encoding the antigen polypeptide. In some embodiments, the polynucleotide comprises two or more 5′UTRs or functional fragments thereof, each of which have the same or different nucleotide sequences. In some embodiments, the polynucleotide comprises two or more 3′UTRs or functional fragments thereof, each of which have the same or different nucleotide sequences.

In some embodiments, the 5′UTR or functional fragment thereof, 3′ UTR or functional fragment thereof, or any combination thereof is sequence optimized.

In some embodiments, the 5′UTR or functional fragment thereof, 3′ UTR or functional fragment thereof, or any combination thereof comprises at least one chemically modified nucleobase, e.g., 5-methoxyuracil.

UTRs can have features that provide a regulatory role, e.g., increased or decreased stability, localization and/or translation efficiency. A polynucleotide comprising a UTR can be administered to a cell, tissue, or organism, and one or more regulatory features can be measured using routine methods. In some embodiments, a functional fragment of a 5'UTR or 3'UTR comprises one or more regulatory features of a full length 5' or 3' UTR, respectively.

By engineering the features typically found in abundantly expressed genes of specific target organs, one can enhance the stability and protein production of a polynucleotide. For example, introduction of 5′UTR of liver-expressed mRNA, such as albumin, serum amyloid A, Apolipoprotein A/B/E, transferrin, alpha fetoprotein, erythropoietin, or Factor VIII, can enhance expression of polynucleotides in hepatic cell lines or liver. Likewise, use of 5′UTR from other tissue-specific mRNA to improve expression in that tissue is possible for muscle (e.g., MyoD, Myosin, Myoglobin, Myogenin, Herculin), for endothelial cells (e.g., Tie-1, CD36), for myeloid cells (e.g., C/EBP, AML1, G-CSF, GM-CSF, CD11b, MSR, Fr-1, i-NOS), for leukocytes (e.g., CD45, CD18), for adipose tissue (e.g., CD36, GLUT4, ACRP30, adiponectin) and for lung epithelial cells (e.g., SP-A/B/C/D).

In some embodiments, UTRs are selected from a family of transcripts whose proteins share a common function, structure, feature or property. For example, an encoded polypeptide can belong to a family of proteins (i.e., that share at least one function, structure, feature, localization, origin, or expression pattern), which are expressed in a particular cell, tissue or at some time during development. The UTRs from any of the genes or mRNA can be swapped for any other UTR of the same or different family of proteins to create a new polynucleotide.

In some embodiments, the 5’UTR and the 3’UTR can be heterologous. In some embodiments, the 5'UTR can be derived from a different species than the 3'UTR. In some embodiments, the 3'UTR can be derived from a different species than the 5'UTR.

Co-owned International Patent Application No. PCT/US2014/021522 (Publ. No. WO/2014/164253, incorporated herein by reference in its entirety) provides a listing of exemplary UTRs that can be utilized in the polynucleotide of the present invention as flanking regions to an ORF.

Exemplary UTRs of the application include, but are not limited to, one or more 5′UTR and/or 3′UTR derived from the nucleic acid sequence of: a globin, such as an α- or β-globin (e.g., a Xenopus, mouse, rabbit, or human globin); a strong Kozak translational initiation signal; a CYBA (e.g., human cytochrome b-245 α polypeptide); an albumin (e.g., human albumin7); a HSD17B4 (hydroxysteroid (17-β) dehydrogenase); a virus (e.g., a tobacco etch virus (TEV), a Venezuelan equine encephalitis virus (VEEV), a Dengue virus, a cytomegalovirus (CMV) (e.g., CMV immediate early 1 (IE1)), a hepatitis virus (e.g., hepatitis B virus), a sindbis virus, or a

PAV barley yellow dwarf virus); a heat shock protein (e.g., hsp70); a translation initiation factor (e.g., elF4G); a glucose transporter (e.g., hGLUT1 (human glucose transporter 1)); an actin (e.g., human α or β actin); a GAPDH; a tubulin; a histone; a citric acid cycle enzyme; a topoisomerase (e.g., a 5′UTR of a TOP gene lacking the 5′ TOP motif (the oligopyrimidine tract)); a ribosomal protein Large 32 (L32); a ribosomal protein (e.g., human or mouse ribosomal protein, such as, for example, rps9); an ATP synthase (e.g., ATP5A1 or the β subunit of mitochondrial H+-ATP synthase); a growth hormone e (e.g., bovine (bGH) or human (hGH)); an elongation factor (e.g., elongation factor 1 α1 (EEF1A1)); a manganese superoxide dismutase (MnSOD); a myocyte enhancer factor 2A (MEF2A); a β-F1-ATPase, a creatine kinase, a myoglobin, a granulocyte-colony stimulating factor (G-CSF); a collagen (e.g., collagen type I, alpha 2 (Col1A2), collagen type I, alpha 1 (Col1A1), collagen type VI, alpha 2 (Col6A2), collagen type VI, alpha 1 (Col6A1)); a ribophorin (e.g., ribophorin I (RPNI)); a low density lipoprotein receptor-related protein (e.g., LRP1); a cardiotrophin-like cytokine factor (e.g., Nnt1); calreticulin (Calr); a procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (Plod1); and a nucleobindin (e.g., Nucb1).

In some embodiments, the 5'UTR is selected from the group consisting of a β-globin 5’UTR; a 5′UTR containing a strong Kozak translational initiation signal; a cytochrome b-245 α polypeptide (CYBA) 5'UTR; a hydroxysteroid (17-β) dehydrogenase (HSD17B4) 5'UTR; a Tobacco etch virus (TEV) 5'UTR; a Venezuelen equine encephalitis virus (TEEV) 5'UTR; a 5' proximal open reading frame of rubella virus (RV) RNA encoding nonstructural proteins; a Dengue virus (DEN) 5'UTR; a heat shock protein 70 (Hsp70) 5'UTR; a eIF4G 5'UTR; a GLUT1 5'UTR; functional fragments thereof and any combination thereof.

In some embodiments, the 3'UTR is selected from the group consisting of a β-globin 3’UTR; a CYBA 3'UTR; an albumin 3'UTR; a growth hormone (GH) 3'UTR; a VEEV 3'UTR; a hepatitis B virus (HBV) 3'UTR; α-globin 3′UTR; a DEN 3'UTR; a PAV barley yellow dwarf virus (BYDV-PAV) 3'UTR; an elongation factor 1 α1 (EEF1A1) 3'UTR; a manganese superoxide dismutase (MnSOD) 3'UTR; a β subunit of mitochondrial H(+)-ATP synthase (β-mRNA) 3'UTR; a GLUT1 3'UTR; a MEF2A 3'UTR; a β-F1-ATPase 3'UTR; functional fragments thereof and combinations thereof.

Wild-type UTRs derived from any gene or mRNA can be incorporated into the polynucleotides of the invention. In some embodiments, a UTR can be altered relative to a wild type or native UTR to produce a variant UTR, e.g., by changing the orientation or location of the UTR relative to the ORF; or by inclusion of additional nucleotides, deletion of nucleotides, swapping or transposition of nucleotides. In some embodiments, variants of 5′ or 3′ UTRs can be utilized, for example, mutants of wild type UTRs, or variants wherein one or more nucleotides are added to or removed from a terminus of the UTR.

Additionally, one or more synthetic UTRs can be used in combination with one or more non-synthetic UTRs. See, e.g., Mandal and Rossi, Nat. Protoc. 2013 8(3):568-82, and sequences available at addgene.org/Derrick_Rossi/, the contents of each are incorporated herein by reference in their entirety. UTRs or portions thereof can be placed in the same orientation as in the transcript from which they were selected or can be altered in orientation or location. Hence, a 5′ and/or 3′ UTR can be inverted, shortened, lengthened, or combined with one or more other 5′ UTRs or 3′ UTRs.

In some embodiments, the polynucleotide comprises multiple UTRs, e.g., a double, a triple or a quadruple 5'UTR or 3'UTR. For example, a double UTR comprises two copies of the same UTR either in series or substantially in series. For example, a double beta-globin 3′UTR can be used (see US2010/0129877, the contents of which are incorporated herein by reference in its entirety).

In some embodiments, the polynucleotides of the invention comprise a 5'UTR and/or a 3'UTR selected from any one of the UTRs disclosed herein. The polynucleotides of the invention can comprise combinations of features. For example, the ORF can be flanked by a 5′UTR that comprises a strong Kozak translational initiation signal and/or a 3′UTR comprising an oligo(dT) sequence for templated addition of a poly-A tail. A 5′UTR can comprise a first polynucleotide fragment and a second polynucleotide fragment from the same and/or different UTRs (see, e.g., US2010/0293625, herein incorporated by reference in its entirety).

Other non-UTR sequences can be used as regions or subregions within the polynucleotides of the invention. For example, introns or portions of intron sequences can be incorporated into the polynucleotides of the invention. Incorporation of intronic sequences can increase protein production as well as polynucleotide expression levels. In some embodiments, the polynucleotide of the invention comprises an internal ribosome entry site (IRES) instead of or in addition to a UTR (see, e.g., Yakubov et al., Biochem. Biophys. Res. Commun. 2010 394(1):189-193, the contents of which are incorporated herein by reference in their entirety). In some embodiments, the polynucleotide comprises an IRES instead of a 5'UTR sequence. In some embodiments, the polynucleotide comprises an ORF and a viral capsid sequence. In some embodiments, the polynucleotide comprises a synthetic 5'UTR in combination with a non-synthetic 3'UTR.

In some embodiments, the UTR can also include at least one translation enhancer polynucleotide, translation enhancer element, or translational enhancer elements (collectively, "TEE," which refers to nucleic acid sequences that increase the amount of polypeptide or protein produced from a polynucleotide. As a non-limiting example, the TEE can be located between the transcription promoter and the start codon. In some embodiments, the 5'UTR comprises a TEE.

In one aspect, a TEE is a conserved element in a UTR that can promote translational activity of a nucleic acid such as, but not limited to, cap-dependent or cap-independent translation.

In one non-limiting example, the TEE comprises the TEE sequence in the 5′-leader of the Gtx homeodomain protein. See Chappell et al., PNAS 2004 101:9590-9594, incorporated herein by reference in its entirety.

In some embodiments, the polynucleotide of the invention comprises one or multiple copies of a TEE. The TEE in a translational enhancer polynucleotide can be organized in one or more sequence segments. A sequence segment can harbor one or more of the TEEs provided herein, with each TEE being present in one or more copies. When multiple sequence segments are present in a translational enhancer polynucleotide, they can be homogenous or heterogeneous. Thus, the multiple sequence segments in a translational enhancer polynucleotide can harbor identical or different types of the TEE provided herein, identical or different number of copies of each of the TEE, and/or identical or different organization of the TEE within each sequence segment. In one embodiment, the polynucleotide of the invention comprises a translational enhancer polynucleotide sequence.

In some embodiments, a 5'UTR and/or 3'UTR comprising at least one TEE described herein can be incorporated in a monocistronic sequence such as, but not limited to, a vector system or a nucleic acid vector.

In some embodiments, a 5′UTR and/or 3′UTR of a polynucleotide of the invention comprises a TEE or portion thereof described herein. In some embodiments, the TEEs in the 3′UTR can be the same and/or different from the TEE located in the 5′UTR.

In some embodiments, the spacer separating two TEE sequences can include other sequences known in the art that can regulate the translation of the polynucleotide of the invention, e.g., miR sequences described herein (e.g., miR binding sites). As a non-limiting example, each spacer used to separate two TEE sequences can include a different miR sequence (e.g., miR binding site).

In some embodiments, a polynucleotide of the invention comprises a miR and/or TEE sequence. In some embodiments, the incorporation of a miR sequence and/or a TEE sequence into a polynucleotide of the invention can change the shape of the stem loop region, which can increase and/or decrease translation. See e.g., Kedde et al., Nature Cell Biology 2010 12(10):1014-20, herein incorporated by reference in its entirety).

Lipid Nanoparticles (LNPs)

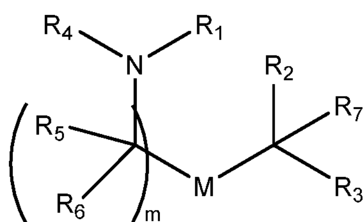
The mRNA vaccines described herein are superior to current vaccines in several ways. In some aspects the vaccine is formulated in a lipid nanoparticle (LNP). The use of LNPs enables

the effective delivery of chemically modified or unmodified mRNA vaccines. Both modified and unmodified LNP formulated mRNA vaccines are superior to conventional vaccines by a significant degree. In some embodiments the mRNA vaccines of the invention are superior to conventional vaccines by a factor of at least 10 fold, 20 fold, 40 fold, 50 fold, 100 fold, 500 fold or 1,000 fold.

In some aspects the vaccine is formulated in a lipid nanoparticle (LNP). The use of LNPs enables the effective delivery of chemically modified or unmodified mRNA vaccines. Both modified and unmodified LNP formulated mRNA vaccines are superior to conventional vaccines by a significant degree. In some embodiments the mRNA vaccines of the invention are superior to conventional vaccines by a factor of at least 10 fold, 20 fold, 40 fold, 50 fold, 100 fold, 500 fold or 1,000 fold.

In one set of embodiments, lipid nanoparticles (LNPs) are provided. In one embodiment, a lipid nanoparticle comprises lipids including an ionizable lipid (such as an ionizable cationic lipid), a structural lipid, a phospholipid, and mRNA. Each of the LNPs described herein may be used as a formulation for the mRNA described herein. In one embodiment, a lipid nanoparticle comprises an ionizable lipid, a structural lipid, a phospholipid, and mRNA. In some embodiments, the LNP comprises an ionizable lipid, a PEG-modified lipid, a phospholipid and a structural lipid. In some embodiments, the LNP has a molar ratio of about 20-60% ionizable lipid: about 5-25% phospholipid: about 25-55% structural lipid; and about 0.5-15% PEG-modified lipid. In some embodiments, the LNP comprises a molar ratio of about 50% ionizable lipid, about 1.5% PEG-modified lipid, about 38.5% structural lipid and about 10% phospholipid. In some embodiments, the LNP comprises a molar ratio of about 55% ionizable lipid, about 2.5% PEG lipid, about 32.5% structural lipid and about 10% phospholipid. In some embodiments, the ionizable lipid is an ionizable amino or cationic lipid and the phospholipid is a neutral lipid, and the structural lipid is a cholesterol. In some embodiments, the LNP has a molar ratio of 50:38.5:10:1.5 of ionizable lipid: cholesterol:DSPC: PEG2000-DMG.

The ionizable lipids described herein (e.g. those having any of Formula (I), (IA), (II), (IIa), (IIb), (IIc), (IId), (IIe), (III), (IV), (V), or (VI) may be advantageously used in lipid nanoparticle compositions for the delivery of vaccines to mammalian cells or organs. In some embodiments, the ionizable lipids have the Formula (I)



(I),

wherein

R_1 is selected from the group consisting of C_{5-30} alkyl, C_{5-20} alkenyl, -R*YR", -YR", and -R"M'R';

R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, C_{2-14} alkenyl, -R*YR", -YR", and -R*OR", or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, $-CQ(R)_2$, and unsubstituted C_{1-6} alkyl, where Q is selected from a carbocycle, heterocycle, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-N(R)_2$, $-C(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-N(R)R_8$, $-O(CH_2)_nOR$, $-N(R)C(=NR_9)N(R)_2$, $-N(R)C(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, -N(R)C(O)OR, -N(OR)C(O)R, $-N(OR)S(O)_2R$, -N(OR)C(O)OR, $-N(OR)C(O)N(R)_2$, $-N(OR)C(S)N(R)_2$, $-N(OR)C(=NR_9)N(R)_2$, $-N(OR)C(=CHR_9)N(R)_2$, $-C(=NR_9)N(R)_2$, $-C(=NR_9)R$, -C(O)N(R)OR, and $-C(R)N(R)_2C(O)OR$, and each n is independently selected from 1, 2, 3, 4, and 5;

each R_5 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R_6 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, $-S(O)_2-$, -S-S-, an aryl group, and a heteroaryl group;

R_7 is selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

R_8 is selected from the group consisting of C_{3-6} carbocycle and heterocycle;

R_9 is selected from the group consisting of H, CN, NO_2, C_{1-6} alkyl, -OR, $-S(O)_2R$, $-S(O)_2N(R)_2$, C_{2-6} alkenyl, C_{3-6} carbocycle and heterocycle;

each R is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R' is independently selected from the group consisting of C_{1-18} alkyl, C_{2-18} alkenyl, -R*YR", -YR", and H;

each R" is independently selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl;

each R* is independently selected from the group consisting of C_{1-12} alkyl and C_{2-12} alkenyl;

each Y is independently a C_{3-6} carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13,

or salts or stereoisomers thereof.

In some embodiments, a subset of compounds of Formula (I) includes those in which

R_1 is selected from the group consisting of C_{5-20} alkyl, C_{5-20} alkenyl, -R*YR", -YR", and -R"M'R';

R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, C_{2-14} alkenyl, -R*YR", -YR", and -R*OR", or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, $-CQ(R)_2$, and unsubstituted C_{1-6} alkyl, where Q is selected from a carbocycle, heterocycle, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-N(R)_2$, $-C(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, and $-C(R)N(R)_2C(O)OR$, and each n is independently selected from 1, 2, 3, 4, and 5;

each R_5 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R_6 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, $-S(O)_2-$, an aryl group, and a heteroaryl group;

R_7 is selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R' is independently selected from the group consisting of C_{1-18} alkyl, C_{2-18} alkenyl, -R*YR", -YR", and H;

each R" is independently selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl;

each R* is independently selected from the group consisting of C_{1-12} alkyl and C_{2-12} alkenyl;

each Y is independently a C_{3-6} carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13,

or salts or stereoisomers thereof, wherein alkyl and alkenyl groups may be linear or branched.

In some embodiments, a subset of compounds of Formula (I) includes those in which when R_4 is $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, or $-CQ(R)_2$, then (i) Q is not $-N(R)_2$ when n is 1, 2, 3, 4 or 5, or (ii) Q is not 5, 6, or 7-membered heterocycloalkyl when n is 1 or 2.

In another embodiments, another subset of compounds of Formula (I) includes those in which

R_1 is selected from the group consisting of C_{5-30} alkyl, C_{5-20} alkenyl, -R*YR", -YR", and -R"M'R';

R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, C_{2-14} alkenyl, -R*YR", -YR", and -R*OR", or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, $-CQ(R)_2$, and unsubstituted C_{1-6} alkyl, where Q is selected from a C_{3-6} carbocycle, a 5- to 14-membered heteroaryl having one or more heteroatoms selected from N, O, and S, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-C(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-CRN(R)_2C(O)OR$, $-N(R)R_8$, $-O(CH_2)_nOR$, $-N(R)C(=NR_9)N(R)_2$, $-N(R)C(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, -N(R)C(O)OR, -N(OR)C(O)R, $-N(OR)S(O)_2R$, -N(OR)C(O)OR, $-N(OR)C(O)N(R)_2$, $-N(OR)C(S)N(R)_2$, $-N(OR)C(=NR_9)N(R)_2$, $-N(OR)C(=CHR_9)N(R)_2$, $-C(=NR_9)N(R)_2$, $-C(=NR_9)R$, -C(O)N(R)OR, and a 5- to 14-membered heterocycloalkyl having one or more heteroatoms selected from N, O, and S which is substituted with one or more substituents selected from oxo (=O), OH, amino, and C_{1-3} alkyl, and each n is independently selected from 1, 2, 3, 4, and 5;

each R_5 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R_6 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, $-S(O)_2-$, -S-S-, an aryl group, and a heteroaryl group;

R_7 is selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

R_8 is selected from the group consisting of C_{3-6} carbocycle and heterocycle;

R_9 is selected from the group consisting of H, CN, NO_2, C_{1-6} alkyl, -OR, $-S(O)_2R$, $-S(O)_2N(R)_2$, C_{2-6} alkenyl, C_{3-6} carbocycle and heterocycle;

each R is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R’ is independently selected from the group consisting of C_{1-18} alkyl, C_{2-18} alkenyl, -R*YR”, -YR”, and H;

each R” is independently selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl;

each R* is independently selected from the group consisting of C_{1-12} alkyl and C_{2-12} alkenyl;

each Y is independently a C_{3-6} carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13,

or salts or stereoisomers thereof.

In another embodiments, another subset of compounds of Formula (I) includes those in which

R_1 is selected from the group consisting of C_{5-30} alkyl, C_{5-20} alkenyl, -R*YR”, -YR”, and -R”M’R’;

R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, C_{2-14} alkenyl, -R*YR”, -YR”, and -R*OR”, or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, $-CQ(R)_2$, and unsubstituted C_{1-6} alkyl, where Q is selected from a C_{3-6} carbocycle, a 5- to 14-membered heteroaryl having one or more heteroatoms selected from N, O, and S, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-C(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-CRN(R)_2C(O)OR$, and a 5- to 14-membered heterocycloalkyl having one or more heteroatoms selected from N, O, and S which is substituted with one or more substituents selected from oxo (=O), OH, amino, and C_{1-3} alkyl, and each n is independently selected from 1, 2, 3, 4, and 5;

each R_5 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R_6 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

M and M’ are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R’)-, -N(R’)C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR’)O-, $-S(O)_2-$, an aryl group, and a heteroaryl group;

R_7 is selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R' is independently selected from the group consisting of C_{1-18} alkyl, C_{2-18} alkenyl, -R*YR", -YR", and H;

each R" is independently selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl;

each R* is independently selected from the group consisting of C_{1-12} alkyl and C_{2-12} alkenyl;

each Y is independently a C_{3-6} carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13,

or salts or stereoisomers thereof.

In yet another embodiments, another subset of compounds of Formula (I) includes those in which

R_1 is selected from the group consisting of C_{5-20} alkyl, C_{5-20} alkenyl, -R*YR", -YR", and -R"M'R';

R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, C_{2-14} alkenyl, -R*YR", -YR", and -R*OR", or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, $-CQ(R)_2$, and unsubstituted C_{1-6} alkyl, where Q is selected from a C_{3-6} carbocycle, a 5- to 14-membered heterocycle having one or more heteroatoms selected from N, O, and S, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-C(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-CRN(R)_2C(O)OR$, $-N(R)R_8$, $-O(CH_2)_nOR$, $-N(R)C(=NR_9)N(R)_2$, $-N(R)C(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, -N(R)C(O)OR, -N(OR)C(O)R, $-N(OR)S(O)_2R$, -N(OR)C(O)OR, $-N(OR)C(O)N(R)_2$, $-N(OR)C(S)N(R)_2$, $-N(OR)C(=NR_9)N(R)_2$, $-N(OR)C(=CHR_9)N(R)_2$, $-C(=NR_9)R$, -C(O)N(R)OR, and $-C(=NR_9)N(R)_2$, and each n is independently selected from 1, 2, 3, 4, and 5; and when Q is a 5- to 14-membered heterocycle and (i) R_4 is $-(CH_2)_nQ$ in which n is 1 or 2, or (ii) R_4 is $-(CH_2)_nCHQR$ in which n is 1, or (iii) R_4 is -CHQR, and $-CQ(R)_2$, then Q is either a 5- to 14-membered heteroaryl or 8- to 14-membered heterocycloalkyl;

each R_5 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R_6 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, $-S(O)_2-$, -S-S-, an aryl group, and a heteroaryl group;

R_7 is selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

R_8 is selected from the group consisting of C_{3-6} carbocycle and heterocycle;

R_9 is selected from the group consisting of H, CN, NO_2, C_{1-6} alkyl, -OR, $-S(O)_2R$, $-S(O)_2N(R)_2$, C_{2-6} alkenyl, C_{3-6} carbocycle and heterocycle;

each R is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R' is independently selected from the group consisting of C_{1-18} alkyl, C_{2-18} alkenyl, -R*YR", -YR", and H;

each R" is independently selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl;

each R* is independently selected from the group consisting of C_{1-12} alkyl and C_{2-12} alkenyl;

each Y is independently a C_{3-6} carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13,

or salts or stereoisomers thereof.

In yet another embodiments, another subset of compounds of Formula (I) includes those in which

R_1 is selected from the group consisting of C_{5-20} alkyl, C_{5-20} alkenyl, -R*YR", -YR", and -R"M'R';

R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, C_{2-14} alkenyl, -R*YR", -YR", and -R*OR", or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, $-CQ(R)_2$, and unsubstituted C_{1-6} alkyl, where Q is selected from a C_{3-6} carbocycle, a 5- to 14-membered heterocycle having one or more heteroatoms selected from N, O, and S, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-C(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-CRN(R)_2C(O)OR$, and each n is independently selected from 1, 2, 3, 4, and 5; and when Q is a 5- to 14-membered heterocycle and (i) R_4 is $-(CH_2)_nQ$ in which n is 1 or 2, or (ii) R_4 is $-(CH_2)_nCHQR$ in which n is 1, or (iii) R_4 is -CHQR, and $-CQ(R)_2$, then Q is either a 5- to 14-membered heteroaryl or 8- to 14-membered heterocycloalkyl;

each R_5 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R_6 is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, $-S(O)_2-$, an aryl group, and a heteroaryl group;

R_7 is selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R is independently selected from the group consisting of C_{1-3} alkyl, C_{2-3} alkenyl, and H;

each R' is independently selected from the group consisting of C_{1-18} alkyl, C_{2-18} alkenyl, -R*YR", -YR", and H;

each R" is independently selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl;

each R* is independently selected from the group consisting of C_{1-12} alkyl and C_{2-12} alkenyl;

each Y is independently a C_{3-6} carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13,

or salts or stereoisomers thereof.

In still another embodiments, another subset of compounds of Formula (I) includes those in which

R_1 is selected from the group consisting of C_{5-30} alkyl, C_{5-20} alkenyl, -R*YR", -YR", and -R"M'R';

R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, C_{2-14} alkenyl, -R*YR", -YR", and -R*OR", or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle;

R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, $-CQ(R)_2$, and unsubstituted C_{1-6} alkyl, where Q is selected from a C_{3-6} carbocycle, a 5- to 14-membered heteroaryl having one or more heteroatoms selected from N, O, and S, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-C(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-CRN(R)_2C(O)OR$, $-N(R)R_8$, $-O(CH_2)_nOR$, $-N(R)C(=NR_9)N(R)_2$, $-N(R)C(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, -N(R)C(O)OR, -N(OR)C(O)R, $-N(OR)S(O)_2R$, -N(OR)C(O)OR, $-N(OR)C(O)N(R)_2$, $-N(OR)C(S)N(R)_2$, $-N(OR)C(=NR_9)N(R)_2$,

-N(OR)C(=CHR$_9$)N(R)$_2$, -C(=NR$_9$)R, -C(O)N(R)OR, and -C(=NR$_9$)N(R)$_2$, and each n is independently selected from 1, 2, 3, 4, and 5;

each R$_5$ is independently selected from the group consisting of C$_{1-3}$ alkyl, C$_{2-3}$ alkenyl, and H;

each R$_6$ is independently selected from the group consisting of C$_{1-3}$ alkyl, C$_{2-3}$ alkenyl, and H;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, -S(O)$_2$-, -S-S-, an aryl group, and a heteroaryl group;

R$_7$ is selected from the group consisting of C$_{1-3}$ alkyl, C$_{2-3}$ alkenyl, and H;

R$_8$ is selected from the group consisting of C$_{3-6}$ carbocycle and heterocycle;

R$_9$ is selected from the group consisting of H, CN, NO$_2$, C$_{1-6}$ alkyl, -OR, -S(O)$_2$R, -S(O)$_2$N(R)$_2$, C$_{2-6}$ alkenyl, C$_{3-6}$ carbocycle and heterocycle;

each R is independently selected from the group consisting of C$_{1-3}$ alkyl, C$_{2-3}$ alkenyl, and H;

each R' is independently selected from the group consisting of C$_{1-18}$ alkyl, C$_{2-18}$ alkenyl, -R*YR", -YR", and H;

each R" is independently selected from the group consisting of C$_{3-14}$ alkyl and C$_{3-14}$ alkenyl;

each R* is independently selected from the group consisting of C$_{1-12}$ alkyl and C$_{2-12}$ alkenyl;

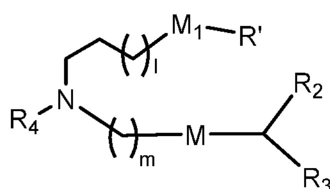
each Y is independently a C$_{3-6}$ carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13,

or salts or stereoisomers thereof.

In certain embodiments, a subset of compounds of Formula (I) includes those of Formula (IA):

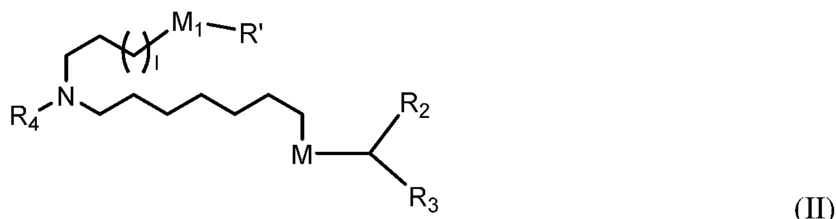


(IA),

or a salt or stereoisomer thereof, wherein l is selected from 1, 2, 3, 4, and 5; m is selected from 5, 6, 7, 8, and 9; M$_1$ is a bond or M'; R$_4$ is unsubstituted C$_{1-3}$ alkyl, or -(CH$_2$)$_n$Q, in which Q is OH, -NHC(S)N(R)$_2$, -NHC(O)N(R)$_2$, -N(R)C(O)R, -N(R)S(O)$_2$R, -N(R)R$_8$, -NHC(=NR$_9$)N(R)$_2$, -NHC(=CHR$_9$)N(R)$_2$, -OC(O)N(R)$_2$, -N(R)C(O)OR, heteroaryl, or

heterocycloalkyl; M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -P(O)(OR')O-, -S-S-, an aryl group, and a heteroaryl group; and R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, and C_{2-14} alkenyl.

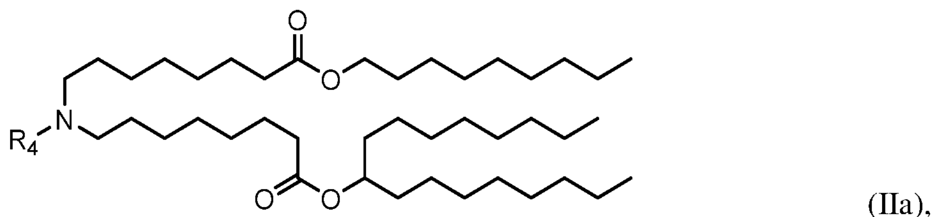
In certain embodiments, a subset of compounds of Formula (I) includes those of Formula (II):



(II)

or a salt or stereoisomer thereof, wherein l is selected from 1, 2, 3, 4, and 5; M_1 is a bond or M'; R_4 is unsubstituted C_{1-3} alkyl, or $-(CH_2)_nQ$, in which n is 2, 3, or 4, and Q is OH, $-NHC(S)N(R)_2$, $-NHC(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)R_8$, $-NHC(=NR_9)N(R)_2$, $-NHC(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, -N(R)C(O)OR, heteroaryl, or heterocycloalkyl; M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -P(O)(OR')O-, -S-S-, an aryl group, and a heteroaryl group; and R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, and C_{2-14} alkenyl.

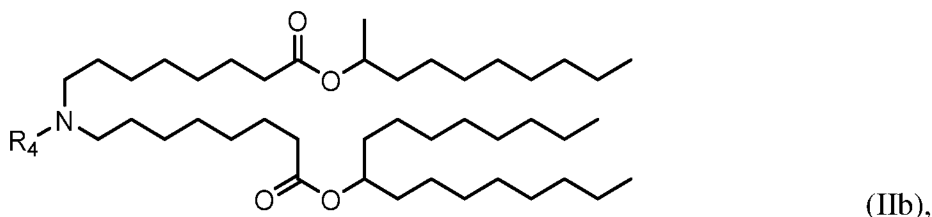
In some embodiments, the compound of formula (I) is of the formula (IIa),



(IIa),

or a salt thereof, wherein R_4 is as described above.

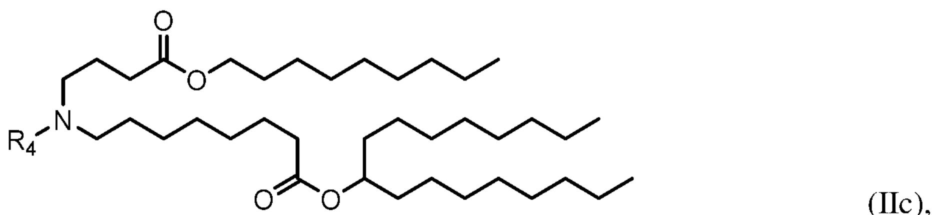
In some embodiments, the compound of formula (I) is of the formula (IIb),



(IIb),

or a salt thereof, wherein R_4 is as described above.

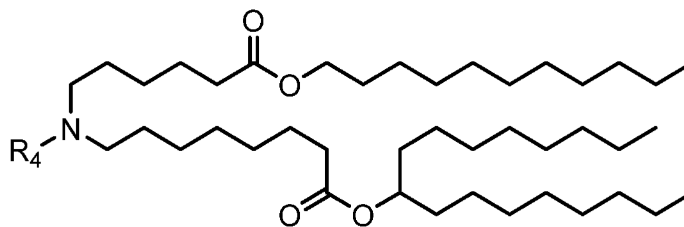
In some embodiments, the compound of formula (I) is of the formula (IIc),



(IIc),

or a salt thereof, wherein R_4 is as described above.

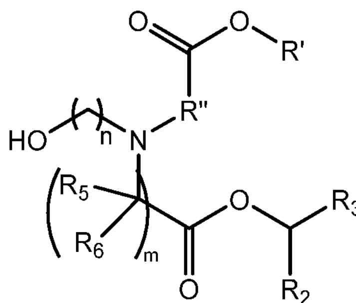
In some embodiments, the compound of formula (I) is of the formula (IIe):



(IIe),

or a salt thereof, wherein R_4 is as described above.

In some embodiments, the compound of formula (I) is of the formula (IId),



(IId),

or a salt thereof, wherein R_2 and R_3 are independently selected from the group consisting of C_{5-14} alkyl and C_{5-14} alkenyl, n is selected from 2, 3, and 4, and R', R'', R_5, R_6 and m are as defined above.

As used herein, the term "alkyl" or "alkyl group" means a linear or branched, saturated hydrocarbon including one or more carbon atoms (e.g., one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty, or more carbon atoms).

The notation "C_{1-14} alkyl" means a linear or branched, saturated hydrocarbon including 1-14 carbon atoms. An alkyl group can be optionally substituted.

As used herein, the term "alkenyl" or "alkenyl group" means a linear or branched hydrocarbon including two or more carbon atoms (e.g., two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty, or more carbon atoms) and at least one double bond.

The notation "C_{2-14} alkenyl" means a linear or branched hydrocarbon including 2-14 carbon atoms and at least one double bond. An alkenyl group can include one, two, three, four, or more double bonds. For example, C_{18} alkenyl can include one or more double bonds. A C_{18} alkenyl group including two double bonds can be a linoleyl group. An alkenyl group can be optionally substituted.

As used herein, the term "carbocycle" or "carbocyclic group" means a mono- or multi-cyclic system including one or more rings of carbon atoms. Rings can be three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or fifteen membered rings.

The notation "C_{3-6} carbocycle" means a carbocycle including a single ring having 3-6 carbon atoms. Carbocycles can include one or more double bonds and can be aromatic (e.g., aryl groups). Examples of carbocycles include cyclopropyl, cyclopentyl, cyclohexyl, phenyl, naphthyl, and 1,2-dihydronaphthyl groups. Carbocycles can be optionally substituted.

As used herein, the term "heterocycle" or "heterocyclic group" means a mono- or multi-cyclic system including one or more rings, where at least one ring includes at least one heteroatom. Heteroatoms can be, for example, nitrogen, oxygen, or sulfur atoms. Rings can be three, four, five, six, seven, eight, nine, ten, eleven, or twelve membered rings. Heterocycles can include one or more double bonds and can be aromatic (e.g., heteroaryl groups). Examples of heterocycles include imidazolyl, imidazolidinyl, oxazolyl, oxazolidinyl, thiazolyl, thiazolidinyl, pyrazolidinyl, pyrazolyl, isoxazolidinyl, isoxazolyl, isothiazolidinyl, isothiazolyl, morpholinyl, pyrrolyl, pyrrolidinyl, furyl, tetrahydrofuryl, thiophenyl, pyridinyl, piperidinyl, quinolyl, and isoquinolyl groups. Heterocycles can be optionally substituted.

As used herein, a "biodegradable group" is a group that can facilitate faster metabolism of a lipid in a patient. A biodegradable group can be, but is not limited to, -C(O)O-, -OC(O)-, -C(O)N(R')-, -N(R')C(O)-, -C(O)-, -C(S)-, -C(S)S-, -SC(S)-, -CH(OH)-, -P(O)(OR')O-, -$S(O)_2$-, an aryl group, and a heteroaryl group.

As used herein, an "aryl group" is a carbocyclic group including one or more aromatic rings. Examples of aryl groups include phenyl and naphthyl groups.

As used herein, a "heteroaryl group" is a heterocyclic group including one or more aromatic rings. Examples of heteroaryl groups include pyrrolyl, furyl, thiophenyl, imidazolyl, oxazolyl, and thiazolyl. Both aryl and heteroaryl groups can be optionally substituted. For example, M and M' can be selected from the non-limiting group consisting of optionally substituted phenyl, oxazole, and thiazole. In the formulas herein, M and M' can be independently selected from the list of biodegradable groups above.

Alkyl, alkenyl, and cyclyl (e.g., carbocyclyl and heterocyclyl) groups can be optionally substituted unless otherwise specified. Optional substituents can be selected from the group consisting of, but are not limited to, a halogen atom (e.g., a chloride, bromide, fluoride, or iodide group), a carboxylic acid (e.g., -C(O)OH), an alcohol (e.g., a hydroxyl, -OH), an ester (e.g., -C(O)OR or -OC(O)R), an aldehyde (e.g., -C(O)H), a carbonyl (e.g., -C(O)R, alternatively represented by C=O), an acyl halide (e.g., -C(O)X, in which X is a halide selected from bromide, fluoride, chloride, and iodide), a carbonate (e.g., -OC(O)OR), an alkoxy (e.g., -OR), an acetal (e.g., -$C(OR)_2$R"", in which each OR are alkoxy groups that can be the same or different and R"" is an alkyl or alkenyl group), a phosphate (e.g., $P(O)_4^{3-}$), a thiol (e.g., -SH), a sulfoxide (e.g., -S(O)R), a sulfinic acid (e.g., -S(O)OH), a sulfonic acid (e.g., -$S(O)_2$OH), a thial (e.g., -C(S)H), a

sulfate (e.g., $S(O)_4^{2-}$), a sulfonyl (e.g., $-S(O)_2-$), an amide (e.g., -C(O)NR_2, or -N(R)C(O)R), an azido (e.g., $-N_3$), a nitro (e.g., $-NO_2$), a cyano (e.g., -CN), an isocyano (e.g., -NC), an acyloxy (e.g., -OC(O)R), an amino (e.g., $-NR_2$, -NRH, or $-NH_2$), a carbamoyl (e.g., -OC(O)NR_2, -OC(O)NRH, or -OC(O)NH_2), a sulfonamide (e.g., $-S(O)_2NR_2$, $-S(O)_2NRH$, $-S(O)_2NH_2$, $-N(R)S(O)_2R$, $-N(H)S(O)_2R$, $-N(R)S(O)_2H$, or $-N(H)S(O)_2H$), an alkyl group, an alkenyl group, and a cyclyl (e.g., carbocyclyl or heterocyclyl) group.

In any of the preceding, R is an alkyl or alkenyl group, as defined herein. In some embodiments, the substituent groups themselves can be further substituted with, for example, one, two, three, four, five, or six substituents as defined herein. For example, a C_{1-6} alkyl group can be further substituted with one, two, three, four, five, or six substituents as described herein.

The compounds of any one of formulae (I), (IA), (II), (IIa), (IIb), (IIc), (IId), and (IIe) include one or more of the following features when applicable.

In some embodiments, R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, and $-CQ(R)_2$, where Q is selected from a C_{3-6} carbocycle, 5- to 14- membered aromatic or non-aromatic heterocycle having one or more heteroatoms selected from N, O, S, and P, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-N(R)_2$, $-C(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, and $-C(R)N(R)_2C(O)OR$, and each n is independently selected from 1, 2, 3, 4, and 5.

In another embodiment, R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, and $-CQ(R)_2$, where Q is selected from a C_{3-6} carbocycle, a 5- to 14-membered heteroaryl having one or more heteroatoms selected from N, O, and S, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-C(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-C(R)N(R)_2C(O)OR$, and a 5- to 14-membered heterocycloalkyl having one or more heteroatoms selected from N, O, and S which is substituted with one or more substituents selected from oxo (=O), OH, amino, and C_{1-3} alkyl, and each n is independently selected from 1, 2, 3, 4, and 5.

In another embodiment, R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, and $-CQ(R)_2$, where Q is selected from a C_{3-6} carbocycle, a 5- to 14-membered heterocycle having one or more heteroatoms selected from N, O, and S, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-C(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-C(R)N(R)_2C(O)OR$, and each n is independently selected from 1, 2, 3, 4, and 5; and when Q is a 5- to 14-membered heterocycle and (i) R_4 is $-(CH_2)_nQ$ in which n is 1 or 2, or (ii) R_4 is $-(CH_2)_nCHQR$ in which n is 1, or (iii) R_4 is -CHQR, and $-CQ(R)_2$, then Q is either a 5- to 14-membered heteroaryl or 8- to 14-membered heterocycloalkyl.

In another embodiment, R_4 is selected from the group consisting of a C_{3-6} carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, $-CHQR$, and $-CQ(R)_2$, where Q is selected from a C_{3-6} carbocycle, a 5- to 14-membered heteroaryl having one or more heteroatoms selected from N, O, and S, -OR, $-O(CH_2)_nN(R)_2$, -C(O)OR, -OC(O)R, $-CX_3$, $-CX_2H$, $-CXH_2$, -CN, $-C(O)N(R)_2$, -N(R)C(O)R, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-C(R)N(R)_2C(O)OR$, and each n is independently selected from 1, 2, 3, 4, and 5.

In another embodiment, R_4 is unsubstituted C_{1-4} alkyl, e.g., unsubstituted methyl.

In certain embodiments, the disclosure provides a compound having the Formula (I), wherein R_4 is $-(CH_2)_nQ$ or $-(CH_2)_nCHQR$, where Q is $-N(R)_2$, and n is selected from 3, 4, and 5.

In certain embodiments, the disclosure provides a compound having the Formula (I), wherein R_4 is selected from the group consisting of $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, -CHQR, and $-CQ(R)_2$, where Q is $-N(R)_2$, and n is selected from 1, 2, 3, 4, and 5.

In certain embodiments, the disclosure provides a compound having the Formula (I), wherein R_2 and R_3 are independently selected from the group consisting of C_{2-14} alkyl, C_{2-14} alkenyl, -R*YR”, -YR”, and -R*OR”, or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle, and R_4 is $-(CH_2)_nQ$ or $-(CH_2)_nCHQR$, where Q is $-N(R)_2$, and n is selected from 3, 4, and 5.

In certain embodiments, R_2 and R_3 are independently selected from the group consisting of C_{2-14} alkyl, C_{2-14} alkenyl, -R*YR”, -YR”, and -R*OR”, or R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle.

In some embodiments, R_1 is selected from the group consisting of C_{5-20} alkyl and C_{5-20} alkenyl.

In other embodiments, R_1 is selected from the group consisting of -R*YR”, -YR”, and -R”M’R’.

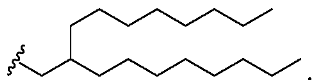
In certain embodiments, R_1 is selected from -R*YR” and -YR”. In some embodiments, Y is a cyclopropyl group. In some embodiments, R* is C_8 alkyl or C_8 alkenyl. In certain embodiments, R” is C_{3-12} alkyl. For example, R” can be C_3 alkyl. For example, R” can be C_{4-8} alkyl (e.g., C_4, C_5, C_6, C_7, or C_8 alkyl).

In some embodiments, R_1 is C_{5-20} alkyl. In some embodiments, R_1 is C_6 alkyl. In some embodiments, R_1 is C_8 alkyl. In other embodiments, R_1 is C_9 alkyl. In certain embodiments, R_1 is C_{14} alkyl. In other embodiments, R_1 is C_{18} alkyl.

In some embodiments, R_1 is C_{5-20} alkenyl. In certain embodiments, R_1 is C_{18} alkenyl. In some embodiments, R_1 is linoleyl.

In certain embodiments, R_1 is branched (*e.g.*, decan-2-yl, undecan-3-yl, dodecan-4-yl, tridecan-5-yl, tetradecan-6-yl, 2-methylundecan-3-yl, 2-methyldecan-2-yl, 3-methylundecan-3-

yl, 4-methyldodecan-4-yl, or heptadeca-9-yl). In certain embodiments, R_1 is



In certain embodiments, R_1 is unsubstituted C_{5-20} alkyl or C_{5-20} alkenyl. In certain embodiments, R' is substituted C_{5-20} alkyl or C_{5-20} alkenyl (*e.g.*, substituted with a C_{3-6} carbocycle such as 1-cyclopropylnonyl).

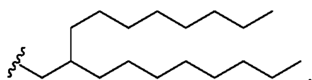
In other embodiments, R_1 is -R"M'R'.

In some embodiments, R' is selected from -R*YR" and -YR". In some embodiments, Y is C_{3-8} cycloalkyl. In some embodiments, Y is C_{6-10} aryl. In some embodiments, Y is a cyclopropyl group. In some embodiments, Y is a cyclohexyl group. In certain embodiments, R* is C_1 alkyl.

In some embodiments, R" is selected from the group consisting of C_{3-12} alkyl and C_{3-12} alkenyl. In some embodiments, R" adjacent to Y is C_1 alkyl. In some embodiments, R" adjacent to Y is C_{4-9} alkyl (*e.g.*, C_4, C_5, C_6, C_7 or C_8 or C_9 alkyl).

In some embodiments, R' is selected from C_4 alkyl and C_4 alkenyl. In certain embodiments, R' is selected from C_5 alkyl and C_5 alkenyl. In some embodiments, R' is selected from C_6 alkyl and C_6 alkenyl. In some embodiments, R' is selected from C_7 alkyl and C_7 alkenyl. In some embodiments, R' is selected from C_9 alkyl and C_9 alkenyl.

In other embodiments, R' is selected from C_{11} alkyl and C_{11} alkenyl. In other embodiments, R' is selected from C_{12} alkyl, C_{12} alkenyl, C_{13} alkyl, C_{13} alkenyl, C_{14} alkyl, C_{14} alkenyl, C_{15} alkyl, C_{15} alkenyl, C_{16} alkyl, C_{16} alkenyl, C_{17} alkyl, C_{17} alkenyl, C_{18} alkyl, and C_{18} alkenyl. In certain embodiments, R' is branched (*e.g.*, decan-2-yl, undecan-3-yl, dodecan-4-yl, tridecan-5-yl, tetradecan-6-yl, 2-methylundecan-3-yl, 2-methyldecan-2-yl, 3-methylundecan-3-yl, 4-methyldodecan-4-yl or heptadeca-9-yl). In certain embodiments, R' is



In certain embodiments, R' is unsubstituted C_{1-18} alkyl. In certain embodiments, R' is substituted C_{1-18} alkyl (*e.g.*, C_{1-15} alkyl substituted with a C_{3-6} carbocycle such as 1-cyclopropylnonyl).

In some embodiments, R" is selected from the group consisting of C_{3-14} alkyl and C_{3-14} alkenyl. In some embodiments, R" is C_3 alkyl, C_4 alkyl, C_5 alkyl, C_6 alkyl, C_7 alkyl, or C_8 alkyl. In some embodiments, R" is C_9 alkyl, C_{10} alkyl, C_{11} alkyl, C_{12} alkyl, C_{13} alkyl, or C_{14} alkyl.

In some embodiments, M' is -C(O)O-. In some embodiments, M' is -OC(O)-.

In other embodiments, M' is an aryl group or heteroaryl group. For example, M' can be selected from the group consisting of phenyl, oxazole, and thiazole.

In some embodiments, M is -C(O)O- In some embodiments, M is -OC(O)-. In some embodiments, M is -C(O)N(R')-. In some embodiments, M is -P(O)(OR')O-.

In other embodiments, M is an aryl group or heteroaryl group. For example, M can be selected from the group consisting of phenyl, oxazole, and thiazole.

In some embodiments, M is the same as M'. In other embodiments, M is different from M'.

In some embodiments, each R_5 is H. In certain such embodiments, each R_6 is also H.

In some embodiments, R_7 is H. In other embodiments, R_7 is C_{1-3} alkyl (*e.g.*, methyl, ethyl, propyl, or i-propyl).

In some embodiments, R_2 and R_3 are independently C_{5-14} alkyl or C_{5-14} alkenyl.

In some embodiments, R_2 and R_3 are the same. In some embodiments, R_2 and R_3 are C_8 alkyl. In certain embodiments, R_2 and R_3 are C_2 alkyl. In other embodiments, R_2 and R_3 are C_3 alkyl. In some embodiments, R_2 and R_3 are C_4 alkyl. In certain embodiments, R_2 and R_3 are C_5 alkyl. In other embodiments, R_2 and R_3 are C_6 alkyl. In some embodiments, R_2 and R_3 are C_7 alkyl.

In other embodiments, R_2 and R_3 are different. In certain embodiments, R_2 is C_8 alkyl. In some embodiments, R_3 is C_{1-7} (*e.g.*, C_1, C_2, C_3, C_4, C_5, C_6, or C_7 alkyl) or C_9 alkyl.

In some embodiments, R_7 and R_3 are H.

In certain embodiments, R_2 is H.

In some embodiments, m is 5, 7, or 9.

In some embodiments, R_4 is selected from $-(CH_2)_nQ$ and $-(CH_2)_nCHQR$.

In some embodiments, Q is selected from the group consisting of -OR, -OH, $-O(CH_2)_nN(R)_2$, -OC(O)R, $-CX_3$, -CN, -N(R)C(O)R, -N(H)C(O)R, $-N(R)S(O)_2R$, $-N(H)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(H)C(O)N(R)_2$, -N(H)C(O)N(H)(R), $-N(R)C(S)N(R)_2$, $-N(H)C(S)N(R)_2$, -N(H)C(S)N(H)(R), $-C(R)N(R)_2C(O)OR$, a carbocycle, and a heterocycle.

In certain embodiments, Q is -OH.

In certain embodiments, Q is a substituted or unsubstituted 5- to 10- membered heteroaryl, *e.g.*, Q is an imidazole, a pyrimidine, a purine, 2-amino-1,9-dihydro-6*H*-purin-6-one-9-yl (or guanin-9-yl), adenin-9-yl, cytosin-1-yl, or uracil-1-yl. In certain embodiments, Q is a substituted 5- to 14-membered heterocycloalkyl, *e.g.*, substituted with one or more substituents selected from oxo (=O), OH, amino, and C_{1-3} alkyl. For example, Q is 4-methylpiperazinyl, 4-(4-methoxybenzyl)piperazinyl, or isoindolin-2-yl-1,3-dione.

In certain embodiments, Q is an unsubstituted or substituted C_{6-10} aryl (such as phenyl) or C_{3-6} cycloalkyl.

In some embodiments, n is 1. In other embodiments, n is 2. In further embodiments, n is 3. In certain other embodiments, n is 4. For example, R_4 can be $-(CH_2)_2OH$. For example, R_4 can be $-(CH_2)_3OH$. For example, R_4 can be $-(CH_2)_4OH$. For example, R_4 can be benzyl. For example, R_4 can be 4-methoxybenzyl.

In some embodiments, R_4 is a C_{3-6} carbocycle. In some embodiments, R_4 is a C_{3-6} cycloalkyl. For example, R_4 can be cyclohexyl optionally substituted with *e.g.*, OH, halo, C_{1-6} alkyl, etc. For example, R_4 can be 2-hydroxycyclohexyl.

In some embodiments, R is H.

In some embodiments, R is unsubstituted C_{1-3} alkyl or unsubstituted C_{2-3} alkenyl. For example, R_4 can be $-CH_2CH(OH)CH_3$ or $-CH_2CH(OH)CH_2CH_3$.

In some embodiments, R is substituted C_{1-3} alkyl, *e.g.*, CH_2OH. For example, R_4 can be $-CH_2CH(OH)CH_2OH$.

In some embodiments, R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle. In some embodiments, R_2 and R_3, together with the atom to which they are attached, form a 5- to 14- membered aromatic or non-aromatic heterocycle having one or more heteroatoms selected from N, O, S, and P. In some embodiments, R_2 and R_3, together with the atom to which they are attached, form an optionally substituted C_{3-20} carbocycle (*e.g.*, C_{3-18} carbocycle, C_{3-15} carbocycle, C_{3-12} carbocycle, or C_{3-10} carbocycle), either aromatic or non-aromatic. In some embodiments, R_2 and R_3, together with the atom to which they are attached, form a C_{3-6} carbocycle. In other embodiments, R_2 and R_3, together with the atom to which they are attached, form a C_6 carbocycle, such as a cyclohexyl or phenyl group. In certain embodiments, the heterocycle or C_{3-6} carbocycle is substituted with one or more alkyl groups (*e.g.*, at the same ring atom or at adjacent or non-adjacent ring atoms). For example, R_2 and R_3, together with the atom to which they are attached, can form a cyclohexyl or phenyl group bearing one or more C_5 alkyl substitutions. In certain embodiments, the heterocycle or C_{3-6} carbocycle formed by R_2 and R_3, is substituted with a carbocycle groups. For example, R_2 and R_3, together with the atom to which they are attached, can form a cyclohexyl or phenyl group that is substituted with cyclohexyl. In some embodiments, R_2 and R_3, together with the atom to which they are attached, form a C_{7-15} carbocycle, such as a cycloheptyl, cyclopentadecanyl, or naphthyl group.

In some embodiments, R_4 is selected from $-(CH_2)_nQ$ and $-(CH_2)_nCHQR$. In some embodiments, Q is selected from the group consisting of -OR, -OH, $-O(CH_2)_nN(R)_2$, -OC(O)R, $-CX_3$, -CN, -N(R)C(O)R, -N(H)C(O)R, $-N(R)S(O)_2R$, $-N(H)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(H)C(O)N(R)_2$, -N(H)C(O)N(H)(R), $-N(R)C(S)N(R)_2$, $-N(H)C(S)N(R)_2$, -N(H)C(S)N(H)(R),

and a heterocycle. In other embodiments, Q is selected from the group consisting of an imidazole, a pyrimidine, and a purine.

In some embodiments, R_2 and R_3, together with the atom to which they are attached, form a heterocycle or carbocycle. In some embodiments, R_2 and R_3, together with the atom to which they are attached, form a C_{3-6} carbocycle, such as a phenyl group. In certain embodiments, the heterocycle or C_{3-6} carbocycle is substituted with one or more alkyl groups (*e.g.*, at the same ring atom or at adjacent or non-adjacent ring atoms). For example, R_2 and R_3, together with the atom to which they are attached, can form a phenyl group bearing one or more C_5 alkyl substitutions.

In some embodiments, the LNP has an ionizable amino lipid selected from any of Compounds 1- 232 disclosed in PCT publication WO/2017/049245 published on March 23, 2017 and salts or stereoisomers thereof.

Ionizable lipids can be selected from the non-limiting group consisting of 3-(didodecylamino)-N1,N1,4-tridodecyl-1-piperazineethanamine (KL10), N1-[2-(didodecylamino)ethyl]-N1,N4,N4-tridodecyl-1,4-piperazinediethanamine (KL22), 14,25-ditridecyl-15,18,21,24-tetraaza-octatriacontane (KL25), 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLin-DMA), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLin-MC3-DMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), (13Z,165Z)-N,N-dimethyl-3-nonydocosa-13-16-dien-1-amine, 2-({8-[(3β)-cholest-5-en-3-yloxy]octyl}oxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yl oxy]propan-1-amine (Octyl-CLinDMA), (2R)-2-({8-[(3β)-cholest-5-en-3-yloxy]octyl}oxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Octyl-CLinDMA (2R)), and (2S)-2-({8-[(3β)-cholest-5-en-3-yloxy]octyl}oxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Octyl-CLinDMA (2S)). In addition to these, an ionizable amino lipid can also be a lipid including a cyclic amine group.

The lipid composition of the pharmaceutical composition disclosed herein can comprise one or more phospholipids, for example, one or more saturated or (poly)unsaturated phospholipids or a combination thereof. In general, phospholipids comprise a phospholipid moiety and one or more fatty acid moieties.

A phospholipid moiety can be selected, for example, from the non-limiting group consisting of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl serine, phosphatidic acid, 2-lysophosphatidyl choline, and a sphingomyelin.

A fatty acid moiety can be selected, for example, from the non-limiting group consisting of lauric acid, myristic acid, myristoleic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, alpha-linolenic acid, erucic acid, phytanoic acid, arachidic acid, arachidonic acid, eicosapentaenoic acid, behenic acid, docosapentaenoic acid, and docosahexaenoic acid.

Particular phospholipids can facilitate fusion to a membrane. For example, a cationic phospholipid can interact with one or more negatively charged phospholipids of a membrane (e.g., a cellular or intracellular membrane). Fusion of a phospholipid to a membrane can allow one or more elements (e.g., a therapeutic agent) of a lipid-containing composition (e.g., LNPs) to pass through the membrane permitting, e.g., delivery of the one or more elements to a target tissue.

Non-natural phospholipid species including natural species with modifications and substitutions including branching, oxidation, cyclization, and alkynes are also contemplated. For example, a phospholipid can be functionalized with or cross-linked to one or more alkynes (e.g., an alkenyl group in which one or more double bonds is replaced with a triple bond). Under appropriate reaction conditions, an alkyne group can undergo a copper-catalyzed cycloaddition upon exposure to an azide. Such reactions can be useful in functionalizing a lipid bilayer of a nanoparticle composition to facilitate membrane permeation or cellular recognition or in conjugating a nanoparticle composition to a useful component such as a targeting or imaging moiety (e.g., a dye).

Phospholipids include, but are not limited to, glycerophospholipids such as phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines, phosphatidylinositols, phosphatidy glycerols, and phosphatidic acids. Phospholipids also include phosphosphingolipid, such as sphingomyelin.

In certain embodiments, a phospholipid useful or potentially useful in the present invention is an analog or variant of DSPC.

In certain embodiments, a phospholipid useful or potentially useful in the present invention comprises a modified phospholipid head (*e.g.*, a modified choline group). In certain embodiments, a phospholipid with a modified head is DSPC, or analog thereof, with a modified quaternary amine.

In certain embodiments, a phospholipid useful or potentially useful in the present invention comprises a modified tail. In certain embodiments, a phospholipid useful or potentially useful in the present invention is DSPC, or analog thereof, with a modified tail. As described herein, a "modified tail" may be a tail with shorter or longer aliphatic chains, aliphatic chains with branching introduced, aliphatic chains with substituents introduced, aliphatic chains wherein one or more methylenes are replaced by cyclic or heteroatom groups, or any combination thereof.

In certain embodiments, an alternative lipid is used in place of a phospholipid of the invention.

The LNPs disclosed herein can comprise one or more structural lipids. As used herein, the term “structural lipid” refers to sterols and also to lipids containing sterol moieties.

Incorporation of structural lipids in the lipid nanoparticle may help mitigate aggregation of other lipids in the particle. Structural lipids can be selected from the group including but not limited to, cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, tomatine, ursolic acid, alpha-tocopherol, hopanoids, phytosterols, steroids, and mixtures thereof. In some embodiments, the structural lipid is a sterol. As defined herein, “sterols” are a subgroup of steroids consisting of steroid alcohols. In certain embodiments, the structural lipid is a steroid. In certain embodiments, the structural lipid is cholesterol. In certain embodiments, the structural lipid is an analog of cholesterol. In certain embodiments, the structural lipid is alpha-tocopherol.

In one embodiment, the amount of the structural lipid (e.g., an sterol such as cholesterol) in the lipid composition of a pharmaceutical composition disclosed herein ranges from about 20 mol % to about 60 mol %, from about 25 mol % to about 55 mol %, from about 30 mol % to about 50 mol %, or from about 35 mol % to about 45 mol %.

In one embodiment, the amount of the structural lipid (e.g., an sterol such as cholesterol) in the lipid composition disclosed herein ranges from about 25 mol % to about 30 mol %, from about 30 mol % to about 35 mol %, or from about 35 mol % to about 40 mol %.

In one embodiment, the amount of the structural lipid (e.g., a sterol such as cholesterol) in the lipid composition disclosed herein is about 24 mol %, about 29 mol %, about 34 mol %, or about 39 mol %.

In some embodiments, the amount of the structural lipid (e.g., an sterol such as cholesterol) in the lipid composition disclosed herein is at least about 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 mol %.

The lipid composition of a pharmaceutical composition disclosed herein can comprise one or more a polyethylene glycol (PEG) lipid.

As used herein, the term “PEG-lipid” refers to polyethylene glycol (PEG)-modified lipids. Non-limiting examples of PEG-lipids include PEG-modified phosphatidylethanolamine and phosphatidic acid, PEG-ceramide conjugates (e.g., PEG-CerC14 or PEG-CerC20), PEG-modified dialkylamines and PEG-modified 1,2-diacyloxypropan-3-amines. Such lipids are also referred to as PEGylated lipids. For example, a PEG lipid can be PEG-c-DOMG, PEG-DMG, PEG-DLPE, PEG-DMPE, PEG-DPPC, or a PEG-DSPE lipid.

In some embodiments, the PEG-lipid includes, but not limited to 1,2-dimyristoyl-sn-glycerol methoxypolyethylene glycol (PEG-DMG), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)] (PEG-DSPE), PEG-disteryl glycerol (PEG-DSG), PEG-dipalmetoleyl, PEG-dioleyl, PEG-distearyl, PEG-diacylglycamide (PEG-DAG), PEG-dipalmitoyl phosphatidylethanolamine (PEG-DPPE), or PEG-l,2-dimyristyloxlpropyl-3-amine (PEG-c-DMA).

In one embodiment, the PEG-lipid is selected from the group consisting of a PEG-modified phosphatidylethanolamine, a PEG-modified phosphatidic acid, a PEG-modified ceramide, a PEG-modified dialkylamine, a PEG-modified diacylglycerol, a PEG-modified dialkylglycerol, and mixtures thereof.

In some embodiments, the lipid moiety of the PEG-lipids includes those having lengths of from about C_{14} to about C_{22}, preferably from about C_{14} to about C_{16}. In some embodiments, a PEG moiety, for example an mPEG-NH_2, has a size of about 1000, 2000, 5000, 10,000, 15,000 or 20,000 daltons. In one embodiment, the PEG-lipid is PEG_{2k}-DMG.

In one embodiment, the lipid nanoparticles described herein can comprise a PEG lipid which is a non-diffusible PEG. Non-limiting examples of non-diffusible PEGs include PEG-DSG and PEG-DSPE.

PEG-lipids are known in the art, such as those described in U.S. Patent No. 8158601 and International Publ. No. WO 2015/130584 A2, which are incorporated herein by reference in their entirety.

In one embodiment, PEG lipids useful in the present invention can be PEGylated lipids described in International Publication No. WO2012/099755, the contents of which is herein incorporated by reference in its entirety. Any of these exemplary PEG lipids described herein may be modified to comprise a hydroxyl group on the PEG chain. In certain embodiments, the PEG lipid is a PEG-OH lipid. As generally defined herein, a “PEG-OH lipid” (also referred to herein as “hydroxy-PEGylated lipid”) is a PEGylated lipid having one or more hydroxyl (–OH) groups on the lipid. In certain embodiments, the PEG-OH lipid includes one or more hydroxyl groups on the PEG chain. In certain embodiments, a PEG-OH or hydroxy-PEGylated lipid comprises an –OH group at the terminus of the PEG chain. Each possibility represents a separate embodiment of the present invention.

In one embodiment, the amount of PEG-lipid in the lipid composition of a pharmaceutical composition disclosed herein ranges from about 0.1 mol % to about 5 mol %, from about 0.5 mol % to about 5 mol %, from about 1 mol % to about 5 mol %, from about 1.5 mol % to about 5 mol %, from about 2 mol % to about 5 mol %, from about 0.1 mol % to about 4 mol %, from about 0.5 mol % to about 4 mol %, from about 1 mol % to about 4 mol %, from about 1.5 mol % to

about 4 mol %, from about 2 mol % to about 4 mol %, from about 0.1 mol % to about 3 mol %, from about 0.5 mol % to about 3 mol %, from about 1 mol % to about 3 mol %, from about 1.5 mol % to about 3 mol %, from about 2 mol % to about 3 mol %, from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 1.5 mol % to about 2 mol %, from about 0.1 mol % to about 1.5 mol %, from about 0.5 mol % to about 1.5 mol %, or from about 1 mol % to about 1.5 mol %.

In one embodiment, the amount of PEG-lipid in the lipid composition disclosed herein is about 2 mol %. In one embodiment, the amount of PEG-lipid in the lipid composition disclosed herein is about 1.5 mol %.

In one embodiment, the amount of PEG-lipid in the lipid composition disclosed herein is at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, or 5 mol %.

In some aspects, the lipid composition of the pharmaceutical compositions disclosed herein does not comprise a PEG-lipid.

The lipid composition of a pharmaceutical composition disclosed herein can include one or more components in addition to those described above. For example, the lipid composition can include one or more permeability enhancer molecules, carbohydrates, polymers, surface altering agents (e.g., surfactants), or other components. For example, a permeability enhancer molecule can be a molecule described by U.S. Patent Application Publication No. 2005/0222064. Carbohydrates can include simple sugars (e.g., glucose) and polysaccharides (e.g., glycogen and derivatives and analogs thereof).

A polymer can be included in and/or used to encapsulate or partially encapsulate a pharmaceutical composition disclosed herein (e.g., a pharmaceutical composition in lipid nanoparticle form). A polymer can be biodegradable and/or biocompatible. A polymer can be selected from, but is not limited to, polyamines, polyethers, polyamides, polyesters, polycarbamates, polyureas, polycarbonates, polystyrenes, polyimides, polysulfones, polyurethanes, polyacetylenes, polyethylenes, polyethyleneimines, polyisocyanates, polyacrylates, polymethacrylates, polyacrylonitriles, and polyarylates.

The ratio between the lipid composition and the polynucleotide range can be from about 10:1 to about 60:1 (wt/wt).

In some embodiments, the ratio between the lipid composition and the polynucleotide can be about 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 16:1, 17:1, 18:1, 19:1, 20:1, 21:1, 22:1, 23:1, 24:1, 25:1, 26:1, 27:1, 28:1, 29:1, 30:1, 31:1, 32:1, 33:1, 34:1, 35:1, 36:1, 37:1, 38:1, 39:1, 40:1, 41:1, 42:1, 43:1, 44:1, 45:1, 46:1, 47:1, 48:1, 49:1, 50:1, 51:1, 52:1, 53:1, 54:1, 55:1, 56:1, 57:1, 58:1,

59:1 or 60:1 (wt/wt). In some embodiments, the wt/wt ratio of the lipid composition to the polynucleotide encoding a therapeutic agent is about 20:1 or about 15:1.

In one embodiment, the lipid nanoparticles described herein can comprise polynucleotides (e.g., mRNA) in a lipid:polynucleotide weight ratio of 5:1, 10:1, 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, 55:1, 60:1 or 70:1, or a range or any of these ratios such as, but not limited to, 5:1 to about 10:1, from about 5:1 to about 15:1, from about 5:1 to about 20:1, from about 5:1 to about 25:1, from about 5:1 to about 30:1, from about 5:1 to about 35:1, from about 5:1 to about 40:1, from about 5:1 to about 45:1, from about 5:1 to about 50:1, from about 5:1 to about 55:1, from about 5:1 to about 60:1, from about 5:1 to about 70:1, from about 10:1 to about 15:1, from about 10:1 to about 20:1, from about 10:1 to about 25:1, from about 10:1 to about 30:1, from about 10:1 to about 35:1, from about 10:1 to about 40:1, from about 10:1 to about 45:1, from about 10:1 to about 50:1, from about 10:1 to about 55:1, from about 10:1 to about 60:1, from about 10:1 to about 70:1, from about 15:1 to about 20:1, from about 15:1 to about 25:1,from about 15:1 to about 30:1, from about 15:1 to about 35:1, from about 15:1 to about 40:1, from about 15:1 to about 45:1, from about 15:1 to about 50:1, from about 15:1 to about 55:1, from about 15:1 to about 60:1 or from about 15:1 to about 70:1.

In one embodiment, the lipid nanoparticles described herein can comprise the polynucleotide in a concentration from approximately 0.1 mg/ml to 2 mg/ml such as, but not limited to, 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml, 1.0 mg/ml, 1.1 mg/ml, 1.2 mg/ml, 1.3 mg/ml, 1.4 mg/ml, 1.5 mg/ml, 1.6 mg/ml, 1.7 mg/ml, 1.8 mg/ml, 1.9 mg/ml, 2.0 mg/ml or greater than 2.0 mg/ml.

In some embodiments, the pharmaceutical compositions disclosed herein are formulated as lipid nanoparticles (LNP). Accordingly, the present disclosure also provides nanoparticle compositions comprising (i) a lipid composition comprising a delivery agent such as a compound of Formula (I) or (III) as described herein, and (ii) a polynucleotide encoding an antigen polypeptide. In such nanoparticle composition, the lipid composition disclosed herein can encapsulate the polynucleotide encoding an antigen polypeptide.

Nanoparticle compositions are typically sized on the order of micrometers or smaller and can include a lipid bilayer. Nanoparticle compositions encompass lipid nanoparticles (LNPs), liposomes (e.g., lipid vesicles), and lipoplexes. For example, a nanoparticle composition can be a liposome having a lipid bilayer with a diameter of 500 nm or less.

Nanoparticle compositions include, for example, lipid nanoparticles (LNPs), liposomes, and lipoplexes. In some embodiments, nanoparticle compositions are vesicles including one or more lipid bilayers. In certain embodiments, a nanoparticle composition includes two or more concentric bilayers separated by aqueous compartments. Lipid bilayers can be functionalized

and/or crosslinked to one another. Lipid bilayers can include one or more ligands, proteins, or channels.

In one embodiment, a lipid nanoparticle comprises an ionizable lipid, a structural lipid, a phospholipid, and mRNA. In some embodiments, the LNP comprises an ionizable lipid, a PEG-modified lipid, a phospholipid and a structural lipid. In some embodiments, the LNP has a molar ratio of about 20-60% ionizable lipid: about 5-25% phospholipid: about 25-55% structural lipid; and about 0.5-15% PEG-modified lipid. In some embodiments, the LNP comprises a molar ratio of about 50% ionizable lipid, about 1.5% PEG-modified lipid, about 38.5% structural lipid and about 10% phospholipid. In some embodiments, the LNP comprises a molar ratio of about 55% ionizable lipid, about 2.5% PEG lipid, about 32.5% structural lipid and about 10% phospholipid. In some embodiments, the ionizable lipid is an ionizable amino lipid and the phospholipid is a neutral lipid, and the structural lipid is a cholesterol. In some embodiments, the LNP has a molar ratio of 50:38.5:10:1.5 of ionizable lipid: cholesterol: DSPC: PEG lipid.

In some embodiments, the LNP has a polydispersity value of less than 0.4. In some embodiments, the LNP has a net neutral charge at a neutral pH. In some embodiments, the LNP has a mean diameter of 50-150 nm. In some embodiments, the LNP has a mean diameter of 80-100 nm.

As generally defined herein, the term “lipid” refers to a small molecule that has hydrophobic or amphiphilic properties. Lipids may be naturally occurring or synthetic. Examples of classes of lipids include, but are not limited to, fats, waxes, sterol-containing metabolites, vitamins, fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides, and prenol lipids. In some instances, the amphiphilic properties of some lipids lead them to form liposomes, vesicles, or membranes in aqueous media.

In some embodiments, a lipid nanoparticle (LNP) may comprise an ionizable lipid. As used herein, the term “ionizable lipid” has its ordinary meaning in the art and may refer to a lipid comprising one or more charged moieties. In some embodiments, an ionizable lipid may be positively charged or negatively charged. An ionizable lipid may be positively charged, in which case it can be referred to as “cationic lipid”. In certain embodiments, an ionizable lipid molecule may comprise an amine group, and can be referred to as an ionizable amino lipids. As used herein, a “charged moiety” is a chemical moiety that carries a formal electronic charge, e.g., monovalent (+1, or -1), divalent (+2, or -2), trivalent (+3, or -3), etc. The charged moiety may be anionic (i.e., negatively charged) or cationic (i.e., positively charged). Examples of positively-charged moieties include amine groups (e.g., primary, secondary, and/or tertiary amines), ammonium groups, pyridinium group, guanidine groups, and imidizolium groups. In a particular embodiment, the charged moieties comprise amine groups. Examples of negatively- charged

groups or precursors thereof, include carboxylate groups, sulfonate groups, sulfate groups, phosphonate groups, phosphate groups, hydroxyl groups, and the like. The charge of the charged moiety may vary, in some cases, with the environmental conditions, for example, changes in pH may alter the charge of the moiety, and/or cause the moiety to become charged or uncharged. In general, the charge density of the molecule may be selected as desired.

It should be understood that the terms “charged” or “charged moiety” does not refer to a “partial negative charge" or “partial positive charge" on a molecule. The terms “partial negative charge" and “partial positive charge" are given their ordinary meaning in the art. A “partial negative charge" may result when a functional group comprises a bond that becomes polarized such that electron density is pulled toward one atom of the bond, creating a partial negative charge on the atom. Those of ordinary skill in the art will, in general, recognize bonds that can become polarized in this way.

In some embodiments, the ionizable lipid is an ionizable amino lipid, sometimes referred to in the art as an “ionizable cationic lipid”. In one embodiment, the ionizable amino lipid may have a positively charged hydrophilic head and a hydrophobic tail that are connected via a linker structure.

In addition to these, an ionizable lipid may also be a lipid including a cyclic amine group.

In one embodiment, the ionizable lipid may be selected from, but not limited to, an ionizable lipid described in International Publication Nos. WO2013/086354 and WO2013/116126; the contents of each of which are herein incorporated by reference in their entirety.

In one embodiment, the lipid may be a cleavable lipid such as those described in International Publication No. WO2012/170889, herein incorporated by reference in its entirety. In one embodiment, the lipid may be synthesized by methods known in the art and/or as described in International Publication Nos. WO2013086354; the contents of each of which are herein incorporated by reference in their entirety.

Nanoparticle compositions can be characterized by a variety of methods. For example, microscopy (e.g., transmission electron microscopy or scanning electron microscopy) can be used to examine the morphology and size distribution of a nanoparticle composition. Dynamic light scattering or potentiometry (e.g., potentiometric titrations) can be used to measure zeta potentials. Dynamic light scattering can also be utilized to determine particle sizes. Instruments such as the Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK) can also be used to measure multiple characteristics of a nanoparticle composition, such as particle size, polydispersity index, and zeta potential.

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The size of the nanoparticles can help counter biological reactions such as, but not limited to, inflammation, or can increase the biological effect of the polynucleotide.

As used herein, “size” or “mean size” in the context of nanoparticle compositions refers to the mean diameter of a nanoparticle composition.

In one embodiment, the polynucleotide encoding an antigen polypeptide are formulated in lipid nanoparticles having a diameter from about 10 to about 100 nm such as, but not limited to, about 10 to about 20 nm, about 10 to about 30 nm, about 10 to about 40 nm, about 10 to about 50 nm, about 10 to about 60 nm, about 10 to about 70 nm, about 10 to about 80 nm, about 10 to about 90 nm, about 20 to about 30 nm, about 20 to about 40 nm, about 20 to about 50 nm, about 20 to about 60 nm, about 20 to about 70 nm, about 20 to about 80 nm, about 20 to about 90 nm, about 20 to about 100 nm, about 30 to about 40 nm, about 30 to about 50 nm, about 30 to about 60 nm, about 30 to about 70 nm, about 30 to about 80 nm, about 30 to about 90 nm, about 30 to about 100 nm, about 40 to about 50 nm, about 40 to about 60 nm, about 40 to about 70 nm, about 40 to about 80 nm, about 40 to about 90 nm, about 40 to about 100 nm, about 50 to about 60 nm, about 50 to about 70 nm, about 50 to about 80 nm, about 50 to about 90 nm, about 50 to about 100 nm, about 60 to about 70 nm, about 60 to about 80 nm, about 60 to about 90 nm, about 60 to about 100 nm, about 70 to about 80 nm, about 70 to about 90 nm, about 70 to about 100 nm, about 80 to about 90 nm, about 80 to about 100 nm and/or about 90 to about 100 nm.

In one embodiment, the nanoparticles have a diameter from about 10 to 500 nm. In one embodiment, the nanoparticle has a diameter greater than 100 nm, greater than 150 nm, greater than 200 nm, greater than 250 nm, greater than 300 nm, greater than 350 nm, greater than 400 nm, greater than 450 nm, greater than 500 nm, greater than 550 nm, greater than 600 nm, greater than 650 nm, greater than 700 nm, greater than 750 nm, greater than 800 nm, greater than 850 nm, greater than 900 nm, greater than 950 nm or greater than 1000 nm.

In some embodiments, the largest dimension of a nanoparticle composition is 1 μm or shorter (e.g., 1 μm, 900 nm, 800 nm, 700 nm, 600 nm, 500 nm, 400 nm, 300 nm, 200 nm, 175 nm, 150 nm, 125 nm, 100 nm, 75 nm, 50 nm, or shorter).

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

EXAMPLES

Methods: A human patient was treated with a mRNA encoding a personalized concatemeric cancer vaccine having several neoantigens in a LNP. Blood was collected from the patient at a baseline, day zero, and 7 days after administration of the fourth dose of vaccine construct. Data on the antigen specific activation of T cells was generated using each of the three assays summarized in Fig. 1. T cell activation may be assessed using known techniques such as ELIPOT and flow cytometry. Significantly increased responses were observed with the DC : T cell co-culture method when T cells have undergone IVS as compared to previously reported data using ex vivo T cells. The IVS T cell population was *in vitro* stimulated for 14 days, allowing for the expansion of neoantigen specific T cell clones.

Time points assessed in this assay were baseline and 7d post fourth dose of vaccine. Two DC conditions were tested in this assay, peptide pool pulsed DCs and DCs pulsed with peptides corresponding to individual neoantigens. Parameters such as LOD and LLOQ would be difficult to establish for an assay of this complexity; a 3-examining fold change over baseline will be used to indicate positive results. % Freq. CD8+IFNγ+ results after restimulation with individual neoantigen pulsed DCs are presented in Figures 3A-B.

Results:

Increases in % freq. $CD8^{+}IFN\gamma^{+}$ ranging from 2-16.4x over baseline were observed in the PBMC sample taken at 7 days following the 4th dose of vaccine after restimulation of T cells with DCs pulsed with neoantigen peptide pools (Figures 2A-B and Table 1 below), with peptide pool 16-20 having the greatest % freq. of $CD8^{+}IFN\gamma^{+}$ at C4D8 (27.1% at C4D8 vs. 6.17% at baseline).

Table 1 – Summary % freq. $CD8^+IFN\gamma^+$ T cells to DC stimulation and fold change of C4D8 over baseline

Target Response	DC stimulation	% Freq. $CD8^+IFN\gamma^+$		Fold Change
		Baseline	post-dose 4	
n/a	unpulsed	0.63	0.57	0.9
Class I	neoag 3*	0.58	5.67	9.8
	neoag 4*	0.6	6.51	10.9
	neoag 5*	0.94	9.5	10.1
	neoag 6	0.94	1.55	1.6
	neoag 7	0.48	1.03	2.1
	neoag 8	0.62	1.42	2.3
	neoag 9*	0.79	7.82	9.9
	neoag 10*	0.5	2.54	5.1
	neoag 11	0.85	0.34	0.4
	neoag 12	0.45	0.14	0.3
	neoag 13*	1.1	3.79	3.4
	neoag 14	0.5	0.19	0.4
	neoag 15	0.17	0.23	1.4
	neoag 16*	6.11	24.9	4.1
	neoag 17*	2.08	2.45	1.2
Class I & II	neoag 1*	1.63	11.1	6.8
	neoag 2*	0.75	5.91	7.9
	neoag 18*	0.75	2.9	3.9
Class II	neoag 19	1.22	2.13	1.7
	neoag 20	3.05	2.71	0.9
Pools	neoags 1-5*	1.05	7.32	7.0
	neoags 6-10*	0.41	6.74	16.4
	neoags 11-15	0.95	1.91	2.0
	neoags 16-20*	6.17	27.1	4.4

These results are the first time T cell responses at the individual neoantigen level have been interrogated in patient samples, providing enormous insight into vaccine design and therapeutic manipulation. 18 out of the 20 neoantigens included in the vaccine administered to the patient were predicted to elicit a class I (CD8) T cell response, 3 of which also have predicted class II affinity (thus predicted to potentially stimulate CD4 T cells). 10 out of the 18 predicted class I neoantigens had at least a 3x increase in the % freq. of neoantigen specific $CD8^+IFN\gamma^+$ cells at this time point as compared to baseline (denoted by * in Figures 3A-B and Table 1). As expected, the two predicted class II neoantigens did not have increases in % freq. $CD8^+IFN\gamma^+$ at C4D8 as compared to baseline.

All the neoantigens that drove an increase in the % freq. of $CD8^+IFN\gamma^+$ cells $\geq$ 3x over baseline at this time point had predicted binding of <500nM, whereas the 2 of 8 predicted class I neoantigens that did not produce a neoantigen specific $CD8^+IFN\gamma^+$ cells $\geq$ 3x over baseline at the same time point were not predicted to bind <500nM (Table 2 below). Additionally, there were twice as many neoantigens with multiple predicted binders that produced neoantigen specific $CD8^+IFN\gamma^+$ cells $\geq$ 3x over baseline at C4D8 than neoantigens that produced neoantigen specific

$CD8^+IFN\gamma^+$ cells < 3x over baseline at C4D8 (4 vs. 2, Table 2). The correlation of neoantigen features included in the vaccine (predicted binding, variant RNA expression) with the ability of the neoantigens to drive T cell responses may help us learn what qualities define the best neoantigens to include in patient vaccines in the future.

Table 2 – Predicted binding of neoantigens that resulted in $CD8^+IFN\gamma^+ \geq$ 3x C4D8/baseline or < 3x C4D8/baseline

	NetMHCpan 3, IC50		Number of neoantigens predicted to yield > 1 predicted binders (<500nM)
	Strong binding (<50nM)	Weak binding (<500nM)	
$CD8^+IFN\gamma^+ \geq$ 3x C4D8/baseline	5/10	10/10	4
$CD8^+IFN\gamma^+ <$ 3x C4D8/baseline	5/8	6/8	2

EMBODIMENTS

The following paragraphs encompass various aspects and embodiments of the invention:

1. A method for detecting antigen specific T cell activation in a population of T cells, comprising:

in vitro stimulation (IVS) of a population of T cells, wherein the IVS involves culturing the T cells in an enriched media, stimulation of the cultured T cells with neoantigen matured autologous dendritic cells (DCs), and expanding the stimulated T cells to produce a population of expanded T cells;

restimulation of the expanded T cells with neoantigen matured autologous DCs; and

analyzing the restimulated T cells to detect antigen specific T cell activation.

2. The method of paragraph 1, wherein the enriched media includes IL-2, IL-7, or IL-2 and IL-7.

3. The method of paragraph 2, wherein the T cells are cultured in the enriched media for about 24 hours before stimulation with neoantigen matured autologous DCs.

4. The method of paragraph 1, wherein the stimulated T cells are expanded for 12-16 days.

5. The method of paragraph 1, wherein the stimulated T cells are expanded for 14 days.

6. The method of paragraph 5, wherein the stimulated T cells are expanded while cultured in a media comprising IL-2 and IL-7 for 2 days and then in a media comprising IL-2 for 12 days.

7. The method of any one of paragraphs 1-6, wherein the restimulated T cells are analyzed using flow cytometry.

8. The method of paragraph 1, wherein the population of T cells is a sample of pan T cells purified from a patient's PBMCs.

9. The method of paragraph 8, wherein the patient's PBMCs are obtained from patient apheresis at baseline of a putative therapeutic treatment.

10. The method of paragraph 8, wherein the patient's PBMCs are obtained from patient apheresis at 7 days post-dose of a putative therapeutic treatment.

11. The method of any one of paragraphs 9-10, wherein the putative therapeutic treatment is a personalized cancer vaccine.

12. The method of paragraph 11, wherein the personalized cancer vaccine is an mRNA having one or more open reading frames encoding 3-50 peptide epitopes, wherein each of the peptide epitopes are personalized cancer antigens, formulated in a lipid nanoparticle formulation.

13. The method of paragraph 1, wherein the antigen specific T cell activation is measured as a percent frequency (% freq) of CD8+IFNγ+ cells.

14. The method of paragraph 13, wherein a % freq of CD8+IFNγ+ cells greater than or equal to 3x over baseline indicates that a T cell population exceeds a threshold level of T cell activation.

15. The method of any one of paragraphs 1-14, wherein the analysis of T cell activation is performed on a patient receiving a personalized cancer vaccine and wherein the personalized cancer vaccine is reformulated based on the analysis and the patient is administered the reformulated personalized cancer vaccine.

16. The method of paragraph 15, wherein the reformulated personalized cancer vaccine includes at least one neoantigen that is not in the personalized cancer vaccine initially administered to the patient.

17. The method of any one of paragraphs 1-15, wherein the analysis of T cell activation is performed on a patient receiving a therapeutic treatment with a cancer vaccine and wherein the therapeutic treatment is modified based on the analysis.

18. The method of paragraph 17, wherein a dose of the therapeutic treatment is modified.

19. The method of paragraph 17, wherein the administration schedule of the therapeutic treatment is modified.

20. The method of paragraph 17, wherein a co-therapy is administered to the patient.

21. A personalized cancer vaccine comprising

an mRNA having one or more open reading frames encoding 8-50 peptide epitopes, wherein each of the peptide epitopes are neoantigens, formulated in a lipid nanoparticle formulation, wherein at least 8 of the neoantigens demonstrated an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells as compared to baseline greater than 3x in an in vitro stimulation (IVS) assay.

22. The vaccine of paragraph 21, wherein the IVS assay is a method of any one of paragraphs 1-20.

23. The vaccine of paragraph 21, wherein at least 80% of the neoantigens demonstrated an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells as compared to baseline greater than 3x in an in vitro stimulation (IVS) assay.

24. The vaccine of paragraph 21, wherein at least 90% of the neoantigens demonstrated an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells as compared to baseline greater than 3x in an in vitro stimulation (IVS) assay.

25. The vaccine of paragraph 21, wherein all of the neoantigens demonstrated an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells as compared to baseline greater than 3x in an in vitro stimulation (IVS) assay.

EQUIVALENTS

While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, *i.e.*, elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with “and/or” should be construed in the same fashion, *i.e.*, “one or more” of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to “A and/or B”, when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, *i.e.*, the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (*i.e.* “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase “at least one,” in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase “at least one” refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, “at least one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently “at least one of A and/or B”) can refer, in

one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," "composed of," and the like are to be understood to be open-ended, *i.e.*, to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03. It should be appreciated that embodiments described in this document using an open-ended transitional phrase (*e.g.*, "comprising") are also contemplated, in alternative embodiments, as "consisting of" and "consisting essentially of" the feature described by the open-ended transitional phrase. For example, if the disclosure describes "a composition comprising A and B", the disclosure also contemplates the alternative embodiments "a composition consisting of A and B" and "a composition consisting essentially of A and B".

CLAIMS

What is claimed is:

1. A method for detecting antigen specific T cell activation in a population of T cells, comprising:

in vitro stimulation (IVS) of a population of T cells, wherein the IVS involves culturing the T cells in an enriched media, stimulation of the cultured T cells with neoantigen matured autologous dendritic cells (DCs), and expanding the stimulated T cells to produce a population of expanded T cells;

restimulation of the expanded T cells with neoantigen matured autologous DCs; and

analyzing the restimulated T cells to detect antigen specific T cell activation, wherein the analysis of T cell activation is performed on a patient receiving a personalized cancer vaccine and wherein the personalized cancer vaccine is reformulated based on the analysis and the patient is administered the reformulated personalized cancer vaccine.

2. The method of claim 1, wherein the reformulated personalized cancer vaccine includes at least one neoantigen that is not in the personalized cancer vaccine initially administered to the patient.

3. The method of any one of claims 1-2, wherein the analysis of T cell activation is performed on a patient receiving a therapeutic treatment with a cancer vaccine and wherein the therapeutic treatment is modified based on the analysis.

4. The method of claim 3, wherein a dose of the therapeutic treatment is modified.

5. The method of claim 3, wherein the administration schedule of the therapeutic treatment is modified.

6. The method of claim 3, wherein a co-therapy is administered to the patient.

7. The method of any one of claims 1-6, wherein the enriched media includes IL-2, IL-7, or IL-2 and IL-7, and optionally wherein the T cells are cultured in the enriched media for about 24 hours before stimulation with neoantigen matured autologous DCs.

8. The method of any one of claims 1-7, wherein the stimulated T cells are expanded for 12-16 days.

9. The method of any one of claims 1-8, wherein the restimulated T cells are analyzed using flow cytometry.

10. The method of any one of claims 1-9, wherein the population of T cells is a sample of pan T cells purified from a patient's PBMCs.

11. The method of claim 10, wherein the patient's PBMCs are obtained from patient apheresis at baseline of a putative therapeutic treatment.

12. The method of claim 10, wherein the patient's PBMCs are obtained from patient apheresis at 7 days post-dose of a putative therapeutic treatment.

13. The method of any one of claims 11-12, wherein the putative therapeutic treatment is a personalized cancer vaccine.

14. The method of claim 13, wherein the personalized cancer vaccine is an mRNA having one or more open reading frames encoding 3-50 peptide epitopes, wherein each of the peptide epitopes are personalized cancer antigens, formulated in a lipid nanoparticle formulation.

15. The method of any one of claims 1-14, wherein the antigen specific T cell activation is measured as a percent frequency (% freq) of CD8+IFNγ+ cells.

16. The method of claim 15, wherein a % freq of CD8+IFNγ+ cells greater than or equal to 3x over baseline indicates that a T cell population exceeds a threshold level of T cell activation.

17. A personalized cancer vaccine comprising

an mRNA having one or more open reading frames encoding 8-50 peptide epitopes, wherein each of the peptide epitopes are neoantigens, formulated in a lipid nanoparticle formulation, wherein at least 8 of the neoantigens demonstrated an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells as compared to baseline greater than 3x in an in vitro stimulation (IVS) assay.

18. The vaccine of claim 17, wherein the IVS assay is a method of any one of claims 1-16.

19. The vaccine of claim 17, wherein the IVS assay comprises culturing a population of T cells from a patient in an enriched media, stimulation of the cultured T cells with neoantigen matured autologous dendritic cells (DCs), and expanding the stimulated T cells to produce a population of expanded T cells; restimulation of the expanded T cells with neoantigen matured autologous DCs; and analyzing the restimulated T cells to detect antigen specific T cell activation.

20. The vaccine of claim 17, wherein at least 80% of the neoantigens demonstrated an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells as compared to baseline greater than 3x in an in vitro stimulation (IVS) assay.

21. The vaccine of claim 17, wherein at least 90% of the neoantigens demonstrated an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells as compared to baseline greater than 3x in an in vitro stimulation (IVS) assay.

22. The vaccine of claim 17, wherein all of the neoantigens demonstrated an increase in the % freq. of neoantigen specific CD8+IFNγ+ cells as compared to baseline greater than 3x in an in vitro stimulation (IVS) assay.

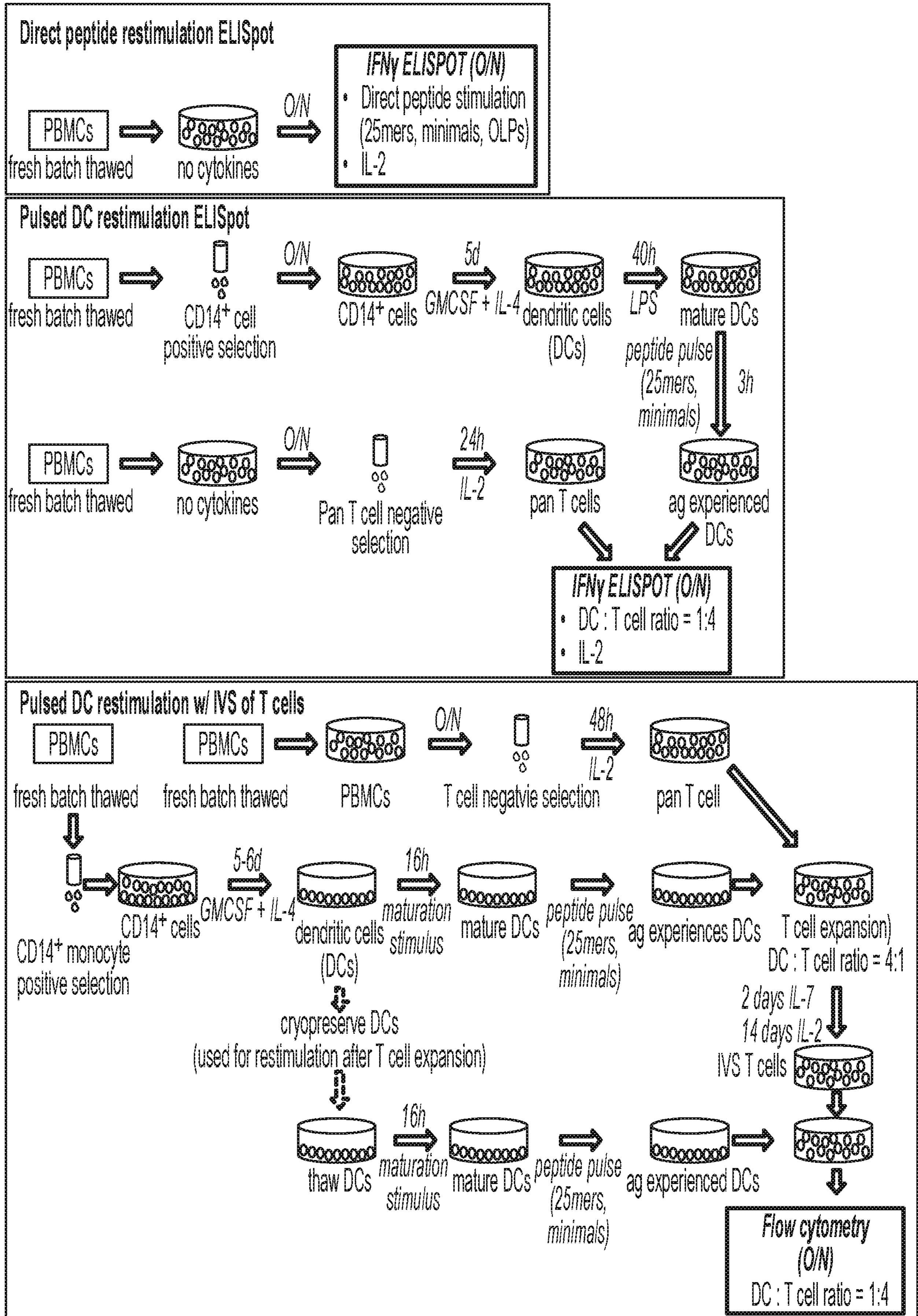

FIG. 1

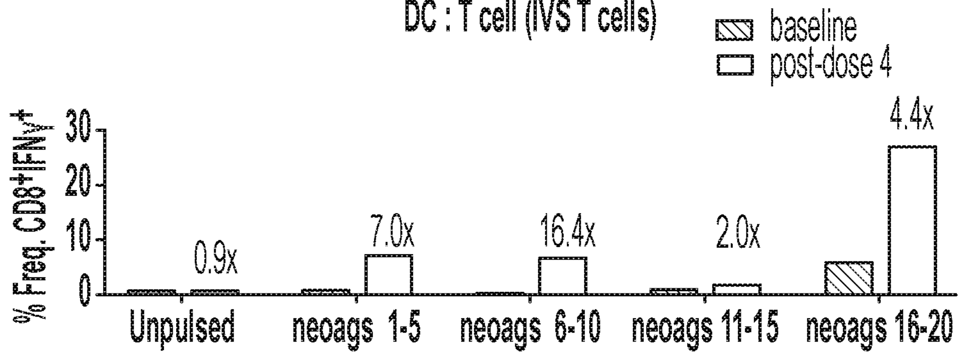

FIG. 2A

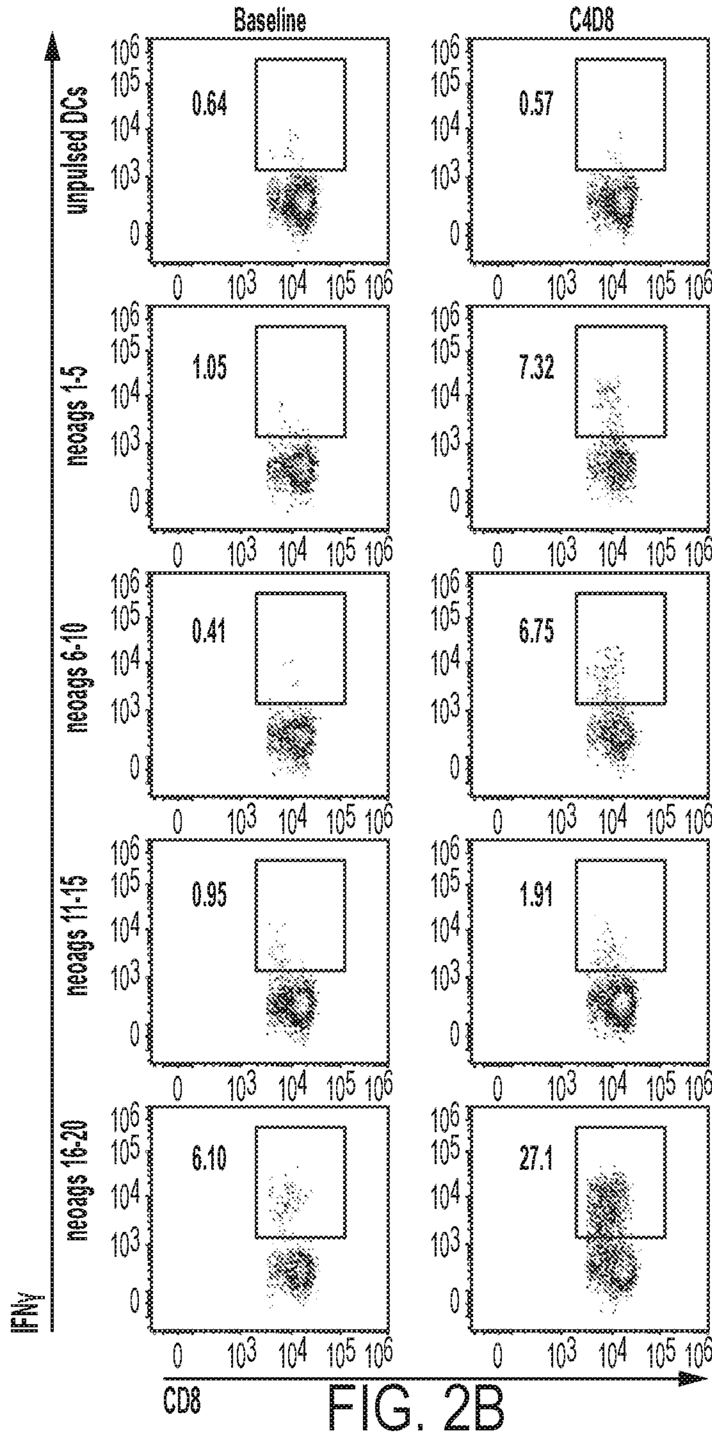

FIG. 2B

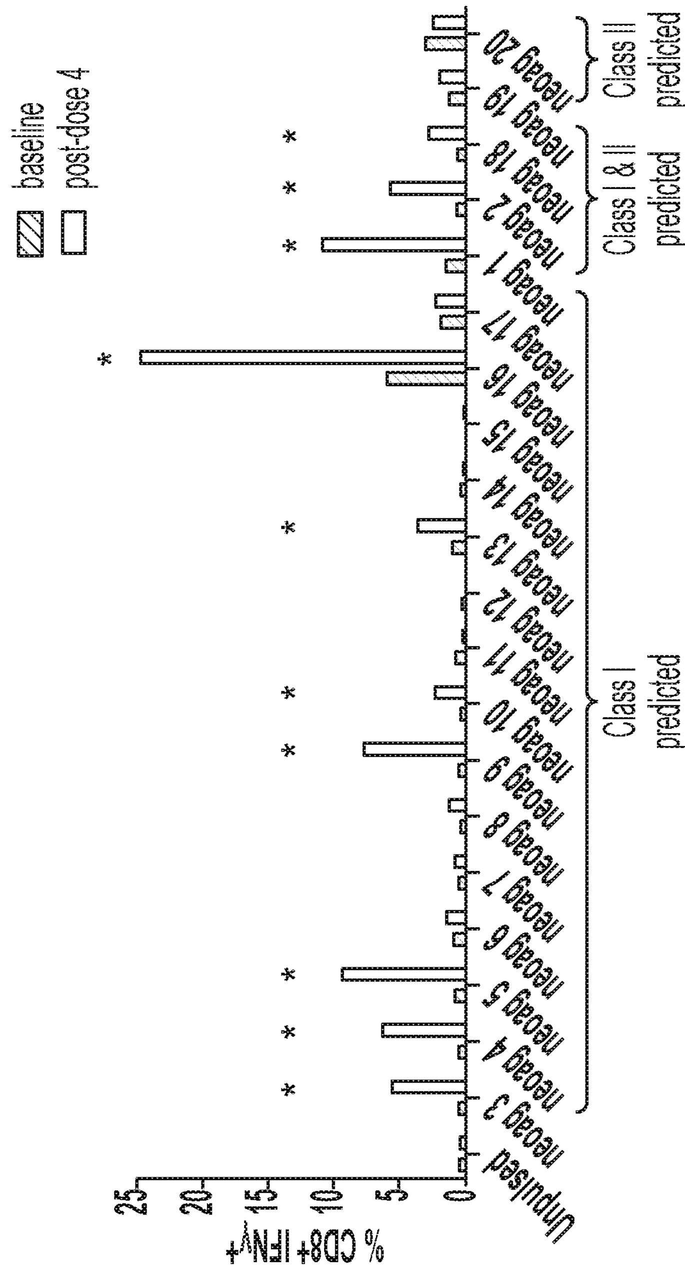

FIG. 3A

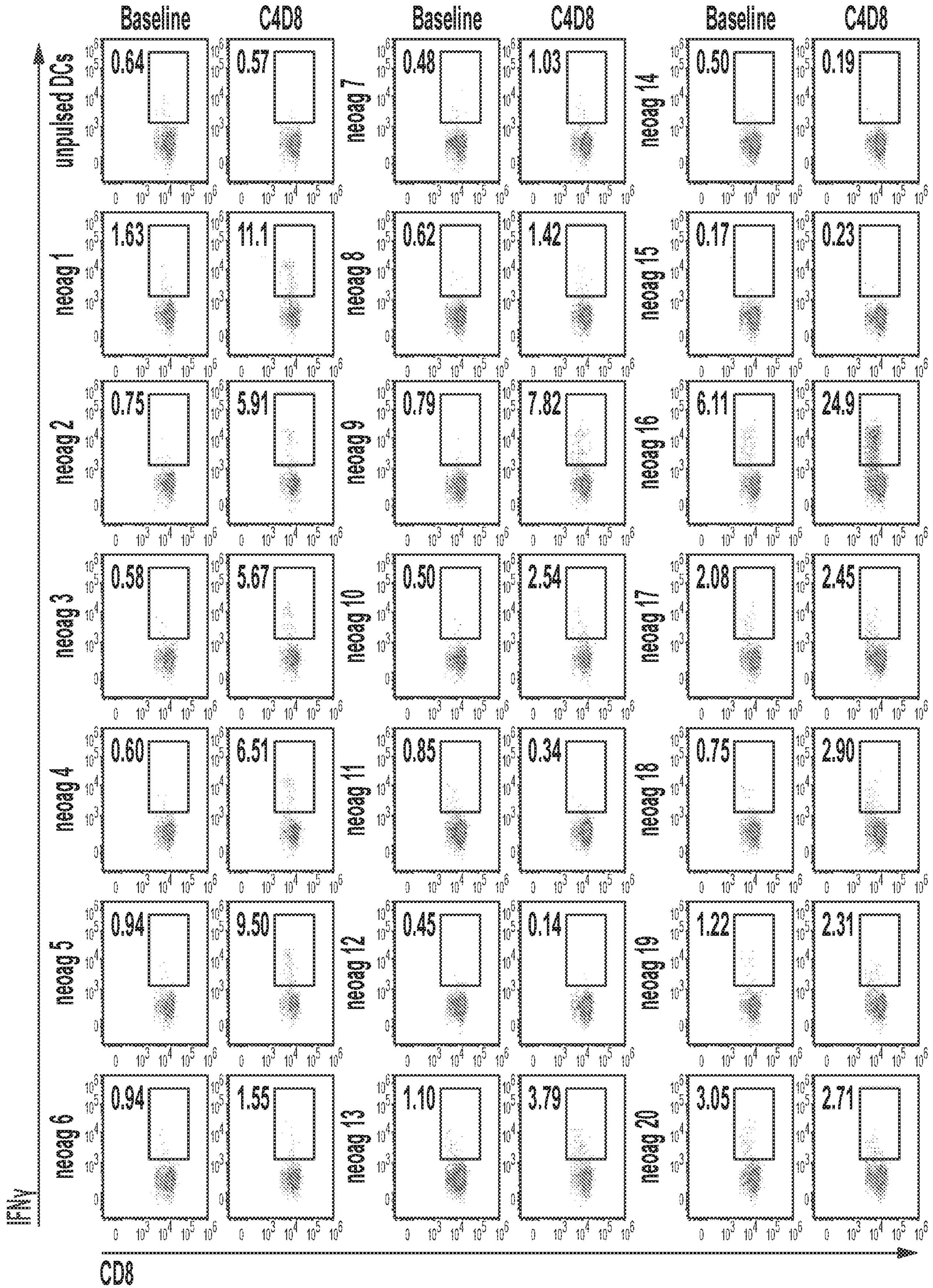

FIG. 3B

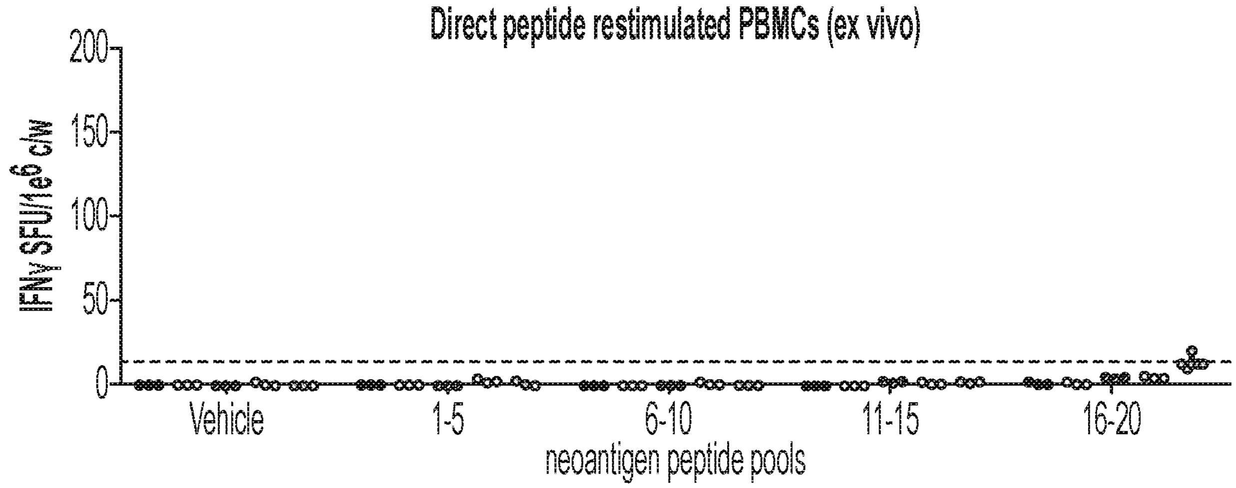

FIG. 4A

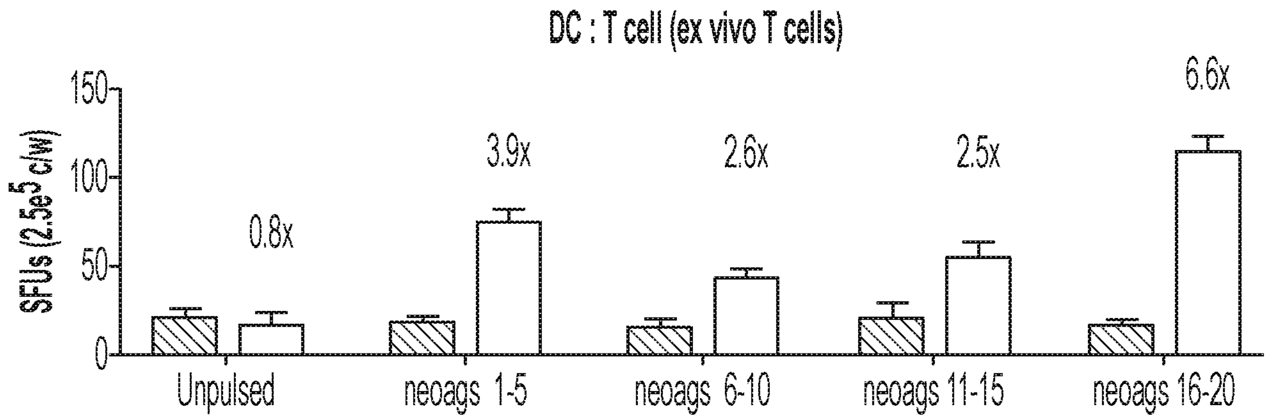

FIG. 4B

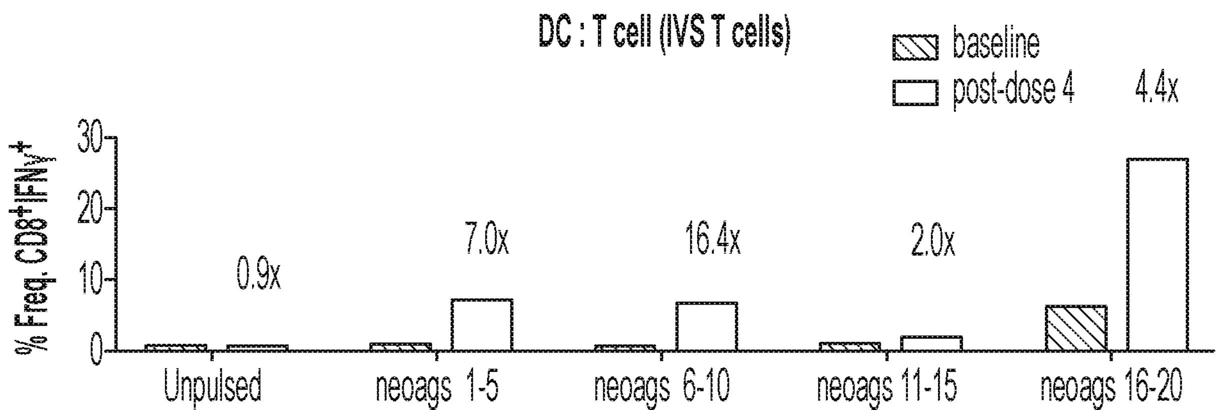

FIG. 4C