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Intratumoral delivery of IL-12 and IL-27 mRNA using lipid nanoparticles for cancer immunotherapy

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Abstract

Cytokines are important immunotherapeutics with approved drugs for the treatment of human cancers. However, systemic administration of cytokines often fails to achieve adequate concentrations to immune cells in tumors due to dose-limiting toxicity. Thus, developing

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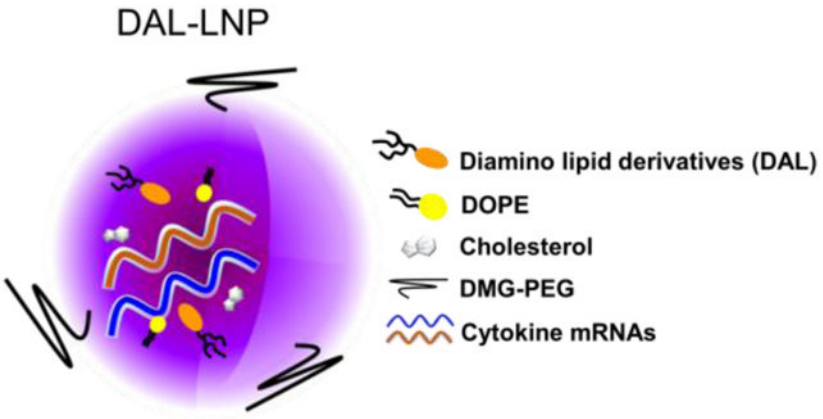
localized therapy that directly delivers immune-stimulatory cytokines to tumors may improve the therapeutic efficacy. In this study, we generated novel lipid nanoparticles (LNPs) encapsulated with mRNAs encoding cytokines including IL-12, IL-27 and GM-CSF, and tested their anti-tumor activity. We first synthesized ionizable lipid materials containing di-amino groups with various head groups (DALs). The novel DAL4-LNP effectively delivered different mRNAs *in vitro* to tumor cells and *in vivo* to tumors. Intratumoral injection of DAL4-LNP loaded with IL-12 mRNA was most potent in inhibiting B16F10 melanoma tumor growth compared to IL-27 or GM-CSF mRNAs in monotherapy. Furthermore, intratumoral injection of dual DAL4-LNP-IL-12 mRNA and IL-27 mRNA showed a synergistic effect in suppressing tumor growth without causing systematic toxicity. Most importantly, intratumoral delivery of IL-12 and IL-27 mRNAs induced robust infiltration of immune effector cells, including IFN-γ and TNF-α producing NK and $CD8^+$ T cells into tumors. Thus, intratumoral administration of DAL-LNP loaded with IL-12 and IL-27 mRNA provides a new treatment strategy for cancer.

Graphical Abstract



Keywords

Diamino lipid-derived nanoparticles (DAL-LNPs); cytokines; mRNA therapeutics; cancer immunotherapy

Introduction

Cytokines are one of the first immunotherapeutics applied for treating human cancers. In 1986, IFN-α was first approved by the FDA for the treatment of hairy cell leukemia[1]. IL-2 was later approved for metastatic renal cell cancer and advanced melanoma in 1992 and 1998, respectively[2]. Currently, many cytokines are explored in clinical trials, including IL-12, IL-15, IL-21, and GM-CSF[3]. Despite efforts to develop systemic cytokine monotherapy for cancers, the delivery of cytokines hinders the initial excitement due to a narrow therapeutic margin. Cytokines act in a paracrine or autocrine fashion and have a relatively short half-life. Therefore, large quantities of cytokines must be systemically administered to achieve a sufficient concentration within the tumor microenvironment (TME), which is often associated with severe toxicities[4–6]. Due to dose-limiting toxicities,

lower doses of cytokines are often used in clinical trials, which result in low concentrations of the cytokines in the TME. Thus, developing localized therapy that directly delivers immune-stimulatory cytokines to tumors may be a promising strategy to overcome this dilemma.

IL-12, IL-27, and GM-CSF have shown dynamic anti-tumor activities in various animal models. IL-12 demonstrates potent anti-tumor activity[7] via enhanced Th1/Tc1 response[7, 8] and T cell recruitment to tumors[9]. However, systemically delivered recombinant IL-12 can lead to fatal consequences[5, 6]. IL-12-induced systemic toxicity can only be relieved if IL-12 expression is confined to the tumor site[17]. To avoid systemic toxicity, researchers have designed various strategies[10–16] to target or restrict IL-12 to tumors. IL-27 is also an anti-inflammatory cytokine that exhibits robust anti-tumor effects. Preclinical models in mice have indicated that both endogenous[18–21] and exogenous[22–24] IL-27 inhibits tumor growth *in vivo*. IL-27 enhances the T cell survival in TME and promotes the generation of memory T cells by programming $CD8^+$ T cells into a unique T effector phenotype, characterized by increased secretion of IFN-γ and IL-10[22, 25]. Likewise, prior studies found that mice inoculated with live or irradiated tumor cells expressing high levels of GM-CSF (GVAX) resulted in the recruitment of antigen-presenting cells such as dendritic cells in tumor sites, which led to induction of anti-tumor immunity and tumor rejection[26, 27]. The efficacy of autologous and allogeneic GVAX was later reported either as a single drug or in combination with other immunomodulators in various animal models and clinical studies[28–30]. GM-CSF has also been included in two clinically approved anti-tumor vaccines, i.e. Sipuleucel-T[31] and T-VEC[32]. Systemically delivered GM-CSF has shown potential clinical benefits including reduced toxicity when used in combination with ipilimumab[33, 34 35].

Potent induction of anti-tumor immunity impeded by systemic toxicity of cytokines strongly justifies the development of clinically relevant, cytokine-based localized therapy. Recently, we found that intratumoral delivery of AAV-IL-27 could repress tumor growth and induce anti-tumor immunity[36]. However, AAV-mediated IL-27 delivery poses potential pitfalls as there is no termination of IL-27 production when the biological activity of IL-27 is sufficient. Therefore, novel approaches that can adeptly deliver immunostimulatory cytokines to tumors are necessary. In this context, we consider that using lipid nanoparticles to deliver mRNAs of immune stimuli to TME is a highly feasible approach. LNPs possess unique features including (i) easy preparation and chemical synthesis for large-scale production; (ii) efficient encapsulation and delivery of mRNA; (iii) transient, mRNA-induced expression of protein; and (iv) low potential systemic toxicity via intratumoral administration.

In this study, we examined the local administration of LNPs encapsulating cytokine mRNA combinations that have not yet been tested in a mouse melanoma model. Intratumoral administration of IL-12 and IL-27 mRNAs by DAL-LNP promoted sustained inhibition of B16F10 melanoma growth without causing significant toxicity. Mechanistically, robust infiltration of immune effector cells, including NK and $CD8^+$ T cells, into tumor tissues was observed after intratumoral delivery of IL-12 and IL-27 mRNA. In summary, we discovered a new mRNA delivery LNPs formulation and a cytokine combination that can be used to

expand current cancer treatments. Such delivery platform merits further development as a novel cancer therapeutic.

Materials and Methods

Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted. DOPE was purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA) and DMG-PEG_{2000} was purchased from NOF America Corporation (White Plains, NY, USA).

Synthesis of DALs

Compounds **b**, **c** and **DAL1** were synthesized according to methods reported previously[37].

Synthesis of **DAL2**: **DAL1** (250 mg, 0.35 mmol) was first hydrolyzed by NaOH aqueous (1 M) in a mixture of THF and MeOH at 70 °C for 3 hrs. Next, 100 mL CH_2Cl_2 was added, which was dried with $MgSO_4$. Then, the solvent was evaporated and 10 mL anhydrous THF was added to the residue. NHS (125 mg, 1.05 mmole) and DCC (230 mg, 1.05 mmole) were added to the solution. The resulting mixture was stirred at room temperature overnight. Tert-butylamine (80 mg, 1.05 mmole) and TEA (150 μL, 1.2 mmole) were added to the above reaction mixture. The resulting mixture was stirred at room temperature overnight. After the solvent was removed under reduced temperature, the residue was purified by column chromatography using a CombiFlash Rf system with a RediSep Gold Resolution silica column (Teledyne Isco) with gradient elution (CH_2Cl_2 and ultra) from 100% CH_2Cl_2 to 80% CH_2Cl_2 (ultra, CH_2Cl_2/MeOH/NH_4OH =75/22/3 by volume) to generate 96 mg of oil like **DAL2**. Using different head groups, **DAL3-7** were synthesized following the same procedure as used for the synthesis of **DAL2**. The amine used for synthesizing **DAL4** was 5-Fluoro-2-aminomethylphenylboronic acid, pinacol ester. The pinacol ester was hydrolyzed during purification through column chromatography to yield pure **DAL4**. The ^{1}H NMR spectra of DAL2–7 are provided in Supplementary Fig. 1.

mRNA synthesis

IL-12, IL-27, and GM-CSF plasmids were purchased from InvivoGen (San Diego, CA, USA) and were amplified to generate templates for *in vitro* transcription. DNA sequences of the cytokines used in this study are listed in supplementary data. mRNA transcripts were synthesized as reported previously[37, 38]. mRNAs were synthesized with full substitution of UTP by pseudouridine-5'-triphosphate (TriLink, USA) using AmpliScribe T7-Flash Transcription Kit (Lucigen, USA) following the manufacturer's instruction. The resulting mRNAs were then purified by RNA Clean & Concentrator (Zymo, USA) and capped using Vaccinia Capping System (NEB, USA) and Cap 2´-O-Methyltransferase (NEB, USA). After one final round of purification, mRNA concentrations were measured using a NanoDrop 2000 Spectrophotometer (ThermoFisher, USA) and stored at −80°C for future use.

Preparation and characterization of LNPs-mRNA

LNPs-mRNA were prepared by mixing lipid materials dissolved in ethanol and mRNA diluted in 10 mM citrate buffer, pH=3[39]. The molar ratio of MC3 LNPs was MC3: DSPC:

Cholesterol: DMG-PEG=50:10:38.5:1.5. For DAL-LNPs, the molar ratio was DALs: DOPE: Cholesterol: DMG-PEG=20:30:40:0.75. LNPs-mRNA were prepared by rapidly blending the aqueous and ethanol phases with a pipette for *in vitro* studies. LNPs-mRNA were prepared by a microfluidic device for *in vivo* studies (Precision NanoSystems, Vancouver, BC, Canada) and dialyzed in PBS (Slide-A-Lyzer™ Dialysis Cassettes, 3.5K MWCO, ThermoFisher, USA). The parameters of microfluidic device: flow rate: total 12mL/min, aqueous phase/ethanol phase = 3/1, room temperature. The size and zeta potential of DAL-LNPs were measured by Zetasizer (Malvern, USA). The mRNA encapsulation efficiency (EE%) was determined by the RiboGreen assay (Invitrogen, USA). Cryo-EM image of DAL4-LNP-IL-12+IL-27 was obtained by Glacios Cryo Transmission Electron Microscope (ThermoFisher, USA) using a similar method reported before[37].

In vitro delivery of cytokine mRNAs LNP to B16F10 melanoma cells

B16F10 cells were originally purchased from ATCC and were maintained in RPMI 1640 Medium (Corning) with fetal bovine serum 10% (v/v). To quantify the delivery efficacy and production of mRNA encoded cytokines in MC3-LNP or DAL4-LNP, we treated the cultured B16F10 cells in a 96 well plate with MC3-IL-12/IL-27/GM-CSF, or DAL4-LNP-IL-12/IL-27/GM-CSF with a dose of 50 ng mRNA of each cytokine mRNA. After 18 hrs incubation, the supernatants of cell cultures were collected, and respective cytokines were quantified by ELISA following standard procedures.

In vivo anti-cancer efficacy of cytokine mRNA LNPs

All animal experiments were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of The Ohio State University and were approved by the Animal Ethics Committee of Institutional Animal Care and Use Committee (IACUC). To establish the mice tumor model, 1×10^5 B16F10 cells in 100 μL PBS were subcutaneously injected in female C57BL/6 mice. Mice were randomly grouped (n = 5–7 in each group) when the tumor reached about 50 mm^3. Tumor volume is calculated by length × $(width)^2/2$. For the biodistribution study, intratumoral injection of GFP mRNA in DAL4-LNP (10 μg mRNA/injection) was performed one-time. GFP expression in the tumor microenvironment was analyzed at 24 hrs. For treatment efficacy studies, intratumoral injections of cytokine mRNA LNPs (2 μg mRNA/injection in Fig. 4; 2 μg IL-12 mRNA/injection or 6 μg IL-27 mRNA/injection in Supplementary Fig. 2) were performed every other day for six doses in total. For toxicity studies, intratumoral treatment with cytokine mRNA LNPs (2 μg mRNA/injection) was performed every other day for three times in total. The tumor size was checked every other day. The body weight was checked on days 8, 12, and 14 after tumor inoculation. Paraffin-embedded tissues (heart, lung, liver and kidney) were prepared from DAL4-LNP-IL-12 + IL-27 mRNA treated mice (n=5) or control-treated mice (n=5). Hematoxylin and eosin (H.E.) stained slides were evaluated under a microscope. Representative images were photographed and are provided in Supplementary Fig 4.

Antibodies and flow cytometry

Fluorescence labeled monoclonal antibodies to mouse CD45 (30-F11), CD11b (M1/70), F4/80 (745–2342), NK1.1 (Pk136), CD3 (145–2c11), CD4 (GK1.5), CD8α (53–6.7), CD19 (1D3), IFN-γ (XMG1.2), TNF-α (XT22), and isotype control antibodies were purchased

from BD Biosciences or Biolegend. Mononuclear cells from tumors were prepared as described[40, 41]. For staining cell surface markers, cells were incubated with antibodies in staining buffer (0.1 M PBS, 1% FCS and 0.1% sodium azide) on ice for 30 minutes. Cells were washed three times after staining and fixed in 1% paraformaldehyde. For intracellular staining of IFN-γ and TNF-α, cells were first stimulated with cell stimulation cocktail (Invitrogen, USA) for 4 hrs. The cells were then stained for the cell surface markers (CD3/4/8/NK1.1) followed by a standard intracellular cytokine staining procedure. Stained cells were harvested using a Celesta flow cytometer (BD) and data was analyzed using FlowJo software (Tree Star, Inc., OR).

Statistical analysis

Mann-Whitney test and one-way ANOVA with Dunnett's multiple comparisons were used to analyze *in vitro* data (GraphPad Prism, CA, USA); Two-way ANOVA with repeated measurements was used to analyze tumor volume and body weight data (R3.4.3, The R Foundation); the survival of tumor-bearing mice was analyzed using log-rank tests (GraphPad Prism). All tests were two-tailed and $P < 0.05$ was considered statistically significant.

Results

Synthesis of diamino lipid derivatives

We first designed and synthesized a library of seven di-amino lipid materials (DALs) with the same diamino core and carbon chains with different head groups (Fig. 1A). These DAL compounds provide beneficial characteristics. For example, various functional groups can be installed on the phenyl ring such as boronic acid, hydroxyl group, and halogen. These groups enable us to fine-tune several physicochemical properties such as size, hydrophobicity, and charge of the lipids and their corresponding nanoparticles. Fig. 1B displays a representative synthetic route. Compound **a** underwent a substitution reaction with **b** to yield intermediate **c**. Subsequently, a reductive amination reaction between **c** and **d** produced **DAL1**[37]. Removal of ethyl group on **DAL1** formed **e**, which was further reacted with 5-Fluoro-2-aminomethylphenylboronic acid, pinacol ester and hydrolyzed to synthesize **DAL4**. We confirmed the structures of all these DALs using both ^{1}H NMR and mass spectrum (MS) (Supplementary Data and Supplementary Fig. 1).

Formulations of DAL lipid nanoparticles (DAL-LNPs) for *in vitro* mRNA delivery and cytokine expressions

Next, we studied the mRNA delivery efficiency of DAL lipid nanoparticles (DAL-LNPs) using luciferase mRNA in B16F10 melanoma tumor cells *in vitro*. We formulated DALs with 1,2-dioleoylsnglycero-3-phosphoethanolamine (DOPE), cholesterol (Chol), 1,2-dimyristoyl-racglycero-3-methylpolyoxyethylene (DMG-PEG$_{2000}$, PEG) (molar ratio: DAL/DOPE/Chol/PEG = 20/30/40/0.75), and luciferase mRNA to prepare the nanoparticles (DAL-LNP-Luc) as described previously[37]. The mRNA encapsulation efficiency (Fig. 2A), formulation size distribution, (Fig. 2B) and polydispersity index (PDI) (Fig. 2B) were also determined. The mRNA encapsulation efficiency of DAL-LNPs ranged from 2.6% in DAL1-LNP-Luc to 90.3% in DAL4-LNP-Luc (Fig. 2A). These nanoparticles had a size

of 150–200 nm and PDI less than 0.2 as measured by dynamic light scattering (Fig. 2B). After treating the B16F10 cells with DAL-LNPs for 18 hours, the luciferase mRNA delivery efficiency was quantified by a bioluminescence reporter assay. As shown in Fig. 2C, DAL4-LNP induced the highest luminescence signal in cultured cells when compared with other DAL-LNPs and Lipofectamine™ 3000. DAL4-LNP displayed a spherical morphology when visualized by cryo-EM microscopy (Fig. 2D). Thus, we chose DAL4-LNP as a lead candidate to encapsulate cytokine mRNAs for further testing.

We investigated the cytokine mRNA delivery and protein expression *in vitro* by an ELISA assay. We treated B16F10 cells with DAL4-LNP encapsulating either IL-27 mRNA (DAL4-LNP-IL-27), IL-12 mRNA (DAL4-LNP-IL-12), or GM-CSF mRNA (DAL4-LNP-GM-CSF) for 18 hours and collected culture supernatants. The results (Fig. 2E–G) showed that each of the cytokine proteins can be expressed and secreted in the supernatants of cell culture, which was more efficient than MC3 formulated LNPs, an FDA approved LNP formulation. These results indicated that the DAL4-LNP can effectively deliver cytokine mRNA in cells.

DAL4-LNP can effectively deliver mRNA into tumors for protein expression

After selecting DAL4-LNP from *in vitro* assays, we tested if DAL4-LNP could be used to deliver mRNA to established tumors for protein expression *in vivo*. For this purpose, we generated DAL4-LNP loaded with mRNA encoding GFP and i.t. injected DAL4-LNP-GFP to established B16F10 tumors. 12 hours after injection of LNP-GFP mRNA, GFP^+ cells were readily detected in tumors, ranging from 3–15% of total tumor cells by flow cytometry (Fig. 3A). Among GFP^+ cells, approximately 66.7% were $CD45^-$ non-immune cells which are primarily tumor cells and fibroblasts, and 31.9% of cells were $CD45^+$ immune cells (Fig. 3B). Among GFP^+ immune cells, about 98% were $CD19^+$ B cells, and 2% were monocyte or macrophages, while GFP^+ T cells were barely detected (Fig. 3B). Thus, DAL4-LNP can serve as an efficient vehicle for mRNA delivery to tumors for protein expression.

Intratumoral delivery of LNP-IL-12/IL-27 inhibits tumor growth

Cytokines such as IL-12[7], IL-27[22–24] and GM-CSF[26, 27] have demonstrated anti-tumor activity when delivered systemically or locally. To compare the therapeutic potential of each cytokine mRNA in DAL4-LNP, we first probed the treatment efficacy in a subcutaneous B16F10 mouse tumor model by delivering a single cytokine mRNA with DAL4-LNP via intratumoral injection every other day for six doses. DAL4-LNP loaded with IL-12 mRNA (DAL4-LNP-IL-12) exhibited the strongest tumor inhibitive effect when compared to DAL4-LNP encapsulated with IL-27 mRNA (DAL4-LNP-IL-27) or GM-CSF mRNA (DAL4-LNP-GM-CSF) with slower tumor growth and prolonged survival (Fig. 4A–B).

Previous gene therapy based on the systemic application of IL-27 and IL-12 has shown significant synergy of these two cytokines in tumor inhibition[42]. To determine if intratumoral injections of cytokine mRNA nanoparticles could induce stronger tumor growth inhibition, we tested IL-12 mRNA in combination with IL-27 and/or GM-CSF mRNA in DAL4-LNP. DAL4-LNP-IL-12+IL-27 outperformed other combinations by retarding tumor growth (Fig. 4C) and extending the survival of tumor-bearing mice (Fig. 4D). Interestingly, the combination of all three cytokine mRNAs did not induce better tumor inhibition or

prolong survival compared to the IL-12+IL-27 combination. To further calibrate the dosage of DAL4-LNP-IL-12+IL-27, we changed the IL-27 mRNA dose from 2 μg to 6 μg per mouse for six doses while the IL-12 mRNA dose remained at 2 μg (Supplementary Fig. 2). We observed that the increased dosage of DAL4-LNP-IL-27 also showed significant tumor inhibition compared to the control group, and DAL4-LNP-IL-12+IL-27 resulted in 100% survival on day 35. To assess potential systemic toxicity, we comprehensively monitored body weight changes in addition to overall survival during the treatment process and analyzed histology of major organs after DAL4-LNP treatments. No significant body weight changes were observed as compared with control mice (Supplementary Fig. 3). Histology analysis of major organs (heart, lung, liver and kidney) from five treated mice demonstrated no observed inflammatory changes (Supplementary Fig. 4) as compared to control mice. Consequently, intratumoral delivery of DAL4-LNP-IL-12+IL-27 mRNA did not cause noticeable systematic toxicity within the dosages tested.

Intratumoral delivery of DAL4-LNP-IL-12 + IL-27 mRNA induces robust infiltration of immune effector cells into tumors

Both IL-12 and IL-27 have been shown to be potent cytokines capable of inducing T and NK cell responses in tumor models[7, 22–24]. We hypothesized that intratumoral injection of DAL4-LNP-IL-12 + IL-27 mRNA can induce potent immune responses that lead to the infiltration of immune effector cells into tumors. To test this hypothesis, we analyzed tumors from mice receiving the treatment regimen and examined the density of immune cells in tumors by flow cytometry. DAL4-LNP-IL-12 + IL-27 mRNA-treated tumors resulted in a nearly 10-fold increase of total $CD45^+$ leukocytes compared to control-treated tumors (Fig. 5A). The percentages of almost every subtype of leukocytes among the total tumor mononuclear cells, including $CD19^+$ B cells, $F4/80^+$ macrophages, $CD4^+$ T cells, $CD8^+$ T cells, and NK cells, increased when compared to the control group. Additionally, tumor-infiltrating T and NK cells from DAL4-LNP-IL-12 + IL-27 mRNA treated tumors significantly increased the number of IFN-γ (Fig. 5B) and TNF-α (Fig. 5C) producing cells. Thus, intratumoral delivery of DAL4-LNP-IL-12+IL-27 mRNA induced robust infiltration of immune effector cells into tumors.

Discussion

In this study, we tested the strategy of using novel lipid nanoparticles (LNPs) to deliver cytokine mRNAs to TME. Our results suggested that DAL4-LNP can efficiently deliver mRNAs both *in vitro* and *in vivo* for cytokine expressions. Additionally, intratumoral delivery of dual IL-12 + IL-27 mRNAs demonstrated the most potent inhibition of tumor growth, which was reflected by enhanced infiltration of immune effectors into the tumor vasculature and activation of effector cells to secrete downstream anti-tumor signaling molecules.

Based on the interesting chemical structures of diamines, we first synthesized seven new ionizable lipids containing di-amino groups and various head groups. Next, we formulated these ionizable lipids with phospholipid, cholesterol, and PEG-lipid to encapsulate luciferase mRNA or single or multiple cytokine mRNAs. The DAL-LNP-mRNA used in this study

was prepared as previously reported[43, 44]. The electrostatic complexation between mRNAs and LNPs was ensured by the charge interactions between the negatively charged phosphate groups in mRNA backbones and the protonated amine groups in ionizable DAL lipids. We screened and characterized the mRNA formulation by delivering luciferase mRNA in B16F10 melanoma cells *in vitro*. DAL4-LNP showed the highest luciferase mRNA delivery efficiency with a size of ~130 nm and mRNA encapsulation efficiency of around 90%. By comparing different DALs based LNPs used in this study, there is no obvious correlation between the properties of LNPs, including size, PDI, or encapsulation efficiency (Fig. 2A, B), and their mRNA delivery efficiency (Fig. 2C). We speculate that the head group of DAL4 might facilitate particle formulation and its interactions with cell and endosome membranes[43, 45]. However, the mechanism needs to be further uncovered.

The *in vivo* delivery experiments suggest that DAL4-LNP mRNA delivered by intratumoral injection is an efficient approach for protein expression in mouse tumors. The results showed that 12 hrs after DAL4-LNP-GFP mRNA injection, up to 15% of total tumor cells were GFP^+. Among GFP^+ cells, about 70% were $CD45^-$ cells (presumably tumor cells and fibroblasts) and 30% were immune cells. One interesting observation is that DAL4-LNP could selectively target mRNA to $CD19^+$ B cells but not T cells (Fig. 3B). The data suggest that DAL4-LNP may display some unique epitopes that can selectively bind to B cells. DAL4-LNP may have the potential to be developed as therapeutics to address B cell malignancies.

We observed that local therapy using DAL4-LNP-IL-12 and DAL4-LNP-IL-27 mRNAs demonstrated a strong synergistic effect in inhibiting tumor growth. Our approach of localized cytokine delivery to the tumor displayed that IL-12 is the most potent anti-tumor agent amongst the three considered, which is consistent with previous studies that identified the role of IL-12 to promote Th1/Tc1 response[7, 8] and to enhance T cell trafficking to tumors[9]. IL-27 is a member of the IL-12 family, and thus, promotes similar anti-tumor effects as IL-12[46, 47]. Despite similar capacities, IL-12 and IL-27 activate T cells and NK cells through different mechanisms: IL-12 activate T and NK cells via Stat4 pathway and IL-27 activates through Stat1 and Stat3 pathways. In addition to promoting Th1/Tc1 responses, we previously reported that IL-27 could enhance T cell survival in tumor microenvironment[47, 48]. These different properties of IL-27 and IL-12 explain the commensal effects of these two cytokines in both systemic therapy[42] and localized therapy observed in this study. Another notable observation in this study is that the addition of GM-CSF does not further improve the antitumor effects of IL-12 and IL-27 mRNA LNPs. Although this observation requires further investigation, less effectiveness may be related to GM-CSF-mediated expansion of myeloid-derived suppressor cells[49, 50], which could inhibit IL-12/IL-27-mediated anti-tumor immune responses and thereby promote tumor growth instead.

Our analysis indicates that intratumoral delivery of DAL4-LNP-IL-12 + IL-27 mRNA induced compelling anti-tumor immune responses. Notably, we observed a nearly 10-fold increase of total leukocyte infiltration within the TME after DAL4-LNP-IL-12 + IL-27 mRNA treatment. Furthermore, INF-γ and TNF-α producing T and NK cell effectors significantly increased in DAL4-LNP-IL-12 + IL-27 treated tumors, signaling an activation

or stimulation of the recruited effector cells to secrete anti-tumor signaling molecules to further boost the anti-tumor response (Fig. 5). Sustained tumor inhibition after stopping treatment suggests that immune memory responses were established in treated mice. Currently, the lack of T lymphocyte infiltration[51] has been considered to be a major factor responsible for low responding rates to anti-PD-1 therapy. Hence, it is tempting to hypothesize that our current therapeutic modality can potentially be used in combination with existing checkpoint inhibitors for enhanced efficacy.

A critical problem for cytokine-based cancer therapy is its narrow therapeutic window limited by severe adverse side effects[3]. Previously, the adoption of IL-12 therapy in clinics has been hindered by fatal toxicity[5, 6]. However, in this study, we establish that intratumoral injection of DAL4-LNP-IL-12 + IL-27 mRNA did not induce significant systemic toxicity *in vivo*, which is reflected by no significant body weight loss in treated mice or increase in overall fatality as compared to control-treated mice. Moreover, histology examination showed no inflammatory signatures were detected in major organs after DAL4-LNP-IL-12 + IL-27 mRNA administration. We speculate that two factors may play a role in mitigating systemic toxicity. First, the intratumoral injection approach may confine cytokine production to the TME with low systemic release. In the TME, the produced cytokines can be rapidly utilized by nearby immune cells. Indeed, previous studies have shown that intratumoral injection of LNPs can not only improve the local expression of the protein of interest but also reduce the systemic exposure of cargo[16, 52, 53] Second, IL-27 is an anti-inflammatory cytokine that induces IL-10 production[54–56]. This property of IL-27 can potentially reduce the toxic inflammation caused by IL-12 alone. Therefore, dual IL-12+IL-27 mRNA DAL4-LNP delivery platform exhibits increased potency as well as decreased systemic toxicity.

Taken together, we disclose a new LNPs mediated mRNA delivery formulation and a cytokine combination that can induce robust tumor infiltration of immune effectors and inhibit tumor growth with reduced toxicity. The therapeutic modality can be further applied to expand current immunotherapy. Additionally, the delivery platform merits further development as a novel immunotherapeutic against cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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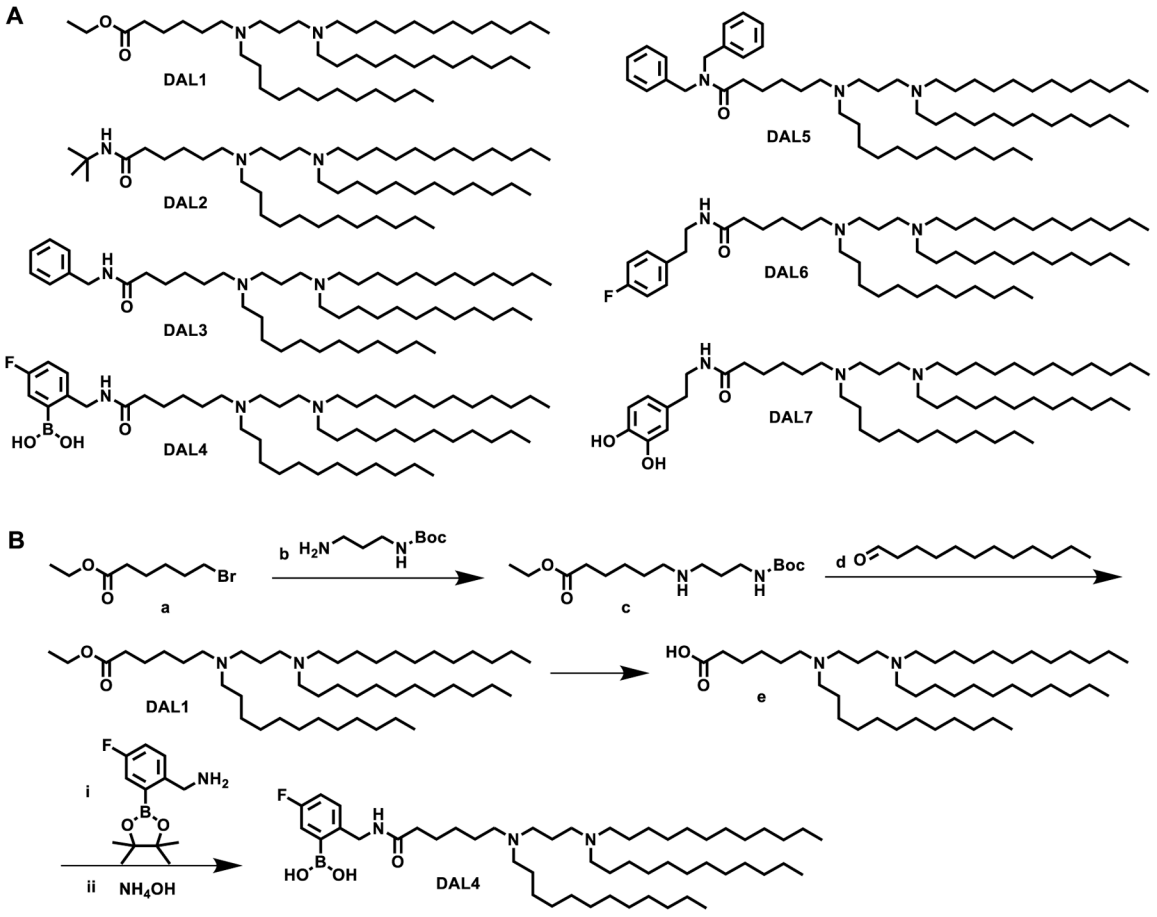

Fig. 1. Synthesis of diamino lipid derivatives (DALs).

(A) The structures of DALs. (B) Synthetic routes of DAL1 and DAL4.

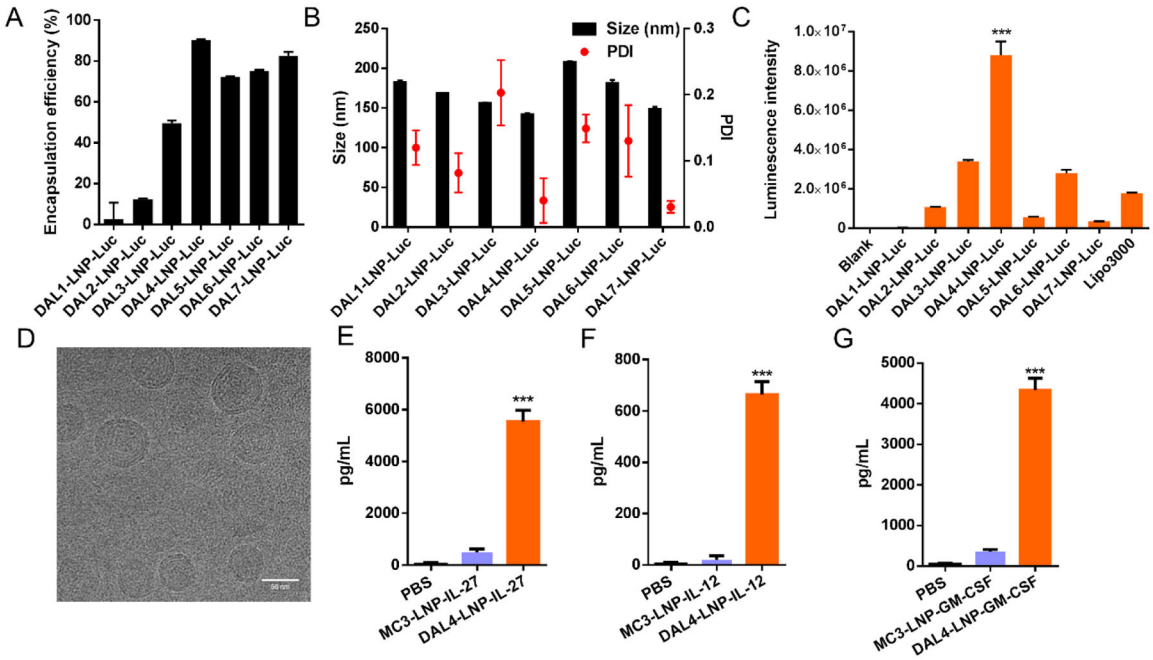

Fig. 2. DAL-LNPs delivery of mRNA *in vitro*.

(A) Encapsulation efficiency, (B) size distribution and polydispersity index (PDI) of DAL-LNPs encapsulating luciferase mRNA (DAL-LNP-Luc). (C) *In vitro* delivery of DAL-LNP-Luc in B16F10 cells. N=3. (D) Cryo-EM image of DAL4-LNP co-encapsulating IL-12 mRNA and IL-27 mRNA. Bar = 50 nm. (E-G) *In vitro* delivery of MC3-LNP or DAL4-LNP encapsulating either IL-27 mRNA (E), IL-12 mRNA (F), or GM-CSF mRNA (G) in B16F10 cells. N=3–4. The concentrations of cytokines in the supernatants were determined by ELISA. All data are presented as the mean ± S.D. Statistical significance in C, E, F, and G were analyzed using one-way ANOVA with Dunnett's multiple comparisons test. ***$P < 0.001$.

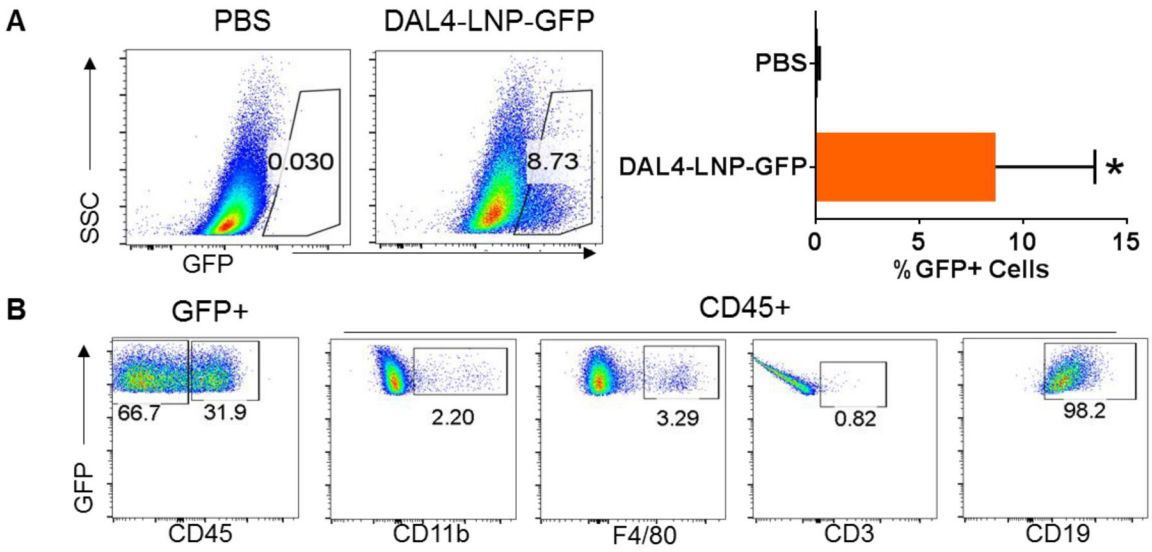

Fig. 3. DAL4-LNP for mRNA delivery to tumors.

Flow cytometry was used for quantifying GFP+ cells (A) and their subtypes (B). **P*<0.05 by Mann-Whitney test.

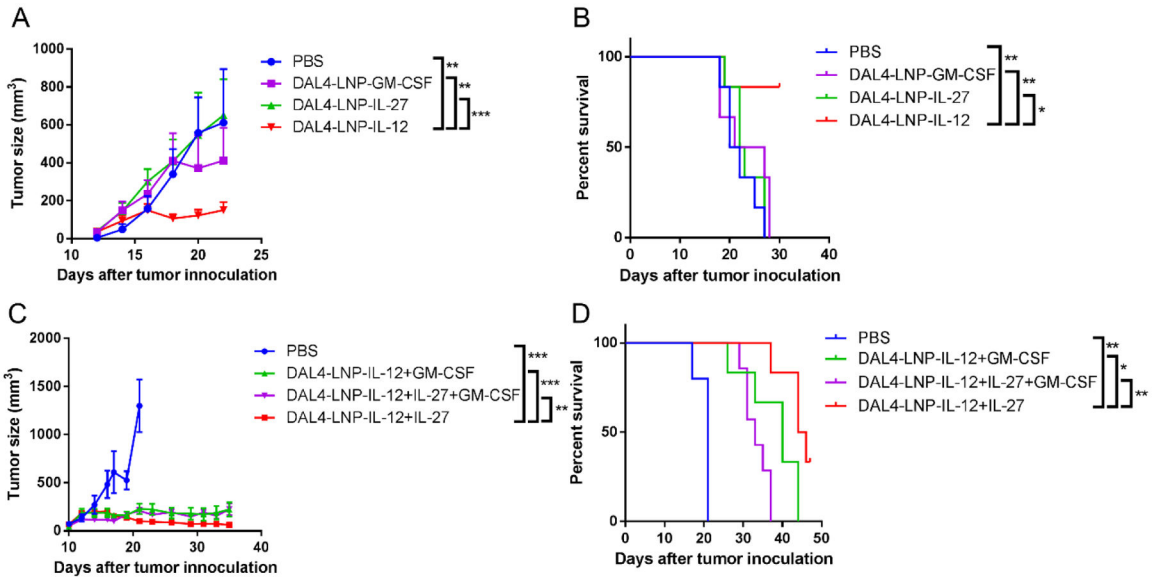

Fig. 4. *In vivo* anti-cancer activity of DAL4-LNP encapsulating single or multiple cytokine mRNAs.

(A) B16F10 tumor size after treatment of single cytokine mRNA in DAL4-LNP (n = 6). (B) Overall survival of B16F10 tumor-bearing mice. (C) B16F10 tumor size after treatment with DAL4-LNP encapsulating two or three cytokine mRNAs (n = 5–7). (D) Overall survival of B16F10 tumor-bearing mice. Data in (A) and (C) are presented as the mean ± S.E.M. Statistical significance in (A) and (C) was analyzed using two-way ANOVA with repeated measurements. Statistical significance in (B), (D) were analyzed using the log-rank (Mantel-Cox) test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

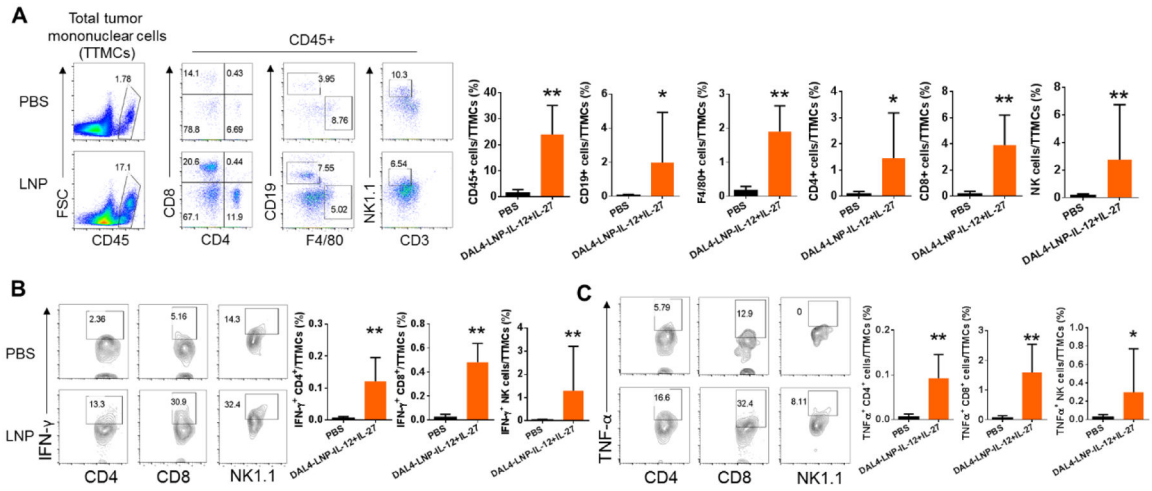

Fig. 5. Intratumoral injection of DAL4-LNP-IL-12 + IL-27 mRNA induces robust infiltration of immune effector cells into tumors and stimulates the production of anti-tumor signaling molecules.

(A) Flow cytometry was used for quantifying CD45+ leukocytes and its subtypes. (B-C) Intracellular staining and flow cytometry were used for quantifying IFN-γ (B) and TNF-α (C) producing T and NK cells. **P*<0.05, ***P*<0.01 by Mann-Whitney tests.