Inhibition of neutrophil elastase prevents neutrophil extracellular trap formation and rescues mice from endotoxic shock

Emeka B. Okeke[a,b], Cameron Louttit[b,c], Chris Fry[d], Alireza Hassani Najafabadi[a,b], Kai Han[a,b], Jean Nemzek[d,*], James J. Moon[a,b,c,e,**]

[a] Department of Pharmaceutical Sciences, University of Michigan, Ann Arbor, MI, 48109, United States
[b] Biointerfaces Institute, University of Michigan, Ann Arbor, MI, 48109, United States
[c] Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI, 48109, United States
[d] Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI, 48109, United States
[e] Graduate Program in Immunology, University of Michigan, Ann Arbor, MI, 48109, United States

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ABSTRACT

Neutrophil elastase (NE) is a serine protease stored in the azurophilic granules of neutrophils and released into the extracellular milieu during inflammatory response or formation of neutrophil extracellular traps (NETs). Neutrophils release NETs to entrap pathogens by externalizing their cellular contents in a DNA framework decorated with anti-microbials and proteases, including NE. Importantly, excess NETs in tissues are implicated in numerous pathologies, including sepsis, rheumatoid arthritis, vasculitis, and cancer. However, it remains unknown how to effectively prevent NET formation. Here, we show that NE plays a major role during NET formation and that inhibition of NE is a promising approach for decreasing NET-mediated tissue injury. NE promoted NET formation by human neutrophils. Whereas sivelestat, a small molecule inhibitor of NE, inhibited the formation of NETs *in vitro*, administration of free sivelestat did not have any efficacy in a murine model of lipopolysaccharide-induced endotoxic shock. To improve the efficacy of sivelestat *in vivo*, we have developed a nanoparticle system for delivering sivelestat. We demonstrate that nanoparticle-mediated delivery of sivelestat effectively inhibited NET formation, decreased the clinical signs of lung injury, reduced NE and other proinflammatory cytokines in serum, and rescued animals against endotoxic shock. Collectively, our data demonstrates that NE signaling can initiate NET formation and that nanoparticle-mediated inhibition of NE improves drug efficacy for preventing NET formation.

Sepsis is defined as a systemic inflammatory response due to infection, and its spectrum of diseases (severe sepsis and septic shock) affects around 1.5 million Americans every year [1]. In addition to mortality, the morbidity of sepsis is significant as many survivors of sepsis face severe limitations in performing their daily activities [2,3]. Moreover, sepsis places a considerable burden on the healthcare expenditure and is known as the most expensive condition treated in the U.S. hospitals costing $23 billion in 2013 [1,4]. Sepsis is driven by the propagation of hyper-inflammatory responses to infection [5–7], and currently, there is no specific treatment for sepsis. Clinical interventions include anti-inflammatory therapies, such as corticosteroids, administration of antibiotics, fluid resuscitation, and mechanical ventilation, and recent clinical trials for sepsis have all failed [8]. Anti-inflammatory therapies are utilized in sepsis because bacterial components are major drivers of the inflammatory response. For example, lipopolysaccharide (LPS), the endotoxin on the outer membrane of Gram-negative bacteria, is a potent activator of the acute inflammatory response [9,10]. Although LPS-induced endotoxic shock does not adequately mimic human sepsis [11], it is a very good model to study the pathophysiological features of the systemic inflammatory response that accompanies sepsis and could reveal novel therapeutic targets in this regard [12].

Neutrophils are the first responders to infection and play a critical role in host immune defense [13–15]. During bacterial infection, neutrophils can undergo programmed cell death, termed NETosis, by externalizing their cellular contents in a DNA framework decorated with antibacterial proteins and serine proteases [16]. These DNA-protein architectures extruded from neutrophils are called neutrophil

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extracellular traps (NETs), which mainly provide a physical fibrous network to entangle bacteria or other pathogens and enhance neutrophil antimicrobial activity [16–18]. However, NETs have been described as a double-edged sword [19] because excess NETs produced by overtly activated neutrophils have been implicated in promoting various human pathologies, including sepsis [20], rheumatoid arthritis [21], diabetes [22], vasculitis [23], and cancer [24]. Therefore, there have been recent efforts to prevent NET formation or eliminate excess NETs as potential therapeutic approaches [25–27].

Neutrophil elastase (NE) is a proteolytic enzyme stored in the azurophilic granules of neutrophils whose name is derived from its ability to degrade the extracellular matrix protein elastin. In addition to elastin, NE also degrades other cellular matrix proteins, including collagen and fibronectin [28,29]. The ability of elastase to destroy the extracellular matrix, particularly in the lungs, has been well known for over three decades, and deficiency of the endogenous NE inhibitor, $\alpha 1$-antitrypsin is associated with the development of lung emphysema [30]. NE is also known to induce cell proliferation and activate several cytokine and chemokine signaling pathways [31–34]. Endogenous proteinase inhibitors, such as α-1 antitrypsin, Elafin, and secretory leucocyte protease inhibitor (SLPI), are readily mobilized to counter NE activity. However, studies have shown that NE released from azurophil granules can bind tightly to the plasma membrane with its catalytic activity preserved, thereby shielding it from the activity of circulating endogenous inhibitors [35]. Additionally, it has been shown long before NETs were discovered that NE bound to DNA is insensitive to protease inhibitors [36,37]. Although the molecular mechanisms leading to NETs formation are not completely understood, recent studies have shown that reactive oxygen species (ROS) can initiate the translocation of NE from the granules to the nucleus where NE processes histones, leading to NET release into the extracellular space [38–40]. Hence, NE is pivotal in the process of NET formation, and inhibition of NE activity may provide a viable approach for preventing NETs formation. DNAse can degrade away the DNA backbone of NETs, but DNAse does not inhibit the activity of proteins decorated on NETs [41].

Several pharmaceutical companies have been investigating different NE inhibitors for use in the clinic [42]. However, there is currently no NE inhibitor approved by the FDA, indicating the challenge in demonstrating the clinical efficacy of NE inhibitors. For example, a highly selective and fully reversible oral inhibitor of NE produced by Astrazeneca (AZD9668) failed to show improvement in a recently conducted phase II clinical trial of patients with chronic obstructive pulmonary disease (COPD) – a disease in which NE has been implicated [43]. Sivelestat is a second-generation NE inhibitor discovered by ONO pharmaceuticals in Japan and is clinically available in Japan and South Korea for patients with acute lung injury (ALI) associated with systemic inflammatory response. A multi-national clinical trial of sivelestat on patients with ALI was deemed unsuccessful, and efforts to expand the use of sivelestat to other countries have failed [44]. Thus, there is a need for a new approach to improve the efficacy of sivelestat.

Nanoparticles have emerged as a veritable approach for the delivery of therapeutics to cells with the goal of achieving specificity and reducing systemic toxicity [45]. Since NE is stored in the granules of neutrophils, we hypothesized that nanoparticle-mediated delivery of NE inhibitor to neutrophils would improve its therapeutic efficacy. We previously described Interbilayer-Crosslinked Multilamellar Vesicles (ICMVs) as a new class of lipid-based nanoparticles with attractive features for targeted drug delivery [46]. Here, we show that ICMVs loaded with sivelestat (ICMV-Sive) are readily taken up by neutrophils and effectively inhibit NET formation *in vitro*. We also demonstrate that ICMV-Sive inhibits NET formation and extend animal survival in an *in vivo* model of endotoxic shock. Taken together, our data highlights the significance of NE in NET formation and suggests that nanoparticle-mediated delivery of sivelestat is a promising strategy for preventing NET formation in the context of endotoxic shock.

1. Materials and methods

1.1. Animal experiments

The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Michigan, Ann Arbor. Four to six-week old female BALB/c mice were purchased from The Jackson Laboratory. Mice were maintained at the University of Michigan Animal Care facility under specific pathogen-free conditions. Mice were allowed to acclimatize for one week before experiments were started. LPS Escherichia coli 0111:B4 (Sigma Aldrich) was administered by intraperitoneal route (20 mg/kg). Mice were injected with 50 mg/kg sivelestat (Cayman Chemical) 1 h after LPS challenge in the form of free drug or ICMV loaded drug. In some animals, blank ICMV were injected as a control. Animals challenged with LPS were monitored periodically for clinical signs and were assigned scores to indicate disease severity. Mice were monitored for movement, body condition, and alertness. Disease severity was scored in a semi-quantitative fashion as previously described [47] as follows: 0, = no abnormal clinical sign; 1, = ruffled fur but lively; 2, = ruffled fur, moving slowly, hunched, and sick; 3, = ruffled fur, squinted eye, hardly moving, down and very sick; 4, = moribund; and 5, = dead. A clinical score of 4 was used as the humane endpoint.

For biodistribution studies, groups of mice were injected with LPS (20 mg/kg, i.p). After 1 h, animals were injected with DiR-labelled ICMV-Sive intraperitoneally. Mice were sacrificed after 12 h, and major organs, including heart, liver, spleen, lungs and kidney, were harvested and imaged using the IVIS optical imaging system. For biochemical analysis of animal serum, LPS-challenged mice injected with free sivelestat or ICMV-Sive were sacrificed after 12 h. Blood was collected by cardiac puncture, and the levels of aspartate aminotransferase (AST) and creatinine in animal serum were measured.

1.2. Neutrophil isolation

Blood was collected from the peripheral vein of healthy volunteer donors into heparin tubes, and neutrophils were obtained by first spinning heparinized blood on Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) then subjecting the red blood cell (RBC) layer to 1.5% dextran sedimentation, followed by hypotonic lysis as previously described [48]. Isolated neutrophils were washed with PBS before use. Murine bone marrow-derived neutrophils were isolated, as previously described [49]. Briefly, femur and tibia were obtained, freed of muscle tissue and flushed with supplemented Hanks' balanced saline solution (HBSS; 1X HBSS, 0.5% fetal bovine serum (FBS) and 20 mM HEPES) using 10 ml syringe and 30-gauge needle. Cells were pipetted up and down to obtain a single-cell suspension and centrifuged at 300 g for 5 min. RBCs were lysed as above, and cells were then centrifuged on a discontinuous gradient of 52%, 69%, and 78% Percoll (GE Healthcare) diluted in HBSS (100% Percoll = 9 parts Percoll and 1 part 10X HBSS) and centrifuged (1500 g, 30 min, without brake). Neutrophils from the 69%/78% interface were collected, washed in PBS, and resuspended in complete RPMI medium for use.

1.3. NET production, quantification, and microscopy

NETs were generated and quantified as previously described [16,50,51]. Briefly, to generate NETs, isolated neutrophils were resuspended in RPMI 1640 medium supplemented with 3% fetal bovine serum (NET medium) and 2×10^6 neutrophils per well were seeded on 6 well plates. The cells were activated for 4 h with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) at 37 °C. The supernatant was carefully removed, and the smear on the bottom of the wells was collected by vigorous agitation with fresh 2 ml of media. Samples were centrifuged at 100 g, and the NET supernatant was collected. The DNA content of the NET was quantified using a Take3 Trio micro-volume

plate (Biotek Instruments) according to the manufacturer's instructions. To quantify NETs, 0.1×10^6 neutrophils were plated in 96 well plates. The cells were incubated in NET medium in the presence or absence of free sivelestat or ICMV-Sive at the indicated concentration. NET formation was induced with 100 nM PMA or 5 μM recombinant human NE for 4 h at 37 °C. NET was quantified by measuring the fluorescence intensity of extracellular DNA released in culture using 1 μM Sytox Green. The release of NE was also determined using 0.5 mM of the fluorogenic elastase substrate (Z-Ala-Ala-Ala-Ala)2Rhodamine110 (Cayman Chemical). Fluorescence intensity was measured using a fluorescent plate reader (Synergy Neo, BioTek Instruments). For fluorescence microscopy analysis, neutrophils were plated 20,000 cells/well and cultured as above to induce NETs. Cells were fixed with 4% paraformaldehyde (PFA), stained with 1 μM Sytox Green for 15 min, and images were captured using a Nikon TiU microscope with attached CCD camera. In some experiments, images were analyzed using a size-restricted circle finding algorithm based on MATLAB script developed in-house. The area of each object in a given field of view was calculated. NET formation was also visualized using confocal microscopy. Neutrophils were seeded on 18 mm coverslips coated with 0.001% poly-L-lysine (Sigma-Aldrich). Neutrophils were then incubated for 4 h at 37 °C as described above in the presence/absence of sivelestat (20 μM) or DNAse (20 units/ml) with 100 nM PMA. Cells were fixed with 4% PFA and blocked with 10% FBS. DNA was stained with Sytox Green, and immunohistochemistry was performed with anti-NE IgG conjugated to Alexa Fluor 647 (Santa Cruz Biotechnology). The coverslips were mounted onto slides using Prolong Diamond Anti-fade media (Fisher Scientific), and images were acquired using a Nikon A1 confocal microscope.

1.4. Treatment of endothelial cell with NETs and flow cytometry

Human umbilical vein endothelial cells (HUVECs) were obtained from Angio-Proteomie. Cells were cultured in T75 flasks until confluent. Thereafter, 0.1×10^6 cells were seeded unto 12 well plates until 90% confluent. The cells were washed with PBS and cultured with NET supernatant containing 20 μg/ml DNA in the presence or absence of sivelestat (20 μM) for 12 h. The cells were trypsinized, washed twice with fluorescence-activated cell sorting (FACS) buffer containing 1% bovine serum albumin (BSA). The cells were incubated with anti-CD16/32 blocking antibodies and stained with CD54 (ICAM-1) antibody conjugated to allophycocyanin (APC) on ice for 20 min. Cells were washed and resuspended in FACS buffer containing 1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) and analyzed by flow cytometry. Flow cytometry data was analyzed using Flowjo software (Tree Star Inc, Ashland, OR).

1.5. Synthesis and characterization of ICMVs

ICMVs loaded with sivelestat were synthesized as previously reported with slight modifications [46,52]. Briefly, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] sodium salt (MPB) (Avanti Polar Lipids) were mixed in 1:1 M ratios and dried under vacuum to produce thin films. In some experiments, 0.2 M percent of the lipophilic fluorophores 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt (DiD, Fisher Scientific) or 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR, Fisher Scientific) was added. The dried lipid films were hydrated in the presence of sivelestat to facilitate drug encapsulation. To induce vesicle fusion and crosslinking, $CaCl_2$ (40 mM) and dithiothreitol (DTT; 15 mM) were added. The resulting ICMVs were centrifuged at $18000 \times g$ for 5 min at 4 °C to pellet the particles, and the supernatant containing unloaded sivelestat was removed. Finally, the ICMV pellets were washed with DNA grade water (Fisher Scientific) and resuspended in PBS. To determine the amount of drug loaded in ICMVs,

the particles were dissolved with methanol, and the drug concentration was determined by High Performance Liquid Chromatography (HPLC). Particle diameter and zeta potential were measured by dynamic light scattering (DLS) using a Malvern ZetaSizer Nano ZSP.

1.6. Assessment of neutrophil particle uptake

Isolated neutrophils were activated with 10 ng/ml TNF-α for 30 min and incubated with ICMVs labelled with DID in 96 well plates or 18 mm slides coated with 0.001% poly-L-lysine for 1 h. After incubation, cells were fixed with 4% PFA and washed twice to remove free particles. For flow cytometry analysis, cells were incubated with anti-CD16/32 blocking antibodies and stained with Ly6G antibody conjugated to Phycoerythrin (PE) on ice for 20 min. For analysis by confocal microscopy, cells were incubated with Hoechst 33342 after fixing and observed under a confocal microscope.

1.7. Histology studies

For histopathological analysis, the right lung lobes were fixed in 4% PFA and embedded in paraffin. Five-micron sections were placed onto glass slides and stained with hematoxylin and eosin (H&E) for microscopy analysis. To identify NET formation in lungs *in vivo*, 5 μm sections of paraffin-embedded mouse lungs were prepared and mounted on glass slides. After dewaxing, samples were permeabilized with 0.1% Triton X-100 for 10 min and blocked with PBS containing 1% BSA and 0.1% Tween-20. The sections were incubated with primary antibodies – anti-citrullinated-histone H3 (1:100; Abcam) and anti-NE (1:50; Abcam), followed by detection with Alexa Fluor 488 goat anti-rat (1:500; Abcam) and Alexa Fluor 568 goat anti-rabbit (1:500; Abcam) secondary antibodies for 1 h at room temperature. Samples were also stained with DAPI for DNA detection.

1.8. Statistical analysis

All data were plotted and analyzed using GraphPad Prism software version 5.0 (La Jolla, CA). A Kaplan Meier survival curve plot was used for the survival data, and the *P* values were determined using Mantel-Cox test. Differences between data sets were analyzed by performing one-way ANOVA followed by Tukey's multiple-comparisons test. Differences were considered significant if $P \leq 0.05$.

2. Results

2.1. Sivelestat inhibits NET formation

As NETs are implicated in numerous human pathologies, there is significant interest to inhibit NET formation. We investigated the ability of sivelestat to inhibit NET formation. Activation of human neutrophils with phorbol 12-myristate 13-acetate (PMA) resulted in the formation of NETs as measured by extracellular DNA content with the cell impermeable dye, Sytox green (Fig. 1A). As expected, addition of DNAse to PMA-activated neutrophils resulted in a decreased Sytox green signal (Fig. 1A). Importantly, human neutrophils treated with both PMA and sivelestat exhibited a significantly decreased Sytox green signal, compared with the PMA control group ($P < 0.001$, Fig. 1A), suggesting strong inhibition of NET formation by sivelestat. We also quantified the release of NE during NET formation with a fluorogenic elastase substrate. NET formation by PMA-activated neutrophils resulted in a high elastase signal (Fig. 1B), but sivelestat significantly decreased the elastase signal from PMA-activated neutrophils ($P < 0.001$, Fig. 1B). To confirm these results, we visualized human neutrophils undergoing NET formation with confocal microscopy. As expected, human neutrophils activated with PMA released extracellular DNA, which appeared as fibrous strands (Fig. 1C). Sivelestat treatment effectively decreased these extracellular NET structures (Fig. 1C, Fig. S1). These
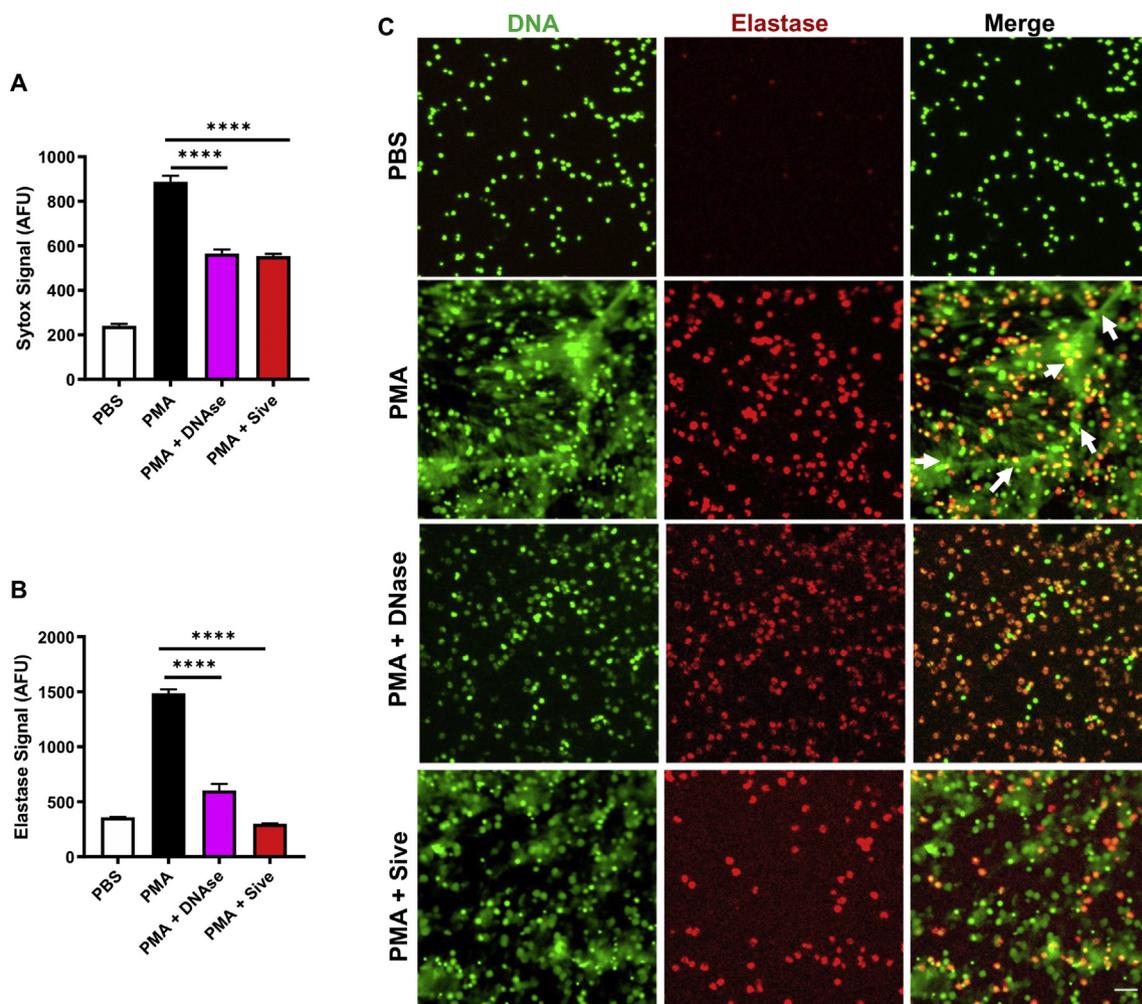
Fig. 1. Inhibition of neutrophil elastase prevents NET formation. Neutrophils were purified from human peripheral blood and activated with PMA in 96 well plates in the presence or absence of sivelestat (10 μM) or DNAse (20 units/ml) for 4 h. The release of extracellular DNA or neutrophil elastase was quantified by the addition of 1 μM Sytox Green and 0.5 mM of the elastase substrate (Z-Ala-Ala-Ala-Ala)2Rhodamine110, respectively, and analyzed using a fluorescent plate reader (A, B). Cells were seeded on microscopic slides, and immunofluorescence stain of NET formation was analyzed by confocal microscopy as the colocalization of extracellular DNA and neutrophil elastase (C). White arrows depict NETs. The data presented are representative of 3 independent experiments with similar results. ****, $P < 0.0001$ analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparisons test. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

results are in line with previous studies showing prevention of NET formation in NE knockout mice [38,41].

Different signaling pathways have been shown to induce NETs, including PMA, LPS, IL-8, and microbes [53]. Given the broad-spectrum activities of NE and its ability to activate various cellular signaling pathways [31], we examined whether NE could induce NET formation. We cultured human neutrophils in the presence of recombinant human NE and quantified NET release by measuring the extracellular DNA content using the Sytox green assay. Indeed, we found that elastase treatment triggered robust NET formation (Fig. 2A). Importantly, addition of sivelestat during elastase treatment effectively reduced NET formation ($P < 0.001$, Fig. 2A and B). To further investigate the role of NE in NET formation, we isolated neutrophils from the bone marrow of wild type (WT) and NE knockout (KO) mice and activated them to form NETs. Consistent with previous reports [41], NE KO mice demonstrated impaired ability to form NETs, compared to WT mice (Fig. 2C and D). Overall, these results suggest that NE released during NET formation can induce *de novo* NETs, thereby constituting a feed-forward loop for the propagation of NET formation.

2.2. Sivelestat reduces NET-associated cytotoxicity and inflammatory responses

Cytotoxicity of NETs to various cells, including endothelial and epithelial cells, is a major factor in NET-associated pathologies [54,55]. We investigated whether sivelestat could reverse NET-induced endothelial damage. NETs were harvested from activated human neutrophils and cultured with a monolayer of human umbilical vein endothelial cells (HUVECs) in the presence or absence of sivelestat. As expected, NETs induced endothelial cell death (Fig. 3A and B). Interestingly, NET-induced endothelial damage was reversed by sivelestat treatment ($P < 0.0001$, Fig. 3A and B). Additionally, NETs increased the expression of neutrophil adhesion molecule – Intercellular Adhesion Molecule - 1 (ICAM-1) on HUVECs, which was reversed by treatment with sivelestat although this was not statistically significant (Fig. 3C). The upregulation of ICAM-1 by NETs suggests that NETs can propagate the inflammatory response of endothelial cells. Therefore, we investigated the ability of sivelestat to inhibit inflammatory responses in neutrophils activated by another NET inducer – LPS. We isolated neutrophils from murine bone marrow and activated them with LPS in the presence or absence of sivelestat. LPS induced the production of NE and other pro-inflammatory cytokines and chemokines, including G-CFS,
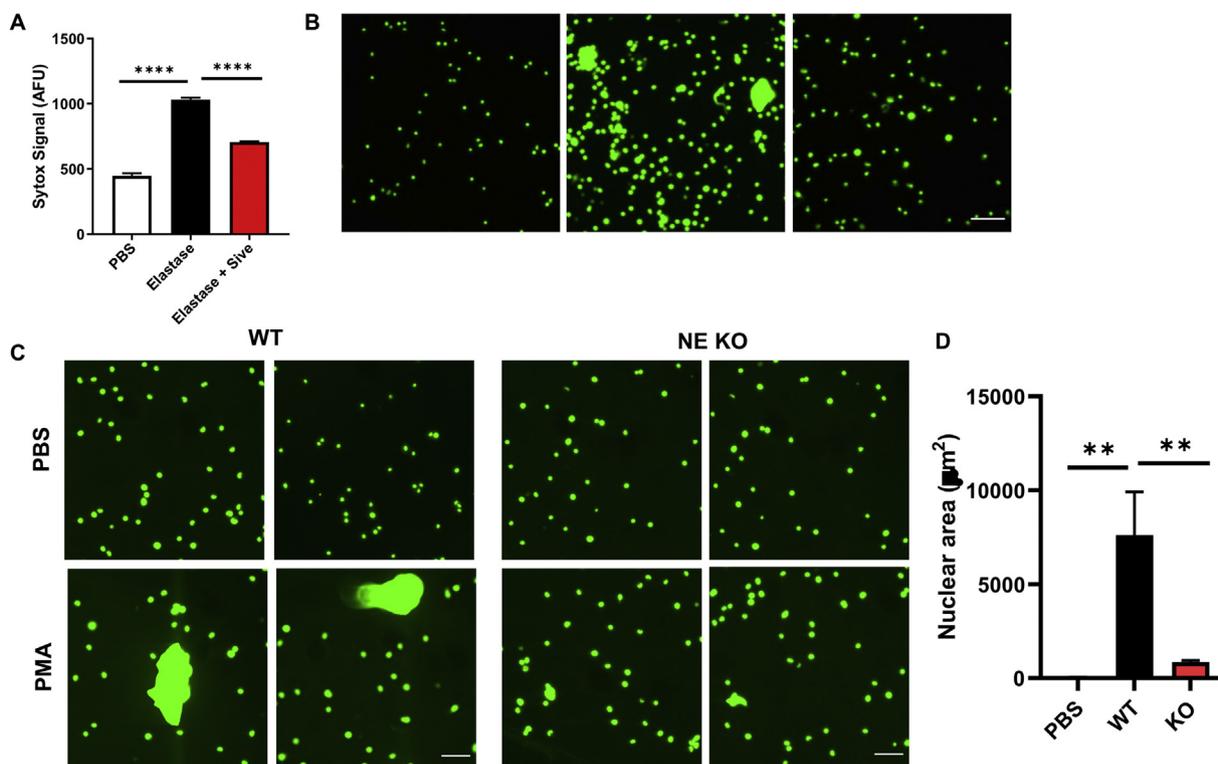
Fig. 2. Neutrophil elastase induces NETs. Human neutrophils were cultured in the presence of 5 μM recombinant human elastase for 4 h in the presence or absence of sivelestat. The release of extracellular DNA was quantified by the addition of 1 μM Sytox Green and analyzed using a fluorescence plate reader (A). For fluorescence microscopy, cells were fixed with 4% PFA before the addition of Sytox Green (B). Murine bone marrow-derived neutrophils from WT or NE KO mice were cultured with PMA to induce NETs. After 4 h, cells were fixed with 4% PFA and visualized under microscopy (C). Area of each object in microscopic field of view was calculated using an algorithm developed in-house (D). The data presented are representative of 3 independent experiments with similar results. **, $P < 0.01$; ****, $P < 0.0001$ analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparisons test. Scale bar = 60 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
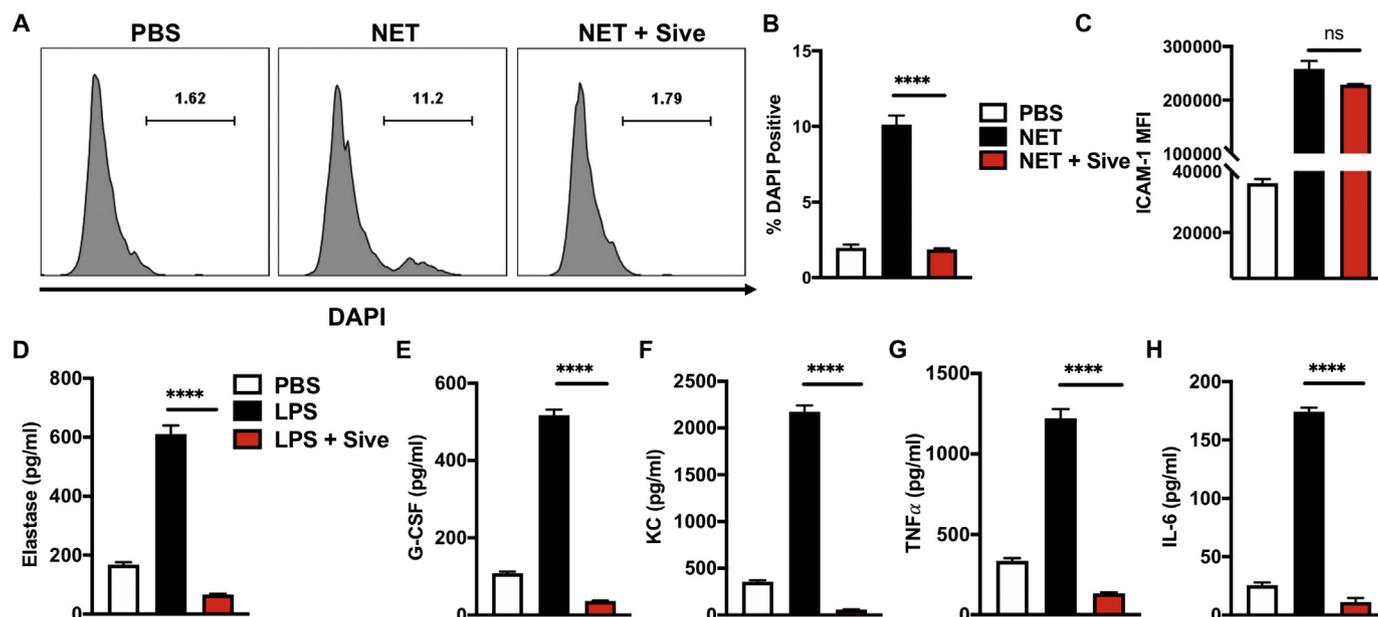

Fig. 3. Sivelestat inhibits NETs-induced endothelial damage and proinflammatory cytokine production. Human umbilical vein endothelial cells (HUVECs) were cultured with NET supernatant derived from human neutrophils containing 20 μg/ml DNA in the presence or absence of sivelestat for 12 h. Cell viability (A, B) and the upregulation of endothelial adhesion molecule ICAM-1 (C) were analyzed by flow cytometry. Murine bone marrow-derived neutrophils were cultured with LPS 100 ng/ml in the presence or absence of sivelestat for 12 h. The levels of proinflammatory cytokines in the culture supernatant were analyzed by ELISA (D–H). The data presented are representative of 3 independent experiments with similar results. ****, $P < 0.0001$ analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparisons test.
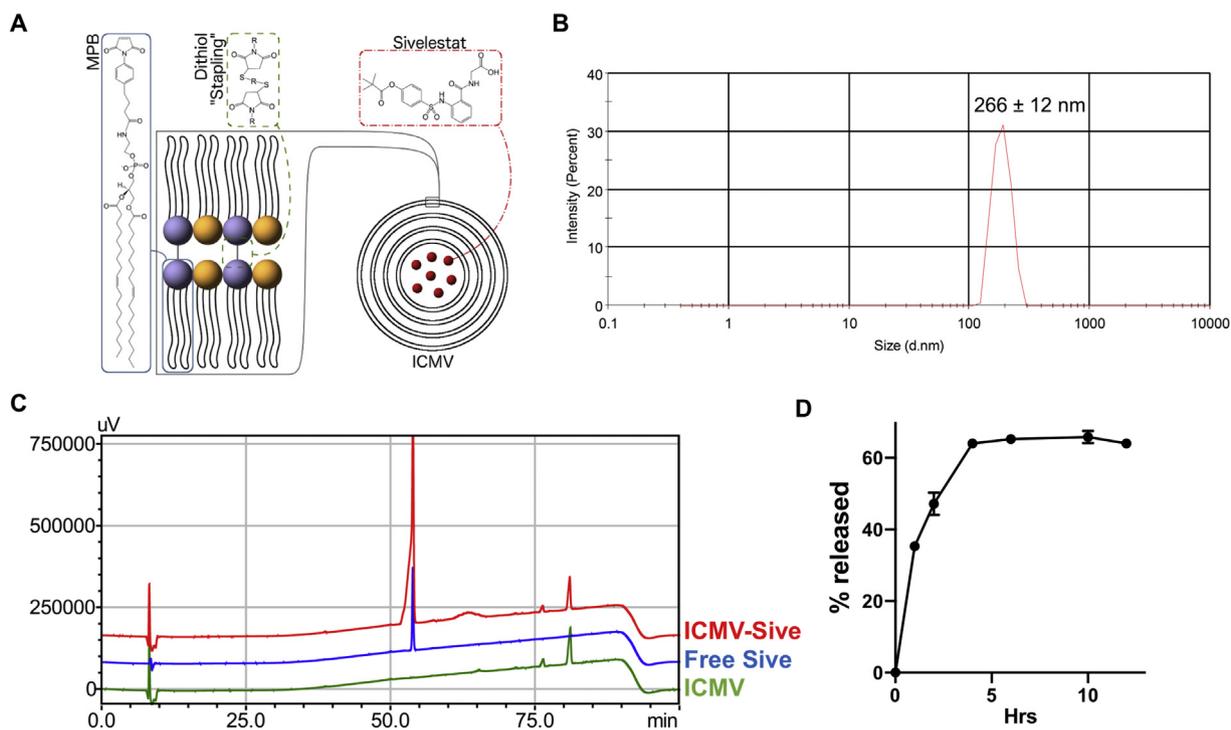
Fig. 4. Synthesis and characterization of sivelestat loaded ICMVs. ICMVs were synthesized following the scheme shown in A. The size of sivelestat-loaded ICMVs was determined by dynamic light scattering analysis (B). Quantification of sivelestat encapsulated in ICMVs was analyzed by HPLC (C). Kinetics of sivelestat release from ICMVs was studied in media containing 10% FBS, and the drug release was quantified using LC-MS (D). The data presented are representative of 3 independent experiments with similar results.

KC, TNF-α, and IL-6 (Fig. 3D–H). Importantly, sivelestat treatment significantly inhibited the production of NE, and inflammatory cytokines and chemokines from LPS-treated neutrophils ($P < 0.0001$, Fig. 3D–H). Taken together, our data show sivelestat prevents NET formation and NET-associated inflammatory responses.

2.3. ICMVs delivering sivelestat inhibits NETs in vivo

Although Sivelestat is clinically available in Japan and Korea for the prevention of acute lung injury in patients, a recently concluded clinical trial aimed to expand its use in North America and Europe was declared unsuccessful [44]. Since sivelestat has poor pharmacokinetics and requires continuous infusion in human and animal models [56,57], we reasoned that targeted delivery of sivelestat to neutrophils would improve its efficacy in prevention of ALI and NET formation. We sought to use ICMVs for delivery of sivelestat to neutrophils. ICMVs encapsulating sivelestat were synthesized as previously described [46,52]. Briefly, dried lipid films containing DOPC, the anionic maleimide-headgroup lipid MPB and sivelestat dissolved in methanol were hydrated to make simple liposomes. After Ca^{2+}-mediated vesicle fusion, dithiothreitol (DTT) was added to the vesicle suspension to crosslink maleimide headgroups of juxtaposed membranes and form ICMVs (Fig. 4A). Sivelestat was incorporated into ICMVs with an encapsulation efficiency of 60 ± 5% as determined by high-performance liquid chromatography (HPLC) (Fig. 4B). ICMVs loaded with sivelestat (ICMV-Sive) exhibited a homogenous hydrodynamic size of 266 ± 12 nm and a polydispersity (PDI) of 0.20 ± 0.04, as measured by dynamic light scattering (Fig. 4C) with a zeta potential of −41.8 ± 7.1 mV. ICMV-Sive incubated at 37 °C in 10% FBS released ~65% of sivelestat within 12 h (Fig. 4D).

To examine the ability of neutrophils to internalize ICMVs, murine neutrophils were incubated with fluorescently tagged ICMVs for 1 h. Samples were then centrifuged and the supernatant discarded to remove free particles. Neutrophils readily phagocytosed ICMVs with >

65% of neutrophils associating with ICMVs within 1 h of incubation (Fig. 5A). Confocal microscopy confirmed that ICMVs were internalized into neutrophils (Fig. 5B). Based on the efficient internalization of ICMVs by neutrophils, we hypothesized that ICMV-Sive would show an increased efficacy to inhibit NET formation, compared with free sivelestat. Previous studies have shown that activation of neutrophils increases their internalization ability [58]. To increase the uptake of the nanoparticles by neutrophils, cells were pretreated with TNF-α for 30 min before incubation with ICMV-Sive or free drug for only 10 min. Samples were then washed twice and the supernatant discarded to allow for the sole presence of internalized particles. NET formation was then induced with PMA for 4 h. Pre-treatment with ICMV-Sive significantly reduced the release of extracellular DNA ($P < 0.05$) and NE ($P < 0.001$) from PMA-treated neutrophils, compared to the free sivelestat treatment (Fig. 5C and D).

To demonstrate the efficacy of ICMV-Sive *in vivo*, we used an LPS model of endotoxic shock [12]. LPS was injected into mice intraperitoneally, and after 1 h of LPS injection, animals were administered i.p. with free sivelestat, blank ICMV, or ICMV-Sive. Animals were monitored for clinical signs and survival. At sacrifice, peritoneal lavage, blood, and lungs were collected for analysis. Strikingly, ICMV-Sive showed greater efficacy in the reduction of clinical signs ($P < 0.001$, Fig. 6A) and improvement in survival of mice ($P < 0.05$, Fig. 6B), compared to the free sivelestat control group. To investigate the ability of ICMV-Sive to inhibit NET formation *in vivo*, we performed immunofluorescence staining of paraffin-embedded mouse lung sections. NETs were identified by the co-localization of extracellular DNA, with NE and citrullinated-histone H3 (Cit-H3). ICMV-Sive showed greater reduction in NETs formation, compared to the free drug (Fig. 6C). Concomitantly, ICMV-Sive reduced lung injury as evidenced by reduced infiltration of inflammatory cells to the lungs, hemorrhage, and interstitial edema (Fig. 7A). This was accompanied by greater reduction of NE and other proinflammatory cytokines in the serum of the animals (Fig. 7B–D). To investigate the biodistribution and biosafety of ICMV-
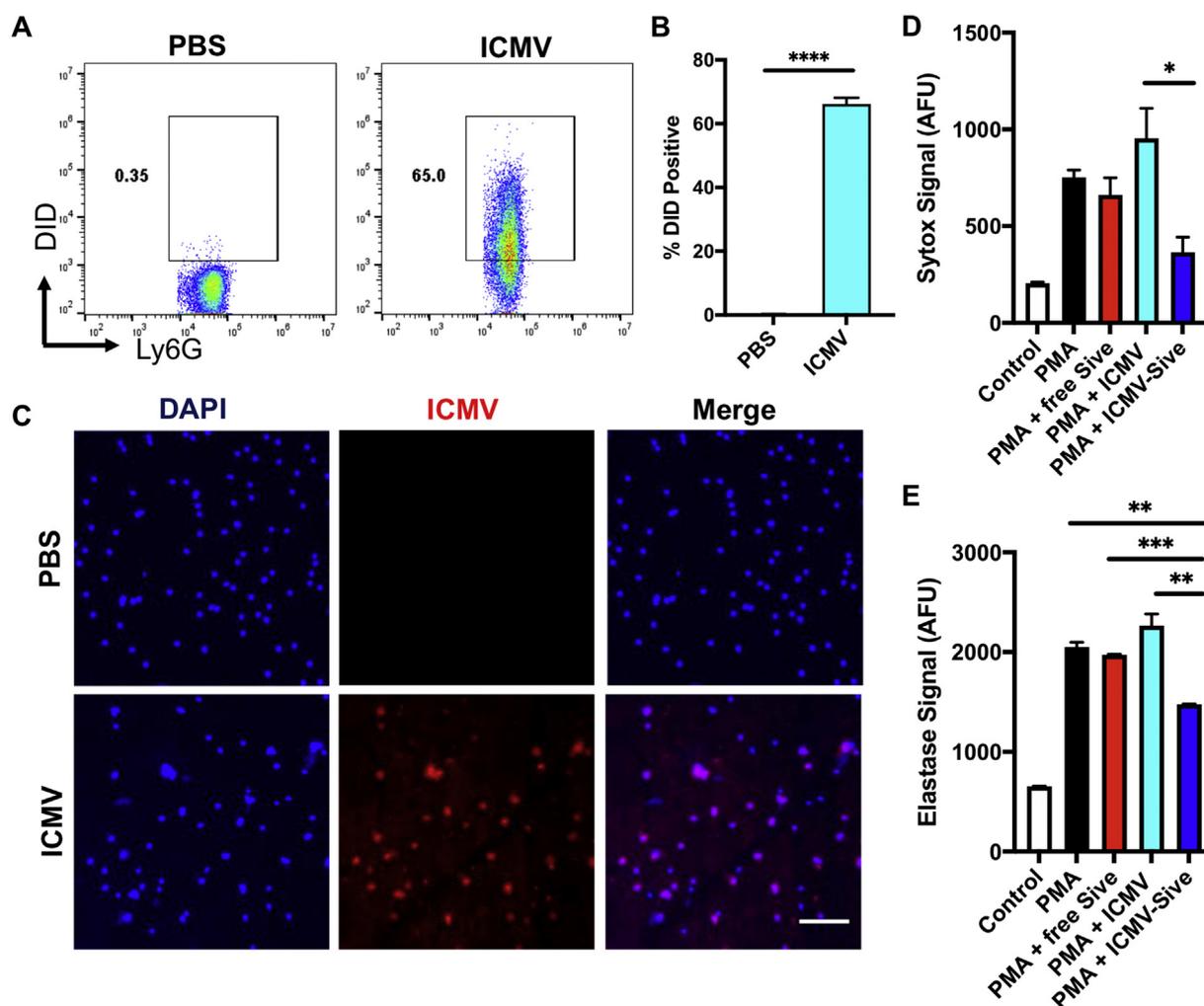
Fig. 5. Inhibition of NET formation by ICMV-sivelestat. Activated murine neutrophils were incubated for 1 h with ICMVs labelled with DID. Cells were fixed with 4% PFA, washed twice and particle uptake was analyzed by flow cytometry (A, B) or confocal microscopy following staining with Hoechst 33342 (C). Activated neutrophils were cultured for 10 min with blank ICMV, ICMVs loaded with sivelestat or free sivelestat. Cells were washed twice, followed by activation with 100 nM PMA for 4 h. The release of extracellular DNA or neutrophil elastase was quantified by the addition of 1 μM Sytox Green and 0.5 mM of the elastase substrate (Z-Ala-Ala-Ala-Ala)2Rhodamine110, respectively, and analyzed using a fluorescence plate reader (D, E). The data presented are representative of 3 independent experiments with similar results. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparisons test. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Sive, mice were challenged with LPS and 1 h later, fluorescently labelled ICMV-Sive was administered. After 12 h, biodistribution of ICMV-Sive in major organs was examined using an *in vivo* imaging system (IVIS). ICMV-Sive mostly accumulated in the liver and spleen (Fig. 7E and F). Serum analysis indicated that injection of free sivelestat or ICMV-Sive did not elevate serum levels of AST and creatinine, compared with PBS-treated mice, indicating no major toxicity or abnormal liver and kidney functions in treated animals (Fig. 7G and H). Interestingly, ICMV-Sive also led to decreased neutrophil infiltration in the peritoneum (Fig. S2). Overall, our data indicated that nanoparticle-mediated delivery of sivelestat effectively decreased NET formation and clinical signs of lung injury in a murine model of LPS-induced endotoxic shock.

3. Discussion

The formation of NETs by neutrophils was discovered as a novel mechanism of inhibiting microbial function [16]. However, excess NET formation has been implicated in the pathologies of several diseases from inflammation to cancer. Hence, there is significant interest to inhibit NET formation in order to limit bystander tissue injury. DNAse I

was one of the first inhibitors of NETs formation that was described and is indeed clinically available for the treatment of cystic fibrosis, a disease in which lung damage is mediated by NET formation [59,60]. It is now known that DNAse does not inhibit the functions of NET-associated proteases and NETs treated with DNAse can still induce tissue injury [41,50]. Thus, it remains unknown how to effectively target and inhibit NETs.

Before NETs were discovered, Tkalcevic et al., reported that NE knockout mice are resistant to LPS-induced endotoxic shock [61]. Our findings are in line with the work of Tkalcevic et al. and further highlights NETs formation as a major contributor to mortality in endotoxic shock. Consistent with the results of this study, Nakamura et al. showed that mice deficient in SLP1, the endogenous inhibitor of neutrophil elastase are more susceptible to LPS-induced endotoxic shock [62]. Furthermore, Kolaczkowska et al., showed that NE knockout mice do not form NETs in a sepsis model [41]. Overall, the overwhelming evidence indicates that genetic and pharmacological inhibition of NE prevents NET formation.

NET formation has been shown to be triggered by different stimuli, including microbes, PMA, LPS, and cytokines. Indeed, the degree and kinetics of NET formation are dependent on the originating stimulus
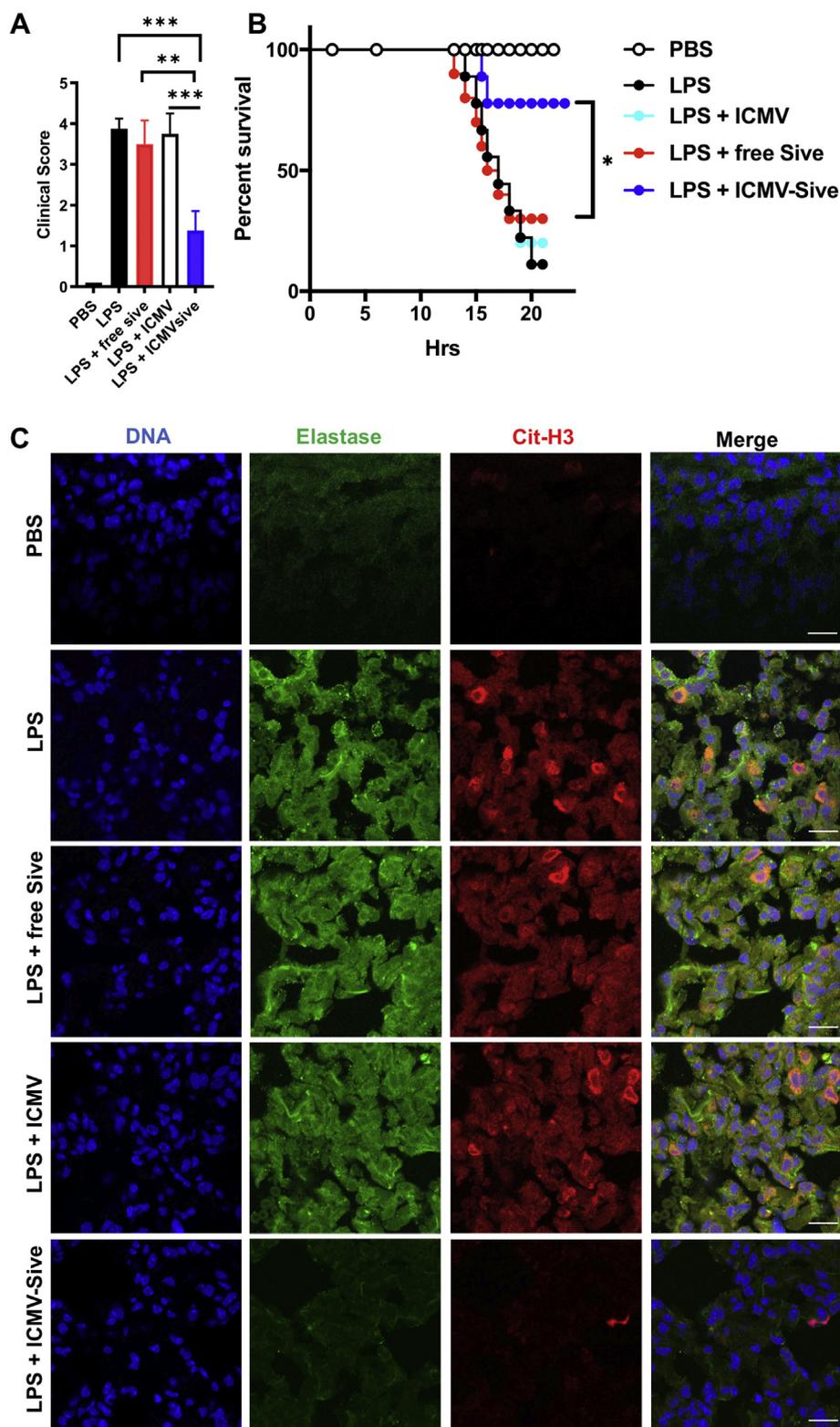
Fig. 6. ICMV-sivelestat rescues mice from LPS-induced mortality. Groups of mice (n = 10) were injected with LPS (20 mg/kg) or PBS. After 1 h, mice were administered i.p. with blank ICMV, ICMVs loaded with sivelestat or free sivelestat and monitored for clinical signs and survival (A, B). In a separate experiment, immunofluorescence staining was performed on lung sections from mice (n = 5) and analyzed by confocal microscopy. Staining depicts DAPI (blue), NE (green) and Cit-H3 (red). Colocalization of all three markers indicate NET formation. The data presented are representative of 3 independent experiments. (A) ***, $P < 0.001$ analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparisons test. (B) *, $P < 0.05$, analyzed by log rank (Mantel−Cox) test. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and the signaling pathway initiated [53,63]. Our data showed that NE induces NET formation in human neutrophils. Hence, NET formation induced by one stimulus can lead to the production of NE, which in turn promotes *de novo* NET formation and exacerbates tissue damage. This is in line with recent reports that NE signaling constitutes a feed-forward loop that drives NET formation [64]. It is speculated that NE contributes to the NET formation by activating the membrane pore-forming protein gasdermin D (GSDMD) [64]. Activated GSDMD perforates the

granule membrane, increasing the release of NE and enabling the translocation of NE to the nucleus, where it processes histones and allows nuclear expansion [40,64].

Based on these findings, here we have sought an alternative strategy of inhibiting NET formation by targeting NE. We have shown that inhibition of NE signaling hinders the NET formation, reduces NET-mediated vascular damage, and alleviates the production of inflammatory cytokines. Since NE inhibition prevents the release of
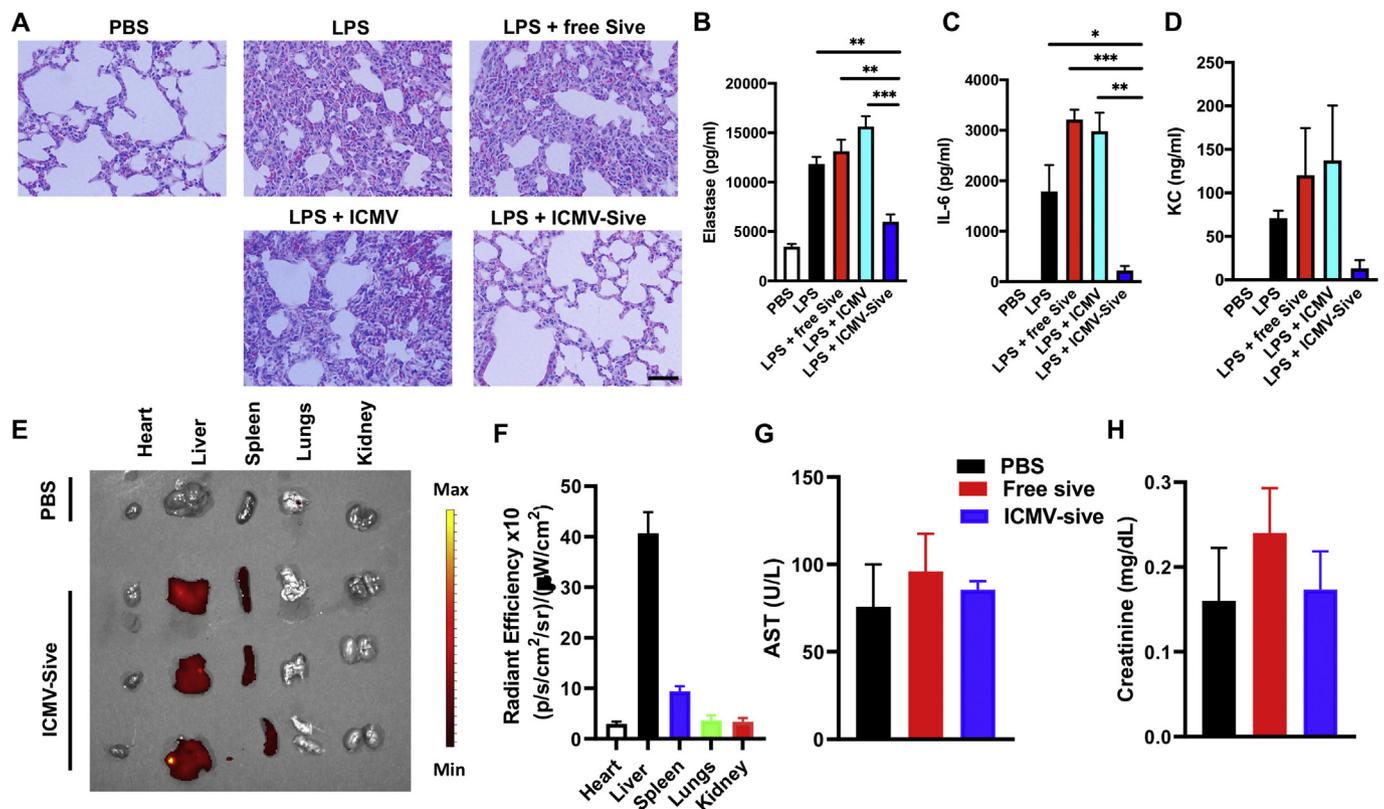
Fig. 7. ICMV-sivelestat prevents lung damage and proinflammatory cytokine production. Groups of mice (n = 5) were injected with LPS (20 mg/kg) or PBS. After 1 h, mice were administered i.p. with blank ICMV, ICMV- sivelestat, or free sivelestat. Animals were sacrificed after 18 h and H&E staining done on lung sections (A) (400x). The levels of NE, IL-6 and KC in animal serum were analyzed by ELISA (B–D). LPS-challenged mice were injected with ICMV-Sive labelled with DiR. Animals were sacrificed after 12 h, and the level of fluorescence in the major organs, including heart, liver, spleen, lungs and kidney, were analyzed using IVIS imaging system (E, F). The levels of AST and creatinine in animal serum were also measured (G, H). The data presented are representative of 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparisons test. Scale bar = 5000 μm.

extracellular DNA and inhibits the function of NE, which is a major protease on NETs, we argue that NE inhibition is a promising approach for reducing NET-mediated tissue injury. Indeed, the role of NE in disease pathology has been well-documented [65,66]. For example, *P aeruginosa*, the most common pathogen in the lung of cystic fibrosis patients, has been shown to propagate tissue destruction by release of elastase [67,68]. In addition, sepsis is associated with higher levels of NE in serum [69,70]. However, despite concerted efforts for the development of NE inhibitors, none has been successful in clinical trials. Notable challenges in the clinical utility of NE inhibitors include the fact that NE bound to DNA in NETs is resistant to inhibitor activity [36]. Additionally, like sivelestat, most NE inhibitors function extracellularly and inhibit the action of NE released into the extracellular space.

To address the poor efficacy of NE inhibitors, we utilized ICMVs for neutrophil-targeted delivery of sivelestat, a potent NE inhibitor. We have previously shown that ICMVs have attractive features for drug delivery; compared to other lipid delivery systems such as liposomes, ICMVs exhibit greater encapsulation efficiency and greater retention of drug cargo in serum conditions [46]. In this study, we show that ICMV-mediated delivery of sivelestat promoted drug uptake by neutrophils and significantly improved the efficacy of sivelestat to inhibit NET formation, compared with free drug. In addition, we have shown that nanoparticle-mediated delivery of a NE inhibitor effectively rescued mice from LPS-induced endotoxic shock. Thus, our data suggest that nanoparticle-mediated delivery of NE is a viable strategy to inhibit NET formation and may significantly improve the efficacy of NE inhibitors.

In conclusion, we have demonstrated that inhibition of NE prevented NET formation and rescued animals from LPS-induced endotoxic shock. Further research is warranted to explore the therapeutic potential of NE inhibitors for not only sepsis but also other diseases, such as cancer, rheumatoid arthritis, and systemic lupus erythematosus, where NETs are known to play crucial pathogenic roles.

Data availability

The data supporting the findings of this study are available within the article and its Supplementary Information files. All relevant data can be provided by the authors upon reasonable request.

Declaration of competing interest

The authors declare no competing financial interest.

Acknowledgment

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2020.119836.

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