**Supplementary Document**

**Virus-mimic mRNA Vaccine for Cancer Treatment**

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Materials and Methods

Cell lines and cell culture: DC2.4 murine dendritic cell line was purchased from ATCC (Manassas, VA). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. B16OVA cells were provided by Dr. Kenneth Rock at Dana Farber Cancer Institute. Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cell culture was maintained in a 37°C incubator supplied with 5% CO₂.

Antibodies and other Reagents: The following antibodies were purchased from BioLegend: anti-CD11c-PerCP/Cy5.5, anti-MHCII-BV711, anti-CD40-FITC, anti-CD80-PE-Cy5, anti-CD86-APC, anti-H-2Kb bound to SIINFEKL-PE. The following antibodies were obtained from eBioscience: anti-CD45-APC-Cy7, anti-CD3-PerCP/Cy5.5, anti-CD4-FITC, anti-CD8-BV510, anti-CD44-APC, anti-IFN-γ-PE. Anti-H-2Kb (SIINFEKL)-PE for antigen specific T cells was a product from ImmuDex. Anti-mouse PD-1 monoclonal antibody was from BioXcell. The following antibodies were purchased from BioLegend and were used in CyTOF analysis: Pr141-CD3, Nd142-MHCII, Nd143-CD11b, Nd144-Ly6C, Nd145-Ly6G, Sm147-CD11c, Nd148-CTLA4, Sm149-CD69, Eu151-CD206, Sm152-CD62L, Eu153-CD103, Gd155-PD-L1 (CD274), Gd158-CD64, Tb159-KLRG1, Gd160-Foxp3, Dy162-CD8a, Dy163-Ki67, Dy164-CD25, Ho165-IFN-γ, Er166-CD44, Er167-CD86(B7-2), Er168-CD80, Tm169-PD-1(CD279), Er170-B220, Yb171-MHCI, Yb172-GATA3, Yb173-T-bet, Yb174-CD4, Yb176-F4/80, Y89-CD45. Sm154-iNOS and Gd156-Arginase-1 were purchased from eBioscience. Phospholipids 1,2-dioleoyl-sn-glycerol-3-ethylphosphocholine (EDOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidyl-ethanolamine (DOPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000 (DSPE-PEG2k) were obtained from Avanti Polar Lipids (Birmingham, Alabama, USA). They were dissolved in ethanol at a final concentration of 2 mg/mL (DSPE-PEG2k) or 20 mg/mL (EDOPC and DOPE). Protamine sulfate was a product of Sigma Aldrich (P4020-5G), and was dissolved in RNase-free water. OVA mRNA (L-7210), eGFP mRNA (L-7601), and luciferase mRNA (L-7202) were products from TriLink. They were all dissolved in 1 mM sodium citrate RNase-free solution (pH 6.4) at a final concentration of 1 mg/mL. TLR9 agonist CpG (tlrl-1826), and TLR7 agonist CL307 (tlrl-c307) were purchased from Invivogen, and were dissolved in RNase-free water. TLR7 agonist imiquimod (INN, tlrl-imq) was also from Invivogen, and was dissolved in dimethyl sulfoxide (DMSO).
**Characterization of mRNA-packaged vaccine particles**: Size distribution and zeta potential of mRNA particles were measured with a Malvern dynamic light scattering Zetasizer. mRNA encapsulation efficiency was determined with a Quant-iT™ RiboGreen™ RNA assay kit (Fisher Scientific, R11490) following a previously described protocol (1). Briefly, particles packaged with 0.5 μg mRNA in 50 μL PBS were diluted 1:1 with a Tris-EDTA buffer (10 mM Tris-HCL, 1 mM EDTA, pH 7.5) to measure unencapsulated mRNA or with Tris-EDTA-Triton-X100 buffer (Tris-EDTA buffer plus 2% Triton X-100) to measure total mRNA in a 96-well plate. RiboGreen was added to each well and fluorescent intensity was measured with a plate reader. Encapsulation efficiency was calculated as (intensity from total mRNA - intensity from unencapsulated mRNA)/intensity from total mRNA (2). Transmission electron microscopy (TEM) of mRNA NP and VLVP was performed following a previously described procedure (3).

**Preparation of bone marrow-derived dendritic cells (BMDCs)**: BMDCs were prepared by incubating bone marrow cells from C57BL/6 mice with granulocyte-macrophage colony-stimulating factor (GM-CSF) as previously described (4). Briefly, bone marrow cells from the femur and tibia were flushed out with 2% FBS-containing PBS using a syringe with a 25-26 gauge needle. Cells were centrifuged at 400 x g for 4 minutes, and treated with ACK lysis buffer (Lonza Inc) to remove red blood cells. Two million cells were resuspended in 10 ml RPMI-1640 culture medium supplemented with 10% FBS, 55 μM β-mercaptoethanol, 1% penicillin/streptomycin, 20 ng/mL GM-CSF, and seeded onto a 10-cm petri-dish. Another 10 ml of fresh growth medium was added on day 3, and 10 ml of growth medium was replaced on days 6 and 8. Non-adherent dendritic cells were harvested on day 10.

**Cytokine measurement**: BMDCs were seeded at a density of 5x10^5 cells/well onto a 24-well plate. Two hours later, cells were treated with mRNA NP, VLVP or a control. Supernatant was collected 18 hours later and cytokine levels (IL-6, TNF-α, IL-12 and IFN- β) were measured with enzyme-linked immunosorbent assay (ELISA) following manufacturer suggested protocols.

**RNA sequencing analysis**: RNeasy Plus Micro Kit (Qiagen) was applied to extract RNA from BMDCs treated with VLVP or control. Duplicated RNA samples were submitted to Novogene for sequencing analysis. Data analysis was performed by the company.

**Flow cytometry**: To measure in vitro transfection efficiency, DC2.4 cells were seeded at 2.6×10^5 cells/well onto a 24-well plate. Cells were treated with eGFP mRNA-packaged mRNA NP, VLVP
or controls, and harvested 18 hours later. Cells were washed once with PBS supplemented with 2% FBS, and resuspended in the same solution before they were analyzed with a BD Accuri C6 flow cytometer (BD Bioscience, San Jose, CA). To measure maturation and antigen presentation, BMDCs were stained for 30 minutes with antibodies specific for CD11c, MHC-II, CD40, CD80, or CD86 for DC maturation, and OVA257-264 (SIINFEKL)-H-2Kb complex for antigen presentation. To detect antigen specific T cells, spleens and popliteal lymph nodes (LNs) were harvested 5 days after the second vaccination. Cells were disseminated into single-cell suspensions and counted. Two million cells were stained with antibodies specific for CD45, CD3, CD4, CD8, or OVA257-264-MHC I dextramer (ImmuDex) following manufacturer’s instructions. To assess intracellular IFN-γ level, 3x10^6 splenocytes or cells isolated from popliteal LNs and tumors were stimulated ex vivo with 10 μg/mL OVA257-264 peptide in complete RPMI 1640 medium supplemented with 55 μM β-mercaptoethanol and 1x protein transport inhibitor cocktail (eBioscience) for 18 hours, and then stained with surface antibodies. Cells were then fixed and permeabilized using a fixation/permeabilization solution kit (BD). They were stained with an anti-IFN-γ antibody, detected on a BD LSR II flow cytometer, and analyzed using FlowJo software.

To process samples for CyTOF analysis, tumor samples were harvested 4 days after the second vaccination. Tumor fragments were digested in RPMI 1640 containing 1% penicillin/streptomycin, 400 U/ml type D collagenase (Worthington Biochemical) and 25 U/ml DNase I (Sigma). The digestion mixture was spun down at 500 rpm for 5 minutes, and the pellet was resuspended in PBS. Tumor-infiltrated hematopoietic cells were enriched through density gradient centrifugation using 50/80% Percoll as described previously (5). Briefly, 3 mL cell suspension was transferred to a 15 mL centrifuge tube, and 5 mL 50% Percoll was added to the bottom of single-cell suspension followed by addition of 5 mL 80% Percoll to the bottom. The sample was centrifugated at 1800xg for 30 minutes. The layer between 50% and 80% Percoll was collected and cells were washed with PBS containing 2% FBS before they were applied for CyTOF analysis.

For CyTOF analysis, single cell suspension was stained with metal-tag viability dye for 5 minutes and then washed with cell staining buffer (Fluidigm). They were then stained for surface and intracellular markers following a company-provided protocol for Foxp3 staining (eBioscience). Cells were then stained with Cell ID Intercalator Ir (Fluidigm) at 4°C overnight. Cells were washed
with cell-staining buffer the next day and prepared for acquisition with Helios (Fluidigm). Results were analyzed with a CyTOF acquisition software and uploaded to Cytofbank for analysis. Briefly, data was normalized using a bead-based normalization algorithm and beads and dead cells were gated out. CD45+ cell population was selected to perform spanning-tree progression analysis for density normalized events (SPADE). Putative cell populations on the resulting SPADE trees were manually annotated based on expression of key canonical markers. Sub-populations were analyzed with FlowJo X. viSNE analysis was performed to generate tSNE plots. The viSNE plot was gated based on the gating strategy (Figure S3). Heat map was plotted using z score to show the relative expression levels of individual markers: 
\[ z = \frac{(x - \bar{x})}{s}, \]
where x is the mean value of marker expression, \( \bar{x} \) is the average of the marker expression among all samples, and s is the standard deviation.

**ELISpot assay:** IP Filter Plates (Millipore Sigma) were pre-rinsed with 35% ethanol and then washed three times with PBS. The plates were then coated with anti-IFN-γ capture antibodies and incubated at 4 °C overnight. On the next day, the plates were blocked with complete medium supplemented with 55 μM β-mercaptoethanol for 2 hours at 37 °C. Cells (1x10^5 splenocytes/well, 0.5x10^5 cells from popliteal LN/well, or 0.1x10^5 cells from tumor/well) were seeded into a 96-well plate and stimulated for 24 - 36 hours with 10 μg/mL OVA257-264 peptide in cell growth medium. Cells were washed 4 times with PBS containing 0.05% Tween-20 and once with PBS only. Anti-IFN-γ detection antibody was added after the last wash, and the plate was incubated for 2 hours at room temperature. The antibody-containing solution was discarded, and the plate went through another round of wash. Avidin-HRP was added and the plate was incubated for 45 minutes. The plate was washed again, and AEC substrate (Fisher Scientific) was added. The reaction was stopped, and the plate was scanned using a CTL ImmunoSpot SeriesS five Versa ELISpot Analyzer (S5Versa-02-9038). Result was analyzed with the ImmunoCapturev.6.3 software.

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Supplementary Figures

**Figure S1. Characterization of mRNA NP.** a) Schematic view of mRNA NP preparation procedure. b-d) Characterization of NPs prepared with eGFP- and OVA-encoding mRNA molecules based on particle size, zeta potential, and encapsulation efficiency. e) TEM image of mRNA NP. The mRNA core was densely stained and a thin layer of phospholipid surrounding the core was lightly stained. Scale bar: 100 nm. f) Fluorescent microscopic image of DC2.4 cells treated with mRNA NP carrying eGFP-encoding mRNA. Scale bar: 200 μm. g) Flow cytometry analysis on transfection efficiency after BMDCs were incubated with mRNA NP or control for 16 hours. h) Flow cytometry analysis on surface maturation markers after BMDCs were co-incubated with mRNA NP or controls for 16 hours. MFI: mean fluorescent intensity. i-j) ELISA assay
measurement of IL-12p70 and TNF-α levels in BMDC cell culture after cells were treated with particles on controls for 18 hours. Lipopolysaccharide served as the positive control. Error bars represent the mean +/- s.e.m.
Figure S2. Dose-dependent cytokine expression by BMDCs. BMDCs were treated with increasing doses of CL307, CpG, or imiquimod (INN), and cell growth media were collected 16 hours later for ELISA measurement of IL-12p70 and TNF-α levels. Samples were triplicated. Data are presented as mean +/- s.e.m.
Figure S3. Gating strategy for CyTOF analysis.
Figure S4. CyTOF analysis of tumor infiltrated immune cells. 

a) Quality control of samples for CyTOF analysis. 
b) Levels of Arg-1+CD11b+ DCs in post-treatment tumor samples. 
c) Levels of maturation markers CD86, CD80 and MHCI in CD8+ and CD103+ DCs. 
d-e) Percentage of Ly6C+CD8+ T cells in post-treatment tumor samples. 
f) CTLA4 expression in T cell subpopulations. 
g) Percentage of CTLA4+ Treg cells.