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(54) Title: MIR-124 COMPOSITIONS FOR IMMUNE MODULATORY THERAPEUTICS

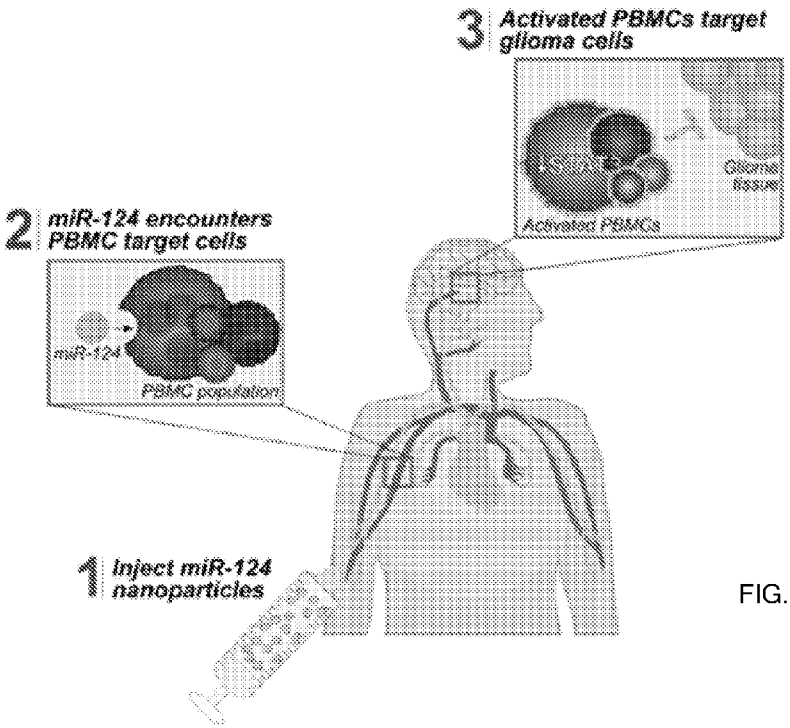

FIG. 13

(57) Abstract: This invention provides methods for preventing, ameliorating or treating a disease associated with glioma in a subject in need, the method comprising administering to the subject an effective amount of a nanoparticle composition comprising miR-124. The methods provide for activating immune system cells against glioma, to make compositions with activated immune system cells. Included are methods for activating immune response against glioma in a subject, and selectively delivering miR-124 to an immune system compartment. In addition, the methods provide for inducing immunological memory against a glioma.

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— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

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MIR-124 COMPOSITIONS FOR IMMUNE MODULATORY THERAPEUTICS

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 62/196,518, filed July 24, 2015, entitled MIR-124 COMPOSITIONS FOR IMMUNE MODULATORY THERAPEUTICS; the contents of which is herein incorporated by reference in its entirety.

REFERENCE TO THE SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled ARC2334WO_SL.txt, created on July 19, 2016 which is 1057 bytes in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] This invention relates to the fields of biopharmaceuticals and therapeutics having at least in part active ingredients comprising one or more micro-RNA structures for gene regulation and modulation, including immune system modulation. The invention also embraces micro-RNA compositions and their methods of use and delivery to cells, tissues and organisms using lipids or lipid nanoparticles.

BACKGROUND OF THE INVENTION

[0004] The use of micro-RNAs (also known as miRNAs and miRs) as therapeutics is appealing owing to robust preclinical data demonstrating their therapeutic efficacy, broad biological activity, and ability to shut down signaling networks at multiple nodes along various pathways, including alternative ligand activation. Therapeutic translation of miRNAs has been limited by technical challenges concerning delivery, stability of the miRNAs, and avoidance of activating immune responses. In general, endogenous microRNAs remain stable when secreted into the circulation if they are enclosed in microvesicles, such as exosomes (Camussi et al., 2010, Kidney Int. 78:838-48, 2010).

[0005] A drawback of nanoparticle chemotherapeutics is that none has been devised to modulate the immune system. For example, nanoparticle delivery of micro-RNAs for intracellular immune targeting has not been achieved.

[0006] Moreover, tumor-mediated immunosuppression is a major barrier to therapeutic approaches. Tumors can inhibit immune responses by several mechanisms. In some cases, a tumor can express various antigens and/or attract immunosuppressive lymphocytes (Chen et al., PLoS One. 6:e24671 2011). In another mechanism, tumors can secrete immunosuppressive cytokines (Orleans-Lindsay et al., Clin Exp Immunol. 126:403-11, 2001). In other cases, a tumor can express surface molecules, which inhibit immune responses (Mann et al., Br J Cancer. 79: 1262-69, 1999).

[0007] Tumors can establish an immunosuppressive environment acting like an invisibility cloak, allowing the tumor to grow while the immune system remains "blind" to the threat. However, microRNAs (miRs) can reverse this situation.

[0008] What is needed are compositions for selective delivery of microRNAs to various compartments of the immune system, such as immune system cells.

[0009] There is an urgent need for nanoparticle formulations of miRNAs, which can be used to treat solid malignancies, as well as tumors within the CNS.

[0010] What is needed are compositions and methods for exploiting the immune system to mediate antitumor immune effects. There is a long-standing need for nanoparticle formulations of miRNAs which can be used to overcome tumor-mediated immunosuppression.

SUMMARY OF THE INVENTION

[0011] This invention provides nanoparticle formulations of miRNAs, which can be used to treat solid malignancies and other tumors. The compositions of the invention can be used as active pharmaceutical formulations for ameliorating, preventing or treating solid malignancies and tumors, particularly gliomas or glioblastomas, in various anatomical locations, e.g., cells, tissues and organs.

[0012] Compositions of this invention may reduce the growth of tumors or malignancies. In certain embodiments, compositions of this invention may cause regression of a malignancy or tumor size.

[0013] Embodiments of this invention include compositions that advantageously provide enhanced effectiveness against tumors or malignancies, and which can provide clinical agents.

[0014] In some embodiments, this invention provides nanoparticle-encapsulated miR-124 mimics, whether single or double stranded. The nanoparticle-encapsulated miR-124 mimic compositions provide surprisingly enhanced delivery of an effective amount of miR-124 to the immune system that thereby prevents glioma growth.

[0015] In further embodiments, compositions of this invention provide increased therapeutic efficacy and immune compartment delivery of nanoparticle-encapsulated miR-124 mimics.

[0016] In some aspects, a nanoparticle-encapsulated miR-124 mimic composition of this invention can provide increased immunomodulatory effects, including reversal of tumor-mediated immunosuppression.

[0017] In certain aspects, this invention can provide miR-124 mimic compositions that can induce immunological memory. By modulating the immune system to create memory, compositions of this invention can induce the immune system to mediate antitumor immune effects over long time periods.

[0018] In some embodiments, the present invention includes methods for preventing, ameliorating or treating a disease associated with a glioma in a subject by administering to the subject an effective amount of a nanoparticle composition comprising miR-124 mimics. The administration can reduce the growth of a glioma, or induce regression of a glioma. The glioma can be located in the brain or spine of the subject. The administration may be intravenous.

[0019] In some embodiments, the nanoparticles encapsulate the miR-124 mimic. The nanoparticles can comprise one or more cationic lipids and one or more helper lipids.

[0020] Lipid nanoparticles for use in this invention can be any lipid nanoparticles known in the art. The nanoparticles may include one or more cationic lipids and one or more helper lipids, as are known in the art. In some embodiments, lipid nanoparticles can include one or more lipids selected from cationic lipids, anionic lipids, cholesterol, sterols, pegylated lipids, and any combination of the foregoing.

[0021] This invention further contemplates methods for activating immune system cells against glioma. The immune system cells can be contacted with a nanoparticle composition containing miR-124 mimics. The immune system cells can be contacted with the nanoparticle composition *in vivo* or *ex vivo*.

[0022] In general, the nanoparticles can encapsulate the miR-124 mimics.

[0023] Embodiments of this invention further include compositions containing immune system cells that are activated against a glioma. The cells may express activated STAT3 (signal transducer and activator of transcription 3), as compared to cells that are not activated. The cells may have increased activity against glioma when the STAT3 activation is reduced with miR-124.

[0024] This invention includes methods for activating immune response against glioma in a subject, by administering to the subject a nanoparticle composition containing miR-124 mimics. The nanoparticle composition may selectively deliver the miR-124 mimic to an immune system compartment in the subject. In some embodiments, the immune system compartment can be peripheral blood mononuclear cells (PBMCs). In certain embodiments, the immune system compartment can be monocytes and macrophages. In such methods, after delivery, the level of miR-124 in the monocytes and macrophages can be at least five-fold greater than in the monocytes and macrophages of a subject to which the composition was not administered.

[0025] In further embodiments, the immune compartment can be B cells. In these methods, after delivery, the level of miR-124 in the B cells can be at least five-fold greater than in the B cells of a subject to which the composition was not administered.

[0026] In general, a glioma can be located in the brain or spine of a subject.

[0027] The administration of a nanoparticle composition can be intravenous.

[0028] Additional embodiments of this invention contemplate methods for inducing immunological memory against a glioma in a subject. In such methods, the nanoparticle formulation can be administered to the subject containing an effective amount of miR-124 mimic. The immunological memory can include persistence of anti-glioma immune effects over a period of at least 5 days, or at least 15 days, or at least 50 days, or at least 100 days.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] FIG. 1: FIG. 1 shows that nanoparticle formulations can enhance the effect of miR-124 mimic. Four lipid-based nanoparticle formulations encapsulating miR-124 mimic were designed and tested in the same murine GL261 glioma model. Of the test articles prepared, nanoparticles in NB5-55-2 (nanoparticle formulation LUNAR-301) showed the greatest effect relative to LIPOFECTAMINE, and produced a median survival time that could not be calculated because >60% of mice were long-term survivors. As taught throughout, LUNAR-301 comprises ATX-002:DSPC:Chol:DMG-PEG at a molar ratio of 55:10:33.5:1.5 with an N/P ratio of 6; where, ATX-002 is an ionizable lipid; DSPC is 1,2-distearoyl-sn-glycero-3-phosphocholine; Chol is Cholesterol; and DMG-PEG is 1,2-Dimyristoyl-rac-glycero-3-methyl-polyoxyethylene. N is the ionizable lipid Nitrogens (N) and P is the Phosphate groups (P) in the miRNA or mimic.

[0030] FIG. 2: FIG. 2 shows that nanoparticle formulations can be selectively delivered to the immune compartment. Samples from peripheral blood mononuclear cells (PBMCs), serum, and liver were taken at 0 and 15 minutes, as well as at 1, 4, 8, and 24 hours. Quantitative PCR was used to assess the miR-124 level in each compartment. Nanoparticle formulation LUNAR-301 showed surprisingly more effective delivery of miR-124 to the PBMC compartment than LIPOFECTAMINE.

[0031] FIG. 3: FIG. 3 shows that nanoparticle formulations can be selectively delivered to the immune compartment. Circulating PBMCs were isolated from C57BL/6 mice after a single treatment with nanoparticle formulation LUNAR-301 at the peak distribution window of 15-20 minutes post injection. T cells (CD3+), B cells (CD19+), NK cells (NK1.1+), and monocytes/macrophages (CD11b+) were sorted by flow cytometry into subset populations, and the amount of miR-124 within each population was quantified by PCR. Compared with untreated control mice, the delivery of miR-124 was highest in the monocyte/macrophage population, and second highest in the B cells.

[0032] FIG. 4: FIG. 4 shows that nanoparticle formulations can inhibit immune suppression, an immunomodulatory property. To assess the immune modulatory properties of nanoparticle formulation LUNAR-301, C57BL/6 mice with subcutaneously implanted GL261 gliomas were treated 5 times with either miR-124 mimic +

LIPOFECTAMINE(TM) or nanoparticle formulation LUNAR-301. Thereafter, the glioma-infiltrating T cells were isolated and assessed for intracellular p-STAT3 and FoxP3 (forkhead box P3). p-STAT3 and FoxP3 levels were significantly lower after treatment with miR-124 mimic + LIPOFECTAMINE (P=0.0032 and P=0.0213, respectively) and nanoparticle formulation LUNAR-301 (P=0.0001 and P=0.0223, respectively) than in the untreated group. Two sample t-test was used for pair-wise comparison. Error bars, mean ± SD.

[0033] FIG. 5: FIG. 5 shows that nanoparticle formulations can induce immunological memory. Mice with initially implanted gliomas who survived for more than 70 days after intracerebral implantation were rechallenged with gliomas implanted in the contralateral brain. These mice had been previously treated with the therapeutic doses of miR-124 mimic + LIPOFECTAMINE and nanoparticle formulation LUNAR-301 (n=2 and n=5, respectively). On day 74, they were re-implanted with tumors, and survival was monitored; they received no further treatment. All but one mouse in the nanoparticle formulation LUNAR-301 group was still alive and healthy at 200+ days after initial intracerebral implantation.

[0034] FIG. 6: FIG. 6 shows that nanoparticle formulations can induce long term immunological memory. Long-term surviving rechallenged mice in FIG. 5 were subsequently rechallenged a third time with intracerebrally implanted gliomas in the original brain hemisphere, again with no further treatment. All but one of the mice previously treated with nanoparticle formulation LUNAR-301 were protected from tumor recurrence, and the mouse that had a recurrence survived for longer than the median survival time of untreated control mice (33 days).

[0035] FIG. 7: FIG. 7 shows that LUNAR-301 does not cause weight loss. Non-tumor-bearing C57BL/6 mice were treated with either nanoparticle formulation LUNAR-301 (LUN), seed sequence control (SSC), or PBS for a total of 9 doses. Their body weights were measured throughout the course of treatment and did not differ among treatment groups. Error bars, mean ± SD.

[0036] FIG. 8: FIG. 8 shows that LUNAR-301 does not induce clinically significant hepatomegaly or splenomegaly. At the conclusion of the study of FIG. 7, liver and spleen weights were measured. A modest but statistically significant increase in liver

weight was found in the nanoparticle formulation LUNAR-301-treated (LUN) mice relative to the SSC-treated ($P = 0.018$) and PBS-treated ($P = 0.014$) groups. Two sample *t*-test was used for pair-wise comparison. Each box-whisker plot represents 5-6 mice per treatment arm.

[0037] FIG. 9: FIG. 9 shows that LUNAR-301 does not induce clinically significant increases in liver transaminases. Just before the final (9^{th}) dose, serum was collected from all mice, liver function tests (LFTs) were performed, and aspartate aminotransferase (AST) was found to be modestly elevated in the nanoparticle formulation LUNAR-301 group (LUN) relative to the SSC ($P = 0.007$) and PBS ($P = 0.012$) groups (ALT = alanine aminotransferase). Two sample t-test was used for pair-wise comparison. Each box-whisker plot represents 5-6 mice per treatment arm.

[0038] FIG. 10: FIG. 10 shows that nanoparticle formulations can be selectively delivered to the immune compartment for activity in a canine model. Expanding to a second model system, purpose-bred non-tumor-bearing beagles were used (n=5). Canines were treated with a single escalating dose of nanoparticle formulation LUNAR-301 (301) or with nine doses at 0.5 mg/kg. Blood samples were drawn pretreatment and at various time points up to 72 hours post treatment. Sera and PBMCs were isolated, and miR-124 was quantified by PCR for each compartment. In all cases, miR-124 was elevated in the sera and PBMCs of canines treated with nanoparticle formulation LUNAR-301 relative to control canines receiving only empty nanoparticles. Within the target PBMC population, canines receiving higher doses of nanoparticle formulation LUNAR-301 had greater levels of miR-124 quantified, and the increases in miR-124 levels were sustained with multi-dosing.

[0039] FIG. 11: FIG. 11 shows that nanoparticle formulations can be selectively delivered to the immune compartment for activity in a canine model. Looking at the multi-dose study in greater resolution over the time course, miR-124 was quantified at doses 1, 4, 7, and 9. Up to a 50-fold increase in miR-124 in the serum and 14-fold increase in miR-124 within the PBMCs was observed relative to pretreatment baselines. Additionally, in the PBMC compartment, there was a dose accumulation effect with each subsequent dosing over the treatment period.

[0040] FIG. 12: FIG. 12 shows that PBMCs from canines were also isolated from fresh blood and evaluated for effector cytokine production. There appeared to be a dose-dependent response in the production of IL-2, INFα, and INFγ after nanoparticle formulation LUNAR-301 treatment; the highest INFγ production was achieved with multi-dosing.

[0041] FIG. 13: FIG. 13 shows a schematic of a proposed mechanism of nanoparticle formulation LUNAR-301 action. While not wishing to be bound by any one particular theory, nanoparticle formulation LUNAR-301 (miR-124 mimic within lipid nanoparticles) is delivered to the patient intravenously and immediately come in contact with circulating PBMCs, most notably monocytes/macrophages and B cells. The nanoparticles are engulfed and rapidly degraded via a pH-mediated process within the endosome, thereby delivering the miR-124 mimic payload inside the cell. MiR-124 subsequently inhibits the STAT3 pathway, facilitating reversal of tumor-mediated immune suppression and resultant immune activation, allowing resident and peripheral immune cells to target the CNS tumor.

[0042] FIG. 14: FIG. 14 shows modifications of nanoparticle formulation dosing and schedule. C57BL/6 mice with established intracerebrally implanted gliomas were treated with either LIPOFECTAMINE alone, empty nanoparticles, or miR-124 mimic + LIPOFECTAMINE on a Monday, Wednesday, Friday schedule at a dose of 1 mg/kg, or with nanoparticle formulation LUNAR-301 on a Monday and Thursday schedule at a dose of 1 or 2.5 mg/kg. Nanoparticle formulation LUNAR-301 at 1 mg/kg was therapeutically equivalent to miR-124 mimic + LIPOFECTAMINE at 1 mg/kg (median survival times were 32.5 and 31.5 days, respectively; $P = 0.813$). Mice treated with nanoparticle formulation LUNAR-301 at 2.5 mg/kg had a median survival time (27 days) comparable with the LIPOFECTAMINE and empty nanoparticle controls (22 days [P=0.930] and 24 days [P=0.904], respectively). All the P-values were calculated using log-rank test. An increase in the dose or a decrease in the schedule frequency was associated with diminished therapeutic effect.

[0043] FIG. 15: FIG. 15 shows in Table 1: Murine *in vivo* pharmacokinetics of nanoparticle formulation LUNAR-301 relative to miR-124 mimic + LIPOFECTAMINE. Pharmacokinetic parameters were calculated for nanoparticle formulation LUNAR-301

and miR-124 mimic + LIPOFECTAMINE using the non-compartmental model (NCA). Parameters shown are the observed maximal concentration (Cmax), time at observed maximal concentration (Tmax), apparent elimination half-life estimated from the terminal time-concentration curve (t½λz), first-order rate constant associated with the terminal portion of the time-concentration curve (λz), area under the curve from time zero to the last sample time (AUClast), area under the curve from time zero extrapolated to infinity (AUCinf_obs), percent AUC extrapolated from last sample time to infinity (AUC % extrap), area under the moment curve from time zero to last sample time (AUMClast), area under the moment curve from time zero extrapolated to infinity (AUMCinf_obs), mean residence time based on time zero to last sample time (MRTlast), and mean residence time based on time zero extrapolated to infinity (MRTinf_obs).

DETAILED DESCRIPTION OF THE INVENTION

[0044] This invention provides a range of novel agents and compositions to be used as therapeutics against glioma in a subject.

[0045] More particularly, this invention encompasses microRNAs (miRNAs) directed to preventing or treating glioma in a subject.

[0046] In general, miRNAs are short (20-24 nt) non-coding RNAs that are involved in post-transcriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of mRNAs. miRNAs can be transcribed by RNA polymerase II as part of capped and polyadenylated primary transcripts (pri-miRNAs) that can be either protein-coding or non-coding. The primary transcript is cleaved by the Drosha ribonuclease III enzyme, which can produce an approximately 70-nt stem-loop precursor miRNA (pre-miRNA). The precursor can be further cleaved by the cytoplasmic Dicer ribonuclease to generate the mature miRNA and antisense miRNA star (miRNA*) products. The mature miRNA can be incorporated into a RNA-induced silencing complex (RISC), which recognizes target messenger RNAs (mRNAs) through certain base pairing with the miRNA, and can result in translational inhibition or destabilization of the target mRNA.

[0047] MIR124-1, which is microRNA 124-1 (*Homo sapiens*, human), is found at NC_000008.11 (9903388..9903472, complement), and NC_000008.10 (9760898..9760982, complement).

[0048] This invention provides a range of compositions containing miR-124 that are useful for providing therapeutic effects because of their activity in modulating expression of a gene, as well as immune effects. The compositions of this invention provides gene regulating or gene silencing activity in vitro and in vivo.

[0049] Embodiments of this invention provide compositions for use as therapeutic agents against glioma. The compositions can be used as pharmaceutical agents for ameliorating, preventing or treating a glioma in a subject.

[0050] A microRNA of this invention can be modified with non-natural nucleotides, or modified nucleotides, or chemically-modified nucleotides, including any such nucleotides known in the art.

[0051] Examples of non-natural, modified, and chemically-modified nucleotide monomers include any such nucleotides known in the art, for example, 2'-O-methyl ribonucleotides, 2'-O-methyl purine nucleotides, 2'-deoxy-2'-fluoro ribonucleotides, 2'-deoxy-2'-fluoro pyrimidine nucleotides, 2'-deoxy ribonucleotides, 2'-deoxy purine nucleotides, universal base nucleotides, 5-C-methyl-nucleotides, and inverted deoxyabasic monomer residues.

[0052] Examples of non-natural, modified, and chemically-modified nucleotide monomers include 3'-end stabilized nucleotides, 3'-glyceryl nucleotides, 3'-inverted abasic nucleotides, and 3'-inverted thymidine.

[0053] Examples of non-natural, modified, and chemically-modified nucleotide monomers include locked nucleic acid nucleotides, 2'-O,4'-C-methylene-(D-ribofuranosyl) nucleotides, 2'-methoxyethoxy (MOE) nucleotides, 2'-methyl-thio-ethyl, 2'-deoxy-2'-fluoro nucleotides, and 2'-O-methyl nucleotides.

[0054] Examples of non-natural, modified, and chemically-modified nucleotide monomers include 2'-amino nucleotides, 2'-O-amino nucleotides, 2'-C-allyl nucleotides, and 2'-O-allyl nucleotides.

[0055] Examples of non-natural, modified, and chemically-modified nucleotide monomers include N^6-methyladenosine nucleotides.

[0056] Examples of non-natural, modified, and chemically-modified nucleotide monomers include nucleotide monomers with modified bases 5-(3-amino)propyluridine, 5-(2-mercapto)ethyluridine, 5-bromouridine; 8-bromoguanosine, or 7-deazaadenosine.

[0057] Examples of non-natural, modified, and chemically-modified nucleotide monomers include 2'-O-aminopropyl substituted nucleotides.

[0058] Examples of non-natural, modified, and chemically-modified nucleotide monomers include replacing the 2'-OH group of a nucleotide with a 2'-R, a 2'-OR, a 2'-halogen, a 2'-SR, or a 2'-amino, where R can be H, alkyl, alkenyl, or alkynyl.

[0059] Examples of non-natural, modified, and chemically-modified nucleotide monomers locked nucleic acid monomers (LNA).

[0060] Some examples of modified nucleotides are given in Saenger, Principles of Nucleic Acid Structure, Springer-Verlag, 1984.

Modes of action

[0061] In some aspects, this invention provides methods for preventing, ameliorating or treating a disease associated with glioma in a subject. The methods can include intravenous administration to the subject an effective amount of a nanoparticle composition comprising miR-124 mimic. The nanoparticle composition can deliver miR-124 mimic for therapeutic effects in reducing the growth of a glioma, or inducing regression of a glioma. The nanoparticles can encapsulate the miR-124 mimic and provide endosomolytic access of the miR-124 mimic to cells.

[0062] In further aspects, methods of this invention include activating immune system cells against glioma. The immune system cells can be contacted with a nanoparticle composition containing miR-124 mimic. The contacting of cells with the nanoparticle composition can be performed in vivo or ex vivo.

[0063] This invention provides compositions containing immune system cells that are activated against glioma. The compositions may have immune system cells that have reduced levels of STAT3 with the miR-124 as compared to cells that are not activated. Such compositions can have increased activity against glioma.

[0064] In some modes, this invention provides methods for activating an immune response against glioma in a subject. A nanoparticle composition of this invention can selectively deliver miR-124 mimic to an immune system compartment in the subject. Immune system compartments can include myeloid lineage cells, lymphocytes, monocytes, peripheral blood mononuclear cells, or macrophage thereof. By activating

the immune system compartment, the methods provide surprising therapeutic effects in reducing the growth of a glioma, or inducing regression of a glioma.

[0065] In some variations, the immune system compartment can be PBMCs, or can be monocytes and macrophages. In these variations, after delivery of the nanoparticle composition, the level of miR-124 in the monocytes and macrophages can be at least five-fold greater than in the monocytes and macrophages of a subject to which the composition was not administered. In further variations, the immune system compartment can include B cells. In these variations, after delivery of the nanoparticle composition, the level of miR-124 in the B cells can be at least five-fold greater than in the B cells of a subject to which the composition was not administered.

[0066] Additional modalities of this invention include methods for inducing immunological memory against a glioma in a subject. In these modalities, administering to the subject an effective amount of a nanoparticle composition containing miR-124 mimic provide immune system cells that are activated against a glioma and create immunological memory against a glioma. The immunological memory and persistent immunotherapeutic effects in vivo can include persistence of anti-glioma immune effects over a long period of time, from days, to weeks, to months, and longer.

Methods for treating glioma

[0067] Methods and compositions of this invention can be used for treatment, amelioration, or prevention of diseases in mammalian subjects associated with glioma. A subject can be a human or mammal. In the methods of this invention, a subject in need of treatment or prevention can be administered an effective amount of a nanoparticle composition of this invention. In certain embodiments, the nanoparticles can encapsulate a nucleic acid agent, such as miR-124 mimic. As used herein, the term "nucleic acid agent" and "nucleic acid" refer to any nucleic acid polymer composed of either polydeoxyribonucleotides (containing 2-deoxy-D-ribose), or polyribonucleotides (containing D-ribose), or any other type of polynucleotide which is an N glycoside of a purine or pyrimidine base, or modified purine or pyrimidine bases. There is no intended distinction in length between the term "nucleic acid agent" and "nucleic acid" as these terms will be used interchangeably. These terms refer only to the primary structure of the

molecule. Thus, these terms include double- and single-stranded DNA, as well as double- and single stranded RNA.

[0068] An effective amount of miR-124, single or double-stranded, can be a dose ranging from 0.001 mg/kg to 50.0 mg/kg. In some embodiments, a dose of double-stranded miR-124 of 1 mg/kg can be administered. The dose can be administered once per week, or 2 times per week, or three times per week, or daily, or twice daily, or 3-5 times per day.

Nanoparticle compositions

[0069] In some aspects, this invention provides pharmaceutical compositions containing an nucleic acid agent and a pharmaceutically acceptable carrier.

[0070] A pharmaceutical composition can be capable of local or systemic administration. In some aspects, a pharmaceutical composition can be capable of any modality of administration. In certain aspects, the administration can be intravenous, subcutaneous, pulmonary, intramuscular, intraperitoneal, dermal, oral, or nasal administration.

[0071] Embodiments of this invention include pharmaceutical compositions containing miR-124 in a lipid or lipid nanoparticle formulation.

[0072] Lipid nanoparticles for use in this invention can be any lipid nanoparticles known in the art.

[0073] In some embodiments, a pharmaceutical composition may comprise one or more lipids selected from cationic lipids, anionic lipids, sterols, pegylated lipids, and any combination of the foregoing.

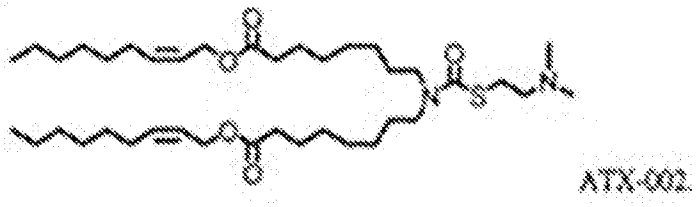
[0074] In certain embodiments, a pharmaceutical composition can be substantially free of liposomes.

[0075] In further embodiments, a pharmaceutical composition can include liposomes, lipid nanoparticles or nanoparticles.

[0076] Some examples of lipids and lipid compositions for delivery of an active molecule of this invention are given in WO/2015/074085, which is hereby incorporated by reference in its entirety.

[0077] In further aspects, this invention includes nanoparticle compositions that can encapsulate and deliver miR-124 to cells with surprisingly advantageous potency. The

nanoparticles can be formed with lipid molecules, for example, any one or more of the compounds ATX-001 to ATX-032 disclosed in WO/2015/074085, the contents of which are incorporated herein by reference in their entirety. In certain embodiments, lipid nanoparticles of this invention can be formed with compound ATX-002 (Compound I), as disclosed in WO/2015/074085, and the formulation is referred to herein as LUNAR-301, which encapsulates miR-124.



Compound I

[0078] A pharmaceutical composition of this disclosure may include carriers, diluents or excipients as are known in the art. Examples of pharmaceutical compositions are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro ed. 1985).

[0079] Examples of excipients for a pharmaceutical composition include antioxidants, suspending agents, dispersing agents, preservatives, buffering agents, tonicity agents, and surfactants.

EXAMPLES

Example 1: **Preparation of nanoparticles**.

[0080] Analogous to LIPOFECTAMINE 2000 (Invitrogen, Carlsbad, CA), a liposomal transfection reagent made from cationic lipids, DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (Roche, Basel, Switzerland) was combined with miR-124. DOTAP:cholesterol nanoparticles with miR-124 were created by Dr. Jack Roth (MD Anderson, Houston, TX). Chitosan miR-124 nanoparticles were prepared on an ionic gelation of anionic tripolyphosphate by Dr. Anil Sood (MD Anderson, Houston, TX). A bi-lipid delivery nanoparticle formulation was used to make four test articles containing miR-124: NB5-55-1, NB5-55-2 (using compound ATX-002 and designated LUNAR-301), NB5-55-3, and NB5-55-4.

[0081] The lipid nanoparticles (LNPs) were prepared by mixing appropriate volumes of lipids in ethanol with an aqueous phase containing miRNA duplexes, employing a NANOESSEMBLER microfluidic device, followed by downstream processing.

[0082] For the encapsulation of miRNA, the desired amount of double-stranded RNA was dissolved in 5 mM citric acid buffer, pH 3.5. Lipids at the desired molar ratio were dissolved in ethanol. The mol% ratio for the constituent lipids is 58% ATX-002 (ionizable amino lipids), 7% DSPC (1,2-dioctadecanoyl-sn-glycero-3-phosphocholine), 33.5% cholesterol, and 1.5% DMG-PEG (1,2-Dimyristoyl-sn-glycerol, methoxypolyethylene glycol, PEG chain Molecular weight: 2000). At a flow ratio of 1:3 ethanol:aqueous phases, the solutions were combined in the microfluidic device (Precision NanoSystems Inc., Vancouver, Canada) using two HPLC prep pumps (AZURA P 2.1L, Knauer, Berlin, Germany). The total combined flow rate was 12 mL/min, per microfluidics chip. From one to four microfluidics chips were utilized, in a custom unit for parallelization (Precision NanoSystems), allowing a variable throughput for different batch sizes. The microfluidics chips employed a herringbone micromixer for extremely quick mixing times, yielding high encapsulation and narrow particle size distribution. The mixed material was then diluted 3x with deionized water after leaving the micromixer outlet, reducing the ethanol content to 6.25%. The diluted LNP slurry was concentrated on a TFF with hollow fiber membranes (mPES Kros membranes, Spectrum Laboratories, Inc., Rancho Dominguez, California), and then diafiltration was performed with modified DPBS, without magnesium or calcium (HyClone, Logan, Utah). A total of 10 diavolumes were exchanged, effectively removing the ethanol.

[0083] Particle size was determined by dynamic light scattering (ZEN3600, Malvern Instruments, UK). Encapsulation efficiency was calculated by determining unencapsulated siRNA content by measuring the fluorescence upon the addition of RiboGreen (Molecular Probes, Eugene, OR) to the LNP slurry (Fi) and comparing this value to the total siRNA content that is obtained upon lysis of the LNPs by 1% Triton X-100 (Ft), where % encapsulation = $(Ft - Fi)/Ft \times 100$.

[0084] The miR-124 duplex used for all formulations comprised the following sense and anti-sense strands:

[0085] sense: 5'-UAAGGCACGCGGUGAAUGCCA-3' (SEQ ID NO: 1)

[0086] antisense: 3'-UAAUUCCGUGCGCCACUUACG-5' (SEQ ID NO: 2)

[0087] which was synthesized by Avetra Bioscience (Mountain View, CA) and hybridized under standard conditions. All constructs tested herein were in the duplex form.

Example 2: **Intracerebral glioma murine model**.

[0088] C57BL/6 mice were intracerebrally implanted with 5 x 10^4 GL261 glioma cells in 5 μL of matrigel. Mice were randomly assigned to control or treatment groups (n=6-10/group) three days post tumor implantation. The animals were treated intravenously for three weeks; thereafter the final endpoint was survival. Treatment groups included: various therapeutic miRNAs individually and in combination + LIPOFECTAMINE, double-stranded-miR-124 mimic + DOTAP, double-stranded-miR-124 mimic in chitosan nanoparticles, double-stranded-miR-124 mimic in DOTAP:cholesterol nanoparticles, and double-stranded-miR-124 mimic in LUNAR-301 nanoparticles (NB5-55-1, NB5-55-2 [also designated LUNAR-301], NB5-55-3, and NB5-55-4).

[0089] All animals were treated with a single formulation at a dose of 1 mg/kg administered Monday, Wednesday, and Friday. A supplementary study was conducted to evaluate nanoparticle formulation LUNAR-301 dosing at 2.5 mg/kg on a different treatment schedule (Monday and Thursday administration). Toxicity to mice was monitored on a regular schedule with no toxicity observed including evaluating weight and performing neurological assessments of "stepping and placing" reflexes. All animal experiments were conducted in compliance with the guidelines for animal care and use established by The University of Texas M. D. Anderson Cancer Center under protocol 08-12-09031 (00001176-RN00). To determine whether immunological memory was induced, surviving mice were rechallenged by implanting 5 x 10^4 GL261 glioma cells intracerebrally in the contralateral hemisphere. Mice were subsequently observed for continued survival.

[0090] In general, a GL261 glioma is a carcinogen-induced mouse syngeneic glioma model, which is a brain tumor model developed in immunocompetent animals that has growth characteristics similar to human glioblastoma multiform (GBM).

Example 3: **Murine pharmacokinetic analysis**.

[0091] The pharmacokinetics of nanoparticle formulation LUNAR-301 comprising the miR-124 duplex *in vivo* were compared with those of double-stranded miR-124 + LIPOFECTAMINE in non-tumor-bearing C57BL/6 mice at a 1 mg/kg i.v. dose. The mice (n=3/group/time point) were treated once and subsequently sacrificed at 0 minutes, 15 minutes, and at 1, 4, 8, and 24 hours post-delivery. At each time point, the serum, liver, and PBMCs were fractioned for total RNA extraction. The miR-124 concentration in each compartment was assessed by quantitative RT-PCR using a standard curve containing a series of miR-124 duplex dilutions. A noncompartmental analysis was performed in mice using industry-standard software (WinNonLin 6.3, Pharsight) to estimate the pharmacokinetic parameters for each individual animal, using drug concentrations observed in serum, liver, and PBMCs. The following parameters were estimated for each animal and tissue: time of peak serum drug concentration (Tmax), peak drug concentration (Cmax), apparent elimination half-life (T1/2, calculated as ln(2)/lambda z, lambda z being the first order rate constant associated with the terminal portion of the time-concentration curve, as estimated by linear regression of time vs. log concentration), area under the time-concentration curve from time zero to the last observed concentration (AUC0-obs, calculated by the linear trapezoidal rule), and area under the time-concentration curve from time zero extrapolated to infinity (AUC0-inf, calculated by adding the last observed concentration divided by lambda z to the AUC0-obs). Mean parameters were then calculated from individual mouse estimates.

Example 4: Murine immune functional studies.

[0092] C57BL/6 mice were subcutaneously implanted with 2 x 10^6 GL261 cells in matrigel to have a tumor sufficiently large for analysis of the infiltrating immune population. Two weeks after implantation, mice were randomized by tumor size and treated with empty nanoparticles (with exactly the same composition as LUNAR-301, but without the double-stranded miR-124 double-stranded miR-124 mimic + LIPOFECTAMINE, or formulation LUNAR-301 (n=5 per group) for 5 doses on Monday, Wednesday, and Friday at 1 mg/kg. The control group of mice was untreated (n=5). For glioma-infiltrating T-cell isolation, subcutaneous gliomas were homogenized in cold MACS buffer (1x PBS, 2% FBS, 2nM EDTA) to make a single-cell suspension.

[0093] Splenocytes were also harvested and homogenized in cold MACS buffer. Red blood cells were removed with red blood cell lysing buffer (Sigma-Aldrich) to form a single-cell suspension of splenocytes that were cocultured with Dynabeads CD3/CD28 (Life Technologies) and supplemental IL-2 for seven days to activate T cells.

[0094] For intracellular phosphorylated Signal transducer and activator of transcription 3, (p-STAT3) and FoxP3 detection, glioma-infiltrating T cells were fixed and permeabilized (eBioscience) for 1 hour at 4°C. Cells were then stained with PE-conjugated anti-pSTAT3 (Y705) antibody (eBioscience) or PE-conjugated anti-FoxP3 antibody (eBioscience) for 30 minutes at 4°C. Activated splenocytes were stained with PE-conjugated anti-granzyme B antibody (eBioscience) for one hour at room temperature. Flow cytometry acquisition was performed with a FACS Calibur (Becton Dickinson, San Diego, CA) and data analyzed using FlowJo software (TreeStar, Ashland, OR).

Example 5: **Murine toxicity study**.

[0095] Non-tumor-bearing C56BL/6 mice were dosed with formulation LUNAR-301 or empty nanoparticles at 1 mg/kg Monday, Wednesday, and Friday for 3 weeks (n=8 per group). A control group of mice remained untreated (n=8). Toxicity to mice was monitored on a regular schedule, evaluating weight and performing neurological assessments that consisted of "stepping and placing" reflexes. After completion of the treatment regimen, mice were euthanized and organs including the spleen, thymus, lung, heart, kidney, brain, liver, and gastrointestinal tract were harvested, formalin fixed, and cryotome sectioned. Tissue sections were stained using H & E. A pathologist blinded to the treatment regimen performed the histologic examination.

[0096] In a second toxicity study conducted by a contract research organization (BTS Research, San Diego, CA), an empty lipid nanoparticle (LNP) formulation, referred to as 11-99-02, was evaluated. Mice were treated with 2 formulations; 11-99-01 and 11-99-02;

[0097] 11-99-01 is comprised of ATX-002: DSPC: Chol: DMG-PEG at a molar ratios of 58:7:33.5:1.5 and a non-targeting control siRNA at an siRNA to total lipid ration of 0.062. ATX-002 is an ionizable lipid; DSPC is 1,2-distearoyl-sn-glycero-3-phosphocholine; Chol is Cholesterol; and DMG-PEG is 1,2-Dimyristoyl-rac-glycero-3-methyl-polyoxyethylene.

[0098] 11-99-02 is an empty lipid nanoparticle (meaning no RNA payload) comprised of ATX-002: DSPC: Chol: DMG-PEG at a molar ratios of 58:7:33.5:1.5, where, ATX-002 is an ionizable lipid; DSPC is 1,2-distearoyl-sn-glycero-3-phosphocholine; Chol is Cholesterol and DMG-PEG is 1,2-Dimyristoyl-rac-glycero-3-methyl-polyoxyethylene.

[0099] Male and female CD-1 mice were treated once on Day 0 by injection into the left lateral tail vein at doses of 5, 10, 20 or 30 mg/kg at a volume of approximately 6 mL/kg. Control groups received only the vehicle, sterile PBS. Body weights of the mice were monitored on Day 0 and at the time of sacrifice (Day 2, approximately 48 hours post treatment), and terminal blood samples were collected for assessment of clinical chemistry and hematology parameters after an overnight fast. The wet weights of the spleen and liver were also measured and recorded on Day 2 (i.e., 48 hours post treatment).

Example 6: **Canine model**.

[00100] Purpose-bred beagles (n=5) were utilized for a second-species analysis. A dose escalation starting at 0.25 mg/kg was selected using an allometric scaling calculator and derived from the effective therapeutic dose (1 mg/kg) of LUNAR-301 comprising the miR-124 duplex mimics in glioma-bearing mice (http://home.fuse.net/clymer/minor/allometry.html).

[00101] Dogs were allowed to acclimate to the surroundings for 14 days prior to initiating the treatment. Serial physical examinations as well as measurement of body temperature, pulse and respiratory rate were performed. Blood sampling in the dogs was performed via jugular venipuncture. Three days before (Day -3) and immediately prior to the delivery of nanoparticle formulation LUNAR-301 (Day 1) baseline physical examination and clinical observations were performed, and blood samples (12 mL) were collected for complete blood counts (CBCs), serum chemistry profiles, and coagulation profiles. Blood samples (3 mL) for analysis of the native wild type single stranded miR-124 concentration in serum and in PBMCs were collected at 10, 20, 30, 45, and 60 minutes, and at 2, 4, 6, 12, 24, 48, and 72 hours post-LUNAR-301 delivery.

[00102] Immune activation status was sampled at 0 and 24 hours. At 72 hours, blood was obtained for CBC, serum chemistry profile, and the coagulation profile. RT-PCR was utilized to quantify wild type miR-124 in canine PBMCs and sera. Nanoparticle

formulation LUNAR-301 and empty nanoparticle administration (i.v.) was through the cephalic vein. Canines were subsequently euthanized and received a complete necropsy, with gross and histological examination. Portions of the drug delivery site, heart, liver, spleen, brain, lung, intestine, kidney, lymph node, thymus, and bone marrow were fixed in formalin and then paraffin embedded. Four-micrometer sections were mounted on glass slides and stained with H&E. Slides were microscopically evaluated by an anatomic pathologist who was blinded to the treatment regimen.

Example 7: **Canine flow cytometry**.

[00103] PMBCs were isolated from fresh blood using Histopaque (Sigma). For FoxP3 staining, PBMCs were transferred to 96-well round-bottom plates and washed with FACS buffer (1x PBS, 2% FBS, 0.09% NaN3), then fixed and permeabilized (eBioscience). PBMCs were stained for thirty minutes at 4°C with 1:30 Anti-Mouse/Rat FoxP3 APC (eBioscience). For cytokine staining, PBMCs were transferred to 96-well flat-bottom plates and cultured for six hours in RMPI 1640 (Gibco) complete medium with 50 ng/mL PMA (Sigma-Aldrich), 500 ng/mL ionomycin (Sigma-Aldrich), and 1:500 Protein Transport Inhibitor Cocktail (eBioscience) for T-cell activation. Activated PBMCs were transferred to 96-well round-bottom plates and washed, fixed, and permeabilized. Cells were incubated with 1:100 Biotin-conjugated anti-canine IL-2, INFγ, or TNFα antibody (RD Systems) for 20 minutes at 4°C. APC-conjugated Streptavidin (BD) 1:50 was used as the secondary antibody. Flow cytometry acquisition was performed with a FACS Calibur (Becton Dickinson, San Diego, CA), and data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Example 8: **Statistical analysis**.

[00104] Overall survival (OS), defined as the time (in days) the animal received treatment to the date of death, was estimated using the method of Kaplan and Meier. The log-rank test and a stratified Cox proportional hazards regression model were used to explore the survival differences between individual treatment groups. A one-sided Fisher's exact test was used for comparison of the cure rates between nanoparticle formulation LUNAR-301 and other treatment groups. The longitudinal mixed-effects model ENREF_16 was employed to assess the tumor growth rate (on a natural logarithmic scale). Statistical software R V-3.0.2 (with packages survival v2.37-4 and

nlme v3.1-111) was used for data analysis. *P* values less than or equal to 0.05 were considered statistically significant.

Example 9: **Nanoparticle formulations can enhance the therapeutic effect of a double-stranded miR-124 mimic, and it can be delivered to the immune compartment**.

[00105] Double stranded miR-124 mimic was encapsulated with an efficiency of >95% and a polydispersity index <0.05 using a GMP-ready process of a NANOESSEMBLER microfluidic device, followed by downstream processing. This produced an 80 nm particle with long-term stability (>4 months) and no variation in particle size. Four test articles (nanoparticles containing double-stranded miR-124 mimics) with modifications of the lipid formulation were devised.

[00106] Therapeutic equivalency studies were conducted in C57BL/6 mice with intracerebral GL261 gliomas. Of the test articles prepared, NB5-55-2 (formulation LUNAR-301) demonstrated superiority, with a median survival time that could not be calculated because >60% of mice were long-term survivors.

[00107] As shown in FIG. 1, the three remaining double-stranded miR-124 mimic-nanoparticle formulations produced median survivals of 30.5, 43.5, and 6 days for NB5-55-1, NB5-55-3, and NB5-55-4, respectively. Significant toxicity was observed in the NB5-55-4 group, and necropsy showed the deaths did not result from intracranial tumors.

Example 10: **Frequency of drug administration**.

[00108] To test whether dose or frequency of drug administration could improve efficacy, formulation LUNAR-301 (dosed at 1 mg/kg or 2.5 mg/kg of the double-stranded miR-124 mimic inside the formulation) was evaluated on a Monday and Thursday treatment schedule. Mice with intracerebral GL261 gliomas were treated, and survival was compared with the traditional dosing schedule of Monday, Wednesday, and Friday (1 mg/kg miR-124 + LIPOFECTAMINE), with all mice treated for three weeks. Formulation LUNAR-301 at 1 mg/kg performed similarly to miR-124 + LIPOFECTAMINE at 1 mg/kg (median survival times for the two groups: 32.5 and 31.5 days, respectively, with no significant difference, P=0.813).

[00109] FIG. 14 shows that mice treated with LUNAR-301 at 2.5 mg/kg showed a median survival (27 days) comparable with and not significantly different from

LIPOFECTAMINE and empty nanoparticle controls (22 days [*P*=0.930] and 24 days [*P*=0.904], respectively).

Example 11: **Nanoparticle formulations can be selectively delivered to the immune compartment**.

[00110] To ascertain the kinetics and concentrations of miR-124 within the PBMCs achievable with formulation LUNAR-301, non-tumor-bearing C57BL/6 mice were dosed with LUNAR-301 and compared with controls (double-stranded miR-124 mimic + LIPOFECTAMINE). The serum, liver, and PBMCs were examined by quantitative RT-PCR to assess the miR-124 level in each compartment. FIG. 3 shows that at all time points and compartments, formulation LUNAR-301 achieved higher concentrations of miR-124 than were delivered with LIPOFECTAMINE. Due to reticuloendothelial system clearance, as expected, greater retention of miR-124 was measured in the liver with LUNAR-301.

[00111] miR-124 kinetics were further analyzed in the PBMC compartment because this is the desired delivery compartment (e.g., immune cells). As shown in FIG. 15, Table 1, after single dosing, peak concentrations were achieved at 15 minutes, and miR-124 persisted for ≤4 hours. The PBMC immune subsets were fractionated, and analyzed for the delivery of miR-124. It was found that formulation LUNAR-301 preferentially targeted monocytes, as denoted by CD11b+ in FIG. 3, consistent with their biological function of phagocytosis and representing an important site of STAT3 regulation, given the innate monocyte/macrophage interface with adaptive immune responses.

Example 12: **Nanoparticle formulation immunomodulatory properties**.

[00112] To verify that formulation LUNAR-301 maintains the immunomodulatory properties documented for double-stranded miR-124 mimic + LIPOFECTAMINE, *in vivo* immune functional studies were performed. C57BL/6 mice subcutaneously implanted with GL261 cells (to achieve sufficient tumor volume to analyze) were treated with double-stranded miR-124 mimic + LIPOFECTAMINE or LUNAR-301, each at 1 mg/kg of double-stranded miR-124 mimic for 5 doses. Thereafter, glioma-infiltrating T-cells were isolated and assessed for intracellular p-STAT3 and FoxP3. As shown in FIG. 4, significant reductions in p-STAT3 and FoxP3 were observed with both double-stranded miR-124 mimic + LIPOFECTAMINE (*P*=0.0032 and *P*=0.0213, respectively)

and formulation LUNAR-301 (P=0.0001 and P=0.0223, respectively) treatments compared with the untreated group. To ascertain if the LUNAR-301 could protect against tumor recurrence, mice surviving initial GL261 glioma implantation after treatment with double-stranded miR-124 mimic + LIPOFECTAMINE or formulation LUNAR-301 were rechallenged with a second intracerebral glioma implanted in the contralateral hemisphere. As shown in FIG. 5, subsequently, only one mouse died in the LUNAR-301 group (survival >200 days post initial tumor implantation). These mice were again rechallenged with a third intracerebral tumor, with no subsequent treatment with formulation LUNAR-301.

[00113] As shown in FIG. 6, 3 of 4 of the previously treated mice again survived, indicating induction of immunological memory. Without wishing to be bound by any one particular theory, immunological memory may not be a general property of anti-STAT3 agents, because small molecular inhibitors of STAT3 may not generate immunological memory in murine melanoma models.

Example 13: **Nanoparticle formulation toxicity**.

[00114] Toxicity was tested by treating mice for two weeks with either formulation LUNAR-301 or empty nanoparticles, followed by necropsy. Microscopic analysis of spleen, thymus, lung, heart, kidney, brain, liver, and GI tract, showed no treatment-related abnormalities. Sporadic vascular/perivascular inflammation was observed in the lungs, probably from foreign-body (i.e., hair) injection during i.v. administration. Additional murine toxicity studies by a contract research organization (BTS Research, San Diego, CA) found that single i.v. administration of empty lipid nanoparticles at doses up to 30 mg/kg of the double-stranded miR-124 mimic in LUNAR-301 were well tolerated, with no unusual observations in either sex. Clinical chemistry and hematologic analysis revealed a few significant intergroup differences; however, these were not dose-related and/or were numerically close to control values.

Example 14: **Immune activation resulting from miR-124**.

[00115] To test that immune activation resulted from delivery of double-stranded miR-124 mimic, and as further controls for the toxicity studies, seed sequence modifications were made at the 5' end of a double-stranded miR-124 mimic.

[00116] Specifically, the sequence of miR-124, 5'-UAAGGCACGCGGUGAAUGCCA-3' (SEQ ID NO: 1) was modified at the six nucleotides of the 5' terminus to 5'-UGGCAAACGCGGUGAAUGCCA-3' (SEQ ID NO: 3). This kept the base composition of the guide strand of the miR-124 mimic the same, but changed the seed sequence to disrupt any potential pairing with miR-124 targets such as STAT3. The passenger strand contained the compensatory changes to keep it a perfectly matched duplex with two base 3' overhangs.

[00117] The duplex resulting from annealing these two oligonucleotides was packaged in the LUNAR nanoparticle formulation and designated seed sequence control (SSC), which was verified to not exert any therapeutic activity in mice harboring intracerebral GL261 (median survival, 16 days). To assess toxicity, non-tumor-bearing C57BL/6J mice (n=5-6 per group) were dosed with formulation LUNAR-301 (1mg/kg), SSC (1mg/kg), or i.v. PBS for three weeks on a Monday, Wednesday, and Friday schedule. Five hours after the 9^{th} dose, the animals were terminated, their spleens and livers examined, and sera assessed for nonspecific immune activation (i.e., cytokine storm). As shown in FIG. 7, body weights were maintained throughout dosing, but the liver weight of the LUNAR-301-treated group was found to be modestly increased (FIG. 8) along with aspartate aminotransferase (AST) levels (FIG. 9), but to levels significantly less than are seen in other industry standards of nanoparticle therapeutics.

[00118] Relative to PBS, nonspecific immune activation was not triggered with the LUNAR nanoparticles, as detected by serum cytokine analysis, with the sole exceptions of G-CSF with formulation LUNAR-301 (P=0; 9^{th} dose), MCP-1 with LUNAR-301 (P=0.009; 1^{st} dose), and SSC (P=0.04; 1^{st} dose).

Example 15: **Nanoparticle formulation delivery in canines**.

[00119] To test formulation LUNAR-301 in a second species, purpose-bred non-tumor-bearing beagles were utilized. Single-dose escalations of LUNAR-301 (0.25, 0.5, and 1 mg/kg, i.v.) or multiple doses of LUNAR-301 (0.5 mg/kg x 9) were given intravenously to the dogs. Pre-delivery (0 h) physical exams were normal (including pulse and respiration) in all dogs and were not altered post administration. But occasionally, mild sedation and emesis were observed (Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Event: grade I) that were not dose related. No

abnormalities attributable to drug formulation or dosage were observed on the CBCs, chemistry panels, or in coagulation. A pattern of mild clinically insignificant fibrinogen elevation was seen with repeated sampling, probably owing to blood withdrawal. During necropsy, microscopic evaluation of systemic organs revealed no significant abnormalities except in one dog receiving empty nanoparticles, who had a probably incidental mild multifocal lymphoplasmacytic choroid plexitis.

Example 16: **Nanoparticle formulations can be selectively delivered to the immune compartment for activity in a canine model**.

[00120] The distribution of miR-124 delivered by formulation LUNAR-301 in canines was determined longitudinally in the PBMC and serum compartments using quantitative RT-PCR. During dose escalation (double stranded miR-124 mimic at 0.25, 0.5 or 1.0 mg/kg), the range of resulting miR-124 levels in the immune compartment was between 0.6 to 1.46 x 10^{13} fold above pretreatment baselines. As shown in FIG. 10, there was no direct linear relationship between administered dose of LUNAR-301 and delivery concentration of miR-124. As shown in FIG. 11, during multidosing of formulation LUNAR-301, miR-124 appeared to accumulate in the PBMC compartment. As shown in FIG. 12, flow cytometry of target immune cells demonstrated a dose-dependent response of IL-2, TNFα, and IFNγ effector cytokines, intracellularly, after LUNAR-301 treatment.

Example 17: **Nanoparticle delivery for intracellular immune targeting**.

[00121] FIG. 13 shows a schematic of a proposed mechanism of formulation LUNAR-301 action. While not wishing to be bound by any one particular theory, LUNAR-301 (miR-124 within lipid) nanoparticles are delivered to the patient intravenously and immediately come in contact with circulating PBMCs, most notably monocytes/macrophages and B cells. The nanoparticles are engulfed and rapidly degraded via a pH-mediated process within the endosome, thereby delivering the double-stranded miR-124 mimic payload inside the cell. MiR-124 subsequently inhibits the STAT3 pathway, facilitating reversal of tumor-mediated immune suppression and resultant immune activation, allowing resident and peripheral immune cells to target the CNS tumor.

Example 18: **Advantages of nanoparticle formulations**.

[00122] LUNAR nanoparticles have several advantages over conventional methods of delivery, including: 1) higher dose-limiting toxicity; 2) long-term stability (>4 months) with minimal variation in particle size; 3) lack of immunogenicity (e.g., cytokine storm), thereby negating the requirement for antihistamine therapy; and 4) biodegradability (without accumulation after multiple dosing). Initial dose escalations of LUNAR-301 in canines and the delivery of double-stranded miR-124 mimic to the immune effector compartment suggest that allometric scaling may not apply.

[00123] Formulation LUNAR-301 has characteristics that make it similar to drugs that have successfully navigated the FDA approval system, is safe in multiple species, is GBM-ready, and can be rapidly translated to patients, because human and mouse miR-124 are 100% homologous.

Example 19: **Combination of STAT3 inhibition and radiation enhances the therapeutic effect against murine brain metastasis**.

[00124] Melanoma patients that develop brain metastasis have a median survival of 6 months (Sampson et al., J. Neurosurg. 88:11-20, 1998). Radiation, a standard of care for local control of melanoma, has been reported to have immune stimulatory capacities. Therefore, the combinatorial effect of inhibitors of the signal transducer and activator of transcript 3 (STAT3) pathway, a potent mediator of immune suppression, and whole-brain radiation in a murine brain metastasis model was investigated.

[00125] C57BL/6 mice were intracerebrally implanted with B16 melanoma, irradiated with 7.5 Gy, and treated with LUNAR-301 which inhibits the STAT3 pathway, or WP1066, a cell permeable AG 490 tyrphostin analog small molecule inhibitor of STAT3. Mice surviving 130+ days after tumor implantation were subcutaneously rechallenged with tumor.

[00126] Monotherapy (LUNAR-301, WP1066, or radiation alone) demonstrated longer survival relative to untreated control mice; combination therapies further extended survival compared to single treatment agents. Median survival for mice receiving radiation combined with LUNAR-301 or WP1066 was 65 days and 45.5 days, respectively, compared to 15 days for untreated mice and 19 – 40 days for monotherapy. While no untreated mice (control) survived over 20 days, the survival rate at day 130 for mice receiving combination therapy was almost 40%. Flow cytometry analysis showed

higher IL-2 production in T cells from tumor-draining lymph nodes in mice treated with radiation plus STAT3 inhibitor.

[00127] These results indicate the combination of STAT3 inhibition and radiation enhances the therapeutic effect against established murine brain metastasis.

Example 20: **Serum cytokine analysis of formulation LUNAR-301**.

[00128] Non-tumor-bearing C57BL/6 mice were treated with nine doses of LUNAR-301 (LUN) or SSC at the therapeutic dose of 1 mg/kg (based on the weight of the double-stranded RNA), or treated with the same volume of PBS. Serum samples were obtained five hours after both the first dose and the 9th dose for evaluation of serum cytokine changes to identify potential nonspecific immune activation secondary to i.v. nucleic acid treatment. The serum cytokines were measured using the Bio-Rad Bio-Plex Pro assay. Only MCP-1 (Monocyte chemoattractant protein-1) at dose 1 in both the formulation LUNAR-301- and SSC-treated mice showed a statistically significant increase above the level in the PBS group (P = 0.009 and P = 0.04, respectively). At dose 9, only G-CSF in the LUNAR-301-treated mice was significantly elevated compared with mice that received PBS (P=0). Both MCP-1 and G-CSF are implicated in monocyte/macrophage activation and recruitment. All the P-values were calculated using linear regression after Box-Cox transformation with parameters λ_1=0 and λ_2=1 and adjusted by Holm's approach. Holm, S. (1979), *Scandinavian Journal of Statistics* **6**, 65–70. Error bars, mean ± SD. The assay was negative for IL-9 and IL-17.

Example 21: Canine clinical trial

Protocol

[00129] Spontaneous brain tumors are reported commonly in canines, where malignancy types are similar to those seen in humans but incidence is more frequent, especially in certain breeds. In vivo analysis can be performed in a canine model using pet dogs with naturally occurring glioma to validate therapies that are promising in murine models prior to initiation of human clinical trials.

[00130] Accordingly, LUNAR-301 is administered to pet dogs with naturally occurring gliomas diagnosed by brain biopsy with owner consent. Tumor volume is measured by using magnetic resonance imaging (MRI) before therapy and at various time points after drug treatment to determine if the drug is shrinking the tumors. Additionally, blood is

obtained to ensure that dogs are tolerating the drug and developing expected immune responses. Animals receive supportive medical care for brain tumors (for example, steroids and anti-seizure medications) in addition to the experimental drug LUNAR-301.

[00131] Results of administration of LUNAR-301 are used to assess the safety of drug delivery in glioma-bearing dogs; evaluate immunological responses; determine response to treatment based on lesion volume as measured by magnetic resonance imaging (MRI); and establish the overall survival time in dogs treated with this therapeutic strategy.

[00132] Eligible subjects include dogs with: 1) either a pathological diagnosis of high-grade glioma or imaging features consistent with a high-grade glioma (See: Young et al Vet Radiol Ultrasound 2011); 2) measurable parenchymal contrast enhancement of tumor detected on brain MRI; 3) age greater than 1 year; 4) a modified Glasgow coma score greater than 14 that are ambulatory at the time of enrollment; 5) normal organ and bone marrow function; and 6) owner understanding and willing to sign a written informed consent document.

[00133] Exclusion criteria include dogs: 1) currently receiving any other type of anti-cancer therapy (except corticosteroids at the lowest tolerable amount) and 2) having leptomeningeal disease only or imaging/ histopathologic evidence of gliomatosis cerebri. The following clinical observations are monitored: 1) complete blood count, serum biochemistry, coagulation parameters, and immune assays (effector function, Treg inhibition, microRNA quantification etc.); 2) daily physical and neurological examination; and 3) characterization and grading of adverse events (AEs) daily using a validated system (Veterinary Cooperative Oncology Group-common terminology criteria for adverse events; VCOG CTACE v1.1).

[00134] After eligibility for this study is determined, owners are permitted to opt for clinicians to perform stereotactic image-guided brain biopsy to histologically determine glioma grade. This procedure has been studied in cadavers at Texas A&M University (Taylor Vet Radiol Ultrasound 2013) and has been utilized in the veterinary clinic for the past 5 years (e.g., Clark J Small Anim Pract 2012).

[00135] The canine model trial is divided into two phases: Phase I - three glioma bearing dogs are used for an inter-canine dose escalation study, and Phase II - up to 21 dogs with glioma are used for a clinical efficacy study.

[00136] Phase I of the trial proceeds according to an accelerated intercanine dose escalation design. A three dog cohort is chosen to proceed with dose escalation after a one week delay following the first treatment. Each dog receives a single test dose of LUNAR-301 at 0.5 mg/kg (doses used in healthy dogs ranged from 0.25-1 mg/kg) delivered through an intravenous catheter followed by a one week observation period. Given no occurrences of adverse events of a grade $\geq$3 which are severe and prolong hospitalization but are not life-threatening, treatment proceeds with dosing at 0.5 mg/kg for two weeks administered Monday, Wednesday, and Friday. During Phase I, the following are observed: 1) daily physical and neurological examination; 2) complete blood count (CBC), serum biochemistry, coagulation profile (6 mL), and urinalysis immediately prior to enrollment and at 24 hours following the delivery of the first dose of LUNAR-301 and final dose of LUNAR-301; and 3) standardized adverse events recording accomplished using Veterinary Cooperative Oncology Group-common terminology criteria for adverse events (VCOG CTACE v1.1). Additionally, effector immune responses of the dogs are monitored. Blood is collected (6 mL) before the first dose, 24 hours after the first dose, immediately before the last dose, and 24 hours after the last dose for analysis of peripheral blood mononuclear cells (PBMCs) via flow cytometry for various markers/cytokines (i.e. Foxp3, TNFα, IL-2, IFNγ). This cohort is excluded from formal radiographic evaluation for progression free survival as these dogs receive sub-therapeutic doses.

[00137] During Phase II of the trial, a maximum of 21 dogs are enrolled for the full therapeutic dosing cohort (9 intravenous deliveries at 0.5 mg/kg LUNAR-301 administered over 3 weeks on a Monday, Wednesday, Friday schedule). Dogs in Phase II that had an MRI performed at another institution are required to have an MRI to permit accurate comparison of pre- and post-treatment volume measurement. The dog is placed under general anesthesia by using a combination of standard premedications and utilizing inhalant gas anesthesia for maintenance of anesthesia, and then placing the dog in a 3T Siemens MRI located at a diagnostic imaging center. Time of anesthesia is typically about 1 hour to acquire standard T1, T2, T2 FLAIR, T2*, and post-contrast images. All dogs in this trial arm are identically monitored to that described for Phase I dogs and also have immune responses assayed in an identical manner. In addition, blood (6 mL) is

banked immediately prior to the first LUNAR-301 dose and 10 minutes following drug delivery for doses 3, 6, and 9. This banked blood is used for miR-124 quantification and potentially molecular profiling. The 10 minutes post-delivery time point was selected as this is when miR-124 peak concentrations were found in the blood and PBMC compartments in healthy dogs.

Adverse events/study withdrawal

[00138] The study begins with a small inter-canine dose escalation study (n=3; Phase I). The purpose of this small cohort is to obtain additional safety and dose optimization data from dogs with glioma. A 21 dog (maximum number of enrollees) efficacy study (Phase II) follows the Phase I study. During Phase II, detection of an adverse event of greater than or equal to grade 3, which are severe conditions requiring prolonged hospitalization but are not life-threatening, that were attributed to the drug results in cessation of drug delivery to that animal. Alternatively, a 25% dose reduction was made at the principal investigator's discretion in the setting of grade 3 toxicities. Dogs had LUNAR-301 administered within 24 hours after missing a dose; however, if more than 24 hours elapsed, then that dose was held and the next dose resumed on the designated schedule. Dogs requiring a delay of more than 2 weeks or dogs requiring more than 2 dose reductions were withdrawn from the study. If posterior probability of a greater than or equal grade 3 event greater than 30% was above 90%, as determined by Bayesian model of averaging continual reassessment methods (BMA-CRM), a dose reduction of 25% was applied. Due to the unique study population (i.e., dogs with CNS malignancy), there are several toxicities that are anticipated in this study population based on the underlying disease. A toxicity grade greater than or equal to 3 in severity was considered a dose-limiting toxicity with the following exceptions: seizure, thromboembolism, syndrome of inappropriate antidiuretic hormone (SIADH), and tumor progression.

[00139] Statistical analysis using BMA-CRM determines whether to stop the trial early based on the presence or absence of efficacy and is used to predict a maximum sample size required to meet a decision endpoint. The trial is stopped if there is a 90% probability for any of 3 events: (1) complete response (CR) greater than 15%, (2) complete response (CR) + partial response (PR) greater than 40%, or (3) complete response (CR) + partial response (PR) + stable disease greater than 85%. Alternatively,

the canine trial is terminated due to futility if there is a greater than 90% probability that progressive disease rate would be above 25% at a three-month follow-up.

Determination of radiographic responses

[00140] Dogs in the Phase II study have an MRI follow-up at 6-8 weeks (optional time point observed if clinical worsening/progression was evident), 3 months, 6 months, 9 months, and 12 months following initiation of LUNAR-301 delivery using protocols identical to those outlined for MRI obtained pre-delivery. Response and progression is evaluated in this study using the new international criteria proposed by the revised Response Evaluation Criteria in Solid Tumors (RECIST) guidelines (v1.1) and/or volumetric software. The enhancing lesion is measured bi-dimensionally on pre- and post-treatment studies. Lesion size is the product of its two largest perpendicular diameters. For multi-centric lesions with discrete foci of enhancement, each enhancing lesion is measured and then the sum is calculated. A non-measurable lesion includes foci of enhancement that are less than 5 mm, non-enhancing lesions seen on T2-weighted or T2 FLAIR images, hemorrhagic lesions, predominantly cystic lesions, necrotic lesions, and leptomeningeal disease. Hemorrhagic lesions often have intrinsic T1-weighted hyperintensity that could be misinterpreted as enhancing tumor, and for this reason, the pre-contrast T1-weighted image is examined to exclude baseline or interval sub-acute hemorrhage.

Determination of progression free survival

[00141] Dogs included in the Phase II component are serially tracked following drug delivery to determine survival time. The progression free survival (PFS) is defined based on MRI as less than a 25% increase in tumor volume on T1-weighted post-contrast MRI scans compared with the MRI obtained prior to treatment. If progression is suspected but there is uncertainty regarding whether imaging changes represent true tumor progression, imaging is repeated in 6-8 weeks or the subject is withdrawn from the study, at which time additional diagnostics or intervention are pursued as indicated. If the repeat imaging or additional procedures show progression, then the PFS is declared at the time progression is first suspected. At the time of death/euthanasia, samples from the CNS are obtained to evaluate immune infiltration, tumor response, and RNA expression to ascertain mechanisms of treatment resistance/failure.

[00142] For Treg and immune effector evaluation, the percent of PBMCs expressing STAT3/Foxp3 and T cell effector responses (TNFα, IL-2 and IFNγ) is compared before and after treatment using paired *t* tests or Wilcoxon signed-rank tests, as appropriate. The longitudinal analysis for all of these markers is similar, i.e., using generalized linear mixed-effects models (GLMMs) to assess the significance of the changing values with time and estimating the trajectory.

Blood, tissue, and other samples from the animals

[00143] Blood is collected from living animals by IV puncture in 6 mL aliquots for CBC/CHEM/COAG three times: prior to dose, 24 hours following 1st dose, and 24 hours after final dose. Blood is also collected by IV puncture in 6 mL aliquots for FACS staining 4 times: enrollment, 24 hours after 1st dose, 24 hours before last dose, and 24 hours after last dose. Lastly, blood is collected by IV puncture in 6 mL aliquots for miR-124 quantification during Phase II only: prior to 1st dose and 10 minutes after doses 3, 6, and 9. A canine brain biopsy is performed to diagnose dogs as having glioma prior to inclusion in study and drug delivery. Canine urine cystocentesis is performed in 5mL aliquots at enrollment and 24 hours after 1st and last dose.

Results of Phase I-Overview

[00144] The aims of the Phase I clinical trial were to establish that LUNAR-301 can safely be given systemically to dogs with spontaneous intracranial gliomas, and to establish that LUNAR-301 induces immune effector changes in these dogs similar to those seen in healthy Beagles.

[00145] The study enrolled 3 canines in this phase of the trial. The first patient experienced anaphylactoid reaction to treatments 4 and 5 of the dose-escalation study, and he was removed from the study before completion.

[00146] Subsequently, the treatment protocol was altered to include pre-treatment with diphenhydramine and famotidine to minimize the risk of anaphylaxis. While additional steroids have not been given for this purpose, all 3 enrolled patients have been receiving 0.5-1.0mg/kg prednisone daily for treatment of tumor-associated cerebral edema.

[00147] The second and third canine enrollees in this phase of the trial have completed their series of 7 infusions of LUNAR-301 at 0.5mg/kg per infusion without major adverse events.

[00148] Both remaining patients are currently stable, with survival times to date of 9 months and 94 days from diagnosis. While the latter (94 days) is still within the median survival time for dogs with supratentorial intracranial masses treated palliatively (5.9 months; Rossmeisl et al, *JAVMA* 2013), the former has already exceeded that benchmark.

Results-Detailed

[00149] Shown in Table 3 are the canine patient metrics for the study participants.

Table 3 Patient metrics

Patient	**1: Castrated Male Boston Terrier**	**2: Castrated Male French Bulldog**	**3: Castrated Male Boston Terrier**
Diagnosis	left frontal grade III astrocytoma, diagnosed by image-guided biopsy	right temporal lobe glioma, presumed from MRI	right frontal lobe high---grade glioma, presumed from MRI
Medications	7.5 mg prednisone q24 PO 150mg levetiracetam q8h PO	5mg prednisone q24h PO 100mg zonisamide q12h PO 375mg levetiracetam q8h PO 25mg metronidazole q12h PO 13-34 lb dose Denamarin q24h PO	64.8mg phenobarbital q12h PO 400mg levetiracetam q8h PO 100mg gabapentin q12h PO 8.75mg prednisone q24h PO 100mg zonisamide q12h PO (added 5/12/16 due to continued frequent seizures)

Cytokine Expression

[00150] Canine PBMCs were isolated by density gradient centrifugation and then stained with antibodies for cytokine expression or cell surface markers and analyzed by flow cytometry as taught herein.

[00151] Results indicated that LUNAR-301 treatment leads to an increase of effector cytokine expression in total PBMCs and an increase of CD4+ and CD8+ T cells and a decrease in CD11b+ cells. Further, LUNAR-301 treatment leads to an increase of effector cytokine expression in CD4+ and CD8+ T cells as well as in CD11b+ cells.

[00152] For patient 1, who was removed from the study, treatment did lead to an increase in effector cytokines, IL-2, TNFα and IFNγ.

[00153] For patient 2, no baseline cytokine expression was obtained due to hemolysis during blood collection and there were no changes throughout the study.

[00154] For patient 3, LUNAR-301 lead to an increase in CD4+ T cells of 0.9 fold over baseline just after dose 1 and a 1.2 fold increase after dose 7; an increase in CD8+ T cells

of 3.5 fold over baseline just after dose 1 and a 2.2 fold increase after dose 7; and a decrease in CD11+ cells of 2.5 fold below baseline just after dose 1 and a 2.0 fold decrease after dose 7. There was an increase in effector cytokines in all three cell types.

[00155] In conclusion, the results of evaluation of peripheral blood immune cells from these three patients showed responses to LUNAR-301 similar to those found in healthy dogs in a previous phase of the trial.

[00156] All publications, patents and literature specifically mentioned herein are incorporated by reference in their entirety.

[00157] It is understood that this invention is not limited to the particular methodology, protocols, materials, and reagents described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be encompassed by the appended claims.

[00158] It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. As well, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms “comprises,” and "comprising", can be used interchangeably as can “containing,” "including", and "having".

[00159] Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

[00160] All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose.

WHAT IS CLAIMED IS:

1. A method for preventing, ameliorating or treating a disease associated with glioma in a subject in need, the method comprising administering to the subject an effective amount of a nanoparticle composition comprising single or double-stranded miR-124 mimic.
2. The method of claim 1, wherein the administration reduces growth of a glioma.
3. The method of claim 1, wherein the administration induces regression of a glioma.
4. The method of claim 1, wherein the glioma is located in the brain or spine of the subject.
5. The method of claim 1, wherein the administration is intravenous.
6. The method of claim 1, wherein the nanoparticles encapsulate the single or double-stranded miR-124 mimic.
7. The method of claim 1, wherein the nanoparticles comprise one or more cationic lipids and one or more helper lipids.
8. The method of claim 1, wherein the nanoparticles comprise compound ATX-002.
9. A method for activating immune system cells against glioma, the method comprising contacting the cells with a nanoparticle composition comprising miR-124.
10. The method of claim 1, wherein the immune system cells are contacted with the nanoparticle composition in vivo or ex vivo.
11. The method of claim 1, wherein the nanoparticles encapsulate the single or double-stranded miR-124 mimic.
12. The method of claim 1, wherein the nanoparticles comprise one or more cationic lipids and one or more helper lipids.
13. The method of claim 1, wherein the nanoparticles comprise compound ATX-002.
14. A composition comprising immune system cells that are activated against glioma.
15. The composition of claim 14, wherein the cells have reduced levels of STAT3 as compared to cells that are not activated.
16. The composition of claim 14, wherein the cells have increased activity against glioma.

17. A method for activating immune response against glioma in a subject in need, the method comprising administering to the subject a nanoparticle composition comprising single or double-stranded miR-124 mimic and selectively delivering the miR-124 mimic to an immune system compartment in the subject.
18. The method of claim 17, wherein the immune system compartment comprises PBMCs.
19. The method of claim 17, wherein the immune system compartment comprises monocytes and macrophages.
20. The method of claim 17, wherein after delivery, the level of miR-124 in the monocytes and macrophages is at least five-fold greater than in the monocytes and macrophages of a subject to which the composition was not administered.
21. The method of claim 17, wherein the immune compartment comprises B cells.
22. The method of claim 17, wherein after delivery, the level of miR-124 in the B cells is at least five-fold greater than in the B cells of a subject to which the composition was not administered.
23. The method of claim 17, wherein the glioma is located in the brain or spine of the subject.
24. The method of claim 17, wherein the administration is intravenous.
25. The method of claim 17, wherein the nanoparticles encapsulate the single or double-stranded miR-124 mimic.
26. The method of claim 17, wherein the nanoparticles comprise one or more cationic lipids and one or more helper lipids.
27. The method of claim 17, wherein the nanoparticles comprise compound ATX-002.
28. A method for inducing immunological memory against a glioma in a subject in need, the method comprising administering to the subject an effective amount of a nanoparticle composition comprising single or double-stranded miR-124 mimic.
29. The method of claim 28, wherein the immunological memory includes persistence of anti-glioma immune effects over a period of at least 5 days.
30. The method of claim 28, wherein the immunological memory includes persistence anti-glioma immune effects over a period of at least 15 days.

31. The method of claim 28, wherein the immunological memory includes persistence anti-glioma immune effects over a period of at least 50 days.
32. The method of claim 28, wherein the immunological memory includes persistence anti-glioma immune effects over a period of at least 100 days.
33. The method of claim 28, wherein the glioma is located in the brain or spine of the subject.
34. The method of claim 28, wherein the nanoparticles encapsulate the single or double-stranded miR-124 mimic.
35. The method of claim 28, wherein the nanoparticles comprise one or more cationic lipids and one or more helper lipids.
36. The method of claim 28, wherein the nanoparticles comprise compound ATX-002.
37. A composition comprising miR-124 encapsulated in lipid nanoparticles, wherein the lipid nanoparticles comprise an ionizable amino lipid compound, 1,2-dioctadecanoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and 1,2-Dimyristoyl-sn-glycerol (DMG-PEG, methoxypolyethylene glycol, PEG chain molecular weight 2000).
38. The composition of claim 37, wherein the miR-124 contains a non-natural, modified, or chemically-modified nucleotide.
39. The composition of claim 37, wherein the ionizable amino lipid compound is from 50-65 mol% of the nanoparticles.
40. The composition of claim 37, wherein the ionizable amino lipid compound is any one of ATX-001 to ATX-032.
41. The composition of claim 37, wherein the ionizable amino lipid compound is ATX-002.
42. The composition of claim 37, wherein the 1,2-dioctadecanoyl-sn-glycero-3-phosphocholine is from 5-10 mol% of the nanoparticles.
43. The composition of claim 37, wherein the cholesterol is from 28-38 mol% of the nanoparticles.
44. The composition of claim 37, wherein the 1,2-Dimyristoyl-sn-glycerol is from 0.5-4 mol% of the nanoparticles.

45. The composition of claim 37, wherein the lipid nanoparticles comprise 58% mol% of ATX-002, 7 mol% of 1,2-dioctadecanoyl-sn-glycero-3-phosphocholine (DSPC), 33.5 mol% cholesterol, and 1.5 mol% 1,2-Dimyristoyl-sn-glycerol (DMG-PEG, methoxypolyethylene glycol, PEG chain molecular weight 2000).

FIG. 1

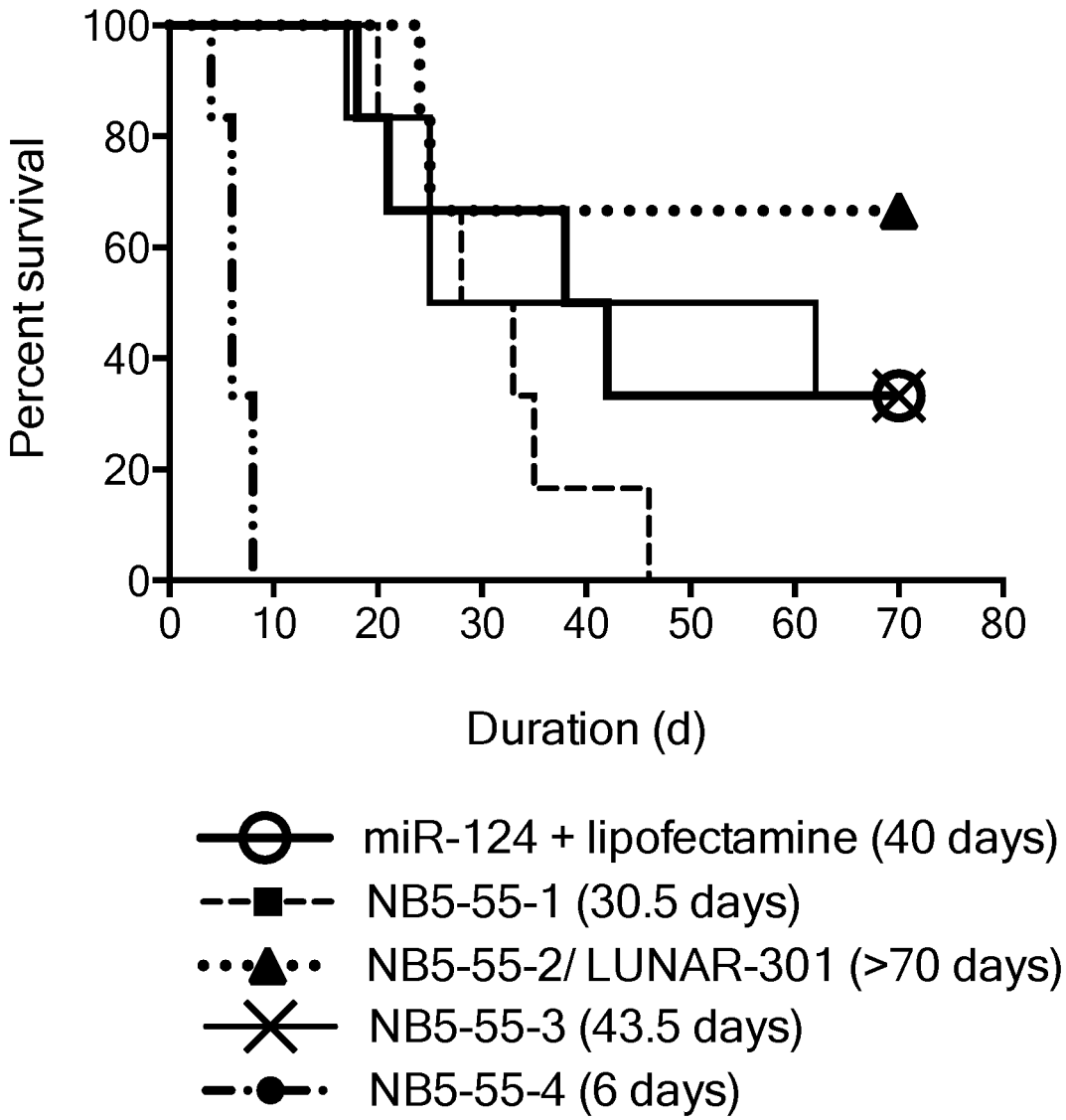

100
80
60
40
20
0
Percent survival
0
10
20
30
40
50
60
70
80
Duration (d)
miR-124 + lipofectamine (40 days)
NB5-55-1 (30.5 days)
NB5-55-2/ LUNAR-301 (>70 days)
NB5-55-3 (43.5 days)
NB5-55-4 (6 days)

FIG. 2

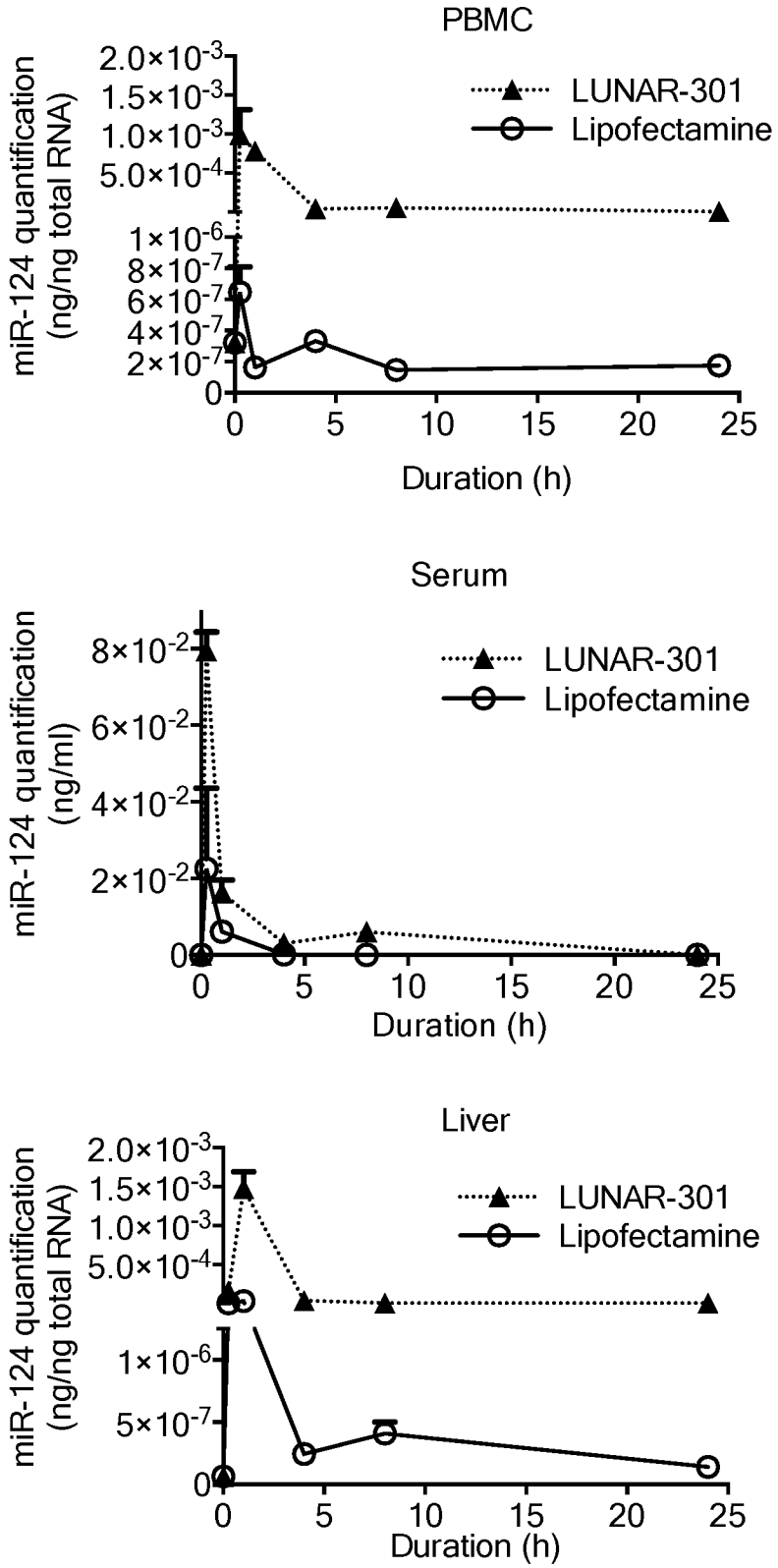

PBMC
miR-124 quantification (ng/ng total RNA)
2.0×10-3
1.5×10-3
1.0×10-3
5.0×10-4
1×10-6
8×10-7
6×10-7
4×10-7
2×10-7
0
0 5 10 15 20 25
Duration (h)
LUNAR-301
Lipofectamine
Serum
miR-124 quantification (ng/ml)
8×10-2
6×10-2
4×10-2
2×10-2
0
0 5 10 15 20 25
Duration (h)
LUNAR-301
Lipofectamine
Liver
miR-124 quantification (ng/ng total RNA)
2.0×10-3
1.5×10-3
1.0×10-3
5.0×10-4
1×10-6
5×10-7
0
0 5 10 15 20 25
Duration (h)
LUNAR-301
Lipofectamine

FIG. 3

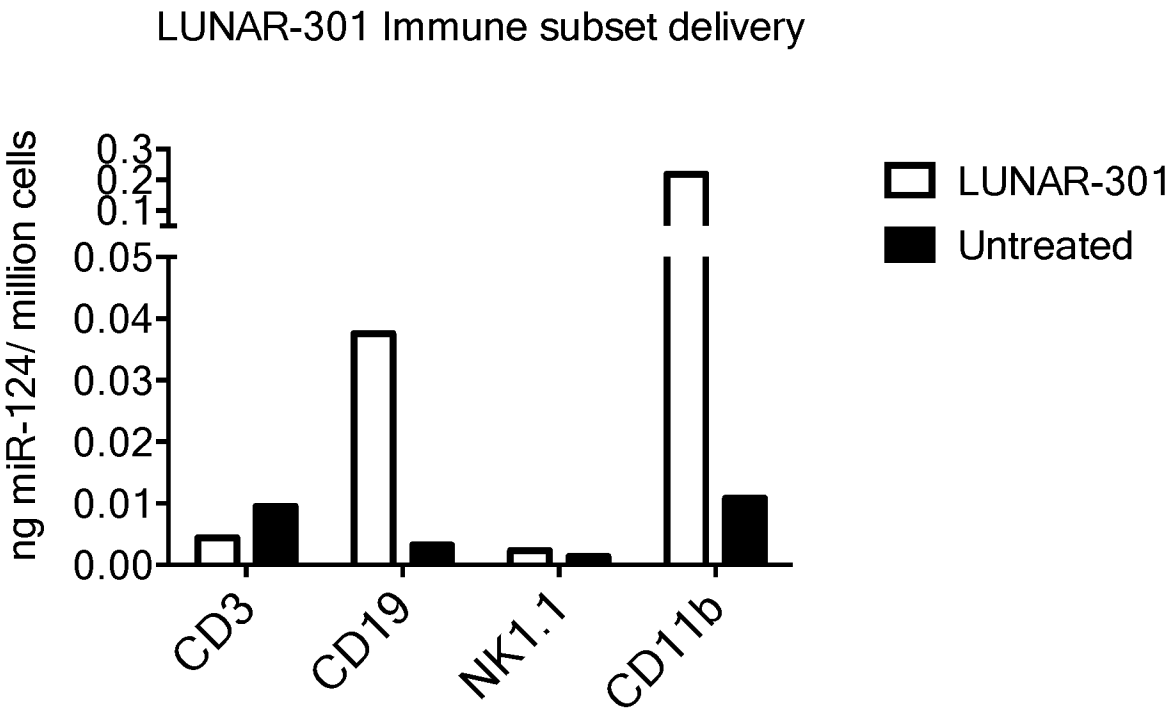

LUNAR-301 Immune subset delivery
ng miR-124/ million cells
0.3
0.2
0.1
0.05
0.04
0.03
0.02
0.01
0.00
CD3
CD19
NK1.1
CD11b
LUNAR-301
Untreated

FIG. 4

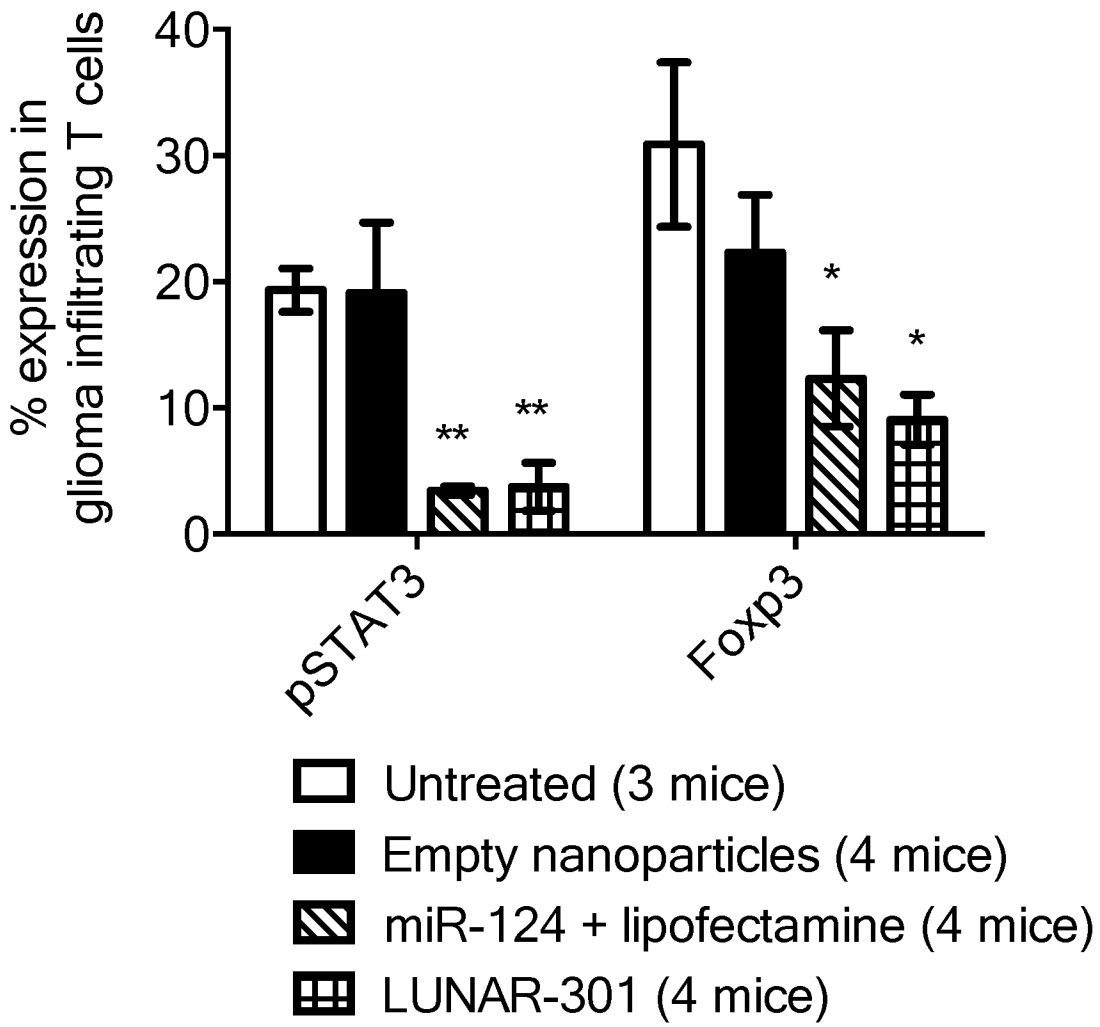

% expression in glioma infiltrating T cells
40
30
20
10
0
**
**
*
*
pSTAT3
Foxp3
Untreated (3 mice)
Empty nanoparticles (4 mice)
miR-124 + lipofectamine (4 mice)
LUNAR-301 (4 mice)

FIG. 5

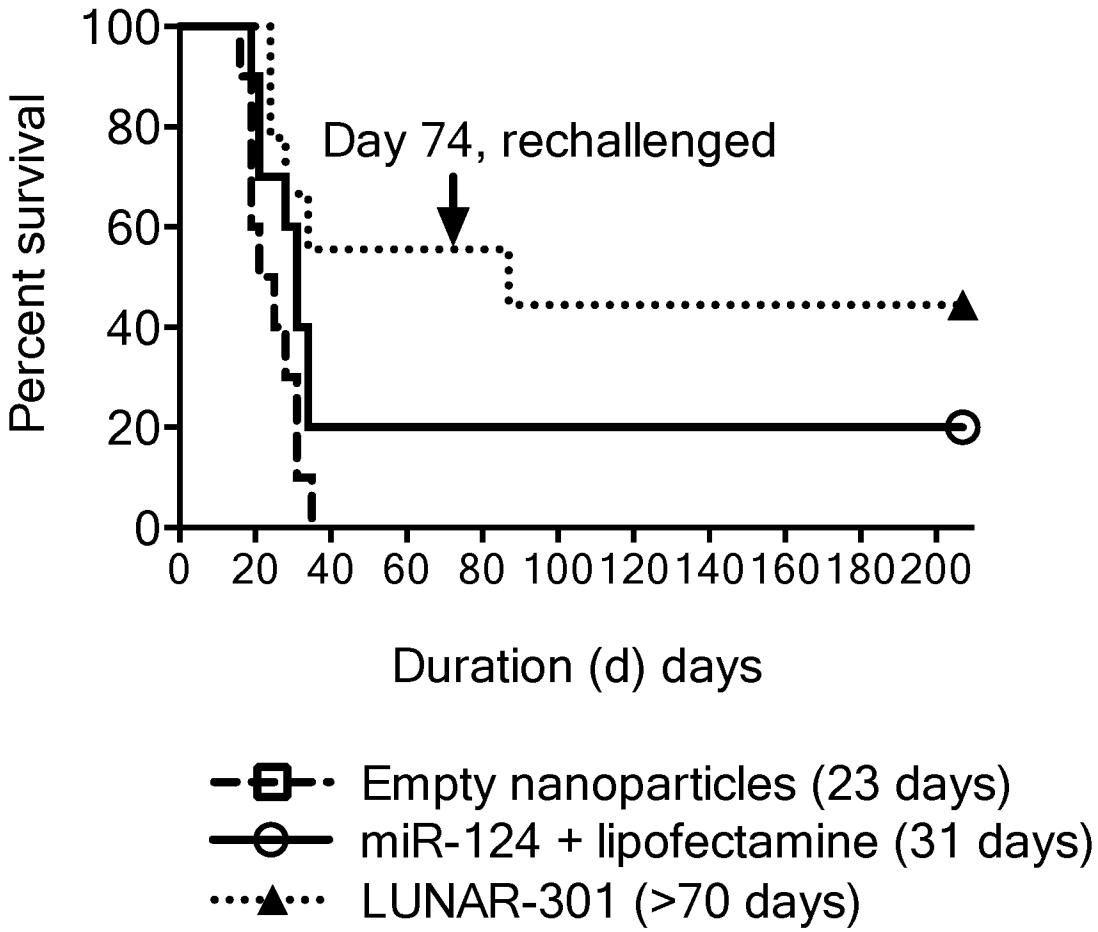

100
80
60
40
20
0
Percent survival
Day 74, rechallenged
0 20 40 60 80 100 120 140 160 180 200
Duration (d) days
Empty nanoparticles (23 days)
miR-124 + lipofectamine (31 days)
LUNAR-301 (>70 days)

FIG. 6

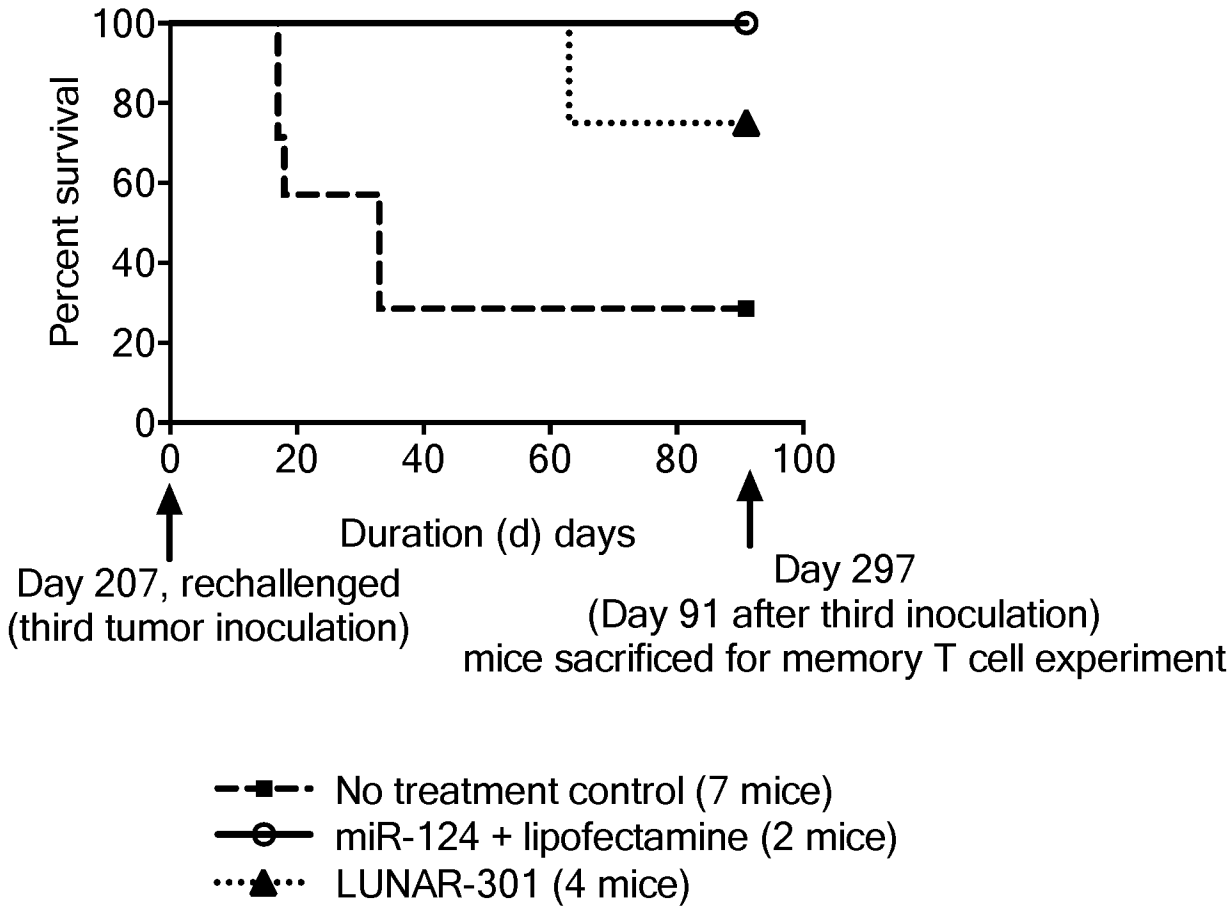

100
80
60
40
20
0
Percent survival
0
20
40
60
80
100
Duration (d) days
Day 207, rechallenged
(third tumor inoculation)
Day 297
(Day 91 after third inoculation)
mice sacrificed for memory T cell experiment
No treatment control (7 mice)
miR-124 + lipofectamine (2 mice)
LUNAR-301 (4 mice)

FIG. 7

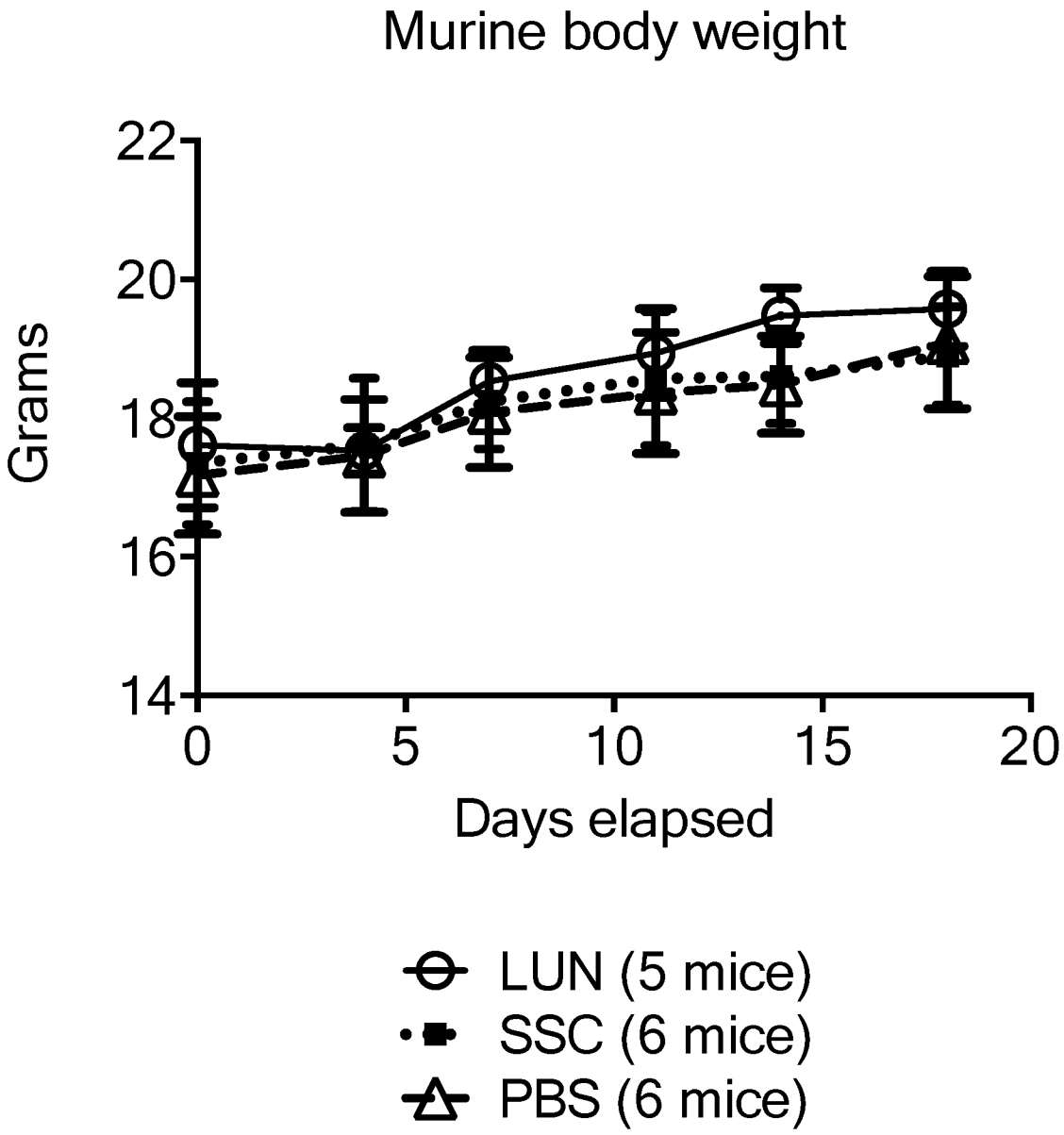

Murine body weight
Grams
22
20
18
16
14
0
5
10
15
20
Days elapsed
LUN (5 mice)
SSC (6 mice)
PBS (6 mice)

FIG. 8

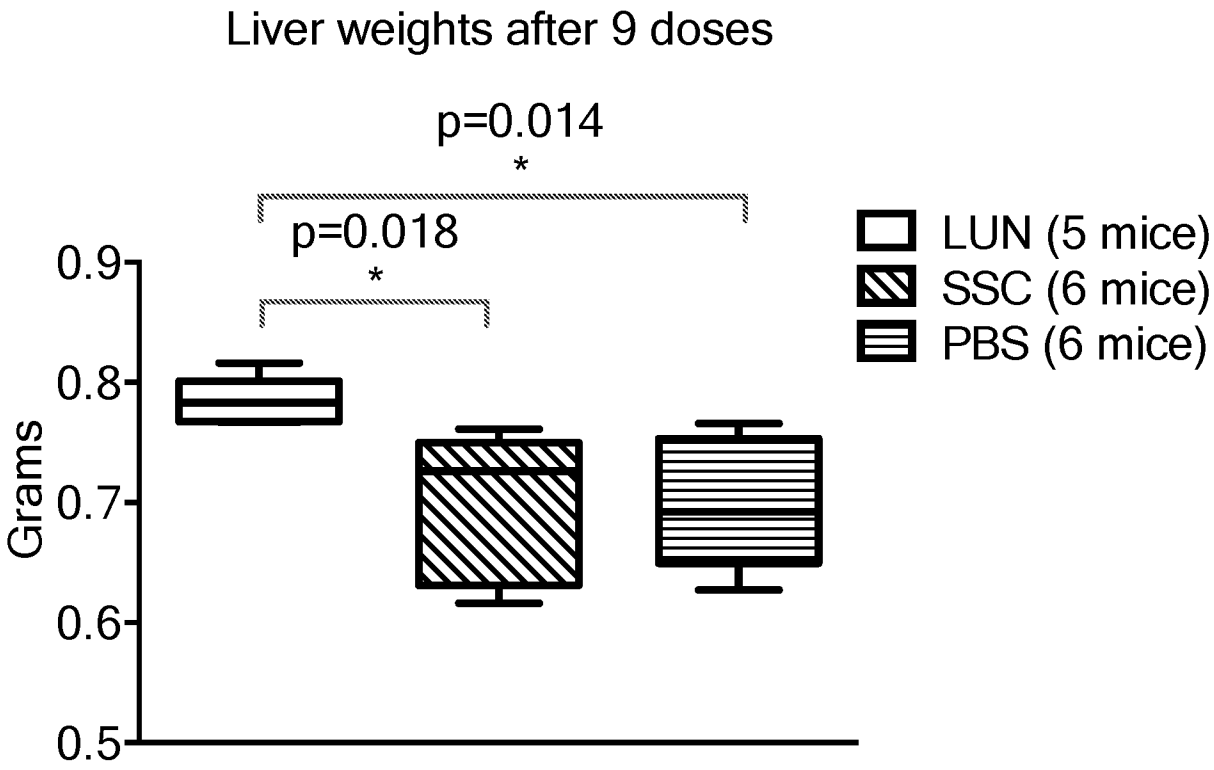

Liver weights after 9 doses
p=0.014
*
p=0.018
*
0.9
0.8
0.7
0.6
0.5
Grams
LUN (5 mice)
SSC (6 mice)
PBS (6 mice)

FIG. 9

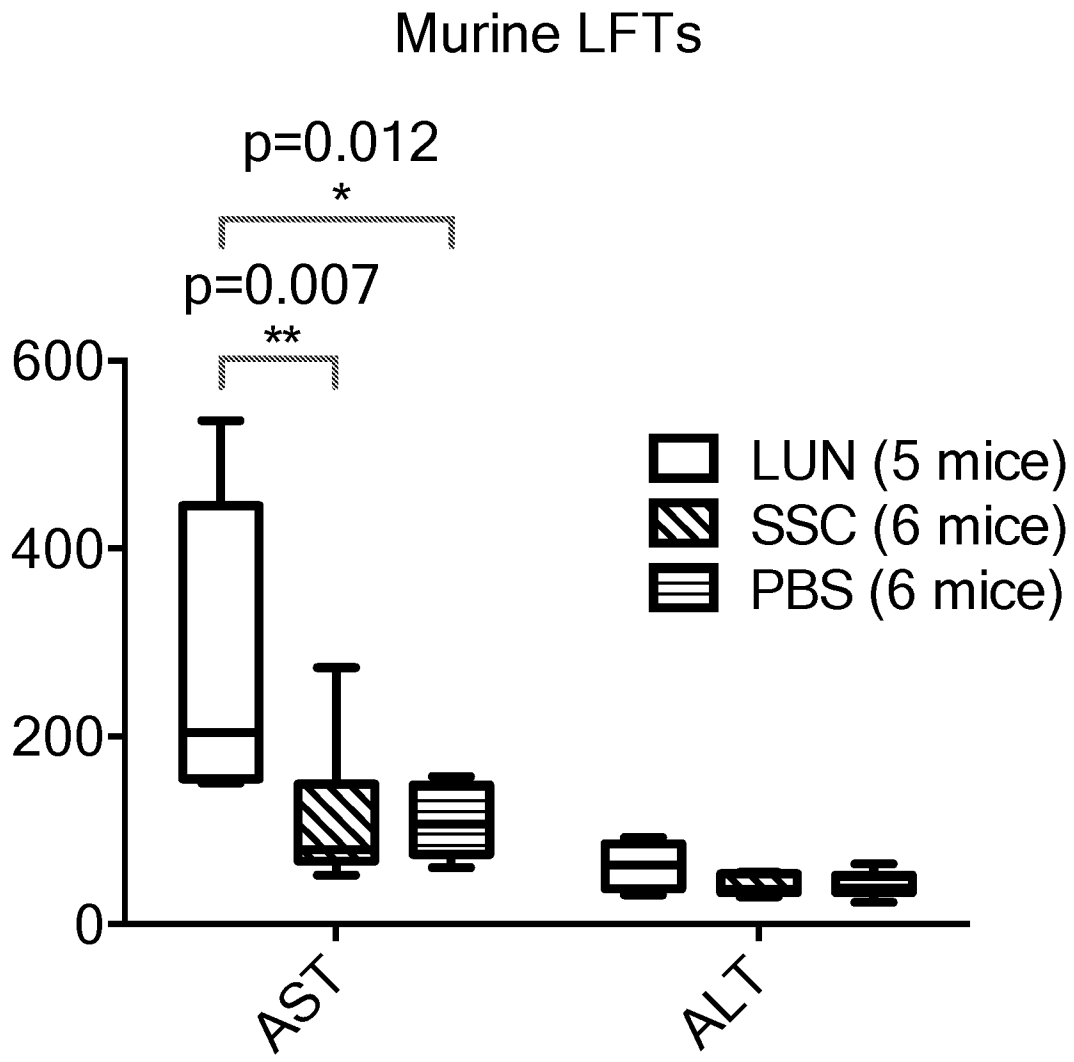

Murine LFTs
p=0.012
*
p=0.007
**
600
400
200
0
LUN (5 mice)
SSC (6 mice)
PBS (6 mice)
AST
ALT

FIG. 10

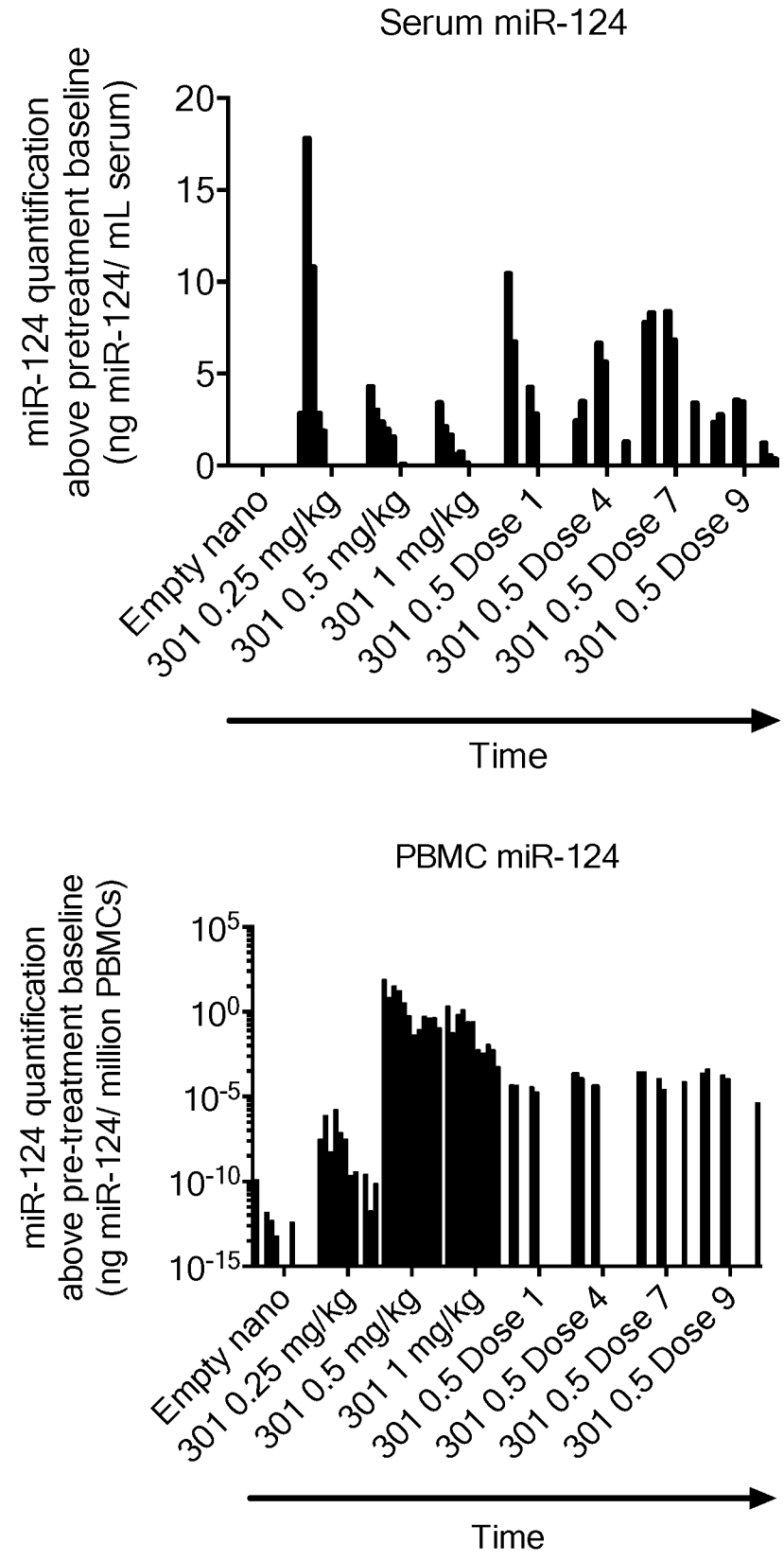

Serum miR-124
miR-124 quantification above pretreatment baseline (ng miR-124/ mL serum)
20
15
10
5
0
Empty nano
301 0.25 mg/kg
301 0.5 mg/kg
301 1 mg/kg
301 0.5 Dose 1
301 0.5 Dose 4
301 0.5 Dose 7
301 0.5 Dose 9
Time
PBMC miR-124
miR-124 quantification above pre-treatment baseline (ng miR-124/ million PBMCs)
10^5
10^0
10^-5
10^-10
10^-15
Empty nano
301 0.25 mg/kg
301 0.5 mg/kg
301 1 mg/kg
301 0.5 Dose 1
301 0.5 Dose 4
301 0.5 Dose 7
301 0.5 Dose 9
Time

FIG. 11

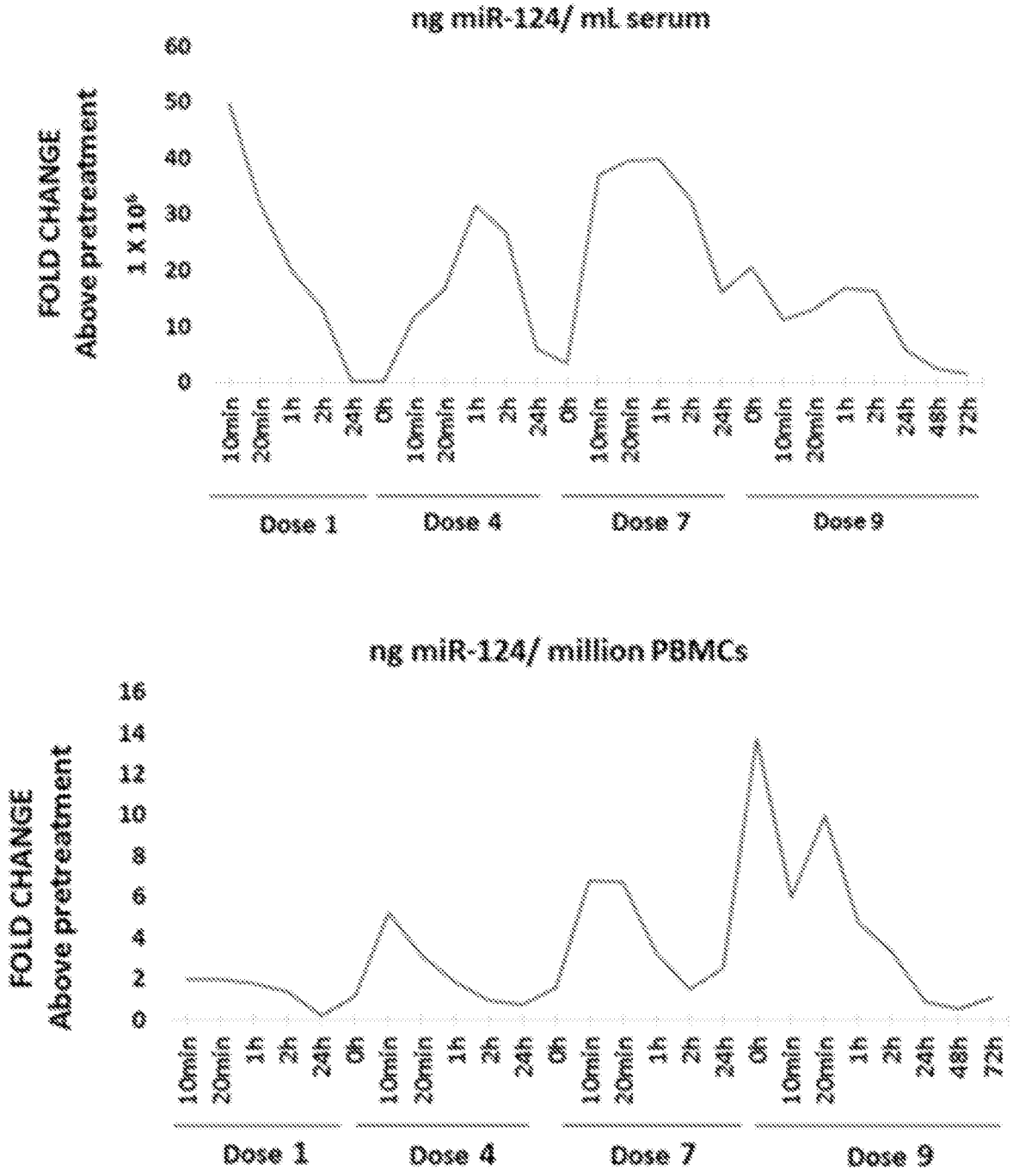

ng miR-124/ mL serum
FOLD CHANGE
Above pretreatment
1 X 10⁶
60
50
40
30
20
10
0
10min
20min
1h
2h
24h
0h
10min
20min
1h
2h
24h
0h
10min
20min
1h
2h
24h
0h
10min
20min
1h
2h
24h
48h
72h
Dose 1
Dose 4
Dose 7
Dose 9
ng miR-124/ million PBMCs
FOLD CHANGE
Above pretreatment
16
14
12
10
8
6
4
2
0
10min
20min
1h
2h
24h
0h
10min
20min
1h
2h
24h
0h
10min
20min
1h
2h
24h
0h
10min
20min
1h
2h
24h
48h
72h
Dose 1
Dose 4
Dose 7
Dose 9

FIG. 12

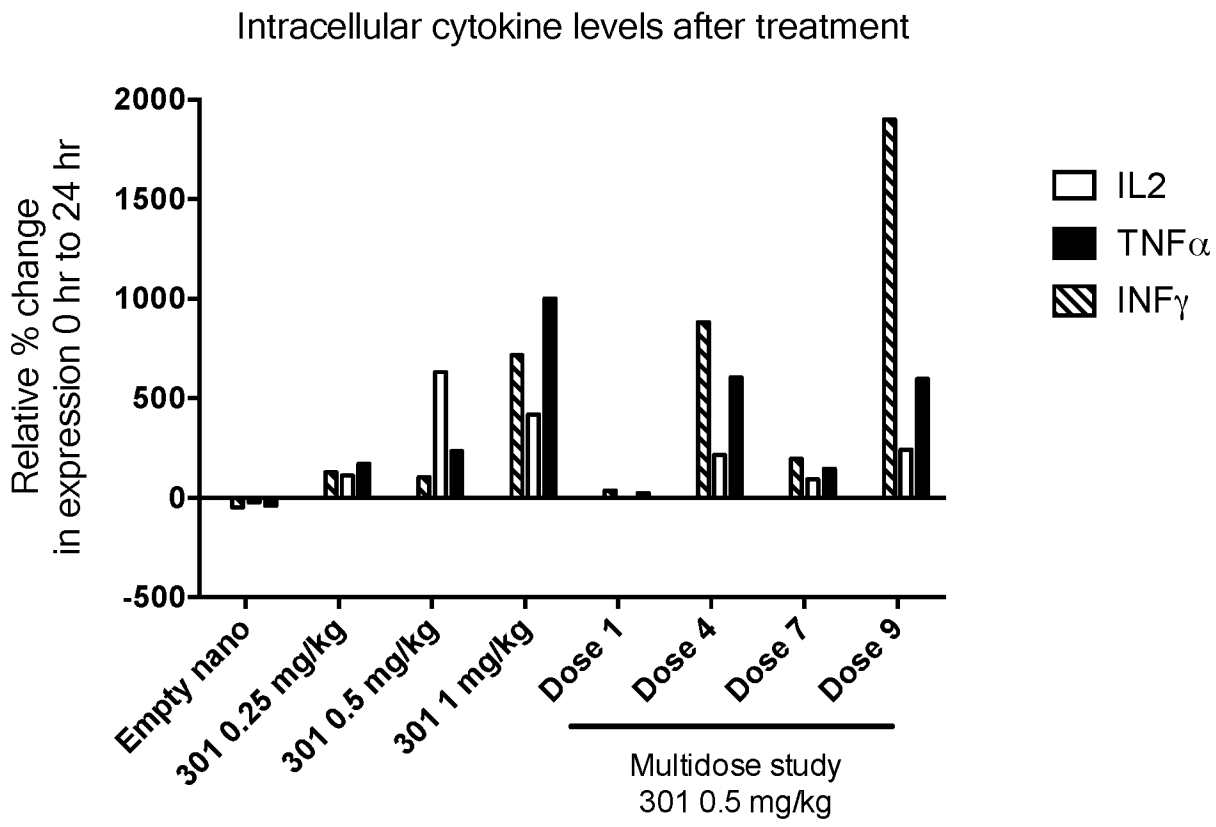

Intracellular cytokine levels after treatment
Relative % change in expression 0 hr to 24 hr
2000
1500
1000
500
0
-500
Empty nano
301 0.25 mg/kg
301 0.5 mg/kg
301 1 mg/kg
Dose 1
Dose 4
Dose 7
Dose 9
Multidose study
301 0.5 mg/kg
IL2
TNFα
INFγ

FIG. 13

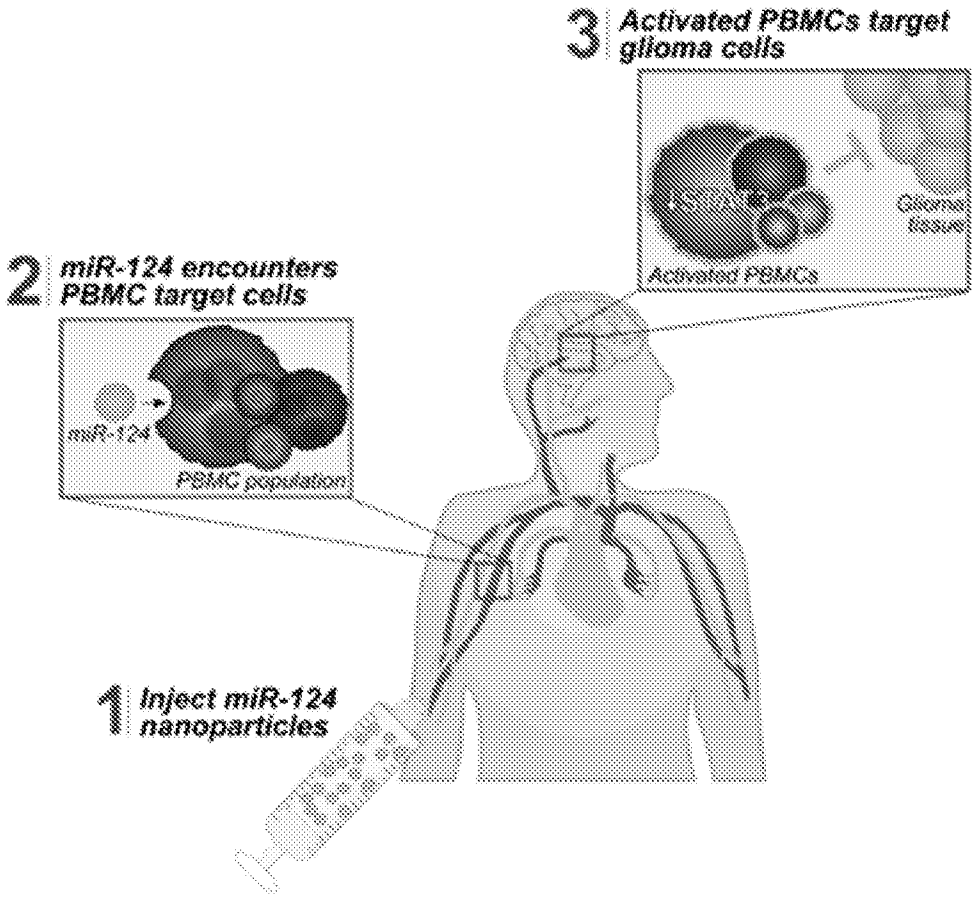

3 Activated PBMCs target glioma cells
Glioma tissue
Activated PBMCs
2 miR-124 encounters PBMC target cells
miR-124
PBMC population
1 Inject miR-124 nanoparticles

FIG. 14

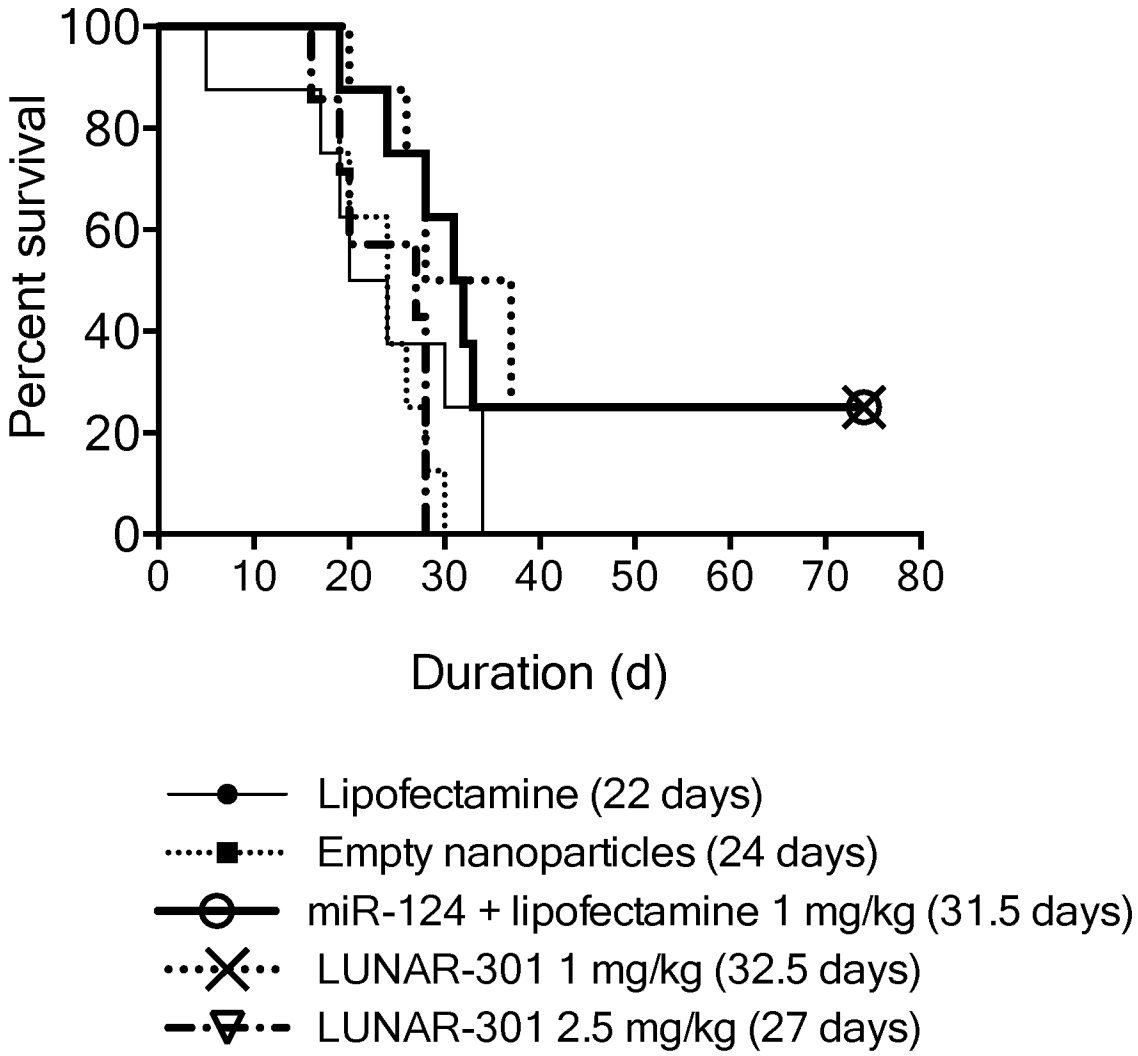

Percent survival
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80
Duration (d)
Lipofectamine (22 days)
Empty nanoparticles (24 days)
miR-124 + lipofectamine 1 mg/kg (31.5 days)
LUNAR-301 1 mg/kg (32.5 days)
LUNAR-301 2.5 mg/kg (27 days)

FIG. 15

Table 1

Parameters		PBMCs		Serum		Liver	
		Lunar-301	Lipofectamine	Lunar-301	Lipofectamine	Lunar-301	Lipofectamine
C_{max}	*	4.6×10^{-7}	4.0×10^{-10}	4.8×10^{-9}	1.3×10^{-9}	6.0×10^{-7}	2.6×10^{-8}
T_{max}	h	0.75	0.25	0.25	0.5	1	1
λz	1/h	0.17	0.01	0.33	0.32	0.03	0.02
$t_{½\lambda z}$	h	4.1	77	2.1	2.2	22.6	383
AUC_{last}	**	1.4×10^{-6}	3.3×10^{-9}	7.1×10^{-9}	2.2×10^{-9}	1.2×10^{-6}	5.3×10^{-8}
AUC_{INF_obs}	**	1.4×10^{-6}	1.7×10^{-8}	7.1×10^{-9}	2.2×10^{-9}	1.4×10^{-6}	8.0×10^{-8}
AUC % Extrap	%	0.5	76.2	0.019	0.008	8.7	29.9
$AUMC_{last}$	***	3.9×10^{-6}	3.7×10^{-8}	2.2×10^{-8}	4.3×10^{-9}	2.1×10^{-6}	7.3×10^{-8}
$AUMC_{INF\_obs}$	***	4.1×10^{-6}	2.5×10^{-6}	2.2×10^{-8}	4.3×10^{-9}	8.8×10^{-6}	3.3×10^{-5}
MRT_{last}	h	2.8	11.1	3.1	2	1.7	1.4
MRT_{INF_obs}	h	2.9	111.2	3.1	2	6.6	316.4

*Units are ng/million cells for PBMCs, ng/ml for serum, and ng/mg for liver.

**Units are h*ng/million cells for PBMCs, h*ng/ml for serum, and h*ng/mg for liver.

***Units are h*h*ng/million cells for PBMCs, h*h*ng/ml for serum, and h*h*ng/mg for liver.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/043607

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 9/127; A61K 31/7088; A61K 31/7105; A61P 35/00; C12N 15/88 (2016.01)
CPC - A61K 9/127; A61K 9/1272; A61K 31/7088; A61K 31/7105; C12N 15/113; C12N 15/88 (2016.08)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC - A61K 9/127; A61K 31/7088; A61K 31/7105; A61P 35/00; C12N 15/88
CPC - A61K 9/127; A61K 9/1272; A61K 31/7088; A61K 31/7105; C12N 15/113; C12N 15/88; C12Q 2600/178

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 514/44A; 514/44R; 424/450; 536/24.5 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Patents, Google Scholar.
Search terms used: miRNA-124, dimyristoyl, glycerol, polyethylene, glycol, PEG, DMG, DSPC, amino, cationic, ionizable, lipid, nanoparticle, glioma, glioblastoma, macrophage, monocyte, B cell

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2014/152932 A1 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 25 September 2014 (25.09.2014) entire document	1-7, 9-12, 14-20, 23-26 --- 8, 13, 21, 22, 27-36
X -- Y	US 2013/0156845 A1 (MANOHARAN et al) 20 June 2013 (20.06.2013) entire document	37-39, 42-44 --- 40, 41, 45
Y	WO 2015/074085 A1 (ARCTURUS THERAPEUTICS, INC.) 21 May 2015 (21.05.2015) entire document	8, 13, 27, 36, 40, 41, 45
Y	HUSSAIN et al. "The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses," Neuro Oncol. 14 June 2006 (14.06.2006), Vol. 8, No. 3, Pgs. 261-79. entire document	21, 22
Y	YIN et al. "Interleukin 7 up-regulates CD95 protein on CD4+ T cells by affecting mRNA alternative splicing: priming for a synergistic effect on HIV-1 reservoir maintenance," J Biol Chem. 19 November 2014 (19.11.2014), Vol. 290, No. 1, Pgs. 35-45. entire document	28-36
Y	MUHAMMAD et al. "Study of the efficacy, biodistribution, and safety profile of therapeutic gutless adenovirus vectors as a prelude to a phase I clinical trial for glioblastoma," Clin Pharmacol Ther, 17 February 2010 (17.02.2010), Vol. 88, No. 2, Pgs. 204-13. entire document	29-32
A	WEI et al. "miR-124 inhibits STAT3 signaling to enhance T cell-mediated immune clearance of glioma," Cancer Res. 01 May 2013 (01.05.2013), Vol. 73, No. 13, Pgs. 3913-26. entire document	1-45

☐ 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
"E" earlier application or patent but published on or after the international filing date
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"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
21 September 2016	20 OCT 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774