

# Ventilator-induced Lung Injury Is Mediated by the NLRP3 Inflammasome

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## ABSTRACT

**Background:** The innate immune response is important in ventilator-induced lung injury (VILI) but the exact pathways involved are not elucidated. The authors studied the role of the intracellular danger sensor NLRP3 inflammasome.

**Methods:** NLRP3 inflammasome gene expression was analyzed in respiratory epithelial cells and alveolar macrophages

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## What We Already Know about This Topic

- The molecular mechanisms for ventilator-induced injury are not clearly delineated

## What This Article Tells Us That Is New

- Bronchial brush samples and cells from bronchoalveolar lavages done on 40 normal patients undergoing elective surgery for more than 5 h and who received tidal volumes of 12 ml/kg or 6 ml/kg ideal body weight documented that there is a probable role of a NLRP3 inflammasome dependent pulmonary response due to mechanical ventilation
- Mouse experiments confirmed this observation

obtained from ventilated patients ( $n = 40$ ). In addition, wild-type and NLRP3 inflammasome deficient mice were randomized to low tidal volume (approximately 7.5 ml/kg) and high tidal volume (approximately 15 ml/kg) ventilation. The presence of uric acid in lung lavage, activation of caspase-1, and NLRP3 inflammasome gene expression in lung tissue were investigated. Moreover, mice were pretreated with interleukin-1 receptor antagonist, glibenclamide, or vehicle before start of mechanical ventilation. VILI endpoints were relative lung weights, total protein in lavage fluid, neutrophil influx, and pulmonary and systemic cytokine and chemokine concentrations. Data represent mean  $\pm$  SD.

**Results:** Mechanical ventilation up-regulated messenger RNA expression levels of NLRP3 in alveolar macrophages ( $1.0 \pm 0$  vs.  $1.70 \pm 1.65$ ,  $P$  less than 0.05). In mice, mechanical ventilation increased both NLRP3 and apoptosis-associated speck-like protein messenger RNA levels, respectively ( $1.08 \pm 0.55$  vs.  $3.98 \pm 2.89$ ;  $P$  less than 0.001 and  $0.95 \pm 0.53$  vs.  $6.0 \pm 3.55$ ;  $P$  less than 0.001), activated caspase-1, and increased uric acid levels ( $6.36 \pm 1.85$  vs.  $41.9 \pm 32.0$ ,  $P$  less than 0.001). NLRP3 inflammasome deficient mice displayed less VILI due to high tidal volume mechanical ventilation compared with wild-type mice. Furthermore,

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treatment with interleukin-1 receptor antagonist or glibenclamide reduced VILI.

**Conclusions:** Mechanical ventilation induced a NLRP3 inflammasome dependent pulmonary inflammatory response. NLRP3 inflammasome deficiency partially protected mice from VILI.

**M**ECHANICAL ventilation (MV) is often used during general anesthesia and as a lifesaving intervention in patients with acute respiratory failure. However, MV can aggravate preexisting lung injury and may even induce lung injury in previously healthy lungs.<sup>1–3</sup> The Acute Respiratory Distress Syndrome Network convincingly demonstrated the clinical relevance of ventilator-induced lung injury (VILI) by showing that the use of lower tidal volumes ( $V_T$ s) in patients with acute lung injury reduced mortality and morbidity.<sup>3</sup> However, the exact mechanisms underlying this outcome are still incompletely understood. An important role for the innate immune response in VILI pathogenesis is proposed.<sup>4,5</sup> Despite use of low tidal volume ventilation, the injured lung is still at risk for overdistension.<sup>6</sup> Some lung areas are collapsed and fluid filled, placing the open recruitable lung regions at risk for hyperinflation injury. Injured tissue can release endogenous molecules that activate innate immune receptors and initiate or propagate inflammation.<sup>7</sup> These molecules are termed alarmins or damage-associated molecular patterns (DAMPs). Adenosine triphosphate (ATP), uric acid crystals, heat shock proteins, S100A8/A9, hyaluronan, and high-mobility group box-1 are DAMPs and recognized by a diverse repertoire of pattern recognition receptors, including the Toll-like receptor and the receptor for advanced glycation end-products.<sup>7,8</sup> High-mobility group box-1, hyaluronan, and ATP are found in bronchoalveolar lavage fluid (BALF) of previously healthy animals subjected to injurious MV.<sup>9–11</sup> An important role for Toll-like receptors in VILI has been demonstrated.<sup>4</sup> Less is known about the recently identified intracellular pattern recognition receptors, the nucleotide-binding and oligomerization domain-like receptors in VILI.<sup>12</sup> The best understood family member is NLRP3 (or cryopyrin or NALP3). NLRP3 assembles upon activation by pathogen-associated molecular patterns or specific DAMPs such as ATP, uric acid, and hyaluronan, with the adaptor apoptosis-associated speck-like protein (ASC) and procaspase-1, forming the NLRP3 inflammasome.<sup>12,13</sup> Caspase-1 is activated by the formation of this multiprotein complex and cleaves prointerleukin-1 $\beta$  (pro-IL-1 $\beta$ ) and pro-IL-18 into their active forms. Previous preclinical and clinical research demonstrated the important role of the proinflammatory cytokine IL-1 $\beta$  in VILI.<sup>14–17</sup> However, the molecular mechanisms by which the IL-1 $\beta$  pathway becomes activated during VILI are largely unknown. The important role of NLRP3 inflammasome in the innate immune response associated with cellular injury and the demonstrated release of the NLRP3 activators ATP and hyaluronan in BALF during in-

jurious MV<sup>10,11</sup> makes the NLRP3 inflammasome a likely candidate to be activated in VILI pathogenesis.

The objective of this current study was to test the hypothesis that NLRP3 inflammasome signaling is involved in the inflammatory response induced by MV.

## Materials and Methods

### Patients

We used bronchial brush samples and BALF cells from a previously published randomized controlled trial in which patients without preexisting lung injury were mechanically ventilated.<sup>18</sup> The Medical Ethics Committee of the Academic Medical Center, Amsterdam, the Netherlands approved the study protocol and informed consent was obtained from all patients. Adult patients scheduled for elective surgery with an estimated duration of 5 h or longer were eligible for this study. Exclusion criteria were history of any lung disease, recent infections, use of immunosuppressive medication, previous thromboembolic disease, and recent ventilatory support.

### Study Design

All patients received anesthesia consisting of 2–3 mg/kg propofol (thereafter 6–12 mg/kg/h), fentanyl 2–3  $\mu$ g/kg, and rocuronium administered intravenously together with epidurally administered bupivacaine (0.125%)-fentanyl (2.5  $\mu$ g/ml). The ventilatory protocol consisted of volume-controlled MV, at an inspired oxygen fraction of 0.40, inspiratory-to-expiratory ratio of 1:2, and a respiratory rate adjusted to achieve normocapnia. Patients were randomly assigned to MV with either  $V_T$ s of 12 ml/kg ideal body weight (high  $V_T$  [HV $_T$ ]) and 0 cm H $_2$ O positive end-expiratory pressure or 6 ml/kg (low  $V_T$  [LV $_T$ ]) and 10 cm H $_2$ O positive end-expiratory pressure. Bronchial brushes and BALF were collected during a bronchoscopic procedure that was performed twice in each patient: the first directly after induction of anesthesia and start of MV in the right middle lobe or lingula, and the second performed in the contralateral lung 5 h thereafter, either perioperatively or directly postoperatively. BALF was obtained and processed as previously described.<sup>18</sup> BALF cells were resuspended in ice-cold phosphate-buffered saline. The resuspended cells were partially used for absolute cell counts and for differential counting using Giemsa-stained cytopsin preparations. The remaining cells were dissolved in RNAlater (Ambion, Austin, TX) and stored at  $-80^{\circ}\text{C}$ . Bronchial brushes were directly placed into RNAlater. After several minutes and thorough vortexing, the brush was removed. RNA was purified using RNeasy mini kit system (Qiagen, Valencia, CA) according to the manufacturer's protocol. Lung brush samples and BALF cells were used to determine messenger RNA (mRNA) expression levels of NLRP3, ASC, and hypoxanthine-guanine phosphoribosyl transferase. Analysis only included patient samples in which paired measurements of both time points was possible and the data from both ventilation strategies was combined.

## Mice

The Animal Care and Use Committee of the Academic Medical Center of the University of Amsterdam, the Netherlands approved this study. Animal procedures were carried out in compliance with Institutional Standards for Human Care and Use of Laboratory Animals.

The generation of NLRP3 and ASC knockout (KO) mice has been described.<sup>19</sup> Seven- to 12-week-old male NLRP3 KO and ASC KO mice, backcrossed 9 times to a C57BL/6 genetic background, were bred in the animal facility of the Academic Medical Center (Amsterdam, the Netherlands). C57BL/6 age- and sex-matched wild-type (WT) mice were purchased from Charles River (Maastricht, the Netherlands). The animals were housed in rooms with a controlled temperature and a 12-h light-dark cycle. They were acclimatized for 1 week before the experiments, and received standard rodent chow and water *ad libitum*.

## Experimental Groups

WT, NLRP3, and ASC KO mice were randomized to a low  $V_T$  or high  $V_T$  ventilation strategy; nonventilated mice served as a control group ( $n = 6$ – $9$ /group). Two sets of experiments were performed. In the first set of experiments we used the right lung for BALF and the left lung for wet weight. In the second set of experiments we used the left lung for histopathology and the right lung for Western blot analysis and to extract RNA as previously described.<sup>20</sup> In both sets of experiments blood was sampled from the carotid artery. In addition, experiments with two pharmacologic blockers were performed. To study the IL-1 $\beta$  pathway, WT mice received 50 mg/kg recombinant human IL-1 receptor antagonist (Biovitrum AB, Stockholm, Sweden) or vehicle (saline) 1 h before ventilation ( $n = 9$ /group), control animals were nonventilated mice receiving IL-1ra or vehicle ( $n = 6$ /group).<sup>21</sup> Here we used the right lung for BALF and the left lung for wet weight, and blood was sampled from the carotid artery. Glibenclamide has previously been used to block the NLRP3 inflammasome.<sup>22</sup> Therefore, we pretreated WT mice with 50 mg/kg glibenclamide or vehicle (10% dimethyl sulfoxide) 1 h before start of high tidal ventilation ( $n = 6$  for controls,  $n = 9$  for ventilated mice). To determine whether the protective effect of glibenclamide is indeed related to NLRP3 inflammasome blockage, and not due to off-target effects, we pretreated HV $_T$  ventilated NLRP3 KO mice with glibenclamide or vehicle ( $n = 9$  per group). In these experiments we used the right lung for BALF and the left lung for wet weights.

## Instrumentation and Anesthesia

Methods used in this VILI model were published in detail previously.<sup>23</sup> In short, mice received an intraperitoneal bolus of 1 ml normal saline 1 h before the start of randomization. In the ventilated mice, a tracheotomy was performed and an Y-tube connector (1.0 mm OD and 0.6 mm ID, VBM Medizintechnik GmbH, Sulz am Neckar, Germany) was

surgically inserted into the trachea under general anesthesia with an injection of “induction”-mix: 7.5  $\mu$ l per 10 g body weight of 1.26 ml 100 mg/ml ketamine, 0.2 ml 1 mg/ml medetomidine, and 1 ml 0.5 mg/ml atropine in 5 ml normal saline. Maintenance anesthesia consisted of 10  $\mu$ l per 10 g body weight of “maintenance”-mix: 0.72 ml 100 mg/ml ketamine, 0.08 ml 1 mg/ml medetomidine, and 0.3 ml 0.5 mg/ml atropine in 20 ml normal saline. Maintenance mix was hourly administered *via* an intraperitoneal catheter (PE 10 tubing, BD, Breda, the Netherlands), every 30 min 0.2 ml sodium carbonate (200 mM NaHCO<sub>3</sub>) was administered *via* the same catheter. Throughout the experiments rectal temperature was maintained between 36.5–37.5°C using a warming pad. In the glibenclamide experiments blood glucose levels and systolic blood pressure were measured directly after the start of ventilation, after 2.5 h and 5 h of MV. Blood pressure was monitored using a murine tail-cuff system and data were recorded on a data acquisition system (PowerLab/4SP, ADInstruments, Spence, Germany). Glucose concentrations were determined using a glucometer (Abbott freestyle glucometer, Hoofddorp, the Netherlands).

## MV Strategies

Animals were placed in a supine position and connected to a ventilator (Servo 900 C; Siemens, Solna, Sweden). Mice were pressure controlled ventilated for 5 h with either an inspiratory pressure of 10 cm H<sub>2</sub>O (resulting in lung-protective  $V_T$  approximately 7.5 ml/kg; low  $V_T$ , LV $_T$ ) or an inspiratory pressure of 18 cm H<sub>2</sub>O (resulting in injurious  $V_T$  approximately 15 ml/kg; high  $V_T$ , HV $_T$ ).<sup>23</sup> Respiratory rate was set at 110 breaths/min and 70 breaths/min with LV $_T$  and HV $_T$ , respectively. Positive end-expiratory pressure was set at 2 cm H<sub>2</sub>O with both MV strategies. The fraction of inspired oxygen was kept at 0.5 and inspiration to expiration ratio was set at 1:1. A sigh (sustained inflation with 30 cm H<sub>2</sub>O) for 5 breaths was performed every 30 min. At the end of the experiment mice were sacrificed by withdrawing blood from the carotid artery, which was used for blood gas analysis.

## Sampling

BALF was harvested from the right lung by instilling three times 0.5 ml aliquots of saline by a 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland) into the trachea. Cell counts were determined using a Coulter cell counter (Beckman Coulter, Fullerton, CA); differential cell counts were performed on cytopsin preparations stained with Giemsa stain. Supernatant was stored at –20°C for total protein level and cytokine measurements. The left lung was weighed immediately after harvesting and lung/body weight ratio, a parameter of lung edema, was calculated.<sup>24</sup>

In the second set of experiments left lungs were fixed in 4% formalin and embedded in paraffin, and 4- $\mu$ m sections were stained with hematoxylin and eosin and analyzed by a pathologist who was blinded for group identity. To score lung injury, we used a modified VILI histology scoring sys-



tem.<sup>25</sup> In short, four pathologic parameters were scored on a scale of 0–4: (1) alveolar congestion, (2) hemorrhage, (3) leukocyte infiltration; and (4) thickness of alveolar wall/hyaline membranes. A score of 0 represents normal lungs; 1, mild, < 25% lung involvement; 2, moderate, 25–50% lung involvement; 3, severe, 50–75% lung involvement and 4, very severe, > 75% lung involvement. The total histology score was expressed as the sum of the score for all parameters. Right lungs were homogenized in 4 volumes of saline and 50  $\mu$ l was transferred in Tripure (Roche, Woerden, the Netherlands) for mRNA analysis. The remaining homogenate was diluted 1:1 in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM MgCl<sub>2</sub>·H<sub>2</sub>O, 1 mM CaCl<sub>2</sub>, 1% Triton x-100, 100  $\mu$ g/ml Pepstatin A, leupeptin, and aprotinin, pH 7.4) and incubated at 4°C for 30 min. Cell-free supernatants were obtained by centrifugation and stored at –80°C.

### Assays

Total protein levels in BALF were determined using a Bradford Protein Assay Kit (OZ Biosciences, Marseille, France) according to manufacturers' instructions. IL-6, IL-1 $\beta$ , and KC levels were measured by enzyme-linked immunosorbent assay (R&D Systems Inc., Minneapolis, MN). Detection limits were 51 pg/ml for IL-6, 13 pg/ml for IL-1 $\beta$ , and 254 pg/ml for KC. Uric acid concentrations were measured in BALF samples using Amplex Red Uric Acid Assay Kit (Molecular Probes, Eugene, OR) with a detection limit of 1.3  $\mu$ M.

### mRNA Expression Analysis

Complementary DNA synthesis from human and murine RNA was performed by a reverse transcription reaction using oligo dT (Invitrogen, Grand Island, NY) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative polymerase chain reactions were performed using LightCycler®SYBR green I master mix (Roche, Mijdrecht, the Netherlands) and measured in a LightCycler 480 (Roche) apparatus using the following conditions: 5 min 95°C hot-start, followed by 40 cycles of amplification (95°C for 10 s, 60°C for 5 s, 72°C for 15 s). For quantification, standard curves were constructed by polymerase chain reactions on serial dilutions of a concentrated complementary DNA sample, and data were analyzed using LightCycler software. Gene expression is presented as a ratio of the expression to the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase for human and murine analysis. The following human primer sequences were used: NLRP3 forward primer 5'-cttctctgatgaggcccaag-3' and reverse primer 5'-gcagcaactggaaggaag-3'; ASC forward primer 5'-ctctgtacgggaaggctctg-3' and reverse primer 5'-tcctccaccaggttagactg-3'; hypoxanthine-guanine phosphoribosyl transferase forward primer 5'-cggctcgttatggcgacc-3' and reverse primer 5'-ccccttgacacacagaggct-3'. Murine primer sequences were: NLRP3 forward primer 5'-ccacagtgtacttcgagaagc-3' and reverse primer 5'-gggtgtgaagtctgtgtgg-3'; ASC forward primer 5'-aaagaaagtctggagctgtgg-3' and reverse primer 5'-gcaatgagtcttgc-

ctgt-3'; hypoxanthine-guanine phosphoribosyl transferase forward primer 5'-tcctctcagaccgctttt-3' and reverse primer 5'-cctggttcacatcgtaac-3'.<sup>26</sup>

### Caspase-1 Western Blot

Electrophoresis of proteins was performed using the NuPAGE system (Invitrogen) according to the manufacturer's protocol. Separated proteins on the NuPAGE gel were transferred to a polyvinylidene fluoride membrane by electroblotting. To detect caspase-1 a rabbit polyclonal antimouse caspase-1 p10 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used.<sup>27</sup>

### Statistical Analysis

Data are expressed as mean  $\pm$  SD unless stated otherwise. Wilcoxon signed-rank test was used for paired human mRNA lung brush and BALF cell samples comparing  $t = 0$  h *versus*  $t = 5$  h. For differences between mechanical ventilation groups, NLRP3 and ASC KO *versus* WT mice, as well as treated *versus* nontreated groups one-way analysis of variance with Bonferroni correction as *post hoc* analysis or a Kruskal-Wallis test with Mann-Whitney U test as *post hoc* analysis was used, depending on data distribution. For analysis of data obtained from the glibenclamide-treated or nontreated NLRP3 KO mice we used Student  $t$  test or Mann-Whitney U test depending on data distribution. Statistical analyses were carried out using GraphPad Prism version 5 (GraphPad Software; San Diego, CA). All  $P$  values are two-sided and  $P$  less than 0.05 is considered to be statistically significant.

## Results

### Patients

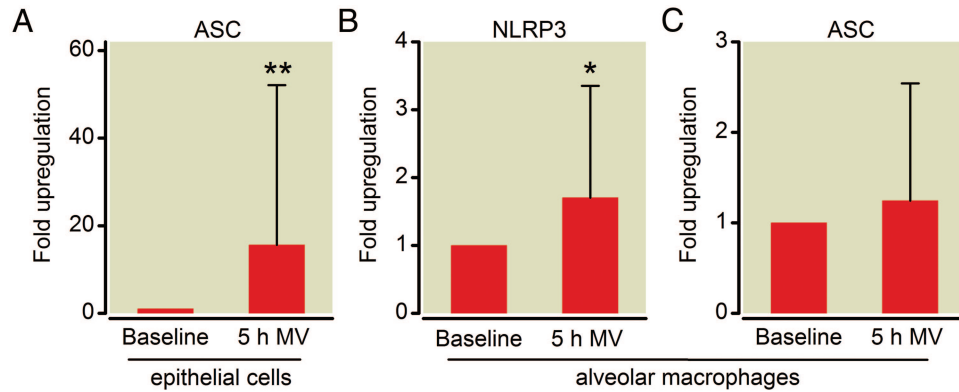
Baseline characteristics, perioperative parameters, and patient characteristics were described in detail previously.<sup>18</sup> In short, from December 2003 through March 2005, 74 patients scheduled for an elective surgical procedure were screened. In total, 28 patients were excluded and 40 patients completed the study protocol; 21 patients were assigned to lung-protective MV and 19 patients to the conventional ventilation strategy. No major differences in baseline characteristics or duration of MV were found between both randomization groups.<sup>18</sup>

### Mice

The characteristics of the physiologic VILI model used were published in detail previously.<sup>23</sup> All animals survived 5 h of MV after which they were sacrificed. Blood gas analysis showed adequate gas exchange, confirming our previous study results,<sup>23</sup> with no differences between WT and KO mice or treated *versus* nontreated groups (data not shown).

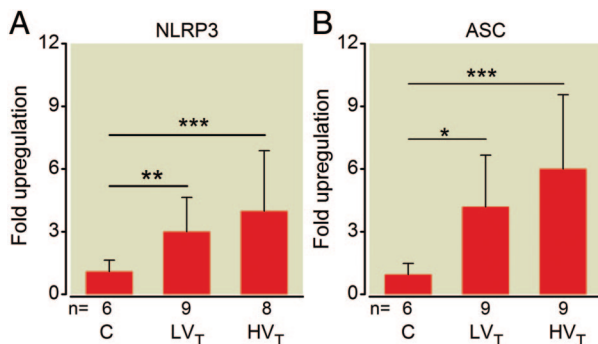
### MV Increases Relative mRNA Expression of NLRP3 and ASC Protein in the Lung

ASC mRNA levels were higher in lung brush samples after 5 h of MV ( $n = 23$  pairs) (fig. 1A), whereas NLRP3 mRNA levels were not detectable in epithelial cells.



**Fig. 1.** Relative messenger RNA expression levels of apoptosis-associated speck-like protein (ASC) (A, C) in human epithelial cells and alveolar macrophages and NLRP3 (B) in alveolar macrophages. Epithelial cells and alveolar macrophages were obtained from brush and lung lavage samples, respectively, at baseline and after 5 h of mechanical ventilation (MV). Gene expression was normalized to the house-keeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT). Samples were included for analysis when paired measurement of both time points was possible. Data represent mean (SD) of  $n = 23$  pairs for ASC in epithelial cells,  $n = 29$  pairs for NLRP3 in alveolar macrophages, and  $n = 34$  pairs for ASC in alveolar macrophages. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

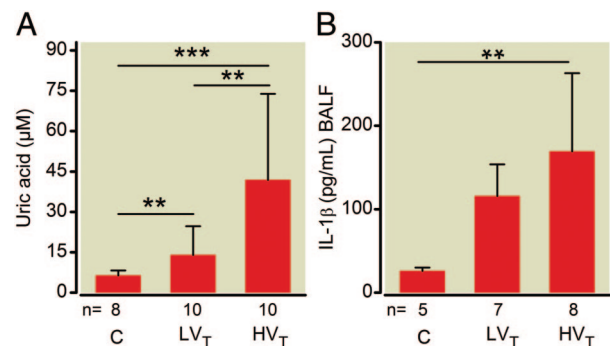
BALF cells before and after MV consisted for more than 99% of macrophages.<sup>18</sup> MV increased relative NLRP3 mRNA expression levels in alveolar macrophages ( $n = 29$  pairs) (fig. 1B). Relative ASC mRNA expression levels in BALF cells were not significantly up-regulated ( $n = 34$  pairs) (fig. 1C). To study if MV in our murine VILI model had the same effect, we determined relative NLRP3 and ASC mRNA expression levels in lung tissue homogenates derived from healthy control mice and healthy WT animals ventilated with LV<sub>T</sub> or HV<sub>T</sub>. Both ventilation strategies significantly increased NLRP3 and ASC mRNA expression in whole lungs compared with the nonventilated group (both  $P$  less than 0.05) (fig. 2A and B); no significant differences between the two ventilation strategies were found.



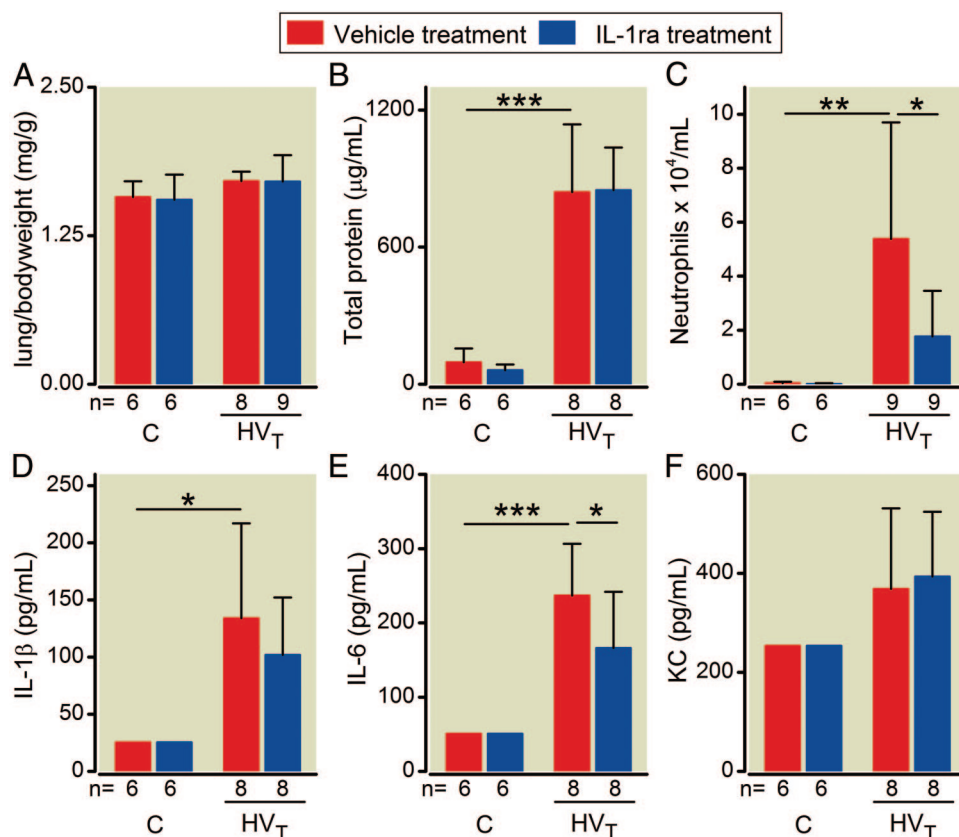
**Fig. 2.** Wild-type mice ventilated for 5 h with low tidal volumes (LV<sub>T</sub>) (approximately 7.5 ml/kg) or high tidal volumes (HV<sub>T</sub>) (approximately 15 ml/kg). Spontaneously breathing mice (C) served as the control group. NLRP3 (A) and apoptosis-associated speck-like protein (ASC) (B) gene expression was measured in lung homogenates and normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT). Data represent mean (SD), number (n) of mice per group is specified in the figure. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

### MV Releases Ligands for NLRP3 and the End Product of NLRP3 Inflammasome Activation, IL-1 $\beta$ in BALF

Upon ligand recognition, NLRP3 assembles together with ASC and caspase-1 to form the NLRP3 inflammasome. Known ligands are uric acid, ATP, and hyaluronan.<sup>12,13</sup> To determine whether, in addition to ATP<sup>10</sup> and hyaluronan,<sup>11</sup> there are other NLRP3-activating DAMPs that are released due to MV, we measured uric acid levels in BALF from WT mice ventilated with LV<sub>T</sub> or HV<sub>T</sub> and non-ventilated animals served as control animals. Significantly more uric acid was released during MV by both ventilation strategies when compared with nonventilated control mice (control *vs.* LV<sub>T</sub>  $P$  less than 0.01, control *vs.* HV<sub>T</sub>  $P$  less than 0.001) (fig. 3A). The end product of NLRP3 inflammasome activation, the proinflammatory cytokine IL-1 $\beta$  was found in BALF. HV<sub>T</sub> ventilation of WT mice significantly increased IL-1 $\beta$  protein



**Fig. 3.** Uric acid (A) levels and IL-1 $\beta$  protein (B) concentrations in bronchoalveolar lavage fluid (BALF). Wild-type mice were ventilated for 5 h with low tidal volumes (LV<sub>T</sub>) (approximately 7.5 ml/kg) or high tidal volumes (HV<sub>T</sub>) (approximately 15 ml/kg). Spontaneously breathing mice (C) served as the control group. Data represent mean (SD), number (n) of mice per group is specified in the figure. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .



**Fig. 4.** Relative lung weights (A), total protein levels in bronchoalveolar lavage fluid (BALF) (B), neutrophil influx (C), interleukin (IL) -1 $\beta$  (D), IL-6 (E), and keratinocyte-derived chemokine (KC) (F) levels in BALF. IL-1 receptor antagonist (IL-1ra) (50 mg/kg; blue bars) treated and vehicle treated wild-type (WT) mice (red bars) were ventilated for 5 h with high tidal volume (HV<sub>T</sub>) (approximately 15 ml/kg) and spontaneously breathing mice served as the control group (C). Data represent mean (SD), number (n) of mice per group is specified in the figure. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

levels in BALF when compared to unventilated mice ( $P$  less than 0.01) (fig. 3B).

Having demonstrated that cytokine IL-1 $\beta$  is released due to HV<sub>T</sub> MV we decided to study the effect of IL-1 $\beta$  signaling in our VILI model. Treatment with IL-1 receptor antagonist attenuated neutrophil influx and BALF IL-6 levels compared with vehicle-treated ventilated mice (fig. 4). Systemic IL-6 levels were reduced ( $P = 0.05$ ) compared with the vehicle treated group (table 1).

#### NLRP3 and ASC KO Mice Display Reduced Lung Injury by Injurious MV

The LV<sub>T</sub> strategy did not result in significant differences between KO and WT mice. The HV<sub>T</sub> strategy however, demonstrated clear differences (fig. 5). Alveolar barrier dysfunction reflected by relative lung weights and total protein concentrations in HV<sub>T</sub> ventilated NLRP3 KO mice were reduced compared with HV<sub>T</sub> WT mice (fig. 5A and B) (both  $P$  less than 0.05). The influx of neutrophils into the pulmo-

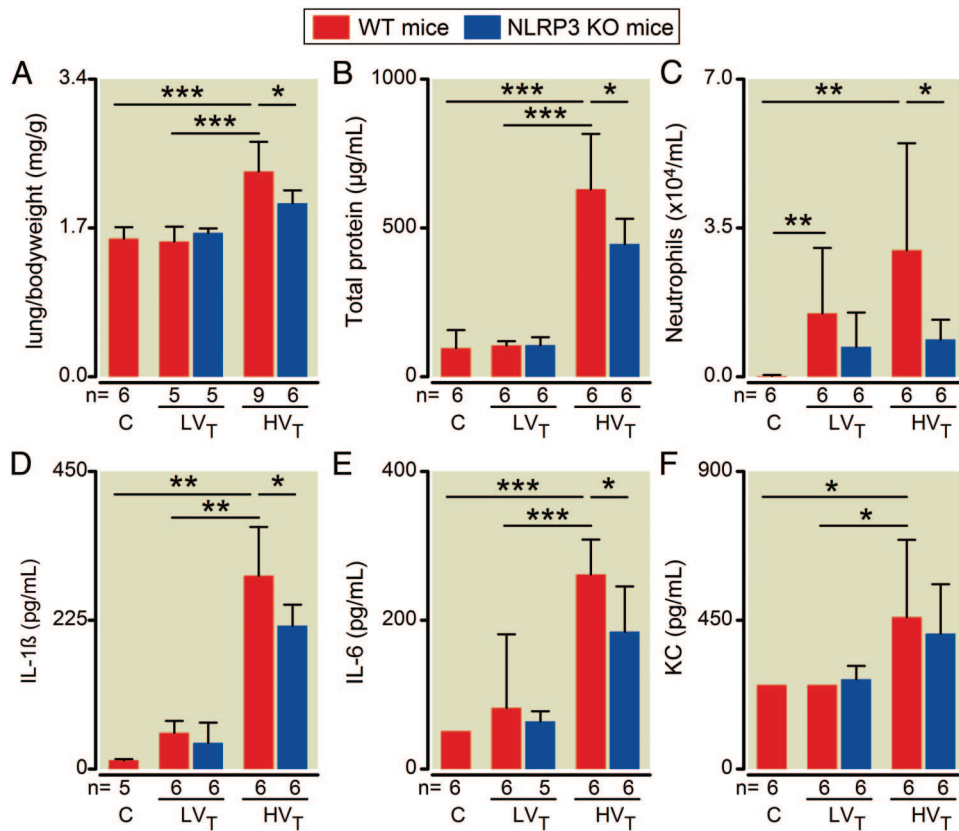
**Table 1.** The Effect of the NLRP3 Inflammasome on Systemic Cytokine Levels

	Control C	High Tidal Ventilation					
		NLRP3 KO Experiment		ASC KO Experiment		IL-1ra Experiment	
		WT	NLRP3 KO	WT	ASC KO	Vehicle	IL-1ra
IL-1 $\beta$	0.01 [0.00]	0.54 [0.35]	0.47 [0.26]	0.28 [0.13]	0.10 [0.05]**	0.31 [0.17]	0.24 [0.11]
IL-6	0.05 [0.00]	0.84 [0.18]	0.52 [0.24]*	0.78 [0.25]	0.50 [0.08]*	1.34 [0.55]	0.76 [0.47]
KC	0.27 [0.03]	2.45 [0.97]	2.29 [0.81]	5.03 [2.11]	5.18 [7.45]	5.09 [6.83]	2.22 [0.70]

Cytokine levels in serum (in ng/ml) in WT, NLRP3 KO, ASC KO, vehicle, or IL-1ra treated mice. Control, spontaneously breathing mice and high tidal volume (HV<sub>T</sub>) mice ventilated for 5 h with a V<sub>T</sub> of approximately 15 ml/kg. Values represent mean [SD] of 6 control mice and 8 ventilated mice per group (n = 6 for ASC KO IL-1 $\beta$ , n = 7 for IL-1ra-treated IL-6, n = 9 for ASC WT and KO KC).

\*  $P < 0.05$  \*\*  $P < 0.01$  WT HV<sub>T</sub> vs. KO HV<sub>T</sub> or vehicle vs. IL-1ra.

ASC = apoptosis-associated specklike protein; C = control; IL = interleukin; IL-1ra = IL-1 receptor antagonist; KC = keratinocyte-derived chemokine; KO = knockout; WT = wild-type.



**Fig. 5.** Relative lung weights (A), total protein levels in bronchoalveolar lavage fluid (BALF) (B), neutrophil influx (C), interleukin (IL)-1 $\beta$  (D), IL-6 (E) and keratinocyte-derived chemokine (KC) (F) levels in BALF. NLRP3 knockout (KO) (blue bars) and wild-type (WT) mice (red bars) were ventilated for 5 h with low tidal volumes (LV<sub>T</sub>) (approximately 7.5 ml/kg) or high tidal volumes (HV<sub>T</sub>) (approximately 15 ml/kg) and spontaneously breathing WT mice (C) served as the control group. Data represent mean (SD), number (n) of mice per group is specified in the figure \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

nary compartment was diminished in HV<sub>T</sub> NLRP3 KO mice ( $P$  less than 0.05) (fig. 5C). Moreover, BALF of HV<sub>T</sub> ventilated WT animals contained significantly more IL-6 and IL-1 $\beta$  protein than HV<sub>T</sub> ventilated NLRP3 KO mice (fig. 5D and E) (both  $P$  less than 0.05). In line, systemic IL-6 levels were reduced in the HV<sub>T</sub> ventilated NLRP3 KO mice ( $P$  less than 0.05) (table 1). No differences in KC levels were found.

When we compared ASC KO with WT mice, a similar pattern as in NLRP3 KO mice was observed (fig. 6). Although we found no differences in the LV<sub>T</sub> strategy, the HV<sub>T</sub> strategy resulted again in differences between KO and WT mice. Relative lung weights and neutrophil influx in BALF were significantly lower in the HV<sub>T</sub> ventilated ASC KO compared with the HV<sub>T</sub> WT group (all  $P$  less than 0.05) (fig. 6A and C). Systemic IL-6 and IL-1 $\beta$  levels were also reduced in these ASC KO mice compared with the HV<sub>T</sub> WT group (table 1).

Histopathologic changes in animals from the HV<sub>T</sub> group were mild and no significant differences in lung injury scores between WT and KO groups were found (see figure, Supplemental Digital Content 1, <http://links.lww.com/ALN/A840>, which demonstrates the lung injury scores of HV<sub>T</sub> ventilated and nonventilated control mice).

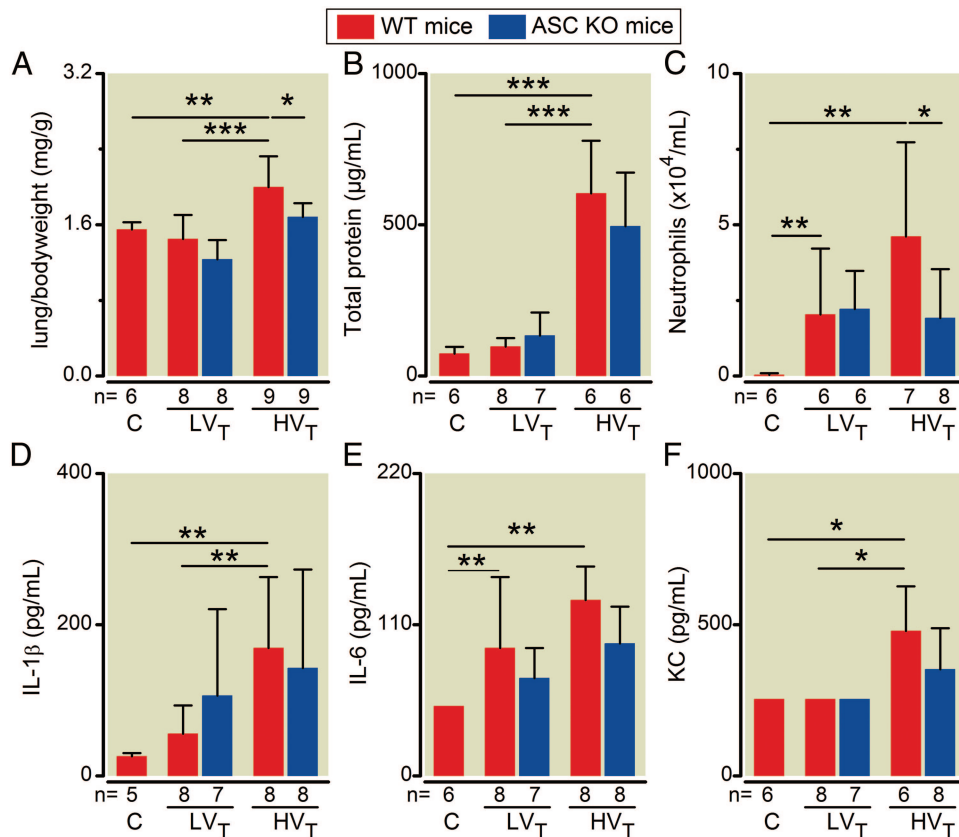
#### MV Results in NLRP3-Dependent Activation of Caspase-1

The NLRP3 inflammasome serves as a platform for the activation of caspase-1, which involves autocatalytic processing of the 45-kDa pro-caspase-1 to generate two subunits, p20 and p10. To obtain direct evidence that caspase-1 is activated in an NLRP3-dependent fashion during MV, we performed immunoblots of lung homogenate samples of ventilated WT and NLRP3 KO mice (fig. 7). In contrast with the nonventilated control group, LV<sub>T</sub> and HV<sub>T</sub> ventilation induced the activation of caspase-1 as detected by the appearance of the p10 subunit of caspase-1. HV<sub>T</sub> ventilated NLRP3-deficient mice did not display active caspase-1, indicating that caspase-1 activation in the WT animals occurred *via* a NLRP3-dependent manner.

#### VILI Is Attenuated in Glibenclamide-treated Mice

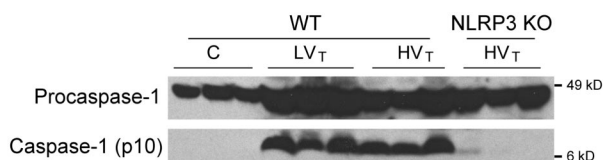
As expected, glibenclamide treatment reduced glucose levels compared with those of vehicle-treated mice; blood pressure levels did not differ between the groups (data not shown). VILI was attenuated in mice treated with glibenclamide (fig. 8). Relative lung weights and total protein levels in BALF were reduced compared with those of vehicle-treated mice ( $P$  less than 0.05) (fig. 8, A and B). Moreover, pulmonary





**Fig. 6.** Relative lung weights (A), total protein in bronchoalveolar lavage fluid (BALF) (B), neutrophil influx (C), interleukin (IL)-1β (D), IL-6 (E) and keratinocyte-derived chemokine (KC) (F) levels in BALF. Apoptosis-associated speck-like protein (ASC) knockout (KO) (blue bars) and wild-type (WT) mice (red bars) were ventilated for 5 h with low tidal volumes (LV<sub>T</sub>) (approximately 7.5 ml/kg) or high tidal volumes (HV<sub>T</sub>) (approximately 15 ml/kg) and spontaneously breathing WT mice (C) served as the control group. Data represent mean (SD), number (n) of mice per group is specified in the figure. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

neutrophil influx and BALF IL-6 levels were significantly lower ( $P$  less than 0.05) (fig. 8, C and E). It is possible that the protective effect of glibenclamide treatment was attributable to effects of glibenclamide apart from inhibiting the NLRP3 inflammasome. Therefore we treated NLRP3 KO mice with glibenclamide. The protective effect disappeared: no statistical significant differences were found in relative lung weights, total protein levels, neutrophil influx, or cytokine and chemokine levels in BALF (table 2).



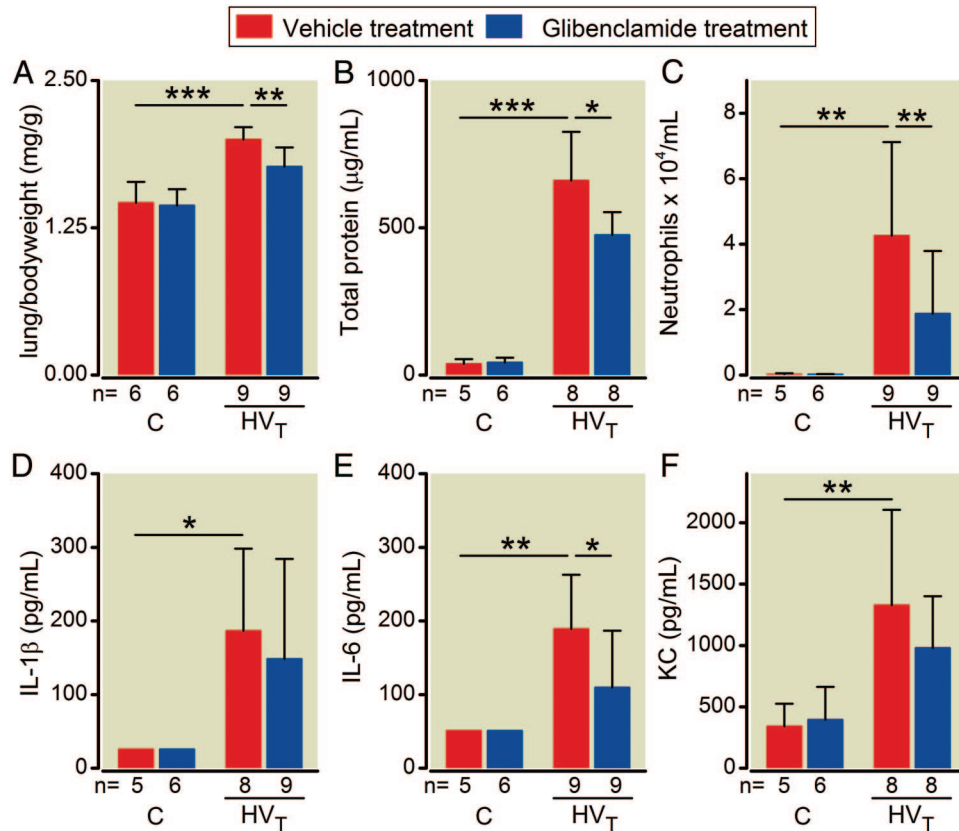
**Fig. 7.** Lung homogenate samples of control (C) wild-type (WT) mice, low tidal volume (LV<sub>T</sub>) (approximately 7.5 ml/kg) ventilated WT mice, and high tidal volume (HV<sub>T</sub>) (approximately 15 ml/kg) ventilated WT and HV<sub>T</sub> ventilated NLRP3 knockout (KO) mice were immunoblotted with antibodies against the caspase-1 and the p10 subunit of caspase-1. Data depict three mice per group.

## Discussion

This study is the first to reveal involvement of NLRP3 inflammasome signaling in inflammation induced by MV. Using human lung brush samples and BALF cells, a murine VILI model ventilating WT, NLRP3 KO, and ASC KO mice and by blockade of the IL-1β or the NLRP3 pathway in HV<sub>T</sub> MV, we demonstrate that (1) NLRP3 is up-regulated in human alveolar macrophages by MV, that (2) NLRP3 and ASC mRNA are up-regulated in lung tissue by MV in mice, that (3) NLRP3 ligand uric acid and the end product of NLRP3 inflammasome activation, IL-1β, are found in BALF of ventilated mice, that (4) MV activates caspase-1 and (5) VILI induced by short-term HV<sub>T</sub> is attenuated, but not abolished, in NLRP3 and ASC KO mice, and that (6) pharmacologic inhibition of the IL-1β and NLRP3 inflammasome pathway results in reduced VILI.

BALF IL-1β levels increased due to injurious ventilation, which confirms the previously demonstrated role for the IL-1β pathway in VILI.<sup>14–17</sup> IL-1β binding to its signaling receptor results in activation of nuclear factor-κB<sup>28</sup>, an important factor perpetuating the inflammatory response. To release IL-1β, a two-step stimulation process is necessary. Step one involves NF-κB-dependent production of pro-





**Fig. 8.** Relative lung weights (A), total protein in bronchoalveolar lavage fluid (BALF) (B), neutrophil influx (C), interleukin (IL)-1β (D), IL-6 (E), and keratinocyte-derived chemokine (KC) (F) levels in BALF. Vehicle-treated (red bars) and glibenclamide-treated mice (50 mg/kg, blue bars) were ventilated for 5 h with high tidal volumes (HV<sub>T</sub>) (~15 ml/kg), spontaneously breathing mice served as the control group (C). Data represent mean (SD), number (n) of mice per group is specified in the figure. \**P* < 0.05, \*\* *P* < 0.01, \*\*\* *P* < 0.001.

IL-1β *via*, for example, TLR-signaling. Involvement of the TLR4 pathway in VILI has previously been established by Vaneker *et al.*<sup>4</sup> This group also reported reduced IL-1β concentrations in lung homogenates of the TLR4 KO mice.

The second step requires the activation of NLRP3 by pathogen-associated molecular patterns or DAMPs and will lead to the cleavage of pro-IL-1β into mature IL-1β by active

caspase-1. Many studies elaborate on tissue injury causing sterile inflammation *via* the release of endogenous danger molecules. It has been shown that injurious MV leads to ATP and hyaluronan release in BALF.<sup>10,11</sup> Extracellular ATP binds to purinergic receptor P2X7 and it thereby activates the NLRP3 inflammasome.<sup>12</sup> Hyaluronan is an important structural component of the extracellular matrix. When released from injured tissue hyaluronan can associate with CD44 and stimulate TLR4, leading to increased pro-IL-1β production. Moreover, endocytosis of CD44-hyaluronan and subsequently the processing of hyaluronan into small fragments can activate NLRP3.<sup>13</sup> Our study adds the release of the DAMP uric acid in BALF due to MV. Previous research demonstrated uric acid release in BALF in a bleomycin-induced lung injury model.<sup>29</sup> Moreover, locally administered uric acid crystals induced NLRP3 inflammasome-dependent acute lung inflammation.<sup>29</sup> Hence, two independent processes are involved in the production and secretion of mature IL-1β and both processes can be activated during MV. In line, activation of caspase-1 due to MV is demonstrated by the presence of the p10 subunit in lung homogenate samples of ventilated WT mice. Our data reveal that caspase-1 activation in HV<sub>T</sub> MV in mice is dependant on NLRP3.

**Table 2.** The Effect of Glibenclamide in High Tidal Ventilated NLRP3 KO Mice

Parameters	High Tidal Ventilation NLRP3 KO Mice	
	Vehicle	Glibenclamide
Relative lung weights (mg/g)	1.97 [0.11]	2.16 [0.33]
Total protein (mg/ml)	0.42 [0.06]	0.41 [0.10]
Neutrophils (%)	36.8 [18.0]	24.4 [28.0]
IL-1β (ng/ml)	0.30 [0.22]	0.17 [0.13]
IL-6 (ng/ml)	0.21 [0.04]	0.16 [0.06]
KC (ng/ml)	0.34 [0.14]	0.24 [0.05]

IL = interleukin; KC = keratinocyte-derived chemokine; KO = knockout.

Granulocytes, monocytes, dendritic cells, T and B cells, epithelial cells, and osteoblasts all express NLRP3, suggesting an important role for NLRP3 in primary defense mechanisms of the body.<sup>30,31</sup> To further specify cell type and tissue that express NLRP3 inflammasome we analyzed human bronchial epithelial cells and alveolar macrophages. NLRP3 mRNA levels were not detectable in epithelial cells, suggesting that no functional NLRP3 inflammasome can be formed. ASC mRNA levels in epithelial cells were significantly up-regulated ( $P$  less than 0.01) due to 5 h of MV. ASC, not only involved in signaling of the NLRP3 inflammasome, is also an important adaptor protein for the NLRP1, NLRC4, and AIM2 inflammasomes.<sup>32</sup> NLRP1 senses anthrax lethal toxin, NLRC4 detects virulence factors from gram-negative pathogens, and AIM2 is activated by cytosolic double-stranded DNA.<sup>32</sup> Which member besides NLRP3 pairs with ASC in VILI pathogenesis is an interesting topic for future research. Previous research demonstrated strong staining of NLRP1, and not NLRP3, in respiratory epithelium.<sup>30</sup> In our VILI model, we found that MV resulted in enhanced NLRP3 and ASC mRNA levels in whole lung homogenates. This increase could be due to influx of inflammatory cells. However, we demonstrated in patients that MV induces an up-regulation of NLRP3 mRNA in alveolar macrophages. This suggests that local, immune cell influx-independent, increased NLRP3 protein expression is possible.

The absence of NLRP3 or ASC both attenuated VILI during HV<sub>T</sub> ventilation in our model. However, the reduction of inflammation was stronger in NLRP3 KO mice than the reduction observed in the ASC KO mice. Differences in protection between mice lacking NLRP3 or ASC were previously shown in an *in vivo* renal ischemic acute tubular necrosis model.<sup>26</sup> Moreover, a more recent study demonstrated an inflammasome-independent role for NLRP3 in renal ischemia-reperfusion injury.<sup>33</sup> These studies suggest that NLRP3 may have an ASC-independent role in inflammation associated with tissue injury.

Interestingly, the lack of NLRP3 inflammasome not only attenuated IL-1 $\beta$  but also IL-6 levels. Reduced IL-6 levels in NLRP3 KO and ASC KO mice were previously demonstrated in a bleomycin-induced lung fibrosis model.<sup>29</sup> IL-6 is a pleiotrophic cytokine with an important role in regulating the immune response; inflammation and hematopoiesis levels are increased in (pre-) clinical VILI studies.<sup>2,17,34,35</sup> Crestani *et al.* showed in an *in vitro* model that IL-6 production by rat alveolar type II cells and human pulmonary epithelial-derived cells (A549 cell line) highly increased with IL-1 $\beta$  stimulation.<sup>36</sup> IL-6 concentrations are sensitive to IL-1 $\beta$  protein and infusion of IL-1 $\beta$  in humans led to increasing IL-6 plasma levels in a dose-dependent fashion.<sup>28</sup> This previously described direct control of IL-6 by IL-1 $\beta$  could partly explain the effect on IL-6 levels shown in our study.

A strong effect on neutrophil influx in BALF was demonstrated in NLRP3 and ASC KO mice; IL-1ra-treated animals also showed a reduced neutrophil influx. However, we did

not detect significant differences in chemokine levels in BALF. This discrepancy could be explained by reduced transendothelial migration of neutrophils due to attenuated levels of adhesion molecules. IL-1 $\beta$  has been shown to up-regulate intercellular adhesion molecule-1.<sup>37</sup>

Glibenclamide is widely used to treat type 2 diabetes. It blocks K<sub>ATP</sub> channels in pancreatic  $\beta$  cells leading to insulin secretion. Recent studies indicate that glibenclamide also exerts antiinflammatory effects: glibenclamide treatment was associated with a survival benefit during lipopolysaccharide-induced lethality in mice<sup>22</sup> and also in patients with gram-negative sepsis.<sup>38</sup> Lamkanfi *et al.* demonstrated an inhibitory effect of glibenclamide on NLRP3 inflammasome activation.<sup>22</sup> In our VILI model, glibenclamide reduced lung edema, neutrophil influx, and IL-6 levels in BALF, findings consistent with the KO experiments. Unexpectedly, we did not detect reduced IL-1 $\beta$  concentrations by glibenclamide treatment, a finding that we cannot explain. In addition to inhibiting the inflammasome, glibenclamide reduces glycemia and influences cardiovascular parameters. Although blood pressure levels were not significantly different between the groups, glibenclamide treatment did affect glycemia. To exclude “off-target” effects such as reduction of glycemia, we treated NLRP3 KO mice with glibenclamide: the protective effect of glibenclamide vanished in NLRP3 KO mice, suggesting that the antiinflammatory effect is in part dependent on NLRP3 activation.

Involvement of the NLRP3 inflammasome pathway was clearly present in the HV<sub>T</sub> group, in which VILI evidently was induced. Although V<sub>T</sub>s have declined gradually in the past decade, there is still underuse of low tidal ventilation in hypoxic and acidotic patients.<sup>39</sup> Moreover, ventilation with LV<sub>T</sub> may still lead to overdistension of healthy lung areas in patients with acute lung injury.<sup>6</sup> Therefore, our HV<sub>T</sub> group still reveals relevant information on lung injury caused by MV. Mice ventilated with LV<sub>T</sub> also developed VILI, although to a lesser extent. This finding is in accordance with previous animal studies, where short-term LV<sub>T</sub> ventilation resulted in inflammation<sup>4</sup> and severely affected the pulmonary extracellular architecture.<sup>40</sup> LV<sub>T</sub> ventilation may promote development of atelectasis and subsequently atelectrauma, which could explain the inflammation seen in murine setting. Activation of the NLRP3 inflammasome during short-term LV<sub>T</sub> ventilation was demonstrated by active caspase-1. However, our data suggest that this pathway is less relevant for the minimal inflammatory changes seen in these mice.

Our study has several limitations. First, lung brushes were obtained from the right middle lobe or lingula. Because most VILI is thought to occur in distal airways, these samples therefore may not be a perfect representative. Second, we used BALF and lung brush samples obtained from a previously published study that was not powered to detect differences in mRNA levels between both ventilation groups. Therefore, we analyzed the effect of MV, irrespective of which ventilation strategy was used. Third, we used a short-

term murine MV model; we cannot extrapolate what the long-term effects would be. Fourth, the anesthesia mix we used may have immunomodulating effects.<sup>41</sup> In our VILI model all animals, except the control mice, received the same amount of anesthesia. A control group of sham-operated, intratracheally intubated, spontaneously breathing mice during anesthesia would be ideal but is not possible because severe hypoventilation and deep respiratory acidosis will result. Fifth, as stated in the figures, some murine measurements were not collected due to technical reasons.

In conclusion, our study offers a novel mechanism that in part explains how MV modulates the innate immune response in the lung. We demonstrate the participation of the intracellular danger sensor NLRP3 inflammasome in VILI. Further studies examining the role of innate immunity in the regulation of VILI are warranted.

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