Hemorrhage-induced acute lung injury is TLR-4 dependent

Katherine A. Barsness,1 John Arcaroli,2 Alden H. Harken,1 Edward Abraham,2 Anirban Banerjee,1 Leonid Reznikov,1 and Robert C. McIntyre1

1Department of Surgery, 2Division of Pulmonary and Critical Care Medicine, and 3Division of Infectious Diseases, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Barsness, Katherine A., John Arcaroli, Alden H. Harken, Edward Abraham, Anirban Banerjee, Leonid Reznikov, and Robert C. McIntyre. Hemorrhage-induced acute lung injury is TLR-4 dependent. Am J Physiol Regul Integr Comp Physiol 287: R592–R599, 2004.—Toll-like receptor 4 (TLR-4), initially identified as an LPS receptor, is critical to the signaling of a variety of danger signals, including heat shock protein 60, fibrinogen, and fibropectin. Recent data also suggest that TLR-4 plays a role in determining survival in both endotoxemia and hemorrhagic shock. We hypothesized that a functional TLR-4 would be required for hemorrhage and endotoxin-induced acute lung injury. Hemorrhage- and endotoxin-induced lung TNF-α mRNA and protein production, neutrophil accumulation, and protein permeability were dependent on a functional TLR-4. Hemorrhage-induced nuclear factor (NF)-κB activation was independent of functional TLR-4, whereas endotoxin-induced activation of NF-κB requires a functional TLR-4 for full response. Therefore, we conclude that 1) hemorrhage-induced acute lung injury is TLR-4 dependent and 2) hemorrhage has a different and distinct TLR-4-dependent intracellular activation mechanism compared with endotoxemia.

mediated lung injury after endotoxin and lung ischemia-reperfusion (28, 35). TLR-4 is the dominant mammalian signaling receptor for bacterial LPS (24, 33, 34). Whereas TLR-4 is widely recognized as the LPS receptor, other ligands of TLR-4 were recently identified, including heat shock protein (HSP) 60, fibrinogen, fibropectin, soluble heparin sulfate, and elastase (19, 30, 31, 46). The mechanisms by which a receptor classically described as the LPS receptor recognizes ligands with widely divergent protein sequences and structures remains to be elucidated. However, TLR-4 does appear to be a more promiscuous receptor for detecting widely divergent stimuli. These newly discovered ligands for TLR-4 comprise a growing list of endogenous ligands that may initiate a systemic inflammatory response in the absence of infection (18, 19). Despite these data on endogenous ligands, no study has yet determined a role for TLR-4 in a noninfectious, systemic, proinflammatory insult such as pancreatitis or hemorrhagic shock.

Significant insight into TLR-4 has come from a spontaneously occurring endotoxin hyporesponsive strain of mice. These endotoxin-resistant mice (C3H/HeJ) have a mutation in the LPS gene causing a critical amino acid substitution (proline 741 to histidine) in the cytoplasmic tail of TLR-4 commonly referred to as TLR-4/d/d (34). In addition to being hyporesponsive to endotoxin, the C3H/HeJ strain exhibits an unexpected survival advantage in hemorrhagic shock (12). Specifically, there was a 65% reduction in hemorrhage-induced mortality in the C3H/HeJ strain compared with the wild type (WT). This C3H/HeJ survival advantage after hemorrhage was also accompanied by an inability to detect systemic TNF-α.

Given these observations that several endogenous ligands released in stress states can activate TLR-4 and that the TLR-4/d/d mutation confers a survival advantage in hemorrhage, we hypothesized that inflammatory lung injury after hemorrhagic shock and endotoxemia would require a functional TLR-4. The purposes of this study were to determine the role of a functional TLR-4 after hemorrhagic shock or endotoxin on 1) NF-κB activation, 2) TNF-α mRNA and protein production, 3) neutrophil accumulation, and 4) protein permeability in the lung. We found that both hemorrhage- and endotoxin-induced acute lung injury were TLR-4 dependent. In addition, hemorrhage has a distinct and different TLR-4-dependent intracellular activation mechanism compared with endotoxemia.

MATERIALS AND METHODS

Animals. Male C3H/HeN mice, 8–14 wk of age, were obtained from Charles River Laboratory. Male C3H/HeJ mice, 8–14 wk of age,

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Preceding factors for acute lung injury include hemorrhagic shock, sepsis, pancreatitis, and transfusion (23, 50). Activation of resident macrophages leads to increased expression of inflammatory cytokines and chemokines that recruit neutrophils to the lung (9, 10, 49). Neutrophils play a critical role in both hemorrhage- and endotoxin-induced acute lung injury (2, 45). However, there may be important pathophysiological differences in lung injury associated with different risk factors. The mechanisms leading to experimental acute inflammatory lung injury after hemorrhage differ from those associated with endotoxemia. Intracellular signaling pathways initiated by hemorrhage lead to increased expression of proinflammatory cytokines in lung neutrophils that are distinct from those associated with endotoxemia. In particular, xanthine oxidase-derived reactive oxygen intermediates appear to be involved in hemorrhage-induced signaling pathways but not in those initiated by endotoxemia (1, 37, 39).

The nuclear factor (NF)-κB family of transcription factors regulates gene induction of a variety of cytokines, chemokines, and adhesion molecules that ultimately lead to acute lung injury. NF-κB activation is an early event after both endotoxemia and hemorrhage (38, 42) and can be found in alveolar macrophages from patients with acute respiratory distress syndrome (26, 36). Inhibition of NF-κB abrogates neutrophil-
were obtained from Jackson Laboratory. The mice were kept on a 12:12-h light/dark cycle with free access to food and water. All experiments were performed in accordance with institutional review board-approved protocols.

Materials. LPS (Escherichia coli serotype 055:B5) came from Sigma (St. Louis, MO). The Coomassie-Plus protein assay reagent was obtained from Pierce (Rockford, IL). A recombinant murine TNF-α ELISA kit was purchased from R&D Systems (Minneapolis, MN). A TransAM NF-κB p65/p50 Transcription Factor Assay kit was obtained from Active Motif (Carlsbad, CA).

Hemorrhage and endotoxin models. For the hemorrhage model, 30% of the calculated blood volume (−0.55 ml in a 20-g mouse) was withdrawn over 60 s from ketamine-xylazine-anesthetized mice by subxiphoid cardiac puncture. The mortality rate with this hemorrhage protocol was 14%, with all deaths occurring within the first several minutes of hemorrhage. This technique decreases mean arterial blood pressure to ~40 mmHg, with restoration to control levels over the 60 min after hemorrhage (37). Endotoxin was administered as an intra-peritoneal injection of 1 mg/kg of E. coli LPS. These specific injury models were chosen because we previously found that a 30% hemorrhage compared with a dose of 1 mg/kg of intraperitoneal E. coli will produce the same degree of lung damage as assessed by wet-to-dry ratios (2).

Preparation of nuclear extracts. Isolation of nuclear extracts was performed as previously described (41). Briefly, lung samples were snap frozen in liquid nitrogen and stored at −70°C until time of assay. Lung tissue was homogenized with 300 μl of buffer A for 30 s. The homogenate was centrifuged at 2,750 rpm for 6 min at 4°C. The supernatant (cytoplasmic content) was discarded. The pellet was resuspended in 50 μl of buffer C and placed on ice for 15 min. The sample was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected and stored in aliquots at −70°C. Protein concentration of the nuclear extract was determined using Coomassie Plus protein reagent standardized to bovine serum albumin according to manufacturer’s protocol.

Electrophoretic mobility shift assay and NF-κB ELISA. Activation of the transcriptional factor NF-κB was determined by electrophoretic mobility shift assay (EMSA) analysis, as previously described (41). The consensus sequence for the immunoglobulin-κ gene was used. Synthetic DNA sequences (with enhancer motifs underlined) were annealed, forming double-strand DNA probes with single-strand ends consisting of sequences of four thymines, allowing the ends to be labeled by base pairing with α[32P]dATP using Sequenase DNA polymerase: κB, 5′-TTTTCGAGCTGGGACTTCC-GAGC-3′, and 3′-GCTCGAGGCTAGAAGGCTGTTT-5′.

DNA binding reaction mixtures of 20 μl contained 10 μg of nuclear extract, 10 mM Tris-Cl, pH 7.5, 50 mM EDTA, 0.5 mM dithiothreitol, 1 mM MgCl2, 4% glycerol, 0.08 μg of poly(dI-dC)-poly(dI-dC), and 0.7 fmol of 32P-labeled double-stranded oligonucleotide. After samples were incubated at room temperature for 20 min, they were loaded onto a 4% polyacrylamide gel (acrylamide-bis-acrylamide, 80:1; 2.5% glycerol in Tris-borate-EDTA) at 10 vol/cm. Each gel was then dried and subjected to autoradiography.

Translocation of the p65 subunit to the nucleus was measured by a TransAM NF-κB p65 Transcription Factor Assay kit. Results were determined by the spectrophotometric absorbance at 655 nm on a microplate reader and expressed as optical density (OD655) per sample.

Polymerase chain reaction for TNF-α mRNA. One hour after endotoxin or hemorrhage, the lung was harvested and homogenized in Tri-reagent. The RNA was sequentially isolated after chloroform extraction and isopropanol precipitation. To evaluate yield and the quality of RNA, an aliquot of the preparation was electrophoresed in agarose in the presence of ethidium bromide. The reasonable estimation of yield was made by running 10 μl of the RNA sample (∼0.5–1.0 μg) in agarose with a known amount of dilute standard RNA solution prepared from concentrated RNA stock solution that has been quantified by UV absorbance. Samples were separated on a 1% gel containing 0.5× TBE (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA pH 8.3) with 0.5 μg/ml ethidium bromide visualized by UV illumination and photographed.

To prepare cDNA, 1–3 μg of total RNA was reverse transcribed by using random primer in a final concentration of 5.5 mM MgCl2, 50 mM KCl, and 10 mM Tris-HCl (pH 8.3), 0.5 μM of each dNTPs, 20 μl of M-MLV reverse transcriptase (Perkin Elmer). The reaction was incubated at room temperature for 10 min, 37°C for 60 min, and then terminated by a 95°C incubation for 5 min.

To ensure that the RNA isolated from lung tissue and the synthesized template for PCR were of sufficient quality for use in real-time RT-PCR, the amplification with primers and probes designed for 18S and β-actin were performed for each sample using ABI Prism 7700. Samples that failed to reach the threshold within 14 cycles for 18S or 21 cycles for β-actin were excluded from the study as they did not pass the quality control.

TaqMan real-time quantitative PCR was performed by amplifying mixtures containing 100 nmol of selected probes, 200 nM of primers, and target cDNA template at 94°C for 20 s and 62°C for 1 min for 50 cycles using ABI PRISM 7700 sequence detector (Applied Biosystems) according to the manufacturer’s protocol. During each PCR cycle, TaqMan probe was cleaved by 3′ exonuclease activity of DNA polymerase cleaves the TaqMan probe, thereby increasing the fluorescence of the reporter dye at the appropriate wavelength. The increase in fluorescence is proportional to the concentration of template in the PCR. The threshold was calculated according manufacturer’s protocol for the TaqMan PCR kit. The number of PCR cycles required for a particular gene amplification to reach this threshold is represented as “CT.” To normalize this value using an internal standard, the CT value representing 18S amplification is subtracted from CT corresponding to amplification of target gene. The result is represented as ΔCT.

To calculate the relative gene expression, ΔCT corresponding to a target gene was subtracted from the ΔCT of the gene selected as a calibrator (18S ribosomal RNA). The result was represented as 2−ΔΔCT. The relative number of amplified target oligonucleotide copies was then calculated as 2−ΔΔCT. Results are expressed as nanograms TNF-α mRNA per nanogram ribosomal RNA.

TNF-α ELISA. Lung tissue was homogenized with 4 vol of homogenate buffer and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was collected for ELISA determination of TNF-α as previously described. The detection limit was 5.1 pg/ml for TNF-α. Absorbance of standards and samples was determined spectrophotometrically at 450 nm using a microplate reader (Bio-Rad, Hercules, CA). Results were plotted against the linear portion of the standard curve. TNF-α levels are expressed as picograms per gram of protein as determined using Coomassie Plus protein assay.

Myeloperoxidase assay. Four hours after a 30% hemorrhage, neutrophils accumulate in the parenchyma (interstitium) of the lung. Interestingly, neutrophil accumulation after systemic LPS reveals a predominantly intravascular adherence pattern of neutrophils (48). This adherence is firm and therefore not capable of being flushed from the lungs before fixation or imaging or processing for myeloperoxidase (MPO) assays. Despite the intravascular location, the neutrophil has an important role in endotoxin-mediated tissue injury. This role is revealed by neutrophil depletion, which prevents the development of lung edema (2, 44). The method for MPO assay has been described previously with minor modifications (45). Tissue was homogenized for 30 s in 10× volume 20 mM potassium phosphate buffer (pH 7.4). The homogenate was centrifuged at 24,000 rpm for 30 min at 4°C. The pellet was resuspended and sonicated on ice for 90 s in 2× volume hexadecyltrimethylammonium bromide buffer (HTAB; 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate, pH 6.0). Samples were incubated in water bath (56–60°C) for 2 h and then centrifuged at 5,000 g for 10 min. The supernatant was collected for assay of MPO activity as determined by measuring the
H2O2-dependent oxidation of 3,3',5,5'-tetramethylbenzidine at 460 nm. All assay measurements were corrected for total grams protein in each sample, as determined by Coomassie Plus protein reagent and expressed as optical density (OD605) per sample.

Evaluation of lung protein permeability. We evaluated lung permeability using the previously published method of Evans blue dye extravasation (32). Evans blue dye binds tightly to albumin and is a sensitive marker of vascular permeability. Evans blue dye was injected into the mice via femoral vein (cut down performed under anesthesia) 3 h after endotoxin or cardiac puncture. The vein was ligated after injection and the skin was closed. One hour after the injection of Evans blue dye, the lung tissue was flushed in situ through the right ventricle with 10 ml normal saline to clear intravascular dye. The lung tissue was homogenized in formamide (1:5 dilution) and then allowed to incubate at 37°C for 18 h. The homogenate was centrifuged, and the resultant supernatant was evaluated for spectrophotometric absorbance at 655 nm on a microplate reader. Results are expressed as optical density (OD655) per sample.

Statistical analysis. Data are expressed as means ± SE. An ANOVA was performed with Statview 4.0 statistical analysis software (SAS Institute, Cary, NC), and a difference was accepted as significant if the P value was equal to, or smaller than, 0.05 as verified by the Bonferroni-Dunn post hoc test.

RESULTS

NF-κB in TLR-4dd after hemorrhage and endotoxemia. NF-κB activation is a proximal step in hemorrhage- and endotoxin-induced lung injury (38, 42). To determine if hemorrhage- or endotoxin-induced NF-κB activation required a functional TLR-4, we used a p65 ELISA to examine the amount of the p65 subunit of NF-κB in the nuclear fraction of lung tissue in WT and TLR-4 d/d mice 1 h after injury. As depicted in Fig. 1, hemorrhage-induced NF-κB translocation is equivalent in WT and TLR-4 d/d mice. Endotoxin induced a dramatic increase in NF-κB translocation in WT (P < 0.01 vs. control). This effect was attenuated in TLR-4 d/d (P < 0.01 vs. WT). We also measured NF-κB-DNA binding with EMSA (Fig. 2), and the results were concordant with the p65 ELISA.

TNF-α production in TLR-4dd after hemorrhage and endotoxemia. We previously showed that TNF-α is required for hemorrhage- and endotoxin-induced lung injury (3, 10, 47). Therefore, we evaluated hemorrhage- and endotoxin-induced TNF-α mRNA and protein production in WT and TLR-4 d/d lung tissue 1 h after injury. As shown in Fig. 3A, hemorrhage induced an increase in TNF-α mRNA in WT that was not seen in TLR-4 d/d (P < 0.05 vs. control and TLR-4 d/d). Endotoxin (Fig. 3B) induced an increase in TNF-α mRNA in WT and TLR-4 d/d; however, there was a 10-fold greater increase in WT compared with TLR-4 d/d (P < 0.05 vs. TLR-4 d/d). TNF-α protein production roughly paralleled mRNA production with increased TNF-α in hemorrhaged WT lung tissue but not in hemorrhaged TLR-4 d/d (Fig. 3C). In endotoxin-treated mice, WT had a dramatic increase in TNF-α production that was significantly reduced in TLR-4 d/d (Fig. 3D).

Lung neutrophil accumulation in TLR-4dd after hemorrhage and endotoxemia. We evaluated neutrophil accumulation in WT and TLR-4 d/d lung tissue by MPO assay 4 h after injury (Fig. 4). Hemorrhage increased lung neutrophil recruitment in WT (P < 0.05 vs. control). There was no increase in neutrophil recruitment in TLR-4 d/d (P < 0.05 vs. WT). Similarly, endotoxin increased neutrophil recruitment in WT (P < 0.05 vs. control) but not TLR-4 d/d (P < 0.05 vs. WT).

Lung permeability in TLR-4dd after hemorrhage and endotoxemia. We previously determined that 30% hemorrhage or 1 mg/kg of intraperitoneal endotoxin induces equivalent degrees of acute lung edema in mice as assessed by an increased lung wet-to-dry weight ratio (4, 39, 42). To assess whether end organ damage was abrogated in TLR-4 d/d, we evaluated lung protein permeability by measurement of extravasation of Evans blue dye 4 h after hemorrhage or endotoxin. As depicted in Fig. 5, both hemorrhage and endotoxemia caused an increase in lung leak in WT 4 h after injury (P < 0.05 vs. controls). However, neither hemorrhage nor endotoxemia caused an increase in lung leak in TLR-4 d/d (P = 0.72 vs. control).

DISCUSSION

TLR-4 has an intracellular toll-IL-1 receptor (TIR) domain that recruits the adapter protein MyD88 (8, 25, 51). The TLR-4 bound MyD88 then recruits a signaling kinase, IL-1 receptor associated kinase ( IRAK), for subsequent downstream signaling cascades (27, 51). The C3H/HeJ (TLR-4 d/d) strain used in this experiment has a naturally occurring TLR-4 mutation with the defect in the intracellular TIR domain (33, 34). The principal function of the TIR domain is to mediate homotypic protein-protein interactions in the signal transduction process.
The defective TLR-4 is thus unable to recruit MyD88 with resultant interruption of the dominant signaling cascade for the TLR-4 receptor (52). Despite the inability of the TLR-4d/d to recruit MyD88, this strain still exhibits hemorrhage- and endotoxin-induced NF-κB activation (13). However, only in the WT strain was NF-κB activation able to induce TNF-α protein production. Another study documented LPS-induced TNF-α protein production both systemically and in lung tissue, albeit with different kinetics than that found in the WT mice (29). These findings all suggest that MyD88-independent pathways leading to NF-κB activation are intact in the TLR-4d/d, yet a functional TLR-4 is required for full LPS-induced activation of the NF-κB transcriptional complex and resultant TNF-α production. Our data are consistent with these previous findings.

Fig. 3. TNF-α mRNA (by RT-PCR) and protein production (by ELISA) 1 h after hemorrhage (HS) or endotoxemia (LPS). Hemorrhage induced an increase in TNF-α mRNA (A) and protein (C) in WT (*P < 0.01 vs. Ct) but not in TLR-4d/d. Endotoxin induced TNF-α mRNA (B) and protein (D) in WT (*P < 0.01 vs. Ct). There was decreased endotoxin-induced TNF-α mRNA and protein in TLR-4d/d (†P < 0.01 vs. WT).

Fig. 4. Lung neutrophil accumulation as assessed by myeloperoxidase (MPO) activity 4 h after hemorrhage (HS) or endotoxemia (LPS). Hemorrhage and endotoxin increased neutrophil accumulation in WT (*P < 0.01 vs. Ct). There was no increase in lung neutrophil accumulation after hemorrhage or endotoxin in TLR-4d/d (†P < 0.01 vs. WT).

Fig. 5. Acute lung leak as assessed by extravasation of Evans blue dye (EBD) 4 h after hemorrhage (HS) or endotoxemia (LPS). Hemorrhage and endotoxemia induced an increase in lung leak in WT (*P < 0.01 vs. Ct). There was no increase in lung leak after hemorrhage or endotoxin in TLR-4d/d (†P < 0.01 vs. WT).
NF-κB was similar between the TLR-4\textsuperscript{−/−} and WT after hemorrhage. Although it was decreased compared with WT, there was still NF-κB activation in the TLR-4\textsuperscript{−/−} after endotoxin compared with baseline. Thus MyD88-independent pathways may still activate NF-κB in the TLR-4\textsuperscript{−/−} strain. However, the TLR-4-MyD88 pathway is required for a full expression of TNF-α.

The role of MyD88-independent signaling is further highlighted in recent studies in MyD88 knockout mice. Kawai et al. (21) found that endotoxin-induced NF-κB and mitogen activated protein kinase (MAPK) activation in MyD88-deficient mice, albeit with different kinetics from WT mice. Despite delayed activation of NF-κB and MAPK, MyD88 knockout mice did not produce TNF-α mRNA or protein. Recent evidence suggests that at least some of the MyD88-independent signaling occurs through two alternate adapter molecules, TIR domain-containing adapter protein (TIRAP) and TIR domain-containing adapter inducing IFN-β (TRIF) (17, 53). Interestingly, TIRAP knockout mice also exhibit delayed activation of NF-κB in response to endotoxin (17). Therefore, whereas MyD88 is the dominant TLR-4 signaling pathway and is required for downstream cytokine production, there are MyD88-independent pathways that can continue to activate transcription factors in TLR-4\textsuperscript{−/−}.

We also evaluated endotoxin- and hemorrhage-induced TNF-α message and protein levels in WT and TLR-4\textsuperscript{−/−}. Similar to what others found (20), the relative reduction of endotoxin-induced NF-κB activation in TLR-4\textsuperscript{−/−} correlated with the reduction in TNF-α mRNA and protein production. Despite equivalent hemorrhage-induced activation of NF-κB in WT and TLR-4\textsuperscript{−/−}, there was no increase in TNF-α mRNA or protein production in TLR-4\textsuperscript{−/−}. This finding suggests that there is hemorrhage-induced TLR-4-dependent regulation of the intranuclear NF-κB transcriptional complex. Regulation of the transcriptional activity of NF-κB is a complex combination of direct protein modifications and intranuclear protein-protein interactions, all of which work in concert to create an active transcriptional complex. An early protein modification of NF-κB is phosphorylation of the p65 subunit by protein kinase A, Casein kinase II, or IκB kinase. Beyond direct p65 modification, there are numerous coactivators that are required for initiation or potentiation of the transcriptional complex, including CBP and p300 (43). Finally, NF-κB transcriptional regulation occurs at the level of histone acetylation. Therefore, the regulation of NF-κB transcriptional activation is an extremely complex area with numerous levels of regulation that may individually or in summation affect the transcriptional activity of NF-κB after both hemorrhage and endotoxemia. Any one of these mechanisms may contribute to the observed decrease in TNF expression despite NF-κB activation in the TLR-4\textsuperscript{−/−} strain.

The final common effect of both hemorrhage- and endotoxin-induced cytokine and chemokine production is the recruitment of neutrophils into organ systems resulting in tissue injury (Fig. 6). In our study, neither hemorrhage nor endotoxemia caused lung neutrophil recruitment or acute lung injury in TLR-4\textsuperscript{−/−}. TNF-α is believed to be one of many cytokines involved in the evolution of lung injury after hemorrhage and endotoxemia (3, 10, 47). We previously showed that even a very low level of lung TNF-α is critical to the development of acute lung injury after hemorrhagic shock (47). Similarly, TNF-α is also involved in endotoxin-induced lung injury as demonstrated by the lack of injury in TNF receptor-1 knockout mice (10). Furthermore, TNF-binding protein prevents endotoxin-induced acute lung injury (44). Although the absolute levels of TNF-α were proportionately different after hemorrhage and endotoxin, WT lung injury as assessed using Evans blue dye extravasation was equivalent in magnitude in these two injuries. These data on the extent of hemorrhage- or endotoxin-induced lung injury were consistent with our previous findings that 30% hemorrhage compared with 1 mg/kg of intraperitoneal endotoxin results in widely divergent TNF-α production, yet similar lung neutrophil recruitment and wet-to-dry ratios (2). We chose to evaluate lung TNF-α on the basis of its important role in lung injury after both endotoxin and hemorrhage, as detailed above (3, 10, 47). Furthermore, DeMaria et al. (12) found that serum TNF was decreased after hemorrhage in TLR-4\textsuperscript{−/−} compared with WT. However, TNF-α is just one of many potential factors that have been implicated in lung neutrophil recruitment and the development of acute lung injury. Therefore, the protection against neutrophil recruitment and lung injury seen in TLR-4\textsuperscript{−/−} after hemorrhage and endotoxin is unlikely to be explained only by reduction in TNF-α production.

\begin{figure}
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\caption{Schematic of hemorrhage (HS) and endotoxin (LPS)-induced intracellular pathways leading to acute lung injury. Endpoints for the study are in the boxes to the right. EMMA, electromobility shift assay; MPO, myeloperoxidase assay; TIRAP, toll-IL-1 receptor (TIR) domain-containing adapter protein; TRIF, TIR domain-containing adapter inducing IFN-β.}
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Bacterial translocation may be a confounding factor in hemorrhagic shock. Translocation of gut-derived bacteria across the intestinal barrier was previously demonstrated in a variety of hemorrhagic shock models. Deitch et al. (11) reported a 61% incidence of bacterial translocation in rats 24 h after hemorrhage and resuscitation. Similarly, Baker et al. (7) demonstrated enteric bacteria in a variety of organs 24 h after rats had been subjected to varying degrees of shock. However, this phenomenon has not been conclusively shown to play a role in the expression of cytokines or tissue injury in hemorrhaged mice. Ayala et al. (6) determined that hemorrhage-induced serum TNF-α levels were not associated with systemic endotoxemia in mice. Similarly, Groetz et al. (16) did not find any endotoxin in portal or systemic blood after hemorrhage in the rat. We previously demonstrated that our nonresuscitated, 30% hemorrhage model does not induce endotoxemia (as determined by limulus assay) (3, 37, 40). Our data demonstrate a divergence between hemorrhage- and endotoxin-induced NF-κB activation and TNF-α production. If the findings were due strictly to endotoxemia (bacterial translocation) after hemorrhage, we would have expected similar activation patterns throughout all of the experiments. Thus these current data suggest that translocation and endotoxemia do not contribute to the pathophysiology of hemorrhage-induced lung injury in keeping with both our own and Ayala et al.’s (6) previous observations.

Other investigators evaluated TLR-4 expression and function in a variety of injury models. DeMaria and colleagues (12) performed an approximate 45% hemorrhage on C3H/HeJ (TLR-4<sup>−/−</sup>) mice and determined that they had increased survival and decreased systemic TNF-α compared with hemorrhaged controls. In a two-hit model of hemorrhage followed by intratracheal endotoxin, TLR-4 expression was controlled both transcriptionally and posttranscriptionally via altered mRNA stability (14). Furthermore, the first hit of shock/resuscitation appeared to regulate TLR-4 gene expression. Finally, Ayala and colleagues (5) evaluated the role of TLR-4 in a two-hit model of hemorrhage followed by cecal ligation and puncture (CLP). In this study, hemorrhage-induced neutrophil priming for subsequent CLP-induced lung injury required a functional TLR-4. These results closely parallel our own that hemorrhagic shock induces subsequent inflammatory responses through TLR-4 signaling mechanisms.

The innate immune system can identify and initiate a response to a wide variety of invading microorganisms through the various toll-like receptors. However, TLR-4 is unique in its ability to recognize both exogenous (foreign or non-self) and endogenous (self) proteins. The endogenous TLR-4 ligands, including heat shock proteins (60 and 70), the extra domain A of fibronectin, oligosaccharides of hyaluronic acid, polysaccharide fragments of heparan sulfate, elastase, and fibrinogen, are all proteins that are either released or produced by cells undergoing stress or abnormal cell death. Therefore, TLR-4’s role in innate immunity extends far beyond its initial description as a pattern recognition receptor for exogenous bacterial products. Rather, TLR-4 is emerging as a more comprehensive receptor for danger signals. Whether the initiating ligand originates from gram-negative bacteria, the abnormal release of intracellular proteins, the production of extracellular matrix proteins or the more global stress of hemorrhagic shock, a functional TLR-4 is required for subsequent inflammatory signaling. A more descriptive classification of TLR-4 as a danger signal receptor would be consistent with the “danger model” of innate immunity as proposed by Matzinger (22). In the danger model, the innate immune system is less concerned with identifying non-self antigens and more concerned with sensing danger from the surrounding tissues. As such, the endogenous danger ligands are those products released from cells in response to stress or undergoing abnormal death. These danger ligands then initiate a host immune response. The danger model thereby allows for, or explains, the activation of the innate immune system by factors other than foreign antigens. Our data on hemorrhagic shock support the danger model and the hypothesis that TLR-4 is more of a danger signal receptor than just merely a pattern recognition receptor. This emerging role for TLR-4 as a danger signal receptor may or may not extend to other toll-like receptors. Currently, only TLR-2 has been demonstrated to signal for an endogenous protein (HSP70) and this is not entirely certain, as recent investigations have revealed bacterial contamination as a potential confounder in HSP70 signaling (15). Interestingly, whereas contemporary literature supports the role of TLR-4 in deleterious danger signaling, these data do not necessarily exclude a beneficial role for TLR-4 signaling in as yet to be determined signaling pathways.

In summary, we determined that hemorrhage and endotoxin induce NF-κB activation, TNF-α mRNA and protein production, lung neutrophil accumulation, and acute lung injury. Furthermore, all of these findings are attenuated in endotoxin-treated TLR-4<sup>−/−</sup> mice. Interestingly, hemorrhaged TLR-4<sup>−/−</sup> mice have attenuated TNF-α mRNA and protein, lung neutrophil accumulation, and acute lung injury but no attenuation in NF-κB activation. Therefore, we conclude that 1) TLR-4 is an important receptor in hemorrhage-induced acute lung injury and 2) hemorrhage-induced TLR-4 signaling occurs by a distinct and different activation pattern than endotoxemia.

**GRANTS**

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