Supporting Information

Of

Lipid nanoparticle-based mRNA candidates elicit potent T cell responses

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Methods

Chemicals

All lipids, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dimyristoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2K) were purchased from Avanti Polar Lipids, DLin-MC3-DMA was purchased from Biorbyt company (Cambridge, England), Hoechst 33342, cholesterol and β-sitosterol was purchased from Sigma-Aldrich. Triton™ X-100 was purchased from Acros Organics. QuantiT™ RiboGreen® RNA reagent and rRNA standards were purchased from Life Technologies. Clean cap EGFP-mRNA (5moU) and OVA-mRNA (5moU) were purchased from Trilink biotechnology. C12-200 and cKK-E12 lipids were synthesized according to the literatures.[29, 30] The following antibodies were used for flow cytometry: anti-Thy1.2 PeCy7, anti-CD8 efluor450, anti-CD25 APC, Live/Dead stain and purchased from BD Bioscience. CD4 and CD8 T-cell isolation kits were purchased from Miltenyi (Leiden, Netherlands).

Cell culture

THP-1, RAW264.7, HeLa, Calu-3 cells were obtained from ATCC. DC2.4 is a murine dendritic cell line kindly provided by Kenneth Rock, University of Massachusetts Medical School, Worcester, MA. Cells were maintained either in DMEM or RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin antibiotic (Gibco). Cell culture and all biological experiments were performed at 37 °C in 5% CO2 conditions in a cell culture incubator.

6 to 8-week-old female mice (C57BL/6 mice were used to isolate BMDCs. Briefly, the femur and tibia from mice hind legs were collected and bone marrow cells were flushed out with PBS using a syringe. Cells were resuspended into RPMI1640 medium with 10% FBS, antibiotics, and β-mercaptoethanol (55 μM, Gibco). Cells were grown with a supplement of recombinant murine granulocyte-macrophage–colony-stimulating factor (GM-CSF) (20 ng/mL, Peprotech). The cell culture medium was refreshed every other day.

Mouse experiments

C57BL/6, OT-I transgenic mice on a C57BL/6 background were purchased from Jackson Laboratory (CA, USA), bred in-house under standard laboratory conditions, and provided with food and water ad libitum. All animal experiments were performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Experiments were approved by the Ethics Committee for Animal Experiments of Leiden University.

LNP-mRNA preparation and characterization

Lipids were combined at the desired molar ratios and concentrations from stock solutions dissolved in chloroform. Solvents were evaporated under a nitrogen flow and residual solvent was removed in vacuo for at least 30 minutes. The lipid film was dissolved in absolute ethanol (total lipids was 0.4 μmol) and used for the assembly. A solution of mRNA was made by diluting mRNA (EGFP-mRNA, OVA-mRNA) in 50 mM citrate buffer (pH = 4, RNase free). The solutions were loaded into two separate syringes and connected to a T-junction microfluidic mixer. The solutions were mixed in a 3:1 flow ratio of mRNA against lipids (1.5 mL/min for mRNA solution, 0.5 mL/min for lipids solution, lipids: mRNA (wt/wt) =40:1). After mixing, the solution was directly loaded in a 20 kDa MWCO dialysis cassette (Slide-A-Lyzer™, Thermo Scientific) and dialyzed against 1 x PBS overnight. The size and zeta potential of LNPs were measured using dynamic light scattering (DLS, Malvern) and Zetasizer (Malvern). Long term stability of LNPs was assessed by measuring the hydrodynamic radius using DLS for 1 month.

The encapsulation efficiency (EE) of mRNA was determined by Quant-iT™ RiboGreen™ RNA Assay Kit (Invitrogen). For the determination of non-encapsulated mRNA, LNPs after dialysis were diluted with 1 x TE buffer (RNase free) and treated with the RiboGreen™ reagent. For the determination of the total amount of mRNA, LNPs after dialysis were treated with 1% Triton X-100 in TE buffer (RNase free) and incubated for 5 minutes followed by dilution with TE buffer and treatment with the RiboGreen™ reagent. The supplied RNA standards were used to generate a standard curve and changes in fluorescence was measured in 96-well plates using a TECAN Infinite M1000 Pro microplate reader. The percentage of mRNA encapsulation (EE%) was determined using the fraction of $(F_{total}$ RNA $- F_{free}$ RNA$)/F_{total}$ RNA * 100%.

***In vitro* GFP protein expression of LNPs**

Briefly, HeLa, and Calu-3, DC2.4, THP-1 and RAW264.7 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured at 37 °C in 5% CO_2 overnight. Then cells were transfected with LNPs containing different concentrations of EGFP-mRNA overnight (0.1 μg/mL, 0.25 μg/mL, 0.5 μg/mL, 1 μg/mL). The expression of GFP protein was quantified by flow cytometry. Data analysis was performed using the FlowJo Software version 7.6. For confocal microscopy, DC2.4 cells were

seeded on the 8-well confocal slide at a density of $5*10^4$ cells/well and cultured overnight, then LNPs were added and incubated overnight (1 μg/mL), after that Hoechst 33342 (5 μM) was added and incubated for 1 h before confocal microscopy imaging (Leica TCS SP8 confocal laser scanning microscope).

BMDC transfection

Bone marrow-derived dendritic cells (BMDCs) were isolated from murine tibia and femurs of C57BL/6 mice. Bone marrow cells were stimulated for 10 days with 20 ng/mL GM-CSF in complete IMDM (supplemented with 100 U/mL PenStrep, 2 mM glutaMAX and 10% FCS). After 10 days of culture, 20,000 BMDCs were plated in 96-well plates (Greiner Bio-One B.V., Alphen aan den Rijn, Netherlands) and different LNPs encapsulating EGFP-mRNA were added at varying concentrations and incubated with BMDC overnight (0.25 μg/mL, 0.5 μg/mL, 1 μg/mL). Cells were analyzed by flow cytometry (CytoFLEX S, Beckman Coulter, CA, USA). Data were analyzed by using FlowJo software (Treestar, OR, USA). For confocal microscopy, BMDCs were seeded on the 8-well confocal slide at a density of $5*10^4$ cells/well and cultured overnight, then LNPs were added and incubated overnight (0.5 μg/mL), then followed with confocal microscopy measurement (Leica TCS SP8 confocal laser scanning microscope).

BMDC activation

Bone marrow-derived dendritic cells (BMDCs) were isolated and cultured described as above. After 10 days, different LNPs containing OVA-mRNA were added to the BMDC cells and incubated overnight (2 ng/mL, 20 ng/mL, 0.25 μg/mL 0.5 μg/mL, 1 μg/mL). After 24 h of incubation, cells were collected and followed immunostaining of CD40 and CD86 to quantify the expression by flow cytometry. Data analysis was performed using the FlowJo Software version 7.6. The supernatant was collected for measuring the expression level of cytokine (IL12-p70) by ELISA.

***Ex vivo* T-cell expansion**

Wild-type (WT) BMDCs were cultured as described above, after 10 days, the BMDCs (20,000 cells per well) were exposed to the different LNP formulations with various concentrations (20 pg/mL, 200 pg/mL, 1 ng/mL, 2 ng/mL, 5 ng/mL, 20 ng/mL, 200 ng/mL). Meanwhile, spleens were removed from OT-I mice and strained through a 70-μm cell strainer to obtain a single-cell suspension. Erythrocytes were lysed with Ammonium-Chloride-Potassium (ACK) lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA; pH 7.3). CD8+ T cells were isolated using a CD8+ T cell isolation kit (Miltenyi Biotec B.V., Leiden, Netherlands) according to the manufacturer's protocol. After 4 h of LNP incubation, the BMDCs were centrifuged, the supernatant medium was removed, and replaced with 60,000 $CD8^+$ T-cells to obtain a number ratio of 3:1 CD8+ T cells:BMDCs. Co-cultures were cultured for 72 h in complete RPMI 1640 medium supplemented with 2 mM glutamine, 10% FCS, 100 U/mL penicillin/streptomycin, and 50 μM β-mercaptoethanol. After 72 h, the cell suspension was stained for anti-Thy1.2 PeCy7, anti-CD8 efluor450, anti-CD25 APC, Live/Dead stain indicating cell viability, and then analyzed by flow cytometry (CytoFLEX S, Beckman Coulter, CA, USA). The supernatant was collected for measuring the expression levels of cytokines by ELISA.

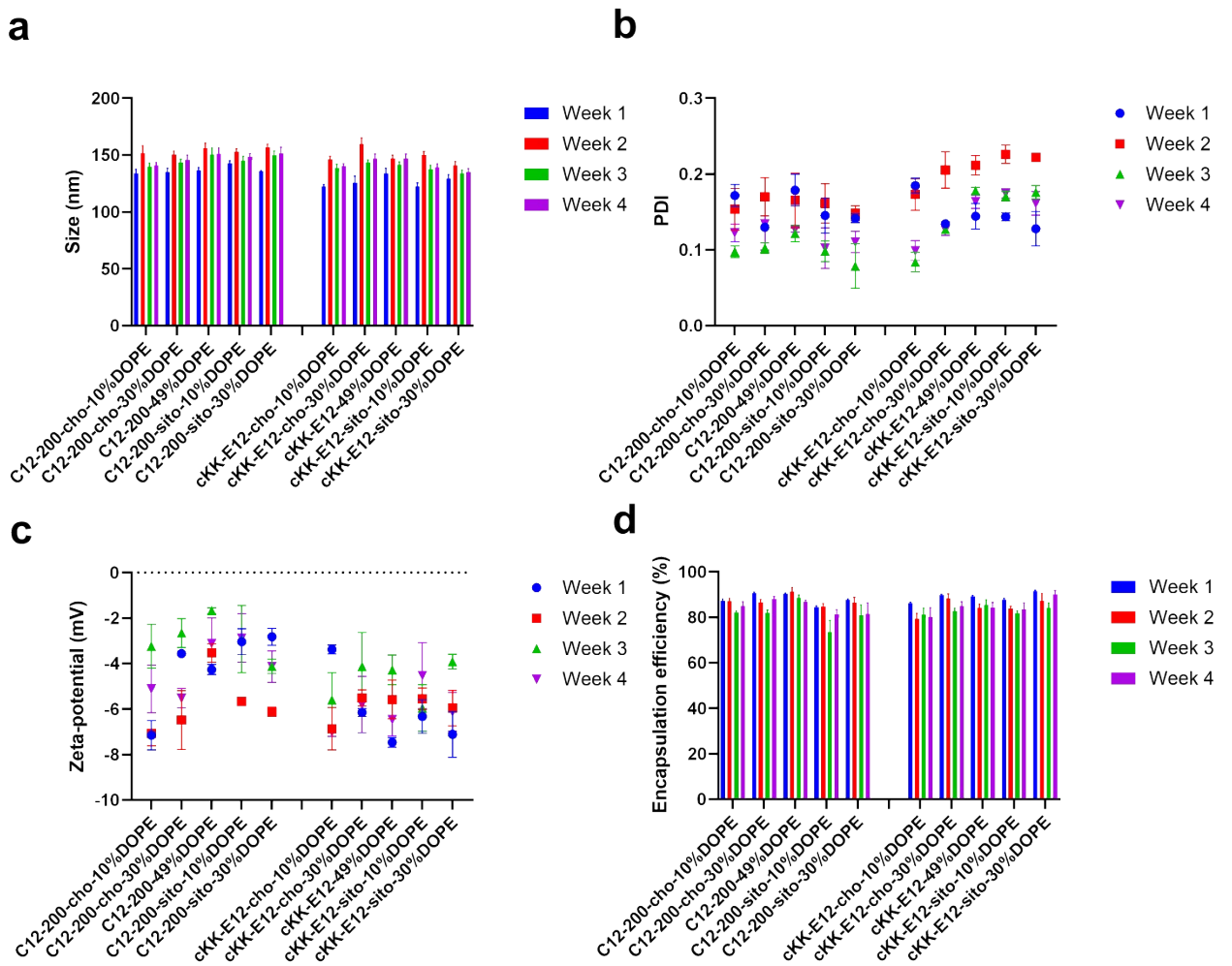
ELISA measurements of OT-I T cell supernatant

Cytokines (IL-2, IFN-γ, and TFN-α) from OT-I T cell supernatants were detected by individual cytokine ELISA kits according to the manufacturer's instructions (BD Biosciences). In brief, the

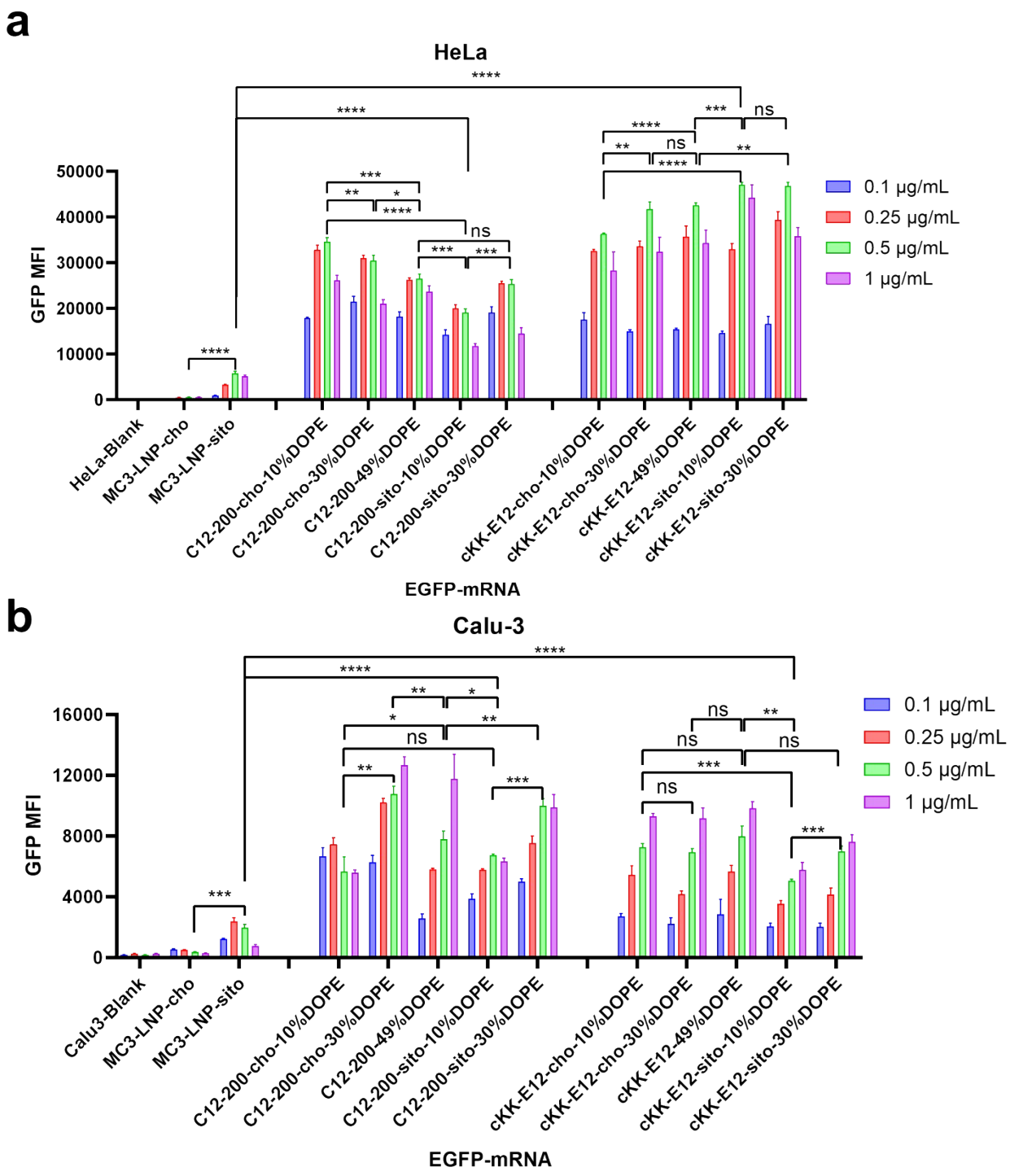
assay plate was coated with 50 µl/well of capture antibody (IL-2, IFN-γ, and TFN-α, respectively) in coating buffer, covered and incubated overnight at 4 °C. Next, the plate was washed (3x) with wash buffer (PBS with 0.05% Tween-20) and blocked with 100 µl/well of assay diluent (PBS with 10%FCS) and incubated for 1 hour at RT. The plate was washed (3x) with wash buffer and 50 µL/well (diluted) samples/standard/blank was added incubated for 2 hours at RT. Next, the plate was washed (3x) with wash buffer and 50 µL/well of the working detector (Detection AB + Sav-HRP reagent) was added and incubated for another 1 hour at RT. After washing the plate (5X) with wash buffer, 50 µL/well substrate solution was added and incubate for 15-30min at RT in the dark. Finally, 25 µl/well stop solution was added and the absorbance at 450 nm was measured within 30 minutes.

Statistical analyses

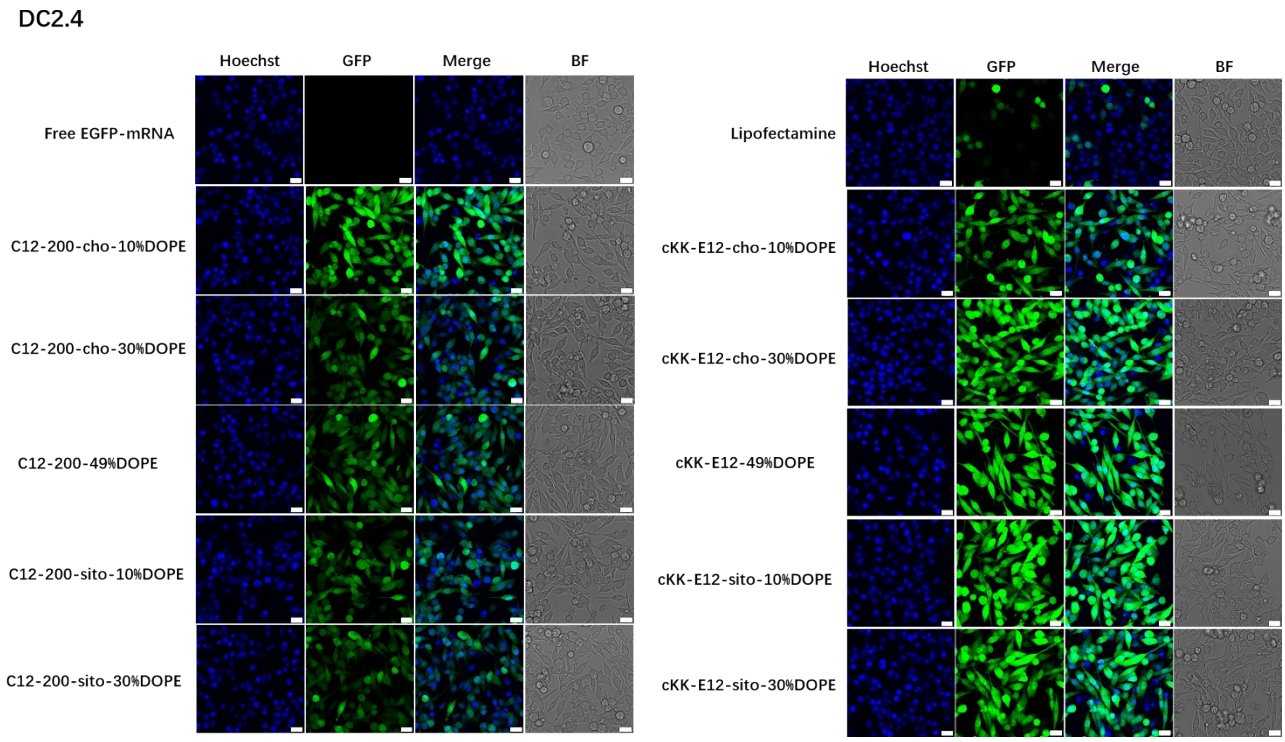
Statistical analyses were performed with Prism 8 (GraphPad). Data were compared using unpaired student t-test analysis. (****, $P < 0.0001$, ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$, ns, no significant difference, n = 3)



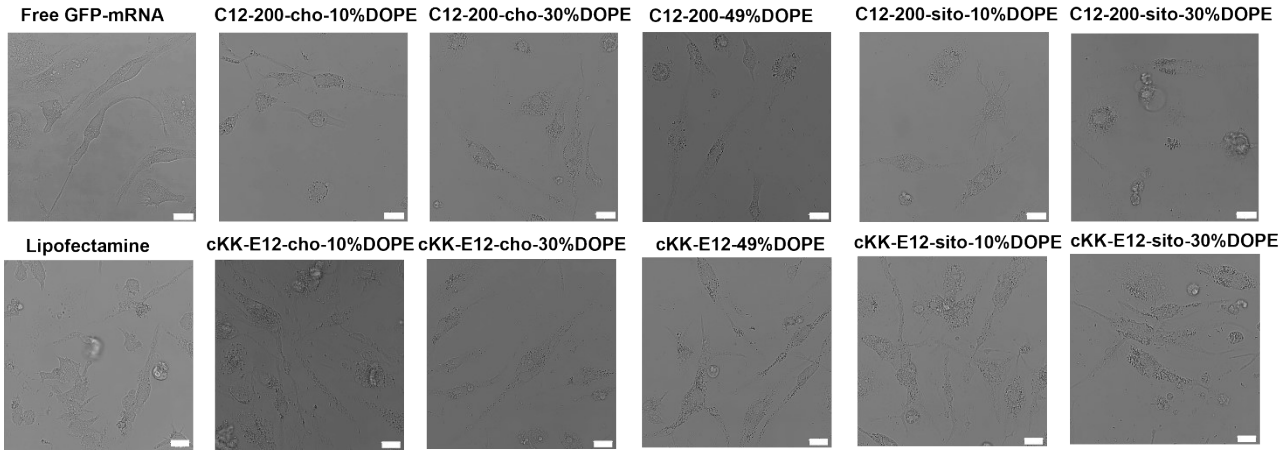
SI Figure 1. Stability of LNPs (stored at 4°C). (**a**) Sizes, (**b**) PDI, (**c**) Zeta potential, (**d**) Encapsulation efficiency of LNPs over 1 month.



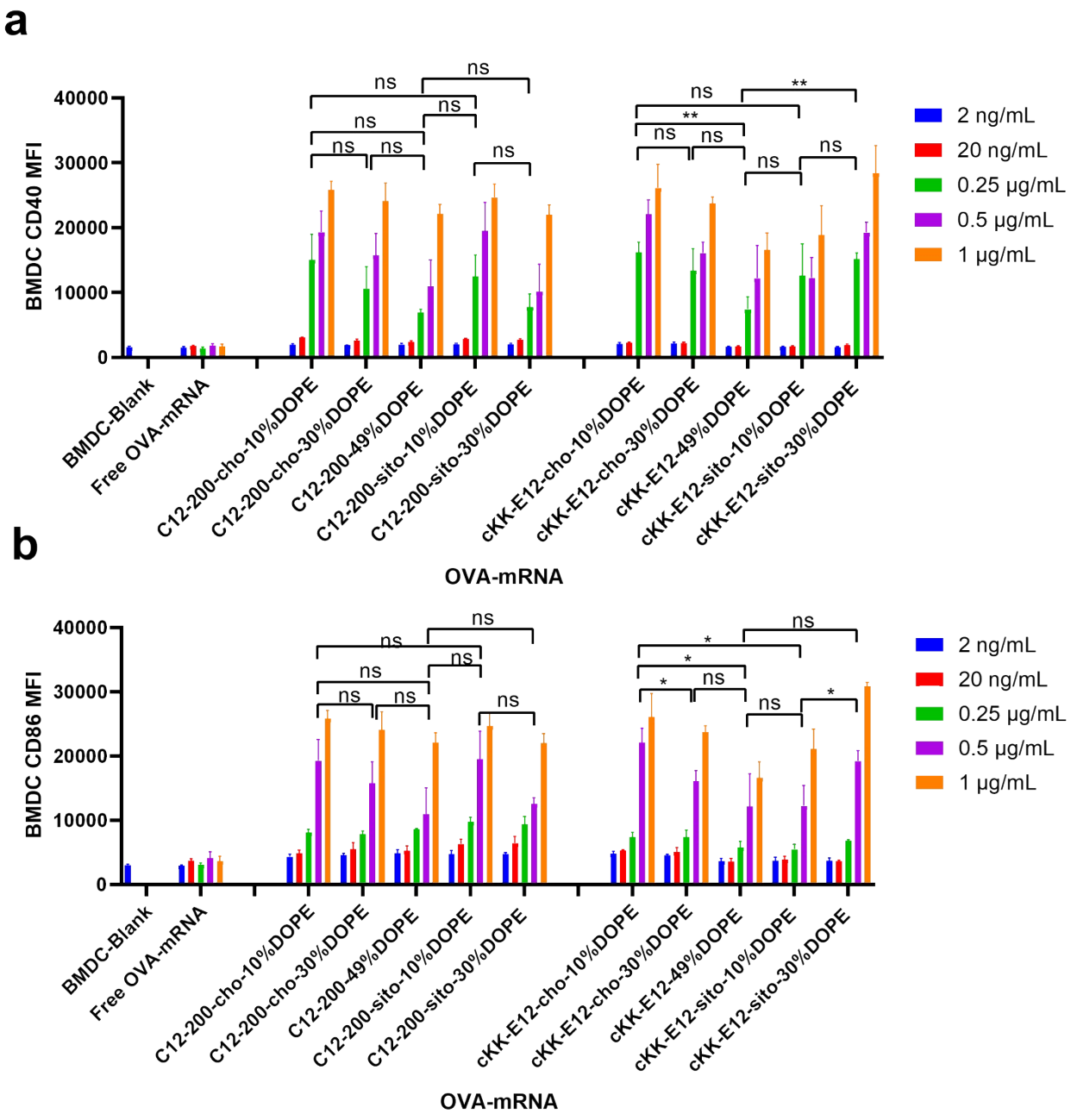
SI Figure 2. Transfection efficiency (mean fluorescence intensity, MFI) after encapsulating EGFP-mRNA within (**a**) HeLa cells and (**b**) Calu-3 cells. Data are presented as mean ± sd. Statistical significance was calculated by unpaired student t-test on 0.5 μg/mL. (****, $P < 0.0001$, ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$, ns, no significant difference, n = 3)



SI Figure 3. Confocal images of the EGFP-mRNA transfection of LNPs on DC2.4 cells (EGFP-mRNA, 1 μg/mL, 24 h). Scale bar is 20 μm.



SI Figure 4. Brightfield channel of confocal images of the EGFP-mRNA transfection of LNPs on BMDC cells, EGFP-mRNA concentration was 0.5 μg/mL, incubated 24 h. Scale bar is 20 μm.



SI Figure 5. The mean fluorescence intensities (MFI) of cellular marker CD40 (**a**) and CD86 (**b**) after BMDCs activation by the different concentrations of LNPs. Data are presented as mean ± sd. Statistical significance was calculated by unpaired student t-test on 0.25 μg/mL (**a**) and 0.5 μg/mL (**b**). (****, $P < 0.0001$, ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$, ns, no significant difference, n = 3)