Toll-like Receptor 4-Myeloid Differentiation Factor 88 Signaling Contributes to Ventilator-induced Lung Injury in Mice

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ABSTRACT

Background: The mechanisms of ventilator-induced lung injury, an iatrogenic inflammatory condition induced by mechanical ventilation, are not completely understood. Toll-like receptor 4 (TLR4) signaling via the adaptor protein myeloid differentiation factor 88 (MyD88) is proinflammatory and plays a critical role in host immune response to invading pathogen and noninfectious tissue injury. The role of TLR4-MyD88 signaling in ventilator-induced lung injury remains incompletely understood.

Methods: Mice were ventilated with low or high tidal volume (HTV), 7 or 20 ml/kg, after tracheotomy for 4 h. Control mice were tracheotomized without ventilation. Lung injury was assessed by: alveolar capillary permeability to Evans blue albumin, wet/dry ratio, bronchoalveolar lavage analysis for cell counts, total proteins and cytokines, results of histopathological examination of the lung, and plasma cytokine levels.

Results: Wild-type mice subjected to HTV had increased pulmonary permeability, inflammatory cell infiltration/lung edema, and interleukin-6/macrophage-inflammatory protein-2 in the lavage compared with control mice. In HTV, levels of inhibitor of κB α decreased, whereas phosphorylated extracellular signal-regulated kinases increased. TLR4 mutant and MyD88−/− mice showed markedly attenuated response to HTV, including less lung inflammation, pulmonary edema, cell number, protein content, and the cytokines in the lavage. Furthermore, compared with wild-type mice, both TLR4 mutant and MyD88−/− mice had significantly higher levels of inhibitor of κB α and reduced extracellular signal-regulated kinase phosphorylation after HTV.

Conclusions: TLR4-MyD88 signaling plays an important role in the development of ventilator-induced lung injury in mice, possibly through mechanisms involving nuclear factor-κB and mitogen-activated protein kinase pathways.
ventilator-induced lung injury (VILI). Although the exact underlying mechanisms of VILI remain unclear, emerging evidence suggests that mechanical ventilation may activate an inflammatory response in the lung that may contribute to VILI. A critical role for the Toll-like receptor (TLR) in mediating the effects of mechanical ventilation on lung inflammation and injury has recently been reported.

TLRs are pattern recognition receptors and are considered key mediators in inflammation. They play an essential role in innate and adaptive immune responses. TLR4 is the first identified and one of the most studied TLR family members. TLR4 recognizes both pathogen-associated molecular patterns (e.g., lipopolysaccharide) and damage-associated molecular patterns (e.g., high-mobility group box 1 and heat shock proteins). Upon stimulation, TLR4 signals through two downstream pathways: myeloid differentiation factor 88 (MyD88) and Toll/interleukin (IL)-1 receptor domain containing adaptor-inducing interferon-β (TRIF-β) dependent pathways, ultimately leading to the activation of nuclear factor-κB (NF-κB) and the production of proinflammatory cytokines. It has been suggested that activation of NF-κB and activator protein 1 controls inflammatory responses through the induction of proinflammatory cytokines, whereas NF-κB activation is associated with phosphorylation of inhibitor of κB α (IκBα), and activator protein 1 activation depends upon activation of mitogen-activated protein kinases (MAPKs). The TLR4-TRIF pathway has been identified as a key genetic pathway in acid aspiration-induced lung injury. More recently, the role of the TLR4-TRIF signaling pathway has been suggested in a mouse model of VILI. Given that MyD88-dependent signaling mediates early phase activation of NF-κB and is a major proinflammatory pathway, we sought to test the hypothesis that, in addition to the TLR4-TRIF pathway, the TLR4-MyD88-dependent pathway plays a key role in a mouse model of VILI and to further explore the role of NF-κB and MAPKs in TLR4-MyD88 signaling pathway.

Materials and Methods

**Animals**

TLR4-functional C3H/HeOuJ (TLR4-wild-type [WT]), TLR4-inactive mutated C3H/HeJ (TLR4-mutant), and MyD88-sufficient (C57BL/6), the background strain, MyD88-WT) mice were purchased from the The Jackson Laboratory (Bar Harbor, ME). MyD88-knockout or null (MyD88-KO or MyD88−/−, respectively) mice were generated by Kawai et al. and were backcrossed more than 10 generations into the C57BL/6 strain. All mice were female, 8–12 weeks of age, and weighed 20–30 g. Mice were fed with normal diet and water ad libitum and were housed in accordance with guidelines from the American Association for Laboratory Animal Care. All experimental animal protocols were performed in accordance with guidelines approved by the Animal Care and Use Committee at the University of Pittsburgh, Pittsburgh, Pennsylvania.

**VILI Animal Model**

Mice were anesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg) via intraperitoneal injection. Anesthesia was maintained by supplementing with one third of the initial dose of anesthetic agents approximately every 45 min during the experimental period. The mice were placed in the supine position on an adjustable warming pad (FHC Inc., Bowdoinham, ME) to maintain at 37 ± 1°C by continuously monitoring with a rectal temperature probe. The trachea was exposed with a neck midline incision under sterile conditions and a 20-gauge, 1-inch-long sterilized metal catheter with smooth beveled tip was inserted and sutured, then connected to a small animal ventilator (Inspira ASV; Harvard Apparatus, Holliston, MA). Mice with mechanical ventilation were ventilated either at 7 ml/kg (low tidal volume [LTV]) with 140 breaths/min or at 20 ml/kg (high tidal volume [HTV]) with 100 breaths/min, 0 positive end-expiratory pressure for 4 h after tracheotomy, whereas control mice (CON) underwent tracheotomy but breathed spontaneously. Both spontaneous and ventilated mice were supplemented with oxygen (approximately 35–45% the fraction of inspired oxygen, as determined by an oxygen monitor, and always maintained at less than 50%). During ventilation, saline (0.01 ml/g body weight) was administered intraperitoneally at 45-min intervals to maintain intravascular volume status. The oxygen saturation and heart rate in the anesthetized mice were continuously monitored with MouseOx (Starr Life Science Corp., Oakmont, PA) and maintained within physiologic range with supplement of oxygen and intraperitoneal administration of glycopyrrolate (0.01 μg/g body weight). Animals were given a lethal dose of the anesthetic agent at the end of 4-h mechanical ventilation before harvesting samples.

**Evans Blue Albumin Permeability Measurement**

Lung injury was assessed by the alveolar capillary permeability to Evans blue albumin (EBA) as described previously. In brief, Evans blue (EB; Sigma–Aldrich, St. Louis, MO) was dissolved in Ca2+–Mg2+ free phosphate-buffered saline (In-vitrogen, Carlsbad, CA) in a concentration of 0.5% (5.2 mM). EB dye conjugated to albumin (EBA) was prepared by adding bovine serum albumin (Fraction V; Sigma, St. Louis, MO) to 0.5% EB to a final concentration of 4% (0.6 mM). After dissolving thoroughly by gently stirring with a magnet bar, this EBA solution was then filtered steriley through a 0.22-μm syringe filter and stored in aliquots at −80°C until use. Each aliquot was used only once for each animal to prevent cross-contamination. To evaluate the alveolar capillary barrier function, EBA (20 mg/kg) was administered via the internal jugular vein 1 h before euthanasia and tissue harvesting. At the termination of each experiment, all animals were euthanized, and blood samples were obtained via the right ventricle for plasma EBA measurement. The pulmonary vasculature was then flushed with phosphate-buffered saline to remove blood-borne elements. The right lung was ligated at the level of the right mainstem bronchus, ex-
cisced, and weighed and stored in liquid nitrogen for subsequent EB assay. After freeze/thaw, the lung tissue was homogenized in 2 ml phosphate-buffered saline and incubated with additional 2 ml formamide (Sigma) at 60°C for 18 h. Formamide extracts were centrifuged (Beckman TLX; Beckman Coulter, Fullerton, CA) at 15,000 g for 30 min at 4°C, and the centrifuged supernatants were collected to quantify lung EBA content by a dual-wavelength spectrophotometric method (model Du-640; Beckman Coulter) at 620 nm and 740 nm. EBA absorbance was calculated by subtracting the corrected pulmonary tissue EBA absorbance at 620 nm per gram of lung tissue by the corrected plasma EBA absorbance at 620 nm.

Lung Wet/Dry Weight Ratio
Lung wet- to dry-weight ratio was used as an index of pulmonary edema formation. At the end of the experimental period, mice were sacrificed, and the right ventricle was flushed with phosphate-buffered saline. Left lungs were weighed immediately after removal (wet weight) and again after drying in an oven at 65°C for 48 h (dry weight).

Lung Histopathologic Examination
Lungs were fixed by inflation with 10% formalin through intratracheal instillation. After fixation, the lungs were embedded in paraffin, 4-µm sections were stained with hematoxylin and eosin, and sections were examined at the light microscopy level.

Bronchoalveolar Lavage Analysis
Lung inflammation was evaluated by cell counts and protein content in bronchoalveolar lavage (BAL) fluid. The BAL procedure was performed with instillation of 1 ml sterile phosphate-buffered saline with four replicates. Approximately 80% of the instilled volume was recovered. All samples were kept on ice until processed. Recovered BAL fluids were centrifuged (5 min, 2,000 rpm, 4°C). The pellet was resuspended in phosphate-buffered saline, and total cell numbers were kept on ice until processed. Recovered BAL fluids were centrifuged (5 min, 2,000 rpm, 4°C). The pellet was resuspended in phosphate-buffered saline, and total cell numbers were counted using a hemocytometer. The supernatants were frozen immediately on dry ice and stored at −80°C for total protein concentration measurement with BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL) and analysis for cytokines.

Cytokine Analysis in BAL Fluid and Plasma
Tumor necrosis factor-α, IL-6, IL-1 β, IL-10, macrophage-inflammatory protein-2 (MIP-2) from BAL fluid and plasma were analyzed by enzyme-linked immunosorbent assay according to the instructions of commercial kits (R&D Systems, Minneapolis, MN). The detection threshold of the assay was as follows: tumor necrosis factor-α, 5.1 pg/ml; IL-6, 1.6 pg/ml; IL-1β, 3.0 pg/ml; IL-10, 4.0 pg/ml; MIP-2, 1.5 pg/ml.

Protein Extraction and Immunoblotting
Frozen lung tissues were thawed and suspended in 10 µl/mg ice-cold radioimmunoprecipitation assay buffer with protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA), homogenized, and centrifuged at 13,000 rpm for 10 min at 4°C to remove insoluble materials. Supernatant protein concentrations were determined with BCA Protein Assay kit. After addition of 2x sodium dodecyl sulfate loading buffer (Invitrogen), equivalent amounts of protein were separated by electrophoresis using 10% sodium dodecyl sulfate-polyacrylamide gels (Invitrogen). The proteins were then transferred onto a polyvinylidene difluoride membrane (Invitrogen). Blots were blocked overnight at 4°C with Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk, then incubated with primary antibody for 1 h followed by horseradish peroxidase-conjugated secondary antibody for chemiluminescent visualization using enhanced chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA). Blots were stripped with stripping buffer (Pierce Chemical Co.) and reprobed with β-actin (Sigma). Films were scanned at 600 dots per inch in 16-bit grayscale on an Epson Precision 4180 flatbed scanner (Epson America, Long Beach, CA). Densitometric analysis was performed using Image J software from the National Institutes of Health (Bethesda, MD). Antibodies against extracellular signal-regulated kinase (ERK), phosphorylated ERK, and IκBα were purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical Analysis
Data were presented as mean ± SD from four to six animals for each experimental condition as indicated in the legends. Two-way or one-way analysis of variance was used to compare differences within groups followed by pairwise multiple comparison procedure (Holm-Sidak method). SigmaPlot statistical software ver. 10.0 (Systat Software, Inc., San Jose, CA) was used for statistical analysis. Statistical significance was defined as P less than 0.05. All P values were two-tailed.

Results
TLR4-Mutant Mice Prevent Increased Pulmonary Permeability and Edema after HTV
As illustrated in figure 1A, in TLR4-WT mice subjected to HTV (20 ml/kg), but not those subjected to LTV (7 ml/kg), there was a marked increase in permeability compared with tracheotomized and spontaneously breathing mice. In contrast, in TLR4-mutant mice, there were no differences in EBA permeability among the three groups. Likewise, there was less pulmonary edema (as assessed by excess lung water measured by wet/dry weight ratio) in TLR4-mutant mice compared with TLR4-WT mice after HTV (fig. 1B).

TLR4-Mutant Mice Elicit Attenuated Inflammatory Cell Infiltration and Cytokine Levels after HTV
Figure 2 shows representative results from lung tissue histologic examination after VILI that illustrate a marked increase...
inflammatory cell infiltration, alveolar septal thickening, and pulmonary edema in the lung subjected to HTV in TLR4-WT mice compared with TLR4-mutant mice. Although both BAL total protein concentration and total cell counts were significantly higher in both TLR4-WT and TLR4-mutant mice in response to HTV mechanical ventilation compared with LTV and CON, TLR4-mutant mice showed significantly attenuated elevation of protein content and cell counts in BAL compared with TLR4-WT mice during HTV (table 1). As shown in table 1, in TLR4-WT mice, BAL fluid levels of IL-6 and MIP-2 increased significantly after 4 h of HTV compared with unventilated CON. In contrast to TLR4-WT mice, TLR4-mutant mice elicited significant but much less pronounced increase in IL-6 and MIP-2 after 4 h of HTV. LTV did not affect BAL levels of IL-6 and MIP-2. It is noteworthy that 4 h of LTV and HTV also induced a significant increase in plasma IL-6 but not MIP-2 levels in both TLR4-WT and TLR4-mutant mice.

Fig. 1. Toll-like receptor 4 (TLR4) is a susceptible gene of increased pulmonary permeability and edema in ventilator-induced lung injury. (A) Pulmonary permeability, as quantified by the amount of Evans Blue albumin (EBA) fluxing across pulmonary vascular barriers, by using mouse lung turbidity correction factor and expressed as permeability index calculated by dividing the EBA absorbance in lung tissue over plasma (see Materials and Methods). In TLR4-wild-type (WT) mice, high tidal volume ventilation (HTV) elicited significant increase in permeability compared with both control (CON) and low tidal volume ventilation (LTV) mice. In contrast, in TLR4-mutant mice, HTV did not increase pulmonary permeability compared with both LTV and CON mice groups. (B) Pulmonary edema, as measured by lung wet/dry ratio, was significantly elevated in TLR4-WT mice after 4 h of HTV compared with LTV and unventilated CON mice. In contrast, HTV increased wet/dry ratio in TLR4-mutant mice was significantly attenuated compared with HTV in TLR4-WT mice. All data are presented as mean ± SD. * P < 0.05 compared with CON; # P < 0.05 compared with LTV. n = 5 in all groups.

Fig. 2. Results of histopathologic examination of lung tissue in Toll-like receptor 4 (TLR4) wild-type (WT) and TLR4-mutant mice after ventilator-induced lung injury. Representative photomicrographs of hematoxylin-and-eosin staining (× 20 objective) of lungs from TLR4-WT (A–C) and TLR4-mutant (D–F) mice subjected to unventilated and tracheotomized control (CON), low tidal volume ventilation (LTV), and high tidal volume ventilation (HTV). HTV illustrates a marked increase in inflammatory cell infiltration, alveolar 2 septal thickening, and pulmonary edema in the lung in TLR4-WT mice (C) compared with TLR4-mutant mice (F).
Table 1. BAL Cell Counts, Protein Content, IL-6, and MIP-2 Levels in VILI

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<th>TLR4-WT</th>
<th>TLR4-Mutant</th>
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<td></td>
<td>CON</td>
<td>LTV</td>
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<tr>
<td>Protein content (μg/ml)</td>
<td>378.9 ± 71.4</td>
<td>386.0 ± 18.1</td>
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<td>Cell counts (×10⁶)</td>
<td>26.4 ± 2.2</td>
<td>27.5 ± 2.6</td>
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<td>BAL IL-6 (pg/ml)</td>
<td>133.4 ± 2.4</td>
<td>321.1 ± 103.9</td>
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<tr>
<td>MIP-2 (pg/ml)</td>
<td>16.5 ± 6.0 (n = 6)</td>
<td>20.9 ± 1.8</td>
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<td>Plasma IL-6 (pg/ml)</td>
<td>30.6 ± 9.8</td>
<td>83.7 ± 13.8‡</td>
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<td>MIP-2 (pg/ml)</td>
<td>1.5§</td>
<td>1.6 ± 0.3</td>
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Data are presented as mean ± SD, n = 5 in all groups except as indicated. Levels of macrophage inflammatory protein 2 (MIP-2) in plasma were under detectable limits in unventilated control mice.

* P < 0.05 compared with strain-matched control (CON) and low tidal volume ventilation (LTV). † P < 0.05 compared with high tidal volume ventilation (HTV). ‡ P < 0.05 compared with strain-matched CON. § The detection threshold of the enzyme-linked immunosorbent assay.

TLR4-Mutant Mice Attenuate NF-κB and MAPK Activation Induced by HTV

Because most TLR signaling pathways culminate in activation of the transcription factor NF-κB, we examined whether HTV would lead to NF-κB activation and whether TLR4 status affected such a change. Under normal conditions, NF-κB and IkBα form NF-κB/IkBα complexes in the cytoplasm that prevent NF-κB from translocating to the nucleus, thereby rendering it inactive. Phosphorylation and degradation of IkBα result in release of NF-κB from the complexes, enabling NF-κB to move into the nucleus, where it activates the induction of proinflammatory cytokines. Thus, IkBα functions as an inhibitor of NF-κB, and NF-κB activation is associated with IkBα degradation.²⁰⁻²¹ We therefore used IkBα degradation as an index of NF-κB activation. As shown in figure 3A, the lung IkBα protein level was decreased in response to HTV in TLR4-WT mice but maintained at the same level in TLR4-mutant mice. Moreover, the MAPK-ERK in lung tissue became significantly more phosphorylated in TLR4-WT mice in response to HTV. It is noteworthy that phosphorylated ERK was significantly lower in TLR4-mutant mice compared with TLR4-WT mice after 4 h of HTV.

MyD88⁻/⁻ Mice Have Attenuated Permeability and Pulmonary Inflammation

As shown in table 2, WT mice showed a modest but significant increase in pulmonary permeability compared with the unventilated CON, and there was no significant change in

Fig. 3. Activation of nuclear factor-κB and mitogen-activated protein kinase (MAPK) in Toll-like receptor 4 (TLR4) wild-type (WT) mice after high tidal volume ventilation (HTV). A representative Western blot of expression of inhibitor of κB α (IkBα) (A) and MAPK-extracellular signal-regulated kinase (ERK) phosphorylation, phosphorylated ERK (B) in lungs isolated from TLR4-WT and TLR4-mutant mice from three independent experiments 4 h after spontaneous breathing with tracheotomy (control [CON]), mechanical ventilation with low tidal volume (LTV; 7 ml/kg), and HTV (20 ml/kg). All data are presented as mean ± SD. * P < 0.05 compared with CON in TLR4-WT mice. RDU = relative densitometry units.
alveolar permeability in MyD88−/− mice. Likewise, lung histology (fig. 4) showed that HTV elicited marked pulmonary edema, alveolar septal thickening, and increased inflammatory cell infiltration in MyD88-WT mice compared with MyD88−/− and CON. Furthermore, HTV increased BAL protein content and cell number significantly in MyD88-WT mice compared with control. In contrast, MyD88−/− mice did not increase either protein content or total cell number in BAL after 4 h of HTV compared with control spontaneously unventilated mice. Finally, MyD88−/− mice had much lower levels of cytokines in BAL compared with WT mice subjected to HTV (table 2).

**MyD88−/− Mice Attenuate NF-κB and MAPK Activation after HTV**

Figure 5A shows that there was minimal degradation of IκBα, and figure 5B shows MAPK-ERK phosphorylation, phosphorylated ERK in MyD88−/− mice, whereas there was significant degradation of IκBα and phosphorylated ERK in MyD88-WT mice after 4 h of HTV compared with CON,

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**Table 2.** EBA Permeability and BAL Cell Counts, Protein Content, IL-6 and MIP-2 Levels in VILI

<table>
<thead>
<tr>
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<th>CON</th>
<th>MyD88-WT</th>
<th>MyD88-KO</th>
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<tr>
<td>EBA Permeability</td>
<td>0.18 ± 0.01 (n = 4)</td>
<td>0.22 ± 0.02* (n = 6)</td>
<td>0.19 ± 0.02†</td>
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<tr>
<td>Protein Content (μg/ml)</td>
<td>412.1 ± 74.8 (n = 6)</td>
<td>636.0 ± 99.0*</td>
<td>469.4 ± 3.4†</td>
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<tr>
<td>Cell Counts (× 104)</td>
<td>17.9 ± 0.9 (n = 6)</td>
<td>47.0 ± 3.3*</td>
<td>21.9 ± 6.9† (n = 4)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>542.5 ± 58.1 (n = 6)</td>
<td>1,010.2 ± 97.5*</td>
<td>556.1 ± 215.3† (n = 4)</td>
</tr>
<tr>
<td>MIP-2 (pg/ml)</td>
<td>8.5 ± 2.5</td>
<td>86.3 ± 8.8*</td>
<td>39.8 ± 10.9*† (n = 4)</td>
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Data are presented as mean ± SD. n = 5 in all groups except as indicated.
* P < 0.05 compared with control (CON). † P < 0.05 compared with high tidal volume ventilation (HTV) (myeloid differentiation factor 88 wild type [MyD88-WT]).

BAL = bronchoalveolar lavage; EBA = Evans blue albumin; IL-6 = interleukin 6; KO = knockout; MIP-2 = macrophage inflammatory protein 2; VILI = ventilator-induced lung injury.
suggesting both NF-κB and MAPK are involved in MyD88 signaling pathway in VILI.

**Discussion**

In the present study, we tested the role of TLR4-MyD88 signaling in the development of VILI. In a mouse VILI model, HTV at 20 ml/kg led to a series of pathologic changes consistent with VILI. These included increased alveolar permeability (fig. 1) with increased cells and protein content in alveolar space (table 1), significant inflammatory cell infiltration and cytokine production in lung tissues (table 1), and activation of the proinflammatory NF-κB and MAPK pathways (fig. 3). We demonstrate that TLR4 is essential in the development of VILI and that the critical role of TLR4 is most likely mediated via MyD88 signaling because MyD88−/− mice have attenuated pulmonary inflammation and were protected from increased pulmonary vascular permeability (table 2). These results suggest that TLR4-MyD88 signaling pathway may be critical for the development of VILI.

A hallmark of acute lung injury (ALI) is structural impairment in the alveolar-capillary membrane barrier with subsequent increased pulmonary vascular permeability and inflammation. Although extensive research with numerous animal models has attempted to identify effective therapeutic strategies for ALI for almost 35 yr, at present, research on the effects of HTV mechanical ventilation has had perhaps the most significant impact on clinical practice, and mechanical ventilation with a reduced tidal volume ventilation strategy is the only definitive treatment modality to improve survival in patients with ALI.3,22,23 However, emerging evidence suggests that mechanical ventilation, even at protective LTV, may activate an inflammatory response in the lung and may cause or predispose to VILI.6–8,16 For example, data from computed tomography demonstrate that even current protective LTV may produce tidal hyperinflation in patients with acute respiratory distress syndrome because of its anatomical heterogeneity of damaged lung,24,25 from which VILI may be considered a regional phenomenon, and thus additional information on the effect of high (and low) tidal volume mechanical ventilation in experimental animals remains an important translational effort. Further improvements of this model may involve additional injurious stimuli to mechanical ventilation, as suggested by the importance of the “two-hit” animal model of ALI that has been emphasized by the National Institutes of Health,26 to simulate comorbidities and complexities of ALI. Nonetheless, the standard VILI model that we adapted has been an important contributor to insights into VILI. It is noteworthy that we took great care to continuously monitor oxygen saturation and heart rate in the anesthetized mice. In addition, mice were supplemented with oxygen under anesthesia with a fraction of inspired oxygen of less than 50% to prevent possible hypoxia while purposefully minimizing chances of hyperoxia.

Lung inflammation, induced by alveolar overdistention during injurious mechanical ventilation, is believed to contribute to VILI.27,28 Toll-like receptors have long been recognized to play a crucial role in innate immune response and adaptive immune response to pathogens and to noninfectious tissue injury.10–14 TLR4 is unique among the TLRs because it is the only known TLR able to activate both MyD88-dependent and TRIF-dependent signaling pathways.11,28 More importantly, TLR4 has been demonstrated to be activated after mechanical ventilation with LTV ventilation7 and plays a critical role in ALI induced by HTV ventilation,9 lipopolysaccharide,30 acid aspiration,15 hemorrhage,31 and ischemia and reperfusion injury.32 It is generally believed that TLR4 simultaneously induces both MyD88-dependent and TRIF-dependent downstream signaling pathways from the plasma membrane.13,14 Upon binding to endogenous activators, TLR4 forms a dimer and recruits the downstream adaptor molecule MyD88, ultimately leading to the activation of NF-κB and activator protein 1, inducing transcription of proinflammatory genes, and resulting in cytomembrane heterogeneity of damaged lung,24,25 from which VILI may be considered a regional phenomenon, and thus additional information on the effect of high (and low) tidal volume mechanical ventilation in experimental animals remains an important translational effort. Further improvements of this model may involve additional injurious stimuli to mechanical ventilation, as suggested by the importance of the “two-hit” animal model of ALI that has been emphasized by the National Institutes of Health,26 to simulate comorbidities and complexities of ALI. Nonetheless, the standard VILI model that we adapted has been an important contributor to insights into VILI. It is noteworthy that we took great care to continuously monitor oxygen saturation and heart rate in the anesthetized mice. In addition, mice were supplemented with oxygen under anesthesia with a fraction of inspired oxygen of less than 50% to prevent possible hypoxia while purposefully minimizing chances of hyperoxia.

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**Fig. 5.** Activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) in myeloid differentiation factor 88 (MyD88)-wild-type (WT) mice after high tidal volume (HTV). A representative Western blot of expression of inhibitor of NF-κB (IκBα) (A) and MAPK- extracellular signal-regulated kinase (ERK) phosphorylation, phosphorylated ERK (B) in lungs isolated from MyD88-WT and MyD88 knockout (KO) mice 4 h after spontaneous breathing with tracheotomy (control [CON]) and mechanical ventilation with HTV from three independent experiments. All data are presented as mean ± SD. * P < 0.05 compared with CON mice. RDU = relative densitometry units.
tokine production (fig. 6). However, a recent study also showed that TLR4 may activate these two signaling pathways in a sequential manner; the MyD88 pathway is induced from the plasma membrane, whereas the TRIF pathway is induced from endosomes. Vaneker et al. demonstrated that lung inflammation is induced by mechanical ventilation with an LTV (8 ml/kg) and that the effect is TRIF-dependent. In general, we detected no significant pulmonary inflammation with our LTV protocol (7 ml/kg), but it is noteworthy that we instrumented (e.g., tracheostomy) our spontaneously breathing CON, whereas Veneker et al. used uncatheterized spontaneously breathing mice as a control.

As such, it is possible that the tracheostomy itself produced a sufficient inflammatory response to obscure the effect of LTV and/or to predispose the mice to injury from additional insult such as HTV mechanical ventilation. Nevertheless, LTV mechanical ventilation did not change pulmonary function (such as EBA permeability, BAL cell count, and protein content) or TLR4 downstream signaling pathways (such as degradation of IκBα and MAPK/ERK phosphorylation). However, there was a two-fold increase in plasma level of IL-6 with LTV compared with spontaneously tracheotomized CON, a finding similar to that of a recent study that used mechanical ventilation at a tidal volume of 10

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**Fig. 6.** Toll-like receptor 4 (TLR4)-myeloid differentiation factor 88 (MyD88) signaling pathway after ventilator-induced lung injury (VILI). Two pathways are involved: TLR4-MyD88 pathway and TLR4-Toll/interleukin-1 receptor-domain containing adaptor-inducing interferon (TRIF) pathway. Upon binding to endogenous activators (e.g., oxidized phospholipids, high mobility group box1, fragmented hyaluronan, heat shock proteins), the adaptor molecule MyD88, which is associated with Toll/interleukin-1 receptor-domain-containing adaptor protein, is recruited to the TLR4 complex. Binding of MyD88 promotes association with interleukin-1 receptor-associated kinases (IRAKs) and tumor necrosis factor receptor-associated factor 6 (TRAF6). The complex IRAKs/TRAF6 dissociates from the receptor and then interacts with another complex, transforming growth factor activated kinase 1 (TAK1). TAK1 results in phosphorylation and degradation of inhibitor of κBα (IκBα), consequent release of nuclear factor-κB (NF-κB), translocation of NF-κB into the nucleus, and transcription of pro-inflammatory genes, inducing cytokine production. In the meantime, TAK1 also leads to activation of mitogen-activated protein kinases (MAPK) such as extracellular signal-regulated kinases (ERK) that activates activator protein 1 (AP-1) transcription factor, which induces the transcription of inflammatory cytokines. In addition to MyD88, other adaptor proteins, such as Toll/interleukin-1 receptor-domain containing adaptor-inducing interferon-β (TRIF), which is associated with TRIF-related adaptor molecule (TRAM), mediate induction of interferon regulatory factor 3 (IRF3), which in turn phosphorylates IκBα, leading to release of NF-κB to the nucleus. Unregulated proinflammatory cytokines may predispose for VILI. RIP1 = receptor interacting protein-1.
ml/kg for 6 h, indicating that a relatively small increase of systemic levels of IL-6 may not correlate to pulmonary function. In the current study, we also demonstrated that MyD88−/− mice elicited marked attenuation in the lung injury, including pulmonary capillary leak, pulmonary protein and cell emigration, and IL-6 production after HTV mechanical ventilation. Moreover, we found that both TLR4-mutant and MyD88−/− mice had attenuated NF-κB and MAPK/ERK activation in the lung, suggesting that the TLR4-MyD88 signaling contributes to the pro-inflammatory response during VILI. Taken together, these data suggest that pulmonary TLR4-MyD88 signaling may play a pivotal role in the pathogenesis of VILI and is responsible for the inflammatory changes observed in the injured lung.

It is unclear how TLR4-MyD88 (and TLR4-TRIF) signaling is activated in response to VILI. Lipopolysaccharide itself, the pathogenic ligand of TLR4, is responsible for TLR4 activation in Gram-negative bacterial infection, and might have been a contributing factor. Although its potential role was minimized in a recent study in which Vaneker et al. 16 carefully monitored the presence of lipopolysaccharide, we are uncertain of any contaminating levels during our protocols. Nonetheless, all our surgical procedures were done in a sterile environment. In addition, the sham CON underwent the same surgical intervention and were used to offset the possible effect of lipopolysaccharide in the animal group that underwent mechanical ventilation. In addition to lipopolysaccharide, recent evidence suggests an important role of endogenous ligands released in the setting of noninfectious tissue in activating TLR4. Examples of these potential endogenous ligands include high-mobility group box 1 protein released from necrotic cells, oxidized phospholipids from local generation of reactive oxygen species, low molecular weight hyaluran and fibrinogen from degraded extracellular matrix, heat shock proteins from necrotic cells, and surfactant protein-A. 34–42 In light of some controversy between the detrimental effects of oxidized phospholipids via intratracheal administration, 15 and the protective effects of oxidized phospholipids via an intravenous route 13,44 in VILI, it may be particularly fruitful to clarify the role of oxidized phospholipids as an endogenous ligand and a potential future therapeutic agent, especially through intratracheal administration. It is possible that the extent and duration of cyclic stretch by mechanical ventilation may trigger different quantitative and qualitative oxidized phospholipids that may produce either anti-inflammatory or pro-inflammatory effects to regulate pulmonary permeability.

In the Acute Respiratory Distress Syndrome Network study, 3 plasma IL-6 levels had a positive correlation to patients' mortality when comparing patients with ALI supported by mechanical ventilation with LTV to those with HTV. In addition, lower tidal volume ventilation was associated with a decrease in plasma IL-6 levels. 45 In our study, we confirmed the clinical studies as HTV elicited significant increase in IL-6 levels in both BAL and plasma after 4 h of HTV (table 1). The extent of IL-6 elevation correlates to increased BAL cell counts, indicating greater neutrophil migration and accumulation in the airspace. In the current study, we also found that HTV increased MIP-2 production in the BAL fluid (tables 1 and 2). MIP-2 has been reported to be increased in VILI and shown to augment migration of neutrophils into the alveoli. 46 Although our data showed that LTV did not increase pulmonary permeability and pulmonary edema, LTV did not prevent increase of IL-6 in BAL and plasma, indicating that IL-6 may not be a causative factor for VILI, and increased levels of IL-6 may be an adaptive host defense to potential injurious MV. Indeed, in a recent study, 47 IL-6 actually played a protective role in VILI, and VILI is considered a neutrophil-dependent process. Data from our group 48 support such a notion in that absence of neutrophil elastase decreased neutrophil migration but increased VILI severity.

In the current study, we did not try to distinguish between the magnitude of contributions of downstream pathways of TLR4 (e.g., MyD88- or TRIF-dependent) signaling in VILI, because previous studies 16 have also shown the role of TLR4-TRIF pathway in the development of inflammatory response in VILI. The strain of mouse used is a major confounding variable to contrast studies performed in different laboratories, and we have systematically described this with respect to VILI. 16 In this regard, C57BL/6J are relatively resistant to HTV-induced lung injury (table 2). Nonetheless, within this strain (with MyD88-null mice being backbred on C57BL/6J background), it was apparent that MyD88−/− mice were resistant to VILI-induced increases in permeability, histopathologic damage, and cytokine production, consistent with an important role for this limb of TLR4 signaling.

The role of gender in VILI remains unclear and may have contributed to any differences from other existing reports. We chose female mice because they have been reported to have increased sensitivity to ALI induced by hyperoxia, 49 bleomycin, 50 and/or ozone. 51

No single animal model reproduces all of the characteristics of ALI, and discrepancies exist between effective therapeutic approaches in animal studies and unsuccessful therapies in human clinical trials. Small animals, such as mice, are a very powerful research tool because they can be genetically modified to facilitate the detailed mechanistic study of complex pathways. It is technically challenging, however, to reproduce long-term mechanical ventilation in mice 52 (compared with human patients); thus, in this regard, results from most animal studies need to be interpreted with caution. Nonetheless, short-term mechanical ventilation in rodents remains the most common model used, and information derived from these studies has been useful in influencing clinical practice in humans. 53,54 The mechanical ventilation model we used here combined HTV ventilation and tracheotomy to reproduce clinical characteristics of ALI in humans.
(i.e., increased pulmonary permeability and inflammation), although other factors may influence the magnitude of lung injury, such as anesthetic use (e.g., sevoflurane, ketamine), variability of pressure support, and positive end-expiratory pressure. The insights gained from the study may still have some important clinical implications. Although lung-protective ventilation strategies using LTV with limited airway pressure and appropriate positive end-expiratory pressure have been proposed as a current recommendation for the management of critically ill patients, a world-wide survey has found that it is not uncommon for critically ill patients to still be ventilated with HTV.

In addition, recent computed tomography images for patients with lung injury have demonstrated nonhomogeneous distribution of pulmonary aeration; thus, lung regions that normally receive relatively small aeration may receive the largest contribution of pulmonary aeration; thus, lung regions that normally receive relatively small aeration may receive the largest contribution of pulmonary aeration. From a clinical point of view, the HTV mechanical ventilation mouse model is still clinically relevant. The emerging evidence of clinical trials and animal models of VILI, including our current study, has shown that routine use of LTV seems beneficial in all patients requiring mechanical ventilation especially for those patients with preexisting lung injury and inflammation.

In summary, our results demonstrate a critical role of TLR4 and its adaptor MyD88 in the development of VILI. The TLR4-MyD88 signaling pathway may possibly act via the mechanisms involving activation of NF-κB and MAPK. Strategies to modulate NF-κB and MAPK activation and routine use of LTV mechanical ventilation may have potential therapeutic benefits in patients suffering from VILI.

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