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Inhibitory Receptor Paired Ig-like Receptor B Is Exploited by *Staphylococcus aureus* for Virulence

Masafumi Nakayama,* Kenji Kurokawa,†,‡ Kyohei Nakamura,*§ Bok Luel Lee,‡ Kazuhiisa Sekimizu,‡ Hiromi Kubagawa,† Keiichi Hiramatsu,‖ Hideo Yagita,# Ko Okumura,# Toshiyuki Takai,** David M. Underhill,†,‡ Alan Aderem,†,‡ and Kouetsu Ogasawara*

The innate immune system has developed to acquire a wide variety of pattern-recognition receptors (PRRs) to identify potential pathogens, whereas pathogens have also developed to escape host innate immune responses. ITIM-bearing receptors are attractive targets for pathogens to attenuate immune responses against them; however, the in vivo role of the inhibitory PRRs in host–bacteria interactions remains unknown. We demonstrate in this article that *Staphylococcus aureus*, a major Gram-positive bacteria, exploits inhibitory PRR paired Ig-like receptor (PIR)-B on macrophages to suppress ERK1/2 and inflammasome activation, and subsequent IL-6 and IL-1β secretion. Consequently, PIR-B−/− mice infected with *S. aureus* showed enhanced inflammation and more effective bacterial clearance, resulting in resistance to the sepsis. Screening of *S. aureus* mutants identified lipoteichoic acid (LTA) as an essential bacterial cell wall component required for binding to PIR-B and modulating inflammatory responses. In vivo, however, an LTA-deficient *S. aureus* mutant was highly virulent and poorly recognized by macrophages in both wild-type and PIR-B−/− mice, demonstrating that LTA recognition by PRRs other than PIR-B mediates effective bacterial elimination. These results provide direct evidence that bacteria exploit the inhibitory receptor for virulence, and host immune system counter-balances the infection. *The Journal of Immunology*, 2012, **189**: 000–000.

*Staphylococcus aureus*, a Gram-positive bacteria, is a major source of mortality in medical facilities (1, 2). Although *S. aureus* often causes skin infections, it can also spread to the bloodstream and lead to life-threatening sepsis. Because *S. aureus* infections cause relatively mild inflammation compared with Gram-negative infections (3, 4), anti-inflammatory therapies do not improve survival, and can have a detrimental effect on Gram-positive sepsis outcomes (5). Thus, Gram-negative infections are more difficult to cure than those with Gram-negative bacteria (2, 6).

Among pattern-recognition receptors (PRRs), TLR2 plays a crucial role in sensing *S. aureus* lipoproteins and inducing production of inflammatory cytokines such as IL-6 and TNF-α (7). Further, intracellular NLRP3 inflammasomes are activated by *S. aureus* α-toxins and peptidoglycan to secret IL-1β (8, 9). In addition to these sensors, several other PRRs directly bind whole *S. aureus* bacteria and contribute to their elimination in vivo. For example, the class B scavenger receptor CD36, class A scavenger receptor (SR-A), and mannose-binding lectin (MBL) recognize lipoteichoic acid (LTA) and facilitate phagocytic clearance of *S. aureus* (10). Recently, human MBL has been shown to bind *S. aureus* wall teichoic acid (WTA) to activate complement pathway (11).

To survive in the host, *S. aureus* evades host immune responses using several mechanisms, including resistance to specific antimicrobial peptides, neutralization of reactive oxygen species, inactivation of complement, inhibition of neutrophil migration, and evasion of phagocytosis (12, 13). Given that *S. aureus*-induced sepsis produces lower levels of inflammatory cytokines than does Gram-negative sepsis (3, 4), *S. aureus* must have additional strategies to dampen host inflammatory responses.

We have recently identified murine paired Ig-like receptor (PIR)-B (14, 15) as a novel macrophage receptor for *S. aureus* (16). PIR-B has four ITIMs in the cytoplasmic domain and inhibits activating signals by surface receptors (14, 15). PIR-B transcript is detected in spleen and bone marrow (14), and the cell surface expression is observed on various hematopoietic cells including macrophages, monocytes, granulocytes, B cells, and dendritic cells (15). Given that PIR-B suppresses TLR-mediated proinflammatory cytokine production in vitro (16), we hypothesized that *S. aureus* specifically targets the inhibitory PIR-B to dampen inflammatory responses. Likewise, *Moraxella catarrhalis* and *Neisseria meningitidis* bind ITIM-bearing carcinoembryonic Ag-
related cell adhesion molecule 1 to suppress TLR2-mediated inflammatory responses in human pulmonary epithelial cells (17). Group B Streptococcus also exploits ITIM-bearing Sia-recognizing Ig superfamily lectin-5 or -9 to suppress phagocytosis and oxidative burst in human leukocytes (18). These findings support the current hypothesis that pathogens may have evolved to target inhibitory receptors to survive in the host (19, 20). However, it remains unknown whether inhibitory PRRs are indeed beneficial for bacterial survival in vivo. Alternatively, these inhibitory PRRs may fine-tune inflammatory responses to perfectly balance bacterial clearance, whereas limiting tissue damage caused by excessive inflammation.

In this study, we sought to determine the in vivo role of PIR-B in host defense against S. aureus infection. Moreover, by screening various S. aureus mutant strains with defects in cell-wall components, we revealed the machinery underlying the interaction between PIR-B and this bacterium.

Materials and Methods

Mice

Six-week-old female C57BL/6 mice were obtained from Charles River Japan (Yokohama, Japan). PIR−/− mice (129/Sv/c57BL/6 background) (21) were backcrossed for at least 10 generations with C57BL/6 mice. β2-microglobulin (β2m)-deficient mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions and used according to the guidelines of the Institutional Animal Care and Use Committee established at Juntendo University and Tohoku University.

Bacterial mutant construction

In S. aureus T363 strain, the lgt gene, which encodes preprolipoprotein diacylglycerol transferase, has been disrupted in RN4220 cells by replacement with a Phelo resistance gene by double-crossover recombination as described previously (22) but using the pKOR1 plasmid to select for a recombinant mutant in S. aureus (23). In a brief, a DNA fragment upstream or downstream of the lgt gene was amplified using primers of lgt-Pla (5′-CTGGATCTACGAGACGCGCCTTAGAGATG-3′) and lgt-Pb (5′-GTC-TAATGAGATCTAAGTGACAGAGGAAC-3′) or lgt-Pl (5′-GTCGACGATC- CATGCGTCTCCATGCGAAACAAA-3′) and lgt-P4 (5′-ACGGACTTCG-CAGTGTCAATATTCCACTT-3′), respectively. The Phelo resistance gene was amplified with Phelo-P2 (5′-GGATCCATATGACGGAGTG-3′) and Phelo-P3 (5′-GAGAAGGACTGAGATACGAATTAGA-3′), containing sequences complementary to lgt-Plb and lgt-P3, respectively. Next, three amplified fragments were connected by joining PCR, and the resultant fragment was inserted into the KpnI and EcoRI sites of pKOR1 plasmid. The resultant plasmid was introduced into RN4220 cells, and double-crossover homologous recombinants were selected and named T363. Deletion of the chromosomal lgt gene was confirmed by PCR. S. aureus and Escherichia coli strains were grown in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) containing, where appropriate, 100 μg/ml ampicillin, 12.5 μg/ml chloramphenicol, 20 μg/ml phosphomycin, or 0.1 μg/ml anhydrotetracycline at the appropriate temperature.

Bacterial culture and fluorescent labeling

The strains of bacteria and plasmids used in this study are listed in Table I. The strains deficient in the expression of genes responsible for the synthesis of cell-wall components were derived of the wild-type (WT) strain RN4220 (24). RN4220, T363, and M0875 (25) were cultured in LB medium at 37 °C. T174 (26), M0674/pM101 (22), and M0674/pM101-ltaS (22) were cultured in LB medium supplemented with the appropriate antibiotics at 30 °C. M0793 (26), M0875 (26), and M0875 (26), Ni-1 (27), N143 (28), and JT1304 (29) were cultured in LB medium supplemented with appropriate antibiotics at 37 °C. After overnight growth, all S. aureus strains were diluted 1:50 (v/v) and were grown for 5 h. Then S. aureus were harvested and washed with PBS. The concentration of bacterial CFU was calculated as: OD600nm = 1, CFU = 4.5 × 109/ml. Bacteria were labeled with TAMRA (Invitrogen, Carlsbad, CA; 50 μg/ml in PBS) at 37 °C for 30 min and then washed with 5% FCS in PBS five times.

Measurement of cells recognizing S. aureus in vivo

Mice (n = 3/group) were i.v. injected with TAMRA-labeled S. aureus. One hour later, mice were sacrificed and splenocytes were prepared with col-lagenase digestion as described previously (30). TAMRA fluorescence intensity in CD11b+ F4/80+ (28), CD8+ CD11c+, CD8+ CD11c+, CD5+, and B220+ cells were analyzed by flow cytometry using FITC–anti-FITC1b, allopolyccyanin–anti-F4/80, allopolyccyanin–anti-CD8a, FITC–anti-CD11c, PE–anti-CD3, and allopolyccyanin-B220 mAbs (BioLegend, San Diego, CA).

Bacterial infection in vivo

Female WT and Pir−/− mice were i.v. injected with the indicated dose of bacteria and survival was monitored for 15 d. Mice were monitored daily and were sacrificed when moribund to avoid pain and suffering. For measurement of cytokine and bacterial CFU in blood, mice were i.v. injected with the indicated CFU of bacteria and sacrificed on day 1 or 2 postinfection. Blood was diluted 10-fold step with PBS containing 0.2% Triton X-100. Bacterial CFU were determined by plating dilutions on LB agar plates and culturing for 24 h. Serum was prepared from blood, and the amounts of IL-6 and IL-1β in serum was measured by ELISA.

Measurement of cytokines

The amount of IL-6 and IL-1β in culture supernatants and mouse serum were measured by the ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturers’ instructions.

Immunoblot

Bacterial infection in vivo

Bone marrow–derived macrophages (BMDMs) infected with S. aureus for the indicated periods were stabilized in RIPA buffer (1% Nonidet P-40, 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% deoxycholate, and 10% SDS, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM PMSF, aprotinin [1 μg/ml], and leupeptin [1 μg/ml]). Cell lysates were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA), followed by detection with Abs against MAPK family members (Cell Signaling, Boston, MA) or IκB-α (Santa Cruz, CA). For immunoprecipitation of PIR-B, cell lysates were precleared with protein G (GE Healthcare Piscataway, NJ), then incubated with 6C1 (31), followed by protein G. Phosphorylation levels of PIR-B were analyzed using 4G10 (Millipore, Bedford, MA) and anti-PIR Abs (R&D Systems). For analysis of IL-1β and caspase-1 processing, immunoblots were performed as described previously (9) with minor modifications. In brief, WT and Pir−/− BMDMs were seeded on six-well plates and cultured overnight. After replacing the media with serum-free media containing M-CSF, cells were cultured with S. aureus RN4220 (10 multiplicity of infection [ moi]) for the indicated periods of time. Culture supernatants and total cell lysates were pooled and then clarified by centrifugation. Proteins were precipitated with Stratatecin resin (Strategene, La Jolla, CA) and detected by immunoblot with anti-IL-1β Ab (R&D systems), anti-caspase-1 Ab (Santa Cruz), and β-actin mAb (Bio-Legend).

Cell lines

HEK293 cells and NIH3T3 cells (American Type Culture Collection, Rockville, MD) were maintained in complete DMEM supplemented with 10% FBS and 2 mM glutamine. The coding region of PIR-B was amplified using 4G10 (Millipore, Bedford, MA) and anti-PIR Abs (R&D Systems). For analysis of IL-1β and caspase-1 processing, immunoblots were performed as described previously (9) with minor modifications. In brief, WT and Pir−/− BMDMs were seeded on six-well plates and cultured overnight. After replacing the media with serum-free media containing M-CSF, cells were cultured with S. aureus RN4220 (10 multiplicity of infection [ moi]) for the indicated periods of time. Culture supernatants and total cell lysates were pooled and then clarified by centrifugation. Proteins were precipitated with Stratatecin resin (Strategene, La Jolla, CA) and detected by immunoblot with anti-IL-1β Ab (R&D systems), anti-caspase-1 Ab (Santa Cruz), and β-actin mAb (Bio-Legend).

Table I. S. aureus strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotypes and Characteristics</th>
<th>References</th>
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<tbody>
<tr>
<td>RN4220</td>
<td>NCTC8325-4, restriction mutant</td>
<td>(24)</td>
</tr>
<tr>
<td>T174</td>
<td>RN4220 tagC:pT0702</td>
<td>(26)</td>
</tr>
<tr>
<td>M0674/pM101</td>
<td>RN4220 ltaS:phleo harboring</td>
<td>(22)</td>
</tr>
<tr>
<td>M0674/pM101-ltaS</td>
<td>RN4220 ltaS:phleo harboring</td>
<td>(22)</td>
</tr>
<tr>
<td>M0793</td>
<td>RN4220 dltA:pT0793</td>
<td>(26)</td>
</tr>
<tr>
<td>M0875</td>
<td>RN4220 ypfP:pT0875</td>
<td>(26)</td>
</tr>
<tr>
<td>NI-1</td>
<td>RN4220 mprF:erm</td>
<td>(27)</td>
</tr>
<tr>
<td>N043</td>
<td>RN4220 sle1:pMsle1</td>
<td>(28)</td>
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<td>T1304</td>
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<td>M0587</td>
<td>RN4220 sVc:phleo</td>
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</tr>
<tr>
<td>T363</td>
<td>RN4220 lgt:phleo</td>
<td>This Study</td>
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were obtained in at least three independent experiments.

In brief, HEK293 cells, PIR-B/HEK293 cells, and CD36/NIH3T3 cells were incubated with the indicated dose of the fluorescently labeled S. aureus for 30 min at 37°C. After incubation with S. aureus, cells were washed with PBS twice, then harvested and analyzed with a FACS-Canto II (BD Biosciences). For competitive inhibition assays, cells were pretreated with the indicated dose of LTA (InvivoGen, San Diego, CA) for 30 min at 37°C.

Measurement of cells recognizing S. aureus in vitro

Binding of S. aureus to PIR-B was analyzed as described previously (16). In brief, NIH3T3 cells, PIR-B/NIH3T3 cells, and mutant form (PSYDR-G119E, P210A) of PIR-B/NIH3T3 cells were incubated with the indicated dose of the fluorescently labeled S. aureus for 30 min at 37°C. After incubation with S. aureus, cells were washed with PBS twice, then harvested and analyzed with a FACS-Canto II (BD Biosciences). For competitive inhibition assays, cells were pretreated with the indicated dose of LTA (InvivoGen, San Diego, CA) for 30 min at 37°C.

Binding of LTA to PIR-B

NIH3T3 cells, PIR-B/NIH3T3 cells, and CD36/NIH3T3 cells were incubated with or without 50 μg/ml LTA (InvivoGen) for 30 min at 4°C. Cells were then stained with anti-LTA mAb (HyCell Biotech, Uden, The Netherlands), followed by biotinylated anti-mouse IgG3 mAb (BioLegend) and streptavidin-PE (BioLegend), and analyzed on a FACS-Canto II (BD Biosciences).

Statistical analyses

Statistical significance was analyzed with two-tailed Student's t test. Data for survival were analyzed according to the Kaplan–Meier method, and the univariate comparison of survival for control versus knockout group was tested using a log-rank test. The p values <0.05 were considered significantly different between comparing samples.

Results

S. aureus targets PIR-B for virulence in vivo

To address the in vivo role of PIR-B in the recognition of bacteria, we injected WT or Pirb−/− mice i.v. with fluorescently labeled live S. aureus. These bacteria are preferentially recognized by F4/80+ CD11b+ splenic macrophages in WT mice, and macrophage rec-

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ogition of *S. aureus* was significantly impaired in *Pirb*<sup>−/−</sup> mice (Fig. 1A, 1B). These results suggest that splenic macrophages effectively access bacterial particles from the passing bloodstream, and that PIR-B plays a role in the direct recognition of *S. aureus* in vivo. To address whether PIR-B has the capacity to internalize bacteria, we performed an in vitro phagocytosis assay using a 293 cell reconstitution system (30). Expression of PIR-B on 293 cells enabled these cells to bind, but not internalize, bacteria (Supplemental Fig. 1), suggesting that PIR-B is a binding, but not a phagocytic, receptor for *S. aureus*.

Although PIR-B negatively regulates inflammatory cytokine production in response to heat-killed *S. aureus* in vitro (16), it remains unknown whether the innate immune suppression by PIR-B is beneficial to the host or advantageous for pathogen survival. Thus, we next addressed this issue. Because *S. aureus* could spread to the bloodstream and lead to life-threatening sepsis (6), WT and *Pirb*<sup>−/−</sup> mice were i.v. infected with various doses of *S. aureus*, and their survival was monitored. When infected with 7 × 10<sup>7</sup> CFU *S. aureus*, all WT and *Pirb*<sup>−/−</sup> mice survived to day 15 (data not shown). Interestingly, on infection with 2 × 10<sup>8</sup> CFU of the bacteria, ∼30% of WT mice died, whereas all *Pirb*<sup>−/−</sup> mice survived to day 15 (Fig. 2A). A more distinct survival difference was observed when mice were infected with a high dose of bacteria (6 × 10<sup>8</sup> CFU); only 15% of WT mice survived, whereas >80% of *Pirb*<sup>−/−</sup> mice survived to day 15 (Fig. 2B). Although no significant difference was observed in blood bacterial number between WT and *Pirb*<sup>−/−</sup> mice at the early time point (5 h) postinfection, *Pirb*<sup>−/−</sup> mice showed effective clearance of bacteria in blood (Fig. 2C, 2D) and spleen (data not shown) on d postinfection, consistent with the low mortality of *Pirb*<sup>−/−</sup> mice. In addition, IL-6 and IL-1β levels were increased in the serum of *Pirb*<sup>−/−</sup> mice infected with *S. aureus* (Fig. 2E). Thus, the loss of PIR-B resulted in enhanced proinflammatory cytokine production. On i.p. infection with 3 × 10<sup>9</sup> CFU *S. aureus*, all WT and *Pirb*<sup>−/−</sup> mice survived to day 15, but *Pirb*<sup>−/−</sup> mice showed the effective clearance of bacteria in peritoneal fluid on day 1 postinfection (data not shown). Taken together, the lack of negative feedback via PIR-B may accelerate bacterial clearance. These results suggest that PIR-B, an inhibitory PRR, is exploited by *S. aureus* for its survival and virulence.

Enhanced ERK1/2 and caspase-1 activation in *Pirb*<sup>−/−</sup> macrophages postinfection with live *S. aureus*

We next addressed intracellular signaling in BMDMs infected with live *S. aureus*. Consistent with in vivo results, BMDMs from *Pirb*<sup>−/−</sup> mice produced higher amounts of IL-6 and IL-1β in response to live *S. aureus* (Fig. 3A). Although ITIMs of PIR-B are constitutively phosphorylated in macrophages (34), infection with live *S. aureus* constitutively phosphorylated in macrophages (34), infection with live *S. aureus* resulted in enhanced phosphorylation of ERK1/2 (Fig. 3B). Because PIR-B has been reported to bind MHC class I molecules (15), we next examined whether loss of MHC class I affects the interaction between PIR-B and *S. aureus*. We first verified that lack of cell surface expression of MHC class I H-2K<sup>b</sup> or PIR-B did not affect each other’s expression on BMDMs (Fig. 4A). We further observed no substantial difference in *S. aureus* recognition (Fig. 4B) or cytokine production (Fig. 4C) in response to *S. aureus* between WT BMDMs and β2m-deficient BMDMs. These results indicate that MHC class I molecules do not affect the PIR-B–mediated manipulation of inflammatory cytokine release in macrophages infected with *S. aureus*.

* S. aureus binds PIR-B through the LTA and suppresses macrophage inflammatory responses to bacterial lipoprotein

To explore the molecular mechanism for the interaction between PIR-B and *S. aureus*, we screened various *S. aureus* mutant strains...
FIGURE 4. MHC class I molecules do not affect the interaction between S. aureus and PIR-B. (A) WT, Pirb<sup>−/−</sup>, or β<sub>m</sub><sup>−/−</sup> BMDMs were stained with isotype control mAb (thin histogram) or mAb against the indicated cell surface protein (thick histogram) and analyzed by flow cytometry. (B) WT, Pirb<sup>−/−</sup>, or β<sub>m</sub><sup>−/−</sup> BMDMs were cultured with fluorescently labeled S. aureus (0.2, 1, or 5 moi) for 30 min at 4°C. Cells were then analyzed by flow cytometry. (C) WT, Pirb<sup>−/−</sup>, β<sub>m</sub><sup>−/−</sup> BMDMs were infected with S. aureus at 3 or 10 moi for 12 h at 37˚C. Induction of IL-6 and IL-1β was measured by ELISA. Similar results were obtained in two (A, B) and three (C) independent experiments.

We next addressed macrophage responses to the LTA-deficient mutant strain, ΔltaS. PIR-B phosphorylation was not enhanced in response to this mutant strain (Fig. 5E), which is consistent with the defect in PIR-B binding by ΔltaS (Fig. 5B). Notably, WT BMDMs secreted higher amounts of IL-6 and IL-1β when infected with ΔltaS versus the parental RN4220; IL-6 and IL-1β production in ΔltaS-infected WT BMDMs were equivalent to those observed in Pirb<sup>−/−</sup> BMDMs (Fig. 5F). To confirm that the inhibitory effect on WT BMDMs was ascribed to LTA, we expressed the ltaS gene in our ΔltaS mutant strain (ΔltaS/ΔltaS<sup>+</sup>) and found that reconstitution of ltaS expression suppressed cytokine production by WT BMDMs (Fig. 5F). Consistent with this, the ΔltaS/ΔltaS<sup>+</sup> strain enhanced PIR-B phosphorylation in BMDMs (Fig. 5E), further indicating that LTA is required for PIR-B interaction. LTA deficiency did not affect cytokine production from Pirb<sup>−/−</sup> macrophages. Consistent with increased IL-1β secretion, the ΔltaS mutant induced comparable caspase-1 activation and IL-1β processing in WT and Pirb<sup>−/−</sup> BMDMs, as seen by bands of similar intensity for the cleaved caspase-1 p10 subunit and mature IL-1β (p17) (Fig. 5G). These results suggest that LTA is an essential cell-wall component of S. aureus that binds to PIR-B and consequently suppresses inflammatory responses in BMDMs.

LTA has been considered to be a ligand for TLR2; however, recent studies have questioned an inflammatory role for LTA from S. aureus, because a considerable amount of the activity in LTA preparations has been assigned to lipoprotein contamination, even...
in highly purified samples (10, 25, 37). We and others have recently reported that lipoprotein lipidation-deficient (Δlgt) S. aureus is unable to stimulate TLR2, indicating that lipoprotein is a major TLR2 ligand (25, 37). Consistent with these reports, Δlgt S. aureus failed to induce inflammatory cytokine production either from WT or Pirb^-/- BMDMs (Fig. 6). Taken together, these

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**FIGURE 5.** S. aureus binds PIR-B through LTA and suppresses macrophage inflammatory responses. (A) The strains of S. aureus used in this study and their phenotypes. (B) NIH3T3, PIR-B/NIH3T3, and PIR-B mutant/NIH3T3 cells were cultured with the indicated live TAMRA-labeled S. aureus strains (moi 50 each) for 30 min at 37˚C. S. aureus recognition was analyzed by flow cytometry. Percentage of recognition was quantitated, and data are represented as the mean + SD of triplicates. (C) NIH3T3, PIR-B/NIH3T3, and CD36/ NIH3T3 cells were cultured with (thick histograms) or without (thin histograms) LTA (50 μg/ml) for 30 min at 4˚C. LTA binding to cells was analyzed by flow cytometry using anti-LTA mAb. (D) NIH3T3 cells expressing PIR-B, SR-A, or CD36 were pretreated with the indicated dose of LTA, then cultured with TAMRA-labeled S. aureus as described in (B). The recognition (relative to recognition without LTA) was quantified by flow cytometry. Data are represented as the mean + SD of triplicates. (E) PIR-B phosphorylation in WT BMDMs cultured with the indicated S. aureus strain (moi 10 each) was analyzed as described in Fig. 3B. (F and G) BMDMs were cultured with the indicated S. aureus strain (moi 3 or 10 [F]; moi 10 [G]) for 12 h (F, G) or 24 h (G) at 37˚C. Production of IL-6 and IL-1β was analyzed by ELISA (F). Maturation of IL-1β and caspase-1 were analyzed by immunoblot (G). Similar results were obtained in at least three independent experiments.
results suggest that *S. aureus* targets PIR-B via LTA, which negatively regulates lipoprotein-mediated proinflammatory cytokine production.

Uncontrolled LTA-deficient *S. aureus* growth in both WT and Pirb−/− mice in vivo

We next compared the susceptibility of WT and Pirb−/− mice to ΔltaS infection. Although Pirb−/− mice were resistant to WT *S. aureus* infection (Fig. 2A–D), no significant difference was observed in susceptibility to ΔltaS infection (1 × 10^8 or 3 × 10^8 CFU) between WT and Pirb−/− mice (Fig. 7A, 7B). These data suggest that the interaction between bacterial LTA and host PIR-B affects mouse susceptibility to *S. aureus* infection. Although we hypothesized the ΔltaS mutant to be less pathogenic because of diminished ligation of the inhibitory PIR-B, WT mice were unexpectedly more susceptible to ΔltaS than ΔltaS/ΔltaS+ (Fig. 7C). Further, the ΔltaS mutant survived more than ΔltaS/ΔltaS+ in the blood of WT mice (Fig. 7D). To uncover the mechanism for this heightened virulence, we examined recognition of the ΔltaS mutant by splenic macrophages. WT splenic macrophages recognized this mutant poorly, at levels less than those observed for recognition of the ΔltaS/ΔltaS+ strain by Pirb−/− macrophages (Fig. 7E, 7F).

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![Diagram](http://www.jimmunol.org/)

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7F). This is probably due to the fact that \textit{S. aureus} lacking LTA failed to be recognized by not only PIR-B, but also a wide variety of PRRs such as SR-A and MBL that accelerate the elimination of bacteria (10). Indeed, SR-A failed to recognize the \( \Delta \text{ltas} \) mutant (Fig. 7G). The highly virulent phenotype of \( \Delta \text{ltas} \) therefore appears to be caused by escape from host innate immune recognition in mice, a conclusion supported by studies in \textit{Drosophila} (38). Collectively, these results suggest that \textit{S. aureus} targets PIR-B through LTA to acquire virulence, whereas the host defense system also targets LTA by using a wide variety of PRRs, in addition to the inhibitory PIR-B, to bring about effective bacterial clearance and overcome infection (Fig. 7H).

Discussion

We demonstrate in this study that \textit{S. aureus} exploits PIR-B, an inhibitory PRR, to dampen inflammatory responses and survive in the host. Our findings reinforce the current hypothesis that pathogens target paired inhibitory receptors to dampen immune responses against them (19, 20). We further identified LTA as an essential cell-wall component of \textit{S. aureus} that binds PIR-B and, subsequently, modulates inflammatory responses. Furthermore, we showed that a lipoprotein lipidation-deficient \( \Delta \text{ltg} \) mutant failed to induce inflammatory cytokine release either from WT or \( \text{Pirb}^{-/-} \) BMDMs. Collectively, these results indicate that \textit{S. aureus} targets PIR-B through LTA to dampen bacterial lipoprotein-mediated inflammatory responses and thus increase virulence. Given that PIR-B does not bind to \textit{Listeria monocytogenes} or \textit{Bacillus subtilis} (16) even though their cell walls contain LTA, and that the affinity of purified LTA to PIR-B is low, we cannot rule out the possibility that PIR-B may require not only LTA but also simultaneous recognition of some other \textit{S. aureus} components for binding.

Contrary to our hypothesis that the LTA-deficient mutant would be less pathogenic because of impaired stimulation of PIR-B, the LTA-deficient mutant exhibited a highly virulent phenotype. Given that LTA is the bacterial ligand for a wide variety of PRRs (10), the highly virulent phenotype of the \( \Delta \text{ltas} \) mutant may be caused by escape from phagocytic clearance. Therefore, LTA is a key molecule in the host–microbe interaction where two conflicting immune reactions intersect.

Interestingly, \( \text{Pirb}^{-/-} \) BMDMs showed enhanced caspase-1 activation, suggesting that PIR-B negatively regulates inflammatory activation in response to \textit{S. aureus} infection. Notably, it has been reported that an ITAM receptor activates inflammatory signaling through Syk (39, 40). Although ITAM receptors capable of recognizing nonopsonized \textit{S. aureus} remain to be identified, PIR-B may negatively regulate the signaling through such an activating receptor. Besides macrophages, monocytes and neutrophils, which play an important role for bacterial clearance in blood, express PIR-B on their cell surface (12, 13). Thus, PIR-B may also suppress these immune cell functions upon \textit{S. aureus} bloodstream infections.

Is the inflammatory response beneficial for host defense against bacterial infection? This study using PIR-B-deficient mice showed that enhanced inflammation was effective for the clearance of WT \textit{S. aureus}, but not for the \( \Delta \text{ltas} \) mutant strain that escaped macrophage recognition. Therefore, as long as bacteria are normally recognized and phagocytosed by macrophages, enhanced inflammation could be beneficial for host defense, at least in the case of Gram-positive sepsis that causes relatively mild inflammation. Consistent with this notion, TLR2- and CD36-deficient mice develop less inflammation and are more susceptible to \textit{S. aureus} infection than are WT counterparts (41, 42). Conversely, in Gram-negative infections that cause marked inflammation, these robust inflammatory responses must be regulated to prevent host damages. Indeed, Roger et al. (43) have reported that mice deficient for TLR4 or MyD88 are resistant to Gram-negative \textit{E. coli}–induced septic shock. Although the inhibitory receptor CD200R is not a PRR, mice deficient for its ligand CD200 produce enhanced inflammatory cytokines in response to Gram-negative \textit{Neisseria meningitidis} infection, and these mice succumb to infection (44). PIR-B–deficient mice also show enhanced inflammatory responses and are susceptible to Gram-negative \textit{Salmonella} infection (45). Thus, the immune system must fine-tune the level of inflammation to achieve effective clearance of bacteria without causing host damage.

Interestingly, it has also been proposed that pathogens exert pressure that drives the evolution of paired receptors (19). Notably, although PIR-A has only a short cytoplasmic region, three ITIM-like motifs are preserved as relics in the 3′-untranslated region (Supplemental Fig. 2), suggesting that inhibitory PIR-B was the original receptor, and that multiple forms of PIR-A might have evolved from an ancestral PIR-B in an effort to try to buffer control immune responses.

In this study, we have shown that \textit{S. aureus} exploits PIR-B for virulence. PIR-B, which is not expressed in humans, has been proposed to be a human ortholog of ITIM-bearing Ig-like transcript (ILT)/leukocyte Ig-like receptor family members, based on similarities in structure, expression profiles, and genomic location (46). We have previously reported that the ectopic expression of ILT2 or ILT5 on \( \text{NH}3 \text{T}3 \) cells enables these cells to recognize \textit{S. aureus} (16), and we further observed the expression of both ILT2 and ILT5 on human peripheral monocytes that efficiently recognize \textit{S. aureus} (data not shown). However, it remains unknown whether these ILT receptors are involved in \textit{S. aureus} recognition by monocytes. It also remains unknown whether another ITIM-bearing ILT such as ILT3, ILT4, and leukocyte Ig-like receptor 8 contribute to the recognition, and/or collaborate with each other in the efficient recognition of bacteria. Further human study using neutralizing mAbs or small interfering RNA will be required to address these issues. If \textit{S. aureus} targets these ITIM-bearing receptors on human macrophages and monocytes for virulence, these inhibitory receptors could be novel therapeutic targets for sepsis.

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Disclosures

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