

Single-cell transcriptomics-guided inhalable neutrophil-mimetic nanoplatform restores NET homeostasis through a “block-and-clear” strategy in acute lung injury

Juan Hu

Shanghai Pulmonary Hospital

Fulong Man

Shanghai Pulmonary Hospital

Qiao Li

Shanghai Pulmonary Hospital

Tian Jin

Shanghai Pulmonary Hospital

Enmin Cai

Shanghai Pulmonary Hospital

Jian Yang

Shanghai Pulmonary Hospital

Yuying Jin

Shanghai Pulmonary Hospital

Yongyong Li

Shanghai Pulmonary Hospital

Xin Lv

xinlv@126.com

Shanghai Pulmonary Hospital

Research Article

Keywords: acute lung injury, neutrophil extracellular traps, NET homeostasis, mesoporous polydopamine, DNase I, pulmonary delivery

Posted Date: October 1st, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11059111/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background

Persistent accumulation of neutrophil extracellular traps (NETs) amplifies inflammation and alveolar–capillary barrier injury in acute lung injury (ALI), yet the mechanisms sustaining pathological NET accumulation remain incompletely understood. Here, integrated single-cell transcriptomic and functional analyses uncovered a previously underappreciated formation–disposal imbalance in ALI, in which enhanced NET formation outpaced post-release processing and degradation, disrupting NET homeostasis. Guided by this mechanistic insight, we developed DNase I@mPDA@NM, an inhalable neutrophil-mimetic nanoplatform that coordinates suppression of ongoing NET formation with degradation of extracellular NETs through a “Block-and-Clear” strategy.

Results

Single-cell RNA sequencing revealed increased neutrophil oxidative-stress/NET-formation programs without a matched increase in monocyte/macrophage NET-processing programs. Consistently, LPS-induced ALI exhibited increased pulmonary NET burden together with slower degradation of preformed NETs in bronchoalveolar lavage fluid, supporting a formation–disposal imbalance in vivo. Mechanistically, DNase I@mPDA@NM addressed both sides of this imbalance: mPDA attenuated intracellular ROS and NETosis-associated citrullinated histone H3 responses, whereas DNase I efficiently degraded preformed extracellular NETs. The mPDA-based formulation preserved approximately 70% of DNA-degrading activity under mildly acidic and ALI-relevant conditions, while neutrophil-membrane functionalization enhanced neutrophil association and prolonged pulmonary retention after inhalation. In an endothelial–epithelial barrier model, DNase I@mPDA@NM reduced NET accumulation and preserved barrier integrity, whereas NET add-back partially reversed this protection, supporting a functional contribution of extracellular NET removal to barrier preservation. In LPS-induced ALI, inhaled DNase I@mPDA@NM reduced pulmonary NET accumulation, neutrophilic inflammation, alveolar–capillary leakage, pulmonary edema, and histopathological injury. Whole-lung transcriptomics further revealed attenuation of neutrophil activation, degranulation, and NF- κ B-associated inflammatory programs, together with directional reversal of the ALI-associated reduction in phagocytosis-related programs, providing complementary transcriptomic support for rebalancing NET formation and post-release disposal.

Conclusions

Persistent pulmonary NET accumulation in ALI is associated with a formation–disposal mismatch that disrupts NET homeostasis. By translating this mechanism into a coordinated therapeutic architecture, inhaled DNase I@mPDA@NM restrains ROS-associated ongoing NET formation while eliminating extracellular NETs after release, thereby restoring NET homeostasis and protecting the alveolar–capillary barrier. This single-cell transcriptomics-guided “Block-and-Clear” strategy provides a mechanism-based

framework for NET-directed therapy and a localized nanotherapeutic approach for acute inflammatory lung injury.

1. Background

Acute lung injury (ALI), which can progress to acute respiratory distress syndrome (ARDS), remains a major cause of acute respiratory failure and mortality in critically ill patients [1–4]. A defining pathological feature of ALI is disruption of the alveolar–capillary barrier, in which inflammatory injury to the alveolar epithelium and pulmonary microvascular endothelium increases vascular permeability, promotes protein-rich pulmonary edema, and ultimately compromises gas exchange [1,4–6]. Despite advances in lung-protective ventilation and supportive care, pharmacological strategies capable of directly interrupting the self-sustaining cycle of inflammation and barrier injury remain limited [1–3,6–8]. Neutrophils are central to this pathological amplification [9]. Following rapid recruitment to the injured lung [10], excessive neutrophil activation drives oxidative injury, protease release, and formation of neutrophil extracellular traps (NETs), extracellular chromatin structures decorated with histones and granular proteins such as myeloperoxidase (MPO) and neutrophil elastase (NE) [9,11,12]. Although transient NET formation contributes to antimicrobial defense, excessive and persistent NET accumulation amplifies thromboinflammation, damages epithelial and endothelial cells, and perpetuates alveolar–capillary barrier dysfunction [9,11–13]. Persistent pulmonary NET burden therefore represents not merely a marker of neutrophilic inflammation, but a potentially actionable driver of ALI progression [9,11,13].

Tissue NET burden is intrinsically dynamic and reflects not only ongoing NET formation but also post-release disposal through extracellular degradation, cellular uptake, and intracellular processing [11,14,15]. Excessive generation may overwhelm disposal capacity, whereas insufficient degradation or processing may allow released NET material to persist and continuously propagate inflammatory and barrier injury [11,13,14]. Pathological NET accumulation may therefore reflect disruption of NET homeostasis, rather than excessive NETosis alone [11,14]. This raises a central question: whether persistent pulmonary NET accumulation during ALI reflects enhanced NET formation alone or a mismatch between increased formation and post-release disposal. To address this question, we integrated lung single-cell RNA sequencing with tissue-level NET measurements and functional extracellular NET-degradation assays. Single-cell transcriptomic analysis revealed enhanced oxidative stress-, ROS-, and NET-formation-associated programs in neutrophils without a commensurate increase in monocyte/macrophage programs involved in extracellular-material uptake, phagolysosomal processing, and DNA degradation. This transcriptional mismatch was accompanied by increased pulmonary NET burden and slower degradation of preformed NETs in ALI bronchoalveolar lavage fluid, supporting a formation–disposal mismatch underlying disrupted NET homeostasis (Fig. 1). Notably, the prominent ROS-associated neutrophil response further implicated oxidative activation as a disease-relevant driver of the excessive formation arm [11,16–18]. These findings defined a specific therapeutic requirement: restoration of NET homeostasis would require coordinated restraint of continued ROS-associated NET formation together with elimination of extracellular NETs that had already accumulated.

Guided by this mechanistic insight, we designed an inhalable “Block-and-Clear” nanoplatfrom capable of intervening across these two biologically distinct stages of NET dynamics. ROS-associated NETosis occurs within activated neutrophils and continuously supplies new NET material [11,16], whereas previously released NET scaffolds persist extracellularly and continue to propagate tissue injury [11,13]. Mesoporous polydopamine (mPDA) was therefore selected not merely as a carrier but as the bioactive “Block” component. Its catechol- and quinone-rich chemistry confers ROS-scavenging activity capable of attenuating ROS-associated neutrophil activation and continued NET formation, while its mesoporous architecture enables loading and protection of therapeutic macromolecules [19–21]. DNase I serves as the complementary “Clear” component by cleaving the extracellular DNA scaffold that maintains pre-existing NET architecture [22]. Although inhaled recombinant human DNase I provides clinical precedent for pulmonary nuclease administration [23], free DNase I primarily removes extracellular NETs without restraining their continued generation, while its limited stability and short in vivo half-life constrain sustained nuclease activity within the inflammatory pulmonary environment [24–26]. Loading DNase I into mPDA therefore creates an active carrier–cargo architecture in which mPDA restrains the ROS-associated NET formation while simultaneously providing a protective matrix for DNase I-mediated extracellular NET degradation. To support localized and sustained implementation of both functions, DNase I@mPDA was further coated with neutrophil membrane to enhance its association with inflammatory neutrophils and prolong pulmonary retention, and administered by inhalation to achieve direct lung deposition and high local exposure [22,27,28].

Here, we identified a formation–disposal mismatch associated with disrupted NET homeostasis in ALI and translated this insight into a single-cell transcriptomics-guided, inhalable “Block-and-Clear” strategy (Scheme 1). DNase I@mPDA@NM combined mPDA-mediated suppression of ROS-associated ongoing NET formation with DNase I-mediated degradation of preformed extracellular NETs, while preserving DNA-degrading activity under ALI-relevant conditions. Neutrophil-membrane functionalization enhanced neutrophil association and prolonged pulmonary retention following inhalation. In an endothelial–epithelial barrier model, DNase I@mPDA@NM reduced NET accumulation and preserved barrier integrity; NET add-back partially reversed this protection, supporting a functional contribution of extracellular NET removal to barrier preservation. In LPS-induced ALI, inhaled treatment reduced pulmonary NET burden, neutrophilic inflammation, and alveolar–capillary injury. Whole-lung transcriptomics further showed attenuation of neutrophil activation, degranulation, and inflammatory programs, together with directional reversal of the ALI-associated reduction in phagocytosis-related programs. Collectively, these findings support coordinated targeting of NET formation and post-release disposal as a mechanism-guided nanotherapeutic approach to restoring NET homeostasis and protecting the alveolar–capillary barrier, providing a preclinical basis for the translational development of inhaled NET-directed therapies for ALI.

2. Materials and Methods

2.1. Study design

The study comprised analysis of a public lung single-cell RNA-sequencing dataset (GSE276682), nanoplatform preparation and characterization, mechanistic evaluation in primary neutrophils and an alveolar–capillary Transwell model, and in vivo assessment in LPS-induced ALI mice. Doses were normalized by active-component equivalence. DNase I constituted approximately 30% (w/w) of DNase I@mPDA and 15% (w/w) of the purified DNase I@mPDA@NM formulation. Accordingly, component-equivalent concentrations of free DNase I, free mPDA, DNase I@mPDA, and DNase I@mPDA@NM were 7.5, 17.5, 25, and 50 $\mu\text{g mL}^{-1}$, respectively, in vitro, and nominal nebulizer-loaded doses were 1.5, 3.5, 5, and 10 mg kg^{-1} , respectively, in vivo. Thus, 50 $\mu\text{g mL}^{-1}$ DNase I@mPDA@NM corresponded to 7.5 $\mu\text{g mL}^{-1}$ DNase I and 17.5 $\mu\text{g mL}^{-1}$ mPDA, whereas 10 mg kg^{-1} corresponded to 1.5 mg kg^{-1} DNase I and 3.5 mg kg^{-1} mPDA. Unless otherwise stated, in vivo experiments used 8-week-old male C57BL/6J mice. ALI was induced by intratracheal administration of LPS (1 mg kg^{-1}). Treatments were administered 2 h later by nebulized inhalation using an Aerogen Solo vibrating-mesh nebulizer, with 200 μL loaded per mouse and aerosolized to completion. Tissues and biological samples were collected 24 h after LPS challenge. All animal procedures were approved by the Animal Ethics Committee of Tongji University School of Medicine (TJAA07721106).

2.2 Single-cell RNA sequencing analysis (GSE276682)

Six 10x Genomics mouse lung single-cell RNA-seq datasets (Control, $n = 3$ biological replicates; LPS, $n = 3$ biological replicates) were downloaded from Gene Expression Omnibus (accession GSE276682) and analyzed as independent biological replicates, with all samples included before outcome assessment. Raw UMI count matrices were processed using Seurat v5 (R v4.4). Cells with ≥ 500 UMIs and ≥ 200 detected genes were retained; cells exceeding 20% mitochondrial transcripts were excluded. Within each sample, cells exhibiting abnormally high UMI counts or detected gene numbers ($> \text{median} + 3 \times \text{median absolute deviation}$) were regarded as putative multiplets and excluded. Data were log-normalized (scale factor = 10,000), 3,000 highly variable genes were selected using variance-stabilizing transformation (vst), and principal component analysis was performed using the first 30 principal components. Batch effects between samples were corrected using Harmony based on sample_id prior to UMAP visualization and graph-based clustering (resolution = 0.5). Cell identities were assigned according to canonical lineage markers and cluster-average expression profiles; major populations comprised epithelial cells, endothelial cells, fibroblasts, monocytes/macrophages, neutrophils, lymphocytes, and pericyte/smooth-muscle cells. Biological-replicate identity was verified within each annotated cluster.

2.3 Module scores, pseudobulk analysis, and NET homeostasis imbalance

Module scores were calculated for individual cells as the mean log-normalized expression of the detected genes in each predefined gene set. Formation-associated program scores were subsequently averaged within neutrophils, whereas processing-associated program scores were averaged within monocytes/macrophages, separately for each biological replicate.

For pseudobulk analysis, raw UMI counts from all neutrophils within each biological replicate were aggregated into one count matrix per sample and analyzed using edgeR quasi-likelihood models together with competitive gene-set testing (camera) to compare LPS versus Control. FDR was controlled at 0.05.

Monocyte/macrophage NET-processing-associated programs (NET uptake, DNA degradation, phagolysosomal processing, and inflammatory resolution) were summarized at the sample level using module scores. Effects were estimated as LPS – Control with 95% confidence intervals derived from the same model structure.

Sample-level Formation and Processing scores were calculated using all six biological replicates (Control, n = 3; LPS, n = 3). Each predefined module score was independently standardized across the six samples by subtracting its across-sample mean and dividing by its sample standard deviation. The Formation score was defined as the equal-weight arithmetic mean of the standardized neutrophil ROS-production, oxidative-stress-response, and NETosis module scores. The Processing score was defined as the equal-weight arithmetic mean of the standardized monocyte/macrophage DNA-degradation, NET-uptake, phagolysosomal-processing, and resolution module scores. Formation and Processing scores were examined jointly in a two-dimensional state-space representation.

Supplementary Figures S1 and S2 show sample-level pseudobulk expression of predefined neutrophil ROS/NETosis-associated and monocyte/macrophage NET-processing-associated gene sets, respectively. The neutrophil gene set comprised 15 genes (Cybb, Ncf1, Ncf2, Ncf4, Rac2, Padi4, Mpo, Elane, Ctsg, Prtn3, Gsdmd, S100a8, S100a9, Camp and Nos2), whereas the monocyte/macrophage gene set comprised 34 genes related to extracellular-material recognition and uptake, phagosome–lysosome processing, DNA degradation, lysosomal regulation and inflammatory-resolution-associated programs. Expression values were calculated as logCPM and Z-scaled within each gene across samples for heatmap visualization.

2.4 BALF MPO–DNA levels and degradation kinetics of preformed NETs

Primary mouse neutrophils were stimulated with PMA (100 nM) for 4 h to generate NETs. NET-containing preparations were collected and centrifuged at 450 × g for 10 min to remove intact cells and cellular debris, and endogenous MPO–DNA concentrations in BALF were measured by ELISA as an index of pulmonary NET-associated burden. The resulting cell-free NET-containing supernatant was pooled, thoroughly mixed, and aliquoted equally across experimental conditions to ensure an identical starting NET load. BALF collected from control and LPS-induced ALI mice was centrifuged at 500 × g for 10 min to obtain cell-free BALF. Equal volumes of preformed NET substrate and cell-free BALF were incubated at 37 °C, with aliquots collected at 0, 15, 30, 60, and 120 min for kinetic quantification of residual dsDNA using a PicoGreen dsDNA assay according to the manufacturer's instructions. The fluorescence signal at 0 min for each individual BALF sample was defined as 100%, and the remaining signal at subsequent

time points was expressed as residual NET-derived dsDNA (%). Preformed NET substrate incubated with PBS under identical conditions served as a nonenzymatic decay control.

2.5 Preparation of DNase I@mPDA

Synthesis of mPDA. Mesoporous polydopamine nanoparticles were synthesized via a 1,3,5-trimethylbenzene (TMB)/Pluronic F127 emulsion-templated oxidative polymerization method. Briefly, Pluronic F127 (1.0 g) and dopamine hydrochloride (0.5 g) were dissolved in 100 mL of ethanol/water (1:1, v/v) and stirred at room temperature for 15 min to yield a clear solution. Under continuous stirring (500 rpm), TMB (2.0 mL) was added dropwise, and the mixture was stirred for a further 30 min to form a white emulsion. Aqueous ammonia (5.0 mL) was then added dropwise, and the reaction was maintained at 20–25°C for 2 h, with the reaction vessel covered with perforated cling film. The precise reaction duration was determined by monitoring nanoparticle size to achieve the desired particle diameter. The resulting black mPDA dispersion was centrifuged at $16,304 \times g$ for 15 min; the pellet was washed twice with ethanol/water (1:1, v/v) by ultrasonic dispersion and centrifugation, followed by two washes with acetone, and finally resuspended in deionized water. The nanoparticle concentration was determined by UV absorbance or lyophilization of a known volume.

DNase I loading. DNase I was loaded onto mPDA by magnetic stirring. mPDA-to-DNase I mass ratios of 4:1, 3:1, 2:1, and 1:1 were evaluated. The mixtures were centrifuged ($8,944 \times g$, 10 min), and the pellets were washed twice with deionized water to remove unbound DNase I. Encapsulation efficiency and loading capacity were determined from the difference between the initial and unbound DNase I amounts, quantified using a BCA protein assay. Based on the encapsulation-efficiency and loading-capacity profiles, a 2:1 mPDA-to-DNase I mass ratio was used for formulation preparation.

2.6 Neutrophil-membrane coating

Bone marrow neutrophils were isolated from C57BL/6J mice either left untreated or pretreated with LPS (3 mg kg^{-1} , intraperitoneally, 6 h) using a mouse bone marrow neutrophil isolation kit (Solarbio, P8550) according to the manufacturer's instructions. Plasma membrane fractions were isolated from the purified neutrophils using a plasma membrane protein and cell fractionation kit (Minute™ SM-005; Invent Biotech, Beijing, China) following the manufacturer's protocol. Membranes isolated from neutrophils obtained from LPS-pretreated mice were designated LPS-NM, whereas those isolated from neutrophils obtained from untreated mice were designated NM. Both membrane preparations were used for comparative membrane-protein characterization. Unless otherwise specified, LPS-NM was used for subsequent nanoparticle coating.

The protein content of the isolated LPS-NM used for coating was quantified using a BCA protein assay and used to define the membrane input. LPS-NM and DNase I@mPDA were mixed at a 1:1 membrane protein-to-DNase I@mPDA mass ratio. The mixture was sonicated in an ice bath at 100–150 W for 10 min using an intermittent cycle of 20 s on/40 s off to promote membrane–particle interaction. The resulting suspension was then repeatedly extruded through a 400-nm polycarbonate membrane using a liposome extruder to facilitate uniform membrane coating of DNase I@mPDA. For consistency with the

formulation nomenclature used throughout the study, the resulting LPS-NM-coated nanoparticles are referred to as DNase I@mPDA@NM.

After membrane coating, the concentration of DNase I@mPDA@NM was determined gravimetrically from the dry mass of the final recovered formulation. Briefly, a known volume of the purified nanoparticle suspension was lyophilized to constant weight, and the formulation concentration was calculated from the recovered dry mass and the corresponding suspension volume. Accordingly, DNase I@mPDA@NM concentrations reported throughout the study refer to the total dry-mass concentration of the purified final formulation, rather than to the initial DNase I@mPDA mass or membrane protein input.

2.7 Physicochemical characterization

Hydrodynamic diameter and zeta potential were measured for mPDA, DNase I@mPDA, and DNase I@mPDA@NM by dynamic light scattering. Colloidal stability was assessed by monitoring size and zeta potential over six days. Particle morphology was examined by transmission electron microscopy. CXCR2 (1:2,000; Bioss, USA), CXCR4 (1:2000; ELK Biotechnology, Wuhan, China) and Histone H3 (1:1,000; Cell Signaling Technology, USA) were examined in unstimulated neutrophils, LPS-stimulated neutrophils, isolated membrane preparations, and material formulations by immunoblotting.

2.8 DNase I release and catalytic activity

DNase I release. DNase I release from free DNase I, DNase I@mPDA and DNase I@mPDA@NM was assessed by dynamic dialysis. Formulations were sealed in dialysis bags and incubated in PBS at 37 °C with magnetic stirring. At predetermined intervals, aliquots of the release medium were withdrawn and the released DNase I was quantified by the BCA protein assay. Cumulative release was normalized to the total loaded amount.

DNA-degrading activity. DNase I-mediated DNA degradation was evaluated in phosphate-buffered saline (PBS, pH 7.4), under mildly acidic conditions (pH 6.0), and in bronchoalveolar lavage fluid (BALF) collected from mice with LPS-induced ALI. pBR322 plasmid DNA (1 µg; Beyotime Biotechnology, D2301-100 µg) was incubated with PBS, free DNase I, mPDA, DNase I@mPDA, or DNase I@mPDA@NM at 37 °C for 10 min in the indicated media. mPDA served as a DNase I-free carrier control, while matrix-only samples containing pBR322 DNA without DNase I-containing formulations were included to account for background DNA loss in each medium. Reaction products were resolved on 1% agarose gels containing GelRed in TBE buffer at 100 V for 40 min and visualized using an iBright imaging system. Residual plasmid DNA was quantified by densitometric analysis, and DNA degradation was calculated relative to the corresponding matrix-only control, with PBS alone, pH-matched buffer alone, or ALI BALF alone defined as 0% degradation.

2.9 Radical-scavenging activity and preliminary biocompatibility

Cell-free radical-scavenging activity was assessed using DPPH and ABTS assays. For DPPH, the reagent (Sangon Biotech, D799007-0050) was dissolved in ethanol to a final concentration of 0.1 mmol L⁻¹.

The radical-scavenging activities of the indicated treatments were evaluated at the working concentrations described in Section 2.1. DNase I@mPDA@NM, prepared using an mPDA-to-DNase I feed mass ratio of 2:1, was mixed with an equal volume of DPPH working solution in a 96-well plate, incubated at 37 °C for 30 min in the dark, and measured at 515 nm. The DPPH scavenging rate was calculated as $[1 - (A_{\text{sample}} - A_{\text{sample blank}}) / A_{\text{blank}}] \times 100\%$, where $A_{\text{sample blank}}$ accounts for the intrinsic absorbance of each sample. For ABTS (Shanghai Acme Biochemical Technology, AC10743), the working solution was prepared by mixing ABTS substrate with reaction buffer according to the manufacturer's instructions and incubating in the dark at room temperature until stable. Samples were mixed with the ABTS working solution, incubated at room temperature for 6 min in the dark, and measured at 405 nm. The ABTS scavenging rate was calculated as $[1 - (OD_{\text{sample}} / OD_{\text{blank}})] \times 100\%$.

Cell viability was evaluated using the CCK-8 assay. DNase I@mPDA@NM was tested at total formulation concentrations of 0–100 µg mL⁻¹, defined on the basis of the dry mass of the purified final formulation. Hemolysis was determined after incubation with mouse erythrocytes; PBS and Triton X-100 served as negative and positive controls.

For the preliminary in vivo safety assessment, 8-week-old male C57BL/6J mice were randomly assigned to the PBS, mPDA, DNase I@mPDA, and DNase I@mPDA@NM groups (n = 3 mice per group). The PBS group served as the control group. The formulations were administered once by nebulized inhalation using an Aerogen Solo vibrating-mesh nebulizer, with 200 µL of formulation loaded per mouse and aerosolized until nebulization was complete. The administered doses were 3.5 mg kg⁻¹ for free mPDA, 5 mg kg⁻¹ for DNase I@mPDA, and 10 mg kg⁻¹ for DNase I@mPDA@NM, based on active-component equivalence. At 24 h after administration, blood and the heart, liver, spleen, lung, and kidney were collected for serum biochemical and histopathological analyses. Preliminary in vivo safety was assessed by histological examination of major organs (heart, liver, spleen, lung, and kidney), serum biochemistry, and hepatic and renal function indices after inhalation administration.

2.10 Primary-neutrophil experiments

Bone marrow neutrophils were freshly isolated from C57BL/6J mice using a mouse bone marrow neutrophil isolation kit (Solarbio, P8550) and were identified by CD11b and Ly6G flow cytometry, with a resulting purity of 85.3%. NETosis-associated activation was induced with phorbol 12-myristate 13-acetate (PMA; 100 nM; MCE, HY-18739) for 4 h. Experimental groups comprised untreated control, PMA, PMA + free DNase I, PMA + mPDA, PMA + DNase I@mPDA@NM, and, for the ROS-rescue experiment, PMA + DNase I@mPDA@NM + H₂O₂.

Free DNase I, free mPDA, and DNase I@mPDA@NM were used at component-equivalent concentrations of 7.5, 17.5, and 50 µg mL⁻¹, respectively. The 50 µg mL⁻¹ dose of DNase I@mPDA@NM was expressed

as the total dry-mass concentration of the purified formulation and corresponded to 7.5 $\mu\text{g mL}^{-1}$ DNase I and 17.5 $\mu\text{g mL}^{-1}$ mPDA.

NETosis-associated activation was induced with phorbol 12-myristate 13-acetate (PMA; 100 nM; MCE, HY-18739). mPDA, DNase I, or DNase I@mPDA@NM was added 30 min after PMA stimulation. Intracellular ROS levels were assessed at 1 h, CitH3 levels in neutrophil lysates were measured at 2 h as a cell-associated NETosis marker, and extracellular NET release was evaluated at 4 h after PMA stimulation by measuring supernatant MPO–DNA levels.

2.11 Neutrophil association with labeled formulations

DNase I@mPDA and DNase I@mPDA@NM were incubated with Cy5.5 (MCE, HY-D0924) under continuous stirring in the dark. Unbound Cy5.5 was removed by centrifugation at $12,000 \times g$ for 15 min at 4 °C, followed by three washes with PBS. The resulting Cy5.5-labeled nanoparticles were resuspended in PBS for subsequent fluorescence imaging experiments. Labeled formulations were incubated with primary mouse bone marrow neutrophils for 2, 4, and 6 h.

2.12 Intracellular ROS and CitH3-associated activation

Intracellular ROS was measured 1 h after PMA stimulation using DCFH-DA (Sigma-Aldrich) freshly diluted to 50 μM . Cells were incubated with the probe at 37 °C in 5% CO_2 for 30 min in the dark, with gentle agitation every 10 min. Fluorescence was quantified by fluorescence microscopy and flow cytometry using identical acquisition settings across all groups.

CitH3 was examined relative to total histone H3 by immunoblotting. After treatment, neutrophils were washed and lysed using lysis buffer containing protease inhibitors. Lysates were separated by SDS-PAGE and transferred to PVDF membranes. Primary antibodies against CitH3 (1:1,000; Abcam, ab281584) and histone H3 (1:1,000; Cell Signaling Technology, 14269) were applied, followed by HRP-conjugated secondary antibodies (goat anti-rabbit IgG-HRP, 1:5,000, Yeasen, 33101ES; goat anti-mouse IgG-HRP, 1:5,000, Yeasen, 33201ES). The CitH3/H3 ratio was calculated after densitometric normalization. Intracellular CitH3 was also quantified in neutrophil lysates by enzyme-linked immunosorbent assay (Citrullinated Histone H3 (Clone 11D3) ELISA Kit; Cayman Chemical, 501620).

For immunofluorescence, cells were fixed and permeabilized using immunofluorescence fixation/permeabilization buffer (Yeasen, ZX201S) for 15 min, blocked with QuickBlock™ blocking buffer (Beyotime, P0260) for 15 min, and stained for CitH3, MPO, and DNA. Slides were mounted with DAPI Fluoromount-G® (SouthernBiotech, 0100 – 20) and imaged by fluorescence microscopy. CitH3-positive neutrophils were quantified from representative fields; field-level measurements were averaged within each biological replicate so that each replicate contributed one data point.

2.13 H_2O_2 dose selection and rescue design

For the ROS rescue experiment, neutrophils were stimulated with PMA (100 nM), followed by DNase I@mPDA@NM treatment at 30 min. H_2O_2 (50 μM), selected based on viability and independent validation

experiments (Supplementary Figure S8), was added at 1 h after PMA stimulation. NETosis-associated endpoints were assessed at 2 h.

2.14 Extracellular DNA, MPO–DNA, and preformed NETs

Extracellular DNA was quantified using SYTOX™ Green nucleic acid stain (5 mM in DMSO; Thermo Fisher Scientific, S7020), freshly diluted to 5 μ M in PBS. Culture medium was gently aspirated along the well-wall to avoid disturbing NET structures on the well bottom. The diluted SYTOX Green solution was added, and cells were incubated at 37°C in 5% CO₂ for 15 min in the dark. Washing was omitted because unbound dye does not interfere with extracellular DNA-specific fluorescence and washing risks dislodging NET structures. Fluorescence was measured at excitation 488 nm / emission 523 nm.

MPO-DNA complexes were quantified using a modified capture ELISA. Microplates were coated with a polyclonal anti-MPO antibody (Invitrogen/Thermo Fisher Scientific, PA5-16672), and MPO-associated DNA was detected using the peroxidase-conjugated anti-DNA monoclonal antibody (Anti-DNA-POD) supplied with the Cell Death Detection ELISA kit (Roche Diagnostics, 11774425001).

For the preformed-NET assay, neutrophils were stimulated with PMA (100 nM, 4 h) to allow NET formation before treatment. The cultures were then exposed to PBS, mPDA, free DNase I, or DNase I@mPDA@NM for 4 h. At each collection point, culture supernatants were transferred immediately into pre-chilled low-protein-binding tubes containing EDTA to achieve a final concentration of 10 mM, thereby chelating the divalent cations required for DNase I activity. Samples were gently mixed, kept on ice, and centrifuged at 500 $\times$ g for 5 min at 4 °C to remove residual cells. The resulting supernatants were aliquoted and stored at – 80°C until MPO–DNA analysis. All experimental groups were processed identically, and repeated freeze–thaw cycles were avoided. Supernatant MPO–DNA was measured as described above.

2.15 Compartment-resolved time-course analysis

Neutrophils were stimulated with 100 nM PMA and subsequently analyzed for intracellular and extracellular NET-associated markers. Samples were collected at 2, 3, 4, and 6 h after PMA stimulation. At each time point, cell pellets were separated from culture supernatants by centrifugation at 500 $\times$ g for 5 min at 4 °C. DNase I activity was inhibited at collection using EDTA. Cells were washed and lysed for intracellular CitH3 measurement, whereas clarified culture supernatants were used for MPO–DNA measurement. CitH3 therefore represents NETosis-associated histone citrullination within neutrophils, while MPO–DNA represents the net extracellular abundance of detectable MPO-bound DNA complexes at each collection point. The two curves are not interpreted as direct formation or clearance rates. All samples within one time-course experiment were processed using matched collection intervals and assay conditions. Separate wells were used for each time point, and results were not normalized to cell number or lysate protein.

2.16 Neutrophil phagocytosis

The effects of DNase I, mPDA, and DNase I@mPDA@NM on primary-neutrophil phagocytic function were examined using monodisperse polystyrene (PS) fluorescent microspheres (green, 500 nm, carboxyl-modified; RIGOR Science) incubated in serum-free medium for 2 h. A 4 °C condition was included as a control for energy-dependent uptake. Phagocytosis was quantified by flow cytometry.

2.17 Alveolar–capillary Transwell barrier model

An alveolar–capillary barrier model was established in Transwell inserts (3.0 µm pore size; Corning) coated with type I collagen from rat tail (MCE, HY-NP010). Mouse pulmonary microvascular endothelial cells (Procell System, CP-M001) were seeded on the underside of the insert membrane and allowed to adhere in the inverted orientation for 4 h. The inserts were then returned to the upright orientation, and mouse type II alveolar epithelial cells (Procell System, CP-M003) were seeded on the upper surface of the membrane. Half of the culture medium was replaced on day 3. Barrier integrity was monitored daily; experiments proceeded on day 5, when barrier function was confirmed by measurement of FITC-dextran (4 kDa) apparent permeability coefficient (P_{app} , $P_{app} = (C_r \cdot 0.6) / (C_d \cdot 0.33 \cdot 3600)$), where C_r is the receiver-compartment FITC–dextran concentration after 60 min, C_d is the initial donor concentration, 0.6 mL is the receiver volume, 0.33 cm² is the effective membrane area, and 3600 s is the sampling interval. P_{app} was expressed in cm s⁻¹ and was significantly lower across the co-culture barrier than across cell-free inserts (Supplementary Figure S12).

Bone marrow-derived neutrophils were added to the lower (basolateral, vascular) compartment, whereas LPS (1 µg mL⁻¹) was applied to the upper (apical, alveolar) compartment. After 0.5 h of LPS exposure, the indicated treatments were added to the upper compartment at the component-equivalent concentrations defined in Section 2.1: free DNase I at 7.5 µg mL⁻¹ (11.25 U mL⁻¹), free mPDA at 17.5 µg mL⁻¹, and DNase I@mPDA@NM at 50 µg mL⁻¹ total formulation concentration, corresponding to 7.5 µg mL⁻¹ DNase I and 17.5 µg mL⁻¹ mPDA.

At 8 h after LPS stimulation, supernatants from the upper (apical) compartment were collected for MPO–DNA and CitH3 measurements. Neutrophils that had transmigrated into the upper compartment were recovered and quantified by flow cytometry as CD45⁺Ly6G⁺ cells. Barrier junctional integrity was assessed by immunoblotting for ZO-1 and occludin and by ZO-1 immunofluorescence. Apoptosis of alveolar epithelial cells was evaluated by Annexin V/propidium iodide staining.

2.18 Preparation of preformed NETs and NET add-back assay

Bone marrow-derived neutrophils were stimulated with PMA (100 nM) for 4 h to generate preformed NETs. The PMA-containing medium was subsequently removed, and the cells were gently washed before NET-associated material was collected in fresh medium. The suspension was centrifuged at 450 × g for 10 min to remove intact cells and large cellular debris, and the resulting NET-rich supernatant was collected.

For NET add-back experiments, following the initial LPS stimulation and treatment phase, medium was removed from both the apical and basolateral compartments and the inserts were gently washed before fresh medium was added. Standardized preformed NETs were added exclusively to the basolateral vascular compartment, with an equal volume of vehicle added to the corresponding control groups. The cultures were incubated for an additional 8 h, after which 4-kDa FITC–dextran permeability and endothelial ZO-1 organization were assessed. Preformed NET preparations were quantified according to their dsDNA content using PicoGreen and standardized before use. NETs were added to the basolateral compartment at a final DNA-equivalent concentration of 1 $\mu\text{g mL}^{-1}$.

2.19 Nebulized inhalation treatment and pulmonary biodistribution

At 2 h after LPS challenge, the indicated formulations were administered by nebulized inhalation using an Aerogen Solo vibrating-mesh nebulizer (Aerogen, Ireland). The nebulizer outlet was coupled to a rodent facemask through a short, low-dead-volume adapter to minimize aerosol deposition within the delivery pathway. Mice were gently restrained and allowed to breathe spontaneously throughout aerosol administration without supplementary carrier-gas flow. For each mouse, 200 μL of freshly prepared formulation was loaded into the nebulizer reservoir and aerosolized until nebulization was complete. The formulation concentration was adjusted according to individual body weight to provide nominal nebulizer-loaded doses of 1.5, 3.5, 5, and 10 mg kg^{-1} for free DNase I, free mPDA, DNase I@mPDA, and DNase I@mPDA@NM, respectively. Because the pulmonary deposition fraction was not directly quantified, these doses are reported as nominal nebulizer-loaded doses rather than measured pulmonary deposited doses. For biodistribution and pulmonary retention studies, Cy5.5-labeled free DNase I, DNase I@mPDA, and DNase I@mPDA@NM were administered using the same nebulized inhalation procedure at the corresponding nominal doses. In vivo fluorescence imaging was performed immediately after administration (0 h) and at 4, 8, 12, and 24 h, with fluorescence intensity in the lung region quantified to assess the pulmonary retention of Cy5.5-labeled DNase. At 24 h, the heart, liver, lungs, and kidneys were harvested for ex vivo fluorescence imaging.

2.20 LPS-induced acute lung injury and treatment

ALI was induced in male C57BL/6J mice (8 weeks of age) by intratracheal instillation of LPS (1 mg kg^{-1} ; Section 2.1). Mice were randomly assigned to the Control, LPS, LPS + mPDA, LPS + free DNase I, LPS + DNase I@mPDA, and LPS + DNase I@mPDA@NM groups ($n = 6$ per group). The LPS group served as the untreated ALI group. At 2 h after LPS challenge, the indicated formulations were administered by nebulized inhalation using an Aerogen Solo vibrating-mesh nebulizer, with 200 μL of formulation loaded per mouse and aerosolized until nebulization was complete.

Free mPDA and free DNase I were administered at component-equivalent doses of 3.5 and 1.5 mg kg^{-1} , respectively. DNase I@mPDA and DNase I@mPDA@NM were administered at total formulation doses of 5 and 10 mg kg^{-1} , respectively; the latter corresponded to 3.5 mg kg^{-1} mPDA and 1.5 mg kg^{-1} DNase I.

Pulmonary NET-associated structures were examined by immunofluorescence for CitH3, MPO, and DAPI, and BALF was assayed for MPO–DNA and CitH3. MPO and neutrophil elastase were evaluated by immunohistochemistry. Lung histopathology was examined by hematoxylin and eosin staining. The levels of inflammatory cytokines (IL-6, IL-1 β , and TNF- α) in BALF were measured by ELISA. BALF neutrophils were quantified by flow cytometry (CD45⁺Ly6G⁺).

Vascular permeability was assessed by Evans blue extravasation. Evans blue (2% in PBS; Sigma-Aldrich) was administered via tail vein at 4 mL kg⁻¹ body weight. After 2 h of circulation, mice were perfused with 10–15 mL of saline via the heart for approximately 15 min until the right atrial effluent became colorless. Lungs were harvested, homogenized in 2 mL PBS, and vortexed for 2 min. Protein was precipitated by adding 2 mL of 60% trichloroacetic acid, incubating at 4 °C for 30 min, and centrifuging at 18,000 $\times$ g for 10 min at 4 °C. The supernatant (250 μ L) was transferred to a microplate and analyzed spectrophotometrically at 620 nm. BALF total protein was determined by the BCA assay. Pulmonary edema was evaluated by the lung wet-to-dry weight ratio after drying at 60°C for 72 h until constant weight.

2.21 Bulk RNA sequencing and pathway analysis

Bulk RNA sequencing was performed on lung tissues from the Control, ALI, and DNase I@mPDA@NM-treated groups (n = 3 mice per group) by Shanghai Majorbio Bio-pharm Technology Co., Ltd. Differential-expression analyses were performed for ALI versus Control and DNase I@mPDA@NM-treated versus untreated ALI lungs. DEGs were determined by p-values < 0.05 and |Log2Fold-change| $\geq$ 2.

Lung tissues collected from the Control, ALI, and DNase I@mPDA@NM-treated groups at 24 h after LPS challenge were analyzed by immunoblotting for phosphorylated p65 (p-p65; Cell Signaling Technology, 1:1000), total p65 (Cell Signaling Technology, 1:1000), and GAPDH, and the p-p65/p65 ratio was quantified by densitometric analysis.

2.22 Statistical analysis

Unless otherwise stated, in vitro experiments used three independent biological replicates, and each replicate contributed one data point (field-level or technical measurements were averaged within each biological replicate before group-level analysis). For Fig. 6, n = 6 mice per group (panels A–L), with each animal serving as the independent experimental unit.

Two-group comparisons were performed using two-sided Welch's t-tests. Multi-group comparisons were performed using one-way ANOVA with Tukey's multiple comparison test. Time-course data were analyzed using two-way ANOVA to assess the effects of treatment, time, and their interaction, followed by Dunnett's multiple-comparisons test against the corresponding reference group at each time point, as specified in the figure legends.

Analyses were performed using R v4.4 and GraphPad Prism 11.0.0. Unless otherwise specified, #, * and + in the figures indicate comparisons with the Control/PBS, ALI/PMA, and ALI/PMA+DNase I groups,

respectively; one, two, three, and four symbols denote $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

3. Results

3.1 Single-cell profiling reveals a formation–disposal mismatch underlying disrupted NET homeostasis in ALI

To define the cell-type-specific transcriptional basis of the formation–disposal mismatch in LPS-induced ALI, we profiled lung single-cell RNA-sequencing data (GEO: GSE276682) from control and LPS-challenged mice ($n = 3$ biological samples per condition; Fig. 1A). Following quality control and dataset integration, the pulmonary atlas resolved the major epithelial, endothelial, stromal, and immune populations, including epithelial cells, endothelial cells, fibroblasts, monocytes/macrophages, neutrophils, lymphocytes, and pericyte/smooth-muscle cells (Fig. 1B). All biological replicates contributed cells to the major populations used for condition-level analyses. LPS challenge increased the relative representation of neutrophils and monocytes/macrophages while reducing the relative contribution of epithelial cells (Fig. 1C), consistent with inflammatory remodelling of the injured lung.

We first interrogated the formation arm of NET homeostasis by scoring predefined neutrophil transcriptional programs associated with ROS generation, oxidative stress, and NETosis. Sample-level scores for all three programs were increased after LPS challenge (Fig. 1D), and pseudobulk competitive gene-set testing at the biological-sample level showed concordant enrichment of prespecified ROS- and NETosis-associated programs in the LPS group (Fig. 1E). Gene-level expression patterns related to NADPH oxidase-dependent ROS production and NET-associated effector functions were broadly consistent with these program-level changes (**Supplementary Fig S1**). Thus, the expanded neutrophil compartment in LPS-challenged lungs exhibited enhanced oxidative activation and a transcriptional state favoring ongoing NET formation. We next interrogated the disposal arm of NET homeostasis by examining monocyte/macrophage transcriptional programs related to extracellular-material uptake, DNA degradation, and phagolysosomal processing. In contrast to the increased formation-associated programs in neutrophils, these disposal-associated scores did not show a commensurate increase and overall tended to be reduced after LPS exposure (Fig. 1F).

To visualize this relative divergence at the biological-sample level, we compared standardized formation- and processing-associated transcriptional scores across the six samples (Fig. 1G, H). Control samples generally exhibited lower formation-associated scores and higher processing-associated scores, whereas LPS-challenged samples shifted toward a state characterized by higher formation-associated scores and lower processing-associated scores, accompanied by corresponding gene-level expression patterns across monocyte/macrophage programs related to extracellular-material recognition and uptake, phagolysosomal processing, DNA degradation, and inflammatory resolution (**Supplementary Fig. S2**). These findings revealed a transcriptional skew toward NET generation without a corresponding increase in programs related to post-release handling. We next examined whether this transcriptional

imbalance was accompanied by increased pulmonary NET burden and impaired extracellular NET degradation (Fig. 1I). Lung immunofluorescence revealed an increased abundance of MPO/CitH3/DAPI-positive NET-associated structures in LPS-challenged lungs, accompanied by increased MPO–DNA complexes in BALF (Fig. 1J, K), providing tissue- and BALF-level evidence of increased pulmonary NET-associated burden.

To functionally assess extracellular NET disposal, BALF from control or ALI mice was incubated with an identical preparation of preformed, cell-free NET substrate under cell-free conditions (Fig. 1L). More residual NET-associated MPO–DNA remained after incubation with ALI BALF than with control BALF (Fig. 1M). Consistently, time-resolved PicoGreen analysis showed delayed degradation of preformed NET-derived dsDNA in ALI BALF compared with control BALF (Fig. 1N). These findings indicate reduced extracellular NET-degrading capacity in the ALI alveolar milieu.

Together, these complementary transcriptional, tissue-level, and functional data provide convergent evidence for a formation–disposal mismatch in LPS-induced ALI, characterized by enhanced NET-formation-associated activity without a commensurate increase in post-release disposal capacity, thereby favoring persistent pulmonary NET accumulation. This imbalance defined a therapeutic requirement to address both sides of NET homeostasis: restraining ongoing NET formation while eliminating extracellular NETs that have already accumulated (Fig. 1O).

3.2 Construction and functional characterization of the DNase I@mPDA@NM nanoplatform

The formation–disposal mismatch and persistent pulmonary NET accumulation identified in ALI established a mechanistic rationale for a dual-function intervention targeting both ongoing NET formation and extracellular NET persistence (Fig. 1). We therefore engineered DNase I@mPDA@NM to integrate mPDA-mediated restraint of ROS-associated NET formation with DNase I-mediated degradation of extracellular NET material after release (Fig. 2A). Bone-marrow-derived neutrophils used as the membrane source reached a purity of 85.3% by flow cytometry (Fig. 2B). DNase I incorporation into mPDA was optimized by varying the mPDA-to-DNase I feed mass ratio from 4:1 to 1:1. An mPDA-to-DNase I ratio of 2:1 yielded an encapsulation efficiency of approximately 80% and a loading capacity of approximately 30% and was therefore selected for subsequent preparation of DNase I@mPDA (Fig. 2C). DNase I@mPDA was subsequently coated with neutrophil membrane to generate the final nanoplatform. DNase I@mPDA@NM exhibited a hydrodynamic diameter of approximately 200 nm and a zeta potential of approximately –30 mV (Fig. 2D). Both parameters remained largely unchanged over 6 days, indicating good colloidal stability under the tested conditions (**Supplementary Fig S3**). DNase I release from both DNase I@mPDA and DNase I@mPDA@NM increased progressively and reached approximately 82% at 24 h, with little additional release up to 48 h (Fig. 2E), indicating a sustained release profile.

The cytocompatibility and preliminary biosafety of DNase I@mPDA@NM were then established. Neutrophil viability remained comparable to untreated controls at concentrations up to 50 $\mu\text{g mL}^{-1}$ but decreased at 75 and 100 $\mu\text{g mL}^{-1}$; accordingly, 50 $\mu\text{g mL}^{-1}$ was selected for subsequent in vitro studies

(Fig. 2F). Here, DNase I@mPDA@NM concentration refers to the total dry mass of the final nanopatform. All nanoparticle formulations exhibited low haemolytic activity relative to the Triton X-100 positive control (Fig. 2G).

Consistent with these in vitro findings, nebulized inhalation treatment caused no evident histopathological abnormalities in the heart, liver, lung, or kidney, while hematological parameters and serum liver- and kidney-function indices showed no apparent treatment-related abnormalities (**Supplementary Fig S4–S6**). Electron microscopy further defined the structural features of the assembled nanopatform. The mesoporous architecture of mPDA became less apparent following DNase I loading, whereas DNase I@mPDA@NM exhibited an additional membrane-like outer layer consistent with neutrophil-membrane coating (Fig. 2H). CXCR2 and CXCR4 were detected in LPS-stimulated neutrophils and in both LPS-NM and NM membrane fractions, whereas Histone H3 was absent from the isolated membranes, indicating effective removal of nuclear components during membrane preparation. Both CXCR2 and CXCR4 remained detectable in DNase I@mPDA@NM but not in mPDA or DNase I@mPDA, supporting retention of neutrophil membrane-associated proteins after coating (Fig. 2I).

We next established whether the assembled formulation preserved the mPDA-mediated “Block” function. In both ABTS and DPPH assays, free mPDA exhibited the highest radical-scavenging activity, whereas DNase I alone showed negligible activity (Fig. 2J–M). Although DNase I@mPDA and DNase I@mPDA@NM displayed lower activity than free mPDA, both formulations retained substantial cell-free radical-scavenging capacity, demonstrating preservation of the intrinsic redox-scavenging activity of mPDA after DNase I loading and membrane coating. These findings established that the mPDA component retained its intrinsic radical-scavenging property after assembly into the final nanopatform, providing the functional basis for subsequent evaluation of ROS-associated NET formation restraint.

We then established whether the DNase I component retained its “Clear” function under conditions relevant to the injured lung. DNA degradation was quantified relative to the corresponding DNase I-free matrix control, with matched PBS, pH-matched PBS, or BALF controls defined as 0% degradation. Under baseline PBS conditions, free DNase I, DNase I@mPDA, and DNase I@mPDA@NM all exhibited DNA-degrading activity (**Supplementary Fig. S7**). Under mildly acidic conditions (pH 6.0), free DNase I showed a marked reduction in activity, whereas both DNase I@mPDA and DNase I@mPDA@NM retained substantial DNA-degrading activity (Fig. 2N, P). A similar pattern was observed in BALF collected from mice with LPS-induced ALI: using the corresponding enzyme-free or matrix control as the 0% degradation reference, both DNase-loaded formulations retained substantial DNA-degrading activity under the tested conditions, whereas free DNase I exhibited only minimal detectable degradation (Fig. 2O, Q). Neutrophil-membrane coating did not produce an evident additional loss of the DNase I activity retained by DNase I@mPDA under the tested conditions. Together, these findings established that DNase I@mPDA@NM preserved the two functional components required for the proposed “Block-and-Clear” strategy: mPDA-mediated radical-scavenging capacity and DNase I-mediated extracellular DNA-degrading activity. The resulting platform therefore provided the functional foundation for

subsequent validation of coordinated restraint of ongoing NET formation and clearance of extracellular NET material.

3.3 DNase I@mPDA@NM enables enhanced pulmonary localization and prolonged retention following inhalation

We first determined whether neutrophil-membrane (NM) coating enhanced the association of DNase I@mPDA with neutrophils, providing a potential basis for inflammatory-site localization. Primary neutrophils were incubated with Cy5.5-labeled DNase I@mPDA or DNase I@mPDA@NM for 2, 4, and 6 h, followed by washing to remove unbound nanoparticles (Fig. 3A). Cell-associated fluorescence increased progressively over time for both formulations and was consistently higher in neutrophils treated with DNase I@mPDA@NM at each time point (Fig. 3B, C). These findings demonstrated enhanced neutrophil association following NM coating, consistent with preservation of functional membrane components identified in Fig. 2.

We next determined whether this enhanced neutrophil association translated into prolonged pulmonary exposure following inhalation. LPS-induced ALI was established by intratracheal LPS challenge, followed 2 h later by nebulized inhalation of Cy5.5-labeled free DNase I, DNase I@mPDA, or DNase I@mPDA@NM. In vivo fluorescence imaging was performed immediately after administration and at 4, 8, 12, and 24 h, with fluorescence intensity in the lung region quantified to assess pulmonary retention (Fig. 3D, E, G). Fluorescence associated with free DNase I declined rapidly and was low at 24 h, whereas both mPDA-containing formulations produced more persistent pulmonary signals. DNase I@mPDA@NM exhibited the strongest pulmonary fluorescence over the measured time course, reached its highest signal at 8 h, and maintained a higher pulmonary signal than the other formulations at 24 h. Ex vivo imaging at 24 h further showed that fluorescence was predominantly localized to the lungs, with lower signals detected in extrapulmonary organs (Fig. 3F, H). Together, these findings demonstrated that NM coating enhanced nanoparticle association with neutrophils and was associated with greater and more persistent pulmonary localization after inhalation, supporting localized pulmonary exposure of DNase I@mPDA@NM.

3.4 DNase I@mPDA@NM implements a “Block-and-Clear” strategy to control NET formation and extracellular persistence

We next determined whether DNase I@mPDA@NM could simultaneously restrain ongoing NET formation and eliminate extracellular NET material after release. To distinguish these two processes, intracellular and extracellular NET-associated responses were assessed as separate readout compartments (Fig. 4A, B). Intracellular ROS and lysate CitH3 were used to evaluate oxidative activation and NETosis-associated histone citrullination, whereas extracellular DNA and supernatant MPO–DNA complexes were used to quantify detectable extracellular NET-associated material. This compartment-

resolved design enabled functional attribution of the effects of mPDA and DNase I to the formation and post-release persistence of NETs, respectively.

We first established the formation-associated “Block” function of mPDA. PMA markedly increased intracellular DCFH-DA fluorescence, whereas mPDA and DNase I@mPDA@NM substantially reduced this increase; DNase I alone produced little effect (Fig. 4C–E), indicating that attenuation of intracellular ROS was associated with the presence of mPDA. Consistent with this effect, PMA increased the CitH3/H3 ratio, total CitH3 abundance in neutrophil lysates, and CitH3-positive neutrophil counts per field at 2 h, whereas mPDA and DNase I@mPDA@NM reduced all three CitH3-associated readouts (Fig. 4F–J). DNase I alone produced little change in these early intracellular responses. To further establish the contribution of ROS to this effect, neutrophils were exposed to 50 μ M H₂O₂, which increased intracellular ROS without reducing cell viability or independently increasing CitH3 or supernatant MPO–DNA under the tested conditions (**Supplementary Fig S8 and S9**). H₂O₂ partially reversed the mPDA-associated reduction in CitH3 readouts (Fig. 4F–J), supporting ROS attenuation as a mechanistic contributor to the inhibition of early NETosis-associated activation by DNase I@mPDA@NM. These findings established the mPDA component as the formation-restraining arm of the platform.

We next established the post-release “Clear” function of DNase I. At 4 h after PMA stimulation, mPDA, DNase I, and DNase I@mPDA@NM each reduced supernatant MPO–DNA relative to PMA alone, with the lowest level observed in the DNase I@mPDA@NM group (Fig. 4K). SYTOX imaging showed a corresponding reduction in extracellular DNA, again with the greatest decrease after DNase I@mPDA@NM treatment (Fig. 4L and **Supplementary Fig S10**). Because these extracellular endpoints integrate both NET release and subsequent degradation, they do not by themselves distinguish suppression of formation from removal of pre-existing NETs. We therefore introduced treatment after NET formation using a preformed-NET model. Under these conditions, mPDA produced little change in extracellular MPO–DNA, whereas DNase I and DNase I@mPDA@NM markedly reduced the pre-existing extracellular signal (**Supplementary Fig S11**), directly supporting DNase I-mediated degradation of extracellular NET material after release. Thus, the DNase I component provided a distinct post-release clearance function that was not reproduced by mPDA alone.

We then examined the temporal relationship between the intracellular formation-associated and extracellular persistence-associated responses by measuring lysate CitH3 and supernatant MPO–DNA in parallel at 2, 3, 4, and 6 h after PMA stimulation (Fig. 4M). PMA maintained elevated intracellular CitH3 throughout the observation period (Fig. 4N). mPDA reduced lysate CitH3 from the earliest time point, with more pronounced differences at 3 and 4 h and values approaching the control group by 6 h, whereas DNase I produced little early effect and a modest reduction at later time points. DNase I@mPDA@NM reduced intracellular CitH3 from the earliest measurement and maintained lower levels thereafter. In contrast, extracellular MPO–DNA showed a delayed and progressive increase in the PMA group, rising sharply between 2 and 3 h and continuing to accumulate through 6 h (Fig. 4O). mPDA attenuated this increase from 3 h onward but did not prevent continued accumulation, whereas DNase I and DNase I@mPDA@NM markedly limited the increase after 2 h and maintained relatively low MPO–

DNA levels from 3 to 6 h. DNase I@mPDA@NM showed a temporal profile combining the early intracellular effect associated with mPDA with the sustained extracellular reduction associated with DNase I. Together, these findings demonstrated that the two components acted at complementary stages of NET dynamics: mPDA restrained ROS-associated intracellular NETosis activation, whereas DNase I eliminated extracellular NET material after release. DNase I@mPDA@NM therefore integrated these temporally and mechanistically distinct activities within a single “Block-and-Clear” platform.

3.5 DNase I@mPDA@NM protects the alveolar–capillary barrier by reducing extracellular NET burden in an endothelial–epithelial model

Before assessing barrier protection, we first established that the treatments did not intrinsically alter basal neutrophil phagocytic activity. Uptake of fluorescent microspheres by primary neutrophils was comparable among untreated cells and cells treated with mPDA, DNase I, or DNase I@mPDA@NM, whereas incubation at 4 °C markedly reduced particle uptake and served as a negative control (Fig. 5A, B). These findings excluded a major treatment-associated alteration in basal neutrophil uptake capacity under the tested conditions.

We next established an endothelial–epithelial Transwell co-culture model to recapitulate key features of the alveolar–capillary interface. Pulmonary microvascular endothelial cells and alveolar epithelial cells were cultured on opposite faces of the insert membrane, and the resulting co-culture exhibited substantially lower permeability to 4-kDa FITC–dextran than cell-free inserts, confirming formation of a restrictive cellular barrier (**Supplementary Fig S12**). Primary bone-marrow-derived neutrophils were then introduced into the vascular compartment, while LPS was applied to the alveolar compartment to induce inflammatory barrier injury (Fig. 5C). LPS markedly increased neutrophil transmigration across the endothelial–epithelial interface and increased MPO–DNA and CitH3 levels in the alveolar-side fluid, indicating enhanced NET-associated burden in the compartment reached by migrating neutrophils. mPDA, DNase I, and DNase I@mPDA@NM each reduced neutrophil transmigration and alveolar-side NET-associated readouts, with DNase I@mPDA@NM producing the lowest number of transmigrated CD45⁺Ly6G⁺ neutrophils and the greatest reductions in MPO–DNA and CitH3 (Fig. 5D–G). Thus, the integrated formulation most effectively limited the accumulation of neutrophils and NET-associated material at the injured alveolar interface.

We next determined whether these changes were accompanied by preservation of endothelial and epithelial barrier integrity. In endothelial cells, LPS reduced ZO-1 and occludin abundance and disrupted the continuous junctional distribution of ZO-1. Treatment with mPDA, DNase I, or DNase I@mPDA@NM increased ZO-1 and occludin abundance and improved the continuity of endothelial ZO-1 junctions (Fig. 5H–J). At the epithelial layer, LPS increased alveolar epithelial-cell apoptosis, which was attenuated by all three treatments, with the lowest proportion of apoptotic cells observed after DNase I@mPDA@NM treatment (Fig. 5K, L). Thus, reduction of alveolar-side NET-associated burden was

accompanied by reduced neutrophil transmigration, preservation of endothelial junctional organization, and attenuation of epithelial cell injury.

To determine whether extracellular NET-associated material directly contributed to the observed barrier protection, we performed a NET add-back experiment using standardized preformed NET-rich material. Following the initial LPS stimulation and treatment phase, the medium was removed, the barrier was washed, and a defined amount of preformed NET material was added to the basolateral vascular compartment for an additional 8 h (Fig. 5M). Addition of preformed NET material increased FITC–dextran permeability and disrupted endothelial ZO-1 continuity, demonstrating that extracellular NET material was sufficient to aggravate barrier dysfunction under the tested conditions. Importantly, DNase I@mPDA@NM reduced LPS-induced permeability and preserved ZO-1 continuity, whereas NET add-back increased permeability and partially reversed the improvement in junctional ZO-1 organization (Fig. 5N, O). Together, these findings demonstrate that DNase I@mPDA@NM preserves endothelial–epithelial barrier integrity in association with reduced extracellular NET burden, while the partial reversal produced by NET add-back supports a causal contribution of extracellular NET material to barrier dysfunction.

3.6 DNase I@mPDA@NM reduces pulmonary NET burden and alleviates alveolar–capillary injury in ALI

We next evaluated DNase I@mPDA@NM in LPS-induced ALI. Mice received pulmonary treatment 2 h after LPS challenge, followed by assessment of pulmonary NET-associated burden, inflammatory injury, and alveolar–capillary barrier function. LPS markedly increased the pulmonary MPO⁺/CitH3⁺ colocalized area and produced stronger MPO and NE immunoreactivity in lung tissue, while also elevating MPO–DNA and CitH3 levels in BALF (Fig. 6A, B, D, E; **Supplementary Fig S13A**). These tissue- and BALF-level NET-associated changes were attenuated by treatment. Among the groups included in the BALF analyses, DNase I@mPDA@NM produced the largest reductions in MPO–DNA and CitH3. In the tissue-level analysis, mPDA and free DNase I partially reduced the MPO⁺/CitH3⁺ colocalized area, whereas DNase I@mPDA and DNase I@mPDA@NM produced larger reductions. Importantly, DNase I@mPDA@NM further reduced pulmonary MPO⁺/CitH3⁺ colocalization compared with uncoated DNase I@mPDA (**Supplementary Fig S13A**). This difference is consistent with the enhanced formulation–neutrophil association and prolonged pulmonary exposure observed in Fig. 3, although these data do not independently establish the mechanism responsible for the improved tissue-level effect.

We next examined whether these NET-associated changes were accompanied by reduced inflammatory and structural lung injury. LPS caused inflammatory-cell infiltration, alveolar-septal thickening, and disruption of lung architecture, together with increased IL-6, IL-1 β , and TNF- α levels (Fig. 6C, F–H). Treatment attenuated the histopathological changes and reduced all three inflammatory mediators, with the lowest cytokine levels observed after DNase I@mPDA@NM treatment. Quantitative histopathological analysis further showed a lower lung-injury score after DNase I@mPDA@NM treatment than after uncoated DNase I@mPDA treatment (**Supplementary Fig S13B**). Thus, NM coating was associated with

greater reductions in tissue-level MPO⁺/CitH3⁺ colocalization and histopathological lung injury than the uncoated formulation.

We then assessed whether these effects translated into preservation of alveolar–capillary barrier function. LPS markedly increased Evans blue extravasation and BALF total protein concentration, indicating enhanced alveolar–capillary permeability (Fig. 6I, J). Both measures were reduced by treatment, with the greatest attenuation observed after DNase I@mPDA@NM. LPS also increased the lung wet-to-dry weight ratio, whereas DNase I@mPDA@NM substantially reduced this increase, indicating reduced pulmonary edema (Fig. 6K). Consistent with these functional measurements, LPS disrupted the continuous junctional distribution of ZO-1 in lung tissue, whereas DNase I@mPDA@NM improved the continuity of ZO-1 staining (Fig. 6L). These findings demonstrated that DNase I@mPDA@NM preserved alveolar–capillary barrier integrity and reduced pulmonary edema *in vivo*.

Together, DNase I@mPDA@NM produced coordinated reductions in pulmonary NET-associated burden, inflammatory and histopathological lung injury, and alveolar–capillary barrier dysfunction in LPS-induced ALI. The greater efficacy of DNase I@mPDA@NM than uncoated DNase I@mPDA further demonstrated the functional benefit of NM coating *in vivo*, linking the enhanced pulmonary localization and exposure established above with improved therapeutic activity. These results establish that the “Block-and-Clear” intervention translates from cellular NET control to reduction of pulmonary NET burden and preservation of lung barrier integrity in ALI.

3.7 Whole-lung transcriptomics supports *in vivo* rebalancing of pathological NET homeostasis following DNase I@mPDA@NM treatment

We next performed whole-lung bulk RNA sequencing to determine whether the therapeutic effects of DNase I@mPDA@NM were accompanied by coordinated transcriptional changes in the injured lung. Principal-component analysis clearly separated control and untreated ALI samples, whereas DNase I@mPDA@NM-treated lungs occupied an intermediate transcriptional space between these two groups (Fig. 7A). Differential expression analysis identified widespread transcriptional changes between ALI and control lungs and between DNase I@mPDA@NM-treated and untreated ALI lungs (Fig. 7B, C). Several genes associated with neutrophil activation, oxidative stress, and inflammatory signalling, including *Cybb*, *S100a8*, *Padi4*, *Nlrp3*, *Lcn2*, and *Tnf*, were increased in ALI and reduced following DNase I@mPDA@NM treatment. Hierarchical clustering further showed that a subset of ALI-associated expression changes shifted towards control-like expression patterns after treatment (Fig. 7D), indicating partial molecular normalization of the injured lung.

We next characterized the biological processes associated with these transcriptional changes. GO enrichment analysis showed that genes increased in ALI were enriched for inflammatory responses, regulation of cytokine production, leukocyte migration, reactive oxygen species metabolic process, neutrophil activation, and regulation of neutrophil degranulation, whereas genes reduced following

DNase I@mPDA@NM treatment were enriched for largely overlapping inflammatory and neutrophil-associated biological processes (Fig. 7E, F). Consistent with these findings, GSEA showed that the neutrophil degranulation program was positively enriched in ALI relative to control lungs and negatively enriched following DNase I@mPDA@NM treatment relative to untreated ALI lungs (Fig. 7G, H). KEGG analysis further showed that several inflammatory pathways enriched among genes upregulated in ALI were also enriched among genes downregulated following DNase I@mPDA@NM treatment, including NOD-like receptor, NF- κ B, TNF, Toll-like receptor, IL-17, and chemokine signalling pathways (**Supplementary Fig. S14A, B**). Thus, the transcriptional programs induced during ALI and attenuated by DNase I@mPDA@NM converged on neutrophil activation and inflammatory processes associated with increased NET formation.

To further determine whether treatment altered inflammatory signalling associated with sustained neutrophil activation, we performed GSEA of the NF- κ B activation program. NF- κ B activation was positively enriched in ALI relative to control lungs but negatively enriched following DNase I@mPDA@NM treatment relative to untreated ALI lungs (Fig. 7I, J). We next examined transcriptional programs associated with the processing arm of NET homeostasis. The phagocytosis gene set was significantly negatively enriched in ALI relative to control lungs (Fig. 7K), whereas this program was significantly positively enriched following DNase I@mPDA@NM treatment relative to untreated ALI lungs (Fig. 7L). These reciprocal changes indicate that ALI was associated with suppression of phagocytosis-associated processing programs, whereas DNase I@mPDA@NM treatment shifted these programs in the opposite direction. Together with the reduced extracellular NET-degrading capacity observed in ALI BALF and enhanced degradation of extracellular NET material by DNase-containing constructs, these findings support restoration of the processing side of NET homeostasis. Consistent with this transcriptional pattern, the increased p-p65/p65 ratio in ALI lungs was reduced following DNase I@mPDA@NM treatment (Fig. 7M, N), further supporting attenuation of NF- κ B-associated inflammatory activation.

Together, the transcriptomic findings revealed coordinated alterations on both sides of pathological NET homeostasis: ALI was characterized by enhanced neutrophil activation, degranulation, ROS-associated inflammatory signalling, and NF- κ B activation linked to excessive NET formation, whereas DNase I@mPDA@NM treatment attenuated these formation-associated programs and restored phagocytosis-associated processing programs. Combined with the reduced pulmonary NET burden, enhanced extracellular NET degradation, and improved alveolar–capillary barrier integrity demonstrated above, these findings provide independent molecular support that DNase I@mPDA@NM rebalances the formation–processing relationship underlying persistent NET accumulation and promotes restoration of disrupted NET homeostasis.

4. Discussion

ALI is characterized by excessive neutrophil activation and persistent pulmonary NET accumulation, but the mechanisms that determine whether NETs are transiently generated or persist within injured tissue

remain incompletely understood [9,11,14]. Our findings support a formation–disposal mismatch as one contributor to this persistent NET burden. In LPS-induced ALI, neutrophils exhibited enhanced transcriptional programs related to ROS production, oxidative stress, and NETosis, whereas monocyte/macrophage programs associated with extracellular-material processing and DNA degradation did not increase proportionally. These transcriptional changes were accompanied by increased pulmonary NET burden and reduced apparent extracellular NET-degrading activity in BALF. Together, these findings suggest that pathological NET accumulation reflects not only excessive formation but also insufficient post-release handling, providing a mechanistic basis for viewing NET burden as a dynamic homeostatic problem rather than a static consequence of NETosis [11,14,29].

This framework provides a rationale for the “Block-and-Clear” strategy implemented by DNase I@mPDA@NM. Conventional DNase I-based approaches primarily target NETs after their release and can therefore reduce pre-existing extracellular NET burden without directly limiting continued NET generation [14,30,31]. In contrast, mPDA and DNase I were assigned complementary functions within our platform. mPDA reduced intracellular ROS and CitH3-associated NETosis activation, with partial reversal by H₂O₂ supporting ROS attenuation as an important contributor to its formation-side effect, whereas DNase I directly degraded preformed extracellular NET material. The mPDA core therefore functions as more than a passive carrier: its intrinsic redox activity addresses the upstream oxidative drive of NET formation while its matrix environment helps preserve DNase I activity under conditions relevant to the injured lung [32,33]. By integrating these functions within a single inhalable formulation, the platform addresses both determinants of persistent NET accumulation rather than targeting extracellular NETs in isolation [22,25,34].

The temporal separation between formation-associated activation and extracellular NET persistence further supports this strategy. Intracellular CitH3-associated activation preceded the progressive accumulation of extracellular MPO–DNA, and the effects of mPDA and DNase I followed corresponding temporal patterns. mPDA exerted an earlier effect on intracellular ROS and CitH3, whereas DNase I predominantly influenced extracellular NET-associated material after its appearance. DNase I@mPDA@NM combined these temporally distinct activities within the same treatment, thereby limiting both the generation of new NET material and the persistence of NETs that had already been released. This distinction is important because reducing NET formation alone does not remove accumulated extracellular chromatin, whereas extracellular degradation alone may be continuously offset by ongoing NET release [14]. The therapeutic value of the platform therefore lies not simply in combining two active components, but in coordinating intervention across sequential stages of NET dynamics [35].

The reduction in NET burden was accompanied by preservation of the alveolar–capillary barrier, providing a functional link between NET regulation and tissue protection [36–38]. In the endothelial–epithelial model, DNase I@mPDA@NM reduced neutrophil transmigration and extracellular NET-associated material while preserving endothelial junctional organization and limiting epithelial apoptosis. Importantly, addition of preformed NET material partially reversed the barrier-protective effects of treatment, supporting a causal contribution of extracellular NET-associated material to barrier

dysfunction. Consistent with these findings, inhaled DNase I@mPDA@NM reduced pulmonary NET burden, inflammatory injury, alveolar–capillary leakage, pulmonary edema, and histopathological damage in LPS-induced ALI. Whole-lung transcriptomics further showed attenuation of neutrophil activation, degranulation, and inflammatory programs, including directional reversal of neutrophil-degranulation and TAK1-dependent IKK/NF- κ B signatures. These molecular and functional changes together support the concept that limiting persistent NET burden can interrupt the broader cycle of neutrophil activation, inflammatory amplification, and alveolar–capillary injury [7,10,39].

Several limitations define the scope of the present NET-homeostasis framework. First, NET fate is more complex than the formation–degradation relationship examined here [40]. NET burden may also be influenced by the mode of NET release, molecular composition, extracellular persistence, nuclease susceptibility, cellular uptake, intracellular trafficking, and disposal of degradation products [11,14,15,40]. The monocyte/macrophage programs used in our single-cell analysis are transcriptional correlates and do not directly establish impaired phagocytic NET uptake or intracellular degradation in ALI. Accordingly, our data identify a relative formation–processing mismatch rather than demonstrating defective macrophage-mediated NET clearance. Future studies incorporating cell-type-resolved tracking of NET fate and quantitative in vivo kinetic measurements will be important for defining these processes more directly. Second, NET homeostasis should not be interpreted as maximal NET suppression. Physiological NET formation contributes to host defense, and the optimal therapeutic window will likely require reduction of pathological accumulation while preserving essential antimicrobial functions [11,40]. The present study demonstrates therapeutic benefit from lowering excessive NET burden but does not define the physiological set point at which NET-directed intervention becomes detrimental.

The active nature of mPDA also warrants consideration. Because mPDA can modulate oxidative and inflammatory processes in multiple cell types, its effects are not necessarily restricted to neutrophil NET formation [41,42]. Although the experimental design supports a major contribution of NET regulation, NET-independent effects on epithelial, endothelial, or other inflammatory cells may contribute to the overall therapeutic response. Future cell-type-resolved and causal studies will therefore be required to distinguish NET-dependent from broader antioxidant and anti-inflammatory effects. In addition, the relative contribution of NETs and the therapeutic window for NET modulation may differ across infectious, inhalational, and ventilator-associated forms of lung injury [15,43–45]. Validation in etiologically distinct models will help determine the generalizability of the formation–disposal framework and define contexts in which coordinated NET regulation provides the greatest therapeutic benefit.

In summary, our findings support a model in which persistent pulmonary NET accumulation during ALI is sustained, at least in part, by a relative mismatch between ongoing NET formation and post-release disposal. DNase I@mPDA@NM addresses this imbalance through coordinated restraint of ROS-associated NET formation and degradation of extracellular NETs after release, while inhalation and neutrophil-membrane coating enhance localized pulmonary exposure. This coordinated intervention reduced NET burden, attenuated inflammatory and barrier injury, and improved pulmonary outcomes in

ALI. More broadly, these findings shift NET-directed therapy from removal of accumulated NETs alone toward stage-coordinated regulation of NET fate, providing a conceptual framework for restoring pathological NET homeostasis rather than simply suppressing NET formation.

5. Conclusions

Our findings support a model in which persistent pulmonary NET accumulation in acute lung injury is associated with a relative mismatch between ongoing NET formation and post-release disposal. In LPS-induced ALI, enhanced neutrophil oxidative and NET-formation-associated activity was not accompanied by a commensurate increase in disposal-associated responses, coinciding with increased pulmonary NET burden and reduced apparent extracellular NET-degrading activity. Guided by this formation–disposal mismatch, we developed an inhalable neutrophil-mimetic DNase I@mPDA@NM nanoplatform that coordinates intervention across two complementary stages of NET dynamics: mPDA-mediated restraint of ROS-associated NET formation and DNase I-mediated degradation of extracellular NETs after release. This “Block-and-Clear” strategy reduced persistent pulmonary NET accumulation, attenuated neutrophilic and inflammatory injury, and preserved alveolar–capillary barrier integrity, resulting in reduced vascular leakage, pulmonary edema, and tissue injury in ALI. Collectively, these findings establish a mechanism-guided framework in which pathological NET accumulation is addressed as a homeostatic imbalance rather than as a consequence of excessive NET formation alone. By coordinating suppression of ongoing NET generation with removal of established extracellular NETs, this strategy shifts NET-directed therapy from single-stage intervention toward restoration of pathological NET homeostasis.

Abbreviations

ARDS
acute respiratory distress syndrome
DLS
dynamic light scattering
ELISA
enzyme-linked immunosorbent assay
ABTS
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ALI
acute lung injury
BALF
bronchoalveolar lavage fluid
CCK-8
Cell Counting Kit-8
CitH3
citrullinated histone H3

CI
confidence interval
CXCR2
C-X-C motif chemokine receptor 2
CXCR4
C-X-C motif chemokine receptor 4
DCFH-DA
2',7'-dichlorodihydrofluorescein diacetate
DPPH
2,2-diphenyl-1-picrylhydrazyl
FDR
false discovery rate
GO
Gene Ontology
GSEA
gene-set enrichment analysis
H&E
hematoxylin and eosin
KEGG
Kyoto Encyclopedia of Genes and Genomes
LPS
lipopolysaccharide
Ly6G
lymphocyte antigen 6 complex
mPDA
mesoporous polydopamine
MPO
myeloperoxidase
MPO–DNA
myeloperoxidase–DNA complex
NE
neutrophil elastase
NETs
neutrophil extracellular traps
NF- κ B
nuclear factor kappa B
NM
neutrophil membrane
PBS
phosphate-buffered saline

PMA
phorbol 12-myristate 13-acetate
ROS
reactive oxygen species
scRNA-seq
single-cell RNA sequencing
TAK1
transforming growth factor- β -activated kinase 1
TEM
transmission electron microscopy
ZO-1
zonula occludens-1
P_{app}
apparent permeability coefficient
W/D
wet-to-dry weight ratio
H₂O₂
hydrogen peroxide.

Declarations

Ethics approval and consent to participate

All experimental procedures involving animals were conducted in accordance with the protocol formulated by the Laboratory Animal Welfare and Ethics Committee of Tongji University, and were approved by the Animal Ethics Committee of Tongji University (Animal Ethics: TJAA07721106).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by National Natural Science Foundation of China (82372155, 82572457, 82402507), Shanghai Pulmonary Hospital 2024 Research-Oriented Physician Talent Program (LYRC202404), the "Three Peaks" Program of Shanghai Pulmonary Hospital (Peak A Talent Grant).

Author Contribution

J.H: Conceptualization, Validation, Investigation, Writing-Original Draft; F.M: Formal analysis, Methodology, Conceptualization; Q. L: Formal analysis, Methodology, Conceptualization; T.J: Funding acquisition, Methodology, Supervision; E.C: Methodology; J.Y: Investigation; Y.J: Methodology; Y.L: Funding acquisition, Writing-Review & Editing; X.L: Project administration, Funding acquisition, Resources.

Acknowledgements

Not applicable.

Availability of data and materials

The publicly available single-cell RNA-sequencing dataset analyzed in this study is available in the Gene Expression Omnibus under accession number GSE276682. Other data generated or analyzed during this study are included in this article and its supplementary information files and are available from the corresponding author upon reasonable request.

Clinical trial registration

Not applicable.

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Figures

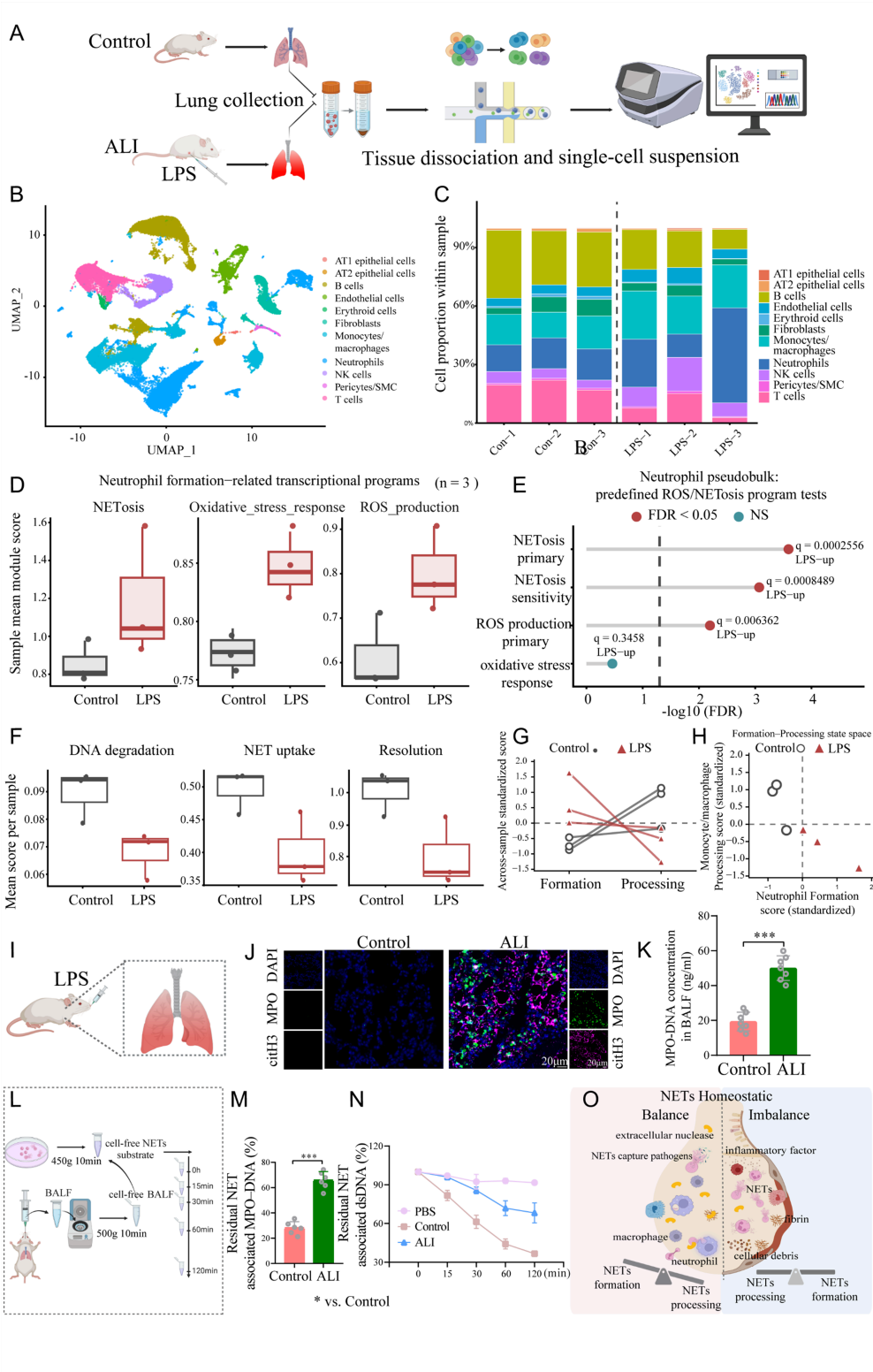


Figure 1

Single-cell profiling identifies divergent NET-formation- and NET-processing-associated responses in LPS-induced ALI. (A) Experimental workflow for lung single-cell RNA sequencing in control and LPS-challenged mice. (B) UMAP of the integrated lung-cell atlas, annotated by major pulmonary cell type. (C)

Cell-type proportions within each biological sample. (D) Sample-level neutrophil NETosis-, oxidative-stress-response- and ROS-production-module scores. (E) Neutrophil pseudobulk competitive tests of prespecified ROS- and NETosis-associated transcriptional programs. The dashed line indicates $q = 0.05$, where q is the false-discovery-rate-adjusted P value. (F) Sample-level monocyte/macrophage DNA-degradation-, NET-uptake- and resolution-module scores. (G) Within-sample Formation and Processing scores, with lines connecting values from the same biological replicate. (H) Formation–Processing state-space plot. Open circles indicate Control, filled triangles indicate LPS. Scores were derived by equal-weight averaging of the standardized predefined formation-related modules in neutrophils and processing-related modules in monocytes/macrophages. (I) Experimental design for LPS-induced ALI. (J) Representative lung immunofluorescence images of CitH3, MPO and DAPI. (K) BALF MPO–DNA concentrations. Panels A–H used $n = 3$ biological samples per condition; panels J–K, M–N used $n = 6$ mice per group. (L) Experimental workflow for BALF-mediated degradation of preformed NETs. (M) Residual NET-associated MPO–DNA after incubation of preformed NETs with cell-free BALF from Control or LPS-induced ALI mice for 2 h at 37 °C. (N) Kinetics of residual NET-associated dsDNA in PBS, control BALF, and ALI BALF. (O) Schematic illustration of NET homeostatic balance and its disruption in ALI. In K and M, Control and LPS groups were compared using Welch’s two-tailed unpaired t -test. In N, time-course data were analyzed by two-way ANOVA followed by multiple-comparisons testing. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus Control; ns, not significant.

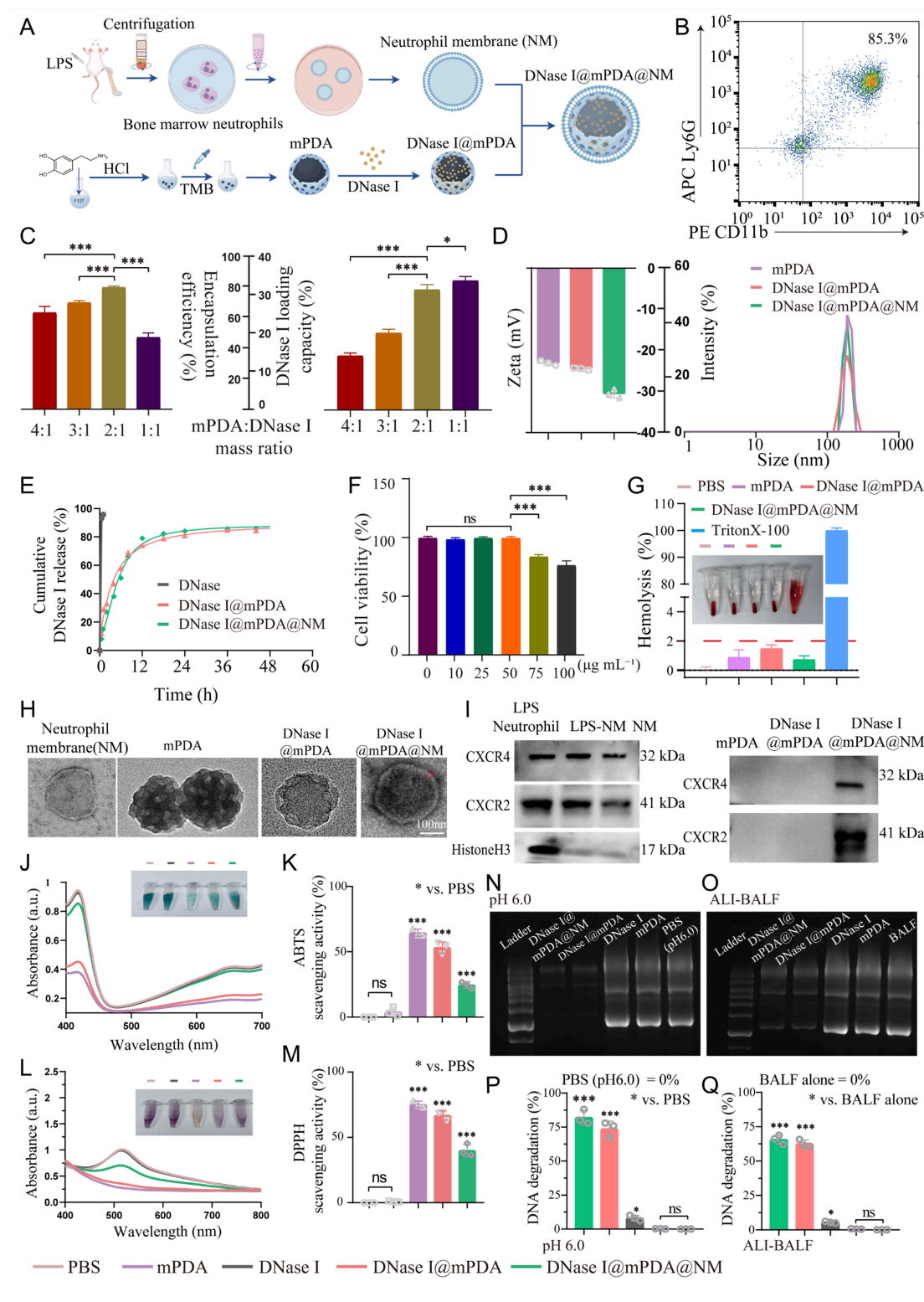


Figure 2

Assembly and functional characterization of the DNase I@mPDA@NM nanoplateform. (A) Schematic of mPDA synthesis, DNase I loading and neutrophil-membrane coating. (B) Flow-cytometric assessment of CD11b+Ly6G+ bone-marrow neutrophil purity. (C) DNase I encapsulation efficiency and loading capacity at mPDA:DNase I mass ratios of 4:1, 3:1, 2:1 and 1:1. (D) Zeta potentials and hydrodynamic-size distributions of mPDA, DNase I@mPDA and DNase I@mPDA@NM. (E) Cumulative DNase I release from

free DNase I, DNase I@mPDA and DNase I@mPDA@NM. (F) Viability of bone marrow-derived neutrophils across the tested material concentrations. (G) Hemolysis of the indicated formulations; Triton X-100 served as the positive control. (H) Transmission electron microscopy of isolated neutrophil membrane, mPDA, DNase I@mPDA and DNase I@mPDA@NM. (I) Immunoblot analysis of CXCR4, CXCR2, and Histone H3 in LPS-stimulated neutrophils, LPS-NM, and NM, and of CXCR4 and CXCR2 in mPDA, DNase I@mPDA, and DNase I@mPDA@NM. (J, K) ABTS absorption spectra and quantified radical-scavenging activity. (L, M) DPPH absorption spectra and quantified radical-scavenging activity. (N, P) DNA degradation under pH 6.0 conditions. (O, Q) DNA degradation in BALF from mice with LPS-induced ALI. DNA degradation (%) was calculated relative to the corresponding enzyme-free matrix control, with PBS (pH 6.0) alone and ALI-BALF alone defined as 0% degradation in (P) and (Q), respectively. $n = 3$. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparisons test. Pairwise comparisons in (C) and (F) are indicated by brackets; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. For (K), (M), and (P), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus PBS; for (Q), the same symbols indicate comparisons versus BALF alone. ns, not significant.

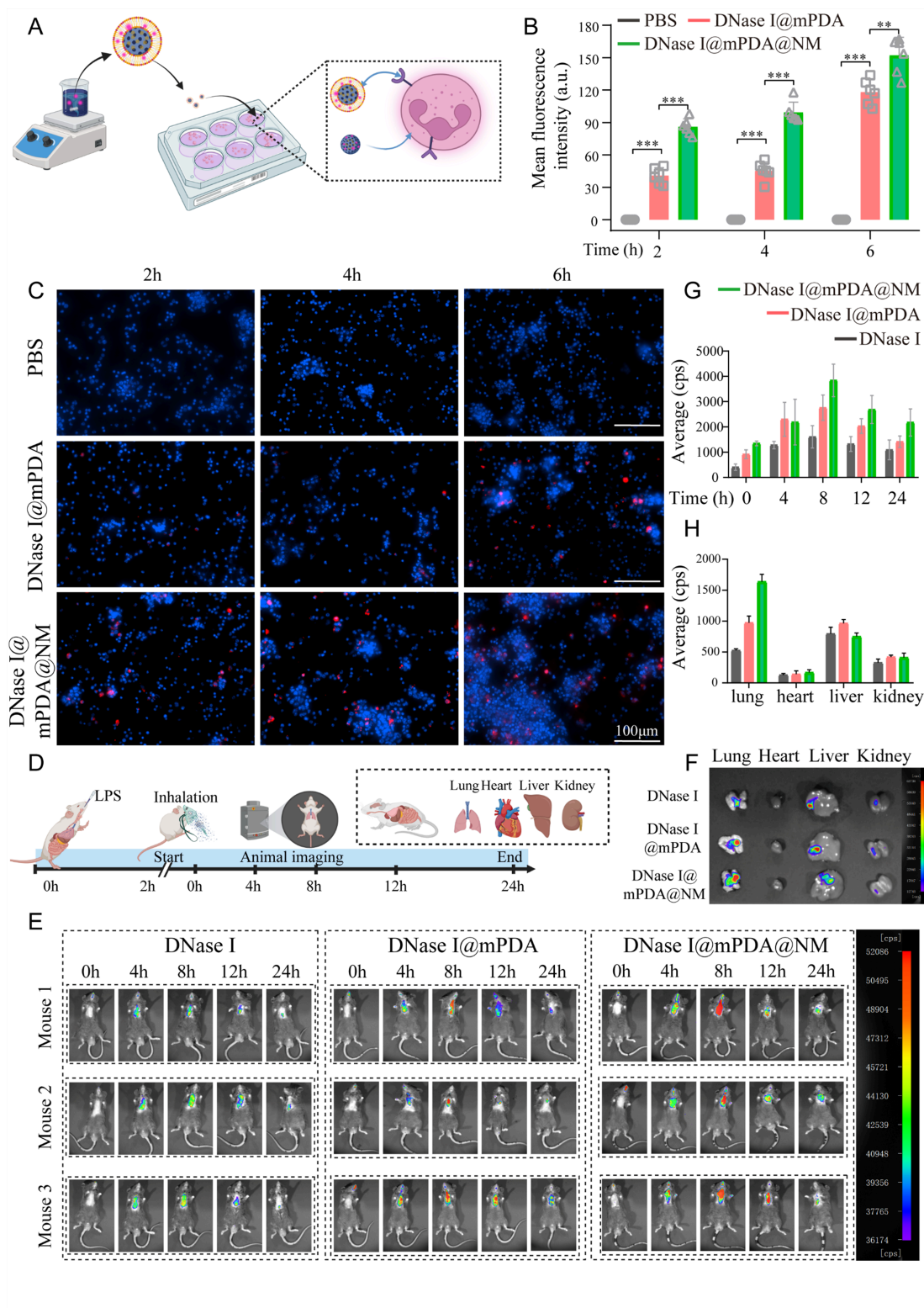


Figure 3

Neutrophil-membrane coating increases formulation–neutrophil association and prolongs pulmonary exposure after inhalation. (A) Schematic of the formulation–neutrophil association assay. (B) Quantification of cell-associated fluorescence after neutrophils were incubated with Cy5.5-labeled DNase I@mPDA or DNase I@mPDA@NM for 2, 4 and 6 h. (C) Representative fluorescence images corresponding to panel B; nuclei are shown in blue and formulation-associated Cy5.5 signal in red. (D) In

vivo imaging design. Cy5.5-labeled free DNase I, DNase I@mPDA or DNase I@mPDA@NM was administered by inhalation 2 h after intratracheal LPS challenge. (E) Representative in vivo fluorescence images showing pulmonary Cy5.5-associated fluorescence at 0, 4, 8, 12, and 24 h after inhalation. (F) Ex vivo fluorescence images of the lung, heart, liver and kidney at 24 h. (G) Quantification of Cy5.5-associated fluorescence intensity in the lung region over time. (H) Quantification of ex vivo organ fluorescence at 24 h. Fluorescence represents labeled formulation-associated signal and does not establish the presence of intact catalytically active DNase I. In vitro association experiments used n = 6 independent biological replicates; in vivo imaging used n = 6 mice per group. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple-comparisons test. *P < 0.05, **P < 0.01, and ***P < 0.001; ns, not significant.

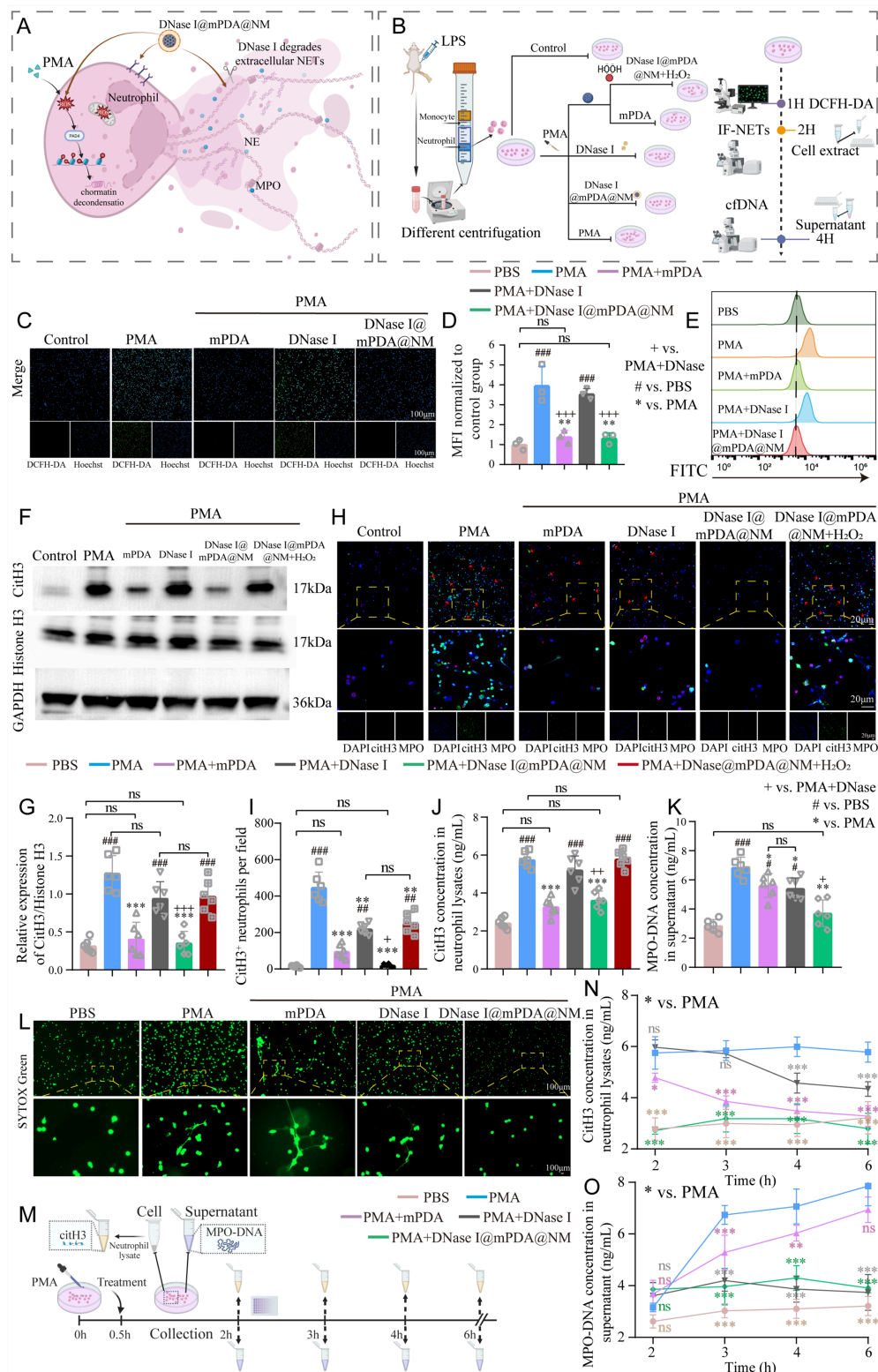
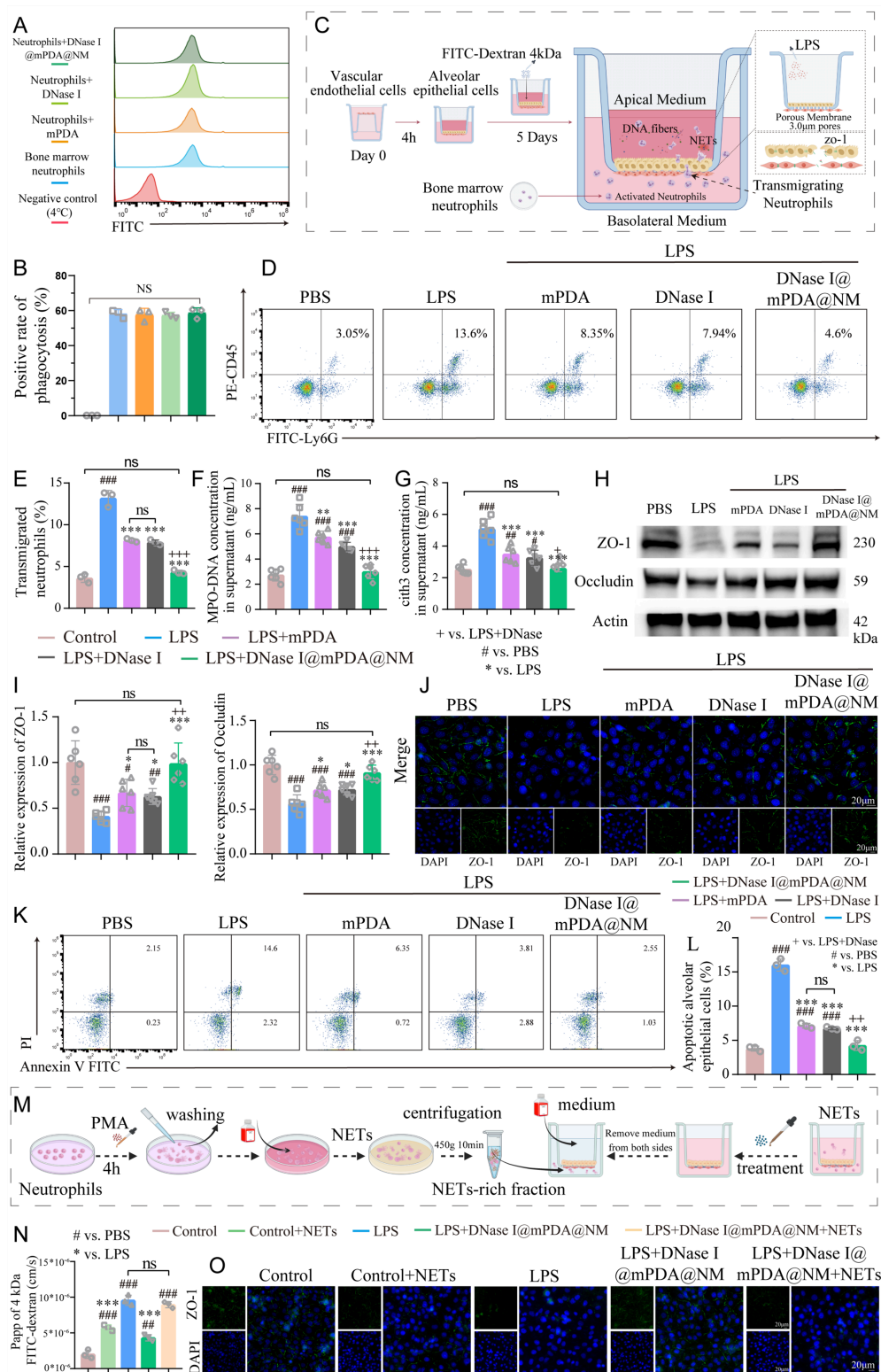


Figure 4

mPDA and DNase I act at complementary stages of NET accumulation. (A) Schematic of mPDA-mediated restraint of intracellular ROS-associated NETosis activation and DNase I-mediated degradation of extracellular NETs. (B) Experimental workflow separating intracellular and extracellular sample fractions. (C, D) Representative DCFH-DA fluorescence images and quantification of intracellular ROS 1 h after PMA stimulation. (E) Representative flow-cytometric DCFH-DA fluorescence distributions. (F, G)

CitH3/H3 immunoblotting and quantification in neutrophil lysates 2 h after PMA stimulation. (H, I) Representative CitH3/MPO/DAPI immunofluorescence images and quantification of CitH3-positive neutrophils. The validation of the H₂O₂-alone condition is shown in Supplementary Figure S9. (J) CitH3 concentrations in neutrophil lysates. (K) MPO–DNA concentrations in culture supernatants 4 h after PMA stimulation. (L) Representative SYTOX images of extracellular DNA. (M) Parallel sampling design for time-resolved analysis of intracellular CitH3 and extracellular MPO–DNA. (N) CitH3 concentrations in neutrophil lysates at 2, 3, 4 and 6 h. (O) MPO–DNA concentrations in culture supernatants at 2, 3, 4 and 6 h. Representative images, flow-cytometry plots, and immunoblots are shown. Quantitative data were obtained from n = 3 independent biological replicates in (D) and n = 6 independent biological replicates for all other quantitative analyses. Data in D, G, I–K were analyzed by one-way ANOVA followed by Tukey's multiple-comparisons test. #P < 0.05, ##P < 0.01, and ###P < 0.001 versus PBS; *P < 0.05, **P < 0.01, and ***P < 0.001 versus PMA; +P < 0.05, ++P < 0.01, and +++P < 0.001 versus PMA + DNase I; ns, not significant. Time-course data in N and O were analyzed by two-way ANOVA with treatment and time as factors, followed by Dunnett's multiple-comparisons test to compare each treatment group with the PMA group at each time point. For N and O, *P < 0.05, **P < 0.01, and ***P < 0.001 versus PMA at the corresponding time point; ns, not significant.



DNase I@mPDA@NM reduces NET-associated burden and protects the multicellular alveolar–capillary barrier. (A, B) Representative flow-cytometric histograms and quantification of fluorescent microsphere uptake by bone marrow-derived neutrophils after the indicated treatments; 4 °C served as a low-temperature control. (C) Schematic of the endothelial–epithelial Transwell model used to assess neutrophil transmigration, NET-associated burden, and barrier integrity. (D, E) Representative flow-

cytometric plots and quantification of transmigrated CD45⁺Ly6G⁺ neutrophils. (F, G) MPO–DNA and CitH3 concentrations in the apical supernatant. (H, I) Representative Western blots and quantification of ZO-1 and occludin in pulmonary microvascular endothelial cells; β -actin served as the loading control. (J) Representative immunofluorescence images of endothelial ZO-1 (green) and DAPI (blue). (K, L) Representative Annexin V-FITC/PI plots and quantification of alveolar epithelial cell apoptosis. (M) Schematic of the NET add-back experiment using standardized preformed NETs generated by PMA stimulation and added to the basolateral compartment after treatment. (N) Apparent permeability coefficient (P_{app}) of 4-kDa FITC–dextran following NET add-back. (O) Representative ZO-1 immunofluorescence following NET add-back. Scale bars, 20 μ m. Data are presented as mean $\pm$ SD. n = 3 independent biological replicates for B, E, L, and N; n = 6 independent biological replicates for F, G, and I. Representative flow-cytometry plots are shown in A, D, and K, and representative immunoblot images are shown in H. Data were analyzed by one-way ANOVA with Tukey's multiple-comparisons test. #P < 0.05, ##P < 0.01, and ###P < 0.001 versus PBS; *P < 0.05, **P < 0.01, and ***P < 0.001 versus LPS; +P < 0.05, ++P < 0.01, and +++P < 0.001 versus LPS + DNase I; ns, not significant.

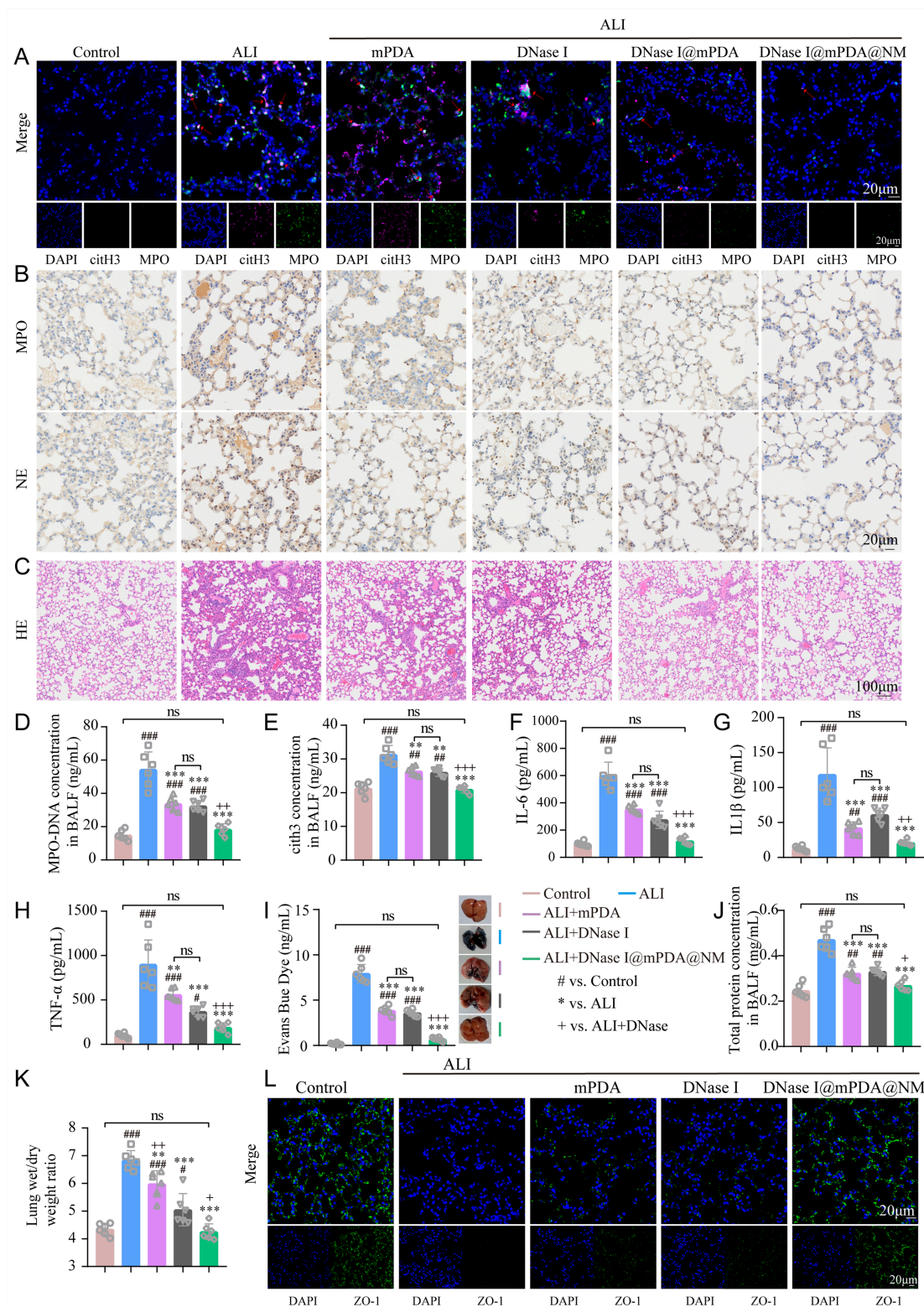


Figure 6

DNase I@mPDA@NM alleviates NET-associated lung injury in LPS-induced ALI. Mice received the indicated pulmonary treatments 2 h after LPS challenge, followed by assessment of pulmonary NET-associated burden, inflammatory injury, and alveolar–capillary barrier integrity. (A) Representative immunofluorescence images of pulmonary CitH3 (magenta) and MPO (green), with nuclei counterstained with DAPI (blue), in the Control, ALI, ALI + mPDA, ALI + DNase I, ALI + DNase I@mPDA, ALI + DNase I@mPDA@NM.

and ALI + DNase I@mPDA@NM groups. Scale bar, 20 μ m. Quantitative analysis is shown in Supplementary Figure S13A. (B) Representative immunohistochemical staining of MPO and NE in lung tissues from the indicated groups. Scale bar, 20 μ m. (C) Representative H&E staining of lung sections from the indicated groups. Scale bar, 100 μ m. Quantitative histopathological analysis is shown in Supplementary Figure S13B. (D, E) MPO–DNA and CitH3 concentrations in BALF. (F–H) IL-6, IL-1 β , and TNF- α levels. (I) Evans blue extravasation, with representative lung images shown alongside the quantitative analysis. (J) Total protein concentration in BALF. (K) Lung wet-to-dry weight ratio. (L) Representative immunofluorescence images of pulmonary ZO-1 (green), with nuclei counterstained with DAPI (blue). Scale bar, 20 μ m. For panels D–L, analyses were performed in the Control, ALI, ALI + mPDA, ALI + DNase I, and ALI + DNase I@mPDA@NM groups. n = 6 mice per group. Data are presented as mean $\pm$ SD. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparisons test. #P < 0.05, ##P < 0.01, and ###P < 0.001 versus Control; *P < 0.05, **P < 0.01, and ***P < 0.001 versus ALI; +P < 0.05, ++P < 0.01, and +++P < 0.001 versus ALI + DNase I; ns, not significant.

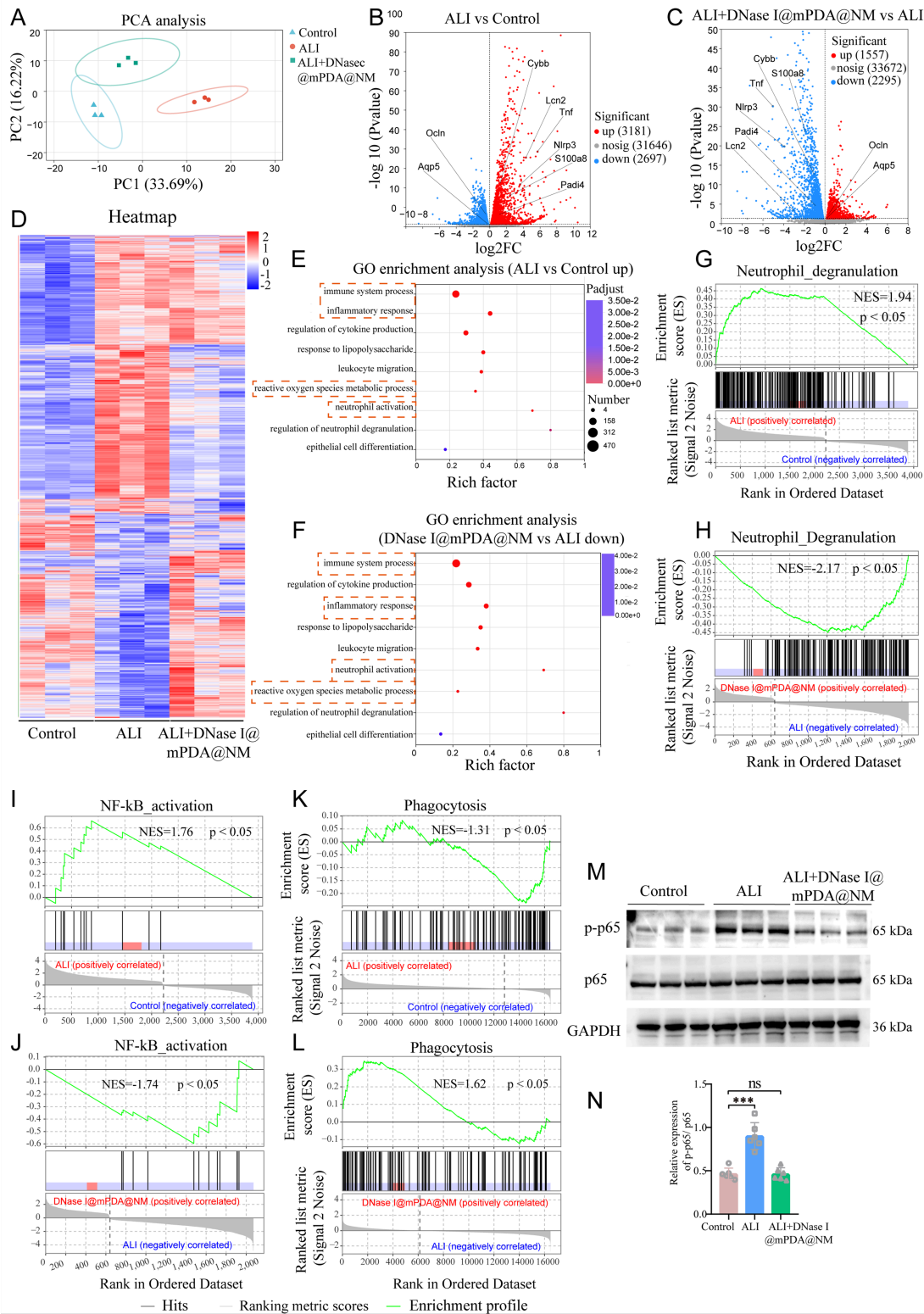


Figure 7

Whole-lung transcriptomics supports in vivo rebalancing of pathological NET homeostasis following DNase I@mPDA@NM treatment. (A) Principal-component analysis of Control, ALI, and ALI + DNase I@mPDA@NM whole-lung transcriptomes. (B) Volcano plot of differentially expressed genes in ALI versus Control lungs. (C) Volcano plot of differentially expressed genes in DNase I@mPDA@NM-treated versus untreated ALI lungs. (D) Hierarchical clustering of differentially expressed genes across the three

groups. (E, F) GO enrichment analysis of genes upregulated in ALI relative to Control and genes downregulated following DNase I@mPDA@NM treatment relative to untreated ALI, respectively. (G, H) GSEA of the neutrophil degranulation gene set in ALI versus Control lungs and DNase I@mPDA@NM-treated versus untreated ALI lungs, respectively. (I, J) GSEA of the NF- κ B activation gene set in the corresponding comparisons. (K, L) GSEA of the phagocytosis gene set in ALI versus Control lungs and DNase I@mPDA@NM-treated versus untreated ALI lungs, respectively. (M) Representative immunoblots of p-p65, p65, and GAPDH in lung tissue. (N) Quantification of the p-p65/p65 ratio. GSEA plots show normalized enrichment scores (NES) and nominal P values as indicated. For transcriptomic analyses, n = 3 mice per group. Data in (N) are presented as mean $\pm$ SD and were analyzed by one-way ANOVA followed by Tukey's multiple-comparisons test. *P < 0.05, **P < 0.01, and ***P < 0.001; ns, not significant.

Supplementary Files

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- [Scheme1.png](#)
- [Additionalfile1.docx](#)