Electronic Supporting Information (ESI)

# A PEG-lipid-free COVID-19 mRNA vaccine triggers robust immune responses in mice

Min Li,[a] Yixuan Huang,[a] Jiacai Wu,[ab] Sanpeng Li,[a] Miao Mei,[c] Haixia Chen,[d] Ning Wang,[ab] Weigang Wu,[a] Boping Zhou,[ab] Xu Tan[c] and Bin Li[*ab]

[a]Department of Infectious Disease, Shenzhen People's Hospital, The First Affiliated Hospital of Southern University of Science and Technology & The Second Clinical Medical College of Jinan University, Shenzhen 518020, China

[b]School of Medicine, Southern University of Science and Technology, Shenzhen, 518055, China

[c]School of Pharmaceutical Sciences, Tsinghua-Peking Center for Life Sciences, Tsinghua University, Beijing 100084, China

[d]Department of Clinical Laboratory, Shenzhen People's Hospital, The First Affiliated Hospital of Southern University of Science and Technology & The Second Clinical Medical College of Jinan University, Shenzhen 518020, China

*Corresponding author. E-mail: libin@mail.sustech.edu.cn

**Experimental Procedures**

**Chemicals and reagents**

tB-UC18 was synthesized in-house according to our previously described methods.[1] 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine phospholipid (DOPE) was purchased from Avanti Polar Lipids. 6-(p-Toluidino)-2-naphthalenesulfonic acid sodium salt was ordered from Sigma-Aldrich. The Laurdan reagent was from Macklin. Cy5-labelled oligo and the SARS-CoV-2 surrogate virus neutralization test kit were ordered from Genscript. Firefly luciferase mRNA (FLuc mRNA) was from APExBIO. Triton X-100, Eagle's Minimum Essential Medium, and Dulbecco's Modified Eagle's Medium (high glucose) were purchased from Sangon Biotech. Firefly luciferase (FLuc) mRNA and enhanced GFP (eGFP) mRNA were obtained from APExBIO. Nuclear staining solution Hoechst 33342, membrane probe DiO, D-luciferin potassium salt, and Cell Counting Kit-8 were obtained from Beyotime. The SARS-CoV-2 RBD ELISA kit was ordered from Elabscience. The SARS-CoV-2 RBD IgG ELISA detection kit was purchased from Vazyme Biotech. HRP-conjugated goat anti-mouse IgG1 and IgG2a were from Abclonal. Mouse IFN-γ, IL-2, IL-4, and IL-10 ELISA kits were obtained from Dakewe Biotech. Human C3a and C5a ELISA kit were from Elabscience. All other chemicals and reagents were used without purification unless otherwise noted.

**Synthesis of SARS-CoV-2 RBD mRNA**

A linearized DNA template containing the 5' untranslated regions, the codon-optimized sequences coding the signal peptide, the SARS-CoV-2 RBD coding sequence, the 3' untranslated regions, and a polyadenosine tail was constructed by APExBIO and used for in vitro transcription. Cap 1 and ψ-modified nucleotide triphosphates were incorporated into mRNA during in vitro transcription. The synthetic mRNA was analyzed by agarose gel electrophoresis and kept at -80°C prior to use.

**Preparation and characterization of formulations**

The RBD-mRNA was formulated into tB-UC18 LLNs using our previously reported procedures.[1] Briefly, tB-UC18 and DOPE were dissolved in ethanol at equimolar ratios. Formulations were prepared by mixing the ethanol phase with the aqueous phase containing the RBD mRNA at a 1:9 volume ratio. The final N:P charge ratio between tB-UC18 and mRNA was set to 3:2. LLNs loaded with other mRNAs were formulated with the same procedure. TT3 LLNs were prepared with previously reported procedures.[2] For in vitro research, formulations were prepared at a final mRNA concentration of 0.02 mg $mL^{-1}$. Formulations were scaled up proportionally for in vivo studies. The resulting formulations were characterized by dynamic light scattering and transmission electron microscopy. Specifically, hydrodynamic size and zeta potential were determined at 25 °C by a Zetasizer (90Plus PALS, Brookhaven). The morphology was obtained by transmission electron microscopy (HT7700, Hitachi) at 80 kV and cryo-electron microscopy (Titan Krios G3i, Thermofisher Scientific) at 300 kV. The p*K*a, the surface polarity, and cytotoxicity were determined using previously described methods.[3,4] Lyophilized tB-UC18 LLNs containing 20% sucrose were prepared as previously described[5] and reconstituted with nuclease-free water after one week of storage at 4 °C to determine the relative luminescence intensity.[5]

**Membrane disruption assay**

The membrane disruption assay was performed by mixing human red blood cells diluted to 4% in PBS (pH 7.4) or citrate buffer (pH 5.4) with an equal volume of nanocarrier with different concentrations. The mixture was incubated at 37°C for 60 min, centrifuged for 5 min in a microcentrifuge (1000 × *g*), and the supernatant was transferred to a new 96-well plate. The absorbance at 540 nm was recorded by a microplate reader (Synergy LX, BioTek). PBS and Triton X-100 (1%) were included as the negative and positive control, respectively. The membrane disruption rate (%) was calculated as follows: $(OD_{540\_sample}-OD_{540\_negative\ control})/(OD_{540\_positive\ control}-OD_{540\_negative\ control})$, and was normalized to that of 1% Triton X-100.

**Cellular internalization**

Human embryonic kidney 293T cells (Stem Cell Bank, Chinese Academy of Sciences) were maintained in minimum essential medium culture medium supplemented with 10% fetal bovine serum. To monitor cellular internalization of mRNA vaccines, RBD mRNA was fluorescently labelled by rapid heating and slow cooling a mixture of mRNA and an eightfold molar excess of Cy5-tagged 17-mer poly(dT). The Cy5-labelled RBD-mRNA encapsulated in tB-UC18 LLNs were added to 293T cells for confocal laser scanning microscopy and flow cytometry. For confocal laser scanning microscopy, cells were incubated with PFTCmvac at

a final mRNA dose of 2 μg $mL^{-1}$ for 4 h. After washing with PBS, cells membranes and nuclei were stained with Dio and Hoechst 33342 for 10 min, respectively. Cells were then imaged using an inverted confocal microscope (TCS SP8, Leica). In the case of flow cytometry, cells were treated with different concentrations of PFTCmvac for 4 h, 8 h, or 24 h. After three washes with PBS, samples were resuspended with PBS and acquired on a NovoCyte flow cytometer (Agilent). For each sample, 10,000 events were analyzed by the NovoExpress software (Agilent).

**Detection of mRNA-encoded protein expression in vitro**

293T cells were seeded in a 96-well white or clear plate at a density of 20,000 cells per well and left to adhere overnight. Cells were then treated with formulation containing 200 ng of FLuc, eGFP, or RBD mRNA for 24 h. Luminescence (for FLuc mRNA) and fluorescence (for eGFP mRNA) signals were detected according to the previous described method.[3] To quantify the expression level of the RBD mRNA-encoded protein, cell culture supernatants were harvested. Meanwhile, cell pellets were washed, resuspended with 100 μL of PBS, and lysed by repeated freeze-thaw cycles and ultrasound for 10 min. Cell lysates were then collected and centrifuged at 1000 g for 10 min. The RBD protein in the cell supernatants and lysates was determined by a SARS-CoV-2 Spike Protein S1 RBD ELISA kit according to the manufacturer's directions.

**Bioluminescence imaging in mice**

The ethical approval was obtained from the ethics committee of Shenzhen People's Hospital. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of Shenzhen People's Hospital. Female C57BL/6J mice aged 6–8 weeks (n = 3) received a single intramuscular (IM) injection of FLuc mRNA-loaded tB-UC18 LLNs at a mRNA dose of 0.25 or 0.5 mg $kg^{-1}$. At the indicated time points, mice were injected with D-luciferin potassium salt intraperitoneally (150 mg $kg^{-1}$) and placed on a small animal imaging system (IVIS Spectrum, PerkinElmer) 8 min post injection for bioluminescence imaging. Luminescence images were acquired and analyzed using the system's software.

**Immunization with mRNA vaccines in mice**

Female BALB/c mice aged 6–8 weeks (n = 5) were randomly grouped and immunized intramuscularly with PFTCmvac at a 1.5 mg $kg^{-1}$ RBD mRNA dose at day 0 and day 14. To assess humoral immune responses, sera from mice at day 28 post initial immunization were isolated and heat-inactivated at 56 °C for 30 min. Meanwhile, the spleens were collected to prepare single-cell suspensions for evaluation of cellular immune responses. Mice were weighed twice a week throughout the study. The hair around the injection site was shaved with an electrical shaver at day 1 and day 28 for evaluation of injection-site reactions. Untreated mice were used as the negative control.

**Detection of binding and neutralizing antibodies in mice immunized with mRNA vaccines**

The SARS-CoV-2 RBD-specific IgG, IgG1 and IgG2a antibodies in mouse sera were detected by a precoated ELISA detection kit. Briefly, 10-fold serially diluted sera were added to a 96-well plate precoated with the recombinant SARS-CoV-2 RBD protein and incubated at 37 °C for 60 min. The plate was washed five times and incubated with HRP-conjugated goat anti-mouse IgG (1:100), IgG1 (1:3000), and IgG2a (1:3000) at 37 °C for 30 min. After washing steps, TMB solution was added and incubated for 10 min at 37 °C in the dark. The reaction was then stopped and the absorbance at 450 nm was measured by a microplate reader (Synergy LX, BioTek). Anti-SARS-CoV-2 neutralizing antibody was evaluated by a SARS-CoV-2 surrogate virus neutralization test kit. In brief, 10-fold serially diluted sera were mixed with an equal volume of HRP-conjugated RBD solution (1:1000) for 30 min at 37 °C. The mixture was then added to the plate precoated with the hACE2 protein for 15 min at 37 °C. Following a wash step, TMB substrate was added to each well for 15 min in the dark at room temperature. Thereafter, the reaction was quenched with stop solution, and the absorbance was recorded at 450 nm using a microplate reader (Synergy LX, BioTek). The neutralization activity was determined according to the following equation: Neutralization (%) = $(1-OD_{450\_sample}/OD_{450\_negative\ control}) \times 100\%$.

**Evaluation of cellular immune responses elicited by PFTCmvac immunization**

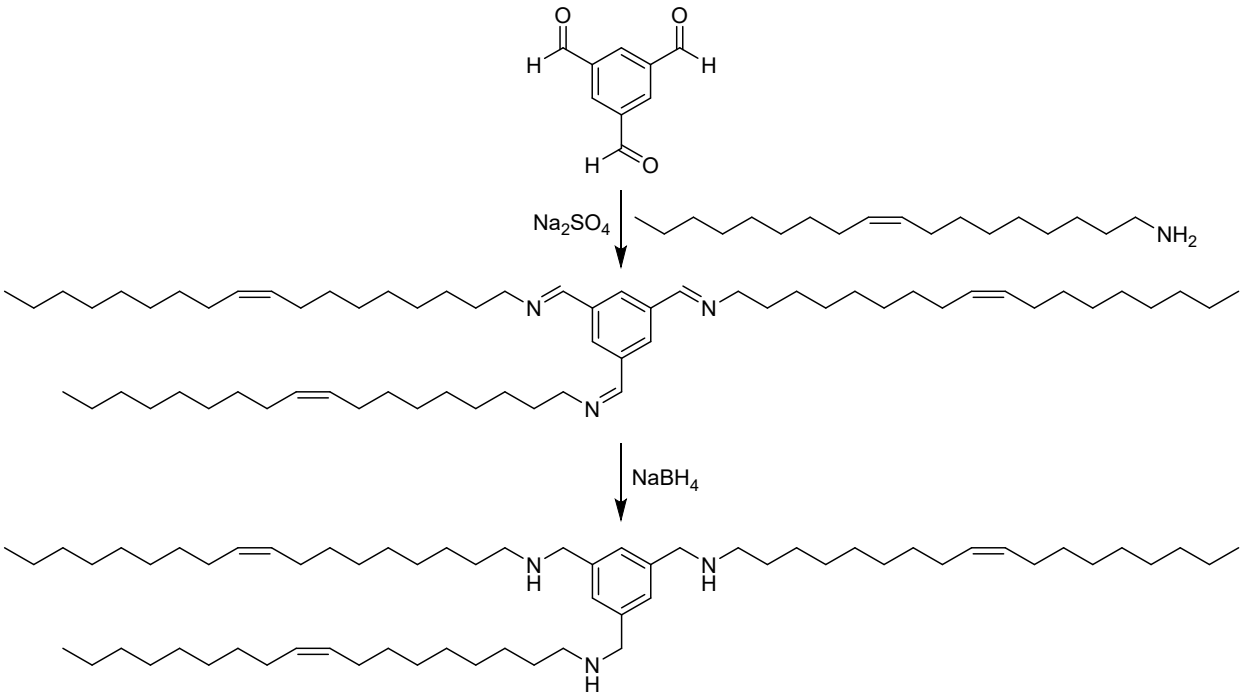
Splenocytes isolated from PFTCmvac-immunized mice were seeded into 96-well plates at a density of $1 \times 10^6$ cells per well and stimulated with the SARS-CoV-2 RBD at a final concentration of 10 μg $mL^{-1}$ for 48 h at 37 °C in 5% $CO_2$. The production of IFN-γ, IL-2, IL-4, and IL-10 in the culture supernatants was titrated by ELISA following the manufacturer's protocols.

**Complement activation assays**

Complement activation of tB-UC18 and TT3 formulations in normal human serum was determined by Human Complement Component 3a and 5a ELISA kits as described previously with minor modifications.[6] Briefly, 10 μL of varying concentrations of formulations was incubated with 40 μL of normal human serum at 37 °C for 60 min. The final concentration of mRNA was in the range of 4 to 64 μg $mL^{-1}$. The C3a and C5a levels were then measured according to the manufacture's protocols.

**Statistical analysis**

Unless otherwise noted, all data were presented as mean ± SEM. Statistical analyses were performed by GraphPad Prism using an unpaired, two-tailed Student's *t*-test. Significant differences were indicated by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.




**tB-UC18**

**Figure S1.** Synthesis of the lipid-like compound tB-UC18. tB-UC18 was synthesized via a two-step reaction including the condensation reaction between benzene-1,3,5-tricarboxaldehyde and oleylamine and the reduction of the Schiff base.

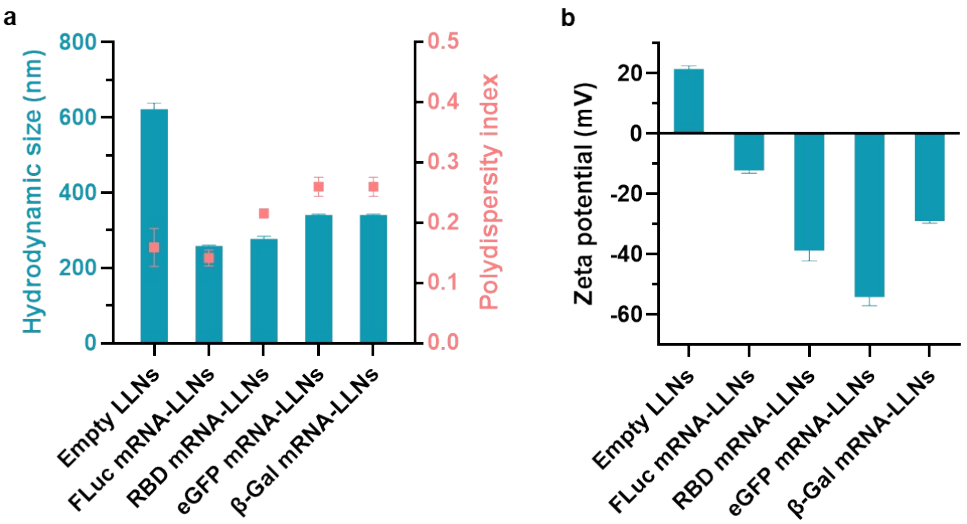



**Figure S2.** Characterization of LLNs encapsulated with various mRNA of varying length. The hydrodynamic size (**a**), polydispersity index (**a**), and zeta potential (**b**) of LLNs were determined by dynamic light scattering.

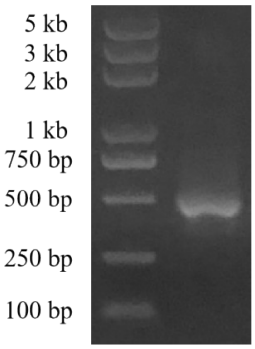



**Figure S3.** Analysis of ψ-modified RBD mRNA on a 1% native agarose gel.

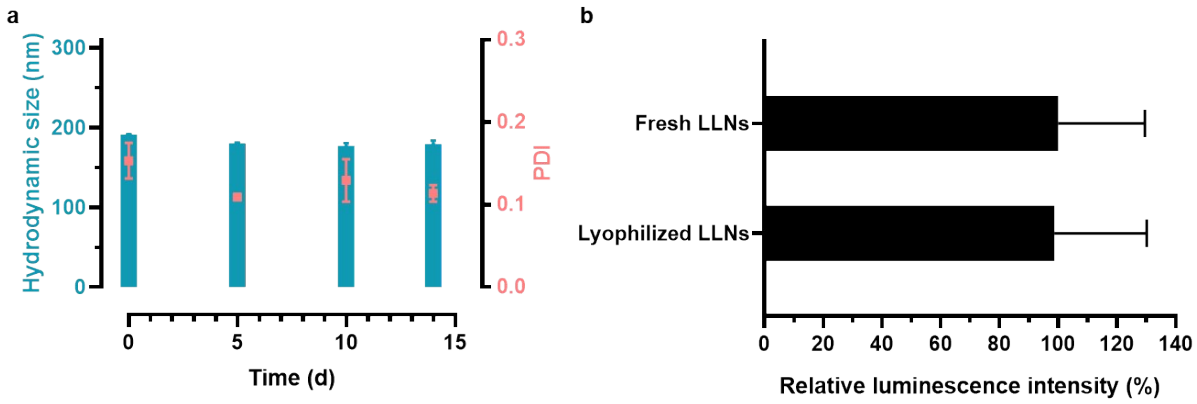



**Figure S4.** The stability of TT3 and tB-UC18 LLNs. (a) The hydrodynamic size changes of TT3 LLNs stored at room temperature was determined by dynamic light scattering. (b) Relative luminescence intensity of lyophilized tB-UC18 LLNs reconstituted with nuclease-free water. The lyophilized formulation containing 20% sucrose were stored at 4 °C for 1 week.

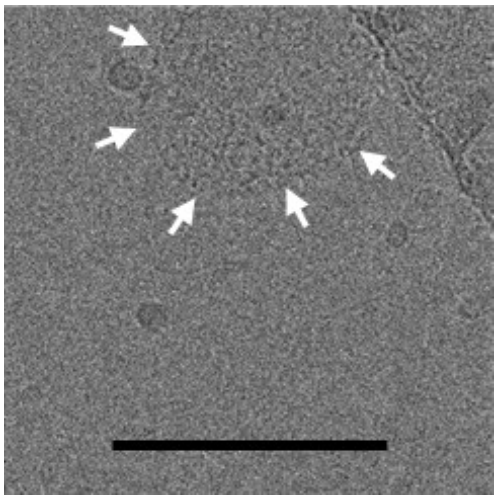


**Figure S5.** A representative cryo-electronic microscopy image of freshly prepared PFTCmvac. The white arrows indicated the edge of a particle. Scale bar, 100 nm.

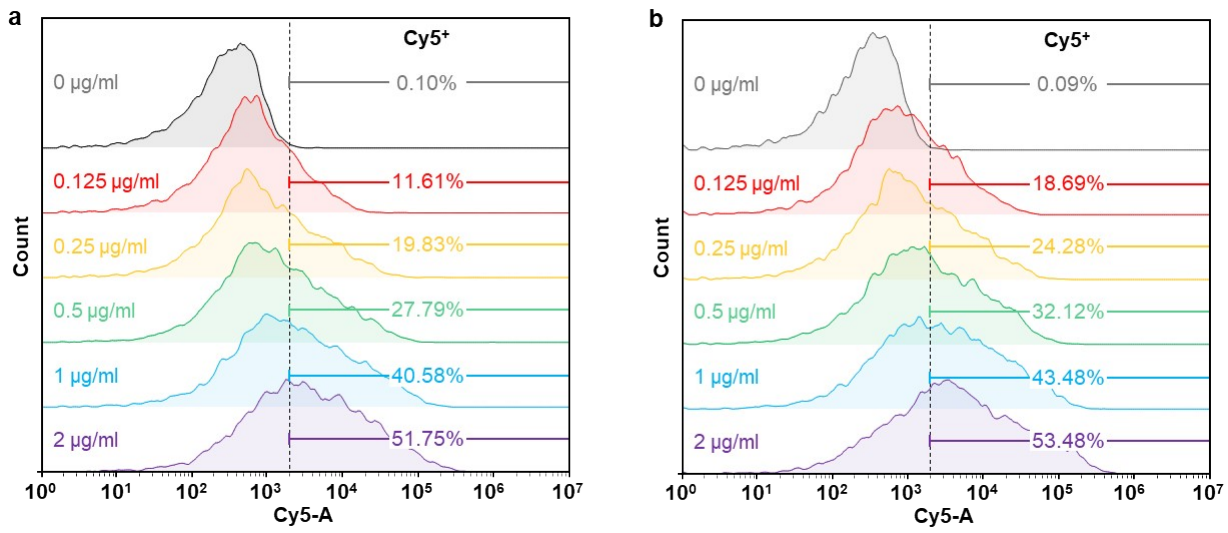



**Figure S6.** Representative flow cytometric analysis of Cy5-positive 293T cells after 8 h (**a**) and 24 h (**b**) of treatment with different concentrations of Cy5-labelled PFTCmvac.

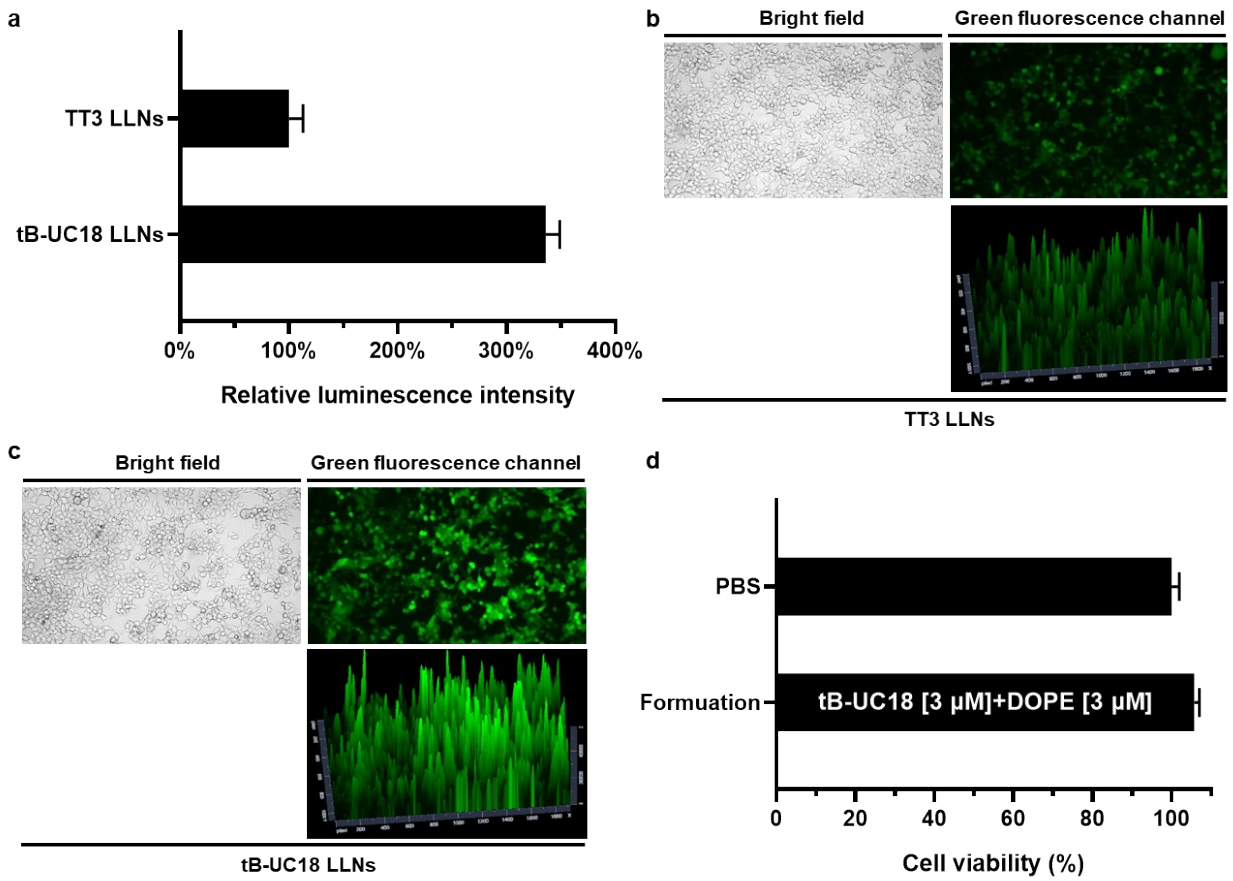



**Figure S7.** Delivery activity and cytotoxicity of tB-UC18 LLNs. (a) Relative luminescence intensity of 293T cells treated with FLuc mRNA-loaded tB-UC18 LLNs was determined by quantification of the expression of luciferase and normalized to that of TT3 LLNs. (b,c) Green fluorescence signals of 293T cells treated with eGFP mRNA-loaded TT3 LLNs (b) or tB-UC18 LLNs (c) was estimated by fluorescence microscope. (d) Cell viability of 293T cells treated with eGFP mRNA-loaded tB-UC18 LLNs was determined by the CCK-8 assay. PBS-treated mice served as the control.

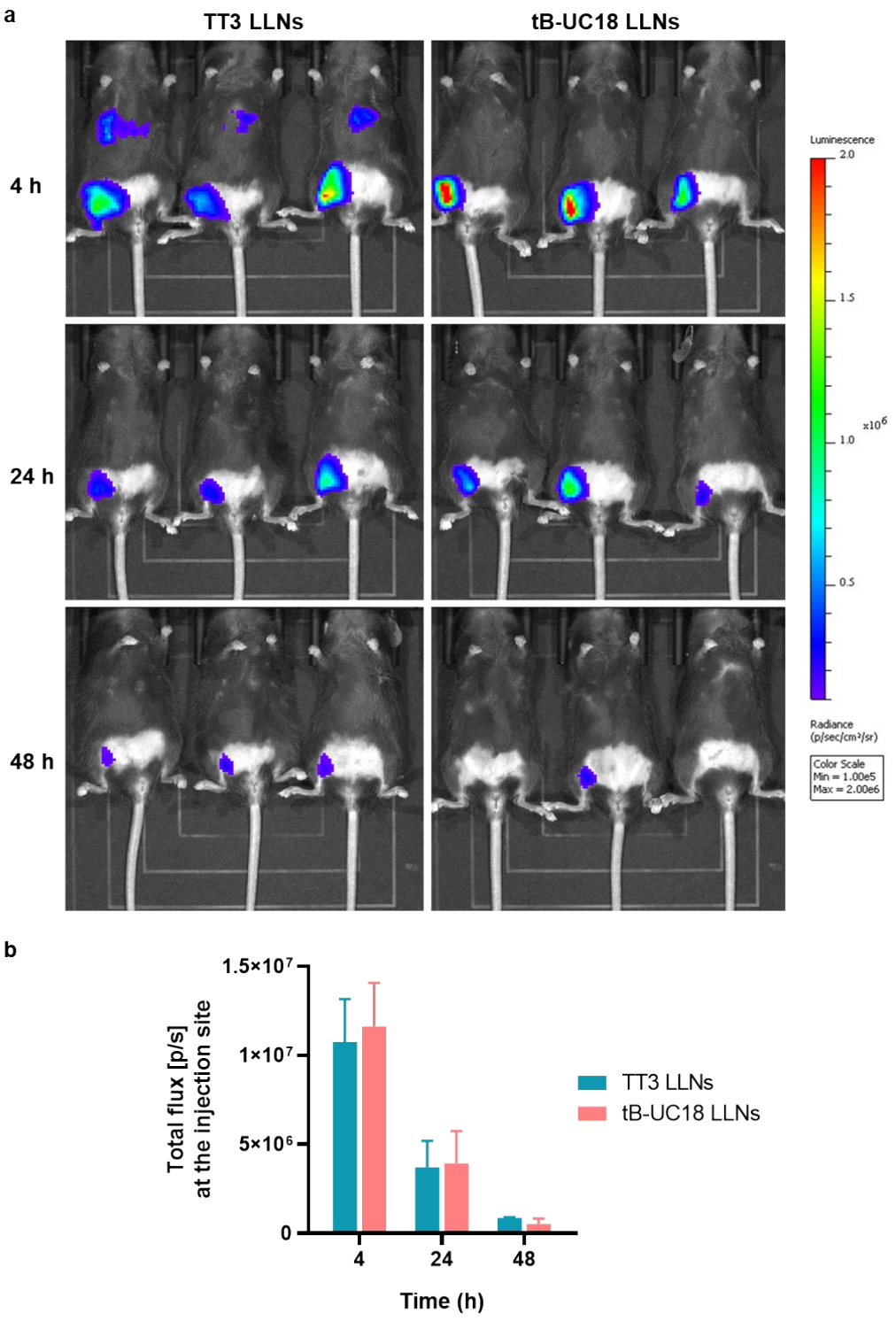



**Figure S8.** Bioluminescent imaging in mice. (a) Whole-body bioluminescent imaging in mice 4 h, 24 h, and 48 h after intramuscular injection of FLuc mRNA-loaded TT3 LLNs or tB-UC18 LLNs at a single mRNA dose of 0.5 mg $kg^{-1}$. (b) Quantification of bioluminescent at the site of injection in (a).

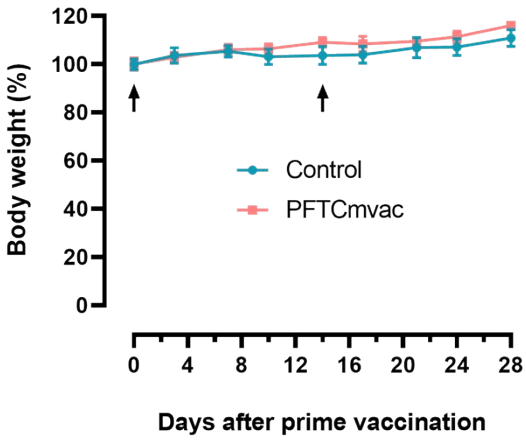



**Figure S9.** Body weight changes in mice immunized with PFTCmvac. Arrows indicate the day of immunization. Untreated mice served as the control.

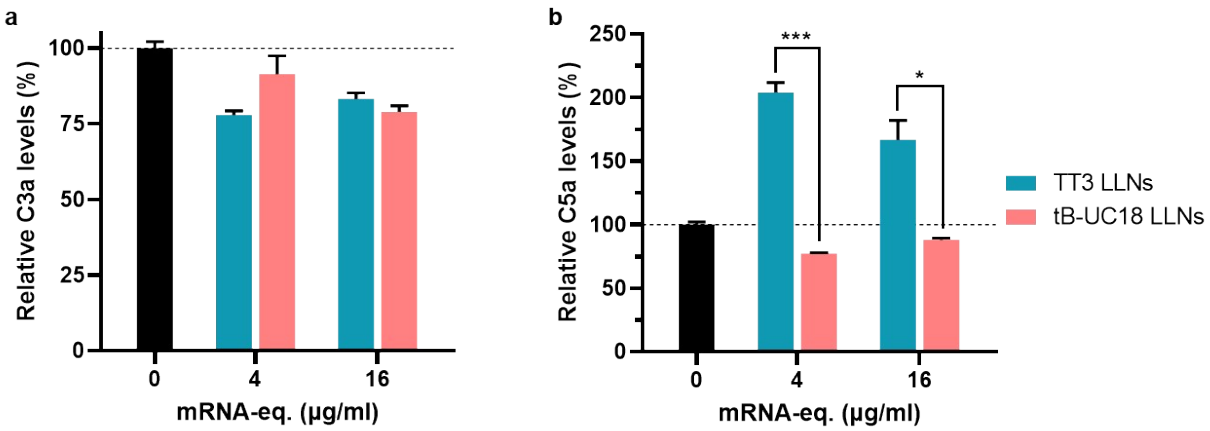



**Figure S10.** Evaluation of complement activation products C3a (a) and C5a (b) in human serum treated with tB-UC18 or TT3 LLNs. Data were normalized to that of PBS-treated serum.

## References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