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Low-tidal-volume Mechanical Ventilation Induces a Toll-like Receptor 4–dependent Inflammatory Response in Healthy Mice

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Background: **Mechanical ventilation (MV) can induce ventilator-induced lung injury. A role for proinflammatory pathways has been proposed. The current studies analyzed the roles of Toll-like receptor (TLR) 4 and TLR2 involvement in the inflammatory response after MV in the healthy lung.**

Methods: **Wild-type (WT) C57BL6, TLR4 knockout (KO), and TLR2 KO mice were mechanically ventilated for 4 h. Bronchoalveolar lavage fluid was analyzed for presence of endogenous ligands. Lung homogenates were used to investigate changes in TLR4 and TLR2 expression. Cytokines were measured in lung homogenate and plasma, and leukocytes were counted in lung tissue.**

Results: **MV significantly increased endogenous ligands for TLR4 in bronchoalveolar lavage fluid and relative messenger RNA expression of TLR4 and TLR2 in lung tissue. In lung homogenates, MV in WT mice increased levels of keratinocyte-derived chemokine, interleukin (IL)-1α, and IL-1β. In TLR4 KO mice, MV increased IL-1α but not IL-1β, and the increase in keratinocyte-derived chemokine was less pronounced. In plasma, MV in WT mice increased levels of IL-6, keratinocyte-derived chemokine, and tumor necrosis factor α. In TLR4 KO mice, MV did not increase levels of IL-6 or tumor necrosis factor α, and the response of keratinocyte-derived chemokine was less pronounced. MV in TLR2 KO mice did not result in different cytokine levels compared with WT mice. In WT and TLR2 KO mice, but not in TLR4 KO mice, MV increased the number of pulmonary leukocytes.**

Conclusions: **The current study supports a role for TLR4 in the inflammatory reaction after short-term MV in healthy lungs. Increasing the understanding of the innate immune response to MV may lead to future treatment advances in ventilator-induced lung injury, in which TLR4 may serve as a therapeutic target.**

MECHANICAL ventilation (MV) facilitates surgical interventions during general anesthesia and is a lifesaving intervention in acute respiratory failure. However, MV can induce lung injury; this has been termed *ventilator-induced lung injury* (VILI).[1] VILI can exacerbate damage in the already injured lung but can also occur in healthy lung.[2] The mechanisms of VILI are incompletely understood, but a role for proinflammatory pathways has been proposed.[3]

Animal models have been used to study the pathophysiology underlying VILI. Traditionally, in these models lungs were preinjured, *e.g.*, by lipopolysacharide.[4] Lipopolysaccharide is a component of the outer membrane of gram-negative bacteria. Upon binding to Toll-like receptor (TLR) 4,[5] lipopolysaccharide induces an intense inflammatory response leading to lung injury.[6–8] TLRs are essential in host defense against pathogens by virtue of their capacity to recognize various microbes and initiate an immune response.[9]

To date, 12 TLRs have been identified in mammals.[5] Recent studies indicate that TLRs recognize not only microbial products, but also endogenous ligands released from damaged tissue, the so-called danger signals.[10,11] Noninfectious lung injury induced by hemorrhage,[12–14] ischemia–reperfusion,[15] contusion,[16] hyperoxia, or administration of bleomycin[17] resulted in inflammatory reactions *via* TLR4 and/or TLR2 signaling, supporting the existence of endogenous ligands. Recently, endogenous ligands for TLR4 and TLR2 were identified, including heat shock protein 60,[18] fibronectin,[19] heparan sulfate proteoglycan,[20] biglycan,[21] hyaluronan,[22] and the myeloid-related proteins 8 and 14.[23]

The concept of TLR-mediated inflammation by endogenous ligands is interesting and may be involved in the development of VILI in the normal lung. Recently, we have shown that low-tidal-volume MV in healthy mice induces a transient inflammatory response without altering the structural integrity of the lung.[24] The aim of the current study was to test the hypothesis that TLR4 and/or TLR2 receptor signaling is involved in the inflammatory response induced by MV in the healthy lung. To test this hypothesis, wild-type (WT), TLR4 knockout (KO), and TLR2 KO mice were mechanically ventilated.

Materials and Methods

All experiments were approved by the Regional Animal Ethics Committee in Nijmegen, The Netherlands,

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and performed under the guidelines of the Dutch Council for Animal Care and the National Institutes of Health. The current study protocol was designed after a pilot study was performed.

Animals

All experiments were conducted in adult male C57BL6 mice (n = 64): TLR4 KO (C57BL6 background) mice (10–12 weeks) (n = 14; body weight 30 [± 3] g) and age-matched WT mice (n = 40; body weight 25 [± 3] g). In a separate set of experiments, TLR2 KO (C57BL6 background) mice (22–24 weeks) (n = 5; body weight 30 [± 2] g) and age-matched WT mice (n = 5; body weight 33 [± 2] g) were used. All KO mice were extensively backcrossed (at least 10 times) and were a gift from Professor Shizuo Akira, M.D., Ph.D. (Osaka University, Osaka, Japan). WT mice were purchased from Charles River (Sulzfeld, Germany).

Mechanical Ventilation in Mice

Mice were anesthetized with an intraperitoneal injection of a combination of ketamine, medetomidine, and atropine as described previously.[24] Mice in the unventilated groups were killed immediately after induction of anesthesia. Mechanically ventilated (MiniVent®; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) animals were orally intubated, ventilated for 4 h, and killed immediately thereafter. The following settings were used during controlled MV: tidal volume, 8 ml/kg body weight; frequency, 150/min; positive end-expiratory pressure, 4 cm H_2O; and fraction of inspired oxygen, 0.4. Rectal temperature was monitored continuously and maintained between 36.0°C and 37.5°C using a heating pad.

Experimental Design

The first set of experiments was performed to investigate the presence of endogenous ligands for TLR4 in bronchoalveolar lavage (BAL) fluid. BAL was performed after 4 h of MV (group V-WT, n = 8) or directly after induction of anesthesia (group C-WT, n = 8).

The second set of experiments was designed to investigate changes in pulmonary TLR4 and TLR2 expression in WT mice and to investigate changes in cytokine profile in WT and TLR4 KO mice after MV. Blood and lungs were harvested after 4 h of MV in WT (group V_{TLR4}WT, n = 8) and TLR4 KO mice (group V_{TLR4}KO, n = 8) or immediately after induction of anesthesia in WT (group C_{TLR4}WT, n = 8) and TLR4 KO mice (group C_{TLR4}KO, n = 6).

In the third set of experiments, the role of TLR2 in MV-induced inflammation was studied. Blood and lungs were harvested after 4 h of MV in WT (group V_{TLR2}WT, n = 5) and TLR2 KO mice (group V_{TLR2}KO, n = 5).

Lipopolysaccharide was measured in air, tubing, ventilator, and BAL fluid from unventilated animals (n = 4) by Limulus Amebocyte Lysate testing (Cambrex Bio Science, Walkersville, MD; detection limit, 0.06 U/ml) to rule out contamination with lipopolysaccharide in our experimental setting.

Tissue Harvesting

Blood was collected by exsanguination, centrifuged at 14,000 rpm (13,000*g*) (Eppendorf 5415 C; Nethler-Hinz GmbH, Hamburg, Germany) for 2 min, and plasma was stored at −80°C. Immediately after exsanguination, heart and lungs were carefully removed *en block via* midline sternotomy. The right upper and lower lobes were snap frozen in liquid nitrogen and stored at −80°C. The right middle lobe was fixed for light microscopy. The left lung was homogenized for measurement of cytokine concentrations.

Bronchoalveolar Lavage and Analysis for the Presence of Endogenous Ligands

For the BAL procedure, 250 μl sterile saline (0.9%) at 37°C was injected and gently aspirated through the endotracheal tube. Lavage fluid was snap frozen in liquid nitrogen and stored at −80°C. Human embryonal kidney (HEK) 293–TLR4 cell lines were purchased from InvivoGen (San Diego, CA) and cultured according to the manufacturer's guidelines. For stimulation, 5.10^4 cells/well were used in flat-bottom 96-wells plates. HEK293-TLR4 cells were exposed to 50 μl BAL fluid and added to 200 μl culture medium for 24 h. Subsequently, interleukin (IL) 8 was measured in culture supernatants using Luminex bead array technology. To verify the presence of endogenous TLR4 ligands, the BAL fluid was incubated with or without 10 μg/ml *Bartonella quintana* lipopolysaccharide as a specific TLR4 antagonist.[25,26] To rule out lipopolysaccharide contamination, we performed the HEK293-TLR4 assay in the presence and absence of polymyxin B (2 μg/ml) with BAL fluid from ventilated WT mice (n = 4).

mRNA Analysis of Lung Homogenates

For polymerase chain reaction (PCR) analysis of messenger RNA (mRNA), the right upper and lower lobes were homogenized with a micro-dismembrator II (Braun, Melsungen, Germany). Total RNA was extracted in 1 ml TRIzol reagent. Subsequently, 200 μl chloroform and 500 μl 2-propanol (Merck, Darmstadt, Germany) were used to separate the RNA from DNA and proteins. After a wash step with 75% ethanol (Merck), the dry RNA was dissolved in 30 μl water.

To obtain double-strain complementary DNA (cDNA), DNase-treated total RNA 1 μg with oligo dT primers (0.01 μg/ml) was reverse transcribed in a reverse transcriptase PCR with a total volume of 20 μl. Subsequently, quantitative PCR was performed using an ABI/PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCRs of glyceraldehyde-3-phosphate dehydrogenase, TLR2 and TLR4 were performed with Sybr Green PCR Master Mix (Applied Biosystems), 5 μl 1/20 diluted cDNA,

and primers in a final concentration of 300 nM in a total volume of 25 μl. The primers were developed using Primer Express® software (Applied Biosystems). Quantification of the PCR signals of each sample was performed by comparing the cycle threshold values, in duplicate, of the gene of interest with the cycle threshold values of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene. TLR2 and TLR4 mRNA expression was expressed as relative expression to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. All primers were validated according to the protocol, and standard curves were all within the tolerable range.

Cytokine Analysis in Lung Homogenates and Plasma

Tumor necrosis factor (TNF) α, IL-6, IL-10, and keratinocyte-derived chemokine (KC) in the homogenized left lung and in plasma were analyzed by enzyme-linked immunosorbent assay (ELISA) (for TNF-α, IL-6, and IL-10: CytoSet, BioSource, Camarillo, CA; for KC: ELISA Kit, R & D Systems, Minneapolis, MN). IL-1α and IL-1β were only assessed in lung homogenates, because of insufficient plasma, using specific radioimmunoassays, as described previously.[27] Lower detection limits were as follows: IL-1α and IL-1β, 40 pg/ml; TNF-α, 32 pg/ml; IL-6, 160 pg/ml; IL-10, 16 pg/ml; and KC, 160 pg/ml.

Histologic Examination

The right middle lung lobe was fixed in 4% buffered formalin solution overnight at room temperature, dehydrated, and embedded in paraplast (Amstelstad, Amsterdam, The Netherlands). Sections of 4-μm thickness were used. The enzyme activity of leukocytes was visualized by enzyme histochemistry using chloracetatesterase staining (Leder staining). Leukocytes were counted manually (20 fields per mouse), and after automated correction for air/tissue ratio, the number of leukocytes/μm^2 was calculated.

Statistical Analysis

Data are expressed as mean (SD) when distributed normally (body weight, endogenous ligands, relative mRNA expression, leukocyte counts) and expressed as median (range) otherwise (cytokine concentrations). Statistical analysis was performed with SAS (SAS Institute Inc., Cary, NC) statistical procedures. Because cytokine concentrations are not normally distributed, Kruskal-Wallis procedures were used, with *post hoc* comparisons of subgroups (Duncan). Data of a particular cytokine concentration variable were ranked, followed by analysis of variance in the General Linear Models procedure using the MEANS procedure with the Duncan option and Bonferroni correction for multiple comparisons. For analysis of endogenous ligands, relative mRNA expression, and leukocyte counts, analysis of variance was used on nonranked data with *post hoc* comparison of group means. The level of significance was set at $P < 0.05$.

Results

Presence of Endogenous Ligands for TLR4 and TLR4/TLR2 mRNA Expression after Mechanical Ventilation

Bronchoalveolar lavage fluid from unventilated WT mice resulted in increased production of IL-8 by HEK293-TLR4 cells compared with the medium. This could only be partly reduced by coincubation with a highly specific TLR4 antagonist, indicating that these cells generate IL-8 in a TLR4-independent manner as well (see Discussion, Role for Endogenous Ligands). BAL fluid from ventilated WT mice induced a significantly higher IL-8 response compared with BAL from unventilated WT mice, and coincubation with a highly specific TLR4 antagonist did significantly reduce the IL-8 response (fig. 1). From the experiments performed in the presence of TLR4 antagonist, it can be derived that the TLR4-dependent response was approximately fivefold higher in ventilated WT mice compared with unventilated WT mice.

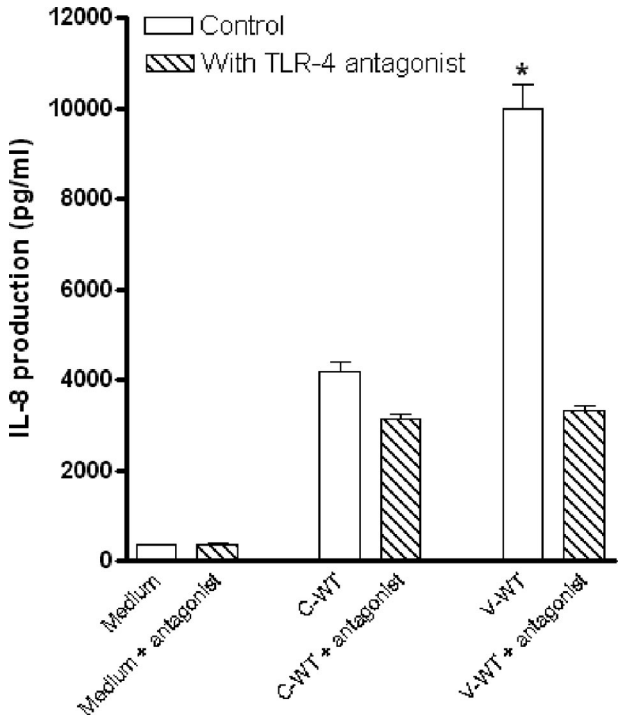

Fig. 1. Presence of endogenous ligands for Toll-like receptor 4 (TLR4) in bronchoalveolar lavage fluid after 4 h of mechanical ventilation. Interleukin-8 (IL-8) production by human embryonal kidney 293–TLR4 reporter cells after exposure to bronchoalveolar lavage fluid. After 4 h of mechanical ventilation, endogenous ligands for TLR4 were enhanced in bronchoalveolar lavage fluid of the ventilated wild-type mice (group V-WT) ($P < 0.0001$) compared with unventilated wild-type mice (group C-WT). Addition of a highly specific TLR4 antagonist decreased the IL-8 production to the level found in group C-WT. Data are expressed as mean (SD). * $P < 0.05$ compared with group C-WT.

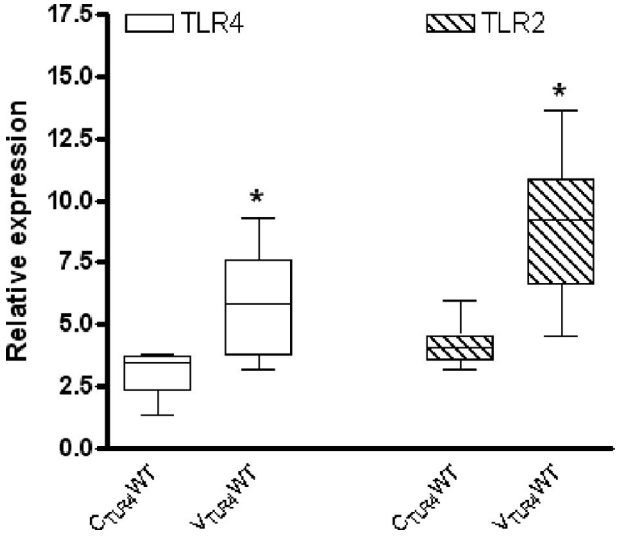

Fig. 2. Pulmonary relative messenger RNA (mRNA) expression of Toll-like receptor (TLR) 4 and TLR2 after 4 h of mechanical ventilation. Mechanical ventilation resulted in enhanced relative mRNA expression of TLR4 ($P = 0.008$) and TLR2 ($P = 0.0006$) in lung tissue of wild-type mice (group V_{TLR4}WT) compared with unventilated wild-type mice (group C_{TLR4}WT). Data are expressed as median with 25th and 75 percentiles (*box*) and range (*whiskers*). * $P < 0.05$ compared with group C-WT.

Mechanical ventilation increased the relative mRNA expression of TLR4 and TLR2 in lung tissue of ventilated WT mice as compared with unventilated WT mice (fig. 2).

Changes in Cytokine Concentration Induced by Mechanical Ventilation

Lung Homogenates. Mechanical ventilation in WT mice increased levels of IL-1α, IL-1β, and KC in lung homogenates (fig. 3). However, in TLR4 KO mice, MV did not increase IL-1β in lung homogenates, and the MV-induced increase in KC was less pronounced. Interestingly, TLR4 KO did not reduce the response of IL-1α to MV. Neither MV nor TLR4 KO affected TNF-α, IL-6, or IL-10 concentrations in lung homogenates.

Plasma. In WT mice, MV was associated with significantly increased levels of IL-6, KC, and TNF-α in plasma (fig. 4). In TLR4 KO mice, MV did not increase plasma levels of IL-6 and TNF-α. MV did result in increased levels of KC, although the response was less pronounced than in WT mice (fig. 4).

Mechanical ventilation in TLR2 KO mice did not result in statistically different cytokine levels in lung tissue homogenates and plasma compared with their age-matched WT ventilated animals (figs. 5 and 6).

Histologic Examination

In WT mice, MV increased the number of pulmonary leukocytes (table 1). In TLR4 KO mice, MV did not affect the number of leukocytes in the lung. Unventilated TLR4 KO mice expressed a higher number of pulmonary leukocytes when compared with unventilated WT mice.

The number of pulmonary leukocytes in ventilated TLR2 KO mice was not different from those in the age-matched ventilated WT mice (table 1).

No lipopolysaccharide contamination was detected in our experimental setting and BAL fluid from unventilated mice.

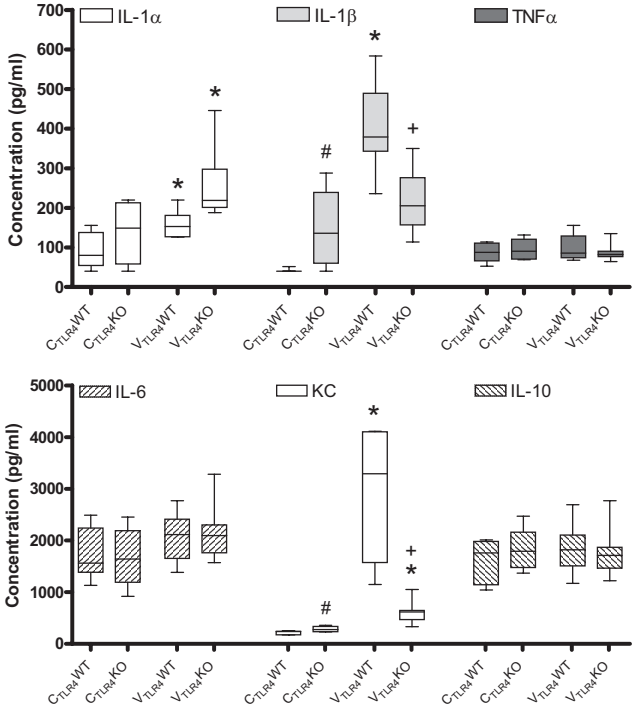

Fig. 3. Cytokine levels in lung homogenates. Levels of interleukin (IL) 6, keratinocyte-derived chemokine (KC), IL-10, IL-1α, IL-1β, and tumor necrosis factor (TNF) α in unventilated (C) and ventilated (V) wild-type (WT) and Toll-like receptor (TLR) 4 knockout (KO) mice. Mechanical ventilation in WT mice (group V_{TLR4}WT) increased KC ($P < 0.0001$), IL-1α ($P = 0.003$), and IL-1β ($P < 0.0001$) in lung tissue homogenates when compared with unventilated WT mice (group C_{TLR4}WT). In TLR4 KO mice (group V_{TLR4}KO), mechanical ventilation did not increase IL-1β in lung homogenates. Mechanical ventilation in TLR4 KO mice (group V_{TLR4}KO) did increase levels of KC ($P = 0.0003$) and IL-1α ($P = 0.003$) when compared with unventilated TLR4 KO mice (group C_{TLR4}KO). Ventilated TLR4 KO mice (group V_{TLR4}KO) showed significantly lower levels of KC ($P < 0.0001$) and IL-1β ($P = 0.0005$) in lung homogenates compared with ventilated WT mice (group V_{TLR4}WT). Between unventilated animals, the levels of KC ($P = 0.0069$) and IL-1β ($P = 0.048$) in lung homogenates were higher in the TLR4 KO mice (group C_{TLR4}KO) compared with WT mice (group C_{TLR4}WT). Data are expressed as median with 25th and 75 percentiles (*box*) and range (*whiskers*). * $P < 0.05$ compared with age-matched unventilated mice. + $P < 0.05$ compared with ventilated WT mice (V_{TLR4}WT). # Compared with unventilated WT mice (C_{TLR4}WT). − = lower detection limit.

Discussion

This study is the first to reveal several key findings regarding the role of TLR4 signaling in the development of MV-induced inflammation in healthy mice. Within the time frame studied, we found that low-tidal-volume MV resulted in increased expression of endogenous TLR4 ligands in BAL and enhanced mRNA levels for TLR4 in

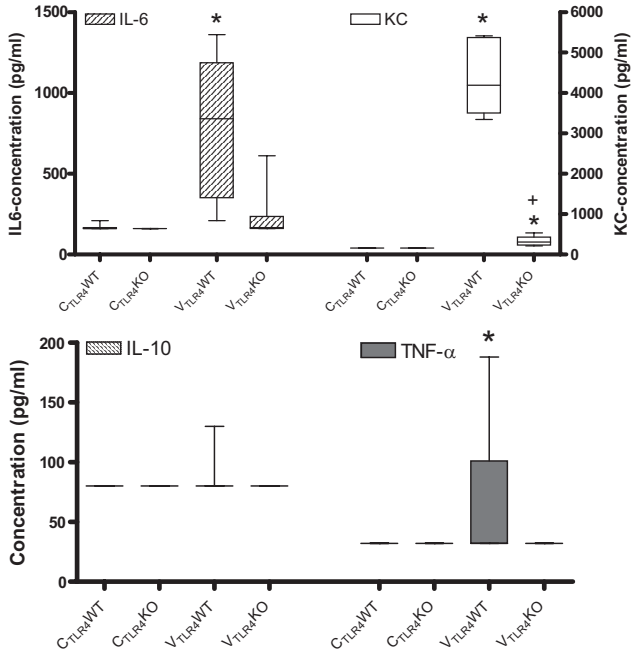

Fig. 4. Cytokine levels in plasma. Levels of interleukin (IL) 6, keratinocyte-derived chemokine (KC), IL-10, and tumor necrosis factor (TNF) α in unventilated (C) and ventilated (V) wild-type (WT) and Toll-like receptor (TLR) 4 knockout (KO) mice. Mechanical ventilation in WT mice (group V_{TLR4}WT) increased IL-6 ($P < 0.0001$), KC ($P < 0.0001$), and TNF-α ($P = 0.04$) in plasma when compared with unventilated WT mice (group C_{TLR4}WT). In TLR4 KO mice (group V_{TLR4}KO), MV did increase KC ($P < 0.0001$) in plasma when compared with unventilated TLR4 KO mice (group C_{TLR4}KO); however, this response was significantly lower ($P = 0.0002$) compared with the ventilated WT mice (group V_{TLR4}WT). No differences were found in the plasma levels of cytokines in the unventilated groups. The median of TNF-α in the V-WT group is not visible because this coincided with the 25th percentile. Data are expressed as median with 25th and 75 percentiles (*box*) and range (*whiskers*). * $P < 0.05$ compared with their own unventilated mice. + $P < 0.05$ compared with ventilated WT mice (V_{TLR4}WT). − = lower detection limit.

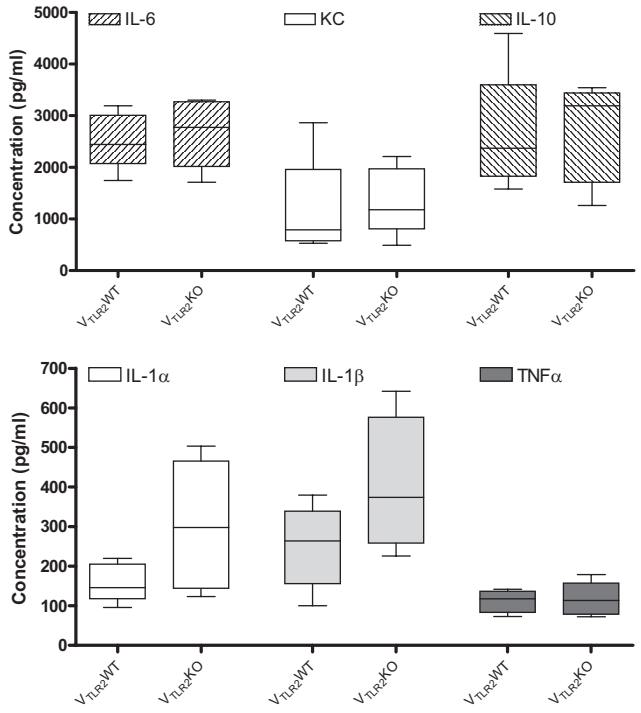

Fig. 5. Cytokine levels in lung homogenates. Levels of interleukin (IL) 6, keratinocyte-derived chemokine (KC), IL-10, IL-1α, IL-1β, and tumor necrosis factor (TNF) α in ventilated (V) wild-type (WT) and Toll-like receptor (TLR) 2 knockout (KO) mice. Mechanical ventilation in TLR2 KO mice did not result in statistically different cytokine levels in lung tissue homogenates compared with age-matched WT ventilated animals.

lung homogenates. In addition, short-term MV resulted in the increase of inflammatory cytokines in the lung and in a systemic inflammatory response in plasma. MV-induced inflammation seemed at least partially TLR4 dependent.

Mechanical Ventilation–induced Inflammatory Response

The current study confirms previous findings regarding changes in cytokine profile, induced by MV, in lungs and plasma of healthy mice.[24] Little has been published about the effects of low-tidal-volume MV on cytokines in lungs and plasma of healthy animals. In accord with our results are an increase of IL-1β mRNA expression in lung tissue,[28] an increase of TNF-α concentration in plasma,[29] and the lack of response of IL-6 and TNF-α in lung homogenates[30,31] after low-tidal-volume MV. The finding of an increased response of IL-6 in plasma but not in lung homogenate can be a time-dependent effect, as shown in a previous study.[24] IL-6 levels peaked in lung homogenates after 2 h of MV and decreased thereafter. However, in plasma, the highest value was reached after 4 h. In contrast to our results, Dhanireddy *et al.*[30] found no increase of KC in lung homogenate and plasma after low-tidal-volume MV, and Takenaka *et al.*[32] did not find an increase of IL-6 or TNF-α in plasma after low-tidal-volume MV. These different results might be partly explained by differences in ventilator settings and experimental setup in these studies.

The current study demonstrates that TLR4 plays a role in development of the inflammatory response after MV in healthy lungs. MV in TLR4 KO did not increase IL-1β in lung homogenates, and increases of KC in lung homogenates were less pronounced compared with WT mice. KC and IL-1β are found to play an early and central role in lung injury induced by hemorrhage.[14,33] IL-1β also is involved in lung injury induced by liver injury.[34] Recently, IL-1α rather than IL-1β was identified as a key mediator in sterile inflammation in response to injured cells.[35] Our results demonstrate that both IL-1α and IL-1β are involved in the inflammatory response after MV, but only the increase in IL-1β was found to be TLR4 dependent. KC is a chemoattractant but also has a direct cytotoxic effect.[36] Jiang *et al.*[17] found KC to be produced by pulmonary epithelial cells in a TLR-dependent manner; however, this was in direct response to bleomycin. More recently, functional TLR4 expression was

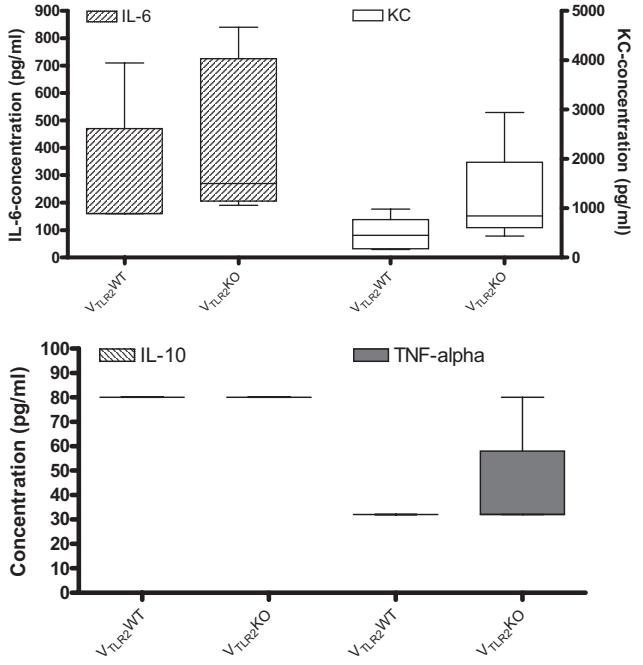

Fig. 6. Cytokine levels in plasma. Levels of interleukin (IL) 6, keratinocyte-derived chemokine (KC), IL-10, and tumor necrosis factor (TNF) α in ventilated (V) wild-type (WT) and Toll-like receptor (TLR2) knockout (KO) mice. Mechanical ventilation in TLR2 KO mice did not result in statistically different cytokine levels in plasma compared with age-matched WT ventilated animals.

found to be critical in the KC increase after hemorrhage.[14] Our results show that KC production in response to MV in healthy lungs seems to be at least partly TLR4 dependent.

The clinical relevance of the cytokine up-regulation by MV is the resulting proinflammatory state. This makes the host more vulnerable to a possible "second hit" (*e.g.*, major surgery).[37] *Vice versa*, MV itself can be the second hit where an already comprised host exists (*e.g.*, MV in the critically ill patient).[38–40] Recently, this "two-hit" hypothesis was linked to TLR4 reactivity.[41] Inhibition of TLR4 may be an effective strategy to prevent or reduce MV-induced pulmonary and systemic inflammation.

Table 1. Leukocyte Counts

Group	Leukocytes $\times 10^{-4}/\mu m^2$, Mean (SD)	Δ due to MV	*P* Value
C_{TLR4}WT	1.9 (2.6)		
V_{TLR4}WT	8.7 (5.1)	6.8	<0.001
C_{TLR4}KO	7.2 (5.1)		<0.001
V_{TLR4}KO	6.1 (4.1)	−1.1	NS
V_{TLR2}WT	3.0 (2.1)		NS
V_{TLR2}KO	4.0 (3.2)	1.0	NS

Values are mean (SD).

Toll-like receptor (TLR) 4: *P* values compared with the unventilated wild-type animals (C_{TLR4}WT group). TLR2: *P* values compared with the ventilated wild-type animals (V_{TLR2}WT group).

C = unventilated; KO = knockout; MV = mechanical ventilation; NS = not significant; V = ventilated; WT = wild type.

Role for Leukocytes

The activation and attraction of leukocytes is an important feature in VILI.[42] Leukocytes are thought to be a principal culprit in sterile inflammation causing tissue damage.[35] KC is a major attractant for leukocytes.[43] In a previous study,[24] we have shown that 4 h of MV in healthy mice preserves alveolar integrity but induces a pulmonary leukocyte influx after the increase of KC in the lung. In the current study, this pulmonary leukocyte influx is confirmed; however, in TLR4 KO mice, MV did not affect the number of pulmonary leukocytes. Recently, Frink *et al.*[14] published a hemorrhage model in which the pulmonary leukocyte influx after hemorrhage was also found to be TLR4 dependent.

In the current study, the number of pulmonary leukocytes was significantly higher in unventilated TLR4 KO mice compared with unventilated WT mice. TLR4 KO mice also showed increased lung homogenate levels of KC and IL-1β compared with WT mice at baseline. Frink *et al.*[14] did not find differences in leukocyte content before hemorrhage; however, in that study, TLR4 mutant mice were used, which have a different background (C3H/HeJ). Because in these mice the defect is not complete, partial responses of TLR4 could be preserved. In the current study, TLR4 KO mice were used. The absence of TLR4 increases their sensitivity to infections.[44] However, before the experiments, these mice did not exhibit clinical signs of discomfort or chronic infection (*i.e.*, abnormal behavior, weight loss). The higher number of pulmonary leukocytes in unventilated TLR4 KO mice is most likely the result of the increased lung homogenate levels of KC.

Role for Endogenous Ligands

Recently, a number of studies suggested that endogenous ligands exists that can bind to TLR4 and induce an inflammatory response.[18–23,45] By using HEK293-TLR4 reporter cells, which produce IL-8 in the presence of TLR4 agonists, we sought for endogenous TLR4 ligands in BAL fluid sampled from unventilated and ventilated WT mice. The IL-8 response in BAL fluid from ventilated mice was remarkably enhanced compared with unventilated mice, and the addition of a highly specific TLR4 antagonist decreased the induction of IL-8, indicating the presence of endogenous TLR4 ligands in BAL fluid from ventilated mice. HEK293-TLR4 cells incubated with BAL fluid from unventilated mice also induced an IL-8 response. This interesting observation could suggest that endogenous ligands for TLR4 are present in the normal (unventilated) lung and released in the air spaces. This might be explained by direct lung injury, induced by performing the BAL procedure as lung lavage is potentially injurious, especially when large volumes are used.[46] We used small volumes for our BAL procedure, but nevertheless cannot exclude that this induces some degree of lung injury and subsequent release of endog-

enous ligands. The fact that the IL-8 response could only partly be prevented by coincubation with a TLR4 antagonist suggests that other non–TLR4-mediated factors play a role. We found that the HEK293-TLR4 cells can also respond to recombinant mouse TNF-α *in vitro* (personal communication, L.A.J. and M.G.N., August 2007). Because small concentrations of TNF-α can be present in BAL fluid of healthy mice,[47] this most likely caused the IL-8 response in the unventilated mice. Most importantly, the IL-8 response was much higher in BAL fluid from ventilated WT mice compared with unventilated WT mice, and coincubation with a TLR4 antagonist prevented this response. This indicates increased concentrations of endogenous TLR4 ligands after MV.

In a noninfectious lung injury model, Jiang *et al.*[17] found hyaluronan, which is released from the extracellular matrix, to act as an endogenous ligand and initiate an inflammatory response in a TLR4-dependent manner. Hillman *et al.*[48] showed that heat shock protein 70, serum amyloid A-3, and TLR2 and TLR4 mRNA were increased in BAL fluid after brief, large-tidal-volume ventilation in fetal sheep. Because the identification of specific endogenous ligands for TLR4 in healthy lungs after MV was beyond the scope of the current study, further investigation is needed.

The current data suggest that TLR2 does not play a role in the MV-induced inflammatory response after short-term MV. Although the relative expression of TLR2 in WT mice was up-regulated, the ventilated TLR2 KO mice did not show differences in cytokine levels compared with age-matched ventilated WT mice after 4 h of MV. This is in accord with preliminary findings in TRIF-deficient mice in which the inflammatory response to MV was clearly suppressed (manuscript in preparation). Because TLR2 signaling is MyD88 dependent and TLR4 is MyD88 or TRIF dependent,[49] it is likely that the TLR4–TRIF pathway is the dominant pathway for the inflammatory response to MV. However, further studies are needed to establish the role of TLR2 in MV-induced inflammation, because we cannot exclude that TLR2 plays a role in the inflammatory response beyond 4 h of MV.

Factors other than MV, possibly affecting TLR4 and 2 signaling, were carefully avoided. Contamination with lipopolysaccharide is suggested to be a confounding factor in many studies.[50] We therefore excluded lipopolysaccharide contamination during the experiments. The possibility of triggering an inflammatory response by invasive procedures (*i.e.*, insertion of an intraarterial line) and subsequent bacterial contamination[37] was eliminated by performing experiments in noninvasively monitored animals. Previously, cardiorespiratory stability in invasively monitored animals has been documented.[24] The possible immune-modulating effects of anesthetics have been studied extensively.[51] Ketamine, for example, is known to have an inhibitory effect on lipopolysaccharide-induced cytokine production,[52–55] possibly by suppressing TLR4 expression.[56] Recently, it has been demonstrated that ketamine alone (without lipopolysaccharide) also attenuated cytokine production in humans in the direct postoperative period after elective abdominal surgery.[57] In the current study, all animals received ketamine. Ideally, an additional control group of spontaneously breathing animals during ketamine, medetomidine, and atropine anesthesia is needed. However, this will result in hypoventilation with severe respiratory acidosis and hemodynamic instability.

Interestingly, a phase III trial is being conducted to investigate the effects of TLR4 inhibition in patients with severe sepsis.‡‡ The current study supports a role for TLR4 in the inflammatory reaction after MV in healthy lungs. Increasing the understanding of the innate immune response to MV and the contribution of MV to the "multiple-hit" concept may lead to future treatment advances in VILI, in which TLR4 may serve as a therapeutic target.

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