

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, PIR-B/LILRB3 may offer a possible target for suppressing macrophage profibrogenic activity in IPF.

CLINICAL RELEVANCE

Alveolar macrophages play a key role in pulmonary fibrosis. However, molecular mechanisms that may counter-regulate their profibrogenic properties have not been identified. Paired immunoglobulin-like receptor-B contributes to the pathogenesis of pulmonary fibrosis via the negative regulation of macrophage effector functions and profibrogenic mediator expression.

Keywords: idiopathic pulmonary fibrosis; macrophage; IL-4; lung; paired immunoglobulin-like receptor-B

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating interstitial lung disease that is currently on the rise (1). Despite intensive research, the pathogenesis of IPF remains unclear, and patients are refractory to current therapies (1).

Although the role of monocytes and macrophages in IPF is controversial, accumulating data highlight the existence of a central IL-4/IL-13-macrophage axis in fibrotic settings, including IPF (2). Recent studies demonstrated divergent and even opposing roles for IL-4/IL-13-activated (i.e., alternatively activated) macrophages (aaMacs) in fibrosis (3–7), and it appears that in diseases such as IPF, aaMacs promote fibrosis (3, 8–10). aaMacs can produce a variety of profibrogenic mediators such as resistin-like molecule- α (Relm- α) and transforming growth factor- β 1 (TGF- β 1), which can directly activate fibroblast properties (11, 12). Furthermore, aaMacs produce matrix metalloproteinases (e.g., MMP-12) and tissue inhibitors of metalloproteinases (e.g., TIMP-1) that regulate inflammatory-cell recruitment and extracellular matrix deposition (13). To date, many of the studies on aaMacs have either focused on the signaling pathways initiated by IL-4/IL-13, such as signal transducer and activator of transcription-6 (STAT6), Type I IL-4 receptor (IL-4R), and Type II IL-4R, or on defining the roles of aaMac-derived products (e.g., Relm- α and arginase-1) in disease settings (13–15). However, pathways that can suppress aaMac polarization have received only limited attention.

The murine paired immunoglobulin-like receptors (PIRs) PIR-A and PIR-B are expressed on many immune cells, including B cells, macrophages, mast cells, dendritic cells, and eosinophils (16–18). PIR-B is a Type I transmembrane glycoprotein with six extracellular immunoglobulin-like domains, a hydrophobic transmembrane segment, and an intracellular polypeptide with four immunoreceptor tyrosine-based inhibitory motifs or immunoreceptor tyrosine-based inhibitory motif-like sequences (17). The recruitment of the Src homology region-2 domain-containing phosphatase-1/2 by PIR-B results in the inhibition of cell activation triggered by activation receptors such as PIR-A, chemokine receptors, innate immune receptors, and adhesion

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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 (LILRB3)/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 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 μ l/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 μ m) 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 [$\pm$ SD], 60 $\pm$ 2 yr) and 10 control subjects (organ donors; four females and six males; mean age, 45 $\pm$ 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 $\pm$ 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 $\pm$ 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-1^{high}/CD11c^{high} cells (R1 in Figures 1C, 1G, and 1K) and Gr-1^{med}/CD11c^{high} 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,

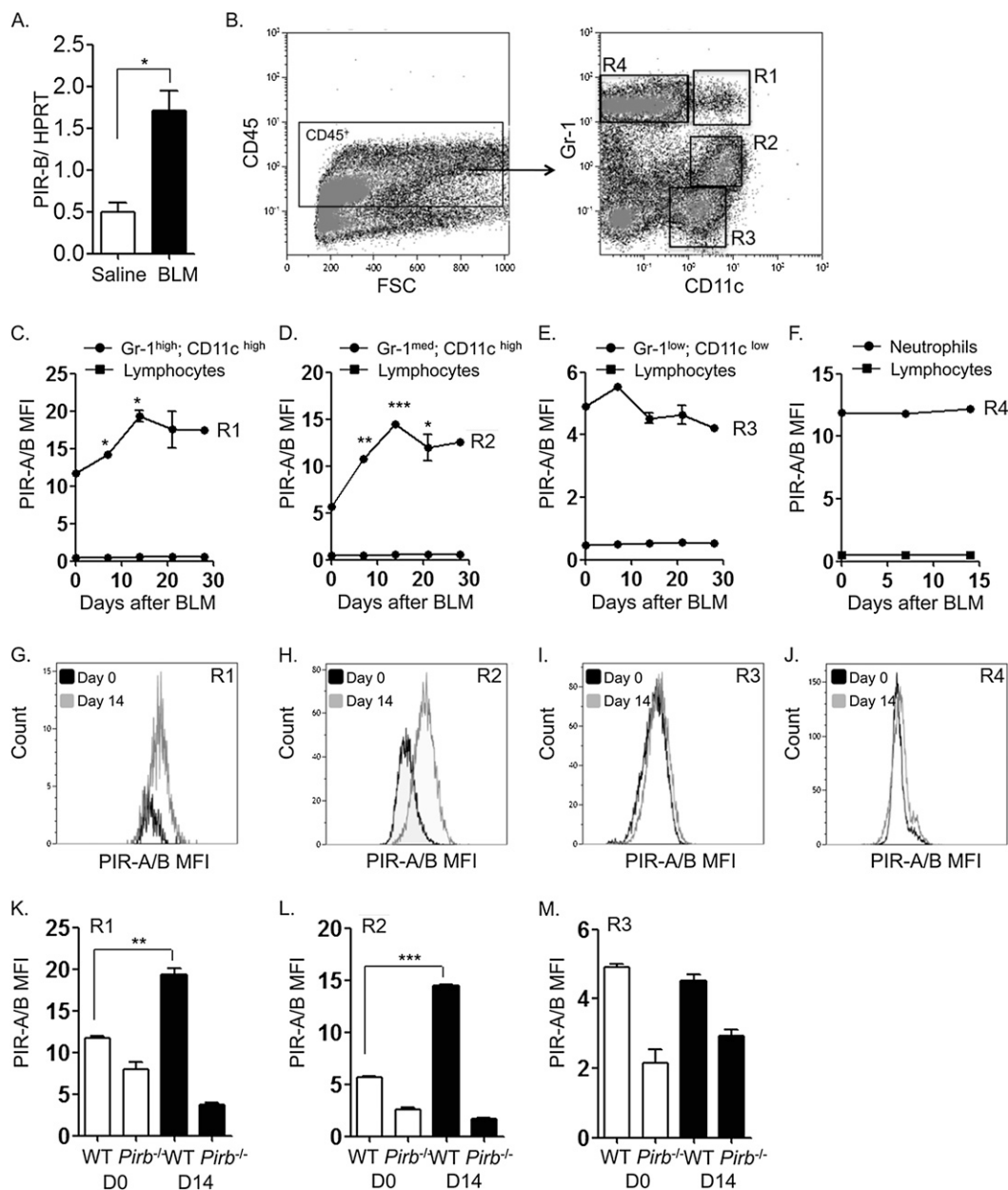


Figure 1. Paired immunoglobulin-like receptor-B (PIR-B) is up-regulated in the lungs of bleomycin (BLM)-treated mice. (A) PIR-B expression was assessed in lung cDNA obtained from saline-treated and BLM-treated wild-type (WT) mice by quantitative PCR analysis, and normalized to the house-keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). (B) The cellular source accounting for PIR-A/B expression was assessed using polychromatic flow cytometric analysis of CD45⁺ cells displaying differential CD11c and Gr-1 expression patterns. Kinetic analysis and representative histogram plots of (C and G) granulocyte receptor-1 (GR-1)^{high}/CD11c^{high}, (D and H) GR-1^{med}/CD11c^{high}, (E and I) GR-1^{low}/CD11c^{low} cells, and (F and J) neutrophils, designated R1, R2, R3, and R4, respectively, are shown. The mean fluorescence intensity (MFI) of PIR-A/B surface expression in (K) GR-1^{high}/CD11c^{high}, (L) GR-1^{med}/CD11c^{high}, and (M) GR-1^{low}/CD11c^{low} cells was obtained from saline-treated (Day 0, D0, open bars) and BLM-treated (Day 14, D14, solid bars) WT and *Pirb*^{-/-} mice. Data are representative of three experiments (3 mice/group per experiment). **P* < 0.05. ***P* < 0.01. ****P* < 0.001.

because PIR-A/B expression was unchanged in Gr-1^{low}/CD11c^{low} cells (R3 in Figures 1E, 1I, and 1M) and Gr-1^{high}/CD11c⁺ 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 *Pirb*^{-/-} mice, which still express PIR-A. To this end, single-cell suspensions of enzymatically digested lungs were obtained from BLM-treated wild-type and *Pirb*^{-/-} mice, and stained with anti-PIR-A/B antibody. PIR-A/B up-regulation was completely diminished in BLM-challenged *Pirb*^{-/-} mice (Figures 1K–1M). Hence, BLM specifically increased PIR-B but not PIR-A expression on the surface of Gr-1^{high}/CD11c^{high} cells (R1 in Figure 1K) and Gr-1^{med}/CD11c^{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 *Pirb*^{-/-} mice were challenged with BLM, and the differential accumulation of inflammatory cells in the BALF was assessed. Interestingly, BLM-treated *Pirb*^{-/-} 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 *Pirb*^{-/-} Mice

PIR-B is a “hallmark” inhibitory receptor capable of suppressing immune-cell activation (17, 31). Thus, we hypothesized that BLM-treated *Pirb*^{-/-} mice will display increased disease severity in comparison with BLM-treated wild-type mice. A histopathological assessment of hematoxylin-and-eosin-stained lung

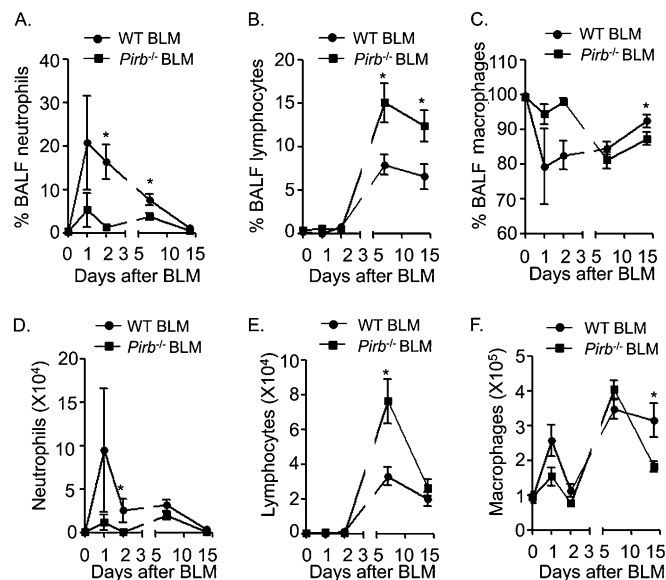


Figure 2. Decreased neutrophilic and increased lymphocytic inflammation in bleomycin (BLM)-treated *Pirb*^{-/-} mice. Bronchoalveolar lavage fluid (BALF) was obtained from saline-treated and BLM-treated WT and *Pirb*^{-/-} mice. The percent (A–C) and total (D–F) differential cell populations were determined by Diff-Quik stain. Data are representative of three experiments (5–11 mice/group). **P* < 0.05.

sections revealed excessive destruction of lung architecture, increased fibrocytic foci, and increased infiltration of mononuclear cells in BLM-treated *Pirb*^{-/-} mice (Figure 3A).

The pathological response to BLM administration includes fibrotic foci consisting of activated myofibroblasts, increased collagen synthesis, and the secretion of extracellular matrix components (32). BLM-challenged *Pirb*^{-/-} 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*^{-/-} mice (Figure 3C). Interestingly, BLM-treated *Pirb*^{-/-} 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*^{-/-} mice revealed that increased α -SMA expression in BLM-treated *Pirb*^{-/-} 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*^{-/-} mice (Figure 3E). Rather, a significant elevation in the percentage of CD45⁺/FSP1⁺/ α -SMA⁺ fibroblasts (a 1.5 $\pm$ 0.2-fold increase) was observed in BLM-treated *Pirb*^{-/-} mice (33) (Figure 3F). Collectively, these results implicate PIR-B as a negative regulator of BLM-induced pulmonary pathology.

Increased BLM-Induced Fibrosis in *Pirb*^{-/-} Mice Correlates with Elevated aaMac Marker Expression

aaMacs 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*^{-/-} 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*^{-/-} mice, respectively. In addition, the expression of MMP-12, TIMP-1, and OPN was markedly increased in the lungs of BLM-treated *Pirb*^{-/-} mice (Figures 4C–4E). Notably, increased Relm- α , MMP-12, TIMP-1, and OPN expression in BLM-treated *Pirb*^{-/-} mice was not attributable to elevated IL-4 or IL-13 concentrations, because BLM-treated *Pirb*^{-/-} mice displayed similar 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 (YM1) 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*^{-/-} 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 ⁺ and MMP-12⁺ Alveolar Macrophages in BLM-Treated *Pirb*^{-/-} Mice

Next, we examined whether the increased expression of aaMac profibrogenic markers in BLM-treated *Pirb*^{-/-} 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⁺/Gr-1⁺/CD11c⁺/CD11b⁺/Siglec-F⁺) (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 ⁺ and MMP-12⁺ 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 ⁺ alveolar macrophages was no different between BLM-treated wild-type and *Pirb*^{-/-} cells, the percentages of Relm- α ⁺ and MMP-12⁺ alveolar macrophages were significantly increased in the lungs of BLM-treated *Pirb*^{-/-} mice in comparison with BLM-treated wild-type mice (Figures 5E and 5I). In fact, BLM-treated *Pirb*^{-/-} mice displayed a 1.5-fold increase in the percentage of Relm- α ⁺ 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-1 β , 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*^{-/-} 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*^{-/-} BM-derived macrophages was not attributable to increased IL-4R levels, because *Pirb*^{-/-} BM-derived macrophages expressed slightly (but statistically significantly) lower levels of IL-4R α 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).

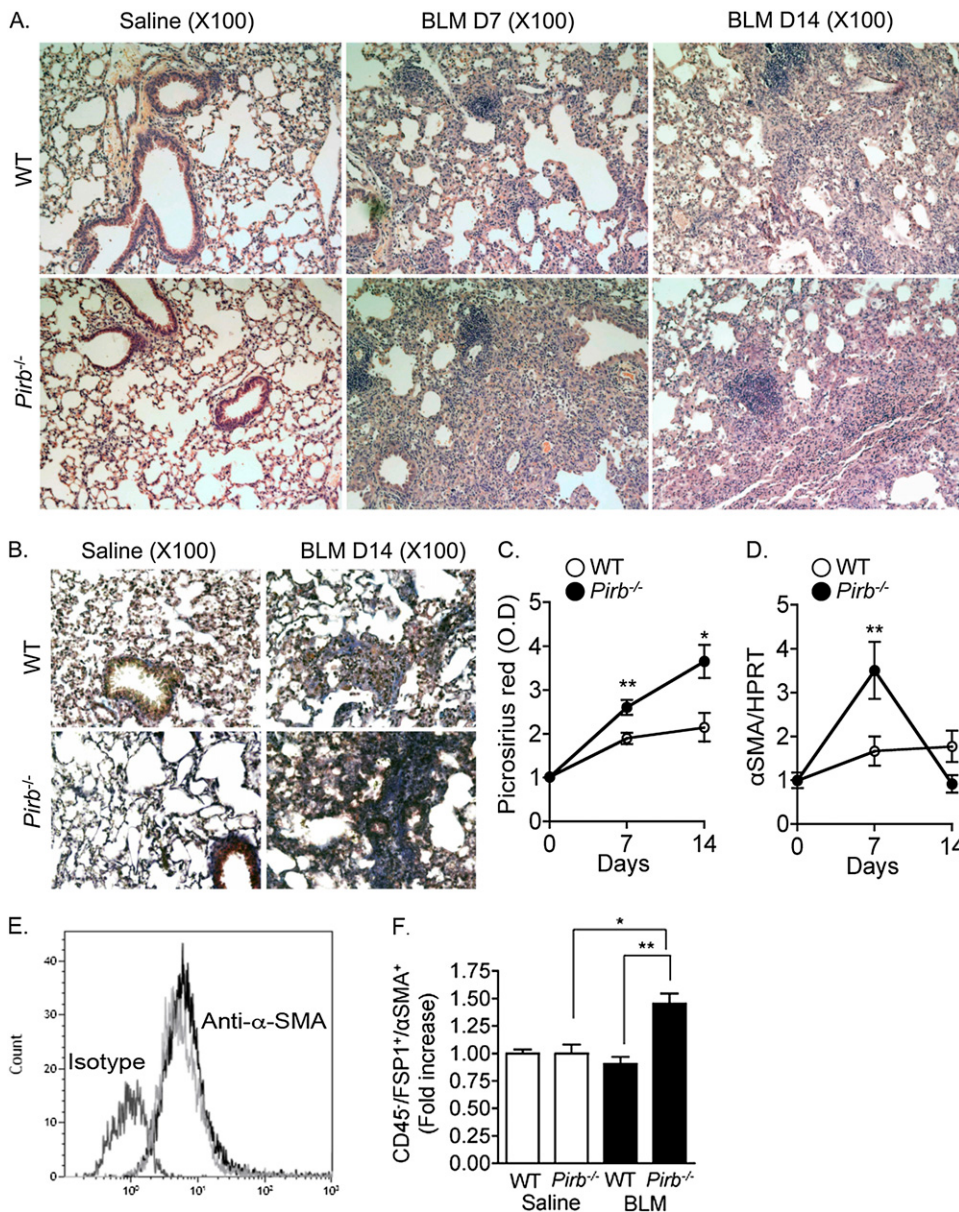


Figure 3. Increased histopathology in bleomycin (BLM)-treated *Pirb*^{-/-} mice. The lungs of saline-treated and BLM-treated WT and *Pirb*^{-/-} mice were obtained 7 days (D7) and 14 days (D14) after treatment. Representative photomicrographs of (A) hematoxylin-and-eosin staining and (B) Masson trichrome staining are shown. In addition, (C) soluble collagen content (as measured by optical density [O.D.] in the bronchoalveolar lavage fluid and (D) quantitative PCR analysis of lung α -smooth muscle actin (α -SMA) concentrations were assessed and normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Enzymatically digested lung cells were stained with anti-CD45, anti-fibroblast specific protein 1 (FSP1), and anti- α -SMA. (E) CD45⁺/FSP1⁺/ α -SMA⁺ cells were gated, and the mean fluorescent intensity of α -SMA protein was determined (black and gray histograms represent BLM-treated WT and *Pirb*^{-/-} mice, respectively). (F) In addition, percentages of cells were assessed using flow cytometric analysis. Data are representative of 2–3 experiments (4–11 mice/group). * $P < 0.05$. ** $P < 0.01$.

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- α 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- α 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- α ⁺ alveolar macrophages were significantly increased (~ 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- α 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).

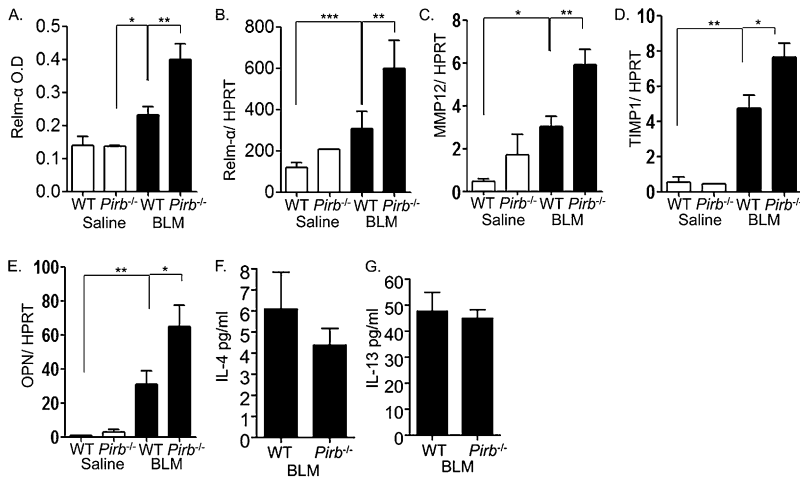


Figure 4. Increased profibrogenic mediators in bleomycin (BLM)-treated *Pirb*^{-/-} mice. The expression of (B) resistin-like molecule-α (Relm-α), (C) matrix metalloproteinase-12 (MMP-12), (D) tissue inhibitor of metalloproteinase-1 (TIMP-1), and (E) osteopontin (OPN) were assessed in the lungs of saline-treated and BLM-treated WT and *Pirb*^{-/-} mice, using quantitative PCR analysis 7 days after treatment. Quantitative PCR analyses were normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Protein concentrations of (A) Relm-α, (F) IL-4, and (G) IL-13 were assessed in bronchoalveolar lavage fluid. Data are representative of three experiments (5–11 mice/group). **P* < 0.05. ***P* < 0.01. ****P* < 0.001.

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-1^{high}/CD11c^{high} and Gr-1^{med}/CD11c^{high} cells. (2) *Pirb*^{-/-} mice display decreased neutrophilic and increased lymphocytic inflammation, accompanied by increased disease severity. (3) PIR-B negatively regulates BLM-induced collagen deposition, α-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 *Pirb*^{-/-} mice is correlated with an increased expression of profibrotic aaMac markers such as Relm-α, MMP-12, TIMP-1, and

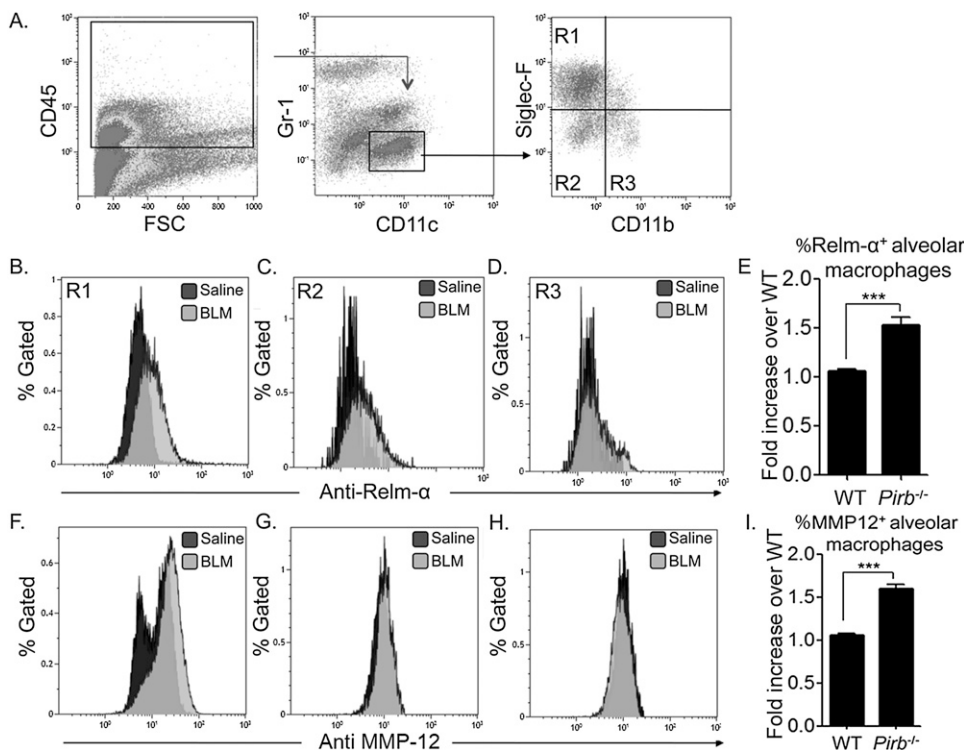


Figure 5. Increased Relm-α⁺ and MMP-12⁺ alveolar macrophages in bleomycin (BLM)-treated *Pirb*^{-/-} mice. WT and *Pirb*^{-/-} mice were treated with saline or BLM. (A) After 7 days, single-cell suspensions from enzymatically digested lungs were obtained, and the expression of Relm-α and MMP-12 was assessed in various lung myeloid cell populations expressing differential Gr-1/CD11c/Siglec-F concentrations (R1, R2, and R3). Representative histogram plots of (B–D) Relm-α-positive and (F–H) MMP-12-positive cells are shown. The fold-increases in the percentages of (E) Relm-α⁺ and (I) MMP-12⁺ alveolar macrophages in BLM-treated WT and *Pirb*^{-/-} mice are depicted. Data are representative of four mice. ****P* < 0.001.

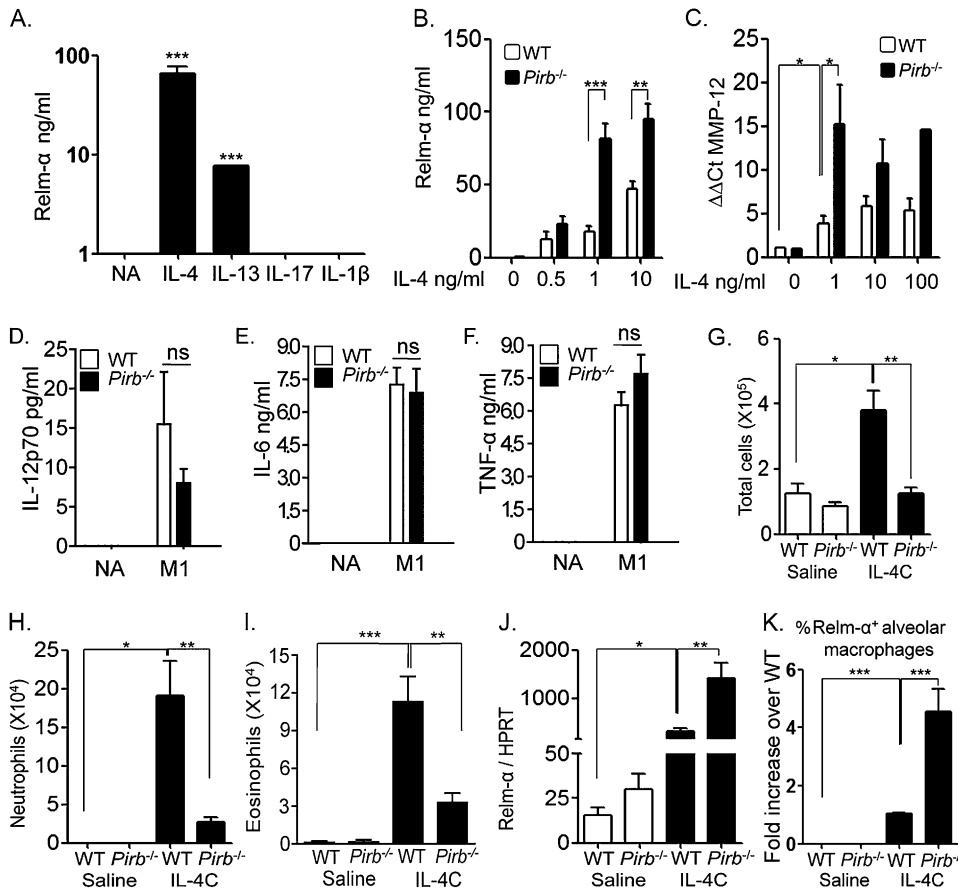


Figure 6. PIR-B regulates IL-4-induced responses *in vivo* and *in vitro*. Bone marrow-derived macrophages from WT (A) and *Pirb*^{-/-} (B and C) mice were obtained and activated with the indicated cytokines for 48 hours. The concentrations of (A and B) Relm-α and (C) MMP-12 were assessed using ELISA 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.

osteopontin (13, 43). 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 *Il4*^{-/-} 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, aaMacs 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 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). Finally, aaMacs 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 an 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 aaMacs 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 the Type 1 and Type 2 IL-4R complexes. Structural cells such as fibroblasts and epithelial cells predominantly express Type 2 IL-4R,

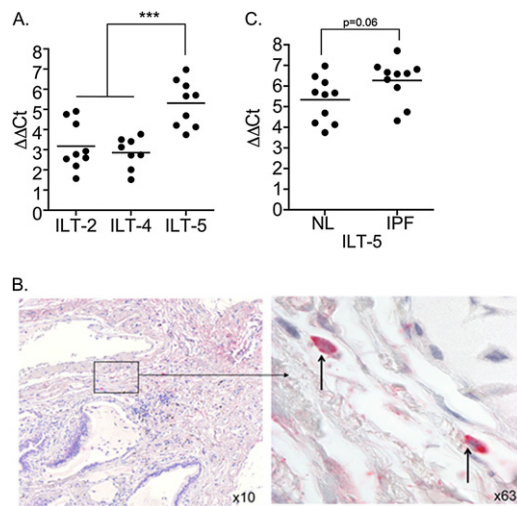


Figure 7. Expression of leukocyte immunoglobulin-like subfamily B member 3 (LILRB3) in human idiopathic pulmonary fibrosis (IPF). (A) The expression of the PIR-B human orthologues LILRB1/immunoglobulin-like transcript (ILT)-2, LILRB4/ILT-4, and LILRB3/ILT-5 was assessed by quantitative PCR analysis in whole-lung biopsies obtained from healthy donors. (B) The cellular source accounting for LILRB3/ILT-5 expression was assessed by immunohistochemistry, using lung biopsies of healthy donors. Arrows indicate LILRB3/ILT-5-positive cells. (C) LILRB3/ILT-5 mRNA expression was assessed in lung biopsies obtained from healthy donors (NL) and patients with IPF (IPF). Each point represents a different donor. *** $P < 0.001$.

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*^{-/-} mice may be attributable to the increased responsiveness of *Pirb*^{-/-} cells to chemotactic stimuli (18, 50).

In an attempt to translate our *in vivo* 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.

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