The Effects of Mechanical Ventilation on Neutrophil Extracellular Trap Formation

by

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Abstract

Mechanical ventilation can physically injure the lung and induce a proinflammatory state. Ventilator induced lung injury (VILI) is associated with neutrophil influx into the lung. Neutrophils can release DNA and granule proteins as cytotoxic neutrophil extracellular traps (NETs). This project aimed to determine if NETs were formed and contributed to injury in a two-hit LPS/VILI mouse model. We found that the combination of LPS and mechanical ventilation induced generation of NETs. High tidal volume mechanical ventilation increased release of HMGB1, an inducer of NETs, over low tidal volume control. The combination of LPS and high tidal volume was the greatest inducer of IL-1β, another potential inducer of NETs. Intratracheal DNase treatment reduced NETs and attenuated the loss of static compliance in the LPS/VILI model. NETosis represents a novel therapeutic target for VILI.
Acknowledgments

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<tbody>
<tr>
<td>ALI</td>
<td>Acute lung injury</td>
</tr>
<tr>
<td>ANCA</td>
<td>Anti-neutrophil cytoplasmic antibodies</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>Activated protein C</td>
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<tr>
<td>ARDS</td>
<td>Adult respiratory distress syndrome</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>C3</td>
<td>Complement component 3</td>
</tr>
<tr>
<td>C5a</td>
<td>Complement component 5a</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>CLP</td>
<td>Cecal ligation and perforation</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CPAP</td>
<td>Continuous positive airway pressure</td>
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<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<td>DAMP</td>
<td>Damage associated molecular pattern</td>
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<tr>
<td>DPI</td>
<td>Diphenylene iodonium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Recombinant human deoxyribonuclease I (Dornase alfa)</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>ECMO</td>
<td>Extra corporeal membrane oxygenation</td>
</tr>
<tr>
<td>EETs</td>
<td>Eosinophil extracellular traps</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FiO₂</td>
<td>Fraction of inspired oxygen</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HFV</td>
<td>High frequency ventilation</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High mobility group box 1</td>
</tr>
<tr>
<td>HNA-3a</td>
<td>Human neutrophil antigen 3a</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>KC</td>
<td>Keratinocyte chemoattractant</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCETs</td>
<td>Mast cell extracellular traps</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
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<tr>
<td>METs</td>
<td>Macrophage extracellular traps</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>--------</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP2</td>
<td>Macrophage inflammatory protein 2</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response 88</td>
</tr>
<tr>
<td>NAVA</td>
<td>Neurally-adjusted ventilator assist</td>
</tr>
<tr>
<td>NE</td>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular trap</td>
</tr>
<tr>
<td>NFkB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cell</td>
</tr>
<tr>
<td>NIV</td>
<td>Non-invasive ventilation</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NOD-like receptor family, pyrin domain containing 3</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOX2</td>
<td>NADPH oxidase 2</td>
</tr>
<tr>
<td>nRDS</td>
<td>Neonatal respiratory distress syndrome</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>Arterial partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PAD4</td>
<td>Peptidylarginine deiminase 4</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Arterial partial pressure of oxygen</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive end expiratory pressure</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>PR3</td>
<td>Proteinase 3</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>Rac2</td>
<td>Ras-related C3 botulinum toxin substrate 2</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation endproducts</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SP-A</td>
<td>Surfactant protein A</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TRALI</td>
<td>Transfusion related acute lung injury</td>
</tr>
<tr>
<td>V&lt;sub&gt;A&lt;/sub&gt;</td>
<td>Alveolar ventilation</td>
</tr>
<tr>
<td>VALI</td>
<td>Ventilator associated lung injury</td>
</tr>
<tr>
<td>VAP</td>
<td>Ventilator associated pneumonia</td>
</tr>
<tr>
<td>VILI</td>
<td>Ventilator induced lung injury</td>
</tr>
<tr>
<td>V&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Tidal volume</td>
</tr>
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</table>
1 Introduction

1.1 Mechanical ventilation

Mechanical ventilation is a key feature of intensive care and inpatient treatments for lung injury. Mechanical ventilators deliver O₂ to the lung, eliminate CO₂, reduce the work of breathing or replace inadequate spontaneous breathing. Early approaches used an iron lung to generate negative pressure on the torso of the patient, and thereby expand the chest wall and lung. The iron lung was lifesaving in many thousands of patients for respiratory paralysis secondary to poliomyelitis.\(^1\) The introduction of positive pressure ventilation allowed for less cumbersome machines but this requires intubation or tracheostomy of patients to directly access the trachea or where lower pressures are used a special facemask.

Mechanical ventilation is common and costly. An analysis from six states in the USA found that almost three percent of hospital inpatients were mechanically ventilated, with these patients making up twelve percent of the hospitals’ costs.\(^2\) Increasing use of mechanical ventilation has been associated with increases in co-morbidities (e.g., diabetes, pulmonary disease and renal disease) and with fewer discharges over the years.\(^3\) Although urban centers are seen to have significantly larger numbers of ventilated patients, this may be due to rural centers sending their sickest patients away to more specialized care facilities. Beyond acute care, increasing use of prolonged mechanical ventilation has led to increasing costs in the ICU as well as reduced discharge home, notwithstanding increased survival after one year.\(^4\) The trends of increasing frequency of mechanical ventilation as well as increased time spent on a ventilator make it essential that improvements are made in standards of care and interventions, in order to keep costs from rising further and yet allow more patients to be discharged home.

1.1.1 Respiratory failure

In adults, respiratory failure regardless of the cause may be an indication for mechanical ventilation. Type 1 respiratory failure is said to occur when levels of hypoxemia reach an arterial partial pressure of oxygen (PaO₂) lower than 60 mmHg (combined with normal or reduced arterial partial pressure of carbon dioxide (PaCO₂)).\(^5\) Mismatch of alveolar ventilation and regional perfusion is the commonest mechanism of hypoxemia (caused by pneumonia, acute
respiratory distress syndrome (ARDS), pulmonary edema, pulmonary embolism etc.), and other mechanisms include low alveolar ventilation ($V_A$), insufficient fraction of inspired oxygen ($FiO_2$), increased alveolar-capillary diffusion gradient, or existence of an intrapulmonary (or extrapulmonary) shunt. Type 2 respiratory failure is said to occur when alveolar ventilation is inadequate, leading to insufficient clearance of $CO_2$ with elevated $PaCO_2 (> 45 \text{ mmHg})$ in addition to lowered $PaO_2$ $^5$. The mechanisms of inadequate $V_A$ include impaired minute ventilation (e.g., airway obstruction, neuromuscular weakness, inadequate respiratory drive) or increased alveolar deadspace (volume of dead space/tidal volume ($V_D/V_T$); e.g., pulmonary embolism, hyperinflation). Respiratory failure can also be caused by acute exacerbations of chronic respiratory disease (e.g., chronic obstructive pulmonary disease (COPD), asthma) versus de novo acute respiratory failure $^6$.

ARDS is a clinical diagnosis and indication for mechanical ventilation made based on rapid onset (within seven days of known clinical injury) of hypoxemia with $PaO_2/FiO_2 \leq 300 \text{ mmHg}$ and bilateral infiltrates on chest X-ray or CT scan, not explained by cardiac failure, fluid overload, lung collapse or effusions. $^7$. Between 15 and 34 of every 100,000 people will be diagnosed with ARDS each year $^8$. Although not part of the consensus definition, neutrophil influx, increased protein leak into the alveoli and reduced lung compliance are important biologic elements of acute lung inflammation $^9$. After a diagnosis of ARDS is made, mechanical ventilation will be considered and if hypoxemia, hypercapnia and/or work of breathing are sufficiently problematic. Even with mechanical ventilation as supportive care the mortality of ARDS is approximately 44% $^{10}$.

Mechanical ventilation can save lives but can also cause harm (see below, section 1.2). Current approaches to mechanical ventilation, while focusing on adequacy of gas exchange and work of breathing, also focus on preventing harm from the ventilator. Use of low tidal volumes to prevent over-distension of the lung and application of positive end expiratory pressure (PEEP) to prevent collapse of alveoli are common practices for safer ventilation $^{11}$. Traditionally high tidal volumes were used to allow for greater oxygenation but were shown to increase mortality.

A different indication for mechanical ventilation is premature birth leading to neonatal respiratory distress syndrome (nRDS). Lacking sufficient surfactant to maintain the surface tension needed to keep alveoli open, these newborns require supportive therapy such as
continuous positive airway pressure (CPAP) and exogenous surfactant, in addition to carefully titrated supplemental O\textsuperscript{12}. When mechanical ventilation is used, care must be taken not to injure the structurally premature lung with high airway pressures or tidal volume ($V_T$).

1.1.2 Types of mechanical ventilation

Conventional mechanical ventilation delivers preselected FiO\textsubscript{2} at a chosen airway pressure and frequency (respiratory rate). During inspiration, the $V_T$ is delivered at specified airway pressure, and during expiration a certain amount of airway pressure, PEEP is used to prevent alveolar collapse at the end of expiration. Different modes of ventilation permit choice of the durations of inspiration and expiration, the initiation and of termination of each breath, and whether the patient will be permitted to breathe spontaneously in addition to the breaths provided by the ventilator\textsuperscript{13}.

Non-invasive ventilation (NIV) is the use of a face mask to deliver air from the machine without intubation or tracheostomy. This has multiple benefits including reduced risk of ventilator associated pneumonia, reduced tracheal damage and less resistance from the endotracheal tube\textsuperscript{14}. The absence of intubation means that sedation needs may be less, creating a more tolerable state for patients as they are better able to communicate, eat and cough. NIV is contraindicated in conditions where the patient’s airway is acutely obstructed, gastrointestinal bleeding is present or the patient is unable to spontaneously breathe. The use of non-invasive positive pressure allows for home use of mechanical ventilation where a patient is suffering from long-term problems such as obstructive sleep apnea or COPD; this can increase convenience and reduce costs through less need for hospitalization.

Neurally-adjusted ventilator assist (NAVA) is a method of using the patient’s own signal to breathe from the brainstem, detected through the phrenic nerve, to control the machine and determine respiratory rate and tidal volume\textsuperscript{15}. As changes in a patient’s blood levels of CO\textsubscript{2}, pH and O\textsubscript{2} alter signaling in the medulla oblongata through their chemoreceptors, respiratory rate and volume are altered depending on the body’s metabolic needs. Mechanoreceptors in the lung then signal to stop inspiration to prevent over inflation of the lung. By tying mechanical ventilation to the body’s natural tendency to breathe, the discrepancy between how much ventilation a patient needs or can handle and how much the machine provides is removed.
The use of high frequency ventilation (HFV) allows for lower tidal volumes and a more stable airway pressure that can protect alveoli from overinflation or collapse. The use of small tidal volumes allows constant dilutions in the lung with new oxygenated gas rather than emptying and filling. This method has been used increasingly in patients diagnosed with ARDS as a therapy as it was shown to reduce in-hospital mortality and improve oxygenation in initial small scale clinical trials \(^\text{16}\). However, recent larger scale clinical trials versus updated standard ventilatory controls reported no benefit and evidence of increased harm \(^\text{17,18}\). In low birth weight infants the use of HFV versus conventional ventilation was associated with improved oxygenation and reduced air leak syndrome, suggesting that nRDS may be an indication for its use \(^\text{19}\).

### 1.1.3 Adjunct therapies to mechanical ventilation

Many techniques are available that may be used as an adjunct therapy to mechanical ventilation when the patient has a poor prognosis or worsening lung injury. Some combination therapies assist mechanical ventilation by providing an alternate method of gas exchange such as extra corporeal membrane oxygenation (ECMO). ECMO provides cardiac assistance by removing blood from the body, exchanging CO\(_2\) and O\(_2\), and returning the oxygenated blood to the body. ECMO therefore allows for resting of the lung, i.e., use of low (non-injurious) VT and appropriate levels of PEEP \(^\text{20}\).

Tracheal gas insufflation, the use of constant gas flow to the endotracheal tube, has also been shown to improve gas exchange in ARDS patients when given with HFV \(^\text{21}\). This method is believed to work by removing CO\(_2\) because it decreases dead space. For methods such as low tidal volumes or HFV, tracheal CO\(_2\) elimination is important to reduce arterial blood levels \(^\text{22}\).

Alternatively one can use conventional ventilation but allow CO\(_2\) to ‘permissively’ build up, so called ‘permissive hypercapnia’, with the primary aim of minimizing injury from mechanical ventilation. However, this leads to a decrease in pH, i.e., hypercapnic acidosis which may attenuate immune activation and in turn reduce injury but could also exacerbate infection \(^\text{23}\). Paralysis and sedation are also given with mechanical ventilation as they are necessary in preventing spontaneous breathing against the machine. They also reduce metabolic demand of the body and thereby reduce the amount of gas exchange required \(^\text{24}\). The use of sedatives has been associated with prolonged ventilation but neuromuscular blocking agents appear to reduce
ARDS-related inflammation and increase oxygenation\(^{25, 26}\). Inducing hypothermia in a patient also reduces metabolism but has additional effects in reducing apoptosis and tissue injury\(^{27}\). Vasodilators (e.g., NO) and smooth muscle relaxants (prostacyclins) can improve ventilation-perfusion matching and help treat hypoxic respiratory failure\(^{28}\). Supportive therapies that directly target inflammation have not yet been established in clinical practice and it is not yet known if targeting an acute inflammatory response may improve outcomes.

Thus, many adjuncts and alternatives to conventional mechanical ventilation have been developed to improve the effectiveness of respiratory support, or perhaps more importantly, to reduce the potential for harm caused by mechanical ventilation. The latter issue is now addressed.

### 1.2 Ventilator associated lung injury

Mechanical ventilation can result in a form of iatrogenic lung injury called ventilator associated lung injury (VALI). The components of VALI include volutrauma, atelectrauma, barotrauma and biotrauma. Cyclic collapse and reflation of alveoli results in atelectrauma where the alveoli may become damaged from shear forces. In patients with more severe ARDS higher levels of PEEP was associated with better survival suggesting alveolar collapse contributes to poor outcome\(^ {29}\). The contribution of atelectasis to VALI remains uncertain as recruitment measures have not been associated with prolonged benefit in gas exchange\(^ {30}\).

Volutrauma is the cyclic overinflation of the lung due to large volumes of air in each tidal volume leading to tissue injury. Pig models have shown that higher tidal volumes result in greater extravascular lung water, increased PaO\(_2\) and decreased PaCO\(_2\)\(^ {31}\). Traditionally higher tidal volumes were used to improve gas exchange but this practice was discarded as it proved to increase mortality\(^ {11}\).

Barotrauma results from high pressure where a volume of air is taken into the lung but limited lung compliance leads to higher pressure rather than greater expansion. It can result in pneumothorax, pneumomediastinum, pneumatocele, or subcutaneous emphysema: all conditions where air is displaced from the lung into a nearby part of the body\(^ {32}\). A barotrauma event is more likely with higher PEEP and underlying lung disease\(^ {33, 32}\). Experiments in rats in
which the thorax was bound to prevent expansion have shown that high volumes are more injurious than higher pressures.\textsuperscript{34}

Finally biotrauma refers to injury due to overactivation of the inflammatory response or release of injurious mediators from dying cells. Increased inflammation leads to the release of cytotoxic mediators from leukocytes and causes further tissue damage to the lung \textsuperscript{35}. These immunogenic or injurious mediators have been shown to fulfill many of Koch’s postulates for attributing cause and effect, as soluble mediators produced by high tidal volume can cause injury when experimentally transferred to a healthy lung \textsuperscript{36}. Cytokines induced during mechanical ventilation may prime the innate immune system which may in turn sensitize the lung to injury.

Another mechanism of injury during mechanical ventilation is impairment in the surfactant lining of the lung. Pulmonary surfactant acts to reduce surface tension at the alveolar air-liquid interface and prevent atelectasis \textsuperscript{37}. Thus, impaired surfactant (quantity or function) will increase surface tension and result in a greater propensity to alveolar collapse. Leak of plasma proteins into the lung lumen can disrupt the properties of the surfactant lining by forming large aggregates of phospholipids \textsuperscript{38}. Hyperoxia will also result in oxidation of surfactant lipids, formation of large aggregates and reductions in compliance as experimentally determined minimum surface tension from these lipids increases \textsuperscript{39}. Peroxynitrite, a reactive species formed during oxidative stress can react with surfactant protein A (SP-A) and disrupt its anti-microbial functions of binding mannose and agglutinating which may result in increased risk of infection \textsuperscript{40}. Surfactant isolated from patients with ARDS or pneumonia shows reduced SP-A and phosphatidylcholine, resulting in increased surface tension \textsuperscript{41}. Disruption of the surfactant layer may cause increased atelectasis and reduced compliance leading to further lung injury during ventilation.

Mechanical ventilation, a physical process, results in important physiological changes in the body. Mechanotransduction is any process by which a cell or tissue can convert a mechanical stimulus into a chemical signal or altered activity. Mechanical ventilation will exert some form of stretch or pressure on the epithelium of the lung and its connective tissues which will eventually result in cytokine production, leukocyte recruitment and inflammation \textsuperscript{42}. The pro-inflammatory state is characterized by recognized increases, among many mediators, in tumor necrosis factor $\alpha$ (TNF$\alpha$), interleukin 1$\beta$ (IL-1$\beta$), IL-8, IL-6 and NO \textsuperscript{43}. Injurious
ventilation increases the secretion of FAS-ligand, a mediator of apoptosis, leading to distant organ injury.\textsuperscript{44} Regulation of the expression of transcription factors such as activator protein 1 (AP-1), cAMP response element-binding protein (CREB) and nuclear factor kappa-light-chain-enhancer of activated B cell (NFκB) contribute to cytokine release but the relationship between mechanical ventilation and such mechanisms is uncertain.\textsuperscript{45}

Overdistension of the lung can open stretch-activated calcium channels on the endoplasmic reticulum and lead to increased concentrations of intracellular calcium.\textsuperscript{46} Experiments using inhibitors of these channels show a reduction in microvascular leak suggesting that increased vascular permeability may be partially an active process through calcium dependent signaling.\textsuperscript{47} Increased vascular permeability and the resulting edema or hemorrhage are major concerns for VALI. Stress failure from high volume and pulmonary hypertension can directly break down the alveolar-capillary barrier making lung injury from ventilation multifactorial.\textsuperscript{48} Cellular membranes can also fail under excessive stress and lead to cell death. Physical stresses can also cause integrins, transmembrane proteins that anchor cells, to bind ligands in the extracellular matrix and alter gene expression.\textsuperscript{49} Finally, an additional potential mechanism whereby mechanical force is translated into downstream injury occurs through the physical stresses placed upon cellular junctions. Alterations in signaling by mechanotransduction can bring about a proinflammatory state that is a major contributor to injury.\textsuperscript{42} Blockade or attenuation of these cascades are potential therapeutics.

1.2.1 Complications with VALI

In clinical practice VALI is difficult to diagnose in the setting of ARDS, as there is usually a primary process (e.g., pneumonia, aspiration pneumonitis or trauma) which injures the lung. This establishes the requirement for mechanical ventilation, but at the same time the ventilation can contribute to the overall lung injury. Thus, mechanical ventilation can be thought of as both a contributor to and a therapy for ARDS. Patients with suspected VALI are ventilated using lung protective methods to ensure adequate blood gas levels, while minimizing the potential for harm.

In addition to mechanical injury, mechanical ventilation puts patients at risk for infections known as ventilator associated pneumonia (VAP). VAP diagnosis involves a suspicion of pneumonia, positive bacterial cultures from bronchoalveolar lavage fluid (BALF) and neutrophil recruitment to the lung lumen. Intubation may put patients at greater risk for
VAP as it creates a passage lacking the body’s protective mechanisms (i.e., mucus, cilia), and prevents productive coughing. As the alveolar-capillary barrier breaks down, decompartmentalization of the lung occurs as there is a less effective barrier separating the alveoli and the circulation. Inflammatory mediators and other signals that were local to the lung are released systemically. Systemic inflammatory response syndrome (SIRS) may result in multiple system organ failure. As injurious ventilation can create a similar proinflammatory response to infectious lung injury, sepsis may occur with or without infection. Increases in IL-6 have been found in patients with VAP, which are accompanied by propensity to septic shock. Microbes and associated inflammatory molecules may also enter the circulation from the lung following alveolar-capillary damage, and thereby induce systemic inflammation.

1.3 Ventilator induced lung injury

1.3.1 Animal models

Many animal models have been used to study VALI, ARDS and other complications of mechanical ventilation, most frequently using mice, rats and rabbits. The term ventilator induced lung injury or VILI is used to differentiate the experimental conditions where injury is purposefully inflicted from the inadvertent clinical condition of VALI. Animals may be mechanically ventilated with high tidal volumes, no PEEP or high FiO\textsubscript{2} in order to injure their lungs. Ventilator settings are made purposely injurious with the intent of replicating injury seen in patients due to overdistension of the lung, alveolar-capillary leakage, reactive oxygen species (ROS) and inflammation.

As there are underlying conditions (e.g., pneumonia, aspiration) in any ventilated patient, animal models of ventilation may replicate these by using a two hit model. A common underlying reason for ARDS and ventilation is sepsis, thus a common model combined with VILI is the use of microbes or their components as a contributor to lung injury. With adequate controls one can separate out the contributions to injury from the ventilator versus the second hit.

Two-hit models of VILI may involve the application of an inflammatory mediator in addition to injurious ventilation. Lipopolysaccharide (LPS) and other components of microorganisms are pathogen associated molecular patterns (PAMPs) that through specific pathogen recognition receptors (PRRs) such as Toll-Like Receptor (TLR) can activate and
chemoattract immune cells. Use of isolated PAMPs allows for understanding basic signaling without confounding elements such as virulence factors. Application of LPS either by endotracheal, peritoneal or intravenous administration can be used to stimulate TLR4 or TLR2 signaling. VILI has been shown to involve signaling through TLR4 as mice with the gene knocked out have attenuated proinflammatory cytokine production when mechanically ventilated. Knockout of MyD88, a downstream adaptor protein for most TLRs, also reduces leukocyte recruitment to the lung and secretion of proinflammatory cytokines induced by mechanical ventilation. Mechanical ventilation itself upregulates TLR4 expression which may prime the lung for exaggerated responses to LPS or gram-negative bacteria. The roles of host factors such as cytokines or damage associated molecular patterns (DAMPs) such as heat shock proteins, hyaluronan, HMGB1 and ATP can also be tested using receptor knockouts. Activation of the immune system is critical to these models as depletion of leukocytes such as neutrophils reduced injury, demonstrating that ‘self-inflicted’ injury from the immune system may be a contributor to VILI.

Infectious models are used as alternatives to single molecule stimulation, as whole microbes are more realistic than isolated PAMPs. In short term experiments, a single PAMP may activate fewer signaling pathways compared to a whole organism which would be composed of multiple PAMPs and potential sources for pathogen recognition or immune activation. Single molecule stimulation models also lessen the possibility of receptor crosstalk in immune cells which may have synergistic effects in activating the cell or attenuating the effect of the microorganism. Killed whole microbes may also be used but lack reproductive and complete cytotoxic activity.

Sepsis is an important clinical condition associated with mechanical ventilation and acute lung injury. In order to model sepsis, experimental animals have systemic inflammation or bacteremia induced. For example, cecal ligation and perforation (CLP) allows a small amount of feces from the cecum into the peritoneal cavity allowing a bacterial burden access to the body from the digestive tract. The large number of microbial species combined with the inability of the immune system to contain the infection results in bacteremia, making this an acceptable model for sepsis. This results in systemic inflammation as indicated by proinflammatory markers (e.g., keratinocyte chemoattractant (KC), IL-1β, TNFα, IL-6 and HMGB1) found in serum, organs and the peritoneal cavity. CLP models have inflammatory profiles similar to
human sepsis and are considered a more realistic alternative to a systemic LPS model. It has been shown that exogenous surfactant administration can reduce injury in two hit CLP/VILI models but not in CLP or injurious ventilation alone, suggesting differential contributions to injury by the two individual components. Use of this model has also demonstrated increased cardiac inflammation from the combination of sepsis and mechanical ventilation irrespective of mode. CLP priming before mechanical ventilation has been shown to enhance inflammation in a greater than additive fashion but similar effects were not seen in lung injury measures. As human injury is assumed to have a large component attributed to direct inflammatory damage these results raise questions about the usefulness of cytokines as surrogate markers.

Pneumonia in the critically ill occurs both as community-acquired pneumonia requiring mechanical ventilation, and as VAP which occurs following intubation and ventilation. To simulate pneumonia, healthy animals can be co-housed with inoculated animals to mimic normal transmission, or live bacteria can be instilled directly. Pneumonia models have shown that nasal colonization of bacteria can lead to infection of the lung following airway damage or obstruction. Inoculation with gastric secretions and bacteria together shows greater mortality than either single ‘hit’ demonstrating that acid-induced lung injury is additive with infection. These results demonstrate that underlying injuries to the lung create higher risks with pneumonia. Clinically relevant models can be used to determine interactions and synergistic effects during multifactorial injury.

In order to be clinically relevant, animal models need to recapitulate clinical features of lung injury including increased vascular permeability, leukocyte recruitment, cytokine production and decreases in compliance. Patients may be ventilated for long stretches of time while laboratory experiments are more likely to last hours at the longest. Many animals can be used to model lung injury including non-human primates, dogs, pigs, rats, rabbits and sheep. Larger animals make blood collection and physiological measurements easier but the costs and complexity of animal procurement, care and procedures are often prohibitive. Mouse models are commonly used for the ease of handling as well as their affordability, and the availability of knockout and transgenic mice provides unique genetic tools. It is important to note that genetic differences between mice and humans in TLR4 will cause it to recognize different structures of LPS. Mice also lack IL-8 but have two similar proteins: macrophage inflammatory protein 2 (MIP-2) and KC. Although these two murine chemokines bind a different receptor than IL-8,
they both function to recruit neutrophils to the lung. Mouse models of mechanical ventilation may not properly reproduce the condition in humans, as experiments have shown that even with high PEEP applied, atelectrauma from alveolar collapse cannot be prevented. As this represents a minority of injury in the human condition, mouse models may have different mechanisms and pathways of injury. Inability of the mouse model to properly match the higher compliance of human lungs suggests that mice should be reserved for knockout experiments while larger animals are better suited for basic studies of VILI and lung function. Nonetheless, PEEP has been shown to be protective in mice as the combination of 0 cm PEEP and high tidal volumes was more injurious than high tidal volume plus 3 cm PEEP. No animal model can fully recapitulate the human clinical condition, but through proper experimental design they can be used to examine specific aspects of disease.

1.3.2 In vitro models

Lung epithelial and endothelial cells can be cultured in vitro as a method to test treatments or isolate signaling pathways. Primary cells or permanent cell lines can be used and are generally grown to confluency to allow monolayers to form both gap junctions and tight junctions. When 100% confluent, the monolayer can be exposed to high stretch, hyperoxic conditions and inflammatory mediators to model VILI. Air-liquid interface models involve culturing the basal surface of the monolayer in contact with media while the apical surface is exposed to air in order to better replicate the pulmonary airway and ensure full differentiation of cultured cells. High stretch of lung epithelial cells grown on flexible membranes has been shown to upregulate a variety of effectors that can contribute to lung injury including ROS production, NF-kB, P-selectin, TLR2, monocyte chemoattractant protein-1 (MCP1), IL-8 and apoptosis. These alterations can lead to greater sensitivity to lung infection and leukocyte recruitment. Many additional signaling pathways have been implicated by use of cell stretch models.

Another in vitro model is the use of isolated blood-free lungs which are perfused with media and ventilated while maintained under temperature and humidity control. This model keeps both the epithelium and endothelium intact but removes any contribution from complement, clotting cascades or leukocytes. Using isolated perfused lungs it has been shown that PEEP loses its protective effects during two hit injury and that mean airway pressure contributes more to injury than tidal volume. The isolated lung model has demonstrated that
BALF MCP-1, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6, are increased even during low tidal volume ventilation with PEEP suggesting that some injury is inherent in any use of mechanical ventilation \(^8^3\).

### 1.4 Neutrophil function in acute lung disease

Neutrophils are one of the mammalian granular leukocytes which play a role in resisting infection from microbes. Neutrophils are characterized by a multi-segmented nucleus and pink staining with hematoxylin and eosin suggesting a neutral pH cytoplasm \(^8^4\). Like all leukocytes, neutrophils derive from a myeloid progenitor but can be identified by the cell surface marker Gr-1 \(^8^4\). Normally found in the blood, neutrophils will travel to and enter tissue sites of infection and injury. They are characteristic of the earliest stage of inflammation. Chemokine gradients, produced by macrophages and non-immune cells following contact with microbial components or other inflammatory mediators, such as IL-8, MIP-2 and KC (mouse protein similar to IL-8) act to help neutrophils home into infected tissues \(^8^5\). These cytokines may also activate or prime cells preparing them to fight infection. Higher concentrations of these cytokines and released proteases from neutrophils are associated with non-recovery from ARDS suggesting a pathogenic role \(^8^6\). Neutrophil extravasation begins after resident macrophages release TNFα, signaling endothelial cells to express P-selectin and E-selectin \(^8^7\). These adhesion molecules act to slow neutrophil movement and promote their rolling along the capillary wall. Signaling through chemokines will cause a change in integrin expression on the neutrophil surface resulting in high-affinity binding and adherence to the endothelial wall. The bound neutrophil must transmigrate across the endothelium and basolateral membrane to enter the tissue, a process requiring cytoskeletal rearrangement. Once in the lung the neutrophil is mobile through use of its pseudopods and can be directed by chemokine signals.

Activated neutrophils can kill microbes through phagocytosis, degranulation or ROS production \(^8^4\). Neutrophils phagocytose opsonized microorganisms through a process requiring receptors for IgG as well as the complement proteins C3b and C4b \(^8^8\). Microbes are engulfed into a phagosome where ROS such as superoxide from NADPH oxidase 2 (NOX2) and hypochlorous acid from myeloperoxidase (MPO, in azurophilic granules) react with lipids, proteins and DNA in the phagosome to kill microbes \(^8^9\). Granular proteins including serine proteases and antimicrobial peptides will also have contact with engulfed microbes when the
phagosome merges with a lysosome or granule. Neutrophil proteases (e.g., cathepsin G, neutrophil elastase) cleave peptide bonds and rapidly degrade microbial proteins while antimicrobial peptides such as defensins act to permeabilize bacteria. Degranulation releases antimicrobial contents of the neutrophil into the extracellular space by fusing granules to the plasma membrane. ROS production can also occur at the plasma membrane releasing them into the extracellular space to act. Neutrophils also produce cytokines themselves upon entering the tissue to recruit other immune cells. Once exhausted, neutrophils must be cleared from the tissue; traditionally this is thought to happen through apoptosis. Without sufficient inflammatory stimulus, or when the cell is senescent, caspase activation and protein degradation occurs within the cell. Macrophages will phagocytose spent neutrophils as the final step to clearing them.

Neutrophil killing functions being either proteolytic or via reactive free radicals may also damage host cells and the extracellular matrix. In the lung, neutrophil alveolitis is believed to be an important contributor to both acute lung injury (ALI) and ARDS. Premature infants show markers of protein damage from MPO activity in the lung suggesting it may be a complicating factor in nRDS. Experimental models show that ROS injure the lung independently of proteases. Neutrophil elastase and ROS have been demonstrated to be synergistic in causing edema in animal experiments, giving proof that neutrophils recruited to the lung are not bystanders in injury. SIRS patients with elevated plasma levels of neutrophil elastase were more likely to develop ALI/ARDS. Patients in this condition when given a neutrophil elastase inhibitor showed reduced mortality and increased rates of weaning and discharge from the ICU. Neutrophil components with known cytotoxicity can be therapeutic targets during lung injury and improve the course of ventilation.

1.5 Neutrophil extracellular traps

1.5.1 Formation

Neutrophil extracellular traps (NETs) are an anti-microbial function of the neutrophil whereby they release genomic DNA coated in granule proteins (Figure 1). NET formation (NETosis) is a mode of cell death independent of apoptosis or necrosis. NETosis is an active process requiring both autophagy and the production of ROS. Both of these processes lead to the inhibition of caspases, excluding apoptosis as a final possible fate for these cells. In apoptosis
the chromatin can be seen to condense before being separated into different vesicles while in NETosis chromatin will decondense to be extruded from the cell. Time courses show that NETosis happens hours earlier than apoptosis. Nuclear blebbing and phosphatidyl serine exposure on the outer leaflet of the plasma membrane, two other markers for apoptosis, are not seen during live imaging of NETosis. Although permeabilized as in necrosis, NETosing neutrophils lack the marker F-actin and display signs that NETosis is an active process.

The traditional view of NETosis as a form of cell death has been challenged by observation of mitochondrial DNA release from neutrophils as well as the visualization of mobile NETosing neutrophils in vivo called cytoplasts. Neutrophils may release mitochondrial DNA following stimulation by GM-CSF and C5a resulting in a still viable cell with the ability to capture pathogens. Mitochondrial NETs seem independent of ROS production but still contain granular proteins. Intra-vital microscopy has shown neutrophils that do release genomic DNA are still able to crawl using pseudopods.

Two pathways of NET formation have been elucidated. In the first, upon stimulation cytoplasmic calcium levels increase through release of endoplasmic reticulum stores, leading to activation of protein kinase C (PKC) (Figure 1). PKC phosphorylates and activates NADPH oxidase 2 (NOX2) leading to its assembly and the production of ROS. This is a required step as patients suffering from chronic granulomatous disease (CGD) with an inactive NOX2 are unable to produce NETs. Diphenylene iodonium (DPI), a chemical inhibitor of NOX2, is also able to prevent NETosis. Singlet oxygen formation by porfimer sodium, a photosensitizer used to generate radicals, is able to induce NETosis in CGD patient neutrophils and healthy controls demonstrating it as a key ROS in the pathway. Rac2 protein, a member of the Rho family GTPases which interacts with NOX2, is also essential for NET generation, and although not necessary, NO can enhance NET release as long as NOX2 is still active. In addition, two signaling proteins ERK and p38 MAPK have been demonstrated to be downstream of ROS production in NETosis and necessary for the pathway. Inhibitors of the Raf-MEK-ERK pathway lead to reduced ROS production suggesting that this pathway may have positive feedback where ROS species lead to ERK activation and a resultant increase in ROS. Granular disruption leads to the release of MPO and neutrophil elastase (NE), with NE knockouts being unable to form NETs. Neutrophil elastase acts to break down histones via proteolytic activity, while peptidylarginine deiminease 4 (PAD4), another calcium activated
protein, converts arginine to citrulline, by deimination, to reduce the positive charge of the histone and its DNA binding affinity\textsuperscript{111}. Citrullination of histones alters the density of the nucleosome structure which may cause chromatin decondensation\textsuperscript{112}. Following the decondensation of chromatin, the nucleus will lose its multilobular shape followed by breakdown of both granular and nuclear membranes. The final stage comprises permeabilization of the plasma membrane and release of the contents into the extracellular space. Although cytoplasmic, cytoskeletal and peroxisomal proteins are also found in NETs, the majority of the protein weight and molar amounts in NETs derives from histones and granular proteins suggesting that adherence to NETs may be specific\textsuperscript{113}.

An alternate pathway for NET formation has been discovered through the observation that certain stimulations induce a process in which blocking ROS cannot prevent NETosis. The protozoan parasite \textit{Leishmania} is able to stimulate neutrophils to activate NETosis in a manner that cannot be inhibited using DPI or catalase to decompose hydrogen peroxide\textsuperscript{114}. Following \textit{Staphylococcus aureus} stimulation \textit{in vitro}, neutrophils will NETose more rapidly than with other inducers (5-60 minutes) in a process independent of ROS\textsuperscript{115}. In these experiments DNA was released extracellularly through vesicles and the plasma membrane was not ruptured. This suggests that depending on stimulation, an alternate pathway of NETosis can occur independently of ROS and may allow for NET release without neutrophil lysis. It has also been shown that ionomycin, a calcium ionophore, does not require ROS production to stimulate NETosis and that while (phorbol 12-myristate 13-acetate) PMA requires MPO to induce NETosis, bacterial stimulation does not\textsuperscript{116}. It is currently unknown if some of the differences in the requirements for NETosis are due to experimental artifacts or represent multiple pathways leading to NET release. Chemical inducers may bypass early steps in the process initiated by membrane receptors.

1.5.2 Inducers of NETosis

Many receptors lie upstream of NETosis including TLRs and cytokine receptors. \textit{In vivo} studies of infection or cytokine signaling have demonstrated that many known activators of neutrophils lead towards NETosis\textsuperscript{117}. Infections or autoimmune disorders with a neutrophil contribution are being re-examined to determine the role of NETosis. NETosis can be induced \textit{in vitro} (with chemicals such as PMA) which activates PKC in a manner similar to diacylglycerol (DAG)\textsuperscript{118}. 
A calcium ionophore can also achieve this by increasing intracellular calcium, as can thapsigargin, an inhibitor of Ca\(^{2+}\) ATPase on the endoplasmic reticulum\(^{119}\). These methods are used for experimental ease and may not recapitulate the process seen in vivo as they do not act at the cell membrane but begin the process downstream in the cytoplasm. Although useful for studying the pathways of NETosis, natural agonists must be used to make true conclusions about disease and their functions in vivo.

As neutrophils are part of the innate immune response, NETosis would be expected following neutrophil activation. Wide diversity is found in microbial inducers of NETosis which include fungi, bacteria, viruses and protozoa. Gram-negative bacteria contain LPS on their outer cell membranes which can activate the immune system through binding primarily to TLR4. Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri* and *Salmonella enterica* have all been shown to induce NETosis presumably through TLR4 signaling\(^{120}\)\(^{121}\)\(^{122}\). Purified LPS has been shown to activate NETosis in adult neutrophils but this process appears delayed in neonate neutrophils\(^{123}\). LPS addition following priming of neutrophils with GM-CSF leads to mitochondrial DNA release\(^{103}\). Instillation of LPS in vivo can recruit neutrophils to the lung to NETose\(^{122}\). LPS signaling can also occur through platelet intermediaries as they can be activated by LPS and in turn activate NETosis\(^{124}\). Some studies show contradictory evidence that LPS cannot induce NETosis but can delay apoptosis or prime for a stronger oxidative burst\(^{103}\)\(^{125}\)\(^{100}\). This suggests that LPS requires an additional signal either through some PAMP, cytokine or a second hit of injury to induce NETosis.

Paracrine signaling from immune cells and other somatic cells following activation through PAMPs can also lead to NETosis. NETosis has been shown following neutrophil stimulation by IL-8, a chemokine for neutrophils typically generated by macrophages, epithelial cells and endothelial cells\(^{126}\). GM-CSF, a cytokine that activates the development of granulocytes, has been shown to induce NETosis only when paired with LPS or C5a, an anaphylatoxin from the complement cascade\(^{103}\). NETosis may also occur downstream of the inflammasome since neutralizing antibodies against IL-1β could prevent NET release in neutrophils treated with gout patient synovial fluid\(^{127}\). NETosis can be seen to arise from signals typically associated with neutrophil development, activation or migration, making NETosis a possible event in conditions of either sterile or infectious inflammation.
1.5.3 Other extracellular DNA traps

Cell types other than the neutrophil have been shown to release their DNA upon activation, leading to the general term ETosis for any DNA extracellular traps formed from immune cells. Eosinophils have been demonstrated to release extracellular DNA with granular proteins in response to stimulation with IL-5, thymic stromal lymphopoietin, LPS and IFNγ. While this form of ETosis is dependent on ROS production it also shows new characteristics of being faster (5-20 minutes) and containing only mitochondrial DNA allowing the cell to survive. These eosinophil extracellular DNA traps (EETs) demonstrate the same toxic activity as NETs towards microorganisms and have a protective effect during CLP-induced sepsis with eosinophilia. EETs have also been observed in noninfectious skin diseases such as allergic dermatitis and Well’s syndrome suggesting that they may serve a role in causing disease.

Both EETs and NETs are found in asthmatic airways but their cause and consequences are unknown. Macrophages and monocytes can also release extracellular DNA traps (METs) in response to bacterial stimulation, cytotoxins, IFNγ and statins. This process in a subset of monocytes exposed to high bacterial loads was characterized by caspase-1 activation, loss of ATP and membrane integrity and may be a final attempt at clearing pathogens by the cell. METs and NETs also appear to play a role in clearing nanoparticles by acting as a physical barrier. Mast cells activated by bacteria, hydrogen peroxide or PMA can also release extracellular traps (MCETs) which allow the cell a phagocytosis-independent method of killing bacteria. As mast cells are typically associated with the allergic response following IgE binding, this may be a unique function in host defense independent of the adaptive immune system. Although degradation of MCETs reduces their anti-microbial activity, MCETs as well as NETs are associated with IL-17 release during psoriasis which exacerbates the disease. As a multitude of innate immune cells have been demonstrated to release DNA extracellularly to kill microbes, these traps may be a frontline defense for the host.

The use of DNA as an extracellular trap and method of killing microorganisms appears to be an evolutionarily conserved function as it can be found in a wide variety of complex organisms. Hemocytes, the phagocytic cell of invertebrates, upon activation by bacteria can release extracellular DNA traps to capture and permeabilize bacteria. Heterophils, an abundant granulocyte of birds, are able to release DNA protein complexes in vitro upon activation. More traditional granulocyte extracellular traps can be seen in other mammals.
such as mice, the animal of choice for studying NETs, as well as cats and cows \(^{117}\). Zebrafish, another commonly used laboratory species, have been shown to form NETs in kidneys when stimulated with synthetic activators, making them a possible choice for in vivo NET production assays but of course cannot be used to study pulmonary NETosis \(^{140}\). Even plants through their root tip cells use extracellular DNA traps to protect from fungal spores \(^{141}\).

1.5.4 NETs and infection

NETs play an important part in innate immunity. Several microorganisms produce DNases whose previously unknown function may be to degrade NETs. For example, *Streptococcus agalactiae* require a released nuclease for full virulence, and knockout of this gene reduces mortality and bacterial burden in a mouse model \(^{142}\). Aged mice with a reduced ability to form NETs have a reduced ability to kill methicillin-resistant *S. aureus* and experience greater bacterial dissemination from a skin infection; therefore deregulation or loss of NETs in the host may increase susceptibility to infection \(^{143}\). In a CLP model of sepsis, mice treated with DNase had higher bacterial numbers, shorter time to death and increased inflammation \(^{144}\). MPO on NETs retains its function in the extracellular space and when provided with hydrogen peroxide is able to produce hypochlorous acid and kill bacteria \(^{145}\). Thus, antimicrobial protein functions may be a method by which NETs confer protection.

1.6 NETs in disease

1.6.1 Cytotoxic effects

NETs containing anti-microbial proteins and histones with known cytotoxicity could be expected to be injurious to the epithelium of the lung and the blood vessel endothelium. Neutrophil granular proteins when extracted are independently cytotoxic to lung-derived cells while histones can increase epithelial cell membrane permeability \(^{146}\) \(^{147}\). Both anti-histone antibodies and activated protein C (APC), which promote histone cleavage, could prevent lethality in mice from experimental sepsis models using LPS, CLP or TNF\(\alpha\) \(^{148}\). It has been demonstrated that activation of endothelial cells in vitro can activate NETosis and result in endothelial cell death \(^{119}\). NET-induced cell death can be prevented with the use of anti-histone antibodies or MPO inhibitors, demonstrating the importance of both the chromatin backbone and granular proteins in the effects of NETs \(^{149}\).
1.6.2 Coagulation

Neutrophils outside of their anti-microbial functions may also serve a role in the formation of blood clots. Coagulation dysfunction and excessive thrombus formation can be injurious by inducing ischemia. As blood passes through the pulmonary circulation, pulmonary embolism is a common risk with procoagulant conditions. Released proteins such as neutrophil elastase and serine proteases, together with externalized nucleosomes, have been found to induce thrombi formation in vivo via proetolysis of the coagulation suppressor, tissue factor pathway inhibitor (TFPI)\(^\text{90}\). Neutrophil proteases such as cathepsin G can also activate platelets leading to greater binding to neutrophils and increased surface expression of clotting receptors\(^\text{150}\). Thus neutrophil activation of clotting may serve a role in trapping bacteria in microvessels but may also lead to thrombotic disease. Histones present in NETs may further promote clotting as histones themselves have been shown to activate platelets and lead to greater thrombus formation\(^\text{151}\). The process of deiminating histones for chromatin decondensation that occurs during NETosis is required for histone-mediated thrombosis as PAD4 knockout mice are seen to have a reduced risk of thrombus formation and fail to form NETs from stimulated neutrophils\(^\text{152}\). The addition of DNA and histones to a forming thrombus increases its tensile strength and increases the time for clot lysis; as clots containing chromatin require both DNase and tissue plasminogen activator to be lysed, future therapies against coagulation may need to target innate immunity as well\(^\text{153}\). Proof that clotting follows NETosis can be seen in experiments where NETs are perfused with blood. Addition of blood to released NETs results in the recruitment of red blood cells, platelets and fibrin\(^\text{154}\). In vivo, stenosis of the inferior vena cava results in the formation of a thrombus with NETs as well as increased extracellular DNA\(^\text{155}\). Either DNase treatment of NETs or neutropenia can prevent thrombus amplification suggesting NETs as an important factor in events such as deep vein thrombosis\(^\text{155}\). The ability of NETs to induce clotting and activated platelets to induce NETosis suggests that the pathways of inflammation and thrombosis are linked through these two cell types.

1.6.3 Autoimmune disease

Interest in NETs has been growing in the field of autoimmune diseases where NETosis is being considered as a possible link between cell death and the formation of self-reactive antibodies. Systemic lupus erythematosus is a disease where antibodies are formed against nuclear antigens leading to pathogenic immune complex formation. Traditionally necrosis, a form of cell death
where the cell contents are released into the extracellular space, was believed to be the source of neutrophil-derived antigens. Recent evidence has shown that NETosis exposes auto-antigens such as LL37 and is associated with greater levels of antibodies to dsDNA. Lupus patients may also exhibit impaired NET degradation associated with anti-NET antibodies, which limit DNase I access to NETs, and the presence of DNase I inhibitors such as serum G-actin. One study has shown that a distinct subset of neutrophils termed low density granulocytes can be isolated from lupus patients and have a greater predisposition to NETosis and proinflammatory signaling. NETs may act as a source of cytoplasmic antigens for autoimmune diseases and may in turn be induced by the proinflammatory response.

In small vessel vasculitis, a disease associated with anti-neutrophil cytoplasmic antibodies (ANCA), ANCAs could induce NET formation which in turn contained the autoantigens MPO and proteinase 3 (PR3). This observation combined with the deposition of NETs in the kidneys suggests that the disease may involve a positive feedback cycle promoting exacerbations. In another autoimmune disorder, rheumatoid arthritis, one target of anti-citrullinated peptide antibodies is citrullinated histone 4, a product of NETosis. NETs also increase production of cytokines such as IL-8 and IL-6 in synovial joint fibroblasts from rheumatoid arthritis patients. Production of antigens for self-reactive antibodies by NETosis may serve as a process that connects the innate and adaptive immune responses in diseases where an early insult of infections is believed to precipitate a chronic inflammatory condition.

The NETs themselves also serve to externalize stimulatory molecules such as IL-17A and TNF-α which can activate an inflammatory response in fibroblasts and may contribute to pathogenesis. Treatment of mice in collagen-induced arthritis with Cl-amidine, a PAD4 inhibitor capable of preventing NETosis, resulted in reduced disease scores suggesting that NETs may be a viable therapeutic target.

1.6.4 NETs in other conditions

Greater understanding of the role of NETs in reproduction has been gained by increased interest in innate immunity and detection of cell-free DNA. Given that placental syncytiotrophoblast microparticles can induce NETosis and increased NETosis is detected in the intervillous space of patients with preeclampsia; it is possible that NETs are a contributor to the reduced perfusion or placental hypoxia of the disease. Sperm-induced NETosis in the female reproductive tract
and resulting fertility loss has been observed in bovines and can be reversed by seminal plasma DNase\(^ {163}\). As neutrophil activation and ROS production is associated with spontaneous fetal loss, it has been hypothesized that NET production or the resulting clotting may be the cause\(^ {164}\).

Neutrophils are known to serve roles in cancer progression and their increased numbers in tumor tissue and peripheral blood of cancer patients have been correlated with poor outcomes\(^ {165}\). NETs have currently been shown to trap circulating tumor initiator cells and increase the number of metastases in a mouse model of CLP and tumor cell injection\(^ {166}\). As NETs can also be detected with tumor associated neutrophils they may also play a contradictory role in immunoediting\(^ {167}\). Cancer has a reciprocal ability to induce NETosis from both malignant and benign neutrophils by an increase blood G-CSF. The increased NETs production in a mouse model occurs with increased lung thrombi and reduced platelet counts suggesting that NET induced thrombi may be a cause of lung injury in this model of solid tumor and LPS\(^ {168}\). NETs may serve a role in both the initiation of secondary tumors and their pathogenicity, making them a possible therapeutic target.

### 1.6.5 Lung injury

Neutrophils have been demonstrated to NETose in the airway following transmigration and in the plasma where they might injure the lung\(^ {122}\)\(^ {169}\). Attention to the possible connection between NETosis and neutrophilic lung injury has been increasing due to the role of cell free DNA in atelectasis, CF and as an injury biomarker\(^ {170}\)\(^ {171}\)\(^ {172}\), histones as mediators of injury and coagulation\(^ {151}\)\(^ {152}\), and serine proteases as mediators of lung injury\(^ {173}\). Targeting of NETs by blocking, degradation or inhibition could attenuate injury due to any of its components.

In a mouse model of trauma, intravenous histone treatment resulted in cytotoxicity, coagulation, cytokine induction and further NETosis\(^ {174}\). The lungs exhibited edema, hemorrhage, thrombosis and high neutrophil recruitment - symptoms associated with histone treatment that could be abrogated with an anti-histone antibody. A trauma patient cohort had plasma levels of circulating histones that associated with respiratory failure, coagulation and endothelial injury\(^ {174}\). Histones are believed to injure cells through non-specific charge-mediated binding to phospholipids. Cell permeabilization may also lead to release of cytoplasmic cytokine and may contribute to inflammation. Histones may also act as an alarmin, signal of sterile injury, through TLR9 to increase inflammation\(^ {175}\). Although NETosis was not explicitly
examined, this article demonstrates the injurious effects of a component of NETs, their potential as a biomarker, as well as a possible therapeutic target through neutralizing antibodies.

A more certain role of NETosis has been determined in mouse models of transfusion related acute lung injury (TRALI), induced by administration of an antibody against mouse major histocompatibility complex (MHC) class 1 or human neutrophil antigen 3a (HNA-3a) which led to increased NETosis. NETosis was dependent on thromboxane production and could be induced by activated platelets. DNase I, antibodies against histones, aspirin, or tirofiban (anti-platelet drug) led to reductions in NETosis, mortality and extravascular lung protein. Numerous interventions against NETosis exist and have shown promise in mouse models of lung injury associated with NETs. These results against both the platelet activator of NETosis and against NETs themselves, solidifies their important pathogenic role in the lung.

As killing foreign microorganisms is the main purpose of NETosis, pneumonia and other lung infections would be expected to induce pulmonary NETosis. *P. aeruginosa* can acquire a mucoid phenotype in cystic fibrosis, escape killing by NETs and cause chronic bacterial infection. Viral infections such as influenza can cause epithelial cells to induce increased superoxide and hydrogen peroxide production leading to NETosis which may result in tissue injury but not necessarily help fight infection. In secondary bacterial pneumonia, NETs were also non-protective and may have resulted in damage to the alveolar-capillary barrier. The ability of certain microorganisms to evade NETosis and the possible role of NETs in lung injury means that NETs may be protective or pathogenic when induced in response to infection.

Many protease components of NETs have been shown to be injurious to the lung by damaging cells or degrading free proteins including LL37, PR3, neutrophil elastase and cathepsin G. Whole NETs are directly cytotoxic to cultures of epithelial and endothelial cells which can be attenuated with antibodies against histones or MPO, emphasizing the possible effects of both the granular and chromatin components in causing lung injury. The effects of NETs in the lung lumen/circulation on mortality or injury are still unknown in both animal models and humans. Based on the cytotoxicity of individual components, NETs would be expected to cause cell death of both microbes and host cells.
1.7 Rationale

Although many versions of lung protective mechanical ventilation have been developed there is still no intervention that acts to block the inflammatory cascade once VALI is suspected. Treating the underlying conditions and providing supportive care represent the main clinical practices with no specific anti-inflammatory treatments given unless indicated by other comorbidities. Inflammation is a major contributor to VALI and ARDS in humans with deadly consequences such as sepsis. As infection is a common complication for patients in the ICU, a careful balance must be met to avoid compromising the patient’s ability to resist infection. Nonetheless, interventions targeting specific components of inflammation induced lung injury could improve survival by preventing the resulting systemic inflammation.

It has been shown previously that depletion of granulocytes in ventilated rabbits attenuates accumulation of extravascular lung protein and decreases in PaO$_2$\textsuperscript{60}. However, a potential therapeutic approach based on this would present unacceptable risk of infection to patients. Patients with VAP treated with G-CSF administration showed increased neutrophil counts and reduced mortality rates supporting the importance of neutrophils in overcoming VAP\textsuperscript{184}. In a mouse model of pneumonia, blocking neutrophil entry into the lung increased mortality\textsuperscript{71}. Thus neutrophil depletion represents an unfeasible treatment for VALI. However, along with their ability to fight infections in the lung, NETs may play an important role in injuring the lung parenchyma and progression of the proinflammatory state in VALI. Recently it has been shown that HMGB1, a DAMP present in plasma from patients with VALI, can induce NETosis\textsuperscript{59}. Two other inducers of NETosis, IL-8 and IL-1β, are also induced by mechanical ventilation\textsuperscript{185,186}. The pathway of NETosis contains potential specific targets which might spare other neutrophil functions such as phagocytosis, degranulation and ROS production. NETosis or the resulting NETs can be inhibited by targeting PAD4, autophagy, extracellular DNA, histones or HMGB1. Inhibition of autophagy can prevent NETosis and has been demonstrated to reduce inflammation in response to VILI\textsuperscript{100,187}. PAD4 inhibition prevents NETosis formation \textit{in vitro} and can prevent NETosis during infection\textsuperscript{111,112}. Direct intervention against chromatin components by an anti-histone antibody or by DNase treatment have been demonstrated to reduce lung injury\textsuperscript{174}.\textsuperscript{176} It has been demonstrated that use of ethyl pyruvate to inhibit HMGB1 results in decreased neutrophil infiltration into the lung as well as reduced autophagy\textsuperscript{188,189}. Despite their potential
contribution to lung injury extracellular DNA and histones in the context of NETosis have not been previously examined in VALI and present a potential novel therapeutic target.

1.8 Hypothesis

Mechanical ventilation with simulated infectious injury can recruit neutrophils to the lung lumen and activate NETosis. NETs in the lung contribute to VILI and represent a target for therapy.

Aim 1: To detect NETosis in a two-hit mouse model (LPS and injurious ventilation) and determine the relative contribution of each hit.

Aim 2: To determine whether DNase 1 administration can decrease NET abundance in the lung and decrease lung injury.
2 Methods

2.1 Animal Approval

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

2.2 Ventilator model

Adult C57BL/6 mice (Charles River, St. Constant, Canada) between 20-25 g were anesthetized using intraperitoneal (IP) injection of 80 mg/kg ketamine (Pfizer, Kirkland, Quebec, Canada) and 20 mg/kg xylazine (Bayer, Toronto, Ontario, Canada). A tracheotomy was done with a cut down 18 gauge needle. Animals were paralyzed with 0.1 mg pancuronium (Sandoz, Boucherville, Quebec, Canada) IP and mechanically ventilated by a computer controlled small animal ventilator (SCIREQ flexivent, Montreal, Canada) using room air and a volume controlled setting (Figure 2). To simulate injurious ventilation, the animals’ respiration rate was set to 45 breaths/minute, a tidal volume of 20 mL/kg and 0 cm PEEP. As a ventilation control the respiration rate was set to 135 breaths/minute, tidal volume of 10 mL/kg and 2 cm PEEP. Ventilated animals had their body temperature maintained by a warm water pad and pump (Stryker, Toronto, Ontario, Canada). Every hour mice were given a supplementary dose of 0.1 mg of pancuronium, 26.67 mg/kg ketamine and 6.67 mg/kg xylazine IP. Hourly deep inflations were given to recruit atelectatic lung prior to hourly measurements of respiratory physiology. Quasi-static compliance was measured using pressure-volume loops and dynamic compliance was measured using a snapshot perturbation. Spontaneous breathing controls were left in their cages following instillation and were sacrificed 6 hours later following anesthesia and intubation. At the end of four hours of ventilation the carotid artery was cut and blood was collected in a capillary tube for analysis by Radiometer ABL 700 (Radiometer, London, Ontario, Canada).

The left atrium was cut and 3 mL of PBS was perfused through the right ventricle and pulmonary circulation. The lungs were lavaged three times with three aliquots of 700 µL of HBSS (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.1 g glucose, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃) (Life Technologies, Burlington, Ontario, Canada) which was spun for 10 min at
400 × g and 4°C. The lavage supernatant (BALF) was then stored at -80 °C. Cells were resuspended and counted on a hemocytometer and 50,000 cells were placed on a slide by cytospin. Slides were stained in the differential dyes of the Hemacolor kit (EMD Millipore, Billerica, Massachusetts, U.S.) before sealing a coverslip on with Permount (Fischer Scientific, Ottawa, Ontario, Canada). Pictures of five fields were taken at 40 × magnification and 100 cells were counted as either macrophages or neutrophils.

2.3 LPS stimulation

Two hours prior to ventilation, mice were anesthetized using an IP injection of 40 mg/kg ketamine and 10 mg/kg xylazine and 5 minutes of 2% (v/v) isoflurane (Abbot, Montréal, Québec, Canada). A 22 gauge angiocath (BD, Mississauga, Ontario, Canada), cut down to 1 cm, was inserted into the trachea through the mouth by guidance of a dissection lamp positioned outside the trachea. 5 µg of LPS from *E. Coli* 0111:B4 (Sigma-Aldrich, Oakville, Ontario Canada) in 50 µL PBS or a vehicle control was instilled followed by 200 µL of room air to ensure deposition throughout each lung.

2.4 DNase treatment

After intubation and placement on the ventilator at low tidal volume the mouse was instilled with either 25 µL of 1 mg/mL Dornase alfa (Pulmozyme®) or its vehicle control (8.77 mg/mL sodium chloride; 0.15 mg/mL calcium chloride) twice through a 24 gauge angiocath (BD, Mississauga, Ontario, Canada) followed by 700 µL of air twice. Each instillation/inflation cycle was followed by five deep inflations to reduce airway pressure.

2.5 Concentration of Proteins

BALF protein samples were mixed with four times their volume of acetone, vortexed briefly and incubated on ice for 20 minutes. The samples were then centrifuged for 15 minutes at 15,000 × g at 4 °C. Supernatants were drained and after allowing pellets to dry for 10 minutes, 25 µL of Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) Bromophenol Blue) was added and samples were heated to 95 °C for ten minutes with shaking.
2.6 Western blot

Samples (preparation above) were separated on a 12.5% (w/v) polyacrylamide gel at 150 V for 1 hour. Proteins were transferred onto PVDF membranes for 1 hour at 100 V. Membranes were washed once in TBS (50 mM Tris Base, 150 mM NaCl, pH 7.6) and blocked in 5% w/v non-fat powdered milk and 0.1% (v/v) Tween-20 for 1 hour. Membranes were washed for 5 minutes, 3 times in TBS + 0.05% (v/v) Tween-20 (TBST) and incubated with primary antibody in 5% (w/v) bovine serum albumin (Roche, Mississauga, Ontario, Canada) in TBST overnight, shaking at 4°C. Primary antibodies used included rabbit polyclonal anti-Histone H3 (citrulline 2 + 8 + 17) (Abcam, Toronto, Ontario, Canada) at 1:5000 dilution, rabbit monoclonal anti-HMGB1 at 1:500, mouse monoclonal anti MPO (Abcam, Toronto, Ontario, Canada) at 1:500 dilution, rabbit polyclonal anti-HMGB1 (Cell Signaling Technology, Whitby, Ontario, Canada) and mouse monoclonal anti β-actin at 1:20000 (Sigma-Aldrich, Oakville, Ontario Canada). Membranes were washed as before in TBST and then incubated for 1 hour in blocking buffer with secondary antibody goat anti-rabbit or goat anti-mouse conjugated to horse radish peroxidase (BD, Mississauga, Ontario, Canada) at a 1:10000 dilution. After final washes, the membrane was coated with enhanced chemiluminescence substrate (PerkineElmer, Woodbridge, Ontario, Canada) for peroxidase to expose radiographic film. Films were developed on a Kodak X-Omat before being scanned with an Epson perfection v700 photo scanner. Densitometry was performed with ImageJ (NIH, Bethesda, Maryland, U.S.).

2.7 Cytokine analysis

Cytokine measurements were performed on BALF supernatant samples using the Milliplex system (Millipore, Toronto, Ontario, Canada). The cytokines analyzed were IL-1β, IL-6, KC, GM-CSF, G-CSF, IFN-γ, MIP-2 and MCP1. Analysis was performed by The Centre for the Study of Complex Childhood Diseases, The Hospital for Sick Children, Toronto, Canada.

2.8 BALF DNA/protein quantification

To quantify BALF supernatant dsDNA a standard curve was generated using Lambda DNA in HBSS and Quant-iT™ Picogreen® (Invitrogen, Burlington, Ontario, Canada) reagent was diluted 1 in 200 as per the manufacturer’s instructions. Samples and standard were added to a 96 well
flat bottom plate (Sarstedt, Montreal, Quebec, Canada) before adding the diluted Picogreen reagent in a 1:1 ratio. The plate was covered from light while shaking for 5 minutes before being read on a POLARstar Omega at excitation wavelength 485nm and emission wavelength 520 nm.

For BALF supernatant protein, bovine serum albumin was used for the standard curve and samples were quantified using the bicinchoninic acid (BCA) protein assay (Fischer Scientific, Nepean, Ontario, Canada). 200 µL of working reagent was added to 25 µL of sample or standard and incubated for 1 hour at 37°C before measuring absorbance at 565 nm.

2.9 Statistics

Statistics were performed using SigmaPlot 12 from Systat. Two group experiments were analyzed using the student’s t-test if data was normal (Shapiro-Wilk, P>0.05). Multiple group experiments were analyzed using a Two-way ANOVA with repeated measures if applicable. Data sets that failed the equal variance test were examined by ANOVA on ranks while data sets that failed the normality test were transformed using the Box-Cox method. Dunn’s post-hoc test was used to follow the ANOVA on ranks and the Holm-Sidak method was used for Two-way ANOVA. A threshold of P<0.05 was used to determine significance.
3 Results

3.1 Two hit model of ALI and VILI

3.1.1 Additive effects of LPS and ventilation on injury

3.1.1.1 Compliance

To test if injurious mechanical ventilation could contribute to NETosis we used a two hit model: LPS (for increased neutrophil recruitment) and injurious mechanical ventilation. Compliance readings were taken to determine increasing rigidity of the lung and therefore injury (Figure 3). Initial static and dynamic compliances showed no differences between the groups showing that the pretreatment with LPS had no effect within 2 hours (Two-way ANOVA). Final dynamic compliance was not affected by ventilation setting, but was significantly decreased in groups given LPS versus the vehicle with low tidal volume ($P<0.05$, Two-way ANOVA). The final static compliance was significantly lower in either high tidal volume group when compared to the vehicle with low tidal volume ($P<0.05$, ANOVA on ranks). Delta compliance was calculated by subtracting the final value at 4 hours from the initial reading (Figure 4). Delta static compliance demonstrated an interaction between the pretreatment and method of ventilation ($P=0.05$, Two-way ANOVA). Delta static compliance was significantly increased for all three injury groups versus the vehicle with low tidal volume ($P<0.01$, Two-way ANOVA, Holm-Sidak). Delta dynamic compliance was significantly increased for the LPS with high tidal volume and the LPS with low tidal volume versus the vehicle with low tidal volume ($P<0.05$, ANOVA on ranks, Dunn’s) (Figure 4). Both LPS instillation or high tidal volume were able to reduce lung function as measured by compliance.

3.1.1.2 BALF protein

Protein in BALF was quantified in order to determine breakdown of the epithelial-endothelial barrier and lung injury (Figure 5). Both LPS pretreatment and method of ventilation had a significant effect on protein concentration ($P<0.001$, Two-way ANOVA). The two hit group was significantly more injured than high tidal volume ventilation alone or either of the other two LPS groups ($P<0.05$, $P<0.001$ and $P<0.05$, respectively, Holm-Sidak post-hoc test). Within vehicle
groups, high tidal volume ventilation was significantly more injurious than either spontaneous breathing or low tidal volume ventilation (P<0.001, P<0.05, respectively, Holm-Sidak post-hoc test). The low tidal volume group was also significantly different compared to the spontaneous breathing group (P<0.05, Holm-Sidak post-hoc test). Protein leak into the lung lumen demonstrates that the addition of mechanical ventilation and increasing tidal volumes can induce lung injury, validating our model of VILI. The addition of LPS further increased injury, creating a working two hit model to study neutrophil recruitment during mechanical ventilation.

3.1.1.3 Leukocyte recruitment to the lung lumen

Leukocyte recruitment to the alveolar space was measured to verify inflammation and as a standard to normalize NET production (Figure 6). LPS significantly increased the total number of cells recruited to the lung lumen but there was no effect from the mode of ventilation (P<0.05, ANOVA on ranks, Dunn’s). The percentage of neutrophils and total neutrophil number found in the BALF pellet was significantly greater with the addition of LPS (P<0.05 ANOVA on ranks, Dunn’s post-hoc test) but was not significantly affected by mode of ventilation (Figures 7).

3.1.1.4 Arterial blood gases

We measured arterial blood gases in order to determine if lung function or metabolism was impaired by either of the injurious treatments (Table 1). pH of arterial blood was dependent on both the pretreatment and mode of ventilation (P<0.05, P <0.001, Two-way ANOVA). High tidal volume ventilation significantly decreased pH versus low tidal volumes (P<0.05) however the spontaneous ventilation was significantly lower than either mechanically ventilated group (P<0.001, P<0.05, Holm-Sidak post-hoc test). LPS significantly increased bicarbonate levels only within the spontaneous breathing group (P<0.05, Holm-Sidak post-hoc test). For ventilation differences the spontaneous ventilation group had a significantly greater bicarbonate concentration than the low tidal volume and high tidal volume groups (P<0.001, Holm-Sidak post-hoc test). There was an interaction found between pretreatment and mode of ventilation for bicarbonate levels (P<0.05, Two-way ANOVA). PaO₂ was measured to determine lung function. Although high tidal ventilation groups trended lower than low tidal volume ventilation controls, only the spontaneous breathing mice had a significant decrease (P<0.001, Holm-Sidak post-hoc test). PaCO₂ measurements were taken as a control for ventilation to make sure alterations in CO₂ levels were not conferring additional injury or protection. PaCO₂ was
significantly elevated in the spontaneous ventilation groups (P<0.001, Holm-Sidak post-hoc test). Spontaneous breathing mice appear more injured as assessed by blood gas measurements; however pH demonstrates a decrease in lung function with increasing tidal volumes.

3.1.2 Combined effect of LPS and ventilation on NETosis

With all LPS groups containing comparable numbers of neutrophils any differences in NETs quantification is the result of mechanical ventilation altering the percentage of neutrophils forming NETs. We quantified BALF supernatant DNA as a marker for NETs release within the lung lumen (Figure 8). LPS with high tidal volume showed significantly greater DNA levels than any other group except LPS with low tidal volumes (P<0.05, ANOVA on ranks). While there was no significant difference in BALF DNA levels between high and low tidal volume within the LPS pretreatment group, there was a strong trend to increased DNA with high tidal volume. LPS with low tidal volume was significantly different compared to control of vehicle pretreatment with spontaneous ventilation (P<0.05, ANOVA on ranks). A Western blot for citrullinated histone-3 was performed to confirm that BALF DNA derived from NETs rather than cell necrosis (Figure 9). Citrullinated histone-3 (17 kD) was detectable in all LPS groups (not in vehicle pretreatment); a sharp increase is seen in the LPS with high tidal volume group. The increases in BALF DNA and citrullinated histone-3 occurred when high tidal volume was applied to LPS treated mice. As all LPS groups had similar numbers of BAL neutrophils (Figure 7B), this demonstrates an increase in NETosis induced by tidal volume.

3.1.3 Potential inducers of NETosis

In order to determine the causative factor for increased NETs release several candidate molecules were measured using the Milliplex system. IL-1β has recently been shown to induce NETosis in gout and was the only molecule to require the two-hit injury for induction; pretreatment and ventilation had an interaction in determining IL-1β production (Figure 10A) (P<0.001, Two-way ANOVA). Within the LPS pretreatment groups, high tidal volume induced significantly greater IL-1β levels than either low tidal volume or spontaneous ventilation (P<0.001, Holm-Sidak post-hoc test). High tidal volume groups were statistically greater with LPS compared to vehicle (P<0.001). Mice treated with the vehicle showed no differences among different modes of ventilation. IL-6 and MCP-1 both had a trend to increase with LPS and low
tidal volume and even further so with LPS with high tidal volume (Figure 10B, C). Although this does follow the pattern of NETosis (Figure 8, 9) neither reached statistical significance (P<0.05, ANOVA on ranks).

G-CSF, KC and MIP-2 were greater among the LPS groups compared to the vehicle controls with no differences detected due to ventilation (Figures 11). These cytokines may be responsible for neutrophil recruitment in this model as BALF cell count follows the same pattern.

Two of the cytokines tested were highest in the LPS with spontaneous ventilation. TNFα was significantly increased in groups pretreated with LPS and trended higher with spontaneous ventilation versus the mechanically ventilated groups (Figure 12 A). GM-CSF only increased in the conditions of LPS and spontaneous breathing making in not related to NETosis in this model (Figure 12 B). GM-CSF and TNFα are greatest in a group not containing NETs markers suggesting they are unrelated to NETosis in our model.

As the alarmin HMGB1 is known to both be released during mechanical ventilation and be an inducer of NETosis it may be the causative factor in this model and was tested by western blot (Figure 13). No interaction between pretreatment and ventilation was detected (P>0.05, Two-way ANOVA). Comparing all low tidal volume mice against high tidal volume mice, there was a significant increase in HMGB1 with higher tidal volumes (P<0.05, one tailed t-test).

### 3.2 DNase intervention against pulmonary NETosis

#### 3.2.1 Reduction in markers of NETosis

In order to determine the effect of NET DNA during mechanical ventilation, mice pretreated with LPS and ventilated with high tidal volumes were randomized to receive either DNase treatment or a vehicle control at the start of ventilation. BALF DNA concentration was measured to verify the effectiveness of the DNase treatment (Figure 14). The DNase treated group showed significantly less cell free DNA than its vehicle control (P=0.001, Mann-Whitney U test). Citrullinated histone-3 and MPO were quantified by protein blot (Figure 15). Citrullinated histone-3 in BALF was significantly decreased with DNase administration demonstrating a decrease in NETs protein components (P<0.05). MPO, a protein found in NETs
but also released upon more general neutrophil activation and found in macrophages was not significantly different between the two groups (Figure 16). As the treatment was effective in reducing NETs accumulation in the lungs any differences in injury may be reasonably attributed to NETs.

3.2.2 Attenuation of lung injury

In order to determine if DNase administration is an effective treatment to reduce VILI, injury was assessed as changes in compliance and in BALF protein, cell counts and cytokine levels. During the 4 hour time course, static compliance trended to be higher at the start in the vehicle group but lower by the end of the experiment (Figure 17 A). Dynamic compliance on the other hand appeared to not differ at any time point (Figure 17 B). Delta static compliance was significantly less in the DNAse treatment group (P<0.05, one tailed t-test) however delta dynamic compliance did not show any difference (Figure 18). BALF protein showed no difference between the two groups (P>0.05, one tailed t-test) (Figure 19). BALF total cell count also showed no significant difference between the groups although there was a trend to reduced neutrophil numbers with DNase (Figure 20). Blood gas measurements showed no significant difference in lung function between the two groups (Table 2). Cytokine measurements as above showed no significant differences between vehicle and DNase treated animals (Table 3).
4 Discussion

Our two hit model has successfully demonstrated the formation of NETs following LPS-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. LPS and high tidal volumes both contributed to lung injury with their effects being additive for BALF protein; compliance trended lowest in the two hit injury group but this difference was not significant. Of the signaling molecules measured that are potential NETosis inducers only HMGB1 and IL-1β increased significantly in the group containing the most NETs. Breakdown of NETs by use of DNase attenuated delta compliance and reduced DNA and citrullinated histone 3 levels. These data suggest that NETosis can occur during VILI and may be a contributor to VALI.

We set out to determine if VILI could induce NETosis either via injurious ventilation alone or in a two hit model with LPS. This model was chosen to mimic clinical manifestations of VALI as well as VAP. LPS 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 over inflation and atelectasis, respectively. Protective ventilation (low tidal volume plus PEEP) was used as a control for any changes induced by the tracheostomy, paralytic or the mechanical ventilator. The spontaneous breathing groups were included to measure baseline parameters of the non-mechanically ventilated mouse lung.

We found that LPS and mechanical ventilation were additive in inducing BALF protein, with high tidal volume being more injurious than low tidal volume (Figure 5). Low tidal volume controls showed an increase in BALF protein compared to spontaneous ventilation indicating that even “protective” ventilation strategies may cause some degree of injury. This is consistent with previous reports that have shown healthy mice ventilated with low tidal volumes will injure 190. Consistent with the literature, both LPS and high tidal volume ventilation appeared to decrease compliance but the combination was not significantly more injured (Figures 2, 3) 55,191. Arterial blood pH was significantly lower with high compared to low tidal volumes (Table 1). Differential cell counts demonstrated that no group differed in macrophage numbers and that all LPS groups had neutrophil recruitment to a similar degree (Figures 6, 7). Some previous mouse models of VILI have demonstrated an increase in neutrophil count with high tidal ventilation and
without LPS. This difference may be due to supplemental O\textsubscript{2} or higher respiratory rates than used in our model.

Citrullinated histone and an increase in BALF DNA were detected in both mechanically ventilated groups with LPS pretreatment (Figures 8, 9). The presence of NETs during VILI is a novel finding that cannot be explained solely by the LPS-induced neutrophil recruitment as all LPS groups had similar neutrophil counts yet the level of NETosis was much higher when injurious ventilation was applied to LPS treated animals. The exact role of LPS for NETosis is unknown in this model as it may only be required to recruit significant numbers of neutrophils into the lung.

In order to determine the trigger for NETosis, we measured cytokines that are known inducers. Additional cytokines were tested as measures of inflammation and injury relevant to VILI. Three of the tested candidates (G-CSF, KC and MIP-2) were increased in all three LPS groups with no differences due to mode of ventilation (Figures 11). As LPS alone induced these changes and cell counts among the LPS groups were not significantly different, these cytokines may have acted to chemoattract neutrophils to the lung but were not sufficient to promote high rates of NETosis. KC and MIP-2 are neutrophil chemoattractants in mice while G-CSF upregulates production of neutrophils. Animal models of pneumonia or endotoxemia with mechanical ventilation have shown synergistic effects of the infectious hit and VILI to increase IL-6, MIP-2, MCP-1, TNFα, IL-1β and KC. A previous model of high tidal volume ventilation has shown increases in the two CXCR2 ligands, MIP-2 and KC, comparable to use of LPS, which we did not observe in high tidal volume ventilation alone. The inability of our model to induce cytokine upregulation without LPS may be due to differences in the sterility of housing conditions causing mice to be exposed to sub-infectious doses in other animal care facilities. Two of the cytokines measured (IL-6 and MCP-1) although not showing significant contribution from mode of ventilation appeared to follow a similar trend to the DNA/protein quantification within the LPS groups (Figures 10 B, C). Within LPS treated groups high tidal volume showed the most DNA/protein with low tidal volume second and spontaneous breathing having the least. This suggests a greater inflammatory state with more NETs/injury and that with greater sample size significant differences may have been detected as these experiments were underpowered.
The chemokine MCP-1 was demonstrated to be upregulated only in mice pretreated with LPS and receiving mechanical ventilation, levels trended higher with higher tidal volume (Figure 10 B). This trend reflects observed increases in NETosis markers DNA and citrullinated histone-3, but did not reach statistical significance. MCP-1 is a monocyte chemoattractant and promotes differentiation to macrophages, although this latter effect (typically 4 days after LPS instillation) would not be expected to play a role in our model. MCP-1 may be produced by activated epithelial cells to recruit monocytes for the late stage of innate immunity and lung inflammation rather than being produced by lung epithelial or endothelial cells and acting on the neutrophils. MCP-1 knockouts do however have reduced neutrophil influx in response to gram-negative bacteria demonstrating that either through direct effects or modulation of other cytokines, MCP-1 does play a role in chemotaxis of neutrophils to the lung. In low birth weight infants increases in MCP-1 and related cytokines were associated with greater O₂ dependence, nRDS and bronchopulmonary dysplasia. MCP-1 may not be relevant to injury seen in this model but is clinically important and may be upregulated following infection and VALI.

One cytokine, IL-1β was significantly greater in the LPS and high tidal volume ventilation versus controls for either LPS or high tidal volume alone (Figure 10 A). IL-1β has been identified to be proinflammatory and an inducer of NETosis. VILI is in part mediated by the inflammasome which produces IL-1β in alveolar macrophages following high stretch in vitro. Knockout of NOD-like receptor family, pyrin domain containing 3 (NLRP3), a gene coding for a critical protein of the inflammasome, or use of IL-1β receptor antagonists reduces lung injury in response to high tidal volumes. In clinical studies, the inflammasome is most commonly associated with familial autoinflammatory syndromes causing fever, inflammation and joint pain. In the lung it mediates immunity following viral challenge, a model not tested in our work. There are however several sterile activators of the inflammasome that could be released by injurious ventilation including ATP, glucose and hyaluronan. Our model, consistent with the literature, demonstrates a greater induction of IL-1β in conditions with increased NETosis.

The DAMP HMGB1 was significantly greater with high tidal volume compared to low tidal volume (Figure 13). HMGB1 is a non-histone structural protein for chromatin which has been identified as an alarmin mediating immune responses to infection and sterile injury.
Interestingly, it has been proven that following endotoxemia, HMGB1 will be released via an inflammasome-dependant pathway. It is possible in our model that the increase of HMGB1 in the LPS with high tidal volume group is associated with the production of IL-1β. HMGB1 has also been demonstrated to be released during high tidal volume mechanical ventilation, as well as in patients who are undergoing long term mechanical ventilation. Immune cells such as macrophages release HMGB1 in an active process following LPS stimulation, and it can also be released from lysed cells. Extracellular HMGB1 acts as a signaling molecule by binding TLR4, TLR2 and receptor for advanced glycation end products (RAGE). HMGB1 may also bind bacterial DNA or LPS to cooperatively signal. Necrotic cells from HMGB1 knockout mice have a reduced ability to induce inflammation, suggesting that this protein plays a role in signaling following cell lysis. As HMGB1 has been detected on NETs, NETosis may serve as an additional method to release HMGB1 and increase inflammation. HMGB1 has also been shown to induce NETosis through TLR4 meaning it may act synergistically with LPS in this model. Clinically HMGB1 has been positively correlated with mortality in ventilated premature infants and was used as a prognostic biomarker for a cardiac event in patients with acute coronary syndrome. Through the use of neutralizing antibodies and a TLR4 knockout mouse, HMGB1 plus LPS has been demonstrated to induce NETosis. HMGB1 is a proinflammatory signaling molecule that may promote NETosis in the LPS and VILI mouse model used here.

Intratracheal DNase treatment was used as an intervention against NETs in mice pretreated with LPS and ventilated with high tidal volumes to test its therapeutic potential and provide evidence that NETs may contribute to lung injury. Pulmozyme® is typically used in CF patients in order to break down the DNA content of sputum to reduce its viscosity, allowing for greater lung function and reduced bacterial trapping. Comparing DNase treated mice with vehicle control, an improvement was seen in delta static compliance but no differences were found in dynamic compliance or BALF protein content (Figures 18, 19). The ability of DNase treatment to attenuate injury in only one measurement of compliance may be due to differences in the method of measurement. Static compliance involves pressure measurements for a greater range of volumes while for dynamic compliance pressure is measured at standard end of inspiration and expiration. Dynamic compliance may be more representative of spontaneous ventilation but the greater range of volumes beyond end inspiratory and end expiratory volumes...
used to calculate static compliance may be more accurate. We also found no detectable differences between the DNase group and its control in arterial blood gas measurements (Table 2), indicating limited benefit of DNase treatment in our model. In contrast, intraperitoneal administration of DNase in a TRALI model with NETs improved blood oxygenation; this difference in efficacy may be due to improved degradation of plasma NETs following this route of administration 176. A retrospective study with inclusion criteria of non-CF pediatric patients treated with DNase, either nebulised or given as droplets endotracheally, has demonstrated improvements in chest X-rays and clinical parameters (chest X-ray score, FIO₂, PaCO₂, respiratory rate) suggesting that clinical lung infiltrates can be partly attributed to DNA and that atelectasis can be reduced by use of DNase 170. Conflicting results were seen in another study of mechanically ventilated adult patients where atelectasis did not improve 215. Both DNA and citrullinated histone-3 were reduced significantly in our study supporting efficacy of the DNase and suggesting successful clearance of NETs when DNA was degraded (Figures 15-17). MPO was expected to be less affected by this intervention since it may be released from both macrophages and neutrophils independent of NETosis, and this appears to have been the case (Figure 16). Although extracellular lung DNA is traditionally thought to come from necrotic leukocytes, the detection of citrullinated histone-3 in this model indicates that we are detecting NETosis, not necrosis. The limited efficacy data we observed together with results from the 6 group experiment calls into question the role of NETosis in this model. In order to determine if NETs are truly pathogenic during VILI, further interventions and measurements of injury must be taken.

There are limitations to the work shown here as there are fundamental differences between laboratory models and clinical VALI. First, we used LPS to model VAP and recruit neutrophils to the lung. During mechanical ventilation, a patient may have neutrophils in the lung without a diagnosis of pneumonia as these cells may be recruited due to sterile lung injury, something not seen in our vehicle-pretreatment groups. During VAP, reductions in inflammation may increase the burden of infectious microbes; with LPS alone reductions in inflammation may show a reduction of injury. Patients may be mechanically ventilated for prolonged durations of days to weeks while this model lasts only hours and may not demonstrate the same effects seen in the long term. The model also uses high tidal volumes and no PEEP for VILI, not clinically relevant settings, while VALI occurs despite lung protective ventilation methods. In addition,
the mouse being a smaller animal has differences in its lung properties such as a lack of turbulent flow in its airways which is common in humans\textsuperscript{216}. Lung mechanics in the mouse have also been shown to be strain specific and some effects seen in a model of VILI may not be reproducible between different mouse strains\textsuperscript{217}. Measurements of BALF protein may not be specific for plasma leak into the lung lumen as increases in cell lysis or protein secretion could be responsible.

A major limitation of the experimental design discovered following data collection is the lung injury in the spontaneous breathing animals. These mice exhibited significantly lower pH when compared to mechanically ventilated mice, suggesting respiratory acidosis due to hypoventilation. This is supported by the relative hypercapnea, hypoxia and high bicarbonate in the spontaneous breathing mice (Table 1). The additional dead space or possibly high resistance of the endotracheal tube may restrict breathing without ventilator assistance. Two cytokines, GM-CSF and TNFα, were highest in the LPS and spontaneous breathing group, suggesting some form of interaction between LPS and increased airway resistance to induce inflammation (Figure 12). Interestingly, TNFα has been demonstrated to induce NETosis\textsuperscript{160} although we failed to detect significant increases in DNA and citrullinated histone 3 in the LPS plus spontaneous breathing group where TNFα levels were highest. GM-CSF, with its costimulatory molecule C5a is not likely responsible for NETosis although the complement system may still be induced by mechanical ventilation\textsuperscript{103, 218}. Due to the lack of surgery and unexpected changes in inflammation and lung function in the spontaneous breathing groups, the low tidal volume groups are more appropriate controls.

Finally as we measured cytokines through a multiplex method there is greater internal error due to cross reactivity between the multiple antibodies, analytes and other BALF proteins\textsuperscript{219}. The more injured LPS groups could have greater background noise preventing statistical significant differences from being detected with our numbers per group. Individual testing of candidates may have demonstrated less variance within groups.

4.1 Conclusion

The work presented here focused on the presence and role of NETs in a mouse model of VILI with LPS pretreatment. Based on our results, it has been determined that NETosis is increased in
the two-hit model, suggesting that a similar phenomenon could occur in VALI, where infection is common, and could be a contributor to lung injury. We have demonstrated two candidate inducers of NETosis in our model which may also be therapeutic targets to reduce injury during VALI. Furthermore we have shown that intratracheal DNase treatment could reduce extracellular DNA, and the NET marker citrullinated histone, suggesting improved clearance of NETs, while attenuating one measure of lung injury. The inability of current protective ventilation strategies to prevent VALI in patients with respiratory failure creates a need for novel therapeutics. NETosis represents a new consideration for the role of neutrophils during pathogenesis of lung injury. Non-intuitive therapeutics for neutrophils such as histone blockers, PAD4 inhibitors and DNase treatment could prevent NET induced lung injury without compromising other functions of innate immunity. Reductions in NETosis may lead to protection from injury similar to but not as great as that seen in animals with neutrophil depletion.

4.2 Future directions

We showed that a two hit lung injury model that induced NETosis also increased production of signaling molecules HMGB1 and IL-1β which are proven inducers of NETosis in other models. Since a proinflammatory state is expected following LPS or injurious ventilation and the previously mentioned signaling molecules can induce NETosis, it is possible that one of these cytokines could be the signal to induce NETosis in our model.

HMGB1 has been implicated as a potential therapeutic target in a variety of proinflammatory states such as sepsis and VALI. Similar approaches could be used to test HMGB1’s role in our mouse model of VILI; for example, blocking antibodies could neutralize HMGB1 and prevent binding to receptors or co-ligands such as LPS. In mouse models, anti-HMGB1 antibodies can attenuate extravascular lung protein content and mortality from LPS administration, while direct instillation of HMGB1 can cause injury and death. The glycoside glycyrrhizin has been shown to bind HMGB1 and prevent its chemoattractant properties making it a potential NETosis inhibitor and therapeutic for VALI. The HMGB1 inhibitor has previously been used in a model of septic shock where it abolished mortality. A pig model of LPS and mechanical ventilation demonstrated reduced inflammation, organ injury and improved oxygenation with glycyrrhizin. Box A peptide of HMGB1, an inhibitor of its
proinflammatory activity, has also been used in mouse models of LPS-induced ALI and sepsis to reduce inflammation and mortality \cite{225,226}. The Box A peptide blocks binding to RAGE, but its effects on TLR4 and NETosis are not yet known \cite{227}. Instillation of either of these molecules with either LPS or mechanical ventilation could be used to determine if HMGB1 is a requirement or contributor for NETosis in VILI.

IL-1β is a product of the inflammasome and its actions could similarly be inhibited through the use of an antibody or the commercially available receptor blocker Anakinra, which is used to reduce inflammation in autoimmune disorders. It has been previously shown that knockout of the IL-1 receptor or use of the receptor antagonist in a mouse model can reduce protein permeability in VILI \cite{228}. The connection between IL-1β and NETosis during VILI has yet to be explored.

Additional known activators of NETosis were not tested for in our VILI model. Platelets released into the lumen following breakdown of the epithelial-endothelial barrier could become activated by LPS and bind neutrophils leading to the release of NETs as demonstrated previously in a sepsis model \cite{125}. If increased platelet numbers or activation was detected in the BALF from the more injured groups, thrombosis formation might provide an additional injury measure relevant to NETosis induction. Activation of clotting by NETs or activation of NETosis by platelets are both possible contributors to injury during VILI.

In order to directly demonstrate NET-induced lung injury it would be optimal to inhibit the NETosis specifically without interfering in other aspects of inflammation. Interfering with DAMPs or cytokines would attenuate multiple inflammatory responses and any subsequent reductions in injury could not be attributed to NETs blockade alone. PAD4, the protein responsible for citrullinating histones, is necessary for NET formation as PAD4 knockouts cannot form NETs and demonstrate increased susceptibility to infection \cite{111}. Use of these mice and their wildtype littermates in our model would be one approach to demonstrate the role of NETosis in VILI. Cl-amidine, a small molecule inhibitor of PAD4, is another option which has been reported to reduce injury in models of colitis and arthritis \cite{229,161}. Preliminary experiments with this inhibitor did not demonstrate efficacy \textit{in vivo or in vitro} (Appendix). Alternatively, NETs isolated from activated neutrophils could be instilled into mouse lungs before ventilation.
to determine if NETs alone can induce injury or show additive effect with moderate ventilation. This method may be the most direct test of their effects.

A more reductionist approach using an *in vitro* model of stretched lung epithelial or endothelial cells could also help isolate effects of NETosis specifically. Isolated NETs could be added to epithelial monolayers in a cell stretch system to determine if injury from the two sources is additive. Injury measures *in vitro* could include cell viability measurements, transepithelial electrical resistance, or permeability to large molecules. Conditioned media from stretched cells could also be examined to detect HMGB1 or other cytokines; the conditioned media could also be tested for the ability to induce NETosis in isolated neutrophils. Different cell types could be used to determine the relative contributions of endothelial versus epithelial cells.

We have not yet verified our results in mechanically ventilated patients. Sputum collected from intubated patients with acute respiratory infections have been shown to contain NETs in amounts that correlate with leukocyte count and chemokine concentration. It has also been demonstrated that mechanical ventilation leads to increased ROS production and elastase release by neutrophils. It is possible that during mechanical ventilation, where infection is common, the increase in ROS leads to NETosis and subsequent release of neutrophil elastase. As BAL is performed for diagnoses of VAP, extra fluid could be used to quantify NETs components. Patients could then be stratified based on diagnoses of ARDS and VAP to demonstrate if increased NETosis correlates with poor clinical outcome. NET components could also be correlated with traditional proteins released from activated neutrophils such as neutrophil elastase and MPO activity to determine what portion of neutrophil associated injury arises from NETosis. If known activators of NETosis are found in patient BALF, the effects of this material on isolated neutrophils from healthy individuals would provide further evidence for mechanisms of NETosis in the injured lung. Future studies of the presence of NETs in patients and their mechanism of injury in experiments, could uncover a novel therapeutic for lung injury.
## Tables

### Table 1. Arterial blood gas measurements.

<table>
<thead>
<tr>
<th></th>
<th>PBS + SV (mean ± SD)</th>
<th>PBS + LV(_T) (mean ± SD)</th>
<th>PBS + HV(_T) (mean ± SD)</th>
<th>LPS + SV (mean ± SD)</th>
<th>LPS + LV(_T) (mean ± SD)</th>
<th>LPS + HV(_T) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.22 ± 0.036 N=11</td>
<td>7.31 ± 0.047 ‡ N=10</td>
<td>7.29 ± 0.022 * ‡ N=7</td>
<td>7.22 ± 0.038 N=11</td>
<td>7.28 ± 0.023 ‡ N=6</td>
<td>7.24 ± 0.049 * ‡ N=8</td>
</tr>
<tr>
<td>pO(_2) (mmHg)</td>
<td>78 ± 11.0 † N=9</td>
<td>108 ± 9.2 N=8</td>
<td>93 ± 9.5 N=5</td>
<td>80 ± 12.5 † N=8</td>
<td>104 ± 10.8 N=4</td>
<td>95 ± 30.8 N=4</td>
</tr>
<tr>
<td>pCO(_2) (mmHg)</td>
<td>67 ± 8.1 N=11</td>
<td>47 ± 9.4 ‡ N=10</td>
<td>49 ± 3.0 ‡ N=7</td>
<td>73 ± 6.0 N=11</td>
<td>45 ± 4.8 ‡ N=6</td>
<td>49 ± 5.5 ‡ N=8</td>
</tr>
<tr>
<td>HCO(_3) (mmol/L)</td>
<td>25 ± 2.8 N=11</td>
<td>22 ± 1.2 ‡ N=10</td>
<td>21 ± 1.8 ‡ N=7</td>
<td>27 ± 2.8 N=11</td>
<td>20 ± 1.2 ‡ N=6</td>
<td>19 ± 3.5 ‡ N=8</td>
</tr>
<tr>
<td>Base Excess (mmol/L)</td>
<td>-2 ± 3.1 N=11</td>
<td>-3 ± 1.8 N=10</td>
<td>-4 ± 1.7 N=7</td>
<td>0 ± 4.3 N=11</td>
<td>-5 ± 2.0 N=6</td>
<td>-6 ± 3.59 N=8</td>
</tr>
</tbody>
</table>

*, P<0.05; compared to other ventilations (Holm-Sidak); †, P<0.05; compared to PBS + LV\(_T\) (ANOVA on ranks, Dunn); ‡, P<0.001; compared to spontaneously breathing mice; **, P<0.05; for LV\(_T\) compared to SV group within the same pretreatment. SV, spontaneous ventilation; LV\(_T\), low tidal volume; HV\(_T\), high tidal volume.
**Table 2. Arterial blood gas measurements in DNase versus vehicle controls.**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (mean ± SD)</th>
<th>DNase (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td>7.17 ± 0.054</td>
<td>7.16 ± 0.068</td>
</tr>
<tr>
<td></td>
<td>N=19</td>
<td>N=19</td>
</tr>
<tr>
<td><strong>pO2 (mmHg)</strong></td>
<td>73 ± 11.7</td>
<td>78 ± 15.4</td>
</tr>
<tr>
<td></td>
<td>N=15</td>
<td>N=15</td>
</tr>
<tr>
<td><strong>pCO2 (mmHg)</strong></td>
<td>52 ± 5.9</td>
<td>50 ± 8.1</td>
</tr>
<tr>
<td></td>
<td>N=19</td>
<td>N=19</td>
</tr>
<tr>
<td>**HCO3 (mmol/L)</td>
<td>14 ± 6.7</td>
<td>16 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>N=19</td>
<td>N=18</td>
</tr>
<tr>
<td><strong>Base Excess</strong></td>
<td>-8 ± 5.9</td>
<td>-10 ± 4.3</td>
</tr>
<tr>
<td><strong>(mmol/L)</strong></td>
<td>N=19</td>
<td>N=19</td>
</tr>
</tbody>
</table>

No differences were found between the treatment group and its vehicle control among the measurements (one tailed t-test).
Table 3. Cytokine measurements for DNase versus vehicle experiment.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (mean ± SD) N=10</th>
<th>DNase (mean ± SD) N=10</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>15696 ± 1821.8</td>
<td>16508 ± 1794.4</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>47 ± 27.6</td>
<td>39 ± 23.9</td>
</tr>
<tr>
<td>TNFα</td>
<td>2526 ± 241.3</td>
<td>2656 ± 278.0</td>
</tr>
<tr>
<td>IL-1β</td>
<td>49 ± 23.9</td>
<td>62 ± 42.8</td>
</tr>
<tr>
<td>IL-6</td>
<td>14549 ± 3331.7</td>
<td>16208 ± 4123.9</td>
</tr>
<tr>
<td>KC</td>
<td>11162 ± 1252.6</td>
<td>11970 ± 2454.5</td>
</tr>
<tr>
<td>MIP-2</td>
<td>10104 ± 2057.0</td>
<td>10895 ± 3422.6</td>
</tr>
<tr>
<td>MCP-1</td>
<td>230 ± 130.8</td>
<td>470 ± 492.3</td>
</tr>
</tbody>
</table>

G-CSF = granulocyte colony stimulating factor, GM-CSF = granulocyte macrophage colony stimulating factor, TNFα = tumor necrosis factor α, IL-1β = Interleukin 1β, IL-6 = interleukin 6, KC = keratinocyte chemoattractant, MIP-2 = macrophage inflammatory protein 2, MCP-1 = monocyte chemoattractant protein 1.
Figure 1. Pathway of NETosis regulation.
Following binding of a proinflammatory ligand neutrophil NETosis can be induced. PKC activation and increases in intracellular Ca\(^{2+}\) will lead to the activation of autophagy, NOX2 and PAD4. PAD4 and neutrophil elastase will enter the nucleus to deiminate histones and decondense the chromatin. Autophagy and ROS production will inhibit caspases and prevent apoptosis. After disintegration of granular and nuclear membranes the granular proteins will bind to the chromatin and the two will be released as NETs. Adapted by permission from Macmillan Publishers Ltd: [Cell Death and Differentiation] (Remijsen Q, Kuijpers TW, Wirawan E, Lippens S, Vandenabeele P, Vanden Berghe T. Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. 2011, 18(4): 581-588.), copyright (2011)
Figure 2. Schematic of the experiments for two hit model of LPS and VILI. Animals were randomized into one of six groups. Pretreatment with either 5 µg LPS in 50 µL or 50 µL PBS for vehicle control was done 2 hours before ventilation. Spontaneous breathing controls were left in their cages and sacrificed 6 hours after pretreatment. Mechanical ventilation was either used at injurious high tidal volumes and no PEEP or protective low tidal volumes and with PEEP for 4 hours with hourly compliance readings. Mice were sacrificed by cutting the carotid artery for blood gas analysis and then given a BAL. SV, spontaneous ventilation; LVₜ, low tidal volume; HVₜ, high tidal volume; PEEP, positive end expiratory pressure; BAL, bronchoalveolar lavage.
Figure 3. The effect of LPS and mechanical ventilation on compliance.
Mechanically ventilated mice were given an hourly snapshot perturbation and pressure volume loop to test lungs for a loss of compliance during ventilation. (A) Significant difference in final static compliance with high tidal volume †, P<0.05; LPS + HV_T (n = 10) vs. PBS + LV_T (n = 10) and PBS + HV_T (n = 9) (ANOVA on ranks, Dunn's). (B) LPS pretreatment had a significant effect on decreasing final dynamic compliance *, P<0.05 (Two-way ANOVA). SV, spontaneous ventilation; LV_T, low tidal volume; HV_T, high tidal volume.
Figure 4. Change in compliance as a result of LPS and mechanical ventilation. Final compliance readings were subtracted from the initial as a measure of lung injury. (A) Pretreatment and method of ventilation had an interaction to effect delta compliance (P=0.013, Two-way ANOVA). *, P<0.05; PBS + LV\textsubscript{T} (n = 10) vs. PBS + HV\textsubscript{T} (n = 9), LPS + LV\textsubscript{T} (n = 10) and LPS + HV\textsubscript{T} (n = 10) (Holm-Sidak posttest). (B) LPS decreased delta compliance. †, P <0.05; LPS + LV\textsubscript{T} and LPS + HV\textsubscript{T} vs. PBS + LV\textsubscript{T} (ANOVA on ranks, Dunn’s) SV, spontaneous ventilation; LV\textsubscript{T}, low tidal volume; HV\textsubscript{T}, high tidal volume.
Figure 5. Effect of LPS and mechanical ventilation on BALF protein.
BALF protein was quantified as a measure of injury. Both pretreatment and method of ventilation had a significant effect on BALF protein (Two-way ANOVA), *P=0.01; LPS + HV_T (n = 10) vs. PBS + HV_T (n = 10), † P<0.05 LPS + HV_T vs. LPS + LV_T (n = 10), ‡ P<0.05; PBS + HV_T vs. PBS + LV_T, (n = 10) ** p<0.001; LPS + LV_T vs. LPS + SV (n = 11) and PBS + LV_T vs. PBS + SV (n = 8) (Holm-Sidak). SV, spontaneous ventilation; LV_T, low tidal volume; HV_T, high tidal volume.
Figure 6. Leukocyte recruitment in two-hit mouse model.
BALF cell count was measured by hemocytometer to determine leukocyte recruitment. Pretreatment had a significant effect on BALF cell count. Method of ventilation showed no significant effect. *, P<0.05; LPS + SV (n = 11) or LPS + HV_T (n = 9) versus PBS + SV (n = 10), PBS + LV_T (n = 9) or PBS + HV_T (n = 9). †, P<0.05; LPS + LV_T (n = 9) versus PBS + SV or PBS + HV_T (ANOVA on ranks, Dunn’s). SV, spontaneous ventilation; LV_T, low tidal volume; HV_T, high tidal volume.
Figure 7. Differential cell counts and neutrophil recruitment.
BALF differential cell count was determined using Hemacolor leukocyte stain. (A) LPS induced an increase in the neutrophil percentage and decrease of macrophage percentage of the BALF cell pellet. (B) LPS induced an increase in total neutrophil number with no change in macrophage number. *, P<0.05; LPS + HV\textsubscript{T} (n = 9) or LPS + SV (n = 11) compared to PBS + SV (n = 10), PBS + LV\textsubscript{T} (n = 9) or PBS + HV\textsubscript{T} (n = 9). †, P<0.05; LPS + LV\textsubscript{T} compared to PBS + SV and PBS + LV\textsubscript{T} (ANOVA on ranks, Dunn). SV, spontaneous ventilation; LV\textsubscript{T}, low tidal volume; HV\textsubscript{T}, high tidal volume.
Figure 8. Effect of LPS and mechanical ventilation on DNA release into the lung lumen.

BALF DNA was measured using Picogreen reagent to determine NETs release into the lung lumen. The combination of mechanical ventilation and LPS treatment induced an increase of cell free DNA with high tidal volumes producing greater amounts. *, P<0.05; LPS + HV\textsubscript{T} (n = 10) compared to PBS + SV (n = 8), PBS + LV\textsubscript{T} (n = 10), PBS + HV\textsubscript{T} (n=10) and LPS + SV (n = 11). † P=<0.05 LPS + LV\textsubscript{T} (n = 10) compared to PBS + SV (ANOVA on ranks, Dunn’s). SV, spontaneous ventilation; LV\textsubscript{T}, low tidal volume; HV\textsubscript{T}, high tidal volume.
Figure 9. Citrullinated histone-3 release as a result of LPS and high tidal volume ventilation.
Citrullinated histone-3 was detected in BALF by western blot using equal volume contribution pooled samples from all mice within a group. SV, spontaneous ventilation; LVₜ, low tidal volume; HVₜ, high tidal volume.
Figure 10. Cytokine production as a result of LPS and mechanical ventilation. Multiplex protein analysis was used to detect potential inducers of NETosis. (A) LPS and high tidal volume ventilation had an interaction in inducing IL-1β production (Two-way ANOVA). *, P<0.001; LPS + HV_T (n = 9) compared to LPS + SV (n = 11) and LPS + LV_T (n = 9) (Holm-Sidak). (B) LPS and mechanical ventilation induced MCP-1. †, P<0.05; compared to PBS + SV (n = 8), PBS + LV_T (n = 10) and PBS + HV_T (n = 10). ‡, P<0.05; PBS + SV vs. LPS + LV_T (ANOVA on ranks, Dunn’s). (C) LPS and mechanical ventilation induced IL-6. **, P<0.05; LPS + HV_T or LPS + LV_T compared to PBS + SV and PBS + HV_T. §, P<0.05; LPS + SV vs. PBS + SV (ANOVA on ranks, Dunn’s). SV, spontaneous ventilation; LV_T, low tidal volume; HV_T, high tidal volume.
Figure 11. Cytokine production as a result of LPS instillation. Multiplex protein analysis was used to detect potential inducers of NETosis. (A) LPS induced greater expression of G-CSF. (B) LPS induced greater expression of KC. (C) LPS induced greater expression of MIP-2. *, P<0.05; LPS + HV$_T$ (n = 9) or LPS + SV (n = 11) compared to PBS + SV (n = 8), PBS + LV$_T$ (n = 10) and PBS + HV$_T$ (n = 9). †, P<0.05; LPS + LV$_T$ (n = 9) or LPS + SV compared to PBS + SV. ‡, P<0.05; LPS + LV$_T$ compared to PBS + SV and PBS + HV$_T$ (ANOVA on ranks, Dunn’s). SV, spontaneous ventilation; LV$_T$, low tidal volume; HV$_T$, high tidal volume.
Figure 12. Cytokine production as a result of LPS instillation with spontaneous ventilation.

Multiplex protein analysis was used to detect potential inducers of NETosis. (A) LPS could induce production of GM-CSF with SV. (B) LPS could induce production of TNFα. *, P<0.05; LPS + SV (n = 11) compared to PBS + SV (n = 8), PBS + LV_T (n = 10) and PBS + HV_T (n = 10) (ANOVA on ranks, Dunn). SV, spontaneous ventilation; LV_T, low tidal volume; HV_T, high tidal volume.
Figure 13. HMGB1 release in two hit mouse model. Western blot on BALF was performed to detect HMGB1 an inducer of NETs. (A) Optical density was quantified and averaged. (B) Representative pooled blot from ventilated groups. Ventilation and pretreatment had a significant effect on HMGB1 with no interaction (Two-way, ANOVA). Pretreatments within mechanically ventilated groups were combined for 2 group analysis. High tidal volume ventilation showed a significant increase in HMGB1 release when compared to its low tidal volume control. *, P<0.05; LV_T (n = 19) compared to HV_T (n = 20) (one tailed t-test). SV, spontaneous ventilation; LV_T, low tidal volume; HV_T, high tidal volume.
Figure 14. BALF DNA reduction with DNase treatment.
Picogreen was used to quantify BALF DNA to verify efficacy of the DNase. DNase treatment (n = 9) significantly degraded cell free DNA in the lung lumen compared to vehicle control (n = 7), demonstrating that the drug was efficacious in this model. *, P<0.05; (one tailed t-test).
Figure 15. NETs citrullinated histone 3 reduction with DNase treatment.
Western blot was used to detect citrullinated histone-3 a protein component of NETs. DNase treatment (n = 9) could significantly reduce the amount of NETs proteins in the lung lumen vs. vehicle control (n = 7). Degradation of DNA may leave histones to be cleared by some mechanism. *, P=0.05; (one tailed t-test).
Figure 16. MPO quantification from BALF.
Western blot was used to detect MPO, a protein of neutrophils released upon activation. (A) DNase treatment was unable to reduce the amount of MPO (n = 9) compared to vehicle control (n = 7) (one-tailed t-test). (B) Representative western blot using pooled samples. (ns = not significant)
Figure 17. Time course of compliance during mechanical ventilation.
Mechanically ventilated mice were given a pressure volume loop or snapshot perturbation to test lungs for a loss of compliance during ventilation. (A) For static compliance, the DNase group trends slightly lower for the starting compliance but has the higher mean by the end of ventilation. (B) For dynamic compliance, there were no noticeable differences between the groups at any time point. ns, not significant.
Figure 18. Change in compliance with DNase pretreatment.
Delta compliance was measured by subtracting the final time point from the initial value. (A) DNase treatment (n = 19) is able to attenuate the reduction of static compliance compared to vehicle control (n = 21) *, P<0.05; (one tailed t-test). (B) No effect of DNase on reduction of dynamic compliance (one tailed t-test). ns, not significant.
Figure 19. BALF protein in DNase compared to vehicle.

BCA was used to quantify BALF protein to measure injury. No significant difference in protein leak into the lumen was found comparing the DNase treatment (n = 9) and its control (n = 9) (one tailed t-test). ns, not significant.
Figure 20. Leukocyte recruitment to the lung lumen.
A hemacytometer was used to count BALF cells and determine if reduction in NETs was due to decreased cell recruitment. No statistical difference was found between the DNase (n = 9) and its vehicle control (n = 11) (one tailed t-test). The cell count did trend lower in the DNase group which could be a sign of attenuated inflammation. ns, not significant.
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8 Appendix

8.1 Cl-amidine inhibition of PAD4

We sought a method of inhibiting NETosis while leaving other functions of the neutrophil intact for use in a model of LPS and high tidal volume ventilation. Previous reports using knockout mice have demonstrated the NETosis is dependent on the activity of the protein PAD4 to deiminate histones before releasing chromatin\(^{111}\). Use of Cl-amidine has been demonstrated to reduce levels of citrullinated peptides \textit{in vivo}\(^{161,229}\). In HL-60 cells it can prevent a NETosis like phenomenon from occurring\(^{112}\). To test its effects in our model, a previously published dose of 25 mg/kg of Cl-amidine or equal volume of DMSO control was injected into mice at the same time as LPS administration\(^{229}\). 2 hours later, mice were then mechanically ventilated with high tidal volumes for 4 hours. This preliminary study showed no changes in BALF DNA or citrullinated histone 3, the product of PAD4’s activity (Figure A1 A, B). This experiment also failed to demonstrate an effect on BALF protein (Figure A1 C). These results indicate the drug was not efficacious \textit{in vivo} for our model. This discrepancy may be due to previous uses of the drug being multiple day time courses while our experiment was one dose and 6 hour response. Before retrying the \textit{in vivo} experiment with multiple doses of Cl-amidine I decided to test its efficacy \textit{in vitro} using human neutrophils isolated from peripheral blood. 250 µM of Cl-amidine was used for 1 hour to pretreat neutrophils that were activated with PMA or a calcium ionophore for 4 hours. Extracellular DNA release was measured using SYTOX Green, a cell impermeable DNA dye and NETosis ratio was determined using a Triton-X 100 control for total DNA content of the cells. In this model Cl-amidine failed to show an effect on NETs release versus its vehicle control in measurements of DNA and by fluorescent microscopy (Figure A2).
Figure A1. BALF analysis in Cl-amidine versus vehicle control.
Cl-amidine treatment in mice with LPS and high tidal volume ventilation. The intervention failed to show an effect on (A) BALF DNA, (B) citrullinated histone 3 western blot or (C) BALF protein (n = 3 per group) (one tailed t-test).
Figure A2. Cl-amidine pretreatment for NETosis assay.
250 µM Cl-amidine was added to 50,000 neutrophils 1 hour prior to activation with (A) PMA or (B) calcium ionophore. Extracellular DNA fluorescence, measured using SYTOX Green showed no apparent differences among positive control, Cl-amidine treatment and vehicle control. Figure is a representative experiment.