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V. 研究成果の刊行物・別刷

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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,[†] Kazuhisa 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, *Pirb*$^{-/-}$ 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 *Pirb*$^{-/-}$ 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 counterbalances the infection. ***The Journal of Immunology*, 2012, 189: 5903–5911.**

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-positive 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-

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The online version of this article contains supplemental material.

Abbreviations used in this article: BMDM, bone marrow–derived macrophage; ILT, Ig-like transcript; LB, Luria–Bertani; LTA, lipoteichoic acid; β_2m, β_2-microglobulin; MBL, mannose-binding lectin; moi, multiplicity of infection; PIR, paired Ig-like receptor; PRR, pattern-recognition receptor; SR-A, class A scavenger receptor; TAMRA, 5-(and-6)-carboxytetramethylrhodamine; WT, wild-type; WTA, wall teichoic acid.

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). *Pirb*$^{-/-}$ mice (129/SvJ/C57BL/6 background) (21) were backcrossed for at least 10 generations with C57BL/6 mice. β_2-*microglobulin* (β_2*m*)–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 Phleo 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 brief, a DNA fragment upstream or downstream of the *lgt* gene was amplified using primers of *lgt*-Pa (5′-CTGAGGTACCAAGACCGGGCTTAGAGATGG-3′) and *lgt*-Pb (5′-GTCTATTGGATCCTAAGTTAAATGCCACAGGA-3′) or *lgt*-P3 (5′-GTTCAGCAATCGCTTCCATGGCCAACAAAA-3′) and *lgt*-P4 (5′-ACCGAATTCGCATGTCCAATTTCCACTT-3′), respectively. The Phleo resistance gene was amplified with Phleo-P2 (5′-GGATCCAATAGACCAGTTGCA-3′) and Phleo-P3 (5′-CGATTGCTGAACAGATTAATAATAGA-3′), containing sequences complementary to *lgt*-Pb and *lgt*-P3, respectively. Next, three amplified fragments were connected by joining PCR, and the resultant fragment was inserted at 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 phleomycin, 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 derivates of the wild-type (WT) strain RN4220 (24). RN4220, T363, and M0587 (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), NI-1 (27), NI43 (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: OD_{600} = 1, CFU = 4.5 × 10^8/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 collagenase digestion as described previously (30). TAMRA fluorescence intensity in $CD11b^+$ $F4/80^+$, $CD8^+$ $CD11c^+$, $CD8^-$ $CD11c^+$, $CD3^+$, and $B220^+$ cells were analyzed by flow cytometry using FITC–anti-CD11b, allophycocyanin–anti-F4/80, allophycocyanin–anti-CD8α, FITC–anti-CD11c, FITC–anti-CD3, and allophycocyanin-B220 mAbs (BioLegend, San Diego, CA).

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

Names	Genotypes and Characteristics	References
RN4220	NCTC8325-4, restriction mutant	(24)
T174	RN4220 *tagO*::pT0702	(26)
M0674/pM101	RN4220 *ltaS*::*phleo* harboring pM101	(22)
M0674/pM101-*ltaS*	RN4220 *ltaS*:: *phleo* harboring pM101-*ltaS*	(22)
M0793	RN4220 *dltA*::pT0793	(26)
M0875	RN4220 *ypfP*::pT0875	(26)
NI-1	RN4220 *mprF*::*erm*	(27)
NI43	RN4220 *sle1*::pM*sle1*	(28)
JT1304	RN4220 *atl*::*cat*	(29)
M0587	RN4220 *sitC*::*phleo*	(25)
T363	RN4220 Igt::phleo	This study

Bacterial infection in vivo

Female WT and *Pirb*$^{-/-}$ mice were i.v. infected 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. infected 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 were 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

Bone marrow–derived macrophages (BMDMs) infected with *S. aureus* for the indicated periods were solubilized 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 *Pirb*$^{-/-}$ 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 Strataclean resin (Stratagene, 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 (BioLegend).

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 subcloned into pEF6V5-TOPO (Invitrogen). The coding regions for FcγRIII, FcRγ, CD36, and SR-A were amplified from C57BL/6 BMDM cDNAs, then inserted into pEF6V5-TOPO or pMXs-IRES-Puro (provided by Toshio Kitamura, University of Tokyo). PIR-B/NIH3T3 cells, and mutant form (PSYDR-G119E, P210A) of PIR-B/NIH3T3 cells, FcRγ/HEK293 cells, CD36/NIH3T3 cells, and SR-A/NIH3T3 cells were generated by retroviral transduction as described previously (16). PIR-B/pEF6V5-TOPO or

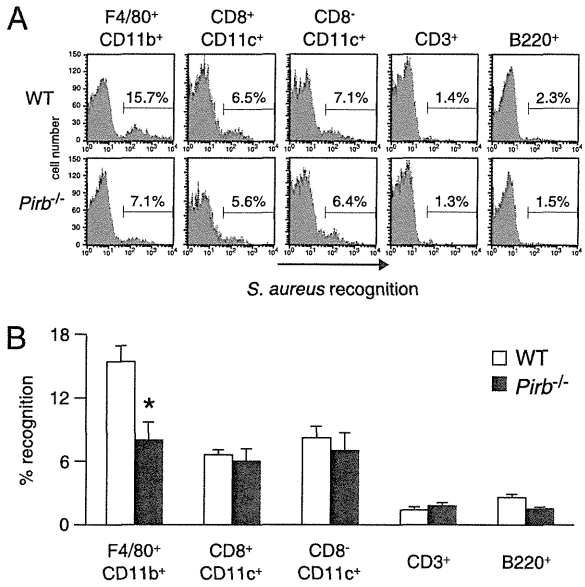

FIGURE 1. *S. aureus* targets PIR-B on splenic macrophages in vivo. (**A** and **B**) WT and $Pirb^{-/-}$ mice (n = 3 per group) were i.v. injected with TAMRA-labeled live *S. aureus* (3×10^8 CFU per mouse). Recognition of bacteria by the indicated splenic cell subsets was analyzed 1 h later (A). Percentage of recognition was quantified, and data are represented as mean + SD in (B). *$p < 0.05$, two-tailed Student t test. Similar results were obtained in at least three independent experiments.

FcγRIII/pEF6V5-TOPO was transfected into HEK293 cells or FcRγ/HEK293 cells, respectively, using Lipofectamine 2000 (Invitrogen).

Phagocytosis assay by trypan blue quenching system

Parental HEK293 cells and 293 cells stably expressing PIR-B or FcγRIII plus Fcγ common chain (10^5 cells/well) were plated onto a poly-L-lysine (Sigma-Aldrich, St. Louis, MO)–precoated 24-well plate 1 d before the phagocytosis assay. Cell lines were incubated with FITC-labeled *S. aureus* (10 moi). For FcR-mediated phagocytosis, FITC-labeled *S. aureus* were opsonized with anti-FITC mAb ($mIgG_{1/k}$; BioLegend). FITC fluorescence intensity was quenched by trypan blue as described previously (32). In brief, the fluorescence of extracellular bacteria was quenched by replacing the medium with 0.2% trypan blue in PBS (pH 5.5); then cells were analyzed on a FACSCalibur (BD Biosciences).

Confocal microscopy

HEK293 cells cocultured with FITC-labeled bacteria were permeabilized with 0.1% Triton X-100 and stained with AF594-phalloidin (0.15 μM; Invitrogen). After fixation with 4% paraformaldehyde, cells were analyzed on a Carl Zeiss confocal laser-scanning microscope LSM510 equipped with ×40 objective lens, as described previously (33).

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, CD36/NIH3T3 cells, and SR-A/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 FACSCanto II (BD Biosciences, San Jose, CA). 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 (Hycult Biotech, Uden, The Netherlands), followed by biotinylated anti-mouse IgG_3 mAb (BioLegend) and streptavidin-PE (BioLegend), and analyzed on a FACSCanto II (BD Biosciences).

Binding of S. aureus *to BMDMs*

WT, $Pirb^{-/-}$, and $\beta_2m^{-/-}$ mouse BMDMs (2×10^5 per well) were seeded on 24-well plate and cultured overnight. BMDMs were cultured with TAMRA-labeled *S. aureus* RN4220 (0.2, 1, or 5 moi) for 30 min at 4°C. Cells were then washed twice with PBS, harvested, and analyzed on a FACSCanto II (BD Biosciences).

Statistical analyses

Statistical significance was analyzed with two-tailed Student 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-

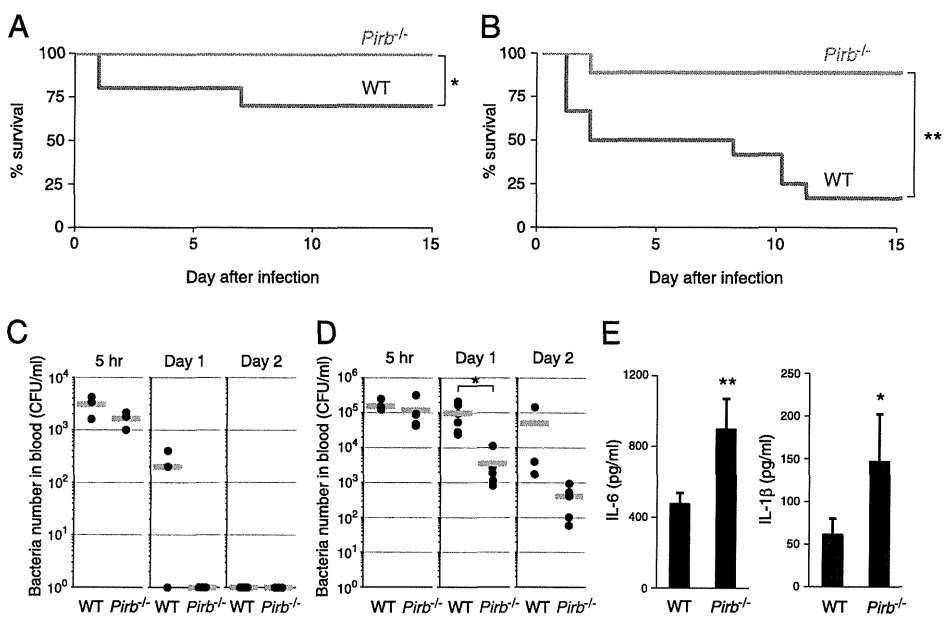

FIGURE 2. $Pirb^{-/-}$ mice are less susceptible to *S. aureus* infection. (**A** and **B**) WT mice (n = 10 [A] or n = 12 [B]) and $Pirb^{-/-}$ mice (n = 10 [A] or n = 9 [B]) were i.v. infected with *S. aureus* (2×10^8 CFU [A] or 6×10^8 CFU [B] per mouse). Mouse survival was monitored for 15 d postinfection. (**C** and **D**) WT mice (n = 3 [C] or n = 6 [D]) and $Pirb^{-/-}$ mice (n = 3 [C] or n = 5 [D]) were i.v. infected with *S. aureus* (1×10^8 CFU [C] or 5×10^8 CFU [D] per mouse). Bacterial number in blood on 5 h, day 1, and day 2 postinfection was determined. Three WT mice were dead on day 2 in (D). (**E**) WT and $Pirb^{-/-}$ mice were i.v. infected with *S. aureus* (5×10^8 CFU per mouse). The following day, IL-6 and IL-1β in blood were measured by ELISA. *$p < 0.05$, **$p < 0.01$, log-rank test (A, B) or two-tailed Student t test (D, E). Similar results were obtained in at least three independent experiments.

ognition of *S. aureus* was significantly impaired in *Pirb*$^{-/-}$ 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*$^{-/-}$ mice were i.v. infected with various doses of *S. aureus*, and their survival was monitored. When infected with 7×10^7 CFU *S. aureus*, all WT and *Pirb*$^{-/-}$ mice survived to day 15 (data not shown). Interestingly, on infection with 2×10^8 CFU of the bacteria, ~30% of WT mice died, whereas all *Pirb*$^{-/-}$ mice survived to day 15 (Fig. 2A). A more distinct survival difference was observed when mice were infected with a high dose of bacteria (6×10^8 CFU); only 15% of WT mice survived, whereas >80% of *Pirb*$^{-/-}$ mice survived to day 15 (Fig. 2B). Although no significant difference was observed in blood bacterial number between WT and *Pirb*$^{-/-}$ mice at the early time point (5 h) postinfection, *Pirb*$^{-/-}$ mice showed effective clearance of bacteria in blood (Fig. 2C, 2D) and spleen (data not shown) on 1 d postinfection, consistent with the low mortality of *Pirb*$^{-/-}$ mice. In addition, IL-6 and IL-1β levels were increased in the serum of *Pirb*$^{-/-}$ mice infected with *S. aureus* (Fig. 2E). Thus, the loss of PIR-B resulted in enhanced proinflammatory cytokine production. On i.p. infection with 3×10^8 CFU *S. aureus*, all WT and *Pirb*$^{-/-}$ mice survived to day 15, but *Pirb*$^{-/-}$ 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$^{-/-}$ *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*$^{-/-}$ 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* enhanced the phosphorylation (Fig. 3B). We next addressed MAPK and NF-κB activation in macrophages infected with live *S. aureus*. Although p38K and NF-κB activation were similar between *Pirb*$^{-/-}$ and WT macrophages, the phosphorylation level of ERK1/2 was markedly enhanced in *Pirb*$^{-/-}$ macrophages (Fig. 3C). We also analyzed pro–IL-1β production and caspase-1 activation, as the latter contributes to the processing of pro–IL-1β to mature IL-1β (35). Interestingly, *Pirb*$^{-/-}$ BMDMs showed not only enhanced pro–IL-1β (p35) production, but also elevated caspase-1 activation, as indicated by increased amounts of the cleaved p10 subunit of caspase-1 (Fig. 3D). Collectively, these results suggest that *S. aureus* targets PIR-B and negatively regulates ERK1/2 activation and inflammasome activation to dampen inflammatory cytokine secretion. Because it has been reported that inflammatory cytokine production depends on phagosome maturation (36), we examined phagosome/lysosome maturation in *Pirb*$^{-/-}$ BMDMs by confocal microscopy. However, we could not see the significant difference in colocalization of fluorescently labeled bacteria and lysosomes between WT and *Pirb*$^{-/-}$ BMDMs (data not shown), suggesting that PIR-B does not regulate phagosome maturation during *S. aureus* infection.

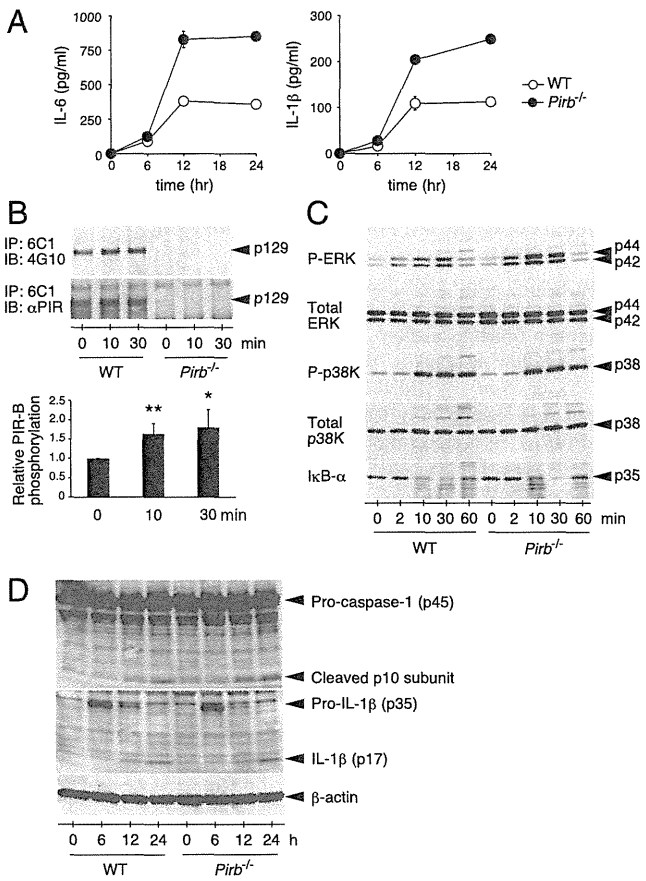

FIGURE 3. *Pirb*$^{-/-}$ macrophages show enhanced inflammatory responses to live *S. aureus*. (**A**) BMDMs from WT and *Pirb*$^{-/-}$ mice were cultured with *S. aureus* (moi 10) for the indicated periods of time. Production of IL-6 and IL-1β was measured by ELISA. (**B**) BMDMs were cultured with *S. aureus* as described in (A) for the indicated periods of time. PIR-B in total cell lysates was immunoprecipitated with 6C1 mAb. Phosphotyrosine and total protein levels of PIR-B were analyzed by immunoblot. Signal intensity of tyrosine-phosphorylated PIR-B proteins was normalized to the intensity of total PIR-B protein and was then compared with the control. $*p < 0.05$, $**p < 0.01$, two-tailed Student *t* test. (**C** and **D**) BMDMs were cultured with *S. aureus* as described in (A) for the indicated period of time. ERK1/2 and p38K phosphorylation, IκB-α degradation, and maturation of IL-1β and caspase-1 were analyzed by immunoblot. Similar results were obtained in at least three independent experiments.

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^b 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 β_2m-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

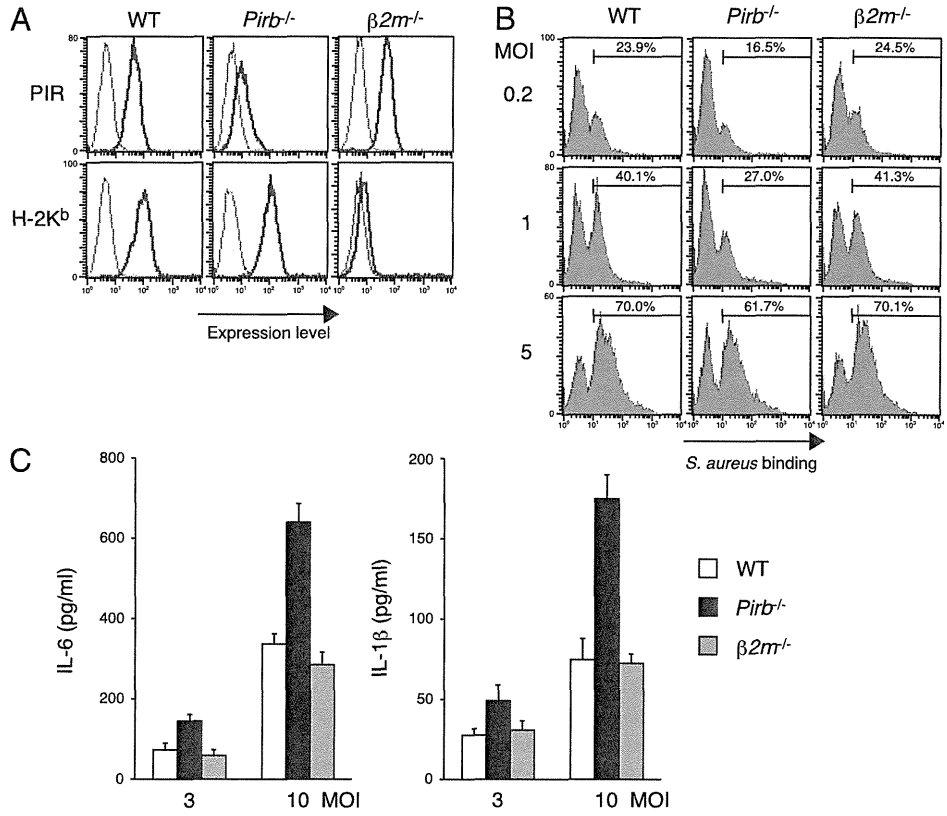

FIGURE 4. MHC class I molecules do not affect the interaction between *S. aureus* and PIR-B. (**A**) WT, $Pirb^{-/-}$, or $\beta_2m^{-/-}$ BMDMs were stained with isotype control mAb (thin histogram) or mAb against the indicated cell surface protein (thick histogram) and analyzed by flow cytometry. (**B**) WT, $Pirb^{-/-}$, or $\beta_2m^{-/-}$ 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, $Pirb^{-/-}$, $\beta_2m^{-/-}$ 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.

(listed in Fig. 5A, Table I) for their ability to bind PIR-B. The panel of mutants includes multiple strains with defects in genes responsible for the synthesis of specific cell-wall components. Each live mutant was labeled with TAMRA. After confirmation that fluorescent labeling did not affect bacterial survival by the CFU assay, and that the fluorescence intensity of each strain was largely equivalent by flow cytometry (data not shown), we analyzed the binding of bacteria to PIR-B. Expression of PIR-B enabled NIH3T3 cells to bind live WT *S. aureus*, and the binding was dramatically abrogated by a mutation of PIR-B in the surface loop of Ig-like domain 2 (Fig. 5B), which is consistent with our previous study using heat-killed bacteria (16). Notably, among the various mutant bacterial strains tested, only the LTA-deficient mutant (Δ*ltaS*) failed to bind PIR-B (Fig. 5B). Staphylococcal LTA consists of ~25 repeating units of poly (1-3)-glycerophosphate linked to a membrane lipid, and the Δ*ltaS* mutation causes a loss in these extracellular repeating units (22). This negatively charged physical feature may be important for the interaction between *S. aureus* and PIR-B. Mutant bacterial strains with defects in WTA (Δ*tagO*), D-alanylation of both WTA and LTA (Δ*dltA*), glycolipids (Δ*ypfP*), or lysylphosphatidylglycerol (Δ*mprF*), as well as the parental RN4220 strain, bound to PIR-B. A loss of expression of *sle1* and *atl*, which code for amidase and an amidase-glucosamidase fusion protein involved in the degradation of peptidoglycan on the bacterial cell surface, respectively, did not affect the bacterial binding to PIR-B (Fig. 5B). The binding of PIR-B to mutant bacteria lacking all lipoprotein lipidation (Δ*lgt*) or lacking a major lipoprotein, SitC (Δ*sitC*), was also comparable with that of the parental strain (Fig. 5B).

We next examined whether purified LTA binds to PIR-B. As shown in Fig. 5C, LTA bound to NIH3T3 cells expressing PIR-B, but the binding activity was weaker than that to CD36, an authentic LTA receptor (10). We further examined the inhibitory effect of purified LTA on the binding of *S. aureus* to PIR-B. A high dose of LTA was required for abrogating the binding of *S. aureus* to not only PIR-B, but also SR-A and CD36 (Fig. 5D). These results suggest that LTA, but not WTA, glycolipids, or lipoprotein lipidation, is required for the binding of live *S. aureus* to the Ig-like domain 2 of PIR-B.

We next addressed macrophage responses to the LTA-deficient mutant strain, Δ*ltaS*. 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 Δ*ltaS* (Fig. 5B). Notably, WT BMDMs secreted higher amounts of IL-6 and IL-1β when infected with Δ*ltaS* versus the parental RN4220; IL-6 and IL-1β production in Δ*ltaS*-infected WT BMDMs were equivalent to those observed in $Pirb^{-/-}$ BMDMs (Fig. 5F). To confirm that the inhibitory effect on WT BMDMs was ascribed to LTA, we expressed the *ltaS* gene in our Δ*ltaS* mutant strain (Δ*ltaS*/*ltaS*$^+$) and found that reconstitution of *ltaS* expression suppressed cytokine production by WT BMDMs (Fig. 5F). Consistent with this, the Δ*ltaS*/*ltaS*$^+$ strain enhanced PIR-B phosphorylation in BMDMs (Fig. 5E), further indicating that LTA is required for *S. aureus*–PIR-B interaction. LTA deficiency did not affect cytokine production from $Pirb^{-/-}$ macrophages. Consistent with increased IL-1β secretion, the Δ*ltaS* mutant induced comparable caspase-1 activation and IL-1β processing in WT and $Pirb^{-/-}$ 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

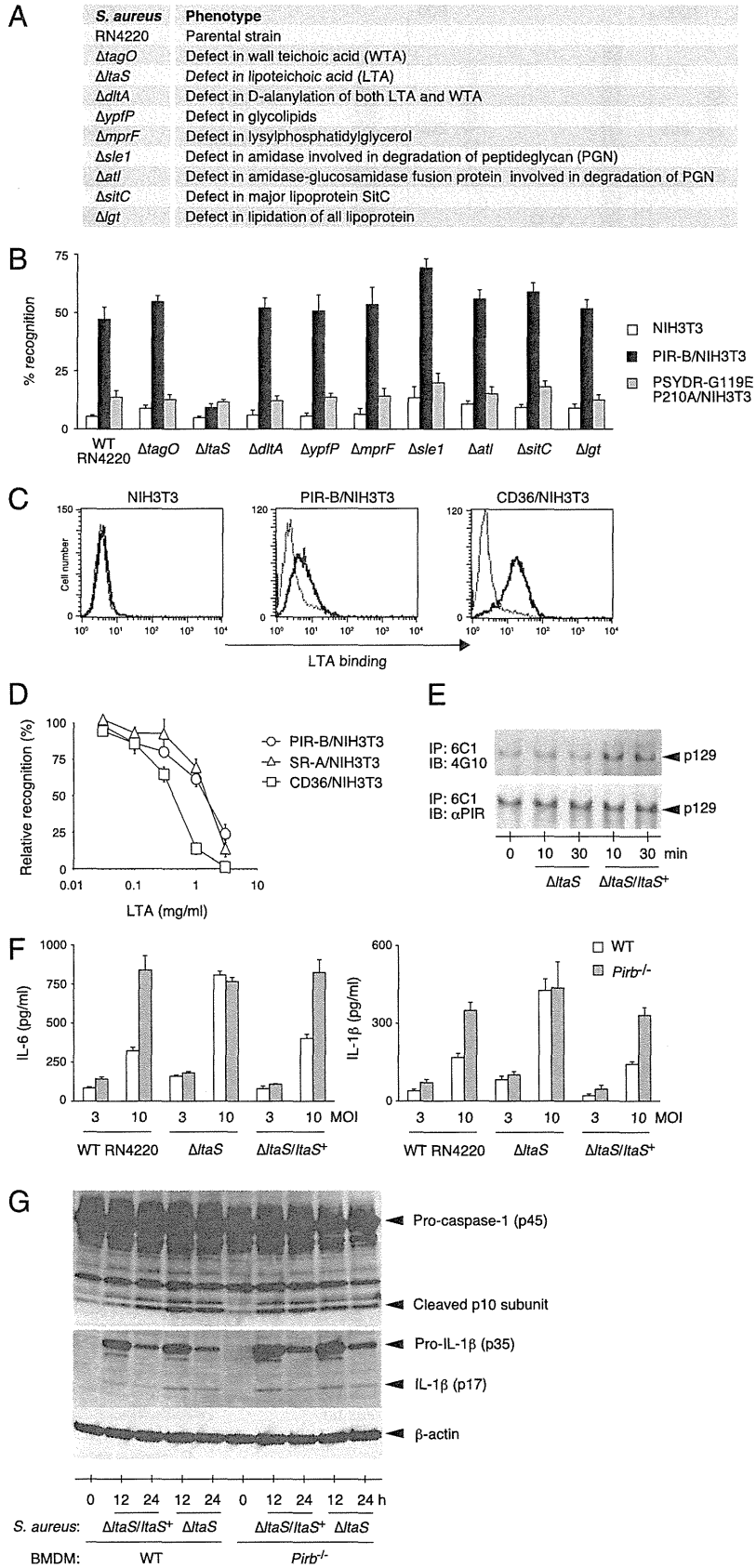

FIGURE 5. *S. aureus* binds PIR-B through LTA and suppresses macrophage inflammatory responses. (**A**) The strains of *S. aureus* used in this study and their phenotypes. (**B**) NIH3T3, PIR-B/NIH3T3, and PIR-B mutant/NIH3T3 cells were cultured with the indicated live TAMRA-labeled *S. aureus* strains (moi 50 each) for 30 min at 37°C. *S. aureus* recognition was analyzed by flow cytometry. Percentage of recognition was quantified, 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.

in highly purified samples (10, 25, 37). We and others have recently reported that lipoprotein lipidation-deficient (Δ*lgt*) *S. aureus* is unable to stimulate TLR2, indicating that lipoprotein is a major TLR2 ligand (25, 37). Consistent with these reports, Δ*lgt* *S. aureus* failed to induce inflammatory cytokine production either from WT or *Pirb*$^{-/-}$ BMDMs (Fig. 6). Taken together, these

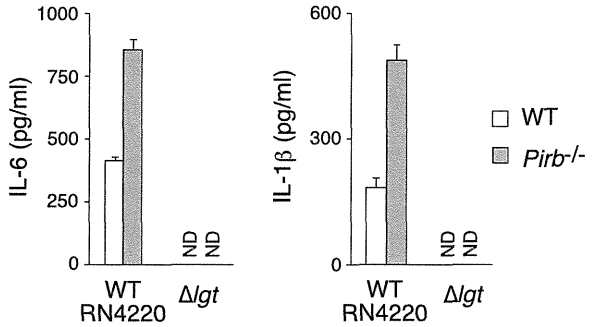

FIGURE 6. PIR-B regulates lipoprotein-mediated inflammatory responses. BMDMs were cultured with the indicated *S. aureus* strain (moi 10) for 12 h at 37°C. Production of IL-6 and IL-1β was analyzed by ELISA. Similar results were obtained in three independent experiments.

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 Δ*ltaS* infection. Although $Pirb^{-/-}$ mice were resistant to WT *S. aureus* infection (Fig. 2A–D), no significant difference was observed in susceptibility to Δ*ltaS* 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 Δ*ltaS* mutant to be less pathogenic because of diminished ligation of the inhibitory PIR-B, WT mice were unexpectedly more susceptible to Δ*ltaS* than Δ*ltaS*/*ltaS*$^+$ (Fig. 7C). Further, the Δ*ltaS* mutant survived more than Δ*ltaS*/*ltaS*$^+$ in the blood of WT mice (Fig. 7D). To uncover the mechanism for this heightened virulence, we examined recognition of the Δ*ltaS* mutant by splenic macrophages. WT splenic macrophages recognized this mutant poorly, at levels less than those observed for recognition of the Δ*ltaS*/*ltaS*$^+$ strain by $Pirb^{-/-}$ macrophages (Fig. 7E,

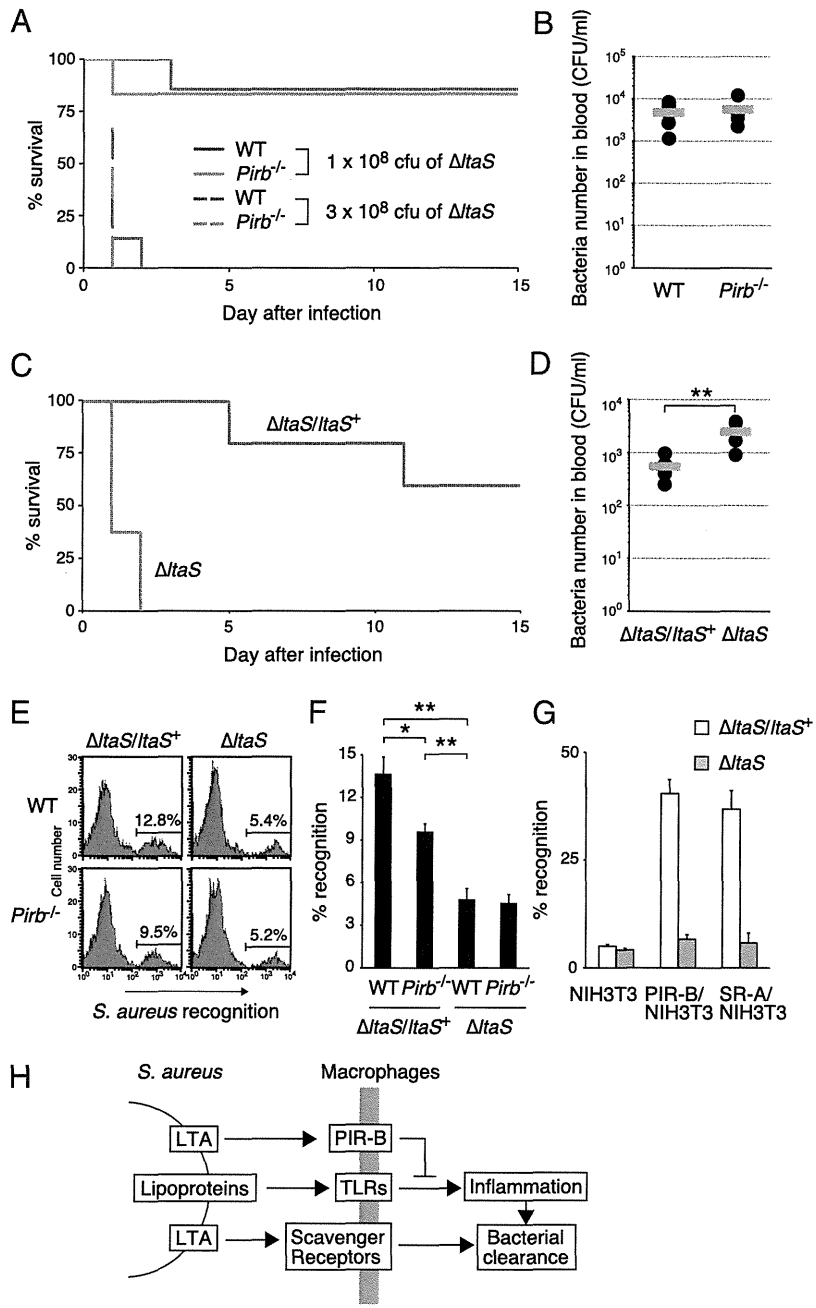

FIGURE 7. LTA-deficient *S. aureus* escapes macrophage recognition. (**A**) Mice were infected with Δ*ltaS* as follows: solid lines represent WT mice (blue; $n = 7$) or $Pirb^{-/-}$ mice (red; $n = 6$) with Δ*ltaS* (1×10^8 CFU per mouse); broken lines represent WT mice (blue; $n = 7$) or $Pirb^{-/-}$ mice (red; $n = 5$) with Δ*ltaS* (3×10^8 CFU per mouse). Survival was monitored for 15 d postinfection. (**B**) WT mice ($n = 6$) or $Pirb^{-/-}$ mice ($n = 4$) were i.v. infected with Δ*ltaS* (1×10^8 CFU per mouse). Bacterial number in blood 1 d postinfection was determined. (**C**) WT mice were i.v. infected with Δ*ltaS*/*ltaS*$^+$ (blue line; $n = 5$) or Δ*ltaS* (gray line; $n = 8$; 2×10^8 CFU each per mouse). Survival was monitored for 15 d postinfection. (**D**) WT mice ($n = 5$) were i.v. infected with Δ*ltaS*/*ltaS*$^+$ or Δ*ltaS* (2×10^8 CFU each per mouse). Bacterial number in blood 1 d postinfection was determined. (**E** and **F**) WT and $Pirb^{-/-}$ mice ($n = 3$ per group) were i.v. injected with the indicated strains of TAMRA-labeled *S. aureus* (3×10^8 CFU per mouse). Recognition of *S. aureus* by F4/80$^+$ CD11b$^+$ splenic macrophages was analyzed 1 h later (E) and represented as mean + SD in (F). (**G**) Indicated NIH3T3 cells were cultured with TAMRA-labeled Δ*ltaS* or Δ*ltaS*/*ltaS*$^+$, and recognition was analyzed as described in Fig. 5B. $^*p < 0.05$, $^{**}p < 0.01$, two-tailed Student *t* test. Similar results were obtained in three (A–F) or two (G) independent experiments. (**H**) Model of the host–bacteria interactions. *S. aureus* targets PIR-B via LTA to suppress lipoprotein/TLR-mediated inflammatory responses and survive in the host. PRRs other than PIR-B also target LTA for the bacterial clearance to overcome the infection.

7F). This is probably due to the fact that *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 Δ*ltaS* mutant (Fig. 7G). The highly virulent phenotype of Δ*ltaS* therefore appears to be caused by escape from host innate immune recognition in mice, a conclusion supported by studies in *Drosophila* (38). Collectively, these results suggest that *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 *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 *S. aureus* that binds PIR-B and, subsequently, modulates inflammatory responses. Furthermore, we showed that a lipoprotein lipidation-deficient (Δ*lgt*) mutant failed to induce inflammatory cytokine release either from WT or $Pirb^{-/-}$ BMDMs. Collectively, these results indicate that *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 *Listeria monocytogenes* or *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 *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 Δ*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, $Pirb^{-/-}$ BMDMs showed enhanced caspase-1 activation, suggesting that PIR-B negatively regulates inflammasome activation in response to *S. aureus* infection. Notably, it has been reported that an ITAM receptor activates inflammasomes through Syk (39, 40). Although ITAM receptors capable of recognizing nonopsonized *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 *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 *S. aureus*, but not for the Δ*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 *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 *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 *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 *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 *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 NIH3T3 cells enables these cells to recognize *S. aureus* (16), and we further observed the expression of both ILT2 and ILT5 on human peripheral monocytes that efficiently recognize *S. aureus* (data not shown). However, it remains unknown whether these ILT receptors are involved in *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 *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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Immune-Related Gene Expression Profile in Laboratory Common Marmosets Assessed by an Accurate Quantitative Real-Time PCR Using Selected Reference Genes

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Abstract

The common marmoset (*Callithrix jacchus*) is considered a novel experimental animal model of non-human primates. However, due to antibody unavailability, immunological and pathological studies have not been adequately conducted in various disease models of common marmoset. Quantitative real-time PCR (qPCR) is a powerful tool to examine gene expression levels. Recent reports have shown that selection of internal reference housekeeping genes are required for accurate normalization of gene expression. To develop a reliable qPCR method in common marmoset, we used *geNorm* applets to evaluate the expression stability of eight candidate reference genes (*GAPDH, ACTB, rRNA, B2M, UBC, HPRT, SDHA* and *TBP*) in various tissues from laboratory common marmosets. *geNorm* analysis showed that *GAPDH, ACTB, SDHA* and *TBP* were generally ranked high in stability followed by *UBC*. In contrast, *HPRT, rRNA* and *B2M* exhibited lower expression stability than other genes in most tissues analyzed. Furthermore, by using the improved qPCR with selected reference genes, we analyzed the expression levels of CD antigens (CD3ε, CD4, CD8α and CD20) and cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12β, IL-13, IFN-γ and TNF-α) in peripheral blood leukocytes and compared them between common marmosets and humans. The expression levels of CD4 and IL-4 were lower in common marmosets than in humans whereas those of IL-10, IL-12β and IFN-γ were higher in the common marmoset. The ratio of Th1-related gene expression level to that of Th2-related genes was inverted in common marmosets. We confirmed the inverted ratio of CD4 to CD8 in common marmosets by flow cytometric analysis. Therefore, the difference in Th1/Th2 balance between common marmosets and humans may affect host defense and/or disease susceptibility, which should be carefully considered when using common marmoset as an experimental model for biomedical research.

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Introduction

The common marmoset (*Callithrix jacchus*) is a New World monkey and is considered potentially useful as an experimental animal model in research fields such as drug toxicology [1,2], neuroscience [3,4], autoimmune diseases [5,6] and infectious diseases [7,8], because of its size, availability and high genetic similarity with humans [9,10]. Compared with mice, common marmosets are more useful as an *in vivo* model to study immune function [11]. However, essential tools and gene information for conducting studies using common marmosets are in short supply or unavailable. For example, monoclonal antibodies specific for common marmosets have been only partially established. Although DNA microarray research for common marmoset brain has been reported [12], sufficient studies have not been performed in other research fields.

Quantitative real-time polymerase chain reaction (qPCR) is the dominant quantitative technique for gene expression analysis due to its broad dynamic range, accuracy, sensitivity, specificity and