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Supporting Information for

Inhaled mRNA nanoparticles dual-targeting cancer cells and macrophages in the lung for effective transfection

Zhongmin Tang[1+], Xinru You[1+], Yufen Xiao[1+], Wei Chen[1], Yongjiang Li[1], Xiangang Huang[1], Haijun Liu[1], Fan Xiao[1,2], Chuang Liu[1], Seyoung Koo[1], Na Kong[1,2], Wei Tao[1*]

1 Center for Nanomedicine and Department of Anesthesiology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

2 Liangzhu Laboratory, Zhejiang University Medical Center, Hangzhou, Zhejiang 311121, China

+ These authors contributed equally.

*Corresponding Author: Wei Tao

Email: wtao@bwh.harvard.edu

Materials and Methods

Materials

Generation 0 dendrimer (G0) of cationic ethylenediamine core-poly(amidoamine) and 1,2-epoxytetradecane were obtained from Sigma-Aldrich. Sodium salt form of hyaluronic acid (HA) with a molecular weight of 1.01M Da - 1.80M Da was purchased from Lifecore Biomedical. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)] (ammonium salt) (DSPE-PEG) with a PEG molecular weight (MW) of 3,000 Da was purchased from Avanti Polar Lipids. DSPE-PEG-NHS with a PEG molecular weight (MW) of 3,400 Da was purchased from Abbexa. Mannosamine was bought from Sigma-Aldrich. Lipofectamine with a molecular weight of 2,000 Da (Lip2k) was obtained from Invitrogen. *EGFP* mRNA and Cyanine 5-labeled *Luciferase* mRNA (Cy 5-labeled *Luc*-mRNA) were purchased from TriLink Biotechnologies. Primary antibodies used for western blot assay and immunofluorescence and immunohistochemistry staining: anti-p53 (Santa Cruz Biotechnology, sc-126; diluted for 1,000 times), anti-BAX (Cell Signaling Technology, #2774; diluted for 1,000 times), anti-PUMA (Santa Cruz Biotechnology, H-136; 1:1,000 dilution), and anti-cleaved Caspase-3 (Cell Signaling Technology, #9661; 1:1,000 dilution). The cationic lipid G0-C14 compound was prepared through the ring-opening reaction between 1,2-epoxytetradecane and G0 dendrimer following our previously depicted approaches. The Mannose-PEG-DSPE was prepared through an amide reaction between DSPE-PEG-NHS ester and mannosamine and then dialyzed against a 1000 Da dialysis tube. Steady-Glo® Luciferase Assay System for monitoring *in vitro* luciferase expression was bought from Promega Corporation. D-Luciferin (potassium salt) for monitoring *in vivo* luciferase expression was bought from Cayman.

Synthesis of mRNA NPs

A modified self-assembly approach was adopted to synthesize various mRNA-encapsulated NPs. First, the following solutions were prepared: G0-C14, HA, and DSPE-PEG or Man-PEG-DSPE were dissolved separately in dimethylformamide (DMF), deionized H_2O and deionized H_2O to form a homogeneous solution at the concentrations

of 0.5, 0.5, and 5 mg/mL, respectively. Then, 20 μg of mRNA (in 20 μL of stored buffer) and 250 μg of G0-C14 (in 500 μL of DMF) were mixed gently (at a G0-C14/mRNA weight ratio of 12.5) to enable the electrostatic complexation between the negatively charged mRNA and positively charged G0-C14. Afterwards, 250 μg of HA pre-dissolved in 500 μL of deionized H_2O) was quickly added to the complexes to achieve a homogeneous solution. Afterwards, the homogeneous mixture was added dropwise to 10 mL of DNase/RNase-free HyClone HyPure water (Molecular Biology Grade) containing 1 mg of DSPE-PEG or Man-PEG-DSPE under magnetic stirring (1,200 rpm). After a 30-min self-assembly reaction, the solution was centrifuged with Amicon tubes [Millipore, catalog no. UFC9100; MW cutoff (MWCO), 100 kDa] to purify the mRNA-encapsulated NPs. After washing 3 times with PBS, the mRNA NPs were collected and re-dispersed in pH 7.4 PBS buffer for further *in vitro* or *in vivo* use or storage at the pre-designated environments. The Cy 5-labeled *Luc*-mRNA with a length of 1,921 nucleotides was chosen as a model mRNA to investigate the effects on NP encapsulation and the subsequent mRNA transfection efficacy. As shown in Fig. S1, different compositions of G0-C14/HA were adjusted to investigate the sizes of targeted mRNA NPs. In addition, various NPs composed of different ratios of G0-C14/HA and DSPE-PEG/Man-PEG-DSPE were also synthesized and compared for *in vitro Luc*-mRNA transfection efficacy. After optimization, the NPs were synthesized by using Man-PEG-DSPE/G0-C14/HA at the ratio of 4:1:1 for the following *in vitro* and *in vivo* studies.

Characterization of the targeted mRNA NPs

The dynamic light scattering (DLS, ZetaPALS, Brookhaven Instruments Corporation) instrument was used to determine the size and the stability of the targeted mRNA NPs in PBS containing 10% fetal bovine serum at 37 °C. The size and morphology of mRNA NPs were characterized by JEOL 1200 EX transmission electron microscope (TEM) at an accelerating voltage of 80 kV. To quantify the mRNA encapsulation efficiency (EE%), Cy 5-labeled *Luc*-mRNA-encapsulated NPs were obtained using the same self-assembly strategy described previously. In brief, the fluorescence intensity of Cy 5-labeled *Luc*-mRNA encapsulated in the targeted NPs was measured by a microplate reader (TECAN, Infinite M200 Pro). The EE of the mRNA in the synthesized NPs was determined to be approximately 50%. The images of treated cells were recorded by a confocal laser scanning microscope (Olympus FV1000). The *in vitro* luminescence was also monitored by a microplate reader (TECAN, Infinite M200 Pro). The *in vivo* luminescence was detected by the *In Vivo* Imaging System (IVIS, PerkinElmer, Lumina LT Series III).

Cell lines

The cervical carcinoma cell lines of HeLa (#CCL-2) and the *p53*-null human non-small cell lung cancer (NSCLC) cell line of H1299 (#CRL-5803) were bought from American Type Culture Collection (ATCC). Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute-1640 (RPMI-1640) bought from ATCC were utilized to cultivate HeLa cells and H1299 cells, respectively. The cell culture medium was supplemented with fetal bovine serum (FBS; Gibco) at the volume ratio of 10% and penicillin/streptomycin (Thermo-Fisher Scientific) at the volume ratio of 1%. Opti-MEM Reduced Serum Medium was bought from Sigma Aldrich for diluting luciferase substrate *in vitro*.

***In vitro* transfection efficiency of targeted *Luc*-mRNA NPs**

To investigate the thermostability and protection efficiency of various formulations of targeted NPs, we used *Luc*-mRNA to study the cellular transfection efficacy in H1299 and HCT116 cells. Firstly, H1299 cells or HCT116 cells were seeded in 24-well plates at a density of 3×10^4 cells per well. After 24 h of cell attachment, cells were transfected with freshly prepared *Luc*-mRNA NPs at two different concentrations of mRNA (0.25, 0.5, and 1 μg/mL) for various time points, and then the bioluminescence intensity was detected.

***In vitro* transfection efficacy of targeted *EGFP*-mRNA NPs**

The *p53*-null H1299 cells were cultured in 96-well plates with a density of 3×10^3 cells per well. After overnight of cell attachment, the cells were treated with targeted *EGFP*-mRNA NPs at two different mRNA concentrations (0.25, or 1.0 μg/mL) for 24 h, followed by the addition of 100 μL of fresh complete medium for incubation for another 24 h. Then, the cells were harvested by trypsinization and were resuspended in PBS. The expression of green fluorescence induced by GFP was measured using flow cytometry (BD Biosystems). Finally, the percentages of cells that expressed GFP were quantified and analyzed by FlowJo software.

Anti-cancer efficacy of targeted *p53*-mRNA NPs

The *p53*-null H1299 cells were cultured in 96-well plates with a density of 3×10^3 cells per well. After overnight of cell attachment, the cells were treated with targeted *p53*-mRNA NPs at various mRNA concentrations (0.13, 0.25, 0.5, or 1 μg/mL) for 24 h, followed by the addition of 0.1 mL of fresh complete medium and incubation for another 24 h to evaluate the cell viability. Afterwards, 100 μL of fresh RPMI 1640 and 10 μL of AlamarBlue reagent were added to each well. Then, the plates were put in an incubator for 20 min. To measure the number of viable cells in each condition, a plate reader (TECAN, Infinite M200 Pro) was used to measure the fluorescence intensity at 590 nm (excitation at 545 nm).

***In vitro* and *in vivo* targeting efficacy of HA-Man-PEG NPs to inflammatory macrophages**

For *in vitro* targeting efficacy evaluation, RAW264.7 cells were firstly treated with 100 ng/mL LPS for 1 h, then types of NPs were incubated with the RAW264.7 cells and the uptake at 0.5, 2, and 6 h were monitored using the confocal image microscope.

For *in vivo* experiments, the Balb/c mice first inhaled the LPS solution (2 mg/Kg). Twenty-four hours later, Cy5-labeled mRNA only and Cy5-labeled mRNA NPs were inhaled by the mice, then the whole lung tissues were firstly collected at 24 h for mRNA only group, 2 h, 4 h, 6 h, 8 h, and 24 h for mRNA NPs group, and then the nucleus was stained with DAPI, the macrophage was stained with Alex 488 for the subsequent imaging.

Animals

All *in vivo* studies were performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee of Brigham and Women's Hospital, and the Institutional Animal Ethics Committee of Zhejiang University. Under strict regulations and pathogen-free conditions, animal studies were conducted in the animal facility of Brigham and Women's Hospital and Zhejiang University. Female BALB/c mice (4- to 6-week-old) were purchased from the Jackson Laboratory, and six-week-old C57BL mice were obtained from Zhejiang Medical Academy Animal Center. and kept for at least 7 days before conducting animal experiments to adapt the mice to the environment and food in animal facilities.

***In vivo* transfection of targeted *Luc*-mRNA NPs**

BALB/c mice with a weight of 18–20 g were treated with targeted *Luc*-mRNA NPs *via* intramuscular injection or inhalation delivery (10 μg of *Luc*-mRNA per mouse, n = 6 per group). At the pre-designated time points (6, 24, and 48 h) after administration, mice were intraperitoneally injected with D-Luciferin at a dose of 150 mg/kg. Ten min later, the bioluminescence images of the mice were recorded using an IVIS (PerkinElmer, Lumina LT Series III). In addition, the *ex vivo* luminescence images of main organs including lung, liver, heart, spleen, and kidneys were also recorded (n = 3) at 24 h after inhalation delivery of targeted *Luc*-mRNA NPs. Moreover, Cy 5-labeled targeted *Luc*-mRNA NPs were utilized to study the biodistribution of the mRNA after inhalation delivery. The main organs including the lung, liver, heart, spleen, and kidneys of mice were collected for *ex vivo* fluorescence imaging 6 h after intravenous injection or inhalation delivery.

***In vivo* transfection efficacy of targeted *EGFP*-mRNA or *p53*-mRNA NPs**

BALB/c mice with a weight of 18–20 g were treated with targeted *EGFP*-mRNA or *p53*-mRNA NPs *via* inhalation delivery (10 μg of *EGFP*-mRNA or *p53*-mRNA per mouse, n = 6 per group) or the equivalent dose of blank NPs. Twenty-four h after inhalation delivery, mice were anesthetized and the lung tissues were harvested for immunofluorescence staining of GFP or p53 to investigate the *in vivo* mRNA transfection efficacy.

***In vivo* mRNA targeted delivery to the orthotopic lung tumor site**

HE staining: To establish the orthotopic lung tumor model, Lewis lung carcinoma cells were used. Six-week-old C57BL mice were obtained from Zhejiang Medical Academy Animal Center. Animal studies were conducted following the protocol approved by the Institutional Animal Ethics Committee of Hangzhou Normal University. The lung tissues, collected for H&E analysis to assess the lung tumor verification from each group, were fixed in 10% buffered formalin, and embedded in paraffin. 5 μm section was obtained from each specimen and stained with H&E to assess morphology.

Immunofluorescence (IF) staining: Lung tissues collected from different groups were placed on glass coverslips,

fixed with 4%methanol, and permeabilized by 0.2% Triton X-100. Then 2.5% bovine serum albumin was used for blocking for 1 hour. Then the samples were incubated with blocking solution (primary antibodies at 1:250) for 1 hour and then incubated in goat anti-rat-Alexa Fluor 647 in blocking buffer (dilution 1:500) at room temperature for another 1 h. Nuclei were counterstained with Hoechst 33342. The samples were imaged using a laser confocal microscope (Leica, Germany).
Fluorescence intensity: To evaluate the biodistribution of Cy 5-labeled luciferase-mRNA in lung tissues, empty NPs or Cy 5-labeled *Luc*-mRNA NPs were inhaled into lung tumor-bearing mice. At 24 or 48 hours post-administration, the fluorescence intensity was measured and imaged by the Biospace-Optima in vivo imaging system.

***In vivo* biocompatibility evaluation**
To evaluate the potential *in vivo* toxicity of the targeted mRNA NPs, the main organs including the heart, liver, spleen, lung, and kidney were collected 24 h after inhalation delivery of control (PBS), blank NPs, targeted *EGFP*-mRNA or *p53*-mRNA NPs at a dose of 10 μg of mRNA per mouse. The tissue section of the main organs was subjected to hematoxylin and eosin (H&E) staining for standard histological examination. Moreover, the blood was retro-orbitally collected 24 h after inhalation delivery of control (PBS), blank NPs, targeted *EGFP*-mRNA or *p53*-mRNA NPs at a dose of 10 μg of mRNA per mouse. Then, the serum level of several cytokines including interleukin-6 (IL-6), IL-12, tumor necrosis factor-α (TNF-α), and interferon (IFN-γ) were measured using an enzyme-linked immunosorbent assay (ELISA).

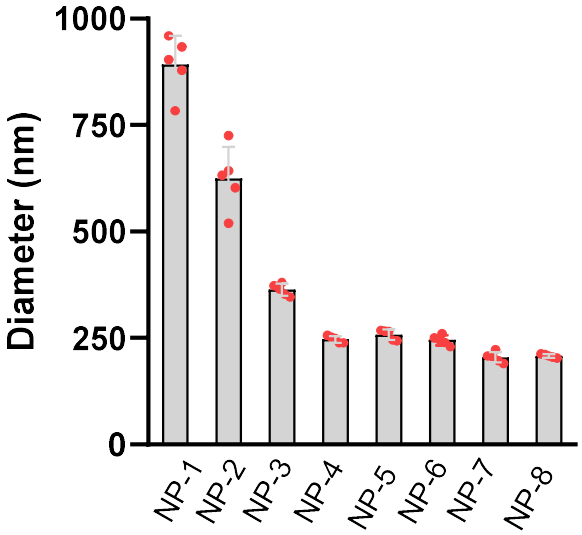

Figure S1. DLS size of NPs synthesized in different conditions (n = 5). The detailed conditions are shown as follows:
NP-1: 100 μL G0-C14 (2.5 mg/mL), 100 μL HA (5 mg/mL) with DSPE-PEG-Man;
NP-2: 100 μL G0-C14 (2.5 mg/mL), 50 μL HA (5 mg/mL) with DSPE-PEG-Man;
NP-3: 100 μL G0-C14 (2.5 mg/mL), 100 μL HA (2.5 mg/mL) with DSPE-PEG-Man;
NP-4: 50 μL G0-C14 (2.5 mg/mL), 50 μL HA (2.5 mg/mL) with DSPE-PEG-Man;
NP-5: 250 μL G0-C14 (1 mg/mL), 250 μL HA (1 mg/mL) with DSPE-PEG-Man;
NP-6: 500 μL G0-C14 (0.5 mg/mL), 250 μL HA (1 mg/mL) with DSPE-PEG-Man;
NP-7: 500 μL G0-C14 (0.5 mg/mL), 500 μL HA (0.5 mg/mL) with DSPE-PEG-Man;
NP-8: 500 μL G0-C14 (0.5 mg/mL), 500 μL HA (0.5 mg/mL) with DSPE-PEG.

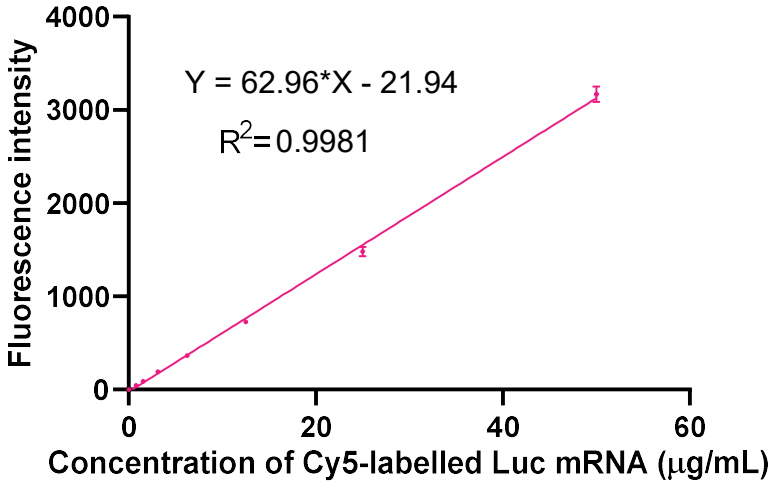

Figure S2. Calibration curve of concentration-dependent fluorescence intensity of naked Cy 5-labeled *Luc*-mRNA.

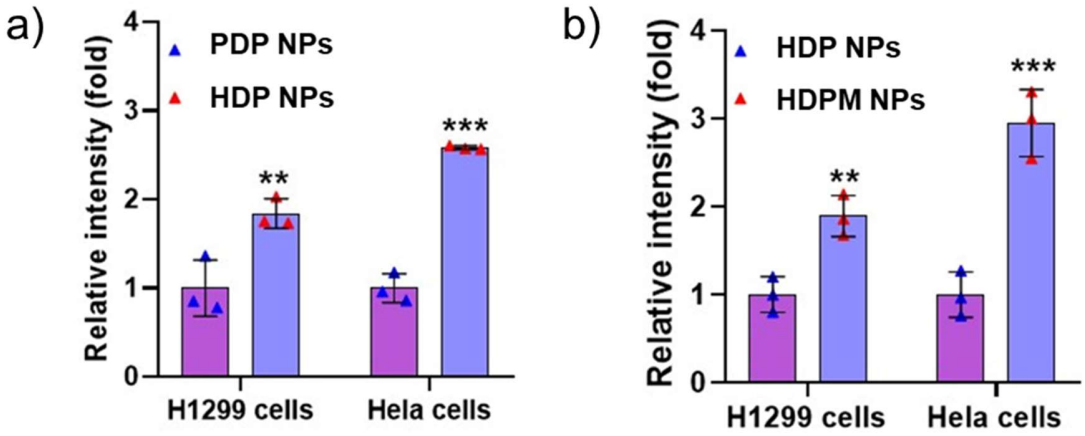

Figure S3. Relative bioluminescence intensity of different mRNA NPs in H1299 cells and HeLa cells (n = 3): (**a**) non-targeted mRNA PDP NPs *v.s.* single-targeted mRNA HDP NPs (average bioluminescence intensity in the PDP group was defined as 1); (**b**) single-targeted mRNA HDP NPs *v.s.* dual-targeted mRNA HDPM NPs (average bioluminescence intensity in the HDP group was defined as 1). Statistical significance was determined using Two-way ANOVA analysis (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001).

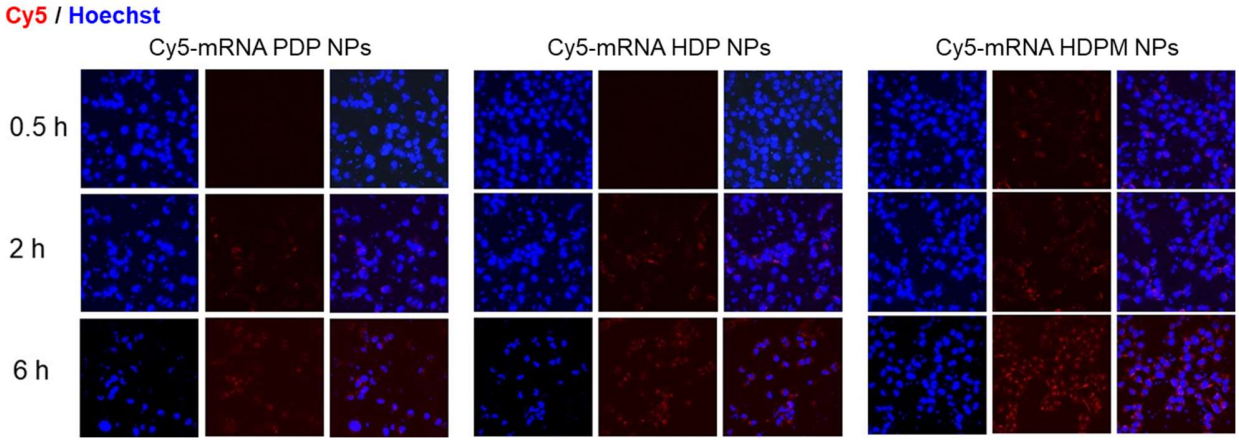

Figure S4. Cellular uptake of different mRNA NPs (non-targeted mRNA PDP NPs, single-targeted mRNA HDP NPs, or dual-targeted mRNA HDPM NPs) in H1299 cells at different time points (0.5, 2, and 6 h), , showing the dual targeting ability of HA and Mannose-containing NPs to the lung cancer cells. The images were captured under 40x magnification.

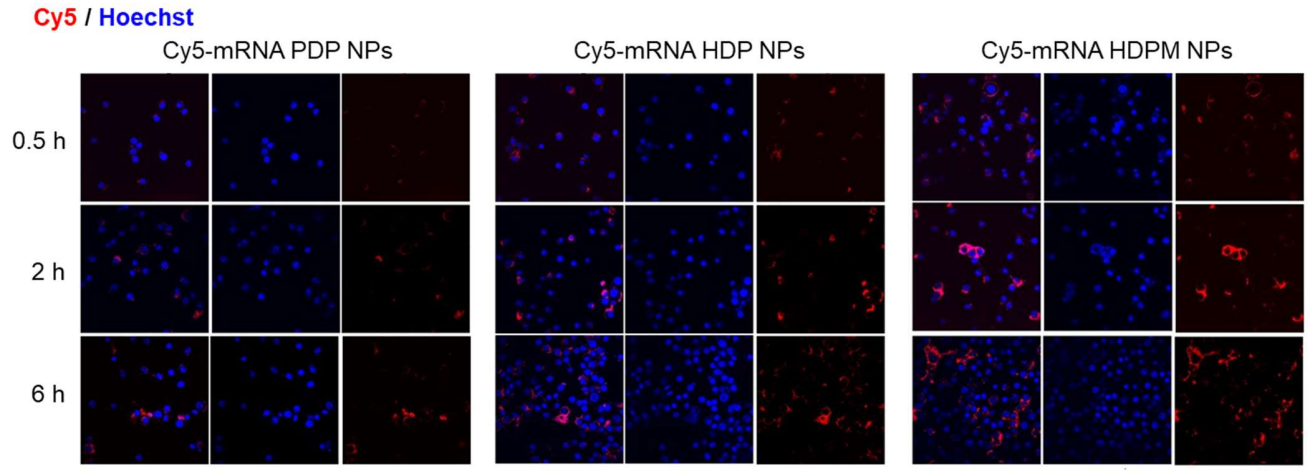

Figure S5. Cellular uptake of different mRNA NPs (non-targeted mRNA PDP NPs, single-targeted mRNA HDP NPs, or dual-targeted mRNA HDPM NPs) in LPS-induced macrophages at different time points (0.5, 2, and 6 h), showing the dual targeting ability of HA and Mannose-containing NPs to the pro-inflammatory macrophages. The images were captured under 60x magnification.

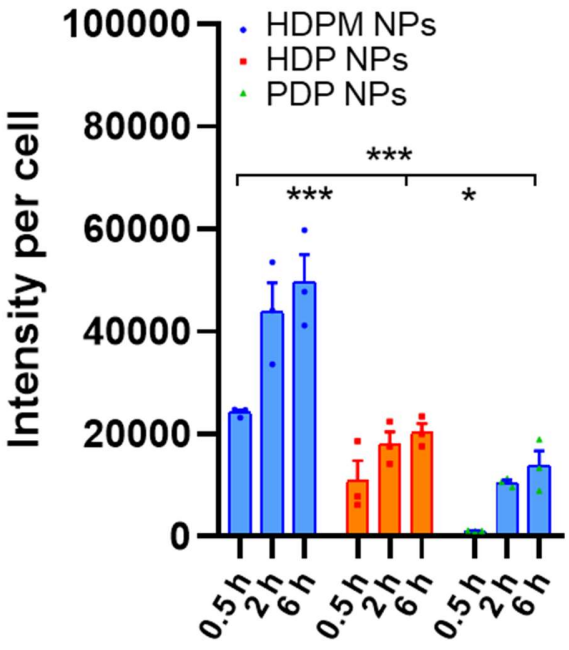

Figure S6. Corresponding quantitative results of the three groups in Figure S5. Statistical significance was calculated using Two-way ANOVA (*** P<0.001, * P<0.05). (n = 3, Mean ± SEM).

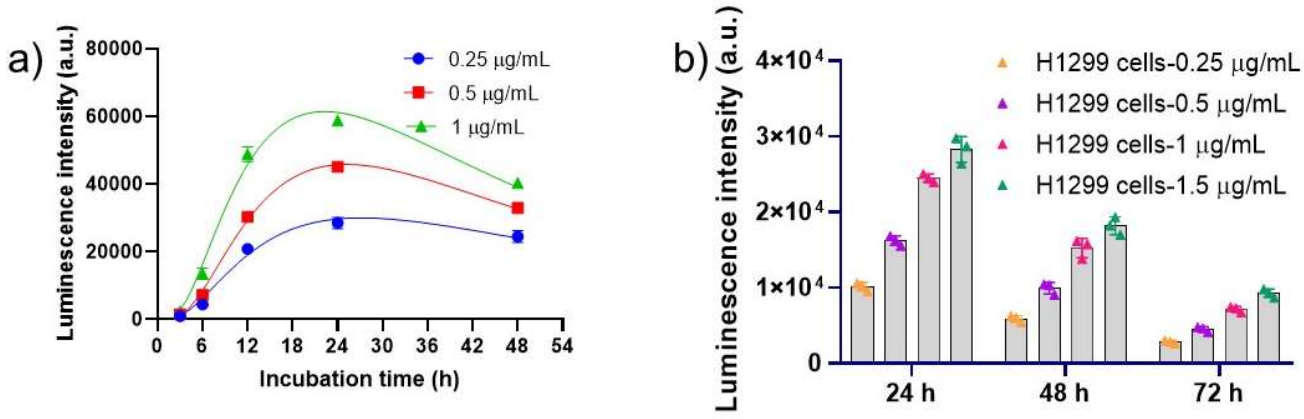

Figure S7. Incubation time- and concentration-dependent luminescence intensity of H1299 cells after the treatment of *Luc*-mRNA HDPM NPs (n = 3, Mean ± SEM).

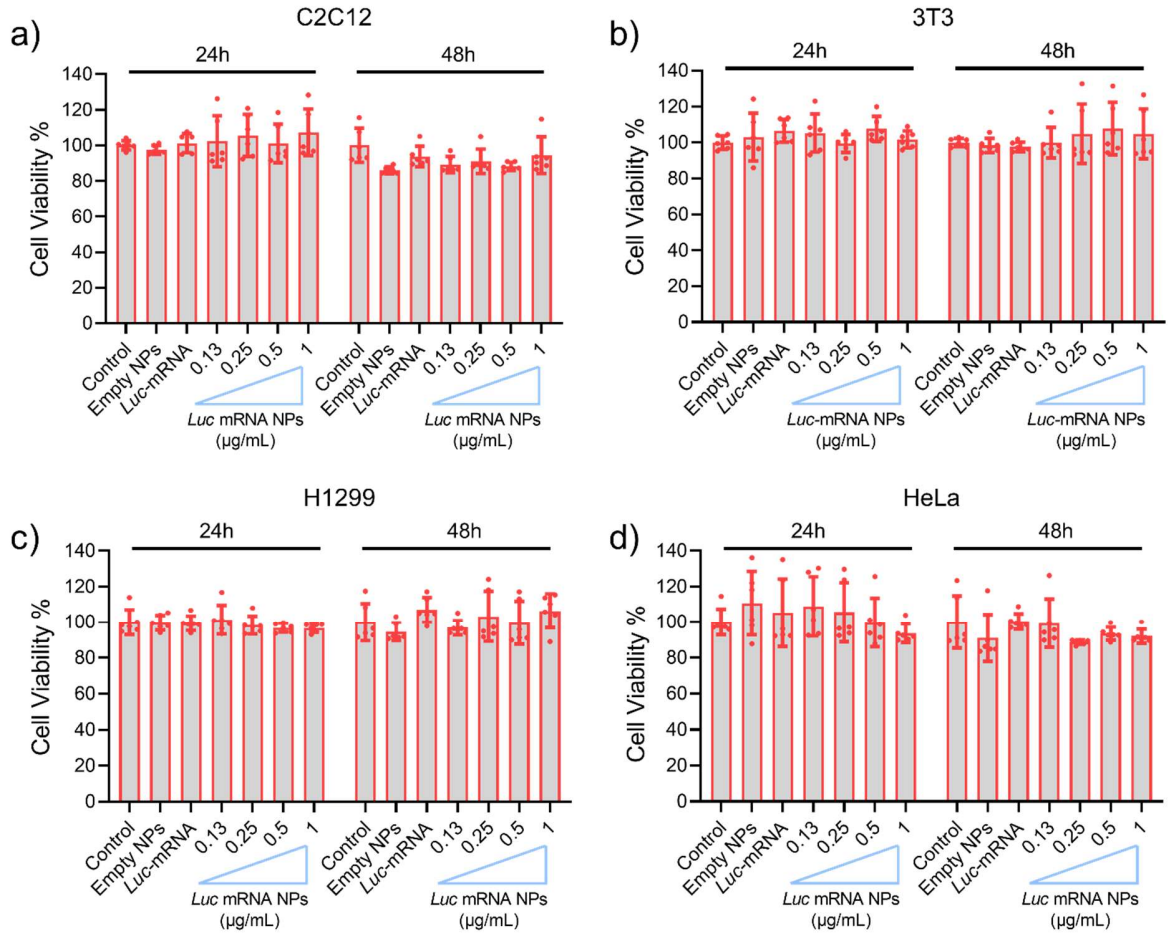

Figure S8. Viability of C2C12 cells (**a**), 3T3 (**b**), H1299 (**c**) and HeLa (**d**) after 24 h or 48 h treatment of empty HDPM NPs, free Luc-mRNA, or Luc-mRNA HDPM NPs at the mRNA concentrations of 0.13, 0.25, 0.5 and 1.0 μg/mL.

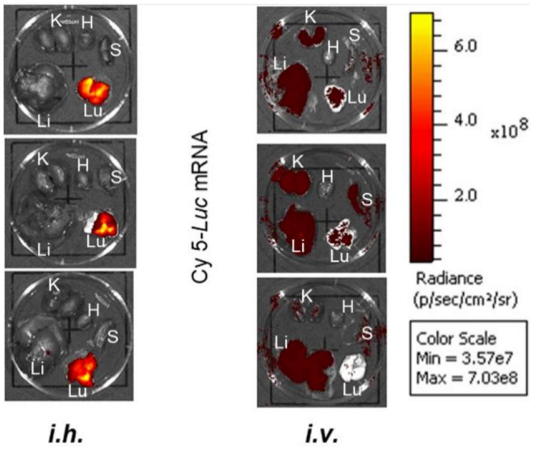

Figure S9. *Ex vivo* biodistribution of Cy5-labeled *Luc*-mRNA HDPM NPs (10 μg *Luc* mRNA/mice) at 6 h after inhalation (*i.h.*) delivery or intravenous (*i.v.*) administration. K: kidney; H: heart; S: spleen; Lu: lung; Li: liver.

Figure S10. Image of spleen isolated from Balb/c mice treated with PBS and *p53*-mRNA HDPM NPs for 7 days (n = 6).

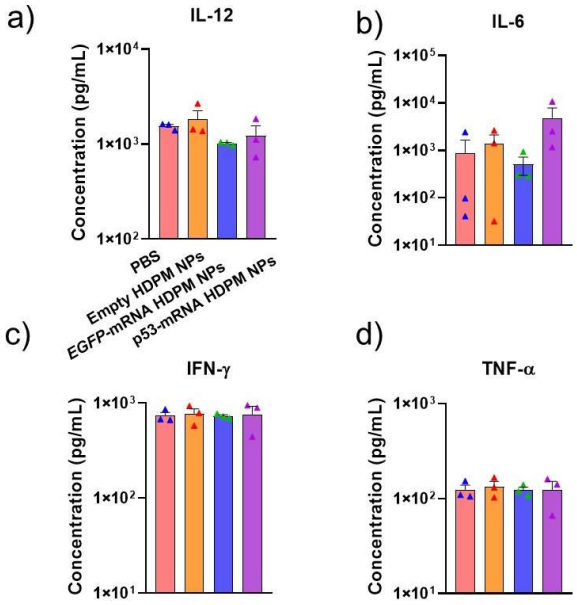

Figure S11. Serum level of various cytokines (IL-6, IL-12, TNF-α, and IFN-γ) of BALB/c mice 24 h after the inhalation delivery of PBS, empty HDPM NPs, *EGFP*-mRNA HDPM NPs, or *p53*-mRNA HDPM NPs (n = 3, mean ± SEM).

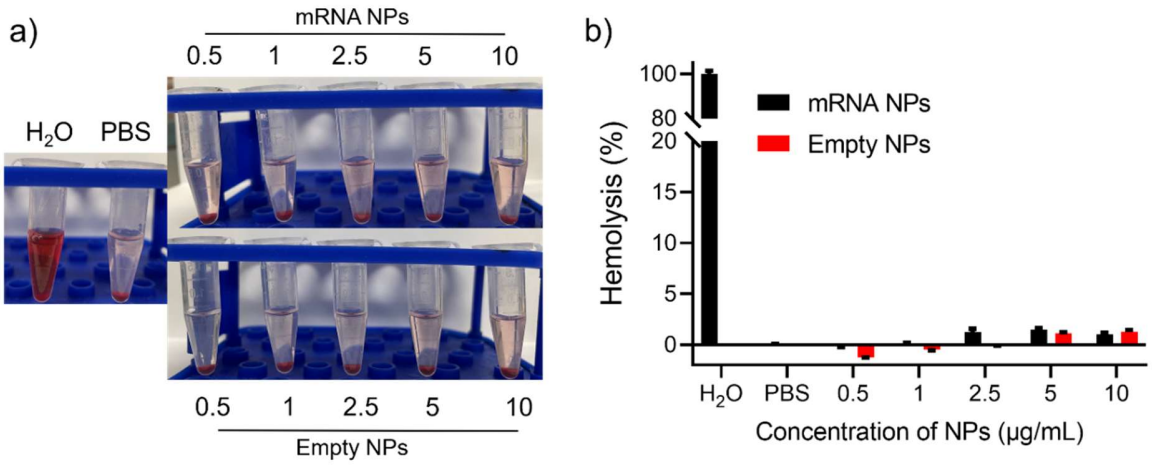

Figure S12. Hemolysis quantification of RBC incubated with *p53*-mRNA HDPM NPs and empty HDPM NPs at different mRNA concentrations for 0.5 h (n = 3, mean ± SEM).

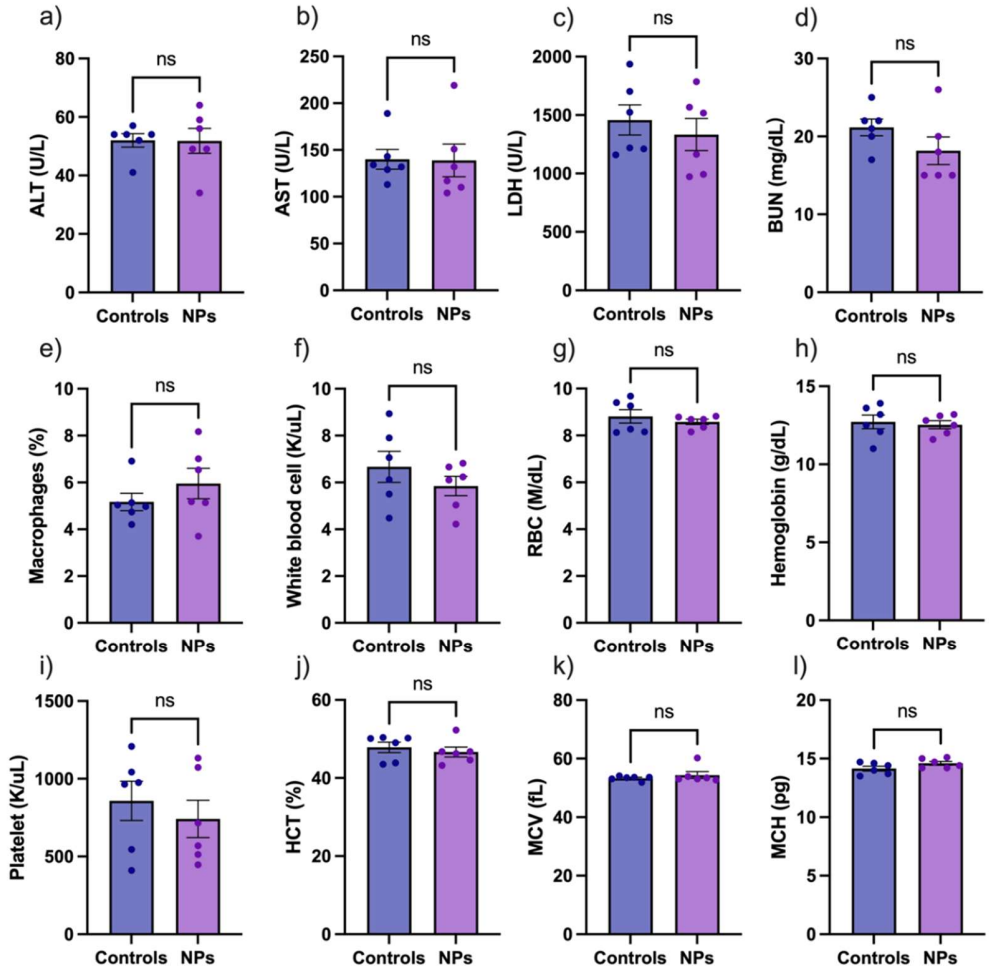

Figure S13. Blood biochemistry analysis (**a**-**d**) and blood hematology analysis (**e**-**l**) of Balb/c mice treated with PBS and *p53*-mRNA HDPM NPs for 7 days (n = 6, mean ± SEM).

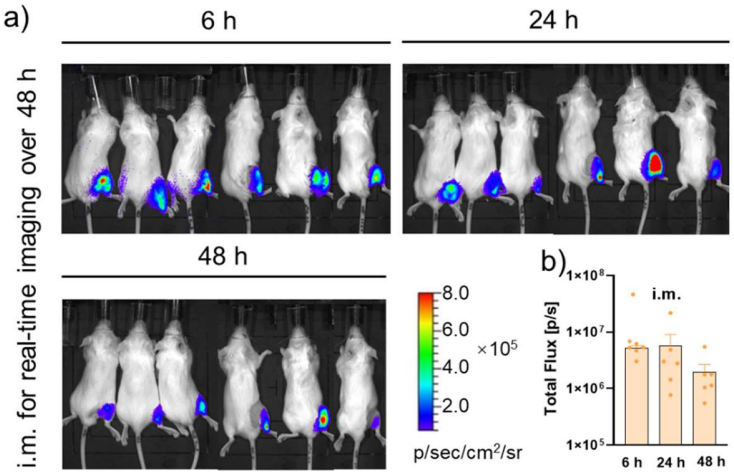

Figure S14. (**a**) *In vivo* bioluminescence images of BALB/c mice at different time points after receiving *Luc*-mRNA NPs via intramuscular (*i.m.*) injection. (**b**) Quantitative analysis of total luminescence flux at different time points of Figure S14a (n = 6, mean ± SEM).