Systemic Inflammation and Remote Organ Damage Following Bilateral Femur Fracture Requires Toll-like Receptor 4


Running Title: TLR4 in Fracture

*Department of Surgery, University of Pittsburgh, Pittsburgh, PA 15213

‡ Department of Critical Care Medicine, University of Pittsburgh, Pittsburgh, PA 15213

† To whom correspondence should be addressed at:
Timothy R. Billiar, MD
Department of Surgery, F-1200 PUH
University of Pittsburgh
200 Lothrop Street
Pittsburgh, PA 15217
Tel: 412-647-1749
Fax: 412-647-3247
Email: billiartr@upmc.edu
ABSTRACT

Extensive soft tissue injury and bone fracture are significant contributors to the initial systemic inflammatory response in multiply injured patients. Systemic inflammation can lead to organ dysfunction remote from the site of traumatic injury. The mechanisms underlying the recognition of peripheral injury and the subsequent activation of the immune response are unknown. Toll-like receptors (TLRs) recognize microbial products but also may recognize danger signals released from damaged tissues. Here we report that peripheral tissue trauma initiates systemic inflammation and remote organ dysfunction. Moreover, this systemic response to a sterile local injury requires toll-like receptor 4 (TLR4). Compared to wild-type (C3H/HeOuJ) mice, TLR4 mutant (C3H/HeJ) mice demonstrated reduced systemic and hepatic inflammatory responses to bilateral femur fracture. Trauma-induced nuclear factor (NF)-κB activation in the liver required functional TLR4 signaling. CD14<sup>−/−</sup> mice failed to demonstrate protection from fracture-induced systemic inflammation and hepatocellular injury. Therefore, our results also argue against a contribution of intestine-derived lipopolysaccharide (LPS) to this process. These findings identify a critical role for TLR4 in the rapid recognition and response pathway to severe traumatic injury. Application of these findings in an evolutionary context suggests that multi-cellular organisms have evolved to utilize the same pattern recognition receptor for surviving traumatic and infectious challenges.

KEYWORDS

Trauma, innate immunity, TLR4, liver injury, fracture, systemic inflammation
INTRODUCTION

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. Over 90% of multiply-injured patients have one or more extremity fractures\textsuperscript{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 multi-system organ failure (MSOF)\textsuperscript{54}.

Traumatic injury may lead to both local and systemic inflammation\textsuperscript{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 (PMN) into injured tissues indicate that a profound inflammatory process occurs following 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\textsuperscript{66}. 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\textsuperscript{37,51,62}. Though many organs are affected by the systemic mediators released during trauma, the liver is a primary site of response\textsuperscript{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 lipopolysaccharide (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. 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. Of these receptors, TLR4 has been recognized as a driver of the innate immune response in situations of sterile inflammation and auto-immunity. Specifically, TLR4 mutant mice demonstrate protection from hemorrhage-induced acute lung and hepatic injury, myocardial reperfusion injury, and hemorrhagic shock related TNF release and mortality.

In light of these considerations, we sought to determine if 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 & METHODS

**Reagents.** All reagents were from Sigma Chemical Co. (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-hour light-dark cycle and free access to standard laboratory feed and water. Male C3H/HeOuJ mice and C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) 8-12 weeks old and weighing 20-30 grams 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 approximately 12 hours prior to experimental manipulation and were acclimatized for 7 days prior to being studied.

**Murine fracture model.** 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 (i.p.) sodium pentobarbital (50 mg/kg) and inhaled isoflurane (Abbott Labs, Chicago, IL). Using sterile technique, 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, Tustin, CA) for continuous mean arterial pressure (MAP) monitoring for the duration of the experiment (6
hours). Bilateral closed mid-shaft femur fracture was then performed using two Hemostats applied to the hind limb region. MAP was maintained above 60 mm Hg throughout the experiment with the administration of Lactated Ringer’s solution (Baxter Corporation, 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’s utilized was 0.008 EU/mL. Sham operated mice underwent anesthesia and femoral cannulation only. All mice were re-anesthetized with i.p. sodium pentobarbital (20 mg/kg) as necessary throughout the experiment. Baseline MAP, total anesthetic dosage, and volume of Lactated Ringer’s administered did not differ between species or experimental groups (sham versus fracture). At the end of 6 hours, mice were sacrificed 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 post mortem blood samples was obtained for cytokine and blood chemistry analysis. Organs were snap frozen in liquid nitrogen for molecular analysis.

**Serum ALT assay.** To assess hepatocellular injury following bilateral femur fracture, serum alanine aminotransferase (ALT) levels were measured using the Opera Clinical Chemistry System (Bayer Co., 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 Inc., Minneapolis, MN).
Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Semi-quantitative reverse transcriptase polymerase chain reaction (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 TRI Reagent (Molecular Research Center, Cincinnati, OH) exactly as directed by the manufacturer. The total RNA was treated with DNase Free (Ambion, Houston, TX) as instructed by the manufacturer using 10 units of DNase I/10 µg RNA. Two micrograms of total RNA were reverse transcribed in a 40 µl reaction volume containing 0.5 µg of oligo (dT)15 (Promega), 1 mM of each dNTP, 15 U AMV reverse transcriptase (Promega), and 1 U/µL of recombinant RNasin ribonuclease inhibitor (Promega) in 5 mM MgCl2, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100 (pH=8.0). The reaction mixtures were preincubated at 21°C for 10 min prior to DNA synthesis. The RT reactions were carried out for 50 min at 42°C and were heated to 95°C for 5 min to terminate the reaction. Reaction mixtures (50 µL) for PCR were assembled using 5 µL of cDNA template, 10 units AdvanTaq Plus DNA Polymerase (Clontech, Palo Alto, CA), 200 µM of each dNTP, 1.5 mM MgCl2 and 1.0 µM of each primer in 1x AdvanTaq Plus PCR buffer. PCR reactions were performed using a Model 480 thermocycler (Perkin Elmer, Norwalk, CT). Amplification of cDNA was initiated with 5 min of denaturation at 94°C. The PCR conditions for amplifying cDNA for TNF and IL-6 were as follows: denaturation at 94°C for 45 s, annealing at 61°C for 45 s, and polymerization at 72°C for 45 s. Amplification of cDNA for IL-10 was carried out by denaturing at 94°C for 30 s, annealing at 62°C for 30 s, and polymerizing at 72°C for 30 s. To ensure that amplification was in the linear range, we empirically determined that 31 was
the optimal number of cycles for TNF and IL-6 cDNA while 34 was the optimal number of cycles for IL-10 cDNA. After the last cycle of amplification, the samples were incubated at 72°C for 10 min and then held at 4°C. The 5' and 3' primers for TNF were GGC AGG TCT ACT TTG GAG TCA TTG C and ACA TTC GAG GCT CCA GTG AAT TCG G, respectively; the expected product length was 307 bp. The 5' and 3' primers for IL-6 were TTC CAT CCA GTT GCC TTC TTG G and TTC TCA TTT CCA CGA TTT CCC AG, respectively; the expected product length was 174 bp. The 5' and 3' primers for IL-10 were CTG CTA TGC TGC CTG CTC TTA and CTG GAG TCC AGC AGA CTC AAT, respectively; the expected product length was 561 bp. 18S ribosomal RNA was amplified to verify equal loading. For this reaction, the 5' and 3' primers were CCC GGG GAG GTA GTG ACG AAA AAT and CGC CCG CTC CCA AGA TCC AAC TAC, respectively; the expected product length was 209 bp. Ten microliters of each PCR reaction were electrophoresed on a 2% agarose gel and scanned at a NucleoVision imaging workstation (NucleoTech, San Mateo, CA).

**Electrophoretic mobility shift assays (EMSA).** NF-κB DNA binding activity was measured by electrophoretic mobility shift assays using nuclear extracts prepared from liver tissue. Livers harvested at the conclusion of the experimental protocol were snap frozen in liquid nitrogen and stored at -80°C. A portion of frozen liver tissue was subsequently homogenized in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol [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 drop-wise fashion. After incubating the nuclei in buffer C plus D for 1 h at 4°C, supernatants were collected by centrifugation at 13,800 x g for 15 min. Double-stranded NF-κB -specific oligonucleotide was end labeled with ³²PATP using T4 polynucleotide kinase (U.S. Biochemicals, Cleveland, Ohio) 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% non-denaturing polyacrylamide gel in 0.5x Tris-borate-EDTA (TBE) buffer. The gels were dried and then subjected to autoradiography.

**Statistical analysis.** Results are expressed as the mean ± SEM. Group comparisons were assessed using the Mann-Whitney Rank Sum Test. The null hypothesis was rejected for p<0.05 (alpha equal to 0.05). Data was 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 while 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. Utilizing 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 to sham operated animals (116.7 ± 8.5 IU/L versus 486.5 ± 325.4 IU/L). Serum ALT levels were significantly lower in TLR4 mutant mice subjected to femur fracture compared to their wild-type counterparts (101.9 ± 18.9 IU/L versus 486.5 ± 325.4 IU/L, p=0.007, Figure 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. In the present study, compared to 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 versus 148 ± 8.3 IU/L, p=0.134, Figure 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 up-regulated 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. Experimental and clinical data suggest that IL-6 and IL-10
levels correlate with the systemic inflammatory response to physiologic insults. Post-injury IL-6 and IL-10 levels are predictive of complications, organ failure and mortality in human trauma patients\textsuperscript{4,43,53}. We therefore employed 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 versus 1408 ± 397.5 pg/mL, p=0.008) and IL-10 (35.7 ± 12.1 pg/mL versus 143.6 ± 38.4 pg/mL, p=0.002) compared to wild-type mice in response to bilateral femur fracture (Figure 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\textsuperscript{−/−} mice compared to their wild-type counterparts (594.4 ± 326.3 pg/mL versus 693.6 ± 368.2 pg/mL [p=0.945], and 115.39 ± 53.4 pg/mL versus 56.3 ± 30.1 pg/mL [p=0.445], respectively; Figure 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\textsuperscript{44}. Semi-quantitative 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 Figure 3, TLR4 mutant mice exhibited lower hepatic levels of IL-6, IL-10, and TNF mRNA following bilateral femur fracture compared to wild-type mice.

TLR4 signaling leads to activation of the transcription factor NF-κB, thereby inducing a variety of pro-inflammatory cytokines and adhesion molecules that contribute to organ injury\textsuperscript{31}. NF-κB activation is a proximal step in hemorrhage and endotoxin-induced
organ injury\textsuperscript{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 (Figure 4).

**DISCUSSION**

Extensive soft tissue injury and bone fractures are significant contributors to the initial systemic inflammatory response in multiply injured patients\textsuperscript{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\textsuperscript{28,36}. Clinically, this is manifested along a spectrum that varies from a mild case of systemic inflammatory response syndrome (SIRS) to the overwhelming and often terminal multiple organ dysfunction syndrome (MODS). 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\textsuperscript{23,47,65}. 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\textsuperscript{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 following severe tissue trauma. The net result of these simultaneous
cascades is amplification 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 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 to 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\textsuperscript{21}. The
lack of protection from fracture-induced systemic inflammation and hepatocellular injury in
CD14\textsuperscript{−/−} 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 mm Hg. 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 I/R and hemorrhagic shock models\textsuperscript{14,32,57,64}. Therefore, it is possible that
both the systemic and local hepatic production of IL-6 and other pro-inflammatory 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 $^{34,49,61}$ and subsequent release and recognition of gut-derived LPS due to bacterial translocation $^{11,16,21}$. This concept of physiologic 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 $^{63}$. In this role, CD14 participates in TLR4/MD2-dependent LPS signaling pathways $^{3,19,25}$. In our study, the lack of protection in CD14$^{-/-}$ 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. TLR4 has been recognized as a driver of the innate immune response in both inflammatory and autoimmune settings. Hemorrhagic shock (HS) and ischemia reperfusion injury (I/R) often complicate traumatic injuries to bone and soft tissue. Both of these conditions result in overt tissue hypoperfusion, the former representing a global insult while the latter typifies a regional insult. Recent reports have implicated that organ injury in both of these models is TLR4-dependent. In comparison, the femur fracture model employed 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. Included among these endogenous TLR4 ligands are heat shock proteins, heparan sulfate, fibronectin, hyaluronic acid, and HMGB-1. Kim et al. recently demonstrated that a neutralizing antibody to HMGB-1 prevented hemorrhage-induced acute lung injury in a murine model. We recently reported that blockade of HMGB-1 protected mice from hepatic I/R injury. 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 utilizing 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.
Acknowledgements. The authors would like to acknowledge the technical help of David Gallo, Jeremy Allen, Hong Liao, and Derek Barclay. CD14⁻/⁻ breeding pairs were provided as a generous gift from Dr. Mason Freeman (Boston, MA).

Grants. This work was supported by National Institutes of Health (NIH) Trauma Center Grant 5P50-GM-053789. JMP and KPM are recipients of an American College of Surgeons Resident Scholarship.

Disclosures. The authors declare no competing financial interests. Correspondence and requests for reprints or other materials should be directed to TRB (billiartr@upmc.edu).
REFERENCES

Reference List


FIGURE LEGENDS

**Figure 1. Hepatocellular injury in mice subjected to bilateral femur fracture requires TLR4.** Serum ALT levels in C3H/HeOuJ versus C3H/HeJ mice (A) and (B) CD14^{−/−} versus CD14 wild-type mice. TLR4 mutant mice demonstrate reduced hepatocellular injury compared to wild-type counterparts (*p=0.011 by Mann-Whitney Rank Sum Test). Data are expressed as mean ± 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 while controls had n=4 mice. [FX denotes bilateral femur fracture].

**Figure 2. Systemic inflammation following bilateral femur fracture is TLR4-dependent.** Serum IL-6 and IL-10 levels C3H/HeOuJ versus C3H/HeJ mice (A,B) and CD14^{−/−} versus 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 to TLR4 competent mice (*p= 0.041 by Mann-Whitney Rank Sum Test). Data are expressed as mean ± 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 while controls had n=4 mice. [FX denotes bilateral femur fracture].

**Figure 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.

**Figure 4. TLR4 mutant mice demonstrate decreased hepatic NF-κB activation by EMSA.**

Hepatic nuclear extracts in C3H/HeOuJ versus C3H/HeJ mice show that TLR4 mutant mice exhibit decreased hepatic NF-κB activation following bilateral femur fracture compared to wild-type counterparts.
Figure 1
Figure 2

A. *Control Sham FX*

B. *Control Sham FX*

C. *Control Sham FX*

D. *Control Sham FX*
A. TNF

HeJ Sham  HeOuJ Sham  HeJ FX  HeOuJ FX

B. IL-6

HeJ Sham  HeOuJ Sham  HeJ FX  HeOuJ FX

C. IL-10

HeJ Sham  HeOuJ Sham  HeJ FX  HeOuJ FX

18S

Figure 3
Figure 4