TLR Cross-Talk Mechanism of Hemorrhagic Shock-Primed Pulmonary Neutrophil Infiltration

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Abstract: Hemorrhage resulted from severe trauma renders patients susceptible to the development of acute lung injury (ALI). The ***accumulation of*** polymorphonuclear neutrophils (PMN) ***in the lung is a critical event in the development of ALI***. PMN migration is a result of a cascade of cellular events, in which PMN, endothelial cells (EC), and macrophages (Mϕ) act in concert. Recent studies explored interrelated novel findings indicating that Toll-like receptors (TLRs) cross-talk mechanisms occurring in PMN, EC, and Mϕ are important determinants for hemorrhage-primed PMN migration. In Mϕ and EC, LPS acts through TLR4 signaling to up-regulate TLR2. Oxidant signaling derived from hemorrhage-activated PMN ***NAD(P)H oxidase*** enhances the TLR2 upregulation through PMN-Mϕ or PMN-EC interaction, resulting in an amplified release of cytokines and chemokines from the Mϕ and expression of adhesion molecules in the EC in response to TLR2 ligands, thereby promoting PMN migration. This review provides an insight of the mechanisms.

Keywords: Acute lung injury, endothelial cells, hemorrhagic shock, HMGB1, macrophages, NAD(P)H oxidase, neutrophils, TLR.

INTRODUCTION

Lung injury is a frequent occurrence in trauma patients and serves as an important component of systemic inflammatory response syndrome (SIRS), developing in up to 50% of patients, depending on the magnitude of the initial trauma. Lung injury is characterized by the clinical picture of hypoxemia, reduced lung compliance, fluffy diffuse infiltrates on the chest radiograph, and the presence of normal pulmonary capillary pressures. Morphologically, lung injury is manifested by alveolar and interstitial fluid accumulation, alveolar hemorrhage, fibrin deposition, and lung neutrophil sequestration [1]. From a pathophysiologic perspective, the accumulation of neutrophils in the lung vasculature, interstitium, and alveolar space is considered a critical event and has been the target of various preventative strategies [1].

PMN migration from the bloodstream to the site of inflammation is a strictly regulated process. The PMN extravasation process can be divided into several sequential steps, including margination, rolling/transient attachment, triggering/integrin activation, stable adhesion and diapedesis/transmigration. Margination is a random, adhesion-independent process that allows the PMN to flow near the endothelium at the margin of postcapillary venules [2, 3]. Margination facilitates PMN rolling, whereby the marginated PMN undergo a series of reversible and low-affinity attachments to the vessel wall. These transient attachments involve interactions between PMN L-selectin and counter-ligands on the activated endothelium such as E-selectin and P-selectin. The successive expression and shedding of L-selectin allows the PMN to loosely adhere to and roll across the endothelium. Rolling continues until local cytokine or chemokine signaling activates PMN integrins and triggers their conformational change. Conformational change promotes a high affinity and irreversible interaction between activated integrins, e.g. intercellular adhesion molecule (ICAM)-1, which stably attaches the PMN to the endothelium. PMN migration or diapedesis across the vessel wall and trafficking is then mediated by an interstitial chemokine gradient between the sites of diapedesis and inflammation [2, 3]. The PMN extravasation process now seems clear. However, it does not answer the question of how hemorrhagic shock (HS) primes for PMN sequestration.

The "two-hit" hypothesis has been appropriately applied to the study of the PMN priming mechanism of post-hemorrhage lung injury in that the hypothesis provides a structural framework for understanding the clinical problem [1]. A simplified animal model of the "two-hit" paradigm, as an example of human diseases, has been used to address the mechanisms of HS-primed PMN migration and lung inflammation [4]. In this model, animals are subjected to a non-severe resuscitated HS (hypotension at 40 mmHg for 1 h), followed by a small dose of intratracheal LPS. While neither shock nor LPS alone induces injury, the combination caused lung PMN accumulation and increased ^{125}I-albumin transpulmonary flux [4]. Findings from this model have suggested that the mechanisms underlying the priming of PMN and inflammation involve complicated receptor cross-talk processes and interaction between PMN and alveolar macrophages (AMϕ) or EC. The studies have shown that the molecular level cross-talk between TLR4 and TLR2 plays a critical role in mediating the cell level interaction between PMN and AMϕ, as well as PMN and EC. As a result, the receptors cross-talk mechanism serves as an important amplifier of inflammation. This review will focus on how HS

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1874-8287/09

amplifies TLR4-TLR2 cross talk in AMϕ and EC and subsequent lung inflammation.

TLRs DEPENDENT MECHANISMS OF INNATE IMMUNITY

The hallmark of innate immunity is the usage of germline-encoded pattern recognition receptors to sense invading pathogens [5]. Although microorganisms have tremendous variety, they possess common structural features. For example, common structural features are shared by LPS from Gram-negative bacteria; peptidoglycan (PGN); lipoteichoic acid (LTA) from Gram-positive bacteria; lipoarabinomannan (LAM) from mycobacterium; unmethylated DNA and bacterial lipoproteins. The pattern recognition receptors sense the presence of these bacterial components and activate common pathways to mediate responses for the elimination of invading microorganisms [6]. TLRs have been defined as key molecules for recognizing bacterial components which evoke inflammatory responses. To date, thirteen mammalian TLR paralogues have been identified (10 in humans and 12 in mice) [7, 8]. This class of receptors is characterized by extracellular leucine-rich repeat (LRR) motifs and a cytoplasmic Toll/IL-1R (TIR) homology domain. LRRs are short protein molecules of 20 to 29 amino acids found in a diverse group of proteins, including CD14, platelet glycoprotein 1b, and RP105 [6]. The cytoplasmic TIR homology of the TLR family is similar to that of IL-1R family proteins, such as IL-1R, IL-18R and T1ST2 [9]. TLRs have unique tissue and cellular distributions, suggesting specialized, non-redundant functions. Some TLRs are broadly expressed while others have very selected expression patterns, for example, TLR7 and TLR8 in B-lymphocytes.

TLR2 is involved in the recognition of components from a variety of pathogens. These include lipoproteins from a number of pathogens, PGN and LTA from Gram-positive bacteria, LAM from mycobacterium, glycosylphophatidylinositol anchors from *Trypanosoma Cruzi*, a phenol-soluble modulin from *Staphylococcus epidermis*, zymosan from fungi, and glycolipids from *Treponema maltophilum* [10, 11]. In addition, TLR2 presumably recognizes atypical LPS from *Leptospira interrogans* or *Porphyromonas gingivalis*, both of which differ from the typical LPS of Gram-negative bacteria in several biochemical and physical properties [12]. TLR2 is predominantly expressed by cells involved in first-line host defense, including monocytes, macrophages, dendritic cells, and neutrophils [13, 14]. Lower expression is observed *in vitro* by endothelial cells and epithelial cells [15]. TLR4 is an essential receptor for LPS signal transduction, though this receptor acts in concert with several additional molecules. LPS binds to LPS-binding protein (LBP) in serum and the LPS-LBP complex is associated with CD14, which is expressed in monocytes/macrophages and PMN [16]. MD2 is a secreted protein that associates with the extracellular portion of TLR4 and enhances its responsiveness to LPS [17].

Upon ligation of TLRs with their cognate ligand, signaling cascades are activated which result in the production of innate effector responses as well as the initiation of an adaptive immune response [18]. Adaptor proteins are first recruited to the cytoplasmic domains of the receptors *via* TIR–TIR interactions. The five adaptors are MyD88, MyD88-adaptor like (Mal, also known as TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF, also known as TICAM1), TRIF-related adaptor molecule (TRAM, also known as TICAM2) and sterile α- and armadillo-motif containing protein (SARM) [19]. MyD88 is utilized by all of the TLRs except TLR3 whereas TRIF signals downstream of TLR3 and TLR4. TLR4 differs from the other TLRs in that it recruits MyD88 and TRIF to its cytoplasmic domain *via* the bridging adaptors, Mal and TRAM, respectively. TLR2 also utilizes Mal to recruit MyD88. SARM, in contrast to the other adaptors, was found to be a negative regulator of TLR signaling. These adaptors (with the exception of SARM) recruit downstream signaling molecules which lead to the activation of NF-κB and members of the IRF family of transcription factors [19, 20]. This ultimately results in the production of pro-inflammatory cytokines and type-1 interferons [20]. It has become apparent that these signaling pathways, which play such a crucial role in host defense, can also cause significant immunopathology if overactivated or insufficiently controlled [21]. Likewise, a defect in TLR signaling can result in severe immunodeficiency [22].

HS-ACTIVATED PMN MEDIATE TLR4 SIGNALING UPREGULATION OF TLR2 IN AMϕ

A recent study demonstrated that LPS-TLR4 signaling upregulates TLR2 expression in AMϕ, and HS-activated PMN play a critical role in the mechanism of the TLR2 upregulation [23]. This cross-talk between TLR4 and TLR2 in AMϕ results in the amplification of expression of cytokines and chemokines in response to the bacterial products LPS and PGN, and subsequently leads to enhanced PMN sequestration in the lung. These findings reveal a novel mechanism underlying HS-primed lung injury, namely that HS-activated PMN that were initially sequestered into the alveoli can instruct AMϕ to upregulate TLR2, thereby sensitizing AMϕ to TLR2 ligands and promoting enhanced lung inflammation (Fig. **1**).

To address the role of antecedent HS in the regulation of TLR2 in AMϕ, the "two-hit" animal model, as described above, was used. Mice were subjected to HS or sham operation, and then given LPS or saline vehicle intratracheally at 1 hour after resuscitation. AMϕ were then recovered from BAL fluid at 1 to 4 hours after LPS or saline for TLR2 mRNA and protein detection. Animals subjected to shock prior to LPS exhibited a marked increase in TLR2 expression as compared to sham and LPS alone groups. To address the role of TLR4 signaling in shock-modulated TLR2 expression, TLR4 mutant C3H/HeJ mice were also subjected to shock followed by LPS intratracheally. LPS failed to upregulate TLR2 mRNA and protein expression in the AMϕ derived from TLR4-mutant mice [23]. These results suggest that modulation of LPS-induced TLR2 upregulation by HS occurs through a TLR4-dependent mechanism.

The role of HS-activated PMN in amplifying TLR4 upregulation of TLR2 in AMϕ was tested by using an *ex vivo* PMN-AMϕ co-culture system. AMϕ from WT mice were co-cultured with PMN that were isolated from either sham operated or HS WT mice in the presence or absence of LPS for up to 6 hours. The alterations in TLR2 mRNA and protein expression in the AMϕ were then detected. Co-culture of

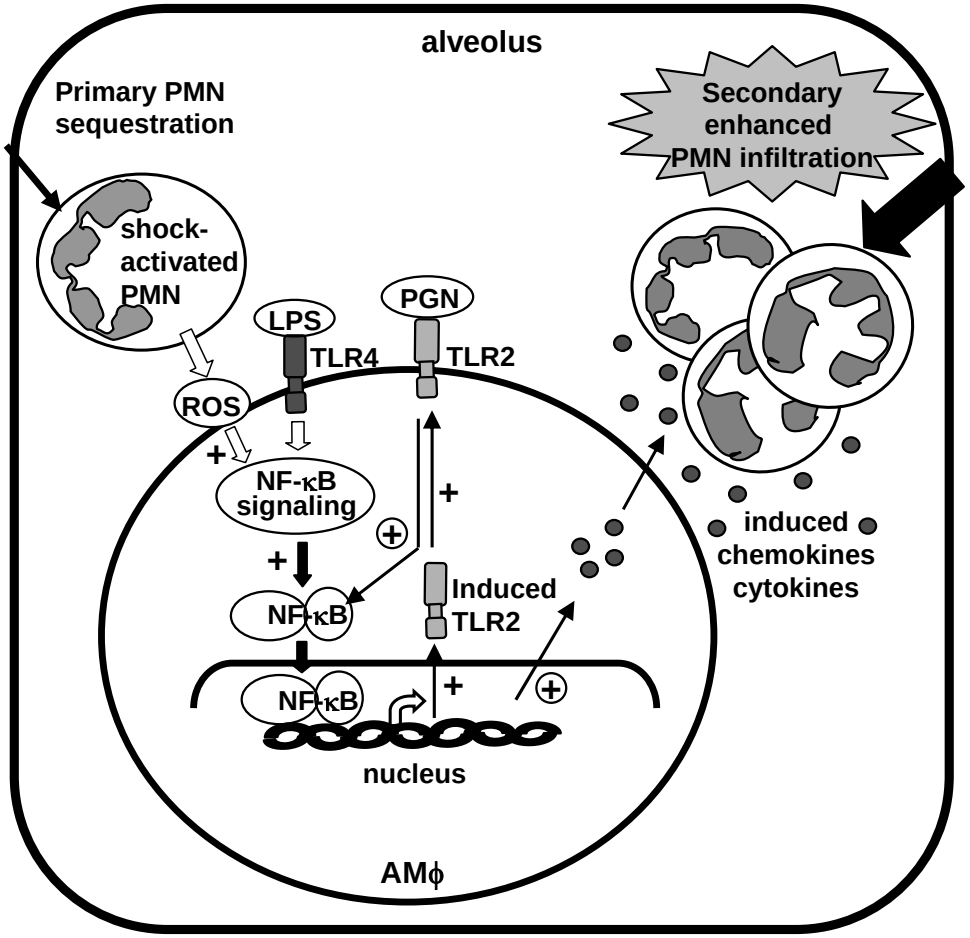

Fig. (1). Model of shock-activated PMN in mediating the TLR4-TLR2 cross-talk in AMϕ and AMϕ priming. Hemorrhagic shock-activated PMN primarily migrate into alveoli in response to a trivial inflammatory stimulus, such as LPS, and interact with AMϕ. The interaction between PMN and AMϕ enhances LPS-induced TLR2 expression (+) in the AMϕ, possibly mediated by PMN derived oxidants and augmented NF-κB activation. The increased TLR2 expression results in the amplified response of the AMϕ to TLR2 agonist (PGN), thereby augmenting cytokines and chemokines expression (circled +) and promoting enhanced PMN transalveolar migration. Thus the shock-activated PMN-mediated TLR4-TLR2 cross-talk activates a positive feedback signal leading to AMϕ priming and exaggerated lung inflammation in response to invading pathogens (adopted from Ref. [23]).

AMϕ with PMN derived from mice subjected to HS caused a significantly higher level of TLR2 expression in the AMϕ in response to LPS at the 6-hour time point as compared to the group co-cultured with PMN isolated from sham animals [23]. These results suggest a dominant role of HS-activated PMN in the response.

To further confirm the role of PMN in amplifying TLR4-induced TLR2 upregulation in AMϕ, circulating PMN were depleted before HS. At 4 hours after LPS challenge, neutropenia induced in mice subjected to HS was associated with a ~58% reduction in TLR2 expression in AMϕ, as compared to mice subjected to HS/LPS with no PMN depletion. In contrast, depletion of PMN did not alter TLR2 expression in sham/LPS treated AMϕ. Repletion with WT PMN isolated from animals subjected to HS restored the expression of TLR2 in AMϕ in response to HS/LPS. However, repletion with PMN derived from sham-operated animals failed to restore TLR2 expression [23]. Interestingly, replenishing the neutropenic sham/LPS mice with PMN obtained from mice subjected to hemorrhagic shock resulted in a ~3.3-fold increase in TLR2 expression in AMϕ compared with non-neutropenic sham/LPS mice. Taken together, these results demonstrate the critical role of shock-activated PMN in the augmented TLR4-induced TLR2 expression in AMϕ[23].

TLR2 UPREGULATION IN AMϕ RESULTS IN ENHANCED CYTOKINE EXPRESSION AND PMN MIGRATION

The pathophysiological significance of the shock-enhanced TLR4 upregulation of TLR2 in AMϕ was first addressed using the *ex vivo* PMN-AMϕ co-culture system. LPS was added to the AMϕ-PMN co-cultures at time 0, followed by removal of the PMN and addition of PGN, the ligand of TLR2, to the AMϕ at 2 hours (the time point at which TLR2 was upregulated in the AMϕ as described above). Chemokine MIP-2, cytokine MIF and TNFα protein levels in the AMϕ were then assessed at 4 hours by Western blotting. PMN-derived cytokines were excluded by removing PMN from the system before addition of PGN. LPS or PGN alone induced a slight increase in MIP-2, MIF, and TNFα in AMϕ that were pre-incubated with PMN isolated from the

mice subjected to either sham or shock. By contrast, the sequential challenges of LPS and PGN caused marked increases in the expression of the inflammatory cytokines in AMϕ that were pre-incubated with HS-activated PMN. These results suggest a priming role vis-à-vis cytokine expression of the upregulated TLR2 in enhancing AMϕ response to bacterial components [23].

To establish a linkage between the increased expression of the cytokines in AMϕ and PMN migration, AMϕ that were co-cultured with HS-activated PMN and sequentially treated with LPS and PGN were recovered and injected into the mouse dorsal air pouch to induce PMN migration. Air pouch lavage fluid was collected at 4 hours after the injection of AMϕ for PMN counts. AMϕ that were pre-incubated with HS-activated PMN and challenged with LPS and PGN induced a significantly higher number of air pouch PMN, as compared to that in the groups pre-incubated with no PMN or the PMN isolated from sham operated mice, respectively. These data paralleled with the changes in MIP-2, MIF, and TNFα expression in the AMϕ[23].

As chemokine-dependent PMN migration is an important determinant of lung PMN infiltration, the role of HS-enhanced LPS upregulation of TLR2 in the formation of alveolar neutrophilia was addressed using the *in vivo* HS model in WT, TLR4 mutant, and $TLR2^{-/-}$ mice. The mice were challenged with HS, and 1 hour after resuscitation LPS plus PGN were administered intratracheally. PMN in BAL were counted at 2-hour and 6-hour time points after LPS-PGN administration to represent PMN infiltration at the initial phase and later phase, respectively. The results showed that amplified AMϕ activation and PMN infiltration induced by PGN are dependent to the augmented up-regulation of TLR2 that resulted from the interaction of PMN and AMϕ. The primarily sequestered PMN seem to be important in priming for consequently enhanced PMN infiltration. As shown in Fig. (**2**), in the early phase, PMN infiltration is dependent on TLR4 signaling, since BAL PMN increased at the 2-hour time point in WT and $TLR2^{-/-}$ mice, but not in TLR4-mutant mice, in response to the treatment of HS/LPS/PGN. LPS alone increased the BAL PMN counts, whereas PGN alone did not. However, in the later phase (by 6 hours), the amplified PMN infiltration is secondary to upregulated TLR2 expression as evident by the facts that (a) BAL PMN was markedly elevated in WT animals, but not in TLR2 deficient or TLR4-mutant animals, which were subjected to HS/LPS/PGN challenges; (b) either LPS or PGN alone failed to induce an amplified PMN infiltration in WT HS mice; and (c) LPS plus PGN failed to increase the PMN counts in WT sham-operated animals (Fig. **2**). These findings explored an important role of the shock activated-PMN and TLR4 signaling in activating a positive-feedback signal leading to exaggerated lung inflammation through the up-regulation of TLR2 expression in AMϕ.

NAD(P)H OXIDASE IS REQUIRED FOR PMN-MEDIATED SENSITIZATION OF AMϕ TO LPS IN HS

How does HS-activated PMN enhance TLR4 upregulation of TLR2 in AMϕ? Studies have shown that reactive oxygen species (ROS) derived from PMN NAD(P)H oxidase play an important role in amplifying the TLR2 upregulation.

The production of superoxide anions (O_2^-) by neutrophils and other phagocytes is an important step in our body's innate immune response. O_2^- is the precursor of a range of chemicals generally referred to as ROS. These act as microbicidal agents and kill invading microorganisms either directly or through the activation of proteases [24-27]. O_2^- is produced by the NADPH oxidase, a highly regulated membrane-bound enzyme complex, which catalyzes the production of superoxide by the one-electron reduction of oxygen using NAD(P)H as the electron donor. The core enzyme is comprised of both membrane-bound (i.e., $gp91^{phox}$ and $p22^{phox}$) and cytosolic (i.e., $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and rac-1/2) components [27, 28]. Upon stimulation, receptor-mediated activation of the oxidase complex activates secondary signaling intermediates, culminating in the phosphorylation and recruitment of the cytosolic components to the membrane-bound components to assemble the active oxidase [27-29]. Studies have suggested that ischemia-reperfusion primes circulating PMN for increased ROS production, therefore augmenting neutrophil-mediated lung injury once the PMN are sequestered in the lung [30, 31]. Though NAD(P)H oxidase has classically been thought of as a part of the antimicrobial armamentarium of phagocytes [32], the role of this enzyme (or its isoforms in other cell types) in signaling has been described [33].

It was observed that in the $gp91^{phox}$ knock-out mice antecedent HS failed to amplify the upregulation of TLR2 expression in response to LPS in the AMϕ[34], suggesting an important role of NAD(P)H oxidase in mediating HS-primed TLR2 upregulation in AMϕ. This observation led to addressing the role for PMN NAD(P)H oxidase in the upregulation of TLR2. In the study, AMϕ from WT mice as well as $gp91^{phox-/-}$ mice were co-cultured with PMN, which were isolated from WT or $gp91^{phox-/-}$ mice subjected to either sham or shock, in the presence or absence of further treatment with LPS for 4 hours. Co-culture of AMϕ with PMN derived from $gp91^{phox-/-}$ mice subjected to HS demonstrated an attenuated TLR2 upregulation in the AMϕ in response to LPS as compared to that in the AMϕ co-cultured with PMN isolated from WT HS animals. Likewise, NAD(P)H oxidase inhibitor, Apocynin (4-hydrocy-3-methoxyacetophenone), significantly attenuated TLR2 expression in WT AMϕ co-cultured with PMN isolated from WT HS mice. Considered together, these results suggest an essential role for PMN NAD(P)H oxidase in mediating TLR2 upregulation in AMϕ[34].

Here is a question that needs to be answered: is AMϕ endogenous NAD(P)H oxidase also involved in the signaling that induces enhanced TLR2 expression? To elucidate this question, AMϕ isolated from $gp91^{phox-/-}$ mice were applied to the co-culture system. The study showed that lacking of endogenous NAD(P)H oxidase in the AMϕ caused a decrease in TLR2 expression in response to LPS stimulation; however the decrease was restored when the AMϕ were co-incubated with PMN isolated from the WT mice subjected to HS [34]. These results indicate that although the endogenous NAD(P)H oxidase in AMϕ is also involved in the signaling, the exogenous oxidants from PMN NAD(P)H oxidase are essential for inducing amplified TLR2 expression in AMϕ in response to LPS.

The role of PMN NAD(P)H oxidase was further confirmed *in vivo* by depleting circulating PMN in the mice sub-

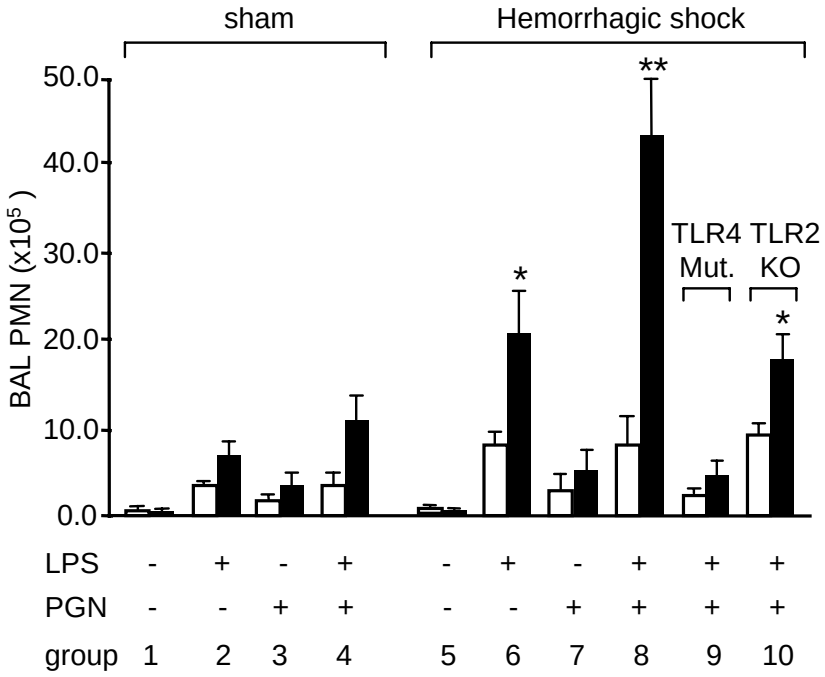

Fig. (2). Effects of combined challenges of LPS and PGN on AMϕ-induced PMN migration. To address the role of TLR2 in regulating transalveolar PMN migration in the lung, WT mice, TLR4 mutant mice, and TLR2$^{-/-}$ mice were challenged with hemorrhage/resuscitation followed by intratracheal injection of LPS (30 μg/Kg BW) plus PGN (300 μg/kg BW) at 1 hour after resuscitation. PMN in BAL fluid were counted at 2-hour (clear bars) and 6-hour (black bars) after LPS/PGN administration. The combination of LPS and PGN induced a 5.2-fold further increase in BAL PMN at 6 hours in WT shocked mice compared with that in the 2-hour group (group 8), but only induced a 2.0-fold further increase in TLR2$^{-/-}$ mice at 6-hour (group 10). The greater difference in BAL PMN between 6-hour and 2-hour points in the WT shock/LPS/PGN-treated animals indicates an enhanced PMN transalveolar migration in the lung following the primary PMN infiltration. ** P<0.01 compared with all other groups; * P<0.01 compared with the groups with no * symbol; (n = 3 per group); (adopted from Ref. [23]).

jected HS/LPS challenge. At 4 h after LPS challenge, neutropenia induced in mice subjected to HS was associated with a ~58% reduction in TLR2 expression in AMϕ, as compared to the group subjected to HS/LPS with no PMN depletion. In contrast, depletion of PMN did not alter TLR2 expression in the AMϕ either from sham/LPS treated WT mice or from shock/LPS treated gp91$^{phox-/-}$ mice. Taken together, these results demonstrate a requirement for PMN NAD(P)H oxidase in HS-enhanced TLR2 upregulation in AMϕ.

The TLR2 gene promoter contains multiple binding sites for transcriptional factors, which include NF-κB, CCAAT/enhancer binding protein, cAMP response element-binding protein, and STAT [35]. Of these, NF-κB has been reported to regulate TLR2 expression in response to cytokines and mycobacterial infection [35, 36]. It has been demonstrated that LPS-TLR4-induced TLR2 upregulation in AMϕ is largely mediated through the NF-κB signaling pathway, since the NF-κB inhibitor IKK-NBD significantly decreased LPS-induced TLR2 expression in AMϕ[23]. Although oxidants are involved in the NF-κB signal transduction pathway [37-39], their molecular targets have not yet been defined. The contribution of redox regulation and location of potential redox-sensitive sites within the NF-κB activation pathway are the subjects of controversy [37]. The upstream events of NF-κB signaling have been investigated to address whether the LPS-TLR4-mediated TLR2 expression depends on MyD88. The study showed that MyD88 is required for both LPS-induced TLR2 upregulation and amplified TLR2 upregulation mediated by HS-activated PMN. This finding led to further elucidate a role of interleukin-1 receptor-associated kinase 4 (IRAK4), a downstream component of the MyD88-dependent pathway following TLR activation, in the oxidant-enhanced upregulation of TLR2 in AMϕ. It was found that co-incubation with shock-activated WT PMN markedly increased IRAK4 activity in AMϕ in response to LPS, and that antioxidant polyethylene glycol (PEG) catalase diminished the increase induced by the WT PMN. Hence, oxidants by themselves seem to be incapable of activating IRAK4, but can amplify LPS-induced IRAK4 activation. IRAK4 may potentially be an important redox-regulated kinase responsible for TLR4-MyD88 signaling. Redox regulation of IRAK4 activity is further suggested by the presence of 10 cysteine residues in this molecule, which may constitute the redox-sensitive sulfhydryl switches [40]. Apparently, further studies will be needed to define the precise mechanisms responsible for the redox-regulation of IRAK4.

HMGB1-TLR4 SIGNALING MEDIATES HS-INDUCED NAD(P)H OXIDASE ACTIVATION IN PMN

The above findings raised an important question: how is PMN NAD(P)H oxidase activated in HS? Studies have shown that High-mobility group box 1(HMGB1) acts through TLR4 signaling to mediate the HS-induced PMN NAD(P)H oxidase activation.

TLR4 sits at the interface of microbial and sterile inflammation by responding to both bacterial endotoxin and multiple other endogenous ligands, including hyaluronic acid [41], heparan sulfate [42], fibrinogen [43], high-mobility

group box 1 (HMGB1) [44, 45], and heat shock proteins [46]. Both inflammation and injury responses in organs subjected to ischemia/reperfusion partially depend on TLR4 [44, 47, 48].

HMGB1 was originally defined as a nuclear protein that functions to stabilize nucleosome formation, and also acts as a transcription factor that regulates the expression of several genes [49]. HMGB1 can be secreted by innate immune cells in response to microbial products or other inflammatory stimuli [50, 51]. HMGB1 is also released by injured cells and is known as one of the main prototypes of the emerging damage-associated molecular pattern molecules (DAMPs) [52-54]. HMGB1 was initially identified as an inflammatory cytokine that is a late mediator of lethality in sepsis [50, 51]. However, recent studies suggest that HMGB1 acts as an early mediator of inflammation contributing to the development of acute lung injury after hemorrhage [55], and hepatic injury after liver ischemia-reperfusion [44].

PMN NAD(P)H oxidase can be activated in HS mice without LPS challenge [56]. TLR4 is important in inducing PMN NAD(P)H oxidase activation in HS, since the HS-induced $p47^{phox}$ phosphorylation and $p47^{phox}$-$pg91^{phox}$ binding in PMN, indicators of NAD(P)H oxidase assembly and activation, were diminished in TLR4-mutant mice. In HS mice, HMGB1 level is significantly increased in the serum, lung, and liver [56]. Pretreatment of the mice with neutralizing antibody against HMGB1 caused a reduction of $p47^{phox}$ phosphorylation and $p47^{phox}$-$pg91^{phox}$ binding in PMN following HS as compared to that in non-specific IgG pretreated group. *In vitro* studies have also shown that direct stimulation of PMN with recombinant HMGB1 resulted in a dose-dependent induction of $p47^{phox}$ phosphorylation and $p47^{phox}$-$pg91^{phox}$ binding in WT PMN, but not in TLR4-mutant PMN. These results exhibited a key role for TLR4 in mediating HMGB1-induced NAD(P)H oxidase activation.

MyD88 is a common adaptor that induces inflammatory response in TLR signaling. The role of MyD88 in mediating HS-induced PMN NAD(P)H oxidase activation was evident by a significant diminution of $p47^{phox}$ phosphorylation in PMN treated with both HMGB1 and MyD88 inhibitor. The role of IRAK4 in MyD88 signaling was also examined by evaluating IRAK4 activity in the PMN. HMGB1 is able to induce a significant increase in IRAK4 activity in WT PMN as compared to the non-HMGB1-treated WT PMN. Pretreatment with MyD88 inhibitor markedly decreased IRAK4 activity in WT PMN. An impaired IRAK4 activation could be observed in TLR4-mutant PMN, in which the HMGB1-induced IRAK4 activity was attenuated dramatically as compared to that in TLR4 WT PMN. Taken together, the results demonstrate a significant role for TLR4-MyD88-IRAK4 signaling in mediating HMGB1-induced PMN NAD(P)H oxidase activation [56].

What is the downstream signaling pathway that mediates the HS-HMGB1-TLR4-induced NAD(P)H oxidase? The answer to this question seems complex. In previous studies, p38 MAPK, extracellular signal-regulated kinase (ERK1/2), protein kinase C (PKC) and Akt have all been suggested as activators of $p47^{phox}$ and $p67^{phox}$ phosphorylation in response to a variety of stimulations, such as TNF, fMLP, and bacteria [57-60]. In the case of HS -induced $p47^{phox}$ phosphorylation, both p38 MAPK inhibitor and Akt inhibitor partially prevented HMGB1-induced phosphorylation of $p47^{phox}$, and a combination of the inhibitors exhibited complete inhibition of $p47^{phox}$ phosphorylation in response to HMGB1 stimulation, therefore suggesting that both p38 MAPK and Akt signaling pathways are involved in the HMGB1-induced NAD(P)H oxidase activation [56]. It is noteworthy that $p47^{phox}$ is phosphorylated on multiple serine residues that could be targeted by different kinases, thus explaining the tight control of NAD(P)H oxidase. It is possible that $p47^{phox}$ phosphorylation by one kinase induces conformational changes that render the other sites of phosphorylation more accessible to other protein kinases. Indeed, it was recently shown that $p47^{phox}$ phosphorylation *in vitro* induces conformational changes of the protein [61, 62]. Thus p38 MAPK and Akt, as downstream components of TLR4-MyD88-IRAK4 signaling, may work in a coordinated manner.

In summary, HS activates the TLR4-MyD88-IRAK4 signaling pathway through HMGB1, and further activates p38 MAPK and Akt pathways to initiate PMN NAD(P)H oxidase activation. PMN NAD(P)H oxidase-derived oxidant, in turn, mediate TLR4-TLR2 cross-talk in AMϕ and sensitize AMϕ response to TLR2 ligands, which acts in a positive feedback manner to amplify pulmonary PMN infiltration and inflammation.

SIGNIFICANCE OF TLR4-TLR2 CROSS-TALK IN INFLAMMATORY RESPONSE

The PMN-mediated amplification of TLR4-TLR2 cross-talk and subsequent exaggeration of local inflammation does not merely occur in a scenario of PMN-AMϕ interaction, but can also be observed when PMN interact with EC. Studies [63] showed that LPS induced TLR2 up-regulation through TLR4- and MyD88-dependent signaling in EC. However, in neutropenic mice, the LPS-induced NF-κB activation and TLR2 expression were significantly reduced, and both responses were restored upon repletion by PMN obtained from WT mice but not by PMN from NAD(P)H oxidase $gp91^{phox-/-}$ mice. These findings were recapitulated in mouse lung vascular endothelial cells co-cultured with PMN, indicating that the augmented NF-κB activation and the resultant TLR2 upregulation in EC were secondary to oxidant signaling generated by PMN NAD(P)H oxidase. The functional relevance of NAD(P)H oxidase in mediating TLR4-induced TLR2 expression in EC was evidenced by markedly elevated and stable ICAM-1 expression as well as augmented PMN migration in response to sequential challenge with LPS and PGN [63]. Thus, PMN NAD(P)H oxidase–derived oxidant signaling is also an important determinant of the cross talk between TLR4 and TLR2 in EC and the control of endothelial cell activation.

The PMN-mediated TLR4-TLR2 cross-talk suggests a highly coordinated, oxidant-mediated upregulation of TLR2 in response to LPS. When one considering the interactions of the innate immune system as microbes are first encountered, the value of such temporal organization is significant. For example, Gram-negative bacteria persist in tissues and, if they are not immediately killed through the activation of PMN, complement, and other antimicrobial factors, they may spill out systemically and result in septic shock. Survival in the face of such infections depends upon the innate immune system, which must be able to monitor and respond to pathogens over a prolonged period of time. Given the

need for a prolonged response to bacterial infection, it has always seemed somewhat surprising that response to LPS is temporally finite. This endotoxin tolerance means that within hours after exposure to LPS, innate immune cells are incapable of responding again to a re-challenge [64, 65]. But it is now clear that as LPS sensitivity wanes, the immune system has at its disposal the capability of marshaling responses *via* oxidative metabolites and their ability to upregulate other TLRs [64]. The subsequent means of responding to bacteria depend upon the ability of the innate immune system to destroy microbes and enhance the release of alternative immune stimuli. The TLRs that are utilized are the ones that bind the constituents of degrading bacteria, such as lipopeptides, PGN, heat shock proteins, and CpG DNA. It seems plausible that activated PMN may even alter the phenomenon of LPS tolerance, at least in a localized context, by setting into action a positive feedback loop at sites to which PMN are chemoattracted [64]. This would enhance inflammatory responses locally and help fight infection. However, in a setting of post-trauma SIRS, the primed PMN activation serves as an amplifier to cause enhanced PMN infiltration and organ injury.

ACKNOWLEDGEMENT

This work was supported by the National Institutes of Health Grant R01-HL-079669 and a VA Merit Award.

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Received: January 9, 2009 Revised: January 26, 2009 Accepted: January 27, 2009