# Monoclonal antibody against Toll-like receptor 4 attenuates ventilator-induced lung injury in rats by inhibiting MyD88- and NF-κB-dependent signaling

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**Abstract.** The mechanisms through which mechanical ventilation causes non-infectious inflammatory diseases and lung injury are poorly understood. Animals models of this type of injury suggest that it involves signaling mediated by Toll-like receptor (TLR)4 and 9. In this study, in order to gain further insight into the involvement of TLR4 in this type of injury, we performed *in vivo* and *in vitro* experiments to determine the mechanisms through which TLR4 triggers inflammation. We also examined whether the use of TLR4 monoclonal antibody (mAb) can alleviate this type of injury. For this purpose, rats were tracheotomized and administered intratracheal injections of anti-TLR4 mAb or saline, and then ventilated for 4 h at a high tidal volume (HTV) of 40 ml/ kg or allowed to breathe spontaneously for the same period of time (controls). Alveolar macrophages (AMs) were isolated from the bronchoalveolar lavage fluid (BALF) of the rats and stimulated for 16 h with tumor necrosis factor (TNF)-α in the presence or absence of anti-TLR4 mAb. Lung injury was assessed by examining lung histopathology, lung wet/dry weight ratio, BALF total protein and cytokine levels in BALF and plasma. The mRNA and protein expression levels of TLR4, TLR9, myeloid differentiation factor 88 (Myd88) and nuclear factor (NF)-κB were measured in cultured macrophages. Compared to the controls (spontaneous breathing), the ventilated rats exhibited greater pulmonary permeability, more severe inflammatory cell infiltration/lung edema, and higher levels of interleukin (IL)-1β, IL-6 and TNF-α in BALF and plasma. The AMs from the ventilated rats expressed higher mRNA and protein levels of TLR4, TLR9, Myd88 and NF-κB compared with the macrophages from the spontaneously breathing rats. The ventilated rats pre-treated with anti-TLR4 mAb exhibited markedly attenuated signs of ventilation-induced injury, such as less lung inflammation and pulmonary edema, fewer cells in BALF, and lower levels of ILs and TNF-α in BALF and plasma. Similarly, the TNF-α-dependent increases in the mRNA and protein expression of TLR4, Myd88 and NF-κB in AMs were attenuated when TNF-α was co-administered with anti-TLR4 mAb than when TNF-α was administered alone. Co-administering anti-TLR4 mAb also reduced the TNF-α-dependent secretion of ILs. On the whole, our data demonstrate that TLR4 contributes significantly to ventilation-induced lung injury by activating the Myd88/NF-κB pathway, and pre-treating rats with anti-TLR4 mAb partially protects them against this type of injury by inhibiting Myd88/NF-κB signaling.

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## Introduction

Acute respiratory distress syndrome (ARDS) and acute lung injury mark the final stages of several respiratory diseases; the traditional supportive treatment for ARDS is mechanical ventilation. Although such ventilation can be lifesaving, it comes with several potential disadvantages and complications (1). High tidal volume (HTV) mechanical ventilation can cause lung edema and activate inflammatory pathways, a process known as ventilator-induced lung injury (2). Mechanical ventilation and inflammation induce the activation of alveolar macrophages at sensitive sites, and these cells in turn help trigger and sustain an acute inflammatory response (3). The macrophages release inflammatory cytokines, including interleukin (IL)-1β and IL-6 (4-6). The combination of mechanical tissue stretching and inflammation lead to lung injury (7).

Toll-like receptors (TLRs) play a critical role in ventilator-induced lung injury (8). In our previous study, the expression of TLR4 and TLR9 were found to be upregulated in animal models of ventilator-induced lung injury, and the most obvious increase in the expression of TLRs was observed following ventilation for 4 h (9). In particular, the activation of the signaling pathway mediated by TLRs, myeloid differentiation factor 88 (MyD88)/nuclear factor (NF)-κB, has been proposed to cause the release of inflammatory cytokines [IL-1β, IL-6 and tumor necrosis factor (TNF)-α], to strengthen the immune response, and increase endothelial permeability, all of which contribute to injury (10,11). This pathway may be activated in alveolar macrophages during mechanical ventilation, but this has never been directly examined *in vivo*, at least to the best of our knowledge. Consistent with this possibility, studies using mice have indicated that TNF-α intensifies the inflammatory response during endotoxic shock (12) and that signaling via the TNF-α receptor helps drive ventilator-induced lung injury (13).

In this study, we used a combination of *in vivo* and *in vitro* approaches in an aim to determine the mechanisms through which TLR4-mediated signaling drives ventilator-induced lung injury. We also examined whether neutralizing receptor activity using an anti-TLR4 monoclonal antibody may alleviate this type of injury.

## Materials and methods

*Animals*. Forty-eight adult male Sprague-Dawley rats weighing 250-300 g were purchased from the Medical Laboratory Animal Center of Guangxi Medical University, China (certificate no. SCXK-Gui-2010-0001). The rats were fed a normal diet with water *ad libitum* and were housed in accordance with the Chinese Regulations for the Administration of Affairs Concerning Experimental Animals. The study protocol was approved by the Animal Care and Use Committee of Guangxi Medical University.

*Rat model of ventilator-induced lung injury*. Rats were anesthetized intraperitoneally with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg) (no. 091127; Fujian Gutian Pharmaceutical Co. Ltd, Ningde, China). Anesthesia was maintained by administering one-third of the initial dose of anesthetic agents approximately every 45 min during experiments. Rats were placed in a supine position on an adjustable warming pad (Alcott Biotech, Shanghai, China) that was maintained at 37±1˚C; animal temperature was monitored continuously using a rectal probe.

According to the relevant cell count in the bronchoalveolar lavage fluid (BALF) extracted from each of the rats ($3\text{-}6\times10^5$ cells) (14), and the protocol of immunohistochemistry and western blot analysis of anti-TLR4 monoclonal antibody, the rats were subjected to tracheal intubation and administered either 200 µl of normal saline (n=8) or 200 µl of anti-TLR4 monoclonal antibody (ab8376; Abcam, Cambridge, USA) at a dose of 8 µg/kg body weight (n=8). After 1 h, both groups of animals were connected to a small animal ventilator (Alcott Biotech) and ventilated for 4 h at 40 ml/kg with 80 breaths/min and 0 positive end-expiratory pressure. The respiratory parameter were based on a previous study (9) and the preliminary results of our research groups. A third group of rats (n=8) was subjected to tracheotomy and the intratracheal administration of saline, and then allowed to breathe spontaneously. All 3 groups of animals breathed ambient air. Oxygen saturation and heart rate were continuously monitored in the anesthetized rats using the MouseOx system (NatureGene Corp., Beijing, China). At the end of the 4-h ventilation period, the animals were administered a lethal dose of anesthetic agents and lung tissues, blood and BALF were harvested.

*Collection of plasma*. Plasma samples were collected from the atrium dextrum of the rats, followed by centrifugation for 15 min at 500 x g at 4˚C to remove red cells, and the plasma was frozen at -20˚C.

*Collection and culture of alveolar macrophages*. Alveolar macrophages were harvested from the BALF of each group of rats into phosphate-buffered saline (PBS) as previously described (15). The cells were resuspended in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and cultured for 2 h in 25-cm$^2$ flasks. The cultures were then washed with fresh RPMI-1640 to remove non-adherent cells. The viability of the remaining macrophages was assayed using trypan blue before conducting the TNF-α stimulation experiments described below.

*In vitro model of the stimulation of alveolar macrophages with TNF-α*. The alveolar macrophages were harvested in BALF of 24 ventilated rats. The macrophage cultures were divided into 3 treatment groups, with each group containing cultures from 24 ventilated rats pre-treated with saline. One set (n=8) of macrophage cultures was incubated for 2 h with anti-TLR4 antibody (group TNF + Ab), and the other 2 sets (n=16 per set) with PBS. The medium for all 3 sets of cultures was then replaced with fresh RMPI-1640 containing 10% FBS, and the cultures pre-treated with anti-TLR4 antibody (TNF + Ab) or PBS (TNF group) were stimulated for a further 16 h with TNF-α (20 ng/ml), and a third set with PBS only (PBS group).

*Pulmonary edema based on the lung wet/dry weight ratio*. At the end of the 4-h ventilation period, the rats were sacrificed and the right lung was flushed with PBS. Following the ligation of left lungs of the rats, the lungs were weighed immediately after removal (wet weight) and again after drying in an oven at 65˚C for 48 h (dry weight). The ratio was calculated to serve as an index of pulmonary edema.

*Lung histopathology*. Tissue from the right lower lobe was removed and processed for transmission electron microscopy as previously described (16). Specimens were examined on a JEOL 8000 transmission electron microscope (Hitachi High-Technologies Corp., Tokyo, Japan) microscope. The animals were then subjected to intratracheal instillation with 10% formalin to fix the lungs. The lungs were then removed and embedded in paraffin. Sections (4-µm-thick) were made using a rotary microtome (HM 355S; Thermo Fisher Scientific, Waltham, MA, USA) and stained with hematoxylin and eosin, and examined under an IX71 light microscope (Olympus, Tokyo, Japan).

*Analysis of BALF*. Lung inflammation was assessed by counting the numbers of cells and assaying the total protein amount in BALF. Each rat was instilled with 1.0 ml of PBS 5 times, and the recovered volumes were kept on ice. The total recovered volume was approximately 80% of the original 5 ml. The recovered BALF was centrifuged for 5 min at 500 x g at 4˚C. The pellet was resuspended in RPMI-1640 containing 10% FBS, and analyzed on a hemocytometer (YA0810; Solarbio, Beijing, China) to determine the total cell number. Supernatants were frozen immediately on dry ice and stored at -80˚C for cytokine assays.

*Cytokine levels in BALF, plasma and culture medium*. Commercial kits based on the enzyme-linked immunosorbent

Table I. Primer sequences used to detect mRNAs encoding TLR4, TLR9, MyD88, NF-κB and GAPDH.

| Gene | Primer sequence (5'→3') | Product size (bp) |
|---|---|---|
| GAPDH | F: GGCACAGTCAAGGCTGAGAATG | 143 |
| | R: ATGGTGGTGAGA CGCCAGTA | |
| TLR4 | F: CATCCAAAGGAATACTGCAACA | 398 |
| | R: GTTTCTCACCCAGTCCTCATTC | |
| TLR9 | F: CAGCTAAAGGCCCTG ACCAA | 160 |
| | R: CCACCGTCTTGAGAATGTTGTG | |
| MyD88 | F: TATACCAACCCTTGCACCAAGTC | 525 |
| | R: TCAGGCTCCAAGTCAGCTCATC | |
| NF-κB | F: GAG GACTTGCTGAGGGTTGG | 148 |
| | R: TGGGGTGGTTGATAAGGAGTG | |

F, forward primer; R, reverse primer; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

assay (ELISA) (R&D Systems, Inc., Minneapolis, MN, USA) were used to assay TNF-α, IL-1β and IL-6 in BALF and plasma from mechanically ventilated or spontaneously breathing rats, as well as in the medium of alveolar macrophage cultures. Each sample was assayed in duplicate following the manufacturer's instructions (rat TNF-α Quantikine ELISA RTA00; rat IL-1β/IL-1F2 Quantikine ELISA RLB00; Rat TNF-α Quantikine ELISA R6000B; R&D Systems).

*Western blot analysis of specific proteins in alveolar macrophage cultures.* Alveolar macrophages ($2x10^6$ cells) were suspended in 50 µl ice-cold RIPA lysis buffer with protease inhibitors (Beyotime Institute of Biotechnology, Haimen, China), homogenized and centrifuged at 12,000 x g for 5 min at 4˚C to remove insoluble material. The total protein concentration in the supernatant was determined using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). The supernatant was mixed with 4X loading buffer (Takara Bio, Dalian, China), and equal amounts of protein were fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (both from Bio-Rad Laboratories, Inc., Hercules, USA). The blots were blocked for 2 h at room temperature with Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat milk, then incubated overnight with primary antibody, followed by horseradish peroxidase-conjugated secondary antibody for chemiluminescent visualization (Beyotime Institute of Biotechnology). Processed membranes were scanned using the ChemiDoc MP system (Bio-Rad Laboratories, Inc.), and densitometry was performed using in-house software developed at the Affiliated Tumor Hospital of Guangxi Medical University (Nanning, China). Antibodies against TLR4 (ab8376) and TLR9 (ab12121) were purchased from Abcam (Cambridge, MA, USA), while antibodies against MyD88 (Cat. no. 4283) and NF-κB (Cat. no. 3033) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary anti-rabbit (SA00001-2) and anti-mouse (SA00001-1) antibodies were purchased from the ProteinTech Group, Inc. (Bioconnect, Wuhan, China).

*Analysis of gene expression in alveolar macrophage cultures by reverse transcription-polymerase chain reaction (RT-PCR).* Total RNA was extracted from the alveolar macrophage cultures using the GeneJET RNA Purification kit (Thermo Fisher Scientific). Reverse transcription was used to generate cDNA encoding TLR4, TLR9, MyD88 and NF-κB, which was then amplified by PCR. Specific primers to target regions of the corresponding genes were designed based on sequences in GenBank (Table I). In parallel, the gene expressing glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control and used to normalize gene expression levels.

*Statistical analysis.* Data are reported as the means ± SD from 8 animals for each experimental condition, unless otherwise indicated in the figures or tables. Inter-group differences were assessed for statistical significance using the Student's t-test and analysis of variance. All statistical tests were performed using SPSS 13.0 software (IBM, Chicago, IL, USA). All P-values were two-tailed, and a value of $P<0.05$ was defined as the threshold of statistical significance.

## Results

*Anti-TLR4 monoclonal antibody reduces ventilation-induced lung edema and injury.* The rats treated intratracheally with anti-TLR4 antibody prior to mechanical ventilation exhibited significantly less pulmonary edema and BALF total protein than the rats treated with saline prior to ventilation by determining the lung wet/dry ratio (Fig. 1). In fact, edema in the rats pre-treated with antibody was similar to that observed in the rats treated with saline that were then allowed to breathe spontaneously. Similarly, the ventilated rats pre-treated with saline exhibited significantly more alveolar septal thickening than the ventilated rats pre-treated with antibody and the spontaneously breathing rats (Fig. 2).

Using electron microscopy to examine alveolar histopathology in greater detail, we found that, as expected, alveolar cells in the tissue of the ventilated rats pre-treated with saline exhibited a disrupted cytoplasmic and nuclear structure, as well as cell membrane discontinuities (Fig. 3). By contrast, tissue from the ventilated rats pre-treated with antibody exhibited a normal cytoplasmic and nuclear structure and continuous cell membrane for types I and II alveolar epithelial cells and for alveolar macrophages.

*Anti-TLR4 monoclonal antibody reduces the ventilation-induced secretion of pro-inflammatory cytokines.* The levels of IL-1β, IL-6 and TNF-α in BALF and plasma were significantly higher in the ventilated rats pre-treated with saline than in the ventilated rats pre-treated with antibody (Fig. 4). In fact, the levels of these cytokines were similar between the antibody-pre-treated rats and the control rats that were not ventilated.

*Anti-TLR4 monoclonal antibody reduces the ventilation-induced activation of NF-κB.* Since most TLR signaling pathways stimulated by mechanical ventilation culminate in the activation of the transcription factor, NF-κB (8,9,17), we examined whether

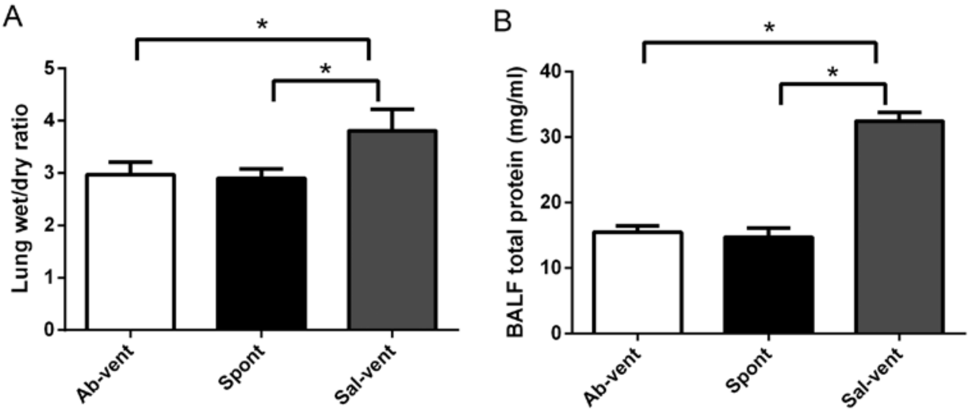


Figure 1. Lung edema and protein content in bronchoalveolar lavage fluid (BALF) in mechanically ventilated rats pre-treated with anti-Toll-like receptor 4 (TLR4) monoclonal antibody (Ab-vent) or saline (Sal-vent). A parallel control group of rats was allowed to breathe spontaneously (Spont). (A) Lung edema was assessed by determining the weight ratio between wet and dry lung. (B) Total protein concentration in BALF. *P<0.05.

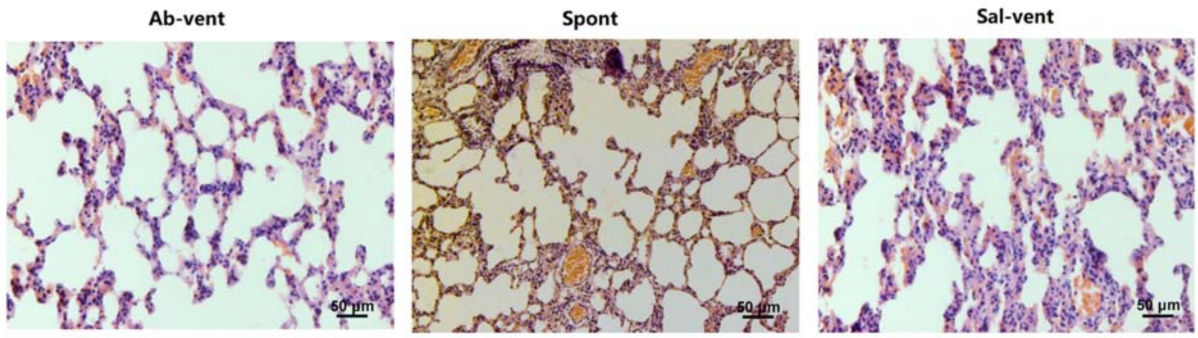


Figure 2. Histology of lung tissue from mechanically ventilated rats pre-treated with anti-Toll-like receptor 4 (TLR4) monoclonal antibody (Ab-vent) or saline (Sal-vent). A parallel control group of rats was allowed to breathe spontaneously (Spont). Tissue sections were stained with hematoxylin and eosin. Lung tissue from saline-pre-treated ventilated rats exhibited patchy areas of hemorrhage and thickened alveolar walls, with inflammatory cell infiltration. Tissue from antibody-pre-treated ventilated rats or spontaneously breathing control rats exhibited much less hemorrhaging and less severe inflammatory cell infiltration. Representative results are shown. Magnification, x200.

pre-treatment with anti-TLR4 monoclonal antibody attenuates NF-κB activation in alveolar macrophages cultured from BALF. Indeed, we found that the protein and mRNA levels of TLR4, NF-κB and MyD88 were significantly higher in the macrophages obtained from the ventilated rats pre-treated with saline than in the macrophages obtained from the ventilated rats pre-treated with anti-TLR4 antibody or in the macrophages from the rats allowed to breathe spontaneously (Fig. 5).

Since TLR9 is upregulated in lung diseases (8,18), we wished to determine whether ventilation-induced injury is associated with changes in the expression of TLR9. The protein and mRNA levels of TLR9 were similar in the ventilated rats, regardless of whether they had been pre-treated with saline or anti-TLR4 antibody, and these levels were higher than in the rats allowed to breathe spontaneously (Fig. 5).

*Anti-TLR4 monoclonal antibody reduces TNF-α-induced cytokine secretion by alveolar macrophages.* Since separate studies have demonstrated that TNF-α is upregulated in ventilation-induced lung injury (8,9,13,19), and that TNF-α can induce the secretion of some inflammatory cytokines (13), we wished to observe directly whether TNF-α stimulates the secretion of IL-1β and IL-6 by alveolar macrophages in our rat model of lung injury. We found that the levels of IL-1β, IL-6 and TNF-α were significantly higher in the macrophages from the high tidal ventilated rats stimulated with TNF-α than in those stimulated with PBS (Fig. 6). Pre-treatment with anti-TLR4 antibody reduced the levels of IL-1β, IL-6 and TNF-α to similar values as in the macrophages stimulated with PBS.

*Anti-TLR4 monoclonal antibody attenuates the TNF-α-induced activation of NF-κB and MyD88 in alveolar macrophages.* Since previous studies have suggested, but not shown directly, that TNF-α may promote inflammation through the TLR/NF-κB/MyD88 pathway (20,21), we examined the protein and mRNA levels of TLR4, NF-κB and MyD88 in alveolar macrophages in the presence and absence of TNF-α stimulation. All levels were significantly higher in the stimulated macrophages than in the mock (PBS)-stimulated macrophages (Fig. 7). The levels in the macrophages stimulated in the presence of anti-TLR4 antibody were similar to those in the PBS-stimulated controls.

## Discussion

In this study, we combined *in vivo* and *in vitro* approaches to clarify several of the molecular steps in ventilation-induced lung injury in rats. We provide evidence to indicate that in

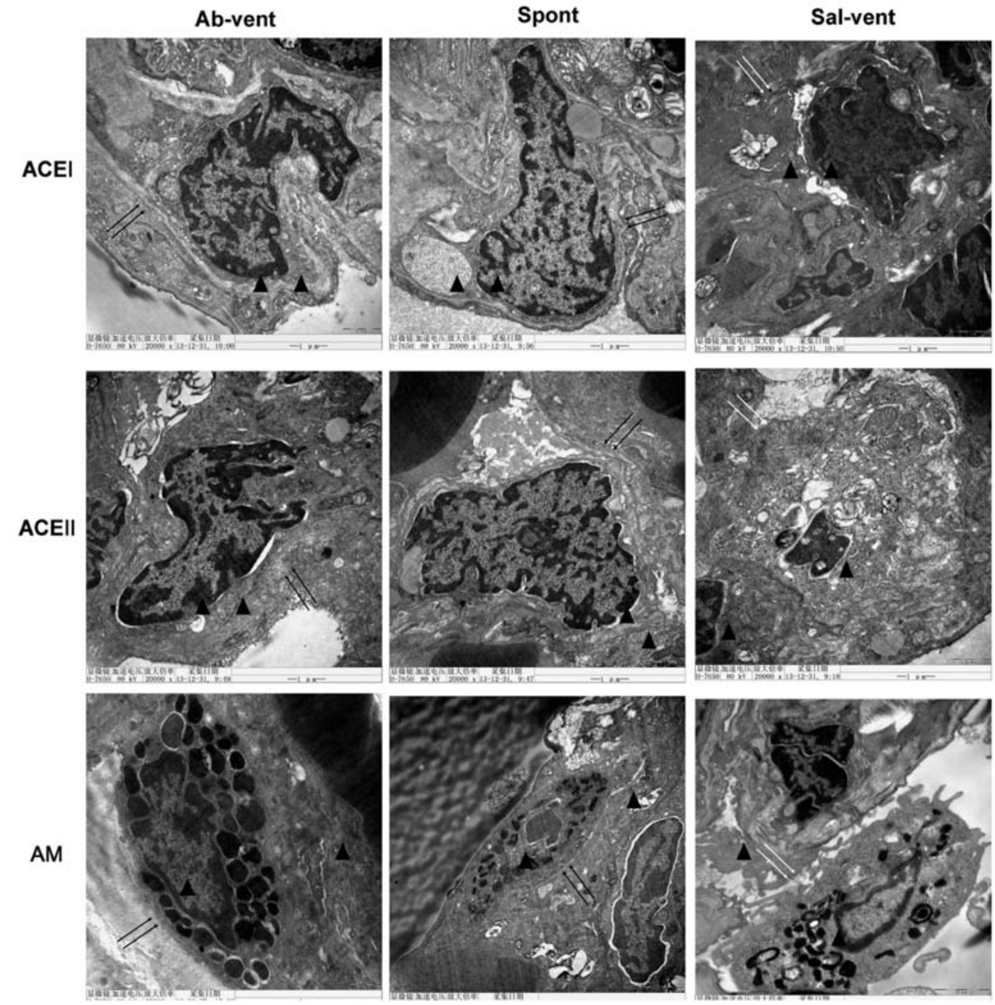


Figure 3. Transmission electron micrographs of alveolar cells from mechanically ventilated rats pre-treated with anti-Toll-like receptor 4 (TLR4) monoclonal antibody (Ab-vent) or saline (Sal-vent). A parallel control group of rats was allowed to breathe spontaneously (Spont). Representative images are shown for types I and II alveolar epithelial cells (ACE I and II) and alveolar macrographs (AMs). Arrows mark areas of continuous membrane and areas marked with triangles indicate cytoplasmic and nuclear structural disorder. Whereas tissue from antibody-pretreated ventilated animals or from spontaneously breathing animals appeared normal, tissue from saline-pre-treated ventilated animals exhibited discontinuous AEC membranes, vacuolization, and cytoplasmic and nuclear structural disorder of AEC and AM membranes. Magnification, x20,000.

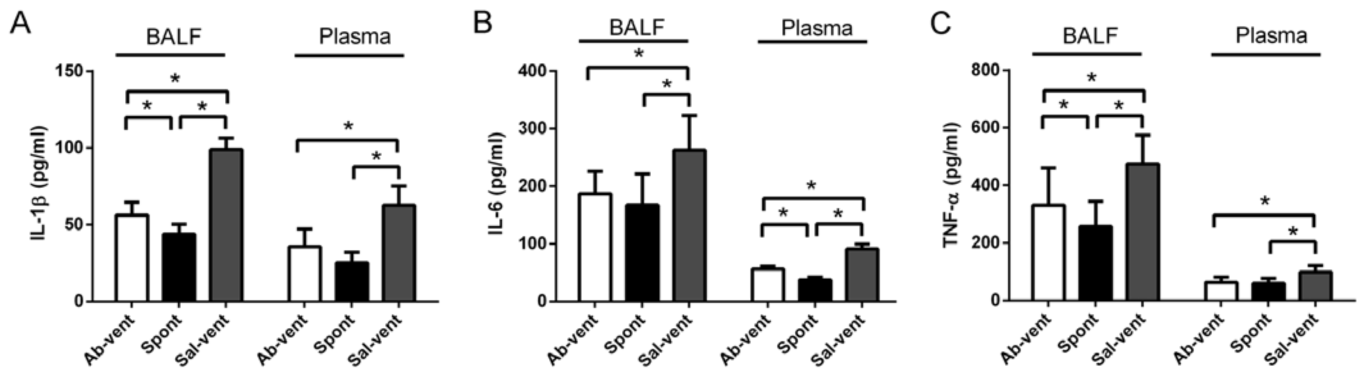


Figure 4. Levels of (A) interleukin (IL)-1β, (B) IL-6 and (C) tumor necrosis factor (TNF)-α in bronchoalveolar lavage fluid (BALF) and plasma from mechanically ventilated rats pre-treated with anti-Toll-like receptor 4 (TLR4) monoclonal antibody (Ab-vent) or saline (Sal-vent). A parallel control group of rats was allowed to breathe spontaneously (Spont). *P<0.05.

response to the stress of mechanical ventilation, TLR4 activates the NF-κB/MyD88 pathway, thereby stimulating alveolar macrophages to secrete the pro-inflammatory cytokines, IL-1β and IL-6. We demonstrated that these events can be triggered by TNF-α, and that the stimulation of alveolar macrophages with TNF-α triggered the upregulation of TNF-α, constituting a positive feedback loop that likely prolongs lung inflammation and exacerbates lung injury. We also demonstrated

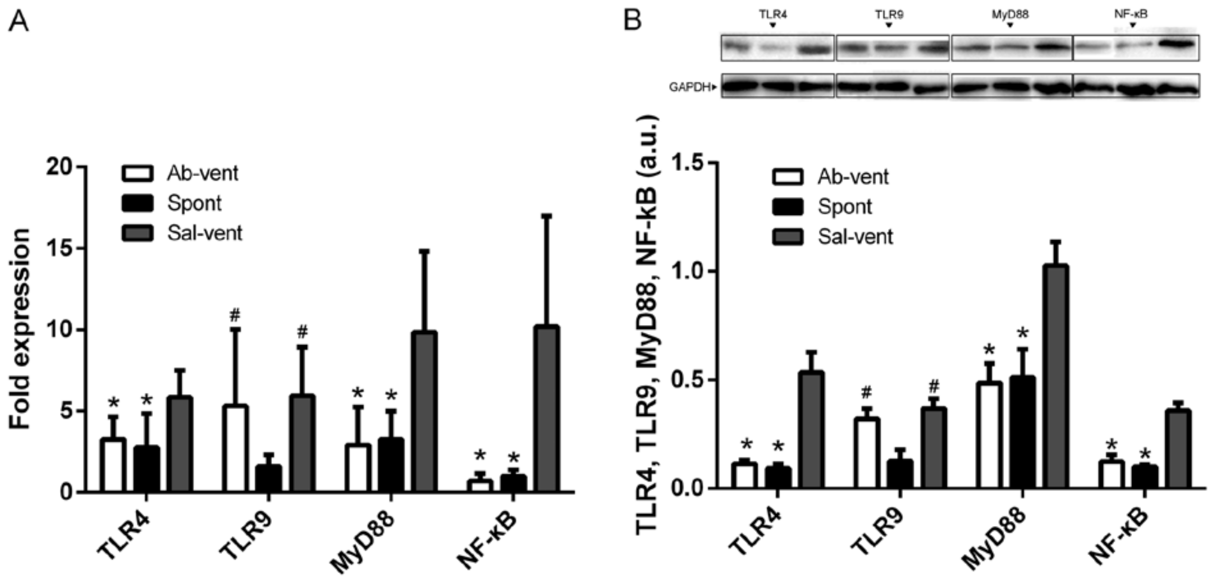


Figure 5. Levels of Toll-like receptors (TLRs) 4 and 9, myeloid differentiation factor 88 (MyD88) and nuclear factor (NF)-κB (A) mRNA and (B) protein in alveolar macrophages from mechanically ventilated rats pre-treated with anti-Toll-like receptor 4 (TLR4) monoclonal antibody (Ab-vent) or saline (Sal-vent). A parallel control group of rats was allowed to breathe spontaneously (Spont). Fold expression for target genes was normalized to that measured for the GAPDH gene. *P<0.05 vs. Sal-vent, #P<0.05 vs. Spont.

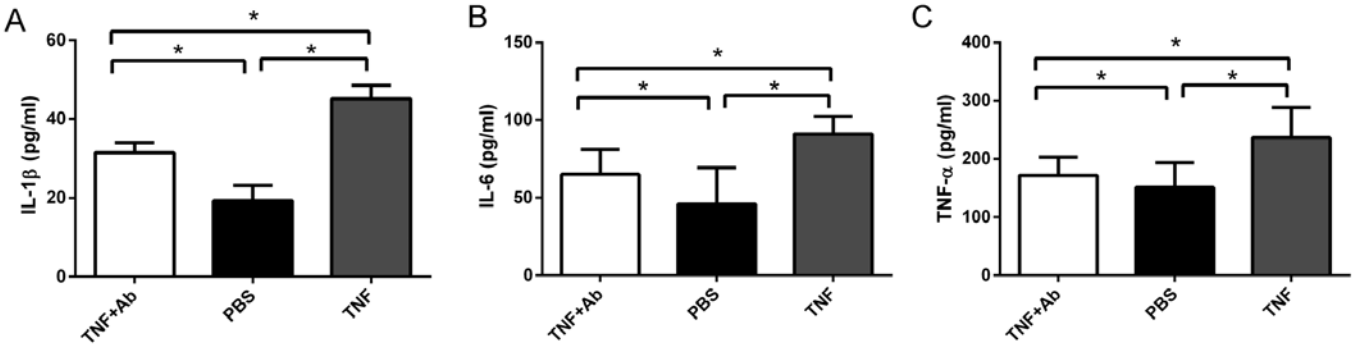


Figure 6. Levels of (A) interleukin (IL)-1β, (B) IL-6 and (C) tumor necrosis factor (TNF)-α in the medium of alveolar macrophage cultures stimulated for 16 h with TNF-α alone (TNF), TNF-α following incubation with anti-toll-like receptor 4 (TLR4) monoclonal antibody (TNF + Ab) or PBS. *P<0.05.

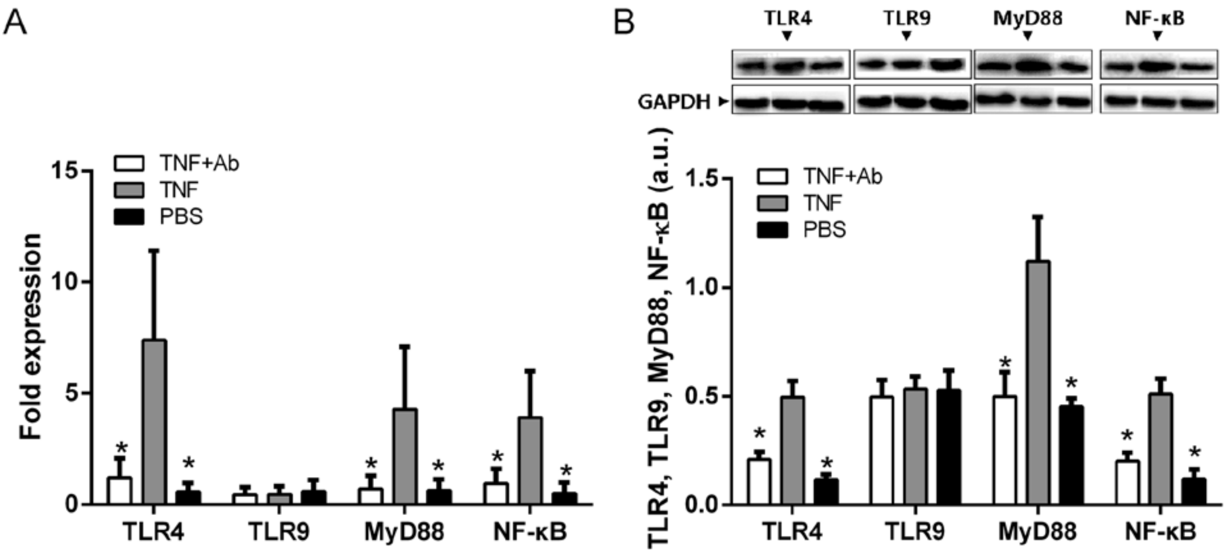


Figure 7. Levels of Toll-like receptors (TLRs) 4 and 9, myeloid differentiation factor 88 (MyD88) and NF-κB (A) mRNA and (B) protein in alveolar macrophage cultures stimulated for 16 h with tumor necrosis factor (TNF)-α alone (TNF), TNF-α following incubation with anti-Toll-like receptor 4 (TLR4) monoclonal antibody (TNF + Ab) or PBS. Fold expression for target genes was normalized to that measured for the GAPDH gene. *P<0.05 vs. PBS.

that pre-treating rats with anti-TLR4 monoclonal antibody prior to mechanical ventilation almost completely eliminated ventilation-induced changes in the production and secretion of cytokines and TNF-α. Stimulating cultures of alveolar macrophages with TNF-α in the presence of anti-TLR4 antibody also eliminated the upregulation and secretion of cytokines and of TNF-α itself, that was observed when macrophages are stimulated with TNF-α alone. Thus, the present study provides several mechanistic insights into ventilation-induced lung injury that can guide future research in this area. Our findings also identify monoclonal antibody targeting of TLR4 as a potential therapy to treat or prevent such lung injury.

A hallmark of acute lung injury is the structural impairment of the alveolar-capillary membrane barrier, leading to increased pulmonary vascular permeability and inflammation (22,23). Our rat model reproduced the clinically important manifestations of ventilation-induced lung injury, including increased alveolar permeability (Fig. 1), overall protein in the alveolar space (Fig. 1), greater inflammatory cell infiltration and cytokine production in BALF and plasma (Fig. 4), and higher pro-inflammatory signaling via NF-κB pathways (Fig. 3) in alveolar macrophages. Mechanical ventilation for 4 h at 40 ml/kg caused the levels of IL-1β, IL-6 and TNF-α to increase almost 2-fold in BALF and plasma, consistent with other studies using rats (8,24). Using this system, we demonstrated that treating rats with anti-TLR4 antibody partially reversed all these ventilation-induced changes. Not only do these findings provide strong evidence for the key role of TLR4 in ventilation-induced lung injury, but they also provide the basis for a molecular targeted therapy.

Non-infectious lung inflammation induced by alveolar over-distention during mechanical ventilation contributes to ventilation-induced lung injury (25,26). TLRs have long been recognized to play a crucial role in both innate and adaptive immune responses to pathogens and to non-infectious tissue injury (27,28). Indeed, both TLR4 and TLR9 have been shown to play critical roles in acute lung injury caused by HTV ventilation, lipopolysaccharide, acid aspiration, hemorrhage, and ischemia and reperfusion (8,29,30). TLRs span the cell membrane and simultaneously activate MyD88-dependent signaling pathways (10,31) and TRIF-dependent pathways. TLR4, for example, dimerizes upon ligand binding, then it recruits the downstream adaptor molecule, MyD88, and ultimately activates NF-κB, inducing the transcription of pro-inflammatory genes, including ones encoding cytokines. Our *in vitro* and *in vivo* findings in the present study are consistent with the hypothesis that TLR4 signaling in response to mechanical ventilation stimulates NF-κB-mediated transcription of pro-inflammatory cytokines. We found that inhibiting TLR4 signaling by treating rats with anti-TLR4 monoclonal antibody reduced ventilation-induced lung injury, reminiscent of how Hayes *et al* were able to reduce the severity of lung injury in their rat model by overexpressing IκBα and thereby inhibiting pulmonary NF-κB (17,32). Indeed, the same pathway may be at work in the present study and in the work of Hayes *et al*, with the only difference being that we inhibited the initial step of TLR4 activation, while Hayes *et al* inhibited the final step of NF-κB activation (32).

The mechanisms through which ventilation-induced lung injury initially activates the TLR4-MyD88 signaling pathway are unclear, since the canonical ligand for TLR4 is lipopolysaccharide due to Gram-negative bacterial infection (11,33). In the absence of infection, it is possible that TLR4 is activated by any of several endogenous ligands, which have been proposed to include high-mobility group box 1 protein released from necrotic cells, oxidized phospholipids arising due to locally generated reactive oxygen species, low-molecular-weight hyaluran and fibrinogen generated during degradation of extracellular matrix, heat shock proteins released from necrotic cells, and surfactant protein A (11,34,35). The cyclic stretching of lung tissue by mechanical ventilation may trigger cell apoptosis and the release of protein contents as well as harmful oxygen species, giving rise to endogenous ligands that may activate TLR4.

Regardless of how lung injury initially triggers the TLR4/MyD88 signaling pathway that activates NF-κB, alveolar macrophages are likely to be the major cells that receive the initial pro-inflammatory signal and transduce it into cytokine signals that prolong and exacerbate injury. Stretching and inflammation are sufficient to cause these macrophages to release inflammatory cytokines (36,37), and it was shown that the ventilation-induced activation of NF-κB in alveolar macrophages induces the secretion of IL-6 and subsequent IL-1β (38). Therefore, we complemented our *in vivo* experiments in rats with *in vitro* experiments using alveolar macrophage cultures.

Although our study focused on TLR4, evidence suggests that TLR9/MyD88 signaling in alveolar macrophages also plays a role in ventilation-induced lung injury (8). We found that mechanically ventilating rats with or without pre-treatment with anti-TLR4 antibody led to mRNA and protein levels of TLR9 in alveolar macrophages that were similar to those in macrophages from spontaneously breathing animals. We also found that TNF-α stimulation in the presence or absence of anti-TLR4 antibody did not significantly alter the levels of TLR9 mRNA or protein in alveolar macrophage cultures. These findings suggest that TLR4 may play a larger role than TLR9 in ventilation-induced lung injury. The fact that we still observed some differences between antibody-pre-treated rats and spontaneously breathing rats, as well as between antibody-treated macrophage cultures and PBS-treated cultures suggests that TLR9 does contribute to lung injury; however, the precise extent and the signaling pathways involved require further investigation. Some studies have suggested that TLR9 in alveolar macrophages plays a role in pathogenesis of VILI (9,38); thus, the role of this receptor should be explored carefully.

A limitation to the present study, common to many animal studies on acute lung injury, is that other factors can affect injury severity, including the type and dose of anesthetic use (e.g., sevoflurane, ketamine or protofol) (39,40), variations in pressure support (41) and positive end-expiratory pressure. Nevertheless, the insights from our study may have important clinical implications (42). For example, our results suggest that strategies to modulate the activation of the TLR4/MyD88 signaling pathway may help treat or even prevent ventilation-induced lung injury. Our findings also highlight the need for more extensive research into the potential involvement of TLR9 in this type of injury.

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