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*J Immunol* 2003; 171:1473-1483; doi: 10.4049/jimmunol.171.3.1473

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Injury Primes the Innate Immune System for Enhanced Toll-Like Receptor Reactivity

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Severe injury causes a dramatic host response that disrupts immune homeostasis and predisposes the injured host to opportunistic infections. Because Toll-like receptors (TLRs) recognize conserved microbial Ags and endogenous danger signals that may be triggered by injury, we wanted to determine how injury influences TLR responses. Using an in vivo injury model, we demonstrate that injury significantly increased TLR2- and TLR4-induced IL-1β, IL-6, and TNF-α production by spleen cells. This influence of injury on TLR reactivity was observed as early as 1 day after injury and persisted for at least 7 days. The outcome of similar studies performed using TLR4-mutant C57BL/10ScN/Cr mice revealed that TLR2 responses remained primed, thus suggesting that injury-induced priming can occur independently of endogenous TLR4 signaling. Increased TLR4 reactivity was also observed in vivo, because LPS-challenged injured mice demonstrated significantly higher cytokine expression levels in the lung, liver, spleen, and plasma. Macrophages and dendritic cells were the major source of these cytokines as judged by intracellular cytokine staining. Moreover, ex vivo studies using enriched macrophage and dendritic cell populations confirmed that T cells did not contribute to the enhanced TLR2 and TLR4 responses. The results of flow cytometry studies using TLR2- and TLR4-MD-2-specific Abs indicated that injury did not markedly alter cell surface TLR2 or TLR4-MD-2 expression. Taken together, these findings establish that injury primes the innate immune system for enhanced TLR2- and TLR4-mediated responses and provides evidence to suggest that augmented TLR reactivity might contribute to the development of heightened systemic inflammation following severe injury. *The Journal of Immunology, 2003, 171: 1473–1483.

Injury disrupts host immunity and in the clinical setting is believed to predispose patients suffering major trauma or burns to opportunistic and nosocomial infections (1–4). The impairment in host defense following injury suggests a defect in cell-mediated immune responses resulting in impaired microbiocidal activity by cells of the innate immune system. However, rather than having suppressed innate immune reactivity against microbial Ags, injured patients and animals demonstrate a prolonged imbalance toward augmented reactivity to bacterial Ags and toxins derived from both Gram-negative and Gram-positive pathogens (5–10). This postinjury inflammatory state has been defined clinically as the systemic inflammatory response syndrome (SIRS)(5) (11). SIRS can often lead to multiple organ dysfunction syndrome (MODS) and death, particularly if infectious complications occur following injury or major surgery (12).

Prior studies have shown that PBMC harvested from control vs severely injured patients produced higher levels of IL-1, TNF-α, or IL-6 in response to Escherichia coli LPS (8, 13, 14). Studies performed using several animal injury models also demonstrated enhanced LPS reactivity by spleen-derived adherent cell populations and Kupffer cells (5, 15, 16). Further investigations into the mechanisms associated with injury-induced perturbations in LPS responses indicated that changes in PGE2 responses, IL-10 reactivity, and pertussis toxin-sensitive signaling pathways were involved (5, 17, 18). As a whole, these observations support the hypothesis that injury augments host inflammatory responses and that enhanced inflammatory reactivity may play a significant role in the development of SIRS and MODS following severe injury.

The recent discovery of a family of mammalian receptors related to Drosophila Toll has provided an explanation for how innate immune cell types can recognize and react against a wide variety of microbial Ags (19). This family of receptors, referred to as Toll-like receptors (TLRs), consists of 10 members that have shared structural homology and shared signaling components (20, 21). In particular, one of the TLRs, TLR4, has been shown to be essential for cellular responsiveness to LPS (22, 23). This was demonstrated by mapping the gene responsible for the LPS-hyporesponsive phenotype (lps) to the TLR4 gene and by controlled cell transfection studies using TLR4 gene expression constructs (22, 24). Ligands for many of the TLRs have been described (25). For example, the major cell wall component of Gram-positive bacteria, peptidoglycan (PGN), is recognized by TLR2 (26, 27); TLR9 binds unmethylated bacterial CpG DNA (28); and lipoteichoic acid recognition has been attributed to both TLR2 (26, 29) and TLR4 (30). Once triggered by these microbial Ags, signaling occurs by a pathway that uses components shared among both TLRs and the IL-1R family, appropriately named the Toll/IL-1R domain.

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Received for publication September 3, 2002. Accepted for publication May 9, 2003.

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1 This research work was supported by grants from the National Institutes of Health (GM57664 and GM35633), the Brook Fund, and the Julian and Eunice Cohen Fund for Surgical Research.

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4 Abbreviations used in this paper: SIRS, systemic inflammatory response syndrome; MODS, multiple organ dysfunction syndrome; TLR, Toll-like receptor; PGN, peptidoglycan; LA, lipid A; HSP, heat shock protein; PAMP, pathogen-associated molecular pattern; IRAK-M, IL-1R-associated kinase-M.
The development of SIRS and MODS after severe injury may involve excessive TLR-induced inflammatory cytokine production that may or may not result from infectious complications. In this study, we used a mouse thermal injury model to determine how injury alters responses to several well-defined TLR2 and TLR4 agonists that represent major antigenic components of both Gram-negative and Gram-positive bacteria. Moreover, we used TLR4-deficient mice to help decipher whether endogenous TLR4 signaling plays an essential role in the development of injury-induced changes in TLR reactivity. We demonstrate that injury leads to augmented TLR2- and TLR4-mediated responses within 24 h and that these heightened responses were maintained for at least 7 days. Furthermore, we show that TLR4 deficiency had no measurable effect on TLR2 responses, suggesting that endogenous or self TLR4 signaling does not mediate the injury-induced augmented reactivity of other TLRs. The effect of injury on TLR4 reactivity was also observed in vivo, because LPS-challenged injured mice demonstrated significantly higher cytokine expression levels in the lung, liver, spleen, and plasma. The results of studies using immunomagnetic cell subset depletion and intracytoplasmic cytokine detection methods demonstrated that the injury-induced enhanced TLR reactivity is mediated principally by macrophages and dendritic cells. Finally, we use TLR2- and TLR4-MD-2-2 specific Abs to demonstrate that injury does not alter cell surface TLR2 or TLR4-MD-2 expression suggesting that changes in TLR2 or TLR4-MD-2 expression density do not explain the injury-induced enhanced TLR2 and TLR4 responses.

Materials and Methods

Mice

Male C57BL/10ScN/Cr mice and age-matched wild-type C57BL/10SnJ controls were obtained from the National Cancer Institute (Frederick, MD) and The Jackson Laboratory (Bar Harbor, ME), respectively. Mice were maintained in an accredited virus-Ab-free animal facility in accordance with the guidelines of the National Institutes of Health and the Harvard Medical School Standing Committee on Animal Research. The mice were acclimated for at least 1 week before being used in experiments at 6–9 wk of age.

Reagents

Diphosphoryl lipid A (L.A) and PGN were obtained from Sigma-Aldrich (St. Louis, MO). LPS from E. coli (B6:O26) was obtained from Difco Laboratories (Detroit, MI). Culture medium for in vitro studies consisted of RPMI 1640 supplemented with 5% heat-inactivated FCS, 1 mM glutamine, penicillin/streptomycin/Fungizone, 10 mM HEPES buffer, 100 μM nonessential amino acids, and 2.5 x 10⁻⁵ M 2-ME, all purchased from Life Technologies (Grand Island, NY).

Mouse injury model

The thermal injury protocol, approved by the National Institutes of Health and the Harvard Medical School Standing Committee on Animal Research, was performed as described previously (31). Four mice per group were anesthetized by i.p. injection of ketamine (125 mg/kg) with xylazine (20 mg/kg). The dorsal fur was shaved, and the animal was placed in an insulated plastic mold to expose 25% total body surface area. This part of the dorsal area was immersed in 90°C (burns) or isothermnic water (shams) for 9 s. All groups were resuscitated with an i.p. injection of 1 ml of 0.9% pyrogen-free saline. This protocol causes a controlled, full-thickness anesthetic injury with a low average mortality of 5%.

Ex vivo studies

Spleens were harvested 3 h, 24 h, or 7 days after injury or sham injury, and cell suspensions were prepared by mincing tissues on wire mesh. Spleen cells were treated with Tris-ammonium chloride solution for 3 min to lyse RBCs and washed twice before suspension in culture medium. Cells were cultured in Costar round-bottom 96-well plates at 2 x 10⁴ cells/well with 1 μg/ml LA, 1 μg/ml LPS, 10 μg/ml PGN, or no additions. The doses selected were shown in a pilot experiment to yield maximum cytokine production. After culture at 37°C in 5% CO₂ for 48 h, supernatants were harvested and stored at -20°C.

In vivo LPS-induced cytokine studies

At 7 days after sham or thermal injury, mice were given 100 μg of LPS by vehicle. Thirty minutes and 2 h later, mice were killed by CO₂ asphyxiation, and blood was collected into heparin-coated 1-ml tuberculin syringes fitted with 27-gauge needles by the cardiac puncture route. Following exsanguinations, lung, liver, and spleen were harvested into icecold PBS containing a protease inhibitor mixture (Complete protease inhibitor cocktail tablets; Roche Applied Science, Indianapolis, IN). Plasma was harvested from blood samples following centrifugation at 3000 g for 20 min. Tissue extracts were prepared by homogenizing tissues for 30 s each on ice using a Tissue-Tearor (BioSpec Products, Bartlesville, OK). Tissue extracts were then clarified by centrifugation at 3000 x g for 20 min. Protein levels in the tissue extracts were measured using the Bradford protein assay reagent according to the manufacturer’s protocol (Sigma-Aldrich). Plasma and tissue extract samples were stored at -20°C before measuring cytokine levels by ELISA.

Cytokine ELISA

IL-1β and TNF-α were measured using ELISA kits purchased from R&D Systems (Minneapolis, MN) according to the manufacturer’s instructions. Briefly, 96-well microtiter ELISA plates (Nunc MaxiSorb; Nalge Nunc International, Rochester, NY) were coated with 50 μl of capture Ab diluted in PBS at 37°C. The plates were blocked with 1% BSA in PBS for at least 15 min and then washed with wash buffer (PBS plus 0.05% Tween 20). Standards and samples were added (50 μl/well) and incubated for 1 h at 37°C. The plates were washed, 50 μl of biotinylated detection Ab was added per well, and the plates were incubated for 1 h at 37°C; then the plates were again washed thoroughly, avidin-HRP was added, and the plates were further washed, 37°C for 30 min. After further washing, the biotin substrate was added; following color development for 10–30 min, the reaction was stopped with 2 M H₂SO₄, and the absorbance was determined with an ELISA plate reader (Molecular Devices, Sunnyvale, CA). IL-6 was measured using capture and biotinylated detection Abs obtained from BD Pharmingen (San Diego, CA) with substitution of extra-avidin alkaline phosphatase detection enzyme and phosphatase substrate (Sigma-Aldrich) for the HRP system. Extrapolation of values for sample data from standard curves was calculated using SoftMax Pro software (Molecular Devices).

FACS analysis of cell surface TLR2 and TLR4-MD-2 expression

At 1 day after injury, spleen cells were prepared from six sham- or burn-injured C57BL/10 SnJ mice. Spleen cells were first incubated with Fc Block (BD Pharmingen) before TLR4-MD-2 staining or with human AB serum (United States Biological, Swampscott, MA) for TLR2 staining to reduce nonspecific background staining, then stained with FITC-labeled anti-F4/80 or anti-CD11c Abs (BD Pharmingen) and PE-labeled anti-TLR4-MD-2 Ab (eBioscience, San Diego, CA) (32). An indirect staining method was used to detect cell surface TLR2 by first staining spleen cells with a monoclonal rat anti-mouse TLR2 Ab (N. Nilsen, U. Nonstä, A. Sundan, T. Espevik, E. Lie; manuscript in preparation) followed by staining with PE-labeled anti-rat IgG (BD Pharmingen) to detect cell surface-bound anti-TLR2 Ab. TLR2-stained spleen cells were then counterstained with FITC-labeled anti-F4/80 or anti-CD11c Abs. An isotype-matched PE-labeled rat IgG was used as a nonspecific control for TLR4-MD-2 staining, and cells stained with PE-labeled anti-rat IgG alone served as a nonspecific control for TLR2 staining. Flow cytometry was performed using the FACS Calibur instrument (BD Biosciences, Mountain View, CA), and the results were analyzed using the accompanying CellQuest Pro software.

Intracellular cytokine detection by FACS

Spleen cells prepared from C57BL/10SnJ or C57BL/10ScN/Cr mice were stimulated with 1 μg/ml LPS in the presence of brefeldin A (10 μg/ml). Six hours later, cells were treated first with Fc Block (BD Pharmingen), surface stained with FITC-labeled anti-CD4, anti-CD8, anti-CD19, anti-F4/80, or anti-CD11c Abs, then fixed for 20 min in 2% paraformaldehyde in PBS (pH 7.4) at 4°C. Following fixation, cells were washed once and then resuspended in 100 μM of permeabilization buffer (PBS (pH 7.4), 0.1% saponin, 1% BSA, and 0.1% sodium azide). Cytokine staining was done by pretreating the fixed and permeabilized cells with 25 μl of a 1 μg/ml solution of an anti-rat IgG (BD Pharmingen) followed by Fc Block and nonspecific anti Ab binding. The fixed and permeabilized cells were then stained with biotin-labeled Abs specific for IL-1β or TNF-α (PeuroTech, Rocky Hill, NJ). After 1-h incubation at room temperature, cells were washed twice in permeabilization buffer, then 25 μl of Cy5-labeled streptavidin (0.2 μg/ml, final; BD Pharmingen) was added. Cells were incubated at room temperature for 30 min and then washed twice by centrifugation. Flow cytometry was again performed using a FACS Calibur instrument.
any significant injury-induced increases or decreases in TLR2- or had also become significantly elevated (Fig. 1). In marked contrast, splenocytes harvested at 3 h after injury did not demonstrate any significant injury-induced increases or decreases in TLR2- or TLR4-stimulated IL-1β, TNF-α, and IL-6 production levels (data not shown). Collectively, these findings demonstrate that injury augmented the cellular responses to TLR2 and TLR4 stimuli by 24 h after injury, and that the enhanced TLR2 and TLR4 reactivity persists for at least 7 days after injury.

TLR4 does not mediate injury-induced effects on TLR reactivity

The linkage of TLR4 gene mutations to the LPS hypersensitivity of the C3H/HeJ and the C57BL/10ScCr mouse strains provided an explanation for the resistance these mice have to LPS-induced shock (24). We tested the hypothesis that endogenous TLR4 signaling triggered by the host response to injury might contribute to the development of augmented TLR reactivity by repeating the ex vivo experiments shown in Fig. 1 using TLR4-deficient C57BL/10ScCr mice. Fig. 2 shows data generated from experiments using C57BL/10ScCr (TLR4-deficient) mice at 24 h and 7 days after injury. As expected, IL-1β, IL-6, and TNF-α production by LPS-stimulated splenocytes from C57BL/10ScCr mice was greatly diminished compared with LPS-stimulated cytokine production by spleen cells from wild-type C57BL/10ScJ mice (Fig. 1). Although the LA response by C57BL/10ScCr spleen cells was undetectable, the TLR2 stimulant, PGN, was able to induce significant IL-1β, TNF-α, and IL-6 production. This finding further supports the specificity of LA for TLR4 and validates using PGN reactivity to study injury effects on TLR2 responses. In these studies using TLR4-deficient mice, the injury-induced increase in TLR2 reactivity as measured by PGN-stimulated spleen cell IL-1β, TNF-α, and IL-6 production was observed at both 24 h and 7 days (Fig. 2). The maintenance of the injury-induced priming for TLR2 reactivity in TLR4-deficient mice supports the idea that the effect that injury has on TLR responses can occur in the absence of TLR4 signaling in vivo. This outcome also suggests that potential endogenous injury-associated TLR4-specific ligands, such as endotoxin, heat shock protein (HSP)60 (33), HSP70 (34, 35), fibronectin A (36), or viral Ags (37) do not measurably influence the injury-induced effect on TLR2 responses.

Injury primes innate immune cell types for enhanced TLR2 and TLR4 reactivity

TLRs are pattern recognition receptors able to detect pathogen-associated molecular patterns (PAMPs) and are a central component of innate immune cell responses. However, because TLR2 and TLR4 can be expressed on a variety of cell types, we wanted to determine which cell types were associated with the injury-dependent augmented TLR2 and TLR4 responses. First, we performed ex vivo studies to determine the effect of depleting T cells on LPS- and PGN-induced cytokine production. We reasoned that this approach would establish whether T cells played a role in mediating the effect of injury on TLR2 and TLR4 responses. As shown in Fig. 3, depleting T cells from the total spleen cell population did not significantly alter the injury-induced enhancement of LPS- or PGN-stimulated IL-1β, IL-6, and TNF-α production. However, we did observe that T cell depletion consistently reduced PGN-stimulated IL-6 production, suggesting that T cells may modulate TLR2-induced IL-6 production. As a whole, these results provide evidence to suggest that injury alters the TLR reactivity of primarily innate immune cell types or B cells.

Next, we used an intracytoplasmic cytokine staining approach to directly determine which cell types expressed IL-1β, IL-6, and TNF-α in response to LPS stimulation and to determine which cells displayed injury-induced changes in cytokine expression levels. Fig. 4A illustrates LPS-stimulated IL-1β, IL-6, and TNF-α expression in FACS-gated CD4+ and CD8+ T cells, B cells (CD19+), macrophages (F4/80+), and dendritic cells (CD11c+) harvested at 7 days after sham or burn injury. Our results indicated that F4/80+ and CD11c+ cells expressed the highest levels IL-1β, IL-6, and TNF-α when stimulated with LPS and that only low levels of these cytokines were detected in T or B cells. Most importantly, we observed that injury increased the percentage of IL-1β-, IL-6-, and TNF-α-expressing F4/80+ and CD11c+ cells, suggesting that the augmented TLR2 and TLR4 reactivity is mediated primarily by macrophages (F4/80+) and dendritic cells (CD11c+) (Fig. 4B). Although of a lower magnitude, we also consistently observed a small injury-induced increase in cytokine-positive B cells. Thus, these findings demonstrate that injury had the greatest influence on the reactivity of macrophages and dendritic cells to TLR2 and TLR4 stimuli.
circulating IL-6 and TNF-α levels; however, IL-1β was not detected in plasma samples prepared from either sham or injured mice (Fig. 5B). Taken together, these findings support the observation that injury causes enhanced TLR4 reactivity and that the augmented in vivo cytokine response elicited by LPS stimulation might be involved in the development of organ failure in critically injured patients that develop opportunistic infections. Unfortunately, attempts to use PGN for similar studies addressing injury effects on TLR2 reactivity in vivo failed due to its low in vivo biological activity.

We wished to investigate whether the injury-induced effect on TLR reactivity involved modulation of cell surface TLR expression. This was accomplished by FACS analysis using a newly developed mAb specific for murine TLR2 and a commercially available mAb specific for the murine TLR4-MD-2 complex (32). Comparing the levels of TLR2 and TLR4-MD-2 expression on
macrophages (F4/80⁺) and dendritic (CD11c⁺) cells harvested from sham- vs burn-injured mice indicated no significant injury-induced influence on TLR2 or TLR4-MD-2 expression (Fig. 6). This finding suggests that marked changes in cell surface TLR2 or TLR4-MD-2 expression levels do not explain the observed injury-induced increase in TLR2 and TLR4 reactivity by macrophages and dendritic cells.

**Discussion**

A major complication of severe injury is the development of opportunistic infections caused by a wide array of pathogens. The inability of the injured host to resist infection combined with the potential for the development of dangerous reactivity against invading pathogens and their toxins can lead to multiple system organ failure and death. These studies were initiated to provide a better understanding of how injury modulates the host response to toxins that may be released by infectious pathogens. Our findings provide several important contributions to our understanding of how injury may modulate innate immune cell responses. First, we demonstrated that injury caused an enhanced reactivity against well-defined TLR2 and TLR4 agonists, LA, LPS, and PGN. This effect occurred as early as 1 day after injury and persisted for at least 7 days after injury. In addition, the results of in vivo studies showed that LPS-challenged injured mice produced significantly higher systemic levels of IL-1β, IL-6, and TNF-α. Second, we demonstrated that TLR4 deficiency did not markedly influence the

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FIGURE 2. IL-1β, IL-6, and TNF-α production by splenocytes harvested from C57BL/10ScN/Cr mice 7 days after injury/sham injury. Spleen cell suspensions were prepared and stimulated in culture for 48 h. Cytokine levels were measured by ELISA. The results represent mean values ± SEM of three independent experiments using three mice per group. *, p < 0.05 using a two-tailed t test to compare sham- vs burn-injured groups.
effects of injury on TLR2 reactivity, suggesting that the innate immune priming effect mediated by injury occurs independently of TLR4 signaling. Third, the injury-dependent increase in TLR2 and TLR4 reactivity was shown to be mediated predominantly by macrophages and to a lesser extent by dendritic cells, as revealed by intracellular cytokine expression in LPS-stimulated spleen cells and by the outcome of ex vivo T cell depletion studies. Finally, we document that the enhanced TLR2 and TLR4 reactivity observed following injury does not involve significant changes in cell surface TLR2 or TLR4-MD-2 expression densities.

Because TLRs have the capacity to recognize PAMPs displayed by a variety of microorganisms, their regulation is fundamental to activation of innate immune responses (21, 38). Ten mammalian TLRs have been molecularly identified, and specific ligands have been found for many of the TLRs (25). In relation to this study, TLR2 is known to bind *Staphylococcus aureus* PGN (26, 39), *Borrelia burgdorferi* outer surface protein A lipoprotein (40), mycobacterial lipoarabinomannan (41), and *Listeria monocytogenes* (42). In contrast, TLR4, as part of a receptor complex comprising MD-2 and CD14, is primarily responsible for LPS recognition and signaling (43–45). We chose to use PGN as a TLR2-specific ligand for these studies, because at the time these studies were initiated, it appeared to be among the least controversial TLR2-specific ligand. We found that spleen cells from TLR4-deficient...
FIGURE 4. Injury effects on TLR-mediated cytokine expression by innate and adaptive immune cell populations. A, Spleen cells harvested at 7 days from sham or injured wild-type C57BL/10SnJ mice were stimulated with LPS (10 μg/ml) in the presence of brefeldin A (10 μg/ml) for 6 h, stained with FITC-labeled Abs specific for the indicated cell surface markers, permeabilized, and then stained with biotin-labeled Abs specific for IL-1β, IL-6, and TNF-α followed by Cy5-labeled streptavidin. The FACS plots shown represent the LPS-induced cytokine staining in the indicated gated cell types. Unstimulated control stains showed <2.3% cytokine staining in all cell types examined. The values shown in the upper right quadrant indicate the percentage of cytokine-positive cells. The results presented are representative of three independent experiments using three mice per group.

B, Plots illustrating IL-1β, IL-6, and TNF-α expression levels in LPS-stimulated gated F4/80+ and CD11c+ cells from three independent experiments using three mice per group. *, p < 0.05 using a two-tailed paired t test.
C57BL/10ScN/Cr mice were as responsive to PGN as cells from sex- and age-matched wild-type C57BL/10SnJ mice, further supporting previous reports demonstrating that PGN recognition is TLR4 independent (27). We also observed that cells from TLR4-deficient C57BL/10ScN/Cr mice were hyporesponsive but not fully unresponsive to LPS stimulation as judged by IL-1β, IL-6, and TNF-α production (Fig. 2). This supports a previous report showing that LPS does not signal exclusively through TLR4 (46). Hence, LA, the bioactive portion of LPS, was used for our studies in addition to LPS to more accurately assess injury effects on TLR4-mediated responses. Accordingly, we observed no measurable response by LA-stimulated cells from TLR4-deficient mice, confirming the TLR4 specificity of our experimental system (Fig. 2).

In addition to finding that injury significantly enhances TLR2- and TLR4-induced IL-1β, TNF-α, and IL-6, we also observed...
some qualitative differences in the pattern of cytokines induced by LPS vs PGN. LPS preferentially induced higher levels of IL-6 than TNF-α, whereas PGN stimulated higher levels of TNF-α than IL-6 (Fig. 1). It was also apparent from our results that injury did not noticeably alter the LPS vs PGN cytokine production patterns, but did markedly augment the quantities produced. This finding was not altogether surprising. Although it is known that TLRs signal via a common pathway using MyD88 and TNFR-associated factor 6, there is evidence that TLRs are capable of activating distinct responses to different PAMPs, perhaps by acting in combination with or via MyD88-independent pathways (47–49). We also found that LA was a much weaker stimulant for IL-1β, IL-6, and TNF-α production than LPS or PGN. This was true for 10 ng/ml to 10 µg/ml doses of LA and LPS tested in preliminary studies (data not shown). Moreover, we found that LA could efficiently stimulate IL-10 production levels that were comparable to levels induced by LPS (data not shown). This indicates that the LA preparation used in these studies was bioactive. Together, these results suggest that, although LA has been demonstrated to be the bioactive component of the LPS molecule, other factors such as CD14, LPS-binding protein, LPS-associated proteins, and polysaccharides, or differences in conformation between LA and LPS, may contribute to the observed difference in bioactivity between LA and LPS (50–52).

Recent evidence has suggested that, in addition to their function as sensors of exogenous or foreign PAMPs, TLRs can recognize and mediate responses to endogenous stimuli (33, 53, 54). This feature of TLR biology is of particular importance to injury research because extensive tissue damage may trigger endogenous TLR stimulation. One example of this is the observation that cell surface expression of the TLR4-MD-2 complex as judged by FACS analysis corresponded to our LPS-induced cytokine staining results in that TLR4-MD-2 was detected on macrophages, dendritic cells, and B cells, but not CD3+CD4+ or CD3+CD8+ T cells (data not shown). Moreover, ex vivo studies examining the influence of depleting T cells on TLR2- and TLR4-mediated IL-1β, IL-6, and TNF-α production demonstrated that T cells did not influence the injury-induced priming for TLR2 and TLR4 responses. Thus, we conclude that injury primes cells of the innate immune system for enhanced TLR2 and TLR4 reactivity.

Because multiple cell types have the capacity to produce IL-1β, TNF-α, and IL-6, we wanted to determine which cell types were responsible for the effect of injury on TLR2 and TLR4 responses. We demonstrated by intracellular cytokine staining that enhanced IL-1β, IL-6, and TNF-α production was attributable primarily to F4/80+ cells (macrophages). Injury also affected LPS-induced cytokine production by CD11c+ cells (dendritic cells) and CD19+ B cells, but to a much lesser degree than what was measured in LPS-stimulated macrophages. Accordingly, we observed that cell surface expression of the TLR4-MD-2 complex as judged by FACS analysis corresponded to our LPS-induced cytokine staining results in that TLR4-MD-2 was detected on macrophages, dendritic cells, and B cells, but not CD3+CD4+ or CD3+CD8+ T cells (data not shown). Therefore, we postulated that TLR4 deficiency might alter the injury-induced effect on TLR2-mediated cytokine production. However, we observed a similar degree of injury-induced priming for PGN (TLR2-mediated) responses in TLR4-deficient mice (Fig. 2) as was observed in wild-type mice (Fig. 1). An exception to this was the observation that PGN-stimulated cells from TLR4-deficient mice produced markedly less IL-6 than wild-type cells. Taken collectively, these results indicate that injury-induced priming for TLR responses can occur independent of TLR4 signaling. Thus, the above-mentioned or any other as-yet-unidentified endogenous TLR4 stimuli are not essential for the injury-induced amplification in TLR2 reactivity.
TLR2 or TLR4 receptor density do not likely contribute to the increased cytokine response displayed by LPS-, LA-, and PGN-stimulated macrophages and dendritic cells. This suggests that changes in TLR signaling might account for the increased TLR2 and TLR4 response. Although many of the TLR signaling components have been described, remarkably little is known about what modulates TLR signaling. However, one recent report defines IL-1R-associated kinase-M (IRAK-M) as a suppressor of TLR-induced cytokine responses, because cells from IRAK-M−/− mice displayed increased TLR reactivity (56). The similarity between the enhanced TLR signaling observed in IRAK-M−/− macrophages and macrophages from injured mice warrants future studies to determine whether injury influences IRAK-M expression or function.

The effect of injury on innate immunity described in this study may be a unique feature of the host response to injury. This up-regulated LPS response has been described in most animal models of injury involving lung injury, hemorrhagic shock, late sepsis, burn injury, and traumatic injury (57–64). Moreover, similar findings have been described in injured patients (13, 65). In contrast, systemic inflammatory responses caused by endotoxin or other related TLR stimuli in uninjured hosts can cause hyporesponsiveness to restimulation (66, 67). Accordingly, we initially believed that TLR responses might be reduced early after injury due to the injury-induced release of TLR agonists from damaged tissue or resident microbes. However, we observed no significant injury-induced effect on TLR responses at 3 h after injury, and as early as 1 day after injury, we observed augmented TLR reactivity rather than suppressed TLR responses. Thus, we conclude from these studies that injury does not induce a hyporesponsive TLR phenotype but causes a pronounced and prolonged TLR hyperresponsive state.

The main finding in these experiments is the observation that injury causes an enhanced TLR-mediated proinflammatory response by cells of the innate immune system. This augmented inflammatory response was established (as measured by IL-1β and TNF-α production) 24 h after injury and maintained through day 7. Our findings addressing injury effects on the innate immune system provide in vivo support for the danger theory in that injury primed the innate immune system for augmented reactivity to innate immune stimuli (55, 68). Moreover, we have demonstrated in this study that injury leads to markedly enhanced LPS reactivity in vivo. In stark contrast, experimental animal models of injury and clinical observations have demonstrated profound impairment of adaptive immune responses and increased susceptibility to invading pathogens (1, 69). There is little doubt that the interplay between the innate and adaptive immune system is essential in controlling microbial infections and host inflammatory responses. The findings presented in this study suggest that, if severe injury impairs host immune defenses and subsequent microbial infections develop, the injured host may become susceptible to pathophysiologic complications resulting from heightened reactivity against microbial-derived toxins. Thus, our future challenge is to better define the complex and contrasting influence that injury has on innate and adaptive immune responses so that therapeutic targets can be developed that will safely restore immune homeostasis.

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