Published in final edited form as:

Shock. 2016 November ; 46(5): 519–526. doi:10.1097/SHK.0000000000000650.

TLR2 on bone marrow and non-bone marrow derived cells regulates inflammation and organ injury in cooperation with TLR4 during resuscitated hemorrhagic shock

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Abstract

Background—Although the role of TLR4 in driving inflammation and organ injury after hemorrhagic shock and resuscitation (H/R) is well established, the role of TLR2 - another receptor for damage-associated molecular pattern (DAMP) molecules - is not. In this study, we used a combination of TLR2^{-/-} and wild type (WT) mice treated with anti-TLR2 and anti-TLR4 neutralizing monoclonal antibodies (mAb) to discern the contribution of TLR2 relative to TLR4 to the systemic inflammatory response in murine H/R.

Material and Methods—WT mice, TLR2^{-/-} and WT mice receiving an anti-TLR2 or an anti-TLR4 mAb (given as a pre-treatment) were sacrificed at 6 or 20 hr post-H/R. Bone marrow TLR2/WT chimeric mice were created to assess the importance of immune and non-immune cell-associated TLR2.

Results—TLR2^{-/-} mice subjected to H/R exhibited significantly less liver damage and lower markers of systemic inflammation only at 20 hr. Bone marrow chimeric mice using combinations of TLR2^{-/-} mice and WT mice demonstrated that TLR2 on non-bone marrow derived cells played a dominant role in the differences at 20 hr. Interestingly, WT mice treated with anti-TLR2 mAb demonstrated a reduction in organ damage and systemic inflammation at both 6 and 20 hrs following H/R. A combination of anti-TLR2 mAb and anti-TLR4 mAb showed that both receptors drive IP-10 and KC levels and that there is cooperation for increases in IL-6, MIG, and MCP-1 levels between TLR2 and TLR4.

Conclusion—These data also support the conclusion that TLR2 and TLR4 act in concert as important receptors in the host immune response to H/R.

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The authors declare that they have no conflict-of-interest in presenting this manuscript for publication.

Keywords

Hemorrhagic shock; TLR2; TLR4; DAMP

Introduction

Hemorrhagic shock with resuscitation (H/R) contributes to the morbidity of severe trauma and other medical conditions involving hemorrhage by activating inflammatory cascades and causing organ injury. It is now well established that a subset of toll-like receptors (TLR) are central to the initiation and propagation of inflammation induced by H/R (1, 2, 3, 4). Endogenous molecules, referred to as damage-associated molecular pattern (DAMP) molecules, such as HMGB1, mitochondrial DNA, histones, and matrix components can activate signaling through TLR4 and TLR9 on both immune and parenchymal cells, leading to systemic inflammation and organ injury (5, 6, 7, 8). TLR2 is also a DAMP sensor; however, its role in the immuno-inflammatory response to H/R is less clear.

There is now ample evidence that TLR2 expressed on multiple cell types can recognize DAMPs, such as HMGB1 (8, 9). Whereas the role of TLR2 in driving inflammation in some models of tissue trauma seems clear (10, 11, 12), results in other injury models or models of ischemia and reperfusion present conflicting results (13, 14, 15, 16). It should be noted that the majority of these studies have relied on TLR2 knockout mice to study the role of this receptor which may have limitation due to alterations or adaptations in baseline responses.

TLR2 expression is increased in the lung following H/R and this upregulation requires cross talk with TLR4 through reactive oxygen species (17, 18). Studies in human trauma show that surface expression of TLR2 on monocytes can either be up- or down-regulated early in the clinical course and that suppression of TLR2 expression is associated with an increased incidence of Gram-positive sepsis (19, 20). In addition, polymorphisms in the human TLR gene correlate with sepsis propensity following trauma in humans (21, 22). Taken together, these results would suggest that TLR2 expression is rapidly modified during H/R and that, while it plays a key role in microbial recognition after trauma, its role in the initiation of inflammatory pathways during H/R remains unresolved.

In this study, we tested the hypothesis that TLR2 contributes to inflammatory pathway activation after H/R using both TLR2^{-/-} mice and a neutralizing anti-TLR2 monoclonal antibody (mAb) in wild type mice. We find that TLR2^{-/-} mice demonstrate a role for TLR2 only at the later time point studied (20 hrs), while experiments using neutralizing anti-TLR2 mAb reveal a role for TLR2 in inflammation and organ injury measured within 6 hrs of H/R. Furthermore, TLR2 appears to have both overlapping and distinct roles with TLR4 in the initial phases of inflammation induced by H/R.

Materials and Methods

Animal Care

All experimental protocols were approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh. Experimental procedures were carried out in

accordance with all regulations regarding the care and use of experimental animals as published by the National Institutes of Health. Male C57BL/6 (WT) mice (Charles River Laboratories International, Wilmington, MA) and TLR2 knock out mouse colonies were maintained at the University of Pittsburgh in autoclaved cages under specific pathogen-free conditions. Experimental mice were used between the ages of 6–8 weeks with weights ranging from 20–30 g. Animals were allowed access to rodent chow and water ad libitum. All procedures were performed at the University of Pittsburgh in a designated animal workroom that is separate from where the mouse colonies were housed.

Antibodies

Anti-TLR2 mouse IgG1 mAb T2.5 was from HyCult Biotechnology (Uden, Netherlands) and has been described previously (23). Anti-TLR4 mouse IgG2a mAb 5E3 was provided by NovImmune SA (Geneva, Switzerland) and has also been described previously (24). The isotype control antibodies for mouse IgG1 (9E10) and IgG2a (W6/32) were provided by NovImmune SA.

Experimental Groups

Hemorrhagic Shock—Mice were assigned to the following main groups:

1. uninjured control (no injection or manipulation; (n=3–6),
2. sham control with or without injection of mAb: (2a) 100 µg of isotype control mAb (9E10 or w6/32) or vehicle only (PBS) or (2b) 100 µg of T2.5 mAb, 100 µg of 5E3 mAb or combination of both antibodies (Novimmune, Geneva, Switzerland) in 150 µl PBS 30 min prior to surgical intervention (n=6–8/gp), and
3. H/R group with or without injection of the above mentioned mAbs 30 min prior to surgical intervention with 2 h of hemorrhagic shock and 3 times shed blood volume as fluid resuscitation within 30 min (n=6–12/gp).

Each subgroup (uninjured control, sham control and H/R groups) were used with wild type, TLR2 knockout mice or chimeric animals as described below. Mice were sacrificed 6 h or 20 h if not mentioned otherwise after start of hemorrhagic shock. Mice in sham and H/R group were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg, Ovation Pharmaceuticals, Deerfield, IL) and inhaled isofluorane (Abbott Labs, Chicago, IL).

The procedure was carried out as previously published (25). Briefly, after bilateral groin dissections, both femoral arteries were cannulated using polyethylene tubing flushed with 2U heparin per animal (Pharmacia & Upjohn, Kalamazoo, MI). Baseline mean arterial pressure (MAP) was determined with one catheter connected to a blood pressure transducer (Micro-Med, Tustin, CA). Then, using the second catheter, hemorrhage was induced to a MAP of 25 mmHg for 2 h followed by fluid resuscitation with Ringer's solution (three times the volume of shed blood) through this catheter. Successful resuscitation was defined by the restoration of normal blood pressure. After resuscitation, catheters were removed, vessels were ligated, and the wounds were closed. Body temperature was measured with a rectal probe and constantly kept at 37°C±0.5°C throughout the experiment using heating lamps. In

the sham control group mice underwent anesthesia with cannulation but without H/R. Uninjured control mice were sacrificed without any procedures performed in order to obtain physiological baseline levels. All animals were sacrificed after a total time course of 6h or 20h.

Chimeric animals

Chimeric mice were generated by adoptive transfer of donor bone marrow (BM) cells into 8 weeks old irradiated recipient animals as described previously, using combinations of TLR2 KO mice and C57BL/6 mice (7). Herewith WT/WT, WT/KO, KO/KO and KO/WT recipient/donor combinations were generated. Chimeric animals were maintained under the same conditions and underwent the same H/R or uninjured control procedure, as described above, 12 weeks after the adoptive transfer.

Blood and Tissue Collection and Plasma analysis

Tissue and plasma were collected as described before (4, 11). In short: anesthetized mice were euthanized by opening the chest cavity and cardiac puncture with blood withdrawal was performed. The collected heparinized blood samples were centrifuged at 1200xg for 10min and supernatant was centrifuged again at 1200xg for 5min. Plasma was stored at -80°C or immediately used for quantification of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Dry-Chem Veterinary Chemistry Analyzer, HESKA, Loveland, CO; slides from Fujifilm Corporation, Asaka-shi Saitama, Japan). Stored aliquots were used to determine plasma cytokine levels by Luminex™ multiplexing bead array platform (Millipore, Billerica, MA) and results were confirmed by ELISA for interleukin IL-6 (R&D, Minneapolis, MN).

After blood withdrawal left liver lobe and left lung frozen in liquid nitrogen and stored at -80°C. Afterwards blood was flushed from the entire animal using PBS by cardiac puncture, followed by perfusion with 2% paraformaldehyde before collecting the right liver lobe and right lung for H&E staining.

H&E staining and brightfield microscopy

Tissues harvested for histological analysis were fixed for an additional 18h in 2% paraformaldehyde followed by 70% ethanol. Fixed sections were processed, embedded in paraffin wax, and sections cut (5µm) and stained with hematoxylin & eosin (H&E). Images of histological sections were obtained in 5 random fields on a Fluoview 1000 confocal scanning microscope (Olympus, Melville, NY). Imaging conditions were maintained at identical settings.

Peritoneal macrophage stimulation

Thioglycollate-elicited peritoneal macrophages were isolated according to Leijh et al 1984 (26). C57/BL6 mice were injected with 1.5ml of Thioglycollate 4% i.p. per mouse three days prior to harvest. On harvest day 3ml of RPMI Media + 10% BSA were injected into the mouse abdomen. The abdominal fluid was collected under sterile conditions and procedure was repeated additional two times. Cell suspension was filtered through a 40µm filter. Cells were counted and plated in a 96 well plate (10^6 cells per ml, BD Biosciences,

San Jose, California). Supernatant was changed at 3h, 2d and 4d after plating. 10min before stimulation, wells were incubated with 10µg/ml of T2.5, 9E10, w6/32 or 5E3 mAb (Novimmune, Geneva, Switzerland) or PBS.

Afterwards cells were stimulated with ultrapure LPS from *E. coli* O111:B4 (2ng/ml, List Biological Labs, Campbell, CA), PAM3CSK (1µg/ml, Invivogen, San Diego, CA) or PBS for 15min, 30min or 45min. Cells were fixed, permeabilized, blocked and stained with primary antibodies and fluorescently conjugated secondary antibodies in appropriate buffers from Cellomics (ThermoFisher, Pittsburgh, PA). NFκB p65 (ThermoFisher, Pittsburgh, PA), p-p38, p-JNK/SAPK (Cell signaling, Danvers, MA) and pERK (Abcam, Cambridge, MA) antibodies were used for measurements of respective fluorescence intensities in 4% thioglycollate-elicited peritoneal macrophages. Cells were imaged with a Cellomics Arrayscan VTi (ThermoFisher, Pittsburgh, PA) as described previously (27).

No difference could be found between the specific control mAb groups (w6/32, 9E10) and the PBS control group. Combination of these data are summarized and displayed as control group. Results were confirmed by Bone Marrow-derived macrophages (data not shown). Approximately over 90% of cells were CD11b positive (CD11b 15-0112, eBioscience, San Diego, CA).

Scoring of Lung Injury

Paraffin embedded lung tissues were stained with hematoxylin and eosin and cut into lung sections. These sections were scored in a blinded manner for interstitial edema, alveolar edema, hemorrhage, and neutrophil infiltrates using 5 digitized photos of each section. Each parameter was scaled from 0 to 4 (0 being none present, and 4 being severe and diffuse). From each section the average was calculated and used for further statistical analysis.

Statistical Analysis

Statistical Analysis was performed by ANOVA (with post-hoc-testing according to Student-Newman-Keuls method) and/or Student's t-test procedures using Sigmaplot 11 software (Systat Software, San Jose, CA). Data are presented as mean ± standard error of mean (SEM) with differences being accepted as statistically significant if the p-value was less than 0.05.

Results

Systemic Inflammation after H/R is Decreased in TLR2^{-/-} mice

Previous studies using TLR2^{-/-} have failed to show an involvement of TLR2 in inflammation or organ injury at early time points following H/R (2). To determine if a role for TLR2 might become apparent at later time points, TLR2^{-/-} mice were subjected to H/R and sacrificed at 6 (early) and 20 hrs (later) after the initiation of H/R.

There were no significant differences in the volume of blood required to be removed to achieve a mean arterial pressure of 25 to 30 mmHg for 2 hrs or in the mean arterial pressures before and after resuscitation between any of the experimental groups (data not shown). IL-6 was used as a marker of systemic inflammation. As published previously (2), no significant

differences in IL-6 plasma levels between TLR2^{-/-} mice and the wild type (WT) mice could be found at 6 hrs (Fig. 1A). IL-6 plasma levels were significantly lower at 20 hrs in TLR2^{-/-} mice compared to the WT group (31 pg/ml $\pm$ 10 pg/ml vs. 163 pg/ml $\pm$ 37 pg/ml; * = p < 0.05, Fig. 1A). Furthermore, plasma levels of the chemokines IP-10 and MCP-1 (Fig. 1B & E) were also significantly lower at 20 hrs in the TLR2^{-/-} group compared to the WT group. MIG and KC plasma levels (Fig. 1C, D), which were markedly elevated 6 hrs in WT and TLR2^{-/-} mice, had decreased by 20 hrs and continued to show no difference between the groups.

To determine if end organ damage followed trends similar to the inflammatory mediators, we assessed liver damage at 6 and 20 hrs following H/R. As shown in Fig. 1F, plasma ALT levels were significantly elevated to a similar degree in both WT and TLR2^{-/-} mice at 6 hrs. By 20 hrs after H/R, levels were significantly lower in the TLR2^{-/-} mice compared to the WT mice.

Inflammation and Organ Damage after H/R are Dependent on TLR2 on Non-Bone Marrow Derived Cells

We next sought to determine if the differences in IL-6, IP-10, MCP1, and liver damage were dependent on TLR2 expression on bone marrow or non-bone marrow derived cells. Therefore, we generated chimeric mice using combinations of WT and TLR2^{-/-} mice as described in the methods. WT/WT, WT/KO, KO/KO, KO/WT recipient/donor mice combinations underwent H/R and were sacrificed at 20 hrs. Plasma ALT levels were significantly lower in the TLR2^{-/-} mice that had received TLR2^{-/-} bone marrow (KO/KO) compared to WT mice that received WT bone marrow (WT/WT) as expected and consistent with previous findings. However, only the TLR2^{-/-} mice that received WT bone marrow (KO/WT) and not the WT mice that received TLR2^{-/-} bone marrow showed significantly lower ALT levels compared to the WT/WT group (Fig. 2A). Thus, the TLR2-dependent liver injury at 20 hrs following H/R depends more on TLR2 on non-bone marrow derived cells.

Elevation in cytokine and chemokine levels also appears to be dependent on TLR2 on non-bone marrow derived cells (Fig. 2B, C, D, E). Levels of IL-6, IP-10, MIG, and MCP1 were as low or lower in the TLR2^{-/-} mice that received WT bone marrow (KO/WT) to that seen in the TLR2^{-/-} mice that received TLR2^{-/-} bone marrow. In contrast, levels of the circulating mediators were not reduced in the WT mice that received TLR2^{-/-} bone (WT/KO) compared to the WT/WT group.

Specificity of Anti-TLR2 and TLR4 mAB Antibodies

TLR2 and TLR4 are both expressed on the surface of multiple cell types, recognize DAMP in common, and signal through MyD88 (28). Whereas a role for TLR4 in H/R is well-established (1, 2, 3, 7), the relative contribution of TLR2 and TLR4 to the systemic inflammatory response is not known. To address this, we utilized monoclonal antibodies (mAB) developed to specifically block ligand-induced TLR2 or TLR4 receptor activation. To confirm the receptor specificity the two mAB we administered a TLR2 agonist (PAM3CSK4), a TLR4 agonist (LPS) or PBS to cultured monocytes with or without mAB

against TLR2 or TLR4. After 15, 30, or 45 minutes of stimulation, cells were harvested and NFkB translocation was used as a marker for cell activation.

After stimulation with PAM3CSK4 (Fig. 3A) the T2.5 mAb (anti-TLR2) treated cells showed significantly lower NFkB translocation compared to the control or anti-TLR4 mAb at all time points. Additionally, T2.5 mAb did not significantly influence NFkB translocation in response to ultra-pure LPS. Also as expected, the anti-TLR4 mAb blocked LPS-induced NFkB activation without suppressing PAM3CSK activity. The combination of both anti-TLR2 and anti-TLR4 mAb (Fig. 3B) blocked both PAM3CSK and LPS-induced NFkB translocation to a similar level seen with the receptor specific mAb alone. Thus, the mABs used for our studies demonstrated the expected specificity.

Anti-TLR2 mAB Attenuates Inflammation and Tissue Damage Following H/R

We first sought to determine if anti-TLR2 neutralizing mAB would recapitulate the findings observed in TLR2^{-/-} mice using liver damage as an endpoint at 6 and 20 hrs after H/R. Whereas TLR2 deletion resulted in protection from liver damage only at 20 hrs, pretreatment with neutralizing anti-TLR2 mAB resulted in significantly lower ALT levels at both 6 and 20 hrs (Fig. 4C). A reduction in liver necrosis was confirmed by histology at the 6 hrs time point in the anti-TLR2 antibody pretreated group (Fig. 4A). To establish if TLR2 neutralization would impact another organ sensitive to H/R-induced injury, we assessed the histologic changes in the lung at 6 hrs following H/R. As shown in Fig. 4B, H/R resulted in septal thickening and inflammatory cell infiltrate in the control mAb treated group. Anti-TLR2 antibody pre-treatment prevented these changes in the lungs. The protective effects of TLR2 neutralization were confirmed by histologic scoring (Fig. 4D).

TLR2 and TLR4 Act Together in the Initiation and Propagation of the Systemic Inflammatory Response to H/R

The data presented above shows that TLR2 plays a role in the early events that lead to end organ damage induced by H/R and that this role is revealed more dramatically using neutralizing mAb in WT mice than with TLR2 gene deletion. This raises the possibility that other pattern recognition receptors can compensate for the permanent deletion of TLR2. As described above, one candidate is TLR4 which has well-established roles in the early phases of inflammation and organ damage in H/R (2, 7, 25, 29, 30). To determine if TLR2 and TLR4 act synergistically in organ injury and systemic inflammation at early or late time points we pretreated WT mice with anti-TLR2 mAB, anti-TLR4 mAB, or a combination of both mAbs and analyzed markers of liver damage and systemic inflammation at 6 and 20 hrs. The results show that TLR2 and TLR4 appear to both act to drive the magnitude of liver injury as well as the circulating levels of IL6, MIG, and MCP1 at both 6 or 20 hrs after H/R (Fig. 5A–D). However, blocking either TLR2 or TLR4 reduced IP-10 and KC levels to a similar degree at 6 hrs after H/R (Fig. 5E, F) with no additional inhibition observed when both mAB were used. Although pretreatment with anti-TLR4 mAb partially blocked MCP1 and IL-10 increases at 6 hrs, this lead to a slight but significant increase in these chemokines at 20 hrs compared to the animals pretreated with control mAB. These data indicate that TLR2 and TLR4 act in concert to drive inflammation in mice subjected to H/R and for some mediators can substitute for each other.

Discussion

It is likely that a subset of TLR capable of recognizing DAMP, including TLR2, 4, and 9, have dominant roles in the activation of inflammatory pathways following H/R. Whereas considerable evidence exists for early involvement of TLR4 and TLR9 (1, 2, 3, 4, 5, 7), experimental evidence for TLR2 in H/R has been limited. This is despite considerable evidence that TLR2 plays a role in other models of sterile inflammation (10, 11, 12). Using strategies that involve not only TLR2^{-/-} mice but also anti-TLR2 neutralizing mAb in wild type (WT) mice, we reveal critical roles for TLR2 in the upregulation of the systemic inflammatory response and in organ injury induced by H/R. TLR2 appears to have distinct as well as overlapping functions with TLR4 in the early immune response following H/R. Furthermore, TLR2 primarily on non-bone marrow derived cells participates in the immune responses to experiment H/R.

TLR2 dependent signaling can be activated by a number of DAMP and has been implicated in the immunopathology in several models of acute tissue injury including lung contusion (10), neurotrauma (12), extremity injury (11), and hind-limb ischemia (31). Its role in I/R appears to vary by model in studies using TLR2^{-/-} mice and has either little effect in liver I/R (31) or a dominant role in kidney and brain I/R (15, 16). Its role in the remote inflammation induced by burn injury appears to be minimal, again based on studies using TLR2^{-/-} mice (14). Here, we use both TLR2^{-/-} mice and anti-TLR2 mAb in wild type mice to show that TLR2 contributes to the systemic inflammatory response and organ injury in a mouse model of H/R. However, our finding with mAb demonstrates a more pronounced role for TLR2 than that seen with TLR2^{-/-} mice at 6 hrs raises the possibility that the knockouts have other compensatory changes that result in less dependency on TLR2 in the initiation phases of inflammation after H/R. These compensatory mechanisms, however, may not suffice over time as clear differences were seen between the WT and TLR2^{-/-} mice by 20 hrs. TLR2 expression is markedly upregulated over time in H/R and the differences seen at 20 hrs could reflect the roles of this upregulated TLR2 expression (17, 18). TLR2^{-/-} mice revealed an important role for this gene in this system, manifested by a reduction in inflammatory markers in organ injury at the later but not early time point studied. That other receptors could compensate for TLR2 deletion early in the course is conceivable. For example, some DAMP known to be involved in H/R, such as HMGB1, can be recognized by both TLR2 and TLR4 (8, 28, 32). Furthermore, TLR2 is only one of several TLR that utilize the myddosome as a dominant post receptor signaling mechanism (33). Another explanation for the overlapping roles for TLR2 and TLR4, and perhaps even for the more prominent role of TLR2 at the later time point, is that TLR2 can be upregulated in ischemic stress through a TLR4-dependent mechanism (35).

Using a combination of anti-TLR4 and anti-TLR2 mAb we provide evidence for cooperative actions between these receptor systems. These studies establish that both receptors contribute in overlapping but also unique aspects of the early inflammatory response measured by circulating inflammatory mediators. Both the current study and our previous studies (18, 32) establish that TLR2 and TLR4 on numerous cell types contribute to the immune response of H/R. In fact, receptor expression on non-bone marrow derived cells appears to play a dominant role in H/R for both TLR2 and TLR4. Whether the cellular

distribution of the receptors is an important part of the inflammatory response that are unique to either TLR2 and TLR4 is suggested but not proven here.

Another important aspect of the cooperation between TLR2 and TLR4 is the cross talk that is known to occur between these two receptors. We (18, 32) have demonstrated that HMGB1-induced TLR4 activation leads to rapid TLR2 upregulation in lung endothelial cells, as well as other cell types, and that this contributes to a hyper-responsive state. This interaction requires ROS production through the NADPH oxidase on both PMN and endothelial cells (17, 34). Our current studies provide proof that both TLR2 and TLR4 are involved in the initial systemic inflammatory response in H/R.

Others have shown that TLR2 can be either upregulated (20) or downregulated (19) on monocytes from human trauma patients. The factors that determine these changes are not known but expression levels do appear to correlate with susceptibility to Gram-positive sepsis post-trauma (19). Furthermore, certain polymorphisms in the TLR2 gene correlate with susceptibility to Gram-positive infections post-injury (21, 22). Although the key polymorphism (TLR2 T-16934ATA) correlates with ex vivo monocyte responses to TLR2 agonist, the presence of the polymorphism was not assessed for impact on circulating SIRS biomarkers in the patients. We have shown that TLR2 does appear to be involved in the suppression of splenocyte responses in experimental systems involving peripheral tissue trauma (11). It is important to be cautious about extrapolating mouse studies to humans but our data would suggest that TLR2 not only regulates immune responses to post-injury infection but also the magnitude of the initial systemic inflammatory response as well as post-injury immunosuppression.

Acknowledgments

This work is supported through grants from NIH grant Molecular Biology of Hemorrhagic Shock (NIH # 5P50GM053789)

We thank Alicia Frank, John Pribis and Derek Barclay for technical help with this work.

Abbreviations

AB	Antibody
ALT	Alanine transaminase
BM	bone marrow
BSA	bovine serum albumin
DAMP	damage associated molecular pattern
HMGB1	High-Mobility-Group-Protein B1
H&E	Hemotoxylin and Eosin
H/R	hemorrhagic shock and reperfusion
IL-6	Interleukin 6

IP-10	CXCL10, interferon inducible protein
KgBW	kilogram body weight
KC	keratinocyte-derived chemokine
KO	knock out
LPS	lipopolysaccharide
mAB	monoclonal Antibody
MCP-1	monocyte chemotactic protein-1 (CCL2)
MIG	Monokine induced by interferon
TLR	toll like receptor
TP	time point
WT	wild type mice (C57/BL6 mice, Charles River)

Reference List

1. Barsness KA, Arcaroli J, Harken AH, Abraham E, Banerjee A, Reznikov L, McIntyre RC. Hemorrhage-induced acute lung injury is TLR-4 dependent. *Am J Physiol Regul Integr Comp Physiol.* 2004; 287(3):R592–R599. [PubMed: 15072965]
2. Prince JM, Levy RM, Yang R, Mollen KP, Fink MP, Vodovotz Y, Billiar TR. Toll-like receptor-4 signaling mediates hepatic injury and systemic inflammation in hemorrhagic shock. *J Am Coll Surg.* 2006; 202(3):407–417. [PubMed: 16500244]
3. Mollen KP, Anand RJ, Tsung A, Prince JM, Levy RM, Billiar TR. Emerging paradigm: Toll-like receptor 4-sentinel for the detection of tissue damage. *Shock.* 2006; 26(5):430–437. [PubMed: 17047512]
4. Gill R, Ruan X, Menzel CJ, Namkoong S, Loughran PA, Hackam DJ, Billiar TR. Systemic inflammation and liver injury following hemorrhagic shock and peripheral tissue trauma involve functional TLR9 signaling on bone marrow-derived cells and parenchymal cells. *Shock.* 2011; 35(2):164–170. [PubMed: 20577143]
5. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, Hauser CJ. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature.* 2010; 464(7285):104–107. [PubMed: 20203610]
6. Yang R, Harada T, Mollen KP, Prince JM, Levy RM, Englert JA, Gallowitsch-Puerta M, Yang L, Yang H, Tracey KJ, Harbrecht BG, Billiar TR, Fink MP. Anti-HMGB1 neutralizing antibody ameliorates gut barrier dysfunction and improves survival after hemorrhagic shock. *Mol Med.* 2006; 12(4–6):105–114. [PubMed: 16953558]
7. Mollen KP, Levy RM, Prince JM, Hoffman RA, Scott MJ, Kaczorowski DJ, Vallabhaneni R, Vodovotz Y, Billiar TR. Systemic inflammation and end organ damage following trauma involves functional TLR4 signaling in both bone marrow-derived cells and parenchymal cells. *J Leukoc Biol.* 2008; 83(1):80–88. [PubMed: 17925504]
8. Tsung A, Tohme S, Billiar TR. High mobility group box-1 in sterile inflammation. *J Intern Med.* 2014; 276(5):425–43. [PubMed: 24935761]
9. Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, Fenton MJ, Tracey KJ, Yang H. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock.* 2006; 26(2):174–179. [PubMed: 16878026]

10. Hoth JJ, Hudson WP, Brownlee NA, Yoza BK, Hiltbold EM, Meredith JW, McCall CE. Toll-like receptor 2 participates in the response to lung injury in a murine model of pulmonary contusion. *Shock*. 2007; 28(4):447–452. [PubMed: 17558351]
11. Darwiche SS, Ruan X, Hoffman MK, Zettel KR, Tracy AP, Schroeder LM, Cai C, Hoffman RA, Scott MJ, Pape HC, Billiar TR. Selective roles for toll-like receptors 2, 4, and 9 in systemic inflammation and immune dysfunction following peripheral tissue injury. *J Trauma Acute Care Surg*. 2013; 74(6):1454–1461. [PubMed: 23694872]
12. Yu ZQ, Zha JH. Genetic ablation of toll-like receptor 2 reduces secondary brain injury caused by cortical contusion in mice. *Ann Clin Lab Sci*. 2012; 42(1):26–33. [PubMed: 22371907]
13. Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, Yang H, Li J, Tracey KJ, Geller DA, Billiar TR. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J Exp Med*. 2005; 201(7):1135–1143. [PubMed: 15795240]
14. Bird MD, Zahs A, Deburghgraeve C, Ramirez L, Choudhry MA, Kovacs EJ. Decreased pulmonary inflammation following ethanol and burn injury in mice deficient in TLR4 but not TLR2 signaling. *Alcohol Clin Exp Res*. 2010; 34(10):1733–1741. [PubMed: 20608903]
15. Li X, Yun Z, Tan Z, Li S, Wang D, Ma K, Chi N, Liu J, Chen F, Gao G. The role of toll-like receptor (TLR) 2 and 9 in renal ischemia and reperfusion injury. *Urology*. 2013; 81(6):1379.e15–20.
16. Wang Y, Ge P, Zhu Y. TLR2 and TLR4 in the brain injury caused by cerebral ischemia and reperfusion. *Mediators Inflamm*. 2013; 2013:124614. [PubMed: 23864765]
17. Fan J, Frey RS, Malik AB. TLR4 signaling induces TLR2 expression in endothelial cells via neutrophil NADPH oxidase. *J Clin Invest*. 2003; 112(8):1234–1243. [PubMed: 14561708]
18. Fan J, Li Y, Vodovotz Y, Billiar TR, Wilson MA. Hemorrhagic shock-activated neutrophils augment TLR4 signaling-induced TLR2 upregulation in alveolar macrophages: role in hemorrhage primed lung inflammation. *Am J Physiol Lung Cell Mol Physiol*. Apr; 2006 290(4):L738–746. [PubMed: 16272176]
19. Lendemans S, Kreuzfelder E, Rani M, Bayeesh E, Schade FU, Flohé SB, Waydhas C, Flohé S. Toll-like receptor 2 and 4 expression after severe injury is not involved in the dysregulation of the innate immune system. *J Trauma*. 2007; 63(4):740–746. [PubMed: 18089999]
20. Pérez-Bárcena J, Regueiro V, Crespi C, Pierola J, Oliver A, Llompert-Pou JA, Ayestarán JJ, Raurich JM, Marsé P, Ibáñez J, Bengoechea JA. Expression of toll-like receptors 2 and 4 is upregulated during hospital admission in traumatic patients: lack of correlation with blunted innate immune responses. *Ann Surg*. 2010; 251(3):521–527. [PubMed: 20134316]
21. Bronkhorst MW, Boyé ND, Lomax MA, Vossen RH, Bakker J, Patka P, Van Lieshout EM. Single-nucleotide polymorphisms in the Toll-like receptor pathway increase susceptibility to infections in severely injured trauma patients. *J Trauma Acute Care Surg*. 2013; 74(3):862–870. [PubMed: 23425749]
22. Chen KH, Gu W, Zeng L, Jiang DP, Zhang LY, Zhou J, Du DY, Hu P, Liu Q, Huang SN, Jiang JX. Identification of haplotype tag SNPs within the entire TLR2 gene and their clinical relevance in patients with major trauma. *Shock*. 2011; 35(1):35–41. [PubMed: 20577149]
23. Meng G, Rutz M, Schiemann M, Metzger J, Grabiec A, Schwandner R, Lippa PB, Ebel F, Busch DH, Bauer S, Wagner H, Kirschning CJ. Antagonistic antibody prevents toll-like receptor 2-driven lethal shock-like syndromes. *J Clin Invest*. 2004; 113(10):1473–1481. [PubMed: 15146245]
24. Shang L, Daubeuf B, Triantafilou M, Olden R, Dépis F, Raby AC, Herren S, Dos Dantos A, Malinge P, Dunn-Siegrist I, Benmkaddem S, Geinoz A, Magistrelli G, Rousseau F, Buatois V, Salgado-Pires S, Reith W, Monteiro R, Pugin J, Leger O, Ferlin W, Kosco-Vilbois M, Triantafilou K, Elson G. Selective antibody intervention of toll-like receptor 4 activation through Fc γ receptor tethering. *J Biol Chem*. 2014; 289(22):15309–15318. [PubMed: 24737331]
25. Korff S, Loughran P, Cai C, Lee YS, Scott M, Billiar TR. Eritoran attenuates tissue damage and inflammation in hemorrhagic shock/trauma. *J Surg Res*. 2013; 184(2):e17–e25. [PubMed: 23777984]
26. Leijh PC, van Zwet TL, ter Kuile MN, van FR. Effect of thioglycolate on phagocytic and microbicidal activities of peritoneal macrophages. *Infect Immun*. 1984; 46(2):448–452. [PubMed: 6500699]

27. Vakkila J, DeMarco RA, Lotze MT. Coordinate NF-kappaB and STAT1 activation promotes development of myeloid type 1 dendritic cells. *Scand J Immunol.* 2008; 67(3):260–269. [PubMed: 18261039]
28. Lorne E, Dupont H, Abraham E. Toll-like receptors 2 and 4: initiators of non-septic inflammation in critical care medicine? *Intensive Care Med.* 2010; 36(11):1826–1835. [PubMed: 20689929]
29. Fan J, Li Y, Levy R, Fan JJ, Hackam DJ, Vodovotz Y, Yang H, Tracey KJ, Billiar TR, Wilson MA. Hemorrhagic shock induces NAD(P)H oxidase activation in neutrophils: role of HMGB1-TLR4 signaling. *Journal of Immunology.* 2007; 178(10):6573–6580.
30. Liu Y, Yuan Y, Li Y, Zhang J, Xiao G, Vodovotz Y, Billiar TR, Wilson MA, Fan J. Interacting Neuroendocrine and Innate and Acquired Immune Pathways Regulate Neutrophil Mobilization from Bone Marrow following Hemorrhagic Shock. *J Immunol.* 2009; 182(1):572–580. [PubMed: 19109190]
31. Sachdev U, Cui X, McEnaney R, Wang T, Benabou K, Tzeng E. TLR2 and TLR4 mediate differential responses to limb ischemia through MyD88-dependent and independent pathways. *PLoS One.* 2012; 7(11):e50654. [PubMed: 23209800]
32. Li Y, Xiang M, Yuan Y, Xiao G, Zhang J, Jiang Y, Vodovotz Y, Billiar TR, Wilson MA, Fan J. Hemorrhagic Shock Augments Lung Endothelial Cell Activation: Role of Temporal Alterations of TLR4 and TLR2. *Am J Physiol Regul Integr Comp Physiol.* 2009; 297(6):R1670–1680. [PubMed: 19828841]
33. Gay NJ, Gangloff M, O'Neill LA. What the Myddosome structure tells us about the initiation of innate immunity. *Trends Immunol.* 2011; 32(3):104–109. [PubMed: 21269878]
34. Fan J, Frey RS, Rahman A, Malik AB. Role of neutrophil NADPH oxidase in the mechanism of tumor necrosis factor-alpha -induced NF-kappa B activation and intercellular adhesion molecule-1 expression in endothelial cells. *J Biol Chem.* 2002; 277(5):3404–3411. [PubMed: 11729200]
35. Xu J, Benabou K, Cui X, Madia M, Tzeng E, Billiar T, Watkins S, Sachdev U. TLR4 Deters Perfusion Recovery and Upregulates Toll-like Receptor 2 (TLR2) in Ischemic Skeletal Muscle and Endothelial Cells. *Mol Med.* 2015; 21(1):605–15. [PubMed: 26181630]

Figure 1.

C57BL/6 mice or TLR2 KO mice underwent H/R procedure and were sacrificed at 6h or 20h TP. (A) IL-6, (B) IP10 (C) KC, (D) MIG, (E) MCP-1, (F) ALT or (G) AST plasma parameters were displayed. n=6–8 per experimental group, line and * represent $p < 0.05$, Data represent mean $\pm$ SEM.

Figure 2. using TLR2 KO and C57/Bl6 mice WT/WT, WT/KO, KO/KO and KO/WT recipient/donor chimeric mice combinations were generated. Chimeric mice underwent H/R procedure and were sacrificed at 20h TP. (A) ALT, (B) IL-6, (C) IP10, (D) MCP-1, and (E) MIG plasma parameters were displayed. n=6–8 per experimental group, * p<0.05, Data represent mean+/-SEM.

Figure 3.

C57/BL6 mice were injected i.p. with thioglycollate and peritoneal macrophages were harvested. Macrophages were stimulated in vitro with (A) PAM3CSK (1μg/ml), (B) ultrapure LPS (2ng/ml) or (C) PBS for 15min, 30min or 45min after incubation with different mAB (T2.5, 5E3, T2.5+5E3 or control, 10μg/ml). Translocation of NFκB p65 were measured and displayed in arbitrary units (n=8 or 24) per experimental group. Blue line represent mean of un-stimulated control (49±4 AU). Black lines represent *p<0.05 for 30min TP comparing groups, data represent mean±SD.

Figure 4.

(A) H&E staining of liver tissue sections from monoclonal TLR2 Antibody T2.5 (labeled as “TLR2”) or corresponding isotype control mAB (labeled as “control”) pretreated animals are displayed. Mice underwent H/R or sham procedure (A1 – A4). (B) H&E staining of paraformaldehyde-fixed and paraffin-embedded lung from anti-TLR2 or isotype control mAB are displayed. Mice underwent H/R or sham procedure (B1 – B4). Plasma ALT levels of untreated control animals (white bars) or ALT plasma levels of isotype control mAB (black bars) or anti-TLR2 mAB (grey bars) treated mice after H/R procedure are displayed. Blue line represent mean of sham group plasma levels of mice receiving isotype control mAB (C). Average of lung injury using interstitial edema, alveolar edema, hemorrhage, and neutrophil infiltrates as marker (scale in arbitrary units from 0=min to 16=max) White bar displays sham mice receiving isotype control or anti TLR2 mAB, grey and black bars displays corresponding H/R group (D). N=6–16 per experimental group, *p<0.05 (C) or *p<0.01 (D), Data represent mean+/-SEM, scale bar 100µm

Figure 5.

C57/Bl6 mice were pretreated with 100µg of control mAB or T2.5 mAB and/or 5E3 mAB 30min before H/R procedure. (A) ALT plasma levels in U/l are displayed, (B–F) IL-6, MIG, MCP-1, IP-10 and KC are shown at two different time points (6h and 20h TP). significant differences between two groups are marked by a line on top of the bars. Blue lines illustrate mean plasma levels of sham control mice, dotted line illustrate uninjured control plasma levels. (n=6–14 per experimental group, *p<0.05, Data represent mean±SEM)