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(54) Title: MULTILAMELLAR RNA NANOPARTICLES AND METHODS OF SENSITIZING TUMORS TO TREATMENT WITH IMMUNE CHECKPOINT INHIBITORS

(57) Abstract: The present disclosure provides methods of increasing sensitivity of a tumor to treatment with an immune checkpoint inhibitor (ICI) in a subject and methods of treating a subject with an immune checkpoint inhibitor (ICI)-resistant tumor. The methods comprise administering to the subject a composition comprising a nanoparticle comprising a positively-charged surface and an interior comprising (i) a core and (ii) at least two nucleic acid layers, wherein each nucleic acid layer is positioned between a cationic lipid bilayer. Also provided are methods of increasing the number of activated plasmacytoid dendritic cells (pDCs) in a subject in need thereof, comprising administering to the subject a composition comprising a nanoparticle comprising a positivelycharged surface and an interior comprising (i) a core and (ii) at least two nucleic acid layers, wherein each nucleic acid layer is positioned between a cationic lipid bilayer.

MULTILAMELLAR RNA NANOPARTICLES AND METHODS OF SENSITIZING TUMORS TO TREATMENT WITH IMMUNE CHECKPOINT INHIBITORS

FIELD OF THE INVENTION

[0001] This application multilamellar nanoparticles and use thereof to sensitize tumors to immune checkpoint inhibitors.

GRANT FUNDING DISCLOSURE

[0002] This invention was made with government support under grant number K08 CA199224 awarded by the National Institutes of Health, and grant number W81XWH-17-1-0510 awarded by the U.S. Army Medical Research Acquisition. The government has certain rights in the invention.

CROSS REFERENCE TO RELATED APPLICATIONS

[0003] This application claims the benefit of priority to U.S. Provisional Patent Application No. 62/978,694, filed February 19, 2020, the disclosure of which is hereby incorporated by reference in its entirety.

[0004] The following applications also are incorporated by reference: International Patent Application No. PCT/US20/42606, filed July 17, 2020; and International Patent Application No. PCT/US21/16925, filed February 5, 2021.

BACKGROUND

[0005] Due to severe and non-specific deleterious effects of radiation and chemotherapy, targeted therapies capable of selectively killing tumor cells in patients with glioblastoma (GBM) are essential. Tumor-specific immunotherapy can be harnessed to eradicate malignant brain tumors with exquisite precision and without collateral damage to normal tissue. Immunotherapy relies on the cytotoxic potential of activated T cells, which scavenge to recognize and reject tumor associated or specific antigens (TAAs or TSAs). Unlike most drug agents, activated T cells can traverse the blood brain barrier (BBB) via integrin (i.e., LFA-1, VLA-4) binding of ICAMs/VCAMs. T cells can be *ex vivo* activated in co-culture with dendritic cells (DCs) presenting TAAs/TSAs or through transduction with a chimeric antigen receptor (CAR). Alternatively, T cells can be endogenously activated using cancer vaccines; but, in a randomized phase III trial for patients with primary GBM, peptide vaccines targeting the tumor specific EGFRVIII surface antigen failed to mediate enhanced survival benefits over control vaccines. The EGFRVIII vaccine's failure to mediate anti-tumor efficacy highlights the challenge

of therapeutic cancer vaccines. While prophylactic cancer vaccines work to prevent malignancies (e.g., HPV vaccine to prevent cervical cancer), the vaccines require several boosts over months to years to confer protection in immune-replete patients. Furthermore, therapeutic cancer vaccines must induce immunologic response much more rapidly against malignancies (e.g., GBM) that are rapidly evolving. Moreover, GBMs are a highly invasive and heterogeneous tumors associated with profound systemic/ intratumoral suppression that can stymie a nascent immunotherapeutic response.

[0006] RNA vaccines have several advantages over traditional modalities. RNA has potent effects on both the innate and adaptive immune system. RNA can act as a toll-like receptor (TLR) agonist for receptors 3, 7, and 8 inducing potent TLR dependent innate immunity. RNA can also stimulate intracellular pathogen recognition receptors (e.g., melanoma differentiation antigen 5 (MDA-5) and retinoic acid inducible gene I (RIG-I)) and culminates in activating both helper-CD4 and cytotoxic CD8 T cell responses. Unlike DNA vaccines mired by having to cross both cellular and nuclear membranes, RNA only requires access to the cytoplasm and carries a significant safety advantage since it cannot be integrated into the host-genome. Unlike many peptide vaccines, which have only been developed for specific HLA haplotypes (e.g., HLA-A2), RNA bypasses MHC class restriction and can be leveraged for the population at large (28). One drawback to RNA is its lack of stability making it difficult to administer 'naked' RNA directly to patients. Since cancer vaccines must localize to antigen presenting cells (APCs) where RNA must be translated, processed and presented on MHC class I and II molecules, degradation continues to be a potent barrier for development of new mRNA technologies.

[0007] The advancement of cellular therapeutics also is fraught with developmental challenges, making it difficult to generate vaccines for the population at large. To circumvent the challenges of cellular therapeutics, nanocarriers have been developed as RNA delivery vehicles but translation of nanoparticles (NPs) into human clinical trials has lagged due to unknown biologic reactivity of novel NP designs. Alternatively, simple biodegradable lipid-NPs have been developed as cationic and anionic cancer vaccine formulations. Cationic formulations have been manufactured to shield mRNA inside the lipid core while anionic formulations have been manufactured to tether mRNA to the particle surface. However, cationic formulations have been mired by poor immunogenicity, and anionic formulations remain encumbered by the profound intratumoral and systemic immunosuppression that may stymie an activated T cell response.

[0008] Additionally, cancer immunotherapy with immune checkpoint inhibitors (ICIs) has shown significant promise against malignancies with immunologically active ("hot") microenvironments, however, this therapy has failed in clinical trials for patients with immunologically inactive ("cold") tumors. Response to ICIs appears to be predicated on the presence of intratumoral CD8+PD-1+ cells and on activated PD-L1+ host-myeloid cells. These cell populations may be naturally increased in patients with high mutational burdens, but absent in those without response.

[0009] In view of the foregoing, there is a need for improved RNA lipid-nanoparticle (NP) vaccines and methods of using these vaccines to treat a tumor or cancer in patients with an immune checkpoint inhibitor (ICI)-resistant tumor, as well as methods for increasing a response to immunotherapy for immunologically inactive ("cold") tumors.

SUMMARY

[0010] The present disclosure provides a nanoparticle comprising a positively-charged surface and an interior comprising (i) a core and (ii) at least two nucleic acid layers, wherein each nucleic acid layer is positioned between a cationic lipid bilayer. In exemplary embodiments, the nanoparticle comprises at least three nucleic acid layers, each of which is positioned between a cationic lipid bilayer. In exemplary aspects, the nanoparticle comprises at least four or five or more nucleic acid layers, each of which is positioned between a cationic lipid bilayer. In various aspects, the outermost layer of the nanoparticle comprises a cationic lipid bilayer. In various instances, the surface comprises a plurality of hydrophilic moieties of the cationic lipid of the cationic lipid bilayer. In exemplary aspects, the core comprises a cationic lipid bilayer. Optionally, the core comprises less than about 0.5 wt% nucleic acid. The diameter of the nanoparticle, in various aspects, is about 50 nm to about 250 nm in diameter, optionally, about 70 nm to about 200 nm in diameter. In exemplary instances, the nanoparticle is characterized by a zeta potential of about +40 mV to about +60 mV, optionally, about +45 mV to about +55 mV. The nanoparticle in various instances, has a zeta potential of about 50 mV. In some aspects, the nucleic acid molecules are present at a nucleic acid molecule:cationic lipid ratio of about 1 to about 5 to about 1 to about 20, optionally, about 1 to about 15, about 1 to about 10 or about 1 to about 7.5. In various aspects, the nucleic acid molecules are RNA molecules, optionally, messenger RNA (mRNA). In various aspects, the mRNA is *in vitro* transcribed mRNA wherein the *in vitro* transcription template is cDNA made from RNA extracted from a tumor cell. In various aspects, the nanoparticle comprises a mixture of RNA which is RNA isolated from a tumor of a human, optionally, a malignant brain tumor, optionally, a

glioblastoma, medulloblastoma, diffuse intrinsic pontine glioma, or a peripheral tumor with metastatic infiltration into the central nervous system.

[0011] A method of increasing an immune response against a tumor in a subject is provided by the present disclosure. In exemplary embodiments, the method comprises administering to the subject the nanoparticle or pharmaceutical composition of the present disclosure. In exemplary aspects, the nucleic acid molecules are mRNA. Optionally, the composition is systemically administered to the subject. For example, the composition is administered intravenously. In various aspects, the nanoparticle or pharmaceutical composition is administered in an amount which is effective to activate dendritic cells (DCs) in the subject. In various instances, the immune response is a T cell-mediated immune response. Optionally, the T cell-mediated immune response comprises activity by tumor infiltrating lymphocytes (TILs).

[0012] The present disclosure provides a method of increasing sensitivity of a tumor to treatment with an immune checkpoint inhibitor (ICI) in a subject. In exemplary embodiments, the method comprises administering to the subject a composition comprising a nanoparticle comprising a positively-charged surface and an interior comprising (i) a core and (ii) at least two nucleic acid layers, wherein each nucleic acid layer is positioned between a cationic lipid bilayer, optionally, wherein the composition is systemically administered to the subject.

[0013] The present disclosure further provides a method of treating a subject with an immune checkpoint inhibitor (ICI)-resistant tumor. In exemplary embodiments, the method comprises administering to the subject (i) a composition comprising a nanoparticle comprising a positively-charged surface and an interior comprising (i) a core and (ii) at least two nucleic acid layers, wherein each nucleic acid layer is positioned between a cationic lipid bilayer, and (ii) an ICI, optionally, wherein the composition is systemically administered to the subject.

[0014] A method of treating a subject with a tumor or cancer also is provided, wherein the method comprises (i) increasing the number of activated plasmacytoid dendritic cells (pDCs) in the subject in accordance with the method described herein, (ii) isolating white blood cells (WBCs) from the subject, (iii) isolating dendritic cells (DCs) from the WBCs, (iv) contacting the DCs with a fusion protein comprising prostatic acid phosphatase (PAP) and GM-CSF, and (v) administering the DCs to subject.

[0015] Additionally, the disclosure provides a method of preparing a dendritic cell vaccine, the method comprising (i) increasing the number of activated plasmacytoid dendritic cells (pDCs) in the subject, (ii) isolating white blood cells (WBCs) from the subject, (iii) isolating dendritic cells

(DCs) from the WBCs, and (iv) contacting the DCs with a fusion protein comprising prostatic acid phosphatase (PAP) and GM-CSF.

[0016] Additional embodiments and aspects of the presently disclosed nanoparticles, pharmaceutical compositions, and methods are provided below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Figure 1A is a series of illustrations of a lipid bilayer, liposome and a general scheme leading to multilamellar (ML) RNA NPs (boxed).

[0018] Figure 1B is a pair of CEM images of uncomplexed NPs (left) and ML RNA NPs (right).

[0019] Figure 2A is an illustration of a general scheme leading to cationic RNA lipoplexes.

[0020] Figure 2B is an illustration of a general scheme leading to cationic RNA lipoplexes.

[0021] Figure 2C is a CEM image of uncomplexed NPs, Figure 2D is a CEM image of RNA LPXs, and Figure 2E is a CEM image of ML RNA NPs.

[0022] Figure 2F is a graph of the % CD86+ of CD11c+MHC Class II+ splenocytes present in the spleens of mice treated with ML RNA NPs (ML RNA-NPs), RNA LPXs, anionic LPXs, or of untreated mice.

[0023] Figure 2G is a graph of the % CD44+CD62L+ of CD8+ splenocytes present in the spleens of mice treated with ML RNA NPs (ML RNA-NPs), RNA LPXs, anionic LPXs, or of untreated mice.

[0024] Figure 2H is a graph of the % CD44+CD62L of CD4+ splenocytes present in the spleens of mice treated with ML RNA NPs (ML RNA-NPs), RNA LPXs, anionic LPXs, or of untreated mice.

[0025] Figure 2I is a graph of the % survival of mice treated with ML RNA NPs (ML RNA-NPs), RNA LPXs, anionic LPXs, or of untreated mice.

[0026] Figure 2J is a graph of the amount of IFN-α produced in mice upon treatment with ML RNA NPs (ML RNA-NPs), RNA LPXs, anionic LPXs, or of untreated mice.

[0027] Figure 3A is a pair of photographs of lungs of mice treated with ML RNA NPs or of untreated mice.

[0028] Figure 3B is a graph of the %central memory T cells (CD62L+CD44+ of CD3+ cells) present in mice treated with ML RNA NPs loaded with tumor specific RNA or with ML RNA NPs with non-specific RNA (GFP RNA) or of untreated mice.

[0029] Figure 3C is a graph of the % survival of mice treated with ML RNA NPs loaded with tumor specific RNA or with ML RNA NPs with non-specific RNA (GFP RNA) or of untreated mice.

[0030] Figure 3D is a graph of the % survival of mice treated with ML RNA NPs loaded with tumor specific RNA or with ML RNA NPs with non-specific RNA (GFP RNA) or of untreated mice. This model is different from the one used to obtain the data of Figure 3C.

[0031] Figure 4A is a graph of the % expression of CD8 or CD44 and CD8 of CD3+ cells plotted as a function of time post administration of ML RNA NPs.

[0032] Figure 4B is a graph of the % expression of PDL1, MHC II, CD86 or CD80 of CD11c+ cells plotted as a function of time post administration of ML RNA NPs.

[0033] Figure 4C is a graph of the % expression of CD44 and CD8 of CD3+ cells plotted as a function of time post administration of ML RNA NPs.

[0034] Figure 4D is a graph of the % survival of canines treated with ML RNA NPs compared to the median survival (dotted line).

[0035] Figure 4E illustrates the percentage of lymphocytes (y-axis) elicited post-administration of ML RNA-NPs (x-axis) in a canine model.

[0036] Figure 4F illustrates interferon-α production (pg/mL; y-axis) in the hours following administration of ML RNA-NPs in a canine model.

[0037] Figure 4G illustrates an increase in CD80+ expression on Cd11c+ cells (% expression, y-axis) in the hours following administration of the ML RNA-NPs (x-axis).

[0038] Figure 4H illustrates expression of CD8 and CD44+CD8+ cells in the hours following administration of the ML RNA-NPs (x-axis) to canine subject.

[0039] Figure 5 is a CEM image of ML RNA NPs and point to examples with several layers.

[0040] Figure 6 is a cartoon delineating the generation of personalized tumor mRNA loaded NPs: From as few as 100-500 biopsied brain tumor cells, total RNA is extracted and a cDNA library is generated from which copious amounts of mRNA (representing a personalized tumor specific transcriptome) can be amplified. Negatively charged tumor mRNA is then encapsulated

into positively charged lipid NPs. NPs encapsulate RNA through electrostatic interaction and are administered intravenously (iv) for uptake by dendritic cells (DCs) in reticuloendothelial organs (i.e. liver spleen and lymph nodes). The RNA is then translated and processed by a DC's intracellular machinery for presentation of peptides onto MHC Class I and II molecules, which activate CD4 and CD8+ T cells.

[0041] Figure 7A is a timeline of the long-term survivor treatment. First and Second tumor inoculations are shown. Figure 7B is a graph of the percent survival of animals after the second tumor inoculation for each of the three groups of mice: two groups treated before 2nd tumor inoculation with ML RNA NPs comprising non-specific RNA (RNA not specific to the tumor in the subject; Green Fluorescence Protein (GFP) or pp65) and one group treated before 2nd tumor inoculation with ML RNA NPs comprising tumor specific RNA or untreated animals prior to 2nd tumor inoculation. Control group survival percentage is noted as "Untreated".

[0042] Figure 8 is a series of images depicting the localization of anionic LPX in mice upon administration.

[0043] Figure 9 is a graph of the percentage of surviving mice of a group treated with ML RNA NPs alone (RNA-NP) or in combination with PDL1 monoclonal antibodies (RNA-NP+PDL1 mAb) as a function of time (days) after tumor implantation. Control groups included untreated mice (Untreated), mice treated with ML NPs without any RNA (NP Alone), and mice treated with PDL1 monoclonal antibodies alone (PDL1 mAb). *$p<0.05$,Gehan–Breslow–Wilcox.

[0044] Figures 10A-10C are line graphs illustrating tumor volume (mm^3) of melanoma (Figure 10A), percent survival in a sarcoma model (Figure 10B), and percent survival in a metastatic lung model (Figure 10C) at various days post-tumor implantation. The figures demonstrate that the ML RNA-NPs of the disclosure mediate effective anti-tumor immune responses against immunologically cold tumors *in vivo*.

[0045] Figures 11A-11C demonstrate that non-specific ML RNA-NPs of the disclosure mediate significant anti-tumor efficacy that can synergize with ICIs such that an "off the shelf" (i.e., not personalized) construct sensitize cancer to ICIs. Figure 11A: Tumor volumes (mm^3) of C57Bl/6 mice (7-8/group) bearing subcutaneous B16F0 tumors were vaccinated with luciferase RNA-NPs once weekly (x3) or treated twice weekly with PD-L1-mAbs (x3). Figure 11B: Survival plot (% survival; y-axis) of BALB/c mice (8/group) inoculated with K7M2 lung tumors and vaccinated with three weekly GFP RNA-NPs (x3) or twice weekly PD-L1 mAbs. Figure 11C: Non-specific RNA-NPs (luciferase) sensitize response to ICIs in a checkpoint resistant murine

tumor model (B16F0). Tumor volumes (mm^3) provided on y-axis; days after tumor implantation provided on x-axis.

[0046] Figure 12 is a table listing the top 620 genes that are representative of the slow cycling cell (SCC) transcriptome.

DETAILED DESCRIPTION

[0047] The present disclosure relates to nanoparticles comprising a cationic lipid and nucleic acids. As used herein the term "nanoparticle" refers to a particle that is less than about 1000 nm in diameter. As the nanoparticles of the present disclosure comprise cationic lipids that have been processed to induce liposome formation, the presently disclosed nanoparticles in various aspects comprise liposomes. Liposomes are artificially-prepared vesicles which, in exemplary aspects, are primarily composed of a lipid bilayer. Liposomes in various instances are used as a delivery vehicle for the administration of nutrients and pharmaceutical agents. In various aspects the liposomes of the present disclosure are of different sizes and the composition may comprise one or more of (a) a multilamellar vesicle (MLV) which may be hundreds of nanometers in diameter and may contain a series of concentric bilayers separated by narrow aqueous compartments, (b) a small unicellular vesicle (SUV) which may be smaller than, e.g., 50 nm in diameter, and (c) a large unilamellar vesicle (LUV) which may be between, e.g., 50 and 500 nm in diameter. Liposomes in various instances are designed to comprise opsonins or ligands in order to improve the attachment of liposomes to unhealthy tissue or to activate events such as, but not limited to, endocytosis. In exemplary aspects, liposomes contain a low or a high pH in order to improve the delivery of the pharmaceutical formulations. In various instances, liposomes are formulated depending on the physicochemical characteristics such as, but not limited to, the pharmaceutical formulation entrapped and the liposomal ingredients, the nature of the medium in which the lipid vesicles are dispersed, the effective concentration of the entrapped substance and its potential toxicity, any additional processes involved during the application and/or delivery of the vesicles, the optimization size, polydispersity and the shelf-life of the vesicles for the intended application, and the batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products.

[0048] In exemplary embodiments, the nanoparticle comprises a surface and an interior comprising (i) a core and (ii) at least two nucleic acid layers, optionally, more than two nucleic acid layers. In exemplary instances, each nucleic acid layer is positioned between a lipid layer, e.g., a cationic lipid layer. In exemplary aspects, the nanoparticles are multilamellar comprising

alternating layers of nucleic acid and lipid. In exemplary embodiments, the nanoparticle comprises at least three nucleic acid layers, each of which is positioned between a cationic lipid bilayer. In exemplary aspects, the nanoparticle comprises at least four or five nucleic acid layers, each of which is positioned between a cationic lipid bilayer. In exemplary aspects, the nanoparticle comprises at least more than five (e.g., 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more) nucleic acid layers, each of which is positioned between a cationic lipid bilayer. As used herein the term "cationic lipid bilayer" is meant a lipid bilayer comprising, consisting essentially of, or consisting of a cationic lipid or a mixture thereof. Suitable cationic lipids are described herein. As used herein the term "nucleic acid layer" is meant a layer of the presently disclosed nanoparticle comprising, consisting essentially of, or consisting of a nucleic acid, e.g., RNA.

[0049] The unique structure of the nanoparticle of the present disclosure results in mechanistic differences in how the multilamellar nanoparticles (ML-NPs) exert a biological effect. Previously described RNA-based nanoparticles exert their effect, at least in part, through the toll-like receptor 7 (TLR7) pathway. Surprisingly, the multi-lamellar nanoparticles of the instant disclosure mediate efficacy independent of TLR7. While not wishing to be bound to any particular theory, intracellular pathogen recognition receptors (PRRs), such as MDA-5, appear more relevant to biological activity of the multi-lamellar nanoparticles than TLRs. This likely allows ML RNA-NPs to stimulate multiple intracellular PRRs (e.g., RIG-I, MDA-5) as opposed to singular TLRs (e.g., TLR7 in the endosome) culminating in greater release of type I interferons and induction of more potent innate immunity. This allows RNA-NPs to demonstrate superior efficacy with long-term survivor benefit.

[0050] In various aspects, the presently disclosed nanoparticle comprises a positively-charged surface. In some instances, the positively-charged surface comprises a lipid layer, e.g., a cationic lipid layer. In various aspects, the outermost layer of the nanoparticle comprises a cationic lipid bilayer. Optionally, the cationic lipid bilayer comprises, consists essentially of, or consists of DOTAP. In various instances, the surface comprises a plurality of hydrophilic moieties of the cationic lipid of the cationic lipid bilayer. In some aspects, the core comprises a cationic lipid bilayer. In various instances, the core lacks nucleic acids, optionally, the core comprises less than about 0.5 wt% nucleic acid.

[0051] In exemplary aspects, the nanoparticle has a diameter within the nanometer range and accordingly in certain instances are referred to herein as "nanoliposomes" or "liposomes". In exemplary aspects, the nanoparticle has a diameter between about 50 nm to about 500 nm,

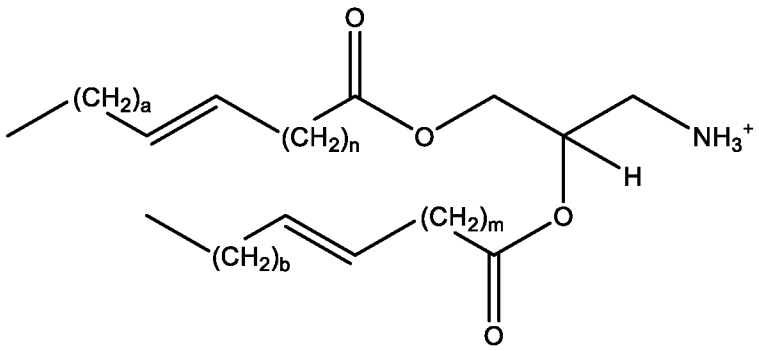
e.g., about 50 nm to about 450 nm, about 50 nm to about 400 nm, about 50 nm to about 350 nm, about 50 nm to about 300 nm, about 50 nm to about 250 nm, about 50 nm to about 200 nm, about 50 nm to about 150 nm, about 50 nm to about 100 nm, about 100 nm to about 500 nm, about 150 nm to about 500 nm, about 200 nm to about 500 nm, about 250 nm to about 500 nm, about 300 nm to about 500 nm, about 350 nm to about 500nm, or about 400 nm to about 500 nm. In exemplary aspects, the nanoparticle has a diameter between about 50 nm to about 300 nm, e.g., about 100 nm to about 250 nm, about 110 nm ±5 nm, about 115 nm ±5 nm, about 120 nm ±5 nm, about 125 nm ±5 nm, about 130 nm ±5 nm, about 135 nm ±5 nm, about 140 nm ±5 nm, about 145 nm ±5 nm, about 150 nm ±5 nm, about 155 nm ±5 nm, about 160 nm ±5 nm, about 165 nm ±5 nm, about 170 nm ±5 nm, about 175 nm ±5 nm, about 180 nm ±5 nm, about 190 nm ±5 nm, about 200 nm ±5 nm, about 210 nm ±5 nm, about 220 nm ±5 nm, about 230 nm ±5 nm, about 240 nm ±5 nm, about 250 nm ±5 nm, about 260 nm ±5 nm, about 270 nm ±5 nm, about 280 nm ±5 nm, about 290 nm ±5 nm, or about 300 nm ±5 nm. In exemplary aspects, the nanoparticle is about 50 nm to about 250 nm in diameter. In some aspects, the nanoparticle is about 70 nm to about 200 nm in diameter.

[0052] In exemplary aspects, the nanoparticle is present in a pharmaceutical composition comprising a heterogeneous mixture of nanoparticles ranging in diameter, e.g., about 50 nm to about 500 nm or about 50 nm to about 250 nm in diameter. Optionally, the pharmaceutical composition comprises a heterogeneous mixture of nanoparticles ranging from about 70 nm to about 200 nm in diameter.

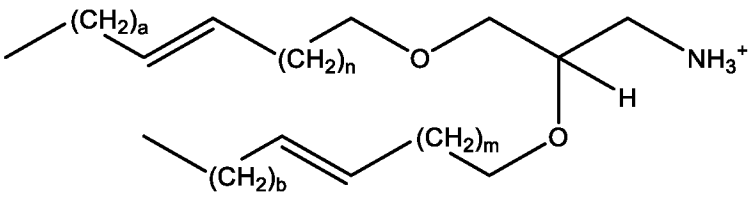
[0053] In exemplary instances, the nanoparticle is characterized by a zeta potential of about +40 mV to about +60 mV, e.g., about +40 mV to about +55 mV, about +40 mV to about +50 mV, about +40 mV to about +50 mV, about +40 mV to about +45 mV, about +45 mV to about +60 mV, about +50 mV to about +60 mV, about +55 mV to about +60 mV. In exemplary aspects, the nanoparticle has a zeta potential of about +45 mV to about +55 mV. The nanoparticle in various instances, has a zeta potential of about +50 mV. In various aspects, the zeta potential is greater than +30 mV or +35 mV. The zeta potential is one parameter which distinguishes the nanoparticles of the present disclosure and those described in Sayour et al., Oncoimmunology 6(1): e1256527 (2016).

[0054] In exemplary embodiments, the nanoparticles comprise a cationic lipid. In some embodiments, the cationic lipid is a low molecular weight cationic lipid such as those described in U.S. Patent Application No. 20130090372, the contents of which are herein incorporated by reference in their entirety. The cationic lipid in exemplary instances is a cationic fatty acid, a

cationic glycerolipid, a cationic glycerophospholipid, a cationic sphingolipid, a cationic sterol lipid, a cationic prenol lipid, a cationic saccharolipid, or a cationic polyketide. In exemplary aspects, the cationic lipid comprises two fatty acyl chains, each chain of which is independently saturated or unsaturated. In some instances, the cationic lipid is a diglyceride. For example, in some instances, the cationic lipid may be a cationic lipid of Formula I or Formula II:



[Formula I]



[Formula II]

wherein each of a, b, n, and m is independently an integer between 2 and 12 (e.g., between 3 and 10). In some aspects, the cationic lipid is a cationic lipid of Formula I wherein each of a, b, n, and m is independently an integer selected from 3, 4, 5, 6, 7, 8, 9, and 10. In exemplary instances, the cationic lipid is DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), or a derivative thereof. In exemplary instances, the cationic lipid is DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium propane), or a derivative thereof.

[0055] In some embodiments, the nanoparticles comprise liposomes formed from 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA) liposomes, DiLa2 liposomes from Marina Biotech (Bothell, Wash.), 1,2-dilinoleyloxy-3-dimethylaminopropane (DLin-DMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), and MC3 (US20100324120; herein incorporated by reference in its entirety). In some embodiments, the nanoparticles comprise liposomes formed from the synthesis of stabilized plasmid-lipid particles (SPLP) or stabilized nucleic acid lipid particle (SNALP) that have been previously described and shown to be suitable for oligonucleotide delivery in vitro and in vivo. The nanoparticles in some aspects are

composed of 3 to 4 lipid components in addition to the nucleic acid molecules. In exemplary aspects, the liposome comprises 55% cholesterol, 20% disteroylphosphatidyl choline (DSPC), 10% PEG-S-DSG, and 15% 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), as described by Jeffs et al., Pharm Res. 2005; 22(3):362-72. In exemplary instances, the liposome comprises 48% cholesterol, 20% DSPC, 2% PEG-c-DMA, and 30% cationic lipid, where the cationic lipid can be 1,2-distearloxy-N,N-dimethylaminopropane (DSDMA), DODMA, DLin-DMA, or 1,2-dilinolenyloxy-3-dimethylaminopropane (DLenDMA), as described by Heyes et al., J. Control Release 2005; 107(2): 276-87.

[0056] In some embodiments, the liposomes comprise from about 25.0% cholesterol to about 40.0% cholesterol, from about 30.0% cholesterol to about 45.0% cholesterol, from about 35.0% cholesterol to about 50.0% cholesterol and/or from about 48.5% cholesterol to about 60% cholesterol. In some embodiments, the liposomes may comprise a percentage of cholesterol selected from the group consisting of 28.5%, 31.5%, 33.5%, 36.5%, 37.0%, 38.5%, 39.0% and 43.5%. In some embodiments, the liposomes may comprise from about 5.0% to about 10.0% DSPC and/or from about 7.0% to about 15.0% DSPC.

[0057] In some embodiments, the liposomes are DiLa2 liposomes (Marina Biotech, Bothell, Wash.), SMARTICLES® (Marina Biotech, Bothell, Wash.), neutral DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) based liposomes (e.g., siRNA delivery for ovarian cancer (Landen et al. Cancer Biology & Therapy 2006 5(12)1708-1713); herein incorporated by reference in its entirety) and hyaluronan-coated liposomes (Quiet Therapeutics, Israel).

[0058] In various instances, the cationic lipid comprises 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA), dilinoleyl-methyl-4-dimethylaminobutyrate (DLin-MC3-DMA), or di((Z)-non-2-en-1-yl) 9-((4-(dimethylamino)butanoyl)oxy)heptadecanedioate (L319), and further comprise a neutral lipid, a sterol and a molecule capable of reducing particle aggregation, for example, a PEG or PEG-modified lipid.

[0059] The liposome in various aspects comprises DLin-DMA, DLin-K-DMA, 98N12-5, C12-200, DLin-MC3-DMA, DLin-KC2-DMA, DODMA, PLGA, PEG, PEG-DMG, PEGylated lipids and amino alcohol lipids. In some aspects, the liposome comprises a cationic lipid such as, but not limited to, DLin-DMA, DLin-D-DMA, DLin-MC3-DMA, DLin-KC2-DMA, DODMA and amino alcohol lipids. The amino alcohol cationic lipid comprises in some aspects lipids described in and/or made by the methods described in U.S. Patent Publication No. US20130150625, herein incorporated by reference in its entirety. As a non-limiting example, the cationic lipid in certain aspects is 2-amino-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-2-{[(9Z,2Z)-octadeca-9,12-dien-1-

yloxy]methyl}propan-1-ol (Compound 1 in US20130150625); 2-amino-3-[(9Z)-octadec-9-en-1-yloxy]-2-{[(9Z)-octadec-9-en-1-yloxy]methyl}propan-1-ol (Compound 2 in US20130150625); 2-amino-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-2-[(octyloxy)methyl]propan-1-ol (Compound 3 in US20130150625); and 2-(dimethylamino)-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-2-{[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]methyl}propan-1-ol (Compound 4 in US20130150625); or any pharmaceutically acceptable salt or stereoisomer thereof.

[0060] In various embodiments, the liposome comprises (i) at least one lipid selected from the group consisting of 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA), dilinoleyl-methyl-4-dimethylaminobutyrate (DLin-MC3-DMA), and di((Z)-non-2-en-1-yl) 9-((4-(dimethylamino)butanoyl)oxy)heptadecanedioate (L319); (ii) a neutral lipid selected from DSPC, DPPC, POPC, DOPE and SM; (iii) a sterol, e.g., cholesterol; and (iv) a PEG-lipid, e.g., PEG-DMG or PEG-cDMA, in a molar ratio of about 20-60% cationic lipid: 5-25% neutral lipid: 25-55% sterol; 0.5-15% PEG-lipid.

[0061] In some embodiments, the liposome comprises from about 25% to about 75% on a molar basis of a cationic lipid selected from 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA), dilinoleyl-methyl-4-dimethylaminobutyrate (DLin-MC3-DMA), and di((Z)-non-2-en-1-yl) 9-((4-(dimethylamino)butanoyl)oxy)heptadecanedioate (L319), e.g., from about 35 to about 65%, from about 45 to about 65%, about 60%, about 57.5%, about 50% or about 40% on a molar basis.

[0062] In some embodiments, the liposome comprises from about 0.5% to about 15% on a molar basis of the neutral lipid e.g., from about 3 to about 12%, from about 5 to about 10% or about 15%, about 10%, or about 7.5% on a molar basis. Examples of neutral lipids include, but are not limited to, DSPC, POPC, DPPC, DOPE and SM. In various aspects, the nanoparticle does not comprise a neutral lipid. In some embodiments, the formulation includes from about 5% to about 50% on a molar basis of the sterol (e.g., about 15 to about 45%, about 20 to about 40%, about 40%, about 38.5%, about 35%, or about 31% on a molar basis. An exemplary sterol is cholesterol. In some embodiments, the formulation includes from about 0.5% to about 20% on a molar basis of the PEG or PEG-modified lipid (e.g., about 0.5 to about 10%, about 0.5 to about 5%, about 1.5%, about 0.5%, about 1.5%, about 3.5%, or about 5% on a molar basis). In some embodiments, the PEG or PEG modified lipid comprises a PEG molecule of an average molecular weight of 2,000 Da. In other embodiments, the PEG or PEG modified lipid comprises a PEG molecule of an average molecular weight of less than 2,000, for example around 1,500 Da, around 1,000 Da, or around 500 Da. Examples of PEG-modified lipids include, but are not

limited to, PEG-distearoyl glycerol (PEG-DMG) (also referred herein as PEG-C14 or C14-PEG), PEG-cDMA (further discussed in Reyes et al. J. Controlled Release, 107, 276-287 (2005) the contents of which are herein incorporated by reference in their entirety).

[0063] In exemplary aspects, the cationic lipid may be selected from (20Z,23Z)—N,N-dimethylnonacosa-20,23-dien-10-amine, (17Z,20Z)—N,N-dimemylhexacosa-17,20-dien-9-amine, (1Z,19Z)—N,N-dimethylpentacosa-1 6, 19-dien-8-amine, (13Z,16Z)—N,N-dimethyldocosa-13,16-dien-5-amine, (12Z,15Z)—N,N-dimethylhenicosa-12,15-dien-4-amine, (14Z,17Z)—N,N-dimethyltricosa-14,17-dien-6-amine, (15Z,18Z)—N,N-dimethyltetracosa-15,18-dien-7-amine, (18Z,21Z)—N,N-dimethylheptacosa-18,21-dien-10-amine, (15Z,18Z)—N,N-dimethyltetracosa-15,18-dien-5-amine, (14Z,17Z)—N,N-dimethyltricosa-14,17-dien-4-amine, (19Z,22Z)—N,N-dimeihyloctacosa-19,22-dien-9-amine, (18Z,21 Z)—N,N-dimethylheptacosa-18,21-dien-8-amine, (17Z,20Z)—N,N-dimethylhexacosa-17,20-dien-7-amine, (16Z,19Z)—N,N-dimethylpentacosa-16,19-dien-6-amine, (22Z,25Z)—N,N-dimethylhentriaconta-22,25-dien-10-amine, (21 Z,24Z)—N,N-dimethyltriaconta-21,24-dien-9-amine, (18Z)—N,N-dimetylheptacos-18-en-10-amine, (17Z)—N,N-dimethylhexacos-17-en-9-amine, (19Z,22Z)—N,N-dimethyloctacosa-19,22-dien-7-amine, N,N-dimethylheptacosan-10-amine, (20Z,23Z)—N-ethyl-N-methylnonacosa-20,23-dien-10-amine, 1-[(11Z,14Z)-1-nonylicosa-11,14-dien-1-yl]pyrrolidine, (20Z)—N,N-dimethylheptacos-20-en-10-amine, (15Z)—N,N-dimethyl eptacos-15-en-10-amine, (14Z)—N,N-dimethylnonacos-14-en-10-amine, (17Z)—N,N-dimethylnonacos-17-en-10-amine, (24Z)—N,N-dimethyltritriacont-24-en-10-amine, (20Z)—N,N-dimethylnonacos-20-en-10-amine, (22Z)—N,N-dimethylhentriacont-22-en-10-amine, (16Z)—N,N-dimethylpentacos-16-en-8-amine, (12Z,15Z)—N,N-dimethyl-2-nonylhenicosa-12,15-dien-1-amine, (13Z,16Z)—N,N-dimethyl-3-nonyldocosa-13,16-dien-1-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]eptadecan-8-amine, 1-[(1S,2R)-2-hexylcyclopropyl]-N,N-dimethylnonadecan-10-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]nonadecan-10-amine, N,N-dimethyl-21-[(1S,2R)-2-octylcyclopropyl]henicosan-10-amine,N,N-dimethyl-1-[(1S,2S)-2-{[(1R,2R)-2-pentylcyclopropyl]methyl}cyclopropyl]nonadecan-10-amine,N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]hexadecan-8-amine, N,N-dimethyl-[(1R,2S)-2-undecylcyclopropyl]tetradecan-5-amine, N,N-dimethyl-3-{7-[(1S,2R)-2-octylcyclopropyl]heptyl}dodecan-1-amine, 1-[(1R,2S)-2-heptylcyclopropyl]-N,N-dimethyloctadecan-9-amine, 1-[(1S,2R)-2-decylcyclopropyl]-N,N-dimethylpentadecan-6-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]pentadecan-8-amine, R—N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-(octyloxy)propan-2-amine, S—N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-(octyloxy)propan-2-amine, 1-{2-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-1-[(octyloxy)methyl]ethyl}pyrrolidine, (2S)—N,N-dimethyl-1-

[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-[(5Z)-oct-5-en-1-yloxy]propan-2-amine, 1-{2-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-1-[(octyloxy)methyl]ethyl}azetidine, (2S)-1-(hexyloxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, (2S)-1-(heptyloxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-(nonyloxy)-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-[(9Z)-octadec-9-en-1-yloxy]-3-(octyloxy)propan-2-amine; (2S)—N,N-dimethyl-1-[(6Z,9Z,12Z)-octadeca-6,9,12-trien-1-yloxy]-3-(octyloxy)propan-2-amine, (2S)-1-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethyl-3-(pentyloxy)propan-2-amine, (2S)-1-(hexyloxy)-3-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethylpropan-2-amine, 1-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, 1-[(13Z,16Z)-docosa-13,16-dien-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, (2S)-1-[(13Z,16Z)-docosa-13,16-dien-1-yloxy]-3-(hexyloxy)-N,N-dimethylpropan-2-amine, (2S)-1-[(13Z)-docos-13-en-1-yloxy]-3-(hexyloxy)-N,N-dimethylpropan-2-amine, 1-[(13Z)-docos-13-en-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, 1-[(9Z)-hexadec-9-en-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, (2R)—N,N-dimethyl-H(1-metoyloctyl)oxyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, (2R)-1-[(3,7-dimethyloctyl)oxy]-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-(octyloxy)-3-({8-[(1S,2S)-2-{[(1R,2R)-2-pentylcyclopropyl]methyl}cyclopropyl]octyl}oxy)propan-2-amine, N,N-dimethyl-1-{[8-(2-oclylcyclopropyl)octyl]oxy}-3-(octyloxy)propan-2-amine and (11E,20Z,23Z)—N,N-dimethylnonacosa-11,20,2-trien-10-amine or a pharmaceutically acceptable salt or stereoisomer thereof.

[0064] In some embodiments, the nanoparticle comprises a lipid-polycation complex. The formation of the lipid-polycation complex may be accomplished by methods known in the art and/or as described in U.S. Patent Publication No. 20120178702, herein incorporated by reference in its entirety. As a non-limiting example, the polycation may include a cationic peptide or a polypeptide such as, but not limited to, polylysine, polyornithine and/or polyarginine. In some embodiments, the composition may comprise a lipid-polycation complex, which may further include a non-cationic lipid such as, but not limited to, cholesterol or dioleoyl phosphatidylethanolamine (DOPE).

[0065] In various aspects, the cationic liposomes optionally do not comprise a non-cationic lipid. Neutral molecules, in some aspects, may interfere with coiling/condensation of multi-lamellar nanoparticles resulting in RNA loaded liposomes greater than 200 nm in size. Cationic liposomes generated without helper molecules can comprise a size of about 70-200 nm (or

less). These constructs consist essentially of a cationic lipid with negatively charged nucleic acid, and may be formulated in a sealed rotary vacuum evaporator which prevents oxidation of the particles (when exposed to the ambient environment). In this embodiment, the absence of a helper lipid optimizes mRNA coiling into tightly packaged multilamellar NPs where each NP contains a greater amount of nucleic acid per particle. Due to increased nucleic acid payload per particle, these multi-lamellar RNA nanoparticles drive significantly greater innate immune responses, which are a significant predictor of efficacy for modulating the immune system.

[0066] In some aspects, the nucleic acid molecules are present at a nucleic acid molecule: cationic lipid ratio of about 1 to about 5 to about 1 to about 25. In some aspects, the nucleic acid molecules are present at a nucleic acid molecule: cationic lipid ratio of about 1 to about 5 to about 1 to about 20, optionally, about 1 to about 15, about 1 to about 10, or about 1 to about 7.5. As used herein, the term "nucleic acid molecule: cationic lipid ratio" is meant a mass ratio, where the mass of the nucleic acid molecule is relative to the mass of the cationic lipid. Also, in exemplary aspects, the term "nucleic acid molecule: cationic lipid ratio" is meant the ratio of the mass of the nucleic acid molecule, e.g., RNA, added to the liposomes comprising cationic lipids during the process of manufacturing the ML RNA NPs of the present disclosure. In exemplary aspects, the nanoparticle comprises less than or about 10 µg RNA molecules per 150 µg lipid mixture. In exemplary aspects, the nanoparticle is made by incubating about 10 µg RNA with about 150 µg liposomes. In alternative aspects, the nanoparticle comprises more RNA molecules per mass of lipid mixture. For example, the nanoparticle may comprise more than 10 µg RNA molecules per 150 µg liposomes. The nanoparticle in some instances comprises more than 15 µg RNA molecules per 150 µg liposomes or lipid mixture.

[0067] In various aspects, the nucleic acid molecules are RNA molecules, e.g., transfer RNA (tRNA), ribosomal RNA (rRNA), messenger RNA (mRNA). In various aspects, the RNA molecules comprise tRNA, rRNA, mRNA, or a combination thereof. In various aspects, the RNA is total RNA isolated from a cell. In exemplary aspects, the RNA is total RNA isolated from a diseased cell, such as, for example, a tumor cell or a cancer cell. Methods of obtaining total tumor RNA is known in the art and described herein at Example 1.

[0068] In exemplary instances, the RNA molecules are mRNA. In various aspects, mRNA is *in vitro* transcribed mRNA. In various instances, the mRNA molecules are produced by *in vitro* transcription (IVT). Suitable techniques of carrying out IVT are known in the art. In exemplary aspects, an IVT kit is employed. In exemplary aspects, the kit comprises one or more IVT reaction reagents. As used herein, the term "in vitro transcription (IVT) reaction reagent" refers

to any molecule, compound, factor, or salt, which functions in an IVT reaction. For example, the kit may comprise prokaryotic phage RNA polymerase and promoter (T7, T3, or SP6) with eukaryotic or prokaryotic extracts to synthesize proteins from exogenous DNA templates. Optionally, the RNA is *in vitro* transcribed mRNA, wherein the *in vitro* transcription template is cDNA made from RNA extracted from a tumor cell. In various aspects, the nanoparticle comprises a mixture of RNA which is RNA isolated from a tumor of a human, optionally, a malignant brain tumor, optionally, a glioblastoma, medulloblastoma, diffuse intrinsic pontine glioma, or a peripheral tumor with metastatic infiltration into the central nervous system. In various aspects, the RNA comprises a sequence encoding a poly(A) tail so that the *in vitro* transcribed RNA molecule comprises a poly(A) tail at the 3' end. In various aspects, the method of making a nanoparticle comprises additional processing steps, such as, for example, capping the *in vitro* transcribed RNA molecules.

[0069] The RNA (e.g., mRNAs) in exemplary aspects encode a protein. Optionally, the protein is selected from the group consisting of a tumor antigen, a cytokine, and a co-stimulatory molecule. Indeed, the protein is, in some aspects, selected from the group consisting of a tumor antigen, a co-stimulatory molecule, a cytokine, a growth factor, a hematopoietic factor, or a lymphokine, including, e.g., cytokines and growth factors that are effective in inhibiting tumor metastasis, and cytokines or growth factors that have been shown to have an antiproliferative effect on at least one cell population. Such cytokines, lymphokines, growth factors, or other hematopoietic factors include, but are not limited to: M-CSF, GM-CSF, TNF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IFN, TNFα, TNF1, TNF2, G-CSF, Meg-CSF, GM-CSF, thrombopoietin, stem cell factor, and erythropoietin. Additional growth factors for use herein include angiogenin, bone morphogenic protein-1, bone morphogenic protein-2, bone morphogenic protein-3, bone morphogenic protein-4, bone morphogenic protein-5, bone morphogenic protein-6, bone morphogenic protein-7, bone morphogenic protein-8, bone morphogenic protein-9, bone morphogenic protein-10, bone morphogenic protein-11, bone morphogenic protein-12, bone morphogenic protein-13, bone morphogenic protein-14, bone morphogenic protein-15, bone morphogenic protein receptor IA, bone morphogenic protein receptor IB, brain derived neurotrophic factor, ciliary neutrophic factor, ciliary neutrophic factor receptor α, cytokine-induced neutrophil chemotactic factor 1, cytokine-induced neutrophil, chemotactic factor 2 α, cytokine-induced neutrophil chemotactic factor 2 β, β endothelial cell growth factor, endothelin 1, epithelial-derived neutrophil attractant, glial cell line-derived neutrophic factor receptor α 1, glial cell line-derived neutrophic factor receptor α 2, growth related protein, growth related protein α, growth

related protein β, growth related protein γ, heparin binding epidermal growth factor, hepatocyte growth factor, hepatocyte growth factor receptor, insulin-like growth factor I, insulin-like growth factor receptor, insulin-like growth factor II, insulin-like growth factor binding protein, keratinocyte growth factor, leukemia inhibitory factor, leukemia inhibitory factor receptor α, nerve growth factor nerve growth factor receptor, neurotrophin-3, neurotrophin-4, pre-B cell growth stimulating factor, stem cell factor, stem cell factor receptor, transforming growth factor α, transforming growth factor β, transforming growth factor β1, transforming growth factor β1.2, transforming growth factor β2, transforming growth factor β3, transforming growth factor β5, latent transforming growth factor β1, transforming growth factor β binding protein I, transforming growth factor β binding protein II, transforming growth factor β binding protein III, tumor necrosis factor receptor type I, tumor necrosis factor receptor type II, urokinase-type plasminogen activator receptor, and chimeric proteins and biologically or immunologically active fragments thereof. In exemplary aspects, the tumor antigen is an antigen derived from a viral protein, an antigen derived from point mutations, or an antigen encoded by a cancer-germline gene. In exemplary aspects, the tumor antigen is pp65, p53, KRAS, NRAS, MAGEA, MAGEB, MAGEC, BAGE, GAGE, LAGE/NY-ESO1, SSX, tyrosinase, gp100/pmel17, Melan-A/MART-1, gp75/TRP1, TRP2, CEA, RAGE-1, HER2/NEU, WT1. In exemplary aspects, the co-stimulatory molecule is selected from the group consisting of CD80 and CD86. In some aspects, the protein is not expressed by a tumor cell or by a human. In exemplary instances, the protein is not related to a tumor antigen or cancer antigen. In some aspects, the protein is non-specific relative to a tumor or cancer. For example, the non-specific protein may be green fluorescence protein (GFP) or ovalbumin (OVA).

[0070] In various aspects, the nucleic acid layers comprise a sequence of a nucleic acid molecule expressed by slow-cycling cells (SCCs). The term "slow-cycling cells" or "SCCs" refers to tumor or cancer cells that proliferate at a slow rate. In exemplary aspects, the SCCs have a doubling time of at least about 50 hours. SCCs have been identified in numerous cancer tissues, including, melanoma, ovarian cancer, pancreatic adenocarcinoma, breast cancer, glioblastoma, and colon cancer. As taught in Deleyrolle et al., Brain 134(5): 1331-1343 (2011) (incorporated by reference herein, particularly with respect to the description of SCCs), SCCs display increased tumor-initiation properties and are stem cell like. Because of their slow proliferation rate, SCCs are also referred to as label-retaining cells (LRCs). In exemplary instances, the nucleic acid molecules are RNA extracted from isolated SCCs or are nucleic acid molecules which hybridize to RNA extracted from isolated SCCs. Optionally, the SCCs are isolated from a mixed tumor cell population obtained from a subject with a tumor (e.g., a

glioblastoma). As used herein, the term "mixed tumor cell population" refers to a heterogeneous cell population comprising tumor cells of different sub-types and comprising slow-cycling cells and at least one other tumor cell type, e.g., fast-cycling cells (FCCs).

[0071] In exemplary instances, the nanoparticle comprises a mixture or plurality of different RNA molecules expressed by SCCs. In certain instances, the mixture or plurality comprises at least 10 (e.g., at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90) different RNA molecules expressed by SCCs. In some aspects, the mixture or plurality comprises at least 100 (e.g., at least 150, at least 200, at least 250, at least 300, at least 350, at least 400, at least 450, at least 500, at least 550, at least 600, or more (e.g., at least 700, at least 800 at least 900)) different RNA molecules expressed by SCCs. In aspects, the nanoparticles comprise a mixture or plurality of RNA molecules which represent at least in part the transcriptome of SCCs. The term "transcriptome" refers to the sum total of all the messenger RNA molecules expressed from the genes of an organism. The term "SCC transcriptome" refers to the sum total of all the mRNA molecules expressed by SCCs. In particular instances, the SCC transcriptome is produced by first isolating total RNA from the tumor cells, which total RNA is then used to generate cDNA by RT-PCR using routine methods. The cDNA may be used to synthesize protected mRNA transcripts (e.g., 7-methyl guanosine capped RNA) using, for example, an Ambion® mMESSAGE mMACHINE® transcription kit. In exemplary aspects, the SCC transcriptome is the sum total of all the mRNA expressed from the genes listed in Figure 12. In alternative or additional embodiments, the nucleic acid molecules of the nanoparticles, e.g., the RNA, are de novo synthesized RNA encoded by at least two (e.g., at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9) different genes listed in Figure 12. In exemplary instances, the nucleic acid molecules are RNA encoded by at least 10 (e.g., at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90) different genes listed in Figure 12. In some aspects, the nucleic acid molecules are RNA encoded by at least 100 (e.g., at least 150, at least 200, at least 250, at least 300, at least 350, at least 400, at least 450, at least 500, at least 550, at least 600, or more (e.g., at least 700, at least 800 at least 900)) different genes listed Figure 12. Exemplary methods of isolating SCCs are described in the Examples.

[0072] In various instances, the RNA molecules are antisense molecules, optionally siRNA, shRNA, miRNA, or any combination thereof. The antisense molecule can be one which mediates RNA interference (RNAi). As known by one of ordinary skill in the art, RNAi is a ubiquitous mechanism of gene regulation in plants and animals in which target mRNAs are

degraded in a sequence-specific manner (Sharp, Genes Dev., 15, 485-490 (2001); Hutvagner et al., Curr. Opin. Genet. Dev., 12, 225-232 (2002); Fire et al., Nature, 391, 806-811 (1998); Zamore et al., Cell, 101, 25-33 (2000)). The natural RNA degradation process is initiated by the dsRNA-specific endonuclease Dicer, which promotes cleavage of long dsRNA precursors into double-stranded fragments between 21 and 25 nucleotides long, termed small interfering RNA (siRNA; also known as short interfering RNA) (Zamore, et al., Cell. 101, 25-33 (2000); Elbashir et al., Genes Dev., 15, 188-200 (2001); Hammond et al., Nature, 404, 293-296 (2000); Bernstein et al., Nature, 409, 363-366 (2001)). siRNAs are incorporated into a large protein complex that recognizes and cleaves target mRNAs (Nykanen et al., Cell, 107, 309-321 (2001)). It has been reported that introduction of dsRNA into mammalian cells does not result in efficient Dicer-mediated generation of siRNA and therefore does not induce RNAi (Caplen et al., Gene 252, 95-105 (2000); Ui-Tei et al., FEBS Lett, 479, 79-82 (2000)). The requirement for Dicer in maturation of siRNAs in cells can be bypassed by introducing synthetic 21 -nucleotide siRNA duplexes, which inhibit expression of transfected and endogenous genes in a variety of mammalian cells (Elbashir et al., Nature, 411: 494-498 (2001)).

[0073] In this regard, the RNA molecule in some aspects mediates RNAi and in some aspects is a siRNA molecule specific for inhibiting the expression of a protein. The term "siRNA" as used herein refers to an RNA (or RNA analog) comprising from about 10 to about 50 nucleotides (or nucleotide analogs) which is capable of directing or mediating RNAi. In exemplary embodiments, an siRNA molecule comprises about 15 to about 30 nucleotides (or nucleotide analogs) or about 20 to about 25 nucleotides (or nucleotide analogs), e.g., 21-23 nucleotides (or nucleotide analogs). The siRNA can be double or single stranded, preferably double-stranded.

[0074] In alternative aspects, the RNA molecule is alternatively a short hairpin RNA (shRNA) molecule specific for inhibiting the expression of a protein. The term "shRNA" as used herein refers to a molecule of about 20 or more base pairs in which a single-stranded RNA partially contains a palindromic base sequence and forms a double-strand structure therein (i.e., a hairpin structure). An shRNA can be an siRNA (or siRNA analog) which is folded into a hairpin structure. shRNAs typically comprise about 45 to about 60 nucleotides, including the approximately 21 nucleotide antisense and sense portions of the hairpin, optional overhangs on the non-loop side of about 2 to about 6 nucleotides long, and the loop portion that can be, e.g., about 3 to 10 nucleotides long. The shRNA can be chemically synthesized. Alternatively, the

shRNA can be produced by linking sense and antisense strands of a DNA sequence in reverse directions and synthesizing RNA in vitro with T7 RNA polymerase using the DNA as a template.

[0075] Though not wishing to be bound by any theory or mechanism it is believed that after shRNA is introduced into a cell, the shRNA is degraded into a length of about 20 bases or more (e.g., representatively 21, 22, or 23 bases), and causes RNAi, leading to an inhibitory effect. Thus, shRNA elicits RNAi and therefore can be used as an effective component of the disclosure. shRNA may preferably have a 3 -protruding end. The length of the double-stranded portion is not particularly limited, but is preferably about 10 or more nucleotides, and more preferably about 20 or more nucleotides. Here, the 3-protruding end may be preferably DNA, more preferably DNA of at least 2 nucleotides in length, and even more preferably DNA of 2-4 nucleotides in length.

[0076] In exemplary aspects, the antisense molecule is a microRNA (miRNA). As used herein the term "microRNA" refers to a small (e.g., 15-22 nucleotides), non-coding RNA molecule which base pairs with mRNA molecules to silence gene expression via translational repression or target degradation. microRNA and the therapeutic potential thereof are described in the art. See, e.g., Mulligan, *MicroRNA: Expression, Detection, and Therapeutic Strategies*, Nova Science Publishers, Inc., Hauppauge, NY, 2011; Bader and Lammers, "The Therapeutic Potential of microRNAs" *Innovations in Pharmaceutical Technology,* pages 52-55 (March 2011).

[0077] In certain instances, the RNA molecule is an antisense molecule, optionally, an siRNA, shRNA, or miRNA, which targets a protein of an immune checkpoint pathway for reduced expression. In various aspects, the protein of the immune checkpoint pathway is CTLA-4, PD-1, PD-L1, PD-L2, B7-H3, B7-H4, TIGIT, LAG3, CD112 TIM3, BTLA, or co-stimulatory receptor ICOS, OX40, 41BB, or GITR. The protein of the immune-checkpoint pathway in certain instances is CTLA4, PD-1, PD-L1, B7-H3, B7H4, or TIM3. Immune checkpoint signaling pathways are reviewed in Pardoll, Nature Rev Cancer 12(4): 252-264 (2012).

[0078] In exemplary embodiments, the NPs of the present disclosure comprise a mixture of RNA molecules. In exemplary aspects, the mixture of RNA molecules is RNA isolated from cells from a human and optionally, the human has a tumor. In some aspects, the mixture of RNA is RNA isolated from the tumor of the human. In exemplary aspects, the human has cancer, optionally, any cancer described herein. Optionally, the tumor from which RNA is isolated is selected from the group consisting of a glioma (including, but not limited to, a glioblastoma), a medulloblastoma, a diffuse intrinsic pontine glioma, or a peripheral tumor with

metastatic infiltration into the central nervous system (e.g., melanoma or breast cancer). In exemplary aspects, the tumor from which RNA is isolated is a tumor of a cancer, e.g., any of these cancers described herein.

[0079] In various aspects, the nanoparticles comprise a nucleic acid molecule (e.g., RNA molecule) comprising a nucleotide sequence encoding a chimeric protein comprising a LAMP protein. In certain aspects, the LAMP protein is a LAMP1, LAMP 2, LAMP3, LAMP4, or LAMP5 protein.

[0080] *Methods of Manufacture*

[0081] The present disclosure also provides a method of making a nanoparticle comprising a positively-charged surface and an interior comprising (i) a core and (ii) at least two nucleic acid (e.g., RNA) layers, wherein each nucleic acid layer is positioned between a cationic lipid bilayer, said method comprising: (A) mixing nucleic acid molecules and liposomes at a nucleic acid (e.g., RNA): liposome ratio of about 1 to about 5 to about 1 to about 25, such as about 1 to 5 to about 1 to about 20, optionally, about 1 to about 15, to obtain nucleic acid- (e.g., RNA-) coated liposomes. The liposomes are made by a process of making liposomes comprising drying a lipid mixture comprising a cationic lipid and an organic solvent by evaporating the organic solvent under a vacuum; and (B) mixing the RNA-coated liposomes with a surplus amount of liposomes.

[0082] In exemplary aspects, the nanoparticle made by the presently disclosed method accords with the descriptions of the nanoparticles described herein. For example, the nanoparticle made by the presently disclosed methods has a zeta potential of about +40 mV to about +60 mV, optionally, about +45 mV to about +55 mV. Optionally, the zeta potential of the nanoparticle made by the presently disclosed methods is about +50 mV. In various aspects, the core of the nanoparticle made by the presently disclosed methods comprises less than about 0.5 wt% nucleic acid and/or the core comprises a cationic lipid bilayer and/or the outermost layer of the nanoparticle comprises a cationic lipid bilayer and/or the surface of the nanoparticle comprises a plurality of hydrophilic moieties of the cationic lipid of the cationic lipid bilayer.

[0083] In exemplary aspects, the lipid mixture comprises the cationic lipid and the organic solvent at a ratio of about 40 mg cationic lipid per mL organic solvent to about 60 mg cationic lipid per mL organic solvent, optionally, at a ratio of about 50 mg cationic lipid per mL organic solvent. In various instances, the process of making liposomes further comprises rehydrating the lipid mixture with a rehydration solution to form a rehydrated lipid mixture and then agitating,

resting, and sizing the rehydrated lipid mixture. Optionally, sizing the rehydrated lipid mixture comprises sonicating, extruding and/or filtering the rehydrated lipid mixture.

[0084] A description of an exemplary method of making a nanoparticle comprising a positively-charged surface and an interior comprising (i) a core and (ii) at least two nucleic acid layers, wherein each nucleic acid layer is positioned between a cationic lipid bilayer is provided herein at Example 1. Any one or more of the steps described in Example 1 may be included in the presently disclosed method. For instance, in some embodiments, the method comprises one or more steps required for preparing the RNA prior to being complexed with the liposomes. In exemplary aspects, the method comprises downstream steps to prepare the nanoparticles for administration to a subject, e.g., a human. In exemplary instances, the method comprises formulating the NP for intravenous injection. The method comprises in various aspects adding one or more pharmaceutically acceptable carriers, diluents, or excipients, and optionally comprises packaging the resulting composition in a container, e.g., a vial, a syringe, a bag, an ampoule, and the like. The container in some aspects is a ready-to-use container and optionally is for single-use.

[0085] Further provided herein are nanoparticles made by the presently disclosed method of making a nanoparticle.

[0086] *Cells and Populations Thereof*

[0087] Additionally provided herein is a cell (e.g., an isolated cell or an *ex vivo* cell) comprising (e.g., transfected with) a nanoparticle of the present disclosure. In exemplary aspects, the cell is any type of cell that can contain the presently disclosed nanoparticle. The cell in some aspects is a eukaryotic cell, e.g., plant, animal, fungi, or algae. In alternative aspects, the cell is a prokaryotic cell, e.g., bacteria or protozoa. In exemplary aspects, the cell is a cultured cell. In alternative aspects, the cell is a primary cell, i.e., isolated directly from an organism (e.g., a human). The cell may be an adherent cell or a suspended cell, i.e., a cell that grows in suspension. The cell in exemplary aspects is a mammalian cell. Most preferably, the cell is a human cell. The cell can be of any cell type, can originate from any type of tissue, and can be of any developmental stage. In exemplary aspects, the cell is an antigen presenting cell (APC). As used herein, "antigen presenting cell" or "APC" refers to an immune cell that mediates the cellular immune response by processing and presenting antigens for recognition by certain T cells. In exemplary aspects, the APC is a dendritic cell, macrophage, Langerhans cell or a B cell. In exemplary aspects, the APC is a dendritic cell (DC). In exemplary aspects,

when the cells are administered to a subject, e.g., a human, the cells are autologous to the subject. In exemplary instances, the immune cell is a tumor associated macrophage (TAM).

[0088] Also provided by the present disclosure is a population of cells wherein at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95% of the population are cells comprising (e.g., transfected with) a nanoparticle of the present disclosure. The population of cells in some aspects is heterogeneous cell population or, alternatively, in some aspects, is a substantially homogeneous population, in which the population comprises mainly cells comprising a nanoparticle of the present disclosure. If cells are intended to be administered to a subject, the cells may be autologous or allogeneic with respect to the subject to be treated.

[0089] *Pharmaceutical Compositions*

[0090] Provided herein are compositions comprising a nanoparticle of the present disclosure, a cell comprising the nanoparticle of the present disclosure, a population of cells of the present disclosure, or any combination thereof, and a pharmaceutically acceptable carrier, excipient or diluent. In exemplary aspects, the composition is a pharmaceutical composition comprising a plurality of nanoparticles according to the present disclosure and a pharmaceutically acceptable carrier, diluent, or excipient and intended for administration to a human. In exemplary aspects, the composition is a sterile composition. In exemplary instances, the composition comprises a plurality of nanoparticles of the present disclosure. Optionally, at least 50% of the nanoparticles of the plurality have a diameter between about 100 nm to about 250 nm. In various aspects, the composition comprises about 10^{10} nanoparticles per mL to about 10^{15} nanoparticles per mL, optionally about 10^{12} nanoparticles ± 10% per mL.

[0091] In exemplary aspects, the composition of the present disclosure may comprise additional components other than the nanoparticle, cell comprising the nanoparticle, or population of cells. The composition, in various aspects, comprises any pharmaceutically acceptable ingredient, including, for example, acidifying agents, additives, adsorbents, aerosol propellants, air displacement agents, alkalizing agents, anticaking agents, anticoagulants, antimicrobial preservatives, antioxidants, antiseptics, bases, binders, buffering agents, chelating agents, coating agents, coloring agents, desiccants, detergents, diluents, disinfectants, disintegrants, dispersing agents, dissolution enhancing agents, dyes, emollients, emulsifying agents, emulsion stabilizers, fillers, film forming agents, flavor enhancers, flavoring agents, flow enhancers, gelling agents, granulating agents, humectants, lubricants, mucoadhesives, ointment bases, ointments, oleaginous vehicles, organic bases, pastille bases, pigments, plasticizers, polishing agents, preservatives, sequestering agents, skin penetrants, solubilizing

agents, solvents, stabilizing agents, suppository bases, surface active agents, surfactants, suspending agents, sweetening agents, therapeutic agents, thickening agents, tonicity agents, toxicity agents, viscosity-increasing agents, water-absorbing agents, water-miscible cosolvents, water softeners, or wetting agents. *See, e.g.,* the *Handbook of Pharmaceutical Excipients*, Third Edition, A. H. Kibbe (Pharmaceutical Press, London, UK, 2000), which is incorporated by reference in its entirety. *Remington's Pharmaceutical Sciences*, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980), which is incorporated by reference in its entirety.

[0092] The composition of the present disclosure can be suitable for administration by any acceptable route, including parenteral and subcutaneous. Other routes include intravenous, intradermal, intramuscular, intraperitoneal, intranodal and intrasplenic, for example. In exemplary aspects, when the composition comprises the liposomes (not cells comprising the liposomes), the composition is suitable for systemic (e.g., intravenous) administration.

[0093] If the composition is in a form intended for administration to a subject, it can be made to be isotonic with the intended site of administration. For example, if the solution is in a form intended for administration parenterally, it can be isotonic with blood. The composition typically is sterile. In certain embodiments, this may be accomplished by filtration through sterile filtration membranes. In certain embodiments, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag, or vial having a stopper pierceable by a hypodermic injection needle, or a prefilled syringe. In certain embodiments, the composition may be stored either in a ready-to-use form or in a form (e.g., lyophilized) that is reconstituted or diluted prior to administration.

[0094] *Use*

[0095] Without being bound to any particular theory, the data provided herein support the use of the presently disclosed RNA NPs for increasing an immune response against a tumor in a subject. Accordingly, a method of increasing an immune response against a tumor in a subject is provided by the present disclosure. In exemplary embodiments, the method comprises administering to the subject the pharmaceutical composition of the present disclosure. In exemplary aspects, the nucleic acid molecules are mRNA. Optionally, the composition is systemically administered to the subject. For example, the composition is administered intravenously. In various aspects, the pharmaceutical composition is administered in an amount which is effective to activate dendritic cells (DCs) in the subject. In various instances, the immune response is a T cell-mediated immune response. Optionally, the T cell-mediated

immune response comprises activity by tumor infiltrating lymphocytes (TILs). In exemplary aspects, the immune response is the innate immune response.

[0096] In various aspects, the tumor is refractory to immune checkpoint therapy prior to administration of the composition comprising RNA-LPs, i.e., one or more ICIs has reduced efficacy in eliciting an immune response against the tumor. Alternatively, the tumor is not refractory, but the method further enhances sensitivity to the immune response such that enhanced tumor cell death is achieved.

[0097] The data provided herein also support the use of the presently disclosed RNA NPs for increasing dendritic cell (DC) activation in a subject. A method of activating DCs or increasing DC activation in a subject is accordingly furthermore provided. In exemplary embodiments, the method comprises administering to the subject the pharmaceutical composition of the present disclosure. In exemplary aspects, the nucleic acid molecules are mRNA. Optionally, the composition is systemically administered to the subject. For example, the composition is administered intravenously. In various aspects, the pharmaceutical composition is administered in an amount which is effective to increase an immune response against a tumor in the subject. In various instances, the immune response is a T cell-mediated immune response. Optionally, the T cell-mediated immune response comprises activity by tumor infiltrating lymphocytes (TILs). In exemplary aspects, the immune response is the innate immune response.

[0098] The present disclosure also provides a method of increasing sensitivity of a tumor to treatment with an immune checkpoint inhibitor (ICI) in a subject. In exemplary embodiments, the method comprises administering to the subject a composition comprising a nanoparticle described herein, e.g., a nanoparticle comprising a positively-charged surface and an interior comprising (i) a core and (ii) at least two nucleic acid layers, wherein each nucleic acid layer is positioned between a cationic lipid bilayer, optionally, wherein the composition is systemically administered to the subject.

[0099] The present disclosure further provides a method of treating a subject with an immune checkpoint inhibitor (ICI)-resistant tumor. In exemplary embodiments, the method comprises administering to the subject (a) a composition comprising a nanoparticle described herein, e.g., a nanoparticle comprising a positively-charged surface and an interior comprising (i) a core and (ii) at least two nucleic acid layers, wherein each nucleic acid layer is positioned between a cationic lipid bilayer, and (b) an ICI. Optionally, the composition is systemically administered to the subject.

[00100] As used herein, an "immune checkpoint inhibitor" or "ICI" is any agent (e.g., compound or molecule) that that decreases, blocks, inhibits, abrogates or interferes with the function of a protein of an immune checkpoint pathway. Proteins of the immune checkpoint pathway regulate immune responses and, in some instances, prevent T cells from attacking cancer cells. In various aspects, the protein of the immune checkpoint pathway is, for example, CTLA-4, PD-1, PD-L1, PD-L2, B7-H3, B7-H4, TIGIT, VISTA, LAG3, CD112 TIM3, BTLA, or co-stimulatory receptor ICOS, OX40, 41BB, or GITR. In various aspects, the ICI is a small molecule, an inhibitory nucleic acid, or an inhibitor polypeptide. In various aspects, the ICI is an antibody, antigen-binding antibody fragment, or an antibody protein product, that binds to and inhibits the function of the protein of the immune checkpoint pathway. Suitable ICIs which are antibodies, antigen-binding antibody fragments, or an antibody protein products are known in the art and include, but are not limited to, ipilimumab (CTLA-4; Bristol Meyers Squibb), nivolumab (PD-1; Bristol Meyers Squibb), pembrolizumab (PD-1; Merck), atezolizumab (PD-L1; Genentech), avelumab (PD-L1; Merck), and durvalumab (PD-L1; Medimmune) (Wei et al., Cancer Discovery 8: 1069-1086 (2018)). Other examples of ICIs include, but are not limited to, IMP321 (LAG3: Immuntep); BMS-986016 (LAG3; Bristol Meyers Squibb); IPH2101 (KIR; Innate Pharma); tremelimumab (CTLA-4; Medimmune); pidilizumab (PD-1; Medivation); MPDL3280A (PD-L1; Roche); MEDI4736 (PD-L1; AstraZeneca); MSB0010718C (PD-L1; EMD Serono); AUNP12 (PD-1; Aurigene); MGA271 (B7-H3: MacroGenics); and TSR-022 (TIM3; Tesaro).

[00101] In various aspects, the ICI is a PD-L1 inhibitor. Programmed death-ligand 1 (PD-L1; also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1)) is a transmembrane protein that functions to suppress the immune system in, e.g., pregnancy, tissue allografts, and autoimmune disease. Binding of PD-L1 to its receptor PD-1 transmits an inhibitory signal that reduces the proliferation and function of T cells and can induce apoptosis. For example, the PD-L1 inhibitor binds to and inhibits the function of PD-L1. In various aspects, the PD-L1 inhibitor is an anti-PD-L1 antibody, antigen binding antibody fragment, or an antibody-like molecule.

[00102] In various aspects, the ICI is a PD-1 inhibitor. "Programmed Death-1" (PD-1), also known as cluster of differentiation 279 (CD279), refers to an immunoinhibitory receptor belonging to the CD28 family. PD-1 is expressed on previously activated T cells in vivo, and binds to two ligands, PD-L1 and PD-L2. The human PD-1 sequence can be found under GenBank Accession No. U64863. For example, the PD-1 inhibitor binds to and inhibits the function of PD-1, e.g., an anti-PD-1 antibody, antigen binding antibody fragment, or an antibody-

like molecule. In various aspects, the PD-1 inhibitor is durvalumab, atezolizumab, or avelumab. In various aspects, the ICI is a PD-L2 inhibitor. For example, the PD-L2 inhibitor binds to and inhibits the function of PD-L2, e.g., an anti-PD-L2 antibody, antigen binding antibody fragment, or an antibody-like molecule.

[00103] Examples of PD-I and PD-L1 inhibitors are described in, e.g., U.S. Patent Nos. 7,488,802; 7,943,743; 8,008,449; 8,168,757; 8,217,149: and PCT Patent Publication Nos. WO03042402, WO2008156712, WO2010089411, WO2010036959, WO2011066342, WO2011159877, WO2011082400, and WO2011161699; which are incorporated by reference herein in their entireties.

[00104] Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152), is a membrane protein expressed on T cells and regulatory T cells (Treg). CTLA-4 binds B7-1 (CD80) and B7-2 (CD86) on antigen-presenting cells (APC), which inhibits the adaptive immune response. In humans, CTLA-4 is encoded in various isoforms; an exemplary amino acid sequence is available as GenBank Accession No. NP_001032720. A representative anti-CTLA-4 antibody is ipilimumab (YERVOY®, Bristol-Myers Squibb).

[00105] As used herein, the term "antibody" refers to a protein having a conventional immunoglobulin format, comprising heavy and light chains, and comprising variable and constant regions. For example, an antibody may be an IgG which is a "Y-shaped" structure of two identical pairs of polypeptide chains, each pair having one "light" (typically having a molecular weight of about 25 kDa) and one "heavy" chain (typically having a molecular weight of about 50-70 kDa). An antibody may be cleaved into fragments by enzymes, such as, e.g., papain and pepsin. Papain cleaves an antibody to produce two Fab fragments and a single Fc fragment. Pepsin cleaves an antibody to produce a $F(ab')_2$ fragment and a pFc' fragment. In exemplary aspects, the ICI is an antigen binding antibody fragment, e.g., a Fab, Fc, $F(ab')_2$, or a pFc'. The architecture of antibodies has been exploited to create a growing range of alternative antibody formats that spans a molecular-weight range of at least or about 12–150 kDa and a valency (n) range from monomeric (n = 1), dimeric (n = 2) and trimeric (n = 3) to tetrameric (n = 4) and potentially higher; such alternative antibody formats are referred to herein as "antibody-like molecules". Antibody-like molecules can be an antigen binding format based on antibody fragments, e.g., scFvs, Fabs and VHH/VH, which retain full antigen-binding capacity. The smallest antigen-binding fragment that retains its complete antigen binding site is the Fv fragment, which consists entirely of variable (V) regions. A soluble, flexible amino acid peptide linker is used to connect the V regions to a scFv (single chain fragment variable) fragment for

stabilization of the molecule, or the constant (C) domains are added to the V regions to generate a Fab fragment [fragment, antigen-binding]. Both scFv and Fab are widely used fragments that can be easily produced in prokaryotic hosts. Other antibody-like molecules include disulfide-bond stabilized scFv (ds-scFv), single chain Fab (scFab), as well as di- and multimeric antibody formats like dia-, tria- and tetra-bodies, or minibodies (miniAbs) that comprise different formats consisting of scFvs linked to oligomerization domains. The smallest fragments are VHH/VH of camelid heavy chain Abs as well as single domain Abs (sdAb). The building block that is most frequently used to create novel antibody formats is the single-chain variable (V)-domain antibody fragment (scFv), which comprises V domains from the heavy and light chain (VH and VL domain) linked by a peptide linker of ~15 amino acid residues. A peptibody or peptide-Fc fusion is yet another antibody-like molecule. The structure of a peptibody consists of a biologically active peptide grafted onto an Fc domain. Peptibodies are well-described in the art. See, e.g., Shimamoto et al., mAbs 4(5): 586-591 (2012). Other antibody-like molecules include a single chain antibody (SCA); a diabody; a triabody; a tetrabody; bispecific or trispecific antibodies, and the like. Bispecific antibodies can be divided into five major classes: BsIgG, appended IgG, BsAb fragments, bispecific fusion proteins and BsAb conjugates. See, e.g., Spiess et al., Molecular Immunology 67(2) Part A: 97-106 (2015). In exemplary aspects, the antibody-like molecule comprises any one of these antibody-like molecules (e.g., scFv, Fab VHH/VH, Fv fragment, ds-scFv, scFab, dimeric antibody, multimeric antibody (e.g., a diabody, triabody, tetrabody), miniAb, peptibody VHH/VH of camelid heavy chain antibody, sdAb, diabody; a triabody; a tetrabody; a bispecific or trispecific antibody, BsIgG, appended IgG, BsAb fragment, bispecific fusion protein, and BsAb conjugate).

[00106] As used herein, the term “inhibit” and words stemming therefrom does not require a 100% or complete inhibition or abrogation. Rather, there are varying degrees of inhibition of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. The ICIs may inhibit the onset or re-occurrence of the disease or a symptom thereof to any amount or level. In exemplary embodiments, the inhibition provided by the methods is at least or about a 10% inhibition (e.g., at least or about a 20% inhibition, at least or about a 30% inhibition, at least or about a 40% inhibition, at least or about a 50% inhibition, at least or about a 60% inhibition, at least or about a 70% inhibition, at least or about a 80% inhibition, at least or about a 90% inhibition, at least or about a 95% inhibition, at least or about a 98% inhibition).

[00107] As used herein “sensitivity” refers to the way a tumor reacts to a drug/compound, e.g., a ICI inhibitor (e.g., PD-L1 inhibitor). In exemplary aspects, “sensitivity” means “responsive

to treatment" and the concepts of "sensitivity" and "responsiveness" are positively associated in that a tumor or cancer cell that is responsive to a drug/compound treatment is said to be sensitive to that drug. "Sensitivity" in exemplary instances is defined according to Pelikan, Edward, Glossary of Terms and Symbols used in Pharmacology (Pharmacology and Experimental Therapeutics Department Glossary at Boston University School of Medicine), as the ability of a population, an individual or a tissue, relative to the abilities of others, to respond in a qualitatively normal fashion to a particular drug dose. The smaller the dose required producing an effect, the more sensitive is the responding system. "Sensitivity" may be measured or described quantitatively in terms of the point of intersection of a dose-effect curve with the axis of abscissal values or a line parallel to it; such a point corresponds to the dose just required to produce a given degree of effect. In analogy to this, the "sensitivity" of a measuring system is defined as the lowest input (smallest dose) required producing a given degree of output (effect). In exemplary aspects, "sensitivity" is opposite to "resistant" and the concept of "resistance" is negatively associated with "sensitivity". For example, a tumor that is resistant to a drug treatment is neither sensitive nor responsive to that drug, and that drug is not an effective treatment for that tumor or cancer cell. In the context of ICI's, a tumor which is insensitive to ICIs is one which does not respond to ICI therapy in a clinically significant way. Improving the sensitivity of a tumor to an ICI encompasses, e.g., any improvement in the clinical responsiveness to ICI therapy, which may be detected by a reduction in tumor volume or increase in tumor cell death, a reduction in the dose of ICI required to achieve a clinically detectable response, an increase in the time interval between ICI doses (requiring less frequent dosing) while maintaining a clinically detectable response, and the like. "Sensitivity" also is used herein with respect to a host immune response. In this respect, a tumor which evades a host immune response is "resistant" (or refractory). A tumor that is "sensitive" to a host immune response is recognized by the host immune system and subject to attack by immune effector cells. A tumor that is "sensitive" to a host immune response is recognized by the host immune system and subject to attack by immune effector cells.

[00108] In the context of the disclosure, administration of the RNA-LP of the disclosure sensitizes a tumor to an ICI, and together the two active agents increase the sensitivity of the tumor to a host immune response. Remarkably, the RNA-LPs of the instant disclosure can transition an immunologically "cold" tumor, e.g., a tumor lacking infiltrating T cells and/or which is not recognized by the immune system, into an immunologically "hot" tumor, i.e., a tumor exhibiting, e.g., lymphocyte infiltration and interferon gamma production in the tumor microenvironment. As explained herein, immunological treatment of "cold" tumors presents a

great challenge due, at least in part, to the absence of an adaptive immune response. Cancers that tend to give rise to immunologically "cold" tumors include, but are not limited to, glioblastomas, ovarian cancer, prostate cancer, pancreatic cancer, and many breast cancers. "Cold" tumors are limited to these cancer types, however; as cancers evolve in a subject, some develop resistance mechanisms that allow evasion of the immune system. Surprisingly, the nanoparticles of the disclosure "reprogram" the tumor to be recognized by the host immune system. The materials and methods of the disclosure represent a significant advancement in the art by providing a means to expand patient populations responsive to ICIs and immunotherapy generally.

[00109] The increase in sensitivity provided by the methods of the present disclosure may be at least or about a 1% to about a 10% increase (e.g., at least or about a 1% increase, at least or about a 2% increase, at least or about a 3% increase, at least or about a 4% increase, at least or about a 5% increase, at least or about a 6% increase, at least or about a 7% increase, at least or about a 8% increase, at least or about a 9% increase, at least or about a 9.5% increase, at least or about a 9.8% increase, at least or about a 10% increase) relative to a control. The increase in sensitivity provided by the methods of the present disclosure may be at least or about a 10% to greater than about a 95% increase (e.g., at least or about a 10% increase, at least or about a 20% increase, at least or about a 30% increase, at least or about a 40% increase, at least or about a 50% increase, at least or about a 60% increase, at least or about a 70% increase, at least or about a 80% increase, at least or about a 90% increase, at least or about a 95% increase, at least or about a 98% increase, at least or about a 100% increase) relative to a control. In exemplary aspects, the control is cancer or tumor or a subject or a population of subjects that was not treated with the presently disclosed pharmaceutical composition or wherein the subject or population of subjects was treated with a placebo. In some aspects, the "control" is the tumor or cancer of the subject prior to FNA-LP therapy.

[00110] Increased sensitivity to an ICI or increased sensitivity to host immune response may be determined in any of a number of ways. For example, administration of the RNA-LP and ICI may increase the number of cytotoxic T cells in a tumor and/or enhance cytotoxic T cell activity. For example, in various embodiments, the method may increase perforin, IFN-gamma, and/or granzyme production by cytotoxic T cells and increase cytolytic activity. Further, the method described herein may enhance T cell survival, promote T cell longevity, and/or restrict loss of replicative potential. Methods of measuring T cell activity and immune responses are known in the art. T cell activity can be measured by, for example, a cytotoxicity assay, such as those

described in Fu et al., PLoS ONE 5(7): e11867 (2010). Other T cell activity assays are described in Bercovici et al., Clin Diagn Lab Immunol. 7(6): 859-864 (2000). Methods of measuring immune responses are described in e.g., Macatangay et al., Clin Vaccine Immunol 17(9): 1452-1459 (2010), and Clay et al., Clin Cancer Res.7(5):1127-35 (2001). In various aspects, the method of the disclosure enhances cytotoxic T cell mediated killing of cancer cells within the tumor.

[00111] The methods of the present disclosure may comprise the above described step(s) alone or in combination with other steps. The methods may comprise repeating any one of the above-described step(s) and/or may comprise additional steps, aside from those described above. For example, the presently disclosed methods may further comprise steps for making or preparing the nanoparticles or compositions of the present disclosure. For instance, the presently disclosed methods further comprise obtaining a sample of the tumor of the subject, optionally, via a biopsy. The methods also may further comprise isolating total RNA from the cells of the tumor, generating cDNA from the total RNA via reverse transcription, and amplifying mRNA from the cDNA. The presently disclosed methods also in some aspects further comprise mixing the mRNA and the cationic lipid at a RNA: cationic lipid ratio of about 1 to about 10 to about 1 to about 20 (e.g., about 1 to about 19, about 1 to about 18, about 1 to about 17, about 1 to about 16, about 1 to about 15, about 1 to about 14, about 1 to about 13, about 1 to about 12, about 1 to about 11). In exemplary instances, the presently disclosed methods further comprise mixing the mRNA and the cationic lipid at a RNA: cationic lipid ratio of about 1 to about 15.

[00112] In exemplary aspects, the method comprises administering an ICI to the subject. In this regard, the present disclosure further provides a method of treating a subject with an immune checkpoint inhibitor (ICI)-resistant tumor. In exemplary aspects, the method comprises administering to the subject (a) a composition comprising a liposome comprising a cationic lipid and nucleic acid molecules, and (b) a PD-L1 inhibitor, wherein the liposome is systemically administered to the subject. The composition and liposome may be any of those described herein. For example, the liposome (liposome nanoparticle) may comprise DOTAP and the nucleic acid molecules may be a mixture of mRNA expressed by the tumor of the subject. In exemplary aspects, the composition comprising the liposome comprises a heterogeneous mixture of liposomes varied in size, though having a diameter within the range of 50 nm to about 250 nm. In exemplary aspects, the liposomes have a zeta potential of about 30 mV to about 60 mV, optionally, about 40 mV to about 50 mV. In exemplary aspects, the PD-L1 inhibitor is a PD-

L1 antibody. PD-L1 inhibitors are known in the art and include, but are not limited to, atezolizumab, avelumab, and durvalumab.

[00113] The disclosure further provides methods of increasing activated plasmacytoid (pDCs) in a subject, comprising administering the nanoparticles (or composition comprising the nanoparticles) described herein to the subject. The disclosed methods are useful, e.g., in settings relating to treatment with and preparation of dendritic cell (DC) vaccines. DC vaccines are reviewed in Pyzer et al., Hum Vaccin Immunother 10(11): 3125-3131 (2014). In exemplary aspects, the presently disclosed methods of increasing activated pDCs in a subject can further comprise isolating the pDCs from the subject. pDCs are distinguished from other DC subsets by expression of surface markers CD303 (BDCA2), CD304 (BDCA4), CD123 (IL-3R), and CD45RA in humans. Musumeci et al., Front. Immunol. 2019, Vol. 10, Article 1222. Methods of obtaining pDCs from a subject are known in the art and include, for example, leukapheresis. The pDCs thus obtained from the subject may be cultured and primed for antigen presentation. Thus pDCs can be loaded with antigen, for example, by pulsing the cells with an antigenic peptide or with whole tumor cell as a source of antigen. Alternatively or additionally, the pDCs may be primed or activated by culturing with a fusion protein comprising prostatic acid phosphatase (PAP) and GM-CSF. The fusion protein may be the same as the one found in PROVENGE® (sipuleucel-T). The pDCs once primed may then be administered to the subject from which they were obtained. In exemplary aspects, the pDCs are intradermally or subcutaneously administered to the subject.

[00114] Accordingly, the present disclosure also provides methods of treating a subject with a tumor or cancer. In exemplary aspects, the method comprises (i) increasing the number of activated plasmacytoid dendritic cells (pDCs) in the subject in accordance with the presently disclosed method of increasing activated pDCs, (ii) isolating white blood cells (WBCs) from the subject, (iii) isolating dendritic cells (DCs) from the WBCs, (iv) contacting the DCs with a fusion protein comprising prostatic acid phosphatase (PAP) and GM-CSF, and (v) administering the DCs to subject. The present disclosure also provides methods of preparing a dendritic cell vaccine. In exemplary aspects, the method comprises (i) increasing the number of activated plasmacytoid dendritic cells (pDCs) in the subject in accordance with the presently disclosed method of increasing activated pDCs, (ii) isolating white blood cells (WBCs) from the subject, (iii) isolating dendritic cells (DCs) from the WBCs, and (iv) contacting the DCs with a fusion protein comprising prostatic acid phosphatase (PAP) and GM-CSF. In exemplary aspects, the DCs are genetically engineered to express a protein. The protein in some aspects is a tumor

antigen. In alternative aspects, the protein is an antigen-presenting molecules, e.g., MHC, fused to a peptide. The WBCs may be isolated by known techniques, including, for example, leukapheresis. Isolation of DCs from WBCs may accomplished through methods known in the art, such as, e.g., fluorescence activated cell sorting (FACS). As used herein, the term "prostatic acid phosphatase" or "PAP" refers to a glycoprotein synthesized by the prostate gland and functions as an acid phosphatase, which hydrolyzes phosphate esters in acidic medium. PAP was identified more than 50 years ago as a marker for prostate cancer.

[00115] With regard to the presently disclosed methods, the nanoparticle in various aspects comprises at least three (e.g., at least four or at least five or more) nucleic acid layers, each of which is positioned between a cationic lipid bilayer. In various instances, the outermost layer of the nanoparticle comprises a cationic lipid bilayer. In exemplary aspects, the surface comprises a plurality of hydrophilic moieties of the cationic lipid of the cationic lipid bilayer. Optionally, the core comprises a cationic lipid bilayer. In various instances, the core comprises less than about 0.5 wt% nucleic acid. In exemplary aspects, the diameter of the nanoparticle is about 50 nm to about 250 nm in diameter, optionally, about 70 nm to about 200 nm in diameter. In various aspects, the nanoparticle comprises a zeta potential of about 40 mV to about 60 mV, optionally, about 45 mV to about 55 mV. Optionally, the nanoparticle comprises a zeta potential of about 50 mV. In exemplary aspects, the nanoparticle comprises nucleic acid molecules and cationic lipid at a ratio of about 1 to about 5 to about 1 to about 20, optionally, about 1 to about 15 or about 1 to about 7.5. In various aspects, the cationic lipid is DOTAP or DOTMA. Optionally, the nucleic acid molecules are RNA molecules. In various instances, the RNA molecules are mRNA. In certain aspects, the mRNA is *in vitro* transcribed mRNA wherein the *in vitro* transcription template is cDNA made from RNA extracted from a tumor cell. In various aspects, the mRNAs encode a protein. The protein in some instances is selected from the group consisting of: a tumor antigen, a cytokine, or a co-stimulatory molecule. Optionally, the protein is not expressed by a tumor cell or by a human. Also, in other instances, the RNA molecules are antisense molecules, optionally siRNA, shRNA, miRNA, or any combination thereof. Optionally, the nanoparticle comprises a mixture of RNA molecules. In various instances, the mixture of RNA molecules is RNA isolated from cells from a human. In certain instances, the human has a tumor and the mixture of RNA is RNA isolated from the tumor of the human, optionally, wherein the tumor is a malignant brain tumor, optionally, a glioblastoma, medulloblastoma, diffuse intrinsic pontine glioma, or a peripheral tumor with metastatic infiltration into the central nervous system. Optionally, the nanoparticles are prepared by mixing the nucleic acid molecules and the cationic lipid at a RNA: cationic lipid ratio of about 1 to about

5 to about 1 to about 20, optionally, about 1 to about 15. In exemplary aspects, the composition is systemically administered via parenteral administration, optionally, intravenous administration. In exemplary aspects, the subject has an immune checkpoint inhibitor (ICI)-resistant tumor. Optionally, the pDCs are PD-L1^{+}/CD86^{+} pDCs.

[00116] As used herein, the term "increase" and words stemming therefrom may not be a 100% or complete increase. Rather, there are varying degrees of increasing of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. In exemplary embodiments, the increase provided by the methods is at least or about a 10% increase (e.g., at least or about a 20% increase, at least or about a 30% increase, at least or about a 40% increase, at least or about a 50% increase, at least or about a 60% increase, at least or about a 70% increase, at least or about a 80% increase, at least or about a 90% increase, at least or about a 95% increase, at least or about a 98% increase). In various aspects, the "increase" is in reference to baseline measurements (e.g., baseline immunity, sensitivity, or activation) in the absence of (e.g., prior to) administering the nanoparticles of the instant disclosure.

[00117] The present disclosure also provides a method of delivering RNA molecules to an intra-tumoral microenvironment, lymph node, and/or a reticuloendothelial organ. In exemplary embodiments, the method comprises administering to the subject a presently disclosed pharmaceutical composition. Optionally, the reticuloendothelial organ is a spleen or liver. Provided herein are methods of delivery RNA to cells of a tumor, e.g., a brain tumor, comprising systemically (e.g., intravenously) administering a presently disclosed composition, wherein the composition comprises the nanoparticles. Also provided herein are methods of delivering RNA to cells in a microenvironment of a tumor, optionally a brain tumor. In exemplary embodiments, the method comprises systemically (e.g., intravenously) administering a presently disclosed composition, wherein the composition comprises the nanoparticle. In some aspects, the nanoparticle comprises an siRNA targeting a protein of an immune checkpoint pathway, optionally, PDL1. In various aspects, the cells in the microenvironment are antigen-presenting cells (APCs), optionally, tumor associated macrophages. The present disclosure also provides methods of activating antigen-presenting cells in a tumor microenvironment. In exemplary embodiments, the method comprises systemically (e.g., intravenously) administering a presently disclosed composition, wherein the composition comprises the NP.

[00118] The present disclosure provides methods of delivering RNA molecules to cells. In exemplary embodiments, the method comprises incubating the cells with the NPs of the present

disclosure. In exemplary instances, the cells are antigen-presenting cells (APCs), optionally, dendritic cells (DCs). In various instances, the APCs (e.g., DCs) are obtained from a subject. In certain aspects, the RNA molecules are isolated from tumor cells obtained from a subject, e.g., a human. In certain aspects, the RNA molecules are antisense molecules that target a protein of interest for reduced expression. In exemplary aspects, the RNA molecules are siRNA molecules that target a protein of the immune checkpoint pathway. Suitable proteins of the immune checkpoint pathway are known in the art and also described herein. In various instances, the siRNA target PDL1.

[00119] Once RNA has been delivered to the cells, the cells may be administered to a subject for treatment of a disease. Accordingly, the present disclosure provides a method of treating a subject with a disease. In exemplary embodiments, the method comprises delivering RNA molecules to cells of the subject in accordance with the above-described method of delivering RNA molecules to cells. In some aspects, RNA molecules are delivered to the cells *ex vivo* and the cells are administered to the subject. Alternatively, the method comprises administering the liposomes directly to the subject. In exemplary embodiments, the method of treating a subject with a disease comprises administering a composition of the present disclosure in an amount effective to treat the disease in the subject. In exemplary aspects, the disease is cancer, and, in some aspects, the cancer is located across the blood brain barrier and/or the subject has a tumor located in the brain. In some aspects, the tumor is a glioma, a low grade glioma or a high grade glioma, specifically a grade III astrocytoma or a glioblastoma. Alternatively, the tumor could be a medulloblastoma or a diffuse intrinsic pontine glioma. In another example, the tumor could be a metastatic infiltration from a non-CNS tumor e.g. breast cancer, melanoma, or lung cancer. In exemplary aspects, the composition comprises the liposomes, and optionally, the composition comprising the liposomes are intravenously administered to the subject. In alternative aspects, the composition comprises cells transfected with the liposome. Optionally, the cells of the composition are APCs, optionally, DCs. In exemplary aspects, the composition comprising the cells comprising the liposome is intradermally administered to the subject, optionally, wherein the composition is intradermally administered to the groin of the subject. In exemplary instances, the DCs are isolated from white blood cells (WBCs) obtained from the subject, optionally, wherein the WBCs are obtained via leukapheresis. In some aspects, the RNA molecules encode a tumor antigen. In some aspects, the RNA molecules are isolated from tumor cells, e.g., tumor cells are cells of a tumor of the subject. Accordingly, a method of treating a subject with a disease is furthermore provided herein. In exemplary embodiments, the method comprises delivering RNA molecules

to cells of the subject according to the presently disclosed method of delivering RNA molecules to an intra-tumoral microenvironment, lymph node, and/or a reticuloendothelial organ. In various aspects, RNA molecules are *ex vivo* delivered to the cells and the cells are administered to the subject. In exemplary embodiments, the method comprises administering to the subject a pharmaceutical composition of the present disclosure in an amount effective to treat the disease in the subject. In various instances, the subject has a cancer or a tumor, optionally, a malignant brain tumor, optionally, a glioblastoma, medulloblastoma, diffuse intrinsic pontine glioma, or a peripheral tumor with metastatic infiltration into the central nervous system.

[00120] As used herein, the term "treat," as well as words related thereto, do not necessarily imply 100% or complete treatment or remission. Rather, there are varying degrees of treatment of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. In this respect, the methods of treating a disease of the present disclosure can provide any amount or any level of treatment. Furthermore, the treatment provided by the method may include treatment of one or more conditions or symptoms or signs of the disease being treated. For instance, the treatment method of the presently disclosure may inhibit one or more symptoms of the disease. Also, the treatment provided by the methods of the present disclosure may encompass slowing the progression of the disease. For example, the methods can treat cancer by virtue of enhancing the T cell activity or an immune response against the cancer, thereby reducing tumor or cancer growth, reducing metastasis of tumor cells, increasing cell death of tumor or cancer cells, and the like

[00121] The term "treat" also encompasses prophylactic treatment of the disease. Accordingly, the treatment provided by the presently disclosed method may delay the onset or reoccurrence/relapse of the disease being prophylactically treated. In exemplary aspects, the method delays the onset of the disease by 1 day, 2 days, 4 days, 6 days, 8 days, 10 days, 15 days, 30 days, two months, 4 months, 6 months, 1 year, 2 years, 4 years, or more. The prophylactic treatment encompasses reducing the risk of the disease being treated. In exemplary aspects, the method reduces the risk of the disease 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, or more.

[00122] In certain aspects, the method of treating the disease may be regarded as a method of inhibiting the disease, or a symptom thereof. As used herein, the term "inhibit" and words stemming therefrom may not be a 100% or complete inhibition or abrogation. Rather, there are varying degrees of inhibition of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. The presently disclosed methods may inhibit the onset or

re-occurrence of the disease or a symptom thereof to any amount or level. In exemplary embodiments, the inhibition provided by the methods is at least or about a 10% inhibition (e.g., at least or about a 20% inhibition, at least or about a 30% inhibition, at least or about a 40% inhibition, at least or about a 50% inhibition, at least or about a 60% inhibition, at least or about a 70% inhibition, at least or about a 80% inhibition, at least or about a 90% inhibition, at least or about a 95% inhibition, at least or about a 98% inhibition).

[00123] The susceptibility of a tumor to an immune response (or ICI) or, put another way, the effectiveness of an immune response (or ICI) against a tumor, can be determined in a variety of ways. Similarly, treatment a subject for cancer may be determined by any of a number of ways. Any improvement in the subject's well being is contemplated (e.g., at least or about a 10% reduction, at least or about a 20% reduction, at least or about a 30% reduction, at least or about a 40% reduction, at least or about a 50% reduction, at least or about a 60% reduction, at least or about a 70% reduction, at least or about a 80% reduction, at least or about a 90% reduction, or at least or about a 95% reduction of any parameter described herein). For example, a therapeutic response would refer to one or more of the following improvements in the disease: (1) a reduction in the number of neoplastic cells; (2) an increase in neoplastic cell death; (3) inhibition of neoplastic cell survival; (5) inhibition (i.e., slowing to some extent, preferably halting) of tumor growth or appearance of new lesions; (6) decrease in tumor size or burden; (7) absence of clinically detectable disease, (8) decrease in levels of cancer markers; (9) an increased patient survival rate; and/or (10) some relief from one or more symptoms associated with the disease or condition (e.g., pain). For example, the efficacy of treatment may be determined by detecting of a change in tumor mass and/or volume after treatment. The size of a tumor may be compared to the initial size and dimensions as measured by CT, PET, mammogram, ultrasound, or palpation, as well as by caliper measurement or pathological examination of the tumor after biopsy or surgical resection. Response may be characterized quantitatively using, e.g., percentage change in tumor volume (e.g., the method of the disclosure results in a reduction of tumor volume by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90%). Alternatively, tumor response or cancer response may be characterized in a qualitative fashion like "pathological complete response" (pCR), "clinical complete remission" (cCR), "clinical partial remission" (cPR), "clinical stable disease" (cSD), "clinical progressive disease" (cPD), or other qualitative criteria. In addition, treatment efficacy also can be characterized in terms of responsiveness to other immunotherapy treatment or chemotherapy. In various aspects, the methods of the disclosure further comprise monitoring treatment in the subject.

[00124] With regard to the foregoing methods, the NPs or the composition comprising the same, in some aspects, is systemically administered to the subject. Optionally, the method comprises administration of the liposomes or composition by way of parenteral administration.

[00125] Parenteral dosage forms of any agent described herein can be administered to a subject by various routes, including, but not limited to, epidural, intracerebral, intracerebroventricular, epicutaneous, intraarterial, intraarticular, intracardiac, intracavernous injection, intradermal, intralesional, intramuscular, intraocular, intraosseous infusion, intraperitoneal, intrathecal, intrauterine, intravaginal administration, intravenous, intravesical, intravitreal, subcutaneous, transdermal, perivascular administration, or transmucosal. For administration to the brain, a pharmaceutical composition can be introduced into tumor tissue using an intratumoral delivery catheter, ventricular shunt catheter attached to a reservoir (e.g., Omaya reservoir), infusion pump, or introduced into a tumor resection cavity (such as Gliasite, Proxima Therapeutics). Tumor tissue in the brain also can be contacted by administering a pharmaceutical composition via convection using a continuous infusion catheter or through cerebrospinal fluid. In various instances, the liposome or composition is administered to the subject intravenously.

[00126] For purposes of the disclosure, the amount or dose of the active agent (i.e., the "effective amount") administered should be sufficient to achieve a desired biological effect, e.g., a therapeutic or prophylactic response, in the subject over a reasonable time frame. For example, one or more doses of the nanoparticles described herein and ICI should be sufficient to, e.g., sensitize a tumor to an immune response (and optionally treat a cancer) in a clinically acceptable period of time e.g., 1 to 20 or more weeks, from the time of first administration. In certain embodiments, the time period could be even longer. By way of example and not intending to limit the present disclosure, the dose of the active agents of the present disclosure can be about 0.0001 to about 1 g/kg body weight of the subject being treated/day, from about 0.0001 to about 0.001 g/kg body weight, or about 0.01 mg to about 1 g/kg body weight.

[00127] Optionally, the composition is systemically administered in an amount effective to increase the number of PD-L1+/CD86+ myeloid antigen presenting cells (APCs) in the tumor periphery and/or in reticuloendothelial organs, increase PD-L1/CD86 expression by plasmacytoid dendritic cells (pDCs) and CD11c+ myeloid cells, increase Type I interferon release by pDCs, activate T-cell responses, or a combination thereof.

[00128] In instances wherein the method comprises administering a nanoparticle of the disclosure and an ICI to a subject, the nanoparticle composition and ICI may be administered

together (in the same formulation or separate formulations administered close in time) or may be administered sequentially (i.e., the nanoparticle composition is administered and the ICI is administered separately at different time points (e.g., hours or days apart)). In this regard, the nanoparticle composition of the disclosure is optionally administered prior to the ICI, e.g., at least about six hours, at least about 12 hours, at least about 18 hours, or at least about 24 hours prior to ICI administration. In this regard, the nanoparticles may be administered at least about three days, one week, two weeks, three weeks, four weeks (i.e., one month), two months, or three months prior to administration of ICI. For example, the method may, in various instances, comprise a first period of nanoparticle treatment followed by a second period of ICI treatment. The second period of ICI treatment may also entail treatment with the nanoparticles to enhance the immune response (e.g., the second period may comprise both ICI administration and nanoparticle administration). The first period of nanoparticle administration may entail multiple doses of nanoparticles administered to the subject over time, e.g., two, three, four, five, or more doses administered over a treatment period of one week, two weeks, three weeks, four weeks, five weeks or six weeks, prior to administration of an ICI. For example, in an exemplary regimen, three doses of RNA-NPs are administered to a subject over the course of one month, after which the subject is treated with ICI (optionally in combination with RNA-NP treatment).

[00129] In various aspects, the NP or composition is administered according to any regimen including, for example, daily (1 time per day, 2 times per day, 3 times per day, 4 times per day, 5 times per day, 6 times per day), three times a week, twice a week, every two days, every three days, every four days, every five days, every six days, weekly, bi-weekly, every three weeks, monthly, or bi-monthly. In various aspects, the liposomes or composition is/are administered to the subject once a week.

[00130] The present disclosure additionally provides kits comprising an immune checkpoint inhibitor (e.g., a PD-1 antigen-binding protein, such as an anti-PD-1 antibody) and nanoparticle composition in containers with instructions for use. In exemplary aspects, the checkpoint inhibitor and nanoparticle composition are provided in the kit as unit doses. “Unit dose" refers to a discrete amount dispersed in a suitable carrier. In exemplary aspects, the unit dose is the amount sufficient to provide a subject with a desired effect, e.g., cancer cell death. In exemplary aspects, the kit comprises several unit doses, e.g., a week or month supply of unit doses, optionally, each of which is individually packaged or otherwise separated from other unit doses. In some embodiments, the components of the kit/unit dose are packaged with instructions for administration to a patient. In some embodiments, the kit comprises one or

more devices for administration to a patient, e.g., a needle and syringe, and the like. In some aspects, components of the kit are pre-packaged in a ready to use form, e.g., a syringe, an intravenous bag, etc. In exemplary aspects, the ready to use form is for a single use. In exemplary aspects, the kit comprises multiple single use, ready to use forms of the components. In some aspects, the kit further comprises other therapeutic or diagnostic agents or pharmaceutically acceptable carriers (e.g., solvents, buffers, diluents, etc.), including any of those described herein.

[00131] *Subjects*

[00132] The subject is a mammal, including, but not limited to, mammals of the order Rodentia, such as mice and hamsters, and mammals of the order Logomorpha, such as rabbits, mammals from the order Carnivora, including Felines (cats) and Canines (dogs), mammals from the order Artiodactyla, including Bovines (cows) and Swines (pigs) or of the order Perssodactyla, including Equines (horses). In some aspects, the mammals are of the order Primates, Ceboids, or Simoids (monkeys) or of the order Anthropoids (humans and apes). In some aspects, the mammal is a human. In some aspects, the human is an adult aged 18 years or older. In some aspects, the human is a child aged 17 years or less. In exemplary aspects, the subject has a DMG. In various instances, the DMG is diffuse intrinsic pontine glioma (DIPG).

[00133] A subject may be one who has been previously diagnosed with or identified as suffering from or having a condition in need of treatment (e.g., cancer) or one or more complications related to such a condition, and optionally, have already undergone treatment for the condition or the one or more complications related to the condition. Alternatively, a subject can also be one who has not been previously diagnosed as having such condition or related complications. For example, a subject can be one who exhibits one or more risk factors for the condition or one or more complications related to the condition. The subject, in various aspects, has previously received a treatment or therapy for the condition (e.g., previously been administered an anti-cancer therapy).

[00134] *Cancer*

[00135] The cancer treatable by the methods disclosed herein may be any cancer, e.g., any malignant growth or tumor caused by abnormal and uncontrolled cell division that may spread to other parts of the body through the lymphatic system or the blood stream.

[00136] The cancer in some aspects is one selected from the group consisting of acute lymphocytic cancer, acute myeloid leukemia, alveolar rhabdomyosarcoma, bone cancer, brain cancer (e.g., glioma), breast cancer (e.g., triple negative breast cancer), cancer of the anus, anal canal, or anorectum, cancer of the eye, cancer of the intrahepatic bile duct, cancer of the joints, cancer of the head, neck, gallbladder, or pleura, cancer of the nose, nasal cavity, or middle ear, cancer of the oral cavity, cancer of the vulva, chronic lymphocytic leukemia, chronic myeloid cancer, colon cancer, esophageal cancer, cervical cancer, gastrointestinal cancer (e.g., gastrointestinal carcinoid tumor), Hodgkin lymphoma, endometrial or hepatocellular carcinoma, hypopharynx cancer, kidney cancer, larynx cancer, liver cancer, lung cancer (e.g., non-small cell lung cancer, bronchioloalveolar carcinoma), malignant mesothelioma, melanoma, multiple myeloma, nasopharynx cancer, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, peritoneum, omentum, and mesentery cancer, pharynx cancer, prostate cancer, rectal cancer, renal cancer (e.g., renal cell carcinoma (RCC)), small intestine cancer, soft tissue cancer, stomach cancer, testicular cancer, thyroid cancer, ureter cancer, and urinary bladder cancer. In particular aspects, the cancer is selected from the group consisting of head and neck, ovarian, cervical, bladder and oesophageal cancers, pancreatic, gastrointestinal cancer, gastric, breast, endometrial and colorectal cancers, hepatocellular carcinoma, glioblastoma, bladder, and lung cancer (e.g., non-small cell lung cancer (NSCLC), bronchioloalveolar carcinoma). In various aspects, the subject has a solid tumor. Optionally, the subject suffers from a malignant brain tumor, such as a glioblastoma, medulloblastoma, diffuse intrinsic pontine glioma, or a peripheral tumor with metastatic infiltration into the central nervous system.

[00137] In some embodiments, the method described herein further comprises administration of one or more other therapeutic agents. In some aspects, the other therapeutic agent aims to treat or prevent cancer. In some embodiments, the other therapeutic is a chemotherapeutic agent. Common chemotherapeutics include, but are not limited to, adriamycin, asparaginase, bleomycin, busulphan, cisplatin, carboplatin, carmustine, capecitabine, chlorambucil, cytarabine, cyclophosphamide, camptothecin, dacarbazine, dactinomycin, daunorubicin, dexrazoxane, docetaxel, doxorubicin, etoposide, floxuridine, fludarabine, fluorouracil, gemcitabine, hydroxyurea, idarubicin, ifosfamide, irinotecan, lomustine, mechlorethamine, mercaptopurine, meplhalan, methotrexate, mitomycin, mitotane, mitoxantrone, nitrosurea, paclitaxel, pamidronate, pentostatin, plicamycin, procarbazine, rituximab, streptozocin, teniposide, thioguanine, thiotepa, vinblastine, vincristine, vinorelbine, taxol, transplatinum, 5-fluorouracil, and the like. In some embodiments, the other therapeutic is an agent used in radiation therapy for the treatment of cancer; indeed, in some embodiments, the method is part of a treatment

regimen that includes radiation therapy. Further, the method of the disclosure can be performed in connection with surgical resection of a tumor, such as a glioma (e.g., glioblastoma).

[00138] The following examples are given merely to illustrate the present invention and not in any way to limit its scope.

EXAMPLES

EXAMPLE 1

[00139] This example describes a method of making nanoparticles of the present disclosure.

[00140] *Preparation of DOTAP Liposomes*

[00141] On Day 1, the following steps were carried out in the fume hood. Water was added to a rotavapor bath. Chloroform (20 mL) was poured into a sterile, glass graduated cylinder. After opening a vial containing 1 g of DOTAP, 5 mL chloroform was added to the DOTAP vial using a glass pipette. The volume of chloroform and DOTAP was then transferred into a 1-L evaporating flask. The DOTAP vial was washed by adding a second 5-mL volume of chloroform to the DOTAP vial to dissolve any remaining DOTAP in the vial and then transferring this volume of chloroform from the DOTAP vial to the evaporating flask. This washing step was repeated 2 more times until all the chloroform in the graduated cylinder was used. The evaporating flask was then placed into the Buchi rotavapor. The water bath was turned on and adjusted to 25 °C. The evaporating flask was moved downward until it touched the water bath. The rotation speed of the rotavapor was adjusted to 2. The vacuum system was turned on and adjusted to 40 mbar. After 10 minutes, the vacuum system was turned off and the chloroform was collected from the collector flask. The amount of chloroform collected was measured. Once the collector flask is repositioned, the vacuum was turned on again and the contents in the evaporating flask was allowed to dry overnight until the chloroform was completely evaporated.

[00142] On Day 2, using a sterile graduated cylinder, PBS (200 mL) was added to a new, sterile 500-mL PBS bottle maintained at room temperature. A second 500-mL PBS bottle was prepared for collecting DOTAP. The Buchi rotavapor water bath was set to 50 ºC. PBS (50 mL) was added into the evaporating flask using a 25-mL disposable serological pipette. The evaporating flask was positioned in the Buchi rotavapor and moved downward until 1/3 of the flask was submerged into the water bath. The rotation speed of the rotavapor was set to 2, allowed to rotate for 10 min, and then rotation was turned off. A 50-mL volume of PBS with DOTAP from the evaporating flask was transferred to the second 500 mL PBS bottle. The steps were repeated (3-times) until the entire volume of PBS in the PBS bottle was used. The final

volume of the second 500 mL PBS bottle was 400 mL. The lipid solution in the second 500 mL PBS bottle was vortexed for 30 s and then incubated at 50 ºC for 1 hour. During the 1 hour incubation, the bottle was vortexed every 10 min. The second 500 mL PBS bottle was allowed to rest on overnight at room temperature.

[00143] On Day 3, PBS (200 mL) was added to the second 500 mL PBS bottle containing DOTAP and PBS. The second 500 mL PBS bottle was placed into an ultrasonic bath. Water was filled in the ultrasonic bath and the second 500 mL PBS bottle was sonicated for 5 min. The extruder was washed with PBS (100 mL) and this wash step was repeated. A 0.45 µm pore filter was assembled into a filtration unit and a new (third) 500 mL PBS bottle was positioned into the output tube of the extruder. In a biological safety cabinet, the DOTAP-PBS mixture was loaded into the extruder, until about 70% of the third PBS bottle was filled. The extruder was then turned on and the DOTAP PBS mixture was added until all the mixture was run through the extruder. Subsequently, a 0.22 µm pore filter was assembled into the filtration unit and a new (third) 500 mL PBS bottle was positioned into the output tube of the extruder. The previously filtered DOTAP-PBS mixture was loaded and run again throughout. The samples comprising DOTAP lipid nanoparticles (NPs) in PBS were then stored at 4 ºC .

[00144] *RNA Preparation*

[00145] Prior to incorporation into NPs, RNA was prepared in one of a few ways. Total tumor RNA was prepared by isolating total RNA (including rRNA, tRNA, mRNA) from tumor cells. In vitro transcribed mRNA was prepared by carrying out in vitro transcription reactions using cDNA templates produced by reverse transcription of total tumor RNA. Tumor antigen-specific and Non-specific RNAs were either made in-house or purchased from a vendor.

[00146] *Total Tumor RNA*: Total tumor-derived RNA from tumor cells (e.g., B16F0, B16F10, and KR158-luc) is isolated using commercially available RNeasy mini kits (Qiagen) based on manufacturer instructions.

[00147] *In vitro transcribed mRNA*: Briefly, RNA is isolated using commercially available RNeasy mini kits (Qiagen) per manufacturer's instructions and cDNA libraries were generated by RT-PCR. Using a SMARTScribe Reverse Transcriptase kit (Takara), a reverse transcriptase reaction by PCR was performed on the total tumor RNA in order to generate cDNA libraries. The resulting cDNA was then amplified using Takara Advantage 2 Polymerase mix with T7/SMART and CDS III primers, with the total number of amplification cycles determined by gel electrophoresis. Purification of the cDNA was performed using a Qiagen PCR purification kit per

manufacturer's instructions. In order to isolate sufficient mRNA for use in each RNA-nanoparticle vaccine, mMESAGE mMACHINE (Invitrogen) kits with T7 enzyme mix were used to perform overnight in vitro transcription on the cDNA libraries. Housekeeping genes were assessed to ensure fidelity of transcription. The resulting mRNA was then purified with a Qiagen RNeasy Maxi kit to obtain the final mRNA product.

[00148] *Tumor Antigen-Specific and Non-Specific mRNA:* Plasmids comprising DNA encoding tumor antigen-specific RNA (RNA encoding, e.g., pp65, OVA) and non-specific RNA (RNA encoding, e.g., Green Fluorescent Protein (GFP), luciferase) are linearized using restriction enzymes (i.e., SpeI) and purified with Qiagen PCR MiniElute kits. Linearized DNA is subsequently transcribed using the mmRNA *in vitro* transcription kit (Life technologies, Invitrogen) and cleaned up using RNA Maxi kits (Qiagen). In alternative methods, non-specific RNA is purchased from Trilink Biotechnologies (San Diego, CA).

[00149] *Preparation of Multilamellar RNA nanoparticles (NPs)*

[00150] The DOTAP lipid NPs were complexed with RNA to make multilamellar RNA-NPs which were designed to have several layers of mRNA contained inside a tightly coiled liposome with a positively charged surface and an empty core (Figure 1A). Briefly, in a safety cabinet, RNA was thawed from -80 °C and then placed on ice, and samples comprising PBS and DOTAP (e.g., DOTAP lipid NPs) were brought up to room temperature. Once components were prepared, the desired amount of RNA was mixed with PBS in a sterile tube. To the sterile tube containing the mixture of RNA and PBS, the appropriate amount of DOTAP lipid NPs was added without any physical mixing (without e.g., inversion of the tube, without vortexing, without agitation). The mixture of RNA, PBS, and DOTAP was incubated for about 15 minutes to allow multilamellar RNA-NP formation. After 15 min, the mixture was gently mixed by repeatedly inverting the tube. The mixture was then considered ready for systemic (i.e. intravenous) administration.

[00151] The amount of RNA and DOTAP lipid NPs (liposomes) used in the above preparation is pre-determined or pre-selected. In some instances, a ratio of about 15 µg liposomes per about 1 µg RNA were used. For instance, about 75 µg liposomes are used per ~5 µg RNA or about 375 µg liposomes are used per ~25 µg RNA. In other instances, about 7.5 µg liposomes were used per 1 µg RNA. Thus, in exemplary instances, about 1 µg to about 20 µg liposomes are used for every µg RNA used.

EXAMPLE 2

[00152] This example describes the characterization of the nanoparticles of the present disclosure.

[00153] *Cryo-Electron Microscopy (CEM)*

[00154] CEM was used to analyze the structure of multilamellar RNA-NPs prepared as described in Example 1 and control NPs devoid of RNA (uncomplexed NPs) which were made by following all the steps of Example 1, except for the steps under "RNA Preparation" and "Preparation of Multilamellar RNA nanoparticles (NPs)". CEM was carried out as essentially described in Sayour et al., Nano Lett 17(3) 1326-1335 (2016). Briefly, samples comprising multilamellar RNA-NPs or control NPs were kept on ice prior to being loaded in a snap-freezed in Vitrobot (and automated plunge-freezer for cryoTEM, that freezes samples without ice crystal formation, by controlling temperature, relative humidity, blotting conditions and freezing velocity). Samples were then imaged in a Tecnai G2 F20 TWIN 200 kV / FEG transmission electron microscope with a Gatan UltraScan 4000 (4k x4k) CCD camera. The resulting CEM images are shown in Figure 1B. The right panel is a CEM image of multilamellar RNA-NPs and the left panel is a CEM image of control NPs (uncomplexed NPs). As shown in Figure 1B, the control NPs contained at most 2 layers, whereas multilamellar RNA NPs contained several layers. Figure 5 provides another CEM image of exemplary multilamellar RNA NPs. Here, the multiple layers of RNA layers alternating with lipid layers are especially evident.

[00155] *Zeta Potentials*

[00156] Zeta potentials of multilamellar RNA NPs were measured by phase analysis light scattering (PALS) using a Brookhaven ZetaPlus instrument (Brookhaven Instruments Corporation, Holtsville, NY), as essentially described in Sayour et al., Nano Lett 17(3) 1326-1335 (2016). Briefly, uncomplexed NPs or RNA-NPs (200 µL) were resuspended in PBS (1.2 mL) and loaded in the instrument. The samples were run at 5 runs per sample, 25 cycles each run, and using the Smoluchowski model.

[00157] The zeta potential of the multilamellar RNA NPs prepared as described in Example 1 was measured at about +50 mV. Interestingly, this zeta potential of the multilamellar RNA NPs was much higher than those described in Sayour et al., Oncoimmunology 6(1): e1256527 (2016), which measured at around +27 mV. Without being bound to any particular theory, the way in which the DOTAP lipid NPs are made for use in making the multilamellar RNA NPs (Example 1) involving a vacuum-seal method for evaporating off chloroform leads to less

environmental oxidation of the DOTAP lipid NPs, which, in turn, may allow for a greater amount of RNA to complex with the DOTAP NPs and/or greater incorporation of RNA into the DOTAP lipid NPs.

[00158] *RNA Incorporation by Gel Electrophoresis:*

[00159] A gel electrophoresis experiment was conducted to measure the amount of RNA incorporated into ML liposomes. Based on this experiment, it was qualitatively shown that nearly all, if not all, of the RNA used in the procedure described in Example 1 was incorporated into the DOTAP lipid NPs. Additional experiments to characterize the extent of RNA incorporation are carried out by measuring RNA-NP density and comparing this parameter to that of lipoplexes.

EXAMPLE 3

[00160] This example demonstrates the *in vivo* sites of localization of RNA-NPs upon systemic administration and that RNA NPs mediate peripheral and intratumoral activation of DCs.

[00161] DOTAP lipid NPs made as essentially described in Example 1 are complexed with Cre recombinase-encoding mRNA to make Cre-encoding RNA-NPs. These multilamellar RNA-NPs are administered to Ai14 transgenic mice, which carry a STOP cassette flanked by loxP. The STOP cassette prevents the transcription of tdTomato until Cre-recombinase is expressed. A week after RNA-NPs are administered, the lymph nodes, spleens and livers of the transgenic mice are harvested, sectioned and stained with DAPI. The expression of tdTomato is analyzed by fluorescent microscopy following the procedures as essentially described in Sayour et al, *Nano Letters* 2018. It is expected that the Cre-mRNA-NPs localize *in vivo* to lymphoid organs, including liver, spleen, and lymph nodes.

[00162] DOTAP lipid NPs made as essentially described in Example 1 are complexed with non-specific RNA (e.g., RNA that was not tumor antigen-specific; ovalbumin (OVA) mRNA) and intravenously injected into C57Bl/6 mice (n=3-4/group) bearing subcutaneous B16F10 tumors. Lymph nodes, spleens, livers, bone marrow and tumors are harvested within 24 hrs and analyzed for expression of the Dendritic Cell (DC) activation marker, CD86, by CD11c cells (*$p<0.05$ Mann-Whitney) test). It is expected that the OVA mRNA-NPs demonstrate widespread *in vivo* localization to the lymph nodes, spleens, livers, bone marrow, and tumors and activated the DCs therein (as shown by the increased expression of the activation marker CD86 on CD11c+ cells). Because activated DCs prime antigen-specific T cell responses, lead to anti-

tumor efficacy (with increased TILs) in several tumor models, we tested the anti-tumor efficacy of the multi-lamellar RNA NPs.

EXAMPLE 4

[00163] This example describes a comparison of the nanoparticles of the present disclosure to cationic RNA lipoplexes and anionic RNA lipoplexes.

[00164] Cationic lipoplexes (LPX) were first developed with mRNA in the lipid core shielded by a net positive charge located on the outer surface (Figure 2A). Anionic RNA lipoplexes (Figure 2B) have been developed with an excess of RNA tethered to the surface of bi-lamellar liposomes. RNA-LPX were made by mixing RNA and lipid NP at ratios to equalize charge. Anionic RNA-NPs were made by mixing RNA and lipid NP at ratios to oversaturate lipid NPs with negative charge. Various aspects of the RNA-LPX and anionic RNA LPX were then compared to the multilamellar RNA NPs described in the above examples.

[00165] Cryo-Electron Microscopy (CEM) was used to compare the structures of the RNA LPX and the multilamellar RNA-NPs prepared as described in Example 1. Uncomplexed NPs were used as a control. CEM was carried out as essentially described in Example 2. Figure 2C is a CEM image of uncomplexed NPs, Figure 2D is a CEM image of RNA LPXs (wherein that mass ratio of liposome to RNA is 3.75:1) and Figure 2E is a CEM image of the multilamellar RNA-NPs (wherein that mass ratio of liposome to RNA is 15:1). These data support that more RNA is held by the ML RNA-NPs. Additional data show that the concentration drops more with ML RNA-NP complexation versus RNA LPX supporting multilamellar formation of ML RNA-NPs not observed by simple mixing of equivalent amounts of RNA and lipid NPs by mass or charge (i.e. RNA-LPX and anionic RNA-LPX respectively). This supports that more RNA is “held” by ML RNA-NPs described herein.

[00166] Also, an experiment was conducted to determine where the anionic LPXs localize upon administration to mice. As shown in Figure 8, anionic LPXs localized to the spleens of animals upon administration, consistent with previous studies (Krantz et al, Nature 534: 396-401 (2016).

[00167] RNA LPX, anionic lipoplex (LPX) or multilamellar RNA-NPs were administered to mice and spleens were harvested one week later for assessment of activated DCs (*$p<0.05$ unpaired t test). The RNA used in this experiment was tumor-derived mRNA from the K7M2 tumor osteosarcoma cell line. As shown in Figure 2F, mice treated with multilamellar RNA NPs exhibited the highest levels of activated DCs.

[00168] Anionic tumor mRNA-lipoplexes, tumor mRNA-lipoplexes, and multilamellar tumor mRNA loaded NPs were compared in a therapeutic lung cancer model (K7M2) (n=5-8/group). Each vaccine was intravenously administered weekly (x3) (**p<0.01, Mann Whitney). The % CD44+CD62L+of CD8+ splenocytes is shown in Figure 2G and the % CD44+CD62L+of CD4+ splenocytes is shown in Figure 2H. Also, Figure 2J shows that multilamellar (ML) RNA-NPs mediate substantially increased IFN-alpha, which is an innate anti-viral cytokine. This demonstrates that ML RNA-NPs allow for substantially greater innate immunity which is enough to drive efficacy from even non-antigen specific ML RNA-NPs. These data also indirectly support that ML RNA-NPs increase the number of activated plasmacytoid dendritic cells (pDCs) which cells are the most important producers of IFN-alpha. Taken together, the data demonstrates the superior efficacy of multilamellar tumor specific RNA-NPs, relative to anionic LPX and RNA LPX.

[00169] Anionic tumor mRNA-lipoplexes, cationic tumor mRNA-lipoplexes and multilamellar tumor mRNA loaded NPs were compared in a therapeutic lung cancer model (K7M2) (n=8/group). Each vaccine was iv administered weekly (x3), *p<0.05, Gehan Breslow-Wilcoxon test. The percent survival was measured by Kaplan-Meier Curve analysis. As shown in Figure 2I, multilamellar tumor specific RNA-NPs mediated superior efficacy, compared to cationic RNA lipoplexes and anionic RNA lipoplexes, for increasing survival.

[00170] The ability of multilamellar RNA-NP to activate the innate immune response *in vivo* also was examined in the glioma tumor microenvironment.

[00171] RNA-NPs localize to perivascular regions of tumors and reprogram the TME in favor of activated myeloid cells. K-luc bearing animals (n=5/group) were vaccinated with tumor RNA-NPs or NPs alone. Tumors were harvested 48h later for RNA-seq analysis. In animals receiving RNA-NPs, a significant upregulation of gene signatures for BATF3, IRFs, and IFN response genes was observed. In particular, the RNA-NP of the invention significantly upregulated expression of BATF3 (associated with effector dendritic cell phenotype), IRF5 and IRF7 (interferon regulatory factors), and ISG15 and IFITM3 (interferon response genes). These genes have been shown to be essential for sensitizing immunotherapeutic responses. As such, the RNA-NPs upregulate critical innate immune gene signatures in the glioma tumor microenvironment that associated with effector immune response, in effect turning tumors from “cold” to “hot,” allowing immune checkpoint inhibitors to be active where they were previously ineffective prior to RNA-NP treatment.

[00172] Herein it is demonstrated that the multilamellar RNA-NP formulation targeting physiologically relevant tumor antigens is more immunogenic (Figures 2F-2H, 2J) and significantly more efficacious (Figure 2I) compared with anionic LPX and RNA LPX. Without being bound to any particular theory, by altering RNA-lipid ratios and increasing the zeta potential, a novel RNA-NP design composed of multi-lamellar rings of tightly coiled mRNA has been developed (Figure 1C), which multi-lamellar design is thought to facilitate increased NP uptake of mRNA (condensed by alternating positive/negative charge) for enhanced particle immunogenicity and widespread *in vivo* localization to the periphery and tumor microenvironment (TME). Systemic administration of these multi-lamellar RNA-NPs localize to lymph nodes, reticuloendothelial organs (i.e. spleen and liver) and to the TME, activating DCs therein (based on increased expression of the activation marker CD86 on CD11c+ cells). These activated DCs prime antigen specific T cell responses, which lead to anti-tumor efficacy (with increased TILs) in several tumor models.

EXAMPLE 5

[00173] This example demonstrates the ability of multilamellar RNA-NPs to systemically activate DCs, induce antigen specific immunity and elicit anti-tumor efficacy.

[00174] The effect of multilamellar RNA NPs were tested in a second model. Here, BALB/c mice (8 mice per group) inoculated with K7M2 lung tumors were vaccinated thrice-weekly with multilamellar RNA-NPs. A control group of mice was untreated. The lungs were harvested one week after the 3rd vaccine for analysis of intratumoral memory T cells ***$p<0.001$, Mann Whitney test. Figure 3A provides a pair of photographs of RNA-NP treated-lungs (left) and of untreated lungs (right). Figure 3B is a graph of the % central memory T cells (CD62L+CD44+ of CD3+ cells) in the harvested lungs of untreated mice, mice treated multilamellar RNA NPs with GFP RNA, and mice treated multilamellar RNA NPs with tumor-specific RNA.

[00175] Also, BALB/c mice or BALB/c SCID (Fox Chase) mice (8 mice per group) were inoculated with K7M2 lung tumors and vaccinated intravenously thrice-weekly with multilamellar RNA-NPs comprising GFP RNA or tumor-specific RNA. A control group of mice was untreated. % survival was plotted on a Kaplan-Meier curve (***$p<0.0001$, Gehen-Breslow-Wilcox). As shown in Figure 3C, the percent survival of BALB/c mice treated with multilamellar RNA NPs with tumor-specific RNA was highest among the three groups. Interestingly, the percent survival of BALB/c SCID (Fox Chase) mice treated with multilamellar RNA NPs with GFP RNA was about the same as mice treated with multilamellar RNA NPs with tumor-specific RNA (Figure 3D).

[00176] Taken together, the data of Figures 3A-3D demonstrate that monotherapy with RNA-NPs comprising GFP RNA or tumor-specific RNA mediates significant anti-tumor efficacy against metastatic lung tumors in immunocompetent animals and SCID mice. In BALB/c mice bearing metastatic lung tumors (Figures 3A-3D), both GFP (control) and tumor specific RNA-NPs mediate innate immunity and anti-tumor activity; however, only tumor specific RNA-NPs mediate increases in intratumoral memory T cells and long-term survivor outcome (Figures 3A-3D). Anti-tumor activity of RNA-NPs in mice bearing intracranial malignancies was also demonstrated (data not shown).

[00177] These data demonstrate that multilamellar RNA-NPs systemically activate DCs, induce antigen specific immunity and elicit anti-tumor efficacy. Figures 3A-3D show that control RNA-NPs elicit innate response with some efficacy, but tumor specific RNA-NPs elicit a more robust response. Compared with untreated mice, no effects of uncomplexed NPs have been observed, but both non-specific (GFP RNA) and tumor-specific RNA when incorporated into multilamellar RNA NPs mediate innate immunity; however only tumor specific RNA-NPs elicit adaptive immunity that results in a long-term survival benefit (Figures 3A-3D).

EXAMPLE 6

[00178] This example demonstrates personalized tumor RNA-NPs are active in a translational canine model.

[00179] The safety and activity of multilamellar RNA-NPs was evaluated in client-owned canines (pet dogs) diagnosed with malignant gliomas or osteosarcomas. The malignant gliomas or osteosarcomas from dogs were first biopsied for generation of personalized tumor RNA-NP vaccines.

[00180] To generate personalized multilamellar RNA NPs, total RNA materials was extracted from each patient's biopsy. A cDNA library was then prepared from the extracted total RNA, and then mRNA was amplified from the cDNA library. mRNA was then complexed with DOTAP lipid NPs, into multilamellar RNA-NPs as essentially described in Example 1. Blood was drawn at baseline, then 2 hours and 6 hours post-vaccination for assessment of, PD-L1, MHCII, CD80, and CD86 on CD11c+ cells. CD11c expression of PD-L1, MHC-II, PDL1/CD80, and PD-L1/CD86 is plotted over time during the canine's initial observation period. CD3+ cells were analyzed over time during the canine's initial observation period for percent CD4 and CD8, and these subsets were assessed for expression of activation markers (i.e., CD44). From these data, it was shown that multilamellar RNA-NPs elicited an increase in 1) CD80 and MHCII on

$CD11c^+$ peripheral blood cells demonstrating activation of peripheral DCs; and 2) an increase in activated T cells

[00181] Interestingly, within a few hours after administration, tumor specific RNA-NPs elicited margination of peripheral blood mononuclear cells, which increased in the subsequent days and weeks post-treatment, suggesting that RNA-NPs mediate lymphoid honing of immune cell populations before egress.

[00182] These data demonstrated that personalized mRNA-NPs are safe and active in translational canine disease models.

[00183] Specific data from canines evaluated in this manner are shown. A 31 kg male Irish Setter was enrolled on study per owner's consent to receive multilamellar RNA-NPs. Tumor mRNA was successfully extracted and amplified after tumor biopsy. Immunologic response was plotted in response to 1st vaccine. The data show increased activation markers over time on CD11c+ cells (DCs) (Figure 4A) The data show increased CD8+ cells that are activated (CD44+CD8+ cells) within the first few hours post RNA-NP vaccine. These data support that the multilamellar RNA-NPs are immunologically active in a male Irish Setter. A male boxer diagnosed with a malignant glioma was enrolled on study per owner's consent to receive RNA-NPs. Tumor mRNA was successfully extracted and amplified after tumor biopsy. Immunologic response is plotted in response to 1st vaccine (Figure 4B). The data show increased activation markers over time on CD11c+ cells (DCs). As shown in Figure 4C, an increase in activated T cells (CD44+CD8+ cells) was observed within the first few hours post RNA-NP vaccine. These data support that the multilamellar RNA-NPs are immunologically active in a male canine boxer. Additional observations from treatment of canines with spontaneous glioma are illustrated in Figures 4E-4H. Figure 4E illustrates the percentage of lymphocytes elicited in the days post-vaccination, which suggests margination for antigen education before egress. Figure 4F illustrates a spike in interferon-α production, and Figure 4G illustrates an increase in CD80 expression in CD11c+ cells, in the hours following administration of the ML RNA-NPs. Figure 4H illustrates expression of CD8+ cells and CD44+CD8+ cells, noting a shift toward a more immunologically "active" environment. The data support the use of ML RNA-NPs to transition toward an immune milieu that is more responsive to immunotherapy.

[00184] After receiving weekly RNA-NPs (×3), the canines diagnosed with malignant gliomas had a steady course. Post vaccination MRI showed stable tumor burdens, with increased swelling and enhancement (in some cases), which may be more consistent with pseudoprogression from an immunotherapeutic response in otherwise asymptomatic canines.

Survival of canines diagnosed with malignant gliomas receiving only supportive care and tumor specific RNA-NPs (following tumor biopsy without resection) is shown in Figure 4D. In Figure 4D, the median survival (shown as dotted line) was about 65 days and was reported from a meta-analysis of canine brain tumor patients receiving only symptomatic management. In a previous study, cerebral astrocytomas in canines has been reported to have a median overall survival of 77 days. The personalized, multilamellar RNA NPs allowed for survival past 200 days.

[00185] Aside from low-grade fevers that spiked 6 hrs post-vaccination on the initial day, personalized tumor RNA-NPs (1x) were well tolerated with stable blood counts, differentials, renal and liver function tests. To date, four canines diagnosed with malignant brain tumors were treated. It is important to highlight that these canines received no other therapeutic interventions for their malignancies (i.e., no surgery, radiation or chemotherapy), and all patients assessed developed immunologic response with pseudoprogression or stable/smaller tumors. One canine was autopsied after RNA-NP vaccines. In this patient there were no toxicities believed to be related to the interventional agent.

[00186] These results suggest safety and activity of tumor specific RNA-NPs in client-owned canines with malignant brain tumors for subjects that did not receive any other anti-tumor therapeutic interventions.

EXAMPLE 7

[00187] This example demonstrates toxicology study of murine glioma mRNA and pp65 mRNA encapsulated in DOTAP liposomes after intravenous delivery to C57BL/6 mice.

[00188] The objective of this study was to evaluate the safety of pp65 mRNA encapsulated by DOTAP liposomes when delivered intravenously in C57BL/6 mice. Experimental procedures applicable to pathology investigations are summarized in Table 1. All interim phase animals were submitted for necropsy on Day 35±1 day. Necropsies were performed by University of Florida personnel. Tissue samples listed in Table 2 were collected and fixed in 10% neutral buffered formalin with the exception of eye and testis tissue, which was fixed in Davidson's solution; tissues from the early death animal were fixed in 10% neutral buffered formalin.

TABLE 1

Group	Treatment a	Total Dose (total mRNA+LP) (mg/kg)	Number of Mice					
			Day 35±1 day		Day 56±2		Day 112±3 days	
			Males	Female	Males	Female	Males	Female
1	Vehicle	0	5	5	5	5	5	5

2	LP	0 + 15.0	5	5	5	5	5	5
3	RNA + LP	0.2 + 3.0	5	5	5	5	5	5
4	RNA + LP	1.0 + 15.0	5	5	5	5	5	5

TABLE 2

Tissue Collection and Examination

Provantis Tissue Term	Protocol Tissue Term	Collect	Microscopic Evaluation
BONE, FEMUR	Femur with bone marrow (R)	X	X
BONE MARROW		X	X
BONE, STERNUM	Sternum	X	X
BRAIN	Brain stem	X	X
	Cerebellum		
	Cerebrum		
EPIDIDYMIS	Epididymis	X	X
ESOPHAGUS	Esophagus	X	X
EYE	Eye with optic nerve (R)	X	X
NERVE, OPTIC		X	X
GLAND, ADRENAL	Adrenal gland (R)	X	X
GLAND, PARATHYROID	Thyroid/parathyroid gland	X	X
GLAND, THYROID		X	X
GLAND, PITUITARY	Pituitary	X	X
GLAND, PROSTATE	Prostate	X	X
GLAND, SALIVARY	Salivary gland (R, mandibular)	X	X
GLAND, SEMINAL VESICLE	Seminal vesicles	X	X
HEART	Heart	X	X
KIDNEY	Kidney (R)	X	X
LARGE INTESTINE, CECUM	Cecum	X	X
LARGE INTESTINE, COLON	Colon	X	X
LARGE INTESTINE, RECTUM	Rectum	X	X
LIVER	Liver	X	X
LUNG	Lungs	X	X
LYMPH NODE, MESENTERIC	Lymph node (mesenteric)	X	X
MUSCLE, DIAPHRAGM	Diaphragm	X	X
MUSCLE, QUADRICEPS	Quadriceps (R)	X	X
NERVE, SCIATIC	Sciatic nerve (R)	X	X
OVARY	Gonad (Ovary, R)	X	X
PANCREAS	Pancreas	X	X
SITE, INJECTION	Tail (injection site)	X	X
SKIN	Skin	X	X
SMALL INTESTINE, DUODENUM	Duodenum	X	X
SMALL INTESTINE, ILEUM	Ileum	X	X

SMALL INTESTINE, JEJUNUM	Jejunum	X	X
SPINAL CORD	Spinal cord, cervical Spinal cord, lumbar Spinal cord, thoracic	X	X
SPLEEN	Spleen	X	X
STOMACH	Stomach	X	X
TESTIS	Gonad (Testis, R)	X	X
THYMUS	Thymus	X	X
TONGUE	Tongue	X	X
URINARY BLADDER	Urinary bladder	X	X
UTERUS	Uterus	X	X
VAGINA	Vagina	X	X
-	Gross lesions	X	X

[00189] Tissues required for microscopic evaluation were trimmed, processed routinely, embedded in paraffin, and stained with hematoxylin and eosin by Charles River Laboratories Inc., Skokie, Illinois. Light microscopic evaluation was conducted by the Contributing Scientist, a board-certified veterinary pathologist on all protocol-specified tissues from all animals in Groups 1 and 4, and any early death animals.

[00190] Tissues that were supposed to be microscopically evaluated per protocol but were not available on the slide (and therefore not evaluated) are listed in the Individual Animal Data of the pathology report as not present. These missing tissues did not affect the outcome or interpretation of the pathology portion of the study because the number of tissues examined from each treatment group was sufficient for interpretation.

[00191] *Gross Pathology*: No test article-related gross findings were noted. The gross findings observed were considered incidental, of the nature commonly observed in this strain and age of mouse, and/or were of similar incidence in control and treated animals and, therefore, were considered unrelated to administration of a 1:1 ratio of pp65 mRNA and KR158mRNA in DOTAP liposomes.

[00192] *Histopathology*: No test article-related microscopic findings were noted. There were a few animals with inflammatory cell infiltrates at the injection site; this finding is common for injection sites and at this point in the study, was considered equivocal. The microscopic findings observed were considered incidental, of the nature commonly observed in this strain and age of mouse, and/or were of similar incidence and severity in control and treated animals and, therefore, were considered unrelated to administration of a 1:1 ratio of pp65 mRNA and KR158mRNA in DOTAP liposomes.

[00193] It was concluded that intravenous injection into the tail vein of mice of 1.0 mg/kg KR158 and pp65 mRNAs + 15.0 mg/kg DOTAP liposome on Study Days 0, 14, and 28 resulted in no gross or microscopic test article-related findings on Study Day 35±1 day. There were small amounts of inflammatory cell infiltrates at the injection site, which is a common finding for injection sites. This finding was equivocal.

EXAMPLE 8

[00194] This example describes a study aimed at determining the impact of pDCs transfected with multilamellar RNA-NPs on antigen specific T-cell priming.

[00195] While pDCs are well-known stimulators of innate immunity and type I IFN, they also mediate profound effects on intratumoral adaptive immunity. They can: 1) directly present antigen for priming of tumor specific T cells; 2) assist adaptive response through chemokine recruitment of other DC subtypes (via chemokines CCL3, CCL4, CXCL10); 3) polarize Th1 immunity through IL-12 secretion; and/or 4) mediate tumor antigen shedding (through cytokine, TRAIL or granzyme B) for DC loading and T cell priming. Despite these effector functions, pDCs may also dampen immunity through release of immunoregulatory molecules (IL-10, TGF-β, and IDO) and promotion of regulatory T cells (Tregs). The purpose of this study was to elucidate the effects of RNA-NP transfected-pDCs on adaptive immunity and antigen specific T cell priming. It was hypothesized that RNA-NP activated pDCs serve as direct primers of antigen specific immunity and assist classical DCs (cDCs) and/or myeloid-derived DCs (mDCs) in promoting effector T-cell response. These experiments shed new light on the activation state of pDCs requisite for RNA-NP mediated immunity and their exhaustion over time that may be co-opted for enhanced immunotherapeutic effect.

[00196] *Statistical Analyses*

[00197] In the study of Example 9.1 where survival is of interest, the log-rank test is used to compare Kaplan-Meier survival curves between treatment groups and control groups. Experience with our tumor models indicates that median overall survival in untreated control mice is approximately 30 days, with survival times following a Weibull distribution with shape parameter k=6. As an example, with 10 mice each in 2 tumor-bearing groups (treated and untreated), comparison of survival curves using a one-sided log-rank test evaluated at 0.05 significance has at least 80% power to detect an improvement in median survival of 8 days in the treated group compared to the untreated group. This effect size was determined by simulating 1000 Weibull-distributed survival datasets with shape parameter k=6 under the

alternative hypothesis effect size and then observed the proportion of log-rank tests of these datasets that were significant at $p<0.05$. In the studies of Examples 9.2-9.4, responses observed at different times are analyzed using a two-way ANOVA model with mutually exclusive groups distributed among treatments and observation times. change in immune response parameters over time are assessed using generalized linear mixed effect models (GLMMs). Response variables for experiments that are completely replicated at least once are analyzed using GLMMs. Experimental replication is modeled as a random effect to account for "batch" or "laboratory day" variability. Treatment and control groups are modeled as fixed effects and compared using ANOVA-type designs nested within the mixed effect modeling framework.

[00198] Example 8.1

[00199] This example describes an experiment designed to determine anti-tumor efficacy of RNA-NPs in wild-type and pDC KO mice.

[00200] Tumorgenicities for KR158b-luc, GL261-luc and a murine H3.3K27M mutant cell line have been set up. KR158b-luc and GL261-luc are both transfected with luciferase so that tumors can be monitored for growth using bioluminescent imaging. Tumorigenic dose of KR158b-luc and the H3K27M mutant line is $1x10^4$ cells. Tumorigenic dose of GL261-luc is $1x10^5$ cells. GL261 and KR158 are injected into the cerebral cortex of C57Bl/6 (3 mm deep into the brain at a site 2 mm to the right of the bregma); H3K27M glioma cells are injected midline. Tumor mRNA is extracted from the parental cell lines (i.e. KR158b without luciferase) for vaccine formulation consisting of an intravenous (iv) injection of 25 μg of tumor specific mRNA complexed with 375 μg of our custom lipid-NP formulation (per mouse). These are compared simultaneously to 10 negative control mice receiving NPs alone and nonspecific (i.e. pp65 mRNA) RNA-NPs. Mice are vaccinated 3 times at 7-day intervals beginning 5 days after tumor implantation. IFN-α levels are assessed from serum of wild-type and pDC KO mice at serial time points (5 d, 12 d, and 19 d). In wild-type mice who develop treatment response, but succumb to disease, the immunologic escape mechanisms in tumors (i.e., expression of checkpoint ligands, IDO, downregulation of MHC class I) and within the tumor microenvironment (i.e., MDSCs, Tregs, and TAMs) are explored.

[00201] Based on preclinical data demonstrating anti-tumor activity of RNA-NPs in these models, it is anticipated that anti-tumor activity is abrogated in pDC KO mice.

[00202] Example 8.2

[00203] This example describes an experiment designed to determine the pDC phenotype and function following activation by RNA-NPs.

[00204] To assess pDC phenotype, KR158b bearing C57Bl/6 mice are vaccinated with TTRNA-NPs composed from 375 μg of FITC labeled DOTAP (Avanti) with 25 μg of TTRNA (derived from KR158b and delivered iv). Twenty-four hours after vaccination recipient mice are euthanized (humanely killed with CO_2) for collection of spleens, tumor draining lymph nodes (tdLNs) and tumors. Organs are digested into a single cell suspension, undergo RBC lysis (PharmLyse, BD Bioscience) before incubation at 37°C for 5 minutes. Ficoll gradients are used to separate WBCs from parenchymal cells. The cells at the interface are collected, washed, and analyzed. pDCs are stained for CD11c, B220 and Gr-1 (ebioscience). Distinct pDC subsets are identified by differential staining for CCR9, SCA1, and Ly49q. Activation state is assessed based on expression of co-stimulatory molecules (e.g., CD40, CD80, CD86) chemokines (e.g., CCL3, CCL4, CXCL10) and chemokine receptors (e.g., CCR2, CCR5, CCR7). Detection secondary antibody is rabbit IgG conjugated with AlexaFlour®488 (ThermoFisher Scientific) for FITC detection. Effector versus regulatory function is determined through intracellular staining for effector (e.g., IFN-I, IL-12) versus regulatory cytokines (e.g., TGF-β, IL-10). Analyses will be conducted by multi-parameter flow cytometry (LSR, BD Bioscience) and immunohistochemistry (IHC).

[00205] Based on our preliminary data showing substantial increases in pDCs in peripheral and intratumoral organs, it is expected to identify FITC positive pDCs in the spleen, tdLNs and intracranial tumors.

[00206] Example 8.3

[00207] This example describes an experiment designed to determine whether RNA-NP transfected pDCs mediate direct or indirect activation of antigen specific T cells.

[00208] While pDCs are well known stimulators of innate immunity and type I IFN, their cumulative effects on antigen specific responses are still being uncovered. Since they express MHC class II, they have APC capacity, but compared to their cDC counterparts, they are believed to be poor direct primers of antigen specific immunity. This experiment is aimed at yielding a better understanding of pDCs, in the context of RNA-NPs, as either direct primers or facilitators of antigens specific immunity. To determine the effects of pDCs on antigen specific T cells, KR158b bearing mice are vaccinated with TTRNA (derived from the murine glioma line KR158b) encapsulated into FITC-labeled NPs (Avanti), and FACSort (BD Aria II) relevant FITC+

pDCs from spleens, tdLNs and intracranial tumors (as indicated above). RNA-NP transfected pDCs are then co-cultured with naïve magnetically separated CD4 and CD8 T cells, and T cells are assessed for proliferation, phenotype (effector vs central memory), function and cytotoxicity. Indirect effects from pDCs are assessed via *ex vivo* co-cultures with TTRNA-loaded DCs (matured ex vivo from murine bone marrow) with naïve CD4 and CD8 T cells. *Ex vivo* co-cultures will be performed in triplicate, for 7 days in a 96 well plate with naïve T cells (40,000 RNA-NP transfected pDCs with 400,000 T cells) labeled with CFSE (Celltrace, Life Technologies). T cell proliferation is determined by measuring CFSE dilution by flow cytometry. Phenotype for effector and central memory populations is determined through differential staining for CD44 and CD62L. These T cells are re-stimulated for a total of 2 cycles before supernatants are harvested for detection of Th1 cytokines (i.e. IL-2, TNF-α, and IFN-γ) by bead array (BD Biosciences). Stimulated T cells are also incubated in the presence of KR158b (stably transfected with GFP) or control tumor (B16F10-GFP) and assessed for their ability to induce cytotoxicity. Amount of GFP in each co-culture, as a surrogate for living tumor cells, are quantitatively measured by flow cytometry.

[00209] The *in vivo* effects of FACSorted RNA-NP transfected pDCs are determined by adoptively transferring these cells (250,000 cells/mouse) to tumor-bearing mice (weekly x3) and harvesting spleens, tdLNs, and tumors one week later for assessment of antigen specific T cells by YFP expression in IFN-γ reporter mice (GREAT mice, B6 transgenic, containing IFN-γ promotor with IRES-eYFP reporter, Jackson labs). In separate experiments, IFN-γ reporter mice are vaccinated with TTRNA-NPs with and without pDC depleting mAbs before harvesting spleens, tdLNs, and intracranial tumors one week later for determination of antigen specific T cells by YFP expression. T cell functional assays are performed as described above.

[00210] It is anticipated that these pDCs are requisite for priming antigen specific T cells through either direct and/or indirect means.

[00211] Example 8.4

[00212] This example describes an experiment designed to determine whether RNA-NP activated pDCs promote antigen specific T cell priming from cDCs and/or mDCs.

[00213] While IFN-I release from pDCs is known to increase activation markers on cDCs and mDCs, the role of pDCs on direct T cell priming from cDCs/mDCs is less clear. This experiment is aimed at elucidating the ability of RNA transfected cDCs and mDCs to prime antigen specific T cells in the presence or absence of activated pDCs. To determine effects of pDCs on other

DC subsets, KR158b bearing C57Bl/6 and pDC knock out (KO) mice (BDCA2-DTR, B6 transgenic mice, Jackson labs) are vaccinated and T cell priming from cDCs and mDCs are assessed. FITC+ cDC and mDC populations are sorted via FACSort within 24h of iv TTRNA-NPs (FITC-labeled) and are evaluated for their ability to prime naïve T cell responses in vitro based on proliferation, functional and cytotoxicity assays. Resident and migratory cDCs are identified by CD11c+CD103+MHCII+cells and CD11c+CD11b+MHCII+cells respectively; mDCs are identified by CD11c+CD14+ MHCII+ cells. cytokines, chemokines and activation markers are analyzed as described in Example 9.1. In vivo effects of these cDC/mDC are carried out in cell transfer experiments as described in Example 9.2. Briefly, FACSorted cDCs and mDCs from TTRNA-NP vaccinated C57Bl/6 mice or pDC KO mice are adoptively transferred (250,000 cells/mouse) to tumor-bearing mice (once weekly x3) before harvesting spleens, tdLNs, and intracranial tumors one week later for assessment of antigen specific T cells by YFP expression in IFN-γ reporter mice. Proliferation, functional and cytotoxicity assays are performed.

[00214] It is expected that ML RNA-NPs activate pDCs which enhance activation phenotype and direct priming of T cells from cDCs and mDCs.

[00215] If a lack of indirect effects from pDCs on cDCs and/or mDCs, pDC□s effects on NK cells are evaluated including their activation state, function, and cytotoxicity.

[00216] Example 8.5

[00217] This example describes an experiment designed to determine how pDCs influence effector/regulatory T cells over time within the intratumoral microenvironment.

[00218] Recruitment of pDCs to tumors is typically associated with a regulatory phenotype characterized by increased IDO, FoxP3+Tregs and secretion of immunoregulatory cytokines. In this experiment, it is determined whether RNA-NP activated pDCs function distinctly by activating T cells over time in the tumor microenvironment. To determine intratumoral effects of pDCs, TTRNA-NPs are administered to KR158b bearing IFN-γ reporter mice with and without pDC depleting mAbs (Bioxcell). Activated and regulatory T cells are assessed over time in the intratumoral microenvironment at serial time points (6h, 1d, 7d, and 21d). Effector T cells are characterized, and Tregs are phenotyped through expression of FoxP3, CD25, and CD4. pDCs from non-depleted animals will be FACSorted from these sites and are phenotyped for expression of cytokines, chemokines, activation markers (e.g., CD80, CD86, CD40), cytolytic markers (e.g., TRAIL, granzyme b) and regulatory markers (e.g., IL-10, TGF-β, IDO).

Immunophenotypic changes by tumor cells are also assessed over time (i.e. MHC-I, PD-L1, SIRPα).

EXAMPLE 9

[00219] This example describes a study aimed at evaluating the role of type I interferons on RNA-NP activated T-cell egress, trafficking and function.

[00220] *Statistical Analysis:* Tumor-bearing mice are randomized prior to receiving interventional treatments. The choice of 10 animals per group should yield adequate power for detecting effects of interest. As an example, within an ANOVA design with 7 treatment groups observed at a particular time, a pairwise contrast performed within the ANOVA framework can detect an effect size equal to 1.27 SD units with 80% power at a 2-sided significance level of 0.05. Immune parameter responses observed in experimental groups at several observation times are analyzed using generalized linear models (GLMs) with normal or negative binomial response errors. Responses are organized in a two-way ANOVA design with mutually exclusive groups distributed among treatments and observation times. Response variables for experiments that are completely replicated at least once are analyzed using GLMMs. Experimental replication is modeled as a random effect to account for "batch" or "laboratory day" variability. Treatment and control groups are modeled as fixed effects and compared using ANOVA-type designs nested within the mixed effect modeling framework.

[00221] Example 9.1

[00222] This example describes an experiment designed to determine the chemokine receptor, S1P1, and VLA-4/LFA-1 expression profile of antigen specific T cells after RNA-NP vaccination.

[00223] IFN-I's effects on sphingosine-1-phosphate receptor 1 (S1P1), which is necessary for T cell egress from lymphoid organs, and integrins (i.e. VLA-4, LFA-1) necessary for T cell traversion across the BBB are assessed. KR158b bearing IFN-γ reporter mice, or IFN-γ reporter mice receiving IFNAR1 blocking mAbs (Bioxcell) are implanted with TTRNA-NPs. RNA-NPs composed from 375 μg of DOTAP (Avanti) with 25 μg of TTRNA (extracted from KR158b and delivered iv) are administered once weekly (x3) and are begun 5 days after implantation. One week after the last vaccine, recipient mice are euthanized (humanely killed with CO_2) and spleens, tdLNs, bone marrow, and intracranial tumors are harvested. Organs are digested, and antigen specific T cells from spleens, lymph nodes, bone marrow and tumors are identified by YFP expression and by differential staining for effector and central memory T cells (i.e. of

CD62L and CD44) at serial time points (7, 14 and 21 days). Th1-associated chemokine receptors (i.e. CCR2, CCR5, CCR7 and CXCR3), S1P1 expression, VLA-4, and LFA-1 expression (ebioscience) from CD4 and CD8 T cells are assessed by multi-para meter flow cytometry and IHC.

[00224] It is expected that LFA-1 and CCR2 are expressed on activated T cells following RNA-NP administration. If no changes in chemokine expression pattern, S1P1 and integrins on activated T cells after IFNAR1 mAbs, RNA-seq analysis is performed on FACS sorted T cells (YFP+ cells) from mice treated with and without IFNAR1 mAbs and assess changes in immune related genes.

[00225] Example 9.2

[00226] This example describes an experiment designed to determine the effects of IFN-I on *in vitro* and *in vivo* migration of RNA-NP activated T cells.

[00227] Based on our data demonstrating increased antigen specific T cells in peripheral organs but lack of anti-tumor efficacy after IFNAR1 blockade, IFN-I's effects on RNA-NP activated T cell migration are determined. KR158b bearing IFN-γ reporter mice, or IFN-γ reporter mice receiving IFNAR1, LFA-1 or CCR2 blocking antibodies are vaccinated with iv TTRNA-NPs once weekly (x3). *In vivo* traversion across the BBB is assessed from percentage and absolute numbers of T cells in intracranial tumors (relative to spleen, lymph nodes and bone marrow) at serial time points (5 d, 10 d, 15 d, 20 d post RNA-NPs).

[00228] The migratory capacity of T cells is also analyzed via in vitro cultures. KR158b tumor bearing naïve, INFAR1, LFA-1 or CCR2 KO animals (B6 transgenic, Jackson) are vaccinated with iv TTRNA-NPs. T cells are FACSorted via a BD Aria II Cell Sorter into a 50-100% FBS solution. These T cells are assessed for migratory capacity in transwell assays (ThermoFisher Scientific). Briefly, T cells are placed in the upper layer of a cell culture insert with a permeable membrane in between a layer of KR158b-GFP tumor cells. Migration is assessed by number of cells that shift between layers. T cells are plated in T cell media with and without IL-2 (1 microgram/mL) at a concentration of 4 $x10^6$ per mL for co-culture with tumor cells ($4x10^6$/mL) (x48hrs) before determination of IFN-γ by ELISA (ebioscience). Amount of GFP in each co-culture, as a surrogate for living tumor cells, is quantitatively measured by flow cytometric analysis.

[00229] It is anticipated that type I IFNs are necessary for activated T cell trafficking across the BBB. If there is an inability to adequately define antigen specific T cells, the response

against a physiologically relevant GBM antigen, pp65, which will be spiked into our tumor mRNA cohort, is tracked in HLA-A2 transgenic mice by overlapping peptide pool re-stimulation assays and through analysis for pp65-HLA-A2 restricted epitope NTUDGDDNNDV by tetramer staining for CD8+ cells in spleens, tdLNs and intracranial tumors.

[00230] Example 9.3

[00231] This example describes an experiment designed to delineate the contribution of IFN-I on antigen specific T cell function following RNA-NPs.

[00232] IFN-Is have been shown to promote Tregs and regulate effector and memory CD8+ cells (56), but they are also essential in promoting activated T cell responses following RNA-NP vaccination. Due to these distinct effects, the contribution of IFN-I on antigen specific T cell function following RNA-NP vaccines is determined. KR158b bearing IFN-γ reporter mice, or IFN-γ reporter mice receiving IFNAR1 mAbs, are vaccinated with iv TTRNA-NPs once weekly (x3). Antigen specific T cells are assessed by YFP+ cells. YFP+ T cells from spleens, lymph nodes, bone marrow and tumor are assessed for their activation status (i.e. CD107a, perforin, granzyme), proliferation (through fluorescent dilution of adoptively transferred cells labeled with CellTrace Violet), differentiation (into effector and central memory subsets, and cytotoxicity. T cell cytotoxicity is determined in the presence of KR158b (stably transfected with GFP) or control tumor (B16F10). It is also expected that type I IFNs enhance T cell proliferation and function within the tumor microenvironment.

[00233] If no changes in migratory capacity or function of antigen specific T cells after blockade of type I IFN, the effects of type I IFN on modulating T cell exhaustion is assessed. the effects of type I IFNs on expression of immune checkpoints (i.e. PD-1, TIM-3, LAG-3) and their ligands on tumor cells and APCs (i.e. PD-L1, galectin-9) is also evaluated.

EXAMPLE 10

[00234] This example demonstrates non-antigen specific multilamellar (ML) RNA NPs mediate antigen-specific immunity long enough to confer memory and fend off re-challenge of tumor.

[00235] An experiment was carried out with long-term surviving mice (e.g., mice that survived for ~100 days) that were challenged a total of two times via tumor inoculation, but treated only once weekly (x3) with ML RNA NPs comprising GFP RNA or pp65 RNA (each of which were non-specific to the tumor) or with ML RNA NPs comprising tumor-specific RNA. The treatment occurred just after the first tumor inoculation and about 100 days before the second tumor

inoculation. Because none of the control mice (untreated mice) survived to 100 days, a new control group of mice were created by inoculating the same type of mice with K7M2 tumors. The new control group like the original control mice did not receive any treatment. The long-time survivors also did not receive any treatment after the second time of tumor inoculation. A timeline of the events of this experiment are depicted in Figure 7A.

[00236] Remarkably, mice in all 3 groups contained long-time survivors that survived the second tumor challenge. As shown in Figure 7B (which shows only the time period following the 2nd inoculation), mice in all 3 groups contained long-time survivors with survival to 40 days post tumor implantation (second instance of tumor inoculation). Interestingly, the percentage of long-time survivor mice that were previously treated with ML RNA NPs comprising non-specific RNA (GFP RNA or pp65 RNA) survived to 40 days post second tumor inoculation, comparable to the group treated with ML RNA NPs comprising tumor specific RNA (treated before second tumor challenge).

[00237] These data support that ML RNA NPs comprising RNA non-specific to a tumor in a subject provides therapeutic treatment for the tumor comparable to that provided by ML RNA NPs comprising RNA specific to the tumor, leading to increased percentage in animal survival.

EXAMPLE 11

[00238] This example demonstrates that the administration of ML RNA NPs in combination with an ICI leads to significantly increased survival in tumor-bearing subjects.

[00239] To test the effect of ML RNA NPs in combination with an ICI, tumor bearing C57Bl/6 mice were treated with ML RNA NPs alone (RNA NPs) or in combination with an anti-PDL1 monoclonal antibody (PDL1 mAb). Control groups included untreated mice, mice treated with nanoparticles not loaded with any RNA (NPs alone) or with just PDL1 mAb. For tumor implantation, ~200,000 MOC-1 cells, which are mouse oral cavity squamous cell carcinoma (OSCC) cells were implanted subcutaneously in C57Bl/6 mice. For the groups receiving nanoparticles (ML RNA NPs alone or in combination with PDL1 mAb or NPs Alone), the NPs were injected intravenously within 24 hours of tumor implantation and then two more times once weekly. For the groups of mice receiving ICI (ML RNA NPs + PDL1 mAb or PDL1 mAb alone), PD-L1 mAbs (400 µg) were injected intraperitoneally followed by 200 µg twice weekly until the third dose of NPs was administered. Surviving mice from each group were monitored over the study period of about 100 days and the percentage of mice in each group surviving was plotted as a function of time post tumor implantation. The results are shown in Figure 9. As shown in

this figure, the percentage of surviving mice treated with ML RNA NPs in combination with an ICI was far greater than those receiving either treatment alone.

EXAMPLE 12

[00240] This Example demonstrates that ML RNA-NPs of the instant disclosure mediates anti-tumor immune responses against immunologically "cold" tumors, i.e. tumors which did not respond to ICIs. As demonstrated in Figures 10A-10C, administration of the ML RNA-NPs of the disclosure with an immune checkpoint inhibitor (here, anti-PD-L1 antibody) resulted in reduced tumor volumes in a melanoma model compared with administration of RNA-NP alone and checkpoint inhibitor alone. Administration of ML RNA-NPs also resulted in enhanced subject survival in a sarcoma model and a metastatic lung model. The data establish that ML RNA-NPs reprogram immunologically "cold" tumors, as well as demonstrate the effectiveness of the ML RNA-NPs over a range of cancers and tumor types.

EXAMPLE 13

[00241] This Example describes an exemplary method for isolating slow cycling cells.

[00242] An exemplary method comprises (a) contacting a mixed tumor cell population with a cell proliferation dye or mitochondrial dye (e.g., MitoTracker™) which binds to cells (e.g., binds to the surface or the interior of the cells) of the mixed tumor cell population; (b) separating the dyed cells into sub-populations based on the intensity of the fluorescence emitted by the cell proliferation dye or mitochondrial dye; and (c) selecting and isolating the sub-population exhibiting the top 1-20% of fluorescence intensity or removing the sub-population exhibiting the bottom 80% of fluorescence intensity, thereby isolating SCCs from the mixed tumor cell population.

[00243] The cell proliferation dye or mitochondrial dye may comprise a thiol-reactive chloromethyl group or amine-reactive group. The cell proliferation dye may bind to the cell interior and comprises carboxyfluorescein succinimidyl ester (CFSE), optionally, CellTrace™ CFSE, CFDA-SE, CFDA, CellTrace™ Violet, Blue, Yellow, Far Red or any wavelengths of the color spectrum. In exemplary aspects, the cell proliferation dye is a cell surface binding dye such as, e.g., CellVue Claret dyes, PKH26 and e-Fluor Proliferation dyes. In exemplary aspects, the mitochondrial dye is a cell mitotracker dye comprising Rosamine-based Mitotracker probes (Orange CMTMRos, Orange CM-H2TMRos, Red CMXRos, Red CM-H2XRos, Deep Red CMXRos, Deep Red CM-H2XRos) and Carbocyanin-based Mitotracker probes (Green FM, Orange FM, Red FM, Deep Red FM).

[00244] Additional dyes that could be used in the \ method of isolating SCCs include, but are not limited to, CellTrace Proliferation dyes (Blue, Violet, CFSE, Yellow, Far Red), CFDA, CFDA-SE, CellVue Claret dyes, PKH26 and e-Fluor Proliferation dyes). The concentration of the dyes may vary from 0.1 uM to 50uM and the labeling time may vary from 1 minute to 1 hour. The labeling solution may be PBS or any serum-free or protein-free medium. The cell density for labeling may be from 0.1 million cells per ml of labeling solution to 20 million cells per ml of labeling solution. A chasing period may need to be performed after labeling. After this chasing period, which varies between 2 days and 8 weeks, the labeling intensity is quantified by flow cytometry.

[00245] The method may comprise a combination of one or more of the aforementioned dyes. For example, the method may comprise contacting a mixed tumor cell population with at least two cell proliferation or mitochondrial dyes, optionally, at least 3, at least 4, at least 5, at least 6, or more cell proliferation or mitochondrial dyes.

[00246] The SCCs selected may be those cells exhibiting the most fluorescence. In exemplary aspects, the SCCs represent the top 1 to 20% of cells having the highest fluorescence intensity. In aspects, FCCs (fast-cycling cells) may be those cells exhibiting the least fluorescence. In exemplary aspects, the FCCs represent the bottom 1 to 20% cells having the lowest fluorescence intensity. Accordingly, a method to isolate SCCs may comprise selecting and isolating the sub-population of cells exhibiting the top 1-20% of fluorescence intensity. For example, the method may comprise selecting and isolating the sub-population of cells exhibiting the top 1%, top 2%, top 3%, top 4%, top 5%, top 6%, top 7%, top 8%, top 9%, top 10%, top 11%, top 12%, top 13%, top 14%, top 15%, top 16%, top 17%, top 18%, top 19% or the top 20% fluorescence intensity. The selection of cells based on fluorescence intensity may be achieved through techniques of flow cytometry and cell sorting, e.g., fluorescence-activated cell sorting (FACS). It is understood that larger isolated fractions may work with less efficacy and smaller fractions may work with less efficiency. SCCs and FCCs are identified based on their respective ability to be stained and retain labeling.

[00247] Optionally, dead cells may be removed from the mixed tumor cell population. In some aspects, the method comprises contacting the cells of the mixed tumor cell population with a dead cell stain agent including but not limited to propidium iodide (PI), non-fixable SYTOX DNA-binding dyes (e.g., SYTOX AADvanced, SYTOX Blue, SYTOX Orange, SYTOX Red or SYTOX Green) and live/dead fixable dyes (e.g., LIVE/DEAD Fixable Dead Cell Stain Blue,

Aqua, Yellow, Green, Red, Far Red, Near-IR). Dead cell stain agents are dyes that enters dead cells and cannot penetrate live cells.

[00248] The isolation of SCCs from the mixed tumor population may be carried out in one of the following ways. In a first method, SCCs are isolated from the mixed population of tumor cells based on proliferation rates. In exemplary aspects, SCCs are isolated based on their capacity to retain CellTrace dyes (Carboxyfluorescein succinimidyl ester-CFSE or Cell Trace Violet-CTV, Invitrogen). The SCCs and FCCs are grouped as CFSE/Violethigh- top 10% and CFSE/Violetlow- bottom 10%, respectively, or FCCs in some aspects are isolated as CFSElow-bottom 85% (Deleyrolle LP, et al. (2011) Brain 134:1331-43). Thus, SCCs in some aspects are isolated by selecting for cells grouped as CFSE/Violethigh- top 10% or by removing CFSElow-bottom 85% (FCCs). In a second method, SCCs are isolated based on mitochondrial content. In various instances, the cell-permeant MitoTracker™ (ThermoFisher Scientific, Waltham, MA) probes containing a mildly thiol-reactive chloromethyl moiety for labeling mitochondria is used to alternatively identify and isolate SCCs. In alternative or additional aspects, the following dyes are used to label live cells: Rosamine-based MitoTracker dyes, which include MitoTracker Orange CMTMRos, a derivative of tetramethylrosamine, and MitoTracker Red CMXRos, a derivative of X-rosamine. Reduced MitoTracker dyes, MitoTracker Orange CM-H2TMRos and MitoTracker Red CM-H2XRos, which are derivatives of dihydrotetramethylrosamine and dihydro-X-rosamine, respectively also are used in various instances. The carbocyanine-based MitoTracker dyes including MitoTracker Red FM, MitoTracker Green FM dye, and MitoTracker® Deep Red FM are additional dyes that are suitable for use to stain mitochondria and identify SCCs. The MitoProbe™ DiIC1(5) (1,1”,3,3,3’3”-hexamethylindodicarbo-cyanine iodide), which penetrates the cytosol of eukaryotic cells and accumulates primarily in mitochondria with active membrane potentials at concentrations below 100 nM, can be used to identify and isolate SCCs, which demonstrated greater mitochondrial membrane potential. Labeling of the cells is performed at 1 nM to 100 nM for 5 minutes to 12 h. SCCs can then be identified by the up to top 50% most brightest cells. In a third method, SCCs are isolated based on lipid content. In exemplary aspects, LipidSpot is used. Live or fixed cells are incubated with LipidSpot dyes, including but not limited to LipidSpot 610 and LipidSpot 488. In other exemplary aspects, LipidTox is used. Fixed cells are incubated with lipidTox dyes including but not limited to LipidTOX Green neutral lipid stain, LipidTOX Red neutral lipid stain or LipidTOX Deep Red neutral lipid stain.

[00249] The dilutions of the dyes may vary from 1/10 to 1/5000 (e.g., about 1/10, about 1/50, about 1/100, about 1/250, about 1/500, about 1/750, about 1/1000, about 1/2000, about 1/3000, about 1/4000, about 1/5000). The concentrations of the dyes in certain aspects range from about 5 nM to 1000 nM. In various aspects, the labeling time ranges from about 1 minute to about 24 hours. The labeling solution may comprise PBS or any buffer. Optionally, the buffer does not comprise a detergent. In various aspects, the buffer is at a neutral pH. The cell density for labeling may be from about 0.1 million cells per ml of labeling solution to about 20 million cells per ml of labeling solution.

[00250] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[00251] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the disclosure (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. If aspects of the invention are described as "comprising" a feature, embodiments also are contemplated "consisting of" or "consisting essentially of" the feature.

[00252] Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range and each endpoint, unless otherwise indicated herein, and each separate value and endpoint is incorporated into the specification as if it were individually recited herein. Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about" as that term would be interpreted by the person skilled in the relevant art.

[00253] All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the disclosure and does not pose a limitation on the scope of the disclosure unless

otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the disclosure.

[00254] Preferred embodiments of this disclosure are described herein, including the best mode known to the inventors for carrying out the disclosure. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the disclosure to be practiced otherwise than as specifically described herein. Accordingly, this disclosure includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the disclosure unless otherwise indicated herein or otherwise clearly contradicted by context.

WHAT IS CLAIMED:

1. A method of increasing sensitivity of a tumor to treatment with an immune checkpoint inhibitor (ICI) in a subject, the method comprising administering to the subject a composition comprising a nanoparticle comprising a positively-charged surface and an interior comprising (i) a core and (ii) at least two nucleic acid layers, wherein each nucleic acid layer is positioned between a cationic lipid bilayer, optionally, wherein the composition is systemically administered to the subject.
2. A method of treating a subject with an immune checkpoint inhibitor (ICI)-resistant tumor, comprising administering to the subject (i) a composition comprising a nanoparticle comprising a positively-charged surface and an interior comprising (i) a core and (ii) at least two nucleic acid layers, wherein each nucleic acid layer is positioned between a cationic lipid bilayer, and (ii) an ICI, optionally, wherein the composition is systemically administered to the subject.
3. The method of claim 1 or 2, wherein the ICI is a PD-L1 inhibitor.
4. The method of claim 3, wherein the PD-L1 inhibitor is a PD-L1 antibody.
5. The method of any one of claims 1-4, wherein the nanoparticle comprises at least three nucleic acid layers, each of which is positioned between a cationic lipid bilayer.
6. The method of claim 5, wherein the nanoparticle comprises at least four nucleic acid layers, each of which is positioned between a cationic lipid bilayer.
7. The method of claim 6, wherein the nanoparticle comprises five or more nucleic acid layers, each of which is positioned between a cationic lipid bilayer.
8. The method of any one of claims 1-7, wherein the outermost layer of the nanoparticle comprises a cationic lipid bilayer.
9. The method of any one of claims 1-8, wherein the surface comprises a plurality of hydrophilic moieties of the cationic lipid of the cationic lipid bilayer.
10. The method of any one of claims 1-9, wherein the core comprises a cationic lipid bilayer.
11. The method of any one of claims 1-10, wherein the core comprises less than about 0.5 wt% nucleic acid.

12. The method of any one of claims 1-11, wherein the diameter of the nanoparticle is about 50 nm to about 250 nm in diameter, optionally, about 70 nm to about 200 nm in diameter.

13. The method of any one of claims 1-12, wherein the nanoparticle comprises a zeta potential of about 40 mV to about 60 mV, optionally, about 45 mV to about 55 mV.

14. The method of claim 13, wherein the nanoparticle comprises a zeta potential of about 50 mV.

15. The method of any one of claims 1-14, wherein the nanoparticle comprises nucleic acid molecules and cationic lipid at a ratio of about 1 to about 5 to about 1 to about 20, optionally, about 1 to about 15 or about 1 to about 7.5.

16. The method of any one of claims 1-15, wherein the cationic lipid is DOTAP or DOTMA.

17. The method of any one of claims 1-16, wherein the nucleic acid molecules are RNA molecules.

18. The method of claim 17, wherein the RNA molecules are mRNA.

19. The method of claim 18, wherein the mRNA is *in vitro* transcribed mRNA wherein the *in vitro* transcription template is cDNA made from RNA extracted from a tumor cell.

20. The method of 18 or 19, wherein the mRNAs encode a protein.

21. The method of claim 20, wherein the protein is selected from the group consisting of a tumor antigen, a cytokine, and a co-stimulatory molecule.

22. The method of 20, wherein the protein is not expressed by a tumor cell or by a human.

23. The method of claim 17, wherein the RNA molecules are antisense molecules, optionally siRNA, shRNA, miRNA, or any combination thereof.

24. The method of claim 17, wherein the nanoparticle comprises a mixture of RNA molecules.

25. The method of claim 24, wherein the mixture of RNA molecules is RNA isolated from cells from a human.

26. The method of claim 25, wherein the human has a tumor and the mixture of RNA is RNA isolated from the tumor of the human, optionally, wherein the tumor is a malignant brain tumor, optionally, a glioblastoma, medulloblastoma, diffuse intrinsic pontine glioma, or a peripheral tumor with metastatic infiltration into the central nervous system.

27. The method of any one of claims 1-26, wherein the nanoparticles are prepared by mixing the nucleic acid molecules and the cationic lipid at a RNA: cationic lipid ratio of about 1 to about 5 to about 1 to about 20, optionally, about 1 to about 15.

28. The method of any one of claims 1-27, wherein the composition is systemically administered via parenteral administration, optionally, intravenous administration.

29. The method of any one of claims 1-28, wherein the composition is systemically administered in an amount effective to increase the number of PD-L1+/CD86+ myeloid antigen presenting cells (APCs) in the tumor periphery and/or in reticuloendothelial organs, increase PD-L1/CD86 expression by plasmacytoid dendritic cells (pDCs) and CD11c+ myeloid cells, increase Type I interferon release by pDCs, activate T-cell responses, or a combination thereof.

30. A method of increasing the number of activated plasmacytoid dendritic cells (pDCs) in a subject in need thereof, comprising administering to the subject a composition comprising a nanoparticle comprising a positively-charged surface and an interior comprising (i) a core and (ii) at least two nucleic acid layers, wherein each nucleic acid layer is positioned between a cationic lipid bilayer, optionally, wherein the nanoparticle is systemically administered to the subject.

31. The method of claim 30, wherein the nanoparticle comprises at least three nucleic acid layers, each of which is positioned between a cationic lipid bilayer.

32. The method of claim 31, wherein the nanoparticle comprises at least four nucleic acid layers, each of which is positioned between a cationic lipid bilayer.

33. The method of claim 32, wherein the nanoparticle comprises five or more nucleic acid layers, each of which is positioned between a cationic lipid bilayer.

34. The method of any one of claims 30-33, wherein the outermost layer of the nanoparticle comprises a cationic lipid bilayer.

35. The method of any one of claims 30-34, wherein the surface comprises a plurality of hydrophilic moieties of the cationic lipid of the cationic lipid bilayer.

36. The method of any one of claims 30-35, wherein the core comprises a cationic lipid bilayer.

37. The method of any one of claims 30-36, wherein the core comprises less than about 0.5 wt% nucleic acid.

38. The method of any one of claims 30-37, wherein the diameter of the nanoparticle is about 50 nm to about 250 nm in diameter, optionally, about 70 nm to about 200 nm in diameter.

39. The method of any one of claims 30-38, wherein the nanoparticle comprises a zeta potential of about 40 mV to about 60 mV, optionally, about 45 mV to about 55 mV.

40. The method of claim 39, wherein the nanoparticle comprises a zeta potential of about 50 mV.

41. The method of any one of claims 30-40, wherein the nanoparticle comprises nucleic acid molecules and cationic lipid at a ratio of about 1 to about 5 to about 1 to about 20, optionally, about 1 to about 15 or about 1 to about 7.5.

42. The method of any one of claims 30-41, wherein the cationic lipid is DOTAP or DOTMA.

43. The method of any one of claims 30-42, wherein the nucleic acid molecules are RNA molecules.

44. The method of claim 43, wherein the RNA molecules are mRNA.

45. The method of claim 44, wherein the mRNA is *in vitro* transcribed mRNA wherein the *in vitro* transcription template is cDNA made from RNA extracted from a tumor cell.

46. The method of 44 or 45, wherein the mRNAs encode a protein.

47. The method of claim 46, wherein the protein is selected from the group consisting of: a tumor antigen, a cytokine, or a co-stimulatory molecule.

48. The method of claim 46, wherein the protein is not expressed by a tumor cell or by a human.

49. The method of claim 43, wherein the RNA molecules are antisense molecules, optionally siRNA, shRNA, miRNA, or any combination thereof.

50. The method of claim 43, wherein the nanoparticle comprises a mixture of RNA molecules.

51. The method of claim 50, wherein the mixture of RNA molecules is RNA isolated from cells from a human.

52. The method of claim 51, wherein the human has a tumor and the mixture of RNA is RNA isolated from the tumor of the human, optionally, wherein the tumor is a malignant brain

tumor, optionally, a glioblastoma, medulloblastoma, diffuse intrinsic pontine glioma, or a peripheral tumor with metastatic infiltration into the central nervous system.

53. The method of any one of claims 30-52, wherein the nanoparticles are prepared by mixing the nucleic acid molecules and the cationic lipid at a RNA: cationic lipid ratio of about 1 to about 5 to about 1 to about 20, optionally, about 1 to about 15.

54. The method of any one of claims 30-53, wherein the composition is systemically administered via parenteral administration, optionally, intravenous administration.

55. The method of any one of claims 30-54, wherein the subject has an immune checkpoint inhibitor (ICI)-resistant tumor.

56. The method of any one of claims 30-55, wherein the pDCs are PD-L1$^+$/CD86$^+$ pDCs.

57. A method of treating a subject with a tumor or cancer, the method comprising (i) increasing the number of activated plasmacytoid dendritic cells (pDCs) in the subject in accordance with the method of any one of claims 30-56, (ii) isolating white blood cells (WBCs) from the subject, (iii) isolating dendritic cells (DCs) from the WBCs, (iv) contacting the DCs with a fusion protein comprising prostatic acid phosphatase (PAP) and GM-CSF, and (v) administering the DCs to subject.

58. A method of preparing a dendritic cell vaccine, the method comprising (i) increasing the number of activated plasmacytoid dendritic cells (pDCs) in the subject in accordance with the method of any one of claims 30-56, (ii) isolating white blood cells (WBCs) from the subject, (iii) isolating dendritic cells (DCs) from the WBCs, and (iv) contacting the DCs with a fusion protein comprising prostatic acid phosphatase (PAP) and GM-CSF.

FIGURE 1A

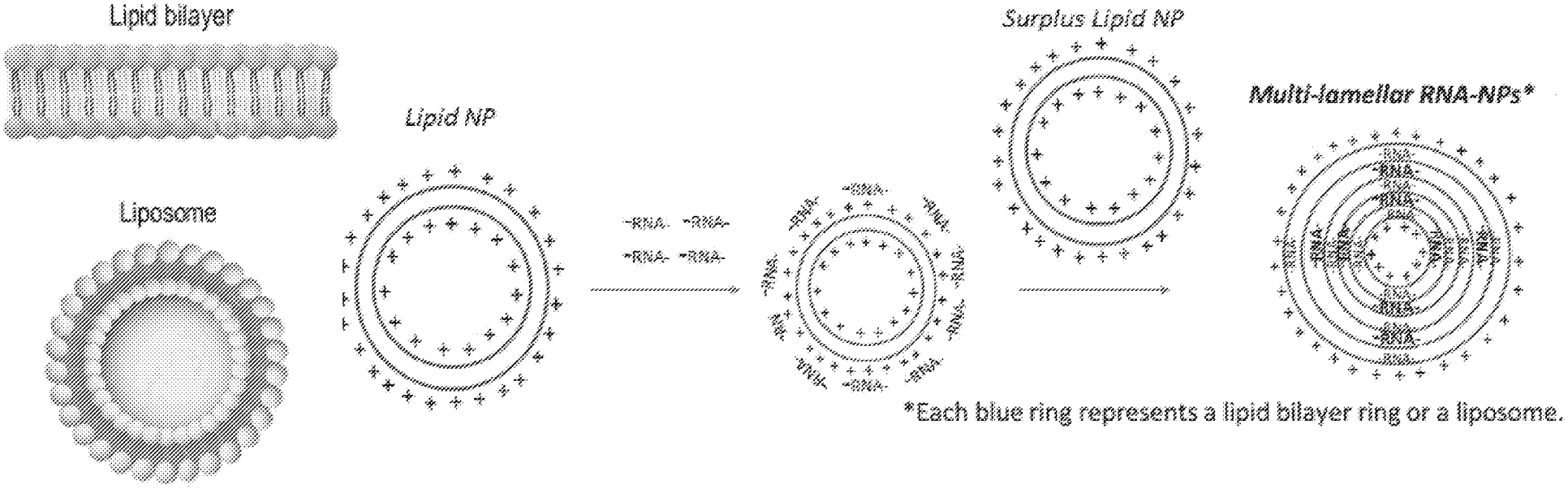


FIGURE 1B

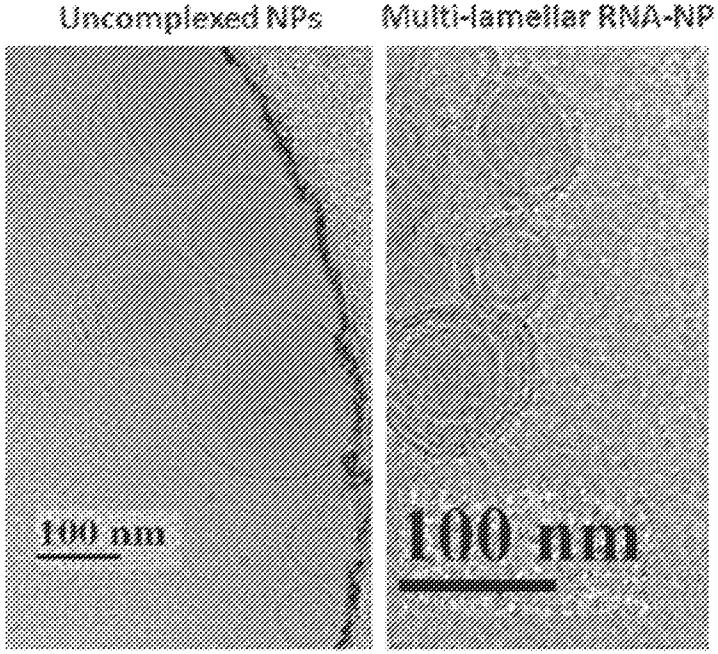


FIGURE 2A

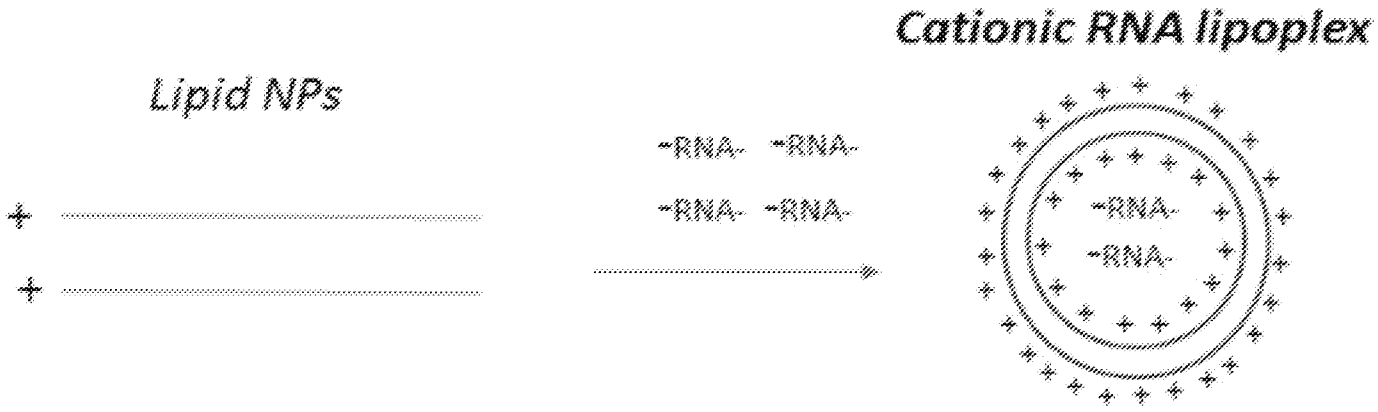


FIGURE 2B

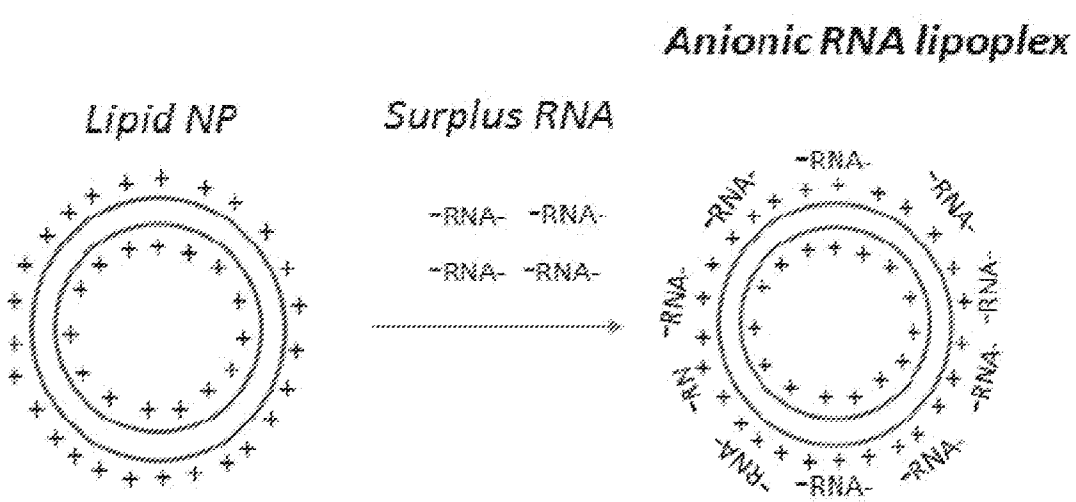


FIGURE 2C - Uncomplexed NPs

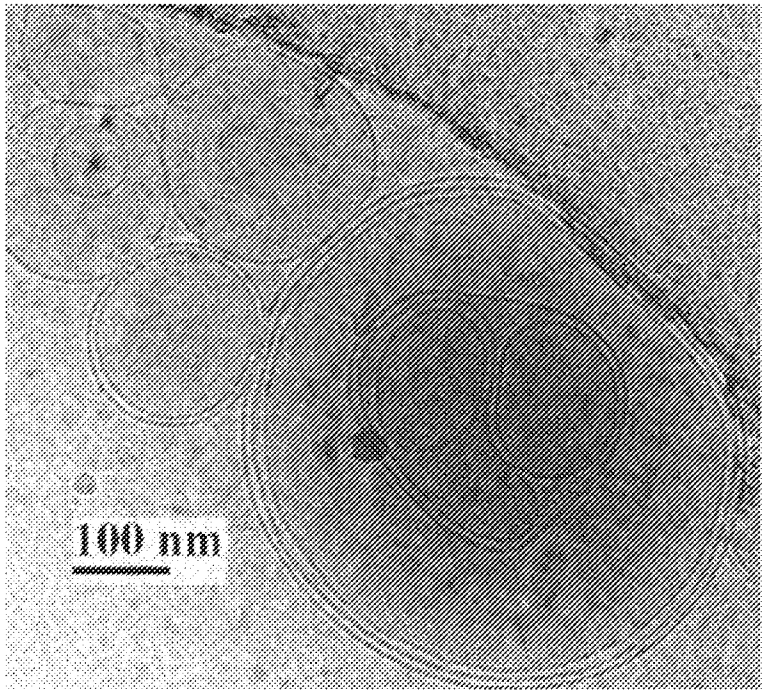


FIGURE 2D - RNA Lipoplexes

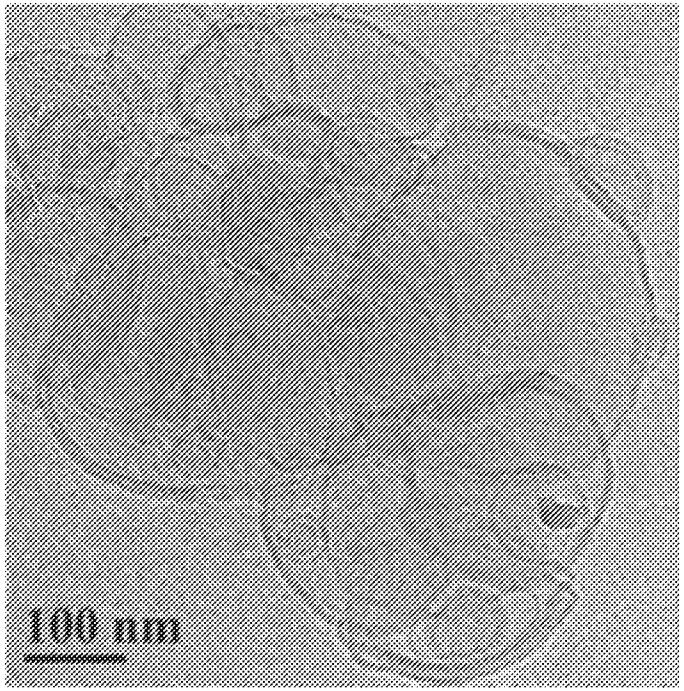


FIGURE 2E – Multi-lamellar RNA-NPs

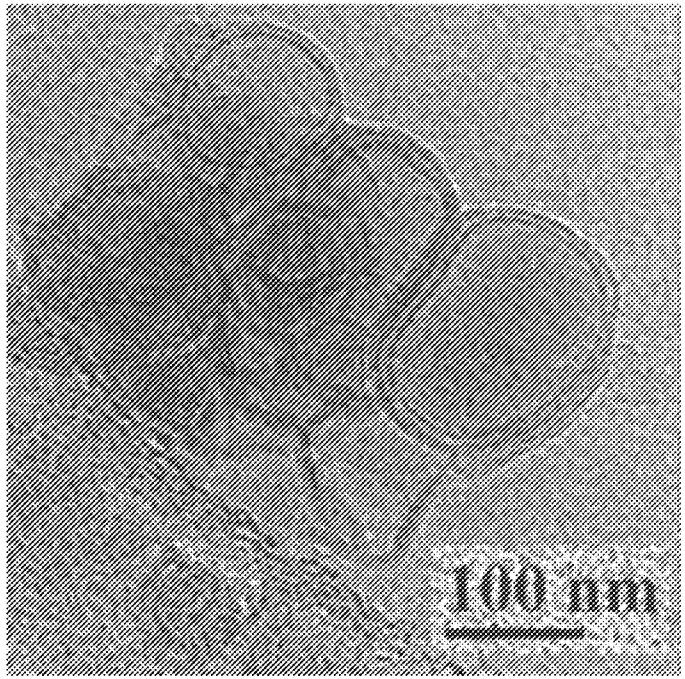


FIGURE 2G

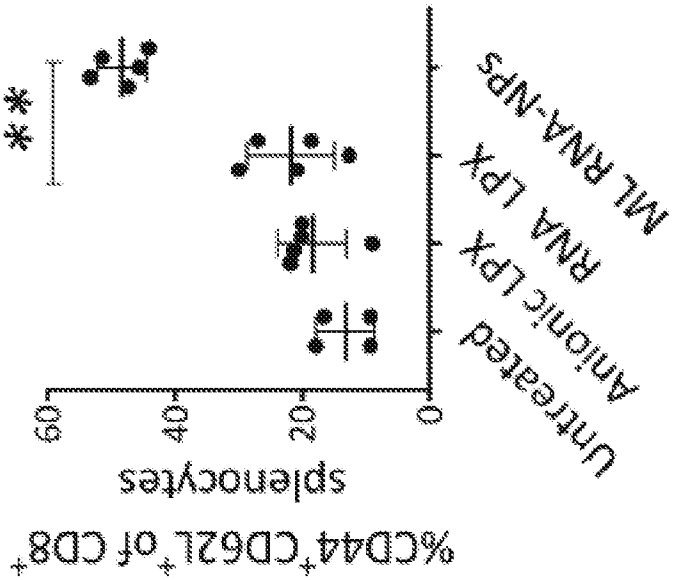


FIGURE 2H

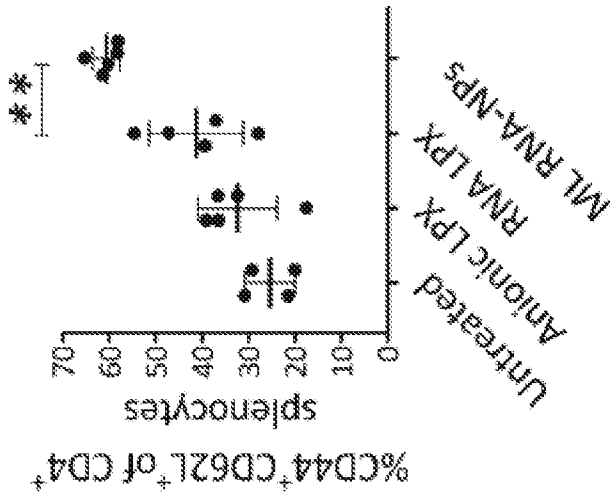


FIGURE 2F

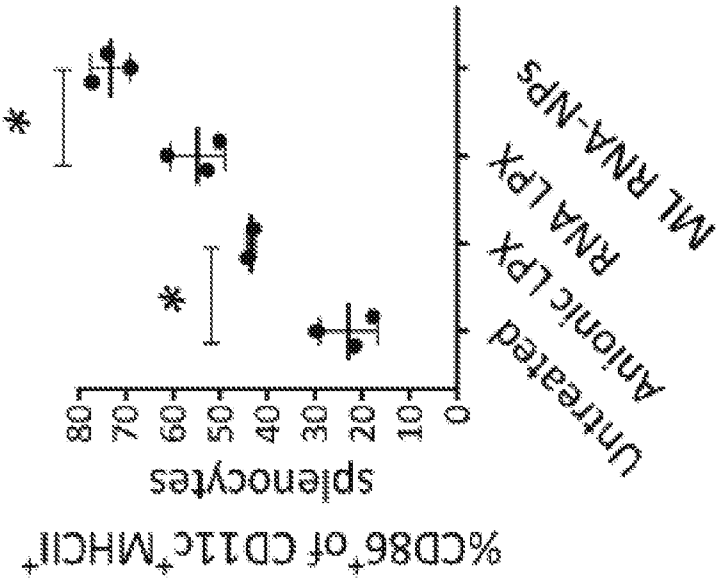


FIGURE 2I

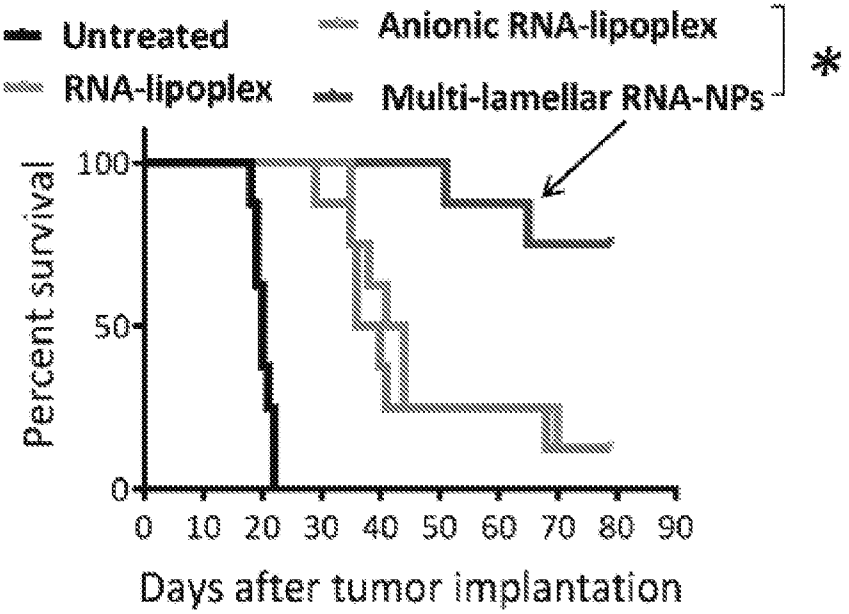


FIGURE 2J

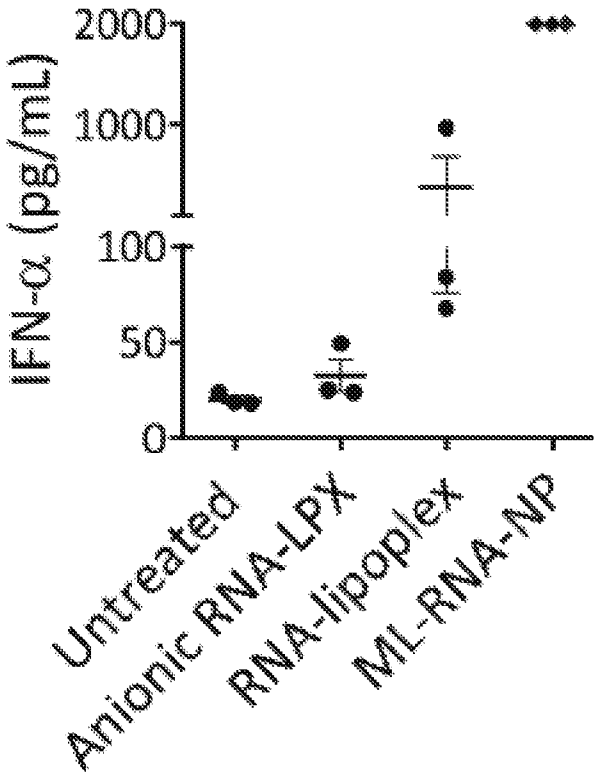


FIGURE 3A

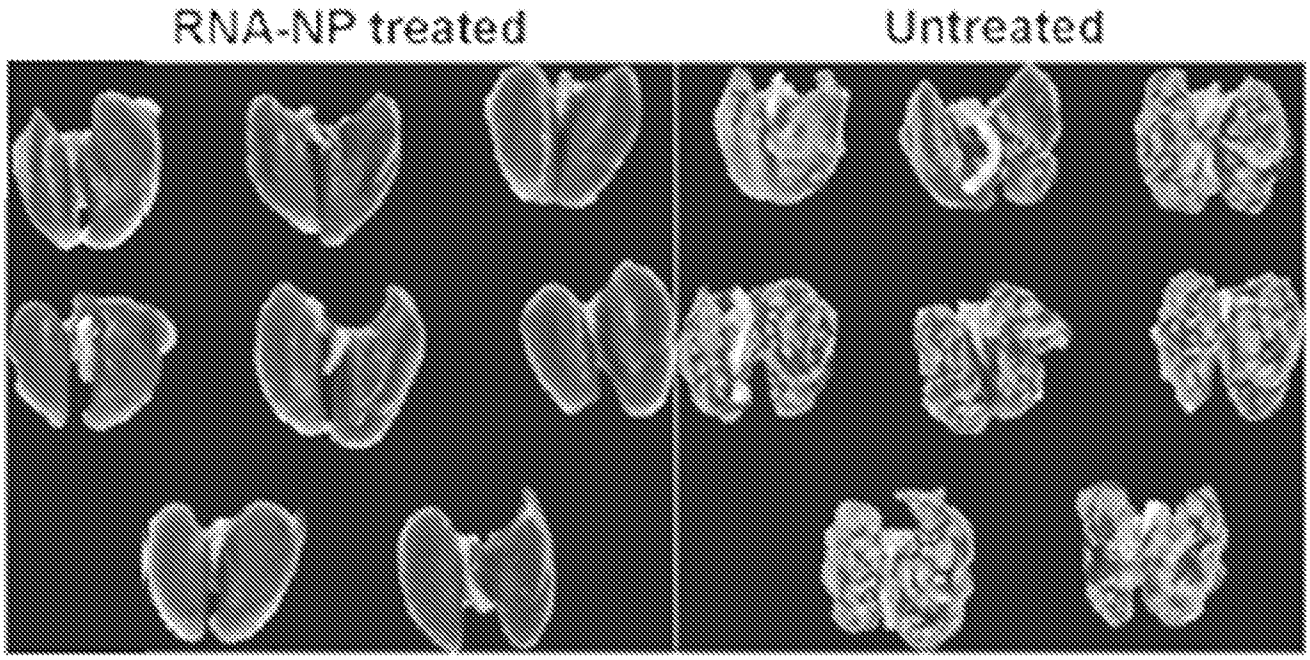


FIGURE 3B

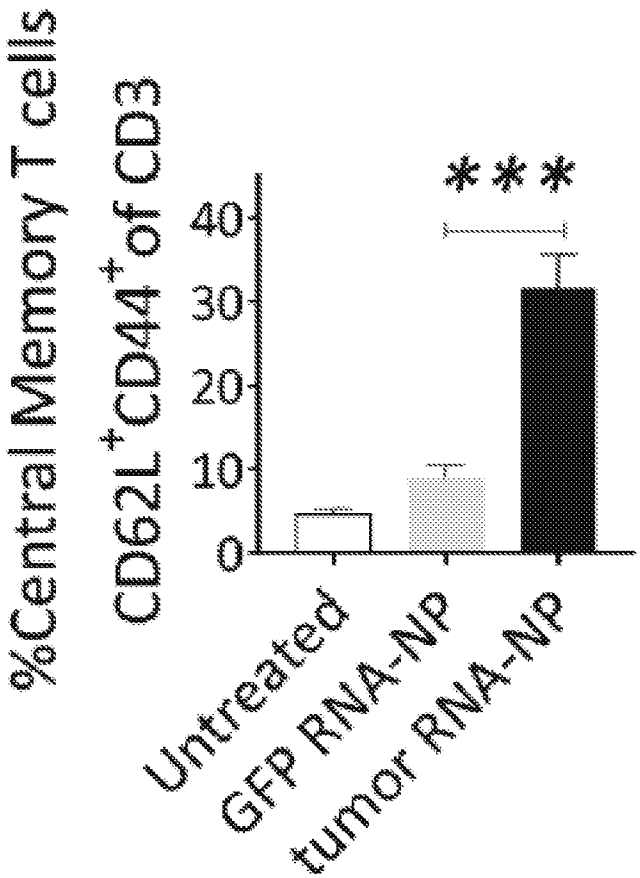


FIGURE 3C

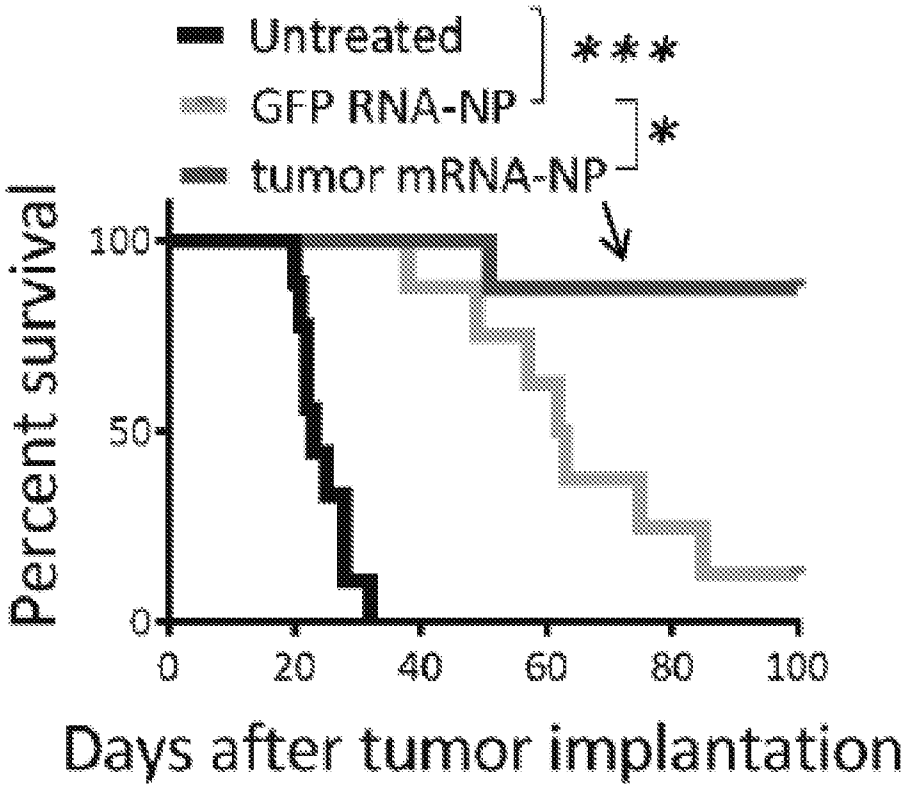


FIGURE 3D

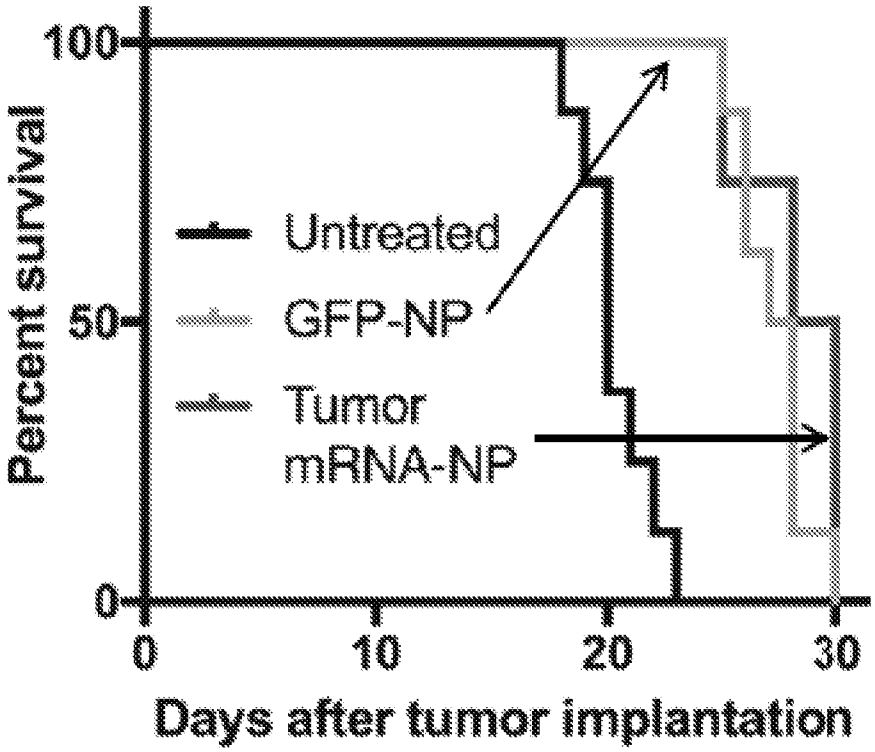


FIGURE 4A

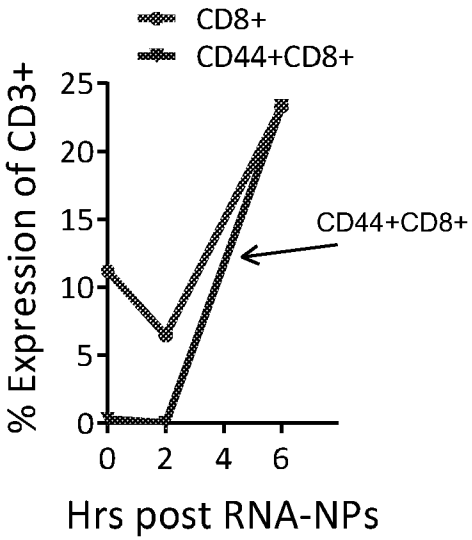


FIGURE 4B

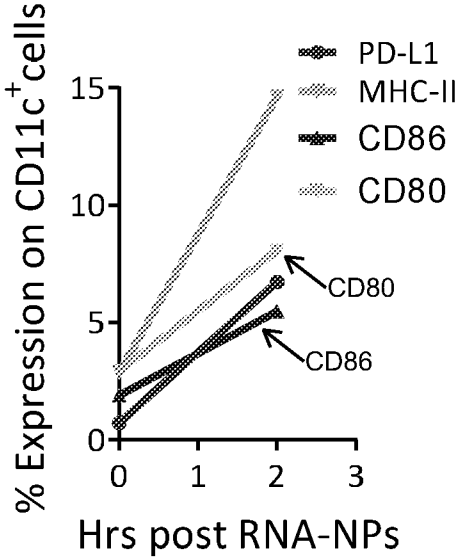


FIGURE 4C

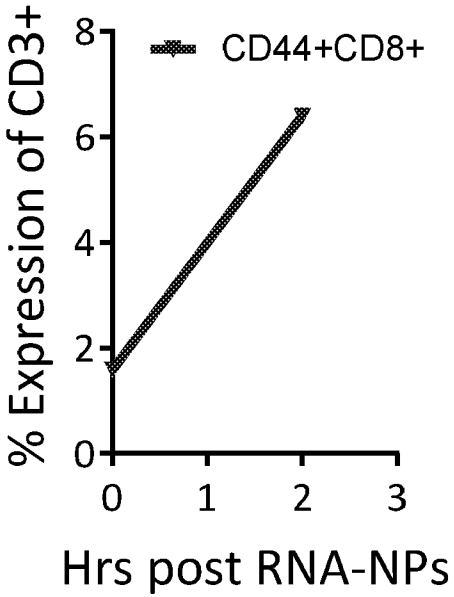


FIGURE 4D

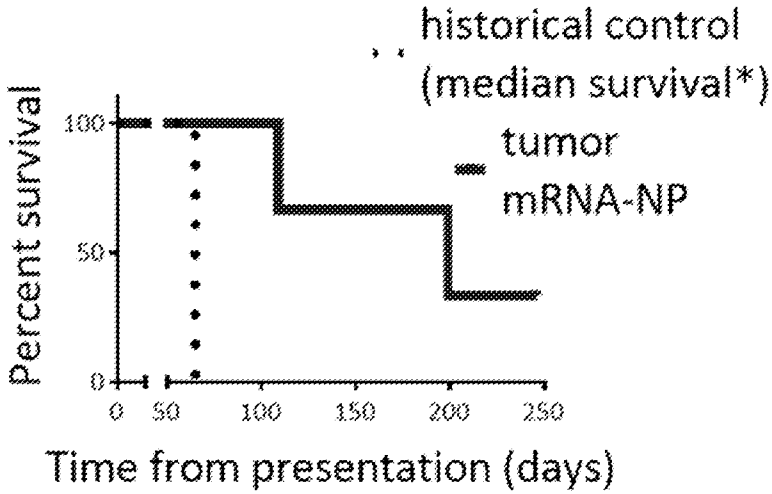


FIGURE 4E

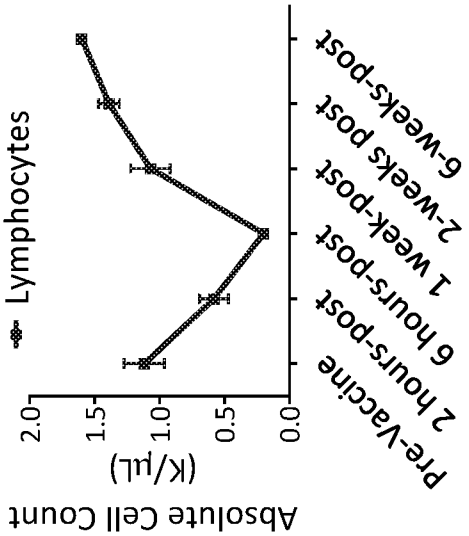


FIGURE 4F

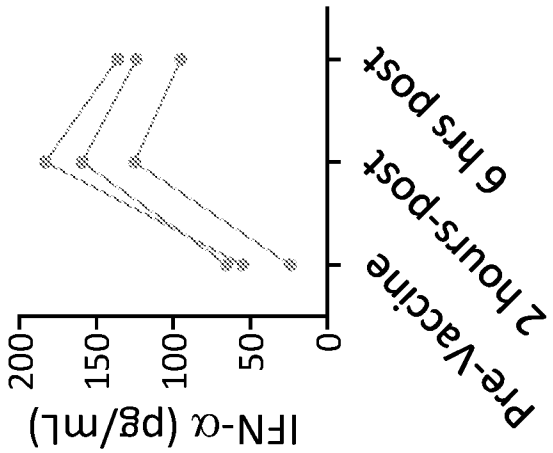
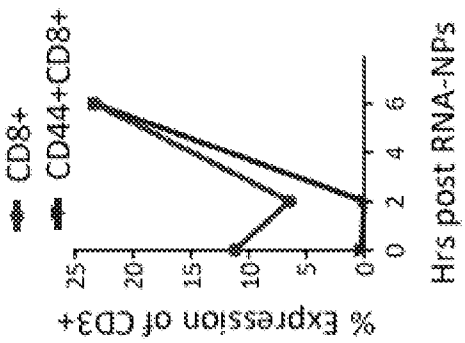


FIGURE 4G

FIGURE 4H



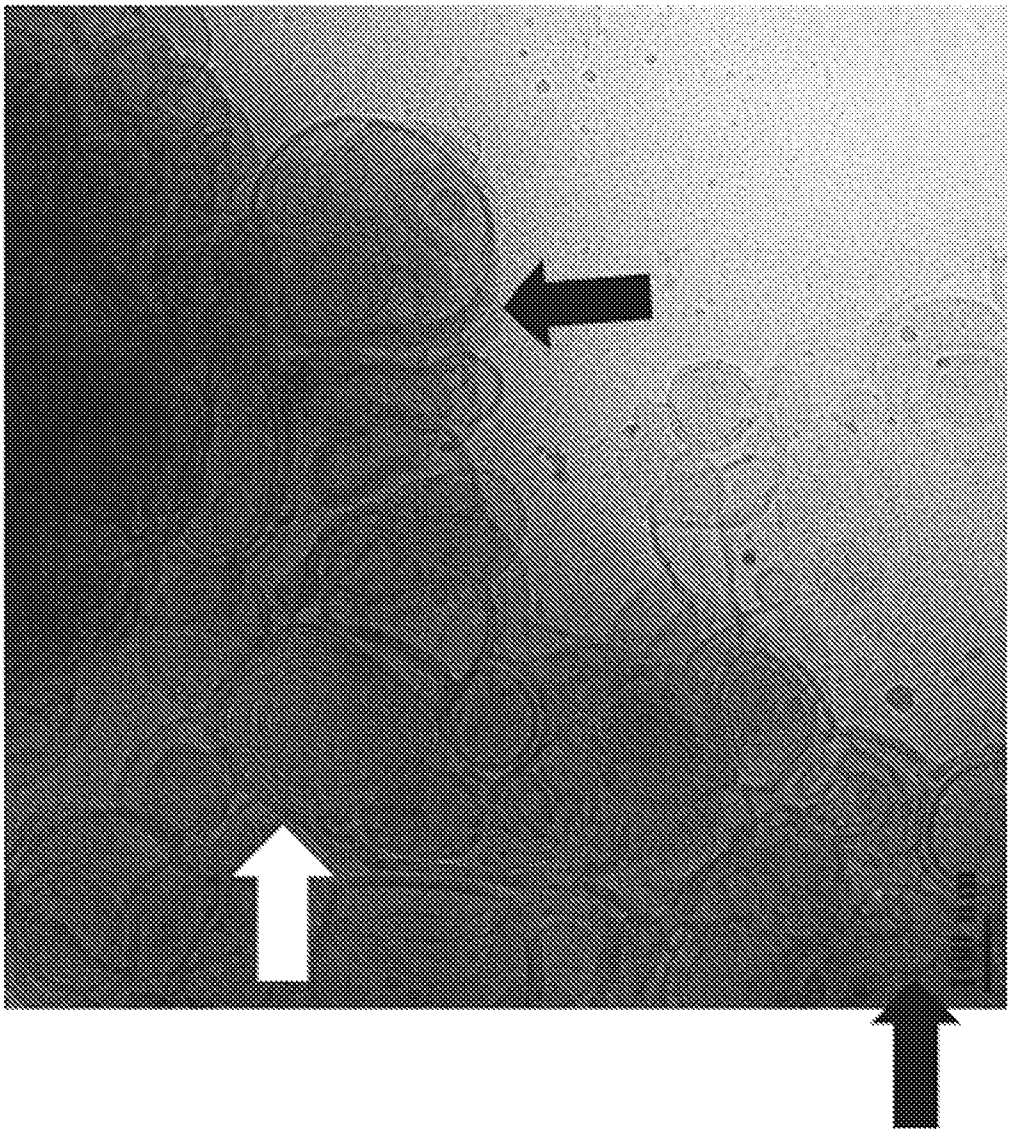


FIGURE 5

FIGURE 6

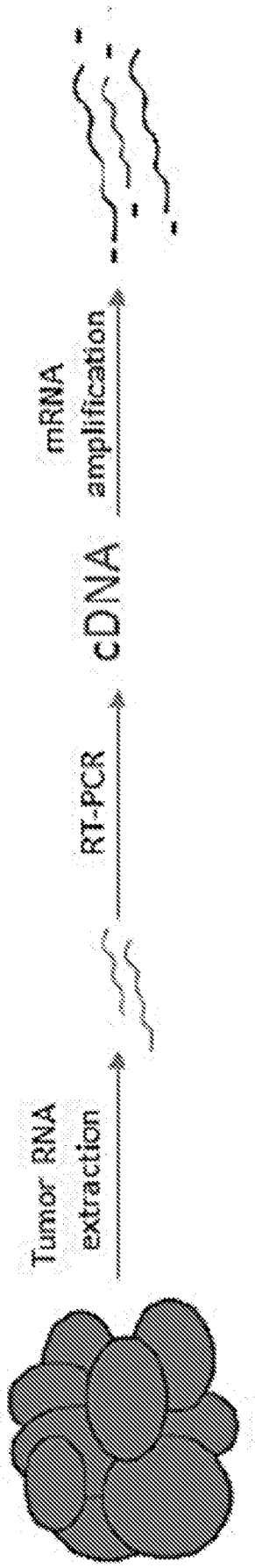


FIGURE 6 Continued

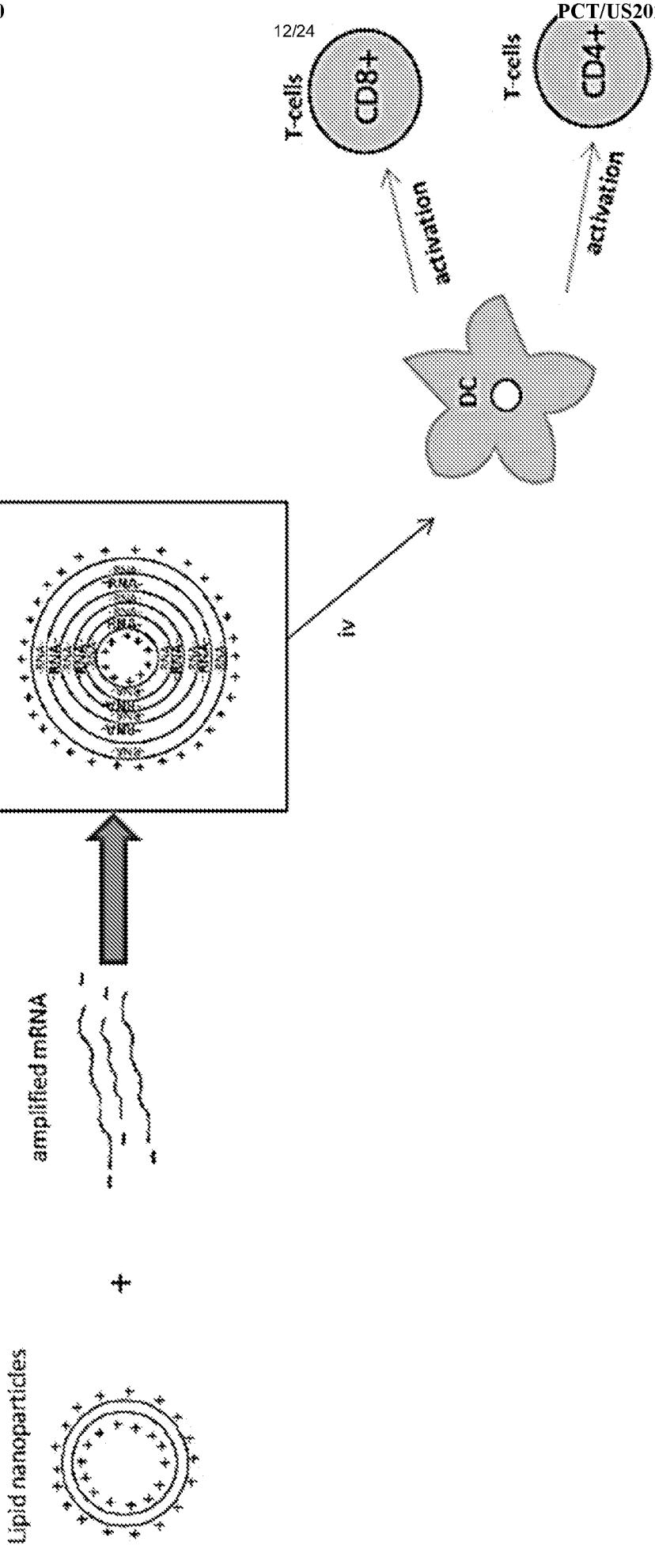


FIGURE 7A

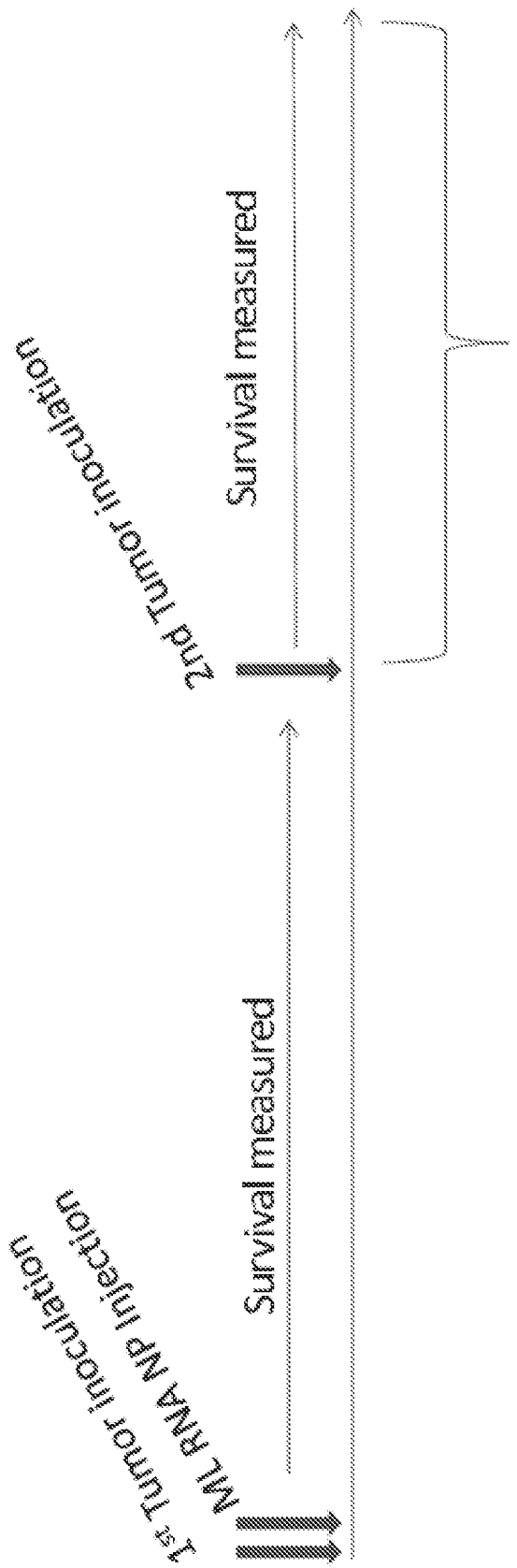


FIGURE 7B

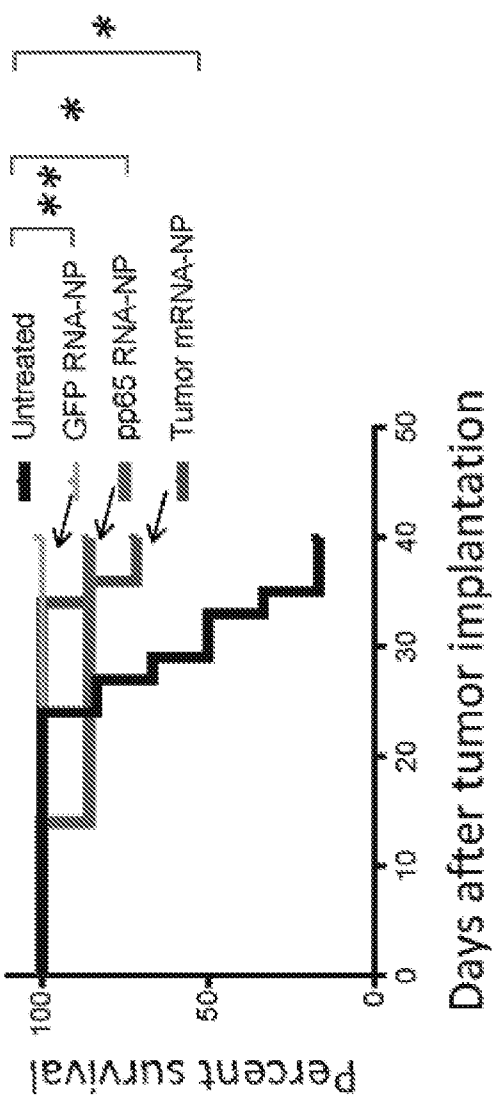


FIGURE 8

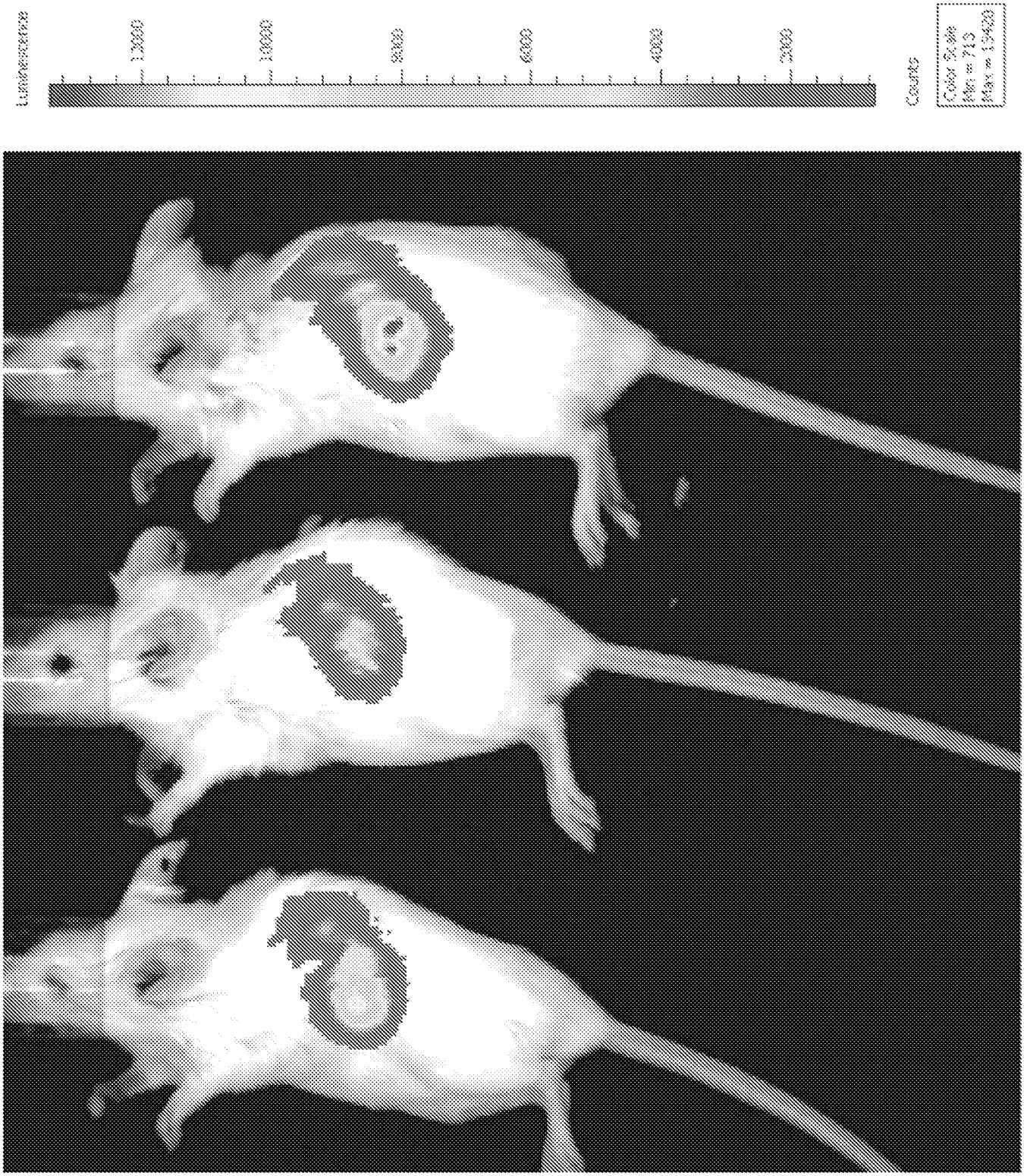

Luminescence
12000
10000
8000
6000
4000
2000
Counts
Color Scale
Min = 713
Max = 13420

FIGURE 9

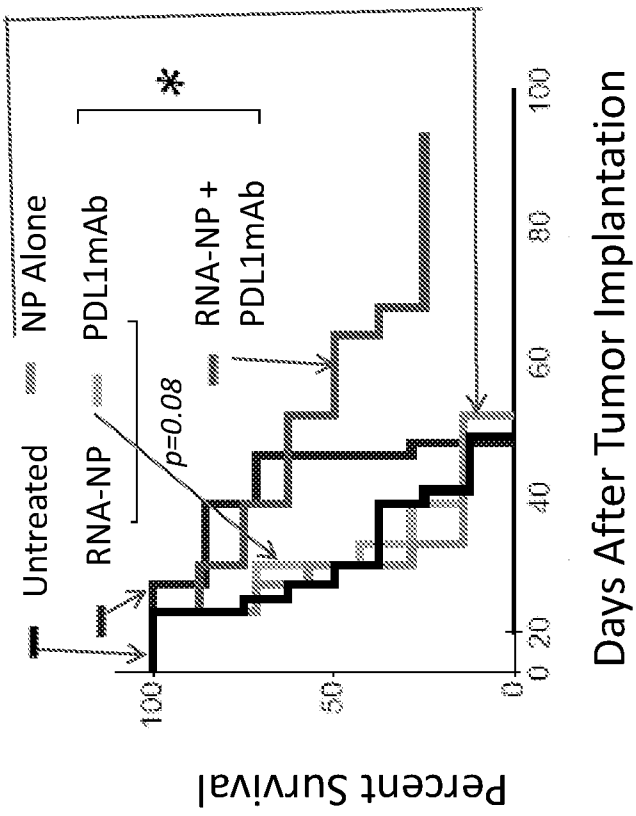

Untreated
NP Alone
RNA-NP
PDL1mAb
RNA-NP +
PDL1mAb
p=0.08
*
Percent Survival
100
50
0
0 20 40 60 80 100
Days After Tumor Implantation

FIGURE 10A

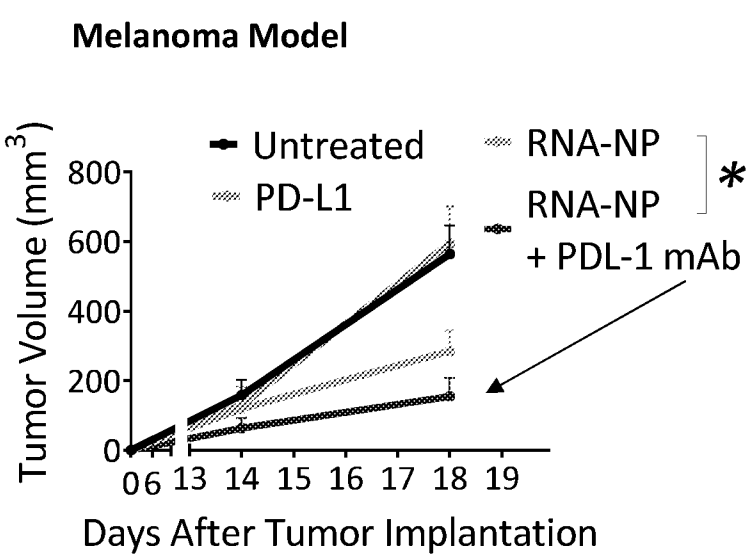


FIGURE 10B

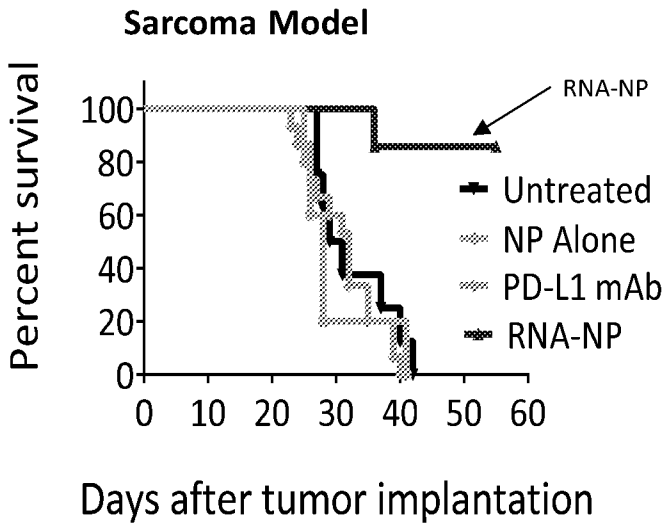


FIGURE 10C

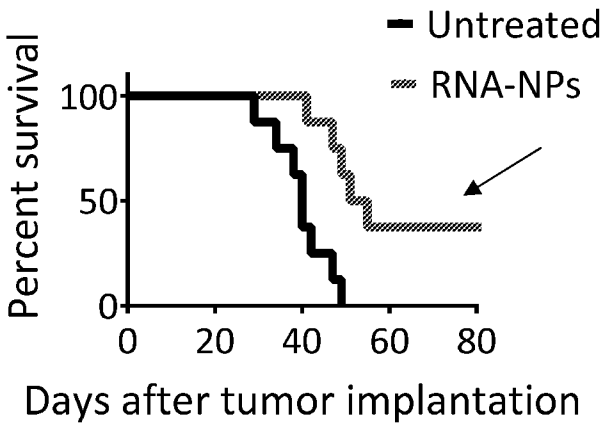


FIGURE 11A

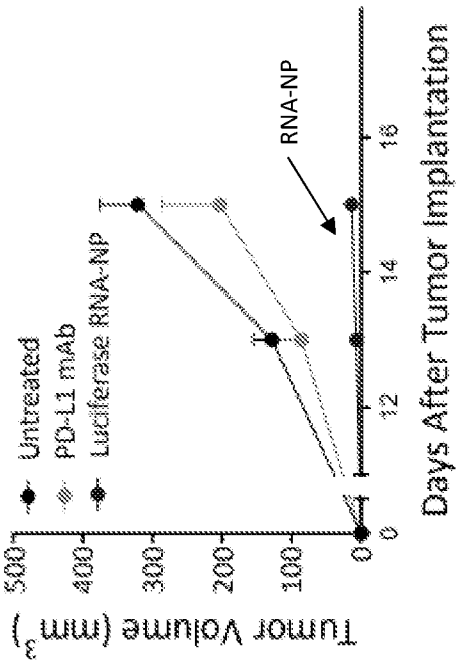


FIGURE 11B

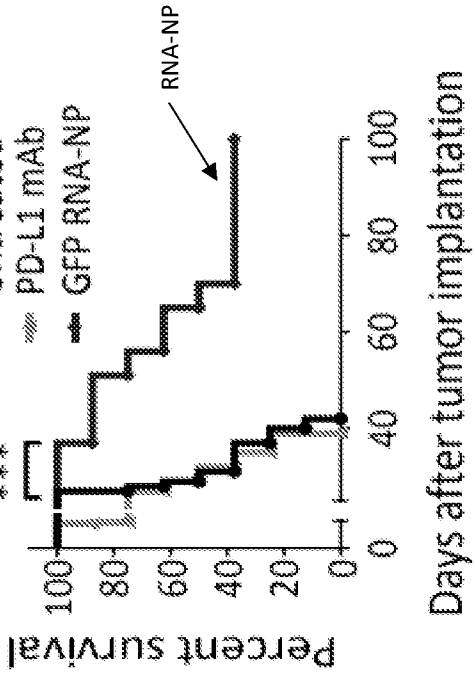


FIGURE 11C

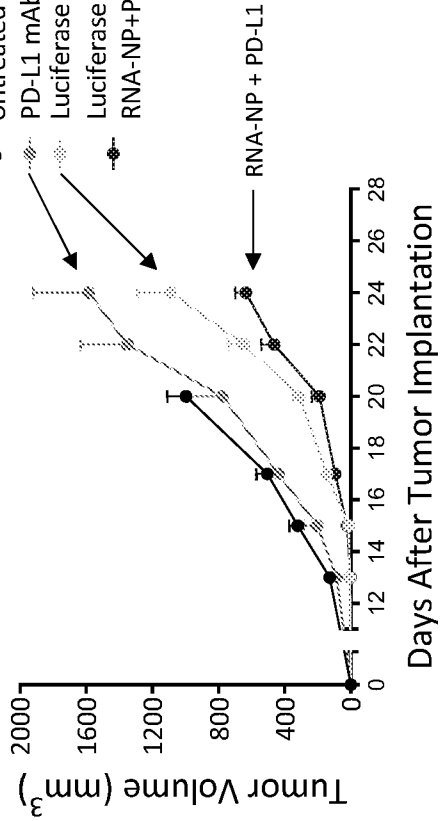


Figure 12

Heme	PRDM16(MEL1)-RPN1; WDR48-PDGFRB; TPM3-PDGFRB;PDE4DIP-PDGFRB; MYC(C-MYC); BCL9-IGH; FCGR2B-IGH; FCRL4-IGH; MUC1/IGH; PBX1-TCF3(E2A); ALK-NPM1; MYC(C-MYC); MLL; RPN1-MECOM(EVI1); GOLGA4-PDGFB; RPL22(EAP)-RUNX1(AML1); AFF1(AF4)-MLL; FGFR3-IGH; PDGFRA -BCR ; HIP1-PDGFRB; CCD6-PDGFRB; ETV6(TEL)-ACSL6; GIT2-PDGFRB; ETV6(TEL)-PDGFRB; NIN-PDGFRB; CCDC88C(KIAA1509)-PDGFRB; TP53BP1-PDGFRB; TLX3(HOX11L2)-BCL11B; CBFB; NPM1(NPM)-RARA; SPECC1-PDGFRB; RABEP1-PDGFRB; DEK-NUP214 (CAN); MLLT4(AF6)-MLL; NUP98-HOXA9; MYC(C-MYC)- IGH; KAT6A(MYST3)-CREBBP(CBP); RUNX1(AML)-RUNX1T1(ETO); MYC(C-MYC)-IGL; MLLT3(AF9)-MLL; PAX5-IGH; ABL1-BCR; CCND1(BCL1)-IGH; MLL-KIAA0284; MLL-CREBBP(CBP); ZBTB16(PLZF)-RARA; BIRC3(API2)-MALT1; MLL-ELL; MLL-MLLT1(ENL); IGH-MAF; IGH-BCL2; IGH-IGL; PML-RARA; MYH11-CBFB; PBX1-TCF3(E2A); RPN1-MECOM(EVI1); MAML2-MLL; TRA-TCL1A(TCL1); MYH11-CBFB; ETV6(TEL-RUNX1(AML1); CRLF2-IGH; TLX3-BCL11B; TAL1-STIL
Heme – Primarily Pediatric	PBX1-TCF3; CEBPD-IGH; TCF3(E2A)-HLF; RBM15-MKL1; MLLT10-MLL; FUS-ERG; CBFA2T3-RUNX1; MLLT10-PICALM; LMO1-TRA; LMO2(Rhom2)-TRAα
RET Fusions	ACBD5-RET; AFAP1L2-RET; AKAP13-RET; ANKRD26-RET; BCR-RET; CDC123-RET; CCDC6-RET (RET/PTC1); CLIP2-RET; CUX1-RET; DLG5-RET; EPHA5-RET; ERC1-RET; ETL4-RET; ETV6-RET; FGFR1OP-RET; FKBP15-RET; FRMD4A-RET; GEMIN5-RET; GOLGA5-RET (RET/PTC5); HOOK3-RET; KHDRBS1-RET; KIAA1468-RET; KIF13A-RET; KIF5B-RET; KTN1-RET (RET/PTC8); MYH10-RET; MYH13-RET; MYO5A-RET; MYO5C-RET; NCOA4-RET (RET/PTC3); PCM1-RET; PDCD10-RET; PICALM-RET; PPFIBP2-RET; PRKAR1A-RET (RET/PTC2); RASGEF1A-RET; RASSF4-RET; RET-RET; RRBP1-RET; RUFY2-RET; SNRNP70-RET; SPECC1L-RET; SQSTM1-RET; TBC1D32-RET; TBL1XR1-RET; TFG-RET; TNIP1-RET; TRIM24-RET (RET/PTC6); TRIM27-RET; TRIM33-RET (RET/PTC7); UEVLD-RET; VCL-RET; WAC-RET; ZNF485-RET
Head and Neck MYB Rearrangements	MYB Fusions; MYB – NFIB; MYB/PDCD1LG2; EWSR1/POU5F1; CHCHD7/PLAG1; TCEA1/PLAG1
MAML2 Fusions	CRTC1–MAML2; CRTC3–MAML2
Ewing's Sarcoma	EWSR1/FLI1; EWSR1/ERG; EWSR1/ETV1;EWSR1/ETV4; EWSR1/FEV; FUSR1/FEV; FUSR1/ERG
Rhabdomyosarcoma (including Alveolar)	PAX3-FOXO1; PAX7-FOXO1; PAX3-NCOA1; FOXO1-FGFR1; PAX3-NCOA2; FOXO1-PAX3; PAX3-FOXO4; MLLT3-KMT2A
Ependymoma	YAP1 fusion; C11orf95-RELA fusions; C11orf95-YAP1; YAP1-MAMLD1; YAP1- FAM118B
Clear Cell Sarcoma	EWS/ATF1
Glioblastoma	FIG-ROS1
Non-Small Cell Lung Cancer	EML4-ALK; SCD5-ROS1; BAG4-FGFR1; C10orf10-ROS1; CCDC6-RET; CCDC6-ROS1; CD74-ROS1; EZR-ROS1; FGFR3-TACC3; HIP1-ALK; IRF2BP2-NTRK1; KDEL-ROS1; KIF5B-RET; LIMA1-ROS1; LRIG3-ROS1; NCOA4-RET; SCD5-ROS1; SDC4-ROS1; SLC34A2-ROS1; SQSTM1-ALK; SQSTM1-NTRK1; TGF-ALK; TPM3-ROS1; TRIM33-RET; TRMT61B-ALK
Glioma	KIAA1549-BRAF; FGFR1-FGFR1; FGFR3-TACC3; C10orf10-ROS1; FGFR1-TACC1; FAM131B-BRAF; AKAP9-BRAF; GGA2-PRKCB; HIF1A-VRK1; WNK1-STK38L; RNF130-BRAF; GGA2-PRKCB; TAOK1-C17orf105; C11orf80-MKNK1; RIMKLB-PIP4K2A; TEX261-AAK1; SRGAP3-SRGAP3-RAF1; MYB-QKI; GOPC-ROS1; SRGAP3-RAF1; AFAP1-NTRK2; EHD1-GRK3; GNAI1-BRAF; SQSTM1-NTRK2; PDGFRA-FIP1L1/LNX1; FXR1-BRAF; EPHB2-PDZD4; MYB-PCDHGA1; QKI-NTRK2; EIF2AK4-PKM; MAP3K13-UTS2B; TYRO3-TTR; MAP3K10-SUPT5H; MAP3K13-CCDC50/UTS2B; ANK2-ALPK1; NRBP1-DROSHA; LRRK2-GXYLT1; CAMK2D-ANK2; QKI-RAF1; SIK2-MEGF11; NLK-ELMO1; PPM1A-COQ8B; VEGFA-STK38; EPHB1-BFSP2; STK39-TMEM245; CCDC6-BRAF; AGK-BRAF; NUAK2-CYB5R1; NACC2-NTRK2; BRAF-MACF1; CLCN6-BRAF; AKT2-ZNF576; NEK11-BDH1; MKRN1-BRAF; RPS6KA2-MCHR2; CTNND2-NRBP1; AKT2-SRRM5
Sarcoma	AAK1-RPL36A; ACTB-FOSB; ACVR2A-SUMO1; ASPSCR1-TFE3; ATF1-EWSR1; BCOR-CCNB3; BCOR-MAML3; BRD4-NUTM1; C11orf95-RELA; C12orf45-CDK7; C5orf22-MAP2K5; CAMKK2-DIP2B; CAMKK2-PMCH; CDK19-LYZ; CDK2-PAN2; CIC-DUX4; CIC-FOXO4; CIC-NUTM1; CLIP1-ROS1; CLK2-

Figure 12 Continued

	MARS; CMKLR1-HNF1A; CRTC1-SS18; CSNK1G2-PDE5A; CYT-SSX1; CYT-SSX2; DYRK2-CNTN2; DYRK2-PKP1; ELK3-CDK17; EPC1-PHF1; EPC2-PHF1; EPHB3-PAX2; ERBB3-CRADD; ERG-EWSR1; ETV4-EWSR1; ETV6-NTRK3; EWSR1-ATF1; EWSR1-CREB1; EWSR1-EIAF; EWSR1-ERG; EWSR1-ETS; EWSR1-ETV1; EWSR1-ETV4; EWSR1-EWSR1-FLI1; EWSR1-EWSR1-POU5F1; EWSR1-FEV; EWSR1-FLI1-FLI1; EWSR1-FLI1-FLI1-FLI1; EWSR1-KCNH2; EWSR1-NFATC2; EWSR1-POU5F1; EWSR1-SMARCA5; EWSR1-SP3; EWSR1-TFCP2; EWSR1-UQCRH; FLI1-NM_13986.3; FN1-ALK; FUNDC2-RPS6KA6; FUS-CREB3L2; FUS-ERG; FUS-FEV; FUS-NCATc2; FUS-NFATC2; FUS-TFCP2; HIC2-TEC; HLA-A-ROS1; IGH-BCL2; IRX2-TERT; JAZF1-BCORL1; JAZF1-JAZF1-PHF1; JAZF1-JAZF1-PHF1-PHF1; JAZF1-PHF1; JAZF1-SUZ12; KDM6A-RPS6KA3; LIMK1-TYW1; LMTK2-RBFOX3; MBTD1-CXorf67; MBTD1-MBTD1-CXorf67; MDM2-SYK; MEAF6-PHF1; MED21-PIP5K1C; MOK-MUCL1; MOK-WARS; MYDGF-MAP2K3; NFATC2-EWSR1; NSD3-NRG1; NUAK1-C12orf65; NUAK1-UHRF1BP1L; PA2G4-MARK1; PAK4-L3MBTL4; PAN3-FLT3; PAX3-MAML3; PCDH15-PCDH15; PDGFRB-FBLN1; PIP5K1C-PPM1H; PKN3-TRIM65; PPFIBP1-ALK; PRKD3-TMEM51; PTAR1-PIP5K1B; PTK7-CNOT2; RAB3B-PKN2; RAP1B-SCYL2; RPA1-GUCY2D; RPS6KA2-KIFC1; RPS6KA3-KDM6A; RREB1-TFE3; Sarcoma; SCYL2-GIT2; SCYL2-LINC01619; SMARCB1-WASF2; SRI-PIP4K2C; SS18-SS18-SSX2; SS18-SSX1; SS18-SSX1-SSX1; SS18-SSX2; SS18-SSX2-SSX2; SS18-SSX4; SS18L1-SSX1; SSX1-SS18; SSX1-SS18L1; SSX1-SSX1; SSX1-SYT4; SSX2-SS18; STK10-GNS; TEAD4-MAP3K12; TET-ETS; TFE3-ASPSCR1; TGFBR2-XPC; TGFBR3-MGEA5; TPM3-NTRK1; TPM4-NTRK3; TRIO-CDH4; TRIO-FBXL7; TRIO-TERT; TRPS1-PLAG1; TTK-TMEM63B; TUFT1-PKN2; UHMK1-DCAF8; VCP-TFE3; VGLL2-NCOA2; YWHAE-NUTM2A; YWHAE-NUTM2B; YWHAE-NUTM2E; ZC3H7B-BCOR; ZNF292-MAP3K4
Neuroblastoma	NF1-NF1; KMT2A-FRYL; PAFAH1B2-FOXR1; NBPF1-ASIC2; TERT-ALK; KMT2A-FOXR1
Retinoblastoma	RB1-RB1
Nephroblastoma	HDAC9-DGKB
Lymphoma	RUNX1-RUNX1T1; ABL1-BCR; AFF1-AFF1; AFF1-CCDC84; AFF1-DSCAML1; AFF1-ELF2; AFF1-FXYD6; AFF1-KMT2A; AFF1-PBX1; AFF1-RABGAP1L; ALK-MSN; ALK-TFG; ATG16L2-KMT2A; ATIC-ALK; BCL11B-NKX2-5; BCL11B-TLX3; BCL11B-TRD; BCL2-KDSR; BCL6-CIITA; BCL6-IKZF1; BCL6-PIM1; BCR-FGFR1; BCR-JAK2; BCR-RALGPS1; CCDC122-VPS36; CCND1-IGLL1; CDK6-SEMA4F; CIITA-BCL6; CIITA-C15orf65; CIITA-CD274; CIITA-PDCD1LG2; CIITA-RALGDS; CIITA-RMI2; CIITA-SNX29; CLIP1-ROS1; CLTC-ALK; CLTC-CLTC-ALK; CLTCL1-ALK; CNTRL-FGFR1; CSF2RA-CRLF2; CTCF-PARD6A; CTNNA3-ARHGAP21; CUX1-FGFR1; DMRT1-BCL6; DSCAML1-KMT2A; EBF1-PDGFRB; EIF4A2-BCL6; ELF2-KMT2A; ESRRG-ACBD3; ETV6-ABL1; ETV6-ARNT; ETV6-FGFR3; ETV6-INO80D; ETV6-JAK2; ETV6-NCOA2; ETV6-RNF217-AS1; ETV6-RUNX1; ETV6-SYK; EWSR1-ZNF384; FGA-RUNX1; FIP1L1-PDGFRA; FOXP1-IGH; FOXP1-IGHA2; FUS-ERG; FXYD6-KMT2A; GAS5-BCL6; GRHPR-BCL6; HLA-A-ROS1; HSP90AA1-BCL6; IGH-BACH2; IGH-BCL10; IGH-BCL2; IGH-BCL3; IGH-BCL6; IGH-BCL9; IGH-CBFA2T3; IGH-CCND1; IGH-CCND2; IGH-CCND3; IGH-CCNE1; IGH-CD44; IGH-CEBPA; IGH-CEBPB; IGH-CEBPD; IGH-CEBPE; IGH-CEBPG; IGH-CNN3; IGH-CRLF2; IGH-DDX6; IGH-EPOR; IGH-ETV6; IGH-FCGR2B; IGH-FCRL4; IGH-FOXP1; IGH-ID4; IGH-IGL; IGH-IL3; IGH-IRF4; IGH-KDM4C; IGH-LHX4; IGH-MALT1; IGH-MIR125B1; IGH-MUC1; IGH-MYC; IGH-MYCN; IGH-NBEAP1; IGH-PAFAH1B2; IGH-PAX5; IGH-RHOH; IGH-SPIB; IGH-TENM2; IGH-TERT; IGH-TRA; IGH-TRD; IGK-BCL10; IGK-BCL2; IGK-BCL6; IGK-CCND2; IGK-CDK6; IGK-KDSR; IGK-MYC; IGK-PVT1; IGK-ZC3H12D; IGL-BCL2; IGL-BCL3; IGL-BCL6; IGL-BCL9; IGL-CCND1; IGL-CCND2; IGL-IRF4; IGL-MYC; IGL-PRDM16; IGL-PVT1; IGL-REL; IKZF1-BCL6; IKZF2-ERBB4; ITK-FER; ITPR3-PNPLA1; JAK2-PCM1; KMT2A-ACTN4; KMT2A-AFDN; KMT2A-AFF1; KMT2A-AFF4; KMT2A-BCL9L; KMT2A-BTBD18; KMT2A-CREBBP; KMT2A-DCP1A; KMT2A-ELL; KMT2A-EPS15; KMT2A-FOXO4; KMT2A-FRYL; KMT2A-GAS7; KMT2A-MAML2; KMT2A-MLLT1; KMT2A-MLLT10; KMT2A-MLLT3; KMT2A-SEPT11; KMT2A-TNRC18; LCP1-BCL6; LIN28B-STX7; LPP-BCL6; LRMP-BCL6; LY75-DCL1; MALT1-BIRC3; MAP4-MALT1; MBNL1-BCL6; MEF2D-DAZAP1; MLLT10-PICALM; MSN-ALK; MSN-MSN-ALK; MYC-BCL7A; MYC-IGH; MYC-IGK; MYC-ZBTB5; MYC-ZCCHC7; NAP1L1-MLLT10; NAPA-BCL6; NDST2-RUNX1; NFKB1-KMT2A; NGB-BCL2; NM_001077493.1-CUEDC2; NOP2-TCF3; NPM1-

Figure 12 Continued

	ALK; NT_079596.2-LYL1; NUP214-ABL1; P2RY8-CRLF2; PAFAH1B2-IGH; PAX5-ASXL1; PAX5-AUTS2; PAX5-DACH2; PAX5-ELN; PAX5-ETV6; PAX5-GOLGA6A; PAX5-IGH; PAX5-JAK2; PAX5-KIF3B; PAX5-LOC392027; PAX5-NCOR1; PAX5-NOL4L; PAX5-SLCO1B3; PAX5-TAOK1; PBX1-KMT2A; PCM1-JAK2; PDE9A-MUS81; PDE9A-REXO1L1P; PDE9A-VWF; PICALM-MLLT10; PIGR-BCL6; PIM1-BCL6; PVT1-ZCCHC7; RAB1A-XPO1; RABGAP1L-KMT2A; RABGAP1L-ZBTB37; RALGPS1-ABL1; RANBP17-TRA; RANBP17-TRD; RARA-KMT2A; RCSD1-ABL1; RHOH-BCL6; RIC3-TRB; RIC3-TRBC2; RNF213-MYC; RUNX1-EVX1; RUNX1-FGA7; RUNX1-PRDM16; RUNX1-RUNX1; SEC31A-ALK; SEC31A-JAK2; SENP6-TOP1; SERINC3-ZCCHC7; SET-NUP214; SNHG5-BCL6; SQSTM1-ALK; SQSTM1-NUP214; SRSF3-BCL6; STIL-TAL1; TAF15-ZNF384; TCF3-HLF; TCF3-PBX1; TCF3-ZNF384; TCTA-TAL1; TEX10-XPA; TFG-ALK; TLX1-RPL4P1; TLX1NB-TRDC; TPM3-ALK; TPR-FGFR1; TRA-CDKN2A; TRA-IRF4; TRA-MYC; TRA-NECTIN2; TRA-NOTCH1; TRA-OLIG2; TRA-TRB; TRB-CCND2; TRB-HOXA@; TRB-HOXA10; TRB-HOXA11; TRB-IRS4; TRB-LCK; TRB-LMO1; TRB-LMO2; TRB-LYL1; TRB-MYB; TRB-NOTCH1; TRB-TAL1; TRB-TAL2; TRB-TLX1; TRD-LMO1; TRD-LMO2; TRD-NKX2-5; TRD-PVT1; TRD-RANBP17; TRD-TAL1; TRD-TLX1; TRD-TLX3; TRG-IGH; TRG-TRB; TRIM46-KRT18P6; VAV1-GSS; VAV1-MYO1F; VAV1-S100A7; VAV1-THAP4; XM-941465.1-BCL2; ZCCHC7-SERINC3; ZMYM2-FGFR1
Osteosarcoma	EWSR1-CREB3L1; BNC2-MTAP
Melanoma	AGK-BRAF; AKAP6-PRKD1; ANKHD1-CYSTM1; ATF1-EWSR1; ATG7-BRAF; CCDC91-BRAF; CCT3-C1orf61; CDK14-TFEC; CLCN6-RAF1; CLIP1-ALK; CSK-TMEM266; CUX1-BRAF; DCLK2-UST; DDR2-FMO4; DYNC1I2-BRAF; ETV6-NTRK3; EVC2-STK32B; EWSR1-ATF1; EWSR1-CREB1; EWSR1-NFATC2; GCN1-PLA2G1B; GGA3-VRK2; GNA12-SHANK2; GRK3-EP300; GTF2I-BRAF; GTF3C2-ALK; GXYLT2-NEK3; HIPK2-ARPC1A; HOXB6-EPHA5; IGFBP3-LIMK1; INPP5F-GRK5; KCTD2-ARHGEF12; LBH-FLT4; LMNA-NTRK1; LMNA-RAF1; LYN-APMAP; MAP3K8-LYZL2; MAPK14-SIN3A; MARK1-RHOU; MARK2-BATF2; MKLN1-CDK14; MPRIP-RAF1; MZT1-BRAF; NARS2-PAK1; NPM1-ALK; OBSCN-DISC1; OXSR1-ATXN7; PAK1-PDGFD; PARP1-MIXL1; PARP14-TTBK1; PDK3-NAV3; PEAK1-NRG4; PEAK1-PSTPIP1; PI4KA-CDH10; PI4KA-LZTR1; PI4KA-MYO18B; PI4KA-PHF21B; PMEL-NRBP1; PMEL-RIPK4; POMK-LSS; PRKAA2-USP24; PRKCE-ADGRG6; PRPF4B-F13A1; PSEN1-STK4; RAD18-BRAF; RAI14-MAPK9; RB1-ITM2B; RECK-ALX3; RPL19-PAK2; RRM2B-STK3; SCAMP2-WDR72; SIK2-RBFOX1; SLC12A7-AAMDC; SLC12A7-BRAF; STK32A-SPINK5; TAL1-RPL4P1; TAOK1-ASGR2; TBK1-GRIP1; TMEM8B-TLN1; TP53-NTRK1; TPR-ALK; TRAF2-NTRK2; TRAK1-RAF1; TRIM24-BRAF; TRIM63-NTRK1; TRIO-ADAMTS12; TRIO-CLIC5; TRIO-CLNK; TRIO-LIFR; TRIO-SLC9A3; VRK3-POU2F2; WASF2-FGR; ZKSCAN1-BRAF; ZNF767P-BRAF
Bladder Cancer	BCKDHB-TTK; BCR-PPIL2; BMPR1A-ZYG11A; CCSER1-BMP2K; CD36-ITK; CDC42BPA-WWOX; CDK6-HEPACAM2; FGFR1-NTM; FGFR3-BAIAP2L1; FGFR3-JAKMIP1; GAK-LUC7L3; GDF15-NRG1; GRK3-AP1B1; GRK3-RHBDL3; IKBKB-UNC5D; KIAA0430-EEF2K; MAP3K5-PERP; MAPK8-RAP1B; MTSS1-ERBB2; NEK4-SETD2; PEAK1-CIB2; PEAK1-VPS33B; PIKFYVE-MLPH; PKN3-CYTH3; RPS6KB2-ST3GAL2; SRPK1-EHMT2; STK24-TMTC4; STK3-MRPL48; STK3-ZNF706; STK38-ZNF184; TLK2-IRAK3; YES1-SCFD2
Breast Cancer (Ductal, lobular) + Invasive	ETV6-RUNX1; ABL1-ADAMTSL2; ABR-CLUH; ABR-STAP2; ADAM9-NRG1; ADCK1-KCNQ2; ADK-PI4K2A; AHNAK-RPS6KA4; AKT2-YIF1B; ANK1-FGFR1; ARFGEF2-SULF2; ARMT1-PDPK1; ARSG-MAP2K6; ATP1B3-PRKAA2; ATR-DBR1; B3GLCT-STK33; BCAS3-PRKAA2; BCAS4-BCAS3; BCR-ANKRD28; BCR-MRVI1; BMP2K-SLC4A4; BMPR1B-NPTX1; BRD3-PIP5KL1; BRD4-NOTCH3; BRSK1-HSPBP1; BRWD1-DYRK1A; BUB1B-CCDC170; CAMK1D-FAM188A; CAMK1D-TSPAN18; CASK-SQRDL; CCDC6-RET; CDK1-WDR11; CDK12-FBXL20; CDK13-PDE1C; CDK13-TAX1BP1; CDK19-PRDM1; CDK6-TAF1D; CDK7-CCNB1; CDK8-IKZF3; CEP170-PRKACB; CFAP47-MAP3K8; CHGA-MAPKAPK2; CLK2-CD244; COG6-MAPK8; COL10A1 NT5DC1-HIPK1; CSF1R-CAP2; DCLK1-SERPINE3; DDX42-RPS6KB1; DEPDC1B-ELOVL7; DSTYK-NAV1; DYRK1A-DSCAM; DYRK1A-KDM4B; DYX1C1-SGK3; EFNA3-PIK3C2G; EIF2AK2-CEBPZ; EIF2AK3-PIEZO1; ENPP1-TESK2; ERBB2-ENO1; ERBB2-GTF2E2/SMIM18; ERBB2-LMNTD1; ERBB2-MED1; ERBB2-PSMB3; ERBB2-SPTBN2; ERC1-PIK3C2G; ERC1-RET; ERLIN2-FGFR1; ESR1-AKAP12; ESR1-CCDC170; ESR1-DAB2;

Figure 12 Continued

	ESR1-NOP2; ESR1-PCDH11X; ESR1-POLH; ESR1-YAP1; ETV6-NTRK3; EVL-LCK; FBXL20-TLK2; FGFR1-ZNF703; FGFR2-AFF3; FGFR2-CASP7; FGFR2-CCDC6; FGFR3-TACC3; FRK-DTNBP1; FRMD3-BRD4; GAK-MAGI1; GRHL2-NRBP2; GRK2-BAIAP2; GRK2-CHGA; GRK2-POLD4; GRK3-SF3A1; GRK6-UIMC1; GSK3B-FRNF217-AS11; HDAC7-IRAK4; IGF1R-PPP4R1; IGF1R-TOLLIP; IGF1R-TRIP4; IGF1R-WDR93; IGF2R-ACVR2A; JAK1-ZC3H7B; KIAA1549-BRAF; KIT-ANKRD11; KIT-PDGFRA; LONP1-MAP2K7; LRRK1-ADAMTS17; MAGI3-AKT3; MAP2K2-CAMKK1; MAP2K4-CD79B; MAP2K4-DNAH9; MAP2K5-ADAMTSL3; MAP2K7-SH3GL1; MAP3K1-AGMO; MAP3K1-PARN; MAP3K2-SUPT3H; MAP4K5-NIN; MAPK1-KCNJ4; MAPK14-MICU2; MAPKAPK2-CDC42BPG; MAPKAPK5-GNPTAB; MARK2-FAM168A; MARK2-RTN3; MARK3-MIA2; MARK3-SLC25A21; MARK4-LYPD5; MARNF217-AS1-SEL1L; MAST2-C6orf52; MAST2-MMEL1; MAST4-LRP1B; MAST4-NLN; MBOAT2-CSNK1G1; MED25-MAPK15; MELK-CFAP537; MFGE8-IGF1R; MGA-EPHA5; MOK-ANKRD66; MOV10-STK25; MRAS-NME9; MTF2-BRDT; MYO3B-IL1RAPL2; NAP1L1-STK38L; NEK7-CRB1; NEK9-TMED10; NLK-BCAS3; NLK-LGALS9; NRBP1-RAB10; NSD3-FGFR1; NVL-NEK2; OBSCN-IGSF21; OSBPL2-ADCK1; PAK1-MYO7A; PAK4-CYP2A6; PDPK1-CCNF; PDPK1-MAPK8IP3; PI4K2A-ZDHHC16; PI4KB-SELENBP1; PIK3CB-PCCB; PIK3CB-TSPO; PIP4K2B-MED24; PIP4K2B-MLLT6; PIP5K1A-MLLT11/CDC42SE1; PIP5K1C-PALM; PKMYT1-CLDN6; POLA1-MAP3K15; POMK-NKAIN3; PPFIA1-MARK2; PPP6R1-AURKC; PRKCD-FANCD2; PRKCE-TRIM13; PRKD2-CKM; PRKDC-CEP290; PRKDC-DEFB103B; PRKDC-GSR; PRKDC-PXDNL; PRKDC-SPIDR; PSPH-PHKG1; PTK2-PPP2R5E; PTPRT-NCOA3; RAB8A-MAP2K2; REBM6-REBM5; RHOT1-FGFR1; RIPK1-BCKDHB; RNF170-YES1; RNF40-PLK1; ROCK1-ABHD3; ROCK1-LRIG3; ROCK2-PPP2CA; RPN2-STK17A; RPS6KA1-RASGRP3; RPS6KA6-HDX; RPS6KB1-EFCAB3; RPS6KB1-RNF213; RPS6KB2-VSIR; RYK-RABL3; SCYL2-ANKS1B; SGK1-PDSS2; SMG1-NDE1/MYH11; SMS-DYRK2; SNTG2-TRPM6; SPINT2-PAK1; SRC-MANBAL; SRPK1-ANKRD42; SRPK1-DDAH1; SRPK2-PUS7; STAG2-EPHB1; STARD3-STRADA; STK10-SH3PXD2B; STK10-SNX2; STK11-KDM4B; STK11-SMARCD2; STK11-TYK2; STK24-ANKS1B; STK24-ATF7; STK24-PIP5K1B; STK3-POLR2K; STK35-CHD6; STK39-MAT1A; STK4-C20orf197; STK4-TSHZ2; STK40-MDM4; STK40-RBBP4; SULF2-PRICKLE; SYNJ2-CDK19; TANC2-STARDA; TAOK1-ACER3; TAOK1-NLK; TAOK1-ZNF207; TAOK2-ADGRG1; TBC1D9-PLK4; TBL1XR1-PIK3CA; TEC-PASD1; TENM4-NRG1; TESK2-GPR37L1; TEX14-CLTC; TEX14-MSI2; TIMP3 SYN3-SCYL3; TLK1-CAT; TLK2-KCNJ16; TLK2-PITPNC1; TLK2-SIGLEC9; TOPBP1-NEK11; TP53RK-ZNF227; TRIB1-SLC25A32; TRIO-IFT52; TTC13-JAK2; TXLNA-SBK1; TXLNG-SYAP1; TYK2-SHC2; UHMK1-PPOX; ULK2-DOCK6; ULK2-UQCR11/MBD3; USP8-TRPM7; UTRN-SGK1; VRK3-PARP4; VTI1A-PIK3CB; WEE2-APOBEC3B; WNK1-KDM5A; WNK1-PRMT8; WNK1-WNT5B; WRB-NLK; XRN1-PIP4K2A; YES1-COL26A1; ZNF37A-PIP5K1B; ZNF587-BRSK1; ZNF791-FGFR1; ZVF577-FGFR1
Colon cancer	ABR-KCNK1; APIP-SLC1A2; BCR-PTPRD; BLK-ACOT11; BMPR2-CADM2; BMPR2-WDTC1; BRWD1-LMTK3; CAMK1D-FUBP1; CCDC6-RET; CDK12-CACNB1; CDK12-CLCN4; CDK13-DIP2C; CDKL1-TSEN2; CHEK1-NTM; CSNK1D-SECTM1; EIF4ENIF1-TEX14; ELOVL6-POMK; EPHA2-HCK; EPHA2-PREX1; ERBB2-DNAJC7; ESCO2-PTK2B; ETV6-NTRK3; EWSR1-NFATC2; FOXN3-CDKL1; GSK3B-TIAM1; IGF1R-CHD7; INSR-RBFOX3; LYN-VPS13B; MAP3K13-NSUN2; NUMB-TAOK3; NUP210L-EEF2K; PANK2-PAK5; PHACTR4-PIP5K1A; POMK-CHRNA6; PTK2-CHKA; ROCK2-BLM; RYK-ITFG1; SCYL1-PACS1; SIK3-UBR1; STK24-SLC35F1; STK4-FOXP1; TAOK1-VMP1; TMC1-ADCK1; TRIM52-DAPK1; TRIO-ASTN1; UBE2H-NEK11; UHMK1-FBXL7; WNK1-MANBA; ZNF436-FLT1
Esophageal Cancer	COL4A5-COL4A6
Lung Cancer	EML4-ALK; AAK1-WFDC10B; ABR-GAS7; ABR-YWHAE; ABRACL-MAP3K5; ACVR2A-CASP8; ADAM2-PRKDC; ADCY9-PRKCB; AFF3-MAP4K4; ANO3-FGFR4; APPL2-CIT; ARMC10-BRAF; ATP1B1-NRG1; ATP1B3-GRK7; BAG4-FGFR1; BAZ1A-NKX2-1; BAZ1A-RNF115; BAZ1A-SFTA3; BAZ2A-ACVR1B; BCR-ANKRD28; BCR-MYH11; BNC2-BNC2; BNC2-CDKN2B-AS1; BTF3L4-BRAF; BUB1B-PCM1; C19orf47-MAP3K10; CAMK1D-ARL3; CAMK2B-SPIDR; CAMK2D-ANK2; CCDC6-RET; CD74-NRG1; CD74-NTRK1; CD74-ROS1; CDC42BPB-LINC01588; CDC42BPB-ZFYVE1; CDK6-FN1; CDK6-STXBP6; CDKL2-CXCL11/ART3; CIT-FAM222A; CLIP1-ROS1; CLTC-ROS1; COQ8B-AKAP8; CPM-PRKAA2; CSNK1D-SCPEP1; CSNK1D-ST6GALNAC1; DAPK1-AGTPBP1; DIP2B-NRG1; DPYSL2-NRG1;

Figure 12 Continued

	DYRK1A-TTC3; ECHDC1-FYN; ECT2-KALRN; EEF2K-C10orf35; EML4-ALK; EPHA10-COL8A2; EPS15-BRAF; ERBB2-CFB; ERBB2-CTTN; ERBB4-AKAP6; EZR-ERBB4; EZR-ROS1; FAM20B-SAMD7; FAM20C-MKRN1; FGFR2-CCAR2; FGFR2-CIT; FGFR3-BAIAP2L1; FGFR3-TACC3; GHR-BRAF; GRK1-CUL4A/PCID2; HIP1-ALK; HIPK2-ADCK2; HIPK2-GRM8; HIPK3-CAT; HIPK3-LARGE2; HLA-A-ROS1; HTT-GRK4; IGF1R-GLTP; IGF2BP3-PRKCA; IKBKB-GOT1L1; IL4I1-ROS1; IRAK1-PIGR; KDELR2-ROS1; KIAA1468-RET; KID5B-RET; KIF13A-RET; KIF5B-ALK; KIF5B-MET; KIF5B-RET; KIT-SLC4A4; KLC1-ALK; KSR1-EFCAB5; LATS1-LACE1; LMTK2-IMMP2L; MAP2K3-CADM2; MAP3K10-C19orf47; MAP3K3-FCHSD2; MAP3K5-NKAIN2; MAP3K8-MROH2B; MAP4K5-JAM3; MAPK1-FAM155A; MAPK1-SSU72; MARK2-BUB1B; MARK3-EIF5; MARK3-FBLN5; MARK3-KIF26A; MAST4-SREK1IP1; MDK-NRG1; MELK-SDK1; MGA-EPHA5; MINK1-NQO2; MLKL-TMEM231; MLLT3-KMT2A; MPRIP-NTRK1; MRPL13-NRG1; MST1R-FNDC3B; NCOA3-SGK2; NEK7-RAB3GAP2; NIPBL-STK24; NLK-NF1; NPM1-ALK; NSD1-FGFR4; NSD2-FGFR3; NUP214-BRAF; PAK1-CNIH4; PAK1-LNP1; PAK2-BDH1; PAK2-ECE2; PARP8-NRG1; PASK-BOK; PI4K2B-C2orf61; PIK3C3-TRPC6; PIK3CB-BFSP2; PIKFYVE-ZNF124; POMK-ST6GAL1; PRKAA1-TBC1D5; PRKCZ-PRDM16; PRKG1-TRIM5/TRIM22; PTK2-ANGPT1; PTK2-ANKRD11; PTK2-CACNG8; PTK2-RNF139; PTK7-DLK2; PTPN3-ALK; RAB3GAP2-NEK7; RBPMS-NRG1; RFWD3-STK31; ROCK1-GATA6; ROCK1-NRG1; ROCK1-RNF38; ROR1-DNAJC6; RPS6KA3-FAM156B; RPS6KC1-TFPI; RUFY2-RET; SDC4-NRG1; SDC4-ROS1; SGK1-NRROS; SHC1-ERBB2; SIK2-CARD17; SIK3-CD3D; SLC34A2-ROS1; SLC3A2-NRG1; SND1-BRAF; SOCS5-ALK; SP3-STK39; SPNS1-PRKCB; SPTBN1-ALK; SRC-GSS; SRPK1-CEP57L1; SRPK2-LRP5; STK11-ARHGAP45; STK24-TRIQK; STK3-SPRR2E/SPRR2B; STK3-SULT1A3; STK32B-SMIM14; STK38-ANKRD28; STRADA-LYZ; TANC2-PRKCA; TAOK1-BCAS3; TBCK-SLC9B2; TEC-HMGCLL1; TEC-NCAM2; TECR-PKN1; TFG-ALK; THSD4-DSTYK; TLK1-CREG2; TMEM127-ZAP70; TMEM87B-MERTK; TNC-NRG1; TOP2A-TTBK2; TPM3-ALK; TPM3-NTRK1; TPR-ALK; TRIM24-BRAF; TRIM24-NTRK2; TRIM33-RET; TRIM4-BRAF; TRIO-DNAH5; TRRAP-TMEM168; TTBK2-TOP2A; TUBD1-RPS6KB1; TUSC5-CRK; ULK1-FBXW8; USP32-EPHA1; VAMP2-NRG1; VKORC1L1-ALK; VPREB1-RPS6KA3; VRK3-PTOV1; WASF2-FGR; WNK1-B4GALNT3; WNK1-RAD52; WNK2-IGF2; ZC3HAV1-BRAF; ZNF253-BAZ1A
Thyroid Cancer	AGGF1-RAF1; AKAP9-BRAF; AP3B1-BRAF; BCL2L11-BRAF; CCDC6-RET; CEP89-BRAF; CREB3L2-PPAR_; CTSB-EIF2AK1; CTSB-PXK; ERC1-RET; ETV6-NTRK3; FAM114A2-BRAF; FGFR2-OFD1; FN1-MKNK2; GOLGA5-RET; GRID1-BAZ1B; HOOK3-RET; IRF2BP2-NTRK1; KLHL7-BRAF; KTN1-RET; LSM14A-BRAF; MAP3K13-VKORC1; MBD1-RET; METTL7A-SRPK1; NCOA4-NCOA4; NCOA4-PCDH15; NCOA4-RET; NFASC-NTRK1; NTRK1-TFG; NUP98-TPR; PAX8-PPAR_; PAX8-PPARG; PAX8-PPARγ1; PCDH15-PCDH15; PCM1-RET; POU2F1-EPHA4; PPL-NTRK1; PRKAR1A-RET; RAF1-AGGF1; RBMS3-BRAF; RBPMS-NTRK3; RET-CCDC6; RET-GOLGA5; RET-KTN1; RET-NCOA4; RET-PCM1; RET-PRKAR1A; RET-RFG9; RET-TRIM24; RET-TRIM33; SQSTM1-NTRK1; SQSTM1-NTRK3; SSBP2-NTRK1; STRN-ALK; TANK-BRAF; TFG-MET; TFG-NTRK1; TG SLA-MINK1; TG-GAK; TG-WNK3; THADA-IGF2BP3; TPM3-NTRK1; TPR-NTRK1; TRIM24-RET; TRIM27-RET; TRIM33-RET; UACA-LTK; VTI1A-PIK3CB; ZC3HAV1-BRAF
Pancreatic Cancer	HACL1-RAF1; ATG7-RAF1; ATP1B1-NRG1; ATP1B1-PRKACA; BNC2-MTAP; CDH1-NRG1; CTRC-NTRK1; EWSR1-NFATC2; GATM-BRAF; HACL1-RAF1; HERPUD1-BRAF; MYRIP-BRAF; SND1-BRAF; ZSCAN30-BRAF
Leukemias	ABL1-BCR; ACAD10-MAPKAPK5; ADAMTS17-RARA; AFDN-KMT2A; AFF1-AFF1; AFF1-CCDC84; AFF1-DSCAML1; AFF1-ELF2; AFF1-FXYD6; AFF1-KMT2A; AFF1-PBX1; AFF1-RABGAP1L; ARID1B-ZNF384; ATG16L2-KMT2A; ATM-ATM; BCL11A-GRIP2; BCL11A-MECOM; BCL11B-NKX2-5; BCL11B-TLX3; BCL11B-TRD; BCL2-IGH; BCL3-MYC; BCL6-IGH; BCR-ABL1; BCR-FGFR1; BCR-JAK2; BCR-PDGFRA; BCR-RALGPS1; BNC2-CDKN2B-AS1; BTG1-MYC; CBFA2T3-GLIS2; CBFB-MYH11; CCDC6-PDGFRB; CCDC94-KMT2A; CDK11B-SLC35E2; CDKN2A-CDKN2B; CDKN2A-TRD; CENPK-KMT2A; CHD1-MTOR; CHIC2-ETV6; CHST11-ATP1B4; CHTOP-FGFR1; CLTC-ALK; CMC4-TRB; CNTRL-FGFR1; CRTC2-CREB3L4; CSF2RA-CRLF2; CTCF-PARD6A; CTNNA3-ARHGAP21; CUL1-DPP6; CUX1-FGFR1; DAB2IP-KMT2A; DDX20-TBX15; DEK-NUP214; DIAPH2-KMT2A; DOCK6-CCDC130;

Figure 12 Continued

	DPM1-GRID1; DSCAML1-KMT2A; DUSP10-PRDM16; EBF1-PDGFRB; EDIL3-MKLN1; ELAVL1-TYK2; ELF2-KMT2A; ELF4-ERG; EP300-ZNF384; ETV1-FLT3; ETV6-ABL1; ETV6-ABL2; ETV6-ACSL6; ETV6-ARNT; ETV6-FRK; ETV6-GOT1; ETV6-INO80D; ETV6-JAK2; ETV6-MECOM; ETV6-NCOA2; ETV6-NTRK3; ETV6-PDGFRB; ETV6-PTPRR; ETV6-RNF217-AS1; ETV6-RUNX1; ETV6-SYK; EWSR1-ZNF384; FAM133B-CDK6; FAM172A-CDC73; FGA-RUNX1; FIG4-SEC63; FIP1L1-PDGFRA; FIP1L1-RARA; FOCAD-CDKN2B-AS1; FOXO4-KMT2A; FOXP1-PPP1R2; FRNF217-AS13-CCND1; FUS-ERG; FXYD6-KMT2A; GAS6-TMEM255B; GATA2-HOXA9; GOSR1-ZNF207; GRB10-SDK1; HOXD13-NUP98; HPRT1-HPRT1; IFNGR2-GART; IGH-BACH2; IGH-BCL10; IGH-BCL11A; IGH-BCL2; IGH-BCL3; IGH-BCL6; IGH-BCL9; IGH-CBFA2T3; IGH-CCND1; IGH-CCND3; IGH-CDK6; IGH-CEBPA; IGH-CEBPB; IGH-CEBPD; IGH-CEBPE; IGH-CEBPG; IGH-CHST11; IGH-CRLF2; IGH-DDX6; IGH-EPOR; IGH-ERVW-1; IGH-ETV6; IGH-FGFR3; IGH-ID4; IGH-IGL; IGH-IL3; IGH-LHX4; IGH-MAF; IGH-MIR125B1; IGH-MYC; IGH-NSD2; IGH-TERT; IGH-TRA; IGH-TRD; IGK-BCL2; IGK-CDK6; IGK-MYC; IGK-PVT1; IGL-BCL2; IGL-BCL6; IGL-CCND2; IGL-CDK6; IGL-MYC; IGL-PVT1; KAT6A-CREBBP; KAT6A-EP300; KAT6A-LEUTX; KAT6A-NCOA2; KAT6B-CREBBP; KDR-PDGFRA; KIA0999-IFT46; KMT2A-ABI1; KMT2A-ACTN4; KMT2A-AFDN; KMT2A-AFF1; KMT2A-AFF4; KMT2A-ARHGAP26; KMT2A-ARHGEF12; KMT2A-ARHGEF17; KMT2A-BCL9L; KMT2A-BTBD18; KMT2A-C2CD3; KMT2A-CBL; KMT2A-CEP170B; KMT2A-CREBBP; KMT2A-CT45A2; KMT2A-DCP1A; KMT2A-DCPS; KMT2A-DIAPH2; KMT2A-ELL; KMT2A-EP300; KMT2A-EPS15; KMT2A-FNBP1; KMT2A-FOXO4; KMT2A-FRYL; KMT2A-GAS7; KMT2A-KNL1; KMT2A-LAMC3; KMT2A-LOC100128568; KMT2A-MAML2; KMT2A-MLLT1; KMT2A-MLLT10; KMT2A-MLLT11; KMT2A-MLLT3; KMT2A-MLLT6; KMT2A-MYH11; KMT2A-MYO1F; KMT2A-NRIP3; KMT2A-SARNP; KMT2A-SEPT11; KMT2A-SEPT2; KMT2A-SEPT5; KMT2A-SEPT9; KMT2A-SH3GL1; KMT2A-SMAP1; KMT2A-SUGP2; KMT2A-TIRAP; KMT2A-TNRC18; KMT2A-VAV1; KMT2A-ZFYVE19; KMT2C-ACTR3B; KPNB1-ACE; KSR2-SETMAR; LCK-NT_079596.2; LINC01565-MECOM; LMO1-LMO2; LRBA-SH3D19; LTBP1-BIRC6; MDM4-DNMT38; MECOM-MECOM; MECOM-RUNX1; MEF2C-KMT2A; MEF2D-BCL9; MEF2D-CSF1R; MEF2D-DAZAP1; MEF2D-HNRNPUL1; MEF2D-SS18; MLLT10-CEP164; MLLT10-CLP1; MLLT10-KMT2A; MLLT10-PICALM; MLLT10-PPP2R1B; MLLT3-KMT2A; MN1-ETV6; MN1-FLI1; MNX1-ETV6; MPO-ZNF296; MTAP-BNC2; MTAP-CDKN2A; MTAP-CDKN2B; MTAP-CDKN2B-AS1; MTAP-LINGO2; MYB-GATA1; MYB-MNX1; MYC-BCL7A; MYC-IGH; MYC-IGK; MYC-KRT18P6; MYC-TcR-alpha; MYH11-CBFB; MYO18A-PDGFRB; NAP1L1-MLLT10; NCOR1-LYN; NCOR2-SCARB1; NDST2-RUNX1; NEK1-CLCN3; NF1-LRRC37B; NFKB1-KMT2A; NIPBL-HOXB9; NM_001077493.1-INA; NOP2-TCF3; NPM1-RARA; NR6A1-OBP2B; NT_079596.2-LYL1; NT5C2-KMT2A; NUP214-ABL1; NUP214-XKR3; NUP98-DDX10; NUP98-HOXA11; NUP98-HOXA13; NUP98-HOXA9; NUP98-HOXC11; NUP98-HOXC13; NUP98-HOXD11; NUP98-HOXD13; NUP98-KMT2A; NUP98-NSD1; NUP98-NSD3; NUP98-PHF23; NUP98-POU1F1; NUP98-PSIP1; NUP98-RARG; NUP98-TOP2B; NUP98-TPR; NVL-FMN2; P2RY8-CRLF2; PAFAH1B2-IGH; PAX5-ASXL1; PAX5-AUTS2; PAX5-DACH2; PAX5-ELN; PAX5-ETV6; PAX5-GOLGA6A; PAX5-JAK2; PAX5-KIDINS220; PAX5-KIF3B; PAX5-LOC392027; PAX5-NCOR1; PAX5-NOL4L; PAX5-SLCO1B3; PAX5-TAOK1; PBX1-KMT2A; PCM1-JAK2; PDE9A-MUS81; PDE9A-REXO1L1P; PDE9A-VWF; PDGFRB-DTD1; PDGFRB-TRIP11; pGEX4T2-TCL1A; PICALM-KMT2A; PICALM-MLLT10; PLAUR-EXOC3L2; PMEL-TAL1; PML-ADAMTS17; PML-CDC6; PML-RAB40B; PML-RARA; PML-RARG; POLR2A-FBN3; PPP2R1B-SIK3; PTPN2-UBLCP1; PVT1-ASAP1; RABGAP1L-KMT2A; RALGPS1-ABL1; RANBP17-TRA; RANBP17-TRD; RANBP2-ALK; RARA-KMT2A; RBM15-MAL; RBM15-MKL1; RCSD1-ABL1; RIC3-TRB; RIC3-TRBC2; RNF213-SLC26A11; RPN1-MECOM; RPN1-PRDM16; RPS11-FLT3LG; RSAD2-ELMOD3; RUNCX1-PRRC1; RUNX1-ADAMTSI9; RUNX1-CBFA2T2; RUNX1-CBFA2T3; RUNX1-CLCA2; RUNX1-CPNE8; RUNX1-EVX1; RUNX1-FGA7; RUNX1-KCNMA1; RUNX1-MECOM; RUNX1-MSD1; RUNX1-NOL4L; RUNX1-PRDM16; RUNX1-PRDX4; RUNX1-RPL22P1; RUNX1-RUNX1; RUNX1-RUNX1T1; RUNX1-USP42; RUNX1T1-RUNX1; SART3-PDGFRB; SERINC3-ZCCHC7; SET-NUP214; SQSTM1-FGFR1; SQSTM1-NUP214; STIL-TAL1; STIM1-NSD1; STYXL1-BCR; TAF15-ZNF384; TAL1-KRT18P6; TAL1-RPL4P1; TBC1D16-RNF213; TBL1XR1-RARA; TCF3-HLF; TCF3-PBX1; TCF3-ZNF384; TCTA-TAL1; TFG-ADGRG7; THADA-MECOM; TLX1-RPL4P1; TLX1-TRA; TLX1NB-KRT18P6; TLX1NB-TRDC; TMEM52B-XIAP; TP53-TP53; TRA-CDKN2A; TRA-MTCP1; TRA-

Figure 12 Continued

	MYC; TRA-NOTCH1; TRA-OLIG2; TRA-TRB; TRB-CCND2; TRB-HOXA@; TRB-HOXA10; TRB-HOXA11; TRB-IRS4; TRB-LCK; TRB-LMO1; TRB-LMO2; TRB-LYL1; TRB-MECOM; TRB-MTCP1; TRB-MYB; TRB-NOTCH1; TRB-TAL1; TRB-TAL2; TRB-TLX1; TRD-LMO1; TRD-LMO2; TRD-NKX2-5; TRD-PVT1; TRD-RANBP17; TRD-TAL1; TRD-TLX1; TRD-TLX3; TRG-IGH; TRG-LYL1; TRG-TRB; TRIP11-PDGFRB; UBR4-ZFP37; USP16-RUNX1; USP22-CA7; USP25-TRAPPC10; VTI1B-RDHI1; WDR18-H2AFX; WSB1-NBR1; XIAP-TENM1; ZBTB16-RARA; ZBTB5-ZBTB5; ZBTB7A-TNK1; ZCCHC7-SERINC3; ZMYM2-FGFR1; ZNF483-GAPVD1; ZNF585B-C2CD2L; BCL11A-GRIP2
Liver Cancer	MYLK-TM7SF2; ABL1-PRDM12; ANXA4-PKN1; ARID1A-PRKCZ; BAZ1B-ABHD11; C1S LPCAT3-MAP3K13; CAMKK2-ANAPC5; CAMKK2-HPD; COMMD9-CDKL2; CUL4B-DAPK2; DNAJB1-PRKACA; ERBB2-PPP1R1B; HSD17B13-CSNK1G2; MAN2A1-FER; MAST4-PLIN2; MYLK-TM7SF2; OXR1-MET; POFUT1-HCK; PRKDC-ATF5; PXK-FLNB; RIOK1-AKR1D1; SPRN CYP2E1-COQ8A; STK19-LY6G6D; STK38-TDRD7; TGFBR1-COL15A1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/18831

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-29, 35-58
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/18831

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 9/127, A61P 37/02, C12N 15/88 (2021.01)

CPC - A61K 47/6915, A61K 47/6911, A61K 9/5123, A61K 9/5015, A61K 9/0019

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)
See Search History document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 2019/0125839 A1 (MODERNA TX, Inc.) 02 May 2019 (02.05.2019); para [0010], [0011], [0091], [0097], [0132], [0442], [1349], [1367], [1380], [1383], [1391], [1417], [1785]	1-4 30-34
Y	SAYOUR et al., Personalized Tumor RNA Loaded Lipid-Nanoparticles Prime the Systemic and Intratumoral Milieu for Response to Cancer Immunotherapy. Nano Letters. 10 October 2018, Vol. 18, No. 10; pages 6195-6206; Title; abstract	30-34

☐ 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	Date of mailing of the international search report
19 April 2021	MAY 10 2021
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300