Supporting information

ROS-responsive poly(β-amino ester) nanoparticle enables targeted delivery of mRNA vaccine to splenic antigen-presenting cells

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1. Materials and Methods

2.1 Materials

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG 2000) were purchased from Bidepharm, and cholesterol was obtained from Shanghai Yuanye Biotechnology Co., Ltd. Firefly luciferase mRNA (mLuc) was sourced from Goldentrans, while cre recombinase (mCre) and OVA (mOVA) mRNAs were acquired from MagicRNA. DiD fluorescent dye was supplied by Beyotime Biotechnology, and D-luciferin substrate was from Bidepharm. Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Advance II 400 MHz spectrometer with deuterated chloroform ($CDCl_3$) and deuterated dimethyl sulfoxide (DMSO-d_6) as solvents.

2.2 Synthesis of TK-COOH

Acetone (0.08 mol, 6 mL) was reacted with thioglycolic acid (0.2 mol, 14 mL) in water at 50 °C for 48 hours. After the reaction, the mixture was cooled to 4 °C to allow the product to precipitate. The resulting precipitate was collected by filtration, washed three times with ethyl acetate, and dried.

2.3 Synthesis of TK-OH

TK-COOH (0.022 mol, 5 g) was dissolved in tetrahydrofuran (THF) under an ice bath. Lithium aluminum hydride (0.067 mol, 2.54 g) was gradually added, and the reaction was then heated to 90 °C for 2 hours. After completion, water was carefully added to quench any residual lithium aluminum hydride. The mixture was filtered, dried over anhydrous magnesium sulfate, and concentrated by rotary evaporation to yield the purified product.

2.4 Synthesis of TKA

TK-OH (0.015 mol, 3 g) was dissolved in dichloromethane (DCM) under an ice bath, and acryloyl chloride (0.034 mol, 2.73 mL) was added dropwise. The reaction proceeded at room temperature for 24 hours. The product was then purified for further application.

2.5 Synthesis of PBAE

A mixture of small molecular amines, dodecylamine, TKA, 1,6-hexanediol diacrylate, and bisphenol A glycerolate diacrylate was combined in specific molar ratios in DMF and heated at 90 °C for 72 hours. Upon cooling to room temperature, an additional small molecular amine was added at a 0.3 molar ratio to the reaction mixture and allowed to react for 24 hours at room temperature to complete the end-capping process.

2.6 Preparation of PBAE nanoparticles

PBAEs were dissolved in DMSO, and cholesterol, DSPC, and DMG-PEG-2000 were dissolved in ethanol at the weight ratio of 16:4:2:1. These solutions were mixed in specific ratios, then added to a 3-fold volume of 25 mM sodium acetate buffer containing mRNA to form PBAE nanoparticles (PBAE NPs). The resulting NPs were dialyzed against distilled water (MWCO = 3500 Da) for purification.

2.7 Transmission electron microscopy

LNP/mRNA samples were prepared by adding 5–10 μL of sample to a copper grid and allowing it to adsorb for 10 minutes. Excess fluid was then removed using filter paper. Next, 5–10 μL of phosphotungstic acid was applied for contrast staining for 3 minutes. After staining, the sample was air-dried at room temperature before imaging with a JEOL JEM-1200EX transmission electron microscope.

2.8 Evaluation of particle size and zeta potential

PBAE NP samples were diluted in ultrapure water to a final volume of 1 mL in a cuvette. The diluted samples were then analyzed using a Zetasizer Nano ZS to determine their hydrodynamic diameter, polydispersity index (PDI), and zeta potential.

2.9 Encapsulation efficiency

RNA quantification was done using the RiboGreen RNA kit (Solarbio). Each LNP formulation was diluted 1:100 in TE buffer in duplicate tubes, with 1% Triton X-100 added to one tube. Samples were analyzed using fluorescence at 492 nm and 540 nm, and RNA content was compared against a standard curve

to calculate encapsulation efficiency using $E=B\times(1-A)\times100$, where A is the RNA content in intact LNPs and B is the RNA content after Triton X-100 treatment.

2.10 Determination of pKa

The apparent pKa of lipid nanoparticles (LNPs) was measured using the fluorescent probe TNS (6-(p-toluidino)-2-naphthalenesulfonic acid, Macklin). Twelve buffer solutions, ranging from pH 4 to 10, were prepared and adjusted using HCl and NaOH as necessary. LNP samples were then mixed with each buffer, and fluorescence intensity was recorded at excitation and emission wavelengths of 322 nm and 431 nm, respectively. A fluorescence versus pH curve was plotted, and the apparent pKa was defined as the pH at which fluorescence indicated 50% protonation of the LNPs.

2.11 Hemolysis assay

Hemolysis assay was conducted as described before.[1] Blood was collected from BALB/c mice via the retro-orbital sinus, and red blood cells (RBCs) were isolated by centrifugation at 3000 rpm for 15 minutes. The RBCs were then washed three times with PBS and suspended in PBS solutions at pH 5.5 and 7.4. A 200 μL RBC suspension was added to each well of a 96-well plate. LNP samples and 1% Triton-X100 (as a positive control) were introduced to the wells, and the plate was incubated at 37°C for 1 hour. After incubation, the plates were centrifuged at 3000 rpm for 5 minutes. From each well, 100 μL of the supernatant was transferred to a clear 96-well plate for analysis. The absorbance at 540 nm was measured using a BioTek Synergy H1 microplate reader (Agilent) to evaluate hemolysis.

2.12 In vivo expression of luciferase mRNA

Animal study was approved by the Animal Experimentation Ethics Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University (AP20240156 and AP20240099). Six-week-old CD-1 mice were administered LNPs containing 4 μg of luciferase mRNA and 80 μg of active lipid via tail vein injection. Six hours post-injection, each mouse received an intraperitoneal injection of 100 μL

luciferin solution (15 mg/mL). After a 10-minute incubation, mice were imaged using a PerkinElmer in vivo imaging system to capture LNP distribution and mRNA expression.

2.13 Determination of mRNA expression in splenic cells

Six-week-old Ai9 mice were injected via the tail vein with 10 μg Cre mRNA formulated with 200 μg active lipid. Forty-eight hours post-injection, tdTomato expression in the spleen was assessed using an in vivo imaging system (IVIS). The spleen was then harvested, and a single-cell suspension was prepared by mechanically dissociating the tissue and filtering it through a 70 μm strainer.

For flow cytometry analysis, the cell suspension (2 × 10^6 cells in 100 μL) was resuspended in Flow Cytometry Staining Buffer (eBioscience) and incubated with fluorophore-conjugated antibodies at 4°C for 1 hour. Data acquisition was performed on a CytoFLEX flow cytometer (Beckman Coulter), and analysis was conducted with FlowJo v10 software. Gating strategy details are provided in Figure S6.

2.14 Vaccination by 93-TKA10-19/mOVA

C57BL/6 mice received two intravenous doses of the vaccine on days 0 and 5, with each dose containing 100 μg of active lipid and 5 μg of OVA mRNA. Untreated mice served as a control group.

Spleen cells were isolated from the immunized mice, and 2 million cells were plated per well in a 48-well plate with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 μL of a stimulation blocker (BD, 550583). After cell concentration adjustment, the cells were incubated at 37°C for 6-8 hours for stimulation. Following incubation, surface markers were stained using fluorophore-conjugated antibodies (listed in Table S) before fixation and permeabilization for intracellular marker staining. The prepared samples were analyzed using a CytoFLEX flow cytometer (Beckman Coulter). Data analysis was conducted using CytoFLEX software or FlowJo v10, with the gating strategy illustrated in Figure S7.

Antibody titers against OVA were measured by indirect ELISA. High-binding ELISA plates (Greiner Bio-One, USA) were coated with 50 μL of OVA (20 μg/mL) in sodium bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed with PBS containing 0.05% Tween-20 and blocked with 2% BSA (Sigma-Aldrich). Serum samples from immunized mice were initially diluted 1:100 and then serially diluted threefold. After adding the diluted serum to the wells, plates were incubated at room temperature for 1 hour. Following additional washes, horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-IgG, IgG1, and IgG2c at 1:50,000 dilution) were added and incubated for 1 hour. Plates were washed again, developed with TMB substrate for 20-30 minutes, and the reaction was stopped with ELISA stop solution. Absorbance was measured at 450 nm using a BioTek plate reader.

2.15 H&E staining

For biosafety examination, major organs including the heart, liver, spleen, lungs, and kidneys, were collected, fixed in paraformaldehyde, embedded in paraffin, and sectioned to a thickness of 10 μm. The sections were deparaffinized, rehydrated, and prepared for further staining. Hematoxylin and eosin (H&E) staining was conducted following the manufacturer's protocol. The stained sections were visualized using an Olympus upright microscope for histopathological assessment.

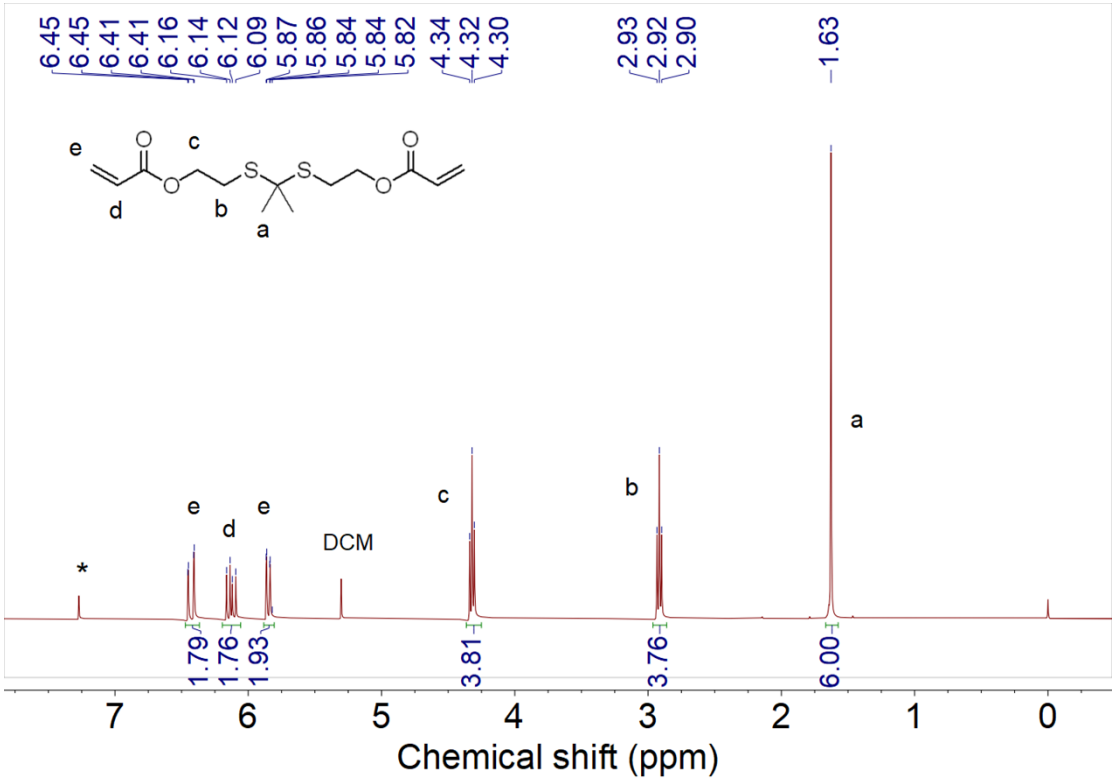

Fig. S1 ^{1}H NMR spectrum of TKA. (400 MHz, $CDCl_3$) δ 6.43 (dd, J = 17.4, 1.4 Hz, 2H), 6.13 (dd, J = 17.3, 10.5 Hz, 2H), 5.85 (dd, J = 10.4, 1.4 Hz, 2H), 4.32 (t, J = 7.0 Hz, 4H), 2.92 (t, J = 7.0 Hz, 4H), 1.63 (s, 6H).

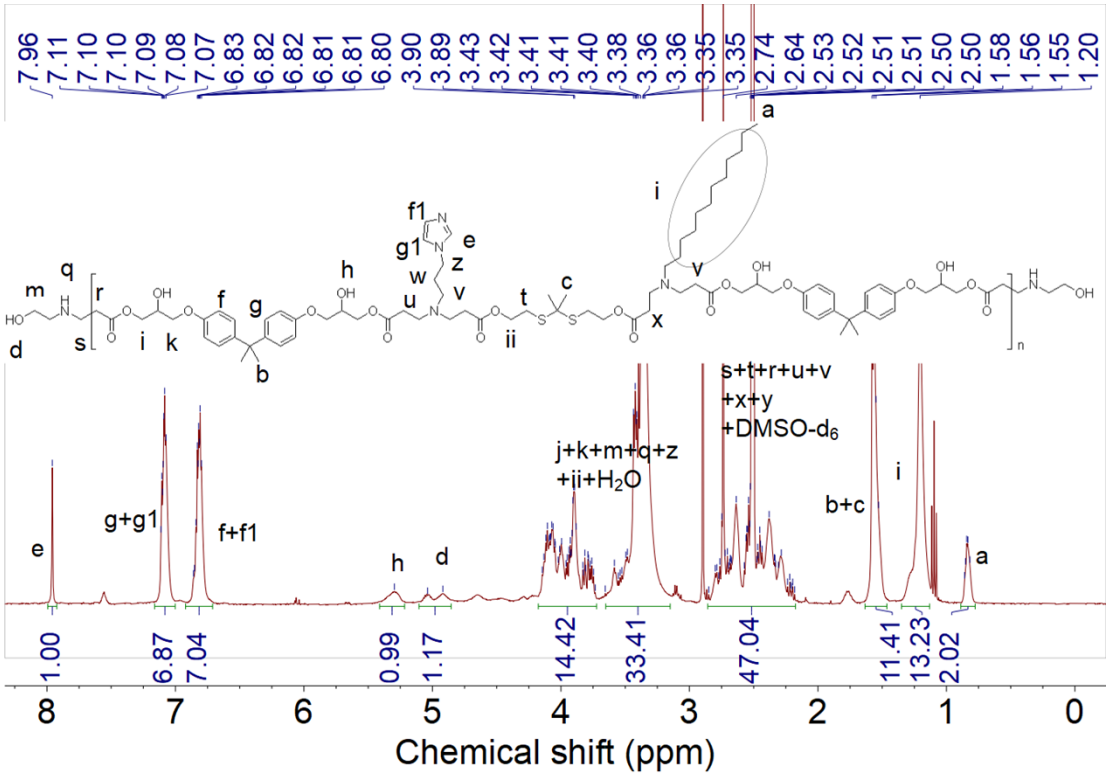

Fig. S2 ^{1}H NMR spectrum of 93-TKA10-19. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.96 (s, 1H), 7.16 – 7.00 (m, 7H), 6.92 – 6.71 (m, 7H), 5.30 (s, 1H), 4.98 (d, J = 47.7 Hz, 1H), 4.18 – 3.72 (m, 14H), 3.65 – 3.15 (m, 33H), 2.86 – 2.17 (m, 47H), 1.56 (dd, J = 13.1, 7.0 Hz, 11H), 1.20 (s, 13H), 0.84 (dt, J = 9.8, 4.6 Hz, 2H).

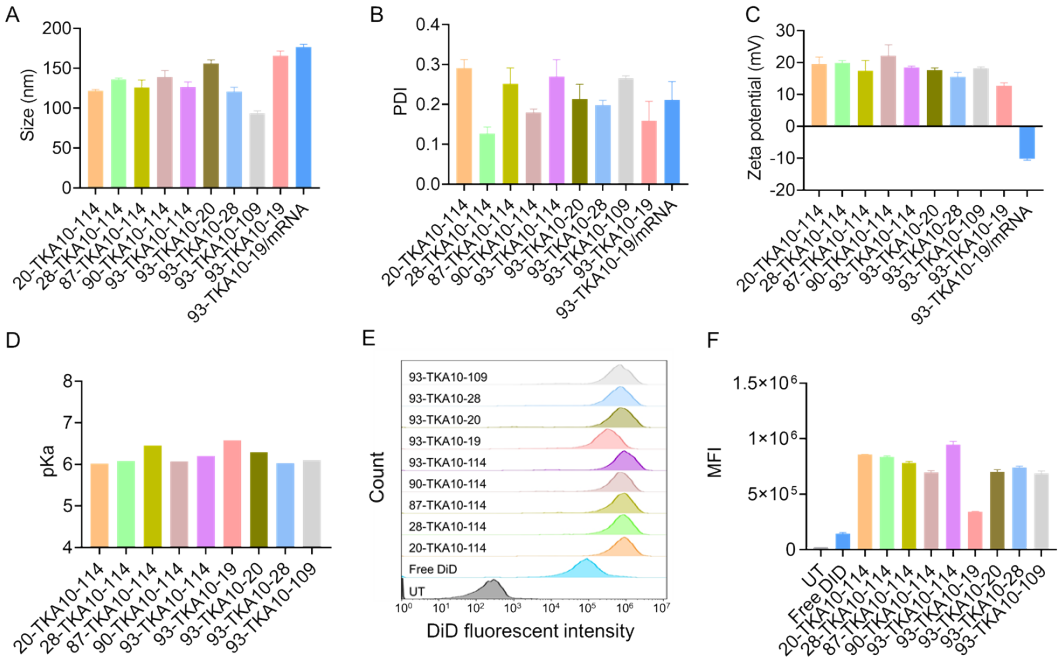

Fig. S3 Characterization of the physicochemical properties of PBAEs with different backbone and end cap amines. (A) Particle sizes of representative PBAE NPs. (B) PDI of representative PBAE NPs. (C) Zeta potentials of representative PBAE NPs. (E) Flow cytometry analysis of cellular uptake of DiD-labeled PBAE NPs. (F) Mean fluorescence intensity (MFI) of cells treated with DiD-labeled PBAE NPs. Data are represented as mean ± SEM.

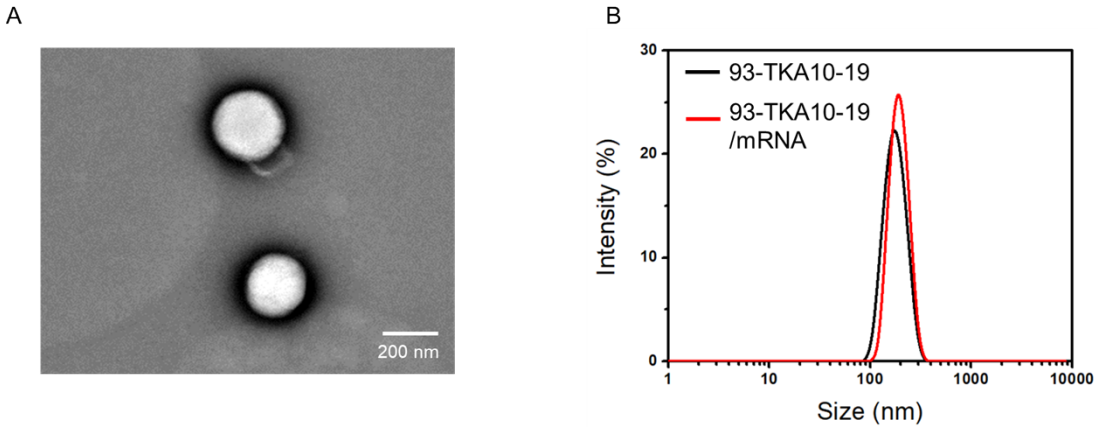

Fig. S4 (A) TEM images of 93-TKA-19 NP. (B) The diameter of 93-TKA-19 NP before and after encapsulation of mRNA.

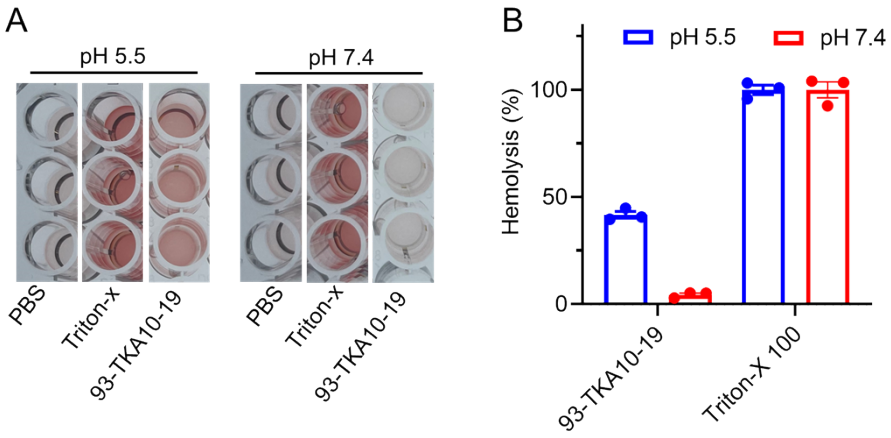

Fig. S5 Hemolysis of RBC after treatment of 93-TKA10-19 under pH 5.5 and 7.4.

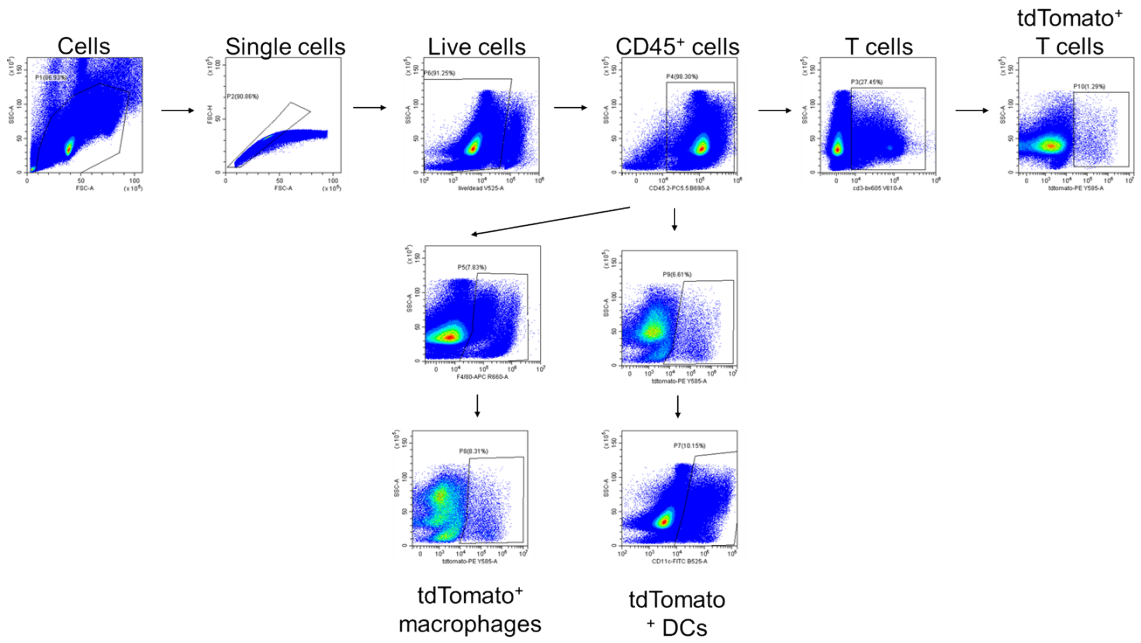

Fig. S6 Gating strategies for determining tdTomato^{+} splenic cells.

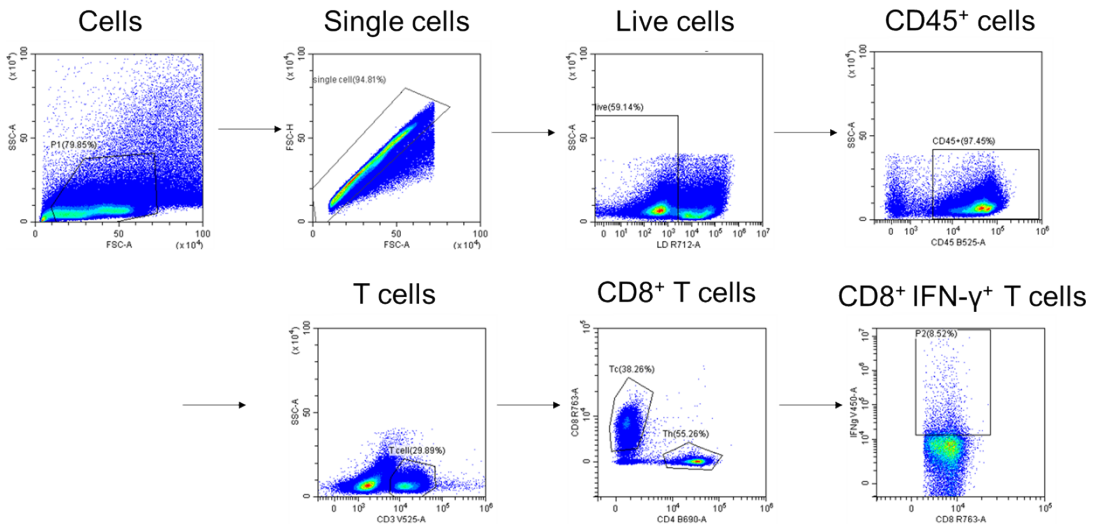

Fig. S7 Gating strategies for determining IFN-γ^+ $CD8^+$ cells.

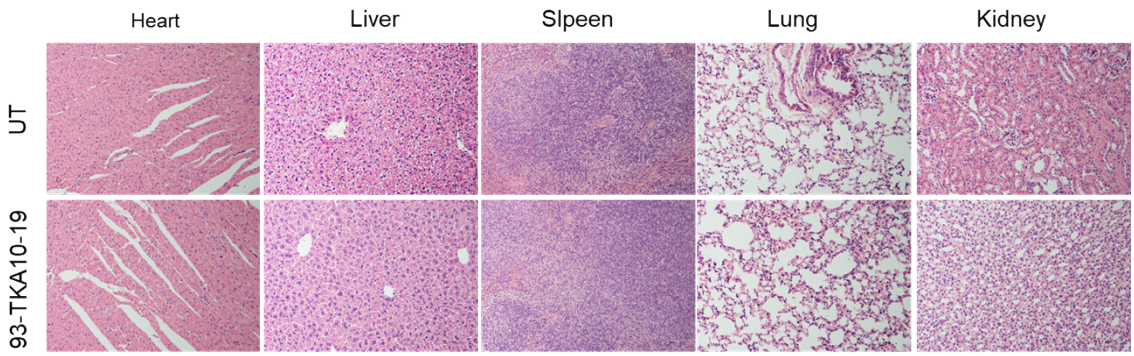

Fig. S8 Histological analysis of heart, liver, spleen, lung, and kidney tissue sections was performed using H&E staining to assess biosafety.

Reference:

1. X. Zhang, K. Su, S. Wu, L. Lin, S. He, X. Yan, L. Shi and S. Liu, *Angewandte Chemie International Edition*, 2024, **63**, e202405444.