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The Role of TLR2 In Vivo following Challenge with Staphylococcus aureus and Prototypic Ligands

Sarah C. Mullaly and Paul Kubes

Based on a wealth of in vitro macrophage studies, immunity to Staphylococcus aureus cell wall-derived peptidoglycan (PGN) and lipoteichoic acid has been attributed to TLR2. We investigated whether the in vitro paradigm of TLR2 dominance would hold true in vivo. Using an experimental peritonitis model, we challenged mice with PGN or lipoteichoic acid and found that only PGN resulted in significant leukocyte (primarily neutrophil) accumulation in the peritoneum at 4 h. PGN-mediated leukocyte recruitment was P-/E-selectin dependent but only partially TLR2 dependent, and also involved the C5aR. Concomitant inhibition of TLR2 and C5aR resulted in a further reduction in PGN-induced peritonitis. Peritoneal neutrophilia was partially mast cell dependent; however, the defect could not be reconstituted with TLR2-/- or C5aR-/- mast cells. Interestingly, macrophage-deficient mice did not have defective neutrophil recruitment. By 24 h, the response to PGN involved primarily monocytes and was TLR2 and C5aR independent. Finally, we challenged mice with live S. aureus and found a similar degree of TLR2 involvement in leukocyte recruitment to that observed with PGN. Most importantly, bacterial clearance from the spleen and peritoneum was not altered in TLR2-/- mice vs wild-type mice. Morbidity was only significantly increased in S. aureus-infected mice treated with a blocking Fab against C5aR. Taken together, these studies indicate that in vivo responses to prototypic TLR2 ligands do not necessarily recapitulate the absolute necessity for TLR2 observed in vitro, and additional receptors contribute, in a significant manner, to PGN and S. aureus-mediated immune responses. The Journal of Immunology, 2006, 177: 8154–8163.

Infection results in the activation of macrophages, mast cells, endothelium, and other cells (1–3). The endothelium can be activated either directly or via the first two cell types, which leads to the critical step of leukocyte recruitment. The first cell type to be recruited is the neutrophil, which engages newly expressed endothelial selectins (E-/P-selectin) to begin rolling, then binds specific chemokines (KC, MIP-2, etc.) to induce adhesion via $\beta_2$ integrins before entering the tissues (4). Monocytes enter the tissues at a later time point using the same selectins, as well as L-selectin ligands and different chemokines (MCP-1, etc.), to induce adhesion via the $\beta_2$ integrins as well as $\alpha_d\beta_1$ integrin, the latter requiring endothelial VCAM-1 expression (5). This well-orchestrated neutrophil and monocyte recruitment is necessary to eradicate the bacterial infection (1, 6).

Germline-encoded transmembrane TLRs are responsible for the detection of microbial infection and the initiation of the innate immune response, including leukocyte recruitment (7–9). TLRs are pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs),3 which are conserved between families of microorganisms. PAMPs are ideal targets for innate immune recognition/activation, as they are produced primarily by microbes and not by host cells. Following the interaction between PAMP and TLR, TLRs signal via their Toll/IL-1R domains (8). The ligation of many TLRs, including TLR2 and TLR4, results in the recruitment of the adaptor protein MyD88 followed by the activation of NF-kB and MAPK. This results in the production of various cytokines, chemokines, adhesion molecules, and other proinflammatory molecules that contribute to leukocyte recruitment and to bacterial clearance.

TLR2 has been identified as the receptor responsible for immunorecognition of the Gram-positive bacteria Staphylococcus aureus as well as the Gram-positive bacterial components, lipoteichoic acid (LTA) and peptidoglycan (PGN) (10–14). Indeed, Takeuchi et al. (10) demonstrated that TLR2-deficient peritoneal macrophages had significantly impaired production of both TNF and IL-6 in response to heat-killed S. aureus, compared with macrophages from wild-type mice. Takeuchi et al. (11) have also shown that thioglycolate-elicited macrophages from TLR2-/- mice were unable to produce IL-6 or NO$_2^-$, and were severely impaired in terms of TNF production in response to PGN. Similarly, Michelsen et al. (12) found that dendritic cells from TLR2-deficient mice were unable to mature, or produce cytokines/chemokines in response to either PGN or LTA. Taken together, these studies indicate that in isolated leukocyte preparations, TLR2 is a dominant receptor for LTA and PGN, and has a significant role in the response to S. aureus.

However, TLR2-independent pathways have also been proposed to participate in S. aureus and PGN-mediated immune responses. Indeed, mice deficient in mannose-binding lectin (15) and/or the C3 complement component (16) display significantly increased...

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3 Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; LTA, lipoteichoic acid; PGN, peptidoglycan; BMMC, bone marrow-derived mast cell; MPO, myeloperoxidase.
mortality following systemic inoculation with \(S. aureus\). Furthermore, PGN can activate the complement cascade in serum, in a cell-independent (and thus, presumably TLR2-independent) manner (17–19). In addition, members of the NOD-leucine-rich repeat family of receptors have been identified as being responsible for intracellular responsiveness to components of PGN (20). These data suggest potentially important TLR2-independent pathways in the recognition of both \(S. aureus\) and of \(S. aureus\)-derived ligands. Finally, aside from macrophages, other cell types, including mast cells and neutrophils, have been shown to respond to PGN and \(S. aureus\) in vitro (21, 22). Clearly, the importance of TLR2 in vivo (where multiple receptors and multiple cell types exist) in response to PGN or \(S. aureus\) may not be as profound as has been reported for TLR2 on macrophages in vitro in response to primarily synthetic ligands or PGN.

More and more, a reductionist approach has been taken to try to elucidate the mechanisms underlying Gram-positive microbial recognition. Initially, PGN was shown to be a TLR2 ligand (11, 13). More recently, extensive purification of PGN has raised the possibility that the very basic moiety is detected by the Nod family of proteins (23), mannose-binding lectin (24), and other receptors (25). We have taken the opposite nonreductionist approach to understand the mechanism(s) by which PGN and Gram-positive bacteria are detected in vivo in a complex multicellular, multireceptor environment to understand the role of TLR2 in the hallmark feature of the immune response to bacteria, namely leukocyte recruitment. A systematic assessment of the role of TLR2 as it pertains to leukocyte recruitment has not been performed to date. This is not trivial, as endothelium, the cell absolutely required for leukocyte recruitment does not express TLR2 to our knowledge (26–28). For TLR2 to be leukocyte recruitment does not express TLR2 to our knowledge (26–28).

To block the C5aR, TLR2−/− mice were treated with Fab of the neutralizing anti-C5aR mAb 20/70 (30, 31) 30 min before PGN challenge. To block P-selectin and/or E-selectin, C57BL/6 mice were treated with Abs (or with isotype control) 30 min before PGN challenge. \(S. aureus\) was provided by Columbia blood agar plates (Dalynn Biologicals), and macrophage was from Apotex. Anti-C5aR mAb was a gift of Dr. J. Alberta, Canada. Purified LPS (\(Escherichia coli\) trihydrate was from Apotex. Abs against P-selectin (RB40.34) and iso-specific binding in the murine system. Mice were injected i.v. with 1 × 105 CFU, in a final volume of 250 μL. At the noted times after injection, mice were orally anesthetized with isoflurane (Bimeda-MTC) and whole blood was collected via cardiac puncture for circulating leukocyte counts. Mice were sacrificed, and peritoneal lavage was performed using 3 mL of PBS. Exudate was recovered following a 60-s gentle manual massage. Cell counts were determined with a hemacytometer, and lavage fluid was cyto spun and stained with Wright-Giemsa. Leukocyte differentials were determined from a count of 200 cells. The percentage of cells was then multiplied by the total cell number in the lavage fluid to obtain the number of peritoneal leukocyte subsets.

Lung myeloperoxidase (MPO) assay

MPO activity was used as a measure of neutrophil recruitment into the pulmonary microvasculature. MPO is a myeloid cell-specific enzyme used extensively to measure neutrophil recruitment into tissue (32, 33). Briefly, lungs were harvested at the end of each experiment and stored at −80°C. Samples were then processed using hexadearyl-trimethylammonium bromide (for enzyme extraction) and dianisidin-H2O2 (as the colorimetric substrate). The change in absorbance at 450 nm in the 96-well plates was determined over 60 s using a kinetic microplate reader (Molecular Devices).

Quantification of P-selectin expression

P-selectin expression was assessed as a measure of endothelial activation using a modified dual-radio-labeled Ab technique (32, 33). RB40.34 (against P-selectin) and A110-1 (rat IgG1) were labeled with either 125I or 131I. The iodine number of the I-label was used as a control for nonspecific binding in the murine system. Mice were injected i.v. with 10 μg of 125I-labeled RB40.34, and a variable dose of 131I-labeled A110-1. Abs were allowed to circulate for 5 min, and then the mice were heparinized. A blood sample was taken via a carotid artery catheter, and the mice were exsanguinated. The mesenteroy, muscle lining the peritoneal cavity, lung, heart, spleen, and brain were harvested and weighed. Both 125I- and 131I-labeled activities were measured in plasma and tissue samples. P-selectin expression was calculated per gram of tissue, by subtraction of the accumulated activity of the isotype control Ab (131I-labeled A110-1) from the accumulated activity of the Ab against P-selectin (125I-labeled RB40.34). Data are represented as the percentage of the injected dose of Ab per gram of tissue. This approach provides reliable quantitative values of adhesion molecule expression, and that radiolabeled-binding Ab can be specifically displaced with sufficient amounts of unlabeled Ab. The technique is sufficiently sensitive that basal levels of P-selectin can be detected in wild-type mice relative to no protein in P-selectin-deficient mice (34).

Bone marrow-derived mast cells (BMMC)s

Bone marrow was isolated from the femurs and tibias of 6- to 8-wk-old male C57BL/6, TLR2-deficient, and C5aR-deficient mice. RBC were lysed with ACK buffer, and the remaining cells were washed and maintained in RPMI 1640 medium with 10% FBS, sodium pyruvate, glutamine, penicillin/streptomycin, 2-ME, 10 ng/ml IL-3, and 12.5 ng/ml SCF. Cells were transferred into new flasks weekly to remove any adherent cells. BMMC were used after a minimum of 6 wk in culture at ~98% purity as determined by flow cytometry and Wright-Giemsa staining. For reconstitution, BMMC (5 × 104 in 250 μl of sterile saline) were injected i.p. into WW mice. Age-matched wild-type littermates were injected with 250 μl of sterile saline. Mice were housed for an additional 4 wk before being challenged with saline or PGN.
Bacterial load measurements

At the indicated time after inoculation with live *S. aureus* 25923, mice were orally anesthetized and whole blood was collected. Mice were then sacrificed and a peritoneal lavage was performed with 3 ml of sterile PBS. Spleens were collected and homogenized in 1 ml of sterile PBS. Bacterial CFU were determined by culturing serial dilutions on Columbia blood agar plates overnight at 37°C.

Statistics

Results are expressed as mean ± SEM, unless otherwise noted. A one-way ANOVA and a t test with Bonferroni correction were used for multiple comparisons. Survival data were analyzed using a log-rank test. Statistical significance was set at *p* < 0.05.

Results

**PGN, but not LTA, induces peritonitis**

To investigate the in vivo responses to prototypic TLR2 ligands, we challenged mice with 5 mg/kg LTA or with increasing doses of PGN (administered i.p.) and, after 4 h, assessed circulating leukocyte counts, as well as leucocyte recruitment into both the peritoneum and lung. Treatment of wild-type mice with LTA or PGN did not induce a significant change in circulating leukocyte counts (Fig. 1A). By contrast, the number of leucocytes in the peritoneal lavage fluid was significantly increased 4 h following challenge with increasing doses of PGN, but not LTA (Fig. 1B). Treatment of mice with the TLR4 ligand, *E. coli* LPS (0.5 mg/kg, i.p. for 4 h), caused a very different leucocyte profile. Significant reduction in circulating leucocyte counts was noted, but no significant alterations in peritoneal leucocyte counts were detected, as we have previously reported (32). The LPS experiments were performed to show the profound difference in responses to TLR4 vs TLR2 ligands. Indeed, the profound blood leucopenia induced by LPS was likely due to neutrophil trapping in the pulmonary microvasculature, measured by MPO assay, suggesting that LPS induces a systemic effect, whereas the effects of PGN remained localized to the peritoneum. Indeed, unlike LPS, treatment of mice with LTA or PGN did not result in significant neutrophil trapping in the lung (Fig. 1C). Because LTA did not induce significant effects on leucocyte recruitment in vivo, it was excluded from subsequent experiments. Fig. 1D demonstrates that endothelial adhesion molecules (P-selectin and E-selectin) were essential for the leucocyte recruitment, suggesting that the endothelium was, in fact, activated. Indeed, we observed an increase in P-selectin in tissues associated with the peritoneal cavity (including mesentery and the muscle lining the peritoneal cavity) but not in heart, lung, spleen, or brain (Table I). The activation was much more subtle than LPS, shown as a comparison but nevertheless increased ~20-fold in mesentery and 7.5-fold in muscle vasculature lining the peritoneum.

**Kinetics of peritoneal leucocyte accumulation**

To investigate the kinetics of leucocyte recruitment into the peritoneal cavity following PGN challenge, we performed a time-course study. Mice were challenged with 5 mg/kg PGN i.p. and, after 0.5, 1, 2, 4, or 24 h; we quantified circulating leucocyte counts, as well as leucocyte recruitment into the peritoneum. Treatment of wild-type mice with PGN did not induce a significant change in circulating leucocyte counts across the various time points (Fig. 2A). There was, however, a progressive increase in leucocyte recruitment into the peritoneum over

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**Table I. Effect of systemic administration of saline, LPS, or PGN on P-selectin expression**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Saline</th>
<th>LPS</th>
<th>PGN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesentery</td>
<td>0.02 ± 0.01</td>
<td>1.97 ± 0.52***</td>
<td>0.39 ± 0.06*</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.02 ± 0.01</td>
<td>0.49 ± 0.07***</td>
<td>0.15 ± 0.03**</td>
</tr>
<tr>
<td>Heart</td>
<td>0.0</td>
<td>1.83 ± 0.50***</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>Lung</td>
<td>0.02 ± 0.01</td>
<td>4.17 ± 1.36***</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.0</td>
<td>1.29 ± 0.25***</td>
<td>0.0</td>
</tr>
<tr>
<td>Brain</td>
<td>0.01 ± 0.01</td>
<td>0.15 ± 0.03***</td>
<td>0.01 ± 0.01</td>
</tr>
</tbody>
</table>

* LPS (50 μg) or PGN (5 mg/kg) were administered i.p. for 4 h. P-selectin expression was determined using the dual-radiolabel technique. Data are presented as the percentage of the injected dose of Ab per gram of tissue. *p < 0.05; **p < 0.01; ***p < 0.001 vs saline-injected mice.
the first 4 h (Fig. 2B), which was not significantly altered at 24 h. As shown in Fig. 2C, there was a 10-fold increase in neutrophil counts as early as 2 h following challenge, reaching significance by 4 h. Neutrophil counts were reduced by ~60% at 24 h, and concomitantly, a significant increase in peritoneal monocyte/macrophage counts was observed (Fig. 2, C and D).

FIGURE 2. Kinetics of PGN-induced leukocyte recruitment into the peritoneal cavity. C57BL/6 mice were challenged i.p. with saline (■) or with 5 mg/kg PGN (▲). After 0.5, 1, 2, 4, or 24 h, blood was drawn by cardiac puncture for circulating leukocyte counts (A). Mice were sacrificed and peritoneal lavage was performed. Total leukocytes (B) as well as the number of neutrophils (C) and macrophages (D) in the exudate were quantified. Results are expressed as the mean ± SEM of at least five mice per group. **, p < 0.01; ***, p < 0.001 vs saline-injected mice.

FIGURE 3. Roles of TLR2 and TLR4 in neutrophil recruitment to the peritoneum. A, C, and E, C57BL/6 (■) or TLR2−/− (▲); B, D, and F, C57BL/6 (■) or TLR4-deficient C57BL/10ScNJ (▲) mice were challenged i.p. with saline or with 5 mg/kg PGN for 4 or 24 h. At the indicated times, mice were sacrificed, peritoneal lavage was performed, and total leukocyte counts (A and B) as well as the number of neutrophils (C and D) and macrophages (E and F) in the exudate were quantified. Results are expressed as the mean ± SEM of at least five mice per group. *, p < 0.05; TLR2−/− vs C57BL/6 mice.
PGN-mediated peritonitis is partially TLR2 dependent at 4, but not 24, h

Based on comprehensive in vitro studies, TLR2 has been identified as the receptor for PGN (11, 13, 35). As such, we were interested in determining whether the in vivo peritonitis response induced by PGN was also TLR2 dependent. To address this question, TLR2-deficient mice were challenged with saline or with 5 mg/kg PGN i.p. for 4 or 24 h. As shown in Fig. 3A, although the 4 h response to PGN was a little 50% TLR2 dependent, the 24 h response was entirely TLR2 independent. A similar trend was manifested in the degree of peritoneal neutrophil accumulation, which was reduced by 60% at 4, but not 24, h, in PGN-challenged TLR2−/− mice compared with wild-type controls (Fig. 3C). TLR2, although important for the early neutrophil recruitment, is not at all important for the subsequent monocyte recruitment (Fig. 3E) to the peritoneum following systemic PGN administration.

Commercial preparations of the TLR2 ligand LTA have previously been shown to be contaminated with LPS (29, 36). LPS produces a very different response to PGN (see Fig. 1), nevertheless, to address whether the TLR2 independence of PGN was due to contaminating LPS, we challenged TLR4-deficient (C57BL/10ScNJ) mice with PGN (5 mg/kg, i.p.) for 4 or 24 h. Neither the total peritoneal leukocyte (Fig. 3B), neutrophil (Fig. 3D), or macrophage numbers (Fig. 3F), following the induction of peritonitis in the C57BL/10ScNJ mice, were significantly different from wild-type controls.

PGN-mediated peritonitis is partially dependent on the C5aR at 4, but not 24, h

Given that the in vivo peritonitis response to PGN was partially TLR2 independent, we determined which receptor was responsible for the residual effects. PGN is known to activate complement in serum (17–19), and C5a functions as the dominant chemoattractant for neutrophils in this pathway (37). As such, we challenged C5aR-deficient mice with 5 mg/kg PGN i.p. for 4 or 24 h. As shown in Fig. 4A, C5aR−/− mice had 40% less neutrophil recruitment into the peritoneal cavity following 4 h PGN challenge compared with wild-type mice, whereas at 24 h, monocyte recruitment (Fig. 4C) was seen in the presence or absence of C5aR.

**FIGURE 4.** Effect of C5aR deletion on neutrophil recruitment to the peritoneum. C57BL/6 (●) or C5aR−/− (□) mice were challenged i.p. with saline or with 5 mg/kg PGN. After 4 or 24 h, mice were sacrificed, peritoneal lavage was performed and total leukocyte counts (A); additionally, the number of neutrophils (B) and macrophages (C) in the exudate were quantified. Results are expressed as the mean ± SEM of at least five mice per group. ***, p < 0.001, C5aR−/− vs C57BL/6 mice.

**FIGURE 5.** Effect of concomitant blockade of TLR2 and C5aR on neutrophil recruitment to the peritoneum. TLR2−/− mice were challenged with saline or with 5 mg/kg PGN in the presence or absence of anti-C5aR Fab (20/70). After 4 or 24 h, mice were sacrificed, peritoneal lavage was performed, and total leukocytes were counted (A). The number of neutrophils (B) and macrophages (C) in the exudate were quantified. Results are expressed as the mean ± SEM of at least four mice per group. ***, p < 0.001, TLR2−/− + 20/70 vs TLR2−/− mice.
PGN-mediated peritonitis is abrogated in the absence of both TLR2 and C5aR

Based on the involvement of both TLR2 and C5aR in PGN-induced peritonitis, we determined whether concomitant inhibition thereof would result in complete abrogation of leukocyte recruitment into the peritoneal cavity. To assess this question, TLR2/−/− mice were pretreated for 30 min with 200 μg of Fab of the blocking anti-C5aR mAb 20/70 (31) and then challenged with PGN (5 mg/kg i.p.) for 4 or 24 h. Fab were used as intact Ab activated complement (J. Zwirner, unpublished observation). As shown in Fig. 5, A and B, blockade of C5aR in TLR2/−/− mice resulted in a marked decrease in peritoneal leukocyte recruitment following 4 h PGN challenge compared with TLR2/−/− mice not receiving the Fab. In fact, at 4 h, peritoneal neutrophil counts were not significantly different between saline-injected TLR2/−/− mice and TLR2/−/− mice which had received anti-C5aR Fab in the presence of PGN. These results were not due to nonspecific effects of the Fab, as circulating and peritoneal leukocyte counts were identical between C57BL/6 mice injected with saline or with anti-C5aR Fab for 4 h (data not shown). By contrast to the profound TLR2 and C5aR dependence at 4 h, by 24 h, PGN-mediated leukocyte recruitment was indistinguishable between TLR2/−/− mice and TLR2/−/− mice pretreated with the anti-C5aR Fab. Indeed, peritoneal neutrophil (Fig. 5B) and peritoneal monocyte (Fig. 5C) counts were not significantly different between the Fab-treated and the untreated TLR2/−/− mice following 24 h PGN. These data suggest that, although concomitant inhibition of TLR2 and C5aR can abrogate almost all early neutrophil recruitment following systemic PGN administration, at later time points, PGN-mediated monocyte recruitment to the peritoneum is completely independent of TLR2 and C5aR. It also suggests that the early neutrophil response is not necessary for the delayed monocyte recruitment.

Macrophage deficiency results in enhanced peritoneal leukocytosis following PGN challenge

Macrophages have been used extensively in vitro in the study of PGN responses via TLR2 (10, 11). Furthermore, a macrophage population is constitutively present in the peritoneum. As such, we...
were interested in determining whether macrophages would play a role in PGN-induced peritoneal neutrophilia. To address this question, macrophage-deficient (op/op) or control (B6C3Fe) mice were challenged with 5 mg/kg PGN i.p. for 4 h. As shown in Fig. 6, A and B, macrophage deficiency resulted in an enhanced, rather than decreased, leukocyte recruitment into the peritoneum compared with controls. Indeed, early peritoneal neutrophilia doubled in the absence of macrophages, an observation previously reported by others (38). Clearly, other cells besides macrophages can mediate the recruitment of circulating neutrophils. Of course, the 24 h monocyte recruitment was not examined as these mice have few circulating monocytes.

Mast cells are involved in PGN-mediated early neutrophil accumulation in the peritoneum

An interesting and consistent observation was that, following PGN challenge, peritoneal mast cells were more difficult to discern as they had become less granular. Furthermore, mast cells are known to express various TLRs (21, 39, 40), as well as C5aR (30, 41), and can be activated by PGN (21, 39). Thus, we were interested in determining whether mast cells could contribute to PGN-mediated peritonitis. WBB6F1/J+/- (control) mice had ~1.3 × 10^5 peritoneal mast cells, whereas no mast cells were found in the peritoneal cavity of W/W^v mice (data not shown). Following PGN challenge, there was a ~50% reduction in the number of leukocytes/neutrophils recruited into the peritoneal cavity in the mast cell-deficient W/W^v mice compared with +/+ controls (Fig. 6, C and D). It is also interesting to note that the number of neutrophils recruited to the peritoneum following PGN challenge in the WBB6F1/J+/- (the background strain for mast cell-deficient mice) was approximately one-half that observed in the C57BL/6 mice.

Because mast cells express TLR2 and C5aR, we examined whether these receptors were responsible for PGN-induced leukocyte recruitment into the peritoneum at 4 h. To assess this, we cultured BMMC from C57BL/6, TLR2-/-, or C5aR-/- mice and used these cells to reconstitute mast cell-deficient (W/W^v) mice. Four weeks after BMMC transfer, mice were challenged with 5 mg/kg PGN (i.p. for 4 h). As shown in Fig. 6, E and F, PGN-challenged mast cell-deficient mice reconstituted with TLR2-/- or C5aR-/- mast cells had similar levels of leukocyte/neutrophil recruitment to mast cell-deficient mice reconstituted with wild-type mast cells, or mast cell-sufficient (WBB6F1/J+/-) mice. The same number of mast cells was seen in mice reconstituted with wild-type, TLR2-/-, or C5aR-/- mast cells (data not shown).

**TLR2 is partially involved in the 4, but not 24, h leukocyte recruitment response following challenge with live S. aureus**

To further increase the complexity, we determined whether leukocyte recruitment to the peritoneum in response to live Gram-positive bacteria would also be partially TLR2 independent. To address this question, TLR2-deficient mice were challenged with saline or with 1 × 10^9 CFU S. aureus (S.a.) for 4 or 24 h. At the indicated times, mice were sacrificed, peritoneal lavage was performed, and total leukocyte counts (A) as well as the number of neutrophils (B) and macrophages (C) in the exudate were quantified. Results are expressed as the mean ± SEM of at least five mice per group. *, p < 0.05, ***, p < 0.001, TLR2-/- vs C57BL/6 mice.

**FIGURE 7.** The role of TLR2 in S. aureus-mediated peritonitis. C57BL/6 (■) or TLR2-/- (□) mice were challenged i.p. with saline or with 1 × 10^9 CFU S. aureus (S.a.) for 4 or 24 h. At the indicated times, mice were sacrificed, peritoneal lavage was performed, and total leukocyte counts (A) as well as the number of neutrophils (B) and macrophages (C) in the exudate were quantified. Results are expressed as the mean ± SEM of at least five mice per group. *, p < 0.05, ***, p < 0.001, TLR2-/- vs C57BL/6 mice.

Effects of TLR2 deficiency on bacterial load/clearance

Based on our observation that leukocyte recruitment into the peritoneal cavity was only partially TLR2 dependent at 4 h challenge with live S. aureus and not at all involved at 24 h, we were interested in determining the role of TLR2 in bacterial clearance over a prolonged period. To assess this, C57BL/6 and TLR2-/- mice were infected i.p. with 1 × 10^9 CFU of live S. aureus 25923. Three and 5 days postinoculation, bacterial titers in the peritoneum and spleen were quantified. As shown in Fig. 8, A and B, there was no significant difference between the number of S. aureus CFU in the C57BL/6 and TLR2-/- mice in either organ. Furthermore, treatment with suboptimal doses of amoxicillin (250 µg/mouse/day via gavage) following inoculation with live S. aureus (to induce release of cell wall PGN and LTA) did not alter bacterial clearance in the TLR2-/- and C57BL/6 mice (data not shown).

**Morbidity is significantly enhanced following S. aureus infection in the absence of C5aR, not TLR2**

Based on our observation that treatment of TLR2-/- mice with a blocking Fab against C5aR abrogated almost all early neutrophil recruitment following systemic PGN administration, we were interested in determining the roles of TLR2 and C5aR in bacterial clearance over a prolonged period. To assess this, TLR2-/- mice were either untreated or pretreated for 30 min with anti-C5aR Fab. Mice were then infected i.p. with 1 × 10^9 CFU of live S. aureus 25923. As shown in Fig. 8C, blockade of C5aR in TLR2-/- mice resulted in a significant increase in morbidity following inoculation with live S. aureus. Whereas 100% survival was observed in TLR2-/- mice that did not receive the Fab, only 40% of TLR2-/-
Contrast, 24 h following PGN challenge the peritonitis response was completely independent of TLR2 and/or C5aR. TLR2 has been identified as a dominant receptor for PGN in vitro (11–13, 35). As it turns out, those studies examining the TLR2-PGN relationship have predominantly used thioglycolate-elicited peritoneal macrophages (11), bone marrow-derived dendritic cells (12) or transfected cell lines (13, 35), given PGN in the absence of plasma. Using a mouse model, we find that both TLR2 and complement are involved at the early critical time for the neutrophil response to PGN. Thus, we would propose that although certain cell types (including macrophages and dendritic cells) demonstrate potent responses to PGN via TLR2 in vitro (11, 12), in a more complex in vivo environment (in the presence of plasma and multiple cell types), various cells and multiple host response mechanisms are responsible for neutrophil recruitment in experimental peritonitis. It should be emphasized that although macrophages are the model system to study TLR signaling in vitro (due to ease of isolation and robust responsiveness), we did not see an essential role for macrophages in vivo in the neutrophil response to PGN. Although most investigators have inferred an essential role for macrophages in vivo in the neutrophil response to PGN, we have previously examined leukocyte recruitment in response to LPS and observed that local administration of LPS into tissue caused a localized recruitment of neutrophils, whereas i.p. administration of LPS caused a very profound systemic response (adhesion molecule expression in all organs) with the majority of neutrophils sequestered into the lungs (32). These responses were entirely dependent upon a single TLR, namely TLR4 (42), suggesting that LPS has only one biological receptor. Unlike the LPS data, however, the in vivo PGN data are not as clear. We have demonstrated robust neutrophil recruitment restricted to the peritoneal cavity, but not the lung, at 4 h, and monocyte recruitment at 24 h following systemic challenge with PGN, but not LTA. The PGN-mediated early neutrophil accumulation is in part, but not absolutely, dependent upon TLR2, whereas the later monocyte response is entirely independent of TLR2. In fact, the C5aR also appeared to play an important role at 4 h, and in its absence, PGN-induced neutrophil influx into the peritoneal cavity was reduced by nearly 50%. Concomitant inhibition of TLR2 and C5aR, using TLR2−/− mice treated with the anti-C5aR blocking Fab 20/70 resulted in a further inhibition of early peritoneal neutrophilia. By contrast, 24 h following PGN challenge the peritonitis response was completely independent of TLR2 and/or C5aR.

A dominant mast cell component has been identified in the peritonitis response following cecal ligation and puncture or Gram-negative bacterial challenge (2, 43). Indeed, mast cells have presynthesized TNF storage pools. Following bacteremia, there is an immediate release of TNF that is entirely mast cell derived (2). Although most investigators have inferred an essential role for mast cells in vivo in the neutrophil response to PGN, we have previously examined leukocyte recruitment in response to LPS and observed that local administration of LPS into tissue caused a localized recruitment of neutrophils, whereas i.p. administration of LPS caused a very profound systemic response (adhesion molecule expression in all organs) with the majority of neutrophils sequestered into the lungs (32). These responses were entirely dependent upon a single TLR, namely TLR4 (42), suggesting that LPS has only one biological receptor. Unlike the LPS data, however, the in vivo PGN data are not as clear. We have demonstrated robust neutrophil recruitment restricted to the peritoneal cavity, but not the lung, at 4 h, and monocyte recruitment at 24 h following systemic challenge with PGN, but not LTA. The PGN-mediated early neutrophil accumulation is in part, but not absolutely, dependent upon TLR2, whereas the later monocyte response is entirely independent of TLR2. In fact, the C5aR also appeared to play an important role at 4 h, and in its absence, PGN-induced neutrophil influx into the peritoneal cavity was reduced by nearly 50%. Concomitant inhibition of TLR2 and C5aR, using TLR2−/− mice treated with the anti-C5aR blocking Fab 20/70 resulted in a further inhibition of early peritoneal neutrophilia. By contrast, 24 h following PGN challenge the peritonitis response was completely independent of TLR2 and/or C5aR.

TLR2 has been identified as a dominant receptor for PGN in vitro (11–13, 35). As it turns out, those studies examining the TLR2-PGN relationship have predominantly used thioglycolate-elicited peritoneal macrophages (11), bone marrow-derived dendritic cells (12) or transfected cell lines (13, 35), given PGN in the absence of plasma. Using a mouse model, we find that both TLR2 and complement are involved at the early critical time for the neutrophil response to PGN. Thus, we would propose that although certain cell types (including macrophages and dendritic cells) demonstrate potent responses to PGN via TLR2 in vitro (11, 12), in a more complex in vivo environment (in the presence of plasma and multiple cell types), various cells and multiple host response mechanisms are responsible for neutrophil recruitment in experimental peritonitis. It should be emphasized that although macrophages are the model system to study TLR signaling in vitro (due to ease of isolation and robust responsiveness), we did not see an essential role for macrophages in vivo in the neutrophil response to PGN. Although most investigators have inferred an essential role for macrophages in vivo in the neutrophil response to PGN, we have previously examined leukocyte recruitment in response to LPS and observed that local administration of LPS into tissue caused a localized recruitment of neutrophils, whereas i.p. administration of LPS caused a very profound systemic response (adhesion molecule expression in all organs) with the majority of neutrophils sequestered into the lungs (32). These responses were entirely dependent upon a single TLR, namely TLR4 (42), suggesting that LPS has only one biological receptor. Unlike the LPS data, however, the in vivo PGN data are not as clear. We have demonstrated robust neutrophil recruitment restricted to the peritoneal cavity, but not the lung, at 4 h, and monocyte recruitment at 24 h following systemic challenge with PGN, but not LTA. The PGN-mediated early neutrophil accumulation is in part, but not absolutely, dependent upon TLR2, whereas the later monocyte response is entirely independent of TLR2. In fact, the C5aR also appeared to play an important role at 4 h, and in its absence, PGN-induced neutrophil influx into the peritoneal cavity was reduced by nearly 50%. Concomitant inhibition of TLR2 and C5aR, using TLR2−/− mice treated with the anti-C5aR blocking Fab 20/70 resulted in a further inhibition of early peritoneal neutrophilia. By contrast, 24 h following PGN challenge the peritonitis response was completely independent of TLR2 and/or C5aR.

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TLR2, and C5aR, the peritonitis response to PGN involves multiple cells and multiple mechanisms. C5aR is found on neutrophils and so direct activation of complement by PGN would cause C5a formation, which would recruit neutrophils, independent of TLR2. In addition, other peritoneal cells could respond to PGN via a TLR2-dependent mechanism to activate mast cells which then release products that could activate endothelium to elicit neutrophil recruitment via various chemoattractants.

Indeed, activation of endothelium is absolutely required to recruit leukocytes into any site of inflammation. Activated leukocytes are unable to bind nonactivated endothelium under physiologic flow in vitro, and removal of adhesion molecules involved in either rolling (selectins) or adhesion (integrins) prevents neutrophil binding to endothelium and subsequent recruitment (45, 46). Therefore, in response to PGN or S. aureus, receptors either directly on endothelium, or indirectly via mast cells would lead to endothelial adhesion molecule expression leading to leukocyte recruitment. It is well-established that neutrophils infiltrate tissues early but by 24 h, monocytes are recruited to clear dying neutrophils and phagocytose any remaining bacteria. Noteworthy, the early neutrophil recruitment was partly TLR2 dependent and partly C5aR dependent, whereas the 24 h monocyte recruitment was independent of TLR2 or C5aR. Clearly, the appropriate endothelial adhesive mechanisms including selectin, chemokine, and integrin ligand expression via TLR2 and/or C5aR are induced to recruit neutrophils. Although the same selectins can recruit monocytes, those cells react to very different chemokines and use not only $\beta_2$ but also $\beta_1$ integrins, requiring expression of VCAM-1 for effective monocyte recruitment. Clearly, this important step does not require TLR2. Although it remains unclear what mechanism recruits the monocytes, we cannot exclude macrophages. Because the macrophage-deficient mice lack monocytes, it is impossible to test this possibility.

Lastly, we have challenged mice with live Staphylococcus aureus, a bacterium whose cell wall contains LTA, PGN, and numerous other molecules, and found a similar pattern of TLR2 involvement in leukocyte recruitment into the peritoneal cavity to that observed with PGN alone. Indeed, although recruitment was partially TLR2 dependent at 4 h, it was entirely TLR2 independent at 24 h. Interestingly, Akira and colleagues (11) reported that while S. aureus cell wall-mediated TNF production in isolated macrophages was entirely via TLR2, TLR2$^{-/-}$ mice had responses to S. aureus (10), entirely consistent with our view that other cell types (besides macrophages) and/or other receptors, aside from TLR2, are involved in S. aureus detection. Furthermore, our results are in agreement with the recent observation of Miller et al. (47) that neutrophil recruitment into the skin 24 h following induction of a localized S. aureus infection was indistinguishable between wild-type and TLR2$^{-/-}$ mice.

Based on our results of neutrophil and monocyte recruitment in TLR2$^{-/-}$ mice, the fact that TLR2 deficiency did not affect peritoneal/splenic bacteremia or survival was, in fact, in line with the recruitment data. In contrast to our findings, Takeuchi et al. (10) found a role for TLR2 in both bacterial clearance and survival. Although at first glance, this seminal study by Takeuchi et al. (10) reporting high susceptibility of TLR2$^{-/-}$ mice to S. aureus infection would suggest profound differences to our findings, careful examination of this study reveals some intriguing similarities. First, TLR2$^{-/-}$ macrophages still responded adequately to heat-killed S. aureus. Second, although there was reduced clearance of S. aureus in the blood of TLR2-deficient mice challenged with $1 \times 10^7$ CFU, in these same mice, there was a significant increase in S. aureus in the kidney in both wild-type and TLR2$^{-/-}$ mice from 2 to 5 days, and the amount of bacteria in the spleen was identical in the two strains of mice. In fact, no detectable difference between wild-type and TLR2$^{-/-}$ mice was detected if a dose of $1 \times 10^6$ CFU was used. Therefore, the only discrepancy between our results and those of Takeuchi et al. (10) was the reduced survival of TLR2$^{-/-}$ mice when $1 \times 10^7$ CFU S. aureus were used. However, a simple explanation may be related to the route of infection or to the bacterial dose. Whereas Takeuchi et al. (10) infected mice i.v., we infected i.p. Indeed, Shi et al. (15) have proposed that redundant mechanisms for bacterial recognition may exist in the peritoneal cavity, but not in the blood, which could explain the observed differences. Furthermore, although Takeuchi et al. (10) observed a significant difference between wild-type and TLR2$^{-/-}$ mice in terms of bacterial load in the blood and kidney on day 2 following infection with $1 \times 10^7$ CFU S. aureus, this difference was not present when mice were challenged with $1 \times 10^6$ CFU bacteria, suggesting that the role for TLR2 may be dose dependent. We used much higher concentrations of bacteria to obtain a more robust signal, but still failed to observe any differences in the absence of TLR2.

Interestingly, our observation that mice that received a blocking Fab against C5aR were no longer resistant to challenge with live S. aureus, suggests that complement functions alongside TLR2 as a dominant player in host defense. Indeed, we would propose that, based on our findings with PGN and S. aureus, loss of TLR2 and C5aR abrogates the immediate neutrophil mobilization which occurs following bacterial challenge. This complete impairment of neutrophil recruitment leads to an overwhelming bacterial infection at 24 and 48 h even though by 24 h the neutrophil response is no longer different between the control and deficient/Fab-treated mice. We would propose that, in addition to inhibiting neutrophil recruitment, inhibition of C5aR could also block oxidant production, degranulation, and phagocytosis to increase likelihood of morbidity. By contrast, when only TLR2 is absent, complement is likely able to facilitate (albeit reduced) neutrophil recruitment, which is sufficient for the containment of infection.

Thus, we conclude from this study that the well-characterized in vitro paradigm of TLR specificity for PGN and S. aureus does not necessarily hold true in vivo. Indeed, peritoneal leukocyte accumulation following challenge with the prototypic ligand PGN is multifactorial. We do not disagree that TLR2 is important, but our data suggest that it is not essential for the immediate neutrophil mobilization following systemic challenge with PGN or live S. aureus. More importantly, we demonstrate a time-dependent monocyte specific recruitment that is entirely TLR2 independent. It would be reasonable that, in the battle for survival, the more mechanisms by which the host can identify bacteria as foreign, the greater likelihood of successful eradication. Indeed, it is well-known that our immune system has evolved receptors to detect flagella, DNA, RNA, and membrane molecules, in addition to opsonins to help identify and kill bacteria (1, 48). Therefore, it stands to reason that the loss of a single detection system is insufficient to subvert the host immune system. Redundancy in detection of bacteria would be protective, such that a single receptor polymorphism is not catastrophic to the host.

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Disclosures

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