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Supporting Information for
Comb-structured mRNA vaccine tethered with short double-stranded RNA adjuvants maximizes cellular immunity for cancer treatment.

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This PDF file includes:

Supporting Information Text
Supplementary Methods
Materials
fLuc and *OVA* mRNA were purchased from TriLink® Biotechnologies (San Diego, CA). mRNA encoding *gLuc* was prepared by IVT using mMessage mMachine T7 kit (Thermofisher, Waltham, MA). DNA templates are prepared by inserting protein-coding sequence of *gLuc* (pCMV-GLuc control vector, New England BioLabs, Ipswich, MA) into pSP73 vectors (Promega, Madison, WI) possessing 120 bp polyA/T sequences downstream of a multiple cloning site. cRNA was purchased from Hokkaido System Bioscience (Hokkaido, Japan) (**Supplementary Table S2**), 5'ppp-RNA was prepared by IVT in the absence of ATP using MEGAshortscript T7 kit (Thermofisher) and DNA template, with sequences listed in **Supplementary Table S1**. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 1 M was purchased from Gibco (Waltham, MA) for cell culturing. HEPES 1 M (pH 7.3) was purchased from Amresco Inc. (Dallas, TX, USA) for using as an mRNA buffer. Sodium chloride 5 M was purchased from Thermo Fisher Scientific, while chloroform was purchased from Wako Pure Chemicals Industries (Osaka, Japan). DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium propane (chloride salt)) and DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) were purchased from NOF Corporation (Tokyo, Japan), while Lipofectamine LTX Plus was purchased from Thermo Fisher Scientific. DC2.4 Mouse Dendritic Cell was purchased from MilliporeSigma (Burlington, MA). RAW-Lucia™ ISG (wild type), RAW-Lucia™ ISG-KO-RIG-I, RAW-Lucia™ ISG-KO-MDA5, HEK-Blue™ Null, HEK-Blue™ hTLR3, and HEK-Blue™ TLR7 were purchased from Invivogen (San Diego, CA). E.G7-OVA and B16F0-Luc were obtained from JCRB Cell Bank (Tokyo, Japan). Normal Human Dendritic Cell was purchased from Lonza Group AG (Basel, Switzerland). RPMI-1640 (Fujifilm Wako Chemicals, Osaka, Japan), DMEM (Sigma-Aldrich, St. Louis, MO), GM-3TM Lymphocyte Growth Medium-3 (LGM-3^{TM}) (Lonza), Opti-MEM Serum-Free medium (Thermofisher), PBS (Fujifilm Wako Chemicals, Osaka, Japan), FBS (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan), penicillin-streptomycin (Sigma-Aldrich), normocin (Invivogen), blasticidin (Invivogen), geneticin^{TM} selective antibiotic (G418 sulfate) (Gibco), granulocyte-macrophage colony-stimulating factor (GM-CSF) 200-15 (Shenandoah BioTech, Warminster, PA). Mercaptoethanol (Thermofisher), Glutamax^{TM} (Gibco), sodium pyruvate 100 mmol/L (Fujifilm), recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN), recombinant human interleukin 4 (IL-4) (R&D Systems), Alpha MEM with nucleosides (StemCell Technologies, Vancouver, Canada) were purchased for cell culture purposes. PE-conjugated anti-mouse CD40 (eBioScience™ 12-0401-81), CD86 (eBioScience™ 12-0862-81), MHC Class I (H-2Kb) Monoclonal Antibody (eBioscience™ 12-5958-82), MHC Class II (I-A) Monoclonal Antibody (NIMR-4) (eBioscience™ 12-5322-81) were purchased from Thermo Fisher Scientific. TruStain FcX™ anti-mouse CD16/32 antibody (Biolegend 101319), FITC anti-mouse CD86 antibody (Biolegend 105005), APC anti-mouse CD11c antibody (Biolegend 117309), human TruStain FcX^{TM} (Biolegend 422301), and PE anti-human CD86 antibody (Biolegend 305405) were purchased from Biolegend (San Diego, CA). Cell staining buffer (BioLegend 420201), Accutase (Sigma-Aldrich), ACK lysing buffer (Thermofisher), CellTrace™ CFSE Cell Proliferation Kit (Thermofisher), luciferin (Summit pharmaceuticals, Tokyo, Japan), IVT RNA kit, SIINFEKL (Thermofisher), Renilla Luciferase Assay System (Promega) Quanti-Luc (Invivogen), Secreted Embryonic Alkaline Phosphatase (SEAP) (Invivogen) were purchased for various cell processing and analysis experiments. Taqman primer/probe mixtures (Applied Biosystems) for mouse IL6 (FAM, Mm00446190), mouse INFB1 (FAM, Mm00439552_s1) and Mouse ACTB (Actin, Beta) Endogenous Control (FAM™ Dye/MGB probe, Non-Primer Limited) (Thermofisher) and Applied biosystems Taqman^{TM} Fast Universal PCR Master Mix (2x) No Amperase^{TM} (Applied Biosystem) were purchased for quantitative PCR experiments.

Mouse IL6 Duoset ELISA kit (R&D systems) and Mouse IFN-β Duoset ELISA kit (R&D systems) were purchased for ELISA experiments. C57BL/6J mice (7 weeks, female) were purchased from Oriental Kobo.

Preparation of comb-structured mRNA

Comb-structured mRNAs were prepared from 10 mM HEPES buffered solution of mRNA, cRNA, and 5'ppp-RNA composed at appropriate molar equivalents. The solution was heated at 65°C for 5 minutes using Takara® PCR Thermal Cycler (Takara Corp., Shiga, Japan), and then gradually cooled down to 30°C over a time span of 10 minutes before settling down to 4 °C. Successful hybridization of dsRNA teeth to mRNA was confirmed by quantifying unreacted Cy5-labeled dsRNA in the flow-through fraction after ultrafiltration. Namely, comb-structured mRNA composed of *OVA* mRNA, cRNA, and Cy5-labeled 5'ppp-RNA was prepared in the solution. Then, the solution was subjected to ultrafiltration using an ultra-0.5 centrifugal filter of MWCO 100K (Merck KGaA, Darmstadt, Germany) under centrifugation at 4 °C for 5 minutes at 10,000 G. The emission at 670 nm of the flow-through was measured using a TECAN plate reader (emission 651 nm) to detect Cy5.

Preparation of anionically-charged mRNA lipoplex

Anionically-charged mRNA lipoplex was prepared according to the previous report (1). First, a cationic liposome was prepared from DOTMA and DOPE (1:1 molar ratio, 6 mM total lipid concentration) using a thin-film hydration and sonication method. Next, mRNA or comb-structured mRNA was dissolved in water containing NaCl and mixed with the liposome suspension at a nitrogen/phosphate (N/P) ratio of 0.5 to obtain anionically-charged mRNA lipoplex with a final NaCl concentration of 150 mM.

Preparation of Lipofectamine LTX with Plus reagent/mRNA lipoplex

Lipofectamine LTX with Plus reagent/mRNA lipoplex was prepared according to the manufacturer's protocol. 3.5 μg of mRNA was dissolved in 350 μL of Opti-MEM® Reduced Serum Medium containing 3.5 μL of Plus™ reagent. This mRNA solution was then added to a 350 μL of Opti-MEM® Reduced Serum Medium containing 8.75 μL of lipofectamine® LTX reagent. Lipofectamine LTX with Plus reagent/comb-structured mRNA lipoplexes were prepared similarly, with the total RNA amount including mRNA, cRNA, and 5'ppp-RNA used for determining mixture ratio of RNA with lipofectamine® LTX and Plus™ reagents.

Preparation of ionizable lipid-based LNP

Ionizable lipid, ALC-0315 (MedChemExpress, Monmouth Junction, NJ), phospholipid, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, Fujifilm Wako, Osaka, Japan), cholesterol (Sigma Aldrich), and PEG-lipid, ALC-0159 (MedChemExpress) were dissolved in ethanol at the molar ratio of 46.3: 9.4: 42.7: 1.6 in molar ratio (total lipid concentration: 35 mM). mRNA was dissolved in 50 mM citrate buffer (pH 3) at the concentration of 459 μg/mL. mRNA and lipid solution were mixed at volume ratio of 2:1 using microfluidics (NanoAssemblr® Spark, Precision Nanosystems, Vancouver, Canada) to prepare LNP at N/P ratio of 6, followed by 40-fold dilution using PBS and ultrafiltration using Amicon Ultra-15-30K centrifugal units (Merck Millipore) for buffer replacement.

Preparation of mRNA polyplex micelles

A block copolymer of poly(ethylene glycol) (Mw: 44 kDa) with poly(N-[N'-(2-aminoethyl)-2-aminoethyl]aspartamide) [PEG-PAsp(DET)] was synthesized as previously reported (2). The polymerization degree of the PAsp(DET) segment was determined to be 59 from the ^{1}H-NMR (400

MHz, JEOL ECS- 400, JEOL, Tokyo, Japan) measurement. PEG-PAsp(DET) was dissolved in 10 mM HEPES buffer. This solution was mixed with mRNA in the same buffer at an N/P ratio of 5 to obtain polyplex micelles (PM). The final concentration of mRNA or comb-structured mRNA was fixed to 300 μg/mL.

Hydrodynamic diameter (D_h) and Zeta potential determination by Zetasizer

DLS measurement was conducted by Zetasizer Nanoseries (Malvern Instruments Ltd., Malvern, UK) to measure D_h of lipoplex and polyplex micelle formulations of *OVA* mRNA with and without dsRNA teeth. The measurement was performed at a detection angle of 173° and a temperature of 25°C. The refractive index (RI) was set to 1.45 (RI of phospholipid) for lipoplex and ionizable lipid-based LNP and 1.59 (RI of polystyrene) for polyplex micelle, respectively. The D_h was obtained by treating the decay rate in the photon correlation function by the cumulant method and applying the Stokes-Einstein equation. The zeta potential was determined by laser-doppler electrophoresis using the same instrument. According to the obtained electrophoretic mobility, the zeta potential of each sample was calculated according to the Smoluchowski equation: $\zeta = (4\pi\eta\mu)/\varepsilon$, where η is the viscosity of the solvent, μ is the electrophoretic mobility, and ε is the dielectric constant of the solvent.

Evaluation of proinflammatory cytokine transcripts induced by comb-structured mRNA in DC2.4 cells

DC2.4 cells were seeded onto 24-well plates at a 2 x 10^5 cells/well density. 24 h later, the culture medium was replaced with serum-free Opti-MEM medium (500 μL) and added with 0.5 μg of *gLuc* mRNA with or without teeth formulated with either Lipofectamine LTX and Plus reagents, anionic liposome, or in vivo-jetPEI® (polyplus transfection, Illkirch, France). mRNA/in vivo-jetPEI® was prepared according to the manufacturer's protocol. In **Figures 2E, F**, **Supplementary Figure S3**, a tooth was annealed to mRNA in "1 tooth" but not annealed in "separate 1 tooth". After 4 h of incubation, cellular mRNA was extracted using RNeasy® Mini Kit (Qiagen N.V., Düsseldorf, Germany), converted to complementary DNA (cDNA) using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo Inc., Osaka, Japan) for quantitative real-time PCR. This experiment used 1,000 ng/mL (500 ng/well) of *gLuc* mRNA and 1,000 ng/mL (500 ng/well) of poly I:C. Tooth RNA doses are 85 ng/mL (43 ng/well) for 24-bp tooth and 126 ng/mL (63 ng/well) for 40-bp tooth in **Figure 2A, B**, 85 ng/mL (43 ng/well) for 2-nt tooth and 96 ng/mL (48 ng/well) for 10-nt tooth in **Figure 2C, D**, 85 ng/mL (43 ng/well) for 1 tooth, separate 1 tooth, and tooth only in **Figure 2E, F**, **Supplementary Figure S3** and 85 ng/mL (43 ng/well) for 1 tooth, 256 ng/mL (128 ng/well) for 3 teeth, and 427 ng/mL (214 ng/well) for 5 teeth in **Figure 2G, H**.

Evaluation of SIINFEKL presentation on H-2Kb of DC2.4

DC2.4 cells was transfected with Lipofectamine LTX with Plus reagent/mRNA lipoplex. The presentation of SIINFEKL on DC2.4's H-2Kb was followed over time by flow cytometry (Cell Analyzer LSRFortessa X-20, Becton Dickinson, Franklin Lakes, NJ, USA) after staining the cells with PE anti-mouse H-2kb bound to SIINFEKL (Biolegend 141603).

Probing of RIG-I and MDA-5 involvement to immunogenicity using RAW-Lucia™ ISG Cells (IFN Reporter RAW 264.7 macrophages)

OVA mRNA (100 ng) with or without teeth formulated with Lipofectamine LTX and Plus reagents in 20 μL Opti-MEM medium was added to each well of 96 plates. Then, RAW-Lucia ISG cells (100,000 cells/well) suspended in 180 μL of DMEM containing 10% FBS and 100 μg/mL penicillin streptomycin

(PS) were added to each well. Of note, RAW-Lucia™ ISG (wild type), RAW-Lucia™ ISG-KO-RIG-I, and RAW-Lucia™ ISG-KO-MDA5 cells were cultured in DMEM containing 10% FBS, 100 μg/mL PS, and 100 μg/mL normocin prior to maintain genetic stability of these transgenic cell lines. In this procedure, cells were incubated with 500 ng/mL of mRNA. Tooth RNA doses were 43 ng/mL for 1 tooth, 128 ng/mL for 3 teeth, and 214 ng/mL for 5 teeth. After 4 h, the medium was collected to measure lLuc expression.

Probing of hTLR3 and TLR7 involvement in immunogenicity using hTLR3-Blue-HEK and TLR7-Blue-HEK293

HEK-Blue™ Null, HEK-Blue™ hTLR3, and HEK-Blue™ TLR7 cells were cultured in a selection medium according to the manufacturer's protocol. *gLuc* mRNA (100 ng) with or without teeth formulated with Lipofectamine LTX and Plus reagents in 20 μL Opti-MEM medium was added to each well of 96 plates. HEK-Blue™ hTLR3 cells (50,000 cells/well) or HEK-Blue™ TLR7 (40,000 cells/well) and HEK-Blue™ Null (50,000 cells/well) were suspended in 180 μL of SEAP medium, respectively, and were added to each well. In this procedure, cells were incubated with 500 ng/mL of mRNA. Tooth RNA doses were 43 ng/mL for 1 tooth, 128 ng/mL for 3 teeth, and 214 ng/mL for 5 teeth. After 16 h of incubation, the plates were examined for absorbance at 630 nm using a TECAN microplate reader. Relative absorbance values are shown in **Figure 5B**.

Gene expression, surface antigen presentation, and cytokines production in primary BMDCs

Primary BMDCs were obtained according to a previous report (3). In brief, the cells were flushed out from the tibia and femur bone marrow of C57BL/6J. The cells were cultured at 37°C in 5% CO_2 in an R10 culture medium containing granulocyte-macrophage colony-stimulating factor (GM-CSF). On day 8, the floating cells in the medium were collected. 400,000 cells suspended in 2 μL of R10 medium was added to each well of a 12-well plate and transfected on the same day with 1 μg/well of *gLuc* mRNA formulated with Lipofectamine LTX and Plus reagents. 10 μL of the medium at several time points was collected for gLuc expression assay using Renilla Luciferase Assay System (Promega) and a luminometer, GloMax-Multi+ Detection System (Promega). After 24 h, both floating and attaching cells were collected, washed, blocked with anti-mouse CD16/31 antibody (1/100 dilution in cell staining buffer), and stained with PE-conjugated antibody mouse CD40 (0.005 mg/mL), CD86 (0.005 mg/mL), MHCI (0.007 mg/mL) and MHCII (0.003 mg/mL) for 20 min on ice. The immunostained cells were then washed, stained with DAPI, and examined by flow cytometry (Cell Analyzer LSRFortessa X-20). In another batch of the experiment, the medium from the transfected cells was collected to quantify the expression of immune molecules at protein levels. Luminex® Multiplex Assays (ThermoFisher Scientific) was performed to quantify expression levels of 24 molecules, including G-CSF, IFN-γ, IL-1α, 1β, 2, 4, 5, 6, 7, 9, 10, 12p40, 12p70, 13, 15, 17, TNF-α, CXCL1, 2, 10, CCL2, 3, 4, 5. Expression of IFN-*β* was quantified using Mouse IFN-*β* Duoset ELISA kit.
In these experiments, cells were incubated with 1,000 ng/mL of mRNA and 1,000 ng/mL of poly IC. Tooth RNA doses were 85 ng/mL (85 ng/well) for 1 tooth, 256 ng/mL (256 ng/well) for 3 teeth, and 427 ng/mL (427 ng/well) for 5 teeth.

Human DC

Human dendritic cell purchased from Lonza was seeded to 12-well plates at 160,000 cells/well containing 2 mL of medium. Cells were transfected with mRNA (0.5 μg/well) using Lipofectamine LTX and Plus reagent. Adherent and non-adherent cells were harvested after 24h for immunostaining. In this procedure, cells were incubated with 500 ng/mL of mRNA and 500 ng/mL of poly IC. Tooth RNA

doses were 43 ng/mL for 1 tooth, 128 ng/mL for 3 teeth, and 214 ng/mL for 5 teeth. Adherent cells were detached from the plate using 400 μL of accutase solution for 5 min, and the reaction was stopped using a culture medium. All cells were centrifuged at 500 g for 5 min at 4°C. Cells were blocked with 100 μL of human TruStain FcX Fc receptor blocking solution (1/250 dilution in cell staining buffer) and kept on ice for 10 min. Then, cells were stained with 100 μL of human PE-CD86 solution (BioLegend) (5 μg/mL) and kept on ice for 20 minutes. After two rounds of washing using 500 μL of cell staining buffer, cells were reconstituted with 500 μL of staining buffer. The cells were analyzed by flow cytometry using BD LSRFortessa™ X-20.

Expression of luciferase mRNA in mice

C57BL/6J mice (7 weeks, female) were intravenously injected with 5 μg/mouse of *fLuc* mRNA formulated with lipoplex. After 24 h, the spleen was extracted for measuring fLuc expression using IVIS Spectrum SP-BFM-T1 (Perkin Elmer) after an intraperitoneal injection of 3 mg of luciferin. fLuc IVIS experiment was also conducted for mice injected with ionizable lipid based LNPs via *i.m.* route, and mRNA PMs *via i.d.* and *i.m* routes (1 μg mRNA/mouse). All animal experiments were conducted under the approval of the animal care and use committees in the Innovation Center of NanoMedicine, Kawasaki Institute of Industrial Promotion (Kanagawa, Japan), and Kyoto Prefectural University of Medicine (Kyoto, Japan).

Examination of surface antigen presentation of dendritic cells in vivo

C57BL/6J mice were intravenously injected with 5 μg of *OVA* mRNA/mouse formulated with lipoplex. After 24 h, splenocytes were extracted from spleens of donor C57BL6 mice and added with ACK lysing buffer to destroy the red blood cells. 5×10^6 cells were taken and centrifuged at 400 G for 5 min at 4 °C, and then resuspended in cell staining buffer. The cells were later blocked with 10 μg/mL CD16/32 antibody in 100 μL solution and kept on ice for 15 min. Thereafter, the cells were stained with a 100 μL mixture of FITC-CD86 (10 μg/mL) and APC-CD11c (25 μg/mL) antibody in cell staining buffer for 20 min on ice. The cells were washed 3 times and then resuspended in 1 mL of cell staining buffer for examination using flow cytometry.

In vivo cytotoxic T lymphocyte (CTL) assay

C57BL/6J mice were vaccinated by intravenous (*i.v.*) injection of 5 μg of *OVA* mRNA/mouse formulated with lipoplex. After 7 days, *in vivo* CTL assay was performed by following a previous protocol (4). In brief, splenocytes were extracted from donor C57BL6 mice and added with ACK lysing buffer to destroy the red blood cells. The remaining cells were washed and divided into two groups. One group was treated with OVA257-264 peptide (SIINFEKL, Toray, Tokyo, Japan) for 1 h in an incubator (37 °C, CO_2 5%), and later dyed with 5 μM CFSE (CellTrace™ CFSE Cell Proliferation Kit - For Flow Cytometry, Molecular Probes, Eugene, OR) (1 mL PBS containing CFSE 5 μM per 3×10^7 splenic cells) for 10 min in a water bath at 37 °C. The other group was left non-treated, incubated for 1 h (37 °C, CO_2 5%), and later dyed with 0.5 μM CFSE (1 mL PBS containing CFSE 5 μM per 3×10^7 splenocytes) for 10 min in a water bath at 37 °C. Both groups were concentrated to 5×10^7 splenocytes per 1 mL PBS and placed on an ice bath before mixing prior to the injection into mice. The splenocytes (1×10^7 cells in 200 μL of suspension) were intravenously injected into vaccinated mice. One day later, the vaccinated mice were sacrificed to check the viability of the injected CFSE-labeled donor splenocytes by flow cytometry (BD LSRFortessa™ X-20).

For iLNP vaccines, C57BL6 mice were vaccinated via *i.m.* injection of 50 μL iLNP solution containing indicated mRNA amount. Seven days after the vaccination, *in vivo* CTL assay as performed as described above.
For mRNA-PM vaccines, C57BL6 mice received the vaccine twice at the interval of 2 weeks with *OVA* mRNA dose set to be 20 μg/mouse for *i.m.* vaccination, and 12 μg/mouse for *i.d.* vaccination, in each of the prime and boost vaccination. In the *i.m.* vaccination, 20 μg mRNA formulated in PM in 100 μL solution was divided into two and injected into the thigh muscle on both left and right sides. In *i.d.* vaccination, 12 μg mRNA formulated in PM in 50 μL solution was divided into five and injected at 5 locations in the dermis of the back of the mouse. On day 6, post-booster dose, mice were subjected to *in vivo* cytotoxicity assay as described above.

Evaluation of CD4 and CD8+ T cells presenting OVA epitopes
C57BL/6J mice were vaccinated one time with i.v. injection of 5 μg of *OVA* mRNA with or without teeth formulated with anionic lipoplex. After 7 days, spleen of mice was excised and extracted for splenocytes. CD4 and CD8 + T cells was collected using CD8+ T and CD4+ T Cell Isolation Kit, mouse (Miltenyi Biotech, Bergisch Gladbach, North Rhine-Westphalia, Germany). Collected CD8+ cell and CD4+ T cells were blocked with TruStain FcX™ anti-mouse CD16/32 antibody and stained by T-select H-2Kb OVA tetramer-SIINFEKL-PE (Medical & Biological Laboratories Co., Ltd, Tokyo Japan) and T-select I-Ab OVA323-339 tetramer- PE (Medical & Biological Laboratories Co., Ltd), respectively. The cells were rinsed using copious volume of cell staining buffer. The percentage of tetramer positive cells over the whole isolated cell population was evaluated using flow cytometry (BD LSRFortessa™ X-20).

Safety analyses
Plasma was collected 24 h after systemic injection of anionic lipoplex for blood chemistry measurement using a DRI-CHEM 7000i (Fujifilm, Tokyo, Japan). For pathological analyses, organs were fixed in 4% PFA, incubated in PBS for 24 h, and immersed in paraffin for sectioning at 4 μm thickness. The sections were stained with hematoxylin and eosin (H&E) for observation using the optical microscope.

Prophylaxis tumor suppression study against subcutaneously implanted lymphoma model
C57BL/6J mice were vaccinated one time with *i.v.* injection of 5 μg of *OVA* mRNA with or without teeth formulated with anionic lipoplex. After 7 days, mice were subcutaneously implanted with 8×10^5 E.G7-OVA cells. The tumor growth was followed for 2 months. The tumor volume was calculated by the formula $(1/2 \times \text{long axis} \times (\text{short axis})^2)$.

Therapeutic tumor suppression study against subcutaneously implanted lymphoma model
C57BL/6J mice were subcutaneously implanted with 1×10^6 E.G7-OVA cells. The therapeutic intervention was started 6 days after the inoculation when the tumor grew to an average size of 185 mm^3 for each group. On this day, and 8 and 15 days later, mice were vaccinated with *i.v.* injection of 5 μg of *OVA* mRNA with or without teeth formulated with anionic lipoplex. The tumor growth was followed as described in the previous section.

Therapeutic tumor suppression study against subcutaneously implanted melanoma model
DNA codon-optimization from Trp2 amino acid sequence (Accession: NP_034154.2) and preparation of plasmid DNA possessing *Trp2* coding sequence under T7 promoter were performed by Genscript Japan (Tokyo, Japan). In PCR-based template DNA preparation from the plasmid by PCR, 80 bp poly A/T

sequence was integrated using a primer possessing 80 nt poly T. IVT from the template was performed using mMessage mMachine T7 kit. C57BL/6J mice were subcutaneously implanted with 2×10^5 B16F0-Luc cells. The therapeutic intervention was started 13 days after the inoculation when the tumor grew to an average size of 30 mm^3 for each group. On this day, and 4, 7, and 11 days later, mice were vaccinated with *i.v.* injection of 10 μg of *Trp2* mRNA with or without teeth formulated with anionic lipoplex.

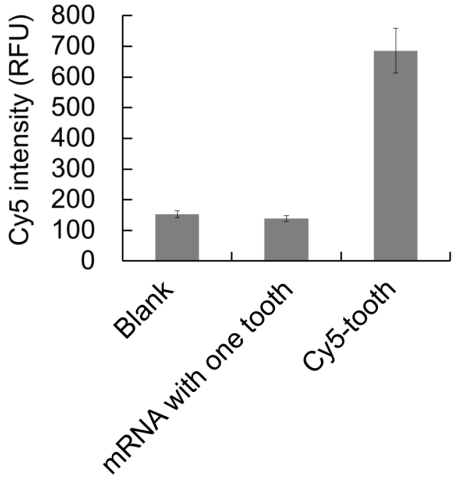
Safety assay by examining IL-6 and IFN-β content in serum

C57BL/6J mice were intravenously injected with 5 μg mRNA with or without teeth formulated in anionic lipoplex. After 6 and 24 hours, mice were sacrificed to obtain serum. The serum was examined for IL-6 and IFN-β content using ELISA.

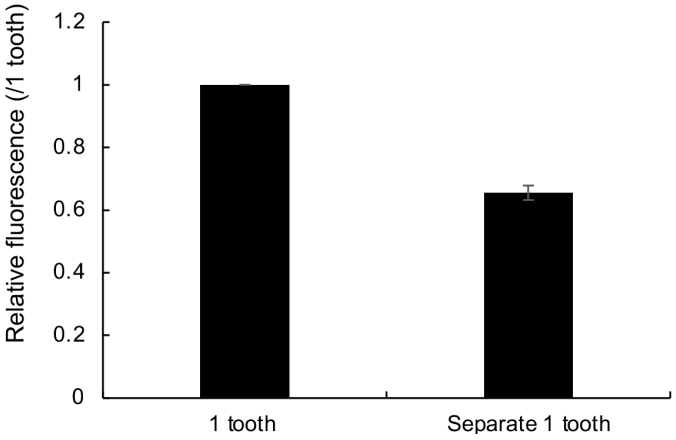
Data presentation

Data are presented as mean ± standard error of the mean (SEM). In **Figures 4B,F,G, 7** statistical analyses are performed using unpaired two-tailed Student's t-test. In **Figures 2, 4C,D, 5, 6, S3, S5, S6, S9, S10** statistical analyses are performed using analysis of variance (ANOVA) followed by Tukey's post hoc test.

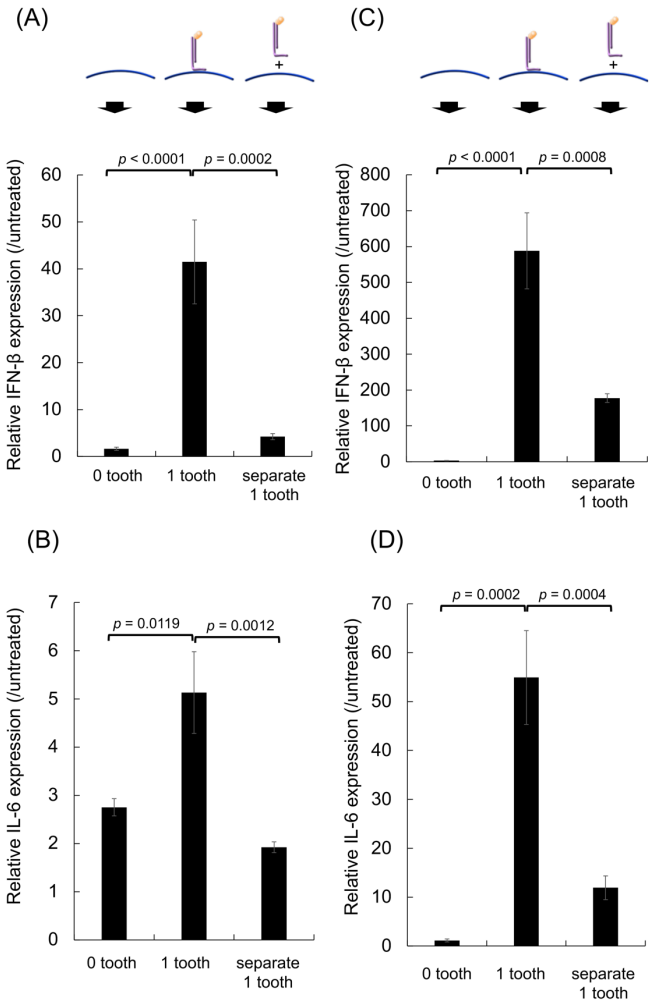
Supplementary Figures



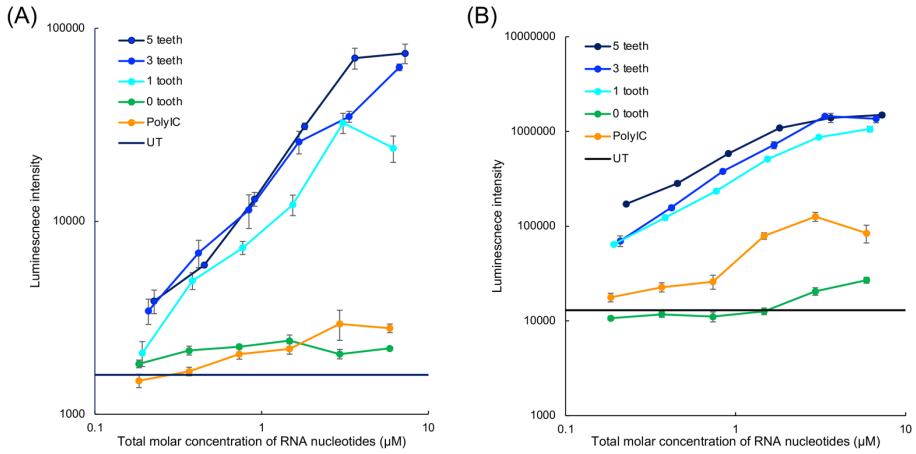
Supplementary Figure S1. Hybridization of the immunostimulatory tooth to mRNA. Comb-structured mRNA with one tooth was prepared from Cy5-labeled 24 nt RNA (GGUGUGUGUGUGUGUGUGUGUG), 43 nt cRNA (with gap sequence of 2 nt) (GGUUCAGGAUGUCCCGCUUCACACACACACACACACACACACC), and *OVA* mRNA (Cy5-tooth + mRNA). After its ultrafiltration with a filter of MWCO 100K, the fluorescence intensity of the flow-through was measured. Cy5-tooth from Cy5-labeled 24 nt 5'ppp-RNA and 43 nt cRNA was used as a control (Cy5-tooth). $n = 4$.



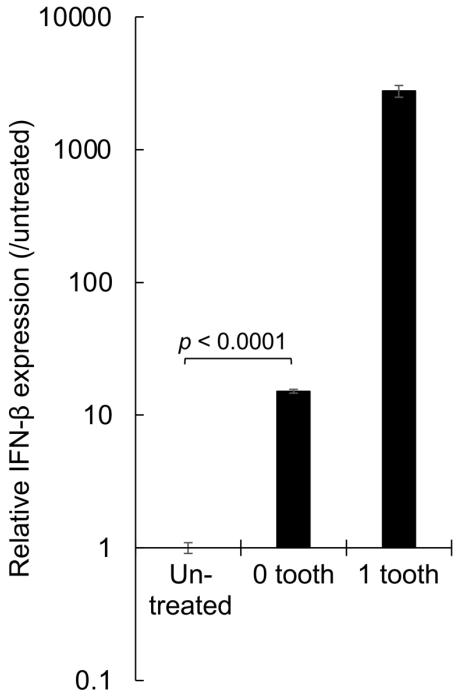
Supplementary Figure S2. Cellular uptake efficiency of dsRNA tooth. mRNA and dsRNA tooth prepared from Cy5-labelled 24 nt GU repeat RNA was introduced into DC2.4 cells in annealed and non-annealed formulations using Lipofectamine LTX. The uptake efficiency of the dsRNA tooth was quantified using flow cytometry 4 h after mRNA introduction. $n = 4$.



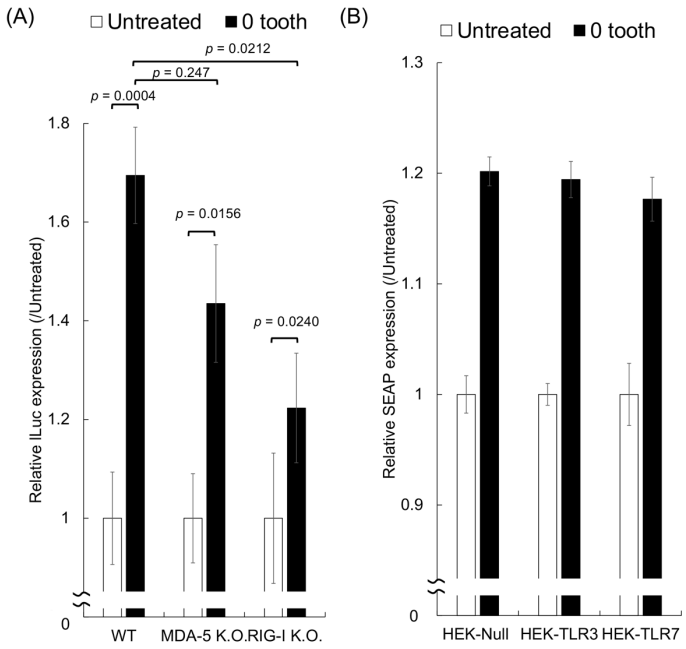
Supplementary Figure S3. Comparison between annealed and non-annealed formulations of 24 nt GU-repeat tooth and mRNA. DC2.4 cells were introduced with mRNA alone (0 tooth), mRNA annealed with 24-nt GU-repeat tooth (1 tooth), and a mixture of mRNA and 24-nt GU-repeat (separate 1 tooth). Transcript levels of interferon (IFN)-β (A, C) and interleukin (IL)-6 (B, D) were measured using quantitative PCR 4 h after mRNA introduction. (A, B) Anionic lipoplex. (C, D) LPEI. $n = 6$.



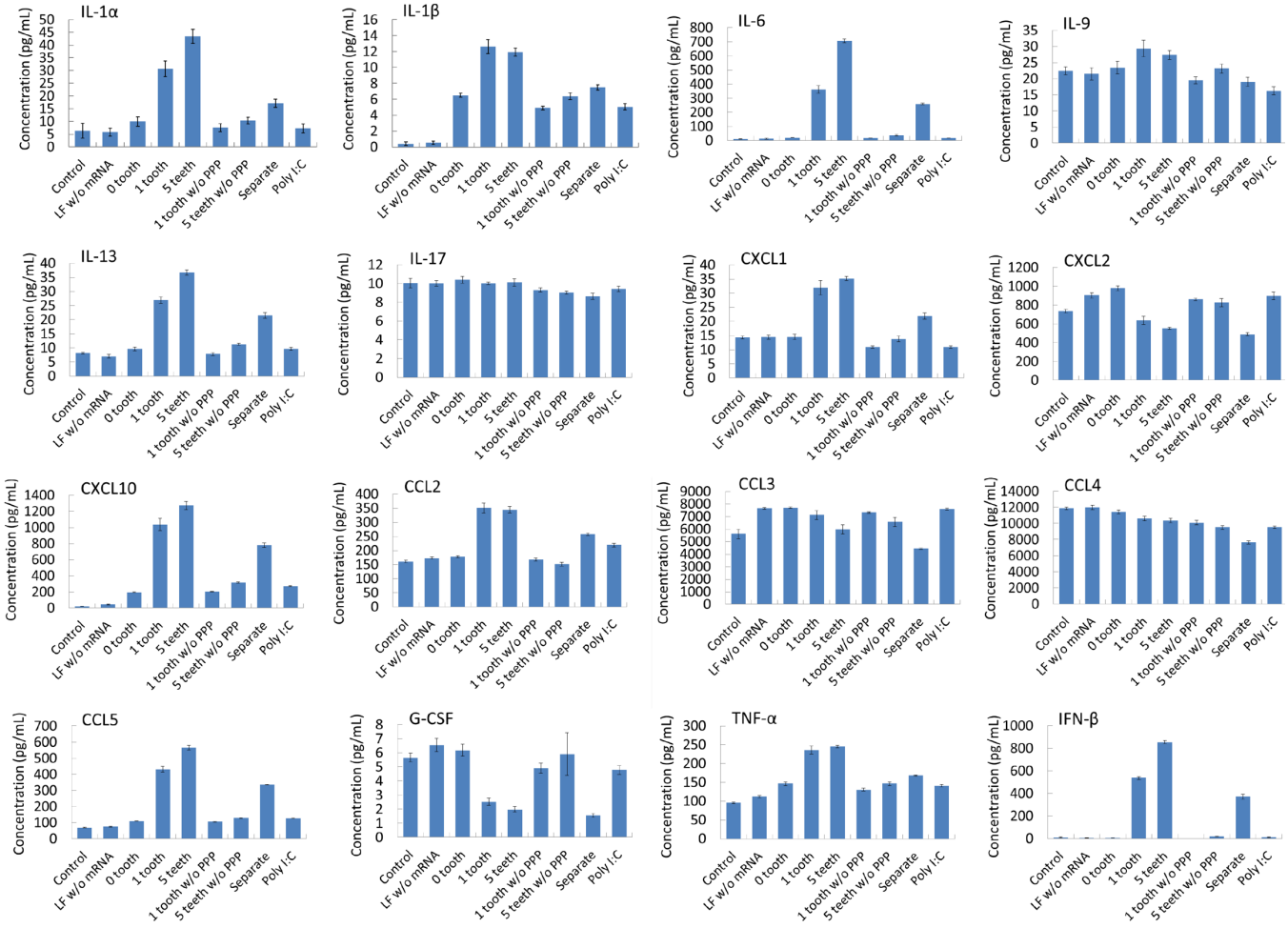
Supplementary Figure S4. Dose-response curves of immunostimulation by comb-structured mRNA. Comb-structured mRNA was added to RAW-Lucia reporter cells. The cells are genetically modified to express Lucia luciferase (lLuc) responding to proinflammatory stimuli under the promoter responsive to interferon regulatory factors. Immunostimulation intensity of each formulation was quantified (A) 4 h and (B) 24 h after mRNA treatment based on lLuc expression. The horizontal axis shows molar concentration of total nucleotides (μM), including mRNA and teeth. $n = 4$.



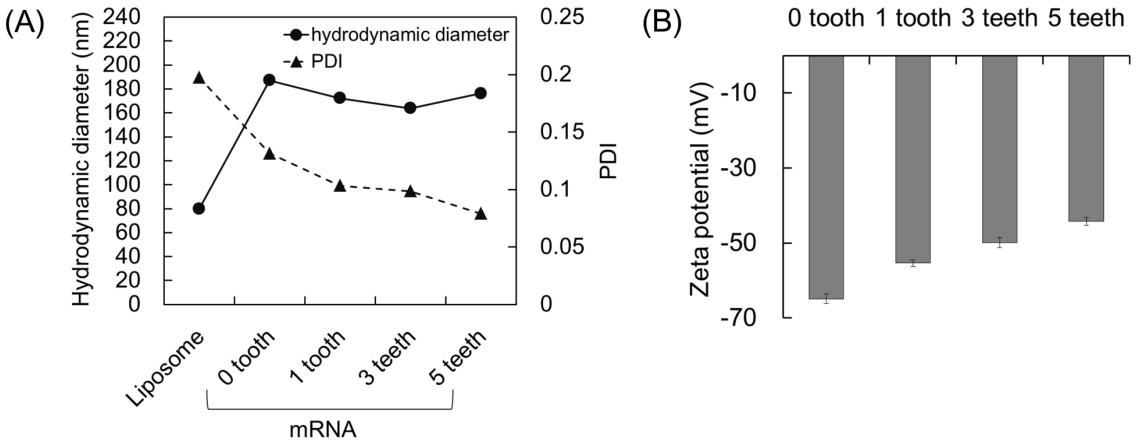
Supplementary Figure S5. Type I interferon responses to mRNA with and without a tooth. *Interferon (IFN)-β* transcript level was measured using quantitative PCR 4 h after mRNA introduction to DC2.4 cells. The vertical axis of Figure 2A is converted to a log scale. $n = 5$.



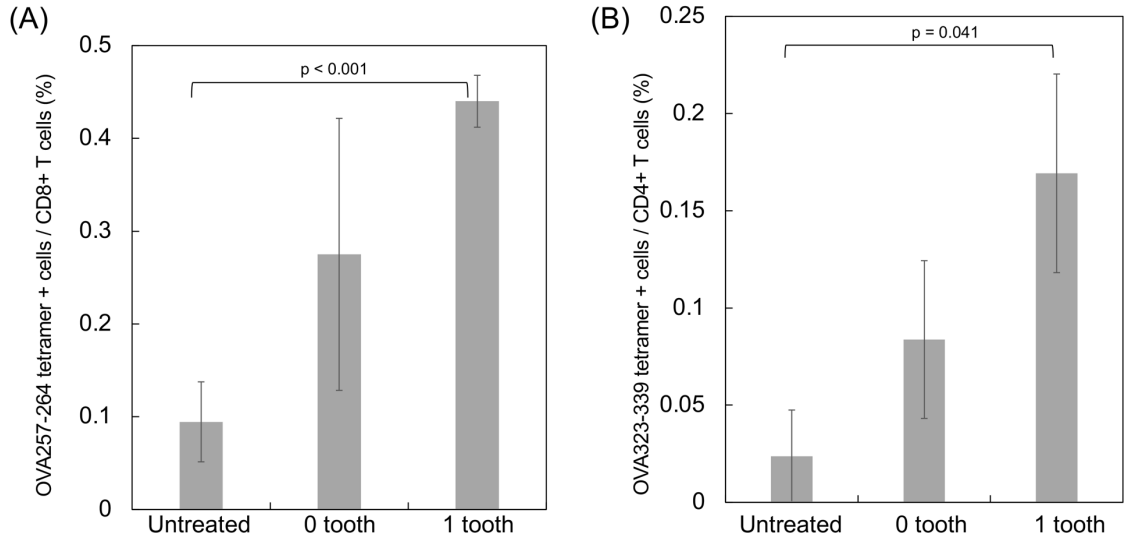
Supplementary Figure S6. Contribution of innate immune receptors for immunostimulation by mRNA without tooth. (**A**) Comb-structured mRNA was added to RAW cells without knockout of immune receptors (WT) and with *MDA-5* or *RIG-I* knockout (MDA-5-KO, RIG-I-KO). The cells were genetically modified to express Lucia luciferase (lLuc) after proinflammatory stimulation for quantifying immunostimulation intensity based on lLuc expression. lLuc amount was measured 24 h after mRNA addition. The same data as used in Figure 6J, K was used in wt and RIG-I K.O. cells. $n = 6$. (**B**) Comb-structured mRNA was added to HEK 293 cells, genetically modified to express TLR3 or TLR7, or without transformation to express TLRs (Null). The cells were transformed to express secreted embryonic alkaline phosphatase (SEAP) reporter after nuclear factor-κB (NF-κB) stimulation. mRNA dose was 5-fold higher than in Figure 5B (2.5 µg/mL, 500 ng/well in a 96-well plate). $n = 6$. Abbr.: PPP, 5' triphosphate. **, $p < 0.01$; ***, $p < 0.001$, n.s., non-significant versus untreated, respectively.



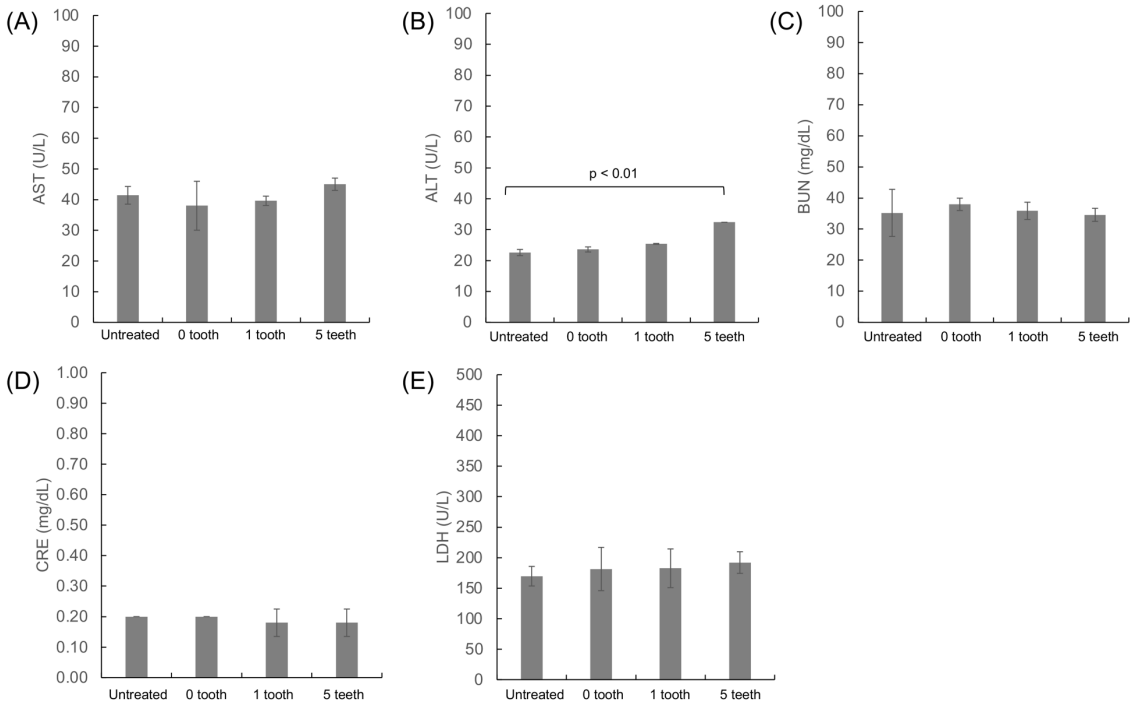
Supplementary Figure S7. Immunological profiling of BMDCs after mRNA treatment. The protein expression profile, shown in **Figure 3**, was presented as the absolute concentration of each protein in the cultured medium. $n = 6$.



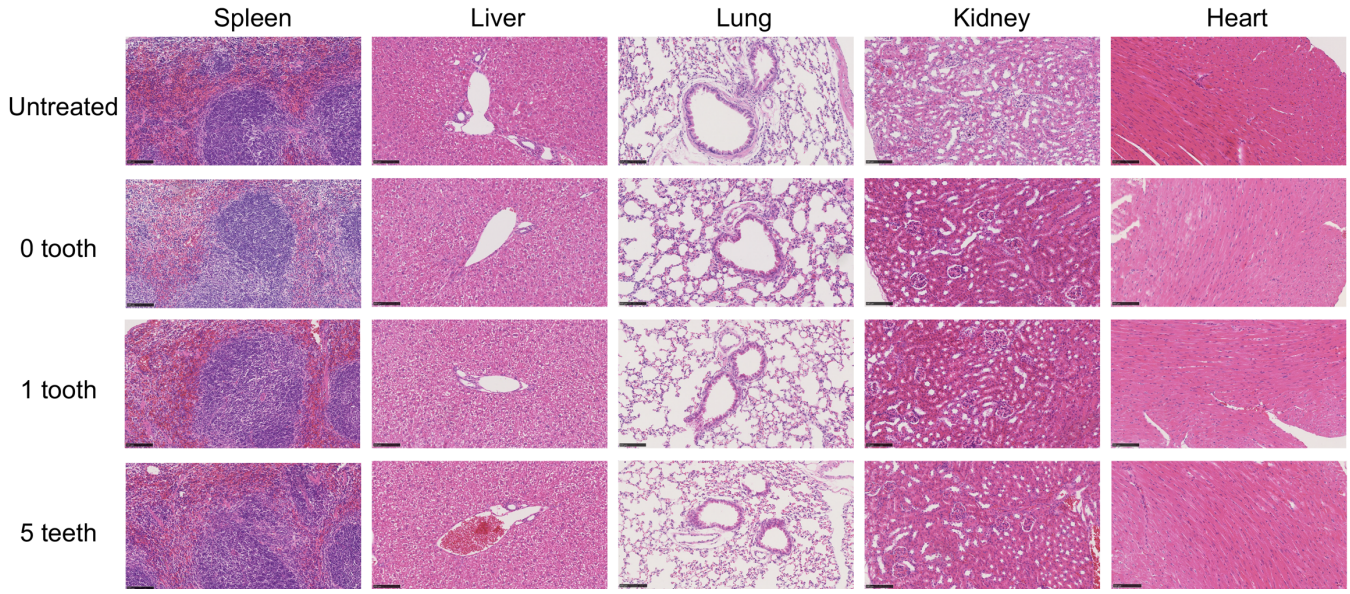
Supplementary Figure S8. Physicochemical characterization of lipoplexes. (**A**) Size and (**B**) ζ-potential of liposome alone and lipoplex loading mRNA with or without immunostimulatory teeth. n = 4.



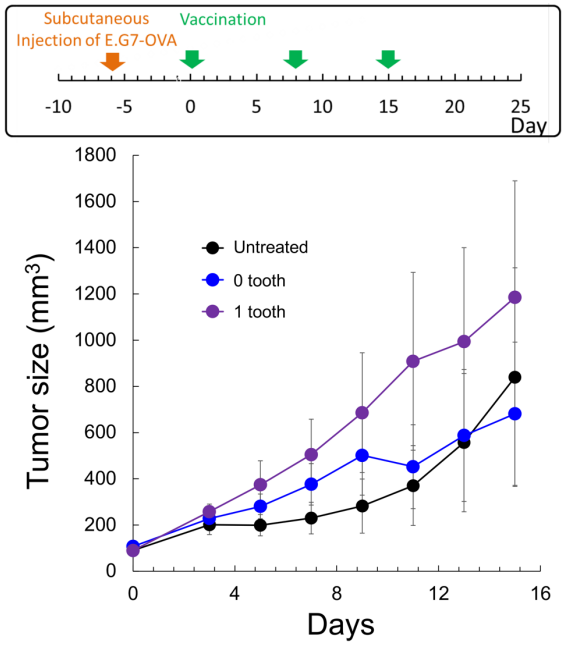
Supplementary Figure S9. MHC/peptide tetramer staining for quantifying antigen-responsive CD8- and CD4-positive splenocytes. Mouse splenocytes were collected 7 d after intravenous administration of *OVA* mRNA lipoplex, followed by separation of CD8- and CD4-positive splenocytes. Then, the cells reactive to OVA epitopes presented on MHC were stained with fluorescent MHC/peptide tetramer for quantification using flow cytometry. n = 4.



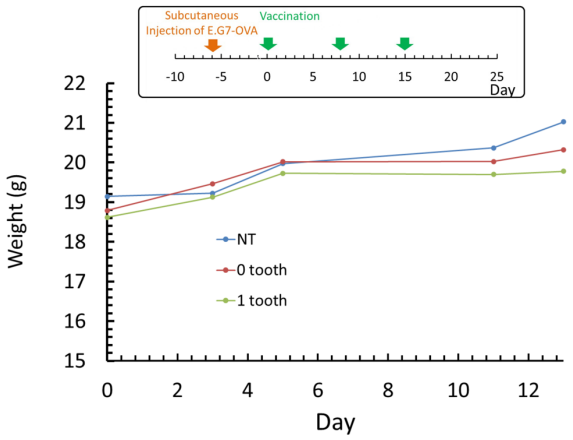
Supplementary Figure S10. Blood biochemistry examination 24 h after systemic injection of anionic lipoplex. (A) Aspartate aminotransferase. (B) Alanine aminotransferase (ALT). (C) Blood urea nitrogen (BUN). (D) Creatinine (CRE). (E) Lactate dehydrogenase (LDH). $n = 5$.



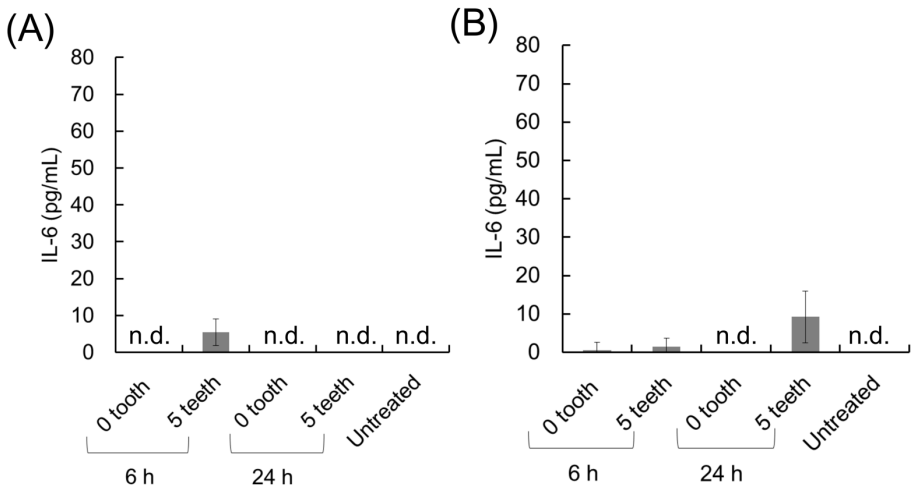
Supplementary Figure S11. Histological observation 24 h after systemic injection of anionic lipoplex. Scale bars: 100 μm.



Supplementary Figure S12. Cancer therapeutic vaccination using control mRNA. After subcutaneous inoculation of lymphoma expressing OVA, the mice were treated with *luciferase* mRNA (1929 nt, Trilink) without tooth and with one tooth. The same tooth dose of 0.23 μg/mouse as in the cancer experiment using *OVA* mRNA (1438 nt) (Figure 4F) and the mRNA dose of 6.7 μg/mouse were used in this experiment. $n = 6$.



Supplementary Figure S13. Weight change in therapeutic cancer vaccination experiment.



Supplementary Figure S14. Safety analyses of PMs in mice. Serum levels of IL-6 were measured using ELISA 6 h and 24 h after *i.m.* (**A**) and *i.d.* (**B**) injection of PMs. $n = 4$. Note that INF-β was undetected in mouse serum at both time points.

Supplementary Tables

Supplementary Table S1 Sequences of 5'ppp-RNA and DNA template for 5'ppp-RNA preparation.

i) 5'ppp-RNA

	Sequences
24 mer GU-repeat	GGUGUGUGUGUGUGUGUGUGUG
40 mer GU-repeat	GGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUG
24 mer random	GGUGUGCCUUCUUGGCGUGCGGUG
40 mer random	GGUCCUGGCUUGCCUGUUCCUUCUUCCGUCCGCUCCGUCG

(ii) DNA template

	Sequences
24 mer GU-repeat	TAATACGACTCACTATAGGTGTGTGTGTGTGTGTGTGTGTG
40 mer GU-repeat	TAATACGACTCACTATAGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
24 mer random	TAATACGACTCACTATAGGTGTGCCTTCTTGGCGTGCGGTG
40 mer random	TAATACGACTCACTATAGGTCCTGGCTTGCCTGTTCCTTCTTCCGTCCGCTCCGTCG

Supplementary Table S2 Sequence of cRNA.

(i) *gLuc* cRNA with 2 nt gap sequence

Location*	Sequence
60	CAAACAGAACUUUGACUAACACACACACACACACACACACC
213	CUUUGAGCACCUCCAGCAACACACACACACACACACACACC
244	CAGCCAGCUUUCCGGGCUACACACACACACACACACACACC
345	ACUCUUUGUCGCCUUCGAUCACACACACACACACACACACC
525	GCGGCAGCCACUUCUUGUACACACACACACACACACACACC

* Locations of the first bases in mRNA hybridized with cRNA are presented as nucleotide numbers from the 5' end. The cRNAs used hybridize to a location 60 in mRNA for 1-tooth mRNA, locations 60, 244, and 525 for 3-teeth mRNA, and locations 60, 213, 244, 345, and 525 for 5-teeth mRNA.

(ii) *gLuc* cRNA with 10 nt gap sequence

Location*	Sequence
244	CAGCCAGCUUUCCGGGCUAAAAAAAAACACACACACACACACACA CACACC

* Locations of the first bases in mRNA hybridized with cRNA are presented as nucleotide numbers from the 5' end.

(iii) *fLuc* cRNA

Location*	Sequence
316	UCGUUGUAGAUGUCGUUUUCACACACACACACACACACACACC
401	CACGUUCAGGAUCUUCUUUCACACACACACACACACACACACC
694	AUGGCGGUGUCGGGGAUUUCACACACACACACACACACACACC
882	GGGUGCUCUUGGCGAAGUUCACACACACACACACACACACACC
1057	UUGUCGUCGCCCUCGGGUUCACACACACACACACACACACACC

* Locations of the first bases in mRNA hybridized with cRNA are presented as nucleotide numbers from the first base of the start codon. The cRNAs used hybridize to a location 316 for 1-tooth mRNA and locations 316, 401, 694, 882, and 1057 for 5-teeth mRNA.

(iv) *OVA* cRNA

Location*	Sequence
252	GGUUCAGGAUGUCCCGCUUCACACACACACACACACACACACC
332	GGGCAGGAUGGGGUACCUUCACACACACACACACACACACACC
563	GUCCUCGUCCUUGAAGGAUCACACACACACACACACACACACC
777	GCUUCUCGAAGUUGAUGUACACACACACACACACACACACACC
1103	GGCGAUGUGCUUGAUGCUUCACACACACACACACACACACACC

* Locations of the first bases in mRNA hybridized with cRNA are presented as nucleotide numbers from the first base of the start codon. The cRNAs used hybridize to a location 252 for 1-tooth mRNA, locations 252, 563, and 1103 for 3-teeth mRNA, and locations 252, 332, 563, 777, and 1103 for 5-teeth mRNA.

(v) *gp100* cRNA

Location*	Sequence
223	GUCGUUGAUCACUCUCAUUCACACACACACACACACACACACC
336	UUGAUGAUUGUGUUGUUUUCACACACACACACACACACACACC
462	CACACGUACACGAAGCUUUCACACACACACACACACACACACC
598	CACGUAAGACUGGGAUCUUCACACACACACACACACACACACC
703	CAGGAAGUGCUUGGUCUUUCACACACACACACACACACACACC

* Locations of the first bases in mRNA hybridized with cRNA are presented as nucleotide numbers from the 5' end. The cRNAs used hybridize to a location 223 for 1-tooth mRNA and locations 223, 336, 462, 598, and 703 for 5-teeth mRNA.

Supplementary Table S3 Dynamic light scattering of iLNPs.

Tooth number	0	1
Size (d, nm)	88.4 ± 0.3	86.3 ± 0.4
Polydispersity index (PDI)	0.18 ± 0.01	0.20 ± 0.01

Supplementary Table S4 Dynamic light scattering of PMs.

Tooth number	0	1	5
Size (d, nm)	78.5 ± 0.8	74.5 ± 0.2	76.4 ± 0.6
Polydispersity index (PDI)	0.23 ± 0.01	0.21 ± 0.00	0.24 ± 0.01

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