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Intestinal Epithelial TLR-4 Activation Is Required for the Development of Acute Lung Injury after Trauma/ Hemorrhagic Shock via the Release of HMGB1 from the Gut

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The mechanisms that lead to the development of remote lung injury after trauma remain unknown, although a central role for the gut in the induction of lung injury has been postulated. We hypothesized that the development of remote lung injury after trauma/hemorrhagic shock requires activation of TLR4 in the intestinal epithelium, and we sought to determine the mechanisms involved. We show that trauma/hemorrhagic shock caused lung injury in wild-type mice, but not in mice that lack TLR4 in the intestinal epithelium, confirming the importance of intestinal TLR4 activation in the process. Activation of intestinal TLR4 after trauma led to increased endoplasmic reticulum (ER) stress, enterocyte apoptosis, and the release of circulating HMGB1, whereas inhibition of ER stress attenuated apoptosis, reduced circulating HMGB1, and decreased lung injury severity. Neutralization of circulating HMGB1 led to reduced severity of lung injury after trauma, and mice that lack HMGB1 in the intestinal epithelium were protected from the development of lung injury, confirming the importance of the intestine as the source of HMGB1, whose release of HMGB1 induced a rapid protein kinase C (PKC)–mediated internalization of surface tight junctions in the pulmonary epithelium. Strikingly, the use of a novel small-molecule TLR4 inhibitor reduced intestinal ER stress, decreased circulating HMGB1, and preserved lung architecture after trauma. Thus, intestinal epithelial TLR4 activation leads to HMGB1 release from the gut and the development of lung injury, whereas strategies that block upstream TLR4 signaling may offer pulmonary protective strategies after trauma. The Journal of Immunology, 2015, 194: 4931–4939.

Trauma is the leading cause of death and disability in patients <54 y of age (1). Of patients who survive their initial injury, a third experience remote organ injury (2), which may be reflective of immune dysregulation in response to the initial insult (3, 4). Of all the organs affected, injury to the lung is one of the leading causes of posttrauma morbidity (5), with an incidence of 79 per 100,000 persons/year, and it carries a mortality rate of 40% (6, 7). Importantly, the mechanisms that mediate the induction of trauma-induced lung injury remain largely unexplained and represent a major gap in our knowledge in the field. In addressing how remote trauma can cause secondary lung injury, previous investigators have postulated that there may be a link between the intestine of the injured host and the development of injury in the lung. However, such a link between the gut and the lung in the pathogenesis of lung injury remains unproven, and the identity of potential molecules involved remains purely speculative.

In seeking to understand the mechanisms that contribute to trauma-induced acute lung injury, we hypothesized that activation of the innate immune system of the intestine after trauma is directly responsible for the development of lung injury. Chief among the potential molecular sensors within the intestine of the injured host that are well positioned to respond to contribute to injury are the TLRs (8). In particular, TLR4, the receptor for LPS (9), and also for a variety of endogenous molecules that are released during trauma (10), is expressed on the intestinal epithelium (11), where it could readily serve as a sensor on the gut during trauma (12). Although TLR4 on the intestinal epithelium has not been linked to the development of secondary lung injury after trauma, it is activated in response to stressors, which leads to the induction of endoplasmic reticulum (ER) stress within the gut epithelium and the loss of mucosal integrity through enterocyte apoptosis (13). Moreover, TLR4 activation leads to the release of damage-associated molecules such as HMGB1, which can cause secondary organ injury through the recruitment of inflammatory cells into the lung parenchyma and/or the disruption of tight junctions between adjacent lung epithelial cells (14).

Based upon these findings, we hypothesize that trauma leads to the development of secondary lung injury through a novel link between TLR4 activation in the intestinal epithelium and the
release of HMGB1, which is required for the lung injury to occur. We further hypothesize that experimental strategies that pharmacologically inhibit TLR4 can attenuate lung injury after trauma. In support of these hypotheses, we provide evidence for a novel link between TLR4-induced ER stress within the intestinal epithelium leading to enterocyte apoptosis, and HMGB1 release, which is required for lung injury, whereas mice that lack TLR4 or HMGB1 in the gut are protected from these events, and the use of a novel TLR4 inhibitor reverses lung injury after trauma in mice.

Materials and Methods

Cell culture and reagents

Human lung alveolar cells were obtained from American Type Culture Collection (HBE 135-E67). Where indicated, cells were treated with LPS (Escherichia coli 0111:B4 purified by gel filtration chromatography (>99% pure; Sigma-Aldrich) for 6 h at a concentration of 25 μg/ml, or HMGB1 (generous gift of Dr. Kevin Tracey, Feinstein Institute, Manhasset, NY) for 6 h at a concentration of 2.5 μg/ml, or with the protein kinase C (PKC) ζ pseudosubstrate (PKC1) and functional inhibitor from Promega at 150 nM for 6 h as described by Warburton and colleagues (15). C34 is a 2-acetamidopyranoside (MW 389) with the Equation C17H27NO9 that we determined recently to be the sole substate (PKCζ) and functional inhibitor from Promega at 150 nM for 6 h as described by Krzyzaniak et al. (17).

To evaluate directly whether the apoptosis of intestinal epithelial cells is required for HMGB1 release, we treated IEC-6 cells with LPS (50 μg/ml for 6 h) in the presence of either saline or the pan-caspase apoptosis inhibitor Z-VAD-FMK (10 μm, 30 min prior; Enzo Life Science). In parallel, cells were also treated with the ER stress inducer thapsigargin (0.5 μM; 6 h; Across Organics). After treatments, media were collected and assessed for the secretion of HMGB1 by ELISA (HMGB1 ELISA kit; IBL International), and the degree of apoptosis by TUNEL (Roche).

Immunohistochemistry

Immunohistochemistry was performed on 5-μm paraffin sections as follows: paraffin sections were first warmed to 56˚C in a vacuum incubator (Isotemp Vacuum Oven; Fisher Scientific), then washed immediately twice in xylene, gradually rehydrated in ethanol (100, 95, 70%, water), and then processed for Ag retrieval in citrate buffer (10 mM pH 6.0)/microwave (1000 W, 6 min). Samples were then washed with PBS, blocked with 1% BSA/5% donkey serum (1 h, room temperature), and then incubated overnight at 4˚C with primary Abs (1:200 dilutions in 0.5% BSA), washed three times with PBS, incubated with appropriate fluorescent-labeled secondary Abs (1:1000 dilution in 0.5% BSA; Life Technologies), as well as the nuclear marker DAPI (Biologiclend), and slides were then mounted using Gelvatol (Sigma-Aldrich) solution before imaging using a Zeiss LSM 710 Confocal microscope (Carl Zeiss, Jena, Germany) under appropriate filter sets.

Quantitative real-time PCR

Total RNA was isolated from whole lung or terminal ileum from mice that were either treated with saline or that had been subjected to experimental trauma/hemorrhagic shock, and euthanized 6 h later. Animals were anesthetized with i.p. pentobarbital sodium (50 mg/kg) and inhaled isoflurane (Abbott Laboratories, Chicago, IL). Under sterile conditions, a left-groin exploration was performed, and the left femoral artery was cannulated with tapered PE-10 tubing and connected to a blood pressure transducer (Micro-Med, Tusit, CA), and continuous mean arterial pressure monitoring for the duration of the experiment (6 h) as we described (12, 20). A bilateral, closed, midsection femur fracture was then performed using two Hemostats applied to the hind-limb region. Where indicated, mice were also subjected to hemorrhagic shock as described previously (21). To do so, after mice recovered from the inhalational anesthesia for 10 min, blood was withdrawn to allow the mean arterial pressure to decline to 25 mm Hg over 5 min, and the blood pressure was maintained at this level for 150 min. The mice were then resuscitated over 10 min with their remaining shed blood plus two times the maximal shed blood in lactated Ringer’s solution (Baxter, Deerfield, IL). Sham-operated mice underwent anesthesia and femoral cannulation only. All mice were reanesthetized with i.p. pentobarbital sodium (20 mg/kg) as necessary throughout the experiment. At the end of 6 h (or after 4 h in cases in which mice underwent hemorrhage), mice were killed under inhalational anesthesia via cardiac puncture technique. Necropsy was performed to verify the presence of bilateral femur fractures and to ensure the absence of fracture-site hematomas. Immediately after euthanasia, the lungs were freshly harvested and either perfused with 4% formalin in a 0.1 M sodium phosphate buffer (pH 7.4) for 60 min before fixation, or kept in a 0.1 M sodium phosphate buffer (pH 7.4) before fixation. The lungs were then fixed in 10% buffered formalin for 72 h, processed for paraffin embedment, and sectioned at 5 μm. Paraffin sections were first warmed to 56˚C in a vacuum incubator, then heated at 60°C on a hot plate for 3 h, and then dehydrated in a concentration gradient of ethanol (30%, 60%, 80%, 95%, and 100%) and then in xylene, gradually redehydrated in ethanol (100, 95, 70%, water), and then processed for Ag retrieval in citrate buffer (10 mM pH 6.0)/microwave (1000 W, 6 min). Samples were then washed with PBS, blocked with 1% BSA/5% donkey serum (1 h, room temperature), and then incubated overnight at 4˚C with primary Abs (1:200 dilutions in 0.5% BSA), washed three times with PBS, incubated with appropriate fluorescent-labeled secondary Abs (1:1000 dilution in 0.5% BSA; Life Technologies), as well as the nuclear marker DAPI (Biologiclend), and slides were then mounted using Gelvatol (Sigma-Aldrich) solution before imaging using a Zeiss LSM 710 Confocal microscope (Carl Zeiss, Jena, Germany) under appropriate filter sets.

Results

Activation of TLR4 in the intestinal epithelium is required for the induction of secondary lung injury after trauma

We first evaluated directly whether the activation of TLR4 within the intestinal epithelium may be required for the development of secondary lung injury after trauma, and if so, sought to evaluate the
potential mechanisms involved. To do so, we subjected wild-type mice and mice that we had generated to lack TLR4 within the intestinal epithelium (TLR4<sup>IEC</sup>) mice (19) to a well-validated model of trauma/hemorrhagic shock as described in Materials and Methods. Mice were then sacrificed at 6 h from the induction of injury. As shown in Fig. 1, wild-type mice subjected to experimental trauma/hemorrhagic shock displayed significant lung injury, which was manifest by thickening of the interalveolar septi and cellular infiltration (Fig. 1A, 1B), by the recruitment of PMNs as revealed by staining for MPO (Fig. 1E, 1F), by apoptosis of pneumocytes as revealed by expression of cleaved caspase 3 within the alveolar epithelium (Fig. 1I, 1J, 1M), and by the increased expression of IL-6 and the chemokine MCP1 in the lung (Fig. 1N, 1O). Importantly, exposure of TLR4<sup>IEC</sup> mice to the same trauma did not cause lung injury, as manifest by reduced histological evidence of inflammation (Fig. 1C), reduced PMN infiltration (Fig. 1G), reduced apoptosis (Fig. 1K, 1M), and reduced IL-6 and MCP-1 expression (Fig. 1N, 1O). These findings together indicate that TLR4 signaling in the gut is required for the induction of lung injury after remote trauma. We next examined the signaling mechanisms within the gut by which these events could occur.

**Trauma/hemorrhagic shock induces ER stress in the intestinal epithelium in a TLR4-dependent manner, causing enterocyte apoptosis and the release of HMGB1**

We recently described that TLR4 activation in the intestinal epithelium induces ER stress within these same cells in the gut (13), which then plays a major role in the induction of epithelial apoptosis through activation of caspase 3 (13). In view of these findings, we next sought to evaluate whether TLR4 signaling in the intestinal epithelium could induce ER stress and intestinal epithelial apoptosis after trauma. As shown in Fig. 2, compared with the normal intestinal epithelial architecture seen under control conditions (Fig. 2A), the induction of trauma/hemorrhagic shock resulted in a marked derangement of the intestinal epithelium and a loss of the intact crypt villus (Fig. 2B). The induction of trauma was also associated with a significant increase in ER stress, as manifest by an increase in the expression of BiP, within the intestinal epithelium (Fig. 2E, 2F), as well as an increase in the expression of XBP1s by quantitative RT-PCR (qRT-PCR; Fig. 2Q). Importantly, the induction of trauma/hemorrhagic shock in TLR4<sup>IEC</sup> mice did not lead to mucosal destruction (Fig. 2C) and did not induce ER stress (Fig. 2G, 2M). An apparent consequence of TLR4-induced ER stress within the intestinal epithelium after trauma was a dramatic increase in apoptosis of the intestinal epithelium in wild-type mice (Fig. 2I, 2J) that was not seen in TLR4<sup>IEC</sup> mice (Fig. 2K, 2R). The destruction in the intestinal mucosa after trauma and induction of apoptosis could both be reversed through pretreatment with the ER stress–reducing agent salubrinal, which has been shown to protect cells from ER stress in other systems (22–24) (Fig. 2D, 2H, 2L, 2Q, 2R). Fig. 2H and 2Q show evidence that ER stress is reduced in the intestinal epithelium after pretreatment with salubrinal. The TLR4-dependent increases in ER stress and apoptosis were associated with an increase in the expression of the damage-associated molecular pattern molecule HMGB1 in the intestinal epithelium, as manifest by immunostaining (Fig. 2M–P) and expression by

**FIGURE 1.** Activation of TLR4 in the intestinal epithelium is required for the induction of secondary remote lung injury after trauma. Representative micrographs showing sections of lung from either wild-type (A, B, E, F, I, and J), TLR4<sup>IEC</sup> (C, G, and K), or wild-type mice pretreated with the ER stress-releasing agent salubrinal [1 mg/kg; (D, H, and L)] that were either untreated or subjected to trauma/hemorrhagic shock as indicated. Sections were stained for H&E (A–D), MPO [(E–H); arrows show MPO<sup>+</sup> pneumocytes], or in (I)–(L) were stained for cleaved caspase 3 (CC3, red), DAPI (blue), or E-cadherin (green); arrows show apoptotic pneumocytes and examined via confocal microscopy. (M) Percentage positive CC3 pneumocytes per high-power field. (N and O) qRT-PCR showing expression of IL-6 (N) or MCP1 (O) in lung lysates in untreated mice (white bars) or mice subjected to trauma/hemorrhagic shock (red bars) under the indicated conditions. (M–O) *p < 0.05 wild-type control versus wild-type trauma, †p < 0.05 wild-type trauma versus TLR4<sup>IEC</sup> trauma, and **wild-type trauma versus wild-type + salubrinal trauma. All data are mean ± SEM; at least six animals per group. Representative of three independent experiments.
qRT-PCR (Fig. 2S), as well as the release of HMGB1 into the serum (Fig. 2T) in a pattern that mirrored the severity of gut inflammation as measured by IL-6 by qRT-PCR (Fig. 2U). The release of HMGB1 into the serum was reduced after pretreatment with salubrinal, suggesting that the increase in HMGB1 release may require the induction of ER stress (Fig. 2P, 2T). Moreover, the pretreatment of wild-type mice with salubrinal significantly attenuated the degree of lung injury in wild-type mice subjected to trauma/hemorrhagic shock, as manifest by reduced gross evidence of lung injury (Fig. 1D), reduced staining for MPO (Fig. 1H), reduced apoptosis (Fig. 1L, 1M), and reduced expression of the proinflammatory cytokine IL-6 (Fig. 1N) and the proinflammatory chemokine MCP-1 (Fig. 1O). Taken together, these findings presented in aggregate in Figs. 1 and 2 suggest that experimental trauma induces TLR4 activation within the intestinal epithelium leading ER stress, resulting in intestinal epithelial apoptosis, and the subsequent increase in HMGB1 expression in the gut and its release into the serum.

Remote lung injury after trauma requires HMGB1 release from the intestinal epithelium

We next sought to determine in greater detail how TLR4 signaling in the intestinal epithelium after trauma could lead to acute lung injury, and investigated whether the TLR4-mediated release of HMGB1 from the intestinal epithelium may be required. To do so, we first generated mice that lack HMGB1 in the intestinal epithelium (HMGB1<sup>−/−</sup>IEC mice). As shown in Fig. 3A and 3B, sections of the intestinal epithelium from untreated wild-type mice show the expression of HMGB1 in the lamina propria and the intestinal epithelium (Fig. 3A), whereas HMGB1 expression was restricted to the lamina propria and not seen in the intestinal epithelium in HMGB1<sup>−/−</sup>IEC mice (Fig. 3B), despite similar histological appearance of the small intestinal villi (Fig. 3C, 3D) and lungs at baseline (Supplemental Fig. 1A, 1B) in both strains. Importantly, HMGB1 release from the intestinal epithelium was found to be required for the induction of lung injury. In support of this conclusion, neutralization of HMGB1 using affinity-purified anti-HMGB1 Ab prevented distal lung injury as manifest histologically (Fig. 3E, 3F), by reduced expression of MPO (Fig. 3I, 3J), by reduced expression of cleaved caspase 3 in the lung (Fig. 3M, 3N, 3Q), and reduced lung IL-6 expression (Fig. 3R). Although HMGB1 may be released from many cell types including leukocytes and epithelial cells (25), HMGB1 release from the intestinal epithelium was critical for the development of remote lung injury, as mice lacking HMGB1 in the intestinal epithelium...
HMGB1(DIEC) were protected from the development of lung injury (Fig. 3G, 3K, 3O, 3Q–S). As anticipated, the induction of trauma in HMGB1(DIEC) mice resulted in reduced circulating HMGB1 in the serum compared with wild-type mice after trauma (Fig. 3T). It is noteworthy that the induction of intestinal epithelial apoptosis is at least partially required for the release of HMGB1, as treatment of intestinal epithelial cells in vitro with either LPS or the ER stress–inducing agent thapsigargin induced apoptosis and the release of HMGB1 into the media, which were both prevented by pretreatment with the pan-caspase inhibitor Z-VAD (Supplemental Fig. 2). To further support a role for HMGB1 in the development of lung injury, injection of rHMGB1 (4 mg/kg body weight) into TLR4(DIEC) mice (which themselves had shown reduced HMGB1 levels in the plasma; Fig. 3T) reversed the protection from trauma-induced lung injury that had been seen in the TLR4(DIEC) mice, which under these conditions displayed sig-

FIGURE 3. Remote lung injury after trauma requires HMGB1 release from the intestinal epithelium. (A and B) Representative confocal micrographs of the intestinal villi of untreated wild-type (A) and intestinal-specific HMGB1 knockout mice (B) HMGB1ΔIEC stained for HMGB1 (red), E-cadherin (green), and DAPI (blue); arrows show HMGB1-stained nuclei in intestinal epithelium and lamina propria in wild-type mice, but in lamina propria only in the HMGB1ΔIEC mice. (C and D) Representative H&E-stained histomicrographs showing sections obtained from the terminal ileum of wild type (C) and HMGB1ΔIEC mice (D). (E–P) Representative micrographs showing sections of lung from either wild-type (E, I, and M), wild-type + exogenous HMGB1 [4 mg/kg; (F, J, and N)], HMGB1ΔIEC (G, K, and O), or TLR4ΔIEC + exogenous HMGB1 [4 μg/g; (H, L, and P)] mice that were either untreated or were subjected to trauma/hemorrhagic shock as indicated. Sections were imaged by bright-field microscopy and stained for H&E (E–H) or MPO [(I–L), brown, arrows show MPO+ cells], or were imaged by confocal microscopy and were stained for cleaved caspase 3 (red, arrows show apoptotic cells), DAPI (blue), or E-cadherin (green) (M–P). (Q and R) Mean ± SEM of percent cleaved caspase 3+ cells/high-power field (Q) and expression of IL-6 (R) and MCP1 (S) by qRT-PCR in wild-type, HMGB1ΔIEC, or TLR4ΔIEC mice that were subjected to trauma/hemorrhagic shock in the presence or absence of exogenous anti-HMGB1 or rHMGB1 (4 μg/g) as indicated. *p < 0.05 wild-type versus HMGB1ΔIEC, *p < 0.05 wild-type versus wild-type + anti-HMGB1, *p < 0.005 TLR4ΔIEC versus TLR4ΔIEC + rHMGB1. All data are mean ± SEM; at least six animals per group repeated in triplicate. (T) Serum HMGB1 by ELISA in mice of the strain indicated subjected to trauma/hemorrhagic shock in the presence of salubrinal (1 mg/kg) or anti-HMGB1 as shown. All groups are significantly different from wild-type by ANOVA, p < 0.05. Representative of three independent experiments.
significant lung inflammation after trauma (Fig. 3H, 3L, 3P–S; the corresponding serum levels of HMGB1 are shown in Fig. 3T). Finally, HMGB1\textsuperscript{DIEC} mice were found to be protected from gut injury after trauma/hemorrhagic shock as manifest by significantly reduced expression of the proinflammatory cytokine IL-6 in the ileum compared with wild-type mice (Supplemental Fig. 1C). Taken together, these findings illustrate that TLR4 signaling in the intestinal epithelium leads to HMGB1 release, which is required for the development of lung injury. We therefore next sought to define in greater detail the mechanisms by which HMGB1 release could lead to lung injury.

**HMGB1 signaling in the intestinal epithelium leads to internalization of the tight junction protein ZO-1 in the pulmonary epithelium**

We next sought to define how HMGB1 release could induce lung injury in the setting of trauma/hemorrhagic shock findings. Previous authors have reported that acute lung injury requires the internalization of the tight junction protein ZO-1, resulting in a loss of the protective barrier (15, 26), which is thought to be an inciting event in the development of lung inflammation (15, 27, 28). These observations raise the distinct possibility that internalization of tight junctions in response to HMGB1 release from the gut after trauma/hemorrhagic shock could participate in the development of lung injury. Consistent with this possibility, we show that exposure of primary human lung epithelial cells to HMGB1 resulted in an internalization of the tight junction protein ZO-1 as compared with untreated cells (Fig. 4A, 4B). Importantly, the pretreatment of cells with PKCi (29, 30) as described in Materials and Methods reversed the internalization of ZO-1 from the cell surface (Fig. 4C), whereas treatment with the inhibitor alone had no effect (Fig. 4D), suggesting a role of PKC-ζ in ZO-1 trafficking in pneumocytes in response to HMGB1. In support of the in vivo significance for a role for HMGB1 in influencing the trafficking of ZO-1 in pneumocytes after trauma/hemorrhagic shock, the expression of ZO-1 on the pulmonary epithelium was found to be reduced on the cell surface compared with saline-treated mice, whereas pretreatment with HMGB1 inhibitory Abs restored the ZO-1 on the cell surface, as revealed by confocal microscopy (Fig. 4E–G, panels E’–G’ correspond to the magnification of areas defined by dotted lines). There was no effect on internalization of ZO-1 after pretreatment with IgG (Fig. 4F, F’). These findings suggest that HMGB1 release leads to the internalization of tight junctions in pneumocytes in the setting of trauma/hemorrhagic shock in which lung injury is seen.

**Novel TLR4 inhibitor C34 inhibits TLR4 and protects against lung injury after trauma in mice**

The earlier findings, in which TLR4 signaling in the intestinal epithelium after trauma/hemorrhagic shock is required for the induction of lung injury, suggest that strategies that inhibit TLR4 could offer novel therapeutic approaches for the prevention of lung injury in trauma. To identify potential TLR4 inhibitors, we recently used a similarity search algorithm using the TLR4 inhibitor Eritoran (E5564) (31) in conjunction with a limited screening approach of small-molecule libraries to identify compounds that could bind to the E5564 site (16). We successfully identified a lead compound, C34, which we recently published to be a 2-acet-
amidopyranoside (MW 389) with the formula C_{17}H_{27}NO_{9} (16). As we recently reported, molecular docking of C34 to the hydrophobic internal pocket of MD-2 demonstrated a tight fit, whereas C34 inhibited TLR4 in vivo and also inhibited TLR4 in the intestine in both mouse and human tissue (16). As shown in Fig. 5, C34 markedly inhibited the degree of lung inflammation that developed in experimental trauma, as measured by a significant reduction in the degree of inflammation within the lung (Fig. 5 A–C) and by the expression of lung IL-6 by qRT-PCR (Fig. 5J). In support of a role for TLR4-induced ER stress in the gut in the process leading to HMGB1 release and lung injury, C34 pretreatment was found to reduce the degree of mucosal injury (Fig. 5D–F) and ER stress within the gut (Fig. 5G–I, 5K), and also to decrease HMGB1 release in the serum (Fig. 5L), and to reduce the expression of TLR4 (Fig. 5M) and HMGB1 (Fig. 5N) within the intestinal mucosa after trauma/hemorrhagic shock. These findings raise the possibility that factors that regulate TLR4 signaling could suppress the degree of lung injury that develops after trauma/hemorrhagic shock, potentially through effects on ER stress and HMGB1 release.

**Discussion**

Three independent lines of evidence support the conclusion that TLR4 activation in the intestinal epithelium is required for the...
induction of secondary lung injury through the release of HMGB1 from the gut. First, mice that lack TLR4 in the intestinal epithelium released less HMGB1 and were protected from the development of lung injury after trauma/hemorrhagic shock, supporting the hypothesis that TLR4 in the intestinal epithelium is required for the development of lung injury. Second, mice that lack HMGB1 in the intestinal epithelium showed reduced concentrations of circulating HMGB1 and were protected from the development of lung injury. Finally, and perhaps most significantly from a therapeutic perspective, treatment of mice with a novel TLR4 inhibitor C34 C17H27NO9 (16) reversed the development of lung injury after systemic trauma/hemorrhagic shock and reduced the extent of HMGB1 release into the circulation (Fig. 5). Taken together, these findings, summarized in the schematic diagram shown in Fig. 6, advance our understanding of how secondary lung injury may occur after remote injury and provide a contextual framework by which novel therapeutic approaches for traumatized patients may be developed.

The current studies build upon a growing body of work that have shed light on the mechanisms that lead to the development of acute lung injury after trauma, and on the role of HMGB1 in particular. For instance, Deitch and colleagues were among the earliest proponents of the importance of the gastrointestinal tract in driving lung injury after trauma (32, 33), and more recently have shown that components of the intestinal lymph play a major role in the development of lung injury in rats (34, 35), although the individual constituents within the lymph fluid that lead to the lung injury remain unknown (34). Further, Abraham and colleagues (36) have shown that HMGB1 plays a critical role in the development of lung injury after trauma (14), as instillation of HMGB1 into the trachea causes lung inflammation, whereas Ab inhibition of HMGB1 attenuates the degree of lung injury and mortality in models of trauma (14, 37, 38). Reino et al. (39) recently showed that TLR4 mutant mice are protected from the development of trauma/hemorrhagic shock–induced lung injury, although the specific TLR4-expressing cell that was responsible for this protection was not shown. The novelty and significance of this work is therefore that our conclusions support these prior observations, but provide additional mechanistic details by showing that TLR4 signaling in the gut leading to intestinal HMGB1 release is critical to the lung injury that was observed, and that the consequences of exaggerated HMGB1 release can be therapeutically reversed by the use of a novel TLR4 inhibitor that we recently identified, namely, C34 (16). Moreover, we have shown that the development of ER stress in the intestinal epithelium in response to TLR4 may be a requirement for lung inflammation to occur, which may explain, in part, why both lymphatic and circulatory routes can contribute to lung injury, as epithelial ER stress would be expected to influence the composition of both of these compartments. We readily acknowledge that although we showed that trauma in mice leads to a significant increase of HMGB1 in the plasma that was not seen in HMGB1Abiec mice, we have not measured HMGB1 in the lymph and cannot therefore exclude a potential role for HMGB1 acting on the lung via the lymphatic system. These studies therefore provide additional details onto the canvas of the “gut hypothesis” of lung injury, and shed light on potential therapeutic possibilities.

One of the more intriguing questions to arise from these studies relates to what may be the source of TLR4 activation in the intestinal epithelium that leads to the downstream consequences that culminate in lung injury. On first inspection, it is tempting to postulate that microbial products present within the intestinal lumen, particularly those that are rich in LPS, may directly activate TLR4. However, it is not known whether TLR4 is on the apical or the basal surface of the pneumocyte, and the poor applicability of the currently available anti-TLR4 Abs for immunolocalization do not readily allow for this determination. It is also plausible that factors within the bloodstream may activate TLR4 within the lung epithelium, which could be either microbial or endogenous in origin. In this regard, a variety of host substrates have been shown in a variety of in vitro and in vivo studies to activate TLR4, including Hsp70, uric acid, S100, heparan sulfate, and HMGB1 itself (40). In support of a role for endogenous molecules as central mediators in the development of trauma-induced lung injury, Deitch and colleagues (34) recently showed that nontuberculous intraluminal molecules were responsible for the development of lung injury in traumatized rats. Notably, both bacterial and nonbacterial activation of TLR4 would be expected to induce ER stress equally within the intestinal epithelium (41–43), a process that we have shown to occur in the setting of intestinal epithelium in the newborn in the pathogenesis of necrotizing enterocolitis (13). The induction of ER stress within the gut is an attractive intermediate pathway to focus on in the release of HMGB1, as a variety of studies including from our group have shown that exaggerated ER stress in response to TLR4 signaling leads to enterocyte apoptosis (13), which may explain why TLR4 signaling so readily induced HMGB1 release from the gut. Using gene array and Ingenuity Pathway Analysis, we have shown that ER stress pathways are rapidly upregulated in the liver in a murine model of hemorrhagic shock plus trauma (44). We now posit that an assessment of the degree of ER stress induction in the gut or other tissues may provide novel biomarkers to determine who may be at risk for the development of further organ injury, and may thus benefit from additional therapeutic intervention. Further studies will be required to explore this in further detail.

In summary, we have shown that development of lung injury after trauma requires the activation of TLR4 within the intestinal epithelium and a gut epithelial source of HMGB1, which is then required for the development of lung injury. We readily acknowledge that although these findings are novel, there are several avenues for further investigation to address some of the questions raised from these studies. These include the determination of the precise agonist that activates TLR4 in the first place as described earlier, as well as an assessment of whether animals that lack TLR4 in other cells may be similarly protected from lung injury after trauma, suggesting that hierarchical TLR4 signaling regulates the lung inflammatory response. It is our hope that by addressing these further questions within the context of the novel pathway that we summarize in Fig. 6 that novel therapeutic approaches may be designed for these injured patients.

**FIGURE 6.** Trauma/hemorrhagic shock induces TLR4 signaling in the intestine leading to lung injury via the release of HMGB1 from the intestine.

**Disclosures**

The authors have no financial conflicts of interest.
References


Supplemental Figure 1. Mice lacking HMGB1 in the intestinal epithelium (HMGB1ΔIEC) are protected from trauma induced intestinal injury. A-B: Representative micrographs of lung from wither wild-type (A) or HMGB1ΔIEC (B) mice stained with H&E. C: qRT-PCR showing the expression of IL-6 in the ileum of wild-type (white bars), HMGB1ΔIEC mice (green bars) that were either untreated (Ctrl) or exposed to trauma/hemorrhagic shock (Trauma). *p<0.05 control vs. trauma; **p<0.05 wild-type trauma vs. wild-type control or HMGB1ΔIEC control; ***p<0.05 wild-type trauma versus HMGB1ΔIEC trauma; Representative of 3 separate experiments with over 5 mice per group.
Supplemental Figure 2. The release of HMGB1 from IEC-6 cells is dependent on apoptosis. A-E: Confluent IEC-6 enterocytes that were treated with either media (A), LPS (50 mg/ml for 6h, B), thapsigargin (Thaps, 0.5 mM, 6h, C), LPS (50 mg/ml for 6h) in addition to the pan-caspase apoptosis inhibitor Z-VAD-FMK (10mM, 30 min prior, D), or Thapsigargin + Z-Vad (E). Slides were stained for TUNEL and DAPI; arrows show apoptotic cells. F-G: Quantification of apoptosis per high power field (F) and concentration of HMGB1 in the media as measured by ELISA (G) under the indicated conditions; *p<0.05 vs. media (i.e. red vs. white bars); **p<0.05 vs. LPS or thapsigargin (i.e. blue vs. red bars); representative of 3 separate experiments with over 100 high power fields examined per experimental condition.