PNAS
www.pnas.org

Supplementary Information for

Delivery of mRNA vaccine with a lipid-like material potentiates antitumor efficacy through Toll-like receptor 4 signaling.

Authors: HongxiaZhang[1†], XinruYou[2†], Xiaojuan Wang[1], Lei Cui[1], Zining Wang[1], Feifei Xu[1], Mengyun Li[1], Zhenggang Yang[3], Jinyun Liu[1], Peng Huang[1], Yang Kang[2], Jun Wu[1,2*], Xiaojun Xia[1*].

*Corresponding author: Xiaojun Xia and Jun Wu.

E-mail: xiaxj@sysucc.org.cn; wujun29@mail.sysu.edu.cn.

This PDF file includes:

Supplementary text
Figures S1 to S8
Table S1

Supplementary Text

Materials and Methods

***In vitro* protein expression of C1-mRNA nanovaccine**

Briefly, DC2.4 cells were seeded in dish (35 mm Glass bottom dish with 10 mm micro-well, Corning) at a density of 5×10^4 cells/well and cultured overnight. Cells were transfected with 400 ng mRNA coding GFP protein. The experiment groups included mRNA only group, Lip2000-mRNA group and C1-mRNA group. The expression of GFP protein was detected by a confocal laser scanning microscopy (LSM880, Zeiss) at 24 hrs post-transfection.

Mechanism of cellular uptake of C1-mRNA nanovaccine

To determine the route of cellular internalization of the C1-mRNA nanovaccine, DC2.4 cells were seeded in a 35mm glass-bottom dish with 10 mm micro-well (Corning) at a density of 5×10^4 cells/well and cultured overnight. The cells were then treated with a macropincytosis inhibitor Amiloride (200nM) or phagycytosis inhibitor Cytochalasin D (5μM) for 1 hr and followed by C1-mRNA-Coumarin6 incubation. Cells were incubated for 4 hrs, then washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were then washed twice with PBS and permeabilized by incubation in 0.2% Triton-X-100 in PBS for 10 min. Finally, cells were washed twice with PBS, counterstained with 500nm 4,6-diamidino-2-phenylindole (DAPI) (Cell Signaling Technology, CST) and applied to determine the uptake mechanism of C1-mRNA nanovaccine by a confocal laser scanning microscopy (LSM880, Zeiss).

Immune activation role of C1-mRNA nanovaccine in BMDCs

BMDCs from WT mice, *Tlr4*$^{-/-}$ mice or *Sting* cKO mice at culture day 4 were treated with a control (medium only), mRNA only, C1 only or C1-mRNA nanovaccine. The concentration of mRNA and lipid were 120 ng/ml and 19.2 μg/ml. After 24 hrs of incubation, cells were collected for flow cytometry to test the expression of CD40, CD80 and CD86 on CD11c+ population. The cell supernatant was collected for IFN-γ ELISA analysis. The cells were washed with FACS buffer, and data were collected on a BD LSR Fortessa X-20. Data analysis were performed using the FlowJo Software version 7.6.

For cytokine analysis, BMDCs from WT mice, *Tlr4*$^{-/-}$ mice or *Sting* cKO mice were seeded in 12-well plate at a density of 2×10^5 cells/well and treated with Control (medium only), mRNA only, C1 only and C1-mRNA nanovaccine. The concentration of mRNA and lipid were 120 ng/ml and 19.2 μg/ml. After 12 hrs of incubation, culture supernatant and RNA of cells were collected for measuring the expression levels of cytokines by ELISA or Real-time PCR.

TLR3/4/7/8/9 inhibition

BMDCs were seeded in a 12-well plate at a density of 2×10^5 cells/well overnight. The cells were then treated with TLR3 inhibitor CU CPT 4a (25 μM), TLR4 inhibitor TKA-242 (5 μM), TLR7/8 inhibitor ODN2088 control (10 μM) or TLR9 inhibitor ODN2088 (10 μM) for 1 hr. Subsequently

mRNA only, C1 only or C1-mRNA was added into the culture, and the cell culture was maintained for another 12 hrs. The cells were collected for RNA extraction to analyze cytokines expression. BMDCs without TLR inhibitor treatment served as the positive control.

Assay of Luciferase activity

DC2.4 cells were seeded on 24-well plates (2×10^5 cells per well) and transfected with NF-κB-luciferase reporter plasmid together with pRL-TK plasmid as an internal control. After 24 hrs, the cells were then treated with or without TLR4 inhibitor TKA-242 (5 μM) for 1 hr, followed by C1 only or C3 only treatment for another 24 hrs. Luciferase activity was measured with the Dual-Luciferase Reporter Assay kit according to the manufacturer's instructions (Promega).

TLR4 signaling measurement and Western blot

BMDCs isolated from wide type mice and *Tlr4*$^{-/-}$ mice were seeded in 6-well plate at a density of 5×10^5 cells/well and treated with lipid-like material C1 or C1-mRNA for 0 min, 15 min, 30 min, 60 min, 120 min and 240 min, respectively. Protein extracts were prepared using lysis buffer (50 mM Tris-HCl (pH 7.5), 0.5% NP-40 substitute, 150 mM NaCl and 12.5 mM NaF) supplemented with Complete Mini EDTA-free protease inhibitor tablets (Roche). Protein concentration was determined with a protein assay kit (Bio-Rad) according to the manufacturer's instructions. Protein samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore LS Immobilon-P, 0.45μm). The blots were blocked with 5% non-fat dry milk in TBST (50nm Tris-HCl at pH7.4 and 150 nm NaCl, and 0.1% Tween 20). Then, PVDF membranes were incubated with appropriated primary antibodies and HRP-conjugated secondary antibodies. The signals were detected using a chemiluminescence (ECL) detection system (Bio-Rad). The following antibodies were used for western blotting analyses: p-IKKα/β(Cell Signaling Technology, #2697，clone: 16A6)、IKKα/β(Cell Signaling Technology, #2682)、p-NF-κB-P65(Cell Signaling Technology, #3033，clone: 93H1)、NF-κB-P65(Cell Signaling Technology, #8242, clone: D14E2)、p-p38 MAPK(Cell Signaling Technology, #4511，clone: D3F9)、p38 MAPK(Cell Signaling Technology, #8690，clone: D13E1)、p-p44/42 MAPK(Erk1/2) (Cell Signaling Technology, #4370，clone: D13.14.4E)、p44/42 MAPK(Erk1/2) (Cell Signaling Technology, #4695，clone: 137F5)、p-SAPK/JNK(Cell Signaling Technology, #4668，clone: 81E11)、SAPK/JNK (Cell Signaling Technology, #9252), β-actin (Sigma, #A5441, clone: AC15). The dilution ratio of β-actin antibody was 1:10000, and the dilution ratios of other antibodies used for Western blot were 1:1000. The strip density was quantified by densitometry using Image J software, and individual target protein levels were normalized with respect to β-actin levels.

Bioplex assay measuring mouse serum levels of cytokines and chemokines

The mouse serum levels of cytokines and chemokines were determined by using Cytokine & Chemokine 26-Plex Mouse ProcartaPlex™ Panel (Invitrogen EPX260-26088-901) and Luminex

200 instrument with xPonent software. Multiplex assays were performed according to manufacturer's instructions. To analyze the data, we set the maximal concentration of the cytokines among all mice as value + 1, and the minimal concentration of the cytokines among all mice as value -1 in the heat map.

Antitumor efficacy of C1-mRNA nanovaccine in vivo

For preventative vaccine, 6 to 8-week old wide type mice or *Tlr4*$^{-/-}$ mice (n=5 for each group) were injected with different vaccines subcutaneously at inguinal region. MC38-OVA tumor cells (1×10^6 cells/mouse) or B16-OVA tumor cells (2×10^5 cells/mouse) were then inoculated subcutaneously into the right flank of the mice. Tumor growth was measured every two days using a digital caliper. Tumor volume was calculated as $0.5\times\text{length}\times\text{width}^2$, where the length was the longer dimension.

To assess the in vivo therapeutic efficacy of C1-mRNA nanovaccine, 6 to 8-week old wide type mice (n=5 for each group) were inoculated subcutaneously with B16-OVA tumor cells (2×10^5 cells/mouse) or B16 tumor cells (2×10^5 cells/mouse) into the right flank of the mice. The animals were then immunized by subcutaneous injection at right inguinal region with C1-$OVA_{257\text{-}264}$ mRNA nanovaccine or C1-Trp2 mRNA nanovaccine. The dose of mRNA used in all experiments was 10 μg per mouse. OVA peptide (200 μg per mouse) and Alum were used in tumor preventive experiment at a volume ratio of 2:1.

Hematological examination and histology analysis

To evaluate the in vivo toxicity of C1-mRNA nanovaccine, the mice blood was drawn and serum was isolated on day 21 after one vaccination. The activities of Alanine aminotransferase (ALT), aspartate aminotransferase (AST), uric acid (UA), creatinine (CR), blood ureanitrogen (BUN), creatine kinase (CK) and lactate dehydrogenase (LDH) levels were measured using individual assay kits. Meanwhile, organs (heart, lung, liver, spleen and kidney) were collected for histological analysis. The organs were then fixed with 4% paraformaldehyde and embedded in paraffin followed by sectioning (3-4μm) and stained with Hematoxylin & Eosin. The slides were assessed using an Olympus IX53 system.

Supplementary figures：

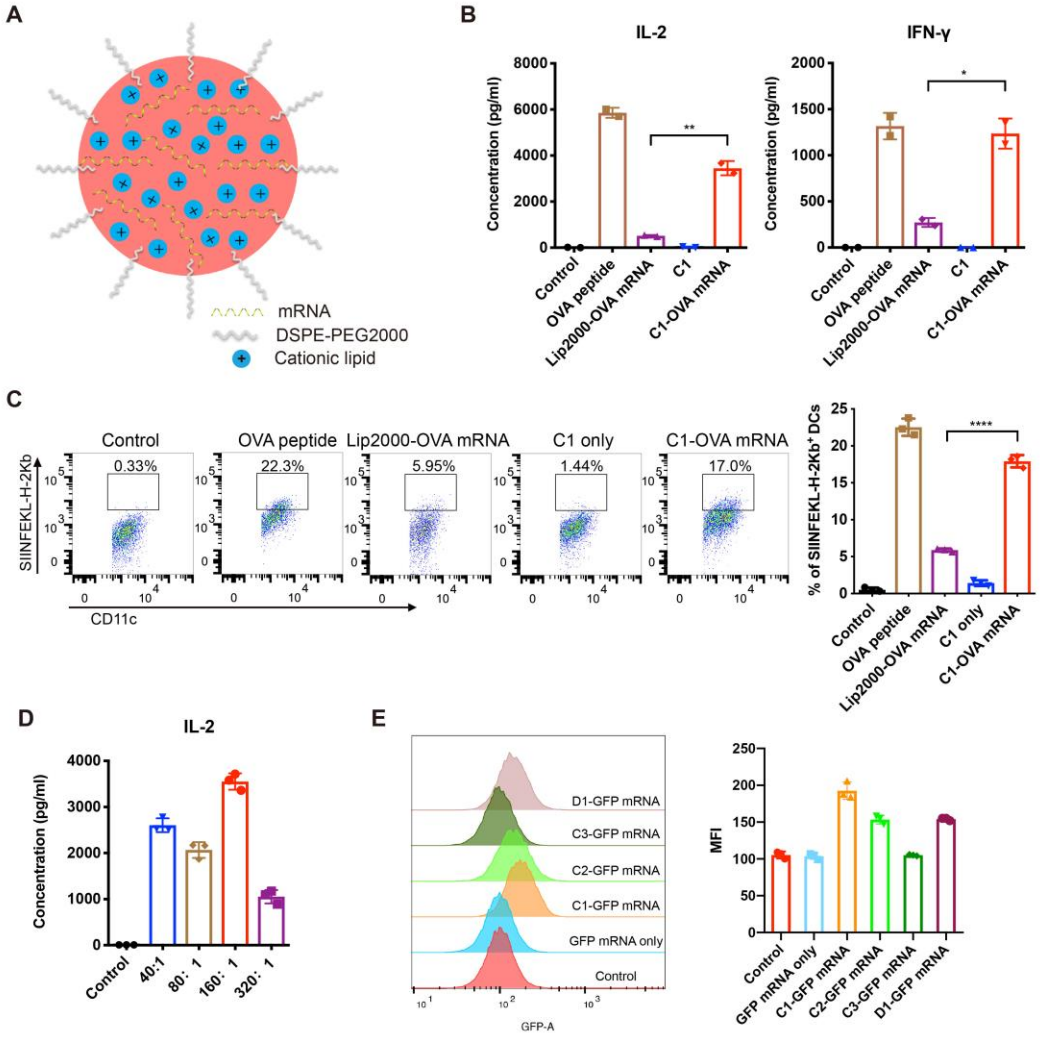

Figure S1. C1-mRNA nanovaccine effect in primary immune cells.
(A) Cartoon illustration of a C1-mRNA nanoparticle.
(B) ELISA result showed the levels of IL-2 and IFN-γ secretion by OT-1 cells after incubation with BMDCs primed with mRNA complexed with Lip2000 or C1. Control: medium only. C1-mRNA dose: mRNA:120ng/ml, C1: 19.2μg/ml; OVA peptide: 200ng/ml.
(C) Flow cytometry result showing the percentage of MHC-I-OVA (SIINFEKL-H2Kb)-positive cells in BMDCs after primed with mRNA complexed with Lip2000 or C1 as in (B). Control: medium only.
(D) ELISA result showed the levels of IL-2 secretion by B3Z cells after incubation with DC2.4 cells primed with mRNA complexed with C1 at different C1/mRNA ratio. The ratio of C1 to mRNA tested was 40:1, 80:1, 160:1 or 320:1, mRNA: 120 ng/ml. Control: medium only.
(E) Flow cytometry measuring GFP protein expression in DC2.4 cells treated by mRNA complexed with different lipids at equivalent doses. Control: medium only. MFI: mean fluorescence intensity.
Data represent the mean ±SD of three independent experiments.
*, P<0.05; **, P<0.01; ****, P<0.0001.

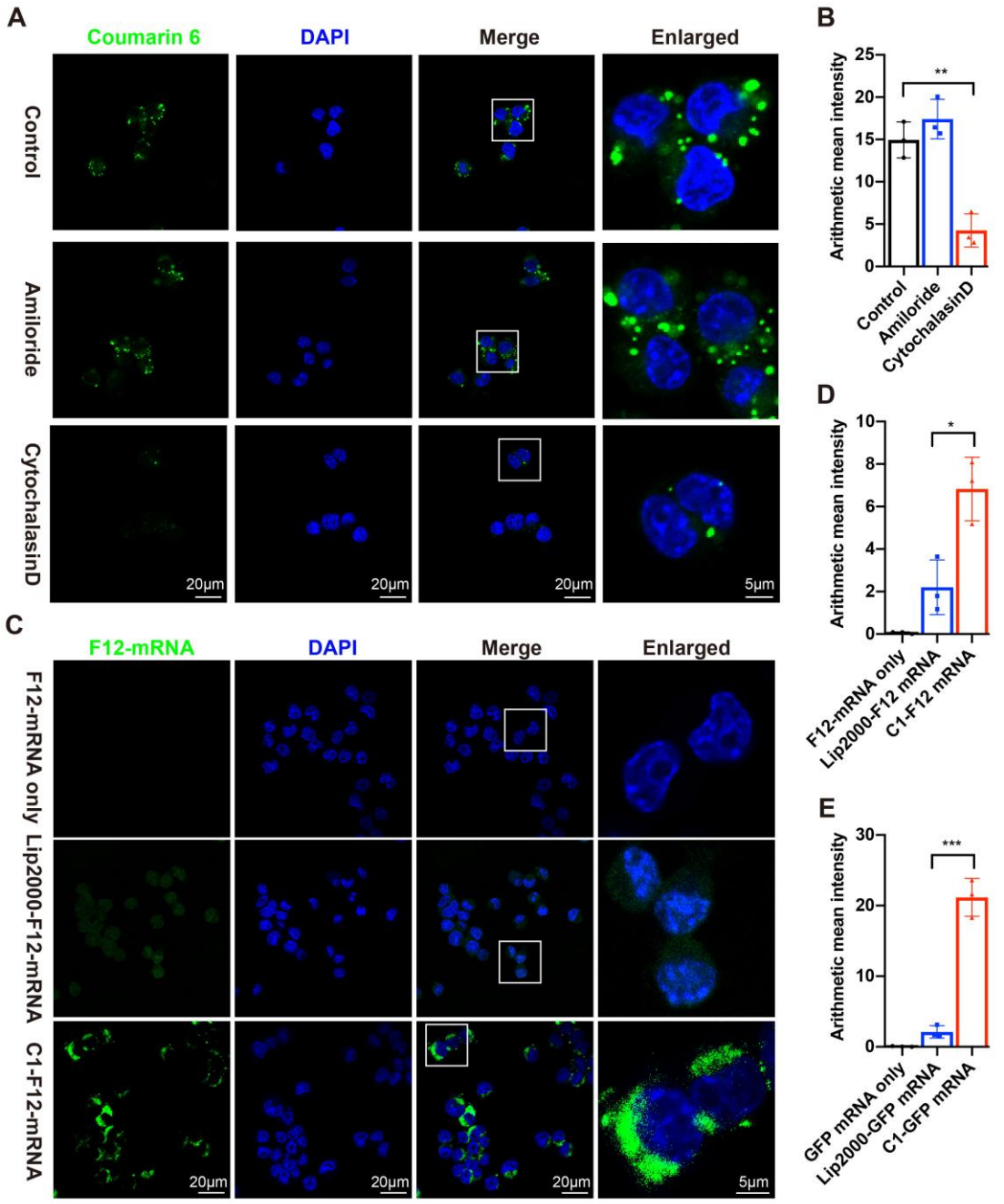

Figure S2. Cellular uptake and intracellular localization of C1-mRNA nanovaccine in DCs.
(A) Immunofluorescent images showing C1 particles labeled with coumarin 6 after co-incubation with BMDCs in medium (Control), or BMDCs pretreated with Cytochasin D or Amiloride. Nuclei were counterstained with DAPI (blue). Control: medium only. Cytochasin D: 5 μM，Amiloride：200 nM, pretreatment time: 1 hr.
(B) Quantified data of the fluorescence intensity in Figure S2A.
(C) Immunofluorescent images of DCs treated with C1-mRNA nanovaccine. DCs were incubated with different groups including F12-conjugated mRNA (F12-mRNA only), Lipfectamine2000-mRNA-F12 (Lip2000-F12-mRNA), and C1-F12-mRNA, then imaged under a fluorescent microscope; Nuclei were counterstained with DAPI (blue).

(D) Quantified result of the fluorescence intensity in Figure S2A.
(E) Quantified result of the fluorescence intensity in Figure 2E.
Data represent the mean ±SD of three independent experiments.
*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

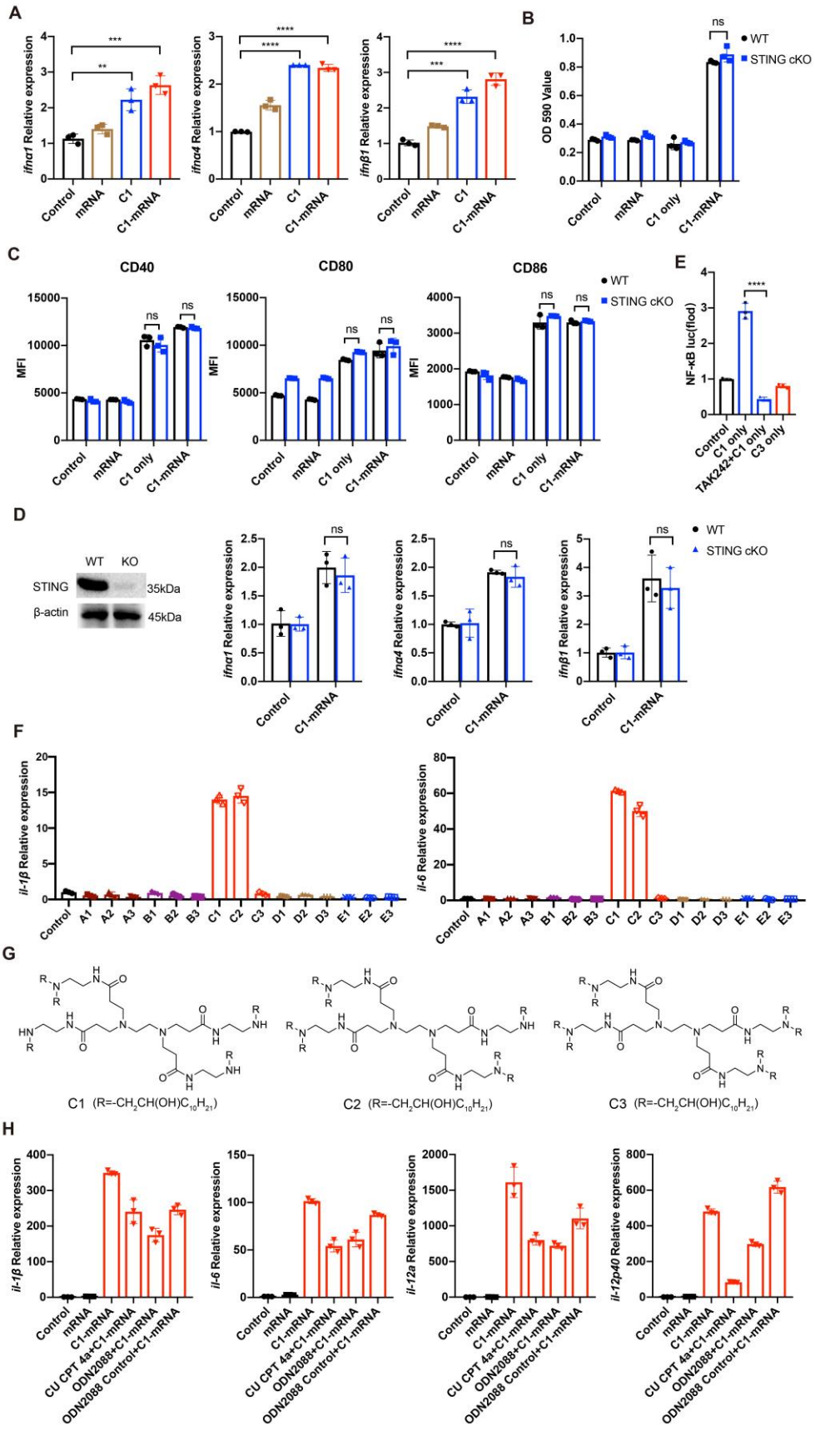
A
ifna1 Relative expression
ifna4 Relative expression
ifnb1 Relative expression
Control
mRNA
C1
C1-mRNA
B
OD 590 Value
WT
STING cKO
ns
C1 only
C
CD40
CD80
CD86
MFI
D
WT
KO
STING
35kDa
β-actin
45kDa
E
NF-κB luc(flod)
TAK242+C1 only
C3 only
F
il-1β Relative expression
il-6 Relative expression
A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3
E1
E2
E3
G
C1 (R=-CH2CH(OH)C10H21)
C2 (R=-CH2CH(OH)C10H21)
C3 (R=-CH2CH(OH)C10H21)
H
il-12a Relative expression
il-12p40 Relative expression
CU CPT 4a+C1-mRNA
ODN2088+C1-mRNA
ODN2088 Control+C1-mRNA

Figure S3. C1-mRNA induced innate immune activation in BMDCs.
(A) PCR result showing up-regulation of *ifn-α1*, *ifn-α4* and *ifn-β* in BMDCs after treatment of C1-mRNA. Control: medium only. C1-mRNA dose: mRNA, 120 ng/ml, C1, 19.2 μg/ml, treatment time: 12 hrs.
(B) The IL2-driven LacZ activity in B3Z cells after co-incubation with WT or STING cKO BMDCs primed with C1-mRNA. Control: medium only.
(C) The surface expression levels of CD40, CD80 and CD86 on BMDCs were measured by antibody staining followed by flow cytometry analysis. Control: medium only. Treatment time: 24 hrs.
(D) Left panel: Western blot confirming the absence of STING protein expression in STING cKO cells. Right panel: PCR result showing up-regulation of *ifn-α1*, *ifn-α4* and *ifn-β* in both WT and STING cKO BMDCs after treatment of C1-mRNA. Control: medium only.
(E) Luciferase reporter assay showed NF-κB reporter activity in DC2.4 cells with indicated treatments. Control: medium only.
(F) Gene expression levels of cytokines *il-1β and il-6* in BMDCs treated by 15 kinds of lipid-like materials. Control: medium only.
(G) The chemical structures of three lipids including C1, C2 and C3.
(H) The effect of TLR3/7/8/9 inhibition on the cytokine gene induction in BMDCs by C1-mRNA nanovaccine. Control: medium only. CUCPT4a: TLR3 inhibitor, 25 μM; ODN2088 Control: TLR7/8 inhibitor, 10 μM; ODN2088: TLR9 inhibitor, 10 μM; pretreatment time: 1 hr.
Data represent the mean ±SD of three independent experiments.
**, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$, ns, not significant.

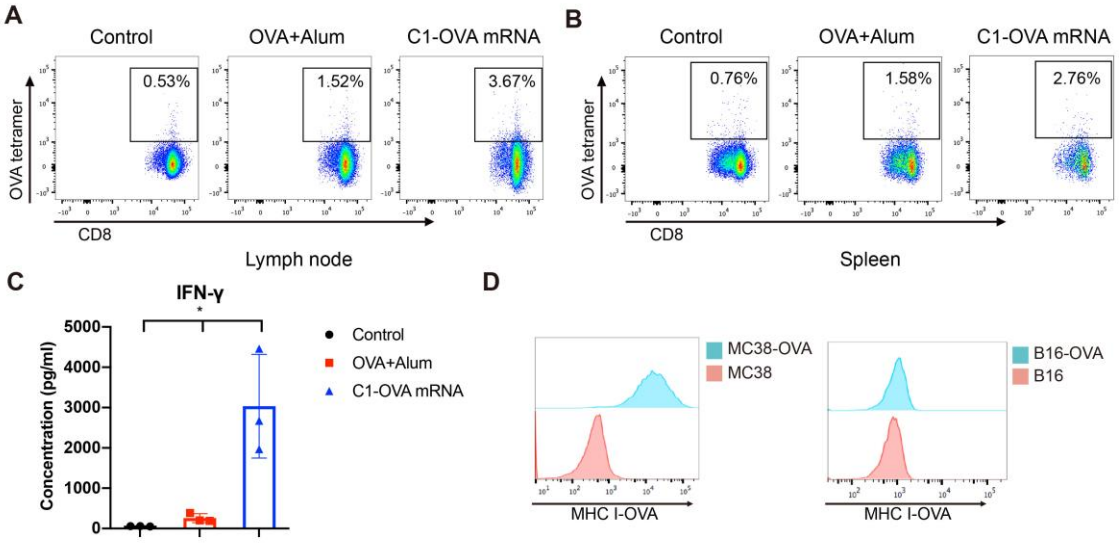

Figure S4. C1-mRNA nanovaccine induced T cell response in vivo.
(A-B) Percentage of OVA(SIINFEKL)-specific $CD8^+$ T cells measured by tetramer staining and flow cytometry analysis in lymph node (A) or spleen (B). Control: PBS injection group.
(C) IFN-γ secretion in lymph node cells isolated from mice in different vaccine groups, after re-stimulation with OVA peptide. Control: PBS injection group.
(D) Flow cytometry confirming OVA protein expression in mouse colorectal cancer MC38-OVA cells and melanoma B16-OVA cells.
Data in (C) represent the mean ±SD of measurements from three mice.
*, $P<0.05$.

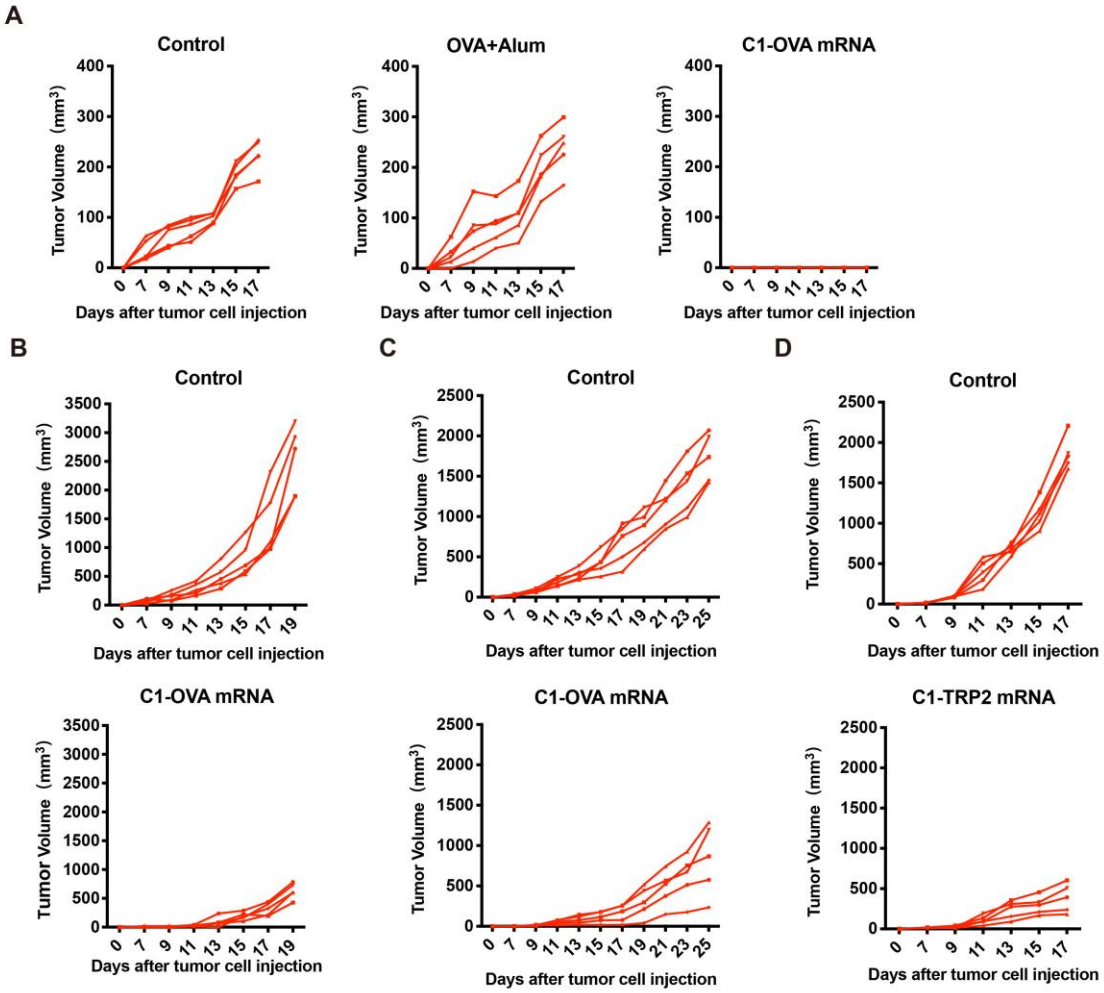

Figure S5. Antitumor efficacy of C1-mRNA nanovaccine.
(A-B) Individual tumor growth curve of each mouse in MC38-OVA tumor model (A), and B16-OVA tumor model (B) in a preventative vaccine setting. Control: PBS injection group. (C-D) Individual tumor growth curve of each mouse in B16-OVA tumor model (C), and B16 tumor model (D) in a therapeutic vaccine setting. Control: PBS injection group.

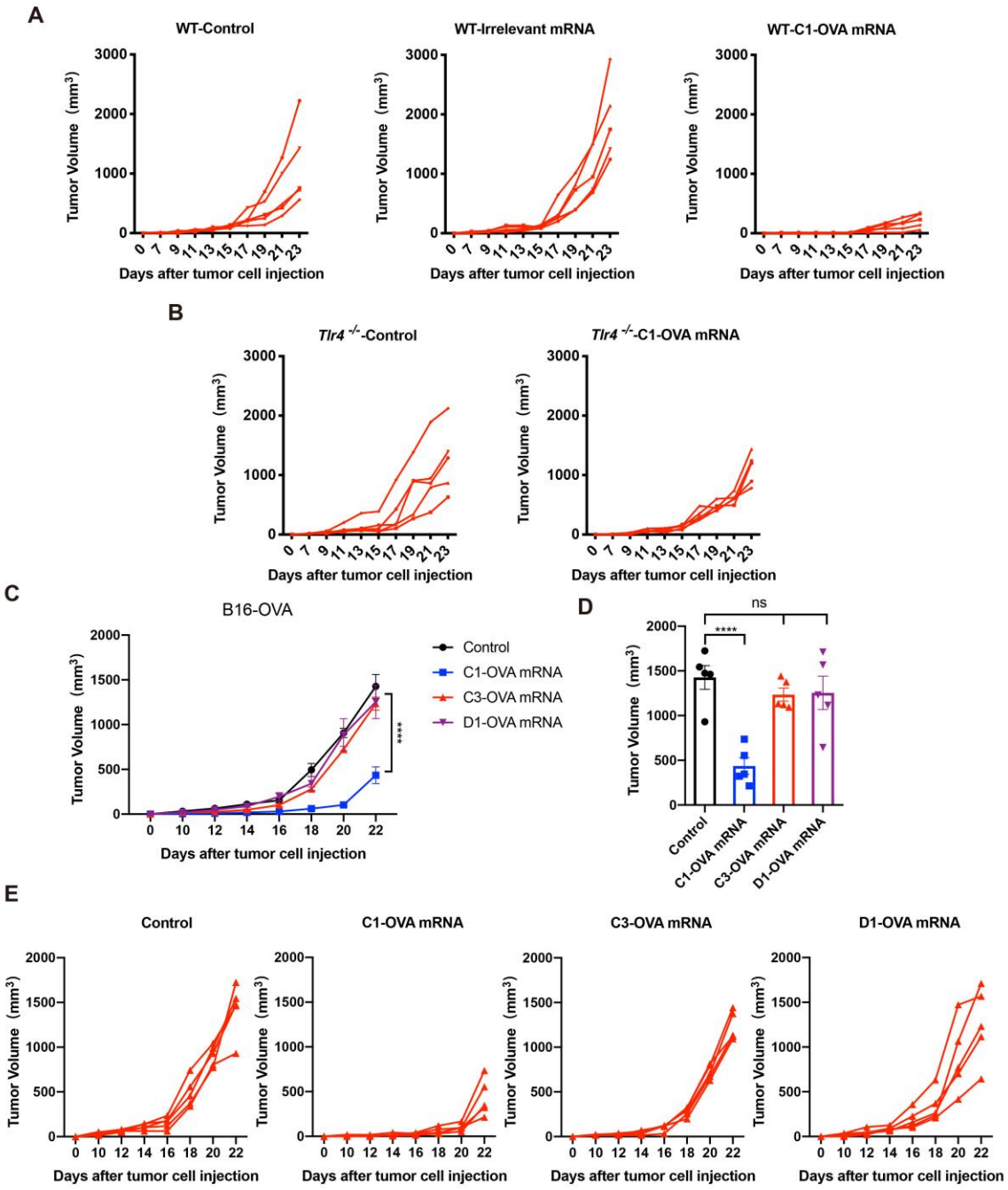

Figure S6. The antitumor efficacy of C1-mRNA nanovaccine is dependent on TLR4 signaling.

(A) Individual tumor growth curve of each mouse in Figure 6B.

(B) Individual tumor growth curve of each mouse in Figure 6C.

(C) The tumor growth curve of B16-OVA tumor-bearing mice treated with different lipid-delivered mRNA nanovaccine. Control: PBS injection group; mRNA nanovaccine dose per mouse: mRNA encoding $OVA_{257-264}$,10 μg; C1/C3/D1, 1.6 mg; n=5 mice per group.

(D) The tumor volume at day 22 in different groups. Control: PBS injection group.

(E) Individual tumor growth curve of each mouse in (C).
Data represent the mean ± SEM.
****, *P*<0.0001, ns, not significant.

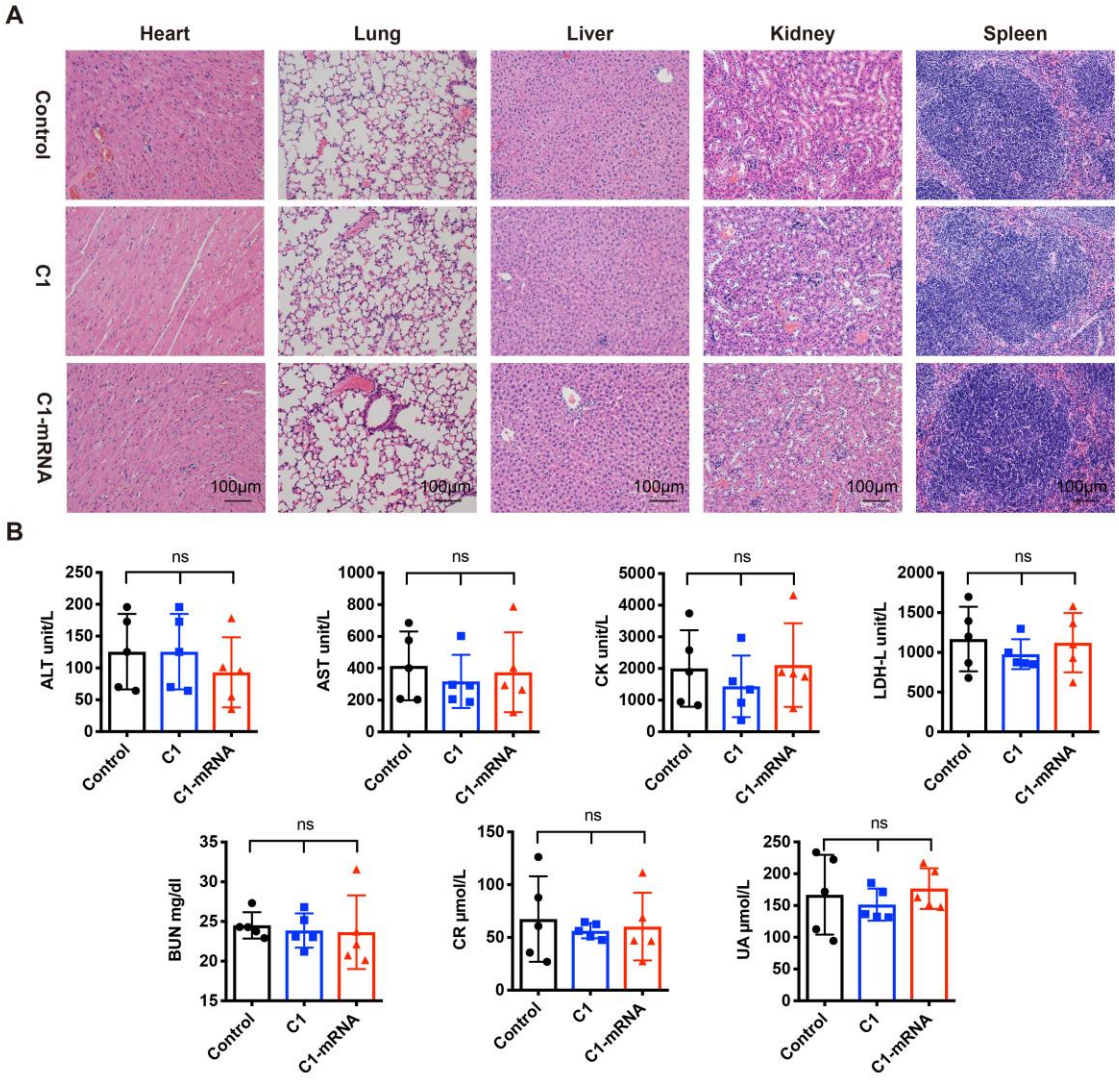

Figure S7. *In vivo* safety profile of C1-mRNA nanovaccine.
(A) Representative images of H&E staining results showing the histology structure of major organs from mice treated with nanovaccine. Control: PBS injection group.
(B) Hematological analysis of liver, kidney and cardiac function of mice received C1-mRNA nanovaccine. ALT: alanine aminotransferase; AST: aspartate aminotransferase; CK: creatine kinase; LDH: lactate dehydrogenase; BUN: blood urea nitrogen; CR: creatinine; UA: uric acid. Control: PBS injection group.
Data represent the mean ± SD, n=5 mice per group.
ns, not significant.

Table S1 Sequences of primers used for RT-PCR

Gene Name	Primer sequences (5'-3')
IL-1β	Forward: GCAACTGTTCCTGAACTCAACT Reverse: ATCTTTTGGGGTCCGTCAACT
IL-6	Forward: TAGTCCTTCCTACCCCAATTTCC Reverse: TTGGTCCTTAGCCACTCCTTC
IL-12A	Forward: CTGTGCCTTGGTAGCATCTATG Reverse: GCAGAGTCTCGCCATTATGATTC
IL-12P40	Forward: TGGTTTGCCATCGTTTTGCTG Reverse: ACAGGTGAGGTTCACTGTTTCT
IFN-α1	Forward: GCCTTGACACTCCTGGTACAAATGA Reverse: CAGCACATTGGCAGAGGAAGACAG
IFN-α4	Forward: TGATGAGCTACTACTGGTCAGC Reverse: GATCTCTTAGCACAAGGATGGC
IFN-β1	Forward: CAGCTCCAAGAAAGGACGAAC Reverse: GGCAGTGTAACTCTTCTGCAT
Tlr4	Forward: ATGGCATGGCTTACACCACC Reverse: GAGGCCAATTTTGTCTCCACA
Actin	Forward: AGTGTGACGTTGACATCCGT Reverse: GCAGCTCAGTAACAGTCCGC