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Activation of NLRP3 Inflammasome in Alveolar Macrophages Contributes to Mechanical Stretch-Induced Lung Inflammation and Injury

Jianbo Wu,*‡,1 Zhibo Yan,‡,5,1 David E. Schwartz,* Jingui Yu, † Asrar B. Malik, ‡ and Guochang Hu*‡,‡†

Mechanical ventilation of lungs is capable of activating the innate immune system and inducing sterile inflammatory response. The proinflammatory cytokine IL-1β is among the definitive markers for accurately identifying ventilator-induced lung inflammation. However, mechanisms of IL-1β release during mechanical ventilation are unknown. In this study, we show that cyclic stretch activates the nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3 (NLRP3) inflammasomes and induces the release of IL-1β in mouse alveolar macrophages via caspase-1– and TLR4-dependent mechanisms. We also observed that NADPH oxidase subunit gp91phox was dispensable for stretch-induced cytokine production, whereas mitochondrial generation of reactive oxygen species was required for stretch-activated NLRP3 inflammasome and IL-1β release. Further, mechanical ventilation activated the NLRP3 inflammasomes in mouse alveolar macrophages and increased the production of IL-1β in vivo. IL-1β neutralization significantly reduced mechanical ventilation-induced inflammatory lung injury. These findings suggest that the alveolar macrophage NLRP3 inflammasome may sense lung alveolar stretch to induce the release of IL-1β and hence may contribute to the mechanism of lung inflammatory injury during mechanical ventilation. The Journal of Immunology, 2013, 190: 000–000.

Mechanical ventilation is necessary to support patients with acute lung injury (ALI) or its most severe form, acute respiratory distress syndrome (ARDS); however, it has also been shown to exacerbate lung injury, the so-called ventilator-induced lung injury (VILI) (1). VILI is characterized by inflammation associated with robust release of proinflammatory cytokines and activation of inflammatory signaling pathways (1). A variety of inflammatory mediators are released into the distal air spaces during ALI (2), and among these is IL-1β, a potent proinflammatory cytokine initiating and amplifying lung inflammation in patients (3). IL-1β can stimulate the production of a variety of chemokines (e.g., IL-8, MCP-1, and MIP-1α) (4). A recent study indicates that IL-1β is critical for the pathogenesis of VILI (5). Activation of the inflammatory response, including increased IL-1 signaling, is a major mechanism of alveolar barrier dysfunction in VILI (5). Studies in patients have demonstrated that IL-1β is among the best markers of ventilator-induced lung inflammation (6). Findings also suggest that IL-1 is a key regulator of inflammation. IL-1β receptor antagonist and anti–IL-1β Ab have been demonstrated to prevent ALI (2, 5, 7).

Alveolar macrophages (AMs) residing in the alveolar space account for 5% of peripheral lung cells (8). Under physiological condition, leukocyte population in the alveolar space is dominated by AMs (comprising >90% of the total cells), with the remainder being mainly dendritic cells and T cells. The lung parenchyma also contains macrophages (9). AMs have a central role in the maintenance of immunological homeostasis and in host defense. In response to inflammatory stimuli, AMs are the primary source of cytokines in lungs. AMs can be rapidly activated by mechanical ventilation and thus may play an important role in the pathogenesis of VILI (10, 11). Depletion of AMs improved alveolar barrier dysfunction and lung inflammatory injury caused by high tidal volume ventilation (5, 11).

The inflammasome mainly consists of nucleotide-binding oligomerization domain-like receptor family members containing pyrin domain (NLRP), the adaptor molecule apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), and caspase-1 (12–14). To date, four distinct inflammasome complexes including NLRP1 (NALP1), NLRP3 (NALP3), IPAF (NLRC4, IL-1–converting enzyme) protease-activating factor, and absent in melanoma 2 (AIM2), have been characterized (2). Among these inflammasome prototypes, NLRP3 is involved in sensing endogenous danger signals, including uric acid crystals and amyloid-β protein (15). In response to danger signals, NLRP3 interacts with procaspase-1 through ASC, which leads to activation of caspase-1. Active caspase-1 promotes cleavage and, therefore, maturation of proinflammatory cytokines (Pro–IL-1β, Pro–IL-18, and IL-33) (15, 16). The production of mature IL-1β is...
however tightly regulated. In macrophages, two signals are needed for the release of biologically active IL-1β. First, transcription of the IL-1β gene and production of cytosolic Pro-IL-1β are dependent on activation of NF-kB via, for example, TLRs. The second signal leads to cleavage of Pro-IL-1β by caspase-1 and release of the mature IL-1β (12–14).

In this study, we addressed the role of lung mechanical stretch in mediating the release of mature IL-1β via activation of the NLRP3 inflammasomes in AMs. We observed that lung cyclic stretch-induced IL-1β release was mediated by mitochondrial reactive oxygen species (ROS). Our findings collectively provide a new insight into the role of NLRP3 inflammasomes in the pathophysiology of VILI and suggest that NLRP3 is a potential therapeutic target for dampening AM activation and thereby preventing VILI.

**Materials and Methods**

*Mouse*

Male C57BL/6, tlr4−/−, and gg9p25−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice used for this study were male, 8–12 wk of age, and weighed 25–30 g. Animal studies were approved by the University of Illinois Institutional Animal Care and Use Committee.

**Isolation of AMs**

AMs were isolated by bronchoalveolar lavage (BAL) as described previously (17). Briefly, mice were anesthetized by i.p. injection of 3 mg/kg xylazine and 75 mg/kg ketamine and then sacrificed by cardiac exsanguination. The lungs and trachea were then excised en bloc, washed in HBSS, and lavaged >10 times with light massaging by slowly instilling and withdrawing 1 ml warm (37°C) Ca2+Mg2+-free HBSS (pH 7.4) containing EDTA (0.6 mM). BAL fluid was collected and then centrifuged at 400 × g for 10 min at 4°C. The cells were then incubated in 100-mm sterilized polystyrene Petri dishes for 2 h at 37°C. The cells adhering to the bottom of dish were collected and replated for further experimental use. The purity of isolated AM was >95% as determined using fluorescently labeled Abs (mAbs) that specifically recognize proteins expressed by mice macrophages (surface Ags F4/80 and CD11b). The viability was >98% as evaluated by trypan blue exclusion.

**Cell culture**

The mouse AMs were cultured at a density of 1 × 105 cells/cm2 on collagen IV–coated dishes in DMEM containing 10% FBS without antibiotics and 2% (v/v) FBS/PBS. Stained cells were determined with a FACSCanto II sample. The mean fluorescence of the cell sample was then normalized to the unstained control group. Cell viability following cyclic stretch was evaluated by measurement of lactate dehydrogenase (LDH) activity in cell-culture supernatants (22). LDH activity was determined by spectrophotometric analysis using the Cytoxicity Detection KitPLUS (LDH; Roche) according to the manufacturer’s instructions. Cellular LDH activity was measured after lysis of the cells with 1% Triton X-100 in PBS. The released LDH activity was expressed as a percentage of total cellular LDH activity.

**Flow cytometric analysis**

Mitochondria-associated ROS levels were measured in AMs by staining cells with MitoSOX (Invitrogen-Molecular Probes) (23, 24). Briefly, cells were incubated for 30 min at 37°C in a 10 μM MitoSOX (Invitrogen-Molecular Probes) solution prepared in 1:1 (v/v) DMEM/FBS. Cells were then rinsed three times with PBS, harvested with 500 μl trypsin-EDTA solution, centrifuged at 2300 × g for 5 min, and resuspended in 3 ml fresh 2% (v/v) FBS/PBS. Stained cells were determined with a FACSCanto II flow cytometer (BD Biosciences) and data analyzed with FlowJo analytical software (Tree Star). Signals from 1 × 104 cells were acquired for each sample. The mean fluorescence of the cell sample was then normalized to the unstained control group.

**Western blotting and immunoprecipitation**

Western blot analysis was carried out on both the cultured medium and cell lysates. The cell-culture media were collected and concentrated by filter centrifugation (Millipore Amicon; cutoff ≤10,000 nominal m.w. limit). The collected medium samples were then concentrated using a commercial protein precipitation kit (2D Clean-up Kit; GE Healthcare). The cells were washed twice with PBS and lysed in a lysis buffer. Protein concentrations of the samples were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Protein samples of concentrated medium or cell lysate supernatants were mixed with an appropriate volume of SDS sample buffer and separated by SDS-PAGE gel (10–12%). The protein bands were then transferred onto nitrocellulose membranes by electro blotting. One blot was incubated with primary Abs overnight, washed, and then incubated with goat anti-rabbit or anti-mouse IgG conjugated to HRP (1:5000–8000) for 60 min. Protein bands were detected using the ECL SuperSignal reagent (Pierce, Rockford, IL). Relative band densities of the
various proteins were measured from scanned films using National Institutes of Health ImageJ Software.

Immunoprecipitation analysis was performed as described previously (21). Macrophages were lysed in buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 mM NaF, 1 mM PMSE, 1 mM Na3VO4, and protease inhibitor mixture. Samples were precleared using 1 mg control IgG together with protein A/G PLUS-agarose beads, and then incubated overnight at 4°C with anti-NLRP3, anti-ASC, or anti-caspase-1 Ab, followed by addition of 25 ml protein A/G PLUS-agarose beads. The resulting immunoprecipitates were dissolved in SDS-PAGE sample buffer for electrophoresis and immunoblot analysis.

Depletion of AMs in mice
Clodronate liposomes were prepared as previously described (11). Briefly, phosphatidylserine, phosphatidylcholine, and cholesterol were mixed at a molar ratio of 1:6:4 in chloroform. The chloroform is removed by rotary evaporation (100 rpm) at 40°C. A clodronate stock solution or PBS was added, and the mixture was placed under nitrogen and sonicated for 3 min. The liposome suspension was filtered through 200-nm filters. The clodronate liposome solution was then delivered to anesthetized (ketamine 90 mg/kg, i.p.) mice by nebulization.

In vivo experimental protocols
A well-established in vivo model of VILI was used as described previously (25, 26). Briefly, wild-type C57BL/6 and and IgG~p~ mice were anesthetized with ketamine (75 mg/kg), underwent tracheotomy, and were intubated at 28 ml/kg for 2 h. Stilled with 100 ml MOPS, some of the animals were pretreated with end expiratory pressure. To maintain a partial pressure of CO2 in arterial blood between 35 and 45 torr (4.7–6.0 kPa), a tidal volume of 28 ml/kg, a respiratory rate of 60 breath/min, and 0 cm H2O end-expiratory pressure. For normal volume ventilation, mice received 7 ml/kg tidal volume, a respiratory rate of 120 breath/min, and 0 cm H2O end-expiratory pressure. For normal volume ventilation, mice received 7 ml/kg tidal volume, a respiratory rate of 120 breath/min, and 0 cm H2O end-expiratory pressure. To maintain a partial pressure of CO2 in arterial blood between 35 and 45 torr (4.7–6.0 kPa), a tidal volume of 25 ml/kg, a respiratory rate of 80 breath/min, and 0 cm H2O end-expiratory pressure. To maintain a partial pressure of CO2 in arterial blood between 35 and 45 torr (4.7–6.0 kPa), a tidal volume of 25 ml/kg, a respiratory rate of 80 breath/min, and 0 cm H2O end-expiratory pressure. To maintain a partial pressure of CO2 in arterial blood between 35 and 45 torr (4.7–6.0 kPa), a tidal volume of 25 ml/kg, a respiratory rate of 80 breath/min, and 0 cm H2O end-expiratory pressure. To maintain a partial pressure of CO2 in arterial blood between 35 and 45 torr (4.7–6.0 kPa), a tidal volume of 25 ml/kg, a respiratory rate of 80 breath/min, and 0 cm H2O end-expiratory pressure.

Assessment of pulmonary vascular permeability and edema formation in mice
Protein concentration in BAL was determined with the bicinchoninic acid method. To determine differences between control and experimental groups, the Student t test was performed for paired samples. Parameter changes between different groups over time were evaluated by a two-way ANOVA with repeated measures. Statistical analyses were performed using SPSS 15.0 statistics software. A p value <0.05 was considered statistically significant.

Results
Cyclic stretch induces the release of IL-1β and IL-18 by mouse AMs
To test the hypothesis that inflammasome sense mechanical stretch in AMs, we first determined the effects of cyclic stretch on IL-1β and IL-18 production, known to depend on inflammasome activation (12, 28). Western blot analysis showed that cyclic stretch induced magnitude- and time-dependent proteolytic cleavage of Pro–IL-1β and Pro–IL-18 and resulted in the release of mature 17-kDa IL-1β and 18-kDa IL-18 in media supernatants (Fig. 1A, 1B, Supplemental Fig. 1A–D). The appearance of IL-1β in the cell-culture medium was verified by ELISA assay (Fig. 1C, 1D). Consistent with previous observations (29, 30), 17-kDa IL-1β and 18-kDa IL-18 were undetected intracellularly (data not shown). Cyclic stretch had no effect on protein expression of Pro–IL-1β and Pro–IL-18. Processing and release of IL-1β has been shown to occur with significant cell death and cell lysis (31, 32). Thus, we also determined the effects of cyclic stretch on cell viability (LDH release). As shown in Fig. 1E, cyclic stretch used in our study did not alter LDH release. Together, these data suggest that cyclic stretch alone is able to induce mature IL-1β and IL-18 production in murine AMs.

Cyclic stretch–induced release of IL-1β is caspase-1 dependent
Inflammasome activation resulted in recruitment and activation of caspase-1, the key regulatory component of the inflammasome multiprotein complex responsible for processing of Pro–IL-1β into the mature IL-1β (18, 33). Because caspase-1 is secreted after inflammasome activation, we analyzed cell lysate from cyclic stretch–stimulated macrophages for the presence of mature caspase-1 using immunoblotting. As shown in Fig. 2A and Supplemental Fig. 1E, active caspase-1 was not detected in control (static) cells. However, we observed the appearance of the p10 product of caspase-1 following cyclic stretch. Consistent with changes in IL-1β release, cyclic stretch also induced magnitude- and time-dependent proteolytic cleavage of procaspase-1 and caspase-1 production (Fig. 2A, 2B, Supplemental Fig. 1E, 1F), indicating that mechanical stretch induced proteolytic processing of caspase-1. We next evaluated the role of caspase-1 in mechanical stretch–induced IL-1β release using a caspase-1–specific inhibitor, Z-YVAD-FMK. As shown in Fig. 2C and Supplemental Fig. 1G, Z-YVAD-FMK in a dose-dependent manner ablated the mechanical stretch–induced increase in IL-1β release. The similar effect of Z-YVAD-FMK on mature IL-1β in the cell-culture medium following stretch was verified by ELISA assay (Fig. 2D). Furthermore, depletion of procaspase-1 with a specific siRNA abolished stretch-induced IL-1β release (Fig. 2E, 2F, Supplemental Fig. 1H). These data suggest that maturation of IL-1β induced by cyclic stretch was mediated via caspase-1 pathway.

Cyclic stretch activates NLRP3 inflammasome pathway
The assembly of the NLRP3 inflammasome complex is an initial step of inflammasome activation requiring a pyrin domain/pyrin domain interaction between ASC and NLRP3 and caspase recruitment domain/caspase recruitment domain interaction between ASC and procaspase-1 for caspase-1 activation and subsequent IL-1β release (14). We observed that the association of the complex consisting of NLRP3, ASC, and caspase-1 in AMs was induced at 1 h after cyclic stretch and further increased between 2 and 4 h.

Statistical analysis
Data are expressed as mean ± SD. One-way ANOVA and Student Newman–Keuls test for post hoc comparisons were used to determine
FIGURE 1. Cyclic stretch induces the release of active forms of IL-1β and IL-18 from mouse AMs. (A) Mouse AMs were cyclically stretched at the levels of 0, 8, 15, and 20% for 4 h. Following cyclic stretch (CS), the release of mature IL-1β and IL-18 was measured in the supernatants (SN) of AMs by Western blot analysis. The levels of Pro–IL-1β and Pro–IL-18 were determined in AM cell lysates (Lys) after cyclic stretch. (B) Mouse AMs were exposed to 20% cyclic stretch for the indicated times. Following CS, the release of mature IL-1β and IL-18 was measured in the SN of AMs by Western blot analysis. The levels of Pro–IL-1β and Pro–IL-18 were determined in AM Lys after cyclic stretch. (C) The levels of IL-1β in the culture medium following different magnitudes of cyclic stretch were detected by ELISA. (D) The levels of IL-1β in the culture medium following 20% CS for different time intervals were detected by ELISA. (E) Mouse AMs were cyclically stretched at the levels of 0, 8, 15, and 20% for 4 h. The concentrations of LDH were measured in supernatants to evaluate cell cytotoxicity. Data are means from three independent experiments. *p < 0.05 versus control group (static).

(Fig. 3A, Supplemental Fig. 2A), suggesting the ability of mechanical stretch to activate NLRP3 inflammasome.

To further assess the contribution of NLRP3 to IL-1β and IL-18 release induced by cyclic stretch in AMs, we knocked down NLRP3 with a specific siRNA. Treatment of AMs with NLRP3-targeted siRNA diminished NLRP3 protein levels by 80% (Fig. 3B). We found that NLRP3 knockdown abolished the effect of cyclic stretch on activation of caspase-1 and subsequent release of mature IL-1β (Fig. 3B, 3C, Supplemental Fig. 2B) and IL-18 (Fig. 3B, Supplemental Fig. 2C), with no effect on protein expression of procaspase-1, Pro–IL-1β, and Pro–IL-18. In cooperation with NLRP3 and IPAF, AIM2 has been shown to play an

FIGURE 2. Cyclic stretch (CS)–induced IL-1β release is caspase-1 dependent. (A) Mouse AMs were cyclically stretched at the levels of 0, 8, 15, and 20% for 4 h. The levels of caspase-1 (Casp-1) were detected by Western blotting. (B) Mouse AMs were exposed to 20% CS for the indicated times. After stretch, the levels of caspase-1 were detected by Western blotting. (C) Effects of Casp-1 specific inhibitor ac-YV AD-FMK (YVAD) on IL-1β release and expressions of Pro–IL-1β, Pro–Casp-1, and mature Casp-1. (D) The levels of IL-1β were detected by ELISA. (E) Effect of Casp-1 siRNA on IL-1β release, Pro–IL-1β, Pro–Casp-1, and mature Casp-1. A scrambled siRNA (si Sc) was used as a negative control. (F) The levels of IL-1β were detected by ELISA. Data are means from three independent experiments. *p < 0.05 versus control group (static), †p < 0.05 versus CS control group (stretched). Lys, Cell lysates; SN, culture supernatants.
important role in activation of caspase-1 during bacterial infection (34). However, in contrast to NLRP3 inflammasome, we observed that siRNA-induced depletion of IPAF and AIM2 had no effect on cyclic stretch–induced caspase-1 activation and subsequent release of mature IL-1β and IL-18 (Fig. 3B, 3C, Supplemental Fig. 2B, 2C), indicating that activation of caspase-1 and the release of IL-1β and IL-18 by mechanical stretch were mediated by NLRP3 inflammasome but not by IPAF and AIM2 inflammasomes.

Mitochondria ROS are required for inflammasome activation induced by cyclic stretch

Because mitochondrial ROS production was found to be important in NLRP3 inflammasome activation (15), we determined whether mitochondrial ROS were generated in response to cyclic stretch. As shown in Fig. 4A, exposure of AMs to cyclic stretch significantly increased mitochondrial ROS fluorescence. Treatment of macrophages with the mitochondria-targeted antioxidant SS-31, a scavenger for mitochondrial ROS (35), reduced the levels of mitochondrial ROS (Fig. 4B, 4C, Supplemental Fig. 2B, 2C). SS-31 in a dose-dependent manner also inhibited cyclic stretch–induced caspase-1 activation and IL-1β production (Fig. 4D, 4E, 4F, Supplemental Fig. 2D). Treatment with the mitochondrial complex I inhibitor rotenone, known to result in ROS generation (23, 36), enhanced caspase-1 activation (Fig. 4E) and IL-1β release (Fig. 4E, 4F, Supplemental Fig. 2E) caused by cyclic stretch in AMs. These data together demonstrate that mitochondrial ROS may be required for the IL-1β release in AMs induced by mechanical stretch.

NADPH-derived ROS were shown to be essential for fatty acid–induced inflammasome activation (28). To determine whether NADPH-derived ROS are involved in NLRP3 inflammasome activation and IL-1β release following cyclic stretch, we isolated AMs from gp91phox−/− mice. Deletion of gp91phox did not inhibit stretch-induced caspase-1 activation and IL-1β release (Fig. 5A, 5B, Supplemental Fig. 3A). Further analysis using flow cytometry showed that gp91phox deletion did not alter mitochondrial ROS production induced by stretch (Fig. 5C, 5D). These results therefore exclude a role for NADPH oxidase–derived ROS in NLRP3 inflammasome activation in AMs. Taken together, mechanical stretch activates NLRP3 inflammasome via a mitochondrial ROS-dependent signaling pathway.

Uric acid released from AMs following cyclic stretch activates NLRP3 inflammasome partially through mitochondrial ROS

Uric acid is present in normal cells and released from dying cells (37). Uric acid crystallizes at concentrations exceeding limits of solubility (~6.8 mg/dL or even lower under conditions of reduced pH or temperature), and it is capable of activating the NLRP3 inflammasome (38). In our study, uric acid was also released from AMs following cyclic stretch (Fig. 6, Supplemental Fig. 4A). Immunoprecipitation with an anti-ASC Ab followed by immunoblotting for NCAM-1, ASC, and caspase-1 cleavage product p10 fragments (Casp-1). The NLRP3 activation was confirmed by using IP with anti-NLRP3 Ab followed by immunoblotting for ASC, NLRP3, and Casp-1. (B) Effects of depletion of NLRP3, IPAF, or AIM2 with respective siRNAs on IL-1β, IL-18, and mature Casp-1 production following 20% CS for 4 h. IL-1β and IL-18 release in the culture supernatants (SN) of AMs and levels of Pro–IL-1β, Pro–IL-18, Pro–Casp-1, and Casp-1 in cell lysate (Lys) were determined by Western blot analysis. A scrambled siRNA (si Sc) was used as a negative control. (C) The levels of IL-1β in cell-culture media were measured by ELISA. The graph shows the mean and SEM from three independent experiments. *p < 0.05 versus the control (static) group, †p < 0.05 versus si Sc + CS group.

FIGURE 3. Cyclic stretch (CS) stimulates IL-1β production via NLRP3-dependent signaling pathway. (A) CS-induced NLRP3 inflammasome activation in AMs. AMs were exposed to 20% CS for the indicated time. The assembly of NLRP3 inflammasome was detected using immunoprecipitation (IP) with anti-ASC Ab followed by immunoblotting (IB) for NLRP3, ASC, and caspase-1 cleavage product p10 fragments (Casp-1). The NLRP3 activation was confirmed by using IP with anti-NLRP3 Ab followed by immunoblotting for ASC, NLRP3, and Casp-1. (B) Effects of depletion of NLRP3, IPAF, or AIM2 with respective siRNAs on IL-1β, IL-18, and mature Casp-1 production following 20% CS for 4 h. IL-1β and IL-18 release in the culture supernatants (SN) of AMs and levels of Pro–IL-1β, Pro–IL-18, Pro–Casp-1, and Casp-1 in cell lysate (Lys) were determined by Western blot analysis. A scrambled siRNA (si Sc) was used as a negative control. (C) The levels of IL-1β in cell-culture media were measured by ELISA. The graph shows the mean and SEM from three independent experiments. *p < 0.05 versus the control (static) group, †p < 0.05 versus si Sc + CS group.
inflammasome following crystallization (18). We observed that uric acid concentration in the medium after stretch was significantly increased (7.8 μg/ml versus 0.2 μg/ml) (Fig. 6A). Allopurinol, the inhibitor of uric acid synthesis, dose-dependently decreased uric acid levels, with complete inhibition of uric acid production occurring at 10 mM (Fig. 6A). This concentration of allopurinol was not cytotoxic (data not shown). Accordingly, 10 mM of allopurinol was chosen and used in the subsequent experiments. Exposure of AMs to allopurinol attenuated, but did not abolish cyclic stretch–induced caspase-1 activation and IL-1β production (Fig. 6B, Supplemental Fig. 3B). Allopurinol had no effects on the release of IL-1β in nonstretched AMs and synthesis of Pro–IL-1β (Fig. 6B, Supplemental Fig. 3B). To determine whether uric acid–induced IL-1β production following stretch was dependent on mitochondrial ROS, we investigated the role of uric acid in mitochondrial ROS generation. Exogenous MSU in a dose-dependent manner (2.5–10 μg/ml) stimulated mitochondrial ROS generation (Fig. 6C, 6D) and IL-1β release (Fig. 6E). Allopurinol did not completely inhibit mitochondrial ROS generation (Fig. 6F), suggesting that mitochondrial ROS production following cyclic stretch was partially dependent on uric acid.

Cyclic stretch–induced release of IL-1β is dependent of TLR4 signaling

Pro–IL-1β biosynthesis and intracellular accumulation depend upon TLR4 signaling activation of NF-κB. To determine whether cyclic stretch–induced inflammasome activation is TLR4 dependent, we mechanically stretched AMs isolated from tlr4−/− mice. Compared to wild-type AMs, tlr4−/− AMs exhibited a dramatic decrease in the protein levels of Pro–IL-1β. Although cyclic stretch caused a slight increase in IL-1β release in tlr4−/− AMs, the concentration of IL-1β following stretch in the medium was much less than that in wild-type AMs (Fig. 7A, 7B, Supplemental Fig. 3C). Flow cytometric data showed that increased mitochondrial ROS generation was reduced in tlr4−/− AMs compared to wild-type AMs (Fig. 7C). These results suggest a role for TLR4 in the production of mitochondrial ROS in response to cyclic stretch.
High tidal volume mechanical ventilation activates NLRP3 inflammasome

To address whether mechanical stretch could activate NLRP3 inflammasome in lungs, we used a well-established mouse model of VILI (25, 26). The assembly of NLRP3, ASC, and caspase-1 in lungs ventilated with high tidal volume was induced at 1 h after mechanical ventilation and further increased within 4 h. Caspase-1 cleavage as well as mature IL-1β and IL-18 were detected at 1 h and further increased within 4 h (Fig. 8A). The IL-1β concentration in BAL fluid, representing IL-1β released from pulmonary cells, was elevated at 1 h after mechanical ventilation (Fig. 8B). Mechanical ventilation-activated NLRP3 inflammasome (Fig. 8A) and release of IL-1β (Fig. 8A, 8B, Supplemental Fig. 4A) and IL-18 (Fig. 8A, Supplemental Fig. 4B) were significantly attenuated by pretreatment of mice with a mitochondrial ROS inhibitor, but not by depletion of NADPH oxidase-derived ROS. To determine the contribution of AMs to IL-1β release in the lung, AMs were depleted using a liposomal clodronate technique (11). Lavageable AM count was reduced by 75% at 4 d with a 10-ml aerosolized dose of 20 mg/ml clodronate liposome solution. Depletion of AMs resulted in significantly reduced NLRP3 inflammasome activation (Fig. 8C) and release of IL-1β (Fig. 8A, 8B, Supplemental Fig. 4A) and IL-18 (Fig. 8A, Supplemental Fig. 4B) attributable to mechanical ventilation. These findings suggest that high tidal mechanical ventilation activates pulmonary NLRP3 inflammasomes and that AMs may play a key role in mechanical stretch–induced IL-1β generation in lungs.

To determine the importance of IL-1β in mechanical ventilation–induced inflammatory lung injury, we investigated the effects of IL-1β neutralization on lung inflammation and injury caused by...
high tidal volume ventilation. Mechanical ventilation at high tidal volume (28 ml/kg) induced an increase in trans-alveolar protein permeability (Supplemental Fig. 4E) and lung edema formation (Supplemental Fig. 4F). These effects were significantly reduced in mice pretreated with IL-1β Ab (Supplemental Fig. 4E, 4F).

**Discussion**

Mechanical ventilation induces the production of IL-1β in lungs of animal models (5) and patients (6). Because IL-1β is a proinflammatory cytokine and a mediator of sterile inflammation that acts through IL-1R (38, 39), it may have a role in the mechanism of lung inflammation and injury induced by mechanical stretch. In the current study, using in vitro model of cyclic stretch and in vivo model of VILI in mice, we demonstrated the central role of mechanical stretch in activating NLRP3 inflammasome in AMs, which in turn mediated IL-1β release via mitochondrial ROS-dependent signaling. These results are in accord with recent studies in Nlrp3<sup>−/−</sup> mice, highlighting the importance of NLRP3 inflammasome in mediating lung inflammation during VILI (40).

Previous studies also showed that genetic deletion of IL-18 or caspase-1 conferred resistance to VILI in mouse models (41). Our results taken in the context of previous studies provide support for the central role of NLRP3 inflammasome activation in AMs secondary to mechanical stretch in the mechanism of lung inflammation and injury.

To address the molecular basis of inflammasome activation in response to mechanical stretch, we focused on ROS generation by AMs. ROS generation has been shown to activate NLRP3 inflammasome (18, 33). Studies have suggested a role for NADPH oxidase-derived ROS in NLRP3 activation (33, 42), whereas other studies pointed to mitochondrial ROS in activating NLRP3 inflammasome in macrophages (23, 24). Macrophages lacking...
functional NADPH oxidase (NOX)1, NOX2, and NOX4 responded newly to NLRP3 stimulation (23). Studies employing inhibition of mitochondrial ROS and analysis of mice lacking gene for gp91phox subunit of NADPH oxidase demonstrated that mitochondria ROS were responsible for NLRP3 inflammasome activation and mediated inflammation induced by mechanical ventilation. Mitochondrial ROS promoted NLRP3 inflammasome complex assembly, caspase-1 activation, and subsequent IL-1β release in AMs and whole lungs. The basis of mitochondrial ROS-induced NLRP3 inflammasome activation is not clear. One tenable mechanism is that mitochondrial ROS can induce translocation of NLRP3 to mitochondria-associated endoplasmic reticulum membranes, where ASC is recruited, thereby activating NLRP3 inflammasome (43). Another possibility is that mitochondrial ROS can promote mitochondrial permeability transition, which may facilitate cytosolic release of mitochondria DNA and stimulate activation of NLRP3 inflammasome, resulting in the production of IL-1β and IL-18 (23, 44).

The production of mitochondrial ROS in response to mechanical stretch has been shown in nonphagocytic cells, including endothelial cells (45) and pulmonary epithelial cells (46). In this study, we showed that mechanical stretch also stimulated mitochondrial ROS generation in AMs. Activation of mitochondrial ROS system may be the result of distention and deformation of mitochondria following stretch and alterations in mitochondrial K+ ATP channel activity (46). Another mechanism may involve stretch-induced uric acid production, which stimulates mitochondrial ROS generation by an as-yet-undefined mechanism.

Uric acid, a product of purine catabolism, is released from damaged cells in response to variety of stresses. At a high concentration, uric acid precipitates and forms crystals that are internalized, resulting in activation of NLRP3 inflammasome (18, 38, 47). The mechanism by which endocytosed uric acid crystals are sensed by the NLRP3 inflammasome is currently not known and neither is it clear whether crystals directly interact with NLRP3 or whether sensing occurs via intermediary proteins (18). Our data, however, showed that cyclic stretch induced uric acid release from AMs. Because in our model, inhibition of mitochondrial ROS completely abolished IL-1β release following mechanical stretch, it appears that uric acid may be released upon stretch, contributing to activation of NLRP3 inflammasome through enhancement of mitochondrial ROS production. Taken together, these findings show that stretch-induced uric acid can activate NLRP3 inflammasome partially through the stimulation of mitochondrial ROS production.

In the current study, we observed that mechanical stretch induced the maturation and secretion of IL-1β and IL-18 in AMs via NLRP3 inflammasome activation. IL-1β and IL-18 are two closely related IL-1 family cytokines that function as key mediators of host immune response (48). Our results demonstrated that IL-1β neutralization significantly reduced mechanical ventilation–induced inflammatory lung injury. A recent study has shown that mechanical ventilation enhanced IL-18 levels in mouse lung and that anti–IL-18–neutralizing Ab treatment or genetic deletion of IL-18 reduces lung injury following mechanical ventilation (41). These findings together with ours suggest that both IL-1β and IL-18 participate in the pathogenesis of lung inflammation and injury associated with VILI secondary to activation of NLRP3 inflammasome.

In summary, this study for the first time, to our knowledge, demonstrates the essential role of NLRP3 inflammasome in AMs in the pathogenesis of VILI. Mechanical stretch stimulates mitochondrial ROS production in AMs, which in turn signals assembly of ASC, NLRP3, and caspase-1 to activate NLRP3 inflammasome, leading to the processing and maturation of Pro–IL-1β into the active IL-1β variant. TLR4 signaling also plays an important role in Pro–IL-1β expression and subsequent IL-1β secretion. Mechanical stretch–induced uric acid production has an additional effect, which may be to enhance mitochondrial ROS generation and thereby amplify NLRP3 inflammasome activation in response to cyclic stretch of AMs (Fig. 9). The results point to potential therapeutic approaches to targeting NLRP3 inflammasome in AMs for treatment of VILI.


