Systemic inflammation and remote organ damage following bilateral femur fracture requires Toll-like receptor 4

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TRAUMA AND ITS SEQUELAE ACCOUNT for 6% of all deaths and remain the leading cause of death in the United States for individuals under the age of 54 years. Injuries involving bone and soft tissue represent a major cause of morbidity and mortality in the trauma population. More than 90% of multiply injured patients have one or more extremity fractures (10). Moreover, the presence of long bone fractures and soft tissue trauma strongly correlates with the development of systemic inflammatory response syndrome (SIRS), sepsis, and multisystem organ failure (54).

Traumatic injury may lead to both local and systemic inflammation (12, 48). Systemic inflammation can lead to multiple organ dysfunction remote from the site of traumatic injury. Increased expression of mediators (cytokines, eicosanoids), upregulation of leukocyte adhesion molecule expression, and influx of polymorphonuclear cells into injured tissues indicate that a profound inflammatory process occurs after traumatic injury. However, the exact molecular events initiating the systemic inflammatory response to local soft tissue and bone injury are unknown. Previous animal and human studies have demonstrated the immunosuppressive effects of blunt traumatic injury (64). The deleterious impact of femur fracture and associated soft tissue injury on remote organ function has been described in regard to splenocyte proliferation, intestinal permeability, and hepatic ischemia (37, 51, 60). Although many organs are affected by the systemic mediators released during trauma, the liver is a primary site of response (18).

The Toll-like receptors (TLRs) are an evolutionarily conserved family of pattern recognition receptors central to the innate immune response to infection. TLRs are responsive to pathogen-associated ligands such as LPS, peptidoglycan, and other microbial components. Recent evidence also suggests that TLRs can recognize endogenous ligands that signal host injury, including hyaluronic acid, heparan sulfate, heat-shock proteins, fibronectin, and biglycan (22, 27, 40, 41, 50, 56). Thus a paradigm of the innate immune response to injury is emerging in which TLRs recognize danger signals initiated by cellular damage independent of infection (30, 38). Of these receptors, TLR4 has been recognized as a driver of the innate immune response in situations of sterile inflammation and autoimmunity. Specifically, TLR4 mutant mice demonstrate protection from hemorrhage-induced acute lung and hepatic injury, myocardial reperfusion injury, and hemorrhagic shock-related tumor necrosis factor (TNF) release and mortality (2, 8, 42, 46).

In light of these considerations, we sought to determine whether a sterile, local tissue injury in the form of bilateral femur fracture causes systemic inflammation and remote organ dysfunction in a TLR4-dependent manner.

MATERIALS AND METHODS

Reagents. All reagents were from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Animals. Mice used in the experimental protocols were housed in accordance with University of Pittsburgh and National Institutes of Health (NIH) animal care guidelines in specific pathogen-free conditions. The animals were maintained in the University of Pittsburgh Animal Research Center with a 12:12-h light-dark cycle and free access to standard laboratory feed and water. Male C3H/HeJ mice and C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME), 8–12 wk old and weighing 20–30 g, were used in experiments. To exclude a role for LPS in trauma-induced inflammation, experiments were repeated with male CD14−/− and CD14 wild-type mice (C57BL/6J, Charles River Laboratories, Wilmington, MA) (13). All animals were
fasted for ~12 h before experimental manipulation and were acclimated for 7 days before being studied.

Reverse transcribed. This research protocol complied with the regulations regarding the care and use of experimental animals published by the NIH and was approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh. Briefly, animals were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg) and inhaled isoflurane (Abbott Labs, Chicago, IL). We used a sterile technique to perform a left groin exploration, and the left femoral artery was cannulated with tapered polyethylene-10 tubing and connected to a blood pressure transducer (Micro-Med, Tustin, CA) for continuous mean arterial pressure (MAP) monitoring for the duration of the experiment (6 h). Bilateral closed midshaft femur fracture was then performed using two hemostats applied to the hindlimb region. MAP was maintained above 60 mmHg throughout the experiment with the administration of lactated Ringer solution (Baxter, Deerfield, IL) through the femoral cannula as needed in 0.1-ml boluses. This served to ensure that the animals were not in a state of circulatory shock. According to the manufacturer, the endotoxin content of the lactated Ringer solution used was 0.008 EU/ml. Sham-operated mice underwent anesthesia and femoral cannulation only. All mice were anesthetized with intraperitoneal pentobarbital sodium (20 mg/kg) as necessary throughout the experiment. Baseline MAP, total anesthetic dosage, and volume of lactated Ringer solution administered did not differ between species or experimental groups (sham vs. fracture). At the end of 6 h, mice were killed under inhalational anesthesia. Necropsy was performed to verify the presence of bilateral femur fractures and to ensure the absence of fracture site hematomas. Serum from postmortem blood samples was obtained for cytokine and blood chemistry analysis. Organs were snap frozen in liquid nitrogen for molecular analysis.

**Serum alanine aminotransferase assay.** To assess hepatocellular injury after bilateral femur fracture, serum alanine aminotransferase (ALT) levels were measured using the Opera Clinical Chemistry System (Bayer, Tarrytown, NY).

**Serum IL-6 and IL-10 assay.** Serum IL-6 and IL-10 levels were used as a means of evaluating systemic inflammation and were quantified with ELISA kits (R&D Systems, Minneapolis, MN). RT-PCR. Quantitative RT-PCR was used to determine hepatic cytokine mRNA levels of IL-6, IL-10, and TNF. Total RNA was extracted from thawed hepatic tissue samples with chloroform and phenoretic mobility shift assays using nuclear extracts prepared from Bcl-13,800 p l u sDf o r1ha t4°C, supernatants were collected by centrifugation at 200 g for 15 min before being vigorously vortexed for 10 s at a maximum speed. Nuclear proteins were extracted by gently resuspending the nuclei with an appropriate volume of buffer C [20 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 0.2 mM PMSF, and 0.5% NP-40] and incubated on ice for 15 min before being vigorously vortexed for 10 s at a maximum speed. Nuclear proteins were extracted by gently resuspending the nuclei with an appropriate volume of buffer C [20 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF] along with buffer D (same as buffer C but has 1.6 M KCl). The ratio of buffer C to buffer D was 3 to 1. Buffer D was added in a dropwise fashion. After incubating the nuclei in buffer C plus D for 1 h at 4°C, supernatants were collected by centrifugation at 13,800 g for 15 min. Double-stranded nuclear factor-κB (NF-κB)-specific oligonucleotide was end-labeled with 32PATP using T4 polynucleotide kinase (U.S. Biochemicals, Cleveland, OH) and purified on a G-50 Sephadex spin column. Nuclear proteins (5 μg per well) were incubated with 50,000 cpm of 32P-labeled oligonucleotide for 30 min at room temperature in a reaction mixture containing 1 μg of poly(dI-dC), 10 mM Tris-HCl (pH 7.5), 10% glycerol, 1.0 mM EDTA, 1% NP-40, 1 mg/ml BSA, and 1.0 mM DTT (final volume 20 μl). The DNA protein complexes were resolved on a 4% nondenaturing polyacrylamide gel in 0.5 × Tris-borate-EDTA (TBE) buffer. The gels were dried and then subjected to autoradiography.

**Statistical analysis.** Results are expressed as the means ± SE. Group comparisons were assessed using the Mann-Whitney Rank Sum Test. The null hypothesis was rejected for P < 0.05 (α = 0.05). Data were analyzed using SigmaStat Version 3.1 (SPSS, Chicago, IL). In the comparison between C3H/HeOuJ and C3H/HeJ mice, control groups consisted of n = 5 mice, sham groups consisted of n = 8 mice, and bilateral femur fracture groups consisted of n = 10 mice. All experimental groups in the comparison between CD14−/− mice and CD14+ WT mice consisted of n = 6 mice, whereas control groups consisted of n = 4 mice.

**RESULTS**

Organ damage and dysfunction may occur remote from the site of traumatic tissue injury. Serum ALT measurements were obtained as a systemic assessment of hepatocellular injury. Using continuous blood pressure monitoring to ensure the absence of circulatory shock, we found that wild-type mice (C3H/HeOuJ) subjected to bilateral femur fracture had increased serum ALT levels compared with sham-operated animals (116.7 ± 8.5 IU/l vs. 486.5 ± 325.4 IU/l). Serum ALT levels were significantly lower in TLR4 mutant mice subjected to femur fracture compared with their wild-type counterparts (101.9 ± 18.9 IU/l vs. 486.5 ± 325.4 IU/l, P = 0.007, Fig. 1).
Sham-operated animals from both strains responded to surgical manipulation and anesthesia with similar ALT levels and therefore manifest comparable hepatocellular injury responses to the sham procedure. Recognition of LPS by the host requires a receptor complex composed of TLR4, CD14, and myeloid differentiation protein-2 (MD2). Recently published work has shown that in vitro responses of liver nonparenchymal cells to nanomolar concentrations of LPS require functional CD14 signaling (52, 55). In the present study, compared with CD14 wild-type mice, CD14−/− mice failed to demonstrate protection from hepatocellular injury following bilateral femur fracture as measured by serum ALT levels (164.5 ± 5.2 IU/L vs. 148 ± 8.3 IU/L, P = 0.134, Fig. 1). This result suggests that the reduced liver damage experienced by TLR4 mutant mice is not due to a lack of sensitivity to gut-derived LPS.

IL-6, IL-10, and TNF are upregulated in the response to injury. Previous work has demonstrated increases in these mediators in human fracture site hematomas, as well as in the systemic circulation (12). Experimental and clinical data suggest that IL-6 and IL-10 levels correlate with the systemic inflammatory response to physiological insults. Postinjury IL-6 and IL-10 levels are predictive of complications, organ failure, and mortality in human trauma patients (4, 43, 53). We therefore used circulating IL-6 and IL-10 levels as markers of the systemic inflammatory response to femur fracture. TLR4 mutant mice demonstrated significantly lower levels of circulating IL-6 (211.3 ± 24.1 pg/ml vs. 1,408 ± 397.5 pg/ml, P = 0.008) and IL-10 (35.7 ± 12.1 pg/ml vs. 143.6 ± 38.4 pg/ml, P = 0.002) compared with wild-type mice in response to bilateral femur fracture (Fig. 2). There were no significant differences in circulating IL-6 or IL-10 levels between TLR4 mutant and TLR4 wild-type sham-operated animals. In further support of a lack of a role for gut-derived LPS in the response to local tissue injury, serum IL-6 and IL-10 levels were similar in CD14−/− mice compared with their wild-type counterparts [594.4 ± 326.3 pg/ml vs. 693.6 ± 368.2 pg/ml (P = 0.945) and 115.39 ± 53.4 pg/ml vs. 56.3 ± 30.1 pg/ml (P = 0.445), respectively] (Fig. 2). These results manifest a pivotal role for TLR4 in the systemic inflammatory response to remote tissue trauma.

The liver plays a central role in regulating the systemic inflammatory response to trauma (44). Semiquantitative RT-PCR was employed to further assess end-organ inflammation in the liver with regard to expression of IL-6, IL-10, and TNF. Total hepatic RNA was isolated, and results were normalized to 18S. As depicted in Fig. 3, TLR4 mutant mice exhibited lower hepatic levels of IL-6, IL-10, and TNF mRNA following bilateral femur fracture compared with wild-type mice. TLR4 signaling leads to activation of the transcription factor NF-κB, thereby inducing a variety of proinflammatory cytokines and adhesion molecules that contribute to organ injury (31). NF-κB activation is a proximal step in hemorrhage and endotoxin-induced organ injury (17). In mice subjected to bilateral femur fracture, increased hepatic NF-κB DNA binding was noted by EMSA in TLR4 wild-type mice. In contrast, TLR4 mutant mice showed a muted hepatic inflammatory response to femur fracture evidenced by decreased NF-κB activity (Fig. 4).

### DISCUSSION

Extensive soft tissue injury and bone fractures are significant contributors to the initial systemic inflammatory response in multiply injured patients (35). Local tissue damage induces both local and systemic responses in the host. The magnitude of the systemic inflammatory response correlates with the severity of injury (28, 36). Clinically, this is manifested along a spectrum that varies from a mild case of SIRS to the overwhelming and often terminal multiple organ dysfunction syndrome. A multitude of both clinical and experimental studies demonstrate alterations in inflammatory and immunological function following trauma. This is characterized by local and systemic release of cytokines and other inflammatory mediators, including hepatic acute phase proteins, arachidonic acid metabolites, complement components, reactive species (oxygen and nitrogen), proteolytic enzymes, and neuroendocrine signals (23, 47, 63). For example, elevated circulating levels of IL-1, IL-6, IL-8, IL-10, TNF, soluble TNF receptor, and sICAM have all been shown to correlate with injury severity, organ failure, and mortality in multiply injured trauma patients (5, 6, 15, 26, 29, 39, 43, 48). The upregulation of leukocyte adhesion molecules, endothelial cell damage, and the resulting influx of inflammatory cells into damaged tissues all indicate that a profound inflammatory process occurs after severe tissue trauma. The net result of these simultaneous cascades is am-
plification of the local injury response and potential remote organ damage.

This study was undertaken to determine the role of TLR4 in the systemic and hepatic inflammatory response to remote tissue trauma. Our findings indicate that even in the absence of circulatory shock, local tissue trauma is capable of inciting a cascade of systemic and remote organ inflammation that culminates in hepatocellular damage. We found diminished hepatic IL-6, IL-10, and TNF mRNA levels in TLR4 mutant mice. Semiquantitative PCR assessment of the hepatic inflammatory response to bilateral femur fracture in C3H/HeOuJ and C3H/HeJ mice. TNF (A), IL-6 (B), and IL-10 (C). All results are normalized to 18S expression. Data shown are representative of three experiments with similar results.

Fig. 3. Decreased hepatic IL-6, IL-10, and TNF mRNA levels in TLR4 mutant mice. Semi-quantitative PCR assessment of the hepatic inflammatory response to bilateral femur fracture in C3H/HeOuJ and C3H/HeJ mice. TNF (A), IL-6 (B), and IL-10 (C). All results are normalized to 18S expression. Data shown are representative of three experiments with similar results.

Fig. 4. TLR4 mutant mice demonstrate decreased hepatic NF-κB activation by EMSA. Hepatic nuclear extracts in C3H/HeOuJ vs. C3H/HeJ mice show that TLR4 mutant mice exhibit decreased hepatic NF-κB activation after bilateral femur fracture compared with wild-type counterparts.

Fig. 2. Systemic inflammation after bilateral femur fracture is TLR4 dependent. Serum IL-6 and IL-10 levels C3H/HeOuJ vs. C3H/HeJ mice (A, B) and CD14-/- vs. CD14 wild-type mice (C, D). TLR4 mutant mice demonstrate reduced serum IL-6 (*P = 0.026 by Mann-Whitney rank sum test) and reduced serum IL-10 compared with TLR4 competent mice (*P = 0.041 by Mann-Whitney rank sum test). Data are expressed as means ± SE, n = 6 mice per group for controls, n = 8 mice per group for shams, and n = 10 mice per group for bilateral femur fracture. For experiments using CD14-/- mice, sham and fracture groups had n = 6 mice, whereas controls had n = 4 mice.
patic injury and a muted hepatic inflammatory cytokine and NF-κB response after femur fracture in TLR4 mutant mice. The systemic inflammatory response was also diminished in TLR4 mutant compared with TLR4 competent mice as measured by circulating IL-6 and IL-10 levels. Thus these results demonstrate that the remote hepatic organ injury and early systemic and hepatic inflammatory responses to femur fracture occur through a TLR4-dependent process. In experimental paradigms of trauma/hemorrhage, previous studies have suggested that inflammation is induced by gut-derived LPS (21). The lack of protection from fracture-induced systemic inflammation and hepatocellular injury in CD14<sup>−/−</sup> mice suggests that the established LPS recognition pathway is not involved in this TLR4-dependent process.

Our results do not define the mechanism underlying the liver damage that occurs with femur fracture. We closely monitored systemic blood pressure throughout the experimental time frame and maintained MAP above 60 mmHg. Although we cannot absolutely rule out local perfusion deficits, this alone seems an unlikely explanation for our findings. Inflammatory mediators such as IL-6 have been shown to contribute to end-organ injury in hepatic ischemia reperfusion (I/R) and hemorrhagic shock models (14, 32, 57, 62). Therefore, it is possible that both the systemic and local hepatic production of IL-6 and other proinflammatory mediators may contribute to the end-organ damage observed in our model.

Several studies have suggested that many of the inflammatory changes resulting from traumatic injury are secondary to increased gut permeability (34, 49, 59), and subsequent release and recognition of gut-derived LPS due to bacterial translocation (11, 16, 21). This concept of physiological stress resulting in impaired gut barrier function and subsequent translocation of bacteria/endotoxin into the systemic circulation and remote organs has been termed the “gut hypothesis” (7). However, both animal and human trauma studies have failed to conclusively demonstrate either the presence of elevated LPS or increased bacterial translocation (1, 9, 45, 49). As a result, a lack of consensus exists surrounding the role of bacterial or endotoxin translocation in the systemic inflammatory response to trauma. Cellular responses to LPS in levels typically measured in the circulation (pg/ml to ng/ml) require the participation of either surface or soluble CD14 (61). In this role, CD14 participates in TLR4/MD2-dependent LPS signaling pathways (3, 19, 25). In our study, the lack of protection in CD14<sup>−/−</sup> mice suggests that LPS is not involved in either the hepatic injury or systemic inflammatory response resulting from bilateral femur fracture. Moreover, LPS levels from fractured mice were all less than 0.25 EU/ml, and no differences were noted between TLR4 wild-type and TLR4 mutant animals (data not shown). This implies that the pathway of recognizing and responding to a peripheral, traumatic insult is distinct from that of typical TLR4-dependent LPS signaling.

The similarity between the SIRS of infection and the SIRS of injury suggests a common response mechanism for the recognition of both infectious agents and tissue injury by the innate immune system. Support for this concept has come from studies showing that activation of immune cells by microbial products, as well as endogenous molecules released by either degraded tissue matrix or necrotic cells, requires signaling through the Toll-like receptor family (22). TLR4 has been recognized as a driver of the innate immune response in both inflammatory and autoimmune settings. Hemorrhagic shock and I/R injury often complicate traumatic injuries to bone and soft tissue. Both of these conditions result in overt tissue hypoperfusion, the former representing a global insult, whereas the latter typifies a regional insult. Recent reports have implicated that organ injury in both of these models is TLR4 dependent (2, 33, 42, 46, 59). In comparison, the femur fracture model used in our study represents a local, peripheral tissue injury. As such, our results extend existing observations by showing that in the absence of shock physiology, trauma-induced systemic inflammation and remote organ dysfunction require functional TLR4 signaling.

Recent evidence has shown that both endogenous cellular and tissue matrix elements can stimulate signaling via TLR4 (20, 22, 40, 50, 56, 58). Included among these endogenous TLR4 ligands are heat shock proteins, heparan sulfate, fibronectin, hyaluronic acid, and HMGB-1. Kim et al. (24) recently demonstrated that a neutralizing antibody to HMGB-1 prevented hemorrhage-induced acute lung injury in a murine model (24). We recently reported that blockade of HMGB-1 protected mice from hepatic I/R injury (60). In that same study, we showed that TLR4 mutant mice were protected from hepatic injury and that anti-HMGB-1 antibody afforded no additional protection in TLR4 mutant animals. In combination with the findings presented here, these studies provide evidence that TLR4 functions to recognize and respond to signals from stressed or injured tissues to initiate inflammatory cascades. Whereas we would speculate that tissue injury stimulates TLR4 signaling through the release of endogenous molecules detected by pattern recognition receptors, our studies have not yet identified the source or nature of these activating substances. At this point, it is unclear whether a single ligand will account for fracture-induced TLR4 activation.

Further work is required to identify the ligands responsible for initiating TLR4 signaling after traumatic injury. It is intriguing to speculate that multicellular organisms have conserved mechanisms to deal with traumatic injury and infections by using the same receptor to monitor their environment for either challenge. Our work begins to provide evidence to support developing strategies directed at the level of the TLR4 receptor in an attempt to mitigate trauma-induced inflammation and remote organ damage.

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REFERENCES


