Paired Immunoglobulin-Like Receptor–B Inhibits Pulmonary Fibrosis by Suppressing Profibrogenic Properties of Alveolar Macrophages

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Macrophages are lung-resident cells that play key roles in fibrosis. Surprisingly, pathways that inhibit macrophage functions, especially in idiopathic pulmonary fibrosis (IPF), receive little attention. The cell-surface molecule paired immunoglobulin-like receptor B (PIR-B) can suppress macrophage activation. However, its role in pulmonary fibrosis remains unknown. We sought to define the role of PIR-B in IPF. The expression of PIR-B was assessed (by quantitative PCR and flow cytometry) after bleomycin treatment. Differential cell counts, histopathology, and profibrogenic-mediator expression, for example, collagen, α-smooth muscle actin, resistin-like molecule–α (Relm-α), matrix metalloproteinase (MMP)–12, and tissue inhibitor of metalloproteinase (TIMP)–1, were determined (by ELISA quantitative PCR and flow cytometry) in the lungs of wild-type and Pirb−/− mice after bleomycin or IL-4 treatment. Bone marrow-derived wild-type and Pirb−/− macrophages were stimulated with IL-4 and assessed for Relm-α and MMP-12 expression. PIR-B was up-regulated in lung myeloid cells after bleomycin administration. Bleomycin-treated Pirb−/− mice displayed increased lung histopathology and an increased expression of collagen and of the IL-4–associated profibrogenic markers Relm-α, MMP-12, TIMP-1, and osteopontin, which were localized to alveolar macrophages. Increased profibrogenic mediator expression in Pirb−/− mice was not attributable to increased IL-4/IL-13 concentrations, suggesting that PIR-B negatively regulates IL-4-induced macrophage activation. Indeed, IL-4–treated Pirb−/− mice displayed increased Relm-α expression and Relm-α–macrophage concentrations. IL-4–activated Pirb−/− macrophages displayed increased Relm-α and MMP-12 induction. Finally, leukocyte immunoglobulin-like receptor subfamily B member 3 (LILRB3)/immunoglobulin-like transcript–5, the human PIR-B orthologue, was expressed and up-regulated in lung biopsies from patients with IPF. Our results establish a key role for PIR-B in IPF, likely via the regulation of macrophage activation. Therefore, Pirb/LILRB3 may offer a possible target for suppressing macrophage profibrogenic activity in IPF.

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molecules (19–22). Based on various similarities between PIR-B and the human leukocyte immunoglobulin-like receptor subfamily B member 3 (LILRB)/immunoglobulin-like transcript (ILT) family of receptors (e.g., structure, ligand binding, and genomic localization), PIR-B is likely the orthologue of human LILRB3/ILT-5 (22). Thus, defining the role of PIR-B in regulating murine immune cell responses is relevant to human immune cell activation. Specifically, the role of PIR-B in the regulation of pulmonary fibrosis remains unknown.

In this study, we demonstrate that Pirb−/− mice display an increased susceptibility to bleomycin (BLM)-induced fibrosis. To this end, bleomycin-treated Pirb−/− mice evidence increased histopathology and collagen production. Furthermore, they exhibit increased concentrations of the profibrogenic markers Relm-α, MMP-12, and osteopontin (OPN). Notably, the increased expression of Relm-α and MMP-12 in bleomycin-treated Pirb−/− mice was localized to alveolar macrophages. Subsequent analyses revealed that PIR-B suppressed IL-4–induced responses in vivo, and inhibited IL-4–induced macrophage activation in vitro. Finally, LILRB3/ILT-5 was readily detected in lung alveolar macrophages, and its expression was increased in lung biopsies of patients with IPF. Collectively, our data demonstrate a key role for PIR-B in IPF, and highlight the PIR-B/macrophage axis as a potential immunopharmacological target in this disease.

MATERIALS AND METHODS

Mice
Male and female 6- to 8-week-old Pirb−/− mice (backcrossed > F9 to C57BL/6) were kindly provided by Dr. Hiromi Kubagawa (University of Alabama, Birmingham, AL) (23). C57BL/6 wild-type mice were obtained from Harlan Laboratories (Rehovot, Israel). In all experiments, age-matched, weight-matched, and gender-matched mice were housed under specific pathogen-free conditions, according to institutional, age-matched, weight-matched, and gender-matched mice were housed under specific pathogen-free conditions, according to institutionally approved protocols of the Animal Care Committee at Tel Aviv University.

BLM-Induced Pulmonary Fibrosis
Mice were anesthetized with xylazine and ketamine, and intratracheally challenged with either bleomycin sulfate (0.03–0.1 U/mouse) or saline (50 μl/mouse). Mice were killed 0–28 days after challenge, and bronchoalveolar lavage fluid (BALF) was obtained as described elsewhere (24).

Real-Time Quantitative PCR
Lung cDNA was subjected to quantitative PCR, as previously described (25). A complete list of primers used in this study is provided in Table E1 in the online supplement.

Flow Cytometry
A detailed description of our flow cytometry procedures is available in the online supplement.

Histopathology
Saline-challenged and BLM-challenged lungs were fixed, paraffin-embedded, and stained with hematoxylin and eosin (Pioneer Research Chemicals, Essex, UK) or Masson’s trichrome reagents (D.D.K, Milan, Italy) (25).

Immunohistochemistry
Lungs were fixed in 4% (wt/vol) paraformaldehyde and paraffin-embedded. Lung sections (3 μm) were cut, subjected to antigen retrieval and the quenching of endogenous peroxidase activity (3% vol/vol H2O2, 20 min), and incubated with anti–human immunoglobulin-like transcript 5/CD85a (Clone 222821; R&D Systems, Minneapolis, MN). Immune complexes were visualized using peroxidase-coupled secondary antibodies (Histostain Plus Kit; Zymed/Invitrogen, Grand Island, NY).

IL-4–Induced and IL-13–Induced Airway Inflammation
IL-4 has a short half-life in vivo. Thus, a long-acting form of IL-4 was produced by mixing recombinant murine IL-4 (Peprotech, Rocky Hill, NJ) with a neutralizing monoclonal antibody (BVD4-1D11) at a 2:1 molar ratio (IL-4C). This procedure increases the half-life and bioactivity of IL-4 in vivo (26). IL-4C was administered every other day for 4 days. IL-13 was administered as previously described (18). BALF was assessed 48 hours after the final challenge for differential cell counts and Relm-α expression, and lungs were obtained for flow cytometric analysis.

Bone Marrow–Derived Macrophage Activation
Bone marrow (BM)–derived macrophages were obtained as previously described (27). A detailed description of macrophage activation is available in the online supplement.

ELISA
A detailed description of our ELISA procedures is available in the online supplement.

Human Tissue
Lung tissue biopsies were obtained from 10 patients with IPF and a usual interstitial pneumonia histological pattern (three females and seven males; mean age [± SD], 60 ± 2 yr) and 10 control subjects (organ donors; four females and six males; mean age, 45 ± 9 yr). Samples were snap-frozen and subjected to quantitative PCR analysis, as described previously (28). The study protocol was approved by the Ethics Committee of the Justus–Liebig University School of Medicine (approval number AZ 31/93). Informed consent was obtained in written form from each subject for the study protocol.

Statistical Analysis
Data were analyzed by ANOVA, followed by the Tukey post hoc test or Student t test, using GraphPad Prism 4 (GraphPad, Inc., San Diego, CA). Data are presented as mean ± SEM, and P < 0.05 was considered statistically significant.

RESULTS

The Expression of PIR-B Is Up-Regulated in Lung Myeloid Cell Subsets after the Induction of BLM-Induced Pulmonary Fibrosis
PIR-B expression was assessed in whole-lung cDNA obtained 7 days after treatment with BLM or saline (Figure 1A). Quantitative PCR analysis revealed a 3.5 ± 0.8-fold up-regulation of PIR-B expression in the lungs of BLM-treated mice, compared with their saline-treated counterparts (Figure 1A). The assessment of PIR-A/B protein expression in the lungs revealed that PIR-A/B was up-regulated in lung CD45+ cells after BLM treatment (Figure E1). We then examined the cellular source for PIR-A/B up-regulation, using polychromatic flow analysis, gating on CD45+ cells differentially expressing granulocyte receptor (Gr-1) and CD11c (29) (Figure 1B). At baseline, PIR-A/B was expressed in various lung CD45+ cells (Figures 1C–1F). Seven days after BLM challenge, PIR-A/B protein concentrations were up-regulated in Gr-1med/CD11chigh cells (R1 in Figures 1C, 1G, and 1K) and Gr-1med/CD11clow cells (R2 in Figures 1D, 1H, and 1L). In these myeloid populations, increased PIR-A/B expression levels were observed on Day 7 and persisted at least up to 14 days after BLM treatment. Importantly, the BLM-induced PIR-A/B up-regulation was cell-specific.
because PIR-A/B expression was unchanged in Gr-1\textsuperscript{low}/CD11c\textsuperscript{low} cells (R3 in Figures 1E, 1I, and 1M) and Gr-1\textsuperscript{high}/CD11c\textsuperscript{high} cells, likely representing neutrophils (R4 in Figures 1F and 1J). T lymphocytes, which do not express PIR-A/B, were used as an internal negative control (Figures 1B–1F).

PIR-A and PIR-B share a similar extracellular domain (17, 30) that is not distinguished by the commercial anti–PIR-A/B antibody (clones 6C1 or 10-1-PIR). To define whether the observed up-regulation of PIR-A/B was attributable to the increased expression of PIR-A, PIR-B, or both, PIR-A expression was examined in BLM-challenged \textit{Pirb}\textsuperscript{2/2} mice, which still express PIR-A. To this end, single-cell suspensions of enzymatically digested lungs were obtained from BLM-treated wild-type (WT) and \textit{Pirb}\textsuperscript{2/2} mice, and stained with anti–PIR-A/B antibody. PIR-A/B up-regulation was completely diminished in BLM-challenged \textit{Pirb}\textsuperscript{2/2} mice (Figures 1K–1M). Hence, BLM specifically increased PIR-B but not PIRA expression on the surface of Gr-1\textsuperscript{high}/CD11c\textsuperscript{high} cells (R1 in Figure 1K) and Gr-1\textsuperscript{med}/CD11c\textsuperscript{high} cells (R2 in Figure 1L).

**PIR-B Regulates BLM-Induced Lung Infiltration by Inflammatory Cells**

The BLM-dependent up-regulation of PIR-B suggested a role for PIR-B in experimental IPF. Therefore, wild type and \textit{Pirb}\textsuperscript{−/−} mice were challenged with BLM, and the differential accumulation of inflammatory cells in the BALF was assessed. Interestingly, BLM-treated \textit{Pirb}\textsuperscript{−/−} mice displayed decreased neutrophil infiltration and a slight (but statistically significant) reduction in monocyte/macrophage accumulation (Figures 2A, 2C, 2D, and 2F). Notably, these mice also displayed significantly elevated accumulations of lymphocytes (Figures 2B and 2E).

**Increased Histopathology in BLM-Challenged \textit{Pirb}\textsuperscript{−/−} Mice**

PIR-B is a "hallmark" inhibitory receptor capable of suppressing immune-cell activation (17, 31). Thus, we hypothesized that BLM-treated \textit{Pirb}\textsuperscript{−/−} mice will display increased disease severity in comparison with BLM-treated wild-type mice. A histopathological assessment of hematoxylin-and-eosin-stained lung
Pirb-challenged wild-type mice (Figure 3B), and soluble collagen increased lung collagen deposition in comparison with BLM-induced pulmonary pathology. Rather, a significant elevation in the percentage of observed in BLM-treated mice was not attributable to an increased expression of these mediators in alveolar macrophages. Thus, enzymatically digested lung cells were stained with various cell-surface markers and alveolar macrophages were electronically gated (CD45<sup>-</sup>/Gr-1<sup>-</sup>/CD11c<sup>-</sup>/CD11b<sup>-</sup>/Siglec-F<sup>-</sup>) (Figure 5A) (35), and the cells were assessed for Relm-α expression per cell, because the mean fluorescent intensity of a substantial decrease in IL-13 and IL-4 concentrations as those of BLM-treated wild-type mice (Figures 4F–4G).

Of note, additional aaMac markers such as T lymphocyte-derived eosinophil chemotactic factor (YMI) were not up-regulated after BLM challenge, even in wild-type mice (data not shown). Furthermore, concentrations of BLM-induced IL-6, chemokine (C-X-C motif) ligand 1, and chemokine (C-C motif) ligand 2 were also similar in BLM-challenged Pirb<sup>−/−</sup> and wild-type mice (Figure E2). Taken together, these data implicate PIR-B as a regulator of profibrogenic (but not proinflammatory) mediator expression in pulmonary fibrosis.

**Increased Relm-α<sup>+</sup> and MMP-12<sup>−</sup> Alveolar Macrophages in BLM-Treated Pirb<sup>−/−</sup> Mice**

Next, we examined whether the increased expression of aaMac profibrogenic markers in BLM-treated Pirb<sup>−/−</sup> mice was attributable to an increased expression of these mediators in alveolar macrophages. Thus, enzymatically digested lung cells were stained with various cell-surface markers and alveolar macrophages were electronically gated (CD45<sup>-</sup>/Gr-1<sup>-</sup>/CD11c<sup>-</sup>/CD11b<sup>-</sup>/Siglec-F<sup>-</sup>) (Figure 5A) (35), and the cells were assessed for Relm-α and MMP-12 expression. Flow cytometric analysis revealed that all of the Relm-α<sup>+</sup> and MMP-12<sup>−</sup> cells were indeed alveolar macrophages (Figures 5B and 5F), whereas other myeloid cells were negative for Relm-α and MMP-12 expression (Figures 5C, 5D, 5G, and 5H). Although the mean fluorescent intensity of Relm-α<sup>+</sup> alveolar macrophages was no different between BLM-treated wild-type and Pirb<sup>−/−</sup> cells, the percentages of Relm-α<sup>+</sup> and MMP-12<sup>−</sup> alveolar macrophages were significantly increased in the lungs of BLM-treated Pirb<sup>−/−</sup> mice in comparison with BLM-treated wild-type mice (Figures 5E and 5I). In fact, BLM-treated Pirb<sup>−/−</sup> mice displayed a 1.5-fold increase in the percentage of Relm-α<sup>+</sup> cells (Figures 5E and 5I). These data suggest that PIR-B regulates profibrogenic mediator expression in alveolar macrophages during IPF.

**PIR-B Regulates IL-4-Induced Responses**

IL-4 is a key cytokine involved in the alternative activation of macrophages, and can directly induce Relm-α expression in macrophages (8, 13, 36). To examine the possibility that PIR-B negatively regulates IL-4-induced effects in lung macrophages, we first determined whether additional cytokines that are involved in IPF, such as IL-17 and IL-1β, increase Relm-α expression in macrophages. As expected, only IL-4 and to a lesser extent IL-13 induced Relm-α production in BM-derived macrophages (Figure 6A). The stimulation of Pirb<sup>−/−</sup> macrophages with IL-4 resulted in increased Relm-α secretion and MMP-12 production, in comparison with IL-4–treated wild-type cells (Figures 6B and 6C). Importantly, increased responsiveness to IL-4 stimulation in Pirb<sup>−/−</sup> BM-derived macrophages was not attributable to increased IL-4R<sub>α</sub> levels, because Pirb<sup>−/−</sup> BM-derived macrophages expressed slightly (but statistically significantly) lower levels of IL-4R<sub>α</sub> than did wild-type cells (Figure E3). Interestingly, the deficiency of Pirb did not alter the secretion of IL-12p70, TNF-α, or IL-6 after the “classic” activation of macrophages (Figures 6D–6F).

**Increased BLM-Induced Fibrosis in Pirb<sup>−/−</sup> Mice Correlates with Elevated aaMac Marker Expression**

aaMac and aaMac-associated gene products, including Relm-α, MMP-12, TIMP-1, and osteopontin, are up-regulated after BLM challenge, and play key roles in fibrosis (13, 34). The myeloid-associated up-regulation of PIR-B and the recently described role of PIR-B in myeloid-derived suppressor cell polarization (20) prompted us to investigate whether BLM-challenged Pirb<sup>−/−</sup> mice display increased aaMac marker expression. Indeed, Relm-α protein and mRNA expression were strongly increased in the BALF (Figure 4A) and lungs (Figure 4B) of BLM-treated Pirb<sup>−/−</sup> mice, respectively. In addition, the expression of MMP-12, TIMP-1, and OPN was markedly increased in the lungs of BLM-treated Pirb<sup>−/−</sup> mice (Figures 4C–4E). Notably, increased Relm-α, MMP-12, TIMP-1, and OPN expression in BLM-treated Pirb<sup>−/−</sup> mice was not attributable to elevated IL-4 or IL-13 concentrations, because BLM-treated Pirb<sup>−/−</sup> mice displayed similar IL-13 and IL-4 concentrations as those of BLM-treated wild-type mice (Figures 4F–4G).

**Sections revealed excessive destruction of lung architecture, increased fibrocytic foci, and increased infiltration of mononuclear cells in BLM-treated Pirb<sup>−/−</sup> mice (Figure 3A).**

The pathological response to BLM administration includes fibroptic foci consisting of activated myofibroblasts, increased collagen synthesis, and the sequestration of extracellular matrix components (32). BLM-challenged Pirb<sup>−/−</sup> mice consistently displayed increased lung collagen deposition in comparison with BLM-challenged wild-type mice (Figure 3B), and soluble collagen content was significantly higher in the BALF of BLM-treated Pirb<sup>−/−</sup> mice (Figure 3C). Interestingly, BLM-treated Pirb<sup>−/−</sup> mice exhibited higher concentrations of α-smooth muscle actin (α-SMA) 7 days after BLM challenge, which returned to baseline 14 days after BLM treatment (Figure 3D). Moreover, consistent with the α-SMA mRNA expression pattern on Day 7 (Figure 3D), a flow cytometric analysis of BLM-treated Pirb<sup>−/−</sup> mice revealed that increased α-SMA expression in BLM-treated Pirb<sup>−/−</sup> mice was not attributable to increased α-SMA protein expression per cell, because the mean fluorescent intensity of α-SMA was similar in BLM-treated wild-type and Pirb<sup>−/−</sup> mice (Figure 3E). Rather, a significant elevation in the percentage of CD45<sup>-</sup>/FSP1<sup>-</sup>/αSMA<sup>-</sup> fibroblasts (a 1.5 ± 0.2-fold increase) was observed in BLM-treated Pirb<sup>−/−</sup> mice (33) (Figure 3F). Collectively, these results implicate PIR-B as a negative regulator of BLM-induced pulmonary pathology.

**PIR-B Regulates IL-4-Induced Responses**

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To determine whether PIR-B regulates IL-4–induced responses in vivo, IL-4C was administered to wild-type and Pirb\(^{-/-}\) mice, and the accumulation of inflammatory cells and the induction of Relm-\(\alpha\) were assessed. IL-4C–treated Pirb\(^{-/-}\) mice displayed a marked reduction in IL-4C–induced total cell counts in the BALF (Figure 6G), and demonstrated decreased neutrophil and eosinophil accumulation in response to IL-4C (Figures 6H and 6I). IL-4C–induced (but not IL-13–induced) total Relm-\(\alpha\) expression was significantly increased in the lungs of IL-4C–treated Pirb\(^{-/-}\) mice (Figures 6J and E4). Furthermore, flow cytometric analysis revealed that the percentages of Relm-\(\alpha\)1 alveolar macrophages were significantly increased (\(\sim 4.5\)-fold) in the lungs of IL-4C–treated Pirb\(^{-/-}\) mice in comparison with BLM-treated wild-type mice (Figure 6G). Taken together, our results establish a key role for PIR-B in regulating IL-4–induced macrophage activation and Relm-\(\alpha\) expression.

Expression of PIR-B Human Orthologues in Biopsies of Patients with IPF

Finally, we assessed the expression levels of several LILRB/ILT family members in lung biopsies obtained from normal, healthy control subjects (22). A quantitative PCR analysis of whole-lung biopsies revealed a significantly higher expression of LILRB3/ILT-5 in comparison with LILRB1/ILT-2 and LILRB4/ILT-4 (Figure 7A). Given the relatively higher LILRB3/ILT-5 expression level, we focused on this receptor. Immunohistochemical staining localized LILRB3/ILT-5 expression mainly to cells with alveolar macrophage morphology (Figure 7B). Next, we assessed whether LILRB3/ILT-5 is up-regulated in the lungs of patients with IPF. Quantitative PCR analysis revealed an elevated expression of LILRB3/ILT-5 in lung biopsies of patients with IPF, and this elevated expression nearly reached statistical significance (\(P < 0.06\)). Thus, similar to PIR-B, LILRB3/ILT-5 likely regulates macrophage function in IPF.

DISCUSSION

IPF is a major cause of morbidity and mortality worldwide, with no effective treatment. Therefore, a better understanding of the pathways capable of regulating fibrogenesis is critical for the development of efficacious therapies for this unmet medical need (37).
In this study, we defined the role of PIR-B, a “prototype” cell-surface inhibitory receptor, in BLM-induced lung fibrosis. We demonstrate that after BLM challenge, (1) PIR-B is specifically up-regulated in lung myeloid cells, including Gr-1high/CD11chigh and Gr-1med/CD11chigh cells. (2) Pirb2/2 mice display decreased neutrophilic and increased lymphocytic inflammation, accompanied by increased disease severity. (3) PIR-B negatively regulates BLM-induced collagen deposition, a-SMA expression, and Relm-α, MMP-12, TIMP-1, and OPN concentrations. Notably, increased Relm-α and MMP-12 expression was specifically localized to alveolar macrophages. (4) PIR-B negatively regulates IL-4–induced Relm-α expression in vivo, and suppresses IL-4–induced macrophage-derived Relm-α and MMP-12 secretion in vitro. Finally, we demonstrated that (5) the PIR-B human orthologue LILRB3/ILT-5 is highly expressed in alveolar macrophages, and is increased in lung biopsies obtained from patients with IPF. Collectively, our data suggest a key role for PIR-B as an integral regulator of profibrogenic macrophage functions in IPF.

Macrophages have been described in various fibrotic diseases in the lung, gastrointestinal tract, kidney, and liver. In these tissues, numerous roles have been attributed to aaMacs (4, 38, 39). Interestingly, recent data highlight the possibility that aaMacs and aaMac-derived products may actually prevent fibrosis and act to resolve tissue damage during parasitic infections (3, 5–7). Conversely, in settings of allergic-airway inflammation, the roles of aaMacs are much less clear, because recent data highlight no contribution of aaMacs or aaMac-derived products (such as Relm-α) in the development of asthma (25, 40). Therefore, the function of aaMacs and the issue of whether they promote or suppress fibrosis are likely disease-dependent, and may rely on additional factors that are present in the inflammatory milieu. Specifically, the roles of aaMacs in IPF have been the subject of much interest, and recent findings indicate a profibrogenic role for aaMacs in human and murine models of IPF (41, 42). Indeed, we show that the increased histopathology in Pirb2/2 mice is correlated with an increased expression of pro-fibrotic aaMac markers such as Relm-α, MMP-12, TIMP-1, and OPN.
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...fibrosis by polarizing macrophages into an alternatively activated state, where they can induce tissue repair and fibrosis. Furthermore, flow cytometric analyses revealed a specific increase in the concentrations of Relm-α and MMP-12−/− alveolar macrophages in the lungs of BLM-treated Pirb−/− mice. Previous studies indicated that Pirb−/− mice display increased Th2 responses (23), thus raising the possibility of increased IL-4 (or IL-13) production in the lungs of BLM-treated Pirb−/− mice. However, we did not find any differences in the concentrations of IL-4 and IL-13 in BLM-treated wild-type versus Pirb−/− mice. Hence, increased aaMac markers in BLM-treated Pirb−/− mice are likely attributable to a loss of negative regulation of IL-4-induced effects by PIR-B. Supporting this notion is our finding that IL-4–stimulated Pirb−/− macrophages secrete elevated concentrations of Relm-α and MMP-12. Surprisingly, the increased responsiveness of Pirb−/− macrophages was specific to IL-4–induced macrophage responses, because PIR-B did not regulate IL-12p70, IL-6, and TNF-α production after classic macrophage activation. The finding that PIR-B dampens IL-4–induced responses in macrophages, but does not regulate classic macrophage activation, is likely attributable to the differential recruitment and involvement of intracellular signaling intermediates in PIR-B signaling (17, 31).

Numerous studies have documented dominant roles for IL-4 and IL-13 in pulmonary fibrosis (2, 8, 44, 45). For instance, the progression of IPF in humans is associated with sustained IL-4 production (46). Directly related to this, BLM-challenged Pirb−/− mice displayed significantly decreased TGF-β, fibronectin, and collagen deposition after BLM challenge (8). Moreover, it was shown that IL-4 induces an indirect pathway to promote tissue fibrosis and collagen synthesis (8). Thus, IL-4 may promote fibrosis by polarizing macrophages into an alternatively activated state, where they can induce tissue repair and fibrosis. Certainly, aaMac products are readily found in the lungs of patients with IPF (9). Furthermore, various in vivo studies have demonstrated a role for aaMac products in IPF (47). For example, the expression of Relm-α, a hallmark of aaMac marker and potent profibrogenic molecule, is up-regulated in IPF and controlled by an IL-4/IL-13-dependent and STAT6-dependent pathway (48). In addition, cationic amino-acid transporter–2, which regulates arginine transport and arginase metabolism, is involved in BLM-induced fibrosis (10). Additionally, aaMac are a source of TGF-β and CCL18 in IPF, and the attenuation of their functions by the administration of serum amyloid P can therapeutically inhibit established disease in mice (42). Thus, increased pathology in BLM-treated Pirb−/− mice is likely attributable to the increased alternative activation of Pirb−/− macrophages. Intriguingly, despite increased concentrations of aaMacs and their related products in bleomycin-treated Pirb−/− mice, the lymphocytic infiltrate in the BALF of these mice was increased. This is surprising, because aaMac can suppress lymphocyte functions (49). Thus, PIR-B likely regulates additional yet indirect pathways that govern lymphocyte accumulation.

Consistent with our findings in BLM-treated Pirb−/− mice, IL-4–treated Pirb−/− mice displayed decreased neutrophil and eosinophil accumulation and increased Relm-α expression. Interestingly, this effect was specific to IL-4, because the administration of IL-13 into the lungs of wild-type and Pirb−/− mice resulted in similarly increased concentrations of Relm-α and chemokine expression (18). This discrepancy in the PIR-B regulation of IL-13–induced and IL-4–induced responses can likely be attributed to the fact that Relm-α can be secreted by epithelial cells and macrophages, which differentially express Type 1 and Type 2 IL-4R complexes. Structural cells such as fibroblasts and epithelial cells predominantly express Type 2 IL-4R,

Figure 6. PIR-B regulates IL-4–induced responses in vivo and in vitro. Bone marrow–derived macrophages from WT (A) and Pirb−/− (B and C) mice were obtained and activated with the indicated cytokines for 48 hours. The concentrations of (A and B) Relm-α and (C) MMP-12 were assessed using ELISA or quantitative PCR (using the ΔΔCt method), respectively. In addition, bone marrow–derived macrophages were activated using IFN-γ and Escherichia coli (termed “M1” macrophages), and the concentrations of IL-12p70 (D), IL-6 (E), and TNF-α (F) were assessed. Murine IL-4 with a neutralizing monoclonal antibody (BVD4-1D11) at a 2:1 molar ratio (IL-4C) was administered twice every other day to WT and Pirb−/− mice. Forty-eight hours after the final challenge, the mice were killed, and the bronchoalveolar lavage fluid was assessed for (G) total cell, (H) neutrophil, and (I) eosinophil cell counts. In addition, Relm-α concentrations in the lungs were assessed by (J) quantitative PCR and (K) flow cytometric analyses of Relm-α alveolar macrophages. Data are representative of 2–3 experiments (6 mice/experiment per group). HPRT, hypoxanthine–guanine phosphoribosyltransferase; NA, nonactivated; ns, no significance. *P < 0.05, **P < 0.01, ***P < 0.001.
and myeloid cells (e.g., macrophages, dendritic cells, and eosinophils) primarily express Type 1 IL-4R (14, 24). Thus, macrophages will predominantly respond to IL-4, and epithelial cells will primarily respond to IL-13 (14). Because the expression of PIR-B in the lungs is restricted to myeloid cells, it is likely that PIR-B mainly regulates IL-4–induced responses in hematopoietic cells. In contrast, the local administration of IL-13 may overcome the regulatory effect of PIR-B by directly stimulating epithelial cells (which express high concentrations of Type 2 IL-4R) to produce Relm-α. In that scenario, increased cellular infiltration in Pirb<sup>−/−</sup> mice may be attributable to the increased responsiveness of Pirb<sup>−/−</sup> cells to chemotactic stimuli (18, 50).

In an attempt to translate our <i>in vivo</i> findings to human disease, we assessed the expression of PIR-B orthologues belonging to the human LILRB family of receptors (51, 52). We demonstrate that under baseline conditions, LILRB3/ILT-5 is highly expressed in the lungs and localized to alveolar macrophages within the lung parenchyma. Importantly, and similar to our findings with PIR-B, LILRB3/ILT-5 expression was higher in biopsies obtained from patients with IPF. Our inability to demonstrate statistically significant elevations of LILRB3/ILT-5 in whole-lung biopsies is likely attributable to the specific expression of LILRB3/ILT-5 in alveolar macrophages. Thus the LILRB3/ILT-5 signal may be diluted by other factors in the whole-lung sample, and may depend on the relative quantity of macrophages in each biopsy. Nonetheless, our data suggest that LILRB3/ILT-5 negatively regulates macrophage function in human disease. The lung is a tightly regulated organ, possessing immune-suppressive mechanisms that prevent uncontrolled proinflammatory reactions toward a plethora of antigens. We speculate that LILRB3/ILT-5 may play a role in the maintenance of lung macrophage immune quiescence or tolerance. The observed difference in LILRB3/ILT-5 expression in the lungs of normal subjects and patients with IPF suggests an active contribution of the PIR-B human orthologue LILRB3/ILT-5 in the regulation of IPF-associated immune responses. Nonetheless, the relative contributions of this family of molecules to the disease remain to be determined.

In conclusion, our results establish a key role for PIR-B in pulmonary fibrosis, likely attributable to its function in negatively regulating IL-4–induced macrophage activation. These findings suggest that strategies aimed at suppressing aaMac functions in IPF may provide new tools to limit the devastating outcomes of this disease, and highlight PIR-B as a potential therapeutic target.

**References**


