Proinflammatory role of neutrophil extracellular traps in abdominal sepsis

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Luo L, Zhang S, Wang Y, Rahman M, Syk I, Zhang E, Thorlacius H. Proinflammatory role of neutrophil extracellular traps in abdominal sepsis. Am J Physiol Lung Cell Mol Physiol 307: L586–L596, 2014. —Excessive neutrophil activation is a major component in septic lung injury. Neutrophil-derived DNA may form extracellular traps in response to bacterial invasions. The aim of the present study was to investigate the potential role of neutrophil extracellular traps (NETs) in septic lung injury. Male C57BL/6 mice were treated with recombinant human (rh)DNAase (5 mg/kg) after cecal ligation and puncture (CLP). Extracellular DNA was stained by Sytox green, and NET formation was quantified by confocal microscopy and cell-free DNA in plasma, peritoneal cavity, and lung. Blood, peritoneal fluid, and lung tissue were harvested for analysis of neutrophil infiltration, NET levels, tissue injury, as well as CXC chemokine and cytokine formation. We observed that CLP caused increased formation of NETs in plasma, peritoneal cavity, and lung. Administration of rhDNAase not only eliminated NET formation in plasma, peritoneal cavity, and bronchoalveolar space but also reduced lung edema and tissue damage 24 h after CLP induction. Moreover, treatment with rhDNAase decreased CLP-induced formation of CXC chemokines, IL-6, and high-mobility group box 1 (HMGB1) in plasma, as well as CXC chemokines and IL-6 in the lung. In vitro, we found that neutrophil-derived NETs had the capacity to stimulate secretion of CXCL2, TNF-α, and HMGB1 from alveolar macrophages. Taken together, our findings show that NETs regulate pulmonary infiltration of neutrophils and tissue injury via formation of proinflammatory compounds in abdominal sepsis. Thus we conclude that NETs exert a proinflammatory role in septic lung injury.

SEVERE CASES OF ABDOMINAL SEPSIS are associated with high morbidity and mortality in intensive care units (19). A common cause of death in patients with sepsis is infectious complications (35, 60). Besides antibiotics, management of patients with sepsis is largely restricted to supportive therapies and poses a major challenge to clinicians, which is partly due to an incomplete understanding of the underlying pathophysiology. Intestinal perforation contaminates the peritoneal cavity with toxins and microbes, triggering local formation of proinflammatory mediators, which subsequently translocate into the circulation, resulting in a systemic inflammatory response (22, 24, 35). The lung is the most sensitive and important target organ in systemic inflammation (57, 59). Neutrophils constitute the first line of defense against bacterial invasion (50). However, excessive activation of neutrophils is a direct cause of tissue damage and organ failure in abdominal sepsis. For example, inhibition of specific adhesion molecules, such as CD11a, CD44, and CD162, not only inhibits neutrophil recruitment, but also protects against septic lung injury (5, 6, 26).

Neutrophils can eliminate invading microorganisms by secreting antimicrobial proteins as well as by phagocytic uptake and intracellular killing (25, 33, 37). In addition, it has recently been described that activated neutrophils can expel nuclear DNA to form web-like structures containing granular proteins, which are referred to as neutrophil extracellular traps (NETs) (10, 58). On one hand, NETs have been shown to exhibit antimicrobial functions by trapping and killing extracellular pathogens in the blood and tissues during infections (10, 32). On the other hand, NET-associated proteases and granular proteins have been shown to damage epithelial and endothelial cells in vitro (48). Moreover, NETs have been reported to disturb microvascular perfusion in the liver and cause hepatic tissue damage in endotoxemia (15). Thus the net effect of NET formation might be context dependent and related to the degree of bacterial invasion. Several studies have observed increased NET formation in the circulation in infection and sepsis (7, 12, 15, 32) although the role of NETs in vivo remains controversial (9, 20, 27, 36). Administration of recombinant human (rh)DNAse, which effectively catalyzes hydrolysis of extracellular DNA, can be used to examine the impact of NET formation in vivo (12, 34). One recent study examined the effect of recombinant human (rh)DNAse on inflammation and bacterial spread in a model of abdominal sepsis based on cecal ligation and puncture (CLP) (34). However, results from that investigation are difficult to interpret because of inconsistencies in inflammatory responses and bacterial counts. For example, Meng et al. (34) reported that pulmonary accumulation of neutrophils were lower 24 h compared with 6 h after CLP, which is in contrast to most studies on CLP-induced neutrophil recruitment (4, 6, 43). Moreover, in that study, it was reported that rhDNAse decreased bacterial counts in the blood but not in the peritoneal cavity, lung, or liver 24 h after CLP induction. Also, Meng et al. (34) observed that rhDNAse increased bacterial counts in the peritoneal cavity and lung but not in the blood or liver 6 h after initiation of CLP. Thus the role of NET formation in terms of inflammatory response and bacterial clearance in abdominal sepsis remain elusive.

On the basis of these considerations, we aimed to reevaluate the impact of inhibiting NET formation on inflammation in septic lung injury. For this purpose, we used CLP to induce abdominal sepsis in mice.

MATERIALS AND METHODS

Animals. All experimental procedures were performed in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden. Male C57BL/6 mice weighing 20–25 g were housed on an animal facility with 12-h:12-h light/dark cycle at 22°C and fed a laboratory diet and water ad libitum. The mice...
were anesthetized with 7.5 mg of ketamine hydrochloride (Hoffman-LaRoche, Basel, Switzerland) and 2.5 mg of xylazine (Janssen Pharmaceuticals, Beerse, Belgium) per 100 g body wt. Experimental protocol of sepsis. Polymicrobial sepsis was induced by CLP as previously described in detail (6). Briefly, animals were anesthetized, and a midline incision was made to expose the cecum, which was filled with feces by milking stool backwards from the ascending colon, and 75% of the cecum was ligated with a 5–0 silk suture. The cecum was soaked with phosphate-buffered saline (pH 7.4, PBS; Sigma-Aldrich, Stockholm, Sweden) and was then punctured twice with a 21-gauge needle on the antimesenteric border. The cecum was returned into the peritoneal cavity, and the abdominal incision was sutured. Animals were treated intraperitoneally (i.p.) with 5 mg/kg rhDNase (Pulmozyme; Roche, Grenzach-Wyhlen, Germany) or 100 µl vehicle (PBS) at 1 h, 4 h, 7 h, 10 h, and 21 h after CLP, as described before (34). Sham mice underwent the identical laparotomy and resuscitation procedures, but the cecum was neither ligated nor punctured. In separate experiments, animals were depleted of neutrophils by pretreatment with a neutrophil-depleting monoclonal antibody (anti-Ly-6G, clone 1A8, 2 mg/kg; Rat-IgG2a; BioXcell, West Lebanon, NH; n = 5). Animals were xenogenized 6 h and 24 h after CLP to collect samples for further analysis.

Visualization and quantification of extracellular DNA. Extracellular DNA was labeled with the cell-impermeable dye Sytox Green nucleic acid stain (5 µM; Invitrogen, Stockholm, Sweden). Briefly, 10 µl of Sytox Green was injected intravenously 10 min before the harvest of the samples. Lung tissue samples were collected in iced tubes and loaded onto 35-mm microdish (MatTek, Martinsried, Germany) with a thin bottom for high-end microscopy. Images were taken in bright field within 30 min of samples collection. Confocal microscopy was performed using Meta 510 confocal microscopy (Carl Zeiss, Jena, Germany) by a ×10 long-distance objective (numeric aperture = 0.3). Sytox Green was excited by 488-nm laser line, and corresponding emission wavelength was collected by the filter of 500–530 nm. The pinhole was ~1 airy unit, and the scanning frame was 512 × 512 pixels. Quantification of extracellular DNA in the lung was determined using ZEN2009 software, and the area of fluorescence per high-power field was given in percentage of the total area.

Quantification of cell-free DNA. Circulating free DNA (cf-DNA) was used to determine levels of NET formation. Levels of cf-DNA in the peritoneal cavity, bronchoalveolar lavage fluid (BALF), and plasma were quantified by use of the Quant-iT Pico Green dsDNA assay (Invitrogen) according to the manufacturer’s instructions. The fluorescence intensity, which reflects the amount of DNA, was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a microplate reader (SpectraMax Gemini; Molecular Devices, Sunnyvale, CA). A standard curve was made by serial dilution of dsDNA (1–1,000 ng/ml).

Systemic leukocyte and platelet counts. Blood was collected from the tail vein and mixed with Turks solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% vol/vol) in a dilution of 1:20 for quantification of polymorphonuclear leukocytes (PMNL) and mononuclear leukocytes (MNL) or with Stromatol solution (Mascia Brunelli spa; Viale Monza, Milan, Italy) in a dilution of 1:500 for identification of platelets in a Burker chamber.

Lung edema and BALF. The left lung was excised, washed in PBS, gently dried using a blotting paper, and weighed. The tissue was then dried at 60°C for 72 h and reweighed. The change in the ratio of wet weight to dry weight was used as an indicator of lung edema formation. BALF was collected by five washes with 1 ml of PBS containing 5 mM ethylenediaminetetraacetic acid (EDTA, Invitrogen) and then centrifuged; the numbers of MNLs and PMNLs were counted in a Burker chamber.

Myeloperoxidase activity. Myeloperoxidase (MPO) levels in the lung tissue were assayed according to a standard protocol (6). Briefly, frozen tissue was thawed and homogenized in 1 ml of 0.5% hexadecyltrimethylammonium bromide. The sample was next freeze-thawed and centrifuged, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H₂O₂ (450 nm, with a reference filter 540 nm, 25°C). Values were expressed as MPO units per gram of tissue.

ELISA. Plasma levels of high-mobility group box 1 (HMGB1), IL-6, CXCL1, and CXCL2, lung levels of IL-6, CXCL1, and CXCL2, as well as cell culture supernatant levels of CXCL2, TNF-α, and HMGB1 by use of commercially available ELISA kits (Chondrex, Redmond, WA, and R & D Systems Europe, Abingdon, Oxfordshire, UK) were measured. Linearity was assessed and confirmed by samples containing high concentrations of recombinant mouse HMGB1, IL-6, CXCL1, and CXCL2 serially diluted with a calibrator diluent. RhDNase levels in plasma were measured by use of ORG 590 DNase Activity Immunomodulatory Enzyme Immunoassay for the Quantitative Determination of DNase Activity (ORGENTEC, Mainz, Germany) according to the manufacturer’s instructions.

Histology. Lung samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Six-micron sections were stained with hematoxylin and eosin. Lung injury was quantified in a blinded manner by adoption of a modified scoring system as described previously (8, 11, 13), including size of alveolar spaces, thickness of alveolar septa, alveolar hemorrhage, and neutrophil infiltration graded on 0 (absent) to 4 (extensive) scales.

Isolation of bone marrow and blood neutrophils. Bone marrow-derived neutrophils were isolated with a modified method as previously described (52). Briefly, male C57BL/6 mice were euthanized, and femurs and tibias were moved from the lower extremities using scissors and careful dislocation of the epiphyses from the bones. The bone marrow was flushed from both ends of the bone with a 25-gauge needle and a 2-ml syringe filled with Roswell Park Memorial Institute medium 1640 (RPMI 1640, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 2 mM EDTA. Following hypotonic lysis (5 ml ice-cold 0.2% NaCl, added for 45 s, and then 5 ml 1.6% NaCl), neutrophils were separated from mononuclear cells by density gradient centrifugation using a Ficoll-Paque gradient (GE Healthcare, Uppsala, Sweden). The neutrophil layer was isolated and washed with RPMI 1640, and cells were resuspended at 4 × 10⁶ cells/ml. Blood samples were collected as described above, and neutrophils were isolated following a similar protocol as the isolation of bone marrow neutrophils.

NET formation in vitro. To induce in vitro formation of NETs, freshly isolated neutrophils (4 × 10⁶ cells/ml) from mouse bone marrow or blood were incubated with 50 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 3 h at 37°C in RPMI 1640. Cell culture supernatants were removed, and fresh media was added to isolate NETs. Residual neutrophils and NETs were then removed through extensive pipetting. The mixture was centrifuged at 1,400 revolution/min for 5 min to remove cellular components, and NETs containing supernatants were collected. The NET-containing supernatants were further co incubated with PBS or rhDNase for 30 min at 37°C, and then the media with or without NETs were retrieved for further use in vitro experiments.

Macrophage secretion of proinflammatory compounds. RAW264.7 cells (ATCC, Manassas, VA), a murine macrophage cell line, were cultured in Dulbecco’s Modified Eagle Medium (Invitrogen) with FBS (10%), L-glutamine (5%), penicillin (100 U/ml), and streptomycin (100 µg/ml). RAW264.7 cells were seeded in 96-well plates 24 h before stimulation with bone marrow neutrophil-derived NETs. Cells were co-incubated with the conditioned media collected from in vitro NET formation experiments in a 37°C, 5% CO₂ incubator. RAW264.7 cells were co-incubated with or without NETs, genomic DNA (extracted from mouse livers by use of TRIzol Reagent following manufacturer’s protocol), and vehicle (PBS) in a volume of 10 µl per well. Cell culture supernatants were collected 24 h after NET challenge, and samples were saved for CXCL2, TNF-α, and HMGB1 quantification by use of ELISA. In separate experiments, alveolar...
Macrophages were isolated as previously described (61) and incubated with blood neutrophil-derived NETs. Briefly, alveolar macrophages were freshly isolated from BALF and seeded on a 96-well plate 1 h before the experiment. Macrophages were coincubated with or without NETs and vehicle in a volume of 10 µl per well. Cell culture supernatants were collected 24 h after NET challenge, and samples were saved for CXCL2, TNF-α, and HMGB1 quantification by use of ELISA.

**Neutrophil expression of Mac-1.** Bone marrow neutrophils were freshly isolated and resuspended in RPMI 1640. The purity of the isolated neutrophil was over 70% as assessed in hemocytometer. Neutrophils were then resuspended in PBS to 10^7 cells/ml and coincubated with 30 ng/ml of recombinant mouse CXCL2 (R & D Systems) for 10 min at 37°C. Neutrophils were preincubated with or without rhDNAse 20 µg/ml, 20 min before challenge with CXCL2. Cells were stained and fixed for flow cytometric analysis of Mac-1 expression.

**Chemotaxis assay.** Freshly isolated neutrophils from bone marrow were preincubated with 20 µg/ml of rhDNAse or PBS for 30 min, and 1.5 × 10^6 neutrophils were loaded in the upper chamber of the Transwell inserts (5-µm pore size; Corning Costar, Corning, NY). Inserts were placed in wells containing medium (control) or medium plus CXCL2 (100 ng/ml, R & D System). Neutrophils were preincubated with or without rhDNAse (20 µg/ml) 20 min before challenge with CXCL2. After 2 h, inserts were removed, and migrated neutrophils were stained with Turks solution. Chemotaxis was determined by counting migrated neutrophils in a Burker chamber (45).

**Flow cytometry.** For analysis of surface expression of Mac-1, bone marrow neutrophils were freshly isolated and resuspended in RPMI 1640 as previously described. Cells were blocked for 10 min at room temperature with an anti-CD16/CD32 (BD Biosciences Pharmingen, San Jose, CA) antibody blocking Fc-γ III/II receptors. Neutrophils were then stained with phycoerythrin-conjugated anti-mouse Ly-6G (clone 1A8, BD Biosciences) and fluorescein isothiocyanate-conjugated anti-mouse CD11b (clone M1/70, BD Biosciences) antibodies. Cells were further fixed, and erythrocytes were lysed for flow cytometry. Flow cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and analyzed with Cell-Quest Pro software (BD Biosciences). A viable gate was used to exclude dead cells and fragmental components.

Fig. 1. Neutrophil extracellular trap (NET) formation in the lung. A–D: intravascular administration of Sytox Green was used to visualize extracellular DNA in the inflamed lung. E: quantification of extracellular DNA in the lung by measuring the total area of fluorescence per high-power field in percentage of the total area. Veh, vehicle; Depl, neutrophil-depleting antibody; F: dot plot showing Ly-6G⁺ neutrophils. Mice underwent cecal ligation and puncture (CLP) or the identical laparotomy and resuscitation procedures, but the cecum was neither ligated nor punctured (Sham). Animals were treated with intraperitoneal injections of the recombinant human (rh)DNAse, a neutrophil-depleting antibody (Anti-Ly6G), or vehicle (Veh) as described in MATERIALS AND METHODS. Samples were collected 24 h after induction of sepsis. SSC, side scatter. Data are presented as means ± SE. *P < 0.05 vs. Sham and #P < 0.05 vs. Veh + CLP 24 h, n = 5.
**RESULTS**

**Increased NET formation in abdominal sepsis.** The cell-impermeable dye, Sytox Green, was used to visualize extracellular DNA in the lung. No extracellular DNA in the lung could be detected in sham animals (Fig. 1A). In contrast, it was found that CLP caused wide-spread deposition of extracellular DNA in the inflamed lung (Fig. 1B). Treatment with rhDNase, which effectively catalyzes hydrolysis of extracellular DNA, can be used to examine the impact of NET formation (32, 34). Notably, administration of rhDNase abolished CLP-induced deposition of extracellular DNA in the lung (Fig. 1C). Quantification of microvascular Sytox Green staining revealed that induction of sepsis caused significant formation of NETs and that administration of rhDNase decreased NET formation by 87% in the inflamed lung (Fig. 1E). Moreover, depletion of neutrophils by use of a neutrophil-depleting antibody against Ly-6G (>95% reduction in circulating neutrophils, Fig. 1F) markedly reduced CLP-induced deposition of extracellular DNA in the lung (Fig. 1D). We next analyzed cf-DNA levels in the peritoneal cavity and plasma 6 h and 24 h after CLP induction. It was found that NET levels were increased by fivefold and sevenfold in the peritoneal cavity 6 h and 24 h after CLP, respectively (Fig. 2A). Moreover, CLP enhanced plasma levels of NETs by threefold 6 h and by fivefold 24 h after CLP (Fig. 2B). Notably, we also observed increased NET formation in the bronchoalveolar space 24 h after induction of CLP (Fig. 2C). We next examined the effect of rhDNase on NET activity. Treatment with rhDNase markedly decreased formation of NETs in the peritoneal cavity 6 h and 24 h after CLP (Fig. 2A). It was found that administration of rhDNase abolished CLP-induced NET activity in the plasma at 24 h but had no significant effect on NET formation in the plasma 6 h after CLP induction (Fig. 2B). In addition, neutrophil depletion markedly decreased cf-DNA levels in the plasma (Fig. 2B). Interestingly, treatment with rhDNase greatly reduced CLP-induced formation of NETs in the bronchoalveolar space 24 h after CLP (Fig. 2C). Quantification of rhDNase levels showed that i.p. administration of rhDNase resulted in significantly elevated levels of rhDNase in plasma (Fig. 2D). These findings suggest that administration of rhDNase is an effective way to inhibit NET activity in CLP animals.

**Neutrophil recruitment and lung injury in abdominal sepsis.** Neutrophil accumulation is a hallmark in septic lung injury (23, 42). Herein, it was observed that CLP increased lung levels of MPO by more than sixfold 6 h after CLP (Fig. 3A). Pulmonary MPO activity was still elevated 24 h after CLP (Fig. 3A). In parallel, the number of alveolar neutrophils was markedly increased 24 h after induction of CLP (Fig. 3B). Administration of rhDNase had no significant effect on pulmonary MPO activity at 6 h but reduced lung activity of MPO 24 h after CLP (Fig. 3A). Moreover, we observed that treatment with rhDNase reduced the number of alveolar neutrophils from 61.4 ± 7.4 × 10^3 cells to 27.1 ± 1.2 × 10^3 cells in the lung, corresponding to a 65% reduction, 24 h after induction of CLP (Fig. 3B). In addition, we found that administration of rhDNase had no significant effect on circulating levels of

**Bacterial cultures.** The number of colony-forming units (CFU) was determined in lung, spleen, and blood. Briefly, 6 h and 24 h after CLP, blood was taken from the interior vena cava. Lung and spleen were harvested and homogenized under aseptic conditions. Subsequently, serial-diluted blood and organ homogenates were plated on trypticase soy agar II with 5% sheep blood (Becton Dickinson, Heidelberg, Germany) and incubated under aerobic conditions at 37°C for 24 h. The number of bacterial colonies were then counted and specified as CFU/ml blood and CFU/g tissue. The broad-based antibiotic imipenem/cilastatin (Tienam, 25 mg/kg; Merck Sharp & Dohme, Sollentuna, Sweden) was injected i.p. 30 min after CLP induction as a comparison.

**Statistics.** Data are presented as means ± SE. Statistical evaluations were performed using the nonparametric Mann-Whitney test for comparisons between groups. \( P < 0.05 \) was considered statistically significant, and \( n \) represents the number of animals.
neutrophils and platelets in CLP mice (Table 1). In separate experiments, we examined whether rhDNAse might exert any direct effects on isolated neutrophils. However, we found that rhDNAse had no effect on CXCL2-induced Mac-1 expression on neutrophils (Fig. 3, C and D). In addition, rhDNAse had no impact on CXCL2-evoked neutrophil chemotaxis (Fig. 3E). CLP caused significant lung injury indicated by the increase in lung edema formation. Thus lung wet:dry ratio increased in CLP animals from 4.5 ± 0.1 to 5.5 ± 0.2 (Fig. 4A).

### Table 1. Systemic blood cell differential counts

<table>
<thead>
<tr>
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<th>MNL</th>
<th>PMNL</th>
<th>Total Leukocyte</th>
<th>Platelet</th>
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<tr>
<td>Sham</td>
<td>5.4 ± 0.3</td>
<td>1.6 ± 0.3</td>
<td>7.0 ± 0.5</td>
<td>758 ± 58</td>
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<tr>
<td>Veh + CLP 6 h</td>
<td>1.8 ± 0.2*</td>
<td>1.2 ± 0.1</td>
<td>3.0 ± 0.3*</td>
<td>928 ± 81*</td>
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<tr>
<td>DNAse + CLP 6 h</td>
<td>2.1 ± 0.4</td>
<td>1.6 ± 0.3</td>
<td>3.8 ± 0.7</td>
<td>822 ± 126</td>
</tr>
<tr>
<td>Veh + CLP 24 h</td>
<td>2.2 ± 0.5*</td>
<td>0.3 ± 0.1*</td>
<td>2.5 ± 0.5*</td>
<td>554 ± 24*</td>
</tr>
<tr>
<td>DNAse + CLP 24 h</td>
<td>3.0 ± 0.6</td>
<td>0.6 ± 0.1</td>
<td>3.4 ± 0.6</td>
<td>319 ± 32</td>
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Values are means ± SE, 10^6 cells/ml. Blood was collected from cecal ligation and puncture (CLP) animals treated with vehicle (Veh) and DNAse for 6 h and 24 h after sepsis induction as well as from sham-operated mice. Leukocytes were identified as monomorphonuclear leukocytes (MNL) and polymorphonuclear leukocytes (PMNL). *P < 0.05 vs. sham, n = 5.

Vueification of rhDNAse reduced lung wet:dry ratio to 4.6 ± 0.2 in CLP mice (Fig. 4B). Thus inhibition of NET activity decreased CLP-provoked lung edema by 90%. Moreover, morphological examination revealed normal lung microarchitecture in sham animals (Fig. 4A), whereas CLP caused clear-cut destruction of the lung tissue structure characterized by interstitial edema, capillary congestion, and neutrophil accumulation (Fig. 4C). Notably, it was found that treatment with rhDNAse decreased CLP-provoked changes of the microarchitecture and neutrophil accumulation in the lung (Fig. 4D). Quantification of the morphological changes revealed that CLP increased the lung injury score and that inhibition of NET formation significantly reduced the lung injury score in CLP animals (Fig. 4E).

**NET activity regulates CXC chemokine formation.** CXC chemokines are potent regulators of neutrophil trafficking into inflamed tissues (28). Herein, we observed that CXC chemokine levels were low in plasma (Fig. 5, A and B) and lungs (Fig. 5, C and D) of sham mice. Plasma and pulmonary levels of CXC chemokines were already elevated at 6 h and further increased 24 h after CLP (Fig. 5, A–D). Notably, injection of rhDNAse reduced levels of CXCL1 in plasma and lung by 64% and 67%, respectively, 24 h after CLP (Fig. 5, A and C).
Moreover, we found that inhibition of NET activity decreased CXCL2 levels in plasma and lung by more than 50% 24 h after induction of CLP (Fig. 5, B and D).

**NET activity regulates systemic inflammation.** Systemic inflammation in patients with sepsis is characterized by increased levels of HMGB1 and IL-6 (21, 38). It was found that plasma levels of HMGB1 were low but detectable in sham animals (Fig. 5E). In contrast, CLP increased plasma levels of HMGB1 by 120-fold, i.e., from 1.6 ± 0.7 ng/ml up to 181.4 ± 13.7 ng/ml (Fig. 5E). Notably, treatment with rhDNase reduced CLP-evoked production of HMGB1 to 61.4 ± 15.7 ng/ml, corresponding to a 66% reduction (Fig. 5E). In addition, we observed that the plasma and pulmonary levels of IL-6 were greatly increased in septic compared with sham animals (Fig. 5, F and G). Interestingly, it was observed that treatment with rhDNase decreased levels of IL-6 by 74% and 72% in the plasma and lungs, respectively, 24 h after CLP induction (Fig. 5, F and G).

**NET-dependent formation of proinflammatory cytokines in macrophages.** NETs were first generated by stimulating bone marrow neutrophils with PMA (Fig. 6A). NETs were coincubated with RAW264.7 cells for 24 h. It was found that NET stimulation of macrophages triggered massive formation of CXCL2, TNF-α, and HMGB1 (Fig. 6, B–D). For example, NET challenge increased macrophage secretion of CXCL2 by 22-fold after 24 h of stimulation (Fig. 6B). Notably, coincubation of NETs with rhDNase abolished NET-induced production of CXCL2, TNF-α, and HMGB1 in RAW264.7 macrophages (Fig. 6, B–D). Genomic DNA isolated from the liver had no effect on RAW264.7 cell formation of CXCL2, TNF-α, and HMGB1 (Fig. 6, B–D). We next generated NETs from blood neutrophils stimulated with PMA (Fig. 6E) and coincubated NETs with isolated alveolar macrophages from the lung. It was found that NETs from blood neutrophils was a potent inducer of CXCL2, TNF-α, and HMGB1 secretion from alveolar macrophages (Fig. 6, F–H). Moreover, coincubation with rhDNase abolished NET-induced formation of CXCL2, TNF-α, and HMGB1 in alveolar macrophages (Fig. 6, F–H).

**Bacterial spread in sepsis.** CLP caused a time-dependent increase in the bacterial load in the lung, spleen, and blood (Fig. 7, A–C). Administration of rhDNase had a small effect on the bacterial load in the lung but had no effect on the...

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**Fig. 4.** NETs regulate CLP-induced lung damage. A: edema formation was determined 24 h after CLP induction. Representative hematoxylin and eosin-stained sections of lung tissue from sham (B) animals and animals treated with vehicle (Veh) (C) or rhDNase (D). E: lung injury score was calculated as described in MATERIALS AND METHODS. Scale bar = 100 µm. Data are presented as means ± SE. *P < 0.05 vs. Sham and #P < 0.05 vs. Veh + CLP, n = 5.

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bacterial load in the spleen and blood 6 h after induction of CLP (Fig. 7, A–C). Treatment with rhDNAse had a small effect on the bacterial load in all these organs 24 h after CLP induction (Fig. 7, A–C). We used a broad-spectrum antibiotic, i.e., imipenem/cilastatin, as a comparison. We observed that administration of imipenem/cilastatin markedly reduced the bacterial load in the lung (Fig. 7A), spleen (Fig. 7B), and blood (Fig. 7C) 24 h after induction of CLP.

DISCUSSION

This study demonstrates that NET formation exerts proinflammatory effects in septic lung injury. Our data show that NETs promote neutrophil recruitment to the lung. One mechanism behind NET-induced pulmonary accumulation of neutrophils might be related to increased formation of proinflammatory compounds. Indeed, we could document that NETs directly triggered CXCL2, TNF-α, and HMGB1 secretion from macrophages. Thus targeting NET formation could be a useful strategy to ameliorate lung damage in abdominal sepsis.

Management of patients with septic lung injury is challenging and largely limited to supportive care. Novel therapeutic options are required to improve clinical outcome of patients with sepsis. Numerous investigations have reported that NET formation is increased in severe infections and sepsis although the impact of NETs in septic lung damage remains elusive (7, 12, 15, 32). Herein, we provide evidence showing that induction of abdominal sepsis causes wide-spread DNA deposition in the inflamed lung. Moreover, it was found that CLP challenge increased cf-DNA levels in plasma, peritoneal cavity, and alveolar compartment. Notably, depletion of neutrophils markedly decreased DNA deposition in the lung as well as cf-DNA levels in the plasma of septic animals, suggesting that neutrophils are the likely source of extracellular DNA in abdominal sepsis. Numerous studies have shown that pharmacological inhibition by use of DNAse is an effective way to block formation of NETs both in vitro and in vivo (32, 34). This study shows that inhibition of NET formation decreases lung edema and injury in abdominal sepsis. Although initial studies reported that NETs were effective in trapping bacteria and play an important role in the host defense against microbial infection under certain circumstances (10, 32, 58), accumulating reports have documented that NETs cause tissue damage in different models of inflammation and infection (14, 36). For example, Narasaraju and colleagues (36) reported that excessive NET formation contributes to lung damage in influenza pneumonitis and others have found that depletion of NETs protects against transfusion-induced lung injury (14). Meanwhile, other studies reported that NET formation might be of beneficial value during infections (15, 32, 41), which makes this issue somewhat controversial, and it is possible that the role of NETs are context and model dependent. Nonetheless, our findings support the concept that NETs cause tissue damage in septic lung injury characterized by excessive neutrophil activation. This notion is also supported by a recent study showing that NETs directly cause pulmonary epithelial and endothelial cell damage and death (48). Indeed, considering

![Figure 5: NETs regulate CLP-induced increase of proinflammatory compounds.](image)

Levels of CXCL1 (A and C) and CXCL2 (B and D) in the plasma and lung were determined 6 h and 24 h after CLP induction. Levels of high-mobility group box 1 (HMGB1) (E) in plasma and IL-6 levels in plasma and lung (F and G) were determined 6 h and 24 h after CLP induction. Mice underwent CLP or the identical laparotomy and resuscitation procedures, but the cecum was neither ligated nor punctured (Sham). Animals were treated with intraperitoneal injections of the rhDNAse or vehicle as described in MATERIALS AND METHODS. Data are presented as means ± SE. *P < 0.05 vs. Sham and #P < 0.05 vs. Vehicle + CLP, n = 5.
that excessive neutrophil activation has been established as a fundamental component in the pathophysiology of septic lung injury (30, 40, 51), it is not surprising that excessive NET formation may also be deleterious in abdominal sepsis. We also studied the impact of NET formation on systemic inflammation in abdominal sepsis. HMGB1 is a potent proinflammatory cytokine and a late mediator in endotoxemia and sepsis (1, 2, 49, 54), as well a predictor of clinical outcome in patients with severe sepsis (21). In line with previous studies (39, 62), we observed that CLP caused a substantial increase in the

Fig. 6. NET induces macrophage formation of proinflammatory compounds. A: freshly isolated bone marrow neutrophils (BMN) were stimulated with phorbol 12-myristate 13-acetate (PMA) for 3 h. Following coincubation with or without rhDNAse, levels of cf-DNA were quantified in the supernatants. Macrophages (RAW264.7 cells) were coincubated with BMN-derived NETs pretreated with vehicle or rhDNAse and levels of CXCL2 (B), TNF-α (C), and HMGB1 (D) and were quantified in the supernatants 24 h after coincubation. E: freshly isolated blood neutrophils (BN) were stimulated with PMA for 3 h. Following coincubation with or without rhDNase, levels of cf-DNA were quantified in the supernatants. Isolated alveolar macrophages were coincubated with BN-derived NETs pretreated with vehicle or DNase and levels of CXCL2 (F), TNF-α (G), and HMGB1 (H) and were quantified in the supernatants 24 h after coincubation. Data are presented as means ± SE. *P < 0.05 vs. Vehicle and #P < 0.05 vs. NETs, n = 5.
plasma levels of HMGB1. Notably, it was found that NET depletion reduced HMGB1 levels in the plasma by 66% in septic animals, indicating a potent proinflammatory role of NETs in polymicrobial sepsis. In this context, it is interesting to note that a recent study showed that HMGB1 potentiates NET formation in systemic inflammation (53). Another indicator of systemic inflammation is IL-6, and a correlation between high IL-6 levels and mortality of patients with sepsis has been demonstrated (38). Herein, we observed that NET inhibition markedly decreased lung and plasma levels of IL-6 in septic mice. Together, these findings suggest that NETs are a potent inducer of systemic inflammation in abdominal sepsis.

It is well established that neutrophil infiltration is a rate-limiting step in septic lung injury (23, 47, 55). It was therefore of great interest to study the effect of NET depletion on neutrophil infiltration in septic lung damage in the present study. We found that inhibition of NET formation reduced pulmonary levels of MPO, a marker of neutrophil recruitment, by more than 50% in 24-h CLP-induced abdominal sepsis. This inhibitory effect correlated well with our observation that NET depletion decreased sepsis-induced neutrophil infiltration in the bronchoalveolar space by 60%, suggesting that NETs promote pathological accumulation of neutrophils in septic lung injury. Meng et al. (34) published that NET depletion increased neutrophil accumulation in septic lung injury. However, these results are difficult to interpret because Meng et al. (34) also reported that neutrophil recruitment was lower at 24 h compared with 6 h after CLP induction, which is in direct contrast to most studies using this model (6, 18, 43). Indeed, our findings are supported by several studies showing that NET depletion reduces leukocyte accumulation in different models of inflammation (14, 36). In this context, it is important to note that DNAse treatment exerted no direct effect on neutrophil functions, such as CXCL2-induced Mac-1 upregulation and chemotaxis. Pulmonary recruitment of leukocytes is a multi-step process, including initial sequestration in microvessels and firm adhesion on the endothelium, followed by transendothelial and transepithelial migration (47, 55, 56). Trafficking of leukocytes in inflamed tissues is coordinated by secreted chemokines (40, 51). Neutrophils are particularly attracted by CXC chemokines, such as CXCL1 and CXCL2, which are murine homologs of human IL-8 (44, 63). In the present study, we observed that NET depletion decreased CLP-induced generation of CXCL1 and CXCL2 by more than 50% in both plasma and lung, suggesting that NETs have a major impact on CXC chemokine formation in abdominal sepsis. Knowing that pulmonary formation of proinflammatory compounds are generated in part by alveolar macrophages (17), we therefore asked whether NETs can stimulate macrophage secretion of CXCL2, TNF-α, and HMGB1. Interestingly, we found that neutrophil-derived NETs markedly enhanced CXCL2, TNF-α, and HMGB1 secretion from macrophages in vitro. Notably, concomitant lysis of NETs with rhDNase abolished the NET-induced generation of CXCL2, TNF-α, and HMGB1 in macrophages. This is in fact the first evidence showing that NETs are potent stimuli for macrophages and might help to explain the regulatory role of NETs in sepsis-induced formation of proinflammatory substances. Considered together, these results suggest that NETs promote sepsis-induced neutrophil accumulation via production of chemokines and cytokines in the lung.

Together with systemic inflammatory response, rapid spread of bacteria is a hallmark in abdominal sepsis (29). We found that CLP caused a prompt increase in bacterial levels in the lung, spleen, and blood at 6 h, which was further increased 24 h after induction of CLP. Administration of DNAse had no effect on the bacterial burden in the blood at 6 h after CLP but caused a small decrease in the bacterial levels in the blood 24 h after CLP. This pattern was similar in the lung and spleen.

Fig. 7. Bacterial counts were quantified in lung (A), spleen (B), and blood (C) as described in MATERIALS AND METHODS. Bacterial counts were determined in fresh collected samples from mice that underwent CLP or the identical laparotomy and resuscitation procedures, but the cecum was neither ligated nor punctured (Sham). Animals were treated with intraperitoneal injections of rhDNase, imipenem/cilastatin (Imip/Cil), or vehicle as described in MATERIALS AND METHODS. CFU, colony-forming units. Data are presented as means ± SE. *P < 0.05 vs. Sham, †P < 0.05 vs. Veh + CLP 6 h and #P < 0.05 vs. Veh + CLP 24 h, n = 5.

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suggesting a minor influence of NET on the systemic spread of bacteria in abdominal sepsis. This notion is also supported by very similar findings in the blood of CLP animals reported by Meng et al. (34). As a comparison to the minor impact of NETs on early bacterial burden in septic blood, we administered a broad-based antibiotic in vivo, which markedly inhibited bacterial levels in the blood of septic animals. Similar to the 24-h results, we observed that rhDNAse decreased bacterial burden in the lung 6 h after CLP induction, which is in contrast to findings in the study by Meng et al. (34) reporting that rhDNAse increase pulmonary levels of bacteria at this time point. One important difference in the data is that Meng et al. (34) did not observe any bacteria at all in the lung 6 h after CLP, whereas we find significantly elevated levels of bacteria in the lung at this time point. Our findings are in line with previous studies showing a clear-cut increase in the bacterial burden in the lung 6 h after CLP (16, 31). The reason for Meng et al. (34) not observing any bacteria in the lung 6 h after CLP is not known, but it might be related to the fact that we only used male mice, whereas Meng et al. used female mice, which are known to be more resistant to the septic insult (3, 46).

Taken together, our results indicate that NETs play a proinflammatory role in abdominal sepsis. Thus these findings show that NETs cause pulmonary edema and tissue damage in septic lung injury. Indeed, we found that NETs stimulate formation of proinflammatory compounds in macrophages and promote neutrophil recruitment in the lungs of septic animals. We conclude that inhibition of NET formation might be a useful strategy to protect against lung damage in abdominal sepsis.

REFERENCES