(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 September 2020 (10.09.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/176984 A1

(51) International Patent Classification:
A61K 47/44 (2017.01) *A61K 9/51* (2006.01)
A61K 39/00 (2006.01) *A61P 35/00* (2006.01)
A61K 47/10 (2017.01) *A61P 37/04* (2006.01)
A61K 47/24 (2006.01) *C12N 15/87* (2006.01)
A61K 9/14 (2006.01) *C12N 5/0784* (2010.01)

(21) International Application Number:
PCT/CA2020/050281

(22) International Filing Date:
02 March 2020 (02.03.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/813,618 04 March 2019 (04.03.2019) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

(54) Title: LIPID NANOPARTICLES

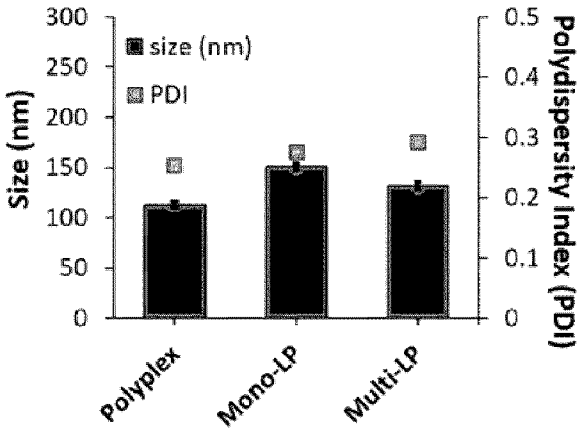

Figure 1

(57) Abstract: A lipid nanoparticle composition is disclosed. The lipid nanoparticle composition includes: a core, and a lipid mixture encapsulating the core. The core includes: (i) a complex of a poly-(beta-amino ester) polymer with DNA or RNA; or (ii) a peptide. The lipid mixture encapsulating the core includes: (a) N1-[2-((1S)-1-[(3-aminopropyl)amino]-4-[di(3-amino-propyl)amino]butylcarboxamido)ethyl]-3,4-di[oleyloxy]-benzamide (MVL5); (b) 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE); and (c) a polyethylene glycol (PEG)-modified lipid. Uses and methods associated with the lipid nanoparticle composition are also disclosed.

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LIPID NANOPARTICLES

FIELD

[0001] The present disclosure relates to lipid nanoparticles formulated for the delivery of DNA, RNA or a peptide.

BACKGROUND

[0002] The following paragraphs are not an admission that anything discussed in them is prior art or part of the knowledge of persons skilled in the art.

[0003] Lipid nanoparticles may be used as non-viral vectors to deliver DNA, RNA and proteins. Lipoplexes are one example of lipid nanoparticles, and refer to lipid-based assemblies of non-covalently associated DNA and RNA, where the association is by charge-charge interactions.

INTRODUCTION

[0004] The following introduction is intended to introduce the reader to this specification but not to define any invention. One or more inventions may reside in a combination or sub-combination of the apparatus elements or method steps described below or in other parts of this document. The inventors do not waive or disclaim their rights to any invention or inventions disclosed in this specification merely by not describing such other invention or inventions in the claims.

[0005] Delivery of DNA, RNA and proteins into a cell is a challenging area of research since cell membranes may act as a barrier to the DNA, RNA or protein.

[0006] Cancer immunotherapy is a therapeutic strategy that exploits the natural ability of the immune system to recognize and kill cancer cells, and is one example of a therapeutic strategy that could benefit from the delivery of DNA, RNA or proteins to a cell, such as a cell in a patient.

[0007] A cancer immunotherapy that delivers the DNA, RNA or protein to a patient in order to stimulate an immune response may be referred to as a vaccine. One challenge associated with the use of mRNA for vaccine development is its sensitivity towards catalytic hydrolysis by ribonucleases. Unprotected mRNA may be degraded under physiological conditions, hence rendering it unsuitable for broad therapeutic applications. In some examples, it is desirable to develop a formulation to stabilize mRNA *in vivo*.

[0008] Although viruses can be used to protect DNA and RNA from catalytic hydrolysis, and to deliver DNA or RNA into a cell, the viruses may induce an undesirable immune response in a mammal if one or more proteins forming the virus are immunogenic. In contrast, non-viral vectors may have lower host immunogenicity.

[0009] The present disclosure provides lipid nanoparticles that may be used for the delivery of DNA, RNA, or proteins to a cell.

[0010] In some examples, the present disclosure provides a lipid nanoparticle composition that includes: a core, and a lipid mixture encapsulating the core. The core includes: (i) a complex of a poly-(beta-amino ester) polymer with DNA or RNA; or (ii) a peptide. The lipid mixture encapsulating the core includes: (a) N1-[2-((1S)-1-[(3-aminopropyl)amino]-4-[di(3-amino-propyl)amino]butylcarboxamido)ethyl]-3,4-di[oleyloxy]-benzamide (MVL5); (b) 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE); and (c) a polyethylene glycol (PEG)-modified lipid.

[0011] Lipid nanoparticles according to the present disclosure may be used to induce an immune response in a mammal, such as an anti-cancer immune response.

[0012] Lipid nanoparticles according to the present disclosure may selectively deliver the DNA, RNA or protein to dendritic cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Embodiments of the present disclosure will now be described, by way of example only, with reference to the attached Figures.

[0014] Figure 1 is a graph illustrating the hydrodynamic size of PbAE 447/mRNA polyplex, mono-LP and multi-LP with the respective PDI. Error bars represent standard deviation (SD) of three measurements (n=3).

[0015] Figure 2 is a graph illustrating the zeta-potential of the PbAE 447/mRNA polyplex, mono-LP and multi-LP. Error bars represent standard deviation (SD) of three measurements (n=3).

[0016] Figure 3 is a graph illustrating the quantification of EGFP-positive BMDCs by flow cytometry 24 or 48 hours after transfection. BMDCs were seeded at density of 3 x 10^5 cells/ml in a 24-well plate and transfected with 0.5 µg of EGFP-mRNA naked (mRNA) or encapsulated into mono-LP and multi-LP. Error bars represent standard error of mean with n=3 per group.

[0017] Figure 4 are fluorescence microscopic images of the dendritic cells of Figure 3 transfected with EGFP-mRNA.

[0018] Figure 5 are bioluminescence imaging of mice 6 hours after i.v. injection of multi-LP loaded with LUC-mRNA (20 µg).

[0019] Figure 6 is a graph illustrating the quantification multi-LP/Cy5-EGFP-mRNA associated APCs in the spleen 24 hours after i.v. or i.d. administration of 20 µg of Cy5-EGFP-mRNA.

[0020] Figure 7 is a graph illustrating the quantification multi-LP/Cy5-EGFP-mRNA associated APCs in the lymph nodes 24 hours after i.v. or i.d. administration of 20 µg of Cy5-EGFP-mRNA.

[0021] Figure 8 is a graph illustrating the quantification mono-LP/Cy5-EGFP-mRNA associated APCs in the spleen 24 hours after i.v. or i.d. administration of 20 µg of Cy5-EGFP-mRNA.

[0022] Figure 9 is a graph illustrating the quantification mono-LP/Cy5-EGFP-mRNA associated APCs in the lymph nodes 24 hours after i.v. or i.d. administration of 20 µg of Cy5-EGFP-mRNA.

[0023] Figure 10 is a graph illustrating the *in vivo* transfection efficiency of APCs in spleen 24 hours after *i.v.* injection of 20 µg of EGFP-mRNA loaded into multi-LP. Error bars represent standard error of mean with n=5 per group.

[0024] Figure 11 is a graph illustrating the *in vivo* transfection efficiency of APCs in lymph nodes 24 hours after *i.v.* injection of 20 µg of EGFP-mRNA loaded into multi-LP. Error bars represent standard error of mean with n=5 per group.

[0025] Figure 12 is an illustration of the flow cytometry analysis of CD69 in spleen NKT, NK and T cells from mice treated with multi-LP loaded with TRP2-mRNA and with or without α-GalCer administrated *i.d.* or *i.v.*

[0026] Figure 13 is a set of graphs illustrating quantification of serum level of IL-1, IL-6, IL-12, and IFN-α 6 hours after i.v. injection of different lipid nanoparticle formulations. Error bars represent standard error of mean with n=3 per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

[0027] Figure 14 is a set of graphs illustrating quantification of serum level of TNF-α, IFN-β, and IFN-γ 6 hours after i.v. injection of different lipid nanoparticle formulations. Error bars represent standard error of mean with n=3 per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

[0028] Figure 15 is a set of flow cytometry plots of CD8+ T cells expressing IFN-γ in peripheral blood.

[0029] Figure 16 is a graph illustrating flow cytometry quantification of SVYDFFVWL+ CD8+ T cells in peripheral blood. The C57BL/6 mice were vaccinated two

times with TRP-2-mRNA with a one-week interval by i.v. administration. All the formulations were prepared with 10 μg of TRP-2-mRNA or TRP-2 (180-188) peptide and with or without 0.5 μg of α-GalCer. Error bars represent standard error of mean with n=5 per group. Error bars represent standard error of mean with n=3 per group. *p < 0.05, **p < 0.01, ***p < 0.001.

[0030] Figure 17 is a graph illustrating OVA-specific IgG antibody titer measured in C57BL/6 mice after 4 weeks from the first vaccination. The mice were vaccinated two times with OVA-mRNA by i.v. administration. All the formulations were prepared with 10 μg of OVA-mRNA and with or without 0.5 μg of α-GalCer. Error bars represent standard error of mean with n=3 per group. *p < 0.05, **p < 0.01, ***p < 0.001.

[0031] Figure 18 is a graph illustrating OVA-specific IgG antibody isotypes induced in immunized mice. Isotypes of antibodies were measured by enzyme-linked immunosorbent assay and data are expressed as the ratio of IgG2c/IgG1 for C57BL/6 mice. All the formulations were injected *i.v.* two times with one-week interval. Error bars represent standard error of mean with n=3 per group. *p < 0.05, **p < 0.01, ***p < 0.001.

[0032] Figure 19 is a graph illustrating the tumor size of C57BL/6 mice that were inoculated with 2×10^5 B16-F10 cells (n = 5-8 per group). The mice were administered the noted lipid nanoparticle formulations or control formulations at days 5, 9 and 13 post-tumor inoculation.

[0033] Figure 20 is a graph illustrating the survival of the C57BL/6 mice administered the noted lipid nanoparticle formulations or control formulations.

[0034] Figure 21 is a graph illustrating the quantification of tumor-infiltrating CD8 and CD4 T-cells by flow cytometry in C57BL/6 mice that were treated by i.v. administration of two doses of the noted lipid nanoparticle formulations or control formulations at days 7 and 14 post-tumor inoculation. Mice were sacrificed on day 17. Error bars represent standard error of mean with n=5 per group. *p < 0.05, **p < 0.01, ***p < 0.001.

[0035] Figure 22 is a graph illustrating the quantification of NK-cells in mice treated by i.v. administration of two doses (at days 7 and 14 post-tumor innoculation) of exemplary lipid nanoparticles, and controls. Mice were sacrificed on day 17 and tumor-infiltrating NK-cells were quantified by flow cytometry. Error bars represent standard error of mean with n=5 per group. *p < 0.05, **p < 0.01, ***p < 0.001.

[0036] Figure 23 is a set of flow cytometry plots quantifying intratumoral SVYDFFVWL+ CD8+ T-cells after treatment with different lipid nanoparticle and control formulations.

[0037] Figure 24 is a graph illustrating the quantification of CD8+ T cells expressing IFN-γ after different prime-boost therapies using TRP2 as the antigen.

DETAILED DESCRIPTION

[0038] Generally, the present disclosure provides a lipid nanoparticle composition that includes: a core, and a lipid mixture encapsulating the core. The core includes: (i) a complex of a poly-(beta-amino ester) polymer with DNA or RNA; or (ii) a peptide. The lipid mixture encapsulating the core includes: (a) N1-[2-((1S)-1-[(3-aminopropyl)amino]-4-[di(3-amino-propyl)amino]butylcarboxamido)ethyl]-3,4-di[oleyloxy]-benzamide (MVL5); (b) 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE); and (c) a polyethylene glycol (PEG)-modified lipid. Lipid nanoparticles where the core includes nucleic acids may be referred to as a "lipopolyplex".

[0039] DOPE exists in either a lammellar or a hexagonal conformation. The transition from lamellar to hexagonal conformation occurs under acidic conditions, such as the low pH of an endosomal compartment of a cell. When a lipid nanoparticle is endocytosed by a cell, the transition from lamellar to hexagonal conformation promotes fusion of the lipid nanoparticle with the endosomal membranes, and release of the materials in the core of the lipid nanoparticle into the cytoplasm.

[0040] Biodistribution and transfection efficacy of a lipid nanoparticle is influenced by the characeristics of the charge of the hydrophilic head of the lipid and by the structure of the hydrophobic tail. MVL5 is a multivalent cationic lipid, whose charge density and hydrophobic tail enable, at least in some examples, endosomal escape, providing dendritic cell-targeting and mRNA delivery, such as after intravenous injection.

[0041] Size and charge are parameters which may be used to passively target APCs. For example, nanoparticles with a size range from ~25 to 200 nm traffic to the draining lymph node (dLN) where they are rapidly taken up by APCs; and positively charged nanoparticles exhibit a faster uptake rate by phagocytic cells than negatively charged or neutral nanoparticles.

[0042] The combination of DOPE and MVL5 may be used to form a lipid nanoparticle with a hydrodynamic diameter of 132.3 ± 3.78 nm and a zeta potential of +33.6 mV, due to the cationic lipid included in the external layer. Dendritic cells (DCs) preferentially uptake nanoparticles having a size from 0.1 μm to 0.5 μm. Positively charged nanoparticles are uptaken faster than neutral or anionic nanoparticles.

[0043] The ratio of polymer : DNA or RNA may be in a range from about 10 : 1 to about 40 : 1 (wt:wt). The ratio of lipid:DNA or RNA molar ratio (mol:mol) may be from about 10:1 to about 20:1, such as about 15:1 or 16:1. The ratio of MVL5 : DOPE : PEG-modified may be from about 10 to about 25 : about 75 to about 90 : about 1 to about 5 (wt:wt:wt). In some preferred examples, the ratio of MVL5 : DOPE : PEG-modified is from about 10 to about 20 : about 80 to about 90 : about 1 to about 2 (wt:wt:wt).

[0044] In some examples, the core includes a complex of a poly-(beta-amino ester) polymer with DNA or RNA, where the DNA or RNA encodes an antigenic peptide. In other examples, the core includes a peptide, where the peptide is antigenic. In either examples, the antigenic peptide may be a tumor associated antigen.

[0045] When the core includes a peptide, the lipid:peptide molar ratio (mol:mol) may be from about 20:1 to about 80:1, such as about 50:1. The lipidnanoparticles may be formulated to deliver from about 100 µg to about 1,000 µg of peptide per kilogram of body weight.

[0046] Nucleic acid-based lipid nanoparticles (i.e. lipid nanoparticles where the core comprises DNA or RNA) provide opportunities for molecular engineering; have the potential to promote both innate and adaptative immune responses; and/or nucleic acid-based lipid nanoparticles expressing complete genes are not *a priori* human leucocyte antigen (HLA)-restricted. In some examples of lipid nanoparticles according to the present disclosure, the DNA or RNA may encode both an antigenic peptide sequence and a costimulatory signal, such as toll-like receptor TLR3, TLR7, or TLR8. Such a costimulatory signal may act as an adjuvant to the antigenic peptide.

[0047] mRNA based lipid nanoparticles provide additional benefits over DNA based lipid nanoparticles since mRNA only needs to gain entry into the cytoplasm of a cell in order to have translation of the encoded peptide occur, and/or mRNA has a reduced risk of integration into the host genome (and therefore has a lower oncogenic potential).

[0048] In lipid nanoparticle compositions that include DNA or RNA, the lipid nanoparticles have the DNA or RNA complexed with a poly-(beta-amino ester) (PbAE) polymer. Such lipid nanoparticles may have a greater transfection efficacy than lipid nanoparticles whose core include uncomplexed DNA or RNA. Such lipopolyplexes may escape from a cell's endosome through ion-pair formation, the proton sponge effect, or both. Precomplexation of DNA or RNA with PbAE may facilitate and enhance loading of the nucleic acid into the lipid-shell.

[0049] PbAEs that may be used in lipid nanoparticles of the present disclosure are discussed in Guerrero-Cázares, Hugo et al. "Biodegradable polymeric nanoparticles show high efficacy and specificity at DNA delivery to human glioblastoma in vitro and in vivo" *ACS nano* vol. 8,5 (2014): 5141-53, which is incorporated herein by reference. One specific example of a PbAE that may be used is the polymeric product of the reaction between 1,4-butanediol diacrylate and 5-amino-1-pentanol, and end-capped with 1-(3-aminopropyl)-4-methylpiperazine. This specific PbAE may be referred to as "PBAE 447", "PBAE 447e", or "PBAE 447e, 1.1:1" based on the nomenclature convention outlined in the Guerrero-Cázares reference. The PBAE 447 polymer has a number average molar mass (MN) of about 10,700 g/mol, a mass average molar mass (MW) of about 38,200 g/mol, and a polydispersity of about 3.58 as measured using gel permeation chromatography.

[0050] Including pegylated lipids in the encapsulating lipid mixture may provide the lipid nanoparticle with one or more beneficial properties over lipid nanoparticles without pegylated lipids. For example, lipid nanoparticles that include pegylated lipids may bypass reticulo-endothelial system and have longer circulation times, resulting in an increased accumulation of the lipid nanoparticles in a tumor. The PEG-modified lipid may be 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-2000PEG, or DSPE-PEG); 1,2-dimyristoyl-rac-glycero-3-methylpolyoxyethylene (DMG-PEG); 1,2-dipalmitoryl-sn-glycero-3-phosphoethanolamine (DPPE) conjugated polyethylene glycol (DPPE-PEG); 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) conjugated polyethylene glycol (DMPE-PEG); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) conjugated polyethylene glycol (DSPE-PEG); C8 or C16 ceramide conjugated polyethylene glycol (ceramide-PEG); or 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) conjugated polyethylene glycol (DOPE-PEG).

[0051] The lipid mixture encapsulating the core may additionally include an immune adjuvanting amount of α-galactosylceramide (α-GalCer), whose poor hydrophilicity makes it difficult to deliver if it is not incoporated into a lipid delivery vehicle. An immune adjuvanting amount of α-GalCer may be an amount from about 50 ng to about 50 μg per kilogram of body weight.

[0052] The α-GalCer may be present in an amount such that the ratio of α-GalCer : DNA, RNA or peptide is in a range of about 1 : about 10 to about 1 : about 75 (wt:wt). Without wishing to be bound by theory, the authors of the present disclosure believe that: α-GalCer may generate more antigen-specific CD8+ T cells compared to TLR-ligands;

and α-GalCer may induce iNKT cells to produce immunostimulatory cytokines, particularly IFN-γ, and may elicit the induction of one or more costimulatory molecules. The authors of the present disclosure believe that these events may promote the activation of antigen presenting cells (APCs), which may release key Th1 cytokines (e.g. IL-12), and may promote the downstream activation of CD4+ and CD8+ T lymphocytes, as well as NK cells and B cells, with important effects on the magnitude and effectiveness of the immune responses.

[0053] The lipid mixture encapsulating the core may additionally include an immune adjuvanting amount of: monophosphoryl lipid A (MLP-A) or an analog thereof, such as phosphorylated hexaacyl disaccharide (PHAD®), monophosphoryl 3-deacyl lipid A (3D-PHAD®), or monophosphoryl hexa-acyl lipid A, 3-deacyl (3D(6A)-PHAD®); a natural Cd1 ligand, such as phosphatidylinositol (PI), isoglobotriosylceramide Gal(α1→3)Gal Glcβ(1→1)Cer (iGb3), GD3, Gg3Cer, sulfatide, phosphatidylcholine (PC), or phosphatidylethanolamine (PE); an α-GalCer analog, such as α-C-GalCer, β-GalCer, (2S,3S,4R)-1-O-(α-D-galactopyranosyl)-N-tetracosanoyl-2-amino-1,3,4-nonanetriol) (OCH), or alpha-GalCer C20:2 (PubChem CID: 11707590); a pathogen-derived Cd1 ligan, such as GSL1, GSL-4, LPG, PIM4, or PPBF; 3M-052, a TLR7/8 agonist; or a methyl-lysophosphatidic acid, such as mLPAs, or leukemia antigen.

[0054] In some examples, the present disclosure provides a lipid nanoparticle composition that includes: a core that includes (i) a complex of PbAE 447 polymer with DNA or RNA; or (ii) a peptide; and a lipid mixture encapsulating the core, wherein the lipid mixture includes: (a) MVL5, (b) DOPE, (c) DSPE-PEG, and (d) an immune adjuvanting amount of α-GalCer; where the ratio of MVL5 : DOPE : DSPE-PEG is from about 10 to about 20 : about 80 to about 90 : about 1 to about 2. In specific examples, the core includes a complex of PbAE 447 polymer with RNA.

[0055] Lipid nanoparticle composition according to the present disclosure may be used to induce an immune response in a mammal. The immune response may include activation of natural killer cells (NK cells), T-cells, or any combination thereof. The T-cells may include natural killer T-cells (NKT-cells).

[0056] When the core of the lipid nanoparticle composition includes (i) a peptide or (ii) DNA or RNA that encodes a peptide, and the peptide is a tumor associated antigen, the immune response may be an anti-cancer immune response.

[0057] The immune response may be induced using the lipid nanoparticle composition in a prime-boost format, such as a heterologous prime-boost format. The lipid nanoparticle composition may be used as a priming vector.

[0058] Lipid nanoparticle composition according to the present disclosure may be used to deliver the DNA, the RNA, or the protein to a dendritic cell. The dendritic cell may express a protein antigen encoded by the DNA or RNA.

[0059] Lipid nanoparticle compositions according to the present disclosure may be formulated for intradermal or intravenous administration.

[0060] Lipid nanoparticle compositions according to the present disclosure may be prepared using a thin-film/rehydration technique where the lipids are dissolved in a suitable solvent and then dried to form a thin film, and then the thin film is subsequently rehydrated in an aqueous solution that includes the DNA, RNA or protein to be encapsulated in the core of the lipid nanoparticle. Alternative techniques known known in the art, such as alcohol-dilution/lyophilization or microfluidic nanoparticle synthesis, may also be used.

[0061] **Examples**

[0062] **Materials and methods**

[0063] Lipids 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EDOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), N1-[2-((1S)-1-[(3-aminopropyl) amino]-4-[di(3-amino-propyl)amino]butylcarboxamido)ethyl]-3,4-di[oleyloxy]-benzamide (MVL5), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG), and α-Galactosyl Ceramide (α-GalCer or KRN700) were purchased from Avanti Polar Lipids.

[0064] Sodium acetate buffer solution (3M, pH 5.2), nuclease-free water and BSA were purchased from Sigma-Aldrich.

[0065] Tyrosinase-related protein 2 (TRP-2, 180-188) peptide (SVYDFFVWL, SEQ ID NO: 1) was purchased from GenScript.

[0066] The following antibodies were purchased from BD bioscience: Anti-TCRβ (Clone H57-597), Anti-CD8a (Clone 53-6.7), anti-CD11b (Clone M1/70), Anti-CD45R/B220 (Clone RA3-6B2), Anti-Mouse CD62L (Clone MEL-14) and anti-CD45 (Clone 30-F11); Biolegend: anti-F4/80 (Clone BM8), anti-CD19 (Clone B4), anti-CD11c (Clone N418), anti-CD69 (Clone H1.2F3), anti-MHC-II (Clone M5/114.15.2), anti-NK1.1 (Clone PK136) and anti-CD44 (Clone IM7).

[0067] Anti-CD8 (Clone KT15) and iTAg Tetramer H-2 Kb TRP2 (SVYDFFVWL, SEQ ID NO: 1) were purchased from MBL international.

[0068] TRP2-mRNA, Enhanced Green Fluorescence (EGFP)-mRNA, Cy5-mRNA, Ovalbumin (OVA)-mRNA and Luciferase (LUC)-mRNA were purchased from TriLink Biotechnologies.

[0069] Six to eight weeks old female C57BL/6 mice were purchased from Charles River (Canada).

[0070] **Synthesis of a poly-(β-amino ester) polymer (PbAE)**

[0071] The PbAE was synthesized in a two-step reaction procedure as described by Persano S, Guevara ML, Li Z, et al. in "Lipopolyplex potentiates anti-tumor immunity of mRNA-based vaccination." Biomaterials. 2017; 125:81-89 (which is incorporated herein by reference). Briefly, in the first step, the base polymer ("B4S4") was synthesized by mixing 1,4-butanediol diacrylate ("B4") (Sigma-Aldrich) with 5-amino-1-pentanol ("S4") (Sigma-Aldrich) at a molar ratio of B4:S4 = 1.2:1. The reaction was maintained at 90 °C and 1,000 rpm of stirring speed for the first 4 hours. After 4 hours, the stirring speed was slowed to 300 rpm and the reaction was maintained at 90 °C for further 12-16 hr. The B4S4 polymer was dissolved in 10 mL of anhydrous tetrahydrofuran (THF) at a final concentration of 100 mg/mL. In a separate glass vial, 1-(3-Aminopropyl)-4-methylpiperazine ("E7") (Alfa Aesar) was dissolved in 40 mL of THF at 0.2 M final concentration. The end-chain capping reaction was performed by mixing the two solutions and leaving it at room temperature, stirring at 400 rpm for 24 hours protected from light. The final product is termed PbAE 447.

[0072] The resulting 447 was purified by precipitation in anhydrous diethyl ether (1:3 v/v THF:diethyl ether) and dried under vacuum for 24 h. The polymer was then dissolved in acetate buffer (10 mM Tris-HCl buffer pH 7.4) and stored at -20°C until use.

[0073] **Preparation and characterization of the PbAE 447 / mRNA polyplex**

[0074] A PbAE 447 / mRNA polyplex was prepared by mixing one volume of the PbAE 447 polymer with one volume of mRNA molecules in acetate buffer using a PbAE 447 / mRNA (w/w) mass ratio = 30. After a 20 min incubation at room temperature, the polyplex was analyzed for size distribution and zeta-potential using a Zetasizer NanoZS dynamic light scattering instrument (Malvern Instruments). The PbAE 447 / mRNA polyplex was also analyzed in a gel retardation assay. Briefly, a polyplex sample containing 250 ng mRNA was loaded into each well and separated by electrophoresis in a 0.7% agarose gel with 1 x TBE buffer (BioRad, Hercules, CA). RNA bands were stained with Gelred nucleic acid gel stain (Biotium) and visualized with a GelDoc system (BioRad).

[0075] Preparation and characterization of a PbAE 447 / mRNA / lipid nanoparticle (a "lipopolyplex") incorporating α-GalCer

[0076] Lipopolyplexes were prepared using EDOPC, DOPE and DSPE-PEG to form a monovalent cationic lipid nanoparticle. A monovalent cationic lipid nanoparticle may also be referred to as a "mono-LP" or "mono-LP nanoparticles" or "mono-LP formulation" or "mono-LP vector". Lipopolyplexes were also prepared using MVL5, DOPE and DSPE-PEG to form a multivalent cationic lipid nanoparticle. A multivalent cationic lipid nanoparticle may also be referred to as "multi-LP" or "multi-LP nanoparticles" or "multi-LP formulation" or "multi-LP vector". The lipid nanoparticle formed using MVL5, DOPE, and DSPE-PEG is one example of a lipid nanoparticle composition according the present disclosure.

[0077] To form either of lipopolyplexes, all the lipids were dissolved in chloroform at a final concentration of 20 mg/mL and used to prepare thin lipid films by rotary evaporation in a Rotavapor (Buchi) under partial vacuum. The thin lipid film was rehydrated with a solution containing the PbAE 447 / mRNA polyplex to prepare the lipopolyplex. The lipid film was formed with 49% EDOPC, 49% DOPE and 2% DSPE-PEG, or with 16% MVL5, 82% DOPE and 2% DSPE-PEG. The lipid film formulations included 0.5 μg of α-GalCer.

[0078] Hydrodynamic size and zeta potential of the lipopolyplexes were measured with a Zetasizer Nano ZS dynamic light scattering instrument (Malvern).

[0079] Preparation of bone marrow-derived dendritic cells (BMDCs)

[0080] BMDCs were prepared from C57BL/6 mice as described in Xia X, Mai J, Xu R, et al. "Porous Silicon Microparticle Potentiates Anti-Tumor Immunity by Enhancing Cross-Presentation and Inducing Type I Interferon Response." Cell Rep. 2015;11(6):957-966 (which protocol is incorporated herein by reference). Briefly, bone marrow cells from the femur and tibia were flushed out with 2% FBS-containing phosphate buffer saline (PBS) using a syringe. Cells were centrifuged at 500 g for 5 min, treated with ACK lysis buffer (Quality Biological) to remove red blood cells and resuspended in RPMI-1640 culture medium supplemented with 10% FBS, 0.1% β-mercaptoethanol, 1% penicillin/streptomycin, 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), and 20 ng/mL interleukin-4 (IL-4). Cells were seeded into 6-well plates at a seeding density of 1 x 10^6 cells/mL; growth medium was changed every other day. The non-adherent dendritic cells were harvested on day 5. BMDCs were stained for CD11b, CD11c and MHC-II and analyzed by flow cytometry (BD Fortessa X-20).

[0081] MTS test with BMDCs

[0082] To test potential cytotoxicity from lipid nanoparticle formulations of the present disclosure, BMDCs were seeded in a 96-well plate at a seeding density of 3 x 10^4 cells/well and treated with 0.1 µg mRNA loaded into mono-LP or multi-LP. Cell viability was measured 24 h later with a tetrazolium-based Cell Titer 96®Aqueous One Solution Cell Proliferation (MTS) assay (Promega) following the manufacturer's instruction.

[0083] Transfection of BMDCs

[0084] Enhanced GFP-mRNA was used as a reporter to test the transfection efficiency in BMDCs. 3 x 10^5 cells/well at day 5 were seeded in 24-well plates and treated in complete RPMI-1640 with mono-LP and multi-LP loaded with 0.5 µg of EGFP-mRNA. The expression of EGFP in cells was determined with a fluorescence microscope (Carl Zeiss) and the percentage of EGFP expressing cells was measured by flow cytometry (BD Fortessa X-20).

[0085] Bioluminescence imaging in live mice

[0086] C57BL/6 mice were injected with multi-LP/LUC-mRNA (20 µg of mRNA) following an intravenous administration route. After 6 hours, the mice were injected intraperitoneally with 200 µl D-Luciferin (15 mg/mL) (Gold Biotechnology) and bioluminescence was measured in a PerkinElmer IVIS® Spectrum imaging system.

[0087] In vivo APCs uptake and transfection

[0088] C57BL/6 mice (n = 5 in each group) were intravenously (*i.v.*) or intradermally (*i.d.*) injected with lipopolyplexes loaded with Cy5-mRNA (20 µg) or EGFP-mRNA (20 µg) for the evaluation of the cellular uptake or transfection efficiency, respectively. After 24 hours, mice were sacrificed and the spleen and draining lymph nodes (dLNs) were harvested and processed into a single cell suspension.

[0089] Single cell suspension was obtained as described in Kranz LM, Diken M, Haas H, et al. Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. Nature. 2016; 534(7607):396-401 (which protocol is incorporated herein by reference). Briefly, spleens or lymph nodes were pressed through a 70 µm cell strainer with the plunger of a 5 mL syringe. Erythrocytes were removed by the addition of ACK lysis buffer (Quality Biological). The cells were stained with anti-B220, anti-CD19, anti-CD11c and anti-F4/80 in order to gate dendritic cells (CD11c+ and F4/80−), Macrophages (CD11c− and F4/80+) and B-cells (CD11c−, B220+ and CD19+) populations. The samples were analyzed by flow cytometry (BD Fortessa X-20). Animal experiments in this study were carried out in accordance with guidelines evaluated and

approved by the ethics committee of University of Ottawa to ensure the humane animal care and use.

[0090] Detection of CD69 in spleen and lymph nodes by flow cytometry

[0091] Mice (C57BL/6, n = 5 in each group) were injected *i.d.* or *i.v.* with multi-LP lipid nanoparticles according to the present disclosure loaded with 10 µg of TRP2-mRNA and with or without 1 µg of α-GalCer. After 24 hours, mice were sacrificed, and spleens were harvested and processed into single cell suspensions.

[0092] Lymphocytes were stained for TCRβ, NK1.1 and CD69 to gate NK cells (TCRβ− and NK1.1+), NKT cells (TCRβ+ and NK1.1+) T-cells (TCRβ+ and NK1.1−). The samples were analyzed by flow cytometry (BD Fortessa X-20).

[0093] Measurement of pro-inflammatory cytokines

[0094] C57BL/6 mice (n = 3 each group) were administered with different lipid nanoparticle formulations according to the present disclosure (Multi-LP/α-GalCer+mRNA, Multi-LP/mRNA) as well as control formulations (Multi-LP/α-GalCer, Free α-GalCer+mRNA, Free α-GalCer). Formulations that included mRNA had 10 µg of TRP-2 mRNA. Formulations that included α-GalCer had 1 µg of α-GalCer.

[0095] After 6 hours, blood was collected by lateral saphenous bleeding and allowed to clot overnight at 4°C; serum was separated by centrifugation at 12000 rpm for 15 min, 4°C. Serum was quickly frozen at −80°C and stored until use. Serum TNF-α, IFN-α, IFN-β, IFN-γ, IL-1β, IL-6 and IL-12 levels were measured by enzyme-linked immunosorbent assay (ELISA) kits (eBioscience).

[0096] Detection of antigen specific T-cell by intracellular and tetramer staining

[0097] Mice (C57BL/6, n = 5 in each group) were injected intravenously with different lipid nanoparticle formulations according to the present disclosure (multi-LP/α-GalCer+TRP2-mRNA, multi-LP/α-GalCer+TRP2-peptide, and multi-LP/TRP2-mRNA) and with control formulations (free α-GalCer+TRP2-mRNA, free α-GalCer+TRP2-peptide, and PBS). The formulations were prepared using, as apporpriate for the formulation: 10 µg of TRP2-mRNA, 10 µg of TRP2-peptide, and/or 0.5 µg of α-GalCer.

[0098] Blood was collected from treated mice by saphenous bleeding and erythrocytes were removed by addition of ACK lysis buffer (Quality Biological). PBMCs were stimulated with 1 µg/mL of TRP2-peptide (SVYDFFVWL, SEQ ID NO: 1) for 6 hours in the presence of protein transport inhibitor, Brefeldin A (BD GolgiPlug™). After incubation, cells were washed and stained with Abs for surface markers-TCRβ, CD8 and V450/viability dye. Cells were then washed, fixed and permeabilized (BD

Fixation/Permeabilization Solution Kit), and stained with anti-IFN-γ. Cells were washed, re-suspended in FACS buffer (1 x PBS and 0.5% BSA) and analyzed by flow cytometry (BD Fortessa X-20).

[0099] SVYDFFVWL-specific tetramer (MBL International) staining was carried out by incubation at room temperature for 10 minutes in FACS buffer. After washing, the PBMCs were stained with anti-TCRβ, anti-CD8 clone KT15, and V450/viability for 30 min at 4°C in FACS buffer. Then, the samples were analyzed by flow cytometry (BD Fortessa X-20).

[00100] **Measurement of antibody titers**

[00101] OVA-specific antibodies titers were measured in the serum of C57BL/6 mice (n = 3 in each group) treated on day 0, 7 and 14 by i.v. injection. Blood was collected by saphenous bleeding on day 28 after priming, and allowed to clot overnight at 4°C. The serum was separated by centrifugation at 10000 rpm for 20 min. Serum was collected and anti-OVA antibody titers were determined by ELISA, as described in Biomaterials (2017) 125:81-89. Briefly, 96-well plates (Nunc-Immuno) were coated by overnight incubation at 4°C with 2 μg/well of OVA in PBS. Plates were blocked with 1% bovine serum albumin in PBS for 2 h, and serial two-fold dilutions of serum samples in PBS were added to the wells. After a 2-h incubation, plates were washed with PBS containing 0.05% Tween 20 and incubated for 1 h at room temperature with HRP-conjugated goat anti-mouse immunoglobulin G (IgG), IgG1 and IgG2c antibodies (Southern Biotechnology Associates). The plates were incubated with tetramethylbenzidine (TMB) substrate and the reaction was stopped by the addition of 1N HCl (Sigma). Absorbance was read at 450 nm with a microplate reader (BioRad). Ab Titers were calculated as the inverse dilution at which the absorbance equaled that of control mice plus 2 standard deviations.

[00102] **Therapeutic efficacy using a subcutaneous B16-F10 tumor model**

[00103] C57BL/6 mice (n = 5 in each group) were inoculated with 2 x 10^5 B16-F10 melanoma tumor cells by s.c. injection to establish subcutaneous tumors, as described in Overwijk WW, Restifo NP, in: John E Coligan, et al. (Eds), B16 as a mouse model for human melanoma. Current Protocols in Immunology, 2001. Chapter 20:Unit 20.1 (which protocol is incorporated herein by reference). Five days after tumor inoculation, mice were intravenously administered with different lipid nanoparicle formulations according to the present disclosure (10 μg of TRP2-mRNA with or without 1 μg of α-GalCer) and boosted at days 9 and 13 post-tumor inoculation. B16-F10 tumor bearing mice were monitored for survival, body weight and tumor growth every two days. Tumor volume was

calculated using the following formula: V = (L x W x W)/2, where V is tumor volume, W is tumor width, L is tumor length.

[00104] Evaluation of tumor infiltrating lymphocytes (TIL) and tetramer-positive CD8+ T-cells in s.c. B16-F10 tumors

[00105] C57BL/6 mice (n = 5 in each group) were inoculated subcutaneously with 2 x 10^5 B16-F10 melanoma tumor cells. Eight days after tumor inoculation, mice were intravenously administered with different lipid nanoparicle formulations according to the present disclosure (10 µg of TRP2-mRNA with or without 1 µg of α-GalCer) and boosted 15 days post-tumor inoculation.

[00106] Tumor cell suspensions were prepared from solid tumors by mechanical disaggregation as described in Pachynski, R. K., Scholz, A., Monnier, J., Butcher, E. C., Zabel, B. A. Evaluation of Tumor-infiltrating Leukocyte Subsets in a Subcutaneous Tumor Model. J. Vis. Exp. (98), e52657, doi:10.3791/52657 (2015). Erythrocytes were removed by the addition of ACK lysis buffer (Quality Biological). The resulting suspension was passed through a 40-µm cell strainer, washed once with PBS and resuspended in FACS buffer for flow cytometric analysis.

[00107] For TIL evaluation, cells were stained with viability dye, anti-CD45, anti-TCRβ, anti-CD8, anti-CD4 and anti-NK1.1 and analyzed by flow cytometry (BD Fortessa X-20). SVYDFFVWL-specific tetramer (MBL International) staining was carried out by incubation at room temperature for 10 minutes in FACS buffer, followed by surface staining with anti-CD45, anti-TCRβ and anti-CD8 clone KT15 for 30 min at 4°C in FACS buffer. The samples were analyzed by flow cytometry (BD Fortessa X-20).

[00108] Germinal center (GC) B cells and central memory CD8+ T-cells (TCM) evaluation

[00109] For examining GC formation *in vivo*, C57BL6 mice (n = 5 in each group) were treated at day 0, 7 and 14 with different formulations prepared in a free or lipid nanoparticle form. After 28 days, spleens were harvested and processed to single cell suspension.

[00110] For flow cytometry analysis, cells were stained with anti-CD45R/B220 and anti-CD19 for B cells and the CD19+ B cell population was further analyzed for two conventional GC markers: GL7 and Fas. For TCM evaluation single cell suspensions were stained with anti-TCRβ, anti-CD8, anti-CD62L anti-CD44. Relative fluorescence intensities were measured by flow cytometry (BD Fortessa X-20).

[00111] Comparison of Ad-priming and priming with peptide or mRNA loaded nanoparticles

[00112] Rhabdoviruses Farmington (FMT), genetically modified with a sequence encoding the full-length melanoma antigen TRP-2, was injected i.v. in 200 µl phosphate-buffered saline at a dose of 10^9 pfu. Multi-LP/α-GalCer+TRP2-peptide and Multi-LP/α-GalCer+TRP2-mRNA were injected i.v. in 100 µl phosphate-buffered saline at a dose of 10 µg of peptide/mRNA and 0.5 µg of α-GalCer. For adenovirus injection, mice were anesthetized in a sealed chamber containing 5% inhalation isoflurane. Adenoviral vector (Ad-TRP2) was administered intramuscularly (i.m.) at a total dose of 1×10^7 pfu in 50 µl phosphate-buffered saline.

[00113] Experimental Results

[00114] Characterization of exemplary lipid nanoparticles with a core comprising mRNA

[00115] An agarose gel retardation assay was performed to assess the mRNA-binding capacity of the cationic PbAE 447 polymer. The gel retardation assay revealed complete mRNA incorporation into the polyplex core at the PbAE/mRNA ratio of 10-40.

[00116] A multi-LP loaded with α-GalCer/mRNA was characterized for physical properties, such as size and zeta-potential (Figures 1 and 2, respectively), exhibiting a hydrodynamic diameter of 132.3 ± 3.78 nm and a strongly positive zeta potential of +33.6 mV, due to the cationic lipid included in the external layer. Whereas the polyplex core alone, prepared using a PbAE 447/mRNA (w/w) ratio of 20, showed a hydrodynamic size of 113 ± 3.16 nm and a zeta-potential of +35.8 mV. A mono-LP prepared using the lipids described above resulted in a nanoparticle with a size of 150.9 ± 3.62 nm and zeta-potential of 38 mV.

[00117] The exemplary multi-LP and mono-LP nanoparticles used in the experiments discussed herein were prepared using two different lipidic formulations, MVL5/DOPE/DSPE-PEG (16:82:2) and EDOPC/DOPE/DSPE-PEG (49:49:2), respectively. To assess the transfection efficiency of the different formulations, the mono-LP and multi-LP were loaded with a mRNA encoding for EGFP and the expression of the reporter gene was detected by flow cytometry (Figures 3 and 4) and fluorescence microscopy (Figure 5). The transfection efficiency was quantified at 24 and 48 hours post-transfection and peak expression was observed at 48 hours. The multi-LP loaded with EGFP-mRNA was highly efficient to transfect BMDCs in vitro, with an efficiency of ~ 50% (Figure 4) while mono-LP showed a lower transfection efficiency; naked mRNA failed to

transfect BMDCs. This data demonstrate that the incorporation of the MVL5 significantly increased the *in vitro* transfection efficiency of mRNA-loaded lipopolyplex platforms.

[00118] Cytotoxicity evaluations performed employing MTS assay indicated that the multi-LP formulation was completely non-toxic in BMDCs.

[00119] Multi-LP nanoparticles loaded with mRNA encoding for luciferase reporter were tested *in vivo*, since transfection systems that work efficiently *in vitro* may fail to function or have serious toxicity *in vivo*. Interestingly, 6 hours after administration a significant bioluminescence signal was detected at the abdominal region, demonstrating successful transfection by mRNA-loaded multi-LP particles (Figure 5).

[00120] Multi-LP nanoparticles did not elicit signs of toxicity in the mice. No changes in the body mass or behavior of mice were detected for up to 4 weeks following a relative high administration dose.

[00121] **Passive DC-targeting in vivo for mRNA delivery**

[00122] APCs are responsible for driving the induction of CD8+ T-cell mediated immune responses. For this study, mice were administered once *i.d.* or *i.v.* with mono-LP and multi-LP nanoparticles loaded with fluorescently labelled mRNA (Cy5-mRNA). 24 hours post treatment, single cell suspensions obtained from spleens and dLNs were stained with an antibody panel directed against common APC markers; DCs (CD11c+ and F4/80−), macrophages (CD11c− and F4/80+) and B cells (CD11c−, B220+ and CD19+).

[00123] At 24 hours after *i.v.* administration, multi-LP nanoparticles were taken up predominately by DCs, while a lower uptake was also observed for B cells and macrophages (Figures 6 and 7). In contrast, Cy5-mRNA loaded multi-LP failed to target APCs and to efficiently release the mRNA into their cytoplasm after *i.d.* injection (Figures 6 and 7).

[00124] On the other hand, the uptake of mono-LP nanoparticles by the different APC populations in both spleen and dLNs was significantly lower than that from multi-LP (Figures 8 and 9). The mono-LP vector showed a certain preference for the *i.d.* administration route, in terms of its ability to target APC populations.

[00125] The *in vivo* transfection efficiency study also confirmed that the expression of EGFP was predominantly observed in DCs both in spleen and dLNs following *i.v.* administration with multi-LP; whereas a minor fraction of EGFP-positive B-cells and macrophages was detected in the spleen.

[00126] Comparison of *i.d.* and *i.v.* routes for the capacity to promote immune cells-activation

[00127] Naïve mice were injected either *i.v.* or *i.d.* with multi-LP nanoparticles loaded with antigen-mRNA with or without α-GalCer. At 24 hours post administration, single cell suspensions obtained from spleens and dLNs were analyzed by flow cytometry in order to measure the up-regulation of the lymphocyte activation marker CD69 on NKT, NK and T cells.

[00128] Administration of multi-LP loaded with α-GalCer/TRP2-mRNA resulted in a significantly stronger activation of NKT, NK and T cells in both spleen and dLNs (see Figure 12), suggesting that α-GalCer and antigen-mRNA may work synergistically to potentiate the immune response particularly after *i.v.* administration.

[00129] Likewise, at 6 hours after *i.v.* administration with multi-LP loaded with α-GalCer and TRP2-mRNA induction of cytokine secretion was observed (Figures 13 and 14). Particularly, the formulation stimulated the release of Type-I interferon, IL-6, IL-12 and IFN-γ. While multi-LP/mRNA alone induced the release of IL-12, the addition of α-GalCer to the lipid nanoparticle formulation resulted in higher levels of IL-12 secretion. The induction of IFN-γ was only attributable to the activation of iNKT. In fact, multi-LP vector loaded with TRP2-mRNA alone did not induce a significant release of IFN-γ while both α-GalCer/TRP2-mRNA and α-GalCer loaded multi-LP nanoparticles induced a comparable level of IFN-γ without a significant difference. Although the levels of Type-I IFNs were higher in the group receiving multi-LP/αGalCer+mRNA compared to the group treated with multi-LP/mRNA, the difference was not significant. Importantly, the formulation without lipid nanoparticles did not induce detectable levels of Type IFNs. The introduction of α-GalCer in the mRNA lipid nanoparticle formulation may be used to stimulate or enhance the release of key cytokines over a corresponding non-adjuvanted mRNA lipid nanoparticle formulation.

[00130] Antigen-specific T-cell response and humoral response after administration

[00131] Mice were intravenously administered with multi-LP/α-GalCer+TRP2-mRNA, multi-LP/α-GalCer+TRP2-peptide, multi-LP/TRP2-mRNA, free α-GalCer+TRP2-mRNA and free α-GalCer+peptide on days 0 and 7. One week after the last administration, the presence of TRP2-specific CD8+ T cells was evaluated by intracellular staining for IFN-γ and TRP2-specific tetramer staining.

[00132] The number of the TRP2(180-188)-specific IFN-γ-secreting CD8+ T-cells was higher in mice immunized with the lipid nanoparticle formulations compared to the

groups immunized with free formulations (Figure 15). Administration of multi-LP/α-GalCer+TRP2-peptide induced relatively high levels of antigen-specific IFN-γ-secreting T cells (Figure 15).

[00133] In addition, the percentage of TRP2-specific IFN-γ-secreting CD8+ T cells was enhanced in the mice that were administered multi-LP/α-GalCer+TRP2-mRNA compared with those administered multi-LP/TRP2-mRNA, demonstrating that by combining α-GalCer with antigen-mRNA it is possible to achieve a stronger antigen-specific CD8+ T-cell immune response (Figure 15). Tetramer staining confirmed the same trend obtained by intracellular IFN-γ staining (Figure 16). In particular, the frequency of TRP2 (180-188) tetramer-positive CD8+ T cells was increased in mice treated with lipid nanoparticle based formulations but not in mice treated with free formulations (Figure 16).

[00134] CD8+ T cells in spleen and dLNs from treated mice were analyzed for the presence of memory T cells (CD62L+ and CD44+ T cells). T cells from mice administered multi-LP/α-GalCer+TRP2-mRNA nanoparticles showed the higher percentage of TCM compared to the groups administered multi-LP/TRP2-mRNA and multi-LP/α-GalCer.

[00135] The induction of humoral immune responses was also evaluated in response to treatment with an exemplary lipid nanoparticle. Multi-LP nanoparticles were prepared using mRNA encoding for ovalbumin protein (OVA-mRNA). The level of OVA-specific IgG in the serum of mice immunized with multi-LP/α-GalCer+OVA-mRNA was higher than that in the multi-LP/OVA-mRNA and multi-LP/α-GalCer groups (Figure 17). Additionally, the level of IgG2c isotype in the serum of mice in the multi-LP/α-GalCer+OVA-mRNA group was higher than the IgG1 isotype levels (Figure 18). These results collectively indicate that the incorporation of α-GalCer increase humoral immune responses, eliciting a strong Th1 profile.

[00136] Germinal center (GC) B cells formation is essential for the production of high affinity antibodies. GC structures support somatic hypermutation, selection of high affinity B cells and their differentiation into plasma and memory cells. Enhanced expression of germinal center (GC) markers (GL7 and FAS) was seen following immunization with multi-LP/α-GalCer+TRP2-mRNA nanoparticles compared with multi-LP/TRP2-mRNA and multi-LP/α-GalCer vaccinations.

[00137] Therapeutic efficacy and characterization of exemplary lipid nanoparticle with α-GalCer/TRP2-mRNA in a B16-F10 melanoma tumor model

[00138] B16-F10 melanoma-bearing mice were *i.v.* administered with exemplary lipid nanoparticles according ot the present disclosure, as well as control formulations, at

days 5, 9 and 13 post-tumor inoculation and the therapeutic efficacy of the combined treatments was evaluated in terms of tumor growth inhibition and survival.

[00139] As shown in Figure 19, treatment with multi-LP/mRNA and multi-LP/α-GalCer alone exhibited a modest inhibitory effect on tumor growth, while multi-LP loaded with an irrelevant mRNA (OVA-mRNA) had no effect, excluding any non-specific immune response. Mice treated with multi-LP/α-GalCer+TRP2-mRNA showed a significantly longer survival compared to mice treated with multi-LP/α-GalCer+TRP2-peptide, multi-LP/TRP2-mRNA, multi-LP/α-GalCer and multi-LP/irrelevant-mRNA (Figure 20).

[00140] Consistent with the above described efficacy studies, administration of multi-LP/α-GalCer+TRP2-mRNA promotes the infiltration of NK and CD8+ T cells within the melanoma subcutaneous tumor (Figure 21 and Figure 22). Furthermore, antigen-specific CD8+ T-cells analysis revealed a higher frequency of TRP2-positive tumor infiltrating CD8+ T-cells in tumor-bearing mice vaccinated with multi-LP/α-GalCer+TRP2-mRNA compared with multi-LP/TRP2-mRNA or multi-LP/α-GalCer (Figure 23).

[00141] Quantification of CD8+ T cells expressing IFN-γ after prime-boost therapies

[00142] Figure 24 is a graph quantifying CD8+ T cells expressing IFN-γ after different prime-boost therapies. The mice were treated with: adenovirus (Ad) as a prime and FMT as a boost (each expressing TRP2); an exemplary lipid nanoparticle of the present disclosure where the core encapsulated TRP2-peptide as a prime and FMT expressing TRP2 as a boost; and an exemplary lipid nanoparticle of the present disclosure where the core encapsulated TRP2-mRNA as a prime and FMT expressing TRP2 as a boost.

[00143] In the preceding description, for purposes of explanation, numerous details are set forth in order to provide a thorough understanding of the examples. However, it will be apparent to one skilled in the art that these specific details are not required. Accordingly, what has been described is merely illustrative of the application of the described examples and numerous modifications and variations are possible in light of the above teachings.

[00144] Since the above description provides examples, it will be appreciated that modifications and variations can be effected to the particular examples by those of skill in the art. Accordingly, the scope of the claims should not be limited by the particular examples set forth herein, but should be construed in a manner consistent with the specification as a whole.

WHAT IS CLAIMED IS:

1. A lipid nanoparticle composition comprising:

a core comprising:

(i) a complex of a poly-(beta-amino ester) polymer with RNA or DNA; or

(ii) a peptide; and

a lipid mixture encapsulating the core, wherein the lipid mixture comprises:

(a) N1-[2-((1S)-1-[(3-aminopropyl)amino]-4-[di(3-amino-propyl)amino]butylcarboxamido)ethyl]-3,4-di[oleyloxy]-benzamide (MVL5),

(b) 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and

(c) a polyethylene glycol (PEG)-modified lipid.

2. The lipid nanoparticle composition according to claim 1, wherein the ratio of polymer : DNA or RNA is in a range from about 10 : 1 to about 40 : 1 (wt:wt).

3. The lipid nanoparticle composition according to claim 1 or 2, wherein the ratio of MVL5 : DOPE : PEG-modified lipid is from about 10 to about 25 : about 75 to about 90 : about 1 to about 5 (wt:wt:wt).

4. The lipid nanoparticle composition according to any one of claims 1 to 3, wherein core comprises a complex of a poly-(beta-amino ester) polymer with DNA or RNA, and the DNA or RNA encodes an antigenic peptide.

5. The lipid nanoparticle composition according to any one of claims 1 to 3, wherein the core comprises a peptide, and the peptide is antigenic.

6. The lipid nanoparticle composition according to claim 4 or 5, wherein the antigenic peptide is a tumor associated antigen.

7. The lipid nanoparticle composition according to any one of claims 1 to 6, wherein the lipid mixture encapsulating the core further comprises an immune adjuvanting amount of α-galactosylceramide (α-GalCer).

8. The lipid nanoparticle composition according to claim 7, wherein the α-GalCer is present in an amount such that the ratio of α-GalCer : DNA, RNA or peptide is in a range of about 1 : about 10 to about 1 : about 75 (wt:wt).

9. The lipid nanoparticle composition according to any one of claims 1 to 8, wherein the PEG-modified lipid is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-2000PEG); 1,2-dimyristoyl-rac-glycero-3-methylpolyoxyethylene (DMG-PEG); 1,2-dipalmitoryl-sn-glycero-3-phosphoethanolamine (DPPE) conjugated polyethylene glycol (DPPE-PEG); 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) conjugated polyethylene glycol (DMPE-PEG); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) conjugated polyethylene glycol (DSPE-PEG); C8 or C16 ceramide conjugated polyethylene glycol (ceramide-PEG); or 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) conjugated polyethylene glycol (DOPE-PEG).

10. The lipid nanoparticle composition according to any one of claims 1 to 9, wherein the poly-(beta-amino ester) polymer is the polymeric product of the reaction between 1,4-butanediol diacrylate and 5-amino-1-pentanol, and end-capped with 1-(3-aminopropyl)-4-methylpiperazine.

11. The lipid nanoparticle composition according to claim 10, wherein the poly-(beta-amino ester) polymer has a number average molar mass (M_N) of about 10,700 g/mol, a mass average molar mass (M_W) of about 38,200 g/mol, and a polydispersity of about 3.58 as measured using gel permeation chromatography.

12. Use of a lipid nanoparticle composition according to any one of claims 1 to 11 for inducing an immune response in a mammal.

13. The use according to claim 12, wherein the immune response comprises activation of natural killer cells (NK cells), T-cells, or any combination thereof.

14. The use according to claim 13, wherein the T-cells comprise natural killer T-cells (NKT-cells).

15. The use according to any one of claims 12 to 14, wherein the core of the lipid nanoparticle composition comprises (i) a peptide or (ii) DNA or RNA that encodes a peptide, the peptide is an tumor associated antigen, and the immune response is an anti-cancer immune response.

16. The use according to any one of claims 12 to 15 wherein the immune response is induced using the lipid nanoparticle composition in a prime-boost format, such as a heterologous prime-boost format.

17. Use of a lipid nanoparticle composition according to any one of claims 1 to 11 for delivering the DNA, the RNA, or the protein to a dendritic cell.

18. The use according to claim 17, wherein the lipid nanoparticle composition is for delivering the DNA or the RNA, and the dendritic cells expresses an encoded protein antigen.

19. The use according to any one of claims 12 to 18, wherein the lipid nanoparticle composition is formulated for intradermal or intravenous administration.

20. A method of inducing an immune response in a mammal, the method comprising administering to the mammal an immune stimulatory amount of a lipid nanoparticle composition according to any one of claims 1 to 11.

21. A method of delivering DNA, RNA or protein to a dendritic cell, the method comprising contacting the dendritic cell with a lipid nanoparticle composition according to any one of claims 1 to 11.

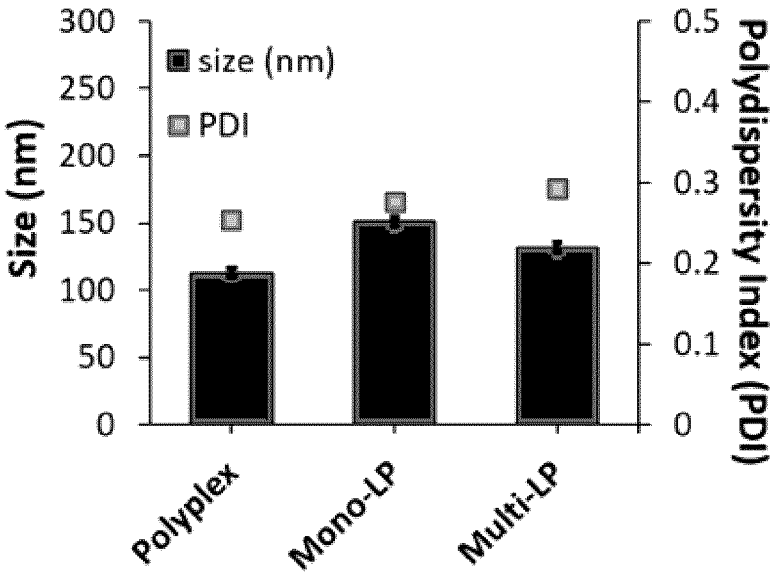

Figure 1

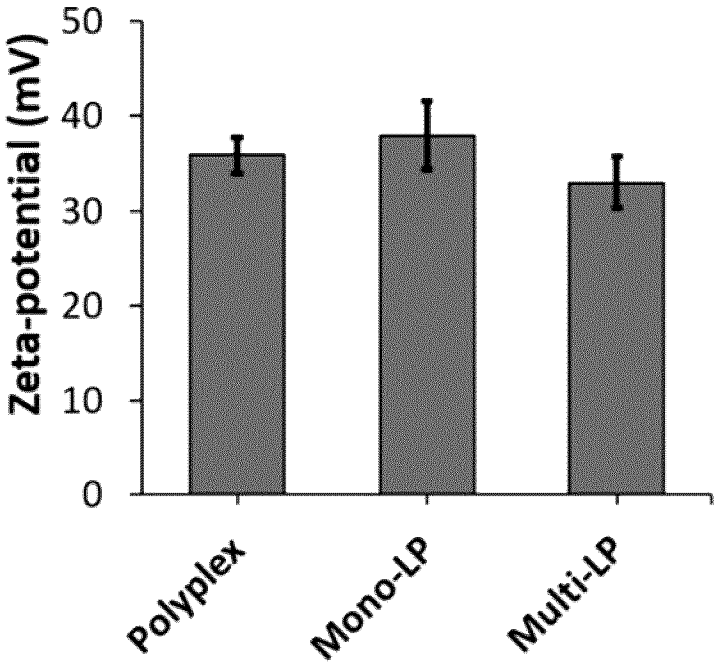

Figure 2

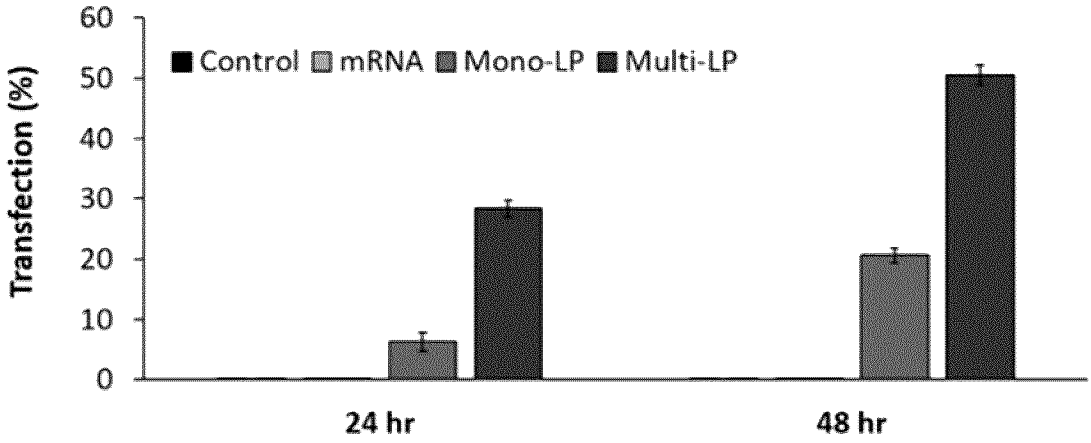

Figure 3

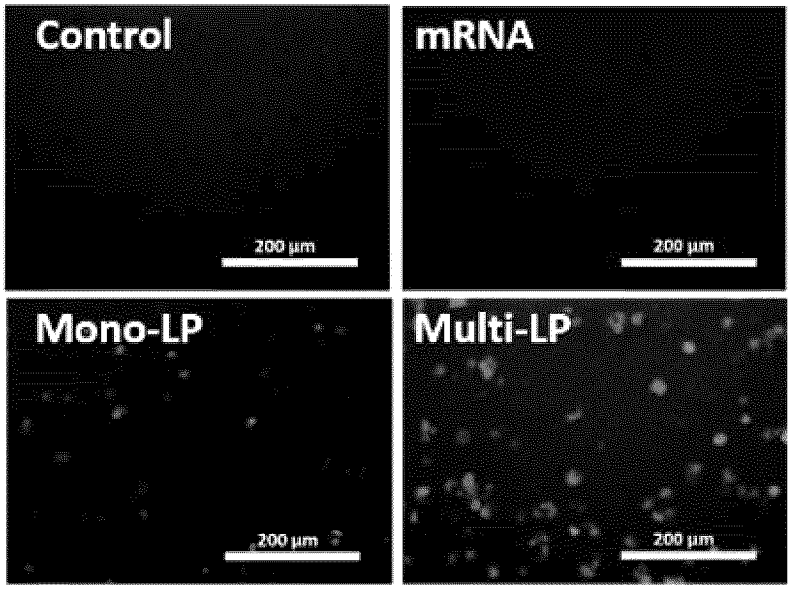

Figure 4

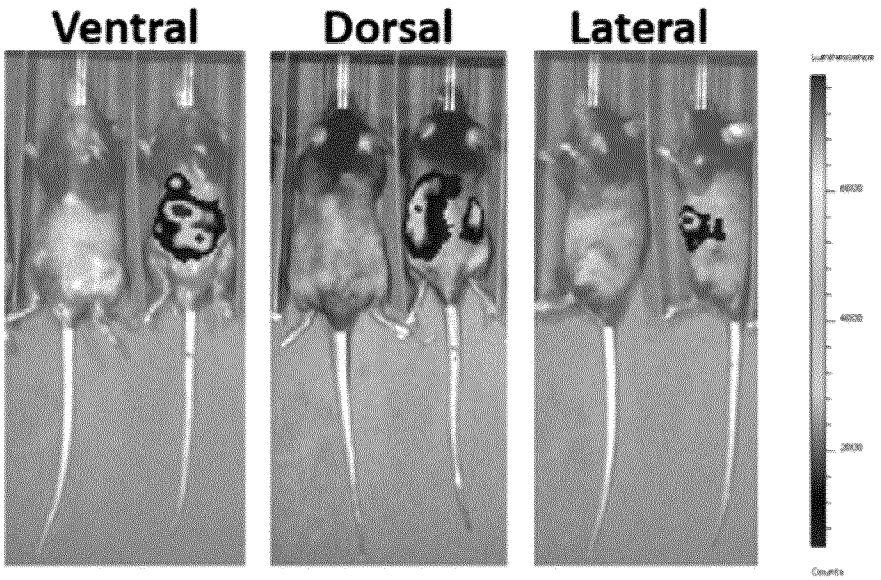

Figure 5

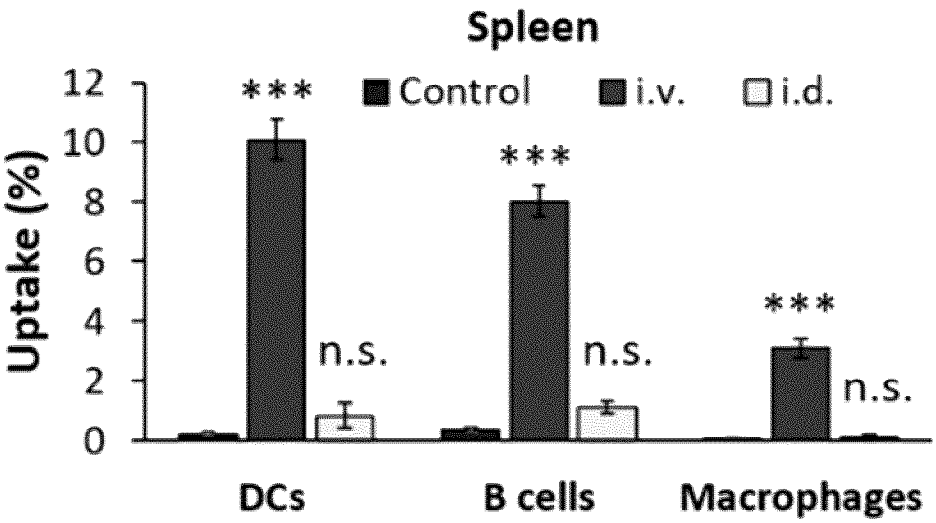

Figure 6

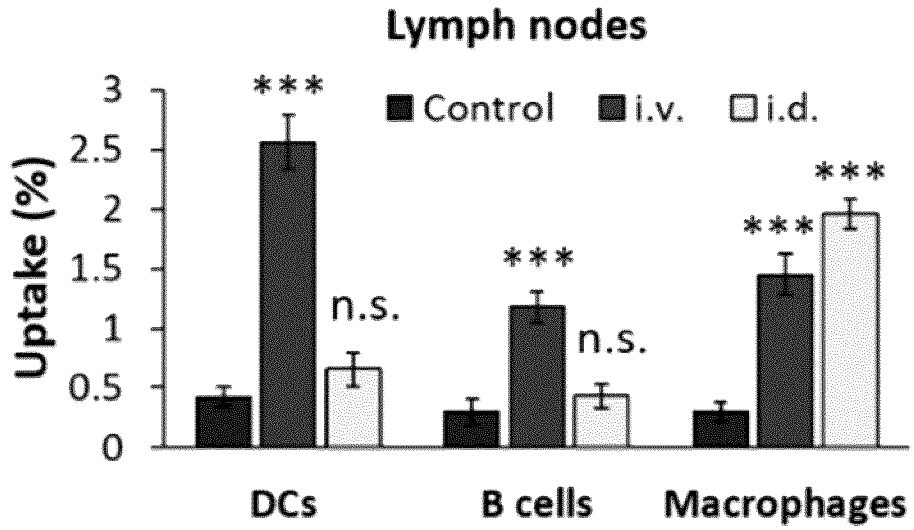

Figure 7

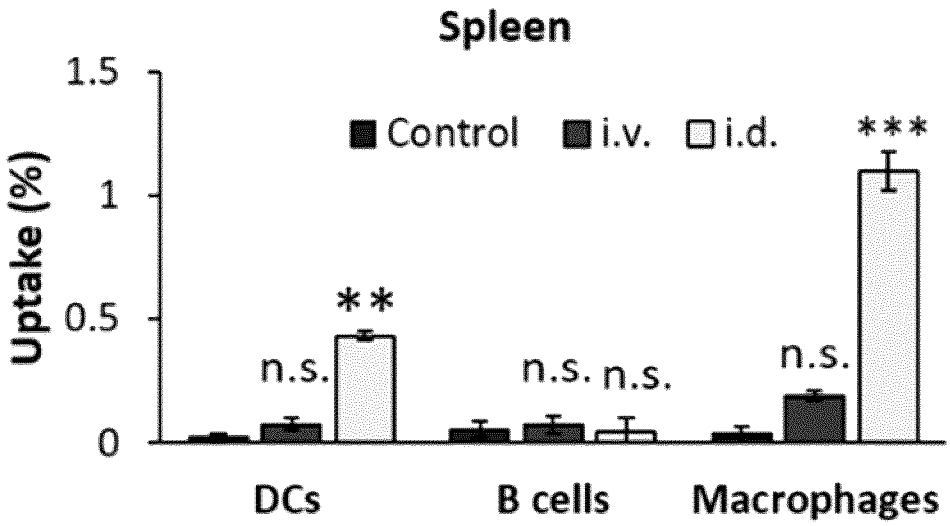

Figure 8

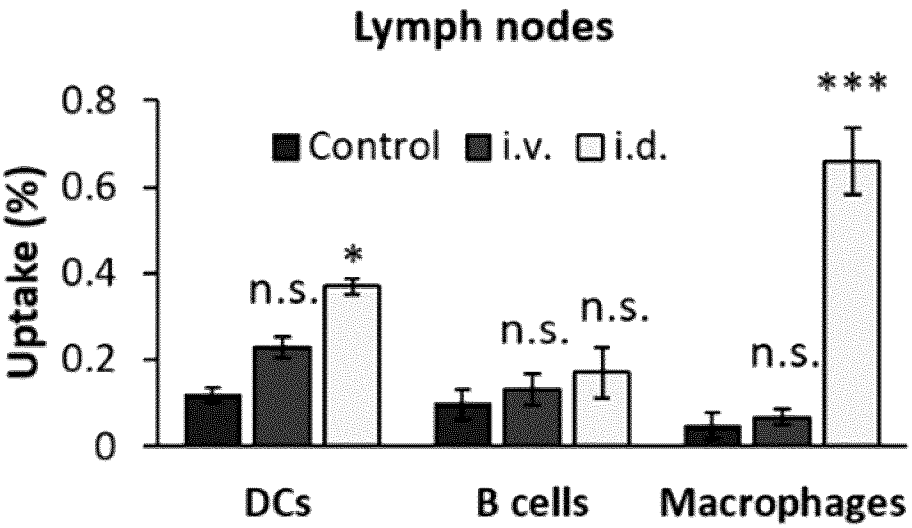

Figure 9

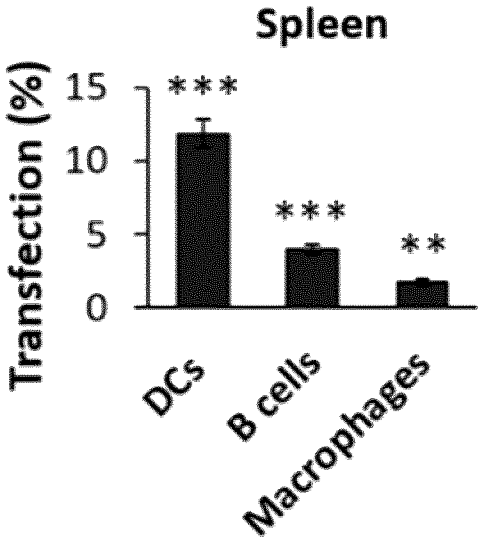

Figure 10

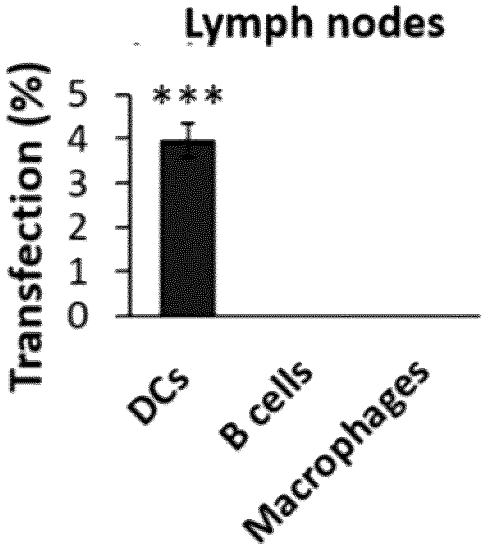

Figure 11

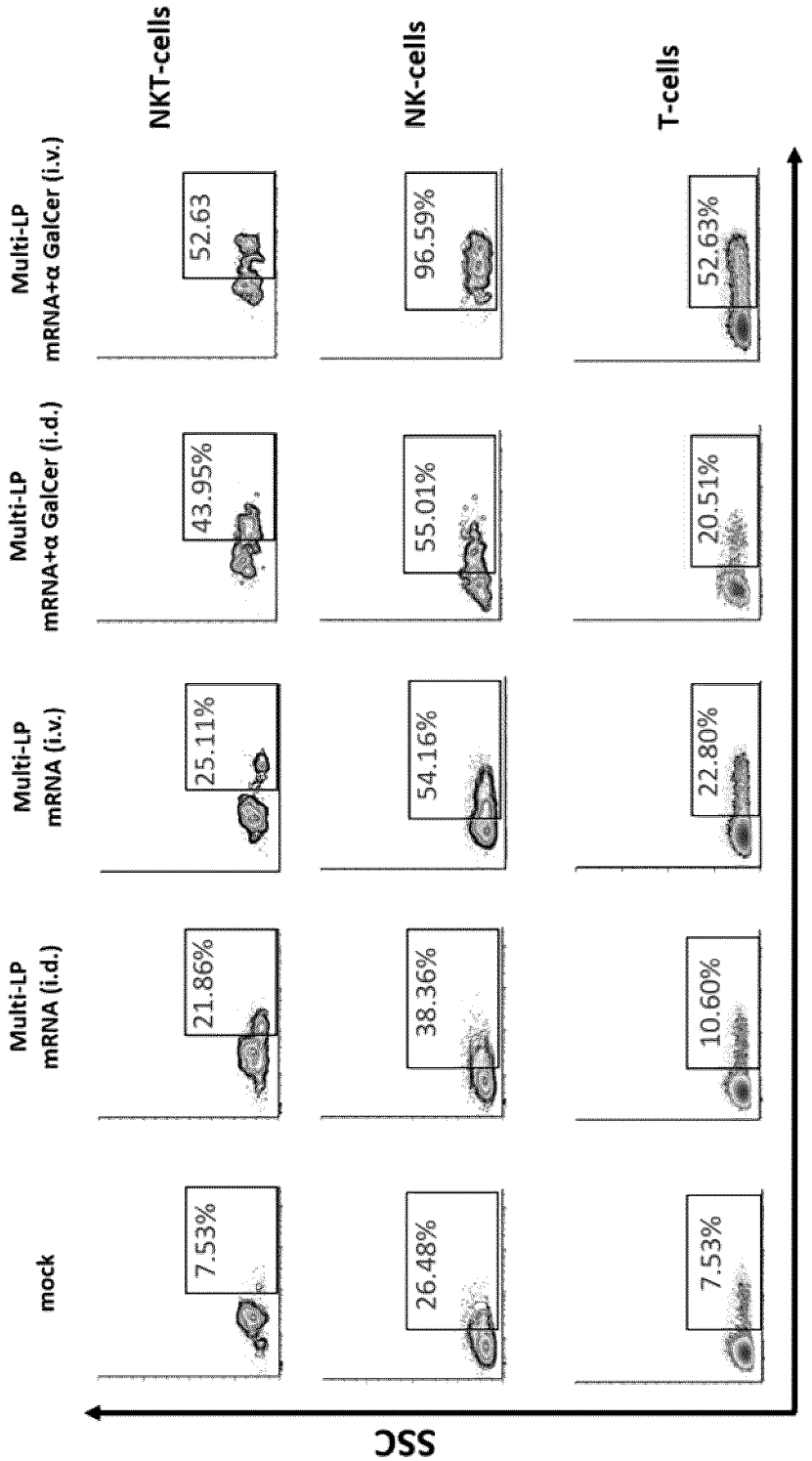
NKT-cells
NK-cells
T-cells
Multi-LP mRNA+α GalCer (i.v.)
Multi-LP mRNA+α GalCer (i.d.)
Multi-LP mRNA (i.v.)
Multi-LP mRNA (i.d.)
mock
52.63
43.95%
25.11%
21.86%
7.53%
96.59%
55.01%
54.16%
38.36%
26.48%
52.63%
20.51%
22.80%
10.60%
7.53%
SSC
CD69

Figure 12

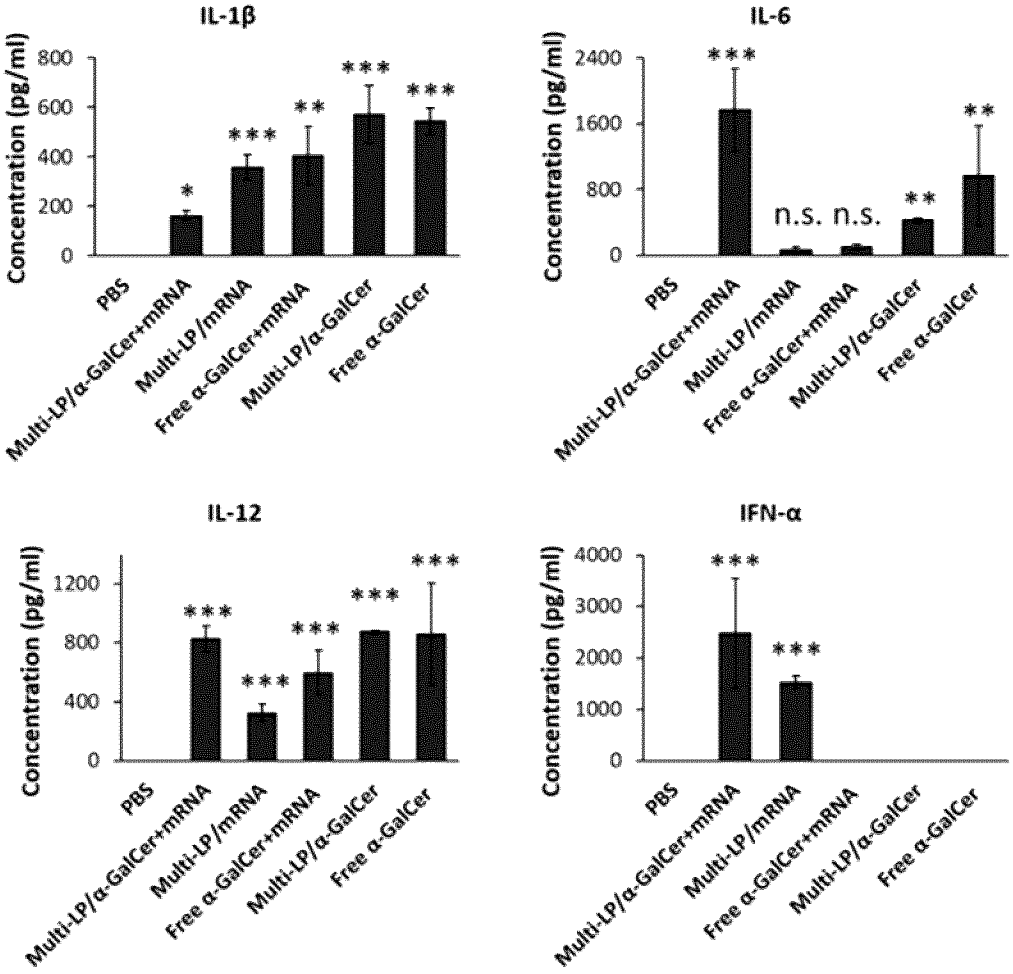
IL-1β
Concentration (pg/ml)
800
600
400
200
0
*
***
**
***
***
PBS
Multi-LP/α-GalCer+mRNA
Multi-LP/mRNA
Free α-GalCer+mRNA
Multi-LP/α-GalCer
Free α-GalCer
IL-6
Concentration (pg/ml)
2400
1600
800
0
***
n.s. n.s.
**
**
PBS
Multi-LP/α-GalCer+mRNA
Multi-LP/mRNA
Free α-GalCer+mRNA
Multi-LP/α-GalCer
Free α-GalCer
IL-12
Concentration (pg/ml)
1200
800
400
0
***
***
***
***
***
PBS
Multi-LP/α-GalCer+mRNA
Multi-LP/mRNA
Free α-GalCer+mRNA
Multi-LP/α-GalCer
Free α-GalCer
IFN-α
Concentration (pg/ml)
4000
3000
2000
1000
0
***
***
PBS
Multi-LP/α-GalCer+mRNA
Multi-LP/mRNA
Free α-GalCer+mRNA
Multi-LP/α-GalCer
Free α-GalCer

Figure 13

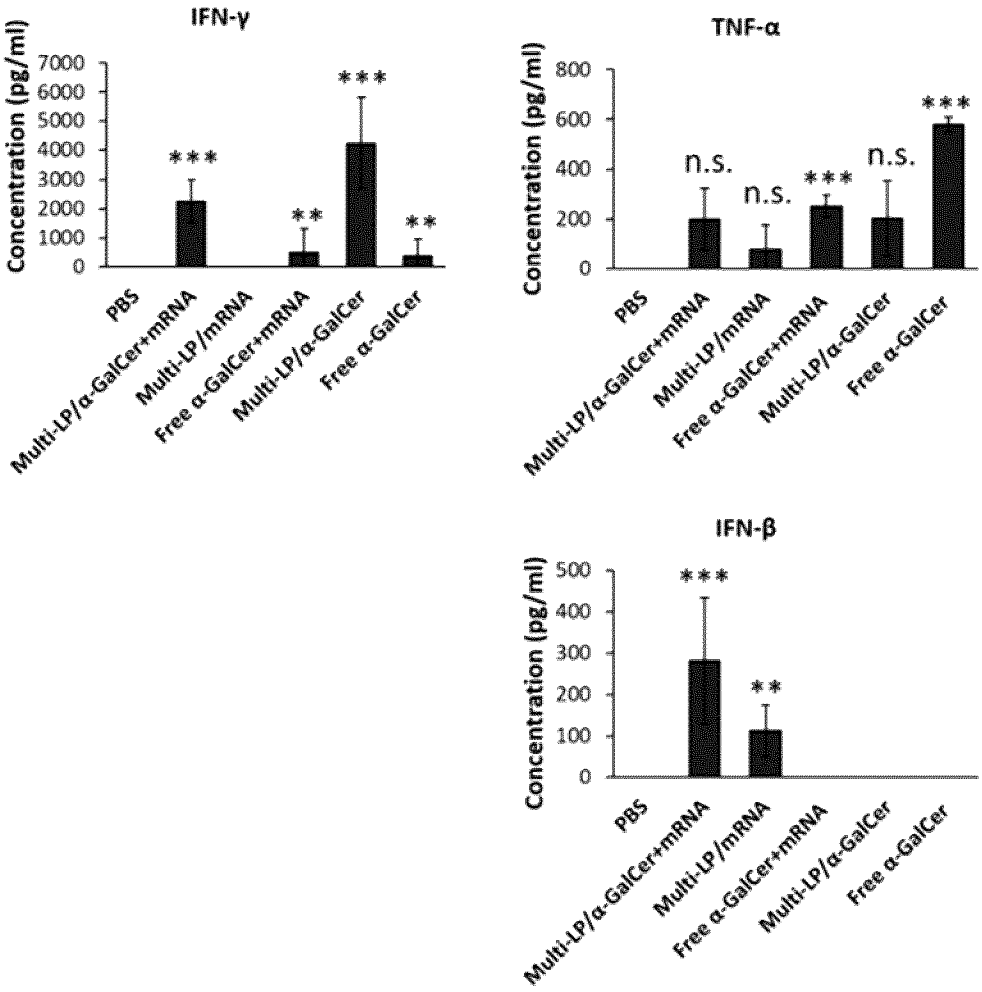
IFN-γ
Concentration (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
***
**
***
**
PBS
Multi-LP/α-GalCer+mRNA
Multi-LP/mRNA
Free α-GalCer+mRNA
Multi-LP/α-GalCer
Free α-GalCer
TNF-α
Concentration (pg/ml)
800
600
400
200
0
n.s.
n.s.
***
n.s.
***
PBS
Multi-LP/α-GalCer+mRNA
Multi-LP/mRNA
Free α-GalCer+mRNA
Multi-LP/α-GalCer
Free α-GalCer
IFN-β
Concentration (pg/ml)
500
400
300
200
100
0
***
**
PBS
Multi-LP/α-GalCer+mRNA
Multi-LP/mRNA
Free α-GalCer+mRNA
Multi-LP/α-GalCer
Free α-GalCer

Figure 14

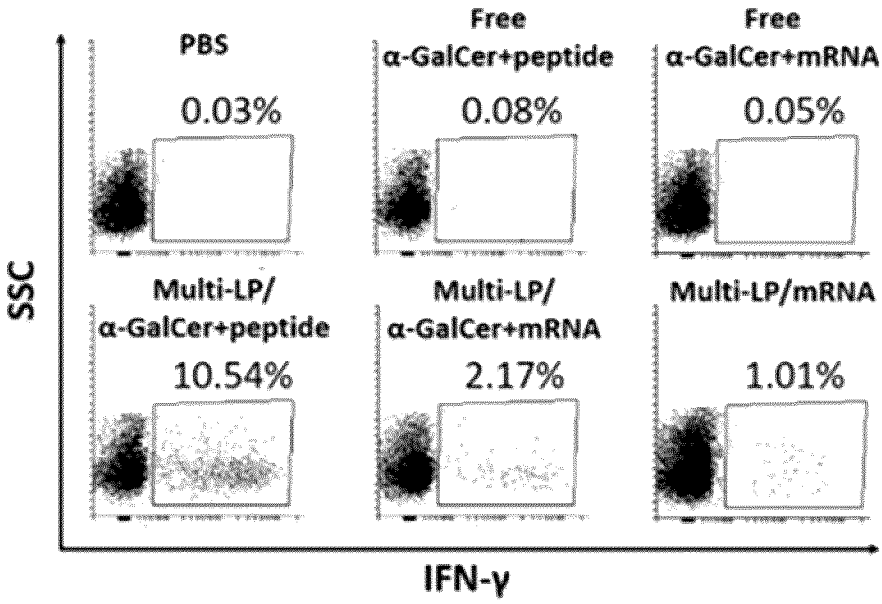

Figure 15

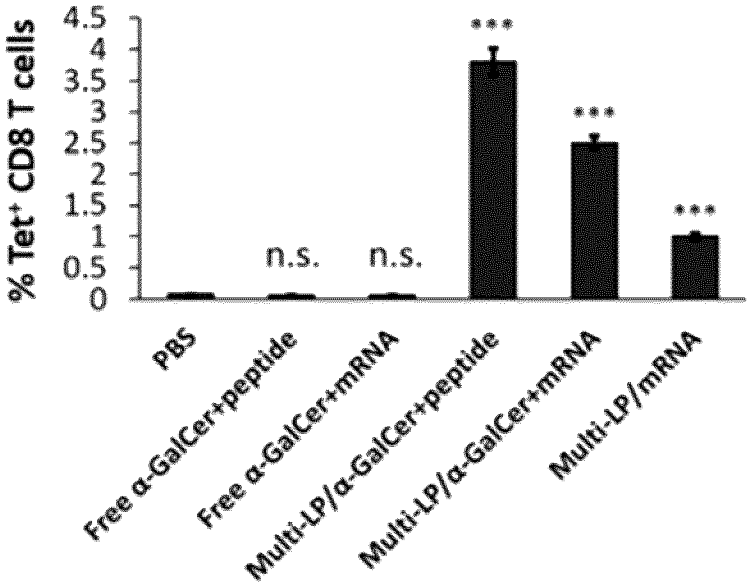

Figure 16

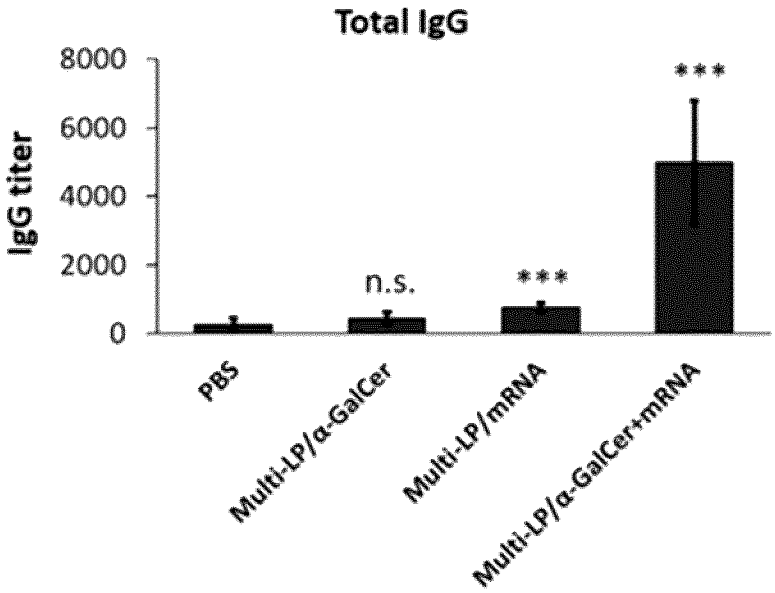

Figure 17

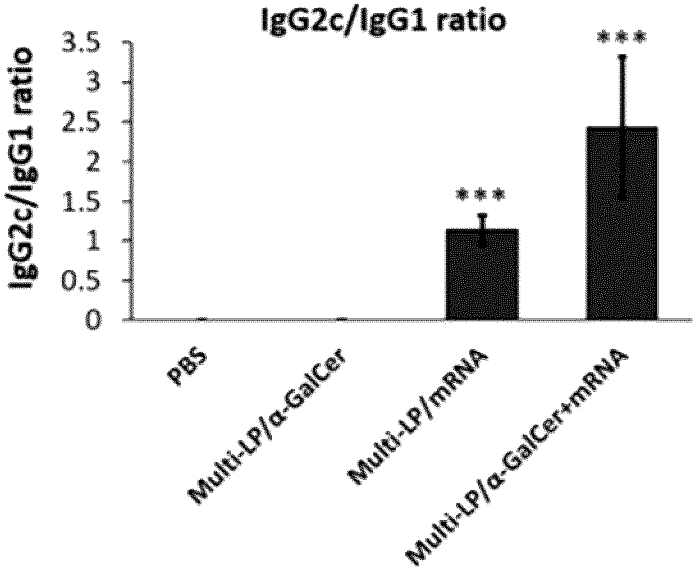

Figure 18

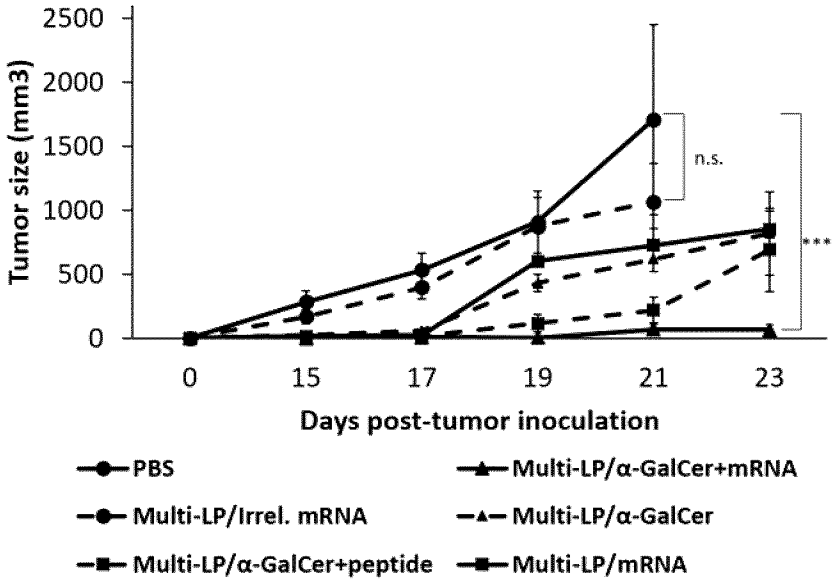

Figure 19

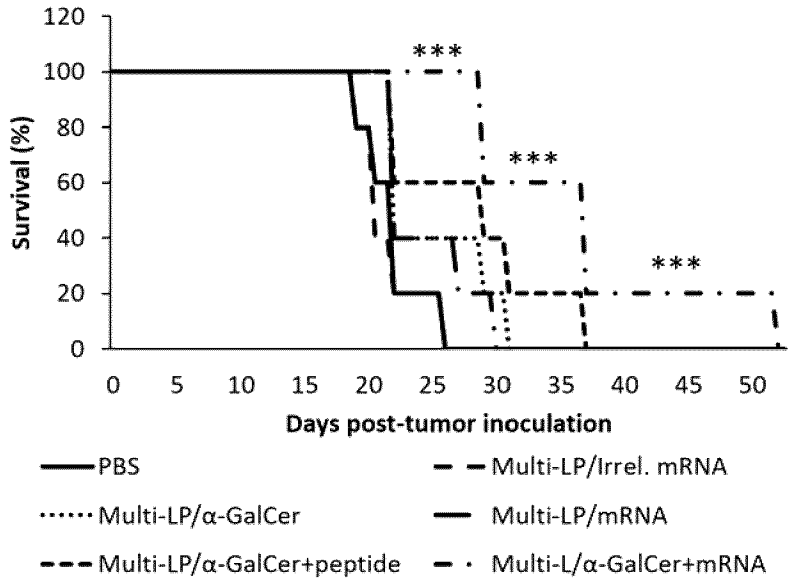

Figure 20

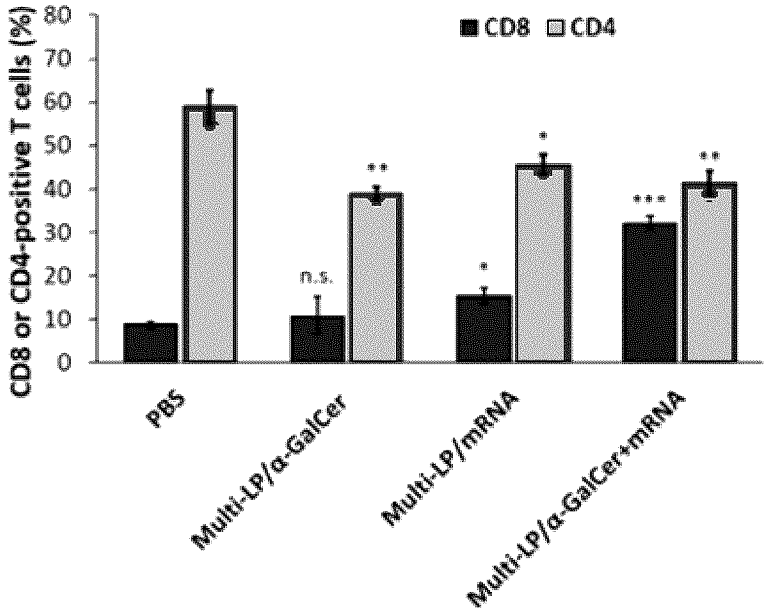

Figure 21

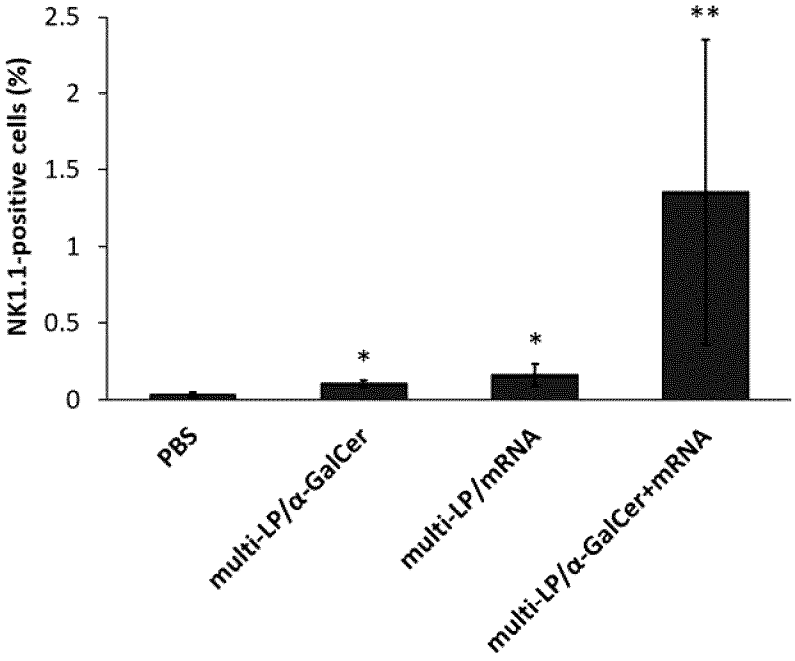

Figure 22

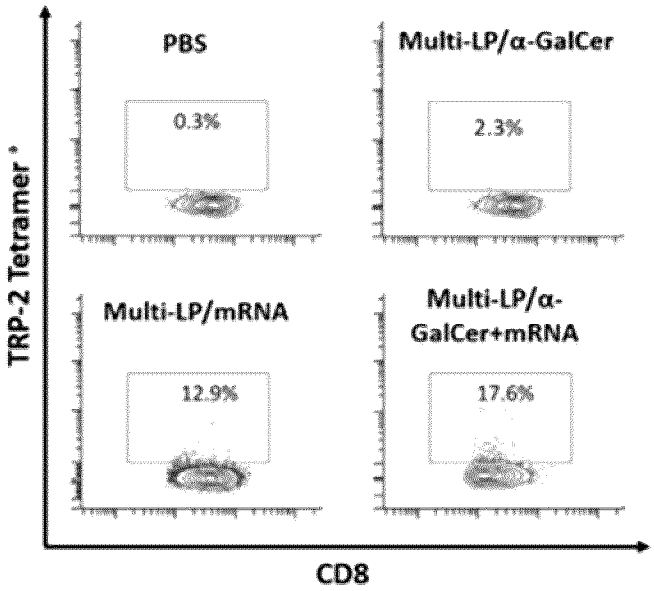

Figure 23

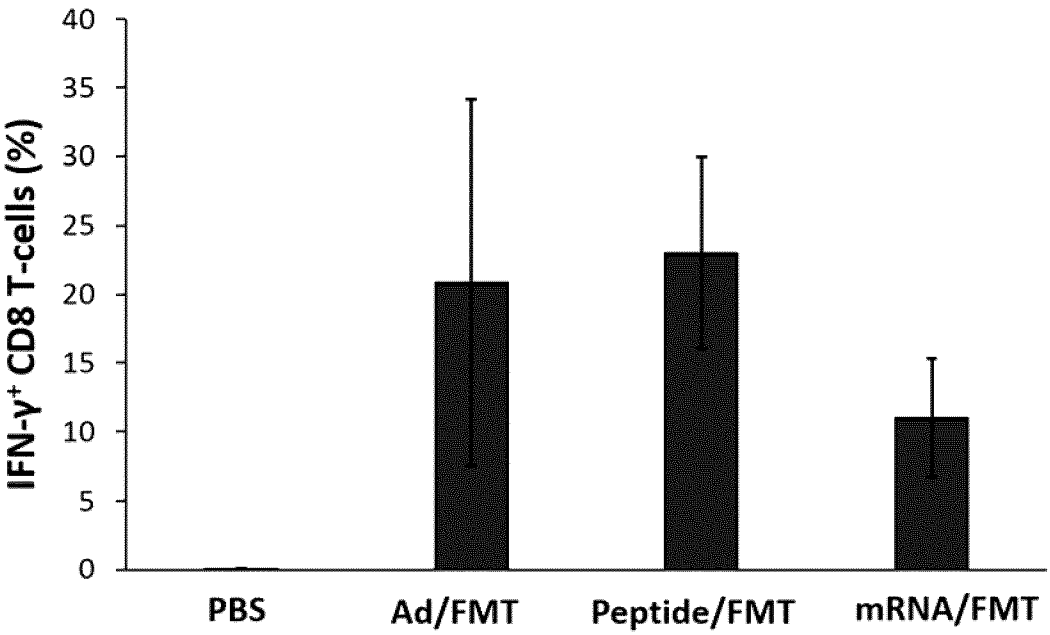

Figure 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2020/050281

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***A61K 47/44*** (2017.01), ***A61K 39/00*** (2006.01), ***A61K 47/10*** (2017.01), ***A61K 47/24*** (2006.01), ***A61K 9/14*** (2006.01), ***A61K 9/51*** (2006.01) (more IPCs on the last page)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: ***A61K 47/44*** (2017.01), ***A61K 39/00*** (2006.01), ***A61K 47/10*** (2017.01), ***A61K 47/24*** (2006.01), ***A61K 9/14*** (2006.01), ***A61K 9/51*** (2006.01) (more IPCs on the last page)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Questel Orbit (FamPat), STNext (Registry, CAPlus), CIPO Library Discovery Tool: Persano, Stojdl, *MVL5*, *DOPE*, *PEG*, nanoparticle, lipid, and related terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	Guevara et al. "Codelivery of mRNA with α-Galactosylceramide Using a New Lipopolyplex Formulation Induces a Strong Antitumor Response upon Intravenous Administration", *ACS Omega* **2019**, *4*, 13015-13026. Published 07 August 2019 (07-08-2019) Entire document	1-21

☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"D"	document cited by the applicant in the international application
"E"	earlier application or patent but published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search 08 May 2020 (08-05-2020)	Date of mailing of the international search report 28 May 2020 (28-05-2020)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 819-953-2476	Authorized officer Samantha Drouin (819) 639-8652

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Persano et al. "Lipopolyplex potentiates anti-tumor immunity of mRNA-based vaccination", *Biomaterials* **2017**, 125, 81–89. Entire document	1-21
A	Wonder et al. "Competition of Charge-Mediated and Specific Binding by Peptide-Tagged Cationic Liposome–DNA Nanoparticles *In Vitro* and *In Vivo*", *Biomaterials* **2018**, 166, 52–63. Entire document	1-21

A61P 35/00 (2006.01) , ***A61P 37/04*** (2006.01) , ***C12N 15/87*** (2006.01) , ***C12N 5/0784*** (2010.01)