

# Mechanical Ventilation Induces a Toll/Interleukin-1 Receptor Domain-containing Adapter-inducing Interferon $\beta$ -dependent Inflammatory Response in Healthy Mice

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**Background:** Mechanical ventilation (MV) can induce lung injury. Proinflammatory cytokines have been shown to play an important role in the development of ventilator-induced lung injury. Previously, the authors have shown a role for Toll-like receptor 4 signaling. The current study aims to investigate the role of Toll/interleukin-1 receptor domain-containing adapter-inducing interferon- $\beta$  (TRIF), a protein downstream of Toll-like receptors, in the development of the inflammatory response after MV in healthy mice.

**Methods:** Wild-type C57BL/6 and TRIF mutant mice were mechanically ventilated for 4 h. Lung tissue and plasma was used to investigate changes in cytokine profile, leukocyte influx, and nuclear factor- $\kappa$ B activity. In addition, experiments were performed to assess the role of TRIF in changes in cardiopulmonary physiology after MV.

**Results:** MV significantly increased messenger RNA expression of interleukin (IL)-1 $\beta$  in wild-type mice, but not in TRIF mutant mice. In lung homogenates, MV increased levels of IL-1 $\alpha$ , IL-1 $\beta$ , and keratinocyte-derived chemokine in wild-type mice. In contrast, in TRIF mutant mice, only a minor increase in IL-1 $\beta$  and keratinocyte-derived chemokine was found after MV. Nuclear factor- $\kappa$ B activity after MV was significantly lower in TRIF mutant mice compared with wild-type mice. In plasma, MV increased levels of IL-6 and keratinocyte-derived chemokine. In TRIF mutant mice, no increase of IL-6 was found after MV, and the increase in keratinocyte-derived chemokine appeared less pronounced. TRIF deletion did not affect cardiopulmonary physiology after MV.

**Conclusions:** The current study supports a prominent role for TRIF in the development of the pulmonary and systemic inflammatory response after MV.

MECHANICAL ventilation (MV) is a life-saving therapy in patients with acute respiratory failure. However, studies have shown that MV can aggravate lung injury and even induce lung injury in the healthy lung.<sup>1</sup> The underlying mechanisms are incompletely understood but a large body of literature indicates that proinflammatory cytokines play an important role in the development of ventilator-induced lung injury.<sup>2</sup>

Toll-like receptors (TLR) are increasingly being recognized as key mediators in inflammation because of their capacity to detect various microbes and initiate an immune response.<sup>3</sup> In addition, TLRs have been shown to recognize endogenous ligands released from damaged tissue, the so-called danger signals.<sup>4</sup> TLR4 is found to play a role in acute lung injury.<sup>5</sup> Endogenous ligands activate TLR4, resulting in an inflammatory response, which is associated with lung injury.<sup>5</sup> MV using clinically relevant tidal volume may limit,<sup>6</sup> but does not prevent, pulmonary inflammation.<sup>7</sup> Recently, we have shown that in healthy mice, MV with clinically relevant tidal volume induces a transient inflammatory response, partly in a TLR4-dependent fashion.<sup>8</sup>

Downstream signaling of TLRs is complex. MyD88 is a universal adaptor protein used by most TLRs.<sup>9,10</sup> In TLR4 a second pathway is involved. This pathway is mediated by Toll/interleukin-1 receptor domain-containing adapter-inducing interferon- $\beta$ , a protein called TRIF.<sup>9,10</sup> Activation of TRIF causes delayed translocation of nuclear factor (NF)- $\kappa$ B into the nucleus and transcription of proinflammatory genes inducing cytokine production.<sup>11</sup> This TRIF pathway has recently been identified as the key signaling pathway in acid-induced lung injury and in hyperoxia-induced lung injury.<sup>5,12</sup> For instance, in TRIF-deleted animals, acid-induced impairment in lung function and development of lung edema was less pronounced.<sup>5</sup> Whether TRIF is involved in the inflammatory response after MV is currently unknown. This is of interest because MV is more clinically relevant than acid- or hyperoxia-induced lung injury.

Accordingly, the aim of the current study was to determine the role of TRIF in the development of the inflammatory response and lung function impairment after MV in healthy mice. We hypothesized that MV-induced inflammation involves a TRIF-dependent path-

This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 9A.

This article is accompanied by an Editorial View. Please see: Curley GF, Kevin LG, Laffey JG: Mechanical ventilation: Taking its toll on the lung. ANESTHESIOLOGY 2009; 111:701–3.

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Received from the Departments of Anesthesiology, Intensive Care, Internal Medicine and the Nijmegen Institute for Infection, Inflammation and Immunity (N4i), Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. Submitted for publication February 2, 2009. Accepted for publication June 19, 2009. Supported by a grant from the Jac. H. de Jong Foundation (Nieuwegein, The Netherlands). Dr. Netea was supported by a grant (Vidi stage) from the Netherlands Organization for Scientific Research, The Hague, The Netherlands. Dr. Halbertsma's current position: Pediatric Intensivist, Maxima Medical Centre, Veldhoven, The Netherlands.

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way. To test this hypothesis wild-type and TRIF mutant mice were mechanically ventilated for 4 h.

## Materials and Methods

All experiments were approved by the Regional Animal Ethics Committee (Nijmegen, The Netherlands) and performed under the guidelines of the Dutch Council for Animal Care and the National Institutes of Health.

### Animals

To test the role of TRIF in our experimental model, studies were conducted by using TRIF mutant mice (C57BL6 background; 10–12 weeks;  $25 \pm 4$  g;  $n = 31$ ). Age-matched wild-type (WT) mice (C57BL6 background;  $26 \pm 3$  g;  $n = 31$ ) were used as controls. TRIF mutant mice were a kind gift from Bruce Beutler M.D., Ph.D. (Professor, Department of Immunology, The Scripps Research Institute, La Jolla, California), who identified and cloned the TRIF gene (called *Lps2*).<sup>13</sup> These TRIF mutant mice have a distal frameshift error in the *Lps2* gene, which has an equivalent gene in humans.<sup>13</sup> WT mice were purchased from Charles River (Sulzfeld, Germany).

### MV in Mice

Mice were anesthetized with an intraperitoneal injection of a combination of ketamine, medetomidine, and atropine (KMA): 7.5  $\mu$ l/g of body weight of induction KMA mix (consisting of 1.26 ml of ketamine, 100 mg/ml; 0.2 ml of medetomidine, 1 mg/ml; 1 ml of atropine, 0.5 mg/ml; and 5 ml of NaCl, 0.9%) was given just before intubation. Animals were orally intubated, mechanically ventilated (Mini-Vent<sup>®</sup>; Hugo Sachs Elektronik-Harvard apparatus, March-Hugstetten, Germany) for 4 h and killed immediately thereafter. The following settings were used during controlled MV: tidal volume 8 ml/kg body weight and frequency 150/min, which is well within the range of measured tidal volume and respiratory rate during spontaneous ventilation in C57BL6 mice.<sup>14</sup> All animals received 4 cm H<sub>2</sub>O positive end-expiratory pressure, and fraction of inspired oxygen was set to 0.4.

To maintain anesthesia, 5.0  $\mu$ l/g of body weight boluses of maintenance KMA mix (consisting of 0.72 ml of ketamine, 100 mg/ml; 0.08 ml of medetomidine, 1 mg/ml; 0.3 ml of atropine, 0.5 mg/ml; and 18.9 ml of NaCl, 0.9%) were given, *via* an intraperitoneally placed catheter, every 30 min. Rectal temperature was monitored continuously and maintained between 36.0°C and 37.5°C by using a heating pad.

### Experimental Design

The first set of experiments was performed to investigate the role of TRIF in MV-induced changes in cytokine profile, leukocyte influx, and NF- $\kappa$ B activity. Blood and lungs were harvested after 4 h of MV in WT mice (group

V-WT,  $n = 8$ ) and TRIF mutant mice (group V-TRIF,  $n = 8$ ) or immediately after induction of anesthesia in WT (group C-WT,  $n = 8$ ) and TRIF mutant mice (group C-TRIF,  $n = 8$ ).

The second set of experiments was designed to assess changes in cardiopulmonary physiology. Continuous intraarterial carotid blood pressure was measured in mechanically ventilated WT mice (group V-WT,  $n = 15$ ) and TRIF mutant mice (group V-TRIF,  $n = 15$ ). Arterial blood gas analysis (iSTAT; Abbott, Birmingham, United Kingdom) was performed after 4 h of MV. We did not include these animals for the cytokine or histopathologic analysis to avoid possible interference with cytokine response resulting from instrumentation (*i.e.*, insertion of arterial catheter) and subsequent bacterial contamination. One TRIF mutant mouse died during instrumentation.

Lipopolysaccharide was measured in experimental circuit by Limulus Amebocyte Lysate testing (Cambrex Bio Science, Walkersville, MD; detection limit 0.06 IU/ml) to rule out contamination with lipopolysaccharide in our experimental setting. Indeed, no lipopolysaccharide could be detected in air, tubing, or ventilator (data not shown).

### Tissue Harvesting

Blood was collected by exsanguination, centrifuged at 14,000 rpm (13,000g) (Eppendorf 5415 C; Nethler-Hinz GmbH, Hamburg, Germany) for 2 min, and plasma was stored at  $-80^{\circ}\text{C}$  for later biochemical analysis. 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  $-80^{\circ}\text{C}$ . The right middle lobe was fixed for light microscopy as described previously.<sup>7</sup> The left lung was homogenized for the measurement of cytokines.

### Biochemical Analysis

Tumor necrosis factor- $\alpha$ , interleukin (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 tumor necrosis factor- $\alpha$ , IL-6, and IL-10: CytoSet, BioSource, CA; for KC: ELISA-Kit, R&D Systems, Minneapolis, MN). Because of insufficient amount of plasma, IL-1 $\alpha$  and IL-1 $\beta$  could only be assessed in lung homogenate using specific radioimmunoassays, as described previously.<sup>15</sup> Lower detection limits: 40 pg/ml for IL-1 $\alpha$  and IL-1 $\beta$ ; 32 pg/ml for tumor necrosis factor- $\alpha$ ; 160 pg/ml for IL-6; 80 pg/ml for IL-10; 160 pg/ml for KC.

NF- $\kappa$ B's DNA-binding activity was determined by electrophoretic mobility shift assay. Nuclear proteins for electrophoretic mobility shift assay were isolated from liquid nitrogen frozen lungs. Lung tissue (20 mg) was homogenized in 5 ml of ice-cold buffer (HEPES 10 mM, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl and 0.6% Nonidet-P40, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulphonylfluo-

ride [Sigma-Aldrich, Zwijndrecht, The Netherlands]) and centrifuged for 30 s at 350g (4°C). The supernatant was then incubated on ice for 5 min and centrifuged for 5 min at 6,000g (4°C). The pellet was resuspended in 200  $\mu$ l of buffer (10 mM HEPES, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 1.2 M sucrose, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulphonylfluoride [Sigma-Aldrich]) and centrifuged for 30 min at 13,000g (4°C). The pellet was then resuspended in 66  $\mu$ l of buffer (HEPES 20 mM, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 420 mM NaCl, 25% glycerol, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulphonylfluoride, 2.0 mM benzamidine, and 5.0  $\mu$ g/ml leupeptine [Sigma-Aldrich]), incubated on ice for 20 min, and centrifuged for 2 min at 6000g (4°C). The supernatants were used as nuclear extracts. Protein concentrations in these extracts were determined by using the Bio-Rad protein assay (Bio-Rad, Veenendaal, The Netherlands).

Double stranded oligonucleotides containing an NF- $\kappa$ B consensus binding site (5'-AGTTGAGGGGACTTCCAGGC-3') were radiolabeled with <sup>32</sup>[P]-adenosine triphosphate by using T4 polynucleotide kinase (Promega, Madison, WI). Labeled NF- $\kappa$ B oligonucleotides were mixed with nuclear extracts (10  $\mu$ g) and incubated at room temperature for 20 min. Then, these samples were loaded on a 4% polyacrylamide gel. After electrophoresis for 45 min, the gel was dried and exposed for 24 h to an X-ray film. The bands on the film were quantified by using optical densitometry software (GeneTools, Syngene, Cambridge, United Kingdom).

#### Messenger RNA Analysis of Lung Homogenates

For Polymerase Chain Reaction (PCR) analysis of messenger RNA, 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  $\mu$ l chloroform and 500  $\mu$ l of 2-propanol (Merck) were used to separate the RNA from DNA and proteins. After a wash step with 75% ethanol (Merck, Darmstadt, Germany), the dry RNA was dissolved in 30  $\mu$ l of water. To obtain double strain complementary DNA, DNase-treated total RNA 1  $\mu$ g with oligo dT primers (0.01  $\mu$ g/ml) was reverse transcribed in a real-time PCR with a total volume of 20  $\mu$ l. Quantitative PCR was subsequently performed by using ABI/PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCRs of glyceraldehyde-3-phosphate dehydrogenase, IL-1 $\beta$  were performed with Sybr Green PCR Master Mix (Applied Biosystems), 5  $\mu$ l of 1/20 diluted complementary DNA and primers in a final concentration of 300 nM in a total volume of 25  $\mu$ l. The primers were developed by using Primer Express<sup>®</sup> software (Applied Biosystems). Quantification of the PCR signals of each sample was performed by comparing the cycle threshold values (Ct), in duplicate, of the gene of interest with the Ct values of the glyceraldehyde-3-phosphate dehydroge-

nase housekeeping gene. IL-1 $\beta$  messenger RNA 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.

#### Leukocytes

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  $\mu$ m-thicknesses were used. The enzyme activity of leukocytes was visualized by enzyme histochemistry by using chloracetate-sterase staining (Leder staining). Leukocytes were counted manually (20 fields per mouse), and after automated correction for air/tissue ratio, the number of leukocytes/ $\mu$ m<sup>2</sup> was calculated.

#### Statistical Analysis

Data are expressed as mean  $\pm$  SD when distributed normally (leukocyte counts, relative messenger RNA expression) and expressed as Box (median, 25th, 75th percentile) and Whiskers (range) otherwise (cytokine concentrations). Statistical analysis was performed with SAS (SAS Institute Inc. Cary NC, version 8.02) statistical procedures. Cytokine concentrations are not normally distributed; therefore, Kruskal Wallis procedures were used with *post hoc* comparisons of subgroups (Duncan). For the analysis of normally distributed data (leukocyte counts and relative messenger RNA expression), ANOVA was used with *post hoc* comparison of group means (Duncan). Two-tailed *P* values are reported, and the level of significance was set to *P* < 0.05.

## Results

#### Inflammatory Response after MV: Role for TRIF

**Lungs.** In WT mice, MV increased levels of IL-1 $\alpha$ , IL-1 $\beta$ , and KC (fig. 1), which is in line with previous data from our lab.<sup>7,8</sup> In contrast, MV in TRIF mutant mice elicited only a minor increase in IL-1 $\beta$  and KC. Levels of IL-1 $\alpha$  after MV were not different between WT and TRIF mutant mice (fig. 1). In addition, MV increased the messenger RNA expression of IL-1 $\beta$  in WT mice, but not in TRIF mutant mice after MV, indicating that TRIF affects IL-1 $\beta$  transcription (fig. 2).

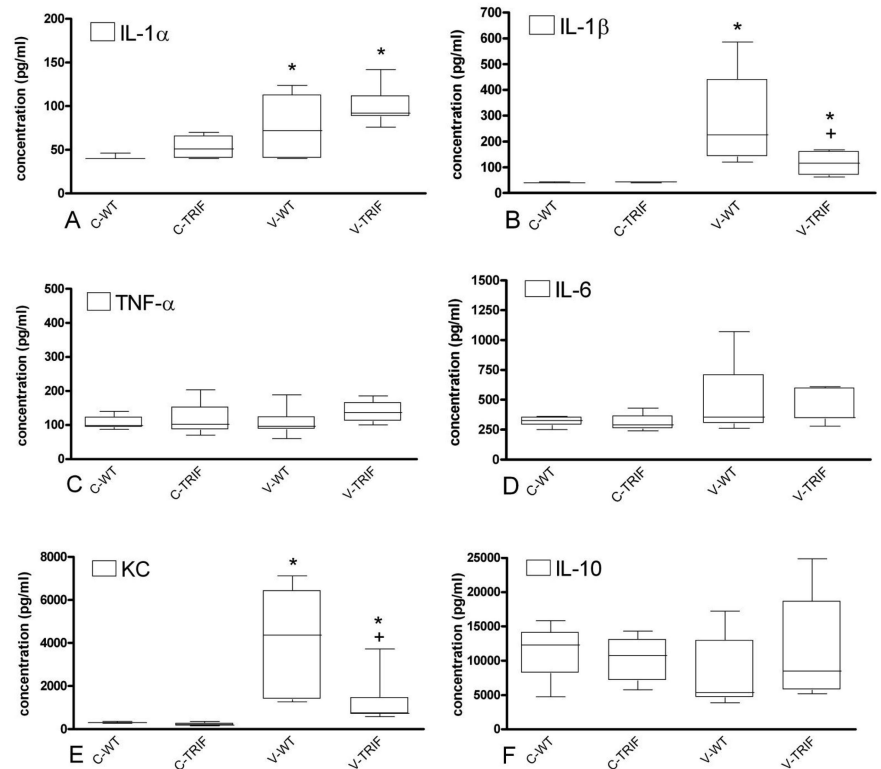
NF- $\kappa$ B activity after MV was significantly lower in TRIF mutant mice compared with WT mice (fig. 3). MV resulted in a pulmonary leukocyte influx in both WT and TRIF mutant mice (fig. 4).

**Plasma.** In WT mice, MV increased levels of IL-6 and KC in plasma (fig. 5). In TRIF mutant mice, no increase of IL-6 was found after MV, and the increase in KC appeared less pronounced compared with WT mice.



## Cytokine levels in lung homogenates

Fig. 1. Cytokine levels in lung homogenates. Levels of interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , tumor necrosis factor- $\alpha$ , IL-6, keratinocyte-derived chemokine (KC), and IL-10 in unventilated (C) and ventilated (V) wild-type (WT) and Toll/interleukin-1 receptor domain-containing adapter-inducing interferon- $\beta$  (TRIF) mutant mice (panels A–F). MV in WT mice (group V-WT) increased IL-1 $\alpha$  ( $P = 0.03$ ), IL-1 $\beta$  ( $P < 0.001$ ), and KC ( $P < 0.001$ ) in lung tissue homogenates when compared with unventilated WT mice (group C-WT). MV in TRIF mutant mice (group V-TRIF) increased levels of IL-1 $\alpha$  ( $P = 0.01$ ), IL-1 $\beta$  ( $P < 0.001$ ), and KC ( $P < 0.001$ ) in lung tissue homogenates when compared with unventilated TRIF mutant mice (group C-TRIF). Ventilated TRIF mutant mice (V-TRIF) showed significantly lower levels of IL-1 $\beta$  ( $P = 0.01$ ) and KC ( $P = 0.01$ ) in lung homogenates after MV compared with ventilated WT mice (group V-WT).  $n = 8$  for all groups. Data are expressed as Box (median, 25th, 75th percentile) and Whiskers (range). \* =  $P < 0.05$  compared with unventilated mice; + =  $P < 0.05$  compared with ventilated WT mice (V-WT). – = lower detection limit.



### Cardiopulmonary Physiology

The animals with an intraarterial cannula exhibited stable hemodynamic variables throughout the experiments. Mean arterial pressure was within normal limits

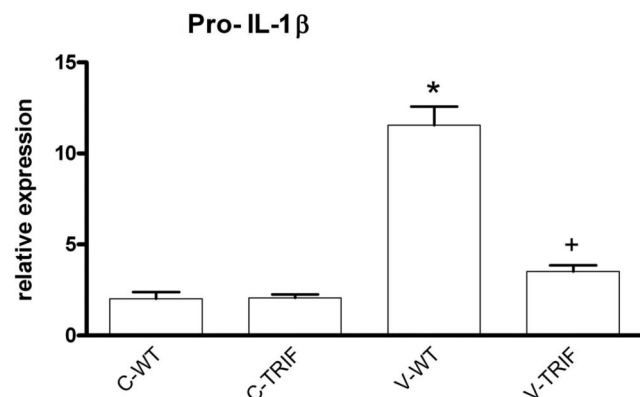


Fig. 2. Pro-IL-1 $\beta$  levels in lung tissue. Prointerleukin (IL)-1 $\beta$  messenger RNA levels in unventilated (C) and ventilated (V) wild type (WT) and Toll/interleukin-1 receptor domain-containing adapter-inducing interferon- $\beta$  (TRIF) mutant mice. Mechanical Ventilation in WT mice (group V-WT) increased pro-IL-1 $\beta$  messenger RNA ( $P < 0.001$ ) in lung tissue when compared with unventilated WT mice (group C-WT). In TRIF mutant mice, no increase in messenger RNA expression of IL-1 $\beta$  was found after mechanical ventilation. Ventilated TRIF mutant mice (V-TRIF) showed significantly lower levels of pro-IL-1 $\beta$  ( $P < 0.001$ ) in lung tissue after mechanical ventilation compared with ventilated WT mice (group V-WT).  $n = 8$  for all groups. Data are expressed as mean (SD). \* =  $P < 0.05$  compared with unventilated mice; + =  $P < 0.05$  compared with ventilated WT mice (V-WT).

and remained above 65 mmHg in all animals, which was in line with previous data from our lab.<sup>7</sup> Blood gas analysis showed pH  $7.30 \pm 0.07$  in WT mice and pH  $7.32 \pm 0.08$  in TRIF mutant mice, arterial oxygen tension of  $146 \pm 23$  mmHg in WT mice and  $157 \pm 20$  mmHg in TRIF mutant mice, arterial carbon dioxide tension of  $41 \pm 6$  mmHg in WT mice and  $39 \pm 5$  mmHg in TRIF

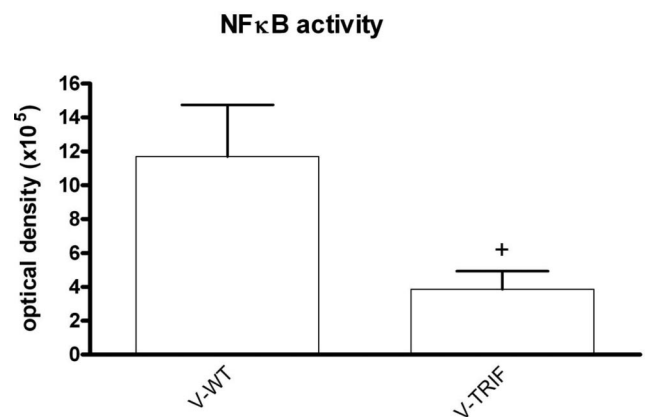
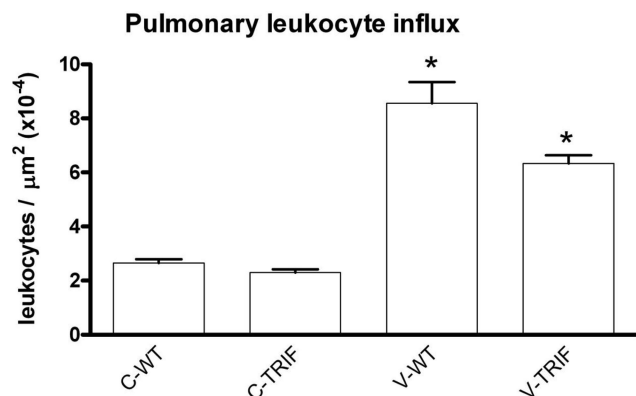


Fig. 3. Nuclear factor (NF)- $\kappa$ B activity in lung tissue. NF- $\kappa$ B activity in lung tissue of ventilated (V) wild type (WT) and Toll/interleukin-1 receptor domain-containing adapter-inducing interferon- $\beta$  (TRIF) mutant mice. In ventilated TRIF mutant mice (group V-TRIF), nuclear factor- $\kappa$ B activity was significantly lower ( $P < 0.001$ ) compared with ventilated WT mice (group V-WT).  $n = 8$  for all groups. Data are expressed as optical densities from electrophoretic mobility shift assay analysis. + =  $P < 0.05$  compared with ventilated WT mice (V-WT).



**Fig. 4. Leukocyte counts.** Pulmonary leukocyte counts in unventilated (C) and ventilated (V) wild type (WT) and Toll/interleukin-1 receptor domain-containing adapter-inducing interferon- $\beta$  (TRIF) mutant mice. Mechanical ventilation resulted in a pulmonary leukocyte influx in WT mice (group V-WT) ( $P < 0.001$ ) compared with unventilated WT mice (group C-WT). In TRIF mutant mice (group V-TRIF), mechanical ventilation also increased pulmonary leukocyte counts ( $P < 0.001$ ) compared with unventilated WT mice (group C-WT). The pulmonary leukocyte number in ventilated TRIF mutant mice appeared lower compared with ventilated WT mice (group V-WT); however, this did not reach statistical significance ( $P = 0.07$ ). \* =  $P < 0.05$  compared with unventilated mice.  $n = 8$  for all groups.

mutant mice, bicarbonate of  $17.4 \pm 3$  mmol/l in WT mice and  $19.1 \pm 3.1$  mmol/l in TRIF mutant mice. Blood gas values,  $\text{PaO}_2/\text{FiO}_2$  ratios, and alveolar-arterial oxygen gradients were not different between WT and TRIF mutant mice after MV.

## Discussion

The current study confirms earlier observations from our laboratory<sup>7,8</sup> and others<sup>1,6,16</sup> that MV using clinical relevant ventilator settings results in a pulmonary and systemic in-

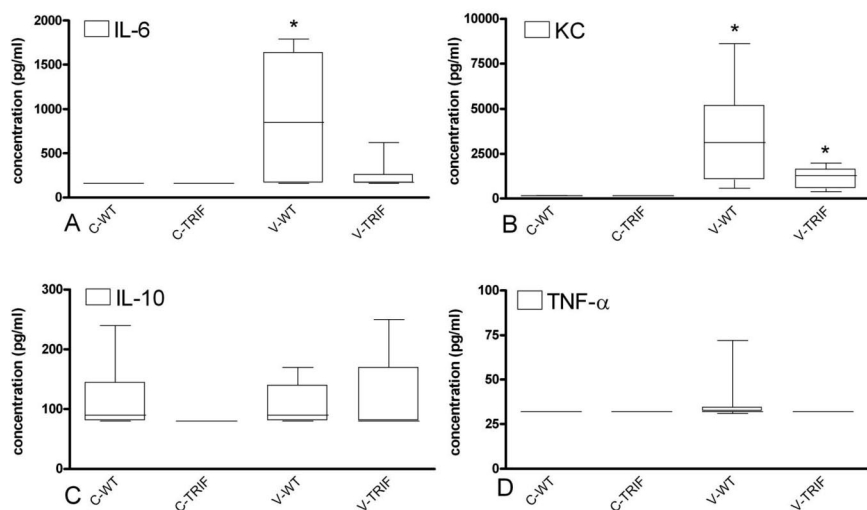
flammatory response. The current study extends these findings by showing that TRIF deficiency attenuates this inflammatory response after MV, by reducing NF- $\kappa$ B activation. TRIF deletion prevented pulmonary pro-IL-1 $\beta$  increase and systemic IL-6 increase after 4 h of MV. Also, pulmonary levels of IL-1 $\beta$  and KC were significantly lower in TRIF-deleted mice lungs compared with WT lungs after MV.

The TRIF pathway is a downstream pathway of TLR4 and TLR3,<sup>9,10</sup> that can cause delayed NF- $\kappa$ B activation.<sup>17</sup> Recently, we have shown the involvement of TLR4 in MV-induced inflammation.<sup>8</sup> The current study extends these findings by showing the involvement of TRIF signaling. We found that the MV-induced increase of KC and IL-1 $\beta$  was TRIF dependent. TRIF is also involved in the downstream signaling of TLR3; therefore, we cannot exclude the involvement of TLR3 in our model. However the results presented here closely resemble the results from our previous TLR4 experiment.<sup>8</sup> Therefore it is likely that in downstream signaling of TLR4 the TRIF pathway is involved in the inflammatory response after MV. Subsequent studies using TLR3 deleted mice are needed to confirm the importance of TLR4 in MV-induced inflammation.

### Potential Relevance of Findings

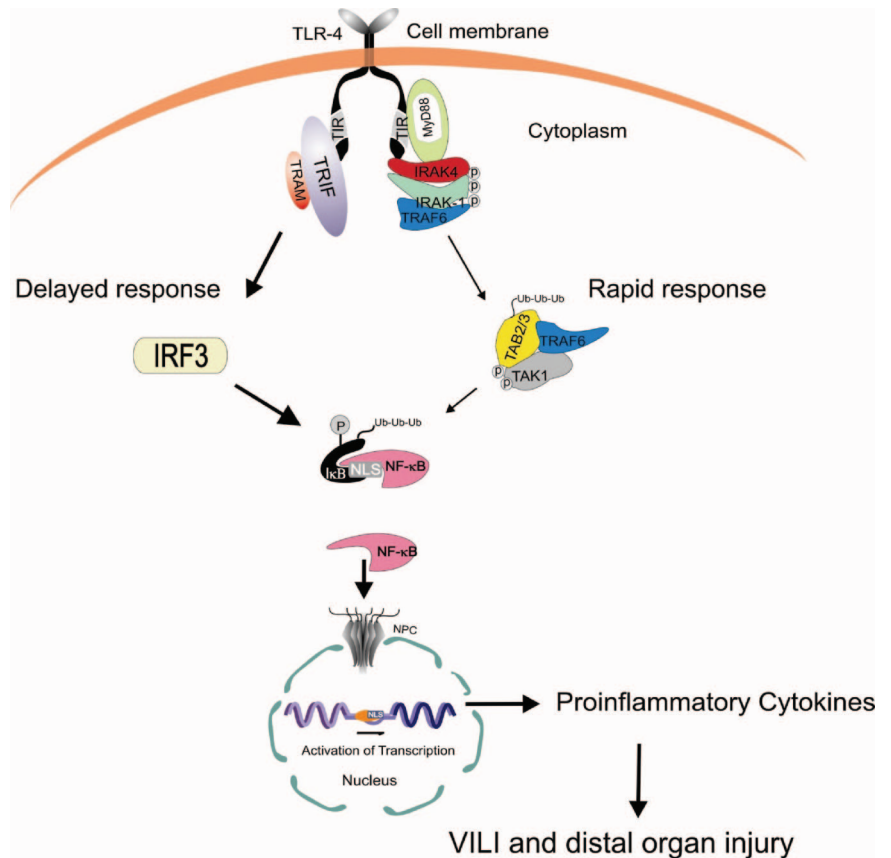
Several studies have pointed out the involvement of the analyzed cytokines in lung injury. IL-1 $\beta$  has been shown to be among the most biologically active cytokines in the lungs,<sup>18,19</sup> it is therefore proposed to play an important role in the pathogenesis of lung injury.<sup>20</sup> In clinical studies, high tidal volume MV results in persistently high plasma levels of IL-1 $\beta$ , which is associated with distal organ failure and mortality.<sup>2,21</sup> In the current study, TRIF deletion prevented IL-1 $\beta$  increase at messenger RNA level and attenuated the increase of IL-1 $\beta$  at

## Cytokine levels in plasma



**Fig. 5. Cytokine levels in plasma.** Levels of interleukin (IL)-6, keratinocyte-derived chemokine (KC), IL-10, and tumor necrosis factor- $\alpha$  in unventilated (C) and ventilated (V) wild type (WT) and Toll/interleukin-1 receptor domain-containing adapter-inducing interferon- $\beta$  (TRIF) mutant mice (panels A-D). Mechanical ventilation in WT mice (group V-WT) increased IL-6 ( $P < 0.001$ ) and KC ( $P < 0.001$ ) in plasma when compared with unventilated WT mice (group C-WT). In TRIF mutant mice (group V-TRIF), no increase in IL-6 was found after mechanical ventilation when compared with the unventilated TRIF mutant mice (group C-TRIF). Mechanical ventilation in TRIF mutant mice (group V-TRIF) did increase levels of KC ( $P < 0.001$ ) when compared with unventilated TRIF mutant mice (group C-TRIF). This was not statistically different from ventilated TRIF mutant mice (group V-TRIF).  $n = 8$  for all groups. Data are expressed as box (median, 25th, 75th percentile) and whiskers (range). \* =  $P < 0.05$  compared with unventilated mice. When no box and whiskers are visible, all levels remained below detection limit.

**Fig. 6.** Downstream signaling of Toll-like receptor 4. Schematic representation of downstream signaling pathways of the Toll-like receptor 4. Two pathways are involved. The rapid Myeloid differentiation primary response protein 88 (MyD88)-dependent response and the delayed Toll/interleukin-1 receptor domain-containing adapter-inducing interferon- $\beta$  (TRIF) response, also known as the MyD88-independent response. Both signaling routes cause translocation of nuclear factor ( $\text{NF-}\kappa\text{B}$ ) into the nucleus, and transcription of proinflammatory genes inducing cytokine production. Our challenging hypothesis is that these cytokines induce a proinflammatory response, which can result in ventilator-induced lung injury and distal organ injury. It should be noticed that activation of Toll-like receptor 3 may also activate TRIF-dependent pathway (not shown in figure). TIR = Toll/interleukin-1 receptor; TRAM = TRIF-related adaptor molecule; IRF3 = interferon regulatory factor 3; IRAK = interleukin-1 receptor-associated protein kinase; TRAF6 = tumor necrosis factor receptor associated factor 6; TAB 2/3 = TAK1-binding protein 2/3; TAK = transforming growth factor  $\beta$  activated kinase 1; I $\kappa$ B = inhibitor of  $\kappa\text{B}$ ; NLS = nuclear localization signal; NPC = nuclear pore complex; VILI = ventilator-induced lung injury.



protein levels, indicating TRIF involvement at the level of transcription. This is interesting because it indicates major involvement of the TRIF pathway and may indicate only minor or no influence of the MyD88 pathway. However this hypothesis needs further investigation.

KC is a chemoattractant, but it also has a direct cytotoxic effect.<sup>22</sup> Jiang *et al.*<sup>23</sup> found KC to be produced by pulmonary epithelial cells in a TLR4-dependent manner in direct response to bleomycin. More recently, functional TLR4 expression was found to be critical in the KC increase after hemorrhage.<sup>24</sup> The current study shows that the increase of KC after MV is at least partly TRIF-dependent.

Our data show that blocking TRIF-dependent pathway prevents the increase in plasma IL-6 after MV. Several studies suggest that IL-6 plays a role in the development of distal organ failure in ventilator-induced lung injury.<sup>2,25</sup> Accordingly, TRIF modulation may attenuate distal organ failure induced by MV.

It should be noticed that our model shows no evidence of either pulmonary dysfunction or distant organ failure, despite the development of an inflammatory response. Apparently, the trigger induced by the ventilator is relatively mild, and the lung is able to cope with the MV-induced inflammatory reaction. This has been demonstrated before in a study from our laboratory<sup>7</sup> and by a clinical study showing that 2 h of MV in healthy children resulted in enhanced cytokine concentrations without

clinical signs of pulmonary dysfunction.<sup>16</sup> In acid-induced lung injury, TRIF deletion indeed diminished impairments in lung function.<sup>5</sup> Therefore, the inflammatory response after MV in healthy lungs may be too subtle to identify changes in cardiopulmonary physiology. However, the inflammatory response after MV is clinically relevant; this forms the basis for the two-hit hypothesis proposing that injury (*e.g.*, the critically ill patient) primes the immune system (first hit) for a lethal inflammatory reaction to a later, otherwise nonlethal, secondary insult (second hit), namely MV.<sup>26-28</sup> This enhanced host response can lead to distal organ failure and is previously linked to TLR4 reactivity,<sup>29</sup> which is interesting because we have shown that TLR4 is involved in the inflammatory response after MV in healthy lungs.<sup>8</sup> Downstream of TLR4, we identified the involvement of TRIF in this response. Therefore, inhibition of TRIF may be an effective strategy to prevent or attenuate MV-induced pulmonary and systemic inflammation. Figure 6 shows a schematic overview of the downstream signaling pathways of TLR4.

KC is a major chemoattractant for leukocytes.<sup>30</sup> In a previous study, we have shown that 4 h of MV in healthy mice induces a TLR4-dependent pulmonary leukocyte influx after the increase of KC in the lung.<sup>7,8</sup> In the current study, this pulmonary leukocyte influx is confirmed. In TRIF mutant mice, leukocyte influx appeared less pronounced; however, this did not reach statistical



significance. This might be explained by the fact that, the increase in KC in plasma in TRIF mutant mice also appeared to be reduced, without reaching statistical significance.

Our study has several limitations. First, all studies were performed in mice. It is unknown whether the response to MV in mice is similar to the response in humans. Second, only the effect of 4 h of MV with clinically relevant tidal volume was tested. In a previous study, we showed that the proinflammatory response is activated within 30 min after initiation of MV and intensifies up to 4 h after initiation of MV.<sup>7</sup> Preliminary observations show that the inflammatory response unaltered after 8 h of MV compared with 4 h of MV. We did not study the effects of TRIF deletion in lungs exposed to high-tidal volume MV, as the use of high-stretch MV is currently avoided in clinical practice. Third, the current study did not evaluate the effect of different ventilatory modes. Interestingly, recent studies indicate that spontaneous ventilatory efforts may improve respiratory function such as diaphragm function, pulmonary gas exchange, and hemodynamics after MV.<sup>31,32</sup>

Factors other than MV possibly affecting TRIF were carefully avoided. Contamination with lipopolysaccharide is suggested to be a confounding factor in many studies.<sup>33</sup> 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)<sup>34</sup> was eliminated by performing experiments in noninvasively monitored animals. Previously, cardiopulmonary stability in invasively monitored animals has been documented.<sup>7</sup> In the current study, mice were slightly acidotic after MV. As hypercapnic acidosis attenuates MV-induced inflammation in healthy mice,<sup>35</sup> this could affect our results. However it is unlikely that the slight metabolic acidosis in the current study significantly affects our data. Indeed, correcting metabolic acidosis does not alter levels of cytokines after MV in healthy mice.<sup>36</sup> The possible immune modulating effects of anesthetics have been studied extensively.<sup>37</sup> Ketamine, for instance, is known to have an inhibitory effect on lipopolysaccharide-induced cytokine production,<sup>38–41</sup> possibly by suppressing TLR4 expression.<sup>42</sup> Recently, it has been demonstrated that ketamine alone (without lipopolysaccharide) also attenuated cytokine production in humans in the direct post-operative period after elective abdominal surgery.<sup>43</sup> In the current study, all animals received ketamine. Ideally, an additional control group of spontaneously breathing animals under ketamine, medetomidine and atropine anesthesia is needed. However, this will result in hypoventilation with severe respiratory acidosis and hemodynamic instability.

The current study supports a role for TRIF 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 ventilator-induced lung injury, in which TRIF may serve as a therapeutic target.

We are grateful for the help of Francien van de Pol, Ing., and Ilona van den Brink, Ing. (Lab technicians, Department of Anesthesiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands) for their expertise in performing the animal experiments. Ineke Verschuieren, Ing. (Lab technician, Nijmegen Centre for Infectious Diseases, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands) for her help with cytokine assays, and Cindy Pigmans, Ing. (Lab technician, Department of Pulmonary Diseases, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands) for her help with analyzing NF- $\kappa$ B activity.

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