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Article

Keywords: Ninjurin-1, acute lung injury, neutrophils, neutrophil extracellular traps

Posted Date: January 13th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8426344/v1>

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Additional Declarations: (Not answered)

NINJ1 plays a vital role in the release of neutrophil extracellular traps in acute lung injury

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Running title: NINJ1 promotes NETs release in ALI.

Keywords: Ninjurin-1, acute lung injury, neutrophils, neutrophil extracellular traps

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37 The total number of tables: 4

38 Total number of figures: 7

39 Supplement figures: 4

40

Abstract

Excessive neutrophil extracellular traps (NETs) formation is a significant contributor to acute lung injury (ALI), making its inhibition a novel therapeutic avenue to improve outcomes. In this study, we revealed that a novel pore-forming protein ninjurin-1 (NINJ1) was predominantly highly expressed in pro-inflammatory neutrophil subpopulations during ALI, using public single-cell RNA sequencing and hotspot analysis. Furthermore, we demonstrated that the NINJ1 oligomerization was essential for the NETs release of neutrophils from both acute respiratory distress syndrome (ARDS) patients and ALI mice. Genetic ablation of *Ninjl* in neutrophils abolished NETs release, thereby attenuating pulmonary dysfunction and reducing ALI-related lethality. Mechanistically, we found that N-glycosylation of NINJ1 at the 60 site was the major contributor to its oligomerization and NETs release. In summary, our findings reveal a novel pore-forming protein-mediated mechanism for NETs release and highlight NINJ1 as a potential therapeutic target for the treatment of ALI/ARDS.

Introduction

Acute lung injury (ALI) is characterized by acute hypoxemia and bilateral pulmonary oedema, resulting from widespread inflammation and increased alveolocapillary permeability, and is associated with high mortality [1]. This condition is triggered by predisposing risk factors such as pneumonia, trauma, inhalation injury, thermal injuries, extrapulmonary sepsis, pancreatitis, massive transfusion, or shock [2]. Although the 2012 Berlin definition reclassified ALI as mild acute respiratory distress syndrome (ARDS), the term ALI continues to be widely used in preclinical research [3]. Advances in critical care management, particularly the adoption of lung-protective ventilation strategies, have markedly improved outcomes in ARDS patients over the past decade [4]. Nevertheless, mortality rates remain unacceptably high, highlighting an urgent need for effective pharmacotherapeutic interventions.

Neutrophils play a pivotal role in the pathogenesis of ARDS [5]. Neutrophil extracellular traps (NETs) are DNA fragments with cytoplasm proteins released from neutrophils, which are involved in the pathogenesis of ALI/ARDS and represent a promising therapeutic target [6]. NETs are web-like structures composed of DNA filaments decorated with cytoplasmic and granular proteins, which are released by neutrophils in response to inflammatory activation. These structures, containing components such as citrullinated histone H3 (citH3), myeloperoxidase (MPO), neutrophil elastase (NE), and cell-free double-stranded DNA (dsDNA), contribute significantly to the pro-inflammatory response and are implicated in the pathogenesis of ALI/ARDS [7]. The process by which neutrophils form NETs is called NETosis. Elevated levels of NETs in peripheral blood, as measured by plasma MPOdsDNA complexes, are positively associated with disease severity and inversely correlated with the PaO₂/FiO₂ ratio in patients with ARDS [8]. Our previous study has suggested that NETs trigger alveolar epithelial cell necroptosis through the cyclic GMP-AMP synthase stimulator of the interferon gene (cGAS/STING) pathway during ALI [9]. Besides, NETs trigger ROS-induced NLRP3 activation, mediating macrophage pyroptosis and triggering endothelial cell ferroptosis in ALI [10]. These

studies indicate that the DNA scaffold of NETs serves as a damage-associated molecular pattern (DAMP), triggering diffuse alveolar damage (DAD), inflammatory cascades, and vascular endothelial injury, thereby disrupting the air-blood barrier. Therefore, targeting NETs release is critical for treating ALI/ARDS.

NETs release occurs through a stepwise and highly orchestrated process, involving sequential stages of cytoskeletal reorganization, plasma membrane vesiculation, chromatin decondensation, nuclear envelope disintegration, and culminating in plasma membrane rupture (PMR) [11]. The NETs scaffold is composed of chromatin filaments (approximately 10-15 nm in diameter) studded with granular and cytoplasmic proteins (forming clusters of 20-50 nm), which assemble into a porous meshwork ranging from 50 to 200 nm. PMR serves as a prerequisite for releasing DNA-based meshwork structures [12]. Previous studies have proposed that lytic cell death-induced PMR is a passive process mediated by pore-forming proteins through osmotic stress [13, [14]. The most well-characterized plasma membrane pore-forming proteins are Gasdermin D (GSDMD) and mixed-lineage kinase domain-like protein (MLKL), which mediate PMR during pyroptosis and necroptosis, respectively [15, [16]. However, a previous study has demonstrated that NETs can release independently of MLKL pores [17]. Furthermore, GSDMD forms small pores (~21 nm diameter) sufficient for releasing pro-inflammatory mediators but restrictive for large DAMPs (including NETs) [18]. For a long time, GSDMD/MLKL pores were considered to drive passive cell rupture *via* nonspecific ion flux and osmotic lysis. However, a recent study has overturned this paradigm [19]. Kayagaki *et al.* identified ninjurin-1 (NINJ1) as an active executioner of PMR, demonstrating its essential role in pyroptotic release of large DAMPs, such as lactate dehydrogenase (LDH, 140 kDa) [20]. This 16-kDa transmembrane protein was initially identified as an adhesion molecule expressed following nerve injury [21]. Its subsequent recognition as a key mediator of PMR in pyroptosis represents a paradigm-shifting discovery. Kayagaki and colleagues showed that NINJ1 oligomerizes into large filaments in the induction of PMR [22]. David *et al.* suggest that NINJ1 oligomerizes into irregular rings to execute PMR by jettisoning membranes like a "cookie-cutter" [23]. However, the precise

99 details of NINJ1's role in NETs release and the molecular mechanism underlying NINJ1 oligomerization are
100 still not precise.

101 Here, we investigated how the pore-forming protein NINJ1-derived NETs release and exacerbate
102 pulmonary injury in ALI. Single-cell RNA sequencing and Hotspot analysis identified neutrophil-specific
103 NINJ1 upregulation and association with NETosis. Genetic depletion of *Ninj1* in neutrophils attenuated NETs
104 release, reduced lung damage, and mitigated histopathology in ALI mice. Mechanistically, Neutrophils from
105 ARDS patients exhibited NINJ1 oligomerization on membranes alongside NETs structures. And N-
106 glycosylation was essential for NINJ1 oligomerization and NETs release. Collectively, our findings unveil a
107 novel role for NINJ1 oligomerization in NET-mediated defense against ALI.

Materials and methods

Ethics statement

All animal experiments described here complied with the guidelines of the Committee on the Use and Care of Animals of Central South University (Changsha, China) and were approved by the IRB of the Xiangya School of Basic Medical Science at Central South University (2023-KT107). Human neutrophils were obtained from ARDS patients undergoing thoracic surgery at Xiangya Hospital of Central South University (Changsha, China). Patients included those diagnosed with ARDS. All patients signed informed consent for their samples to be used for research, and the Clinical Medical Ethics Committee of Xiangya Hospital, Central South University, approved the study (2024030484, Changsha, China).

Mice and induction of ALI

Adult male C57BL/6J mice (8-10 weeks of age, 20-25 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Hunan, China). The mice were maintained in a specific pathogen-free environment at the Central South University Animal Facility. To establish the ALI model, C57BL/6J mice received an intratracheal instillation of lipopolysaccharide (LPS, 5 mg/kg in 50 μ L saline; derived from *E. coli* O111:B4, Sigma-Aldrich) and were euthanized 12 h later. Mice in the Control group were administered an equal volume of saline *via* the same route.

The conditional *Nin1* knockout mouse model (*Nin1*^{fl/fl} on a C57BL/6J background) was generated by Shanghai Model Organisms Center (Cat. No. NM-CKO-200039, China) using CRISPR/Cas9-mediated genome editing. Briefly, a circular donor vector harboring *loxP* sites within intron 1 was constructed. Cas9 mRNA, sgRNAs, and the donor vector were co-microinjected into the cytoplasm of fertilized one-cell C57BL/6J embryos, yielding chimeric founders that were subsequently bred to wild-type C57BL/6J mice to obtain germline-transmitted *Nin1*^{fl/+} offspring. The *Nin1*^{fl/fl} mouse was then crossed with a mouse carrying the Cre recombinase selectively in neutrophils under the control of the endogenous lymphocyte antigen 6

complex (*Ly6G*) promoter/enhancer (official name: *Ly6G*-Cre-2A-tdTomato mice, Cat. No. NM-KI-200219, Shanghai Model Organisms Center, China), to generate the neutrophil conditional *Nin1* knockout (*Nin1^{fl/fl}/Ly6G^{Cre}*, referred as *Nin1^{ΔNeu}* in the manuscript) mice. For all experiments, *Ly6G*-Cre-*Nin1^{fl/fl}* (referred to as *Nin1^{WT}* in the manuscript) littermates were used as controls for *Nin1^{ΔNeu}* mice. Mice were maintained under specific pathogen-free conditions with a 12-hour light/dark cycle and provided food and water ad libitum.

Collection of bronchoalveolar lavage fluid (BALF)

BALF was obtained by performing intratracheal instillation with 0.8 mL of ice-cold saline, a process repeated three times in accordance with established methodology [24]. Following collection, the BALF was centrifuged at 500×g for 5 min at 4 °C. The resulting cell pellet was resuspended in 0.5 mL of PBS. The total protein concentration in the BALF supernatant, which serves as a marker of alveolocapillary barrier integrity, was quantified using a commercially available bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific).

Real-time quantitative polymerase chain reaction (Real-time PCR)

Total RNA was isolated from both tissue samples and cultured cells using RNAiso Plus reagent (Takara, Japan) according to our prior works [25, [26]. RNA purity and concentration were verified by spectrophotometry (A260/A280 ratio >1.8). First-strand cDNA synthesis was performed using the PrimeScript RT reagent kit (Takara) with oligo (dT) primers. Quantitative PCR analysis was conducted on a CFX96 Touch™ system (Bio-Rad Laboratories) using SYBR Green Master Mix (Applied Biosystems) at the Top-Notch Innovation Base of Basic Medicine (Central South University). Gene expression levels were normalized to β-actin and calculated using the comparative $2^{-\Delta\Delta Ct}$ method. All primer sequences were designed using NCBI Primer-BLAST and are detailed in Table 1.

Western blot

Protein extracted from lung tissue, neutrophils, or supernatants was subjected to Western blot analysis

following established protocols from our prior work [24, [27]. In brief, lung tissue or neutrophil samples were lysed using RIPA buffer (Solarbio, Beijing, China) supplemented with protease and phosphatase inhibitors. Total protein content was quantified *via* the BCA method. To concentrate proteins from supernatants, 700 μ L supernatant was combined with 700 μ L 100% methanol and 175 μ L chloroform, followed by vigorous vortexing for 30 s. The mixture was centrifuged at 13,000 $\times$ g for 5 min at 4 °C. After discarding the upper aqueous phase, 700 μ L of 100% methanol was added to the interphase and organic layer, and the sample was recentrifuged under the same conditions. The resulting pellet was air-dried and resuspended in 20 μ L of 10% SDS. Subsequently, 4 μ L of 5 $\times$ SDS-PAGE loading buffer (Beyotime, Jiangsu, China) was added, and the sample was denatured at 95 °C for 10 min. Subsequently, equal quantities of protein (30 μ g per lane) or all proteins from supernatants were separated by electrophoresis on 12% SDS-polyacrylamide gels. Then they transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5% non-fat milk or bovine serum albumin (BSA) for 1.5 h, probed with primary antibodies at 4 °C overnight, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for one hour at room temperature. Band intensities were quantified using Image Lab Analyzer software (Bio-Rad, Hercules, CA, USA). Antibody details are provided in Table 2.

Native and SDS-PAGE of NINJ1

Neutrophils were lysed with native-PAGE lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris, pH 7.5, and 1 $\times$ Complete PMSE, Solarbio, R0030). Following centrifugation at 20,800 $\times$ g for 30 min, lysates were mixed with 5 $\times$ Native Sample Loading Buffer (Sangon Biotech, Shanghai, China, C506032) and resolved using Native PAGE[™] 3-12% Bis-Tris gels (BBI Life Sciences Corporation, C631101-0100). For SDS-PAGE analysis, cells were rinsed with phosphate-buffered saline (PBS) and lysed in RIPA buffer (Beyotime) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Equivalent quantities of native protein were separated under non-reducing conditions using pre-cast 3-12% Bis-Tris gels with Tris-Glycine

native running buffer (pH 8.8, Sangon Biotech) at 4 °C. Equivalent quantities of denatured protein were separated under reducing conditions on a 12% gradient polyacrylamide gel at room temperature. Following electrophoresis, proteins were transferred to PVDF membranes and probed using standard immunoblotting procedures.

Immunofluorescence staining (IF)

IF staining was carried out on 3- μ m paraffin-embedded lung sections or fixed cultured cells. Sections or cells were rinsed with PBS and fixed using 4% paraformaldehyde for 15 min at room temperature. Permeabilization was carried out with 0.1% Tween-20. Nonspecific binding sites were subsequently blocked by incubating the cells for one hour at room temperature in a solution of PBS containing 10% BSA. Finally, samples were incubated overnight at 4 °C with the following primary antibodies: anti-NINJ1 (mouse, Mybiosource, 1:100), anti-MPO (rabbit, 1:200, Proteintech), anti-citH3 (rabbit, 1:200, Abcam), and anti-GM130 (rabbit, 1:100, Proteintech). After washing, samples were treated with species-appropriate secondary antibodies conjugated to fluorophores for one hour at room temperature, protected from light. Nuclei were counterstained with DAPI (Solarbio). Images were captured using a laser scanning confocal microscope (Leica SP8, Germany).

Lung histology and inflammatory injury score

At 12 h post-LPS administration, mice were euthanized, and the left lung lobe was fixed in 4% neutral buffered formaldehyde at 4 °C. Tissue sections (4 μ m) were prepared and stained with hematoxylin and eosin (H&E; Solarbio). Whole-slide imaging was performed using a Panoramic Scan system (3Dhistech, Budapest, Hungary). A rigorous histopathological analysis was conducted in accordance with our previous studies by three independent pathologists (blinded to treatment groups) who employed a standardized scoring matrix (0=normal; 4=severe) to assess: (a) neutrophil-dominant mixed alveolar inflammation, (b) pseudostratified bronchoalveolar hyperplasia, (c) multifocal hemorrhagic foci, (d) eosinophilic lipoproteinosis, and (e)

fibrinous hyaline membranes. Interobserver variability was minimized through consensus training [28, [29].

Survival rate in mice

Mice were administered to mice 1 h after induction of ALI by LPS (25 mg/kg, *ip*). The mortality of the mouse was monitored every 6 h over 84 h, and the percent survival rate was expressed as a Kaplan-Meier survival curve ($n = 16$).

Measurement of LDH

LDH activity was assessed with a commercial assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Quantification was based on absorbance measurements at 450 nm.

Enzyme-linked immunosorbent assay (ELISA)

Levels of tumor necrosis factor-alpha (TNF- α) in both lung tissue homogenates and serum were quantified using commercially available ELISA kits (Invitrogen; Thermo Fisher Scientific) following the supplier's guidelines. Similarly, MPO-dsDNA and citH3-dsDNA complexes were assessed with ELISA kits (JONLNBIO, China) as per the manufacturer's instructions. Absorbance readings taken at 450 nm were converted to concentration values by comparison with a standard curve. Cytokine measurements were normalized to total protein concentration, which was determined using a BCA protein assay kit.

Single-cell transcriptome sequencing data analysis

Single-cell RNA sequencing data were processed with Seurat (v5) [30]. For the GSE224938, GSE36809070 dataset, cells were filtered based on the following criteria: exclusion of those with gene counts greater than 7000 or fewer than 700; removal of cells where mitochondrial UMI counts exceeded 10% or erythrocyte-derived UMI counts comprised more than 20%. Normalization was carried out using the "NormalizeData" function, followed by data scaling *via* "ScaleData". The 2000 most highly variable genes were identified using "FindVariableFeatures". Dimensionality reduction was performed using principal

component analysis (PCA) based on these variable genes, with a resolution parameter of 0.4. Batch effects were mitigated through dataset integration across conditions employing the "RunHarmony" function. Potential marker genes were detected using "FindAllMarkers". Cell type annotation was performed by referencing canonical marker genes. Cellular clusters were visualized using Uniform Manifold Approximation and Projection (UMAP).

Pseudotime analysis

To delineate cellular differentiation trajectories, pseudotime analysis was performed using Monocle2 (v2.26.0) [31]. Genes with a mean expression level above 0.1 were initially selected for inclusion. Subsequently, the "differentialGeneTest" function was applied to filter out genes unsuitable for cell ordering, based on a q-value cutoff of < 0.01 . Dimensionality reduction was carried out *via* the "reduceDimension" method with the DDRTree algorithm. All analyses were conducted using default parameters. Final cell ordering was determined using the "order Cells" function.

Identification of Hotspot gene modules in ALI data

We employed Hotspot (v.0.9.1)²⁰ to detect context-specific gene modules within a single-cell RNA-seq dataset (GSE224938). This algorithm utilizes a user-defined cell-cell similarity metric to construct a k -nearest neighbor (k -NN) graph, upon which it assesses pairwise local correlations between genes within defined cellular neighborhoods. Genes exhibiting significant local autocorrelation are identified, an approach particularly suited to scRNA-seq data, as it remains robust to technical artifacts such as gene dropouts. The resulting gene-gene affinity matrix is subsequently clustered to define discrete gene modules. Unlike global correlation measures (e.g., Pearson's correlation), which assume uniform feature relationships across the entire dataset, Hotspot evaluates dependencies within local contexts of the k -NN graph, thereby capturing spatially or biologically restricted co-expression patterns.

Employing non-negative matrix factorization (NMF) to uncover gene programs has been reported in prior

246 studies [32]. However, as a linear decomposition technique, NMF necessitates a coherent and comprehensive
247 representation of the full dataset, and can exhibit sensitivity to technical artifacts such as batch variation. In
248 contrast, Hotspot identifies gene modules through the analysis of gene-gene covariance, which more
249 effectively captures cooperative gene sets underlying shared biological processes and demonstrates enhanced
250 robustness to batch effects [33]-making it particularly suitable for analyzing heterogeneous neutrophil
251 populations. A distinctive feature of Hotspot is its ability to detect covariances that are spatially restricted to
252 specific cell subpopulations or that display nonlinear, graded patterns across regions of the data manifold. This
253 is especially relevant in ALI, where adaptation to diverse microenvironments-such as disseminating infection-
254 may drive context-specific changes in gene co-expression. Furthermore, Hotspot accommodates both gene
255 pleiotropy and rare cell states, which are salient features of ALI inflammation pathogenesis. Accordingly, we
256 applied Hotspot to dissect the sources of inter-and intra-neutrophil phenotypic heterogeneity in our data, in
257 addition to identifying global, manifold-defining gene modules.

258 To implement Hotspot, we first isolated neutrophils from the dataset ($n = 856$). Genes expressed in fewer
259 than 1% of neutrophils or with zero total expression were filtered out, resulting in a final set of 6,578 genes
260 for downstream analysis. We next identified a subset of genes exhibiting significant spatial autocorrelation
261 within this latent PC space by executing Hotspot with the depth-adjusted negative binomial (danb) observation
262 model and a neighborhood size of 30. The danb model provides a null background distribution for normalizing
263 expression counts, thereby reducing false positives arising from local correlations in cellular library size [34].

264 We retained 1284 genes exhibiting a false discovery rate (FDR) < 0.001 for subsequent local correlation
265 analysis and module clustering (described below). Using the create.modules function in Hotspot-with default
266 parameters aside from a minimum.gene.threshold of 30 and core.only=True-we identified an initial set of 10
267 co-expression modules. Gene assignments for these modules are provided in Supplementary Table 1. Hotspot
268 is implemented as an open-source Python package and is available for use at

<http://www.github.com/Yoseflab/Hotspot>.

Recruitment of healthy controls (HCs) and clinical ARDS patients

Patients diagnosed with ARDS according to the Berlin definition were included in this study. From March 2024 to March 2025, blood samples were collected from 12 ARDS patients and 12 HCs in the Intensive Care Unit of Xiangya Hospital of Central South University. Written informed consent was obtained from all participants or their next of kin. Exclusion criteria included pregnancy or lactation, age under 18 years, active hematologic malignancy or solid tumor cancer, end-stage organ disease, and autoimmune disorders. Age- and sex- matched volunteers undergoing elective surgery, without sepsis, were enrolled as the control group. Demographic and clinical parameters, including age, sex, body mass index (BMI), PaO₂/FiO₂ ratio, Sequential Organ Failure Assessment (SOFA) score, Acute Physiology and Chronic Health Evaluation II (APACHE II) score, white blood cell count (WBC), neutrophil count (NEUT), C-reactive protein (CRP), and procalcitonin (PCT), were collected from the electronic medical records (Table 3).

Neutrophil isolation and culture

Neutrophils were isolated from the bone marrow of 8-10-week-old male C57BL/6J mice using a combination of immunomagnetic separation techniques. Briefly, femurs and tibias were dissected, and bone marrow cells were flushed out with cold PBS using a 26-gauge needle. After lysing red blood cells with red blood cell lysis buffer (Solarbio), the resulting hematopoietic cells were incubated with an Anti-Ly-6G MicroBeads UltraPure (Miltenyi Biotec) and sorted using an LS column and MACS MultiStand (Miltenyi Biotec) cell sorter. For the magnetic isolation procedure, bone marrow cells were labeled with Anti-Ly-6G MicroBeads UltraPure (Miltenyi Biotec). Ly6G⁺ cells were subsequently isolated using an LS column and MACS MultiStand (Miltenyi Biotec) cell sorter, in accordance with the manufacturer's protocols. The purified neutrophil populations were resuspended in complete RPMI-1640 medium (Gibco) containing 10% bovine serum (BCS) and were maintained under standard culture conditions before subsequent experiments.

Peripheral blood samples were collected from HCs following written informed consent, with ethical approval granted by the IRB of the Xiangya School of Basic Medical Science at Central South University (approval reference number 2024030484) in compliance with Helsinki Declaration guidelines. Whole blood was mixed with 0.9% sodium chloride containing 2.0% Dextran T-500 (Solarbio) at a 1.5:1 ratio and incubated for 30 min at room temperature to sediment erythrocytes. The leukocyte-rich supernatant was then carefully layered onto a discontinuous Percoll (Cytiva) gradient prepared by sequentially overlaying 2 mL of 45%, 62%, and 75% Percoll solutions in a 15 mL conical tube using a sterile serological pipette. After centrifugation at 250×g for 30 min with the brake disabled, peripheral blood neutrophils were collected from the interface between the 62% and 75% Percoll layers. The cells were washed with PBS, centrifuged at 250×g for 8 min, and resuspended in RPMI 1640 medium supplemented with 10% BCS. Cell count and purity were determined using flow cytometry before subsequent experiments.

Cell culturing and differentiation

The human promyelocytic leukemia cell line dHL-60 was acquired from the American Type Culture Collection (RRID: CVCL_HL60). All cell lines were verified to be free of contamination. Cells were maintained at 37 °C under a 5% CO₂ atmosphere in RPMI-1640 medium containing L-glutamine and 25 mM HEPES, and supplemented with 10% heat-inactivated BCS and 1% penicillin-streptomycin. To induce neutrophilic differentiation, dHL-60 cells were treated with 1 μM all-trans retinoic acid (ATRA) plus 1% dimethyl sulfoxide (DMSO) for 6 days. Differentiation efficiency was evaluated morphologically using Wright-Giemsa staining. To induce NETosis, differentiated dHL-60 cultures were treated with a working solution of phorbol 12-myristate 13-acetate (PMA) at a final concentration of 100 nM.

To estimate the role of pore-forming proteins in NETs release, we treated cells with GSDMD inhibitor (Disulfiram, 5 μM, TargetMol, China), or RIPK3 inhibitor (GSK872, 1 μM, Medchem Express, USA), or ER stress inducer tunicamycin (TM, 0.25 μg/mL, Cat. No.: HY-100523, Med Chem Express) 30 min before PMA

(100 nM) stimulation.

Plasmids and Transfection

All expression plasmids were commercially sourced from Sangon Biotech. Site-directed mutagenesis was performed to generate the N60A variant of NINJ1. Both the wild-type and mutant coding sequences were amplified by PCR using primers engineered to incorporate 5' Nhe I and 3' Hind III restriction sites. The purified amplicons and the pcDNA3.1-3×FLAG vector were subjected to double digestion with the corresponding restriction endonucleases. The resulting fragments were purified and ligated into the linearized vector backbone using T4 DNA ligase to produce C-terminally 3×FLAG-tagged recombinant constructs.

For transient transfection, LipofectamineTM 3000 (Invitrogen) was used according to the manufacturer's protocol. Briefly, differentiated dHL-60 cells, which had been cultured in differentiation medium for 72 h, were used for transfection. Before transfection, the plasmid DNA and Lipofectamine 3000 reagent were separately diluted in serum-free medium, combined at an appropriate ratio, and incubated for 20 min at room temperature to allow complex formation. The resulting transfection mixture was then added dropwise to the cells. Following gentle agitation to ensure uniform distribution, the medium was replaced with complete growth medium containing serum after 4-6 h. Cells were cultured for an additional 72 h before downstream analysis or functional assays.

RNA sequencing and analysis

Total RNA isolation was performed with RNAiso Plus reagent (Takara) and sequenced on an Illumina NovaSeq 6000 platform (Majorbio, China). Raw sequencing data were obtained in FASTQ format, and an initial quality assessment was conducted with FASTQC. Low-quality reads were filtered out using FASTX-Toolkit. The remaining high-quality reads were aligned to the human reference genome (GRCh38) using TopHat, with gene annotation provided *via* the "-G" option using a human mRNA GTF file. Read counts per gene were generated with HTSeq. Differential gene expression analysis was performed using the DESeq2

338 package in R, comparing the Control and PMA groups. Genes with an adjusted *P*-value of less than 0.05 were
339 considered statistically significantly differentially expressed. Bioinformatics analyses were carried out on the
340 Majorbio Cloud Platform (Majorbio I-Sanger Cloud Platform, www.majorbio.com).

341 **Assay of PMR**

342 Cells were collected and washed twice with HBSS for 5 min each. The cells were resuspended at a density
343 of 1×10^6 /mL and treated with 1 mL of FM 4-64 working solution (MCE, 5 μ M) for 30 min at room temperature.
344 Following incubation, the samples were centrifuged at 400 $\times$ g for 4 min, and the supernatant was aspirated.
345 The pellet was washed twice with HBSS and subsequently stained with DAPI. Finally, cells were resuspended
346 in 1 mL of serum-free medium and examined by a laser scanning confocal microscope.

347 **Quantification of dsDNA**

348 To quantify circulating NETs, levels of dsDNA in plasma, BALF, and cell supernatant were measured
349 using the PicoGreen® dsDNA Quantification Kit (Solarbio). Whole blood collected from mice was transferred
350 into 1.5 mL centrifuge tubes containing EDTA-Na as an anticoagulant. Plasma BALF and cell supernatant
351 were separated by centrifugation at 3000 $\times$ rpm for 15 min at 4 °C. The concentration of dsDNA in the plasma
352 was then quantified using the PicoGreen® dsDNA Quantification Kit, following the manufacturer's
353 instructions.

354 **Scanning electron microscopy**

355 For SEM analysis, the samples were primarily fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer
356 (pH 7.4) for a minimum of 4 hours at 4°C. Following three washes with cold phosphate buffer, the samples
357 were post-fixed in 1% osmium tetroxide for 1-2 hours at room temperature. Subsequently, the samples were
358 dehydrated through a graded series of ethanol solutions, with each step lasting for 15 minutes. Critical-point
359 drying was then performed using liquid CO₂ to preserve the ultrastructural integrity. The dried samples were
360 mounted on aluminum stubs and sputter-coated with a thin layer of gold-palladium to enhance electrical

conductivity. Finally, the specimens were examined under a Zeiss Sigma 300 scanning electron microscope, and representative images were captured.

Statistical analysis

A minimum of three independent biological replicates was performed for each experiment. Data are expressed as mean $\pm$ standard deviation. Statistical analyses and graphical representations were conducted using GraphPad Prism 9 (San Diego, CA, USA). Comparisons between two groups were performed using an unpaired Student's *t*-test, while differences among multiple groups were assessed by one-way ANOVA followed by Tukey's or Dunnett's post hoc test. A *P*-value below 0.05 was considered statistically significant.

Results

NINJ1 is predominantly expressed in neutrophils during ALI

Firstly, we analyzed the expression of NINJ1 in ARDS-related GEO databases GSE32707. Patients diagnosed with ARDS exhibited a significant upregulation in NINJ1 expression, which was evident both at initial presentation (Day 0) and on Day 7 of hospitalization (Figure S1A). Consistently, elevated NINJ1 expression was observed in the LPS-induced ALI mice with lethal endotoxic shock (GSE203340) (Figure S1B). To further investigate NINJ1 expression in the context of ALI pathogenesis, we thus induced murine ALI with LPS and evaluated its pulmonary expression. Both transcriptional and protein levels were found to be significantly upregulated (Figure 1A-D). For single-cell profiling of NINJ1 heterogeneity and spatial distribution among immune cells during ALI, we analyzed a dataset (GSE224938) from LPS-induced ALI mice. Subsequently, identification of 7 major cell subsets was achieved through the t-SNE dimension reduction method, using typical marker genes (Figure 1E), including neutrophils (S100A8, S100A9), macrophages (CD68, CD163), dendritic cell (THBD, CD1C), B cell (CD19, CD79A, MS4A1), plasma cell (CD27, CD38), T cell (PTPRC, CD3D, CD3E), NK cells (NKG7, GNLY). Moreover, Figure 1E right panel, and Figure 1F illustrate the *Ninjl* gene across cell subgroups using FeaturePlot analysis. Results showed that NINJ1 expression was significantly elevated in neutrophils compared to other immune cell types. To elucidate the functional role of NINJ1 in neutrophil biology, the neutrophil population was isolated and subdivided into four distinct subsets (Figure 1G). NINJ1 expression was then systematically evaluated across these subpopulations. Notably, elevated NINJ1 expression was observed in all neutrophil subsets, except the Neu4 cluster (Figure 1H). Pseudotime trajectory analysis was performed using Monocle 2 to reconstruct the temporal dynamics of neutrophil differentiation. The results indicate that the neutrophil populations originate from the Neu4 cluster, progress through the Neu3 cluster, and ultimately terminally differentiate into the Neu1 and Neu2 clusters (Figure 1I-J). To identify key molecular regulators of neutrophil subset diversification,

differential gene expression analysis was conducted on terminally differentiated populations (State 5 and State 6; Figure 1K). Differentially expressed genes were grouped into three co-expression modules. Subsequent GO enrichment analysis demonstrated that State 6, largely composed of the Neu1 subset, was enriched in genes implicated in immune effector mechanisms. Conversely, State 5, mainly consisting of the Neu2 subset, exhibited significant enrichment of genes related to chemotaxis pathways (Figure 1K). Based on the phenotypic characteristics of each cellular subset within the pseudotime trajectory analysis, neutrophils were further annotated into distinct functional states, including resting, active, chemotactic, and inflammatory states (Figure 1L). NINJ1 was highly expressed in active, chemotactic, and inflammatory neutrophil subsets during ALI (Figure 1H, L). Pulmonary cellular distribution analysis reveals that NINJ1 is enriched in neutrophils during ALI in mice.

Hotspot finds modules of the NINJ1 gene with significant autocorrelation within NETosis

To elucidate the molecular functions and interaction networks of NINJ1 in neutrophils during ALI, we employed Hotspot analysis [35], a method designed to detect spatially restricted patterns and nonlinear covariances among genes within cellular subpopulations. This approach is particularly suitable for identifying context-specific co-expression changes within the inflammatory microenvironment of ALI. After the selection of the 1284 significant genes by Hotspot, we ran the module identification procedure on these genes. This approach revealed ten gene modules, among which NINJ1 was significantly enriched in Module 8 (Figure 2A). The functional synergy within Module 8 suggested biological processes and regulatory pathways centered on NINJ1. Notably, this module was upregulated explicitly in the inflammatory neutrophil subset (Figure 2B), implying a role for NINJ1 in neutrophil inflammatory polarization during ALI. Importantly, KEGG pathway enrichment analysis of Module 8 genes revealed significant enrichment in the NETosis pathway, strongly suggesting that NINJ1 may regulate neutrophil extracellular trap formation in ALI. (Figure

2C). Finally, NETosis activity was assessed across neutrophil subsets using a NETosis-related gene set. Significantly increased NETosis scores were observed in both inflammatory and active subpopulations (Figure 2D). Together, our results identify a NINJ1-associated module that controls neutrophil NETosis during ALI.

NINJ1 oligomerization is elevated in neutrophils undergoing NETosis from ARDS patients

To further verify the results from the bioinformatics analysis, this study involved the recruitment of patients meeting the ARDS criteria alongside HCs. We detected the expression of NINJ1 in peripheral blood neutrophils from HCs and ARDS patients (Figure 3A). Compared to neutrophils from HCs, neutrophils from ARDS patients demonstrated a marked increase in the content of NETosis' characteristic components citH3 and MPO (Figure 3B), accompanied by significantly elevated NINJ1 expression (Figure 3C-D). We further observed that NINJ1 formed several speck-like assemblies, consistent with NINJ1 oligomerization in peripheral blood neutrophils from ARDS patients (Figure 3C). The mechanism underlying NINJ1-mediated lytic cell death involves its oligomerization, which is essential for subsequent PMR [36]. Using a biochemical native-PAGE approach that maintains native protein interactions, a band of approximately 180-300 kDa was observed in neutrophils from ARDS patients (Figure 3E). NINJ1 expression is upregulated when neutrophils undergo NETosis (Figure 3E-F). These results suggest that NINJ1 oligomerization contributes to NETosis. In the LPS-induced murine model, neutrophils were isolated from the peripheral blood of both the Control and ALI groups for IF analysis. (Figure 3G). Neutrophils from ALI mice demonstrated a marked increase in the levels of NETosis-specific markers (citH3 and MPO), along with significantly higher NINJ1 level than those from the Control group (Figure 3H-J). NINJ1 speck was observed in neutrophils from ALI mice (Figure 3I). Compared to Controls, both NINJ1 oligomerization and NETosis levels were significantly elevated in the peripheral blood of ALI mice (Figure 3K-L). These results suggest that NINJ1 oligomerization is specifically correlated with NETosis during ALI/ARDS.

439 ***Ninjl* depletion in neutrophils alleviates LPS-induced ALI in mice**

440 To investigate the function of NINJ1 on neutrophils in orchestrating ALI inflammation, neutrophil-
441 specific *Ninjl*-deficient mice were generated by crossing *Ninjl* floxed mice with *Ly6G*-Cre mice (Figure 5A
442 and S2A-B). We confirmed that the NINJ1 expression was specifically deleted in neutrophils isolated from
443 *Ninjl^{fl/fl}/Ly6G^{Cre}* (*Ninjl^{ΔNeu}*) mice, while regular expression was maintained in control *Ly6G*-Cre-*Ninjl^{fl/fl}*
444 mice (*Ninjl^{WT}*) (Figure 5B-C). Furthermore, we verified that the NINJ1 expression was not deleted in *Ly6G*-
445 cells or bone marrow-derived macrophages (BMDMs) isolated from *Ninjl^{ΔNeu}* mice (Figure S2C and S2D).
446 After the LPS challenge, *Ninjl^{WT}* mice exhibited a visibly distressed phenotype, characterized by ruffled and
447 dull fur, reduced locomotor activity, and ocular discharge. In contrast, *Ninjl^{ΔNeu}* mice maintained a healthy
448 appearance with glossy fur and relatively normal levels of activity (Figure S2E). Histological analysis revealed
449 a marked attenuation of lung injury in *Ninjl^{ΔNeu}* mice, characterized by reduced leukocyte infiltration, alveolar
450 congestion, and alveolar barrier thickening compared to *Ninjl^{WT}* mice after LPS challenge (Figure 4D, E).
451 *Ninjl^{ΔNeu}* mice exhibited a significant reduction in areas of segmental consolidation following LPS challenge
452 (Figure 4F). 3D reconstructions further corroborated the structural alterations, revealing markedly enlarged
453 airspace volumes in *Ninjl^{ΔNeu}* mice compared to *Ninjl^{WT}* across all observed time points (Figure 4F).
454 *Ninjl^{ΔNeu}* mice further increased the survival rate of ALI mice (Figure 4G). Additionally, LDH activity in
455 serum was reduced in *Ninjl^{ΔNeu}* mice (Figure 4H), suggesting an attenuation of pulmonary cellular damage.
456 The inflammatory cascade represents a hallmark characteristic of ALI. Our previous studies have identified
457 the triggering receptor expressed on myeloid cells-1 (TREM-1) signaling pathway and the Pyrin domain-
458 containing 3 (NLRP3) inflammasome as a novel mechanism driving inflammatory amplification in ALI [28,
459 [37]. The TNF- α level in serum from ALI *Ninjl^{ΔNeu}* mice was significantly lower than that of *Ninjl^{WT}* mice
460 (Figure 4I). *Ninjl^{ΔNeu}* mice exhibited significant downregulation of key inflammatory mediators associated

with ALI, including *Mcp-1*, *Il-1 β* , *Trem-1*, *Tnf- α* , and *Nlrp3* mRNA (Figure 4J). The protein levels of NLRP3, pro-IL-1 β , and IL-1 β p17 in the lungs were significantly decreased in *Ninj1* ^{Δ Neu} mice (Figure 4K-L). This is interesting and implies a non-cell-autonomous effect: neutrophils fail to release NETs, which in turn leads to reduced activation of inflammasomes in other cells (e.g., macrophages). These results suggest that NINJ1 in neutrophils is a key factor in the pathophysiology of organ dysfunction associated with ALI.

NINJ1 drives NETs release in neutrophils during ALI

We further investigated the role of NINJ1 in NETs release during ALI *in vivo*. ELISA analysis showed that after LPS challenge, the levels of MPO-dsDNA and citH3-DNA complexes, as well as dsDNA, in both BALF and serum were significantly lower in *Ninj1* ^{Δ Neu} mice than in *Ninj1*^{WT} Control (Figure 5A-F). PAD4 catalyzes histone arginine citrullination, promoting chromatin decondensation and cytoplasmic release. This decondensed chromatin complexes with granular enzymes, including MPO and NE, and is subsequently extruded to form NETs. Then, peripheral blood neutrophils were isolated from *Ninj1*^{WT} and *Ninj1* ^{Δ Neu} mice (Figure 5G). We found that the fluorescence intensity of MPO and citH3 in *Ninj1* ^{Δ Neu} mice was significantly diminished compared to that in *Ninj1*^{WT} mice after ALI induction (Figure 5H). Western blot analysis revealed a significant decrease in the levels of NETs protein, such as NE, citH3, PAD4, and MPO in neutrophils from *Ninj1* ^{Δ Neu} mice compared with *Ninj1*^{WT} mice (Figure 5I-J). These results demonstrate that neutrophil-specific *Ninj1* depletion prevents NETs release and pulmonary dysfunction, improving ALI outcomes.

NINJ1 is indispensable for NETosis-related PMR

To identify and characterize the specific pore-forming proteins during NETosis-related PMR, we analyzed an available single-cell RNA sequencing dataset (GSE224938). This analysis revealed that *Ninj1* expression was markedly higher than that of other pore-forming proteins, including *Gsdmd* and *Mkl1*, in human

neutrophils (Figure 6A). To further verify the conclusions from the single-cell RNA sequencing dataset, primary human neutrophils were isolated and stimulated with PMA for RNA sequencing analysis. This analysis revealed the differential expression of pore-forming proteins, including *NINJ1*, *GSDME*, *GSDMB*, *GSDMC*, *GSDMD*, and *MLKL*, in PMA-treated neutrophils. Notably, only *NINJ1* was significantly upregulated during PMA-triggered NETosis (Figure 6B). Subsequent pharmacological inhibition of *MLKL* using GSK872, or of *GSDMD* using disulfiram, failed to suppress extracellular dsDNA release in PMA-treated neutrophils (Figure 6C). We also observed oligomerization and speck formation of *NINJ1* in PMA-treated dHL-60 cells (Figure 6D-F) and primary neutrophils (Figure S3A-B). Morphologically, neutrophils undergoing NETosis developed bubble-like herniations that burst to yield shrunken cellular corpses. However, PMA-treated neutrophils with *NINJ1* shRNA exhibited a persistent 'bubble' morphology (Figure 6G, upper panel). PMA-treated *NINJ1*-silenced neutrophils developed an intact plasma membrane ballooning morphology (Figure 6G, lower panel). Scanning electron microscopy of neutrophils revealed a substantial amount of extracellular net-like structures surrounding PMA-induced NETosis, which were substantially reduced upon *NINJ1* silencing (Figure 6H). *NINJ1* shRNA administration reduced dsDNA and MPO-dsDNA complex levels in the culture supernatants of dHL-60 treated with PMA (Figure 6I-J). Similarly, a significant reduction in the supernatant levels of citH3, MPO, and NE was observed in *NINJ1*-silenced neutrophils (Figure 6K). Together, our data support that neutrophil PMR and subsequent NETs release during NETosis occur in a *NINJ1*-dependent manner.

Aberrant N-glycosylation of *NINJ1* is indispensable for its oligomerization and subsequent NETs release

NINJ1 oligomerization is essential for PMR in lytic cell death, yet the upstream regulatory mechanism underlying *NINJ1* oligomerization remains poorly defined. Protein oligomerization is closely linked to post-translational modifications (PTMs), which modulate protein function by altering conformational stability,

subcellular localization, and multimeric assembly [38]. Based on the structural features of NINJ1 documented in UniProt, bioinformatic analysis using CSSPalm predicted no acetylation or palmitoylation sites in NINJ1, but indicated potential phosphorylation at Thr18, Ser25, Ser46, and Ser50 (Figure S4A). However, phosphoproteomic profiling of PMA-treated neutrophils identified 2303 upregulated phosphoproteins, none of which corresponded to NINJ1 (Figure S4B-C). Further bioinformatic analysis using NetNGlyc predicted that the human NINJ1 protein harbored a conserved N-glycosylation site at asparagine 60 (N60), situated within the α 2-helical domain (residues 58-74, Figure 7A). Sequence alignment revealed a high conservation of NINJ1 between mouse and human (89.5% identity, 95.4% homology). Notably, N-linked glycosylation at N60 was conserved in both rodents and humans, and further analysis demonstrated that this glycosylation motif was widely preserved across vertebrate species (Table 4). IF results showed that NINJ1 was predominantly localized at the plasma membrane, with partial colocalization with the Golgi marker (GM130 marker, Figure 7B), which is the primary site for maturation and N-glycosylation of proteins. Incubation with peptide-N-glycosidase F (PNGase F) to remove N-glycosylation (deglycosylation) demonstrated band separation to a nonglycosylated form (Figure 7C). PNGase F treatment abolished the electrophoretic mobility shift of NINJ1 toward a higher molecular weight in PMA-treated neutrophils (Figure 7D). Subsequently, PMA-treated neutrophils incubated with tunicamycin, a well-known inhibitor of N-glycosylation, also showed results similar to those observed after PNGase F treatment (Figure 7E). We then generated the NINJ1-WT and NINJ1-N60A by substituting asparagine (N) with alanine (A). We found that PMA-treated neutrophils with the N60A mutant exhibited impaired NINJ1 oligomerization (Figure 7F). Consistent with the observation in NINJ1 knockout neutrophils, neutrophils with NINJ1-N60A mutant sustained the ballooned morphology characteristic of NETosis (Figure 7G). Scanning electron microscopy results revealed abundant extracellular net-like structures surrounding PMA-stimulated neutrophils, which were markedly diminished in NINJ1-N60A (Figure 7H). The NINJ1-N60A also reduced levels of MPO-dsDNA complexes and dsDNA in the

530 supernatants after PMA stimulation (Figure 7I-J). A significant reduction in supernatant levels of citH3, MPO,
531 and NE was observed in NINJ1-N60A neutrophils. (Figure 7K). Collectively, these data demonstrate that N-
532 glycosylation of NINJ1 at 60 sites is the major contributor to its oligomerization and NETs release.

Discussion

This study demonstrates that the pore-forming protein NINJ1 mediates NETs extrusion and contributes to pulmonary dysfunction in ALI. Analysis of a public single-cell RNA sequencing dataset indicated that NINJ1 is highly expressed primarily in neutrophils during ALI. Hotspot analysis further connected NINJ1 to a functional module related to NETosis. In LPS-induced ALI models, either genetic deletion of *Ninj1* in neutrophils reduced NETs release, alleviated lung injury, and decreased the extent of segmental consolidation and airspace pathology. Notably, neutrophils from ARDS patients undergoing NETosis showed NINJ1 oligomerization on the plasma membrane and within canonical NETs structures. Mechanistic studies revealed that N-glycosylation of NINJ1 is essential for its oligomerization and subsequent NET release.

Extracellular NETs have been recently identified as a DAMP released during ARDS/ALI. However, the mechanism underlying its release remains enigmatic. We have, for the first time, identified NINJ1 as a key pore-forming protein for NETs release. NETs-associated histones contribute to cytotoxicity by disrupting cell membrane integrity [39, [40]. Other NETs components, such as defensins and elastases, also contribute to cytotoxicity through membrane permeabilization and degradation of intercellular junctions, respectively [41]. PMR serves as a prerequisite for the release of NETs, which form porous networks with 50-200 nm openings. Previous studies suggested that caspase11/GSDMD mediates NETs release in neutrophils during sepsis [42]. Conventionally, the terminal PMR following GSDMD pore formation was regarded as a passive consequence of osmotic imbalance, leading to membrane disintegration [43]. However, the identification of the evolutionarily conserved small membrane protein NINJ1 has led to a paradigm shift, revealing that PMR constitutes an actively executed process. *David* and colleagues observed that NINJ1 rings or disks of heterogeneous sizes up to 180 nm inner diameter were released from macrophages upon induction of pyroptosis [23]. Our results demonstrated that NETs extrusion during ALI is a NINJ1-dependent event. Consistent with this, comparative immunophenotyping and single-cell transcriptomic analyses have identified

NINJ1 as a candidate biomarker of severity in both influenza- and COVID-19–induced lung injury [44]. Functionally, NINJ1 compromises plasma membrane integrity and promotes PMR in a manner dependent on mechanical stress. During lytic cell death, such stress may originate from osmotic imbalance induced by pore-forming proteins such as GSDMD or through lipid peroxidation [45]. Thus, we propose that plasma membrane rupture may require the coordinated action of both small and large pore-forming proteins. Specifically, the alterations in membrane tension resulting from GSDMD-formed small pores may trigger the activation of NINJ1. Our study bridges a critical knowledge gap by identifying NINJ1 as a key regulator of NETs release, a finding supported by *in vitro* evidence using neutrophils from ARDS patients and primary murine cells, as well as by an *in vivo* model of ALI.

NINJ1 mediates PMR in response to multiple lytic cell death inducers, including pyroptosis, PANoptosis, post-apoptosis, and ferroptosis [43]. In contrast, our study demonstrates that during NETosis, the absence of NINJ1 severely impaired plasma membrane disintegration, specifically preventing rupturing in ballooned cells, while blebbing remained unaffected. Notably, *Ninjl*-deficient neutrophils exhibited a persistent ballooned morphology, underscoring a unique and essential role for NINJ1 in the execution of NETosis. Similarly, a recent study demonstrated that NINJ1 has a pivotal role in mediating NETs formation under the stimulation of calcium oxalate [46]. However, that study focused primarily on NETosis formation in myeloid-specific *Ninjl* deletion mice, whereas our investigation more directly elucidates the specific function of NINJ1 in facilitating PMR and subsequent NETs release *in vitro*, using ARDS patient-derived and primary murine neutrophils. Collectively, these findings highlight that NINJ1-mediated PMR extends beyond previously reported death pathways such as pyroptosis and PANoptosis. Additionally, under conditions of MLKL-dependent necroptosis, NINJ1 deficiency only partially reduced the release of LDH and DD-150 from BMDMs, whereas HMGB1 release remained largely unaffected [20]. These observations suggest the operation of an NINJ1-independent pathway mediating PMR during necroptosis, likely attributable to the

direct membrane-disrupting capacity of oligomerized MLKL. Our results revealed that neutrophils undergoing NETosis during ALI exhibit low expression of MLKL. In contrast, NINJ1 expression was most prominent in neutrophils compared to other immune cell types in ALI, with significantly higher levels observed in neutrophils relative to both GSDMD and MLKL. The clinical relevance of our finding is supported by the observation that neutrophils from ARDS patients express more NINJ1 oligomerization and produce high levels of NETs. *Ninjl*^{ΔNeu} mice exhibited diminished pulmonary damage, accompanied by reduced areas of segmental consolidation and attenuated airspace pathology in a mouse model of ALI. Consistent with our results, genetic ablation of *Ninjl* in myeloid cells substantially attenuated oxalate-induced acute kidney injury [46]. An anti-NINJ1 monoclonal antibody attenuates NINJ1-mediated PMR and suppresses inflammatory responses in models of hepatitis [47]. However, these studies neither address ALI nor specifically examine the role of NINJ1 on neutrophils.

NINJ1 oligomerization is crucial for PMR in lytic cell death. The precise mechanisms triggering NINJ1 activation or oligomerization remain elusive. Palmitoylation-mediated activation of GSDMD is critical for its membrane localization and pore formation during pyroptosis [48]. MLKL causes necrotic membrane disruption upon phosphorylation by RIP3 [49]. By analogy, NINJ1 may also undergo post-translational modification during its activation. Based on the structural features of NINJ1 documented in UniProt, CSSPalm-based bioinformatic analysis predicted a lack of acetylation and palmitoylation sites, but indicated potential phosphorylation at Thr18, Ser25, Ser46, and Ser50. However, our phosphoproteomic profiling of PMA-treated neutrophils identified 2303 upregulated phosphoproteins, none corresponding to NINJ1. N-glycosylation has been reported to regulate protein oligomerization [50], with evidence indicating that site-specific N-glycosylation critically determines the pentameric versus hexameric assembly of recombinant human IgM [51]. Our results demonstrate that NINJ1 is N-glycosylated at N60, which is essential for its oligomerization. Abrogation of N-glycosylation markedly impaired multimer formation, underscoring the

critical role of this modification in NINJ1 oligomeric assembly. NINJ1 by reported assembles into homomeric complexes through intracellular cis-interactions and N-glycosylation at Asn60 [52]. N60 within amphipathic helix 2 appeared critically important, as it participated in an extensive hydrogen bonding network, likely facilitated by a water molecule, with a highly conserved triad of polar residues: Ser87 and Gln91 in transmembrane helix 1, and Asn133 in transmembrane helix 2 [53]. We have demonstrated that NINJ1 mediates PMR during NETosis and facilitates NETs release. Furthermore, we have established that N-glycosylation of NINJ1 at N60 regulates its NETs extrusion function in neutrophils. The sequential process of N-glycosylation takes place within the Golgi apparatus. Newly synthesized proteins are transported from the endoplasmic reticulum (ER) to the ER-Golgi intermediate compartment, then to the Golgi complex, and finally to their target destinations [54]. Consistent with our observations, *Kayagaki et al.* also reported co-localization of NINJ1 with the Golgi marker GM130 [20]. Our findings elucidate the functional importance of N-linked glycosylation of NINJ1 in neutrophils and propose the targeting of glycosylated NINJ1 as a potential therapeutic strategy to modulate NETs release.

Several limitations should be considered in this study. First, the precise molecular triggers of NINJ1 oligomerization and the exact mechanism by which it mediates PMR are still critical unanswered questions. NINJ1 oligomerization is a common feature across diverse cell death modalities, suggesting the existence of a conserved upstream signaling pathway that regulates its activation [55]. In multiple lytic inflammatory cell death pathways, such as pyroptosis and ferroptosis, calcium (Ca^{2+}) influx serves as a prerequisite event that is both necessary and antecedent to NINJ1 oligomerization and subsequent PMR [56, [57]. Additionally, Ca^{2+} signaling also represents a principal trigger for NETosis [58]. Whether calcium acts as the key upstream signal that initiates NINJ1-mediated NETs release represents an important direction for future research. Furthermore, our findings demonstrate the functional significance of N-linked glycosylation of NINJ1 in neutrophils. However, it remains unclear whether the attached N-glycan mediates carbohydrate-carbohydrate or

carbohydrate-peptide interactions. Precise characterization of the sugar moieties on NINJ1 will be essential to address this mechanistic question.

Conclusion

Collectively, our study demonstrated that the pore-forming protein NINJ1 is active in neutrophils from ALI mice and humans and plays a crucial role in NETs release and organ dysfunction during ARDS/ALI. Mechanistically, we uncovered a previously unrecognized regulatory pathway wherein NINJ1 N-glycosylation is critically required for its oligomerization and the subsequent release of NETs. This study provides a practical idea for targeting NINJ1 to treat ALI in the future.

Abbreviations

ALI, acute lung injury; ARDS, acute respiratory distress syndrome; APACHE II, Acute Physiology and Chronic Health Evaluation II; BCS, bovine serum; BALF, bronchoalveolar lavage fluid; BCA, bicinchoninic acid; BMI, body mass index; BSA, bovine serum albumin; citH3, citrullinated histone H3; CRP, C-reactive protein; cGAS/STING, the cyclic GMP-AMP synthase stimulator of the interferon gene; dsDNA, double-stranded DNA; DAMP, damage-associated molecular pattern; DAD, diffuse alveolar damage; DMSO, dimethyl sulfoxide; ELISA, Enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FDR, false discovery rate; GSDMD, Gasdermin D; HCs, healthy controls; HRP, horseradish peroxidase; IF, immunofluorescence staining; k -NN, k -nearest neighbor; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; Ly6G, lymphocyte antigen 6 complex; MPO, myeloperoxidase; MLKL, mixed-lineage kinase domain-like protein; NETs, neutrophil extracellular traps; NINJ1, ninjurin-1; NE, neutrophil elastase; NEUT, neutrophil count; NMF, non-negative matrix factorization; NLRP3, Pyrin domain-containing 3; PCT, procalcitonin; PMR, plasma membrane rupture; PCA, principal component analysis; PMA, phorbol 12-myristate 13-acetate; TNF- α , tumor necrosis factor-alpha; TREM-1, the triggering receptor expressed on myeloid cells-1; SOFA, Sequential Organ Failure Assessment; UMAP, Uniform Manifold Approximation and Projection; WBC, white blood cell count.

Acknowledgements

We gratefully acknowledge the Top-Notch Innovation Base of Basic Medicine at Central South University for providing instruments and technical support. We also sincerely thank the anonymous reviewers for their insightful comments and constructive suggestions that significantly improved this manuscript.

Author contributions

W.J.Z., Y.B.L., X.Y.Y., M.R.C., J.B.X., W.F.T., C.Y.Z., and N.S.Y.Y. performed the experiments; W.J.Z.,

Y.B.L., Y.F.Z., and C.Y.Z. analyzed the data. J.X.D., Y.Z., and C.X.G. contributed reagents/materials/analysis tools. W.J.Z. wrote the paper. Y.Z. and J.X.D. conceived, designed the experiments, and critically reviewed the manuscript. All authors had final approval of the submitted versions.

Funding

This work was supported by the National Natural Science Foundation of China (82400108, 82370086), Postdoctoral Fellowship Program of CPSF (GZC20233159), the Natural Science Foundation of Hunan province, China (2025JJ60690), and the Open competition mechanism to select the best candidates for key research projects of Ningxia Medical University (XJKF230104).

Availability of data and material

The datasets supporting the conclusions of this article are included within the article and its additional file. For any further data requests, please get in touch with the corresponding author.

Ethics approval and consent to participate

All animal experiments described here complied with the guidelines of the Committee on the Use and Care of Animals of Central South University (Changsha, China) and were approved by the IRB of the Xiangya School of Basic Medical Science at Central South University (2023-KT107). Human neutrophils were obtained from ARDS patients undergoing thoracic surgery at Xiangya Hospital of Central South University (Changsha, China). Patients included both those diagnosed with ARDS and those without ARDS. Informed consent was obtained from all participants, and the study was approved by the Clinical Medical Ethics Committee of Xiangya Hospital, Central South University (Approval No. 2024030484).

680 **Consent for publication**

681 Not applicable.

682

683 **Competing interests**

684 The authors have declared that no competing interest exists.

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828

830 Table 1. Sequences of the primers used to quantify gene expression.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
<i>Mcp-1</i>	GTCCCTGTCATGCTTCTGG	GCGTTAACTGCATCTGGCT
<i>pro-IL-1β</i>	CAGGCAGGCAGTATCACTCA	AGCTCATATGGGTCCGACAG
<i>Trem-1</i>	CTGTGCGTGTTCTTTGTC	CTTCCCGTCTGGTAGTCT
<i>Tnf-α</i>	CCACCCGCTCTTCTGTCTA	TGGTTTGTGAGTGAGGGT
<i>Nlrp3</i>	TACGGCCGTCTACGTCTTCT	CGCAGATCACACTCCTCAAA
<i>Ninj1</i>	GAGTCGGGCACTGAGGAGTAT	CGCTCTTCTTGTTGGCATAATGG
<i>β-actin</i>	TTCCAGCCTTCCTTCTTG	GGAGCCAGAGCA GTAATC

Table 2. Antibody sources and dilutions.

Antibodies	Source	Catalog	Dilution
Primary antibodies for Western blot			
Anti-NINJ1 monoclonal antibody	Santa Cruz	sc-136295	1: 2000
Anti-MPO polyclonal antibody	Proteintech	66177-1-Ig	1: 2000
Anti-citH3 polyclonal antibody	Abcam	Ab5013	1: 2000
Rabbit-anti-ELANE antibody	Abclonal	A8953	1: 2000
Anti-PAD4 polyclonal antibody	Santa Cruz	sc-365369	1: 2000
Anti-IL-1 β polyclonal antibody	R&D	AF-401-NA	1: 2500
Anti-NLRP3 polyclonal antibody	CST	15101	1: 2000
Anti- β -actin monoclonal antibody	ABclonal	AC026	1: 5000
Primary antibodies for immunofluorescence			
Anti-NINJ1 monoclonal antibody	Mybiosource	MBS249960	1:100
Anti-GM130 polyclonal antibody	Proteintech	11308-1-AP	1: 100
Secondary antibody			
HRP-conjugated goat anti-rabbit IgG	SAB	#L3012-2	1: 5000
Goat anti-Mouse IgG	SAB	L3032	1: 5000
Rabbit anti-Goat IgG	SAB	L3042-2	1: 5000
FITC-conjugated Goat anti-Rabbit IgG (H+L)	Abclonal	AS011	1:400
TRITC-conjugated Goat anti-Mouse IgG	Abclonal	AS026	1:400

Table 3. Demographic and clinical characteristics of the ARDS patients

Demographics	ARDS (<i>n</i> =12)
Age, years	68.42 (9.69)
Male, n (%)	8 (66.7)
BMI, kg/m ²	22.25 (4.32)
PaO ₂ /FiO ₂	161.75 (57.88)
SOFA score	5.42 (2.43)
APACHE II score	17.42 (6.33)
WBC, 10 ⁹ /L	13.98 (4.92)
NEUT, 10 ⁹ /L	12.57 (4.79)
CRP, mg/L	129.42 (51.47)
PCT, ng/ml	1.01 (0.75)

Scientific name	Sequence
NINJ1_HUMAN	MDSGTEEYELNGGLPPGTPGSPDASPARWGWRHGPINVNHYASKKSAAESMLDIALLMAN <u>AS</u> QLKAW
NINJ1_MOUSE	MESGTEEYELNGDLRPGSPGDALPPRWGLRNRPINVNHYANKKSAAESMLDIALLMAN <u>AS</u> QLKAVVE
NINJ1_RAT	MDPGTEEYELNGDLRPGSPGDASPPRWGLRNRPINVNHYANKKSAAESMLDIALLMAN <u>AS</u> QLKAVVE
NINJ1_PIG	MDSRLEDYELNGDLRPVSPSSPDASPPRWG - RNRPINMNHYANKKSAAESMLDIALLMAN <u>AS</u> QLKAVIE
NINJ1_GOAT	MDSRPEEYELNGDLRPS SPGSPDASPSRWG - RNQPINMNHYANKKSAAESMLDIALLMAN <u>AS</u> QLKAVIE
CONS	* : . * : ***** . * * : * . ***** * . * * * * * : * * * : * * * . * :
NINJ1_HUMAN	QGPSFAFYVPLVVLISISLVLQIGVGVLILFLVKYDLNNPAKHAKLDLNNLATGLVFIIVVVNIFITA
NINJ1_MOUSE	QGNDFAFFVPLVVLISISLVLQIGVGVLILFLVKYDLNNPAKHAKLDLNNLATGLVFIIVVVNIFITA
NINJ1_RAT	QGFNEFAFFVPLVVLISISLVLQIGVGVLILFLVKYDLNNPAKHAKLDLNNLATGLVFIIVVVNIFITA
NINJ1_PIG	QG PSFAFFIPLVVLISISLVLOV GVGVLILFLVRYDLNNPAKHAKLDLNNLATGLVFIIVVVNIFITA
NINJ1_GOAT	QG TGFAFFIPLVVLISISLVLQI GVGVLILFLVRYDLNNPAKHAKLDLNNLATGLVFIIVVVNIFITA
CONS	** ***** . ***** * . *
NINJ1_HUMAN	FGVQKPLMDMAPQQ
NINJ1_MOUSE	FGVQKPVMDEVAPRQ
NINJ1_RAT	FGVQKPVMDEVAPRQ
NINJ1_PIG	FGVQKPMMDVAPRQ
NINJ1_GOAT	FGVQKP TMDTAPRQ
CONS	* * * * * * * * * * * *

Amino acid sequences of Ninj1 in various species of vertebrates were collected from UniProt knowledgebase and were aligned by T-Coffee. Then, highly conserved sequences are listed. N-glycosylation motifs are

underlined, and an essential Asn in each species is marked in red. Identical amino acids are marked with an asterisk.

Figures and Figure legends

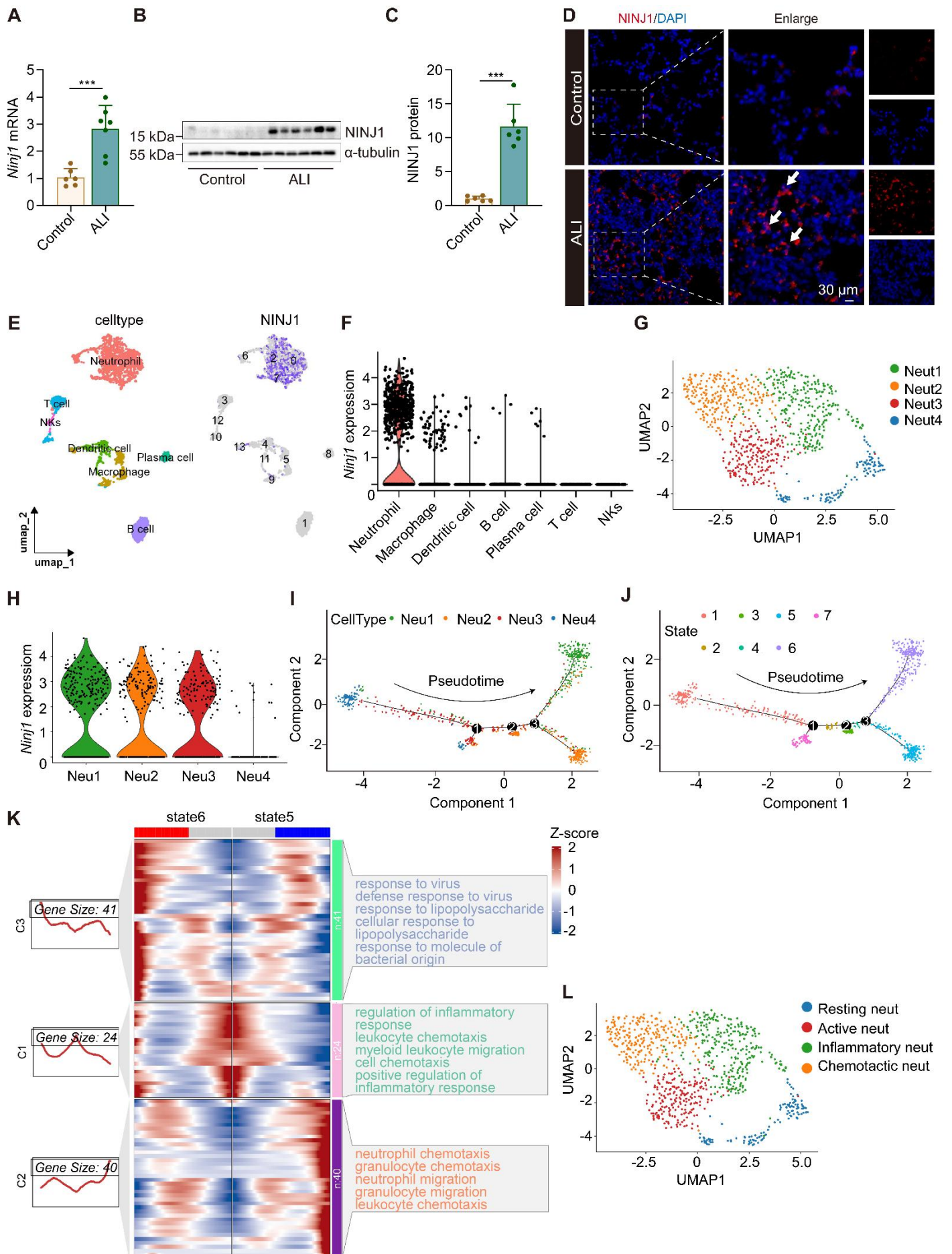


Figure 1. NINJ1 expression is predominantly elevated in activated neutrophils during ALI.

C57BL/6J mice were received an intratracheal instillation of LPS (5 mg/kg, *i.t.*). A-C) Twelve hours later, mouse lungs were excised, and the mRNA and protein expression of NINJ1 were assessed by Real-time PCR and Western blot. D) The fluorescence intensity of NINJ1 protein (red) in lungs from mice with ALI was detected by immunofluorescence, scale bar = 30 μ m. E) Distribution of 7 subtypes of cells by UMAP, coloured by cell types (GSE224938). t-SNE visualizations depict *Ninj1* expression levels, defined by binarized activity thresholds derived from AUC scores. F) The expression of NINJ1 over all immune cells, with each point from an individual sample. G) To elucidate the potential function of neutrophils in this ALI mouse model, a focused analysis of neutrophil populations was conducted, revealing seven distinct neutrophil subsets. H) The expression of *Ninj1* gene signatures was assessed across neutrophil subsets. I) Neutrophil subsets trajectory analysis delineated two divergent cell destinies colored by cluster. The arrow indicated the likely course of evolution within the trajectory. J) Monocle 2 trajectory analysis of 856 neutrophils obtained by 5' gene expression chemistry, annotated by calculated states. K) The heatmaps illustrated the dynamic expression patterns of genes across pseudotime, while the bar plot depicted the functional enrichment profiles of gene clusters in terminal states 5 and 6. L) Neutrophil subpopulations in ALI lung tissues were characterized through pseudotime trajectory analysis.

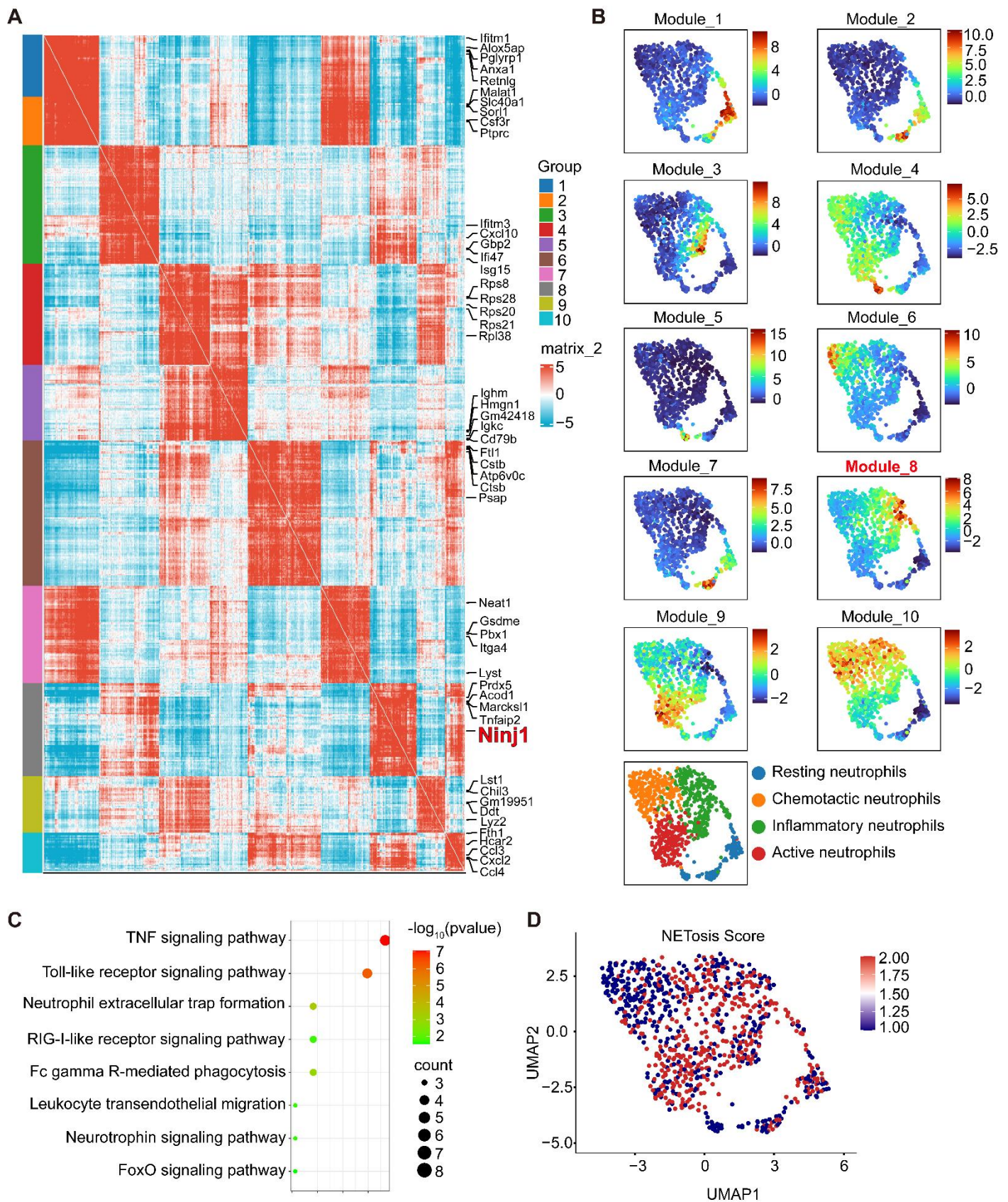


Figure 2. Characterization of transcriptional hotspots in neutrophils using single-cell RNA sequencing.

Hotspot was used to identify spatially relevant genes within a spatial single-cell expression sample from neutrophils. A) 1284 genes selected by Hotspot were grouped into 10 modules based on pairwise spatial correlations. B) Gene modules were visualized with their summary, per bar code, and module scores. Beneath

each module score plot, the expression level of the gene exhibiting the highest autocorrelation was shown for comparative purposes. C) KEGG pathway enrichment analysis was performed on co-expressed genes from Module 8 to investigate neutrophil-relevant biological processes. D) Neutrophil subpopulations were scored utilizing a NETosis-associated gene signature.

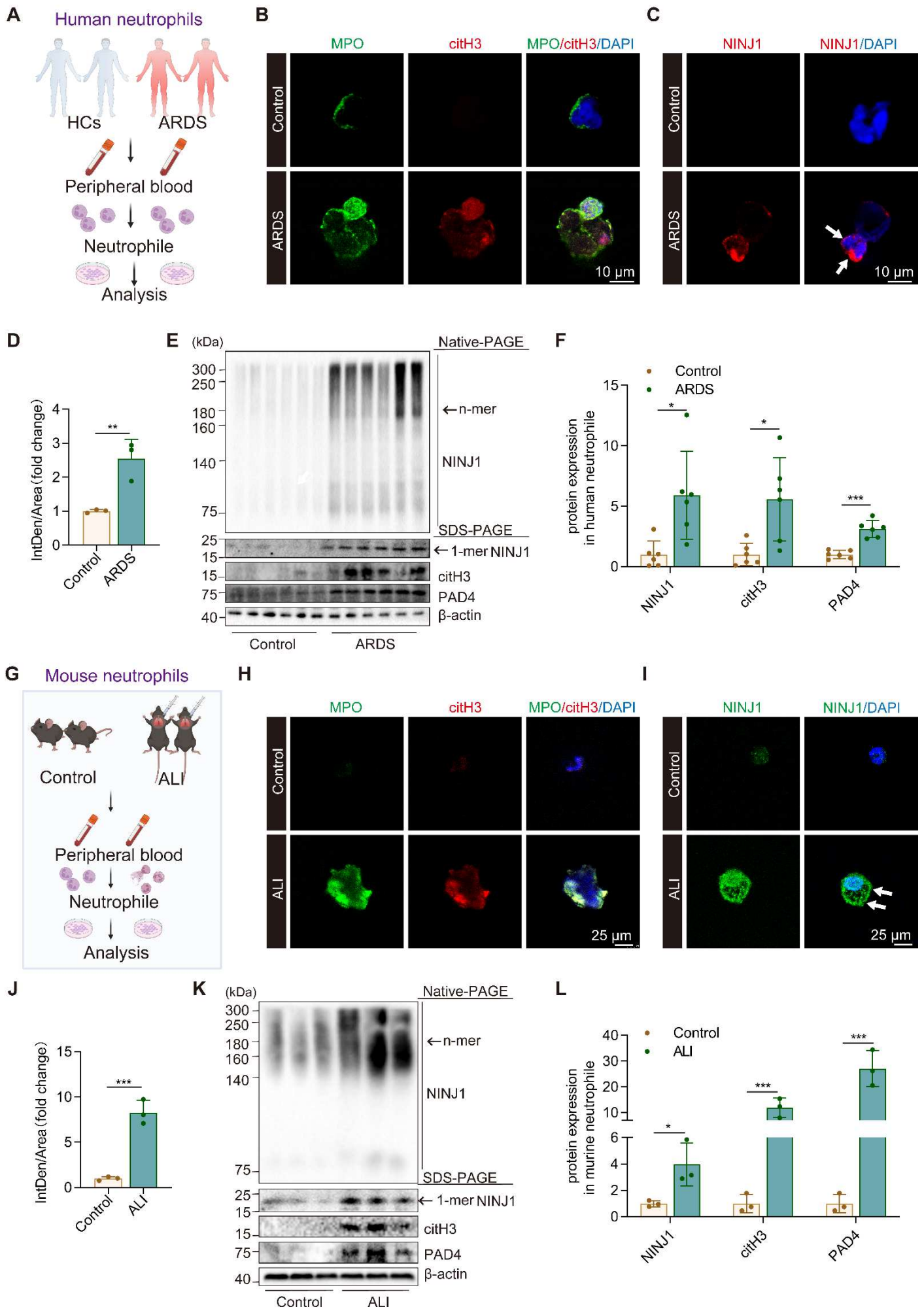


Figure 3. NINJ1 oligomerization was upregulated in clinical neutrophils undergoing NETosis from ARDS patients.

A) Schematic overview of experimental design for B to F. B) Laser-scanning confocal microscopy assessment of NETosis-related protein expression on peripheral neutrophils from HCs ($n = 12$) and ARDS patients ($n = 12$). C) IF of endogenous NINJ1 in circulating neutrophils from ARDS patients and non-ARDS donors. Arrowheads indicated NINJ1 specks. D) Average fluorescent intensity was calculated by NINJ1^+ fluorescence intensity (IntDen)/area of the region (Area) using ImageJ. E-F) Native-PAGE analysis of NINJ1 oligomerization in peripheral neutrophils from HCs and ARDS patients. SDS-PAGE analysis of NINJ1 monomer, citH3, PAD4, and β -actin was used as the internal control. G) Schematic overview of experimental design for H to L. H-I) IF images of NETosis and NINJ1 on peripheral neutrophils from ALI mice ($n = 8$) and non-ALI Control ($n = 8$). J) Quantification of the percentage of NINJ1 specks. K) Native-PAGE analysis of NINJ1 oligomerization in peripheral neutrophils isolated from Control ($n = 8$) and ALI mice ($n = 8$). K-L) SDS-PAGE analysis of NINJ1 monomer, citH3, PAD4, and β -actin was used as the internal control. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

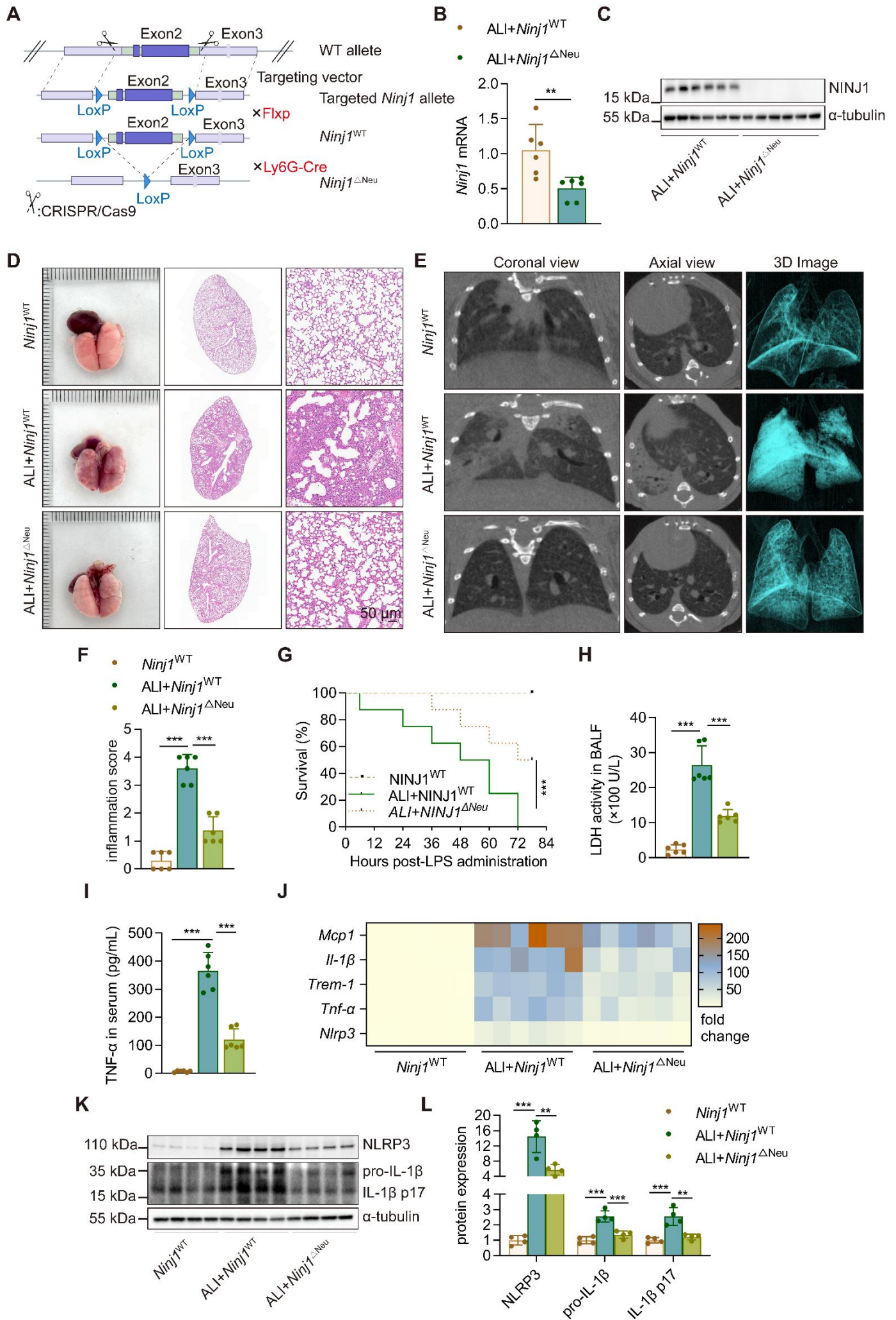


Figure 4. *Ninj1* depletion in neutrophils alleviated LPS-induced ALI *in vivo*.

A) Schematic of neutrophil-specific *Ninj1*-null mice generation. Exon 2 was floxed in the targeted allele and excised in the conditional knockout allele. *Ninj1*^{WT} (*Ly6G-Cre-Ninj1*^{fl/fl}) and *Ninj1*^{ΔNeu} (*Ninj1*^{fl/fl}/*Ly6G*^{Cre}) mice were intratracheally injected with LPS and euthanized 12 h post-treatment (*n*=6). B-C) Real-time PCR and Western blot were used to detect NINJ1 expression in peripheral neutrophils from *Ninj1*^{WT} and *Ninj1*^{ΔNeu} mice. D) Representative images of the mouse lung tissue (Left panel) and the lung histopathology stained with H&E (Right panel), scale bar = 50 μm. E) High-resolution micro-CT was utilized to assess pulmonary structural changes in a murine model of ALI. Multiplanar reconstructions, including dorsal and axial views, were obtained at the level of the T6 thoracic vertebra. Representative 3D reconstructions were generated from *in vivo* acquired images, with voxel intensities thresholded within a Hounsfield unit (HU) range of -1000 to -400. (*n* = 6 mice/group). F) Inflammation score was assessed. G) Kaplan-Meier survival curves. All mice were monitored for 84 h after LPS challenge (25 mg/kg, *n*=16 mice/group). H) The LDH activity in the serum was detected 12 h after the LPS injection. I) The concentration of TNF-α in lungs was detected by ELISA. J) The mRNA expression levels of *Mcp-1*, *Il-1β*, *Trem-1*, *Tnf-α*, and *Nlrp3* in lungs were quantified using Real-time PCR. K-L) The protein levels of NLRP3, pro-IL-1β, and IL-1β p17 fragment in lungs from ALI mice were determined by Western blot (*n*=6). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

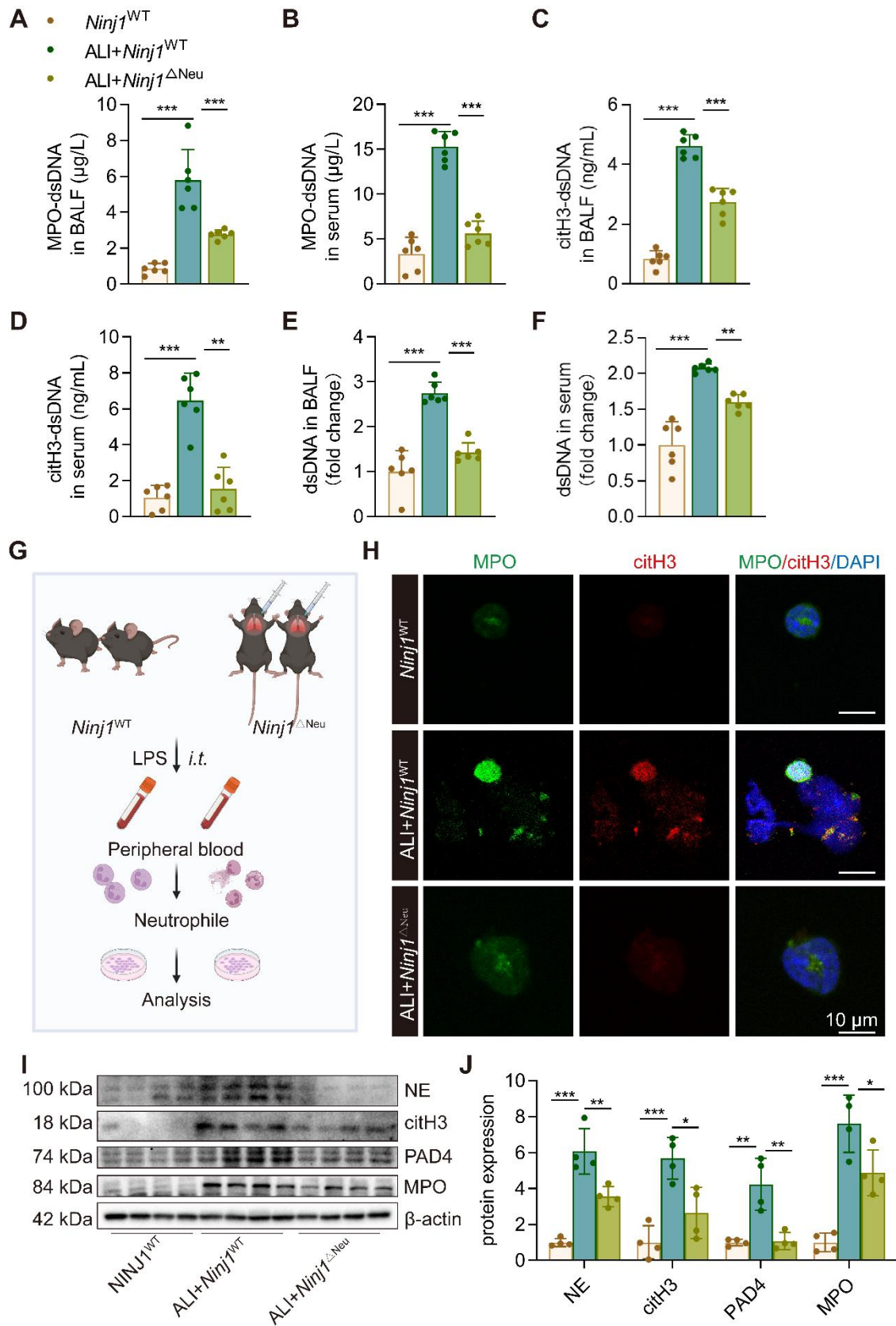


Figure 5. Neutrophil-specific *Ninj1* depletion inhibits NETs release during ALI.

Ninj1^{WT} and *Ninj1*^{ΔNeu} mice were administered with LPS for 12 h (5 mg/kg, *i.t.*). A-D) The levels of NETs components MPO-dsDNA and citH3-dsDNA in BALF and serum detected by ELISA. E-F) dsDNA in BALF and serum was measured using the PicoGreen® dsDNA Quantification Kit. G) Schematic overview of

experimental design for H to J. H) Laser-scanning confocal microscopy assessment of NETosis-related protein expression on peripheral neutrophils from ALI mice. I-J) Western blot was used to detect NE, citH3, PAD4, and MPO expression in neutrophils isolated from *Ninj1*^{WT} and *Ninj1*^{ΔNeu} mice. $n = 6$ mice/group, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

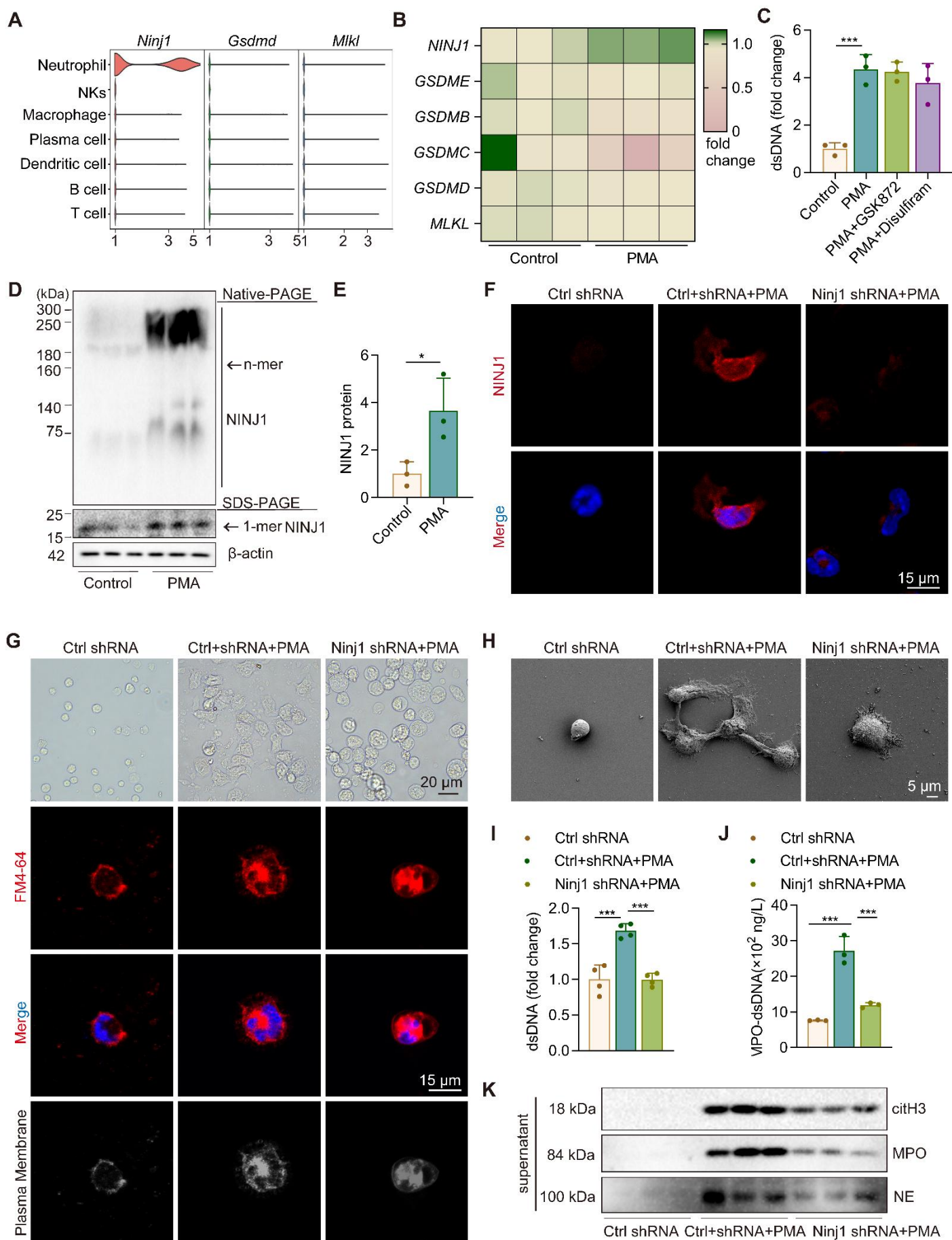


Figure 6. NINJ1 deficiency prevents PMR and NETs release *in vitro*.

A) The expression of pore-forming, including *Ninj1*, *Gsdmd*, and *Mlkl*, in overall immune cells (GSE224938).

B) Neutrophils isolated from human donors were stimulated with PMA for 3 h and subsequently harvested for transcriptomic sequencing analysis. RNA-seq analyses revealed the expression of pore-forming proteins, including *NINJ1*, *GSDME*, *GSDMB*, *GSDMC*, *GSDMD*, and *MLKL*, in neutrophils undergoing PMA-induced NETosis. C) Primary neutrophils were treated with GSK872 (an MLKL inhibitor), or Disulfiram (a GSDMD inhibitor) following PMA induction, and the levels of dsDNA were quantified. D) Native-PAGE analysis of NINJ1 oligomerization in PMA-treated dHL-60 cells. SDS-PAGE analysis of NINJ1 monomer. E) Quantification of NINJ1 monomer protein levels. F) Immunofluorescence images of NINJ1 on PMA-treated dHL-60 cells. G) The corresponding brightfield image was shown in live cells labeled with the plasma membrane dye FM4-64. Assessment of plasma membrane integrity by laser-scanning confocal microscopy in neutrophils transfected with either Ctrl shRNA or *Ninj1* shRNA after the PMA stimulation. H) Representative scanning electron microscopy images of neutrophil morphology in dHL-60 cells. I) dsDNA in supernatant was measured using the PicoGreen® dsDNA Quantification Kit. J) The concentrations of MPO-dsDNA in the neutrophil culture supernatant were determined using an ELISA test. K) The levels of citH3, MPO, and NE in the supernatant were detected by Western blot. $n = 3$, $*P < 0.05$ and $***P < 0.001$.

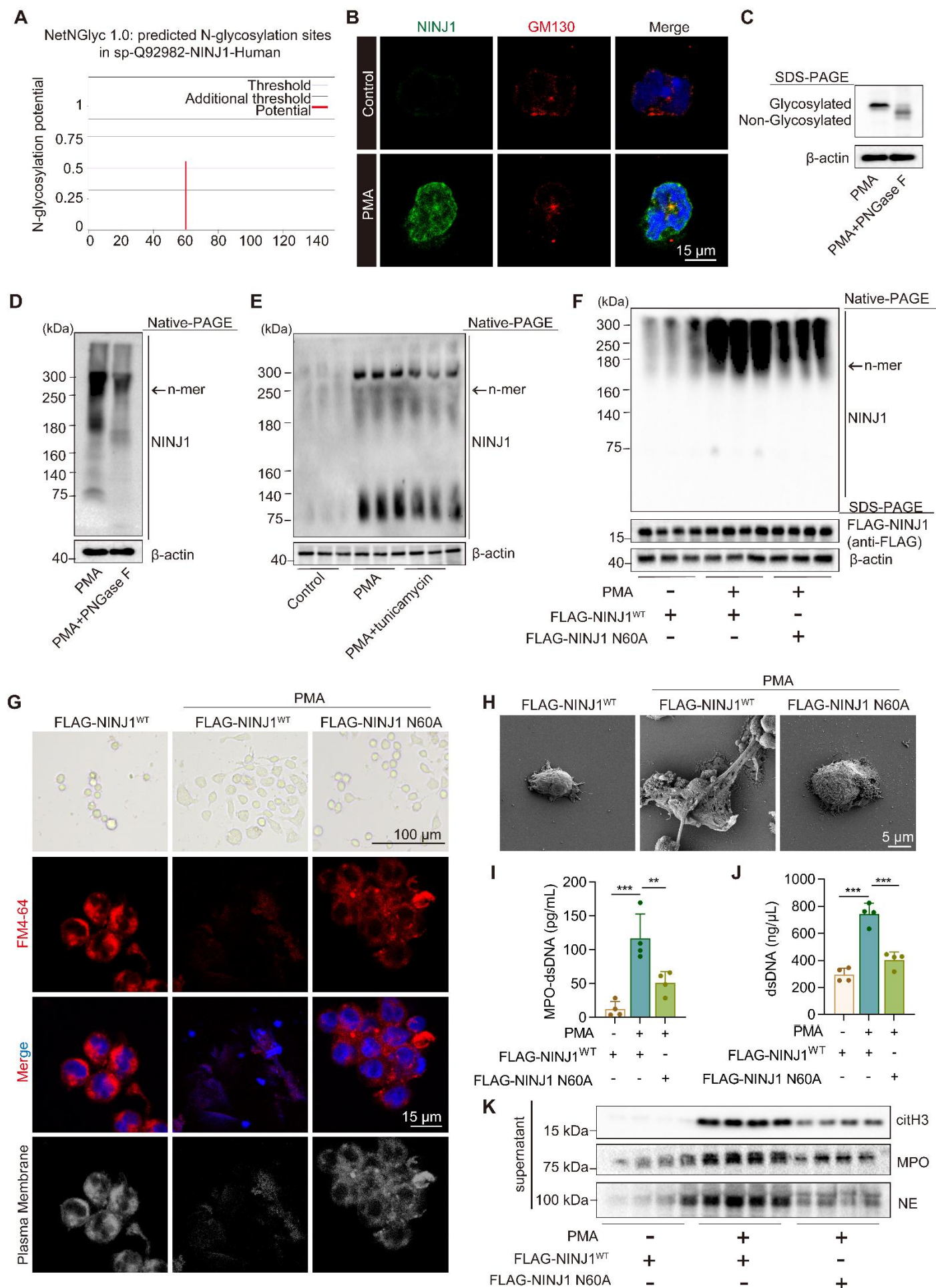


Figure 7. Aberrant N-glycosylation of NINJ1 facilitated NETs release.

A) Schematic representation of the predicted N-glycosylation sites in human NINJ1 was generated using the NetNGlyc server. The WT human NINJ1 protein contains a conserved Asn N-glycosylation site at amino-acid positions 60 (N60), which is located within the α 2-helical domain (amino acids 58-74). B) Immunofluorescence microscopy analysis of NINJ1 co-localization with the Golgi marker GM130. C) Western blot analysis of NINJ1 (glycosylated and nonglycosylated form) in PMA-treated neutrophils pretreated with or without PNGase F (+N). D) Native-PAGE analysis of NINJ1 in PMA-treated dHL-60 pre-incubated with or without PNGase F (+N). E) Western blot analysis of NINJ1 in PMA-treated neutrophils pre-incubated with tunicamycin. F) Potential asparagine N-glycosylation sites (N) were mutated to alanine (A). Native-PAGE was employed to assess NINJ1 oligomerization in neutrophils individually transfected with either NINJ1-WT or the NINJ1-N60A mutant cDNA. G) Corresponding brightfield image of live cells stained with the plasma membrane dye FM4-64. Plasma membrane integrity was further evaluated by laser-scanning confocal microscopy in neutrophils expressing either NINJ1-WT or the NINJ1-N60A mutant after PMA stimulation. H) Scanning electron microscopy images depict the morphological features of neutrophils expressing either NINJ1-WT or the NINJ1-N60A mutant with PMA stimulation. I) Extracellular dsDNA release was quantified using the PicoGreen® dsDNA assay. J) The concentrations of MPO-dsDNA complexes in culture supernatants after 3 h of PMA treatment were measured by ELISA. K) The presence of citH3, MPO, and NE in supernatants was confirmed by Western blot analysis. $n = 4$, $**P < 0.01$, and $***P < 0.001$.

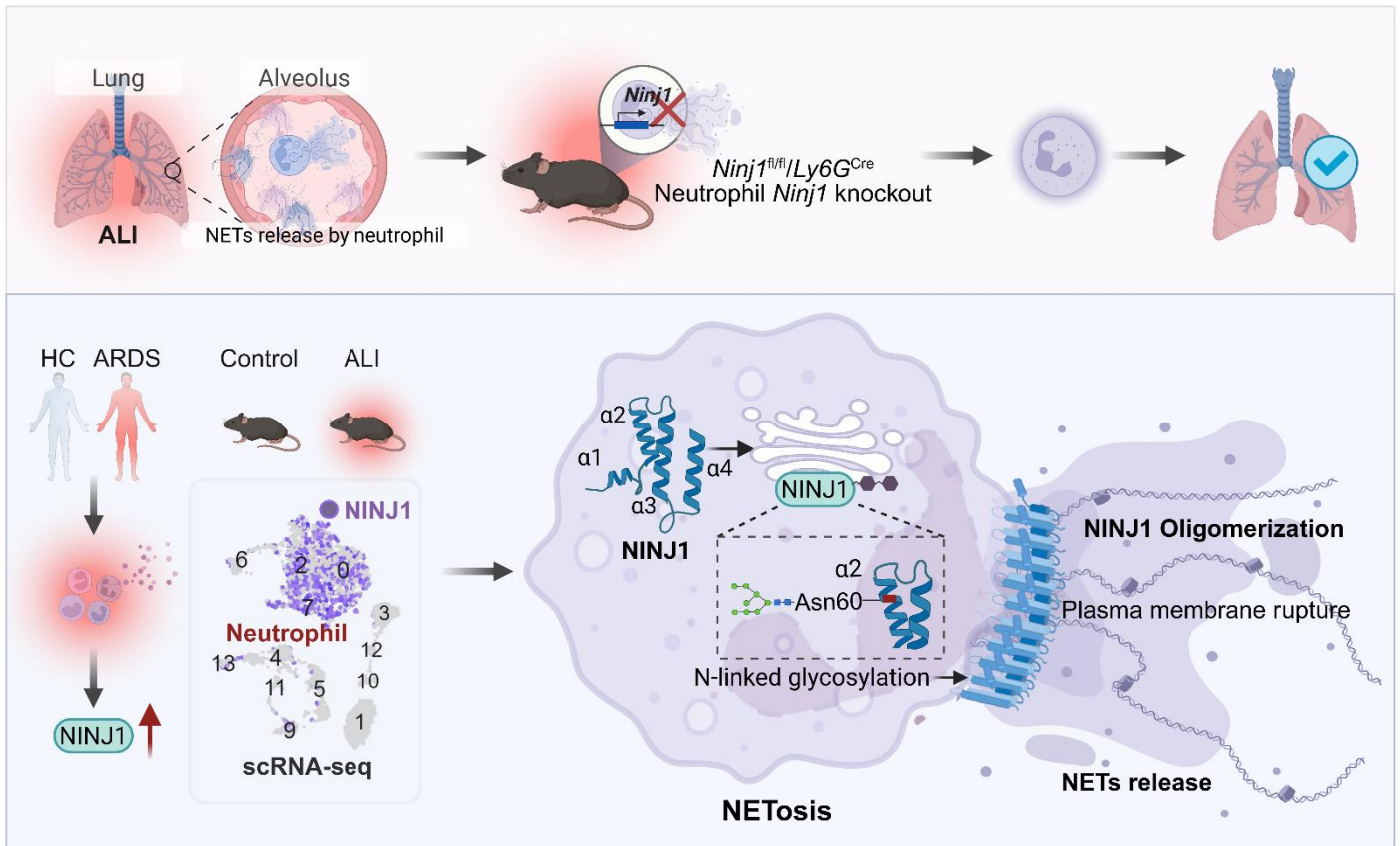


Figure 8. Schematic illustration. The novel pore-forming protein NINJ1 mediates the extrusion of NETs, thereby exacerbating pulmonary injury in ARDS/ALI. This process is dependent on N-glycosylation, which is essential for NINJ1 oligomerization and subsequent NETs release.

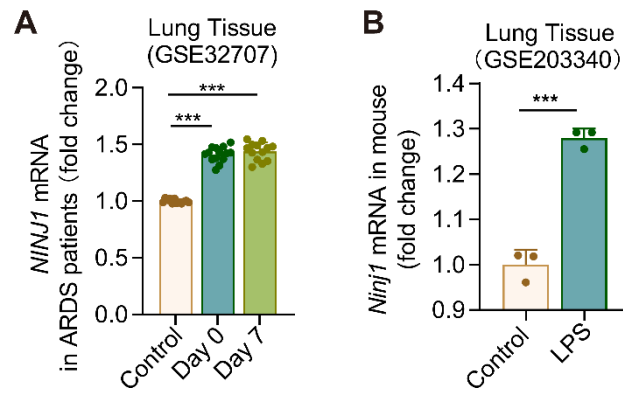


Figure S1. NINJ1 expression is significantly increased in ALI/ARDS.

A) Human *NINJ1* mRNA expression in the lungs from ARDS patients was determined by microarray (GSE32707) ($n=14$). Blood samples were collected from enrolled ARDS patients at the time of hospital admission (Day 0) and on Day 7 thereafter. B) *Ninj1* mRNA expression in the lungs from LPS-induced ALI mice (GSE203340) ($n=3$). *** $P < 0.001$.

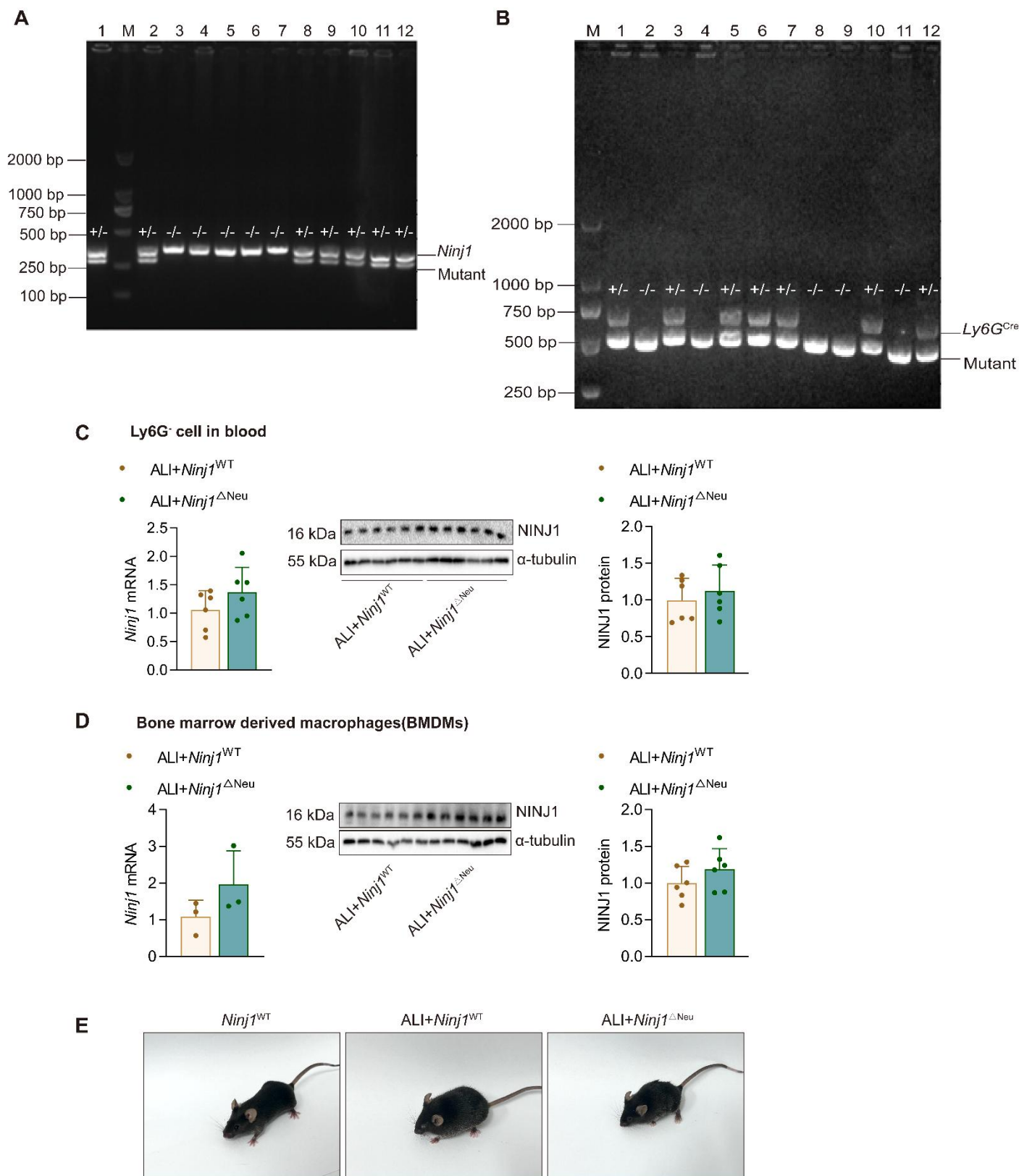


Figure S2. Generation of neutrophil-specific conditional *Ninj1*-null mice.

A) Targeted disruption of the *Ninj1* gene was verified by PCR of genomic DNA isolated from candidate mice.

B) Targeted disruption of the *Ly6G^{Cre}* gene was verified by PCR of genomic DNA isolated from candidate mice. *Ninj1*^{WT} and *Ninj1*^{ΔNeu} mice were intratracheally administered with LPS (5 mg/kg).

C) Real-time PCR and Western blot were employed to detect NINJ1 expression in Ly6G⁻ cells isolated from bone marrow from

Ninjl^{WT} and *Ninjl*^{ΔNeu} mice. D) Detection of NINJ1 expression by Real-time PCR and Western blot in BMDMs from *Ninjl*^{WT} and *Ninjl*^{ΔNeu} mice. E) The physical appearance of *Ninjl*^{WT} and *Ninjl*^{ΔNeu} mice administered with LPS. *n* = 6 mice/group, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

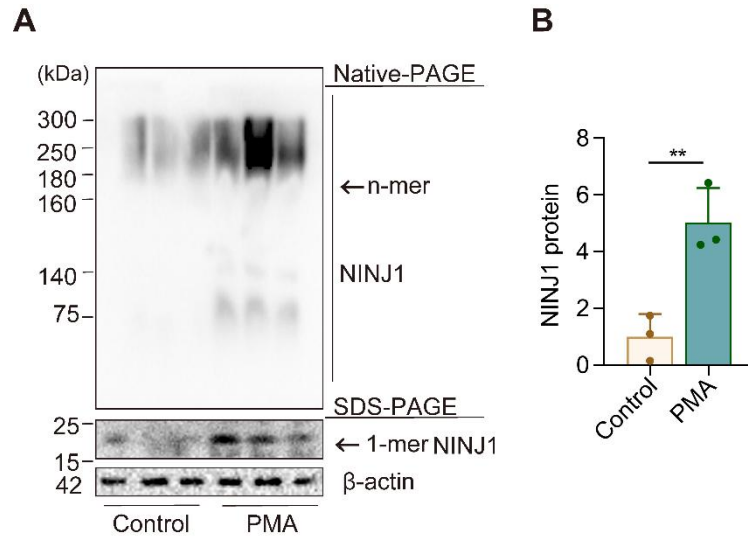


Figure S3. NINJ1 oligomerization is elevated in PMA-induced neutrophils.

Bone marrow neutrophils in mice were treated with PMA (10 nM) for 4 h. A) Native-PAGE analysis of NINJ1 oligomerization in PMA-treated neutrophils. SDS-PAGE analysis of NINJ1 monomer and β -actin was used as the internal control. B) Quantification of NINJ1 monomer protein levels. $n = 3$, $**P < 0.01$.

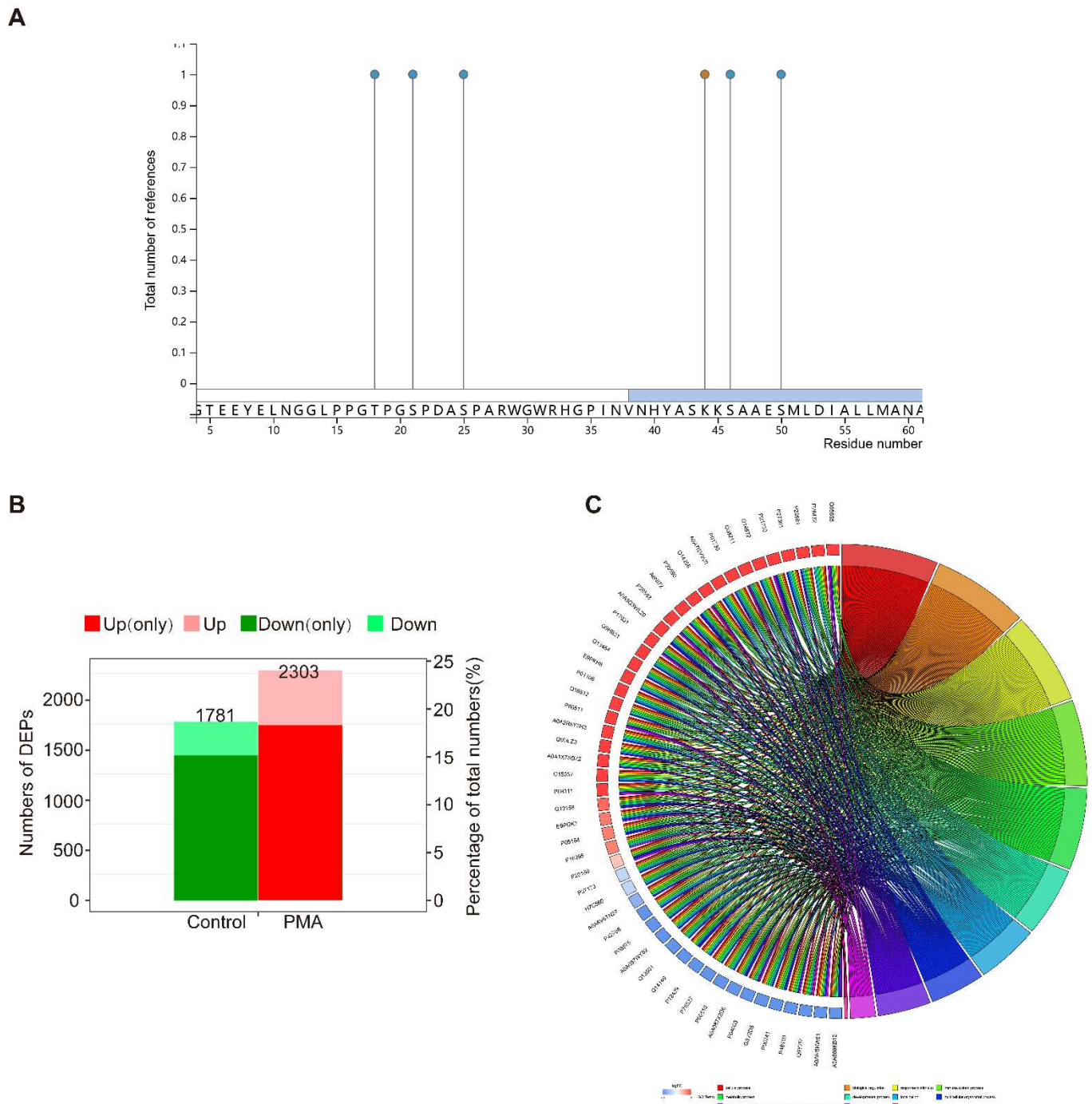


Figure S4. Lack of upregulated NINJ1 phosphorylation upon PMA stimulation.

A) To predict potential phosphorylation sites, Bioinformatic analysis with CSSPalm suggested that NINJ1 contains several putative phosphorylation sites, notably at Thr18, Ser25, Ser46, and Ser50. B) Phosphoproteomic profiling of PMA-treated neutrophils identified 2303 upregulated phosphoproteins, none of which corresponded to NINJ1. C) Phosphoproteomic profiling of PMA-treated neutrophils did not detect any upregulated phosphorylation at the predicted sites on NINJ1, $n = 3$.

Supplementary Files

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