The NLRP3 Inflammasome Is Required for the Development of Hypoxemia in LPS/Mechanical Ventilation Acute Lung Injury

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Abstract

IL-1 β is a potent proinflammatory cytokine that is implicated in the pathogenesis of acute respiratory distress syndrome. We hypothesized that LPS and mechanical ventilation (MV) together could lead to IL-1 β secretion and the development of acute lung injury (ALI), and that this process would be dependent on caspase-1 and the nucleotide binding domain and leucine-rich repeat (NLR) pyrin domain containing 3 (NLRP3) inflammasome activation. The objectives of this study were to determine the specific role of IL-1 β , caspase-1, and the NLRP3 inflammasome in a two-hit model of ALI due to LPS plus MV. We used a two-hit murine model of ALI in which both inhaled LPS and MV were required for the development of hypoxemia, pulmonary neutrophil infiltration, and alveolar leakage. *Nlrp3*-deficient and *Casp1*-deficient mice had significantly diminished IL-1 β levels in bronchoalveolar lavage fluid, and were specifically protected from hypoxemia, despite similar

alveolar neutrophil infiltration and leakage. The IL-1 receptor antagonist, Anakinra, significantly improved the specific development of hypoxemia without significant effects on neutrophil infiltration or alveolar leakage. MV resulted in increased bronchoalveolar lavage extracellular ATP and alveolar macrophage apoptosis as triggers of NLRP3 inflammasome activation. NLRP3 inflammasome activation and IL-1 β production play a key role in ALI caused by the combination of LPS and MV, particularly in the hypoxemia associated with acute respiratory distress syndrome. Blocking IL-1 signaling in this model specifically ameliorates hypoxemia, without affecting neutrophil infiltration and alveolar leakage, disassociating these readouts of ALI. MV causes alveolar macrophage apoptosis, a key step in the activation of NLRP3 inflammasome and production of IL-1 β .

Keywords: acute lung injury; acute respiratory distress syndrome; nucleotide binding domain and leucine-rich repeat pyrin domain containing 3 inflammasome; IL-1 β

Acute respiratory distress syndrome (ARDS), or acute lung injury (ALI), is a clinical syndrome of severe lung failure defined by an acute onset, bilateral opacities on chest radiograph, respiratory failure not fully explained by cardiac failure or fluid overload, and a ratio of arterial oxygen to inspired oxygen of less than 200 mm Hg with positive end-expiratory pressure (PEEP) of greater

than or equal to 5 cm H₂O (1). Mortality rates in ARDS have declined, but even with best supportive care and lung-protective ventilation strategies, the 60-day mortality rate reported by recent ARDS Network clinical trials is approximately 25% (2). Although progress has been made in understanding the mechanisms underlying the pathogenesis of this disease, to date no

therapies, other than low tidal volume ventilation (3), have been shown to improve mortality (4, 5).

There are multiple causes of ARDS, including, most commonly, pneumonia, but also sepsis, aspiration, hemorrhagic shock, and other systemic inflammatory conditions, such as pancreatitis (5). Sepsis is an independent risk factor for the

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Clinical Relevance

A “two-hit” model, in which two separate insults (most commonly, LPS and mechanical ventilation [MV]) are required for the development of acute lung injury (ALI), has been studied extensively. However, the specific mechanisms underlying the development of ALI through synergy of MV and LPS are still unknown. In this study, we show that the two-hit model of ALI generated the two signals required for IL-1 β secretion, which was specifically important for the development of hypoxemia. We found that LPS inhalation followed by MV-induced alveolar macrophage apoptosis and nucleotide binding domain and leucine-rich repeat (NLR) pyrin domain containing 3 (NLRP3) inflammasome activation, triggering IL-1 β production. This chain of events resulted in hypoxemia that was dependent on IL-1 signaling. These data suggest a novel role for IL-1 β and the NLRP3 inflammasome, specifically in the hypoxemia associated with ALI and ARDS.

mechanical ventilation (MV) (6). A “two-hit” animal model—in which two separate insults (most commonly, LPS and MV) are required for the development of ALI—has been studied extensively (7–14). However, the specific mechanisms underlying the development of ALI through synergy of MV and LPS are still unknown. Several hypotheses focus on alterations in transcriptional regulation of inflammatory genes (7, 10, 15–17).

IL-1 β has been widely implicated as having a key role in the development of ALI (18–26). However, the molecular mechanisms activating the IL-1 β pathway in ALI are still unclear. Alveolar macrophages (AMs) are an important source of IL-1 β in ALI; AMs from patients with ARDS secrete much higher levels of IL-1 β (21), and AM depletion results in significantly diminished IL-1 β secretion and injury in animal models of ventilator-induced and ricin toxin-mediated ALI (27, 28).

Production of IL-1 β is tightly controlled and requires the activation of two different pathways. First, the ligation of pattern-

recognition receptors, such as Toll-like receptors by conserved microbial structures, such as LPS, induces production of pro-IL-1 β (the inactive precursor of IL-1 β). Second, pro-IL-1 β is cleaved by active caspase-1 to be released in its active form, IL-1 β . Multiprotein complexes, called “inflammasomes,” are responsible for the activation of caspase-1 (and therefore IL-1 β production) (29). The nucleotide binding domain and leucine-rich repeat (NLR) pyrin domain containing 3 (NLRP3) inflammasome is the most well-characterized inflammasome, and can be activated by any of a diverse number of “danger signals,” including potassium efflux, extracellular ATP (eATP), uric acid crystals, asbestos, and reactive oxygen species (ROS) (29). We recently demonstrated that, in macrophages, mitochondrial dysfunction and subsequent apoptosis is the central hub through which these diverse stimuli lead to NLRP3 inflammasome activation (30). The NLRP3 inflammasome is important both in immune responses to infectious and noninfectious agents (31–35), and in the development of metabolic inflammatory diseases (36–38).

In the current study, we investigated whether a two-hit model of ALI would also generate the two signals required for IL-1 β secretion. We found that LPS inhalation followed by MV-induced AM apoptosis and NLRP3 inflammasome activation. This chain of events resulted in hypoxemia that was dependent on IL-1 signaling. These data suggest a novel role for IL-1 β and the NLRP3 inflammasome, specifically in the hypoxemia associated with ALI and ARDS induced by LPS and MV.

Materials and Methods

Mice

Casp1^{−/−} mice (39) were kindly provided by Dr. Richard A. Flavell (Yale University, New Haven, CT). *Nlrp3*^{−/−} mice (40) were generously provided by Dr. Katherine A. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice used were 8–12 weeks of age and were male.

Administration of Intratracheal Reagents

Mice were anesthetized with isoflurane (Piramal Healthcare, Bethlehem, PA),

suspended from incisors, and LPS or PBS was administered intratracheally. LPS from *Escherichia coli* (0111:B4, ultrapure; InvivoGen, San Diego, CA) was diluted in PBS (Corning, Manassas, VA) to a concentration of 100 μ g/ml, and control mice were given PBS. Both groups received a volume of 2 μ l/g mouse weight, and volumes were in the range of 45–60 μ l for all experiments. In other experiments, the recombinant IL-1 receptor antagonist (IL-1Ra), Anakinra (Kineret, Amgen, Thousand Oaks, CA), was diluted to 10 mg/ml and LPS at 100 μ g/ml in PBS. Control mice received LPS only.

Intubation and MV

Mice to be mechanically ventilated were anesthetized with intraperitoneal injection of a mix of ketamine (Vedco Inc., Saint Joseph, MO) and dexmedetomidine (Pfizer, Irvine, CA) (75 mg/kg and 0.5 mg/kg, respectively). Mice were orotracheally intubated and ventilated using an Inspira volume-controlled small animal ventilator (Harvard Apparatus, Holliston, MA) with a tidal volume of 20 ml/kg and a respiratory rate of 70 breaths per minute, with zero PEEP. Subcutaneous PBS (500 μ l) was administered at the onset of MV and every 3 hours of MV thereafter. Repeat doses of 50 mg/kg of ketamine were administered as subcutaneous injections as needed (usually every 2–3 h). Mice were kept warm on a 38°C heating pad (Hallowell EMC, Pittsfield, MA). Control mice were not anesthetized, and remained in their cages until the end of MV, at which time they were killed together with ventilated mice. In experiments combining both intratracheal agents and MV, mice were first administered intratracheal agents and returned to their cages. After 90 minutes, mice were anesthetized and placed on MV for a total of 4 hours.

Pulse Oximetry

Arterial oxygen saturations were measured in mechanically ventilated mice using the MouseOX pulse oximeter (STARR Life Sciences, Oakmont, PA).

Bronchoalveolar Lavage

The trachea was exposed and cannulated with a 22-G intravenous cannula. PBS (0.5 ml) with 2 mM EDTA was instilled and aspirated twice. Cells were separated from supernatant and immediately

analyzed for cell counts or flow cytometry. Supernatants were stored at -80°C for use in ELISAs.

Statistical Analysis

All data were analyzed with the Prism 4.03 statistical program (GraphPad Software, Inc., La Jolla, CA). To compare differences in bronchoalveolar lavage (BAL) cell numbers, cytokine levels, and BAL/serum protein ratios, the two-tailed Student's *t* test (at 95% confidence interval) was used to compare unpaired samples between experimental groups. For experiments involving three or more groups, we used one-way ANOVA with Tukey's *post hoc* test. For experiments comparing oxygen saturations between groups at different time points, we used the two-way ANOVA with Bonferroni's *post hoc* test. A *P* value less than 0.05 was considered statistically significant.

Ethics Statement

The study was approved by and all animal experiments were conducted according to the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee guidelines.

Results

Intratracheal LPS and MV Trigger IL-1 β Secretion, Increased Neutrophils, and Alveolar Leakage

We sought to replicate conditions under which a two-hit mechanism of sepsis and MV was required for the development of ALI. C57BL/6 male mice were administered LPS intratracheally (200 ng/g body weight) or PBS. Some mice were allowed to continue breathing spontaneously (LPS-SB or PBS-SB). Other mice were orotracheally intubated 90 minutes after intratracheal LPS or PBS and placed on MV with tidal volumes of 20 ml/kg and zero PEEP for 4 hours (LPS-MV or PBS-MV; Figure 1A). We found that the combination of LPS and MV was required in order for ALI to develop in our model. Mice that received both LPS and MV showed increased concentrations of BAL IL-1 β , consistent with our hypothesis that both signals are required for inflammasome activation and IL-1 β secretion. We confirmed this finding by Western blot analysis (Figure 1B), and found a marked increase in mature, secreted IL-1 β only in the setting of both LPS plus MV. In contrast, BAL TNF- α and

IL-6 concentrations increased with LPS alone, and were increased further by the combination of LPS plus MV (Figure 1C). BAL total cells and neutrophils were significantly increased with the combination of LPS plus MV (Figure 1D). Neutrophil chemokines, keratinocyte-derived cytokine (KC) and macrophage inflammatory protein 2 (MIP2), were also significantly increased in mice that received LPS plus MV (Figure 1E). Alveolar leakage, as assessed by BAL/serum total protein ratios, was also significantly elevated by the combination of LPS plus MV (Figure 1F). Neither LPS nor MV alone significantly increased neutrophil infiltration, chemokine concentrations, or alveolar leakage, with the exception of a slight increase in MIP2 with LPS alone. The combination of both treatments was required to generate a significant response, indicating that, in this model, it takes "two hits" to induce ALI.

MV Increases BAL AM Apoptosis

IL-1 β secretion in macrophages requires NF- κ B signaling (usually via Toll-like receptor signaling [i.e., LPS]) and a second signal that initiates the NLRP3 inflammasome formation and activation. We recently demonstrated that, in macrophages, activation of the NLRP3 inflammasome is achieved through the mitochondrial oxidative DNA damage, and is associated with the induction of apoptosis (30). On the basis of these data, we wondered whether MV could be inducing AM apoptosis in our experimental system. BALF cells of mice that underwent MV for 0 or 6 hours were assessed for mitochondrial dysfunction by measuring the membrane potential (a hallmark of apoptosis) by flow cytometry. The mitochondrial membrane potential (Figure 2A) was significantly reduced in AM after 6 hours of MV compared with controls. Further investigation showed a steady increase in AM apoptosis over time on MV, as measured by annexin V staining (Figures 2B and 2C). Importantly, control mice that received only anesthetic for 6 hours did not show increased AM apoptosis (see Figure E1 in the online supplement). It has been suggested that neutrophil infiltration into the lungs during ALI may have an effect on macrophage function (41). However, the increase in macrophage apoptosis was

detected after as few as 2 hours of MV, whereas neutrophil infiltration is measurable only after 4 and 6 hours of MV (Figure 2D). This suggests that MV-induced macrophage apoptosis precedes MV-induced neutrophil infiltration of the lung.

Several stimuli are known to induce macrophage apoptosis, including ROS, uric acid, and eATP (29). Earlier work had suggested that MV could increase BAL eATP (42), and we wondered if we might also find increased eATP in our model. We measured the eATP concentration in BALF from mice after MV and indeed found a significant increase in BAL eATP in comparison to control, nonventilated mice (Figure 2E). These data clearly suggest that MV leads to eATP release, which could then subsequently induce AM apoptosis and trigger NLRP3 activation (30).

The NLRP3 Inflammasome Is Not Involved in Neutrophil Recruitment or Alveolar Leakage in ALI

We hypothesized that activation of the NLRP3 inflammasome and subsequent IL-1 β secretion is important in the development of ALI due to sepsis plus MV. Numerous studies have indicated that neutrophil recruitment is a key functional host defense in response to inflammasome activation during infection (43–46). We predicted that *Nlrp3*-deficient mice treated with LPS plus MV would have decreased BAL neutrophils and alveolar leakage compared with wild-type mice. As expected, we found that *Nlrp3*-deficient mice were significantly defective in IL-1 β secretion in BALF on LPS plus MV (Figure 3A). However, to our surprise, the *Nlrp3*-deficient mice did not exhibit decreased neutrophil migration, chemokine levels, or alveolar leakage after LPS plus MV (Figures 3B–3D). To rule out the potential involvement of other inflammasomes, we performed the same experiment using *Casp1*-deficient mice. As expected, we saw a significant reduction in the amount of secreted IL-1 β in the BAL fluid from *Casp1*-deficient mice (Figure 4A), but this did not correspond to a decrease in neutrophil infiltration, chemokine levels, or alveolar leakage (Figures 4B–4D). Therefore, we conclude that IL-1 β secretion does not correlate directly with the degree of neutrophil infiltration or alveolar leakage in this two-hit ALI model.

Figure 1. LPS plus mechanical ventilation (MV) increase IL-1 β secretion in acute lung injury (ALI). (A) A total of 200 ng/g body weight (intratracheal) LPS or PBS was administered to mice. After 90 minutes, some mice were allowed to continue breathing spontaneously (SB, spontaneous breathing; PBS-SB and LPS-SB) and others were anesthetized and placed on MV with tidal volumes of 20 ml/kg and zero positive end-expiratory pressure (PEEP) for 4 hours (PBS-MV and LPS-MV). (B) Bronchoalveolar lavage (BAL) fluid (BALF) and lung homogenate were assayed for mature IL-1 β and pro-IL-1 β proteins, respectively, by immunoblot ($n = 2$ mice/condition). BALF was assayed for: (C) IL-1 β , TNF- α , and IL-6 concentrations; (D) total cells, neutrophils, and macrophages; and (E) chemokines, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP2). (F) Serum and BAL protein concentrations were measured for determination of BAL/serum protein ratios ($n = 8$ –10/group). Data are representative of three separate experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. PMN, polymorphonuclear cells.

The NLRP3 Inflammasome Is Required for Hypoxemia in LPS-plus-MV-Induced ALI

A cardinal feature of ALI and ARDS is refractory hypoxemia (47). Animals

receiving LPS and MV developed progressive, severe hypoxemia, whereas animals receiving PBS and MV maintained average oxygen saturations above 90% throughout the 4 hours of MV (Figure 5A).

In contrast, mice that received only LPS were not hypoxic when assessed at 5 hours after LPS, but developed hypoxemia when placed on MV (Figure E2). Mice that received PBS did not develop significant

Figure 2. MV causes increased extracellular ATP (eATP) and alveolar macrophage (AM) apoptosis. Mice underwent MV for 0, 2, 4, or 6 hours, and BAL cells were analyzed via flow cytometry. (A) Mitochondrial membrane potential in AMs was significantly reduced after 6 hours of MV. (B and C) AM apoptosis increased over time with MV, as measured by annexin staining. Green indicates Ly6G-positive cells, denoting PMNs, and red indicates F480-positive cells, denoting macrophages. (D) Increases in BAL neutrophils can be detected at 4 and 6 hours. (E) eATP concentrations were increased in cell-free BALF after 6 hours of MV ($n = 6$ –10/group). Data are representative of two separate experiments (A and E) and four separate experiments (B–D). *** $P < 0.001$, * $P < 0.05$.

hypoxemia, with or without MV. To corroborate the noninvasive MouseOx oxygen saturations data, arterial blood gases were obtained from the carotid artery at 4 hours of MV in mice receiving LPS and MV or PBS and MV. Blood gases showed similar results and a high degree of correlation ($R^2 = 0.9303$; $P < 0.0001$) with the MouseOx readings (Figure E4). In light of these data, we investigated whether the induction of the NLRP3 inflammasome during LPS plus MV played a specific role in the development of hypoxemia. Both *Casp1*-deficient and *Nlrp3*-deficient mice were significantly protected from

hypoxemia in LPS plus MV compared with wild-type controls (Figures 5B and 5C). These data strongly suggested that NLRP3 inflammasome activation is specifically required for the development of hypoxemia, but not for neutrophil infiltration or alveolar leakage in our LPS-plus-MV lung injury model.

Because this finding is counterintuitive, we investigated whether neutrophil infiltration into the lungs is required for the development of hypoxemia, and found that neutrophil depletion had no effect on the development of hypoxemia in our model (Figure E3).

Intratracheal IL-1Ra, Anakinra, Significantly Improves the Development of Hypoxemia in ALI

The NLRP3 inflammasome contributes to not only IL-1 β secretion, but also IL-18 secretion. To determine whether the NLRP3 inflammasome-associated hypoxemia was due to IL-1 β or IL-18, we administered the IL-1Ra, Anakinra, intratracheally with LPS and compared the results to LPS administration alone after 4-hour MV (Figure 6A). Both groups developed progressive decreases in oxygen saturation over time on MV, but the desaturations were significantly less in mice treated with Anakinra compared with controls (LPS only; Figure 6B). Consistent with the *Nlrp3*- and *Casp1*-deficient mouse data shown previously here, we did not observe any beneficial effects of the IL-1Ra on other readouts of ALI, including neutrophil migration, chemokine levels in the BALF, and alveolar leakage (Figures 6C–6E). These data indicate that the hypoxemia seen in mice treated with LPS plus MV is IL-1 β dependent.

Discussion

We used an LPS/MV two-hit model of ALI to investigate the synergistic molecular mechanisms involved in the development of ARDS specifically in the setting of sepsis and MV (6). Our key findings are that MV causes AM apoptosis, and that activation of the NLRP3 inflammasome and IL-1 β secretion specifically play a role in the hypoxemia associated with this model of LPS-plus-MV-induced ALI.

Recent data have shown that NLRP3 inflammasome-dependent IL-1 β and IL-18 may be important in the development of ventilator-induced lung injury (VILI) (25, 48–52). Earlier work had suggested that IL-1 directly contributes to the alveolar barrier dysfunction and neutrophil recruitment in VILI induced with injurious volumes (30 ml/kg for 3 h), and that blocking IL-1 signaling in IL-1R-deficient mice or with exogenous IL-1R antagonism ameliorated ALI in this model (24). More recently, Kuipers and colleagues (25) demonstrated a role for the NLRP3 inflammasome in VILI. These researchers demonstrated increased transcription of NLRP3 in both mice and humans after high-tidal volume MV (15 ml/kg for 5 h). In addition, they demonstrated

Figure 3. The NLRP3 inflammasome is not required for neutrophil recruitment into the lung during LPS plus MV. A total of 200 ng/g body weight (intratracheal) LPS was administered to C57BL/6 wild-type (closed circles) and nucleotide binding domain and leucine-rich repeat (NLR) pyrin domain containing 3 (NLRP3)-deficient ($-/-$; open triangles) mice. After 90 minutes, all mice were placed on MV with tidal volumes of 20 ml/kg and zero PEEP for 4 hours. BALF was assayed for: (A) IL-1 β , TNF- α , and IL-6 concentrations; (B) total cells, neutrophils, and macrophages; and (C) chemokines, KC and MIP2. (D) Serum and BAL protein concentrations were measured for determination of BAL/serum protein ratios ($n = 7$ –8/group). Data are representative of two separate experiments. * $P < 0.05$, *** $P < 0.001$.

increased BAL uric acid, a well known trigger for the NLRP3 inflammasome, with MV. In that model of VILI, *Nlrp3*-deficient mice and pharmacologic inhibition of the NLRP3 inflammasome and IL-1 pathways decreased neutrophil infiltration and lung edema, although not consistently across all conditions. Dolinay and colleagues (52) focused on the role of IL-18, another NLRP3 inflammasome-dependent cytokine, and showed that increased IL-18 levels in critically ill patients correlated with mortality. These authors also demonstrated that blockade of IL-18 signaling resulted in diminished neutrophil infiltration and edema in a mouse model of VILI (12 ml/kg

tidal volume for 8 h). These studies brought to light the contribution of the NLRP3 inflammasome in the development of injury related to high-tidal volume MV.

In our study, we focused on the role of the NLRP3 inflammasome using a different, two-hit model of ALI in which both intratracheal LPS and high-tidal volume MV (20 ml/kg $\times$ 4 h) were required to develop signs of injury. We chose this model, because we were interested in studying the specific mechanisms involved in the development of ARDS in patients with severe sepsis or pneumonia who are placed on MV. We were interested in the role of

NLRP3 inflammasome and IL-1 β in this well known clinical scenario, because of the striking similarity between the two “hits” seemingly required for the development of ARDS, and the two-signal control of IL-1 β secretion. We used low-dose LPS (0.2 mg/kg) to ensure that we were modeling the synergy between the two insults rather than the augmentation of one. Other groups have used lower (5–50 μ g/kg) (53) or much higher (1–15 mg/kg) (9, 54) doses of inhaled LPS in two-hit studies. Our Western blot data clearly demonstrate an absence of IL-1 β secretion with the low-dose LPS or MV alone, and a robust secretion of IL-1 β with the combination of MV and LPS,

Figure 4. Caspase-1 is not required for neutrophil recruitment into the lung during LPS plus MV. A total of 200 ng/g body weight (intratracheal) LPS was administered to C57BL/6 wild-type (closed circles) and *Casp1*-deficient (—/—; open squares) mice. After 90 minutes, all mice were placed on MV with tidal volumes of 20 ml/kg and zero PEEP for 4 hours. BALF was assayed for: (A) IL-1 β , TNF- α , and IL-6 concentrations; (B) total cells, neutrophils, and macrophages; and (C) chemokines, KC and MIP2. (D) Serum and BAL protein concentrations were measured for determination of BAL/serum protein ratios ($n = 7$ –8/group). Data are representative of two separate experiments. *** $P < 0.001$.

demonstrating the synergistic effect that we had hypothesized to exist.

We found that NLRP3 activation and IL-1 β production were not associated with neutrophil infiltration and alveolar edema in our model, in contrast to earlier work studying the role of the NLRP3 inflammasome in VILI (24, 25). We believe that this discrepancy can be explained by increased levels of inflammation seen in our two-hit model as compared with VILI models. The degree of neutrophil infiltration seen in our study was 10-fold or more higher than that seen in the VILI studies (24, 25, 52), and is consistent with polymorphonuclear cell (PMN) levels seen in other LPS-plus-MV models (17, 53, 55). Edema is more difficult to compare

between studies; there are a number of well established methods of measuring alveolar edema that prevent accurate comparison between studies. Nevertheless, it is notable that levels of edema seen in VILI studies are decreased by blockade of the NLRP3 inflammasome or IL-1 β under some, but not all conditions (24, 25), and differences in edema can be found even when no differences are detected in IL-1 β levels (25). It was recently reported that allopurinol treatment (to decrease uric acid activation of the NLRP3 inflammasome) attenuated alveolar edema, but not PMN infiltration in a mouse model of VILI (56); this suggests that NLRP3-dependent measures of ALI may vary depending on the model. It is possible that signaling mechanisms for PMN

recruitment and edema may be dependent on the NLRP3 inflammasome in VILI, but that other mechanisms for PMN recruitment and edema predominate in a two-hit LPS-plus-MV model. For example, IL-18, rather than IL-1 β , may play a more prominent role in neutrophil infiltration in the combined LPS-plus-MV (two-hit) model that we used; IL-18 can be produced via inflammasome-independent mechanisms (57, 58), and IL-18 plays an important role in lung injury due to MV and ARDS (52).

In our model, mice that received both LPS and MV developed progressive, severe hypoxemia over time on MV. The progressive desaturation of mice that received LPS and MV suggests that our

Figure 5. *Casp1* knockout (KO) and *Nlrp3* KO mice are protected from hypoxemia in ALI. (A) Mice received either 200 ng/g body weight (intratracheal) LPS (closed circles) or an equivalent volume of intratracheal PBS (open circles). After 90 minutes, mice were anesthetized and placed on MV with tidal volumes of 20 ml/kg and zero PEEP, and oxygen saturation was measured each hour for 4 hours. (B) Wild-type (closed circles) and *Casp1* KO (open squares) mice received 200 ng/g body weight (intratracheal) LPS. After 90 minutes, mice were anesthetized and placed on MV as in (A), and oxygen saturation was measured each hour for 4 hours. (C) Wild-type (closed circles) and *Nlrp3* KO (open triangles) mice received 200 ng/g body weight (intratracheal) LPS. After 90 minutes, mice were anesthetized and placed on MV as in (A), and oxygen saturation was measured each hour for 4 hours ($n = 7$ –8/group). Data are representative of two separate experiments. Values shown are means ($\pm$ SD). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

model is physiologically relevant, and specifically reflects the development of hypoxemia in the setting of both insults. We demonstrated protection from the development of hypoxemia in *Casp1*-deficient and *Nlrp3*-deficient mice. The addition of the Anakinra to LPS treatment blunted the hypoxemic response, suggesting that the development of hypoxemia is specifically related to IL-1, not IL-18. The specific association of NLRP3–IL-1 β axis with a critical aspect of ARDS—hypoxemia—is the main novel finding of this study. Surprisingly, edema and neutrophil infiltration of the lungs, which are other key measures of ALI in this model, were not affected by the absence of caspase-1 or NLRP3, or by the addition of IL-1Ra.

Our finding that animals lacking IL-1 β production could have equivalent alveolar neutrophil infiltration and edema without hypoxemia in our model was unexpected; prior research has clearly established an important role for neutrophils in the development of ALI. Neutrophil depletion decreases lung edema and inflammatory cytokines in many animal models (59–61), and neutrophil stimulation with granulocyte colony-stimulating factor (G-CSF) worsens lung injury (62). Mechanisms of neutrophil-induced lung injury are well established (63–68), and neutrophil infiltration of the lungs and protein-rich alveolar edema are hallmarks of the development of ALI, both in humans and in animal models (5, 47, 69). We asked whether neutrophil infiltration

is required, as would be expected, for the development of hypoxemia in our model. Surprisingly, neutrophil depletion appeared to have no effect on the development of hypoxemia (Figure E3). These results suggest that the development of hypoxemia during ALI is more complicated than previously understood, and may not correlate directly with other measures of ALI. Further work will be required to explain these observations, and may lead to a better understanding of the mechanisms of hypoxemia in ALI. One explanation for how IL-1 might play a role in hypoxemia could involve IL-1-dependent effects on pulmonary blood flow and ventilation–perfusion mismatching. Nevertheless, the improved oxygen saturations with intratracheal IL-1Ra treatment suggest a promising pathway for intervention.

Recent work showed that inflammasome-dependent immune responses can be triggered by cell death (30, 70). Programmed cell death is seen in ARDS (41), suggesting a potential role for inflammasome activation in the development of ARDS. Our group recently discovered that, in macrophages, activation of the NLRP3 inflammasome involves mitochondrial DNA oxidative damage and apoptosis (30). This finding provided a missing link through the common pathway of apoptosis between such diverse danger signals as uric acid, eATP, and ROS and the activation of the NLRP3 inflammasome. Earlier work has suggested that eATP may be an important mediator of

lung injury via the NLRP3 inflammasome (50, 71), and our data, showing that MV increases BALF eATP and that the percent of apoptotic AMs in BALF progressively increased over time on MV, support this hypothesis. These data point strongly toward a mechanism for NLRP3 inflammasome activation in the setting of ALI due to sepsis (LPS) plus MV. We propose that LPS provides the “first signal” required for IL-1 β secretion by inducing pro-IL-1 β transcription, and that MV generates the “second signal” via increasing eATP, initiating apoptosis of AMs, and activating the NLRP3/caspase-1 complex, which then cleaves pro-IL-1 β to mature IL-1 β .

Our work builds on the findings of several other groups examining the LPS/MV two-hit model of ALI (7, 11, 15, 17, 53). A consistent finding in this research is that LPS or MV individually may generate lower levels of inflammation, but that there is a significantly enhanced and synergistic injury when both insults are combined. Others have characterized unique gene expression profiles (7, 10, 14, 16, 53), early changes in lung mechanics (9, 12), systemic effects on distal organs (14, 54), and signaling pathways (7, 11, 13, 15) of LPS-plus-MV-induced ALI. Our data contribute to this field by describing the early onset of AM apoptosis with MV, and by demonstrating a specific role for IL-1 β and the NLRP3 inflammasome in the development of hypoxemia in the two-hit ALI model.

Figure 6. Anakinra significantly improves hypoxemia, but has no effect on other measures of ALI. (A) A total of 200 ng/g body weight LPS or LPS plus 500 μ g Anakinra was administered intratracheally to C57BL/6 wild-type mice. After 90 minutes, all mice were placed on MV with tidal volumes of 20 ml/kg and zero PEEP, and oxygen saturation was measured each hour for 4 hours. (B) Both groups developed progressive decreases in oxygen saturations over time on MV, but desaturations were significantly less in mice treated with Anakinra (open diamonds) compared with control mice that only received LPS (closed circles). Values shown are means ($\pm$ SD). BALF was assayed for (C) total cells, neutrophils, and macrophages and (D) chemokines, KC and MIP2. Serum and BAL protein concentrations were measured for determination of BAL/serum protein ratios (E) ($n = 7$ –8/group). Data are representative of two separate experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

As with all studies, there are potential limitations to our findings. It is possible that MV and LPS may induce other cells, such as alveolar epithelial cells, to secrete IL-1 β . However, experimental macrophage depletion has been shown to be protective in several models of ALI (27, 28), and other researchers have found that airway epithelial cells did not contribute to IL-1 β secretion (72). We have not demonstrated the source of increased eATP found in BALF with MV. In addition, danger signals other than ATP may play a role in precipitating AM apoptosis, including uric acid, ROS, and high motility group box 1 (HMGB1) in the setting of hyperoxia (25, 50, 56). Certainly, it is possible that a combination

of danger signals may be generated during MV that contribute to macrophage apoptosis and NLRP3 activation.

Finally, although we were able to identify a specific link between the NLRP3 inflammasome, IL-1 β , and hypoxemia in LPS-plus-MV-induced ALI, we have not identified the exact mechanism for this relationship. Although we have excluded the necessity of IL-18 signaling in the development of hypoxia, we have not distinguished between IL-1 β versus IL-1 α , and this requires further investigation.

In summary, we developed a two-hit model of ALI in which both LPS and MV were required to generate evidence of injury.

We used this model to demonstrate that IL-1 β is uniquely increased by the combination of LPS and MV, and propose a mechanism of activation of the NLRP3 inflammasome via eATP and macrophage apoptosis with MV. We demonstrated that blocking IL-1 signaling in this model ameliorates hypoxemia, but does not affect other measures of ALI, such as neutrophil infiltration and alveolar leakage. IL-1 β has been widely implicated as having a key role in the development of ALI (18–22); this research adds specific information about the mechanisms by which IL-1 β may be generated, and suggests a more specific role for IL-1 β in

the severe hypoxemia seen in ARDS. These observations suggest that therapeutics targeting the inflammasome pathway and its downstream mediators, such as IL-1 β ,

may hold promise for treatment of hypoxemia during ARDS. ■

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