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PIR-B-Deficient Mice Are Susceptible to Salmonella Infection

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Paired Ig-like receptors of activating (PIR-A) and inhibitory (PIR-B) isoforms are expressed by many hematopoietic cells, including B lymphocytes and myeloid cells. To determine the functional roles of PIR-A and PIR-B in primary bacterial infection, PIR-B-deficient (PIR-B−/−) and wild-type (WT) control mice were injected i.v. with an attenuated strain of Salmonella enterica Typhimurium (WB335). PIR-B−/− mice were found to be more susceptible to Salmonella infection than WT mice, as evidenced by high mortality rate, high bacterial loads in the liver and spleen, and a failure to clear bacteria from the circulation. Although blood levels of major cytokines and Salmonella-specific Abs were mostly comparable in the two groups of mice, distinct patterns of inflammatory lesions were noted. In PIR-B−/− mice vs nodular restricted localization in WT mice. PIR-B−/− bone marrow-derived macrophages (BMMφ) failed to control intracellular replication of Salmonella in vitro, in part due to inefficient phagosomal oxidant production, when compared with WT BMMφ. PIR-B−/− BMMφ also produced more nitrite and TNF-α upon exposure to Salmonella than WT BMMφ did. These findings suggest that the disruption of PIR-A and PIR-B balance affects their regulatory roles in host defense to bacterial infection. The Journal of Immunology, 2008, 181: 4229–4239.

During the last decade, many genes have been identified that encode novel pairs of immunoreceptors which have similar ectodomains but function to produce opposing signals (1, 2). The pairing of activating and inhibitory receptors is thought to be necessary for the initiation, amplification, and termination of immune responses. Paired Ig-like receptors of activating (PIR-A) and inhibitory (PIR-B) isoforms in rodents are among the earliest paired receptors (3, 4). PIR-A associates non-covalently with the Fc receptor common γ-chain, a transmembrane signal transducer containing ITAM motifs in the cytoplasmic tail, to form a cell activation complex (5–8). In contrast, PIR-B contains three functional ITIM motifs in its cytoplasmic tail, which negatively regulate cellular activity via Src homology region 2 domain-containing phosphatase 1 (SHP-1) and 2 (SHP-2) (9–12). PIR-A and PIR-B are expressed by many hematopoietic cell types, including B cells, dendritic cells (DC), monocyte/macrophages, neutrophils, eosinophils, mast cells, and megakaryocyte/platelets (7, 12, 13). PIR are not expressed by T cells, NK cells, or erythrocytes, a feature that distinguishes mouse PIR from the human PIR homologues, leukocyte Ig-like receptors (LILR) (originally called the Ig-like transcripts, monocyte-macrophage Ig-like receptors, or CD85), some of which are expressed by T cells and NK cells as well (14–17). PIR is also expressed by lymphoid progenitors committed to differentiation to T cells, NK cells, and DC, but not to B-lineage cells, suggesting a complex pattern of PIR expression during hematopoietic differentiation (18). In addition to the hematopoietic cells, it has been recently shown that PIR-B is expressed by neurons throughout the brain using in situ hybridization and protein blot analyses (19).

Several interesting findings regarding the PIR ligands and disruption of the PIRb gene (PIR-B−/−) have been demonstrated (20). Like human LILR, both PIR-B and PIR-A react in surface plasmon resonance assays with various MHC class I molecules at relatively high affinity (21). Furthermore, the interaction between PIR and MHC class I is found to occur at cis (i.e., on the same cell) and not at trans (i.e., between different cells; Ref. 22). In addition to endogenous MHC class I, both PIR-B and PIR-A are found to recognize cell wall components of both Gram-positive Staphylococcus aureus and Gram-negative Escherichia coli, suggesting multiple ligand recognition by PIR (23). Although PIR-B−/− mice exhibit normal T and B cell development except for slightly higher levels of peritoneal B-1 cells, PIR-B−/− B cells are hyper-responsive upon BCR ligation. PIR-B−/− mice show significantly augmented IgG1 and IgE responses to T cell dependent Ags and produce more IL-4 and IFN-γ than wild-type (WT) control mice, suggesting an enhanced Th2 response attributable to the immaturity of PIR-B−/− DC (24). PIR-B−/− mice also...
exhibit an exaggerated graft-vs-host (GVH) reaction, possibly due to the interaction between PIR-A on PIR-B<sup>−/−</sup> DC and allogeneic MHC class I on donor T cells that leads to increased proliferation of IFN-γ, a critical cytokine in lethal GVH as well as to increased proliferation of CTLs (21). PIR-B<sup>−/−</sup> phagocytic cells are also shown to be hyperresponsive to integrin ligation (25, 26). In the present study, we have determined the influence of PIR-B deficiency on the pathogenesis of Salmonella infection in mice.

Materials and Methods

Mice

C57BL/6 mice and RAG-2-deficient mice on a C57BL/6 background (RAG<sup>2−/−</sup>) were purchased from The Jackson Laboratory. PIR-B<sup>−/−</sup> mice on a C57BL/6 background were generated by one of us (T.T.; Ref. 23) and were bred and maintained in filter-topped isolator cages at our animal facility. PIR-B<sup>−/−</sup> mice were backcrossed with C57BL/6 mice for at least nine generations. All animals of both sexes were used at 6–12 wk of age. All studies involving animals were conducted in accordance with and after approval of the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Bacterial strain, preparation, and inoculation

WB335, an LT2 strain of Salmonella enterica serovar Typhimurium (S. typhimurium) attenuated due to a spontaneous mutation of rpoS, an RNA polymerase σ factor (27, 28), was used in the present study. This attenuated strain was used because C57BL/6 mice are hypersusceptible to WT Salmonella (27, 28). WB335 was stored at −80°C and a single colony of WB335 from a blood agar plate was picked to LB broth and grown to an OD of 0.5 at 600 nm and the resultant bacterial suspensions were frozen in the presence of 10% glycerol at −80°C as frozen infection stocks. For all infection experiments, the bacterial dose of frozen stocks was re-evaluated by counting colonies on blood agar plates. Visible CFU were determined after overnight incubation at 37°C. All opsonization procedures in this study were performed using noninflamed sera. Erythrocytes in the collagenase-treated splenic cell suspension were lysed in 0.15 M ammonium chloride, pH 7.4. Both cell suspensions with washes to remove tissue debris and subjected to cell surface immunostaining as described previously (7). Briefly, cells were first incubated with Ab93 mAb (rat γ2a isotype) to block the FcγRIII/II (30) and then with the following reagents: FITC- or PE-labeled mAbs specific for mouse CD4 (H129.19, rat CD8, D3S–6.7, rat CD2, CD11c (HL3, hamster γ1), or CD11b (M1/70, rat γ2b) (BD Pharmingen), PE-labeled anti-mouse PIR mAb (6C1, rat γ1c; Ref. 7), or biotin-labeled rat mAbs specific for mouse CD19 (1D3, y2a) (BD Pharmingen) or liver sinusoidal endothelial cell (LSEC; ME-9F1, y2a) (Millenyi Biotech). Streptavidin-allophycocyanin was used as a developing reagent for biotinylated mAbs. Rat anti-mouse endoglin/CD105 mAb (clone 209F7, y2a) (R&D Systems) was labeled with Alexa Fluoro 488 (Invitrogen Molecular Probe). The controls included the isotype-matched irrelevant mAbs labeled with the corresponding fluorochromes or biotin. The stained cells were analyzed by the FACScalibur or FACSsort flow cytometer with CellQuest software (BD Pharmingen).

Phagocytosis analysis of cells

Bone marrow cells were obtained from the femurs of PIR-B<sup>−/−</sup> and WT C57BL/6 mice of the same age and sex. After lysing erythrocytes, bone marrow cells were cultured at 3 x 10<sup>6</sup> cells/ml in RPMI 1640 containing 10% FCS and 10% macrophage CSF (M-CSF) conditioned medium, the culture supernatant of the CMG cell line which was transfected with mouse M-CSF DNA and was kindly provided by Dr. Eric Brown (University of California at San Francisco, San Francisco, CA), for 3 days. After removing nonadherent cells, the adherent cells were cultured in the above fresh medium for an additional two days and harvested to be used as bone-marrow-derived macrophages (BMM<sub>Φ</sub>). Bone marrow polymorphonuclear leukocytes (BMPMN) were enriched by centrifugation at 1200 x g for 30 min at 25°C over a 62% Percoll/38% Percoll (20) gradient (3 x 10<sup>6</sup> cells/ml of 62% Percoll/38% Percoll). BMM<sub>Φ</sub> or BMPMN were incubated with the heat-killed, Alexa 488-labeled, opsonized WB335 at different ratios of bacteria/cell numbers from 10/1 to 1/10 for 30 min with shaking at 37°C. All opsonization procedures in this study were performed using normal fresh mouse sera. After quenching the fluorescence of the extracellular pool with trypan blue at the final concentration of 0.2% for the intracellular fluorescence of phagocytosed WB335 was assessed by FACSot (31, 32).

Intracellular killing assay

For in vitro infection, exponential phase WB335 bacteria, which were opsonized and resuspended in DMEM containing 10% FCS without antibiotics, were added in triplicate at various multiplicity of infection (MOI) into 96-well plates containing 3 x 10<sup>5</sup> BMM<sub>Φ</sub> or BMPMN per well, centrifuged briefly, and incubated at 37°C for 25 min under 5% CO<sub>2</sub>, before addition of gentamicin at the final concentration of 100 mg/ml to kill the extracellular WB335 for 1 h. After replacing the medium with DMEM/10% FCS containing gentamicin (10 μg/ml), infected cells were cultured
for another 2 or 24 h, washed, and lysed in 100 μl Triton X-100 before CFU plate counts (33).

Assays for superoxide, nitrite, and TNF-α release

BMMφ (5 × 10³ cells) or BMPMN (5 × 10³ cells) were resuspended in 250 μl of HBSS containing 10 mM HEPES, 0.5 mM CaCl₂, 1 mM MgCl₂, and 120 μM cytochrome C; plated in triplicate into polystyrene tubes; and stimulated with or without live serum-opsonized WB335 at various MOI or 162 nM PMA for 2 h (for BMMφ) or 15 min (for BMPMN) in 37°C shaking water bath. The respiratory burst reaction as measured by the cytochrome C reduction was stopped by incubation on an ice bath for 10 min, followed by centrifugation at 2000 rpm for 5 min at 4°C and assessment of the supernatant absorbance at 550 nm. The OD values were converted to the nmoles of the reduced cytochrome C by using the extinction coefficient of E550 nm = 2.1 × 10⁴ M⁻¹ cm⁻¹ (34). For nitrite production, BMMφ (10⁵ cells) or BMPMN (5 × 10³ cells) were plated in triplicate into 96-well plates and stimulated with or without heat-killed opsonized WB335 at different concentrations or LPS (1 μg/ml) for 48 h (for BMMφ) or 24 h (for BMPMN), before collection of the supernatants. The concentration of nitrite in the resultant culture supernatants was measured as an index of NO synthase activity by the Griess Reagent system (100–1.56 μM for sensitivity; Promega) according to the manufacturer’s instructions. Labeled bacteria were washed twice, resuspended in 2 ml of HBSS containing 10 mM HEPES, 0.5 mM CaCl₂, 1 mM MgCl₂, and 120 μM cytochrome C; plated in triplicate into polypropylene tubes; and stimulated with or without heat-killed opsonized WB335 bacteria (10⁹ cells) were labeled with the 12 kDa cytochrome C molecule is likely excluded from the interior of the cell due to its size (35). To determine oxidant production inside the phagosome (36, 37), heat-killed WB335 bacteria (10⁷ cells) were labeled with the oxidant sensitive fluorescent dye OxyBURST Green H₂DCFDA (2′,7′-dichlorodihydrofluorescein diacetate; Molecular Probes) according to the manufacturer’s instructions. Labeled bacteria were washed twice, resuspended in PBS and opsonized before incubation with BMMφ at different ratios of bacteria/BMMφ numbers for 2 h at 37°C with shaking. After stopping the reaction by incubation iced bath for 10 min, the fluorescence of BMMφ that engulfed H₂DCFDA-WB335 bacteria was analyzed by FACSort.

Statistical analysis

Data are recorded as the mean ± 1 SD. Differences in group survival were analyzed using Mantel Cox Logrank p test. All other simple comparisons were performed with Student’s t test, with p ≤ 0.05 considered to represent statistical significance.

Results

PIR-B⁻/⁻ mice are more susceptible to Salmonella infection than WT mice

To determine the role of PIR-B in the bacterium/host interaction, we used the Salmonella enteric fever mouse model. S. typhimurium is known as a Gram-negative, facultative intracellular pathogen capable of replication both outside and inside host cells. The WB335 strain of S. typhimurium, which has a mutation of RNA polymerase σ factor (rpoS) resulting in attenuated virulence in susceptible strains of mice such as C57BL/6 (27, 28), was selected for this purpose. Both PIR-B⁻/⁻ and WT C57BL/6 mice of the same age and sex were infected i.v. with various doses of the attenuated WB335 organisms (10³ to 6 × 10⁶ CFU/mouse) in a group of 10–15 mice per each dose and their survival status was monitored for three to four weeks after infection. When inoculated with the high dose of bacteria (6 × 10⁶ CFU/mouse), both groups

FIGURE 1. Survival of mice from Salmonella infection. Age-matched, PIR-B⁻/⁻ (○) and WT (□) C57BL/6 male mice (n = 12 for A and B, n = 10 for C and D, and n = 15 for E) were infected i.v. with the indicated doses of attenuated strain (WB335) of S. typhimurium and survival was monitored for 21 to 30 days. The y-axis indicates survival (%) and the x-axis indicates days after infection.
of mice died within 3 days postinfection (Fig. 1A). When injected with 10- or 100-fold fewer bacteria, all PIR-B\(^{-/-}\) mice and most WT mice died within two weeks after infection (Fig. 1, B and C). However, a small proportion (10–20%) of the WT mice survived for the entire 3 wk test period. A more striking survival difference was observed in the mouse groups receiving lower doses of bacteria (\(5 \times 10^3\) CFU/mouse). With one exception, PIR-B\(^{-/-}\) mice receiving \(5 \times 10^3\) CFU died over a 4-wk period starting at 4 days postinfection, whereas many control mice survived during this time period (Fig. 1D). Furthermore, many of the infected PIR-B\(^{-/-}\) mice showed signs of morbidity much sooner than the infected WT mice during the course of infection. When inoculated with \(10^3\) CFU/mouse, most PIR-B\(^{-/-}\) mice survived for the first 2 wk but started to die in the third week, which resulted in a much sharper slope of the mortality curve than that with \(5 \times 10^3\) CFU (Fig. 1E). Most WT mice survived during this 4-wk period. Although these survival experiments were conducted using male mice, the same results were obtained with female mice, indicating no sexual differences in the susceptibility to Salmonella infection of PIR-B\(^{-/-}\) mice (data not shown). Notably, when the RAG-2\(^{-/-}\) mice, which are totally deficient in mature T and B cells, were infected with the same WB335 strain at \(5 \times 10^5\) CFU/mouse, all five mice survived for the first 14 days after infection and then died during the next 2 days, suggesting that similar to other strains of \(S.\) typhimurium, both innate and adaptive immunity are important for full protection against infection with the attenuated WB335 strain (data not shown). Collectively, these findings indicate that PIR-B\(^{-/-}\) mice are more susceptible than WT mice to infection with \(S.\) typhimurium.

PIR-B\(^{-/-}\) mice are incapable of controlling bacteria replication in vivo

To determine the tissue localization of inoculated bacteria, three to five mice in each dose group were sacrificed during the course of infection and the bacterial loads in various organs were assessed. In mice receiving higher doses of bacteria (\(>6 \times 10^4\) CFU/mouse), both PIR-B\(^{-/-}\) and WT mice exhibited similar bacterial burdens at 2 and 3 days postinfection in all tissues examined including the blood (Fig. 2, A–C). By contrast, in mice receiving low doses of bacteria (\(5 \times 10^3\) CFU), PIR-B\(^{-/-}\) mice were found to have more bacteria than WT mice in the liver and spleen at 7 days postinfection (Fig. 2E). Furthermore, the bacterial burden in those tissues of PIR-B\(^{-/-}\) mice reached ~\(5 \times 10^7\) CFU, close to the maximum number of bacteria recovered from live mice. Bacteremia was detected at 3 days (2/5 mice) and 7 days (2/3 mice) postinfection in PIR-B\(^{-/-}\) mice, but not in WT mice, consistent with the high mortality of PIR-B\(^{-/-}\) mice. In the one PIR-B\(^{-/-}\) mouse that survived for the entire 30 day period, bacteria were undetectable in the blood (Fig. 2F). These findings suggest that the high susceptibility of PIR-B\(^{-/-}\) mice to Salmonella infection is associated with a failure to control bacterial replication in target organs.

Mostly comparable blood levels of cytokines and Abs in both groups of mice

The ability of Salmonella to survive and/or replicate inside macrophages is dependent on the activation state of the host cells that are affected by host cytokines. To determine the role of cytokines in the susceptibility of PIR-B\(^{-/-}\) mice to Salmonella infection, we

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![FIGURE 2.](http://www.jimmunol.org/Downloaded-from/http://www.jimmunol.org/) Bacterial titers in various tissues of mice infected with \(S.\) typhimurium WB335. PIR-B\(^{-/-}\) (○) and WT (○) mice were infected i.v. with the indicated CFU of WB335 organisms. Three (A–C, E, F) or five (D) mice were sacrificed at the indicated days after infection except for one PIR-B\(^{-/-}\) mouse at 30-day postinfection. The bacterial loads (CFU) in the indicated tissues were assessed as described in Materials and Methods. The Log\(_{10}\) values of WB335 CFU per organ represent individual mice. The geometric mean values (―) and the detection limits are indicated (⊥). The total blood volume (ml) was determined as one thirteenth of the body weight (gm).
determined the plasma levels of representative proinflammatory (TNF-α and INFγ) and anti-inflammatory (IL-10) cytokines during the course of infection by ELISA. The concentrations of these cytokines in the blood of infected animals were indistinguishable between PIR-B−/− and WT mice, except for the TNF-α level at 14 days postinfection where PIR-B−/− mice showed significantly higher than WT mice (Fig. 3, A). Notably, there was a large SD of the concentrations of IL-10 in PIR-B−/− mice at 14 days postinfection, suggesting considerable individual variability among the six mice examined. Because Ab is shown to be important for full protection against Salmonella infection, we measured the titer of Abs to the WB335 strain in the plasma at 14 days postinfection. The titers of three major classes of anti-Salmonella Abs did not differ significantly between PIR-B−/− and WT control mice (Fig. 3B). Thus, blood levels of cytokines and Abs could not be the explanation for the susceptibility of PIR-B−/− mice to infection with Salmonella.

Distinct patterns of inflammatory foci and bacteria spreading in liver between PIR-B−/− and WT mice

To obtain further insight into the progression of Salmonella infection in PIR-B−/− and WT mice, we examined the histology of the major organs. Macroscopically, as expected, the spleen size markedly increased following infection and the extent of splenomegaly was comparable in both groups of mice except at 30 days postinfection. The sizes of other organs after infection were also comparable in both groups (Fig. 4A). Microscopically, the white pulp in the spleen was markedly expanded at 1 day postinfection. Numerous nodular inflammatory foci called typhoid nodules, which were characterized by cellular aggregates of PMN (in early stage) and macrophages (in later stage), were scattered predominantly in the red pulp at day 3 of both the WT and PIR-B−/− mice. By day 7, in addition to numerous granulomatous typhoid nodules, we observed the obliteration of follicular margins and the marked proliferation of sinus lining cells and sinus macrophages in both groups of mice (Fig. 4B). These granulomatous typhoid nodules and histological changes in the spleen were still prominent at 14 days and 30 days postinfection in both groups.

In the liver, small typhoid nodules consisting of PMN and Kupffer cells or macrophages were visible in hepatic lobules as early as 1 day postinfection and their incidence increased in the next six days in both groups of mice. Quite distinctive pathological changes were observed, however, at 7 days postinfection. In the WT mice, the inflammatory cellular lesions were localized and exhibited nodular distribution, whereas in the PIR-B−/− mice, in addition to granulomatous typhoid nodules, inflammatory mononuclear cells infiltrated along the sinusoids, resulting in a spreading pattern and the dilatation of sinusoidal spaces accompanied with narrowing of hepatic cords (Fig. 4C). These sinusoidal changes were still observed in most of the PIR-B−/− mice (4/5), but only in a minority of the WT mice (1/5) at 14 days postinfection. At 30 days postinfection the inflammatory foci, including the granulomatous typhoid nodules, became smaller and infrequent in WT mice, but were still frequently observed along with mild sinusoidal infiltration in the surviving PIR-B−/− mouse, indicating that the effects of Salmonella infection were more readily controlled in the WT mice than in the PIR-B−/− mice. In addition to the above changes, fibrin deposition, thrombosis formation, and parenchymal necrosis were also observed in both groups after 3 days postinfection.

To determine the bacterial distribution in livers, we performed immunofluorescence analysis using Salmonella-specific Abs. Salmonella were found more diffusely along the sinusoids in PIR-B−/− mice, but were more localized in the WT mice (data not shown), consistent with the patterns of inflammatory cellular infiltration on H&E stained slides. In lungs and kidneys, interstitial inflammatory cell infiltration was observed in both groups after 7 days postinfection. Collectively, these findings suggest that while the splenic histological changes following Salmonella infection are
FIGURE 4. Pathological changes in mice infected with *S. typhimurium* WB335. A, PIR-B−/− (●) and WT (○) mice infected with WB335 *Salmonella* (5 × 10^3 CFU) were sacrificed at the indicated days of postinfection and the weight index of each organ was estimated as: (the organ weight from infected animals) / (the organ weight from uninfected animals). Results are expressed as arithmetic means ± SD. B and C, H&E staining of spleen and liver at 3 and 7 days postinfection with 5 × 10^3 CFU.
indistinguishable between the PIR-B−/− and WT mice, the liver exhibits quite distinctive inflammatory lesions with diffuse spreading along the sinusoids in PIR-B−/− mice vs nodular localization in WT mice.

Salmonella-infected PIR-B−/− mice have more inflammatory cells in liver, but fewer B cells and CD8+ T cells in spleen than WT infected mice

Because the histological changes of liver were evident at 7 days postinfection (see Fig. 4C), we examined by flow cytometry the infiltrating cells, resident Kupffer cells, and sinusoidal endothelial cells in the 7-day liver samples. As shown in Fig. 5A, the total numbers of Mac-1+/Gr-1+ macrophage/Kupffer cells and of Mac-1+/Gr-1+ PMN in the liver are two- to three-fold greater in PIR-B−/− mice as compared to WT mice. The cell surface PIR levels were also compared among these cell populations using the 6C1 mAb, which recognizes both PIR-A and PIR-B. The surface PIR-A level in the PIR-B−/− macrophage/Kupffer cell, but not PMN, population was enhanced two-fold in comparison with the surface PIR-A and PIR-B levels in the WT cell populations, indicating in turn the enhanced expression of PIR-A on PIR-B−/− macrophage/Kupffer cells (Fig. 5B). The total numbers of CD4+ T, CD8+ T, and CD19+ B cells in the liver were comparable in both groups of mice. Despite the pathological findings of bacterial spreading along the liver sinusoids in PIR-B−/− mice, the sinusoidal endothelial cell population defined by the expression of endoglin/CD105 and LSEC Ag was also indistinguishable between the PIR-B−/− and WT mice.

In spleen, the total numbers of CD19+ B cells, CD4+ T cells, CD8+ T cells, Mac-1+ macrophages, Gr-1+ PMN, and CD11c+ dendritic cells were comparable between PIR-B−/− and WT mice at 7 days postinfection (data not shown). At 14 days postinfection, however, the number of CD19+ B cells and, to a lesser extent, of CD8+ T cells in PIR-B−/− mice did not increase as markedly as in WT mice after Salmonella infection (Fig. 5C). As expected, Mac-1+ macrophages and Gr-1+ PMN were markedly increased in both groups of mice. Other cell subsets were comparable in both groups of mice. Collectively, these findings suggest that systemic infection of Salmonella results in more inflammatory infiltrating cells in liver and/or Kupffer cell reactions, enhanced expression of PIR-A by macrophage/Kupffer cells, and insufficient increase of splenic B cells and CD8+ T cells in PIR-B−/− mice.

PIR-B−/− macrophages are unable to control intracellular Salmonella growth ex vivo

To explore the cellular basis for high susceptibility of PIR-B−/− mice to Salmonella infection, BMMφ and BMPMN from PIR-B−/− and WT mice were examined for their intrrove responses to...
WB335 bacteria. The ability to engulf fluorochrome-labeled, heat-killed WB335 by BMMφ and BMPMN was comparable between PIR-B−/− and WT mice at different ratios of bacteria/phagocytic cells (Fig. 6A). Upon in vitro infection with live WB335, bacterial replication in BMPMN was indistinguishable in both groups of mice at both 2- and 24-h postinfection at different MOI. In the PIR-B−/− BMMφ, however, more bacterial growth was evident at 24 h postinfection with a high MOI (i.e., 10.8) when compared with WT BMMφ. These results show that PIR-B−/− BMMφ are unable to control intracellular Salmonella replication, consistent with the in vivo findings (Fig. 6B). The extracellular superoxide anion release by phagocytes from WT and PIR-B−/− mice upon in vitro infection with heat-killed (not shown) or live WB335 or stimulation with 12-O-tetradecanoylphorbol-13-acetate or fMLP was

FIGURE 6. Ex vivo functions of phagocytes from PIR-B−/− and WT mice. A, Phagocytic activity. BMMφ (left) and BMPMN (right) from PIR-B−/− (■) and WT (□) mice were incubated with heat-killed, Alexa 488-labeled, opsonized WB335 at the indicated ratios of bacteria/phagocytic cells. After quenching the extracellular fluorescence, the intracellular fluorescence of engulfed WB335 was determined by flow cytometry. B, Bactericidal activity. Phagocytes described in A were infected with live opsonized WB335 bacteria at the indicated MOI, and the number of intracellular bacteria was enumerated by CFU plate counts at the indicated time periods. C–F, Cells described in A were incubated with the indicated stimuli and the superoxide anion release (C), the nitrite production (E) and the TNF-α production (F) were assessed as described in Materials and Methods. For phagosomal oxidant production (D), BMMφ from PIR-B−/− and WT mice were incubated with oxidant sensitive dye-labeled WB335 bacteria at the indicated ratios and the fluorescence of BMMφ engulfing the dye-labeled WB335 was analyzed by FACSort. A typical profile at the Salmonella/BMMφ ratio of 100 is shown (left panel) and the values of [100 × (MFI of PIR-B−/− BMMφ/MFI of WT BMMφ)] at different ratios are plotted (right panel). Results are expressed as means ± SD from three different experiments.
similar (Fig. 6C). However, oxidant production inside the phagosome engulfing WB335 was clearly diminished in PIR-B−/− BMMφ compared with WT BMMφ (Fig. 6D). Unlike BMMφ, the phagosomal oxidation in BMPMN was indistinguishable between WT and PIR-B−/− mice (not shown). It should be noted that similar reduction in phagosomal oxidation by PIR-B−/− BMMφ was also observed with other heat-killed microorganisms (e.g., E. coli, S. pneumoniae, S. aureus, and C. albicans), suggesting a generalized phenomenon related to the PIR-B-deficient macrophages and not to a particular pathogen (not shown). These findings are thus consistent with the inability of PIR-B−/− macrophages to control intracellular bacterial growth.

PIR-B−/− BMMφ were also found to produce more nitrite than the WT BMMφ when exposed to heat-killed WB335 at different ratios of bacteria/phagocytes (Fig. 6E). This was also observed with LPS stimulation. Unlike BMMφ, the BMPMN, irrespective of the mouse genotype, did not produce significant amounts of nitrites by either WB335 or LPS stimulation. Furthermore, the PIR-B−/− BMMφ were found to produce more TNF-α upon exposure to the heat-killed WB335 than the WT BMMφ (Fig. 6F). In contrast, BMPMN produced comparable amounts of TNF-α upon stimulation with heat-killed WB335. Collectively, these findings suggest that PIR-B−/− macrophages are unable to control the intracellular growth of Salmonella, but are hyperresponsive to the heat-killed Salmonella as evidenced by enhanced production of nitrite and TNF-α ex vivo.

Discussion

Using a mouse model of Salmonella infection with an attenuated strain of S. typhimurium (WB335), we showed that PIR-B−/− mice were more susceptible to Salmonella infection than the WT mice, as evidenced by increased mortality rate, high bacterial loads in the liver and spleen, and a failure to clear bacteria from the circulation. The blood levels of cytokines (TNF-α, IFN-γ and IL-10) and Salmonella-specific Abs (IgM, IgG, and IgA) were mostly comparable between these two groups of mice. However, distinct bacterial spreading patterns were notable in the liver at 7–14 days postinfection and a diffuse spreading along the sinusoids was observed in PIR-B−/− mice vs a nodular restricted localization in WT mice. PIR-B−/− mice had more inflammatory cells in the liver, but fewer B cells and CD8+ T cells in the spleen than WT mice. These in vivo differences were substantiated by the ex vivo findings where BMMφ in PIR-B−/− mice failed to control intracellular replication of Salmonella due to inefficient phagosomal oxidant production compared with those in WT mice. PIR-B−/− BMMφ also produced more nitrite and TNF-α upon exposure to Salmonella than control macrophages.

Although PIR-A and PIR-B are among the first paired receptors with opposing signaling capabilities to be identified, there are now more than 20 such related immunoreceptors reported, implying that the pairing of activation and inhibition is essential in modulators for initiation, amplification, and termination of immune responses (1, 2). Because PIR is expressed by many hematopoietic cell types (B cells, monocyte/macrophages, DC, PMN, mast cells, and megakaryocyte/platelets), it has been postulated that the disruption of PIR-A and PIR-B balance may affect their regulatory roles in host defense, including humoral immune, inflammatory, Ag-presenting, allergic, and coagulative responses. Indeed, several functional alterations have been reported for the PIR-B−/− mice. These include hyperreactivity of B cells to BCR ligation and to T cell independent Ags (24), enhanced Th2 response due to immature DC (24), exaggerated GVH reactions when sublethally irradiated PIR-B−/− mice receive allogeneic splenocytes (21), and hyperresponsiveness of macrophages and PMN to integrin ligation ex vivo (25, 26). When we initially set up the present experiments, it was difficult to anticipate what the outcomes would be. The expected enhanced inflammatory responses in the PIR-B−/− mice would render them more resistant to Salmonella infection. In contrast, their diminished ability to attenuate the inflammatory reactions might have pathological sequelae.

We have now shown that the PIR-B−/− mice are more susceptible to the primary infection of WB335 than the WT control mice. Interestingly, the slopes of the morbidity curves of 5 × 10³ vs 10³ CFU dose are different; in the former higher dose PIR-B−/− mice died gradually beginning at day 4 postinfection over the next 3 wk, whereas in the latter lower dose most PIR-B−/− mice survived for two weeks but began to die in the third week postinfection, similar to Rag-2−/− mice receiving 5 × 10³ CFU/mouse. It has been shown that the innate immune system can restrict replication of S. typhimurium to a certain degree, but that acquired immunity is essential for effective control and eradication of bacteria (38–40). These survival data suggest that the disruption of the Pirb gene affects both innate and acquired immunity to a primary infection with S. typhimurium.

The high susceptibility of PIR-B−/− mice to Salmonella infection appears to be associated with their inability to control bacterial replication in target organs as evidenced by high bacterial loads in the liver, spleen, and lung tissues; diffuse spreading pattern of bacteria along the liver sinusoids; failure to efficiently clear bacteria from circulation; and more intracellular bacterial growth inside the marrow-derived macrophages ex vivo. It has recently been shown that PIR-B can recognize S. aureus as well as some Gram-negative bacteria such as H. pylori and E. coli, suggesting that PIR-B may recognize pathogen-associated molecular patterns (23). In this regard, we found by immunofluorescence that neither PIR-B nor PIR-A could directly bind live or heat-killed WB335 (S. Oka, unpublished observations).

PIR-B−/− and WT phagocytes ingested opsonized WB335 and subsequently released superoxide anion into culture supernatants at comparable levels. It has been shown that Salmonella survive and replicate in phagocytes within unique Salmonella-containing vacuoles (SCV) inaccessible to the host defense mechanisms (41–43). Delayed acidification of SCV and their incomplete fusion with lysosomes are thought to promote intracellular survival of Salmonella in these vacuoles. Phagosomal oxidant production by PIR-B−/− macrophages was indeed less than by WT macrophages. This reduced production of oxidants may permit increased replication of bacteria inside PIR-B−/− macrophages and leads to the inability to control Salmonella replication in the reticuloendothelial system of PIR-B−/− mice.

The precise mechanism by which the PIR-B deficiency leads to reduction of phagosomal oxidation is presently unknown. However, the extracellular superoxide release by both macrophages and PMN and the phagosomal oxidation by PMN were comparable between PIR-B−/− and WT mice, suggesting that the assembly and activation processes of the NADPH oxidase subunits might not be impaired in PIR-B-deficient phagocytes. It should also be noted that heat-killed bacteria were used for phagosomal oxidation assays because the live bacteria could not be labeled with an oxidation-sensitive dye; hence, the evading strategies of Salmonella from host defense could be excluded from our consideration of the mechanism. Furthermore, the reduced phagosomal oxidation in PIR-B-deficient macrophages was a generalized phenomenon as observed with several other heat-killed microorganisms. Collectively, the processes of phagosomal development and consequent oxidant production appear to be indirectly impaired by disruption of the PIR-A and PIR-B balance in macrophages, possibly through differences in the phagocytosing cellular status, the stimuli via
multiple phagocytic receptors, the cytokine milieu, and the time after onset of exposure to pathogens. Alternatively, PIR-B may have a dual function of both inhibitory and activating activities as suggested by the presence of an additional Src homology 2-binding motif called the immunoreceptor tyrosine-based switch motif (ITSM) "T/SxYxxVII" (where x represents any amino acid) (44). In this regard, it has recently been shown that while PIR-B negatively regulates the eotaxin-dependent eosinophil chemotaxis by recruiting the SHP-1 protein tyrosine phosphatase. PIR-B also positively regulates the leukotriene B4-induced eosinophil chemotaxis by recruiting activating kinases (JAK1, JAK2, Shc, and Crk) (13). It is thus conceivable that PIR-B may recruit some kinases, such as Src family kinases, Syk, and PI3 kinase p85 known to be involved in activation of NADPH oxidase (45, 46), through its ITSM during phagocytosis.

Another clear difference in Salmonella-infected animals is the distinct patterns of inflammatory lesions in the liver after infection. Although in WT mice such lesions were more localized and exhibited nodular distribution, in PIR-B−/− mice inflammatory cells infiltrated diffusely along the sinusoids and exhibited a spreading pattern. PIR-B−/− mice also had more macrophages and PMN in the liver than WT mice, as determined by flow cytometry. These differences were observed as early as 7 days postinfection. Notably, the cell surface PIR-A levels on PIR-B−/− macrophages, but not PMN, were enhanced 2-fold in comparison with those on WT macrophages which were contributed by PIR-A and PIR-B. In our previous study, cell surface levels of PIR on WT splenic B cells and macrophages were found to be up-regulated by ~33% after LPS stimulation for 3 days in vitro (7). The observed enhancement of surface PIR-A levels seen in Salmonella-infected PIR-B−/− mice might result from long exposure to Salmonella, leading to dysregulated macrophage responses against bacterial infection. The increased production of NO and TNF-α by these PIR-B−/− macrophages may have detrimental, rather than protective, effects locally on the surrounding hepatic tissues. Similar association of enhanced PIR-A expression with enhanced host reaction was also demonstrated in DC populations of PIR-B−/− mice that develop an exaggerated acute GVH reaction (21). One of the unique features of PIR is that PIR-B on resting B cells, DC, and myeloid cells is constitutively tyrosine-phosphorylated by Src family tyrosine kinases (Lyn or Fgr and Hck) and associates constitutively with tyrosine phosphatases (SHIP-1 and SHP-2), thereby restraining potential cellular activation via BCR, integrin, and chemokine receptors (25, 26, 47, 48). This tonic inhibition is apparently absent in PIR-B−/− mice and the present studies clearly indicate that the disruption of PIR-A and PIR-B balance also affects their regulatory roles in host defense to bacterial infection.

The finding that the total numbers of splenic B cells and, to a lesser extent, CD8+ T cells in PIR-B−/− mice did not increase as markedly as those in WT mice at 14 days postinfection was unexpected. Septis or endotoxemia is known to accelerate lymphocyte apoptosis in both animals and humans (49, 50). In our studies, LPS-induced apoptosis was frequently observed in the splenic white pulp of PIR-B−/−, but not WT, mice and B cells appeared to be more prone to this cell death (I. Torri and H. Kubagawa, unpublished observations). It is thus reasonable to suggest that PIR-B deficiency leads to increased sensitivity of B cells to LPS-induced apoptosis during Salmonella infection. As T cells do not express PIR, the inability of PIR-B−/− mice to increase CD8+ T cells two weeks after Salmonella infection results most likely from an indirect effect of PIR-B deficiency.

The molecular mechanisms of resistance and susceptibility to Salmonella infection are extremely complex and multifactorial with host innate and adaptive immune responses vs with Salmonella virulence factors or evading systems. Inbred strains of mice vary considerably in resistance or susceptibility to Salmonella infection and several major resistance genes have been identified in mice (51). These include MHC class I and II, natural resistance associated macrophage protein, TLR4, Bruton’s tyrosine kinase, LPB-binding protein and CD14, NADPH oxidase and inducible NO synthase, and cytokines (TNF-α, INF-γ). Many immunodeficient mouse strains are also unable to control the in vivo replication of attenuated Salmonella strains. For example, T cell deficient nu/nu, MHC class II−/−, TCRβ−/−, INF-γ−/−, TNF-α p55R−/−, CD28−/−, CD40L/CD154−/− and MD-2−/− mice all succumb to infection with attenuated strains of Salmonella that are normally eradicated in WT mice (52–55). PIR-A and PIR-B are one pair of the family members of paired immunoreceptors which share similar ectodomains but exhibit opposing signaling capabilities and are postulated to play important regulatory roles in host defense based on their wide cellular distribution. In this study, we show clear evidence that the balance of PIR-A and PIR-B functional activities is important for immune responses to bacterial infection.

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Disclosures
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