Mechanical Ventilation Exacerbates Poly (I:C) Induced Acute Lung Injury: Central Role for Caspase-11 and Gut-Lung Axis

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Research

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Abstract

Background: The mechanisms by which moderate tidal volume ventilation (MTV) may exacerbate preexisting lung injury remain unclear. We hypothesized that in two hit model (polyinosinic-polycytidylic acid (Poly(I:C)), a synthetic analog of dsRNA and MTV), systemic endotoxemia via gut-lung axis would lead to non-canonical (i.e. caspase-11 dependent) and canonical (caspase-1 dependent) inflammasome activation and programmed necrotic cell death (pyroptosis) contributing to acute lung injury (ALI) in intact mice.

Methods: Anesthetized mice were administered Poly(I:C) intratracheally and then 6 h later, they were mechanically ventilated for 4 h with otherwise non-injurious MTV (10ml/kg). Changes in intestinal and alveolar capillary permeability were measured. Further documentation of ALI was assessed by Evans blue albumin permeability, protein and IL-1 family concentration in bronchoalveolar lavage fluid (BALF) or plasma, and histopathology in cohorts of wildtype, whole body genetically ablated caspase-11 (caspase-11^{-/-}), caspase-1/caspase-11 double knockout (caspase-1/11^{-/-}), gasdermin D (GSDMD^{-/-}), and NLRP3^{-/-} mice.

Results: Non-injurious MTV exacerbated mild Poly(I:C) lung injury including disruption of alveolar-capillary barrier and increased levels of IL-6, HMGB1, IL-1 β in BALF and IL-18 in plasma. Combined (Poly(I:C)-MTV) injury was associated with increase in gastrointestinal permeability and endotoxin in plasma and BALF. Poly(I:C)-MTV injury was sensitive to caspase-11 deletion with no further contribution of caspase-1 except for maturation and release of IL-18 (that itself was sensitive to deletion of NLRP3). Combined injury led to large increases in pro-caspase-11 and its cleaved product as well as cleaved product of caspase-1. Genetic ablation of GSDMD attenuated alveolar-capillary disruption and maturation and release of cytokines in combined injury model.

Conclusions: The previously noted TLR-4 independent exacerbation of mild Poly(I:C)-induced ALI by otherwise non-injurious MTV is associated with an increase in gut permeability resulting in systemic endotoxemia. The gut-lung axis resulted in activation of pulmonary non-canonical (cytosolic mediated caspase-11 activation) and canonical (caspase-1) inflammasome (NLRP3) mediated ALI in this two hit model resulting in GSDMD sensitive alveolar capillary barrier disruption, pyroptosis (in alveolar macrophages) and cytokine maturation and release (IL-1 β ; IL-18). Pharmacologic strategies at disrupting communication between gut and lung, inhibition of inflammasomes or effector molecule (GSDMD) in pyroptosis may be useful in ALI.

Introduction

Sepsis is the major underlying cause (~ 75%) of acute respiratory distress syndrome (ARDS) and this often follows the onset of pneumonia [1]. ARDS also occurs in a large number of patients from infections outside the lung and the mechanisms underlying the development of lung injury from remote sources are multifactorial and poorly understood [2]. Both direct (e.g. pneumonia) and indirect (extrapulmonary)

sepsis routinely require mechanical ventilation and it is well known that such lifesaving therapy can exacerbate underlying lung injury in an iatrogenic pathology of ventilator induced lung injury (VILI; [3]). Indeed, minimizing over distension (volutrauma) and/or alveolar collapse and reopening (atelectrauma) by lung-protective ventilation [4] has had the largest impact in reducing morbidity and mortality from ARDS.

In preclinical studies, sensitization of VILI to preexisting acute lung injury (ALI) secondary to pneumonia [5, 6], intratracheal endotoxin [7–10], viral [11] and sterile injury[12–14] is apparent. Although preclinical outcomes vary as a function of magnitude of extrapulmonary septic condition and the nature of mechanical ventilation parameters (tidal volume, onset and duration; positive end expiratory pressure; [15]), sensitization of VILI to events originating in distal site and plasma space including exogenous endotoxin [16, 17] and polymicrobial sepsis [18, 19] has also been documented.

In the current study, we have approached connections of direct and indirect lung injury that are predicted from possible gut-lung interactions and sensitization to subsequent non-injurious moderate mechanical tidal volume ventilation (MTV). We [20] and others [21] have shown that MTV can exacerbate lung injury after intratracheal delivery of polyinosinic-polycytidylic acid (Poly(I:C)), a synthetic analog of dsRNA (that itself can be produced by many viruses during their replicative cycle). The gut plays an important role in indirect lung injury by releasing infectious microbes and inflammatory, injurious mediators directly into the circulation or via interconnecting lymph system secondary to enhanced gut permeability[22]. Endotoxin (lipopolysaccharide (LPS)) derived from gram negative microbes in the gut may thus be liberated in large amounts in the circulation and contribute to lung injury [23, 24]. The canonical detection mechanism of LPS occurs via cell-surface toll receptor-4 (TLR4) but it is noteworthy that neither combined Poly(I:C)-MTV [21] or systemic endotoxemia, per se, [25] is TLR4 sensitive. In this latter comprehensive study [25], the authors noted that indirect lung injury due to systemic endotoxemia involved non-canonical inflammasome caspase-11 mediated pathway and pyroptosis, a necrotic form of cell death, in pulmonary endothelium of intact mice. Murine caspase-11 is the cytosolic receptor for LPS; a pathway of necrotic cell death involving oligomerization of caspase-11 and inflammasome activation leading to cleavage mediated activation of gasdermin D (GSDMD); N-terminal fragment (GSDMD-N) mediated permeabilization has been termed pyroptosis [26].

Accordingly, we: a) confirmed that pretreatment of intact mice with intratracheal (i.t.) Poly(I:C) would lead to sensitization to ALI due to otherwise non-injurious MTV; and b) hypothesized that ALI after Poly(I:C)-MTV was associated with gut derived LPS and caspase-11 non-canonical inflammasome mediated pyroptosis.

Materials And Methods

Experimental protocols

The Animal protocols were approved by the Animal Care and Use Committee and experiments were performed in strict adherence to NIH Guidelines and followed current guidelines for preclinical models in research. Protocols (with brief descriptions below) included: a) MTV enhanced Poly(I:C) induced ALI is associated with increased gastrointestinal permeability and increased endotoxin in plasma and lung; b) MTV exaggerates Poly(I:C) induced acute lung injury through a caspase-11 dependent process; and c) Regulation of caspase-11 expression and activation, its relationship to canonical (NLRP3) mediated caspase-1 activity and gasdermin cleavage dependent pathways in whole lung and isolated macrophages after Poly(I:C), MTV and their combination in intact mice.

In-vivo experimental animal model

C57 BL/6 mice (8–10 weeks old, male) were purchased from Jackson Laboratories. Caspase-1/11^{-/-} mice, caspase-11^{-/-} mice, TLR4^{-/-} mice, NLRP3^{-/-} mice, GSDMD^{-/-} mice were bred and maintained in the University of Pittsburgh animal facility according to NIH animal care guidelines and all procedures were performed according to University of Pittsburgh Animal Research Protocols. The total number of 152 WT mice, 88 Caspase-1/11^{-/-} mice, 88 Caspase-11^{-/-} mice, 32 NLRP3^{-/-} mice, 40 GSDMD^{-/-} mice were prospectively randomized to one of four groups (n = 5–12 per group): a) SHAM: spontaneously breathing and 100 µL endotoxin free water i.t. b) Poly(I:C); 3 mg/kg i.t. Poly(I:C) (tlrl-picw, InvivoGen, USA); c) MTV: connected to animal ventilator 6 h after receiving a volume of 100 µL i.t. endotoxin free water and ventilated with 10 mL/kg, positive end-expiratory pressure of 0 cm H₂O, FiO₂ 0.21, 140 breaths/min); and d) combined Poly(I:C)-MTV: ventilated 4 h after an i.t. dose of 3 mg/kg Poly(I:C). In all groups, ketamine and xylazine were used to maintain anesthesia. Mean arterial blood pressure, heart rate and oxygen saturation were recorded using a mouse STARR system (Life Science Co.). Mice were sacrificed (10 h after starting each protocol) by injecting peritoneal pentobarbitone 300 mg/kg. Additional details are in supplement and were previously described [20].

Additional cohort of wildtype, Caspase-1/11^{-/-} and Caspase-11^{-/-} mice were prospectively randomized to same four groups as above. Lung tissue and freshly cultured primary alveolar macrophages were obtained for determination of pro- and cleaved caspases-11 and -1.

In addition, wildtype mice were prospectively randomized to these same four groups. Water bottles were removed from cages in the morning and 100 mg/mL FITC-D (4 kD) in PBS was administered (44 mg/100 g body weight) by oral gavage 4 hours before sacrifice. Blood or BALF was placed in microtainer tubes and concentration of FITC in serum of BALF determined spectrophotofluorometrically (excitation 485 nm; emission 528 nm). A standard serially diluted FITC-D (0 to 8 µg/mL) was used. Serum from mice not injected with FITC-D was used as blank. In addition, endotoxin was measured (LAL Chromogenic endpoints assay, Hycult biotech, PA, USA) in serum and BALF of wildtype mice in these four groups.

An additional cohort of wildtype were assigned to four protocols above and at time of sacrifice, alveolar macrophages (AMs) were obtained via bronchoalveolar lavage for short term culture and immunofluorescence staining. In brief, mice were bled by cardiac puncture and catheter (20 G) was

inserted into tracheal and connected to 1 mL syringe filled with PBS ($\text{Ca}^{2+}/\text{Mg}^{2+}$ free). A total of 5 mL of PBS was used to wash lungs (10x) and recovered lavage fluid was centrifuged (600 g, 10 minutes), resuspended in RPMI (2×10^6 cells/mL) and transferred to 35 mm petri dishes with 10 mm microwells (Mat Tek corp, Ashland, MA) and placed in incubator. Media was changed and adherent cells (e.g. enriched in alveolar macrophages) were assessed for caspase-1 (FAM-FLICA® Caspase-1 Assay Kit (ImmunoChemistry Technologies, ImmunoChemistry Technologies, LLC), pyroptosis (In Situ Cell Death Detection Kit, TMR red (Sigma-Aldrich), and nuclear staining (Bisbenzimidazole Hoechst 33258) Imaging was observed and recorded with 600 × magnification using a Olympus confocal microscope.

Alveolar-capillary permeability

Evans blue (Sigma-Aldrich) albumin (EBA; 0.5%, 25 mg/kg) was injected intravenously 1 h before euthanasia and lung harvesting. Blood samples and lung tissue were obtained and processed as described previously [18–20] and EBA permeability was calculated by dividing pulmonary EBA absorbance at 620 nm/g lung tissue by plasma EBA absorbance at 620 nm.

Histological examination

For Hematoxylin & Eosin (H & E) staining, the left upper lobe was inflated with 4% paraformaldehyde, embedded in paraffin and assessed via semiquantitative histopathology at light microscopic level including following features: edema, hyperemia and congestion, neutrophil margination and tissue infiltration, intra-alveolar hemorrhage and debris, and cellular hyperplasia [18]. Each feature was graded as absent, mild, moderate, or severe, with a score of 0–3.

Western blot analysis

Cell lysis buffer (cell signaling technology) and a cocktail of protease inhibitors (Sigma-Aldrich) were used to extract protein in lung tissues and alveolar macrophages. 12% SDS gels was used for electrophoresis, Electrophoresis was performed at 80 V for 120 minutes. Then the protein in gels was transferred for 120 minutes at 200 mA to nitrocellulose membranes. 5% milk in 1% Tween-20 in PBS was used to block membranes. The membranes were incubated with a primary antibody (anti-caspase-11 polyclonal antibody, 1:1000, abcam; anti-caspase-1 polyclonal antibody, 1:1000; abcam) at 4 °C overnight and washed three times with PBST (0.1% Tween-20 in PBS). Secondary antibody (1:5000; InvivoGen, USA) was then added and incubated at 37 °C for 1 h.

Cytokines

IL-1 β (ELISA, R&D), IL-6 (ELISA, R&D), HMGB1 (ELISA, Shino-test Corporation) concentrations in BALF and IL-18 in plasma (ELISA, Medical and Biological Laboratories CO., LTD) were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed in GraphPad PRISM 7 (Graph Pad Software Inc.). All data were presented as the means $\pm$ Standard Deviation. Data were analyzed by one-way analysis of variance

(ANOVA) and Student-Newman-Keuls test if normally distributed. Mann-Whitney U-test was used for analyzing nonparametric data. In figures asterisks denote statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

Results

1. MTV enhanced Poly(I:C) induced ALI is associated with increased gastrointestinal permeability and increased endotoxin in plasma and lung

We assessed intestinal and alveolar-capillary permeability by measuring the transmigration of FITC-D (4 kD) from gastrointestinal contents into plasma (Fig. 1A) and BALF (Fig. 1B) spaces, respectively. There were modest increases in both organs after either stimulus alone but the combination of Poly(I:C) followed by MTV led to large increases in both gastrointestinal permeability and alveolar-capillary permeability to FITC-D. We then measured endotoxin in plasma (Fig. 1C) and BALF (Fig. 1D) and noted small increases after either stimulus alone but large increases in endotoxin in plasma and BALF after combined Poly(I:C)-MTV. It is unlikely that endotoxin was due to contamination of Poly(I:C) as the solution tested negative prior to intratracheal instillation. Further refinement and quantitation of lung injury (EBA permeability, BALF protein, histology, cytokine release) was used to assess the nature of interaction of Poly(I:C) and MTV. Increased levels of circulating and intrapulmonary endotoxin after combined injury prompted us to pursue potential role of the intracellular endotoxin receptor caspase-11 (TLR4-independent) in acute lung injury.

2. MTV exaggerates Poly(I:C) induced acute lung injury through a caspase-11 dependent process

Viral infection can activate interferon responses and this can promote the up regulation of the caspase-11 non-canonical inflammasome [27]. Patients with severe respiratory viral infection may require ventilation and Poly(I:C), a double stranded RNA immune stimulant used to mimic the immune activation of viral infections, is known to increase caspase-11 expression in macrophages [27, 28]. We [20] and others [21] have previously shown that Poly(I:C) pre-treatment markedly increases lung injury induced by moderate tidal volume ventilation (MTV = ventilation at 8–10 mL/kg). To determine if up regulation of caspase-11 in the lungs contributed to the pulmonary response to ventilation, we pre-treated wild-type and caspase-11^{-/-} mice with 3 mg/kg intra-tracheal Poly(I:C) followed 6 h later with MTV for 4 h. Caspase-11 activation can promote the activation of the caspase-1 canonical inflammasome [29, 30]. Therefore, to determine the relative contribution of caspase-1 to the injury response we also included mice deficient in both caspase-1 and caspase-11. As shown in Fig. 2 (and consistent with the above findings (Fig. 1) and our previous findings [18–20]), MTV alone for 4 h had no impact on indices of lung injury including leakage of Evans blue dye into the lung (Fig. 2A), accumulation of protein into the BAL fluid (BALF; Fig. 2B), or histologic scoring of lung injury (Fig. 2C, D). While Poly(I:C) treatment alone

induced modest increases in Evans blue dye (Fig. 2A) and protein accumulation (Fig. 2B) in the BALF, the initiation of MTV at 6 h after Poly(I:C) markedly increased the appearance of these large molecular weight species as well as histopathologic quantitative assessment of nature of ALI (Fig. 2D). The deletion of caspase-11 had no impact on the lung injury induced by Poly(I:C) alone but almost completely prevented the exaggerated injury induced by the addition of MTV to Poly(I:C). No further protection was seen in mice deficient in both caspase-11 and caspase-1 consistent with caspase-11 being central to acute lung injury, induced by sequential hits.

To assess the requirement for caspase-11 and caspase-1 on inflammatory mediator production, IL-6, HMGB1 and IL-1 β were measured in BALF (Fig. 3A, B, C) and IL-18 in plasma (Fig. 3D). Similar to the observations made on lung injury, the addition of MTV to Poly(I:C) significantly increased levels of IL-6, IL-1 β and HMGB1 in the BALF and IL-18 in the plasma. Deletion of caspase-11 alone or caspase-11 and caspase-1 together significantly suppressed the increases in these mediators induced by MTV + Poly(I:C). A significant difference in the degree of mediator suppression between the mouse strains was seen for BALF IL-6, IL-1 β levels and plasma IL-18 levels, where deletion of both caspase-11 and caspase-1 lead to an even greater decrease in IL-6, IL-1 β and IL-18 levels than that seen with deletion of caspase-11 alone (Fig. 3D). These findings indicate that caspase-1 contributes to IL-6, IL-1 β and IL-18 release into the circulation consistent with its known role in the maturation and release of this cytokine.

3. Regulation of caspase-11 expression and activation, its relationship to canonical (NLRP3) mediated caspase-1 pathways in whole lung and isolated macrophages after Poly(I:C), MTV and their combination in intact mice

We quantified expression and regulation of caspase-11 and caspase-1 in whole lungs and fresh isolate of a critical immunoregulatory cell (e.g. alveolar macrophage) by Western blot after Poly(I:C) and/or MTV. Procaspace-11 was not affected by MTV and was slightly elevated by Poly(I:C) pretreatment. MTV after Poly(I:C) resulted in a significant increase in procaspase-11 and, large increase in appearance of cleaved caspase-11 in whole lung (Fig. 4A) and alveolar macrophages (Fig. 4B). Overall levels of procaspase-1 were not affected by either stimuli (data not shown). There was a slight increase in cleaved caspase-1 after Poly(I:C) but not MTV and this was greatly increased in combined protocol in both lung (Fig. 4A) and alveolar macrophages (Fig. 4B). Since caspase-11 may affect caspase-1 activity [30], we repeated these experiments in whole lung from caspase-11^{-/-} mice and noted that intrapulmonary cleaved caspase-1 levels were significantly lower in both Poly(I:C) and Poly(I:C) + MTV in caspase-11^{-/-} mice

compared to WT mice (Fig. 4C). Nonetheless, cleaved caspase-1 was still induced in caspase-11^{-/-} mice underscoring the partial interdependence of caspases-1 and -11.

Nucleotide-binding domain leucine-rich repeat-containing protein 3 (NLRP3) is part of a common canonical inflammasome that includes apoptosis-associated speck-like protein (ASC), caspase activation and recruitment domain (CARD) and caspase-1 [21]. Figure 5A shows a significant increase in NLRP3 mRNA level in Poly(I:C) group compared to sham group. There was no difference between the sham and MTV groups. The combination of Poly(I:C) and MTV greatly increased NLRP3 mRNA and this was partially sensitive to genetic ablation of caspase-11 potentially placing caspase-11 upstream of this canonical inflammasome. The canonical pathway was further assessed by measuring IL-6 (Fig. 5B) and IL-1 β (Fig. 5C) secretion in BALF and IL-18 (Fig. 5D) release in plasma of wildtype and NLRP3^{-/-} mice. As expected, increases in these cytokines after Poly(I:C) and combined Poly(I:C)-MTV were sensitive to ablation of NLRP3. These data underscore the interdependence role of caspase-11 and caspase-1 in combined Poly(I:C)-MTV acute lung injury.

4. Caspase-11 cleavage of GSDMD and Poly(I:C)-MTV ALI including pyroptosis in freshly isolated alveolar macrophages from injured lung

Activation of inflammatory caspases-11 and -1 may lead to cleavage mediated activation of GSDMD that in turn is an obligatory step in necrotic cell death (pyroptosis). Accordingly, we repeated experiments with Poly(I:C), MTV and their combination in GSDMD^{-/-} mice and noted GSDMD sensitive Poly(I:C)-MTV mediated lung injury (as determined by Evans blue permeability (Fig. 6A) and protein in BALF (Fig. 6B)) and increased cytokine release (Fig. 6C, D, E, F).

Gasdermin sensitive injury is consistent with necrotic death due to pyroptosis and thus we determined whether pyroptosis occurred in freshly isolated alveolar macrophages from wildtype mice after Poly(I:C), MTV or their combination (Fig. 7). Alveolar macrophages were harvested at end of exposure and stained with Alexa Fluor 488-labeled caspase-1 FLICA, Alexa Fluor 546-labeled in situ cell death reagent-TMR and Hoechst dye (Fig. 7A). Quantitation of colocalization of caspase-1 and TUNEL positive cells showed slight increase in pyroptosis after either Poly(I:C) or MTV and a very large increase after combined exposure in situ (Fig. 7B).

Discussion

We note that otherwise non-injurious moderate tidal volume ventilation exacerbates ALI after i.t. Poly(I:C) in intact mice. Disruption of alveolar-capillary barrier (Figs. 1 and 2) in this two-hit model, as previously reported by Chun et al [21] and us [20], is a central feature of ALI and thus provides a useful preclinical framework in identifying pathways that underscore the major contribution of sepsis to ARDS. Although epidemiologic studies suggest that direct (e.g pneumonia) sepsis comprises a large component of the risk factors in the development of ARDS, a less understood multifactorial extrapulmonary (indirect or systemic) sepsis is also important [2]. In the current study, we note (Fig. 1) that combined Poly (I:C)-MTV enhanced permeability of gastrointestinal tract and introduced endotoxin to the vascular space and lung. The association of systemic sepsis and ALI (via gut-lung axis) was reinforced by intracellular activation of caspase-11 in the lungs (Fig. 4A) and alveolar macrophages (Fig. 4B). Systemic endotoxemia also activated caspase-1 and non-canonical activation of inflammasome (Fig. 5) with synthesis and release (Fig. 3, 5, 6) of IL-1 cytokines (IL-1 β and IL-18) and damage associated molecular pattern molecules (HMGB1). Interactions between caspase-11 and caspase-1 led to GSDMD dependent barrier disruption (Fig. 6) and programmed necrotic pyroptosis in alveolar macrophages (Fig. 7). A schema outlining these pathways is presented in Fig. 8 and underscores the complexities of direct lung injury combined with extrapulmonary sepsis.

Mechanical ventilation is a common clinical strategy to rest injured lung and improve gas exchange in critical care setting [3] and deliver anesthetic agents intraoperatively. The proinflammatory and injurious nature of high tidal volume ventilation has led to adherence to lung-protective ventilation protocols minimizing ventilator induced lung injury (VILI) and greatly reducing morbidity and mortality in ARDS [2–4]. Nonetheless, otherwise non-injurious lower tidal volume ventilation may exacerbate preexisting acute lung injury due to bacterial [5, 6] or viral [11] infection, intratracheal endotoxin [7–10] or sterile injury such as hyperoxia [14] or acid instillation [12, 13]. Sensitization of VILI to events originating at distal sites or plasma space including exogenous endotoxin [16, 17] or polymicrobial sepsis [18, 19] has also been documented. We confirmed [20] that MTV exacerbated modest acute lung injury secondary to intratracheal instillation of Poly(I:C). Poly(I:C), a TLR3 ligand, is a synthetic analog of double stranded RNA that can be produced by many viruses during their replicative cycles [31]. The precise mediators or pathways underlying the synergistic effect of Poly(I:C) and MTV are unknown but are largely independent of TLR4 [21]. In the current study, we add the possibility that extrapulmonary sepsis secondary to enhanced gastrointestinal permeability with Poly(I:C)-MTV underlies this synergistic effect and reveal a role for intracellular LPS mediated caspase-11 activation, a non-canonical inflammasome pathway and interactions of caspase-11 and caspase-1 in lung injury.

The influence of interactions between gut and lung microbiota in respiratory health is firmly established in chronic [32] and acute [23, 24] disease. Evidence of such microbial mingling in ARDS has suggested therapeutic strategies for ARDS of probiotics [33], novel bio-engineered delivery systems [34] and antimicrobial agents [23]. In the current study, combined injury led to an increase in gut permeability with the introduction of endotoxin to the circulation and lung (Fig. 1). We did not attempt to identify mediators

released from lung that might account for this effect on the gut but it is noteworthy that macrophages isolated from Poly(I:C)-MTV treated mice [20] release TNF- α and anti-TNF- α antibodies have been shown to abrogate the increase in gut permeability (and lung edema) in high volume ventilation in rats [35]. We assumed that endotoxemia in our model was secondary to increased gut permeability as Poly(I:C) mixture was endotoxin free and neither intubation nor circuitry for mechanical ventilation introduced significant amounts of endotoxin in lung (Fig. 1D). The identification of systemic endotoxemia in the combined Poly(I:C)-MTV protocol motivated us to pursue caspase-11 mediated pyroptosis in lung as Chun et al [21] reported a TLR-4 independent pathway in this model and Cheng et al [25] noted that introduction of systemic endotoxin caused TLR-4 independent, caspase-11 mediated pyroptosis in mice. HMGB1 is known to deliver extracellular LPS via RAGE to cytosolic caspase-11 [36], therefore, the elevated levels of HMGB1 observed after Poly(I:C)-MTV may contribute to the caspase-11, and LPS dependent future experiments to neutralize systemic endotoxemia or eliminate gut microbes in general (e.g. gnotobiotic mice or combined antibiotic therapies) will help advance gut-lung axis hypothesis beyond associative observations in the current study.

Pyroptosis is an inflammatory programmed cell death pathway activated by murine caspase-1 or caspase-11 (caspase-4 and -5 are human orthologs) and requires cleavage and activation of pore-forming effector protein, GSDMD [27]. It appears to be a key component of innate immunity and teleologically is an effective means of eliminating intracellular pathogens and signaling host via release of inflammatory mediators [25]. Nonetheless, excessive activation is implicated in human diseases including sepsis [37]. For example, dihydromyricetin, an inhibitor of NLRP3, alleviated cecal ligation and puncture-induced lung histopathologic injury in mice [38]. As cell death, per se, and inflammatory mediators are essential components of disruption of alveolar capillary barrier in ALI and ARDS, insight into relevant pathways may be informative of pathogenesis and therapeutic strategies. Caspase-1 activation is well known to be activated via a canonical inflammasome pathway (including but not limited to NLRP3) as well as a caspase-11 mediated non-canonical inflammasome pathway [27]. Canonical activators include dsRNA (and mechanical ventilation [39]) as well as bacteria; non-canonical activators include gram negative bacteria. Accordingly there is interaction of these caspases in the maturation and release of cytokines of IL-1 family (IL-1 β and IL-18), as well as pyroptosis [27, 30]. In the current study, Poly(I:C) is likely to activate the canonical pathway as noted by: a) increase in mRNA of NLRP3 (Fig. 5A) in alveolar macrophages of Poly(I:C)-MTV treated mice that was only partially sensitive in the caspase-11 null mice; and b) synthesis and release of IL-1 β , IL-6 and IL-18 that was sensitive to genetic deletion of NLRP3 (Fig. 5B, C, D). Mechanical ventilation has also been shown [39] to activate NLRP3 inflammasome in alveolar macrophages in a caspase-1 dependent fashion underscoring an additional stimulus of canonical pathway in our combined Poly(I:C)-MTV model. Alveolar-capillary barrier disruption was only partially sensitive to genetic ablation of GSDMD (Fig. 6A, B) as was release of alarmins (HMGB1; Fig. 6D) whereas release of IL-18 to plasma space (Fig. 6F) was highly sensitive to GSDMD deletion in combined injury protocol further underscoring the interaction of these pathways and resultant phenotype (Fig. 8). To the best of our knowledge, in situ identification of cellular components of pyroptosis remains challenging in murine tissue. As such, we utilized an ex vivo strategy involving

isolation and short term culture of murine alveolar macrophages from Poly(I:C)-MTV treated mice (Fig. 7) and quantified pyroptosis via co-expression of caspase-1 and TUNEL. Although macrophages (and precursor monocytes) are prototypic of death by pyroptosis, it is noteworthy that other investigators have utilized primary cultures of murine pulmonary endothelial cells isolated from systemic endotoxemic mice [25] or cultured pulmonary epithelial cells [35] directly exposed, *in vitro*, to reveal presence (and differences) in pyroptosis and release of cytokines. Within the limits of our study, we suggest that combined Poly(I:C)-MTV activates both canonical and non-canonical inflammasome pathways involving both caspase-11, caspase-1 and their interaction and GSDMD dependent pyroptosis in at least alveolar macrophages (Fig. 8). Further cellular origins, aside from alveolar macrophages, awaits improvements in antibody dependent immunohistochemistry in murine lung and pharmacologic separation of caspase-1 and caspase-11 and relevant inflammasome pathways.

Conclusions

By revisiting [25] a two hit model (Poly(I:C)-MTV) of acute lung injury noted to be TLR4 independent, we have detected an additional stimulus, e.g. systemic endotoxemia; as a result of gut-lung axis, both non-canonical caspase-11 (via presumptive cytosolic endotoxemia [28]) and canonical (via NLRP3 inflammasome[38, 39]) and their interactions led to pyroptosis in alveolar macrophages, disruption of alveolar capillary barrier and proinflammatory state within lung. Pharmacologic strategies at disrupting communication between gut and lung, inhibition of inflammasomes or effector molecules (GSDMD) in pyroptosis may be useful in acute lung injury.

Abbreviations

1. MTV: moderate tidal volume ventilation
2. Poly(I:C): polyinosinic-polycytidylic acid
3. ALI: acute lung injury
4. BALF: bronchoalveolar lavage fluid
5. EBA: evans blue albumin
6. HMGB1: high-mobility group box 1
7. GSDMD: gasdermin D
8. NLRP3: nucleotide-binding domain leucine-rich repeat-containing protein 3
9. TLR4: toll like receptor 4
10. ARDS: acute respiratory distress syndrome
11. VILI: ventilator induced lung injury
12. LPS: lipopolysaccharide
13. FITC-D: fluoresceine isothiocyanate dextran
14. AM: alveolar macrophages

- 15. ASC: apoptosis-associated speck-like protein
- 16. CARD: caspase activation and recruitment domain
- 17. PBS: phosphate buffered saline
- 18. RPMI: roswell park memorial institute
- 19. RAGE: Advanced glycosylation end product-specific receptor

Declarations

Ethics approval and consent to participate: Animal protocols are approved by the Animal Care and Use Committee of the University of Pittsburgh and experiments were performed in strict adherence to NIH Guidelines for the Use of Laboratory Animals and followed current guidelines for preclinical models in research.

Consent for publication Not applicable

Availability of data and materials: The datasets generated and/or analyzed during the current study are available in the Dr. Billiar Lab of University of Pittsburgh, and the datasets are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interest.

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Authors' contributions:

SQJ, XBD, TRB, QL, LMZ have made contributions to research concept and design.

SQJ, XBD have made contributions to the acquisition, analysis of data.

SQJ, HL, BRP, TRB, LMZ have made contributions to data interpretation.

CXY, WBL have made contributions to the creation of new software used in the work. SQJ, BRP, TRB, LMZ have made manuscript preparation.

SQJ, BRP, TRB, QL and LMZ have critically revised of the manuscript and approved manuscript final version.

All authors have read and approved the final submitted manuscript version.

All authors have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Figures

Figure 1

Intestinal and alveolar-capillary permeability. Intestinal permeability of mice evaluated by FITC-D levels in plasma (A) and in bronchoalveolar lavage fluid (BALF) (B). 200 μ L FITC-D (30 mg/mL) was instilled through orogastric feeding. Endotoxin levels in plasma (C) and in BALF (D) were measured. All PBS and Poly(I:C) used were tested endotoxin-free. Mice were divided into four groups treated with Sham as control, Poly(I:C), mechanical ventilation with tidal volume of 10mL/kg (MTV) and Poly(I:C) followed with MTV as indicated in the figure. Results are shown as means $\pm$ SEM (n=6) and compared by one-way ANOVA and Student-Newman-Keuls test. *p < 0.05, **p < 0.01, ***p < 0.001.

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Figure 2

Caspase-1 and caspase-11 prevented Poly(I:C)-MTV induced lung injury. Wildtype (WT), Caspase-1/11 null and Caspase-11 null mice (Caspase 1/11 KO and Caspase 11 KO) were treated with four groups as indicated. EBA permeability (n=5) (A), total protein concentration in BALF (n=6 for WT mice, n=12 for caspase-1/11 and caspase-11 KO mice) (B), H&E histology (n=5) (C) of the lung cross section from WT, caspase-1/11 KO, caspase-11 KO mice (Scale bars: 50 μ m) and total histopathologic scores of lung injury (D) were evaluated by two different authors calculated for each animal. Results are shown as means $\pm$

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Caspase-1 and caspase-11 alleviated Poly(I:C)-MTV induced pulmonary inflammatory response. IL-6 (A), HMGB1 (n=5) (B) and IL-1 β (C) in BALF as well as IL-18 levels (D) in plasma were significantly decreased in Caspase 1/11 KO and Caspase-11 KO mice compared to WT mice. In figure 3A, C, D, n=6 for WT mice, n=12 for caspase-1/11 and caspase-11 KO mice. Results are shown as means $\pm$ SEM and compared by one-way ANOVA and Student-Newman-Keuls test. *p < 0.05, **p < 0.01, ***p < 0.001.

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Alterations of protein levels of cleaved caspase-11, procaspase-11 and cleaved caspase-11. MTV following Poly(I:C) instillation resulted in a significant increase in procaspase-11 and, large increase in appearance of cleaved caspase-11 in whole lung (A) and alveolar macrophages (B). Cleaved caspase-1 expression levels were inhibited in caspase-11 KO mice in Poly(I:C)-MTV compared to that in WT mice

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Figure 5

NLRP3 was required for Poly(I:C)-MTV induced lung injury. Poly(I:C)-MTV increased NLRP3 mRNA levels in WT mice but was partially inhibited by caspase-11 KO mice, (n=12) (A). NLRP3 was required for

Poly(I:C)-MTV induced IL-6 (B), IL-1 β release in BALF (C) and IL-18 (D) secretion in plasma. In Fig.5B-D, n=6 of each group for WT mice, n=8 for NLRP3^{-/-} mice. Results are shown as means $\pm$ SEM and compared by one-way ANOVA and Student-Newman-Keuls test. *p < 0.05, **p < 0.01, ***p < 0.001.

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GSDMD was required for Poly(I:C)-MTV induced lung injury. Poly(I:C)-MTV increased EBA permeability, (A), total protein (B), IL-6 (C), HMGB1 (D), and IL-1β (E) in BALF as well as IL-18 levels (F) in plasma in WT but were inhibited in GSDMD KO mice. n=5 of each group for WT mice and GSDMD^{-/-} mice. Results are

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Figure 7

MTV induced alveolar macrophages pyroptosis after Poly(I:C) priming. Alveolar macrophages on and maturation and release of cytokines in combined Poly(I:C)-MTV injury model. The gut-lung axis resulted in activation of pulmonary non-canonical (cytosolic mediated caspase-11 activation) and canonical

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Figure 8

Schematic graph of gut-lung axis. Combined (Poly(I:C)-MTV) insult results in increase in gastrointestinal permeability and endotoxin in plasma and BALF. Poly(I:C)+MTV insult was sensitive to Caspase-11 deletion with no further contribution of caspase-1 but led to large increases in procaspase 11 and its

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