Supplementary Information

Adjuvant incorporated lipid nanoparticles for efficient mRNA-mediated cancer immunotherapy

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Materials & Methods

Lipid nanoparticle (LNP) formulation

Lipid nanoparticles were formulated by rapid mixing of ethanol phase and aqueous phase using the microfluidic device. The lipid mixture containing ionizable lipid (C12-200), DOPE (Avanti Polar Lipids, Alabaster, AL, USA), cholesterol (Sigma Aldrich, St. Louis, MO, USA) and C16-PEG2000 (Avanti Polar Lipids) was dissolved in ethanol phase at a molar ratio of 26.5:20:51:1.5 (ionizable lipid: DOPE: cholesterol: C16-PEG2000). For the preparation of the Pam-LNP, the Pam3CSK4 (Invivogen, San Diego, CA, USA) was mixed with the ethanol phase having lipid mixture at a molar ratio of 26.5:20:50.7:1.5:1.3 (ionizable lipid: DOPE: cholesterol: C16-PEG2000: Pam3CSK4). In addition, desired amount of the OVA mRNA (TriLink BioTechnologies, San Diego, CA, USA) was dissolved in 10mM citrate buffer (pH 3). The molar ratio between the OVA mRNA and the ionizable lipid was 1:10. The prepared ethanol phase and the citrate solution were mixed rapidly in a 3:1 volume ratio with NanoAssemblr Benchtop (Precision Nanosystems, Vancouver, Canada) using a microfluidic chip device. The prepared LNP or the Pam-LNP was dialyzed against 1X PBS using a Slide-A-Lyzer mini dialysis device (MWCO; 3.5k, Thermo Scientific, Waltham, MA, USA). After dialysis, the hydrodynamic sizes of the LNP or the Pam-LNP were assayed by using

dynamic light scattering using a Zetasizer Nano ZS90 (Malvern Panalytical, Malvern, UK). In addition, the mRNA encapsulation efficiencies (EE, %) were measured using Quant-iT RiboGreen RNA reagent (Invitrogen, Carlsbad, CA, USA).

In vivo mRNA delivery efficiency

All *in vivo* experiments were conducted by animal protocols approved by the Institutional Animal Care and Use Committees (IACUC) at Ewha Womans University in accordance with the guidelines of the National Institute of Heath's Guide for the Care and Use of Laboratory Animals (IACUC No. 17-035/18-032). ICR and C57BL/6 mice were purchased from Central lab animal incorporation (Seoul, Republic of Korea). ICR mice were given intramuscular injection of luciferase mRNA. The mRNA encapsulating LNPs (2μg mRNA/mouse) were injected into mice intramuscularly. After 3 hours, luciferin was administered into the LNP injected mice by intraperitoneal injection. 15 minutes after the luciferin injection, the mice were euthanized with isoflurane in a chamber, and the luminescence of the mice was imaged by using IVIS Lumina III (PerkinElmer, Waltham, MA, USA).

Cell culture

DC2.4 Mouse Dendritic Cell Line and E.G7-OVA cell line were obtained from American Type Culture Collection (ATCC) and were cultured in the Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (P/S) (100 U/mL) (Gibco, Grand Island, NY, USA) in 5% CO_2 at 37℃.

Immunization of mice

Female C57BL/6 mice aged 6 wk were purchased from Central lab animal incorporation (Seoul, Republic of Korea). The C57BL/6 mice were intramuscularly injected with formulated LNPs or pam-LNP twice at a weekly interval (20ug of OVA mRNA/mouse).

Blood collection and serum isolation

Blood was collected after 3 hours of first injection for analyzing the proinflammatory cytokines. In addition, a week after the second injection, another blood collection was performed for the measurement of serum IgG levels. After 2 hours of incubation at 20℃,

the collected blood was centrifuged for 15 min at 4,500 revolutions per minute (rpm). The supernatant was centrifuged once more for 5 min at 12,000 rpm at 4℃. The final supernatant was collected and utilized for the cytokine and the serum IgG level measurement.

Levels of Serum Proinflammatory Cytokines (TNF-α, IL-6)

The serum samples isolated after 3 hours of the first injection was utilized for measuring the proinflammatory cytokines (TNF-α, IL-6) using Mouse TNF-α Quantikine ELISA kit and Mouse IL-6 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). In each group, cytokine levels were measured in triplicate.

Measurement of IgG, IgG1, IgG2a

The levels of IgG were measured using serum samples collected 1 week after the second injection. Nunc Maxisorp immune plate was coated with 10ug/ml of Ovalbumin (Invivogen) in 1X ELISA coating buffer (Bio-Rad, Hercules, CA, USA). After the overnight incubation at 4℃, the plate was washed three times with wash buffer (PBS

with 0.05% Tween-20). The washed plate was blocked with 1% bovine serum albumin (BSA) for 1 hr at 20℃, followed by washing three times with wash buffer. Subsequently, the ovalbumine coated, BSA blocked, and washed plate was incubated with the diluted serum (dilution factor: 1:200 in 1% BSA) for 2 hours at 37℃. After washing the plate with wash buffer, the plate was incubated with secondary antibodies; Mouse IgG-heavy and light chain Antibody (cat. A90-116P, Bethyl Laboratories, Montgomery, TX, USA), Mouse IgG1 Antibody (cat. A90-105P, Bethyl) and Mouse IgG2a Antibody (cat. A90-107P, Bethyl) at 1:10000 dilution in 1% BSA for 1 hour at 20℃. After the incubation, the plate was washed five times with wash buffer. TMB substrate solution (Thermo Scientific) was added to the plate and the reaction was stopped with stop solution (Thermo Scientific). Finally, the plate was read at 450 nm absorbance using microplate multi-reader. In each group, antibody titers were analyzed in triplicate.

Enzyme-linked immunosorbent spot (ELISpot)

One week after the second injection, splenocytes were isolated using a 40 μm cell strainer (Falcon, Corning, NY, USA). After the isolation, red blood cells were lysed

using lysing buffer (BD Bioscience) and cell number was counted. After the cell counting, 5.0×10^4 cells were treated with the 4ug of the OVA 257-264 peptide (Invivogen) for 48 hours on Mouse *IFN-γ* ELISpot plate (Mabtech, Nacka Strand, Sweden) at 37℃ in a CO2 incubator. Spots were analyzed using ELISpot reader.

Tetramer assay

One week after the second injection, splenocytes were isolated. The isolated splenocytes were washed with FACS buffer (the mixture of PBS, 0.1% NaN_3 and 0.5% FBS). The washed splenocytes were stained with tetramer in combination of PerCP/Cyanine5.5 anti-mouse CD8a antibody, FITC anti-mouse CD44 antibody, and APC anti-mouse CD45 antibody (BioLegend, San Diego, CA, USA) in FACS buffer after Fc-block for 30 min. After cells staining, the stained cells were washed with FACS buffer. The washed cells were measured by using NovoCyte Flow Cytometer (ACEA Biosciences, San Diego, CA, USA) and analyzed by FlowJo software. 100,000 events were collected per sample.

Tumor cell challenge

Mice injected with samples (PBS, naked mRNA, LNP, Pam-LNP) twice at a weekly interval were inoculated subcutaneously with 1×10^6 E.G7-OVA cells one week after the second injection. Tumor volume and body weight of the mice were monitored twice a week. Tumor size was measured using a digital caliper, and the volumes of the solid tumor was calculated by using an equation $V = 0.5 \times W^2 \times L$ (V; tumor volume, W; tumor width, L; tumor length). The survival of the tumor inoculated mice was estimated by Kaplan-Meier curve plotted by GraphPad. The tumor size measurement was stopped when the tumor size reached 2000 mm^3.

Supporting figures

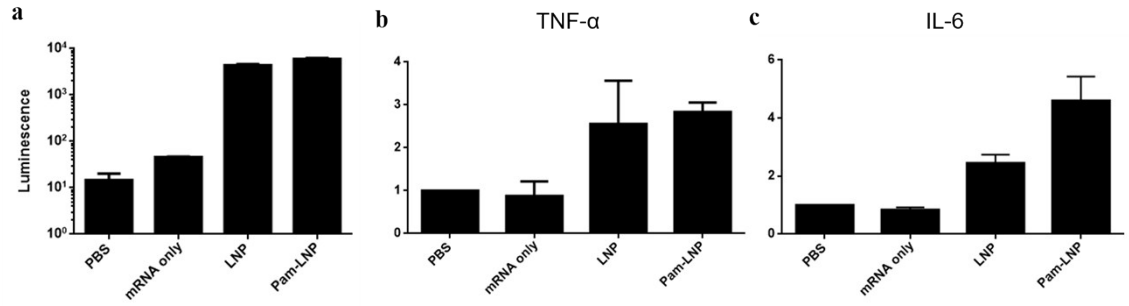

Figure S1. (a) In vitro evaluation of the luciferase expression of Luc mRNA encapsulating LNP or Pam-LNP (b) Intracellular proinflammatory cytokine expression levels (TNF-α, IL-6) after treatment of the mRNA encapsulating mRNA or Pam-LNP, measured by reverse transcription polymerase chain reaction (RT-PCR). Mouse dendritic cell line (DC2.4 cell line) was utilized in this assay.

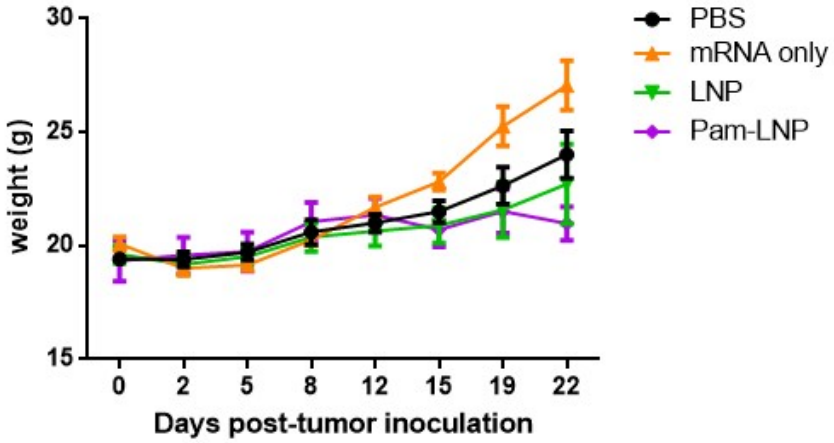

Figure S2. Body weight changes of mice injected with OVA mRNA encapsulating LNP or Pam-LNP (n=5)