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Paired Ig-Like Receptors Bind to Bacteria and Shape TLR-Mediated Cytokine Production

Masafumi Nakayama,* David M. Underhill,† Timothy W. Petersen,2* Bin Li,* Toshio Kitamura,‡ Toshiyuki Takai,§ and Alan Aderem3*

The innate immune system uses a wide variety of pattern recognition receptors including TLRs, scavenger receptors, and lectins to identify potential pathogens. A carefully regulated balance between activation and inhibition must be kept to avoid detrimental and inappropriate inflammatory responses. In this study, we identify murine-paired Ig-like receptor (PIR)-B, and its human orthologs Ig-like transcript 2 and Ig-like transcript 5 as novel receptors for *Staphylococcus aureus*. PIR-B contains four ITIM motifs and is thought to be an inhibitory receptor. Expression of these receptors enables NIH3T3 cells to bind *S. aureus*. In mouse bone marrow-derived macrophages, masking of PIR-B by anti-PIR mAb or genetic deletion of PIR-B shows significantly impaired recognition of *S. aureus* and enhanced TLR-mediated inflammatory responses to the bacteria. These data suggest a novel mechanism for innate immune regulation by paired Ig-like receptor family members. *The Journal of Immunology*, 2007, 178: 4250–4259.

Macrophages initiate innate immune responses by recognizing pathogens, phagocytosing them, and secreting inflammatory mediators. An effective response requires that macrophages recognize pathogen-associated molecular patterns that distinguish the infectious agents from self, and, in addition, discriminate among pathogens (1–4). Recently, the TLRs have emerged as key receptors for effective innate immunity (5–7).

The TLRs are a family of 13 innate immune recognition receptors that recognize a broad range of microbial products including LPS, flagellin, lipoproteins, and bacterial DNA, and that play a vital role in the transcriptional responses of cells of the innate immune system (6, 7). Each TLR has multiple extracellular leucine-rich repeats as well as an intracellular signaling domain that is homologous to the cytoplasmic tail of the IL-1R. Stimulation of TLRs direct activation of NF-κB and production of proinflammatory cytokines (6, 7). Along with the TLRs, many additional innate immune receptors participate in the recognition of microbes, thus fine-tuning the innate immune system’s unique responses to each microbe (4, 8). Activation of these TLR-mediated inflammatory responses must be tightly regulated; too little response leaves the host susceptible to infection, and too much response may lead to lethal systemic inflammation or autoimmunity.

*Staphylococcus aureus*, an extracellular Gram-positive bacteria, is a major source of mortality in medical facilities (9, 10). The primary site of infection is often a breach in the skin that may lead to minor skin and wound infections, but *S. aureus* can also infect any other tissue of the body, causing life-threatening diseases such as osteomyelitis, endocarditis, pneumonia, and sepsis (9, 10). We have previously observed that *S. aureus*-induced inflammatory cytokine production by macrophages is mediated by TLR2 (11, 12), and Takeuchi et al. have reported that TLR2- or MyD88-deficient mice are highly susceptible to *S. aureus* infection (13). Recently, CD36, a class B scavenger receptor, has been reported to facilitate TLR2 activation for effective innate immune responses to *S. aureus*, indicating that accessory receptors can be important for certain TLR-mediated inflammatory responses (14, 15). Compared with infection by Gram-negative bacteria, *S. aureus* triggers lower quantities of inflammatory cytokines (16, 17). The mechanisms by which different types of bacteria elicit different types of inflammatory responses are poorly understood, but they are likely to be controlled by interactions between the different repertoires of pattern recognition receptors that distinguish between various bacteria.

The inhibitory and activating Ig-like receptors, such as FcR and NK receptor, provide negative and positive regulation of immune cells upon recognition of various ligands, thus enabling those cells to elicit a balanced response to extrinsic stimuli (18, 19). Murine-paired Ig-like receptors (PIR)-B (also known as Llrbl3) and PIR-A are expressed on a wide variety of immune cells including B cells, mast cells, macrophages, and dendritic cells (DCs), mostly in a pair-wise fashion (20–22). PIR-B and PIR-A are encoded by a gene cluster, wherein PIR-B is encoded by a single gene and PIR-A is encoded by multiple genes (20–22). Whereas the ectodomains of PIR-B and PIR-A are very similar (>92% aa identity), the cytoplasmic domains differ significantly (22, 23). PIR-B has four ITIMs in the cytoplasmic region and inhibits activating

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signals by surface receptors such as B cell Ag receptors and chemokine receptors (22–24). In contrast, PIR-A contains a short cytoplasmic tail lacking any signal transduction motif. Instead, PIR-A has a basic arginine residue within the transmembrane domain and associates with FcRγ common chain, which contains an ITAM. Cross-linking of the PIR-A/FcRγ complex results in mast cell activation through an ITAM-dependent manner (22, 23). Recently, it has been reported that MHC class I (H-2) molecules are ligands for both PIR-B and PIR-A (25). Therefore, PIR-B and PIR-A appear to provide a homeostatic balance of inhibitory and activating signals to the immune system by interaction with MHC class I. Indeed, in a mouse model of graft-vs-host disease, in which allogeneic splenocytes were transferred into PIR-B-deficient mice, enhanced PIR-A signaling in the host DCs augmented the activation of the Pirb<sup>−/−</sup> donor T cells, resulting in enhanced IFN-γ production by DCs, CD4<sup>+</sup> T cells, and CTLs (25).

In this study, we used a retrovirus-mediated expression-cloning strategy to identify PIR-B as a receptor for <i>S. aureus</i>. Subsequent analysis suggests that at least one specific form of PIR-A (PIR-A1), as well as the PIR-B human orthologs Ig-like transcript (ILT)2 and ILT5 also recognize the pathogen. We propose that these PIRs help fine-tune TLR-mediated inflammatory response to <i>S. aureus</i>, thus providing a novel entry point for therapeutic intervention.

Materials and Methods

Reagents

Anti-PIR mAb (6C1) and recombinant mouse IL-10 were purchased from BD Pharmingen. Anti-mouse IL-10-neutralizing mAb (JES-2A5) and control rat IgG1 mAb were purchased from e Bioscience. Tetramethylrhodamine isothiocyanate (TRITC)-labeled phallolidin was purchased from Molecular Probes. Dextran sulfate was purchased from Amersham. Polyinosinic acid and polycytidylic acid were purchased from Sigma-Aldrich. LPS and the synthetic lipopeptide Pam3CSK4 were purchased from List and EMC Microcollections, respectively. Pepptidegycin and lipoteichoic acid were purchased from Fluka. Murine IL-6, TNF-α, and IL-10 ELISA kits were purchased from R&D Systems.

Mice and bone marrow-derived macrophages

C57BL/6 mice were obtained from The Jackson Laboratory. Pirb<sup>−/−</sup> mice (129/SvJ/C57BL/6 background) (26) were backcrossed for at least 10 generations with C57BL/6 mice. Tlr2<sup>−/−</sup> mice and Myd88<sup>−/−</sup> mice (129/SvJ/C57BL/6 background; provided by S. Akira, Osaka University, Osaka, Japan), were backcrossed for eight generations with C57BL/6 mice. All mice were used according to the guidelines of the institutional animal care and use committee established at the Institute for Systems Biology. Mouse bone marrow-derived macrophages were grown in complete RPMI 1640 (RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM glutamine) with 50 ng/ml recombinant human M-CSF (Chiron). For cytokine measurement, the cells (2 × 10<sup>5</sup>/well) were cultured with 50 ng/ml recombinant human IL-6, TNF-α, and IL-10. For some experiments, the cells were pretreated with the indicated mAb for 1 h at 4°C. The amounts of IL-6, TNF-α, and IL-10 in supernatants were measured by ELISA.

Construction of cDNA library and retroviral expression cloning

Construction of retroviral cDNA library was performed as described previously (28). Briefly, complementary cDNAs were synthesized from poly(A) RNA of C57BL/6 mouse bone marrow-derived macrophages with oligo(dT) primer using the SuperScript Choice System (Invitrogen Life Technologies) according to the manufacturer’s instructions. The synthesized cDNAs were cloned between the BSXI sites of pMX (29) using BSXI adaptors (Invitrogen Life Technologies), generating a retroviral cDNA library. The cDNA library contained ~1 × 10<sup>8</sup> individual clones. For production of retroviruses, culture supernatants of Phoenix-Ampho cells (provided by G. Nolan, Stanford University, Stanford, CA) were harvested 3 days after transfection with the retroviral cDNA library. NIH3T3 cells (4 × 10<sup>6</sup>) were infected with retroviruses expressing this cDNA library in the presence of 8 μg/ml polybrene. Two days after infection, these cells were cultured with TRITC-labeled <i>S. aureus</i> (Wood strain without protein A; Molecular Probes; 10 bacteria per cell) for 30 min at 37°C. After washing with PBS twice, cells were harvested by trypsinization and subjected to cell sorting. After four rounds of sorting, 40 individual clones obtained by limiting dilution were examined on the binding to TRITC-<i>S. aureus</i>. Parental NIH3T3 cells, clone 4S2C37 cells, and NIH3T3 cells infected with retrovirus expressing the cloned PIR-B or PIR-B/A2 711-841 cDNA were cultured with (shaded histogram) or without (open histogram) TRITC-labeled <i>S. aureus</i> (10 bacteria/cell) for 30 min at 37°C. Cell surface binding was quantified by flow cytometry. B. Cells were stained with PE-labeled rat IgG1 (thin histogram) or PE-labeled anti-PIR mAb 6C1 (thick histogram), and analyzed by flow cytometry. C. NIH3T3 cells and PIR-B/NIH3T3 cells were incubated with FITC-labeled <i>S. aureus</i> (10 bacteria/cell) for 30 min at 37°C, and then stained with TRITC-phalloidin. Cells were visualized by immunofluorescence microscopy. D, PIR-B/NIH3T3 cells were reincubated with 5 μg/ml control rat IgG1 or 6C1 mAb, then cultured with (shaded histogram) or without (open histogram) TRITC-<i>S. aureus</i> (10 bacteria/cell) for 30 min at 37°C, and analyzed by flow cytometry. Data are representative of three independent experiments.

Cell lines

NIH3T3 cells (American Type Cell Culture Collection (ATCC) no. CRL-1658) cells were maintained in complete DMEM (DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine). For construction of expression vectors, the coding region for PIR-A1 and PIR-A2 were amplified from RAW264.7 cDNAs, and the coding region for PIR-A2h1 and PIR-A2h2 were amplified from C57BL/6 mouse bone marrow-derived macrophage cDNAs. These fragments were inserted into pMXs-IP. Several mutant forms of PIR-B were prepared by PCR-based mutagenesis using PIR-B/pMXs-IP as a template. The coding region for ILT2 (leukocyte Ig-like receptor (LILR)B1) and ILT5 (LILRB3) were amplified from genomic DNA, and sequenced. The PIR-B cDNA was subcloned into pMXs-IREs-puro (pMXs-IP) (29), generating pMXs-IP-PIR-B.

FIGURE 1. Identification of PIR-B as a receptor for <i>S. aureus</i>. A. Parental NIH3T3 cells, clone 4S2C37 cells, and NIH3T3 cells infected with retrovirus expressing the cloned PIR-B or PIR-B/A2 711-841 cDNA were cultured with (shaded histogram) or without (open histogram) TRITC-labeled <i>S. aureus</i> (10 bacteria/cell) for 30 min at 37°C. Cell surface binding was quantified by flow cytometry. B. Cells were stained with PE-labeled rat IgG1 (thin histogram) or PE-labeled anti-PIR mAb 6C1 (thick histogram), and analyzed by flow cytometry. C. NIH3T3 cells and PIR-B/NIH3T3 cells were incubated with FITC-labeled <i>S. aureus</i> (10 bacteria/cell) for 30 min at 37°C, and then stained with TRITC-phalloidin. Cells were visualized by immunofluorescence microscopy. D, PIR-B/NIH3T3 cells were reincubated with 5 μg/ml control rat IgG1 or 6C1 mAb, then cultured with (shaded histogram) or without (open histogram) TRITC-<i>S. aureus</i> (10 bacteria/cell) for 30 min at 37°C, and analyzed by flow cytometry. Data are representative of three independent experiments.
were selected by puromycin (10 μg/ml; Calbiochem). The expressions of PIR and ILT were confirmed by flow cytometry using 6C1 and HPC4 mAbs, respectively.

**Homology modeling of PIR-B structure**

The three-dimensional structure of PIR-B was predicted through the SWISS-MODEL server (http://swissmodel.expasy.org/) (31). Crystallographic structures of human LIR-1 and FcγRI proteins (1ugn, 1g0x, 1p7q, 1uct, and 1ovz in Research Collaboratory for Structural Bioinformatics protein data bank (www.rcsb.org)) were chosen to be the template structures based on pair-wise sequence alignments (PIR-B and 1ugn shared 49.33% sequence identity).

**Fluorescent labeling of bacteria**

*S. aureus* (Wood 46 strain; ATCC), *Bacillus subtilis* (Marburg strain, ATCC), *Listeria monocytogenes* (strain 10403S; provided by D. Portnoy, University of California, Berkeley, CA), *Helicobacter pylori* (provided by N. Salama, Fred Hutchinson Cancer Research Center, Seattle, WA), *Pseudomonas aeruginosa* (provided by D. Speert, Child and Family Institute, Vancouver, Canada), and *Escherichia coli* (K-12 strain; ATCC) were grown to saturation, then washed three times with PBS. After heat-killing (65°C, 20 min), these bacteria were incubated with PBS containing FITC (50 μg/ml; Molecular Probes) or TRITC (50 μg/ml; Molecular Probes) for 30 min at room temperature. The reaction was stopped by hydroxylamine (150 mM; Sigma-Aldrich), followed by washing five times with PBS. Labeling efficiency was confirmed by flow cytometry. For labeling of live bacteria, the bacterial viability was checked by plating serial dilutions and was typically >90%.

**Measurement of cells recognizing bacteria**

NIH3T3 cells and bone marrow macrophages (2 × 10^5/well) were seeded on 24-well plates and cultured overnight. These cells were incubated with the indicated dose of the fluorescently labeled bacteria for 30 min at 4 or 37°C. In some assay, cells were pretreated with the indicated inhibitor for 30 min at 37°C. After incubation with bacteria, cells were washed with PBS twice, and then harvested and analyzed on a FACSCalibur (BD Biosciences).

**Immunofluorescence microscopy**

This assay was performed as described previously (12). Briefly, cells grown on glass coverslips were treated with FITC-S. aureus for 30 min at 37°C, then washed with PBS, and fixed with 10% formalin in PBS for 15 min. Cells were permeabilized and stained with TRITC-phalloidin. Images were acquired using a Leica SP2 laser scanning confocal microscope equipped with a 63×/1.4 objective lens (Leica). Images were cropped and placed on pages using Adobe Photoshop version 6.0 (Adobe Systems).

**Results**

**Identification of PIR-B as a receptor for *S. aureus***

We previously observed that TLR2 is responsible for inflammatory responses to *S. aureus*, but not for direct binding to the bacteria (11, 12). Recently, we and others have reported that non-TLRs binding whole microbes functionally interact with TLR2 (14, 15, 32). To identify novel receptors that directly bind *S. aureus* and functionally interact with TLR, we used an expression cloning strategy. Bone marrow-derived macrophages from C57BL/6 mice efficiently recognized TRITC-labeled heat-killed *S. aureus*, whereas the binding to mouse fibroblast NIH3T3 cells was minimal, suggesting that bone marrow macrophages, but not NIH3T3 cells, express the receptors that directly bind the bacteria. Thus, we generated a retroviral cDNA expression library from the macrophages, infected NIH3T3 cells, and screened these cells by FACS to select cells that acquired the ability to bind fluorescently labeled *S. aureus*. After four rounds of sorting and amplification, clones were isolated by limiting dilution, resulting in the identification of three single-cell clones, 4S2C37, 4S2C43, and 4S2C46, that bind...
S. aureus (Fig. 1A and data not shown). We sequenced the retrovirus inserts and found that all three clones harbor PIR-B cDNA in the sense orientation. Based on cell surface staining by anti-PIR mAb 6C1 (33), parental NIH3T3 cells lacked PIR-B expression, whereas 4S2C37, 4S2C43, and 4S2C46 cells expressed PIR-B on their cell surface (Fig. 1B and data not shown). To verify that the PIR-B gene, and not a secondary mutation, was responsible for the recognition of S. aureus in these clones, we specifically regenerated NIH3T3 cells stably expressing PIR-B (PIR-B/NIH3T3). These cells expressed PIR-B on their surface and gained the ability to bind S. aureus (Fig. 1A and B). Immunofluorescence microscopy showed that PIR-B/NIH3T3 cells, but not NIH3T3 cells, bound the bacteria efficiently (Fig. 1C). To rule out the possibility that ectopic expression of PIR-B in NIH3T3 cells may be inducing the expression of some other S. aureus-binding molecules (i.e., via its ITIMs), we generated NIH3T3 cells expressing a mutant form of PIR-B lacking all cytoplasmic signaling ITIMs (PIR-A2 homologue 1 (PIR-A2h1), and PIR-A2 homologue 2 (PIR-A2h2)). Association of PIR-A with FcRγ chain is required for efficient expression of PIR-A on the surface of cells such as the LTK fibroblast cell line, macrophages, and splenocytes (33). In contrast, PIR-A presents on the surface of COS7 cells even in the absence of FcRγ chain (34). All forms of PIR-A used in this study were highly expressed on the surface of NIH3T3 cells in the absence of the FcRγ chain (Fig. 3A). Although expression levels of the four PIR-A isoforms were largely equivalent (Fig. 2), we call these PIR-A1, PIR-A2, PIR-A2 homologue 1 (PIR-A2h1), and PIR-A2 homologue 2 (PIR-A2h2). Association of PIR-A with FcRγ chain is required for efficient expression of PIR-A on the surface of cells such as the LTK fibroblast cell line, macrophages, and splenocytes (33).

To further characterize the binding of S. aureus by PIR-B, we tried to compete the interaction with various putative bacterial cell wall ligands. As shown in Fig. 3D, the Gram-positive bacterial cell wall components lipoteichoic and peptidoglycan, and the Gram-negative bacterial cell wall component LPS failed to inhibit the binding. However, the polyanionic reagents dextran sulfate and polyinosinic acid, which interfere with binding of some scavenger receptors to bacteria, blocked PIR-B binding to S. aureus, whereas the polycationic polycytidylic acid did not. The data suggest that PIR-B has scavenger receptor-like binding activity toward S. aureus.

**FIGURE 3.** Recognition of *S. aureus* by PIR. A, Parental NIH3T3 cells and NIH3T3 cells stably expressing the indicated form of PIR were stained with PE-labeled rat IgG1 (thin histogram) or PE-labeled anti-PIR mAb 6C1 (thick histogram), then analyzed by flow cytometry. B, The cells were cultured with TRITC-labeled *S. aureus* (10 bacteria/cell) for 30 min at 37°C, then analyzed by flow cytometry. C, The cells were preincubated with 5 μg/ml control rat IgG1 or 6C1, then cultured with TRITC-labeled *S. aureus* (10 bacteria/cell) for 30 min at 37°C. The recognition (relative to recognition without inhibitor) was quantified by flow cytometry. Data are represented as mean ± SD of triplicates. D, PIR-B/NIH3T3 cells were pretreated with the indicated dose of inhibitors for 30 min, and then incubated with TRITC-labeled *S. aureus* (10 bacteria/cell) for 30 min at 37°C. The recognition (relative to recognition without inhibitor) was quantified by flow cytometry. Data are represented as mean ± SD of triplicates (SD were <10% of the mean; data not shown). Data are representative of two to three independent experiments.
We next addressed the PIR-B recognition site. PIR-B and PIR-A1, which share a higher degree of conservation across Ig-like domain D2 as compared with the other PIR-As (Figs. 2 and 4A), bind *S. aureus* efficiently (Fig. 3B), and the binding was abrogated by polyanionic reagents (Fig. 3D). We hypothesized that charged amino acids in D2 might be important for the binding. We therefore specifically mutated some of the unique amino acid residues in D2 of PIR-B to residues corresponding to the sequence of PIR-A2, and generated stable NIH3T3 cell lines for each resulting mutant. After confirmation of equivalent expression levels of each mutant (Fig. 4B), we examined their binding activities. In the first mutant, we replaced a short segment HNDHK by PSYDR based on the sequence information (Fig. 4A), and also reduced the positive charged amino acids from three to one. In addition, this segment locates on a surface loop in the three-dimensional structural model of PIR-B, which makes it possible to be involved in molecular recognition (Fig. 4C). The substitution, however, did not alter the recognition of *S. aureus* (Fig. 4D). We next generated mutants G119E, P210A, and the double mutant G119E/P210A. These two residues are close to each other and located near the linking area of the D1 and D2 domains, therefore they might help to stabilize the overall structure of PIR-B (Fig. 4C). Although each single substitution did not alter the binding to *S. aureus*, the double mutant did show a slight reduction in binding activity (Fig. 4D). Finally, we tested a construct containing all three substitutions (PSYDR-G119E, P210A-PIR-B/NIH3T3) and found that the binding activity was reduced to a level equivalent to that of PIR-A2 (Fig. 4D). Overall, these mutants provided evidence in support of our hypothesis that the domain D2 of PIR-B is important for the recognition of *S. aureus*.

We next examined whether PIR-B is involved in the binding of *S. aureus* by bone marrow-derived macrophages. As shown in Fig. 5, *Pirb<sup>−/−</sup>* bone marrow-derived macrophages show impaired binding of *S. aureus*

*PIR-B-deficient macrophages show impaired binding of *S. aureus***

We next examined whether PIR-B is involved in the binding of *S. aureus* by macrophages. As shown in Fig. 5, *Pirb<sup>−/−</sup>* bone marrow-derived macrophages show impaired binding of *S. aureus*. We incubated fluorescently labeled *S. aureus* (1 or 10 bacteria/cell) with WT, *Pirb<sup>−/−</sup>* or *Tlr2<sup>−/−</sup>* bone marrow-derived macrophages for 30 min at 4°C. Then these cells were analyzed by flow cytometry. Data are representative of three independent experiments.
PIR-B suppresses TLR-mediated inflammatory response to S. aureus

It has been reported that PIR-B inhibits activation signals by B cell Ag receptor and chemokine receptors such as CCR1 and CXCR2 (22, 24). Thus, we next examined whether PIR-B negatively regulates the macrophage inflammatory response to S. aureus. As shown in Fig. 7, A and B, Pirb<sup>-/-</sup> bone marrow-derived macrophages produced ~2-fold higher levels of the proinflammatory cytokines such as IL-6 and TNF-α in response to S. aureus. In contrast, levels of the anti-inflammatory cytokine IL-10 were suppressed to a similar degree in Pirb<sup>-/-</sup> macrophages (Fig. 7C).

Because it has been reported that PIR binds various MHC class I molecules (25), we considered whether PIR-B exerts a general suppression on TLR signaling in macrophages through its constitutive interaction with MHC class I. However, when we treated WT and Pirb<sup>-/-</sup> macrophages with pure TLR ligands such as PAM3CSK4 lipopeptides (TLR2 ligand) or LPS (TLR4 ligand), we observed no differences in pro- or anti-inflammatory cytokine production (Fig. 7). Also, it has been demonstrated that mAb 6C1 does not block the interaction of PIR-B with MHC class I (25), whereas we have observed that the Ab blocks the interaction of PIR-B with S. aureus (Figs. 1D and 3C). As shown in Fig. 8, A and B, IL-6 production was enhanced and IL-10 production was suppressed in 6C1-pretreated WT macrophages, which is consistent with the results from Pirb<sup>-/-</sup> macrophages. These data demonstrate that the interaction between S. aureus and PIR directly suppresses proinflammatory activation of macrophages.

TLR2-deficient bone marrow-derived macrophages showed impaired IL-6 production (Fig. 7A), indicating that S. aureus-induced IL-6 production is mainly TLR2-dependent, as described previously (11, 12). We next addressed whether the enhancement of IL-6 production by PIR-B deficiency is also TLR2-dependent. As shown in Fig. 8A, 6C1 mAb pretreatment enhanced IL-6 production in WT, but not Tlr2<sup>-/-</sup> or Myd88<sup>-/-</sup> macrophages, suggesting that PIR-B negatively regulates TLR2-mediated inflammatory responses. In contrast, Tlr2<sup>-/-</sup> macrophages produced an equivalent amount of IL-10 as WT macrophages in response to S. aureus (Fig. 7C), and 6C1 suppressed IL-10 production to the same extent in both WT and Tlr2<sup>-/-</sup> macrophages (Fig. 8B). In Myd88<sup>-/-</sup> macrophages, IL-10 production was neither induced by S. aureus nor was it affected by 6C1 treatment (Fig. 8B). These results suggest that S. aureus-induced IL-10 production is TLR2 independent, but is probably dependent on some other TLR, and is enhanced by PIR-B signaling.

Because IL-10 suppresses several proinflammatory cytokines in macrophages (35), we next asked whether enhancement of proinflammatory cytokines is caused by IL-10 suppression in Pirb<sup>-/-</sup> macrophages. Addition of exogenous IL-10 suppressed IL-6 production from Pirb<sup>-/-</sup> macrophages stimulated with S. aureus in a dose-dependent manner, and from WT macrophages to a much lesser extent (Fig. 8C). Furthermore, anti-IL-10-neutralizing mAb JES-2A5 enhanced IL-6 production from WT macrophages stimulated with S. aureus to the equivalent level of that from similarly treated Pirb<sup>-/-</sup> macrophages (Fig. 8D). Taken together, these results suggest that the S. aureus-induced enhancement of inflammatory responses observed in Pirb<sup>-/-</sup> macrophages is largely due to suppression of IL-10 production.
PIRs discriminate between bacteria

We have demonstrated that PIR-B is a novel receptor for S. aureus. We next examined whether PIR-B recognizes other types of bacteria. Although PIR-B/NIH3T3 cells bind S. aureus, they did not efficiently bind other Gram-positive bacteria such as B. subtilis or L. monocytogenese. PIR-B did, however, recognize the Gram-negative bacteria H. pylori and E. coli, but not P. aeruginosa. PIR-A2h1 could not bind any of these bacteria efficiently (Fig. 9A). We also examined IL-6 production from Pirb−/− macrophages in response to these Gram-negative bacteria. As shown in Fig. 9B, Pirb−/− macrophages produced enhanced IL-6 in response to H. pylori, whereas ILT2/NIH3T3 and ILT5/NIH3T3 cells do not. ILT2/NIH3T3 cells also bind E. coli, whereas ILT5/NIH3T3 cells do not. These data suggest that, in humans, PIR family members may also play an important role in innate immune response to bacterial infection.

Human ILTs recognize bacteria

Based on their similarities in structure, expression profiles, and genomic localization, murine PIRs are considered to be the orthologs of human ILT receptors (also called LILRs, or LIRs, and monocyte/macrophage Ig-related receptors (22, 36, 37). Therefore, we asked whether any ILTs recognize a similar array of bacteria as PIR-B. We generated NIH3T3 cells stably expressing ILT2 or ILT5, each of which contain ITIMs like PIR-B, and tested the ability of these cells to recognize various bacteria. As shown in Fig. 9C, both ILT2/NIH3T3 and ILT5/NIH3T3 cells bind S. aureus. It is of interest that PIR-B/NIH3T3 cells bind H. pylori, whereas ILT2/NIH3T3 and ILT5/NIH3T3 cells do not. ILT2/NIH3T3 cells also bind E. coli, whereas ILT5/NIH3T3 cells do not. These data suggest that, in humans, PIR family members may also play an important role in innate immune response to bacterial infection.

Discussion

Paired inhibitory and activating receptors, including FcRs and NK receptors, tightly regulate immune responses (18, 19). In this study, we identify PIR-B as a novel macrophage receptor for S. aureus, demonstrating for the first time that the members of the PIR family act as pattern-recognition receptors for bacteria. Furthermore, we have shown that S. aureus recognition by PIR-B, in conjunction with TLR, directs anti-inflammatory responses to the bacteria.

The inhibitory PIR-B protein is encoded by single gene, whereas the activating PIR-A proteins are encoded by multiple genes. All of the ectodomains, each containing six Ig-like domains, are >92% identical at the amino acid level (20, 21). Therefore, it had been thought that both PIR-B and PIR-A recognize the same ligands. Indeed, both receptors have been recently reported to bind...
PIR-B suppresses TLR-mediated inflammatory responses. A and B, WT, Tlr2−/−, Pirb−/−, or Myd88−/− bone marrow macrophages were pretreated with 5 μg/ml control rat IgG1 or 6C1 for 1 h at 4°C, and then cultured with or without S. aureus (10 bacteria/cell) for 12 h at 37°C. Induction of IL-6 (A) and IL-10 (B) was measured by ELISA. C, WT or Pirb−/− macrophages were cultured with S. aureus (10 bacteria/cell) and the indicated dose of recombinant mouse IL-10 for 12 h at 37°C. Induction of IL-6 was measured by ELISA. D, WT or Pirb−/− macrophages were cultured with or without S. aureus (10 bacteria/cell) in the presence of 5 μg/ml control rat IgG1 or anti-IL-10-neutralizing mAb JES-2A5 for 12 h at 37°C, and induction of IL-6 was measured by ELISA. Data are represented as mean ± SD of triplicates. Data are representative of three independent experiments. n.d., Not detected.

FIGURE 8.
PIRs PROVIDE PATTERN RECOGNITION FOR BACTERIA

therapeutic approaches to combat infections and autoimmune diseases.

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References


