RESEARCH ARTICLE

The atypical chemokine receptor ACKR2 drives pulmonary fibrosis by tuning influx of CCR2⁺ and CCR5⁺ IFNγ-producing γδT cells in mice

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ACKR2; chemokine; interferon-γ; pulmonary fibrosis; γδT lymphocytes

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic lethal disorder characterized by an aberrant immune system and fibroblast activation, excessive extracellular matrix deposition, and irreversible fibrotic changes (58). It is unclear how the early inflammatory phases result in the later fibrotic stage that leads to damage and progressive architectural remodeling (2, 3). However, the involvement of inflammatory processes preceding this late damage suggests that understanding the molecular orchestration of inflammation in IPF could be useful for the development of novel therapeutic interventions (8, 58).

During inflammatory reactions, chemokines are induced following tissue damage or infection (54) and coordinate the recruitment and activation of leukocytes and other cell types (25). As such, chemokines have a role in the pathogenesis of several diseases (47, 48, 51, 58). In the context of IPF and animal models of fibrosis, chemokines and their receptors, including CCR2/CCL2 (30, 61), CCR5/CCL3 (14, 38, 49), CCR3/CCL11 (13), and CXCR2/CXCL1–3 (18, 41), have been implicated in initiating the inflammatory phase and in the perpetuation of inappropriate fibroblast activity. Chemokine signals lead to cell activation and recruitment through their specific receptors. A small family of atypical chemokine receptors (ACKRs) (1) bind to chemokines, do not activate the conventional signaling cascades leading to cell chemotaxis, but trigger pathways of chemokine transport or degradation (26, 40). In particular, ACKR2, previously known as D6, internalizes and degrades CC inflammatory chemokines recognized by CCR1 to CCR5 in a competitive manner (35), thus acting as a chemokine scavenger (10, 26). The predominant sites of ACKR2 expression are placenta (27), lymphatic endothelial cells (34), and some leukocyte subsets (28), where it acts as a negative regulator of immune responses. Most studies show that ACKR2-deficient mice (ACKR2−/−) present exaggerated inflammatory responses in skin (15), placenta (27), and lungs (7), but the role of ACKR2 remains unexplored in the context of fibrosis. Here, we studied ACKR2 expression in lungs and...
whether it contributed to the pathogenesis of pulmonary fibrosis using a murine model of pulmonary fibrosis induced by bleomycin. Our results show that pulmonary injury and inflammation are reduced in the absence of ACKR2, so that pulmonary function was greatly improved and survival due to increased IFNγ-producing γδT-cell influx correlating to reduced Th17 and fibrogenic response induced by bleomycin in mice. These data reveal a previously unappreciated role of receptor ACKR2 in the development of pulmonary fibrosis by fine tuning chemokine-mediated migration of IFNγ-producing γδT cells and immune regulatory function in the lungs.

**MATERIAL AND METHODS**

**Animals.** Wild-type (WT) C57BL/6j mice were purchased from Jackson Laboratory (L’Arbresle Cedex, France). ACKR2-deficient (ACKR2−/−) mice were generated as previously described (15). CCR2−/−, CCR4−/−, and CCR5−/− mice were obtained from Laboratório de Imunofarmacologia/Universidade Federal de Minas Gerais facility. Animals were maintained in a controlled environment under pathogen-free conditions with filtered water and food ad libitum.

**Bone marrow chimeras.** Recipient mice were irradiated, and bone marrow cells from donor mice (4 × 10⁶ per mouse) were injected into each recipient mouse through the retro-orbital venous plexus, as previously described (53). Mice were challenged with bleomycin 4 wk after bone marrow transfer.

**Bleomycin-induced lung injury and fibrosis in mice.** Eight to 10-wk-old male WT and ACKR2−/− mice were used in bleomycin-induced lung injury model performed as previously described (38, 39, 41). Briefly, a single 40-μl injection containing 3.75 mg of bleomycin sulfate (Blenoxane; Bristol-Meyers Squibb) per kg of mice body weight diluted in PBS or PBS only (vehicle) was instilled intranasally. All experiments were conducted conforming to institutional guidelines in agreement with national and international law approved by the ethics committee of the Istituto Clinico Humanitáris and by the Brazilian animal ethics committee Comitê de Ética em Experimentação Animal/Universidade Federal de Minas Gerais (Protocol No. 232/2012).

**Soluble collagen assay.** The total content of lung collagen was measured by the Sircol (Taufkon考定法) soluble collagen assay (Biocolor), following the manufacturer’s instructions.

**Lung histopathology.** The left lung was removed and fixed in 4% neutral phosphate-buffered formalin (pH 7.4) and stained with hematoxylin and eosin or Gomori’s trichrome, as described previously (39). Images of lung sections were captured with a digital camera (Optron). Bone marrow chimeras.

**Assessment of leukocytes in airways and lungs.** Bronchoalveolar lavage (BAL) was performed to obtain leukocytes from alveolar space as previously described (38, 39, 41). Differential counts were obtained from cytospin evaluating the percentage leukocyte populations stained with May-Grünwald-Giemsa and FACS analysis. Lung fragments were incubated for 45 min in RPMI containing 1% fetal bovine serum and collagenase type IV (Sigma-Aldrich) and subsequently passed in Cell Strainer (B&D) to obtain a single cell suspension. CD45+ leukocytes from lung parenchyma were isolated by positive selection using CD45 microbeads (Miltenyi Biotec) and MS columns, according to the manufacturer’s instructions. Leukocytes recovered from BAL and CD45+ cells (1 × 10⁶ cells) from lung parenchyma were stained with fluorochrome-conjugated monoclonal antibodies anti-CD3, -CD4, -CD8, -Ly6G, -Ly6C, -CD11b, -CD11c, -CD115, -MHCII, -DX5, -γδTCR, -CXCR2, -CCR2, -CCR3, -CCR4, -CCR5, -RORγT, -IL-17A, and -IFN-γ or isotype controls (BD Pharmigen). Stained cells were acquired in a FACScanto cytometer (BD Biosciences) and analyzed in BD FACSDiva software (BD Biosciences). The populations of leukocyte and phenotype were calculated by expression of surface or intracellular markers: neutrophils (Ly6G+CXCR2+), eosinophils (Ly6G+CCR3+), macrophages (Ly6C+CD11b+CD11b+), T lymphocytes (CD3+CD4+ and CD3+CD8+), myeloid dendritic cells (MHCII+CD11b+CD11c+), natural killer cells (CD3+DX5+), γδT cells (CD3+γδTCR+), and γδT cell-IFNγ producers (CD3+γδTCR+IFNγ). The expression was calculated using the relative abundance (expressed as 2−ΔΔCT) of mRNA transcripts as follows:

![Table 1. Murine-specific primers for TaqMan gene expression assay](https://physiology.org/journal/ajplung/1009174.202X065/June3-2020)

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<tr>
<th>Name of Target</th>
<th>Abbreviation</th>
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<tr>
<td>18S</td>
<td>18S</td>
<td>Hs09999901_s1</td>
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<td>Bleomycin hydrolase</td>
<td>Blmh</td>
<td>Mm00724431_m1</td>
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<td>Atypical chemokine receptor 2</td>
<td>ACKR2</td>
<td>Mm00455551_m1</td>
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<td>Matrix metalloproteinase 9</td>
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<td>RORγT</td>
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<tr>
<td>SMAD family member 3</td>
<td>SMAD3</td>
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<tr>
<td>C-X-C motif chemokine 10</td>
<td>CXCL10</td>
<td>Mm00445255_m1</td>
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**RNA isolation and real-time RT-PCR.** Total lung RNA was extracted from whole lungs with TRIZol (Invitrogen) according to the manufacturer’s instructions in a gentleMACS Dissociator (MACS; Miltenyi Biotec). First-strand cDNA synthesis (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems) from 2 mg of total RNA was conducted according to the manufacturer’s instructions. A Chromo4 Real-Time PCR Detector (MJ Research) was used for quantitative real-time PCR on cDNA (200 ng) using TaqMan Universal PCR Master Mix (Applied Biosystems) (Table 1). Results represent the relative abundance (expressed as 2−ΔΔCT) of mRNA transcript over the total amount of 18S.
ACKR2 deficiency reduces lung injury and leukocyte influx induced by bleomycin. To characterize the protective effect of ACKR2 deletion on bleomycin-induced lung fibrosis, we first analyzed lungs of WT and ACKR2−/− mice exposed to bleomycin. While at early time points (days 2 and 4) there was no evidence of tissue damage in any genetic background, we found an early gain of lung resistance in WT but not in ACKR2−/− mice 2 days after bleomycin instillation (Table 2). At day 8, WT mice showed multiple inflammatory foci in the lung parenchyma, which developed into extensive areas of inflammation at later time points (~75 and 90% of slices area at days 12 and 16, respectively) with loss of pulmonary architecture (Fig. 3A). Inflammation at day 8 were significantly reduced in lungs of ACKR2−/− mice, which showed a relatively well-preserved pulmonary architecture at later time points (~35% and 50% of area at days 12 and 16, respectively) (Fig. 3A). Consistent with lung tissue damage, bleomycin induced progressive recruitment of leukocyte into the airways of WT mice, with early increase in neutrophils and chronic infiltration of eosinophils, lymphocytes, and monocye/macrophages (Fig. 3B). Similarly, when total lung leukocytes were recovered by CD45 immunomagnetic sorting from collagenase-digested lungs and leukocyte subsets evaluated by FACS analysis, a significant accumulation of neutrophils, eosinophils, macrophages, T lymphocytes, and myeloid dendritic cells in the lung tissue of WT animals instilled with bleomycin was observed (Fig. 3C). Consistent with the protective effect of ACKR2 deletion reported in Fig. 1, in both compartments leukocyte accumulation was significantly reduced in ACKR2−/− mice (Fig. 3, B and C). Thus, in the absence of ACKR2, bleomycin-induced pulmonary inflammation and fibrosis are greatly reduced.

ACKR2-deficient mice show a distinct profile of cytokines and chemokines. As expected, bleomycin instillation was associated with increased levels of proinflammatory and fibrogenic cytokines, such as IL-1β, IL-6, IL-13, and active TGF-β1, in lungs of WT mice, all of which were reduced in ACKR2−/− mice (Fig. 4A). On the contrary, bleomycin induced an early increase (day 2) of IFNγ selectively in the lungs of ACKR2−/− mice (Fig. 4A). Instillation of bleomycin also led to increased levels of several CC and CXC chemokines (Fig. 4B). As compared with WT animals, ACKR2−/− mice had increased pulmonary levels of CCL5, CCL12, and CCL17 at day 2 after bleomycin instillation, while the levels of CCL2, CCL3, CCL11, CXCL2, and CXCL9 were reduced (Fig. 4B).

ACKR2 deficiency impacts on the fibrogenic transcriptional program. As WT and ACKR2−/− mice expressed similar levels of bleomycin hydrolase (data not shown), we next evaluated whether the absence of ACKR2 affected pulmonary expression of genes associated with fibrogenesis, including tissue remodeling markers and Th17-associated genes. In WT mice, bleomycin induced expression of tissue remodeling genes (matrix metalloproteinase-3 and -9/tissue inhibitor of metalloproteinase-1, von Willebrand factor, and α-smooth muscle actin; Fig. 5A), increased expression of profibrogenic Th17-associated genes (IL-17A, IL-22, IL-23, IL-25, and retinoic acid receptor-related orphan receptor; Fig. 5, B and C), and upregulated Treg-associated genes (FoxP3, TGF-β1, and SMAD family member 3; Fig. 5C). There was also enhanced expression of genes associated with negative regulation of Th1 
responses (SOCS1 and SOCS3; Fig. 5C). Overall, the expression of these genes was significantly reduced in ACKR2^{−/−} mice exposed to bleomycin (Fig. 5, A–C).

Lung parenchyma ACKR2 expression contributes to pulmonary fibrosis. To investigate the relative contribution of ACKR2 expression on lymphatic endothelial cells or leukocytes on the protection observed in ACKR2^{−/−} mice exposed to bleomycin, bone marrow chimera experiments were performed (53). Lethality rate (Fig. 6A), weight loss (Fig. 6B), and total lung collagen (Fig. 6C) were similar in WT (Fig. 1) and in chimeric WT→WT and ACKR2^{−/−}→WT mice exposed to bleomycin, while all parameters were decreased in ACKR2^{−/−}.
Fig. 2. ACKR2 deficiency protects mice from pulmonary mechanic dysfunction induced by bleomycin. Mice received a single instillation with PBS or bleomycin (3.75 mg/kg body wt) and the pulmonary mechanics assessed by Buxco Pulmonary Function Tests using forced spirometry at days 8 and 22. A: modification in lung volumes: total lung capacity (TLC), forced vital capacity (FVC), and inspiratory capacity (IC); airway flow: forced expiratory capacity at 50 and 100 ms (FEV50 and FEV100); and pulmonary elasticity: dynamic and chord compliance, and lung resistance were induced by bleomycin in inflammatory phase (day 8) and fibrogenic phase (day 22). B and C: during the fibrogenic phase, at day 22 postbleomycin, the flow × volume (B) and pressure × volume (C) curves were evaluated by spirometry. Bleomycin-instilled wild type (WT) mice (filled squares) or ACKR2−/− mice (gray circles) with respective controls, PBS-treated WT (black circles), and ACKR2−/− mice (open triangles). Results are shown as the means ± SE of 5–8 animals in each group. *P < 0.05 and **P < 0.01, when comparing WT or ACKR2−/− instilled mice with respective PBS-treated controls; #P < 0.05, when comparing WT-given bleomycin-challenged mice with bleomycin-instilled ACKR2−/− mice.

Table 2. Pulmonary resistance in bleomycin-induced acute lung injury day 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Means ± SE</th>
<th>P Value</th>
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<tr>
<td>WT PBS</td>
<td>0.72 ± 0.039</td>
<td></td>
</tr>
<tr>
<td>ACKR2 PBS</td>
<td>0.76 ± 0.056</td>
<td></td>
</tr>
<tr>
<td>WT bleomycin</td>
<td>1.25 ± 0.090</td>
<td>&lt; 0.01, WT PBS vs. WT BLEO</td>
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<tr>
<td>ACKR2 bleomycin</td>
<td>0.94 ± 0.033</td>
<td>&lt; 0.01, ACKR2 BLEO vs. WT BLEO</td>
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WT, wild type; BLEO, bleomycin.

ACKR2 deficiency results in increased recruitment of CCR2+ and CCR5+ IFNγ-producing γδT cells. In WT animals, ACKR2 transcripts peaked at the 2-day time point after (Fig. 1) and in chimeric WT→ACKR2−/− and ACKR2−/−→ACKR2−/− mice exposed to bleomycin (Figs. 6, A–C). Similarly, histopathological analysis confirmed diffuse and dense interstitial lung fibrosis in WT→WT (Fig. 6F) and ACKR2−/−→WT mice (Fig. 6H), while WT→ACKR2−/− (Fig. 6G) and ACKR2−/−→ACKR2−/− mice (Fig. 6I) had reduced lung fibrosis when exposed to bleomycin. We conclude that the protective phenotype observed in ACKR2−/− animals is associated to the expression of ACKR2 by nonhematopoietic (parenchyma) cells.

ACKR2 deficiency results in increased recruitment of CCR2+ and CCR5+ IFNγ-producing γδT cells. In WT animals, ACKR2 transcripts peaked at the 2-day time point after
bleomycin instillation, the same time point when the well-known antifibrogenic cytokine IFNγ (58) was also shown to peak selectively in ACKR2−/− mice (compare Figs. 1A and 4). Consistent with this, FACS analyses at 2 days after bleomycin showed a similar profile of accumulation of neutrophils, eosinophils, macrophages, T lymphocytes, myeloid dendritic cells, and natural killer cells in lung tissue and airways of WT and ACKR2−/− mice (Fig. 7A) but a significant increase in the number of IFNγ+ γδT cells retrieved from lungs and airways of ACKR2−/− mice (Fig. 7, A and B). Finally, when γδT cells were further investigated for the expression of CC chemokine receptors, ACKR2−/− mice showed a significant increase in the number of CCR2+ and CCR5+ γδT lymphocytes mostly IFNγ+, as compared with WT mice, while no difference in
CCR4+ γδT lymphocytes was observed (Fig. 7B). We then used specific gene-targeted mice to identify the CC chemokine receptor responsible for the increased influx of IFNγ+ γδT cells observed in ACKR2-deficient animals exposed to bleomycin. Although CCR2−/−, CCR4−/−, and CCR5−/− mice showed a number of total lymphocytes recruited after bleomycin challenge comparable to WT mice (data not shown), in CCR2−/− and CCR5−/−, but not CCR4−/−, mice there was a significant decrease in total number of γδT cells (Fig. 8A) and IFNγ+ γδT cells (Fig. 8B). Interestingly, CCR2−/− mice showed reduced CCR5+ IFNγ+ γδT cells and CCR5−/− mice exhibited reduced CCR2+ IFNγ+ γδT cells (Fig. 8B). Despite the increased acute accumulation of γδT and IFNγ+ γδT cells in ACKR2−/− mice compared with WT mice at day 2 (Figs. 7B and 9A) and day 4 (Fig. 9A) after bleomycin instillation, we observed that it was not restricted to acute phase but was progressively increased in chronic time points (days 8 and 12) postbleomycin (Fig. 9A). In addition, there was a mixed IFNγ+ γδT cell population expressing CCR2+ and CCR5+ in airways (days 2 and 4) (Fig. 9, B and C), with the predominance of CCR2+ IFNγ+ γδT cells in chronic periods (Fig. 9B) in ACKR2−/− mice (Fig. 9C) challenged with bleomycin. Recently, Segawa et al. (43) demonstrated that IFNγ+ γδT cells suppresses Th17 activity in bleomycin model. Thus we evaluated the kinetic of Th17 lymphocyte influx in airways. We found decreased numbers of Th17 lymphocytes in ACKR2−/− mice compared with WT mice (Fig. 9D), as confirmed by mRNA expression (Fig. 5, B and C). We conclude that after bleomycin instillation the inflammatory CC chemokine receptors control the recruitment of IFNγ+ γδT cells in the airways and the impact of ACKR2 absence is associated with a significant increase of CCR2+ and CCR5+ IFNγ+ γδT cells and related to downregulation of the Th17 lymphocytes.

Depletion of γδT cells reverses the protection observed in the absence of ACKR2. To test whether γδT cells contributed to the protection observed in ACKR2−/− mice exposed to bleomycin, WT and ACKR2−/− mice were treated with a γδTCR-depleting antibody or its isotype control. Isotype-

Fig. 4. ACKR2-deficient mice show a distinct profile of chemokines and cytokines after bleomycin challenge. A and B: mice received a single instillation with PBS or bleomycin (3.75 mg/kg body wt) and the kinetic of pulmonary levels of cytokines IL-1β, IL-6, IL-13, active transforming growth factor-β1 (TGF-β1), and IFN-γ (A) and chemokines CCL2, CCL3, CCL4, CCL5, CCL11, CCL12, CCL17, CCL22, CXCL2, and CXCL9 (B) were evaluated by Luminex/Bio-Plex or ELISA. Results are shown as the means ± SE of 5–8 animals in each group. *P < 0.05 and **P < 0.01, comparing wild-type (WT)-given bleomycin-challenged mice (■) to instilled-ACKR2−/− mice (○).
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Fig. 5. ACKR2 deficiency protects from the fibrogenic transcriptional program induced by bleomycin. Mice received a single instillation with PBS or bleomycin (3.75 mg/kg body wt) and levels of total lung mRNA expression of profibrogenic related genes were monitored using quantitative real time PCR at days 2, 4, 8, 12, and 16 after bleomycin instillation. A: tissue remodeling markers: matrix metalloproteinase-3 and -9 (MMP3 and MMP9), tissue inhibitor of metalloproteinase-1 (TIMP-1), von Willebrand factor (vWF), and α-smooth muscle actin (α-SMA). B: Th17 fibrogenic cytokines: IL-17A, IL-22, IL-23, IL-25, and transforming growth factor-β (TGF-β). C: fibrogenic and Th17-related transcription factors: retinoic acid receptor-related orphan receptor γT (RORγT), forkhead box-P3 (FoxP3), suppressor of cytokine signaling-1 and -3 (SOCS1 and SOCS3), and SMAD family member 3 (SMAD3) were targeted in lungs of wild-type (WT) and ACKR2−/− mice. Results represent the relative abundance (expressed as 2^−ΔΔCT) of mRNA transcript over the total amount of 18S. Results are shown as the means ± SE of 5–8 animals in each group. *P < 0.05 and **P < 0.01, when comparing WT-given bleomycin-challenged mice (●) to instilled-ACKR2−/− mice (○).

DISCUSSION

Although fibrogenesis is clearly associated with increased expression of chemokines (45, 58), the role in lung fibrosis of ACKR2, a scavenger receptor for most inflammatory CC chemokines, has not been previously addressed. ACKR2 is an essential regulatory molecule for inflammatory responses in vivo, as ACKR2−/− mice display enhanced inflammation (5, 7, 10, 15, 56), tumor development (33, 53), and fetal loss (27). However, in other experimental settings, such as colitis (3), spinal cord inflammation and demyelination models (24), and bone remodeling (23), ACKR2 has been associated with a protective effect. Here, we first described the involvement of ACKR2 in pulmonary fibrosis and report experimental evidence indicating that pulmonary fibrosis and dysfunction are attenuated in ACKR2−/− mice. Our data can be summarized as the following explains. First, there was acute, but not chronic, upregulation of lung ACKR2 mRNA after bleomycin instilla-
Fig. 6. Lethality and pulmonary fibrosis induced by bleomycin is dependent of ACKR2 expression by nonhematopoietic (lung parenchyma) cells. A and B: lethality (A) and weight loss (B) from chimerical bone marrow constructs wild-type (WT) BMdo→WT BMre (□), WT BMdo→ACKR2+/− BMre (○), ACKR2−/− BMdo→WT BMre (△), and ACKR2+/− BMdo→ACKR2+/− BMre (○) were monitored daily after bleomycin instillation (3.75 mg/kg of body wt). BMdo, bone-marrow donor; BMre, bone-marrow recipient mice.

C–I: after 21 days of bleomycin instillation the chimerical bone marrow constructs were culled for fibrosis assessment by total lung collagen quantification by Sircol assay (C) and lung histopathology assessment, by representative photomicrography of Gomori’s Trichrome staining (D–I). Bars = 100 μm at ×100 magnification. D and E: PBS-instilled WT mice (D) or ACKR2−/− (E) mice presented normal lung architecture. F–H: histopathology shows a dense and diffuse interstitial lung fibrosis with loss of pulmonary architecture in WT BMdo→WT BMre (F) and ACKR2−/− BMdo→WT BMre (H) bleomycin-challenged mice, whereas there was a focal and less pronounced fibrosis with preserved areas of lung parenchyma in ACKR2+/− BMdo→ACKR2+/− BMre (G) and WT BMdo→ACKR2−/− BMre (I). Results are shown as the means ± SE of 13–16 animals in each group. *P < 0.05 and **P < 0.01 and ***P < 0.001, when comparing WT-given bleomycin-challenged mice to instilled-ACKR2−/− mice. Repeated experiments generated similar data sets.
tion in WT mice. Second, ACKR2−/− mice were protected from lethality, pulmonary inflammation, tissue remodeling, and dysfunction induced by bleomycin. Third, expression of ACKR2 in lung parenchyma cells accounted for the phenotype observed. Fourth, there was early increased IFN-γ expression, mostly by CCR2+ and CCR5+ γδT cells in ACKR2−/− mice exposed to bleomycin. Expression of these cells coincided with increase in the concentration of chemokines active to CCR2 and CCR5. Fifth, accumulation of IFNγ−-producing γδT cells was impaired in CCR2−/− and CCR5−/− mice. Sixth, there was progressive accumulation of CCR2+ and CCR5+ IFNγ-producing γδT cells and reduced Th17 response in airways from ACKR2−/− mice exposed to bleomycin. Seventh, γδT-cell depletion was able to prevent the protection conferred by ACKR2 deficiency. Therefore, ACKR2 controls the early production of CCR2- and CCR5-active chemokines that drive the influx of IFNγ-producing γδT cells, which exert an important control of the fibrogenic response to bleomycin.

ACKR2 binds and scavenges multiple CC chemokines and attenuates lung inflammation induced by *Mycobacterium tuberculosis* infection and allergen challenge in mice (7, 56). In chronic obstructive pulmonary disorder and sepsis patients, ACKR2 is mostly expressed by alveolar macrophages, also expressed by lung lymphatics from smokers (2, 5, 31). We were not able to identify which cell of the parenchyma expresses the ACKR2 receptor because of the limitation of tools to detect murine ACKR2 available in the market. Using bone-marrow chimeras, we found that ACKR2 expression in parenchyma cells was relevant for induction of pulmonary fibrosis in mice. It is possible that ACKR2 can be expressed by alveolar macrophages or lymphatic endothelium. Indeed, the development of lung fibrosis is believed to be associated with leuko-
cyte infiltration and tissue injury that precedes the aberrant remodeling (16, 60). The reduced lung injury associated to impaired leukocyte influx could account for the protection of ACKR2−/− mice. Furthermore, based on our data, and as previously shown (5, 7, 56), the impact of ACKR2 deficiency on progression of pulmonary inflammation and injury is dependent on the precise nature of the challenge.

Pulmonary fibrogenesis is characterized by increased parenchyma remodeling (8, 58) and is critically regulated by chemokine receptors. Th17 numbers in airways. IFN-γ and these are known to act synergistically to induce lung fibrogenic factors, including IL-13 (9), active TGF-β production of fibrogenic factors, including IL-13 (9), early elevation may account for the reduced fibrogenesis and fibrogenesis (29, 43, 57), suggesting that IFN-γ action in the context of pulmonary fibrosis (22). IFN-γ is a cytokine with antifibrotic properties and a negative regulator of TGF-β action in the context of lung injury associated to impaired leukocyte influx could account for the protection of ACKR2−/− mice. Furthermore, based on our data, and as previously shown (5, 7, 56), the impact of ACKR2 deficiency on progression of pulmonary inflammation and injury is dependent on the precise nature of the challenge.

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Recent observations elucidated the ability of γδT cells to produce cytokines that regulate homeostasis and tissue stress (6). γδT cells occur in healthy lungs (55), and their number is
Fig. 9. Increased recruitment of CCR2+ and CCR5+ IFN-γ-producing γδT cells is related to reduced Th17 response after bleomycin instillation in the absence of ACKR2. Mice received a single instillation with PBS or bleomycin (3.75 mg/kg of body wt) and 2, 4, 8, and 12 days after, leukocytes recovered from airway space of WT and ACKR2−/− mice were analyzed by FACS. A–C: the phenotype of IFN-γ-producer γδT lymphocytes in airway space was analyzed by FACS (A) and evaluated by expression of CCR2+ (B) and CCR5+ (C) related to respective populations of CD3+γδTCR+ lymphocytes and CD3+γδTCR+ IFN-γ+. BAL, bronchoalveolar lavage. D: Th17 lymphocyte population was analyzed by FACS using (CD3+CD4+RORγT+IL-17A+). Results are shown as the means ± SE of 5–8 animals in each group. *P < 0.05 and **P < 0.01, when comparing WT-given bleomycin-challenged mice to instilled-ACKR2−/− mice.
Fig. 10. γδT-lymphocyte depletion enhances lethality and pulmonary fibrosis induced by bleomycin and reverts the protection under ACKR2 deficiency. A–H: mice received a single instillation with PBS or bleomycin (3.75 mg/kg body wt) and mouse phenotype was studied during 20 days. Wild-type (WT) and ACKR2−/− mice were injected intraperitoneally with anti-γδTCR (250 μg/mice at days −2 and 0 preceding bleomycin instillation) or control-IgG (250 μg/mice at days −2 and 0 preceding bleomycin). A: lethality after bleomycin instillation in WT mice treated with control-IgG (■), ACKR2−/− mice treated with control-IgG (○), WT mice treated with anti-γδTCR (▲), and ACKR2−/− mice treated with anti-γδTCR (open triangles) were monitored daily after bleomycin instillation. B–H: after 20 days of bleomycin instillation WT and ACKR2−/− mice treated with antibodies were culled for fibrosis assessment by total lung collagen quantification by Sircol assay (B) and lung histopathology assessment, by representative photomicrography of Gomori’s trichrome staining (C–H). C and D: bars = 100 μm at ×100 magnification. PBS-instilled WT mice (C) or ACKR2−/− mice (D) presented normal lung architecture. E–H: histopathology shows a dense and diffuse interstitial lung fibrosis with loss of pulmonary architecture in WT mice treated with control-IgG (E), WT mice treated with anti-γδTCR (G), and ACKR2−/− mice treated with anti-γδTCR (H) challenged with bleomycin, whereas there was a focal and less pronounced fibrosis with preserved areas of lung parenchyma in ACKR2−/− mice treated with control-IgG (F). Results are shown as the means ± SE of 13–16 animals in each group. *P < 0.05 and **P < 0.01 and ***P < 0.001, when comparing WT-given bleomycin-challenged mice to instilled-ACKR2−/− mice.
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reduced in BAL and blood from IPF patients (12, 44), suggesting that they play a role in pulmonary homeostasis and fibrosis. There is evidence that γ6T cells reduce tissue damage and fibrosis associated with pulmonary inflammation and infection (4, 19, 20, 32, 37, 43, 46) by mechanisms that include regulation of IL-22, IL-17A (4), and CXCL10 production (37), although we did not detect increased levels of CXCL10 (data not shown) or IL-22 in lungs of ACKR2−/− mice. Recently, Segawa et al. (43) demonstrated the regulatory role of IFNγ+ γ6T cells suppressing Th17 activity in bleomycin model. In fact, we found an increased number of CCR2+ IFNγ+ γ6T- and CCR5+ IFNγ+ γ6T-cell influx into airways of ACKR2−/− mice challenged with bleomycin, and it was related to decreased numbers of Th17 lymphocytes when compared with WT mice, as confirmed by mRNA expression. Our results show that antibody-mediated depletion of γ6T cells worsened bleomycin-induced lethality and fibrosis in WT and ACKR2−/− mice. It is possible that this may be due to elevated levels of IL-17A in γ6T-cell-depleted mice, as found in γ6−/− mice (43). Finally, the presence of IFNγ-producers γ6T cells could block pulmonary fibrosis in ACKR2−/− mice through the inhibition of Th17 expansion and fibrogenesis (4, 37, 43, 46).

In conclusion, our data demonstrate that ACKR2 is induced during the development of pulmonary fibrosis caused by bleomycin challenge and that its deletion leads to reduced lethality, lung injury, inflammation, and fibrosis. Mechanistically, ACKR2 is determinant for controlling the recruitment of CCR2+ and CCR5+ γ6T cells, which produce IFNγ and influence subsequent development of Th17 response and intensity of fibrosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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