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Double-stranded RNA Interacts with Toll-like Receptor 3 in Driving the Acute Inflammatory Response Following Lung Contusion

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Abstract

Objectives—Lung contusion (LC) is a major risk factor for the development of acute respiratory distress syndrome (ARDS). We set to determine the role of TLR3 and the binding of dsRNA in the pathogenesis of sterile injury following lung contusion.

Methods—TLR3 expression was analyzed in post-mortem lung samples from patients with LC. Unilateral LC was induced in TLR3 (–/–), TRIF (–/–), and WT mice; subsequently, lung injury and inflammation were evaluated. Apoptotic indices, phagocytic activity and phenotypic characterization of the macrophages were determined.

Double stranded (ds) RNA in BAL and serum samples following LC were measured. A TLR3/ dsRNA ligand inhibitor was injected into WT mice prior to LC.

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Measurements and Main Results—TLR3 expression was higher in patients and WT mice with LC. The degree of lung injury, inflammation, and macrophage apoptosis was reduced in TLR3 (−/−), TRIF (−/−), and WT mice with TLR3 antibody neutralization. Alveolar macrophages from TLR3 (−/−) mice had a lower early apoptotic index, a predominant M2 phenotype and increased surface translocation of TLR3 from the endosome to the surface. When compared to viral activation pathways, lung injury in LC demonstrated increased p38 MAPK, ERK1/2 phosphorylation with inflammasome activation without a corresponding increase in NF-κB or Type-1 Interferon production. Additionally, pretreatment with TLR3/dsRNA ligand inhibitor led to a reduction in injury, inflammation, and macrophage apoptosis.

Conclusions—We conclude that the interaction of dsRNA from injured cells with TLR3 drives the acute inflammatory response following LC.

Keywords

Lung contusion; Toll-like receptor 3; TRIF; dsRNA

Introduction

Lung contusion (LC) is the primary cause of mortality following blunt chest trauma and blast explosions and is an independent risk factor for the development of acute respiratory distress syndrome (ARDS) and ventilator-associated pneumonia (VAP)(1, 2). It is critical to delineate the factors responsible for the development of progressive respiratory failure, as 30% of patients with LC progress to ALI/ARDS(1). These conditions have substantial mortality and morbidity, and the resulting economic burden on the healthcare system is considerable.

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that recognize exogenous and endogenous molecules released in response to stress, trauma, and cell damage(3). TLR3 is unique in that it binds a form of nucleic acid called double stranded RNA (dsRNA). Specifically, TLR3 was initially characterized for its responses to viral dsRNA(4), but more recently it has been shown that endogenous nucleic acids from necrotic cells activate immune cells through this TLR(5, 6). While these studies have indicated a role for TLR3, the precise nature of the ligand and the downstream signaling pathways are unclear. Moreover, the precise role of TLR3 in lung contusion, bacterial pneumonia, and other non-viral diseases remains largely unknown. Additionally, TLR3 does not employ the MyD88 (Myeloid differentiation primary response gene 88) signaling pathway but instead signals through a unique adaptor protein called TRIF (TIR-domain-containing adapter-inducing interferon-β).

In the present study, we examine the role of TLR3/TRIF and the downstream regulatory pathways in the pathogenesis of acute inflammation and injury in LC. These models reflect focal and diffuse disease processes that are independent risk factors for the development of ARDS; importantly, while the model of LC is non-lethal, the pneumonia model is lethal(7, 8). We hypothesized that TLR3 activation through RNA from necrotic and injured cells may be responsible for driving the acute inflammatory cascade associated with LC. The data presented here indicate that dsRNA released following lung contusion activates the acute

inflammatory response, rendering TLR3 inhibition a potential new therapeutic strategy to limit lung injury.

Materials and Methods

Human lung-histology and immunofluorescence staining

Human lung contusion specimens were obtained from the Heart and Lung Institute, University of British Columbia, Vancouver, Canada, and access to these tissue specimens was approved by the local research ethics committee. For the lung specimens, slides of lung tissue from autopsy samples were obtained from uninjured lung samples and 15 patients who died following lung contusion.

Histology (Human and Mouse samples)

The formalin-fixed uninjured control and lung-contused sections were paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Histological findings such as peribronchial, parenchymal, and perivascular cell infiltration were semi-quantitatively graded in a blinded manner.

TLR3 immunofluorescence staining (Human)

The sections that represented histological changes consistent with progression of lung contusion in human subjects were further analyzed for immunohistochemical localization of TLR-3. Briefly, 5 μ m sections were de-paraffinized and rehydrated. Following this, heat-induced epitope retrieval was performed in a decloaking chamber containing 1X citrate buffer-pH-6.0 (Dako). Sections were washed and blocked (7% Bovine Serum Albumin + 1% Fetal Calf Serum + 0.05% Azide) for 15 minutes, incubated overnight at 4°C with polyclonal TLR-3 antibody, and incubated with goat anti-rabbit alexa-594 for 60 minutes at RT with intermittent washing. Sections were mounted using ProLong Gold containing DAPI (Invitrogen). All primary and secondary antibodies were diluted in antibody diluent (Dako) containing 2% normal goat serum. (9–11)

TLR3 Immunofluorescence staining-confocal microscopy

Mouse lung sections were prepared and stained as previously described(8).

Animals

Male age-matched (6–8 week) C57/BL6, TLR3 (–/–), and TRIF (–/–) mice (Jackson Laboratories, Bar Harbor, ME) were used. All procedures performed were approved by the Institutional Animal Care and Use Committee at the University of Michigan and complied with state, federal, and National Institutes of Health regulations.

Murine model for lung contusion

Male, 20–25 g C57BL/6 (6–8 wks, bred in-house), TLR-3 (–/–), and TRIF (–/–) mice were anesthetized, and lung contusion was induced. Briefly, after induction of anesthesia, each mouse was placed in a left lateral position and with a cortical contusion impactor the right chest was struck along the posterior axillary line 1.3 cm above the costal margin with a

velocity of 5.8 m/sec adjusted to a depth of 10 mm). The mice were then allowed to recover spontaneously.

Albumin concentrations in BAL

Albumin concentrations in the BAL were measured by ELISA (® Bethyl Laboratories, Inc. Montgomery, TX) as previously described. (7)

Lung pressure-volume (P-V) mechanics

Pulmonary respiratory mechanics were measured immediately after blood samples were obtained and the mice were further exsanguinated by transection of the abdominal inferior vena cava. An 18-gauge metallic cannula was inserted into the trachea through a midline cervical exposure. Animals were then connected to a SCIREQ® Flexivent (Montreal, Quebec, Canada) that allows for simultaneous animal ventilation and data capture. Immediate postmortem ventilation was done with the following parameters: tidal volume 10 ml/kg, respiratory rate 150 breaths per minute and positive end expiratory pressure 2 cm H₂O. With PVRV, a controlled inflation and deflation was performed to measure quasi-static compliance values.

Determination of cytokine levels in BAL

IL-1 β , MIP-2, CCL-2 (MCP-1), CCL-12 (MCP-5), IL-6, KC, CXCL-10, and TNF- α were measured in BAL by ELISA (® R&D Systems, Minneapolis, MN) as previously described. (7)

Determination of interferon's levels in Serum and BAL

Type-1 interferons (α , β) in both serum and BAL samples from TLR3 (–/–) and WT mice were measured in serum and BAL by ELISA (® R&D Systems, Minneapolis, MN).

Immuno-neutralization of TLR-3 in WT mice

TLR-3 was blocked by a single injection of monoclonal murine antibodies against this receptor (R&D Systems, San Diego, CA). The antibody injections were performed intraperitoneal 48 hours prior to LC (antibody concentration was 20 ug/mouse). The BAL and tissues were harvested at 5, 24, 48, and 72 hour time points. The experimental methodology was similar to the one adapted for the knockout animals.

In vitro phagocytosis assay

Phagocytosis assays were performed as previously described (12). Briefly, after lung contusion in WT and TLR-3 knockout mice, alveolar macrophages (AM) were isolated from BAL and plated onto a 2×10^5 cells/well and cultured overnight in DMEM medium. Wells were aspirated and replaced with 50 μ L serum-free medium. Macrophages were then incubated with FITC-labeled, heat-killed *Pseudomonas aeruginosa*. Phagocytosis of FITC-labeled bacteria was measured after quenching of non-ingested bacteria with trypan blue.

TaqMan Quantitative Polymerase Chain Reaction

Total RNA was prepared from alveolar macrophages using by RNeasy mini kit, according to the manufacturer's directions (Qiagen, USA). A total of 1 µg RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen). The cDNA was then amplified by real-time quantitative TaqMan PCR using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). GAPDH was analyzed as an internal control. TaqMan gene expression reagents or SYBR Green Master PCR mix were used to assay Arginase 1 and Fizz 1 (Applied Biosystems). Data are expressed as fold-change in transcript expression. The fold difference in mRNA expression between treatment groups was determined by software developed by Applied Biosystems.

Flow-cytometry: Apoptosis-Annexin V-FITC staining

Apoptosis was performed as previously described(8). Briefly, cells were labeled with Annexin V (BioLegend, San Diego, CA) and incubated for 20 minutes at room temperature. After washing with Annexin V binding buffer, cells were incubated for 10 minutes with LIVE/DEAD stain (Invitrogen, Carlsbad, CA). Cells were washed and blocked with Fc block (CD16/32). The cells were then stained with the following fluorochrome-conjugated mouse antibodies: Ly6C-FITC, Gr-1-PE, CD11c-APCCy7, F4/80-AF488, and CD11b-PE-Cy7 (BioLegend and BD Biosciences, San Jose, CA). Data were analyzed using Flow Jo software.

TLR3 cell-surface expression determination by flow cytometry

Intracellular and extracellular TLR3 expression was determined using flow cytometry. BAL macrophages were obtained from uninjured control and WT mice after LC. Fc binding was blocked with anti-mouse CD16/32 antibody, and the cells were incubated with PE-conjugated anti-F4/80 and pacific blue conjugated anti-CD45 antibody (® BioLegend, San Diego, CA). The cells were then fixed, permeabilized, incubated with polyclonal rabbit anti-mouse TLR3 antibody or rabbit IgG, and stained with FITC conjugated anti-rabbit IgG. The cells were not permeabilized for extracellular staining of TLR3. Data were analyzed using Flow Jo software.

MyD88 staining and Flow cytometry

BAL cells were collected after LC. The cells were fixed, permeabilized, and subjected to intracellular staining. The following primary antibodies were used; MyD88 (R&D system, Minneapolis, MN) CD11c-FITC, TLR3-PE, and F4/80-APC-Cy7 (® BioLegend, San Diego, CA). Data were analyzed using Flow Jo software.

Preparation and isolation of RNA from necrotic cells and co-culture with alveolar macrophages

RNA was isolated from BAL of WT mice 24h following LC. Alveolar macrophages from TLR3 (–/–) and WT mice were harvested from the BAL of uninjured animals. These macrophages were co-cultured in the presence of RNA. At 24h, the medium was collected and measured for pro-inflammatory cytokines by ELISA.

Western Blots

Western blots were performed as previously described (12) (All antibodies from @Cell Signaling Technology, Beverly, MA)

RNA analysis by non-denaturing polyacrylamide gel electrophoresis

To investigate the presence of dsRNA in BAL and serum samples following LC, non-denaturing polyacrylamide gel electrophoresis was performed. The total RNA (50 µg) of the samples and the dsRNA marker (Abnova®, Walnut, CA) were electrophoresed on a 7.5% non-denaturing polyacrylamide gel at 200 V in a 4°C cold room. The gel was run at 18 W for 2–3 h. After electrophoresis, the gel was stained; RNA was identified.

dsRNA immunocytochemistry

Alveolar macrophages were fixed by adding formaldehyde directly to the cells for 15 minutes at room temperature. Cells were washed with PBS, permeabilized with 0.2% Triton X-100 (Sigma-Aldrich), washed twice with PBS, and blocked with PBS containing 1% BSA. MabJ2, which was obtained from English & Scientific Consulting (Bt. Szirák, Hungary), was used to detect dsRNA, and monoclonal anti-TLR-3 polyclonal antibody was used to detect TLR-3. Fixed and permeabilized cells were incubated overnight at 4°C with diluted MabJ2 in PBS/1%BSA (1:500 dilution of the 0.200 mg/ml antibody). Cells were washed twice with PBS and incubated with Alexa-fluor labelled secondary antibodies (Invitrogen) for 1 h. Nuclei were stained with DAPI, the cells on coverslips were mounted in mounting media (Dako), and photomicrographs of the invasive sections were analyzed digitally using Photoshop software version 9.0.2.

RNase treatments

The RNase III (Invitrogen) and RNase A were purchased from Sigma-Aldrich (St. Louis, MO). For dsRNA staining, macrophages were collected after LC, fixed, permeabilized as described above, and incubated for 2 h at 37°C with RNase III (2µg/ml) in reaction buffer or with RNase A (1µg/ml) in PBS. After the incubation period, cells were washed 10 times with washing buffer and processed for immunofluorescence as described above.

Intracellular dsRNA staining and Flow cytometry

For the detection of intracellular staining for dsRNA, BAL cells were collected after LC. The cells were fixed, permeabilized, and subjected to intracellular staining(13). The following primary antibodies were used: J2 (@English & Scientific Consulting, Bt. Szirák, Hungary CD11c-FITC and F4/80-APC-Cy7 (@ BioLegend, San Diego, CA) (14). Data were analyzed using Flow Jo software.

TLR3/dsRNA complex inhibitor administration (in-vivo)

The TLR3/dsRNA complex inhibitor ((R)-2-(3-Chloro-6-fluorobenzo [b] thiophene-2-carboxamido)-3-phenylpropanoic acid, Calbiochem/EMD Millipore, MA®) was dissolved in DMSO and diluted in sterile PBS. The inhibitor (1 mg per mouse) (15) was administered (i.p) into the mice 2h before LC. Mice were subjected to treatment (i.p.) with poly I: C (20 mg/kg body weight) (@Sigma Aldrich, St. Louis, MO) 1h before LC.

TLR3/dsRNA complex inhibitor administration (ex-vivo)

Alveolar macrophages of WT mice were harvested from the BAL 24 and 48 hours following LC. These macrophages were co-cultured in the presence of necrotic RNA (24h after LC), TLR3/dsRNA inhibitor (27 μ m/ml), and/or the synthetic dsRNA molecule poly-I: C (10 μ g/ml). The medium was collected after treatments and measured for pro-inflammatory cytokines by ELISA.

ApoStat

Intracellular caspase activity was measured by apostat (@R&D Systems, Minneapolis, MN). BAL cells were washed and fixed with 1% formalin and reconstituted in 300 μ l of flow buffer. Intracellular caspase activity was measured by an ApoStat kit (R&D Systems) as described by the manufacturer's protocol.

Statistical methods

Data are expressed as mean $\pm$ SEM. Statistical significance was estimated using one-way analysis of variance (ANOVA). Individual inter-group comparisons were analyzed using two-tailed, unpaired t-test with Welch's correction (Graph Pad Prism 6.01). Significance was set at $P < 0.05$.

Results

1. Human and Mouse lung contusion samples show high expression of TLR3

a. Intense TLR3 staining of alveolar macrophages and epithelial cells in post-mortem human lungs—Histopathological evaluation of post-mortem samples from patients with LC reveals a significant degree of severe diffuse alveolitis characterized by influx of macrophages, and rare lymphocytes were seen compared to the normal lungs. Immunofluorescence data demonstrated that the samples from patients with lung contusion have significant expression of TLR3, especially in the alveolar lining and macrophages (Figure 1, Panel a–f).

b. Alveolar macrophage and epithelial cells show high expression of TLR3 in mice following lung contusion—Immunofluorescent images show intense TLR3 signal in alveolar macrophages and epithelial cells (Figure 1, Panel g).

2. TLR3 is functionally important in the development of progressive lung injury following lung contusion

a. TLR3 (–/–) mice exhibited decreased permeability injury and inflammation with LC—We have previously shown that the acute inflammatory response in LC is responsible for deficits in oxygenation and increases in quasi-static pulmonary compliance and severe permeability injury (BAL albumin)(8, 16–19). While there was a significant decrease in the BAL albumin level in TLR3 (–/–) mice (Figure 2a), pulmonary compliance and volumes were significantly lower in WT mice (Figure 2b). Histological evaluation of WT mice showed significantly more injury than TLR3 (–/–) mice at all-time points (Figure 2c). The numbers of macrophages/neutrophils (Figure 2d) and levels of pro-inflammatory

cytokines (interleukin [IL]-1 β , IL-18 and IL-6), monocyte chemotactic protein chemokines (MCP-1 and MCP-5), and macrophage inflammatory protein-2 (MIP-2) were uniformly higher in WT mice (Figure 2f). These molecules reflect the various attributes of acute and sub-acute inflammatory response following LC (16, 20).

b. TLR-3 driven inflammatory response is not mediated by Type-1 Interferons

—In additional experiments, the difference in the elaboration of type-1 interferons (α , β) in both serum and BAL samples from TLR3 ($-/-$) and WT mice yielded low levels with very little difference (Figure 2e). Measurements of transcript levels of type-1 IFNs in alveolar macrophages were elevated at 2 hours following LC in WT mice, but these levels returned to those seen in uninjured mice by 24 hours (data not shown). These results indicate that pathways other than type-1 IFNs are implicated in the TLR3-driven pathogenesis of inflammation in LC.

Additional experiments were performed by administering mouse monoclonal antibody to TLR3 48 hours prior to lung contusion in C57BL/6 mice using IgG and saline control mice ($n=6$). Consistent with the results from TLR3 ($-/-$) mice studies, the degree of injury and inflammation (albumin and BAL cells/cytokines levels) was consistently lower with the administration of monoclonal antibody against TLR3 (Figure 3a). Additionally, we were able to document the intracellular entry of these antibodies (Figure 3b). Taken together, these data demonstrate that the absence of TLR3 confers a protective role as evidenced by the decrease in not only lung permeability injury but also the acute inflammatory response to LC. We conclude that TLR3 plays a role in up-regulating the intensity of acute inflammation following LC.

c. Alteration of alveolar macrophage phenotype, phagocytic activity, apoptosis, and generation of inflammatory cytokines in lung contusion is dependent on TLR3—Macrophage phenotypes can be characterized as pro-inflammatory (M1) or immuno-modulatory and tissue remodeling (M2). Macrophage polarization into M1 or M2 phenotype dictates the nature, duration, and severity of an inflammatory response (7, 21). Since increased susceptibility to bacterial infection during lung injury correlates with defects in alveolar macrophage phagocytosis, we measured ex vivo phagocytosis in an *in vitro* assay using isolated alveolar macrophages from wild type and TLR3 ($-/-$) mice. The relative phagocytic activity was significantly higher after LC in the TLR3 ($-/-$) mice at 24 and 48 hour time points (Figure 4a). The M1 phenotype (also termed as classically activated) is characterized by increased production of oxidative burst and nitric oxide release. Unmitigated expression of M1 phenotype indicates significant injury, particularly in sterile injury such as LC. Conversely, the M2 phenotype (also termed as alternatively activated) is associated with decreased production of pro-inflammatory cytokines, is characterized by an increased upregulation of FIZZ-1/Arginine pathway and is indicative of the reparative process. The TLR3 knockout mice exhibited a protective M2 as evidenced by increased Arginase-1 and Fizz-1 expression, suggestive of a functional alteration and change in polarity of alveolar macrophages (Figure 4b & c). There was significantly increased apoptosis at all-time points in the alveolar macrophages from WT mice following LC compared with TLR3 ($-/-$) mice (Figure 4d).

d. Surface translocation of TLR3 increases following LC—We performed experiments to evaluate whether there is surface translocation of TLR3 with injury. Here, we used flow cytometry of macrophages to demonstrate that there is significant cell surface translocation of TLR3 in WT mice following LC compared to the uninjured control (Figure 4e). These data indicate that LC has a significant impact on TLR3 translocation.

e. Activation of MyD88 in macrophages following lung contusion—Next, we sought to investigate whether the TLR2/4 adaptor Myeloid differentiation primary response gene 88 (MyD88), an activator of all TLRs, has the ability to modulate TLR3 signaling following lung contusion. Previous studies have shown an important role for TLR4 and MyD88 in the evolution of acute inflammatory response following LC (20). We used flow cytometry of macrophages to demonstrate the expression of MyD88 in WT and TLR3 (–/–) mice following LC. Our data showed that TLR3 expression was significantly higher in WT mice, as expected, MyD88 expression was 2-fold higher in TLR3 (–/–) mice compared to the WT mice following lung contusion (Figure 4f). In the absence of studies with dual knockout mice, this data suggest that the TLR3/TRIF-induced inflammation and injury following lung contusion is likely independent of complementary activation of pathways involving TLR2 or 4.

g. Co-culture of RNA from necrotic cells in the BAL with alveolar macrophages—We next sought to determine whether the substances released by necrotic cells were responsible for the inflammatory response. KC, CCL5, and CCL3 were not activated upon addition of RNA from necrotic lung cells (neither at 100 ng nor at 1 µg/ml) in the TLR3 (–/–) mice compared to WT mice (Figure 4f). The positive control in these experiments was the synthetic dsRNA molecule Polyinosinic: polycytidylic acid (poly I:C). These data demonstrate that RNA from necrotic lung cells promotes an inflammatory response via the induction of potent pro-inflammatory mediators and thus represents the ligand for activation of TLR3 in LC.

3. TLR3/TRIF pathway and downstream signaling activation following LC

a. Downstream signaling in LC—To determine TLR3 downstream signaling activation following LC, WT and TLR3 (–/–) mice lung lysates and nuclear extracts were analyzed by Western blot using various antibodies (Figure 5a). While the WT lung lysates showed an increase in phospho-IRF-3, phospho-ERK1/2, and phospho-p38 MAPK, an increased phosphorylation of IRF-3 was not observed in the nuclear isolates. Phosphorylated NF-κB and IκBα activation was not detected in WT or TLR3 mice following LC. Additional immunofluorescence staining for phospho-NF-κB and total NF-κB revealed similar results (data not shown). TLR3 (–/–) mice showed significant reductions in TRAF6 (Figure 5b) and NLRP-3 compared to WT mice (Figure 5a). The TLR3/TRIF signaling pathway activation in LC is therefore independent of NF-κB and mediated through phosphorylation of p38 MAPK and ERK1/2. With significant differences in levels of IL-1β and IL-18 with NLRP-3, it thus appears that inflammasome activation independent of activation of NF-κB and IFN production is unique to TLR3 mediated acute inflammation following LC (Figure 5b and c).

b. TRIF is functionally important in the development of progressive lung injury and inflammation following LC—

We next evaluated the role of TRIF in LC. There was a significant increase in the BAL albumin level (Figure 6a) with histological evidence of increased injury in WT mice at all-time points compared to TRIF (–/–) mice (Figure 6b). Similar results were observed with increased BAL cellularity (Figure 6c) and inflammatory cytokines (IL6, IL-1 β , MCP-5, MIP-2, KC, and TNF α) (Figure 6d) in WT mice. These results suggest that TRIF plays an important role in up-regulating the intensity of acute inflammation following LC.

In total lung lysates and nuclear extracts phosphorylated NF- κ B and I κ B α activation was not detected in WT or TRIF (–/–) mice following LC (data not shown). TLR3 mice showed significant reductions in Activator protein-1 (AP-1) expression (Figure 6e). AP-1 is known to control a number of cellular processes including differentiation, proliferation, and apoptosis(22).

4. Role of dsRNA in lung inflammation following LC

a. RNA expression in WT mice in BAL and serum samples following LC—

TLR3 activation in viral diseases has been shown to be mediated specifically by ligation with double-stranded RNA (dsRNA) components from the infecting virus. However, what activates TLR3 during acute inflammation secondary to LC is unclear. Using gel electrophoresis, we detected the presence of two distinct bands representing dsRNA of 20–30 and 30–50 base pairs in length in both cell-free BAL and serum from animals following LC (Figure 7a). Based on the number of base pairs, the shorter RNAs likely represent endogenous microRNAs and/or siRNAs.

b. Alveolar macrophage show increased expression of dsRNA following lung contusion—

Immunofluorescent imaging demonstrates intense dsRNA signal in alveolar macrophages (J2 Ab(13)) from contused mice compared to corresponding uninjured controls (Figure 7b). Using flow cytometry, we observed a two-fold dsRNA and multifold TLR3 expression increase with LC (Figure 7c). We found that dsRNA staining disappeared completely with RNase III treatment, a dsRNA-specific RNase, but only moderately with the preferably single-stranded RNA cleaving RNase A (23) (Figure 7e), supporting the notion that we are staining dsRNA.

c. TLR3/dsRNA complex inhibitor reduces the injury and inflammation following lung contusion—

With the administration of TLR3/dsRNA complex inhibitor(15) in the peritoneal cavity of animals prior to LC, there was a significant decrease in BAL albumin, total caspase activation in alveolar macrophages (ApoStat™), and cytokine levels of IL-1 β and MCP1 (Figure 7d). TLR3/dsRNA complex inhibitor treatment significantly reduces the injury, inflammation, and apoptosis of the alveolar macrophages following LC.

d. TLR3/dsRNA complex inhibitor attenuates the expression of pro-inflammatory cytokines by cultured macrophages ex-vivo following LC—

Alveolar macrophages from WT injured/control mice were cultured in the presence of RNA

obtained from BAL cells following LC, poly-I:C, and a combination of RNA and poly-I:C in the presence or absence of TLR3/dsRNA complex inhibitor. RNA from the necrotic cells generated significantly higher expression in KC and MIP-2 at all the time points compared to control groups (Figure 7f). The TLR3/dsRNA complex inhibitor significantly reduced pro-inflammatory cytokines levels in all groups. However, the ability of the inhibitor to reduce the activity of cytokine production with poly-I: C was limited. It is known that the activity of poly-I: C is also mediated through RIG-I and MDA-5, two of the most important cytoplasmic sensors for activation of dsRNA (24, 25).

Discussion

The major findings of our study confirm the importance of the TLR3/TRIF pathway in the regulation of the acute inflammatory response in lung contusion. Additionally, we report that dsRNA from injured cells is the ligand that interacts with TLR3 in the context of inflammatory lung injury. There were major differences in the TLR3 activation in LC when compared with the described double stranded RNA viral activation pathways. First, we observed translocation of the TLR3 receptor to the surface of the alveolar macrophage occurs following LC. Second, the downstream signaling is mediated through TRIF, ERK1/2, p38 MAP kinase, and AP-1, but is independent of NF- κ B and does not involve type-1 interferon production. Additionally, the TLR3 dependent activation of the inflammasome characterized by elevated levels of IL-1 β and IL-18 and NLRP-3 is independent of either IFN or NF- κ B activation.

The major limitation of our study is the lack of relevant clinical data for the human lung samples that were subjected to immuno histochemistry. Though these patients had documented lung contusion, it is not possible to confirm the precise nature and cause of death. It is entirely possible that these patients at the time of death had other serious illness including ventilator-associated pneumonia and sepsis with multi-organ dysfunction. Additionally, a full characterization of the nature of the dsRNA was not performed.

In the previously reported swine model (26, 27), the insult is delivered by a captive bolt and the animal is immediately placed on a mechanical ventilator. This places significant restrictions on the duration of the study but has its advantages in terms of easier instrumentation such as study of cardiac output. In comparison the murine model used in the current manuscript, in addition to being a small animal model, has several advantages. We employ a spontaneously breathing mouse model following a closed-chest insult that eliminates the additional insult of VILI, the potential to be evaluated over a period of 7 days and provides ample opportunities to mechanistically study the pathways by the use of genetic knockouts and readily available primers/reagents.

In the present study, we found that lung contusion is a potent trigger for the induction of TLR3 expression in the lungs of both rodent and human samples. These changes are indicative of acute inflammation secondary to local mechanical trauma. These data indicate that alveolar macrophages and epithelial cells are the primary cells involved in the activation of TLR3 in LC. The major cell behind the TLR3 driven acute inflammatory response is the alveolar macrophage through its recruitment, activation, and phenotypic alteration. We show

that in alveolar macrophages following LC there is increased surface translocation of TLR3 and that this exposes the receptor to extracellular dsRNA released from injured cells. We note that recent data suggest that cigarette smoke augments the expression and response of TLR3 in human macrophages, contributing to neutrophilic airway inflammation and parenchymal destruction in the lungs of smokers and patients with COPD (28).

Three distinct mechanisms explain the abrogated inflammatory response in the absence of TLR3 in LC. One feature includes the reduction in apoptosis of the alveolar macrophage, a cell that plays a protective role in the resolution of inflammation(19), and this is a finding similar to observations in hyperoxia(29). The second mechanism involves the TLR3 driven change in polarization of the macrophage. Lung contusion is known to promote increased recruitment of alveolar macrophages to the site of injury(19). While a protective M2 phenotype is beneficial in LC(7). The M1 phenotype (also termed classically activated) is characterized by increased production of oxidative burst and nitric oxide release and secretion of pro-inflammatory cytokines (30). The significant increase in the levels of Arginase-1 and Fizz-1 in the TLR3 (–/–) knockout genotype following LC demonstrate a protective M2 phenotype (alternatively activated), which is similar to CCR2 (–/–) mice following LC(7). The M2 macrophage subpopulations may play a role in suppressing inflammation and initiating wound repair after acute injury(31).

Finally, we report that TLR3 is an endogenous sensor of necrosis and that in its absence there is a reduction in the amplification of the inflammatory response following LC. The current work provides strong evidence that the dsRNA released from necrotic cells is the ligand for activation of TLR3 in LC. This finding has significant therapeutic implications. Other toll-like receptors (e.g. TLR-2/4) have been reported to play a role in the generation of acute inflammatory response following LC (20, 32). However, significant complications from uncontrolled Gram-positive and -negative bacterial infections have been observed when the activities of these TLRs have been blocked (33–36). Currently there are many agents available including monoclonal antibodies that have the ability to abrogate TLR3 without any serious adverse effects. For example IPH33 is a monoclonal antibody targeting the Toll-like Receptor-3 receptor (TLR3) and it has been humanized (Innate Pharma ®). Clinical use of these antibodies in COPD, RA and IBD, have been proposed. Perturbation of this important sensing molecule and its downstream signaling pathways will therefore significantly increase our fundamental knowledge of and further inspire novel methods to restrict the inflammatory pathways associated with the exuberant local and systemic inflammatory activation associated with ARDS.

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Figure 1. Increased TLR3 expression in Lung contusion

Post-mortem lung samples in patients with LC compared to normal lung tissue.

Representative confocal images from normal human lung (a), normal lung stained with anti-TLR3 antibody (b), lung with LC stained with anti-TLR3 antibody (red) (c), and LC-co-localization with antibodies to TLR3 and CD68 alveolar macrophages (green) (d).

Histological analysis of post-mortem lung samples in control (e) and patient with LC (f).

These samples were obtained from the tissue bank at the University of British Columbia. No clinical data correlates other than the diagnosis of the banked specimen was provided. g: WT mice showed predominant TLR3 expression following LC. WT mice on C57BL/6

background were subjected to LC. The lung samples were harvested at 5, 24, 48, and 72h time points, and the samples were subjected to immuno-fluorescent staining with F4/80 for macrophages (green), TLR3 (red), and nuclear staining with DAPI ($n=3$ per group). Overlay composite view is provided.

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Figure 2. Injury and inflammation was reduced in TLR3 (-/-) mice following lung contusion
a: After LC, mice were sacrificed at different time points, and albumin concentration in the BAL were determined by ELISA ($n=16$ per group). **b:** Pulmonary compliance measurements in WT and TLR3 (-/-) mice following LC. “Quasi-static” closed chest P-V behavior was measured at 5h post-injury in WT and TLR3 (-/-) mice. The total lung capacity was higher in the TLR3 (-/-) mice. ** $P < 0.05$ WT vs. TLR3 (-/-) mice. **c:** Histological evaluation of TLR3 (-/-) mice following LC. In WT mice, there were large areas of hemorrhage at 5h with increasing necrosis and inflammation at 24 and 48h compared to TLR3 (-/-) mice.

Unlike the WT, TLR3 mice showed resolution by 72h. **d:** *Macrophage and neutrophil levels in BAL were higher in WT mice following LC.* Cytospin analysis of BAL samples was performed after lung injury. The levels of macrophages and neutrophils were significantly higher at all-time points in WT mice compared to TLR3 (–/–) mice ($n = 16$ per group). **e:** *Increased inflammatory response in WT was not associated with differences in levels of type-1 interferons.* BAL Interferon β , serum interferon β , BAL interferon α , and serum interferon α were measured by ELISA ($n = 6$ per group). **f:** *TLR3 (–/–) mice showed a significant reduction in cytokine response compared to WT following LC.* The levels of IL-6, IL-1 β , MIP-2, TNF- α , MCP-5 (CCL12), MCP-1 (CCL2) and IL-18 in the BAL were significantly higher in WT mice following LC compared to TLR3 (–/–) mice ($n = 16$). Statistical analysis was performed with two-tailed unpaired t-test with Welch's correction. * $P < 0.05$ WT vs. TLR3 (–/–) mice.

TLR3 LysoTrack Nuclei

Figure 3. Functional importance of TLR3 using antibody neutralization in the development of lung injury following LC

a. Monoclonal murine antibodies to TLR3 (or saline and IgG for controls) were administered i.p. 48 hours prior to LC in WT animals. Values with IgG controls were similar to saline and have been omitted for clarity. Albumin, which is representative of permeability injury, and total cells in the BAL fluid were quantified as described in Materials and Methods. IL-1 β , IL-6, MCP-1, CXCL10, KC, and MIP-2 levels in the BAL of WT and TLR3 (-/-) mice were analyzed by ELISA after injury ($n=6$). The total number of

macrophages was higher in WT mice compared to the anti-TLR3 antibody-administered groups (data not shown). The wild type mice treated with anti-TLR3 antibody exhibited significantly less acute neutrophil recruitment at 48 hours in response to lung contusion (data not shown). There was no significant difference in the levels of IL-1 β between the two groups at 5 hours after LC, but there was a significant decrease in IL-1 β levels at the 24h time points in the antibody administered mice compared to the corresponding control. The levels of the cytokines IL-6, MCP-1, CXCL-10, KC, and MIP-2 were lower in the mice treated with anti-TLR3 antibody compared to corresponding control groups. * $P < 0.05$ WT vs. corresponding antibody treated mice. b. To confirm the intracellular entry of the antibodies, the macrophages were co-stained with anti-TLR3 antibody and lysotrack® (lysosomal staining). The overlay composite view is provided.

Figure 4. Phenotypic alteration, characterization, localization of the receptor, and cytokine production of alveolar macrophage following LC

Phagocytosis assay a: BAL Macrophages from WT and TLR3 (-/-) following LC were incubated with FITC-labeled heat-killed *Pseudomonas aeruginosa* ($n=6$). *Quantitative TaqMan PCR analysis:* Arg1 (*b*) and FIZZ1 (*c*) transcript expression was measured in WT and TLR3 (-/-) mice following LC. Real time PCR analysis showed there was a significant increase in the levels of Arginase-1 and Fizz-1, the classic M2 marker genes, at 48 and 72 hours in TLR3 (-/-) mice ($n=5$). ** $P < 0.01$ and * $P < 0.05$ WT vs. TLR3 (-/-). *d:* TLR3 (-/-)

–) mice show reduced apoptosis of alveolar macrophage compared to WT following LC. The BAL cells were stained with fluorochrome-conjugated mouse antibodies. Macrophages and neutrophils were selected for FACS scan analysis. The figure shown here reflects values from a single animal in each group. Values are represented as mean (n=6 per group). *P< 0.05 WT vs. TLR3 (–/–) mice. **e:** *Toll-like receptor 3 (TLR3) expression in BAL macrophages.* Immunofluorescence analyses of TLR3 in macrophages were performed. For intracellular staining, cells were fixed and permeabilized and subsequently incubated with anti-TLR3 Ab. For cell surface staining, cells were fixed and incubated with anti-TLR3 Ab. (n=3). **f:** *Myd88 expression in BAL macrophages.* Flow cytometry analyses of TLR3 and MyD88 in macrophages were performed. For staining, cells were fixed and permeabilized and subsequently incubated with anti MyD88 and anti-TLR3 Ab. (n=3). **g:** *RNA from alveolar macrophages following LC induced more cytokine production in WT mice than TLR3 (–/–) mice.* RNA isolated from alveolar macrophages after LC was co-cultured with uninjured WT and TLR3 (–/–) mice alveolar macrophages respectively in the presence or absence of Poly: IC. The cell culture media collected and the levels of pro-inflammatory mediators MIP-2, KC, and MCP-1 were measured (n=6 per group). *P< 0.05 WT mice vs. corresponding TLR3 (–/–) group.

Figure 5. TLR3/TRIF pathway and downstream signaling activation following LC

Western blots. To determine TLR3 downstream signaling activation following LC, lung lysates and nuclear extracts were analyzed with Western blot using various antibodies ($n=3$ per group). In whole lung lysate, phospho-IRF-3, phospho-ERK1/2, and phospho-p38 MAPK were found to be reduced in TLR3 (-/-) mice, and no difference was found in phosphorylated JNK after LC (a). TLR3 mice showed significant reductions in NLRP-3 (a). Phosphorylated NF-kB and IKBa activation in whole lung lysate was not detected in WT or TLR3 (-/-) mice after LC (b). Additionally, TRIF, TRAF6, and activator protein-1 expression levels in whole lung lysate were not changed in WT or TLR3. In nuclear extracts, phosphorylated NF-kB, IKBa, and IRF3 activation was not detected in WT or TLR3 (-/-) mice following LC. (b). In the nuclear extracts, phosphorylated NF-kB, IKBa, and IRF3 activation was not detected in WT or TLR3 (-/-) mice after LC (c). Densitometry analyses

of the proteins ($n=3$) for each group were performed. Error bars indicate S.E.M. * $P<0.05$ WT versus TLR3 ($-/-$) (two-tailed t -test).

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Figure 6. Functional importance of TRIF in Lung contusion

a: BAL levels of albumin and cytokines: We performed separate experiments using TRIF (-/-) mice and corresponding WT mice and examined them at 5, 24, 48, and 72 hour time points. There was a significant increase in the BAL albumin level in WT mice -. Values are represented as mean ($n=12$ to 16 per group). $**P < 0.01$ and $*P < 0.05$ WT vs. TRIF (-/-) mice. **b: Histological evaluation** of WT mice revealed significantly more inflammation, neutrophil infiltration, and wall edema at all-time points. **c. Macrophage and neutrophil levels in BAL** were lower in TRIF (-/-) mice following LC. There was a significant increase

in the levels of macrophages and neutrophils in WT mice. **d:** *TRIF* ($-/-$) mice showed a significant reduction in cytokine response compared to WT following LC. The IL-6 levels in the BAL were significantly higher at 24 hours in WT mice following LC. The levels of IL-1 β , MCP-5, MIP-2, KC, and TNF- α in the BAL were significantly decreased in *TRIF* ($-/-$) mice ($n=12$). * $P < 0.05$. **e:** *TRIF* downstream signaling activation following LC: The whole lung lysate extracts were analyzed with Western blot using various antibodies. TRAF6 and HMGB1 expression did not vary between WT and TLR3. TLR3 mice showed significant reductions in AP-1 expression at 2 hours compared to WT mice. Densitometry analyses of the proteins ($n=3$ per group) for each group were performed. Error bars indicate S.E.M. * $P < 0.05$ WT vs. *TRIF* ($-/-$) (two-tailed t -test).

Figure 7. Increased dsRNA release from injured cells is the ligand for the TLR3-driven acute inflammatory response following LC

a: dsRNA expression in WT mice in BAL and serum samples following LC: Gel electrophoresis from BAL, lung, and serum samples in WT mice with and without LC. dsRNA bands were observed with BAL and serum samples (red boxes) following LC. **b:** Macrophages show elevated expression of dsRNA following LC. The macrophages from WT mice were harvested after LC and subjected to immuno-fluorescent staining with dsRNA (green), TLR3 (red), and DAPI (blue) (n=3). **c:** dsRNA intracellular expression in BAL cells following LC. C57BL/6 mice were subjected to LC, and the levels of TLR3 and dsRNA molecules on the cell surface were monitored by Flow cytometry (n=3). **d:** TLR3/dsRNA complex inhibitor reduces injury and inflammation following LC. WT mice were administered the complex inhibitor 2 hours before LC, which significantly reduced the levels of albumin, cytokines (IL-1 β and MCP1), and macrophage apoptosis. * $P < 0.05$ dsRNA complex inhibitor versus LC alone. **e:** Effect of RNase on dsRNA staining in macrophages following LC: After LC, macrophages were collected, fixed, permeabilized, and treated with 2 μ g/ml of RNase III or 1 μ g/ml of RNase A for 2h at 37°C. Cells were stained with antibody to dsRNA (green) and DAPI (blue) (n=3). **f:** TLR3/dsRNA complex inhibitor reduced the expression of pro-inflammatory cytokines by cultured macrophages following

LC. Alveolar macrophages were cultured in the presence of RNA (obtained from the BAL cells of injured mice at 24 h), and/or poly-I: C, RNA+ poly I: C with/without TLR3/dsRNA inhibitor. The expression of pro-inflammatory cytokines was measured ($n=4$). *** $P<0.001$, ** $P<0.01$ and * $P<0.05$ between corresponding groups in the presence/absence of dsRNA complex inhibitor.

Figure 8. Proposed signal transduction pathways involving TLR3

Lung contusion induces alveolar disruption and cellular necrosis. Double stranded (ds) RNA released from the cells are the key ligand for activation of the TLR3, specifically located on the surface of the endosome. Additionally, the injury/inflammation causes a significant surface translocation of TLR3, further amplifying the inflammatory process. The TLR3/TRIF signaling pathway activation in LC is independent of NF- κ B and mediated through phosphorylation of p38 MAPK and ERK1/2. The major effect is borne on the alveolar macrophage inducing increased apoptosis and phenotypic alteration. TLR3 recognition of dsRNA (SS) also activates the inflammasome complex via [NLRP-3, IL1 β , IL18] to generate a robust pro-inflammatory response (Figure-8).