Electronic Supplementary Information

A Living Neutrophil Biorobot Synergistically Blocks Multifaceted Inflammatory Pathways in Macrophages to Effectively Neutralize Cytokine Storm

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Experimental

Materials and instruments. 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine (HSPC) and cholesterol were purchased from AVT (Shanghai) Pharmacutical Tech Co., Ltd (Shanghai, China). 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol)2000-mannose (DSPE-PEG2000-mannose) was obtained from Tuoyang Biotech Co., Ltd (Shanghai, China). Lipopolysaccharide (LPS), polyinosinic-polycytidylic acid [poly(I:C)], double-stranded DNA (dsDNA) were purchased from Invivogen (Carlsbad, CA, USA). All the chemicals and solvents were of reagent grade unless otherwise indicated. All the chemicals were purchased from Sigma-Aldrich (St. Louis, USA) and Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) without further purification.

Transmission electron microscope (TEM) images were taken with an H-800 transmission electron microscope (Hitachi, Japan). Dynamic light scattering (DLS) was detected on a Litesizer 500 dynamic light scattering (Anton Paar, Austria). High-performance liquid chromatography mass

spectrometry (HPLC-MS, Shimadzu, Japan) was used for quantification of TPCA-1 and H151. Fluorescent measurements were taken on a FluoroMax-4 Spectrofluorometer (Horiba, Japan). Cytotoxicity assays were performed on an Infinite 200 pro microplate reader (Tecan, Switzerland). Fluorescent images were taken by a FV3000 confocal laser scanning microscopy (CLSM, Olympus, Japan). Intracellular fluorescence was detected by a flow cytometry (BD FACSVerse, USA). *In vivo* images were conducted on a IVIS Spectrum (PerkinElmer, USA).

Cell culture. RAW 264.7 macrophages were purchased from American Type Culture Collection (ATCC, USA), and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and penicillin/streptomycin (1%, w/v). Cells were cultured at 37 °C under an atmosphere of 5% CO_2 and 90% relative humidity.

Animals. Male C57BL/6 mice were purchased from Hangzhou Medical College (Hangzhou, China). All animal researches were performed in accordance with National Institute of Health Guidelines under the protocols, approved by the ethics committee at the Affiliated Drum Tower Hospital of Nanjing University Medical School (SYXK(Su)2019-0056).

Preparation of M-Lip@TH. Mannose decorated-liposome loading TPCA-1 and H-151 (M-Lip@TH) was prepared using the classic thin lipid film hydration method. Firstly, HSPC, cholesterol and DSPE-PEG2000-mannose (20:6:3, w/w) were dissolved in the mixture of chloroform and methanol (3:2, v/v), followed by adding the H-151 (0.05 mg) and TPCA-1 (0.05 mg) dispersed in methanol (1 mL). The mixture was evaporated to form a dried thin film, and the film was hydrated with oscillator at 60 °C. Then, the resulting large liposome vesicles were ultrasonicated at 300 W for 15 min in an ice-water bath using a lab ultrasonic cell pulverizer (Shanghai ZOLLO Industrial Co., Ltd), generating M-Lip@TH. As for control, M-Lip@TH without mannose decoration (named as Lip@TM), M-Lip@TH without loading H-151 (named as M-Lip@T) and M-Lip@TH without loading TPCA-1 (named as M-Lip@H) were also prepared.

Characterization of M-Lip@TH. The size distribution and zeta potential of M-Lip@TH were evaluated by the dynamic light scattering (DLS). The morphology was observed by TEM. The

concentrations of H-151 and TPCA-1 were analyzed by HPLC-MS. For TPCA-1, and the mobile phase was HPLC-grade methanol, ddH_2O (65:35, v/v). The detection was conducted at wavelength of 310 nm and the flow rate was 1 mL/min. For H-151, the flow rate and solvent were as follows: 0.5 mL/min; solvent A, 0.05% formic acid in ddH_2O; solvent B, 0.05% formic acid in acetonitrile; gradient from 10% to 90% solvent B within 6 min. The detection was conducted at wavelength of 254 nm.

Drug loading (DL) rate and encapsulation efficiency (EE) of M-Lip@TH. For investigate the DL and EE of M-Lip@TH, 100 μL of Lip@TH was dissolved in 900 μL DMSO, and then vortexed for 30 s and centrifuged at 15000 rpm for 10 min. The drug concentrations were analyzed by HPLC-MS, and the DL and EE of drug in M-Lip@TH were calculated as follows:

$$DL\ (\%) = {}^{W_d}/_{W_s} \times 100 \quad (1)$$

$$EE\ (\%) = {}^{W_d}/_{W_t} \times 100 \quad (2)$$

where W_d is the total amount of drug in M-Lip@TH, W_s is the total amount of M-Lip@TH, and W_t is the initial amount of drug used to prepare M-Lip@TH.

Physical stability of M-Lip@TH. M-Lip@TH was stored at 4 °C for 14 d, the size and zeta potential were tested every other day by the DLS.

Serum stability of M-Lip@TH. M-Lip@TH incubated with 50% FBS was kept at 37 °C for 24 h, the size of M-Lip@TH was measured at 0, 2, 4, 6, 8, 12 and 24 h by the DLS.

***In vitro* release of H-151 and TPCA-1 in M-Lip@TH.** To determine the drug release profile of M-Lip@TH, 1 mL of M-Lip@TH was added into a dialysis bag (molecular weight cut-off of 2000 Da, Millipore, USA). The dialysis bag was immersed into 50 mL of PBS solution containing 1% Tween-80 at 37 °C with stirring at 50 rpm. Then, 0.1 mL of dialysis medium was withdrawn at different time points (0, 1, 2, 4, 6, 8, 12 and 24 h) with the addition of equivalent volume of release medium, followed by measuring drug concentrations by HPLC-MS.

Preparation of Biorobot. Mature murine neutrophils were isolated from bone marrow in murine femurs and tibias. After lysing erythrocytes, cells from bone marrow were resuspended in PBS, and added into a percoll mixture solution consisting of 55, 65, and 75% Percoll (v/v), followed by centrifuging at 3000 rpm for 30 min. neutrophils were collected at the interface of 65 and 75% fractions and washed twice by PBS. The purity of neutrophils was evaluated using immunofluorescence double staining with fluorescein isothiocyanate (FITC)-conjugated Ly-6G/Ly-6C (Gr-1) antibody and phycoerythrin (PE)-conjugated MAIR-IV (CLM-5) antibody. Subsequently, neutrophils (10^7 cells/mL) were treated with M-Lip@TH (400 μM of TPCA-1 and 400 μM of H-151) at 37 °C for 2 h to generate Biorobot. The morphology of neutrophils and Biorobot stained with Wright-Giemsa were observed with an optical microscope.

The loading efficiency of H-151 and TPCA-1 in Biorobot. For investigating the drug loading efficiency of Biorobot, 100 μL of Biorobot was disrupted by 900 μL DMSO, and then vortexed for 3 min and centrifuged at 15000 rpm for 10 min. The supernatant was collected to determine the drug concentrations by HPLC-MS.

Cytotoxicity of M-Lip@TH against neutrophils. The cytotoxicity of M-Lip@TH was determined by MTT assay. neutrophils were seeded into 96-well plates (2×10^4 cells/well), and treated with M-Lip@TH with different concentrations of H-151 and TPCA-1 for 4 h and 8 h. Subsequently, 20 μL of MTT solution (5 mg/mL) was added into each well and incubated for another 4 h, followed by removing the medium and adding DMSO (150 μL) to dissolve the residue. The absorbance intensity at 570 nm was measured using a microplate reader.

Uptake and distribution of M-Lip@TH in neutrophils. Neutrophils (10^7 cells/mL) were incubated with rhodamine B (RhB)-labeled M-Lip@TH (RhB concentration of 1 μM) at 37 °C for 0.5, 1, 1.5, 2, 2.5 and 3 h. The cells were washed three times with PBS, followed by measuring the intracellular fluorescence intensity using the flow cytometry. Subsequently, neutrophils were incubated with RhB-labeled M-Lip@TH for 1.5 h, and then centrifuged at 800 rpm for 5 min. The cell was then washed three times with PBS, stained with Hoechst 33342 for 15 min at room temperature, and imaged by CLSM.

SDS-PAGE analysis of Biorobot. The proteins in neutrophils and Biorobot were determined using SDS-PAGE. The total protein concentrations of neutrophils and Biorobot were quantified by Pierce™ BCA Protein Assay Kit. After denaturization in 100 °C water bath for 15 min, 20 μg of each sample was loading into 10% SDS-PAGE, followed by running under the voltage of 80 V for 0.5 h then 100 V for 1.5 h. Subsequently, the gel was stained with Coomassie blue for 2 h and washed by deionized water overnight. The image was taken by a camera.

Evaluation of physiological functions of Biorobot. The inflammation-responsive expression of the specific protein CD11b and chemotaxis of Biorobot were evaluated. The neutrophils or Biorobot was incubated with 0, 1, 10, 100 nM of fMLP at 37 °C for 1 h, respectively, and then washed three times with PBS, followed by incubating with PE-conjugated CD11b antibody for 0.5 h at room temperature. The fluorescence intensity was determined using the flow cytometry.

The chemotaxis of Biorobot was evaluated using transwell migration assay (3 μm of pore size and 6.5 mm of diameter). Neutrophils or Biorobot were added to the upper chamber of the transwell, respectively, and the lower chamber of the transwell was filled with DMEM containing different concentrations (0, 1, 10, 100 nM) of fMLP. After 0.5-h incubation, the cells in the lower chamber were collected, observed with an optical microscope and counted with a haemacytometer. The migration ratio was calculated as follows:

$$Migration\ ratio\ (\%) = N_{low}/N_{add} \times 100 \quad (3)$$

where N_{low} and N_{add} are the counted number of neutrophils in the lower chamber and added in the upper chamber, respectively.

Stability of M-Lip@TH in neutrophils. FRET analysis was used to evaluate the stability of M-Lip@TH in neutrophils, which incubated with DiO/DiI-labeled M-Lip@TH for 1.5 h, followed by culturing at 37 °C for 2 h and 4 h, and centrifuged at 800 rpm for 5 min, then monitoring the intracellular fluorescence intensity under the excitation wavelength of 480 nm using the spectrofluorometer and imaging with CLSM. In addition, Biorobot was incubated with DMEM containing PMA (100 nM) for 4 h. The supernatant was collected and the fluorescence intensity was

measured using the spectrofluorometer with the excitation wavelength of 480 nm.

Inflammation triggered drug-release of Biorobot. The *in vitro* release profiles of drugs from Biorobot were analysed in the presence of PMA. Biorobot were seeded into 24-well plates (10^6 cells/well), and then incubated with DMEM containing PMA (100 nM) for 6 h. At different time points, the concentrations of drugs in supernatant were detected using HPLC-MS and calculated the release rate of drugs.

Uptake and distribution of M-Lip@TH in RAW 264.7 cells. RAW 264.7 cells were treated with RhB labeled Lip@TH or M-Lip@TH (RhB concentration of 1 μM) for 12 h, respectively, followed by digesting with trypsin. The intracellular intensity of RhB at 4 h and 8 h was tested by the flow cytometer. For investigating the distribution of M-Lip@TH released from Biorobot in macrophages, RhB labeled Biorobot (10^6 cells/mL) were incubated with RAW 264.7 cells in the presence of PMA (100 nM) at 37 °C for different time points (0, 2, 4, 8, 12 and 24 h). After incubation, RAW 264.7 cells were collected and measured the intracellular fluorescence intensity using the flow cytometry. Furthermore, RAW 264.7 cells were incubated with RhB labeled Biorobot in the presence of PMA (100 nM) at 37 °C for 2 h and 12 h. The RAW 264.7 cells were then washed three times with PBS, stained with Hoechst 33342 for 15 min at room temperature, and imaged by CLSM.

Stability of M-Lip@TH in RAW 264.7 cells. The stability of M-Lip@TH released from Biorobot in RAW 264.7 cells was also evaluated using FRET analysis. RAW 264.7 cells were incubated with DiO/DiI labeled Biorobot in the presence of PMA (100 nM) at 37 °C for 4 h, and then fresh culture medium was replaced. At predetermined time points (2, 6, 12 and 24 h), the culture medium was removed, and RAW 264.7 cells were washed three times with PBS, stained with Hoechst 33342 for 15 min at room temperature, and imaged by CLSM. In addition, RAW 264.7 cells were collected and washed three times with PBS, followed by measuring the intracellular fluorescence intensity using the spectrofluorometer with the excitation wavelength of 480 nm.

Cytotoxicity of Biorobot against RAW 264.7 cells. The cytotoxicity was evaluated by MTT assay. Briefly, RAW 264.7 cells were seeded into 96-well plates (5×10^3 cells/well), and cultured at 37 °C

overnight. Then, cells were incubated with a culture medium containing Biorobot in the presence of PMA (100 nM) for 24 h. MTT solution (20 μL, 5 mg/mL) was added and incubated for another 4 h, followed by removing the medium and adding DMSO (150 μL) to dissolve the residue. The absorbance intensity at 570 nm was measured using the microplate reader.

Cytokine assay. RAW 264.7 cells were seeded into 24-well plates (2×10^4 cells/well), and cultured at 37 °C overnight. Cells were pretreated with LPS, poly(I:C) or dsDNA for 6 h, followed by adding Biorobot (TPCA-1 concentration of 1 μM and H-151 concentration of 1 μM) in the presence of PMA (100 nM). After 24-h incubation, the supernatants were collected to analyse the cytokine levels using enzyme-linked immunosorbent assay (ELISA).

Gene expression of inflammatory factors. RAW 264.7 cells (2×10^4 cells/well) were seeded into 24-well plates, and cultured at 37 °C overnight. Cells were pretreated with LPS for 6 h, and then treated with Biorobot (TPCA-1 concentration of 1 μM and H-151 concentration of 1 μM) in the presence of PMA (100 nM) for 24 h, followed by measuring the gene expression of different inflammatory factors using a qRT-PCR system (StepOnePlus, Thermo Fisher, USA).

Western blot. RAW 264.7 cells (2×10^4 cells/well) were seeded into 24-well plates, and cultured at 37 °C overnight. Cells were pretreated with LPS or dsDNA for 6 h, and then treated with Biorobot (TPCA-1 concentration of 1 μM and H-151 concentration of 1 μM) in the presence of PMA (100 nM) for 24 h. For the analysis of p65 and TBK1 protein expression by western blot, RAW264.7 cells with different treatments were lysed using RIPA buffer supplemented with protease inhibitor cocktail on the ice for 30 min, then the whole proteins were quantified using the Pierce™ BCA Protein Assay Kit. After boiling at 100 °C for 15 min, equivalent amounts of proteins (20 μg) of different treatment groups were analyzed using 10% SDS-PAGE at 80 V for 0.5 h then 100 V for 1.5 h. Then the proteins in SDS-PAGE gel were transferred into PVDF membranes at 250 mA for 2 h, followed by blocking with 5% skim milk for 2 h. Furthermore, the PVDF membranes were incubated with the corresponding anti-p-p65, anti-p65, anti-p-TBK1, anti-TBK1 and anti-GAPDH antibodies at 4 °C overnight. After being washed with TBST buffer thrice, the PVDF membranes were incubated with anti-rabbit-IgG-HRP secondary antibody for 2 h at room temperature. Finally,

the protein bands in PDVF membranes were visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA) and imaged using by Tanon-5200 Chemiluminescence.

***In vivo* inflammation targeting of Biorobot.** The acute lung injury model was performed by LPS challenge in mice. Briefly, C57BL/6 mice were anesthetized by inhalation of isoflurane, and LPS (4 mg/kg) dissolved in sterile saline was instilled intranasally (*i.n.*). Control mice received LPS-free sterile saline. Then the mice were divided into different groups and injected intravenously (*i.v.*) with DiR-M-Lip@TH or DiR-Biorobot. At the predetermined time intervals, mice were imaged using IVIS Spectrum Imaging System. In addition, main organs were harvested after 24 h to evaluate the biodistribution. The inflammation targeting of DiR-M-Lip@TH or DiR-Biorobot in pneumonia mice was investigated. The lung tissues of pneumonia mice were frozen, sliced, fixed with 4% paraformaldehyde and imaged with CLSM.

***In vivo* anti-inflammatory effects of Biorobot.** The model was generated as mentioned above. C57BL/6 mice were challenged *i.n.* with LPS (4 mg/kg) directly into each nostril. After 4 h administration, mice were treated with Biorobot (1 mg/kg TPCA-1 and 1 mg/kg H-151). At predetermined time points, mice were anesthetized, and the tracheas were exposed, and a tracheal cannula was inserted into the lung, followed by flushing the lungs with 4 °C cold sterile saline (1 mL) for three times. Lung bronchoalveolar lavage fluids (BALFs) were collected. The collected BALFs samples were used to evaluate the levels of TNF-α, IL-6, MCP-1, IFN-β and NO by ELISA. The mice were sacrificed at 72 h after the last injection to harvest their lungs for histological assay, immunohistochemical staining and transcriptomic analysis, meanwhile the whole blood was collected for hematological indices measurement.

Transcriptomic analysis. The collected lung tissues were treated with TRIzol™ reagent (Invitrogen, USA) and stored at -80 °C before RNA sequencing. The RNA sequencing process was conducted by Illumina HiSeq X10 (Illumina, USA). The value of gene expression was transformed as $\log_{10}$ [TPM (Transcripts Per Million reads) + 1], and the data were normalized through fragments per kilobases per million reads method. Finally, GO pathway enrichment analysis was processed

using the free online platform of Majorbio Cloud Platform.

Statistical analysis. All the results are presented as mean ± s.d. with at least 6 biological replicates. Error bars represent standard error of the mean from independent samples assayed within the experiments. Statistical analysis was done with GraphPad Prism 6 software. Statistical significance was calculated using unpaired Student's *t*-test, and a p-value < 0.05 was considered to be statistically significant.

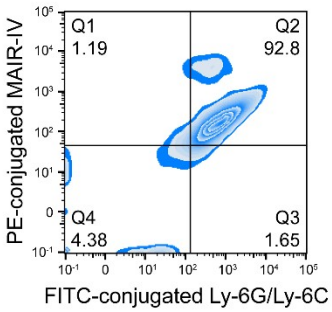

Fig. S1 Flow cytometry analysis of neutrophil purity, utilizing double staining with FITC-conjugated Ly-6G/Ly-6C (Gr-1) antibody and PE-conjugated MAIR-IV (CLM-5) antibody. Within the flow cytometry panel, the quadrants are delineated as follows: the lower-left quadrant corresponds to $FITC^{-}/PE^{-}$ cells, the lower-right to $FITC^{+}/PE^{-}$ cells, the upper-left to $FITC^{-}/PE^{+}$ cells, and the upper-right to $FITC^{+}/PE^{+}$ cells.

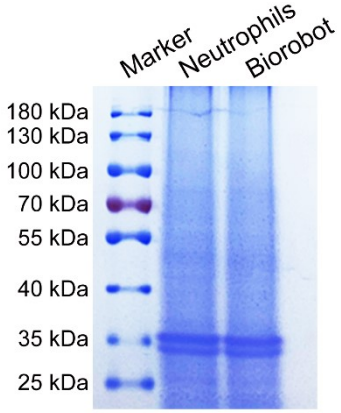

Fig. S2 Comprehensive identification of neutrophil membrane and intracellular proteins in the Biorobot through the SDS-PAGE assay.

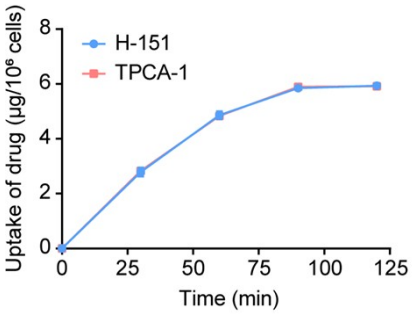

Fig. S3 Cellular uptake of M-Lip@TH by neutrophils. Neutrophils (1×10^6 cells) were incubated with M-Lip@TH (50 μg/mL) for different times, followed by quantifying the drugs in neutrophils.

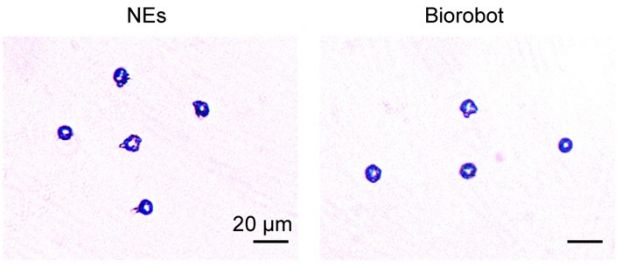

Fig. S4 Morphological images of neutrophils and Biorobot. Scale bar: 20 μm.

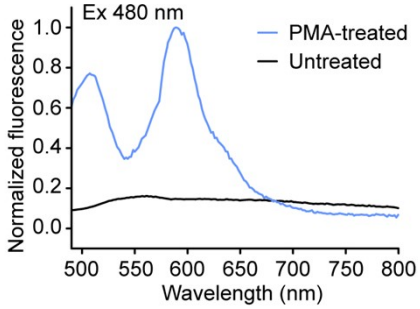

Fig. S5 Identification of the release of DiO/DiI-labeled M-Lip@TH from Biorobot under inflammatory stimulation. DiO/DiI-labeled M-Lip@TH loaded neutrophils were treated with PMA for 4 h, and the emission spectra of cell supernatant were measured under the excitation of 480 nm light.

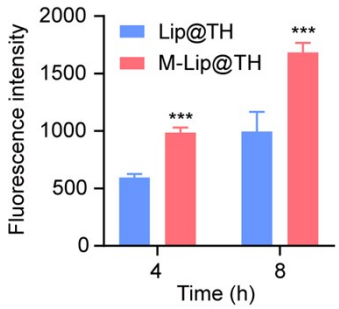

Fig. S6 Celluar uptake of RhB-labeled Lip@TH or M-Lip@TH in RAW 264.7 cells for 4 or 8 h measured by flow cytometry. The data are presented as mean ± s.d. (n = 6).

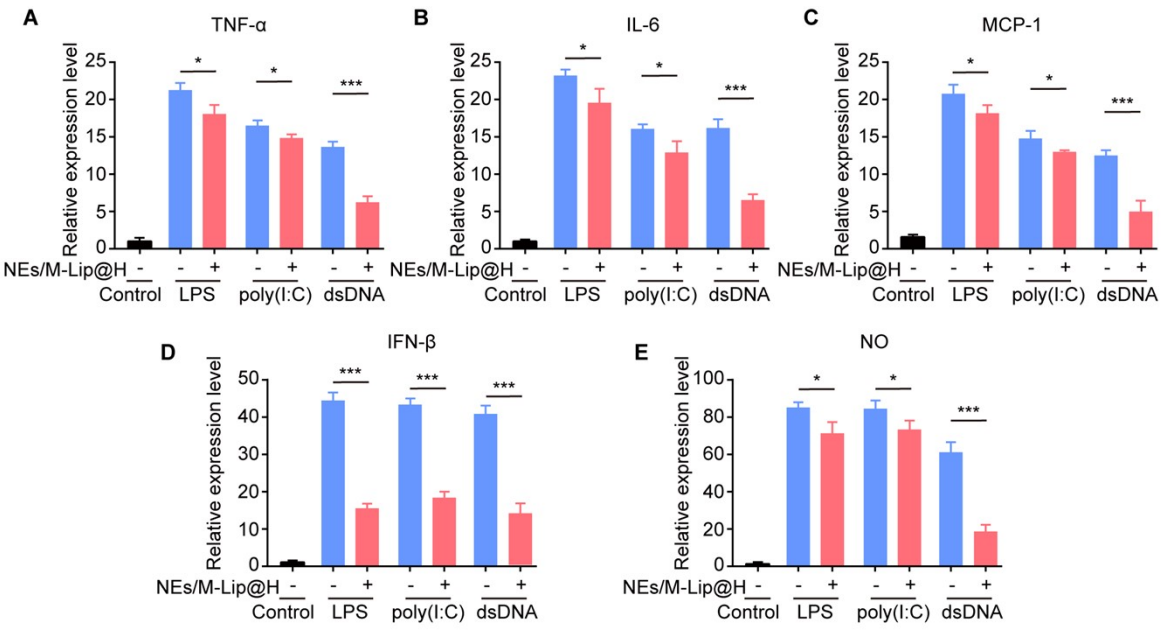

Fig. S7 *In vitro* inhibitory effects of NEs/M-Lip@H on the activation of different signaling pathways. The data are presented as mean ± s.d. (n = 6). *represents $p < 0.05$. ***represents $p < 0.001$.

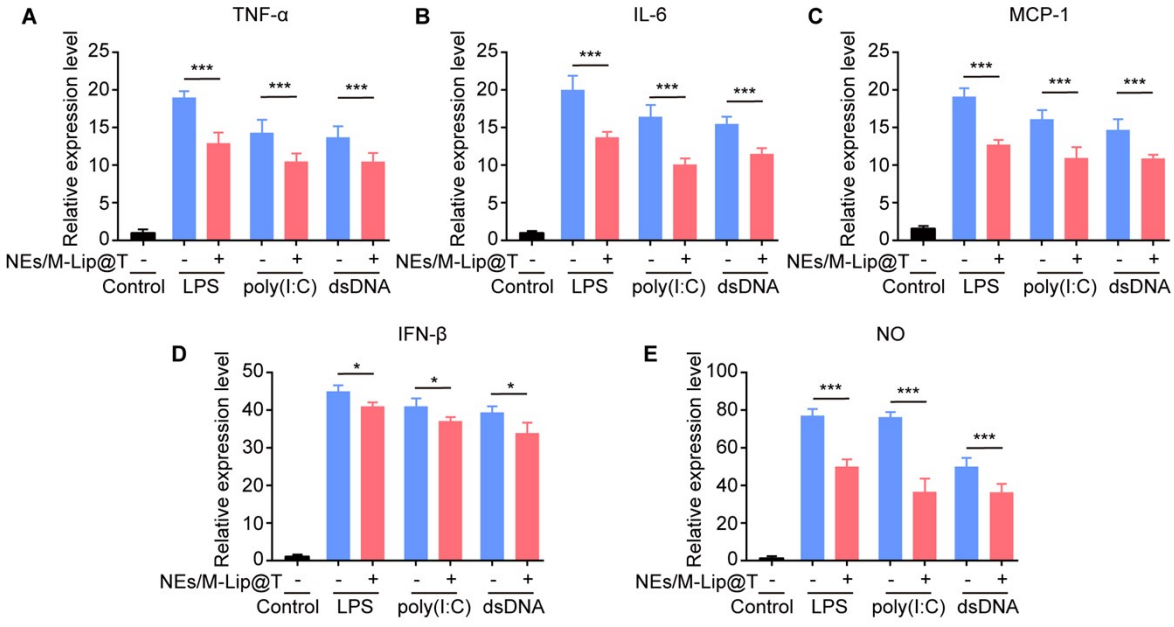

Fig. S8 *In vitro* inhibitory effects of NEs/M-Lip@T on the activation of different signaling pathways. The data are presented as mean ± s.d. (n = 6). *represents $p < 0.05$. ***represents $p < 0.001$.

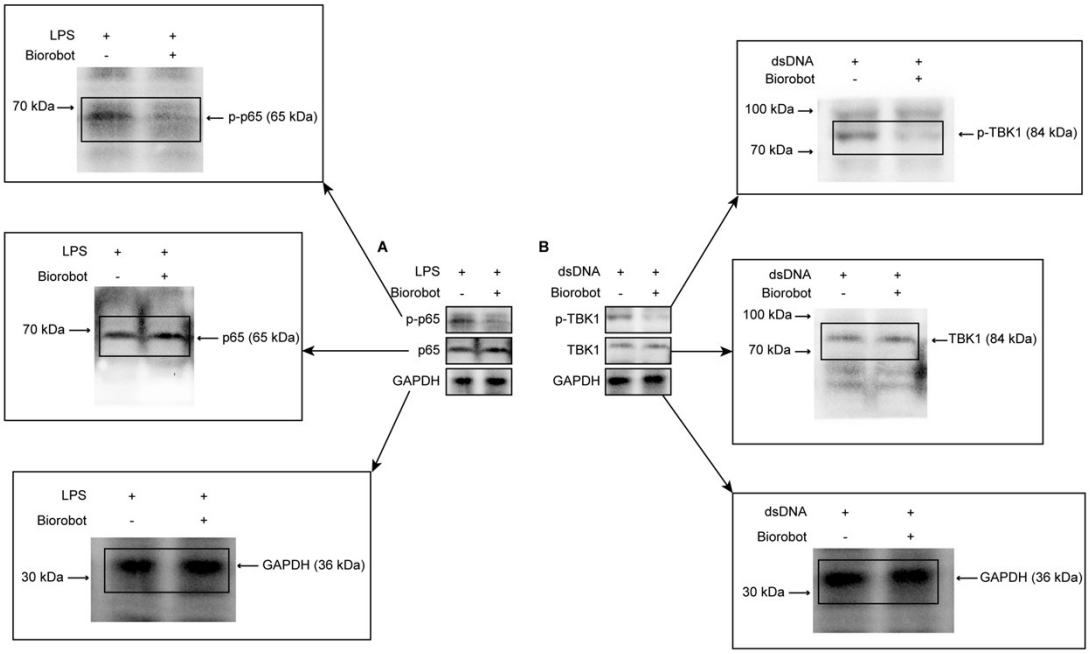

Fig. S9 Western blot analyses depicting the phosphorylation status of key proteins in macrophages post-treatment. (A) Comparative levels of phosphorylated p65 (p-p65) and total p65 following treatment with either PBS or Biorobot. (B) Assessment of phosphorylated TBK1 (p-TBK1) and total TBK1 levels in macrophages treated with PBS or Biorobot.

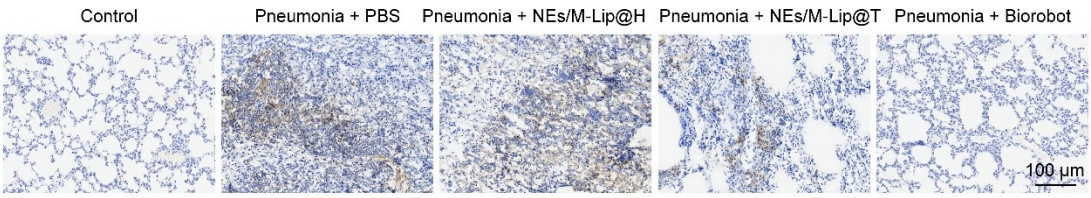

Fig. S10 Immunohistochemical staining for CD86 in lung tissue sections following treatment with Biorobot. A noticeable reduction in CD86-positive M1 macrophages is evident, indicating the immunomodulatory effect of Biorobot. Scale bar: 100 μm.

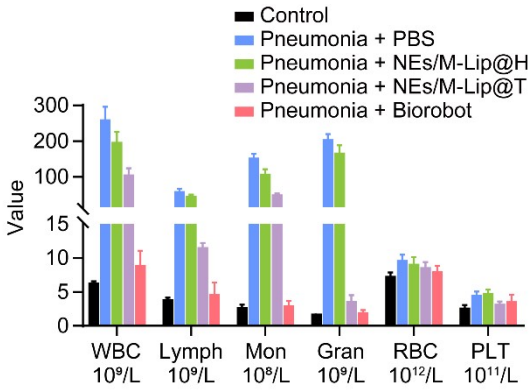

Fig. S11 The hematological profiles of mice post-administration of various treatments. Key hematological parameters include WBC (white blood cells), Lymph (lymphocytes), Mon (monocytes), Gran (granulocytes), RBC (red blood cells), and PLT (platelets).

Table S1 The size distribution, zeta potential, drug loading (DL) rate and encapsulation efficiency (EE) of different formulations. The data are presented as mean ± s.d. (n = 6).

Formulation	Size (nm)	PDI	Zeta potential (mV)	DL(%)		EE(%)	
				H-151	TPCA-1	H-151	TPCA-1
Lip@TH	104.23 ± 1.05	0.16 ± 0.07	-15.33 ± 2.14	2.58 ± 0.26	2.64 ± 0.31	98.60 ± 0.88	98.80 ± 0.96
M-Lip@H	109.52 ± 0.93	0.12 ± 0.08	-12.21 ± 1.96	2.47 ± 0.10	—	98.80 ± 0.72	—
M-Lip@T	110.10 ± 1.14	0.10 ± 0.04	-14.29 ± 0.94	—	2.48 ± 0.09	—	99.20 ± 0.26
M-Lip@TH	112.35 ± 0.38	0.13 ± 0.06	-17.21 ± 1.48	2.46 ± 0.12	2.47 ± 0.11	98.40 ± 0.26	98.90 ± 0.19