

# Mechanical Ventilation Induces Neutrophil Extracellular Trap Formation

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## ABSTRACT

**Background:** Mechanical ventilation can injure the lung and induce a proinflammatory state; such ventilator-induced lung injury (VILI) is associated with neutrophil influx. Neutrophils release DNA and granular proteins as cytotoxic neutrophil extracellular traps (NETs). The authors hypothesized that NETs were produced in a VILI model and may contribute to injury.

**Methods:** In a two-hit lipopolysaccharide/VILI mouse model with and without intratracheal deoxyribonuclease (DNase) treatment or blockade of known inducers of NET formation (NETosis), the authors assessed compliance, bronchoalveolar lavage fluid protein, markers of NETs (citrullinated histone-3 and DNA), and markers of inflammation.

**Results:** Although lipopolysaccharide recruited neutrophils to airways, the addition of high tidal mechanical ventilation was required for significant induction of NETs markers (*e.g.*, bronchoalveolar lavage fluid DNA:  $0.4 \pm 0.07$   $\mu\text{g}/\text{ml}$  [mean  $\pm$  SEM],  $P < 0.05$  *vs.* all others,  $n = 10$  per group). High tidal volume mechanical ventilation increased airway high-mobility group box 1 protein ( $0.91 \pm 0.138$  *vs.*  $0.60 \pm 0.095$ ) and interleukin-1 $\beta$  in lipopolysaccharide-treated mice ( $22.4 \pm 0.87$  *vs.*  $17.0 \pm 0.50$  pg/ml,  $P < 0.001$ ) and tended to increase monocyte chemoattractant protein-1 and interleukin-6. Intratracheal DNase treatment reduced NET markers (bronchoalveolar lavage fluid DNA:  $0.23 \pm 0.038$  *vs.*  $0.88 \pm 0.135$   $\mu\text{g}/\text{ml}$ ,  $P < 0.001$ ; citrullinated histone-3:  $443 \pm 170$  *vs.*  $1,824 \pm 403$ ,  $P < 0.01$ ,  $n = 8$  to 10) and attenuated the loss of static compliance ( $0.9 \pm 0.14$  *vs.*  $1.58 \pm 0.17$  ml/mmHg,  $P < 0.01$ ,  $n = 19$  to 20) without significantly impacting other measures of injury. Blockade of high-mobility group box 1 (with glycyrrhizin) or interleukin-1 $\beta$  (with anakinra) did not prevent NETosis or protect against injury.

**Conclusions:** NETosis was induced in VILI, and DNase treatment eliminated NETs. In contrast to experimental transfusion-related acute lung injury, NETs do not play a major pathogenic role in the current model of VILI. (ANESTHESIOLOGY 2015; 122:864-75)

**M**ECHANICAL ventilation is a key feature of intensive care for patients with respiratory failure but can cause a form of iatrogenic lung injury called ventilator-associated lung injury. When studied in laboratory models, the phenomenon is termed ventilator-induced lung injury (VILI). Although the mechanisms of VILI remain incompletely understood, neutrophil infiltration is key,<sup>1</sup> and a variety of inflammatory mediators and processes are associated with this injury.<sup>2</sup>

It has been shown previously that depletion of granulocytes in ventilated rabbits attenuates the accumulation of extravascular lung protein and increases  $\text{PaO}_2$  levels.<sup>3</sup> Although vital to fight infection, neutrophils could contribute to lung injury *via* several mechanisms, including secretion of proteases (*e.g.*, elastase), production of reactive oxygen species, and release of cytokines and other proinflammatory mediators.<sup>4</sup> Neutrophil depletion is not a viable therapeutic

### What We Already Know about This Topic

- Neutrophil accumulation in the lungs during various forms of injury (infection or aspiration) has been suggested to contribute to the lung injury
- Recent studies show that neutrophils cast their genomic DNA, coated with granule proteins, as neutrophil extracellular traps that can trap and kill pathogens but may also injure lung tissue

### What This Article Tells Us That Is New

- By creating lung injury in mice using intratracheal lipopolysaccharide and mechanical ventilation, the authors documented that neutrophil extracellular traps were formed when lipopolysaccharide was present during high tidal ventilation and could be treated with deoxyribonuclease I
- The treatment cleared the neutrophil extracellular traps and improved lung mechanics, but other measures of lung injury (including increased neutrophil count, increased protein content in lavage fluid, and low oxygenation) persisted, documenting a limited role for the neutrophil extracellular traps in this form of lung injury

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approach to ventilator-associated lung injury given the resulting impairment of immune response, but targeting selective neutrophil functions could hold promise for protection.

Neutrophil extracellular traps (NETs) are antimicrobial structures cast by the neutrophils whereby they release genomic DNA coated with granule proteins. NETs can trap and kill various pathogens, but in certain conditions, NETs are associated with tissue damage<sup>5–8</sup> or autoimmune disease.<sup>9–12</sup> Recently, NETs have been detected in the lung microvasculature in transfusion-related acute lung injury, and targeting NET components was protective in a mouse model of transfusion-related acute lung injury.<sup>13</sup> NETs have also been detected in human allergic asthmatic airways<sup>14</sup> and in mouse models of pneumonia.<sup>15</sup> The potential role of NETs in VILI has not been clearly established.

Neutrophil extracellular trap formation (NETosis) is a mode of cell death independent of apoptosis or necrosis. The mechanism of NETosis is not completely understood but appears to include both reactive oxygen species–dependent and reactive oxygen species–independent pathways, resulting in citrullination of histones (a key marker of NETosis) and allowing chromatin decondensation. Several known inducers of NETosis are increased in models of lung injury associated with mechanical ventilation, including the cytokines interleukin (IL)-8 and IL-1 $\beta$ .<sup>16,17</sup> In addition, high-mobility group box I (HMGB1), a damage-associated molecular pattern recognition molecule, is increased in bronchoalveolar lavage fluid (BALF) by mechanical ventilation in both humans<sup>18</sup> and rat models.<sup>19</sup> In patients with ventilator-associated pneumonia,<sup>18</sup> HMGB1 has recently been shown to promote NETosis.<sup>20</sup> If NETs play a pathological role in VILI, targeting NETosis could potentially spare other neutrophil functions, partially preserving immune protection while providing protection from lung injury.

We hypothesized that NETosis would occur in VILI and that NETs might contribute to lung injury. We used a two-hit mouse model (intratracheal lipopolysaccharide followed by high tidal volume mechanical ventilation) to demonstrate NETs in the airways, with both hits contributing to NET formation. We targeted NETs by treating with deoxyribonuclease (DNase) I to degrade extracellular DNA. This treatment effectively cleared NETs and improved lung mechanics but did not affect other measures of lung injury.

## Materials and Methods

### Animal Approval

Animal experiments were approved by the animal care committee at the Hospital for Sick Children Research Institute, Toronto, Ontario, Canada, in accordance with the Canadian Council on Animal Care guidelines.

### Two-hit Lipopolysaccharide/VILI Model

Adult C57BL/6 mice (Charles River, Canada) between 20 and 25 g were anesthetized (ketamine, xylazine, or isoflurane) and randomized to six groups (n = 10 per group;

sample size based on experience with similar lung injury models): phosphate-buffered saline (PBS)/spontaneous ventilation; lipopolysaccharide/spontaneous ventilation; PBS/low tidal volume (LV<sub>T</sub>) ventilation; PBS/high tidal volume (HV<sub>T</sub>) ventilation; and PBS/HV<sub>T</sub> and lipopolysaccharide/LV<sub>T</sub>. Pretreatment was with 5  $\mu$ g of lipopolysaccharide from *Escherichia coli* 0111:B4 (Sigma-Aldrich, Canada) or a vehicle (PBS) control instilled *via* the trachea, followed by 200  $\mu$ l of air to ensure deposition throughout each lung. Two hours after pretreatment, mice were again anesthetized, tracheotomy was performed, and mechanical ventilation commenced using a computer-controlled small animal ventilator (SCIREQ flexivent, Canada) using room air and a volume-controlled setting.

Injurious ventilation was achieved with the following: respiration rate, 45 breaths/min; tidal volume, 20 ml/kg; and positive end-expiratory pressure (PEEP), 0 cm H<sub>2</sub>O (high tidal volume, HV<sub>T</sub> group). Protective ventilation used the following: respiration rate, 135 breaths/min; tidal volume, 10 ml/kg; and PEEP, 2 cm H<sub>2</sub>O (low tidal volume, LV<sub>T</sub> group). Additional controls (spontaneous ventilation) were allowed to recover after lipopolysaccharide or vehicle instillation and were anesthetized, intubated, and sacrificed 6 h later without any mechanical ventilation.

Normothermia was maintained using a heating blanket to maintain body temperature at 37°C throughout mechanical ventilation. Hydration was maintained by giving a subcutaneous injection of lactated Ringer's solution (300  $\mu$ l) at the start of ventilation. Anesthesia was maintained with hourly supplementary doses of 0.1 mg pancuronium, 26.67 mg/kg ketamine, and 6.67 mg/kg xylazine intraperitoneal. Recruitment maneuvers (deep inflations to 30 cm H<sub>2</sub>O for 5 s) performed hourly. Quasistatic compliance was measured using pressure–volume loops, and dynamic compliance was measured using a snapshot perturbation (SCIREQ flexivent).

At 4 h of ventilation (*i.e.*, 6 h after lipopolysaccharide or vehicle instillation), the carotid artery was cut and the animals exsanguinated under anesthesia; blood was collected in a capillary tube for blood gas analysis (Radiometer ABL 700; Radiometer, Canada). Lungs were perfused and lavaged three times with three aliquots of 700  $\mu$ l of HEPES-buffered saline solution (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.1 g glucose, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, and 4.2 mM NaHCO<sub>3</sub>) (Life Technologies, Canada). BALF was stored at –80°C. BALF cells were recovered, differentially stained (Hemacolor; EMD Millipore, USA), and counted.

### DNase I Treatment

In a second set of animals, mice (n = 20 per group) were randomized to instillation with 25  $\mu$ l of 1 mg/ml Dornase alfa (Pulmozyme®; Genentech Inc., USA) or vehicle control (8.77 mg/ml NaCl; 0.15 mg/ml CaCl<sub>2</sub>) twice through a 24-gauge angiocath (BD, Canada), followed by 700  $\mu$ l of air each time. Both instillations were performed immediately after intubation and placement on the ventilator. All animals

in this series were subjected to the two-hit lipopolysaccharide/VILI model described above.

### **Blockade of HMGB1 and IL-1 $\beta$**

In a third series of animals, mice ( $n = 6$  per group) were randomized to receive either vehicle (saline), glycyrrhizin (10 mg/kg, intraperitoneal, 10 min before the commencement of ventilation), or anakinra (Kineret<sup>®</sup>; Swedish Orphan Biovitrum Inc., USA) 10 mg/kg, intratracheal, at the time of lipopolysaccharide instillation plus 10 mg/kg i.v. at the commencement of ventilation. All animals in this series were subjected to the two-hit lipopolysaccharide/VILI model described above.

### **Quantifying BALF DNA and Protein**

Bronchoalveolar lavage fluid supernatant double-stranded DNA was quantified using Quant-iT Picogreen<sup>®</sup> (Invitrogen, Canada) following the manufacturer's protocol. BALF protein concentration was quantified using the bicinchoninic acid protein assay (Fisher Scientific, Canada).

### **Western Blot**

Bronchoalveolar lavage fluid protein samples (equal volume) were separated on polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Primary antibodies against citrullinated histone-3 (citrulline 2 + 8 + 17), myeloperoxidase (Abcam, Canada), and HMGB1 (Cell Signaling Technology, Canada) were incubated with blocked membranes overnight. Washed blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibody and signal detected by enhanced chemiluminescence.

### **Cytokine Analysis**

Cytokine measurements were performed on BALF supernatant samples using the Milliplex system (Millipore, Canada). The cytokines analyzed were IL-1 $\beta$ , IL-6, keratinocyte chemoattractant (KC), granulocyte macrophage colony-stimulating factor (CSF), granulocyte CSF, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), macrophage inflammatory protein (MIP)-2, and monocyte chemoattractant protein (MCP)-1. Analysis was performed by The Analytical Facility for Bioactive Molecules, The Hospital for Sick Children, Toronto, Ontario, Canada.

### **Preparation of Frozen Lung Sections**

Immediately after the ventilation experiment, the lungs were dissected, cut into small pieces (approximately 2  $\times$  2 mm), embedded in optimal cutting temperature medium (4583; Sakura Finetek, USA), and frozen on dry ice. Contiguous frozen sections (10  $\mu$ m) were cut from different lobes of the lung using a JUNG CM3000 cryostat microtome (Leica Microsystems Nussloch GmbH, Germany) and adhered to poly-L-lysine-coated glass microscope slides. Frozen sections were stored at  $-80^{\circ}\text{C}$  until use.

### **Immunolocalization of Cit-H3 in Lung Sections**

Lung tissue sections from different groups (controls, HV<sub>T</sub> + PBS, and HV<sub>T</sub> + lipopolysaccharide) were used for immunostaining for the NET marker citrullinated histone-3 (Cit-H3). In brief, slides were air dried (10 to 15 min) and fixed in 4% paraformaldehyde for 15 min. To suppress autofluorescence, sections were immersed in Sudan Black B (0.2% in 70% ethanol) for 15 min and washed for the next blocking step of nonspecific antibody interaction using 5% bovine serum albumin for 1 h at room temperature. Rabbit- $\alpha$ -Cit-H3 antibody (ab5103; 1:500 dilution; Abcam, United Kingdom) was incubated overnight at 4 $^{\circ}\text{C}$ . Primary antibody and DNA were detected by goat  $\alpha$ -rabbit immunoglobulin G-Alexa fluor 635 secondary antibody (1:1,000 dilution) and 4',6-diamidino-2-phenylindole dihydrochloride (1:1,000 dilution), respectively. The confocal images were captured using an Olympus IX81 inverted fluorescence microscope equipped with a Hamamatsu C9100-13 back-thinned EM-CCD camera (Quorum Technologies Incorporated, Canada) and Yokogawa CSU X1 spinning disk confocal scan head with Spectral Aurora Borealis upgrade (Carl Zeiss Canada Ltd., Canada). The objectives used were 20x/0.75 or 40x/1.35. The microscope was operated with Volocity software (Perkin Elmer, USA).

### **Histology and Morphometry**

Lungs were perfusion fixed with 10% buffered formalin while inflated with room air at a pressure of 20 cm H<sub>2</sub>O. The trachea was ligated and fixation continued in 10% buffered formalin for 24 h. Lung tissue was processed as previously described,<sup>21</sup> and sections were stained in hematoxylin and eosin. Blinded morphometric analysis was performed as described<sup>21</sup> using four tissue sections per animal and 10 random nonoverlapping images per section. Calculations of tissue fraction and mean linear intercept were as previously described.<sup>21</sup>

### **Statistics**

Data are presented as mean  $\pm$  SEM. Statistics were performed using SigmaPlot 12 (Systat Software Incorporated, USA). Two group experiments were analyzed using the Student  $t$  test if data were normally distributed (or Mann-Whitney U test if not). Multiple group experiments were analyzed using a two-way ANOVA where applicable. Data sets that failed the equal variance test were examined by ANOVA on ranks. ANOVA was followed by *post hoc* testing when significant effects were found (Dunn following ANOVA on ranks, Holm-Sidak for two-way ANOVA). Due to the large number of samples below detection limit in control groups for citrullinated histone-3 immunoblot, these data were categorized as "detectable" or "nondetectable" and subjected to Fisher exact test followed by Bonferroni correction. A threshold of  $P$  value less than 0.05 was used to determine significance.

## Results

### Lipopolysaccharide and VILI

We compared mechanical ventilation at protective (LV<sub>T</sub> group: low tidal volume + moderate PEEP) and injurious (HV<sub>T</sub> group: high tidal volume + zero PEEP) settings in mice that had been randomized to receive lipopolysaccharide instillation *versus* a vehicle (PBS) control 2 h previously. Blood gas measurements (table 1) indicated evidence of hypoventilation in the spontaneous breathing groups (intended controls, Pao<sub>2</sub> = 78 ± 11.0 mmHg) presumably due to ventilation *via* endotracheal tube without mechanical assistance.

Final static compliance was significantly lower in HV<sub>T</sub> + vehicle *versus* LV<sub>T</sub> + vehicle and trended lower in LV<sub>T</sub> group pretreated with lipopolysaccharide (fig. 1A). Both lipopolysaccharide pretreatment and high tidal volume contributed to the decrement in static compliance, and the two factors demonstrated significant interaction (fig. 1B). Similar patterns were seen in dynamic compliance (see fig. 1, Supplemental Digital Content 1, <http://links.lww.com/ALN/B133>). BALF protein measurements also demonstrated that both pretreatment and tidal volume were significant factors increasing leak, but there was no significant interaction on this parameter (fig. 2A). Arterial blood gas readings demonstrated significantly lower oxygenation in HV<sub>T</sub> *versus* LV<sub>T</sub> groups and trended lower in lipopolysaccharide-pretreated animals (table 1).

### Lipopolysaccharide and High Tidal Volume Induce NETosis

We then sought to determine whether mechanical ventilation could induce pulmonary NETosis either alone or with the addition of lipopolysaccharide. Lipopolysaccharide induced neutrophil recruitment to the lung without any change in macrophage numbers (fig. 2B), resulting in a significant increase in total inflammatory cell count and a shift from macrophage to predominantly neutrophil proportions (fig. 2B and see fig. 2, Supplemental Digital Content 1, <http://links.lww.com/ALN/B133>). The effect of HV<sub>T</sub> on neutrophil

recruitment was not significant (fig. 2B); however, Western blot of pooled BALF from the four groups demonstrated that citrullinated histone-3 (Cit-H3) was strongly induced only in the lipopolysaccharide + HV<sub>T</sub> group (fig. 3A). When BALF samples from individual animals were run separately, Cit-H3 was detectable in 9 of 10 lipopolysaccharide + HV<sub>T</sub> animals, but only in 3 of 9 in lipopolysaccharide + LV<sub>T</sub>; it was detected in 1 of 9 vehicle + HV<sub>T</sub> animals and was below the limit of detection in all vehicle + LV<sub>T</sub> animals. Thus, both HV<sub>T</sub> ( $P = 0.002$ ) and lipopolysaccharide ( $P = 0.04$ ) were significant contributors to NETosis (Fisher exact test). BALF DNA was significantly increased in lipopolysaccharide + HV<sub>T</sub> over all other groups (fig. 3B). The release of NETs into distal lung airways is supported by fluorescent microscopy observation. Immunofluorescent staining of Cit-H3 demonstrated increased staining in lung cryosections from lipopolysaccharide + HV<sub>T</sub> mice, which colocalized with DNA in alveolar regions of distal lung (fig. 3C).

### Potential Inducers of NETosis and Lung Injury

As lipopolysaccharide induced neutrophil recruitment but NETs were prominent only with the addition of HV<sub>T</sub>, we examined potential NETosis-inducing factors associated with injurious ventilation. HMGB1 was detectable by Western blot in BALF from all groups and was significantly increased by mechanical ventilation even in the absence of lipopolysaccharide (fig. 4). Multiplex assay of BALF cytokines IL-1 $\beta$ , MCP-1, IL-6, and MIP-2 demonstrated highest levels in lipopolysaccharide + HV<sub>T</sub> *versus* either single hit (fig. 5, A–D). In contrast, TNF $\alpha$ , KC, and granulocyte CSF appear to be induced primarily by lipopolysaccharide with little contribution from HV<sub>T</sub> (fig. 5, E–G), suggesting that they recruit neutrophils but may play little role in inducing NETosis itself under these experimental conditions. Granulocyte macrophage-CSF was significantly increased only by lipopolysaccharide + spontaneous ventilation (see fig. 3, Supplemental Digital Content 1, <http://links.lww.com/ALN/B133>).

**Table 1.** Arterial Blood Gas Measurements—LPS/VILI Model

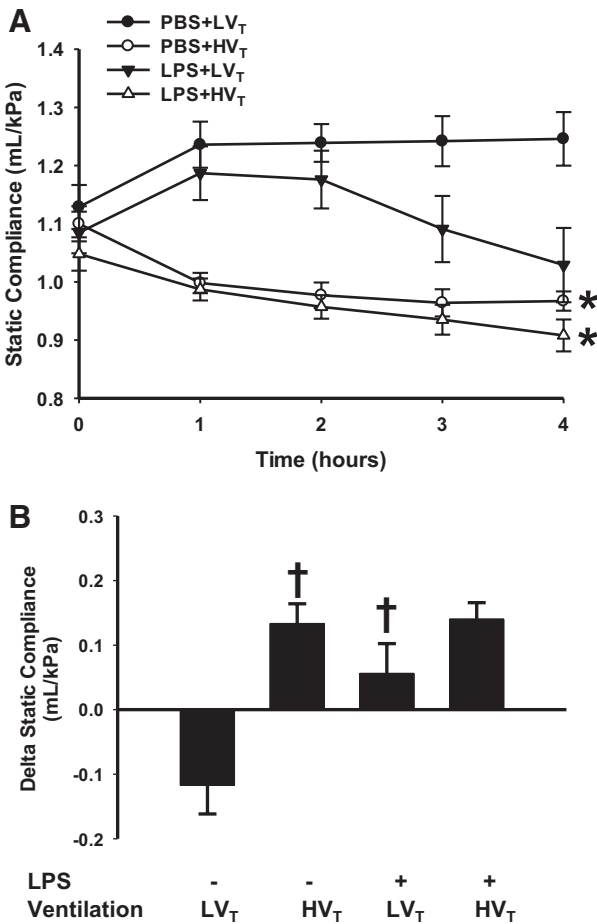
Parameters	PBS + SV	PBS + LV <sub>T</sub>	PBS + HV <sub>T</sub>	LPS + SV	LPS + LV <sub>T</sub>	LPS + HV <sub>T</sub>
pH*	7.22 ± 0.036 N = 11	7.31 ± 0.013 N = 10	7.29 ± 0.015 N = 7	7.22 ± 0.038 N = 11	7.28 ± 0.016 N = 6	7.24 ± 0.014 N = 8
Pao <sub>2</sub> (mmHg)†	78 ± 11.0 N = 9	108 ± 3.2 N = 8	93 ± 4.1 N = 5	80 ± 12.5 N = 8	104 ± 4.5 N = 4	80 ± 5.3 N = 3
Paco <sub>2</sub> (mmHg)†	67 ± 8.1 N = 11	47 ± 2.1 N = 10	49 ± 2.5 N = 7	73 ± 6.0 N = 11	45 ± 2.7 N = 6	49 ± 2.3 N = 8
HCO <sub>3</sub> <sup>-</sup> (mmol/l)‡	25 ± 2.8 N = 11	22 ± 0.7 N = 10	21 ± 0.8 N = 7	27 ± 2.8 N = 11	20 ± 0.9 N = 6	19 ± 0.8 N = 8
Base excess (mmol/l)§	-2 ± 3.1 N = 11	-3 ± 0.8 N = 10	-4 ± 0.9 N = 7	0 ± 4.3 N = 11	-5 ± 1.0 N = 6	-6 ± 0.9 N = 8

Data are mean ± SEM.

\* Two-way ANOVA (LPS  $P < 0.05$ , ventilation  $P < 0.05$ , no interaction); † two-way ANOVA (ventilation  $P < 0.001$ , LPS not significant, no interaction); ‡ two-way ANOVA (ventilation  $P < 0.001$ , LPS not significant, interaction  $P = 0.005$ ); § ANOVA on ranks ( $P < 0.05$ , LPS + SV vs. LPS + HV<sub>T</sub>).

HV<sub>T</sub> = high tidal volume; LPS = lipopolysaccharide; LV<sub>T</sub> = low tidal volume; PBS = phosphate-buffered saline; SV = spontaneous ventilation; VILI = ventilator-induced lung injury.

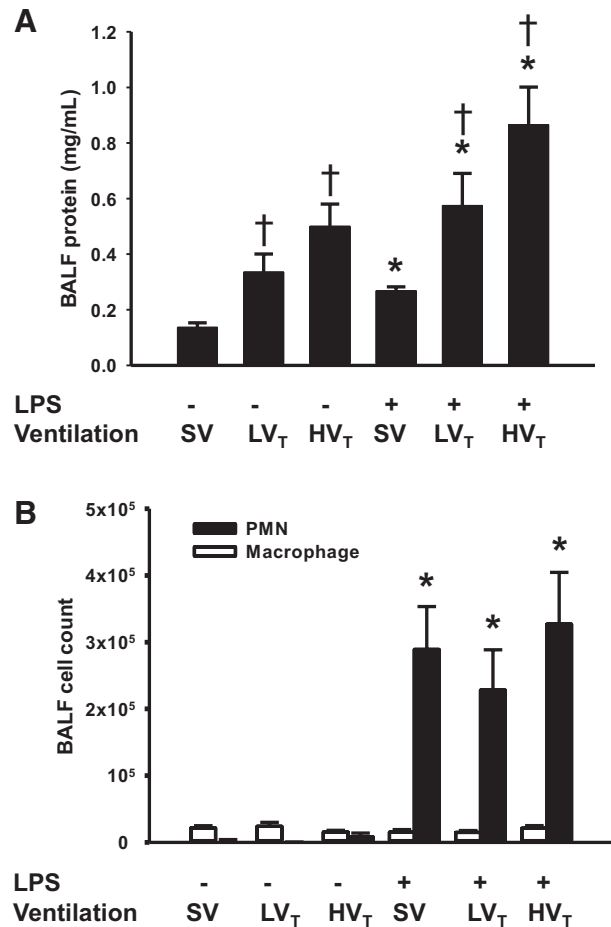




**Fig. 1.** Compliance changes in a two-hit model of lipopolysaccharide (LPS) and mechanical ventilation. (A) Time course of static compliance in mechanically ventilated mice demonstrating a loss of compliance from high tidal volume (HV<sub>T</sub>) ventilation (ANOVA on ranks). (B) Delta static compliance calculated by subtracting final from initial values. Both pretreatment with LPS and tidal volume are significant, and there is a significant interaction (two-way ANOVA). LV<sub>T</sub> = low tidal volume; PBS = phosphate-buffered saline. \**P* < 0.05 versus PBS + LV<sub>T</sub> control; †*P* < 0.05 versus no LPS + LV<sub>T</sub>. N = 10 per group. Note that 1 kPa = 7.5 mmHg.

### DNase Intervention against NETs

To test whether NETosis contributed to injury, we used endotracheal instillation of DNase I to reduce NETs abundance in the lung, in a separate series of experiments. DNase (*vs.* vehicle) reduced the Cit-H3 (*P* = 0.004; fig. 6A) and DNA (*P* = 0.001; fig. 6B). BALF cell counts were not different (fig. 6C), suggesting that DNase was efficacious at attenuating NETs rather than reducing neutrophil recruitment. Both vehicle and DNase-treated animals demonstrate significant injury based on decreased compliance (fig. 6D), blood gas measurements, and BALF protein (table 2). The decrement in static compliance was reduced in the DNase-treated compared with vehicle-treated groups demonstrating that NETs did alter pulmonary mechanics (*P* = 0.003; fig. 6D). However, other measures

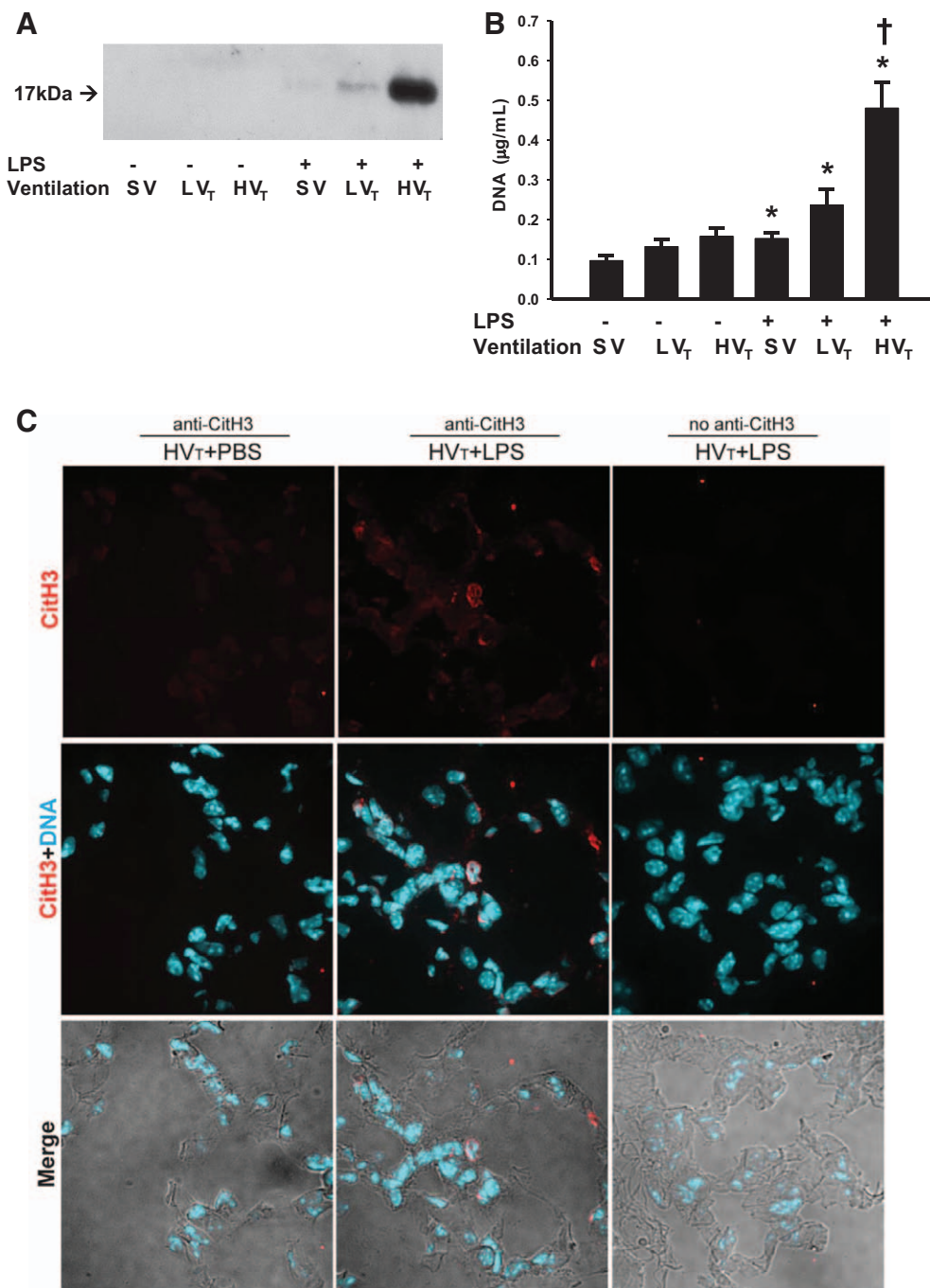


**Fig. 2.** Bronchoalveolar lavage fluid (BALF) protein and cell counts in a two-hit model of lipopolysaccharide (LPS) and mechanical ventilation. (A) BALF protein concentration. Both pretreatment with LPS and tidal volume had significant effects but without significant interaction (two-way ANOVA). (B) BALF cell counts. Macrophage numbers were unchanged among the groups. LPS but not mechanical ventilation induced neutrophil recruitment (ANOVA on ranks). HV<sub>T</sub> = high tidal volume; LV<sub>T</sub> = low tidal volume; PMN = polymorphonuclear cells; SV = spontaneous ventilation. \**P* < 0.05 versus vehicle within same ventilation group; †*P* < 0.05 versus other ventilation modes in same pretreatment group. N = 10 per group.

of lung injury (blood gases, BALF protein levels, and levels of BALF myeloperoxidase) were not reduced by DNase (table 2), and there were no differences between the two groups in BALF cytokine levels (see table, Supplemental Digital Content 1, <http://links.lww.com/ALN/B133>). In addition, morphometric analysis indicated no difference in tissue fraction (table 2) or mean linear intercept (data not shown) among the two groups.

### Pharmacological Intervention against IL-1β and HMGB1

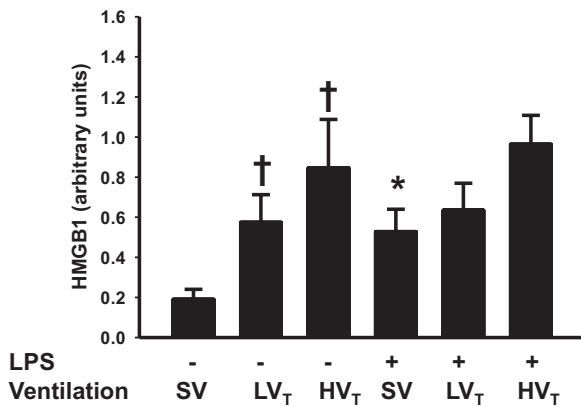
To test whether the increases in IL-1B or HMGB1 were responsible for NET formation, we used pharmacological blockers: anakinra, a recombinant form of IL-1R antagonist, and glycyrrhizin, a small molecule inhibitor of HMGB1.<sup>22</sup>



**Fig. 3.** Pulmonary neutrophil extracellular trap formation in a two-hit model of ventilator-induced lung injury. (A) Western blot for citrullinated histone-3 (Cit-H3) using equal volumes pooled bronchoalveolar lavage fluid. A strong band can be seen only in the lipopolysaccharide (LPS) + high tidal volume (HV<sub>T</sub>) group. (B) Bronchoalveolar lavage fluid DNA concentration was increased in LPS + HV<sub>T</sub> (two-way ANOVA). \**P* < 0.05 versus vehicle within same ventilation group; †*P* < 0.05 versus all other groups. N = 10 per group. (C) Immunofluorescence staining of Cit-H3 on cryofixed lung sections. Immunofluorescence image analyses of alveolar regions show colocalization of Cit-H3 with DNA in HV<sub>T</sub> + LPS frozen tissue sections. Control (HV<sub>T</sub> with no LPS) shows no obvious staining of Cit-H3. The negative control (without primary antibody) rules out the possibility of nonspecific binding of secondary antibody. LV<sub>T</sub> = low tidal volume; PBS = phosphate-buffered saline; SV = spontaneous ventilation.

In contrast to the results with DNase intervention, treatment significantly reduced neither BALF Cit-H3 nor DNA compared with vehicle (see fig. 4, A and B, Supplemental Digital Content 1, <http://links.lww.com/ALN/B133>). BALF cell

counts were similar among the vehicle and treatment groups (see fig. 4C, Supplemental Digital Content 1, <http://links.lww.com/ALN/B133>), and static compliance was not protected (see fig. 4D, Supplemental Digital Content 1, <http://links.lww.com/ALN/B133>).



**Fig. 4.** Bronchoalveolar lavage fluid levels of high-mobility group box 1 (HMGB1). HMGB1 levels in bronchoalveolar lavage fluid from ventilated mice, quantified by western blot analysis of individual samples ( $n = 8$ – $10$  per group). Two-way ANOVA indicated both pretreatment and ventilation were significant but no interaction. \* $P < 0.05$  versus vehicle within same ventilation group; † $P < 0.05$  versus other ventilation modes in same pretreatment group.  $N = 10$  per group. HV<sub>T</sub> = high tidal volume; LPS = lipopolysaccharide; LV<sub>T</sub> = low tidal volume; SV = spontaneous ventilation.

links.lww.com/ALN/B133). No significant differences in blood gas measurements or BALF protein were found (table 3).

## Discussion

We set out to determine whether VILI could induce NETosis either *via* injurious ventilation alone or in a two-hit model with lipopolysaccharide. This model was chosen to mimic clinical manifestations of ventilator-associated lung injury. Lipopolysaccharide recruitment of neutrophils to the lung was included based on previous experience in which mechanical ventilation alone induced minimal neutrophil influx in mouse models. High tidal volumes and no PEEP were used to injure the lung through overinflation and atelectasis, respectively. Protective ventilation (low tidal volume plus PEEP) was used as a control for any changes induced by the tracheostomy and anesthesia.

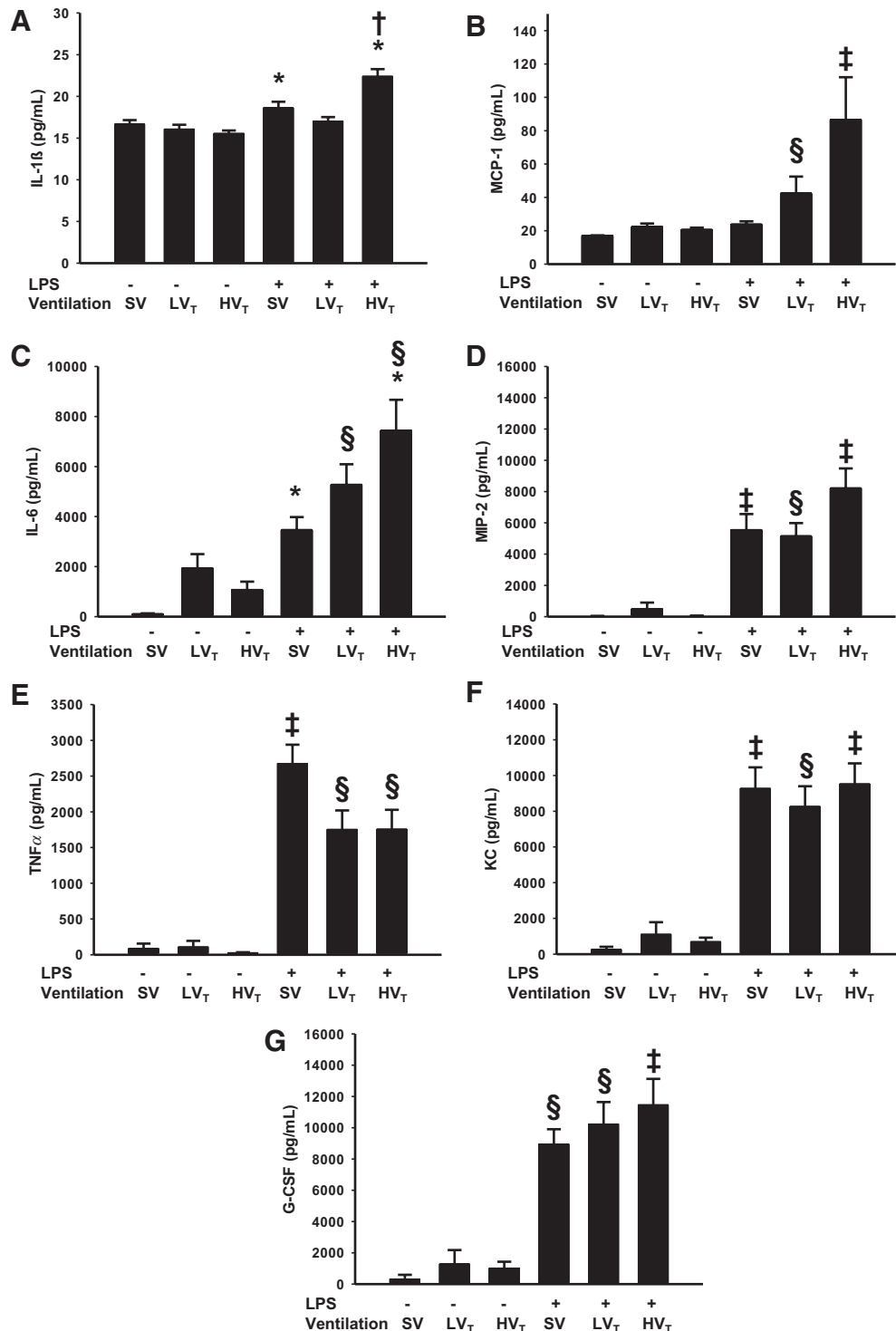
Our two-hit model successfully demonstrated the formation of NETs as evidenced by the detection of free DNA and citrullinated histones in BALF after lipopolysaccharide-induced neutrophil influx into the lung and injurious mechanical ventilation. This model can be used for future studies of the effect of NETosis on mechanical ventilation. Lipopolysaccharide and injurious ventilation (high tidal volumes and absence of PEEP) both contributed to lung injury (reduced lung compliance or increased BALF protein) with a significant interaction on the decrement in static compliance. Although lipopolysaccharide clearly induced neutrophil recruitment to the airspaces and increased several cytokines associated with lung injury and/or NETosis (e.g., TNF $\alpha$ , KC, granulocyte CSF), important markers of NET formation in the BALF (*i.e.*, Cit-H3, DNA) were significantly

increased only in the presence of both lipopolysaccharide and injurious ventilation (*i.e.*, the lipopolysaccharide + HV<sub>T</sub> group). Although extracellular lung DNA may also derive from necrotic leukocytes, the detection of citrullinated histone-3 in our study indicates detection of NETosis—not necrosis. Recovery of NET components (DNA, Cit-H3) in BALF suggests release of NETs into airways; fluorescent microscopy results are consistent with release of NETs into alveolar spaces.

Reduction of NETs by DNase was successful as assessed by both BALF markers (Cit-H3 and DNA), but this treatment attenuated compliance changes (*i.e.*, improved lung mechanics) without significantly protecting other measures of lung injury suggesting that NETosis can occur during VILI and may impact lung mechanics. Although attenuation of NETs was not protective with respect to inflammatory cell infiltration, cytokine release, tissue fraction, or protein leak in this model, improved compliance might be advantageous. We were unable to prevent NET formation using pharmacological inhibitors of two known NET inducers.

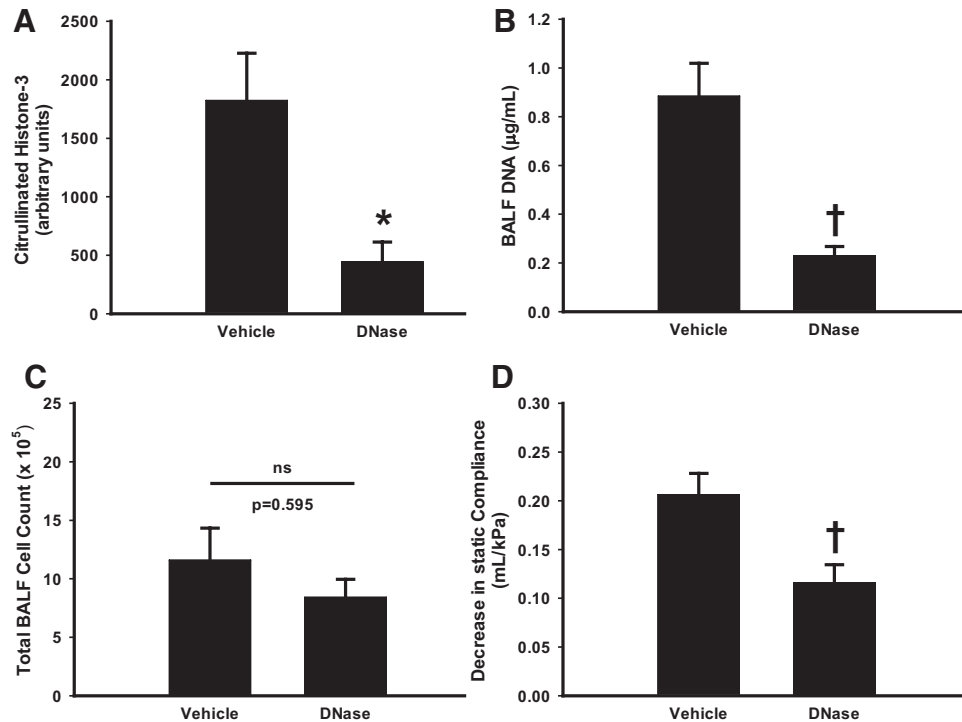
The presence of NETs during VILI is a finding that cannot be explained solely by the lipopolysaccharide-induced neutrophil recruitment because all lipopolysaccharide groups had similar neutrophil counts yet the level of NETosis was far higher when injurious ventilation was applied after lipopolysaccharide pretreatment. The exact role of lipopolysaccharide in formation of NETosis in this model is unknown; lipopolysaccharide may only be required to recruit significant numbers of neutrophils into the lung. Previous studies<sup>15</sup> have shown high levels of pulmonary NETosis after longer (24 h) time courses postlipopolysaccharide instillation in the absence of mechanical ventilation. Rossaint *et al.*<sup>23</sup> have recently detected NETosis in a short-term VILI model without the addition of lipopolysaccharide, in contrast to our findings in the PBS + HV<sub>T</sub> group. This may be due to differences in the models (one hit, peak inspiratory pressure 45 cm H<sub>2</sub>O, 2-h ventilation *versus* two hit, lipopolysaccharide 2-h preexposure followed by 4 h of ventilation with more moderate end-inspiratory pressures of 15 to 20 cm H<sub>2</sub>O). It is not possible to directly compare the levels of NETosis in the latter study *versus* our model due to the differences in approaches for NET detection. Nevertheless, our data suggest that lipopolysaccharide plus injurious ventilation interact to accelerate the production of NETs in the pulmonary airspaces.

We assayed cytokines that are known to be associated with VILI and/or are known to induce NETosis. Animal models of pneumonia or endotoxemia with mechanical ventilation have demonstrated synergistic effects of the infectious—and the ventilator—components of injury in increasing IL-6, MIP-2, MCP-1, TNF $\alpha$ , IL-1 $\beta$ , and KC.<sup>24–26</sup> A previous model of HV<sub>T</sub> has shown increases in the two CXCR2 ligands, MIP-2 and KC, comparable with use of lipopolysaccharide,<sup>27</sup> but we did not observe such increases with HV<sub>T</sub> alone. Three of the cytokines measured (IL-6, MIP-2, and MCP-1) did not show a significant difference between HV<sub>T</sub>



**Fig. 5.** Inflammatory profile of bronchoalveolar lavage fluid. Multiplex protein kit was used to analyze bronchoalveolar lavage fluid concentrations. (A) Only interleukin (IL)-1 $\beta$  was significantly increased in lipopolysaccharide (LPS) + high tidal volume (HV<sub>T</sub>) versus any other group. (B) Monocyte chemoattractant protein-1 (MCP-1), (C) IL-6, and (D) macrophage inflammatory protein-1 (MIP-2) were increased by HV<sub>T</sub> only in the presence of LPS. (E) Tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), (F) keratinocyte chemoattractant (KC), and (G) granulocyte colony-stimulating factor (G-CSF) were primarily increased in response to LPS. LV<sub>T</sub> = low tidal volume; SV = spontaneous ventilation. IL-1 $\beta$  by two-way ANOVA; all others ANOVA on ranks. \* $P$  < 0.05 versus vehicle under same ventilation; † $P$  < 0.05 versus other ventilations under same pretreatment; ‡ $P$  < 0.05 versus all vehicle groups; § $P$  < 0.05 versus PBS + SV. N = 10 per group.





**Fig. 6.** Deoxyribonuclease (DNase) I intervention against pulmonary neutrophil extracellular trap formation: quantification of citrullinated histone-3 (Western blot, A) and DNA (B) in bronchoalveolar lavage fluid (BALF) demonstrates a reduction in neutrophil extracellular traps with intratracheal DNase treatment. (C) No significant change in BALF cell count was detected (Mann–Whitney test). ns = not significantly different. (D) DNase treatment led to an attenuation in the decrement in compliance. \* $P = 0.001$  versus vehicle (Mann–Whitney); † $P < 0.005$  versus vehicle ( $t$  test).  $N = 10$  per group. Note that 1 kPa = 7.5 mmHg.

**Table 2.** Effects of DNase treatment on Lung Injury

Parameters	Vehicle	DNase
pH*	7.17 ± 0.013 N = 19	7.16 ± 0.016 N = 19
Pao <sub>2</sub> (mmHg)	73 ± 3.0 N = 15	78 ± 4.0 N = 15
Paco <sub>2</sub> (mmHg)	52 ± 1.4 N = 19	50 ± 1.9 N = 19
HCO <sub>3</sub> <sup>-</sup> (mmol/l)	14 ± 1.5 N = 19	16 ± 0.7 N = 18
Base excess (mmol/l)	-8 ± 1.4 N = 19	-10 ± 1.0 N = 19
BALF protein (mg/ml)	1.22 ± 0.079 N = 9	1.20 ± 0.107 N = 9
Tissue fraction	0.261 ± 0.0155 N = 10	0.266 ± 0.0149 N = 10
BALF MPO (arbitrary units)	880 ± 137 N = 9	1,140 ± 267 N = 9

Data are mean ± SEM. No significant between-group differences.

\* $P > 0.05$ .

BALF = bronchoalveolar lavage fluid; DNase = deoxyribonuclease; MPO = myeloperoxidase.

versus LV<sub>T</sub> but did appear to follow a similar trend to the BALF DNA/Cit-H3 quantification within the lipopolysaccharide groups (fig. 5, B–D), suggesting that NETs production correlated with a greater overall level of inflammation.

**Table 3.** Effects of Kineret or Glycyrrhizin Treatment on Lung Injury

Parameters	Vehicle	Kineret	Glycyrrhizin
pH	7.13 ± 0.021 N = 6	7.16 ± 0.024 N = 5	7.16 ± 0.022 N = 6
Pao <sub>2</sub> (mmHg)	108 ± 5.8 N = 6	97 ± 5.0 N = 4	102 ± 5.4 N = 4
Paco <sub>2</sub> (mmHg)	45 ± 3.6 N = 6	49 ± 2.0 N = 5	49 ± 1.7 N = 6
HCO <sub>3</sub> <sup>-</sup> (mmol/l)	14 ± 1.2 N = 6	15 ± 0.8 N = 5	15 ± 0.7 N = 6
Base excess (mmol/l)	-13 ± 1.9 N = 6	-11 ± 1.1 N = 5	-10 ± 0.9 N = 6
BALF protein (mg/ml)	0.30 ± 0.036 N = 6	0.30 ± 0.028 N = 6	0.23 ± 0.008 N = 6

Data are mean ± SEM. No significant between-group differences.

BALF = bronchoalveolar lavage fluid.

However, IL-6 and MCP-1 are not linked to NETosis; in fact, IL-6-knockout mice are still able to form NETs.<sup>28</sup> In addition, MIP-2 has been shown to stimulate NET production in murine lung and bone marrow neutrophils *ex vivo*, but this appears to be *via* an alternative, reactive oxygen species-independent mechanism.<sup>29</sup> Finally, TNF $\alpha$ , KC, and granulocyte CSF (fig. 5, E–G) have all been shown to induce NETosis<sup>30–32</sup> yet the levels of these cytokines were similarly

increased in both  $HV_T$  and  $LV_T$  in the presence of lipopolysaccharide, suggesting that they alone are not sufficient to trigger production of high levels of NETs in our model, perhaps due to the brief time frame.

One cytokine, IL-1 $\beta$ , was significantly greater in the lipopolysaccharide and  $HV_T$  *versus* controls for either lipopolysaccharide or ventilation alone (fig. 5A), suggesting that it might be the best candidate cytokine to trigger NETosis in our model. IL-1 $\beta$  has been identified as proinflammatory and an inducer of NETosis.<sup>20,33</sup> VILI is in part mediated by the inflammasome, which produces IL-1 $\beta$  in alveolar macrophages after high stretch *in vitro*,<sup>34</sup> and blockade of this pathway reduces lung injury caused by high tidal volume.<sup>35</sup> Our model, consistent with the literature, demonstrates a greater induction of IL-1 $\beta$  in conditions with increased NETosis.

The damage-associated molecular pattern molecule HMGB1 was significantly greater with high compared with low tidal volume (fig. 4). HMGB1 is a nonhistone structural protein for chromatin which has been identified as an alarmin that mediates immune responses to infection and sterile injury.<sup>36</sup> Immune cells such as macrophages release HMGB1 in an active process after lipopolysaccharide stimulation, and it can also be released from lysed cells.<sup>37</sup> Interestingly, it has been shown that after endotoxemia, HMGB1 is released *via* an inflammasome-dependant pathway,<sup>38</sup> suggesting a possible link between IL-1 $\beta$  and HMGB1 in the current experiments. In addition, experimental high tidal volume<sup>18</sup>—as well as established mechanical ventilation in patients<sup>39</sup>—increases release of HMGB1. Extracellular HMGB1 acts as a signaling molecule by binding toll-like receptor (TLR) 4, TLR2, and receptor for advanced glycation end products; in combination with lipopolysaccharide, HMGB1 has been shown to induce NETosis through TLR4,<sup>20</sup> consistent with our observations.

Of the signaling molecules measured that are potential inducers of NETosis, only HMGB1 and IL-1 $\beta$  increased significantly in lipopolysaccharide +  $HV_T$  *versus* both one-hit groups. This suggested that induction of these mediators by injurious ventilation may be the trigger for NETosis in this model; however, blockade of either of these molecules was insufficient to prevent NET formation despite doses guided by published studies using rodent models of acute tissue injuries. Given the large number of molecules now known to induce NETs, it is probable that blockade of any single one may be insufficient to prevent NETosis in the context of our *in vivo* model.

Intratracheal DNase treatment was used as an intervention against NETs in mice pretreated with lipopolysaccharide and ventilated with high tidal volumes to test its therapeutic potential and provide evidence that NETs may contribute to lung injury. DNase-treated mice demonstrated decreased levels of Cit-H3 and BALF DNA compared with vehicle-treated animals (fig. 6, A and B), despite comparable BALF cell count (fig. 6C). Although DNase pretreatment was associated with lower decrement in static compliance (fig. 6D), we found no impact of DNase on other measures

of lung injury such as oxygenation, levels of BALF protein or myeloperoxidase, or tissue fraction in morphometry (table 2). This isolated improvement in compliance thus may be due to decreases in viscosity of airway secretions rather than protection from VILI.

In contrast to our findings, intraperitoneal administration of DNase in a transfusion-related acute lung injury model with NETs improved oxygenation, possibly because of improved degradation of plasma NETs.<sup>40</sup> In addition, in the mouse VILI model mentioned above,<sup>23</sup> either elastase knockout or treatment of wild-type mice with DNase I provided effective protection, whereas DNase-knockout mice displayed aggravated injury in a model in which injury was induced by high end-inspiratory pressure (45 cm H<sub>2</sub>O) as a “single hit.” However, the limited efficacy of DNase treatment in our two-hit model (lipopolysaccharide preexposure plus milder inspiratory pressures) confirms that NETs are formed in VILI but may have only a limited role in pathogenesis in the context of preexisting inflammatory stimulus (*e.g.*, lipopolysaccharide).

NETosis represents a new consideration for the role of neutrophils during pathogenesis of lung injury, and the mechanism have recently been shown to require both integrin-mediated signaling and activation of G-protein-coupled receptors on neutrophils.<sup>23</sup> Released histones such as those displayed on NETs are toxic to epithelial<sup>41</sup> and endothelial cells and promote lung edema, microvascular thrombosis, and hemorrhage in mouse models of trauma, suggesting that they may be viable therapeutic targets in lung injury<sup>42</sup>; however, their usefulness may be limited in the context of mechanical ventilation or where preexisting infection is present.

## Conclusions

We have established a double-hit lipopolysaccharide/VILI model to study NETs in the airways. In this mouse model, NETs form only during high VT. Increased BALF levels of two known NET inducers—HMGB1 and IL-1 $\beta$ —correlate with NET formation in the airways, and NETs in lipopolysaccharide/VILI can be successfully eliminated by DNase treatment. Although DNase treatment eliminates NETs and improves lung compliance, it does not prevent airway inflammation.

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## Competing Interests

Christopher Yildiz received a University of Toronto Scholarship (Toronto, Ontario, Canada). The other authors declare no competing interests.

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