Am J Physiol Lung Cell Mol Physiol 314: L1010–L1025, 2018.
First published February 22, 2018; doi:10.1152/ajplung.00233.2017.

RESEARCH ARTICLE

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

Remo C. Russo,[1,2,3] Benedetta Savino,[3,4] Massimiliano Mirolo,[3] Chiara Buracchi,[3] Giovanni Germano,[3] Achille Anselmo,[3] Luca Zammataro,[3] Fabio Pasqualini,[3] Alberto Mantovani,[3,5] Massimo Locati,[3,4]* and Mauro M. Teixeira[2]*

[1]*Laboratory of Pulmonary Immunology and Mechanics, Department of Physiology and Biophysics, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil;* [2]*Laboratory of Immunopharmacology, Department of Biochemistry and Immunology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil;* [3]*Humanitas Clinical and Research Center, Rozzano, Italy;* [4]*Department of Medical Biotechnology and Translational Medicine, University of Milan, Milan, Italy; and* [5]*Humanitas University, Rozzano, Italy*

Submitted 31 May 2017; accepted in final form 16 February 2018

Russo RC, Savino B, Mirolo M, Buracchi C, Germano G, Anselmo A, Zammataro L, Pasqualini F, Mantovani A, Locati M, Teixeira MM. The atypical chemokine receptor ACKR2 drives pulmonary fibrosis by tuning influx of CCR2$^+$ and CCR5$^+$ IFNγ-producing γδT cells in mice. *Am J Physiol Lung Cell Mol Physiol* 314: L1010–L1025, 2018. First published February 22, 2018; doi: 10.1152/ajplung.00233.2017.— Chemokines coordinate lung inflammation and fibrosis by acting on chemokine receptors expressed on leukocytes and other cell types. Atypical chemokine receptors (ACKRs) bind, internalize, and degrade chemokines, tuning homeostasis and immune responses. ACKR2 recognizes and decreases the levels of inflammatory CC chemokines. The role of ACKR2 in fibrogenesis is unknown. The purpose of the study was to investigate the role of ACKR2 in the context of pulmonary fibrosis. The effects of ACKR2 expression and deficiency during inflammation and fibrosis were analyzed using a bleomycin-model of fibrosis, ACKR2-deficient mice, bone marrow chimeras, and antibody-mediated leukocyte depletion. ACKR2 was upregulated acutely in response to bleomycin and normalized over time. ACKR2$^{-/-}$ mice showed reduced lethality and lung fibrosis. Bone marrow chimeras showed that lethality and fibrosis depended on ACKR2 expression in pulmonary resident (nonhematopoietic) cells but not on leukocytes. ACKR2$^{-/-}$ mice exhibited decreased expression of tissue-remodeling genes, reduced leukocyte influx, pulmonary injury, and dysfunction. ACKR2$^{-/-}$ mice had early increased levels of CCL5, CCL12, CCL17, and IFNγ and an increased number of CCR2$^+$ and CCR5$^+$ IFNγ-producing γδT cells in the airways counterbalanced by low Th17-lymphocyte influx. There was reduced accumulation of IFNγ-producing γδT cells in CCR2$^{-/-}$ and CCR5$^{-/-}$ mice. Moreover, depletion of γδT cells worsened the clinical symptoms induced by bleomycin and reversed the phenotype of ACKR2$^{-/-}$ mice exposed to bleomycin. ACKR2 controls the CC chemokine expression that drives the influx of CCR2$^+$ and CCR5$^+$ IFNγ-producing γδT cells, tuning the Th17 response that mediated pulmonary fibrosis triggered by bleomycin instillation.

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

* M. Locati and M. M. Teixeira contributed equally to this work.

Address for reprint requests and other correspondence: R. C. Russo, Lab. of Pulmonary Immunology and Mechanics, Dept. of Physiology and Biophysics, Inst. of Biological Sciences, Univ. Federal de Minas Gerais, Avenida Antonio Carlos, 6627, Pampulha, 31270-901, Belo Horizonte, MG, Brasil (e-mail: remo@ufmg.br).

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 (4×10^6 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 and fibrosis 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 conformed to institutional guidelines in agreement with national and international law approved by the ethics committee of the Istituto Clinico Humanitas 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 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 hemotoxylin and eosin or Gomori's trichrome, as described previously (39). Images of lung sections were captured with a digital camera (Optronics DEI-470) connected to a microscope (Olympus IX70) with a magnification of ×40 or 100. Bars = 100 mm for Gomori's trichrome and 200 mm for hemotoxylin and eosin.

Assessment of respiratory mechanic dysfunction. Mice were tracheostomized, placed in a body plethysmograph, and connected to a computer-controlled ventilator (Forced Pulmonary Maneuver System; Buxco Research Systems, Wilmington, NC), as previously described (36, 52). Under mechanical respiration the dynamic compliance and lung resistance were determined by resistance and compliance test. To measure functional residual capacity (FRC), ventilation was stopped at the end of expiration and spontaneous breathing maneuvers with consequent pressure changes were recorded to calculate the FRC by Boyle's law. To measure the chord compliance and inspiratory capacity (IC), the quasistatic pressure-volume maneuver was performed, which inflates the lungs to a standard pressure of +30 cmH_2O and then slowly exhales until a negative pressure of −30 cmH_2O is reached. Chord compliance was determined at the pressure +10 cmH_2O. The total lung capacity was calculated by FRC + IC. Fast-flow volume maneuver was performed, and lungs were first inflated to +30 cmH_2O and immediately afterwards connected to a highly negative pressure to enforce expiration until −30 cmH_2O. The forced vital capacity, forced expiratory volume at 50 and 100 ms, and flow-volume curve were recorded during this maneuver. Suboptimal maneuvers were rejected and for each test in every single mouse at least three acceptable maneuvers were conducted to obtain a reliable mean for all numeric parameters.

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 $2^{-\Delta CT}$) of mRNA transcript over the total amount of 18S.

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 100 U/ml 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 magnetic microbeads (Miltenyi Biotec) and MS columns, according to the manufacturer's instructions. Leukocytes recovered from BAL and $CD45^+$ cells (1×10^6 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 phenotypes were calculated by expression of surface or intracellular markers: neutrophils ($Ly6G^+CXCR2^+$), eosinophils ($Ly6G^+CCR3^+$), macrophages ($Ly6C^+CD115^+CD11b^+$), T lymphocytes ($CD3^+CD4^+$ and $CD3^+CD8^+$), myeloid dendritic cells ($MHCII^+CD11b^+CD11c^+$), natural killer cells ($CD3^+DX5^+$), γδT cells ($CD3^+\gamma\delta TCR^+$), and γδT cell-IFNγ producers ($CD3^+\gamma\delta TCR^+IFN\gamma$), expressing the chemokine receptors CCR2, CCR4, or CCR5 and Th17 lymphocytes ($CD3^+CD4^+ROR\gamma T^+IL\text{-}17A^+$).

Table 1. *Murine-specific primers for TaqMan gene expression assay*

Name of Target	Abbreviation	TaqMan
18S	18S	Hs99999901_s1
Bleomycin hydrolase	Blmh	Mm00724431_m1
Atypical chemokine receptor 2	ACKR2	Mm00445551_m1
Matrix metalloproteinase 3*	MMP3	Hs00233962_m1
Matrix metalloproteinase 9	MMP9	Mm00600163_m1
Tissue inhibitor of matrix metalloproteinase 1	TIMP-1	Mm00441818_m1
von Willebrand factor	vWF	Mm00550379_m1
α-Smooth muscle actin	α-SMA	Mm01546133_m1
Transforming growth factor-β1	TGFβ1	Mm03024053_m1
Interleukin-17A	IL-17A	Mm00439619_m1
Interleukin-22	IL-22	Mm00444241_m1
Interleukin-23	IL-23	Mm00518984_m1
Interleukin-25	IL-25	Mm00499822_m1
Forkhead box P3	Foxp3	Mm00475165_m1
RAR-related orphan receptor-γ	RORγT	Mm00441139_m1
Suppressor of cytokine signaling 1	SOCS1	Mm00782550_s1
Suppressor of cytokine signaling 3	SOCS3	Mm00545913_s1
SMAD family member 3	SMAD3	Mm00489638_m1
C-X-C motif chemokine 10	CXCL10	Mm00445235_m1

Cytokine measurement in lungs. One hundred milligrams of lung tissue were homogenized in 1 ml of PBS with protease inhibitors (Complete tablets, Roche Diagnostic; PMSF, Sigma-Aldrich) and 0.05% Tween 20. Cytokines and chemokines were analyzed simultaneously using a Luminex multiplex bead assay (Bio-Rad) mouse 12-plex multicytokine (CCL2, CCL3, CCL4, CCL5, CCL11, CXCL2, CXCL9, IL-1β, IL-6, IL-10, TNF-α, and VEGF) beads in according to the manufacturer's instructions. The chemokines CCL12, CCL17, CCL22, and cytokines IL-13, active transforming growth factor-β_1 (TGF-β_1), and IFNγ levels were quantified using DuoSet ELISA kits (R&D Systems), in accordance with the manufacturer's instructions (39).

γδT-lymphocyte depletion in vivo. γδT lymphocytes were depleted in WT and ACKR2$^{-/-}$ mice by intraperitoneal injection with 250 μg/day of purified hamster anti-γδTCR (clone UC7-13D5; BD PharMingen) or IgG isotype control (BD PharMingen) in sterile PBS, as previously described (3, 19), at *days −2* and *0* before bleomycin instillation (3.75 mg/kg).

Statistical analysis. All data are presented as the means ± SE and were analyzed by one-way ANOVA and differences between groups were assessed using the Student-Newman-Keuls post hoc test or log-rank test. Graphs and analysis were performed using GraphPad Prism 4 software. Differences were considered significant at $P < 0.05$.

RESULTS

ACKR2 deficiency protects against bleomycin-induced pulmonary fibrosis and dysfunction. In the lung tissue of animals instilled with bleomycin, we observed an acute upregulation of ACKR2 transcripts, which peaked at *day 2* and then decreased over time returning to basal values in the chronic phase (at *day 16*) (Fig. 1*A*). As ACKR2 transcript upregulation preceded the development of pulmonary fibrosis, we investigated its role in this experimental setting comparing WT and ACKR2$^{-/-}$ mice instilled with bleomycin. As compared with WT animals, ACKR2$^{-/-}$ mice showed decreased lethality rate (Fig. 1*B*) and body weight loss (Fig. 1*C*). Consistent with this, the increase in lung collagen content observed in WT animals instilled with bleomycin was significantly reduced in ACKR2$^{-/-}$ mice (Fig. 1*D*). Semiquantitative analyses of lung tissue sections (Figs. 1, *E–H*) revealed dense and diffuse interstitial lung fibrosis with loss of pulmonary architecture in WT mice (Fig. 1*G*), while ACKR2$^{-/-}$ mice showed less pronounced fibrosis and preserved areas of lung parenchyma (Fig. 1*H*). In this experimental setting, pulmonary dysfunction was evident both at *days 8* and *22* after bleomycin instillation and was demonstrated by reduction of lung volumes and airway flow, with marked reduction of total lung capacity and forced vital capacity and progressive loss of IC and forced expiratory volume (Fig. 2*A*). There was also progressive (from *days 8* to *22*) reduction of lung elasticity, as seen by progressive reduction in pulmonary compliance and gain of lung resistance in WT mice with bleomycin (Fig. 2*A*). Overall, all these mechanic functional changes were less intense in ACKR2$^{-/-}$ mice (Fig. 2, *A* and *B*). Indeed, there was reduced airway flow (Fig. 2*B*) and flattened pressure × volume curve (Fig. 2*C*), characteristic of pulmonary fibrosis, 22 days after bleomycin in WT mice but not in ACKR2$^{-/-}$ mice. Collectively, evidence indicates that ACKR2$^{-/-}$ mice are significantly protected from bleomycin-induced lung injury and consequent pulmonary dysfunction, suggesting a nonredundant role of ACKR2 in the pathogenesis of pulmonary fibrosis.

ACKR2 deficiency reduces lung injury and leukocyte influx induced by bleomycin. To characterize the protective effect of ACKR2 deletion on bleomicyn-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. 3*A*). Inflammatory foci 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. 3*A*). 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 monocyte/macrophages (Fig. 3*B*). 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. 3*C*). 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-β_1, in lungs of WT mice, all of which were reduced in ACKR2$^{-/-}$ mice (Fig. 4*A*). On the contrary, bleomycin induced an early increase (*day 2*) of IFNγ selectively in the lungs of ACKR2$^{-/-}$ mice (Fig. 4*A*). Instillation of bleomycin also led to increased levels of several CC and CXC chemokines (Fig. 4*B*). 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. 4*B*).

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. 5*A*), 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-β_1, and SMAD family member 3; Fig. 5*C*). There was also enhanced expression of genes associated with negative regulation of Th1

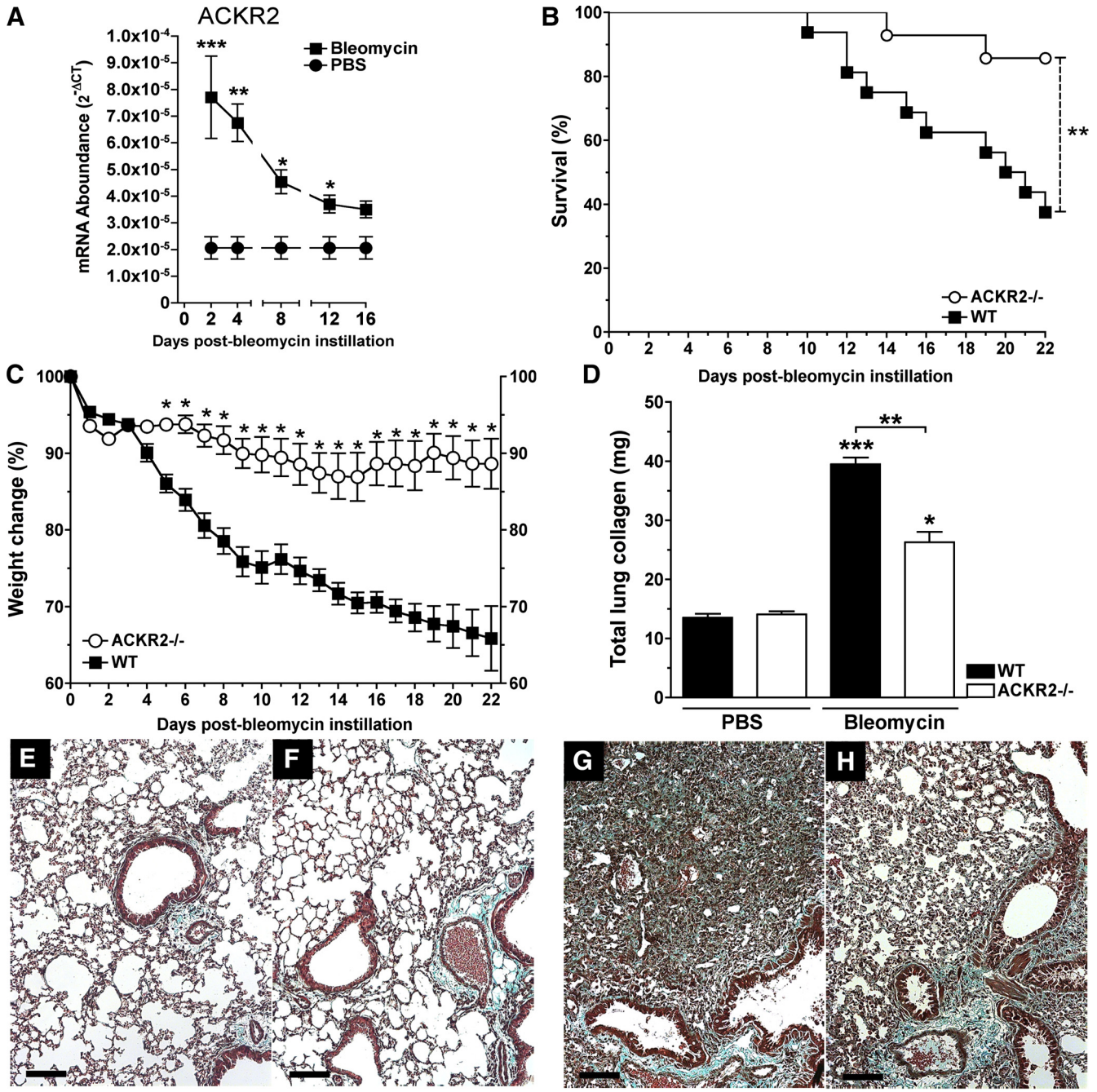

Fig. 1. Expression of ACKR2 in lungs: receptor upregulation in lungs and pulmonary ACKR2 deficiency protects against lethality and pulmonary fibrosis. Mice received a single instillation with PBS or bleomycin (3.75 mg/kg body wt). *A*: kinetic of lung ACKR2 receptor mRNA expression in wild-type (WT) mice by real-time PCR using Taqman probes after PBS (●) or bleomycin instillation (■). *B* and *C*: results represent the relative abundance (expressed as $2^{-\Delta CT}$) of mRNA transcript over the total amount of 18S. WT (■) and ACKR2$^{-/-}$ mice (○) lethality (*B*) and weight loss (*C*) were monitored daily after bleomycin instillation. *D–H*: after 22 days of bleomycin instillation WT and ACKR2$^{-/-}$ mice were culled for fibrosis assessment by total lung collagen quantification by Sircol assay (*D*) and lung histopathology assessment by representative photomicrography of Gomori's Trichrome staining (*E–H*). Bars = 100 μm at ×100 magnification. *E* and *F*: PBS-instilled WT mice (*E*) or ACKR2$^{-/-}$ (*F*) mice presented normal lung architecture. *G* and *H*: histopathology shows a dense and diffuse interstitial lung fibrosis with loss of pulmonary architecture in WT mice (*G*) bleomycin-challenged mice, whereas there was a focal and less pronounced fibrosis with preserved areas of lung parenchyma in ACKR2$^{-/-}$ mice (*H*). 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.

responses (SOCS1 and SOCS3; Fig. 5*C*). 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. 6*A*), weight loss (Fig. 6*B*), and total lung collagen (Fig. 6*C*) 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$^{-/-}$

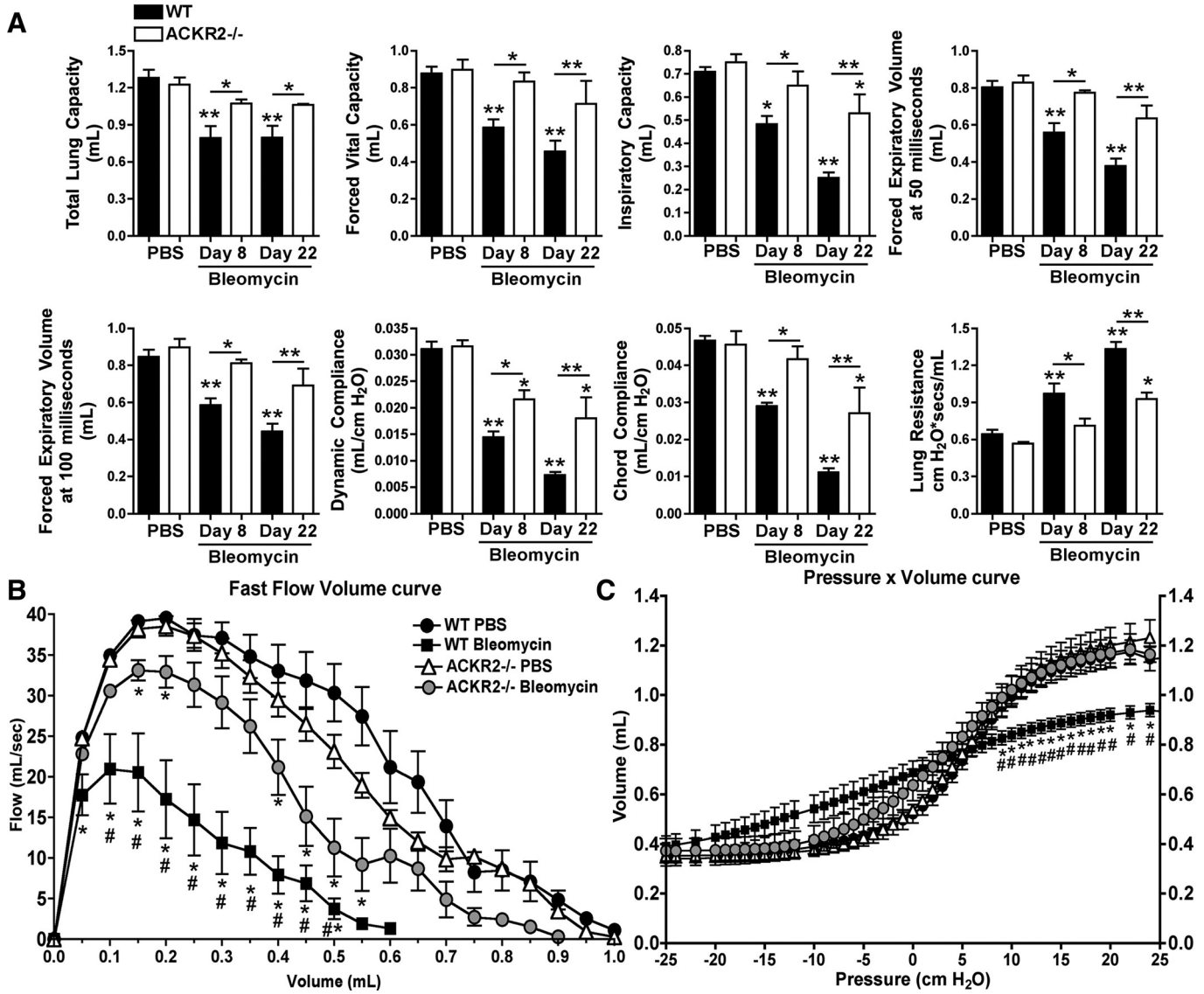

Fig. 2. ACKR2 deficiency protects mice from pulmonary mechanic dysfunction induced by bleomycin. Mice received a single instillation with PBS or bleomycin (3.75 mg/kg body wt) and the pulmonary mechanics assessed by Buxco Pulmonary Function Tests using forced spirometry at *days 8* and *22*. *A*: modification 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 (FEV_{50} and FEV_{100}); 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*

Groups	Means ± SE	*P* Value
WT PBS	0.72 ± 0.039	
ACKR2 PBS	0.76 ± 0.056	
WT bleomycin	1.25 ± 0.090	$P < 0.01$, WT PBS vs. WT BLEO
ACKR2 bleomycin	0.94 ± 0.033	$P < 0.01$, ACKR2 BLEO vs. WT BLEO

WT, wild type; BLEO, bleomycin.

(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. 6*F*) and ACKR2$^{-/-}$→WT mice (Fig. 6*H*), while WT→ACKR2$^{-/-}$ (Fig. 6*G*) and ACKR2$^{-/-}$→ACKR2$^{-/-}$ mice (Fig. 6*I*) 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

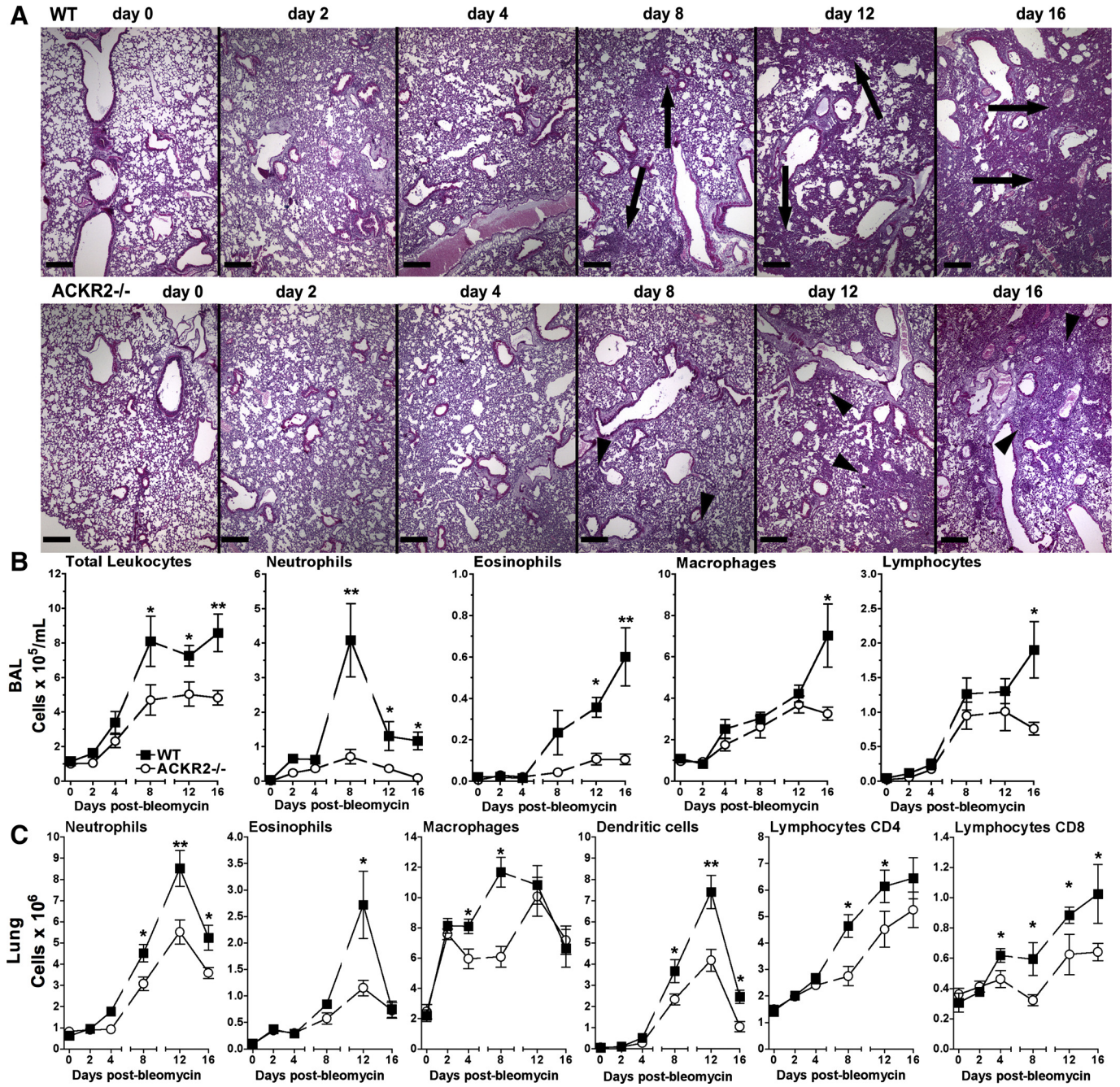

Fig. 3. ACKR2 deficiency reduces leukocyte influx and lung injury induced by bleomycin. *A*: mice received a single instillation with PBS or bleomycin (3.75 mg/kg of body wt), and pulmonary pathology was assessed using hematoxilin & eosin staining at *days 2*, *4*, *8*, *12*, and *16* after bleomycin challenge. Areas of tissue injury and inflammation induced by bleomycin are indicated in wild-type (WT) mice (arrows) and ACKR2$^{-/-}$ mice (arrowhead). Bars = 200 μm at ×40 magnification. *B*: leukocyte influx into the airway space was quantified using Neubauer chamber and cytospin preparations. BAL, bronchoalveolar lavage. *C*: lung leukocyte were recovered from lung parenchyma by CD45+ selection using magnetic beads and stained for FACS analysis: neutrophils (Ly6G$^+$CXCR2$^+$), eosinophils (Ly6G$^+$CCR3$^+$), macrophages (Ly6C$^+$CD115$^+$CD11b$^+$), T-lymphocytes (CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$), and myeloid dendritic cells (MHCII$^+$CD11b$^+$CD11c$^+$). Results are shown as the means ± SE of 5–8 animals in each group. *$P < 0.05$ and **$P < 0.01$, comparing WT-given bleomycin-challenged mice (■) to instilled-ACKR2$^{-/-}$ mice (○).

bleomycin instillation, the same time point when the well-known antifibrogenic cytokine IFNγ (58) was also shown to peak selectively in ACKR2$^{-/-}$ animals (compare Figs. 1*A* 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 natrual killer cells in lung tissue and airways of WT and ACKR2$^{-/-}$ mice (Fig. 7*A*) 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

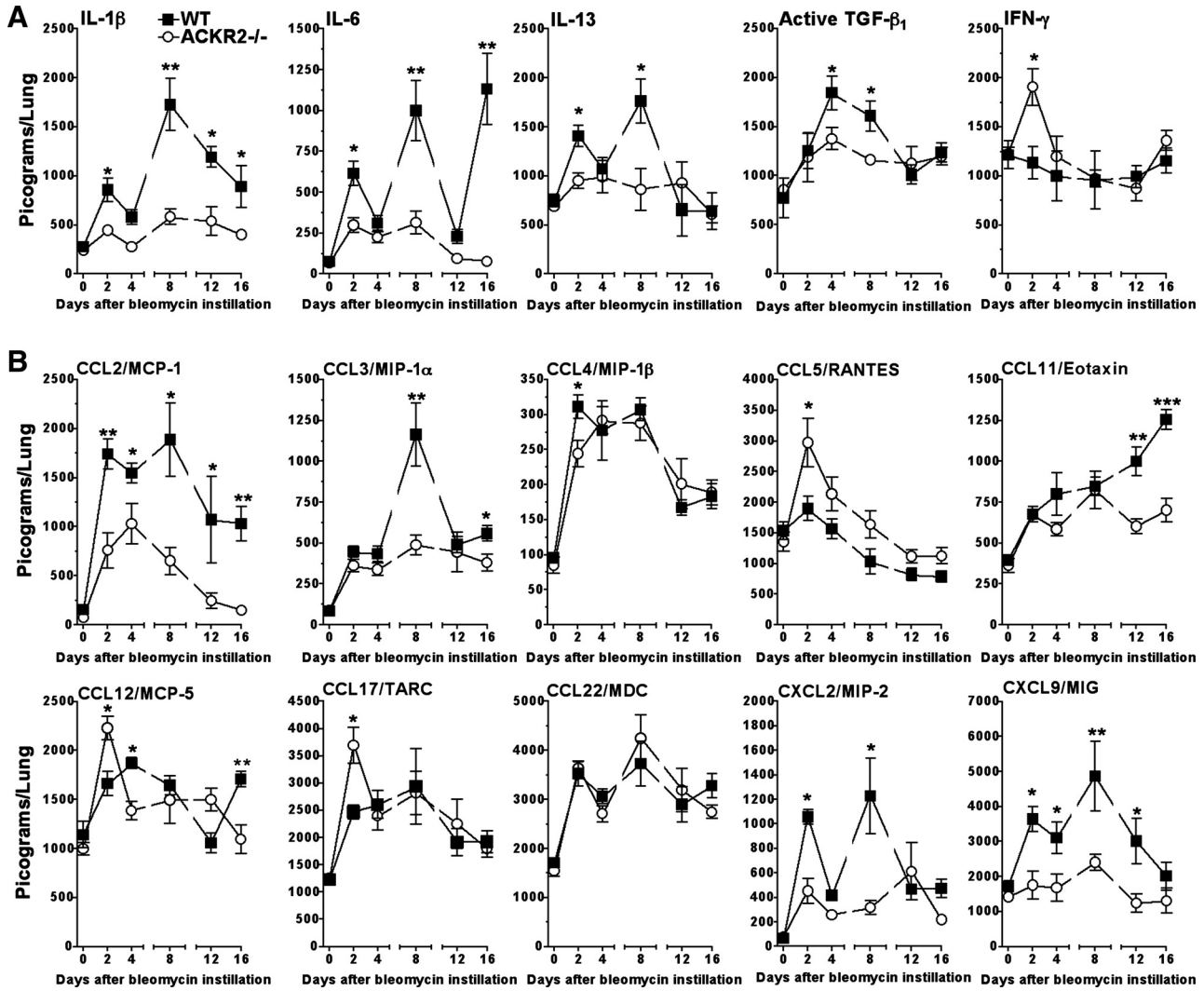

Fig. 4. ACKR2-deficient mice show a distinct profile of chemokines and cytokines after bleomycin challenge. *A* and *B*: mice received a single instillation with PBS or bleomycin (3.75 mg/kg body wt) and the kinetic of pulmonary levels of cytokines IL-1β, IL-6, IL-13, active transforming growth factor-β_1 (TGF-β_1), 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 (○).

CCR4$^+$ γδT lymphocytes was observed (Fig. 7*B*). 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. 8*A*) and IFNγ$^+$ γδT cells (Fig. 8*B*). Interestingly, CCR2$^{-/-}$ mice showed reduced CCR5$^+$ IFNγ$^+$ γδT cells and CCR5$^{-/-}$ mice exhibited reduced CCR2$^+$ IFNγ$^+$ γδT cells (Fig. 8*B*). Despite the increased acute accumulation of γδT and IFNγ$^+$ γδT cells in ACKR2$^{-/-}$ mice compared with WT mice at *day 2* (Figs. 7*B* and 9*A*) and *day 4* (Fig. 9*A*) 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. 9*A*). 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. 9*B*) in ACKR2$^{-/-}$ mice (Fig. 9*C*) 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. 9*D*), 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-

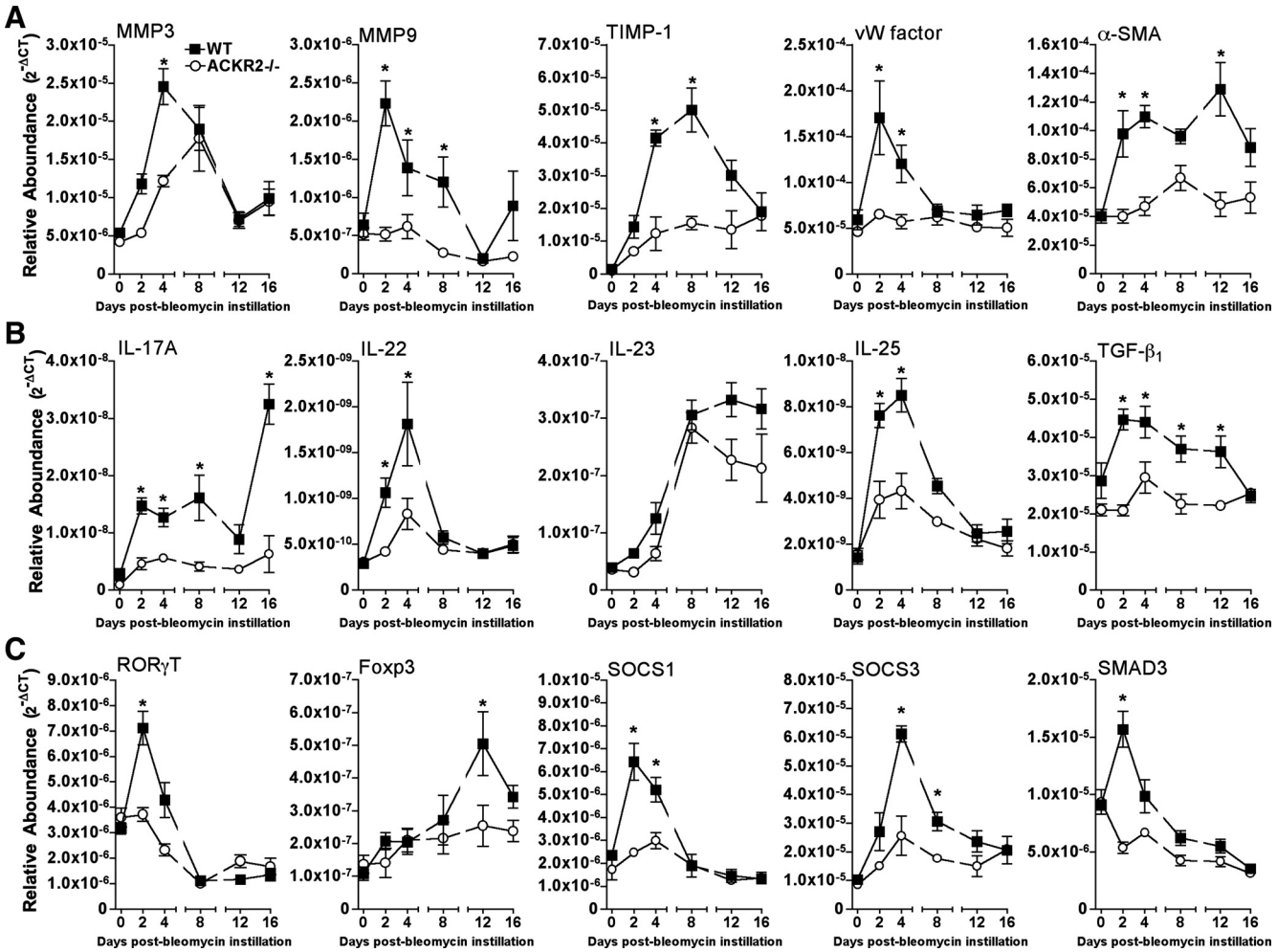

Fig. 5. ACKR2 deficiency protects from the fibrogenic transcriptional program induced by bleomycin. Mice received a single instillation with PBS or bleomycin (3.75 mg/kg body wt) and levels of total lung mRNA expression of profibrogenic related genes were monitored using quantitative real time PCR at *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_1 (TGF-β_1). *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 $2^{-\Delta CT}$) 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 (○).

treated ACKR2$^{-/-}$ mice remained significantly healthier than isotype-treated WT mice when challenged with bleomycin (Fig. 10, *A–C*). ACKR2$^{-/-}$ and WT mice treated with depleting anti-γδTCR antibodies exhibited greater lethality (Fig. 10*A*) and weight loss (Fig. 10*B*) and increased lung fibrosis (Fig. 10*C*), as compared with the isotype control groups. A dense and diffuse interstitial lung fibrosis with loss of pulmonary architecture was observed in isotype-treated WT mice exposed to bleomycin (Fig. 10*F*). Histopathological changes were more severe in WT mice treated with anti-γδTCR (Fig. 10*H*). In isotype-treated ACKR2$^{-/-}$ mice, bleomycin caused focal and less-pronounced fibrosis with preserved areas of lung parenchyma (Fig. 10*G*), but γδT-cell-depleted ACKR2$^{-/-}$ mice displayed diffuse interstitial lung fibrosis (Fig. 10*I*) similar to WT mice (Fig. 10*F*). Taken together, evidence indicates that γδT lymphocytes recruited in the airways after bleomycin instillation are responsible for the resistance to the bleomycin-induced pulmonary fibrosis observed in ACKR2$^{-/-}$ mice.

DISCUSSION

Although fibrogenesis is clearly associated to 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-

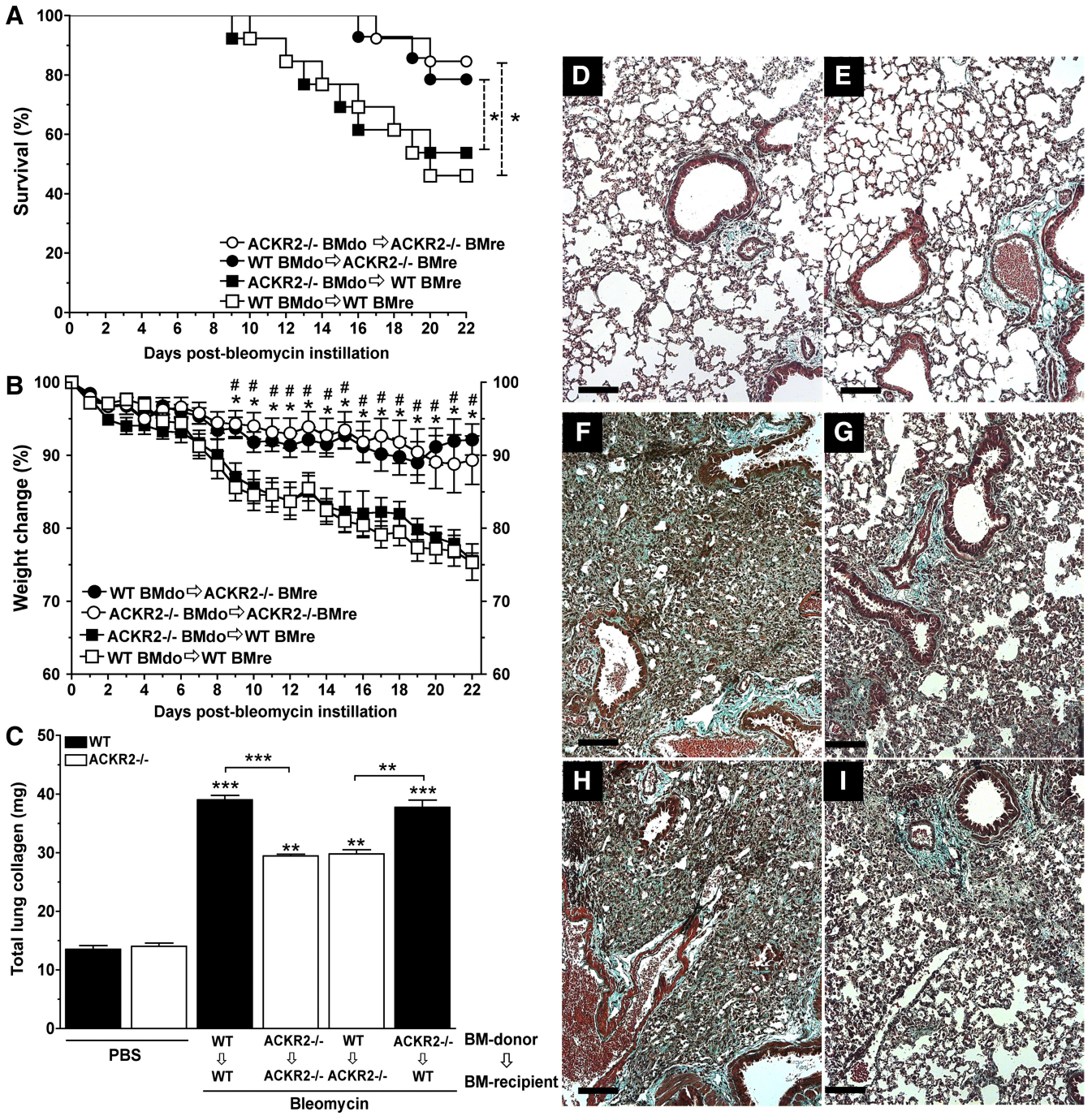

Fig. 6. Lethality and pulmonary fibrosis induced by bleomycin is dependent of ACKR2 expression by nonhematopoietic (lung parenchyma) cells. *A* and *B*: lethality (*A*) and weight loss (*B*) from chimerical bone marrow constructs wild-type (WT) BMdo→WT BMre (□), WT BMdo→ACKR2$^{-/-}$ BMre (●), ACKR2$^{-/-}$ 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.

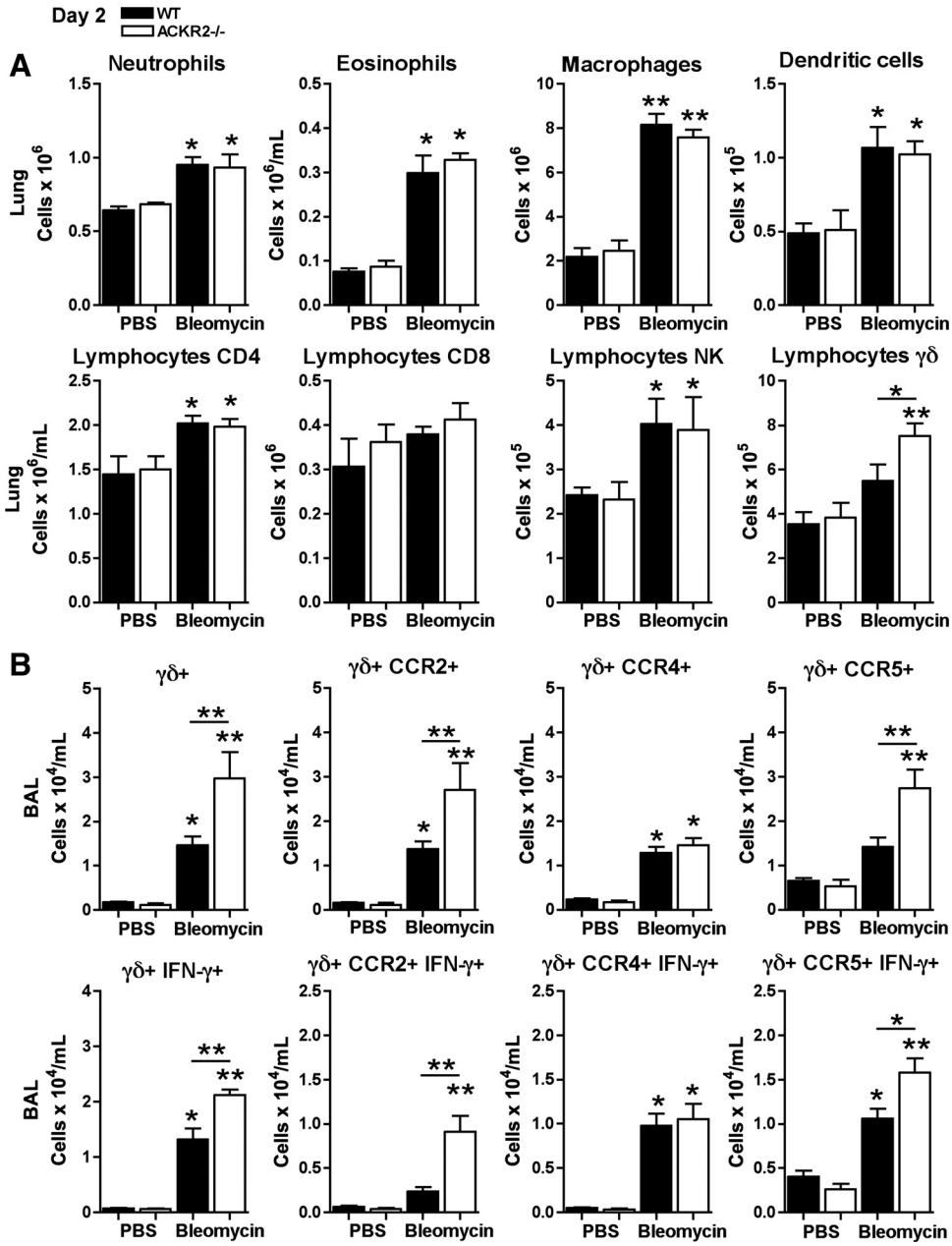

Fig. 7. Increased recruitment of CCR2+ and CCR5+, but not CCR4+, IFN-γ-producing γδT cells after bleomycin instillation in the absence of ACKR2. *A*: mice received a single instillation with PBS or bleomycin (3.75 mg/kg of body wt), and 2 days after lung leukocytes were recovered from lung parenchyma by $CD45^+$ selection using magnetic beads, stained for neutrophils ($Ly6G^+CXCR2^+$), eosinophils ($Ly6G^+CCR3^+$), macrophages ($Ly6C^+CD115^+CD11b^+$), T-lymphocytes ($CD3^+CD4^+$ and $CD3^+CD8^+$), myeloid dendritic cells ($MHCII^+CD11b^+CD11c^+$), natural killer (NK) cells ($CD3^+DX5^+$), and γδT cell ($CD3^+\gamma\delta TCR^+$) analyzed by FACS. *B*: the phenotype of γδT cells recovered in airway space was analyzed by FACS and evaluated by expression of $CCR2^+$, $CCR4^+$ and $CCR5^+$ related to respective populations of $CD3^+\gamma\delta TCR^+$ lymphocytes and $CD3^+\gamma\delta TCR^+IFN\text{-}\gamma^+$ for IFN-γ-producer γδT-lymphocytes. BAL, bronchoalveolar lavage. 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.

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 paremchyma 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 $IFN\gamma^+$ 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-

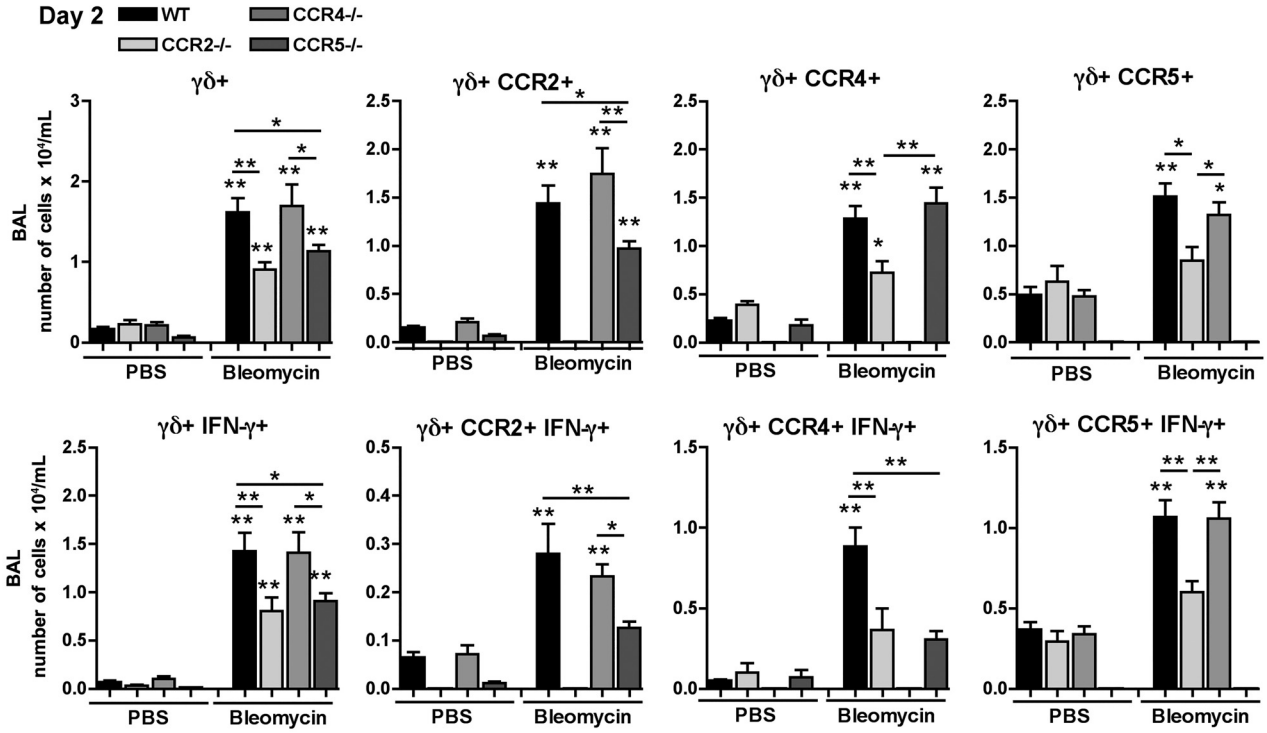

Fig. 8. CCR2 and CCR5, but not CCR4, deficiency impacts the IFN-γ-producing γδT-cell recruitment after bleomycin instillation. Mice received a single instillation with PBS or bleomycin (3.75 mg/kg body wt) and 2 days after, leukocytes recovered from airway space of CCR2$^{-/-}$, CCR4$^{-/-}$, and CCR5$^{-/-}$ mice were analyzed by FACS. *A* and *B*: the phenotype of γδT cells recovered in airway space was evaluated by expression of CCR2^{+}, CCR4^{+}, and CCR5^{+} (*A*) and CD3^{+}γδTCR^{+}IFN-γ^{+} for INF-γ-producer γδT-lymphocytes (*B*). BAL, bronchoalveolar lavage. 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-CCR2$^{-/-}$, CCR4$^{-/-}$, and CCR5$^{-/-}$ mice.

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 chemokines (47, 48, 51, 58) and Th2/Th17 responses (58). We found that ACKR2$^{-/-}$ mice displayed decreased pulmonary fibrosis and dysfunction and these were related to reduced expression of mRNA transcripts of genes associated with fibrogenesis (39, 41, 50, 59) and Th2/Th17 responses, mostly by reduction in Th17 numbers in airways. IFNγ is a cytokine with antifibrotic proprieties and a negative regulator of TGF-β_1 action in the context of pulmonary fibrosis (22). IFNγ was elevated early in ACKR2$^{-/-}$ mice but not in WT mice after bleomycin. This early elevation may account for the reduced fibrogenesis and production of fibrogenic factors, including IL-1β (57), IL-6 (42), IL-13 (9), active TGF-β_1 (9), CCL2 (11, 30, 61), CCL3 (14, 38, 49), and CXCL2 (18, 41). These results suggest that ACKR2 tunes the lung fibrogenesis through modulation of a fibrogenic program. Moreover, ACKR2$^{-/-}$ mice had low levels of lung IL-13, TGF-β_1 and Th17 activity after bleomycin and these are known to act synergistically to induce lung fibrogenesis (29, 43, 57), suggesting that IFNγ activity could block the fibrogenesis dampening the TGF-β_1 and Th17 response in ACKR2$^{-/-}$ mice.

We found that ACKR2 deficiency resulted in elevated levels of CCL5, CCL12, and CCL17 at the same time that ACKR2 expression peaked in WT mice. These chemokines act on CCR5, CCR2, and CCR4, respectively, and hence induce recruitment of γδT after bleomycin challenge. Therefore, ACKR2 deficiency in lungs could modify the local chemokine microenvironment, supporting γδT-cell influx. In fact, CCR2^{+} and CCR5^{+} γδT cells were elevated in ACKR2$^{-/-}$ mice and correlated with the elevation of pulmonary levels of CCL5, CCL12, and IFNγ. γδT cells can exhibit polarized phenotypes, which reflects on the pattern of chemokine receptor expression (17). Th1 γδT cells express CXCR3^{+} and CCR5^{+}, whereas CCR4 is expressed by Th2 γδT cells (17). In addition, Th1 CCR2^{+} γδT cells are potent IFN-γ producers and displayed cytotoxic functions in tumor beds (21). In keeping with this, there was an increase in Th1 IFNγ-producing CCR2^{+} and CCR5^{+} γδT cells in ACKR2$^{-/-}$ mice challenged with bleomycin. In response to bleomycin, there was also an impaired recruitment of IFNγ^{+} γδT cells in CCR2$^{-/-}$ and CCR5$^{-/-}$ but not CCR4$^{-/-}$ mice. Interestingly, CCR2$^{-/-}$ mice showed reduced CCR5^{+} IFNγ^{+} γδT cells and CCR5$^{-/-}$ mice exhibited reduced CCR2^{+} IFNγ^{+} γδT cells. Altogether, these data indicate that in absence of ACKR2 increased levels of CCL12 and CCL5 observed after bleomycin challenge may be responsible for the recruitment of CCR2^{+} and CCR5^{+} IFNγ^{+} γδT cells which protect the mice.

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

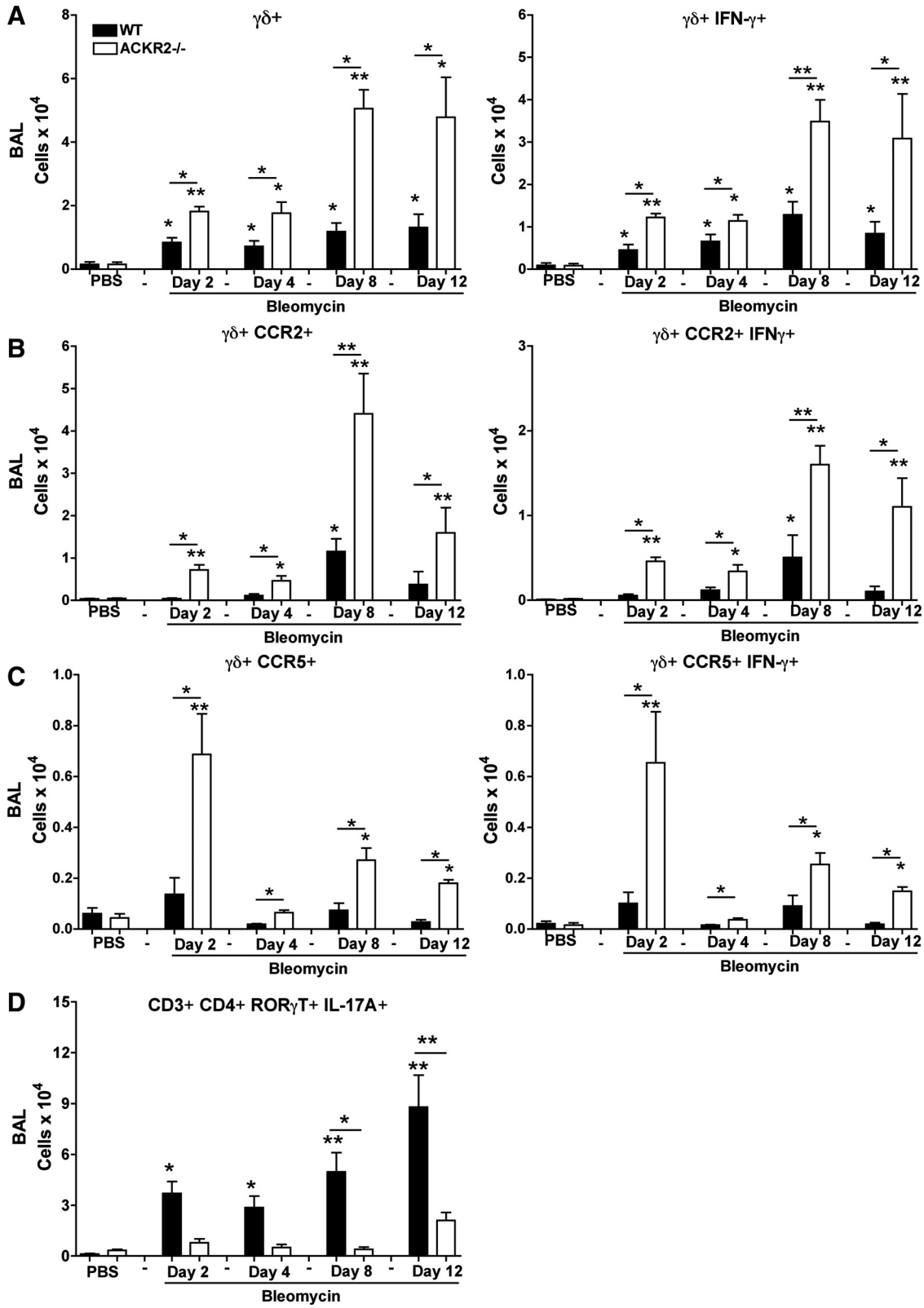

Fig. 9. Increased recruitment of $CCR2^+$ and $CCR5^+$ IFN-γ-producing γδT cells is related to reduced Th17 response after bleomycin instillation in the absence of ACKR2. Mice received a single instillation with PBS or bleomycin (3.75 mg/kg of body wt) and 2, 4, 8, and 12 days after, leukocytes recovered 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 $CD3^+\gamma\delta TCR^+$ lymphocytes and $CD3^+\gamma\delta TCR^+IFN\text{-}\gamma^+$. BAL, bronchoalveolar lavage. *D*: Th17 lymphocyte population was analyzed by FACS using ($CD3^+CD4^+ROR\gamma T^+IL\text{-}17A^+$). 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.

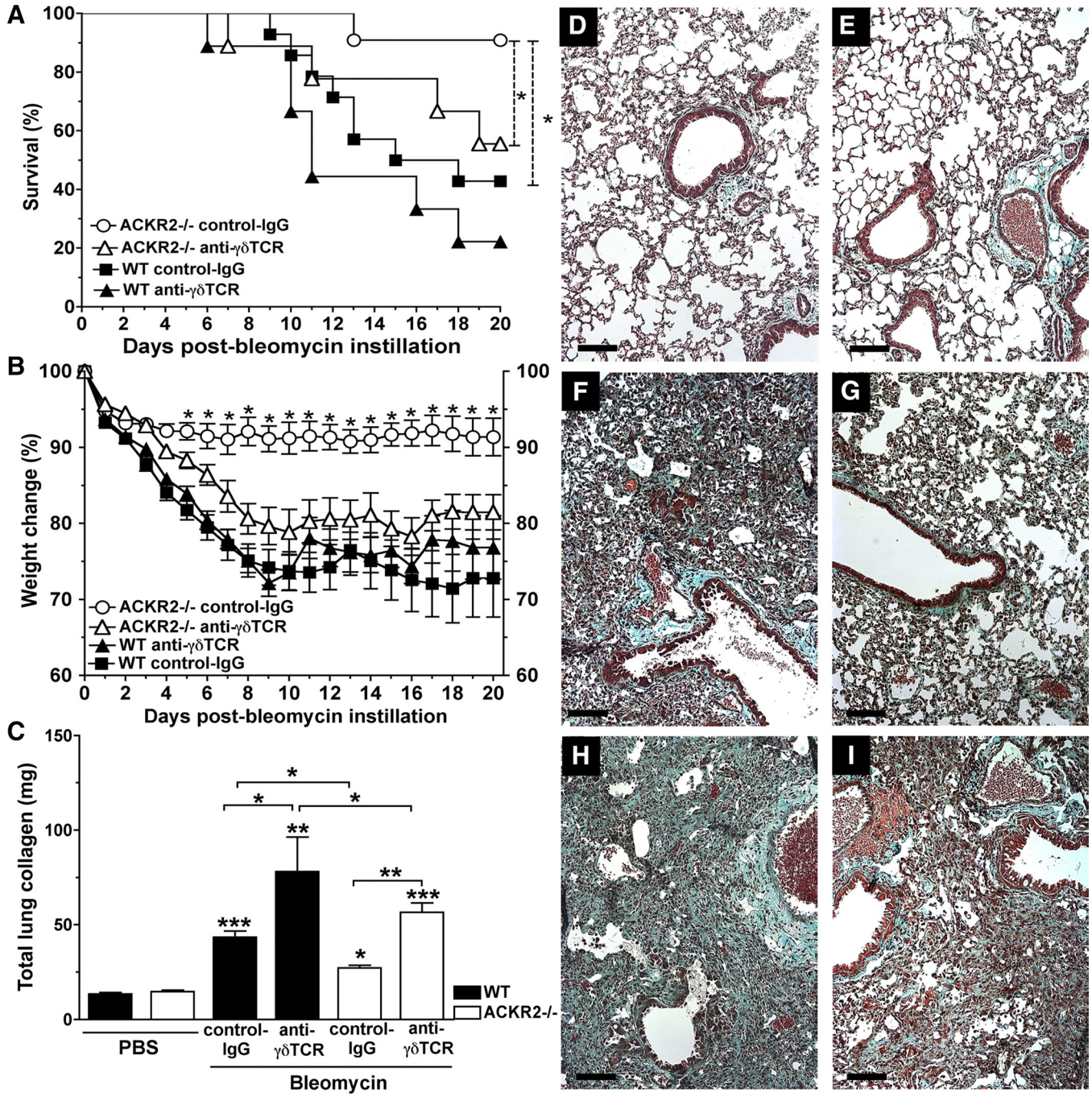

Fig. 10. γδT-lymphocyte depletion enhances lethality and pulmonary fibrosis induced by bleomycin and reverts the protection under ACKR2 deficiency. *A–H*: mice received a single instillation with PBS or bleomycin (3.75 mg/kg body wt) and mouse phenotype was studied during 20 days. Wild-type (WT) and 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.

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 γδT 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^{+} γδT cells suppressing Th17 activity in bleomycin model. In fact, we found an increased number of CCR2^{+} IFNγ^{+} γδT- and CCR5^{+} IFNγ^{+} γδT-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 γδT 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 γδT-cell-depleted mice, as found in γδ$^{-/-}$ mice (43). Finally, the presence of IFNγ-producers γδT 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^{+} γδT cells, which produce IFNγ and influence subsequent development of Th17 response and intensity of fibrosis.

GRANTS

This investigation received financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil) SWE Program under CNPq Grant Agreement No. 201489/2007-4, Edital Universal 14/2011 under CNPq Grant Agreement No. 476071/2011-9, Edital Universal 01/2016 under CNPq Grant Agreement No. 422260/2016-9, and a collaborative grant from CNPq and Italian Foreign Affair Ministry (General Direction for Cultural Cooperation and Promotion, Italy); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/Brazil); Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG/Brazil); Pró-Reitoria de Pesquisa da UFMG/EDITAL PRPq–02/2018; European Union Sixth Framework Program (FP6-2005/2010, INNOCHEM consortium–Innovative Chemokine-based Therapeutic Strategies for Autoimmunity and Chronic Inflammation) under Grant Agreement No. LSHBCT2005518167); and European Union Seventh Framework Program (FP7-2007/2013, TIMER consortium) under Grant Agreement No. HEALTH-F4-2011-281608.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

R.C.R., A.M., M.L., and M.M.T. conceived and designed research; R.C.R., B.S., M.M., C.B., G.G., A.A., L.Z., and F.P. performed experiments; R.C.R., B.S., M.M., C.B., G.G., A.A., L.Z., and F.P. analyzed data; R.C.R., B.S., M.M., C.B., G.G., A.A., L.Z., F.P., A.M., M.L., and M.M.T. interpreted results of experiments; R.C.R. prepared figures; R.C.R., A.M., M.L., and M.M.T. drafted manuscript; R.C.R., A.M., M.L., and M.M.T. edited and revised manuscript; R.C.R., B.S., M.M., C.B., G.G., A.A., L.Z., F.P., A.M., M.L., and M.M.T. approved final version of manuscript.

REFERENCES

1. **Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, Horuk R, Sparre-Ulrich AH, Locati M, Luster AD, Mantovani A, Matsushima K, Murphy PM, Nibbs R, Nomiyama H, Power CA, Proudfoot AE, Rosenkilde MM, Rot A, Sozzani S, Thelen M, Yoshie O, Zlotnik A.** International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol Rev* 66: 1–79, 2013. doi:10.1124/pr.113.007724.
2. **Bazzan E, Saetta M, Turato G, Borroni EM, Cancellieri C, Baraldo S, Savino B, Calabrese F, Ballarin A, Balestro E, Mantovani A, Cosio MG, Bonecchi R, Locati M.** Expression of the atypical chemokine receptor D6 in human alveolar macrophages in COPD. *Chest* 143: 98–106, 2013. doi:10.1378/chest.11-3220.
3. **Bordon Y, Hansell CA, Sester DP, Clarke M, Mowat AM, Nibbs RJ.** The atypical chemokine receptor D6 contributes to the development of experimental colitis. *J Immunol* 182: 5032–5040, 2009. doi:10.4049/jimmunol.0802802.
4. **Braun RK, Ferrick C, Neubauer P, Sjoding M, Sterner-Kock A, Kock M, Putney L, Ferrick DA, Hyde DM, Love RB.** IL-17 producing gammadelta T cells are required for a controlled inflammatory response after bleomycin-induced lung injury. *Inflammation* 31: 167–179, 2008. doi:10.1007/s10753-008-9062-6.
5. **Castanheira FV, Borges V, Sônego F, Kanashiro A, Donate PB, Melo PH, Pallas K, Russo RC, Amaral FA, Teixeira MM, Ramalho FS, Cunha TM, Liew FY, Alves-Filho JC, Graham GJ, Cunha FQ.** The atypical chemokine receptor ACKR2 is protective against sepsis. *Shock* 2017 Aug 24. doi:10.1097/SHK.0000000000000969.
6. **Chien YH, Meyer C, Bonneville M.** γδ T cells: first line of defense and beyond. *Annu Rev Immunol* 32: 121–155, 2014. doi:10.1146/annurev-immunol-032713-120216.
7. **Di Liberto D, Locati M, Caccamo N, Vecchi A, Meraviglia S, Salerno A, Sireci G, Nebuloni M, Caceres N, Cardona PJ, Dieli F, Mantovani A.** Role of the chemokine decoy receptor D6 in balancing inflammation, immune activation, and antimicrobial resistance in Mycobacterium tuberculosis infection. *J Exp Med* 205: 2075–2084, 2008. doi:10.1084/jem.20070608.
8. **du Bois RM.** Strategies for treating idiopathic pulmonary fibrosis. *Nat Rev Drug Discov* 9: 129–140, 2010. doi:10.1038/nrd2958.
9. **Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A.** IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. *Nat Med* 12: 99–106, 2006. doi:10.1038/nm1332.
10. **Fra AM, Locati M, Otero K, Sironi M, Signorelli P, Massardi ML, Gobbi M, Vecchi A, Sozzani S, Mantovani A.** Cutting edge: scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6. *J Immunol* 170: 2279–2282, 2003. doi:10.4049/jimmunol.170.5.2279.
11. **Gharaee-Kermani M, McCullumsmith RE, Charo IF, Kunkel SL, Phan SH.** CC-chemokine receptor 2 required for bleomycin-induced pulmonary fibrosis. *Cytokine* 24: 266–276, 2003. doi:10.1016/j.cyto.2003.08.003.
12. **Henriques A, Silva C, Santiago M, Henriques MJ, Martinho A, Trindade H, da Silva JA, Silva-Santos B, Paiva A.** Subset-specific alterations in frequencies and functional signatures of γδ T cells in systemic sclerosis patients. *Inflamm Res* 65: 985–994, 2016. doi:10.1007/s00011-016-0982-6.
13. **Huaux F, Gharaee-Kermani M, Liu T, Morel V, McGarry B, Ullenbruch M, Kunkel SL, Wang J, Xing Z, Phan SH.** Role of eotaxin-1 (CCL11) and CC chemokine receptor 3 (CCR3) in bleomycin-induced lung injury and fibrosis. *Am J Pathol* 167: 1485–1496, 2005. doi:10.1016/S0002-9440(10)61235-7.
14. **Ishida Y, Kimura A, Kondo T, Hayashi T, Ueno M, Takakura N, Matsushima K, Mukaida N.** Essential roles of the CC chemokine ligand 3-CC chemokine receptor 5 axis in bleomycin-induced pulmonary fibrosis through regulation of macrophage and fibrocyte infiltration. *Am J Pathol* 170: 843–854, 2007. doi:10.2353/ajpath.2007.051213.
15. **Jamieson T, Cook DN, Nibbs RJ, Rot A, Nixon C, McLean P, Alcami A, Lira SA, Wiekowski M, Graham GJ.** The chemokine receptor D6 limits the inflammatory response in vivo. *Nat Immunol* 6: 403–411, 2005. doi:10.1038/ni1182.
16. **Jones HA, Schofield JB, Krausz T, Boobis AR, Haslett C.** Pulmonary fibrosis correlates with duration of tissue neutrophil activation. *Am J Respir Crit Care Med* 158: 620–628, 1998. doi:10.1164/ajrccm.158.2.9711075.

17. **Kabelitz D, Wesch D.** Features and functions of gamma delta T lymphocytes: focus on chemokines and their receptors. *Crit Rev Immunol* 23: 339–370, 2003. doi:10.1615/CritRevImmunol.v23.i56.10.
18. **Keane MP, Belperio JA, Moore TA, Moore BB, Arenberg DA, Smith RE, Burdick MD, Kunkel SL, Strieter RM.** Neutralization of the CXC chemokine, macrophage inflammatory protein-2, attenuates bleomycin-induced pulmonary fibrosis. *J Immunol* 162: 5511–5518, 1999.
19. **King DP, Hyde DM, Jackson KA, Novosad DM, Ellis TN, Putney L, Stovall MY, Van Winkle LS, Beaman BL, Ferrick DA.** Cutting edge: protective response to pulmonary injury requires gamma delta T lymphocytes. *J Immunol* 162: 5033–5036, 1999.
20. **Lahn M, Kanehiro A, Takeda K, Joetham A, Schwarze J, Köhler G, O'Brien R, Gelfand EW, Born W.** Negative regulation of airway responsiveness that is dependent on gammadelta T cells and independent of alphabeta T cells. *Nat Med* 5: 1150–1156, 1999. doi:10.1038/13476.
21. **Lança T, Costa MF, Gonçalves-Sousa N, Rei M, Grosso AR, Penido C, Silva-Santos B.** Protective role of the inflammatory CCR2/CCL2 chemokine pathway through recruitment of type 1 cytotoxic γδ T lymphocytes to tumor beds. *J Immunol* 190: 6673–6680, 2013. doi:10.4049/jimmunol.1300434.
22. **Leask A, Abraham DJ.** TGF-beta signaling and the fibrotic response. *FASEB J* 18: 816–827, 2004. doi:10.1096/fj.03-1273rev.
23. **Lima IL, Silva JM, Rodrigues LF, Madureira DF, Fonseca AC, Garlet GP, Teixeira MM, Russo RC, Fukada SY, Silva TA.** Contribution of atypical chemokine receptor 2/ackr2 in bone remodeling. *Bone* 101: 113–122, 2017. doi:10.1016/j.bone.2017.05.003.
24. **Liu L, Graham GJ, Damodaran A, Hu T, Lira SA, Sasse M, Canasto-Chibuque C, Cook DN, Ransohoff RM.** Cutting edge: the silent chemokine receptor D6 is required for generating T cell responses that mediate experimental autoimmune encephalomyelitis. *J Immunol* 177: 17–21, 2006. doi:10.4049/jimmunol.177.1.17.
25. **Locati M, Otero K, Schioppa T, Signorelli P, Perrier P, Baviera S, Sozzani S, Mantovani A.** The chemokine system: tuning and shaping by regulation of receptor expression and coupling in polarized responses. *Allergy* 57: 972–982, 2002. doi:10.1034/j.1398-9995.2002.02166.x.
26. **Mantovani A, Bonecchi R, Locati M.** Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nat Rev Immunol* 6: 907–918, 2006. doi:10.1038/nri1964.
27. **Martinez de la Torre Y, Buracchi C, Borroni EM, Dupor J, Bonecchi R, Nebuloni M, Pasqualini F, Doni A, Lauri E, Agostinis C, Bulla R, Cook DN, Haribabu B, Meroni P, Rukavina D, Vago L, Tedesco F, Vecchi A, Lira SA, Locati M, Mantovani A.** Protection against inflammation- and autoantibody-caused fetal loss by the chemokine decoy receptor D6. *Proc Natl Acad Sci USA* 104: 2319–2324, 2007. doi:10.1073/pnas.0607514104.
28. **McKimmie CS, Fraser AR, Hansell C, Gutiérrez L, Philipsen S, Connell L, Rot A, Kurowska-Stolarska M, Carreno P, Pruenster M, Chu CC, Lombardi G, Halsey C, McInnes IB, Liew FY, Nibbs RJ, Graham GJ.** Hemopoietic cell expression of the chemokine decoy receptor D6 is dynamic and regulated by GATA1. *J Immunol* 181: 3353–3363, 2008. doi:10.4049/jimmunol.181.5.3353.
29. **Mi S, Li Z, Yang HZ, Liu H, Wang JP, Ma YG, Wang XX, Liu HZ, Sun W, Hu ZW.** Blocking IL-17A promotes the resolution of pulmonary inflammation and fibrosis via TGF-beta1-dependent and -independent mechanisms. *J Immunol* 187: 3003–3014, 2011. doi:10.4049/jimmunol.1004081.
30. **Moore BB, Paine R 3rd, Christensen PJ, Moore TA, Sitterding S, Ngan R, Wilke CA, Kuziel WA, Toews GB.** Protection from pulmonary fibrosis in the absence of CCR2 signaling. *J Immunol* 167: 4368–4377, 2001. doi:10.4049/jimmunol.167.8.4368.
31. **Mori M, Andersson CK, Graham GJ, Löfdahl CG, Erjefält JS.** Increased number and altered phenotype of lymphatic vessels in peripheral lung compartments of patients with COPD. *Respir Res* 14: 65, 2013. doi:10.1186/1465-9921-14-65.
32. **Murdoch JR, Lloyd CM.** Resolution of allergic airway inflammation and airway hyperreactivity is mediated by IL-17-producing gammadeltaT cells. *Am J Respir Crit Care Med* 182: 464–476, 2010. doi:10.1164/rccm.200911-1775OC.
33. **Nibbs RJ, Gilchrist DS, King V, Ferra A, Forrow S, Hunter KD, Graham GJ.** The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. *J Clin Invest* 117: 1884–1892, 2007. doi:10.1172/JCI30068.
34. **Nibbs RJ, Kriehuber E, Ponath PD, Parent D, Qin S, Campbell JD, Henderson A, Kerjaschki D, Maurer D, Graham GJ, Rot A.** The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *Am J Pathol* 158: 867–877, 2001. doi:10.1016/S0002-9440(10)64035-7.
35. **Nibbs RJ, Wylie SM, Pragnell IB, Graham GJ.** Cloning and characterization of a novel murine beta chemokine receptor, D6. Comparison to three other related macrophage inflammatory protein-1alpha receptors, CCR-1, CCR-3, and CCR-5. *J Biol Chem* 272: 12495–12504, 1997. doi:10.1074/jbc.272.19.12495.
36. **Nogueira DS, Gazzinelli-Guimarães PH, Barbosa FS, Resende NM, Silva CC, de Oliveira LM, Amorim CC, Oliveira FM, Mattos MS, Kraemer LR, Caliari MV, Gaze S, Bueno LL, Russo RC, Fujiwara RT.** Multiple exposures to ascaris suum induce tissue injury and mixed th2/th17 immune response in mice. *PLoS Negl Trop Dis* 10: e0004382, 2016. doi:10.1371/journal.pntd.0004382.
37. **Pociask DA, Chen K, Choi SM, Oury TD, Steele C, Kolls JK.** γδ T cells attenuate bleomycin-induced fibrosis through the production of CXCL10. *Am J Pathol* 178: 1167–1176, 2011. doi:10.1016/j.ajpath.2010.11.055.
38. **Russo RC, Alessandri AL, Garcia CC, Cordeiro BF, Pinho V, Cassali GD, Proudfoot AE, Teixeira MM.** Therapeutic effects of evasin-1, a chemokine binding protein, in bleomycin-induced pulmonary fibrosis. *Am J Respir Cell Mol Biol* 45: 72–80, 2011. doi:10.1165/rcmb.2009-0406OC.
39. **Russo RC, Garcia CC, Barcelos LS, Rachid MA, Guabiraba R, Roffê E, Souza AL, Sousa LP, Mirolo M, Doni A, Cassali GD, Pinho V, Locati M, Teixeira MM.** Phosphoinositide 3-kinase γ plays a critical role in bleomycin-induced pulmonary inflammation and fibrosis in mice. *J Leukoc Biol* 89: 269–282, 2011. doi:10.1189/jlb.0610346.
40. **Russo RC, Garcia CC, Teixeira MM.** Anti-inflammatory drug development: broad or specific chemokine receptor antagonists? *Curr Opin Drug Discov Devel* 13: 414–427, 2010.
41. **Russo RC, Guabiraba R, Garcia CC, Barcelos LS, Roffê E, Souza AL, Amaral FA, Cisalpino D, Cassali GD, Doni A, Bertini R, Teixeira MM.** Role of the chemokine receptor CXCR2 in bleomycin-induced pulmonary inflammation and fibrosis. *Am J Respir Cell Mol Biol* 40: 410–421, 2009. doi:10.1165/rcmb.2007-0364OC.
42. **Saito F, Tasaka S, Inoue K, Miyamoto K, Nakano Y, Ogawa Y, Yamada W, Shiraishi Y, Hasegawa N, Fujishima S, Takano H, Ishizaka A.** Role of interleukin-6 in bleomycin-induced lung inflammatory changes in mice. *Am J Respir Cell Mol Biol* 38: 566–571, 2008. doi:10.1165/rcmb.2007-0299OC.
43. **Segawa S, Goto D, Iizuka A, Kaneko S, Yokosawa M, Kondo Y, Matsumoto I, Sumida T.** The regulatory role of interferon-γ producing gamma delta T cells via the suppression of T helper 17 cell activity in bleomycin-induced pulmonary fibrosis. *Clin Exp Immunol* 185: 348–360, 2016. doi:10.1111/cei.12802.
44. **Selman M, Pardo A.** Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers. *Proc Am Thorac Soc* 3: 364–372, 2006. doi:10.1513/pats.200601-003TK.
45. **Selman M, Pardo A, Barrera L, Estrada A, Watson SR, Wilson K, Aziz N, Kaminski N, Zlotnik A.** Gene expression profiles distinguish idiopathic pulmonary fibrosis from hypersensitivity pneumonitis. *Am J Respir Crit Care Med* 173: 188–198, 2006. doi:10.1164/rccm.200504-644OC.
46. **Simonian PL, Wehrmann F, Roark CL, Born WK, O'Brien RL, Fontenot AP.** γδ T cells protect against lung fibrosis via IL-22. *J Exp Med* 207: 2239–2253, 2010. doi:10.1084/jem.20100061.
47. **Smith RE.** Chemotactic cytokines mediate leukocyte recruitment in fibrotic lung disease. *Biol Signals* 5: 223–231, 1996. doi:10.1159/000109194.
48. **Smith RE, Strieter RM, Phan SH, Kunkel SL.** C-C chemokines: novel mediators of the profibrotic inflammatory response to bleomycin challenge. *Am J Respir Cell Mol Biol* 15: 693–702, 1996. doi:10.1165/ajrcmb.15.6.8969262.
49. **Smith RE, Strieter RM, Phan SH, Lukacs NW, Huffnagle GB, Wilke CA, Burdick MD, Lincoln P, Evanoff H, Kunkel SL.** Production and function of murine macrophage inflammatory protein-1 alpha in bleomycin-induced lung injury. *J Immunol* 153: 4704–4712, 1994.
50. **Swiderski RE, Dencoff JE, Floerchinger CS, Shapiro SD, Hunninghake GW.** Differential expression of extracellular matrix remodeling genes in a murine model of bleomycin-induced pulmonary fibrosis. *Am J Pathol* 152: 821–828, 1998.
51. **Takamizawa A, Koyama S, Sato E, Masubuchi T, Kubo K, Sekiguchi M, Nagai S, Izumi T.** Bleomycin stimulates lung fibroblasts to release

neutrophil and monocyte chemotactic activity. *J Immunol* 162: 6200–6208, 1999.
52. **Tavares LP, Garcia CC, Vago JP, Queiroz-Junior CM, Galvão I, David BA, Rachid MA, Silva PM, Russo RC, Teixeira MM, Sousa LP.** Inhibition of Phosphodiesterase-4 during Pneumococcal Pneumonia Reduces Inflammation and Lung Injury in Mice. *Am J Respir Cell Mol Biol* 55: 24–34, 2016. doi:10.1165/rcmb.2015-0083OC.
53. **Vetrano S, Borroni EM, Sarukhan A, Savino B, Bonecchi R, Correale C, Arena V, Fantini M, Roncalli M, Malesci A, Mantovani A, Locati M, Danese S.** The lymphatic system controls intestinal inflammation and inflammation-associated colon cancer through the chemokine decoy receptor D6. *Gut* 59: 197–206, 2010. doi:10.1136/gut.2009.183772.
54. **Viola A, Luster AD.** Chemokines and their receptors: drug targets in immunity and inflammation. *Annu Rev Pharmacol Toxicol* 48: 171–197, 2008. doi:10.1146/annurev.pharmtox.48.121806.154841.
55. **Wands JM, Roark CL, Aydintug MK, Jin N, Hahn YS, Cook L, Yin X, Dal Porto J, Lahn M, Hyde DM, Gelfand EW, Mason RJ, O'Brien RL, Born WK.** Distribution and leukocyte contacts of gammadelta T cells in the lung. *J Leukoc Biol* 78: 1086–1096, 2005. doi:10.1189/jlb.0505244.
56. **Whitehead GS, Wang T, DeGraff LM, Card JW, Lira SA, Graham GJ, Cook DN.** The chemokine receptor D6 has opposing effects on allergic inflammation and airway reactivity. *Am J Respir Crit Care Med* 175: 243–249, 2007. doi:10.1164/rccm.200606-839OC.
57. **Wilson MS, Madala SK, Ramalingam TR, Gochuico BR, Rosas IO, Cheever AW, Wynn TA.** Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A dependent. *J Exp Med* 207: 535–552, 2010. doi:10.1084/jem.20092121.
58. **Wilson MS, Wynn TA.** Pulmonary fibrosis: pathogenesis, etiology and regulation. *Mucosal Immunol* 2: 103–121, 2009. doi:10.1038/mi.2008.85.
59. **Wu Z, Yang L, Cai L, Zhang M, Cheng X, Yang X, Xu J.** Detection of epithelial to mesenchymal transition in airways of a bleomycin induced pulmonary fibrosis model derived from an alpha-smooth muscle actin-Cre transgenic mouse. *Respir Res* 8: 1, 2007. doi:10.1186/1465-9921-8-1.
60. **Zemans RL, Colgan SP, Downey GP.** Transepithelial migration of neutrophils: mechanisms and implications for acute lung injury. *Am J Respir Cell Mol Biol* 40: 519–535, 2009. doi:10.1165/rcmb.2008-0348TR.
61. **Zhang K, Gharaee-Kermani M, Jones ML, Warren JS, Phan SH.** Lung monocyte chemoattractant protein-1 gene expression in bleomycin-induced pulmonary fibrosis. *J Immunol* 153: 4733–4741, 1994.