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Plexin C1 deficiency permits synaptotagmin 7–mediated macrophage migration and enhances mammalian lung fibrosis

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ABSTRACT: Pulmonary fibrosis is a progressive and often fatal condition that is believed to be partially orchestrated by macrophages. Mechanisms that control migration of these cells into and within the lung remain undefined. We evaluated the contributions of the semaphorin receptor, plexin C1 (PLXNC1), and the exocytic calcium sensor, synaptotagmin 7 (Syt7), in these processes. We evaluated the role of PLXNC1 in macrophage migration by using Boyden chambers and scratch tests, characterized its contribution to experimentally induced lung fibrosis in mice, and defined the mechanism for our observations. Our findings reveal that relative to control participants, patients with idiopathic pulmonary fibrosis demonstrate excessive monocyte migration and underexpression of PLXNC1 in the lungs and circulation, a finding that is recapitulated in the setting of scleroderma-related interstitial lung disease. Relative to wild type, PLXNC1$^{-/-}$ mouse macrophages are excessively migratory, and PLXNC1$^{-/-}$ mice show exacerbated collagen accumulation in response to either inhaled bleomycin or inducible lung targeted TGF-β1 overexpression. These findings are ameliorated by replacement of PLXNC1 on bone marrow–derived cells or by genetic deletion of Syt7. These data demonstrate the previously unrecognized observation that PLXNC1 deficiency permits Syt7-mediated macrophage migration and enhances mammalian lung fibrosis.—Peng, X., Moore, M., Mathur, A., Zhou, Y., Sun, H., Gan, Y., Herazo-Maya, J. D., Kaminski, N., Hu, X., Pan, H., Ryu, C., Osafo-Addo, A., Homer, R. J., Feghali-Bostwick, C., Fares, W. H., Gulati, M., Hu, B., Lee, C.-G., Elias, J. A., Herzog, E. L. Plexin C1 deficiency permits synaptotagmin 7–mediated macrophage migration and enhances mammalian lung fibrosis. FASEB J. 30, 4056–4070 (2016). www.fasebj.org

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Pulmonary fibrosis is an incurable process that involves the progressive accumulation of scar tissue in the adult human lung (1). It results from a variety of occupational (2, 3) or therapeutic (4, 5) exposures and is also a feature of diverse autoimmune, inflammatory, and genetic diseases, including scleroderma (6), sarcoidosis (7), and cystic fibrosis (8). Idiopathic pulmonary fibrosis (IPF) is a particularly severe form of this condition (1) that typically leads to death within ~3 yr of diagnosis. Whereas in some situations treatment with antifibrotic agents can attenuate the decline in ventilatory function caused by IPF, benefits are limited, heterogeneous, and accompanied by significant toxicities (9, 10). Thus, further exploration of the mechanisms that drive fibrotic responses remain an important area of investigation in the field of lung biology.

The types of pulmonary fibrosis correspond to the distinction between innate and acquired immunity. Some forms of fibrosis, such as those associated with autoimmune diseases like scleroderma-related interstitial lung disease (SSc-ILD), are at least partially responsive to conventional immunosuppression (11). Other forms, such as IPF, have no obvious primary acquired immune response and respond poorly to immunosuppression (12). However,

ABBREVIATIONS: BAL, bronchoalveolar lavage; BMDC, bone marrow–derived cell; LGRC, Lung Genomics Research Consortium; PBMC, peripheral blood mononuclear cell; PLXNC1, plexin C1; rmSema 7a, recombinant murine semaphorin 7a; Sema 7a, semaphorin 7a; SSc-ILD, scleroderma-related interstitial lung disease; Syt7, synaptotagmin 7; Tg, transgene

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both forms may be dependent on innate immune cells, such as monocytes and macrophages, to orchestrate or amplify fibrosis. Studies performed in several models of experimentally induced fibrosis support the widespread therapeutic potential of interventions that are aimed at modulating macrophage-driven responses (13–15). In most adult tissues, macrophages are believed to derive from circulating monocytic precursors. Therefore, the finding that monocytes obtained from the blood of patients with both IPF and SSc-ILD display scavenger receptor expression and fibroblast-stimulating properties suggests that they may be partially programmed to promote fibrosis before entering the lung. Consequently, therapies that target the trafficking and migration of monocytes and macrophages into or within the lung might be of benefit. Migratory abilities of effector cells, such as fibroblasts, have been extensively characterized in both primary biospecimens from fibrosing human lung conditions (16, 17) and in animal models. To date, however, there exists little information regarding immune cell migration, particularly monocytes and macrophages, in these contexts.

Neuronally active molecules have emerged as a novel area of research in the pathogenesis of fibrosis and remodeling. Among the most extensively studied is the GPI-anchored membrane protein, semaphorin 7a (Sema 7a). Sema 7a stimulates epithelial apoptosis, monocyte and macrophage activation and accumulation (18, 19), lymphocyte activation (20, 21), and TGF-β1 production and activation (18, 22) in an array of fibrosing and inflammatory conditions. Whereas the contribution of Sema 7a and its receptor β1 integrin has been well characterized in fibrotic and remodeling responses that affect the lung and other organs, the contribution of Sema 7a's cognate receptor plexin C1 (PLXNC1) is less described. PLXNC1 is a large transmembrane protein that contains extracellular Sema and PSI (plexin-semaphorin-integrin) domains as well as an intracellular GTPase-activating protein domain (23–25) that functions predominantly as a receptor for Sema 7a (23–25). In the few modeling systems in which it has been studied, engagement of PLXNC1 inhibits Sema 7a–driven processes *via* stabilizing effects on the cytoskeleton (26). As the Sema 7a–PLXNC1 axis has been shown to oppose the actions of β1 integrin interaction by inhibiting migration or generating repulsive guidance cues in multiple cell types, PLXNC1 has been deemed an inhibitory receptor for Sema 7a (23–25, 26). Of interest, whereas a role for PLXNC1 expression by neutrophils in the development of acute lung injury has been recently shown (27), this gene has been only minimally explored in diseases that are characterized by pathologic tissue remodeling, such as pulmonary fibrosis. In addition, contributions of other neuronally active proteins, such as the calcium binding proteins that control neurotransmitter release, have yet to be investigated in these clinical contexts. For example, the exocytic calcium sensor, synaptotagmin 7 (Syt7), is involved in multiple processes that are potentially related to pulmonary fibrosis—including cell migration, phagocytosis, and surfactant recycling—and has been shown to mediate fibrosis of other tissues. However, a role for this protein in the pathogenesis of pulmonary fibrosis has not been explored and a relationship to PLXNC1 has not been defined.

On the basis of the studies noted above, we formulated the hypothesis that PLXNC1 and Syt7 are involved in the regulation of both monocyte–macrophage migration and associated fibrosing processes in the mammalian lung. We characterized expression of these proteins in 2 independent models of experimentally induced pulmonary fibrosis and defined their role in injury and pathologic remodeling. We found that underexpression or absence of PLXNC1 exacerbates macrophage migration and experimentally induced lung fibrosis *via* a previously unrecognized mechanism that involves Syt7. PLXNC1 is underexpressed and Syt7 is overexpressed in the lungs and blood of patients with 2 different forms of pulmonary fibrosis, which indicates that this antagonistic relationship may be a critical regulatory pathway in fibrotic human lung disease.

MATERIALS AND METHODS

Human participants

Studies were carried out with explicit informed consent on protocols that were approved by the Human Investigation Committee at the Yale University School of Medicine. For studies of peripheral blood, participants age ≥21 yr with IPF or SSc-ILD who were followed at the Yale Interstitial Lung Disease Center of Excellence and the Yale Scleroderma Research Center were eligible to enroll (19, 21, 28). Healthy, age-matched controls were recruited from Yale's Program on Aging and from the greater New Haven community (19, 21, 28). Exclusion criteria included current or recent use of immunosuppression; known chronic infection; active cardiovascular, renal, or neoplastic disease; and/or inability to provide informed consent. For microarray studies of IPF lung tissue, deidentified tissues collected by the Lung Genomics Research Consortium (LGRC) were used (29). For studies of SSc-ILD lung tissue, deidentified tissues obtained from the U.S. National Institutes of Health, National Institute of Arthritis and Musculoskeletal and Skin Diseases National Scleroderma Core Center (SSCORE) facility were used. For evaluation of bronchoalveolar lavage (BAL) macrophages, excess BAL fluid—which would otherwise have been discarded—that was obtained from clinically indicated diagnostic procedures was used with patient consent. Deidentified cells and tissues were considered nonhuman participants by the Yale University Institutional Review Board.

Isolation of peripheral blood mononuclear cells and CD14$^+$ monocytes

After informed consent and enrollment, 40 ml heparinized peripheral blood was drawn from study participants, anonymized, and transported to the laboratory. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by using Ficoll paque–based separation (Stem Cell Technologies, Vancouver, BC, Canada) and either stored for future studies or subjected to negative selection of CD14$^+$ monocytes (Miltenyi, San Diego, CA, USA) as previously described (28).

Western blot

Western blot detection of PLXNC1 was performed as previously described (18).

Boyden chamber studies

Isolated human monocytes were placed into modified Boyden chambers (30) in the presence or absence of recombinant human Sema 7a (Abnova, Taipei City, Taiwan). After 18 h, inserts were

harvested and stained with hematoxylin and eosin, and migration was quantified as cells per high-powered field. At least 4 high-powered fields were evaluated per condition.

mRNA analysis

Total cellular RNA from human PBMCs or lung tissue was obtained by using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer instructions. Primer sequences for PLXNC1 (5′–AGAAAACTGGCTGGATATTTCGT–3′/5′–GCTTCCCACCATAGTCACTGTA–3′), Syt7 (5′–ATGGCGTACATCCAGTTGGAA–3′/5′–GGACTCGTAGCACTTCTGACA–3′), and β-actin (5′–GTGGGCCGCTCTAGGCACCA–3′/5′–CGGTTGGCCTTAGGGTTCAGG–3′) were used. Relative expression of genes was performed using the $2\Delta C_t$ method as previously described (31).

Image stream analysis of human cells

PBMCs were stained for flow cytometric detection of CD14 and PLXNC1. Combined flow cytometry and confocal microscopy was performed by using Amnis Image Stream Machine (EMD Millipore, Billerica, MA, USA) in the Yale Rheumatic Disease Research Core Facility. Data were analyzed by using the Ideas software package from EMD Millipore.

Microarray

To evaluate differences in expression levels between candidate genes, we used the Mann-Whitney *U* test for independent samples of the normalized, $\log_2$-transformed microarray expression values. Statistical significance was defined as $P < 0.05$.

Human macrophage isolation

Macrophage isolation from BAL specimens from participants without parenchymal lung disease was performed by using adherence separation as previously described (32).

Animal studies

The PLXNC1$^{-/-}$, TGF-β1 transgene-positive (Tg^{+}), and Syt$^{-/-}$ null mice used in these studies have been previously described (18, 33, 34). Mice were backcrossed onto the C57BL/6 background for >10 generations and wild-type littermates were used as controls. Except where indicated, at least 5 mice were included in each experimental group and end point analysis was performed on mice that survived to the selected time point. Animal experiments were approved by the Yale School of Medicine Institutional Animal Care and Use Committee in accordance with federal guidelines.

Scratch tests

Murine peritoneal macrophages were obtained as previously described (18) and allowed to adhere to the bottom of 12-well tissue culture plates for 2 h. Medium was changed to serum-free OptiMEM (Thermo Fisher Scientific) that was or was not supplemented with 20 nM recombinant murine Sema 7a (rmSema 7a; Abnova), and an artificial wound was induced by using a 200-μm pipette tip. Plates were photographed at baseline and after 72 h. Percentage of wound closure was calculated and experiments were repeated twice.

Bleomycin administration

Sex-matched, 8-wk-old wild-type PLXNC1$^{-/-}$, Syt7$^{-/-}$ or PLXNC1$^{-/-}$ x Syt7$^{-/-}$ mice were exposed to a single bleomycin inhalation (0.8 U/kg; Teva Parenteral Medicines, Irvine, CA, USA) or sterile PBS *via* orotracheal administration. Mice were humanely killed and evaluated at various time points between d 0 and 14 according to Zhou *et al*. (31).

Doxycycline administration

Sex-matched, 6- to 8-wk-old wild-type TGF-β1 Tg^{+} and Tg^{-} mice received 0.5 mg/ml doxycycline in their drinking water for up to 28 d as previously described (33). In selected studies, mice were treated with intraperitoneal injections of a specific PLXNC1 neutralizing antibody as previously described (35).

Tissue harvest

Animals were anesthetized, BAL was performed, median sternotomy was performed, and right heart perfusion was completed with calcium and magnesium-free PBS. Heart and lungs were then processed for the molecular and biochemical assays (31).

Flow cytometric analysis of PLXNC1-expressing cells

Mouse spleens and lungs were digested for flow cytometry. Viable cells were quantified by using trypan blue staining. Antibodies against CD14, F4/80, CD11c (all BD Pharmingen, Franklin Lakes, NJ, USA), PLXNC1 (R&D systems, Minneapolis, MN, USA), EpCAM (eBiosciences, San Diego, CA, USA), and ColIα1^{+} (EMD Millipore) were employed using methods developed and reported by our group (13, 31). Cell sorting was performed by using BD FACSCalibur (BD Pharmingen). Data were analyzed by using FlowJo software (version 7.5; Tree Star, Ashland, OR, USA). For all analyses, isotype control staining was subtracted from true antibody staining to determine the percentage of positive cells. Percentages of live cells that coexpressed PLXNC1 and CD14 (spleen) or PLXNC1 combined with F4/80 and CD11c (lung) were sequentially determined after bleomycin challenge or doxycycline initiation.

TUNEL analysis

End labeling of exposed 3′-OH ends of DNA fragments in paraffin-embedded tissue was undertaken with the TUNEL *in situ* cell death detection kit AP (Roche, Indianapolis, IN, USA) as previously described (31).

Quantification of lung collagen

The lower 3 lobes of the right lung were frozen in liquid nitrogen and stored at −80°C until they underwent quantification of total soluble collagen by using the Sircol Collagen Assay kit (Biocolor; Accurate Chemical and Scientific Corp., Westbury, NY, USA) according to manufacturer instructions (13).

Histologic analysis

Mouse lungs were removed *en bloc*, inflated to 25 cm pressure with PBS that contained 0.5% low melting point agarose gel, fixed, paraffin-embedded, sectioned, and stained. Staining with

hematoxylin and eosin and Mallory's trichrome were performed in the Research Histology Laboratory of the Department of Pathology at the Yale University School of Medicine (31). Modified Ashcroft Scores of mouse lungs were performed as previously described (36). Immunodetection of Syt7 (Abcam, Boston, MA, USA) was performed by using modifications of previously published protocols (31).

Bone marrow transplantation

Bone marrow chimeras were created as previously described (21).

Statistics

All data are presented as means ± SEM unless stated otherwise. Normally distributed data were compared by using Student's *t* test or ANOVA with Bonferroni *post hoc* test where necessary. Non-normally distributed data in 2 groups were compared by using the nonparametric 2-tailed Mann-Whitney *U* test. Prism 5.0 (GraphPad Software, La Jolla, CA, USA) Statistical Analytics Software (SAS Institute, Cary, NC, USA) was used for all of these analyses. A value of $P < 0.05$ after correction for multiple testing, where relevant, was considered to be significant.

RESULTS

Monocyte migration is enhanced in IPF

Monocytes and macrophages present in blood and lungs of patients with IPF display fibrosis-promoting properties. To determine whether these cells are also excessively motile, $CD14^+$ monocytes that were isolated by negative selection from the blood of controls and patients with IPF were subjected to migration assays in modified Boyden chambers. Here, as shown in **Fig. 1*A***, relative to control cells, IPF monocytes displayed 3.25-fold increased baseline migration ($P < 0.05$; Fig. 1*A*) that was further increased by 1.47-fold after stimulation with the chemoattractant, Sema 7a ($P < 0.01$; Fig. 1*A*). These data indicate that unstimulated IPF monocytes display an excessively motile phenotype that is exacerbated by exposure to Sema 7a.

PLXNC1 is underexpressed by IPF monocytes

Cellular migration is controlled by a number of factors, including rearrangement of the cytoskeleton. To determine whether the excessive migration of IPF monocytes described above resulted from reduced expression of PLXCN1, which regulates cytoskeletal dynamics, several experiments were performed. Because PLXNC1 is known to be expressed on circulating monocytes obtained from healthy human volunteers (37), we first evaluated whether differential expression of this gene was seen in the setting of pulmonary fibrosis. To this end, relative expression of PLXNC1 was evaluated by using quantitative real-time PCR on archived PBMCs that were obtained from a well-characterized IPF cohort recruited from the Yale Interstitial Lung Disease Center of Excellence. Participant characteristics are shown in **Table 1**. These studies revealed that relative to control participants ($n = 42$), IPF PBMCs ($n = 38$) showed a 28.9% reduction in relative expression of PLXNC1 ($P < 0.0077$; Fig. 1*B*). In determining the site of this reduced expression, we performed a separate set of studies by using magnetic bead sorting approaches followed by Western blot and found that, in normal participants, PLXNC1 was found to be exclusively expressed by $CD14^+$ monocytes (Fig. 1*C–E*). When expanded to fibrotic participants by using image stream analysis, these studies demonstrated a 3.59-fold reduction in expression of PLXNC1 by IPF monocytes relative to control cells ($P < 0.01$; Fig 1*F*). These data show that PLXNC1 expression in human circulation localizes to cells that express CD14, likely monocytes, and that this gene is relatively underdetected in the clinical setting of IPF.

PLXNC1 is underexpressed by monocytes in SSc-ILD

IPF is a clinical entity that is defined by a lack of an identifiable immune component and a poor response to classical forms of immunosuppression. To evaluate whether our findings are specific to this relatively rare clinical entity or might be present in more widespread forms of fibrosis, PLXNC1 expression was also determined on cells that were obtained from patients with SSc-ILD, an autoimmune form of lung fibrosis in which monocytes are also abundantly migratory (30). Here, a 3.14-fold reduction in PLXNC1 expression by circulating monocytes was also found ($P < 0.0096$; Fig. 1*F*), which indicates that PLXNC1 underexpression in monocytes is not restricted to IPF but is also observed in SSc-ILD. These data suggest that PLXNC1 might play a role in many forms of fibrotic lung disease.

PLXNC1 is underexpressed in IPF and SSc-ILD lung tissue

We next reasoned that if underexpression of PLXNC1 was mechanistically related to pulmonary fibrogenesis, this finding would also be observed in the fibrotic lung. To address this question, we evaluated PLXNC1 expression in a gene expression data set of 219 lung tissue samples, including those from patients with IPF ($n = 123$) and normal lung histology samples from control participants ($n = 96$), that were previously generated by members of our group (29). The complete data set is publicly available in GEO under accession no. GSE47460 and as well as on the LGRC website (*http://www.lung-genomics.org/*). Characteristics of these participants are shown in **Table 2**. Comparing microarray expression levels of PLXNC1 in IPF with those of control samples revealed significant underexpression of this gene in IPF ($U = 4681$; $Z = 2.629$; $P = 0.0086$; Fig. 1*G*), which was confirmed histologically by using immunolocalization on explanted lung tissues (Fig. 1*H* and Supplemental Fig. S1*A*, *B*). PLXNC1 was noted to localize at least partially to cells with the morphology of macrophages, a finding that was confirmed on human macrophages that were obtained *via* BAL (Supplemental Fig. S1*D*). Similarly, reduced detection was also observed in SSc-ILD lung explants that were obtained from the U.S. National Institutes of Health, National Institute of Arthritis and Musculoskeletal and Skin Diseases SSCORE

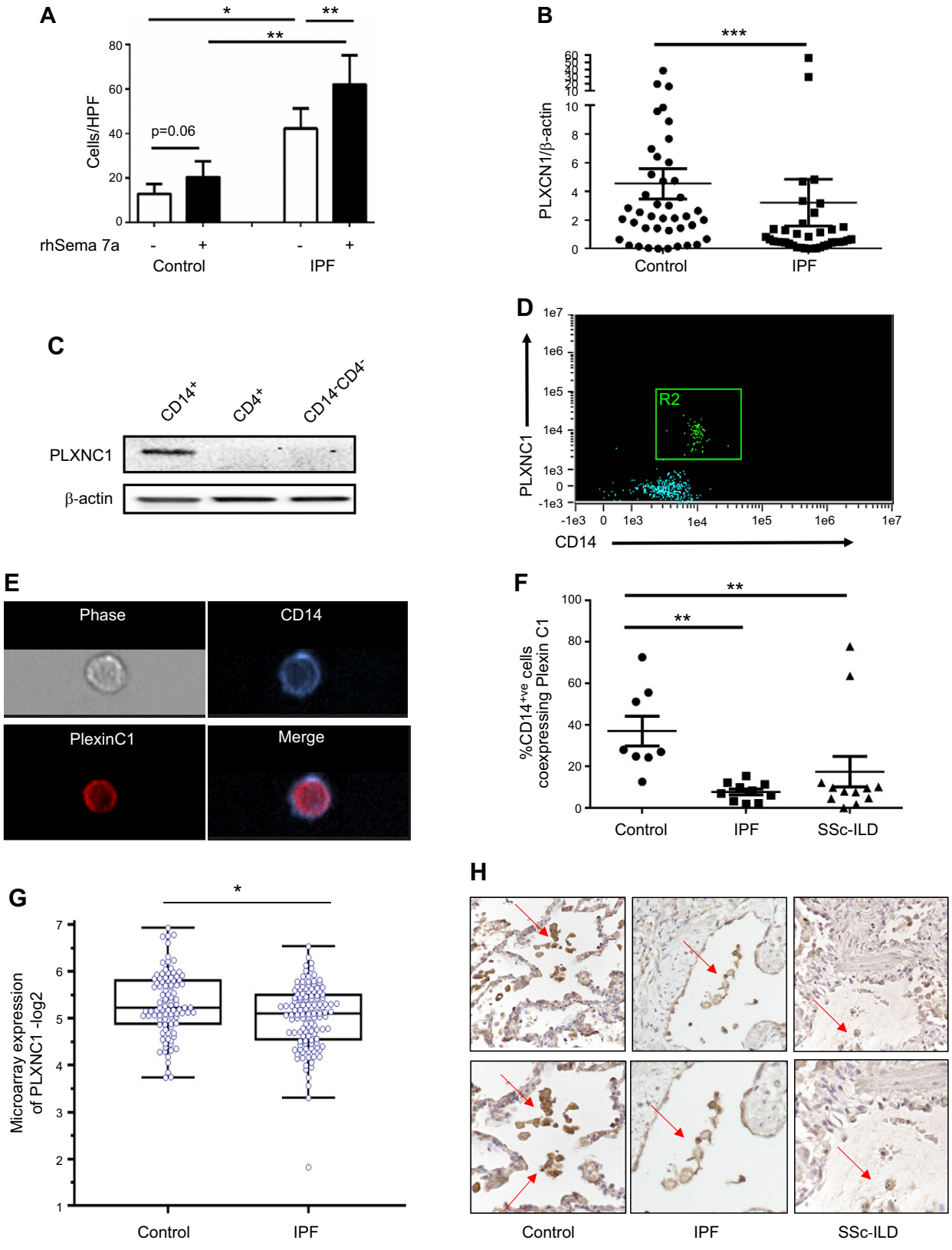

Figure 1. PLXNC1 expression is reduced on monocytes and lung tissue in human IPF and SSc-ILD. *A*) Relative to control (white, $n = 5$), IPF monocytes (black, $n = 9$) show increased migration in modified Boyden chambers at baseline and in response to recombinant human Sema 7a (rhSema 7a). *B*) Relative to control, IPF PBMCs demonstrated reduced relative expression of PLXNC1. *C*) Western blot of $CD14^+$, $CD4^+$, and $CD14^-CD4^-$ PBMCs from controls finds PLXNC1 to be exclusively detected on $CD14^+$ cells. *D*, *E*) Cytometry plot (*D*) of CD14 (*x* axis) and PLXNC1 (*y* axis) and simultaneously performed confocal microscopy (*E*) for CD14 (blue) and PLXNC1 (red) demonstrates that PLXNC1 is expressed by $CD14^+$ cells. *F*) Relative to control, monocytes obtained from both IPF and SSc-ILD display reduced surface expression of PLXNC1. *G*) Normalized expression of

(*continued on next page*)

TABLE 1. *Epidemiologic and clinical characteristics of the participants used for the quantitative real-time PCR–based evaluation of PLXNC1 in PBMCs*

Characteristic	IPF, $n = 38$	Control, $n = 42$	P
Age (yr)	69.76 (59.97–71.30)	65.60 (59.9–71.3)	0.2088
Sex			
Female	8 (21.05)	22 (52.4)	0.0038
Male	30 (78.95)	20 (47.6)	
Race			
White	34 (89.47)	38 (90.5)	0.2850
Nonwhite	4 (10.53)	4 (9.5)	
FVC%	67.05 (62.47–71.63)	N/A	N/A
DLCO%	44.00 (39.13–48.86)	N/A	N/A
On which diagnosis is based			
HRCT	23 (61)	N/A	N/A
Pathology	15 (39)	N/A	
Supplemental oxygen			
No	19 (50)	N/A	N/A
Yes	19 (50)	N/A	

Data are expressed as means with 95% confidence intervals or as numbers with percentages. DLCO %, carbon monoxide diffusing capacity percentage predicted; FVC%, forced vital capacity percentage predicted; HRCT, high-resolution computed tomography; N/A, not applicable.

facility (Fig. 1*H* and Supplemental Fig. S1*C*). The finding that PLXNC1 is underexpressed in 2 distinct forms of pulmonary fibrosis suggests its participation in a fibrotic process that is common to both diseases.

PLXNC1 is underexpressed on extrapulmonary monocytes after bleomycin administration in a mouse model of pulmonary fibrosis

In evaluating the above results, we noted that the relative change in PLXNC1 expression between control and disease samples was small in some cases, which suggested that, if attenuated levels of PLXNC1 indeed contribute to pulmonary fibrosis, additional pathways or mediators might be implicated. We thus turned to *in vivo* modeling to better understand PLXNC1's role in the fibrotic mammalian lung. As we had observed reduced expression of PLXNC1 by monocytes and macrophages in 2 forms of human lung fibrosis, we focused on these populations in the murine model. To this end, we first evaluated the expression of PLXNC1 on monocytes and macrophages after intratracheal bleomycin administration, which is commonly used to simulate salient features of the IPF disease state. When these cells were enumerated at various stages after inhalational administration of bleomycin, it was found that relative to cells that were obtained from PBS-challenged mice at the same time point, PLXNC1 expression on $CD14^{lo}$ cells in the periphery was unchanged (data not shown). In contrast, percentages of $CD14^{hi}$ cells that express PLXNC1 were unchanged 48 h after bleomycin exposure, then decreased by 23.5% within 5 d ($P < 0.05$; **Fig. 2*A***), and then further decreased by 32.6% at 14 d postbleomycin exposure ($P < 0.01$; Fig. 2*A*). These findings suggest that PLXNC1 underexpression on peripheral $CD14^{hi}$ monocytes is associated with lung fibrosis.

PLXNC1 expression is first reduced, and then increased, on $F4/80^{+}CD11c^{+}$ lung macrophages in 2 separate models of pulmonary fibrosis

After injury, the pool of lung resident macrophages is augmented by bone marrow–derived precursors (38). We therefore thought it possible that, similar to extrapulmonary monocytes, lung macrophages might display alterations in PLXNC1 as well. Here, we made the observation that after bleomycin administration, PLXNC1 expression by $F4/80^{+}CD11c^{+}$ lung macrophages demonstrated stability at 2 d, followed by a 51.4% reduction at the 5-d time point ($P < 0.01$; Fig. 2*B*, *C*) and a subsequent rebound increase of 71.1% after 14 d ($P < 0.05$; Fig. 2*B*, *C*). Evaluation of PLXNC1 expression by $CD45^{-}EpCAM^{+}$ cells meeting flow cytometric criteria for epithelia and $CD45^{-}$collagen-I$\alpha1^{+}$ cells meeting flow cytometric criteria for fibroblasts remained unaltered at any of these time points in the bleomycin model (Supplemental Fig. S2*A*, *B*). Similar macrophage findings were observed in a second model of fibrosis that was caused by lung-specific, doxycycline-inducible overexpression of the bioactive form of the human TGF-β1 gene—from here on referred to as TGF-β1 Tg^{+} (Fig. 2*D*). These data indicate that experimental induction of fibrosis using 2 different methods results in temporal alterations in PLXNC1 expression by lung macrophages, with reduced expression seen during the period immediately preceding fibrogenesis

Log_2-transformed PLXNC1 expression in lung tissue microarrays from the LGRC reveals reduced expression in IPF lung tissue compared with control. *H*) Immunodetection of PLXNC1 (brown, denoted with red arrows) in control lung tissue (left), IPF lungs (middle), and SSc-ILD (right) lung explants ($n = 5$ per group). HPF, high-power field. With the exception of microarray data, results are shown as means ± SEM. Original magnification, ×10 and counterstained with hematoxylin. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ after correction for multiple comparisons testing.

TABLE 2. *Epidemiologic and clinical characteristics of the lung microarray cohort*

Characteristic	IPF, $n = 123$	Control, $n = 96$
Age (yr)	64.8 ± 8.3	63.8 ± 11.2
Sex [*n* (%)]		
Male	82 (66.7)	46 (47.9)
Female	41 (33.3)	50 (52.1)
Race (*n*)		
White	113	88
African-American	5	3
Hispanic	1	2
Asian or Pacific Islander	2	1
Native American	0	1
Not disclosed	2	1
Pulmonary function test		
FVC%	65 ± 16	95 ± 13
DLCO%	48 ± 18	83 ± 17
FEV1%	71 ± 16	95 ± 13

Values are means ± SD or as noted. DLCO%, carbon monoxide diffusing capacity percentage predicted; FEV1%, forced expiratory volume in 1 s percentage predicted; FVC%, forced vital capacity percentage predicted.

and increased expression seen at the height of established fibrosis.

PLXNC1-deficient macrophages display enhanced migration in response to Sema 7a

The finding that PLXNC1 expression is reduced on monocytes and macrophages during this critical phase in 2 models led us to explore the potential function of PLXNC1. Because PLXNC1 is known to halt cytoskeletal rearrangement and suppress migration of both tumor cells and melanocytes (25, 26), we suspected that PLXNC1 deficiency would also result in excessive migration of peritoneal macrophages. To test this hypothesis, peritoneal macrophages that were obtained from mice with the PLXNC1 locus intact ($PLXNC1^{+/+}$, wild type) or deleted ($PLXNC1^{-/-}$) were subject to scratch tests performed in the presence or absence of rmSema 7a. Here, it was found that $PLXNC1^{-/-}$ macrophages displayed a 2.14-fold increase in migration relative to control macrophages ($P < 0.05$; Fig. 2*E*, *F*), and this effect was even more pronounced in the presence of rmSema 7a ($P < 0.0001$; Fig. 2*E*, *F*). These data indicate that PLXNC1 likely suppresses macrophage migration, both in the baseline state and in response to rmSema 7a.

Ubiquitous deletion of PLXNC1 exacerbates experimentally induced lung fibrosis

The finding that PLXNC1 inhibits macrophage migration, combined with its reduced expression during the time point typically identified as the interface between injury and fibrosis in both the bleomycin and TGF-β1 fibrosis models, led us to believe that PLXNC1 is antifibrotic and its expression on macrophages during this time period occurs as a protective response. To determine the validity of this hypothesis, we performed loss-of-function studies in which mice with the PLXNC1 locus intact ($PLXNC1^{+/+}$) and deleted ($PLXNC1^{-/-}$) were randomly assigned to receive orotracheal bleomycin or vehicle control and were observed for 14 d. Considering that macrophages are believed to contribute to fibrosis by orchestrating processes that are downstream of injury, we used a low dose of bleomycin (0.8 U/kg) designed to avoid excessive injury and mortality. By using this approach, when surviving mice were humanely killed at the 14-d time point, we detected a 2.2-fold increase in lung inflammation measured by BAL cell counts ($P < 0.0001$; **Fig. 3*A***), which consisted primarily of macrophages in all groups (Supplemental Fig. S2*C*). In addition, there was a 2.25-fold increase in total soluble lung collagen evaluated by Sircol assay ($P < 0.01$; Fig. 3*B*) and a 2.65-fold increase in modified Ashcroft scores ($P < 0.0001$; Fig. 3*C*, *D*) in mice that lacked PLXNC1. Of importance, these findings did not relate to increased structural cell death responses as the percentage of cells that showed TUNEL-positive nuclei was unaltered at any time point during the injury, inflammatory, and fibrotic phases of the bleomycin model ($P > 0.05$ all time points evaluated; Supplemental Fig. S2*D*, *E*). In a separate set of studies, a 1.8-fold increase in collagen accumulation was also seen in TGF-β1 Tg^{+} mice that were crossed with $PLXNC1^{-/-}$ animals ($P < 0.0001$; Fig. 3*E*). These data indicate that widespread absence of PLXNC1 worsens fibrosis-relevant end points in 2 experimental models.

Replacement of PLXNC1 on bone marrow–derived cells is sufficient to attenuate fibrosis and remodeling

We next performed gain-of-function experiments to evaluate whether replacement of PLXNC1 on hematopoietic cells was sufficient to rescue the $PLXNC1^{-/-}$ phenotype. Here, bone marrow chimeras were generated in which PLXNC1 deficiency was specifically replaced on bone marrow–derived cells (BMDCs; $PLXNC1^{+/+}$ donor into $PLXNC1^{-/-}$ host) to be compared with animals containing PLXNC1 in both tissue compartments ($PLXNC1^{+/+}$ donor and host) or neither compartment ($PLXNC1^{-/-}$ donor and host). Successful engraftment was confirmed by flow cytometry,

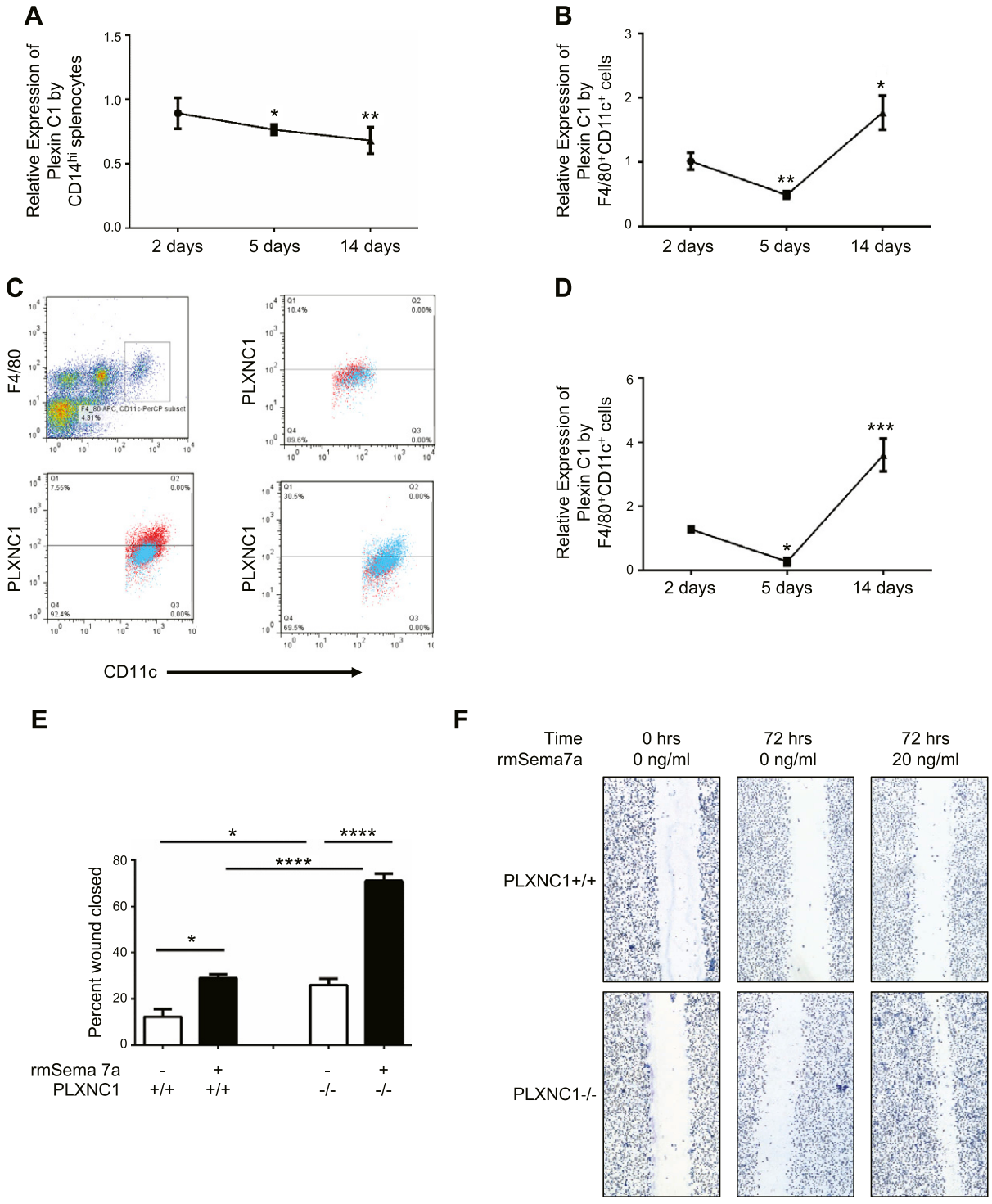

Figure 2. PLXNC1 is reduced on monocytes and macrophages in experimentally induced lung fibrosis, and PLXNC1-deficient monocytes display enhanced migration *in vitro*. *A*) Relative to PBS-treated control, $CD14^{hi}$ splenocytes obtained from bleomycin-treated mice demonstrate reduced expression of PLXNC1 after 5 and 14 d. Splenocytes were evaluated as a concentrated source of peripheral leukocytes. *B*) Relative to control, PLXNC1 expression by $F4/80^{+}CD11c^{+}$ lung macrophages is stable at 48 h, reduced after 5 d, and increased at 14 d after inhaled bleomycin. *C*) Representative FACS plots. $F4/80^{+}CD11c^{+}$ cells were selected within the live cell gate (upper left). Then, coexpression of CD11c (*x* axis) and PLXNC1 (*y* axis) was evaluated as shown in PBS- (red) and bleomycin-treated (blue) lungs; at 48 h (upper right); at 5 d (lower left); and at 14 d (bottom right). *D*) Similar kinetics of PLXNC1 expression by $F4/80^{+}CD11c^{+}$ lung macrophages exists in TGF-β1 Tg^{+} mice. *E*) $PLXNC1^{-/-}$ peritoneal macrophages display enhanced migration in scratch tests at baseline (left) and in response to rmSema 7a (right). *F*) Representative images of scratch tests performed on peritoneal macrophages obtained from $PLXNC1^{+/+}$ (upper) and $PLXNC1^{-/-}$ (bottom) mice. Images are at ×10 magnification. Experiments were performed in triplicate. Data are shown as means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

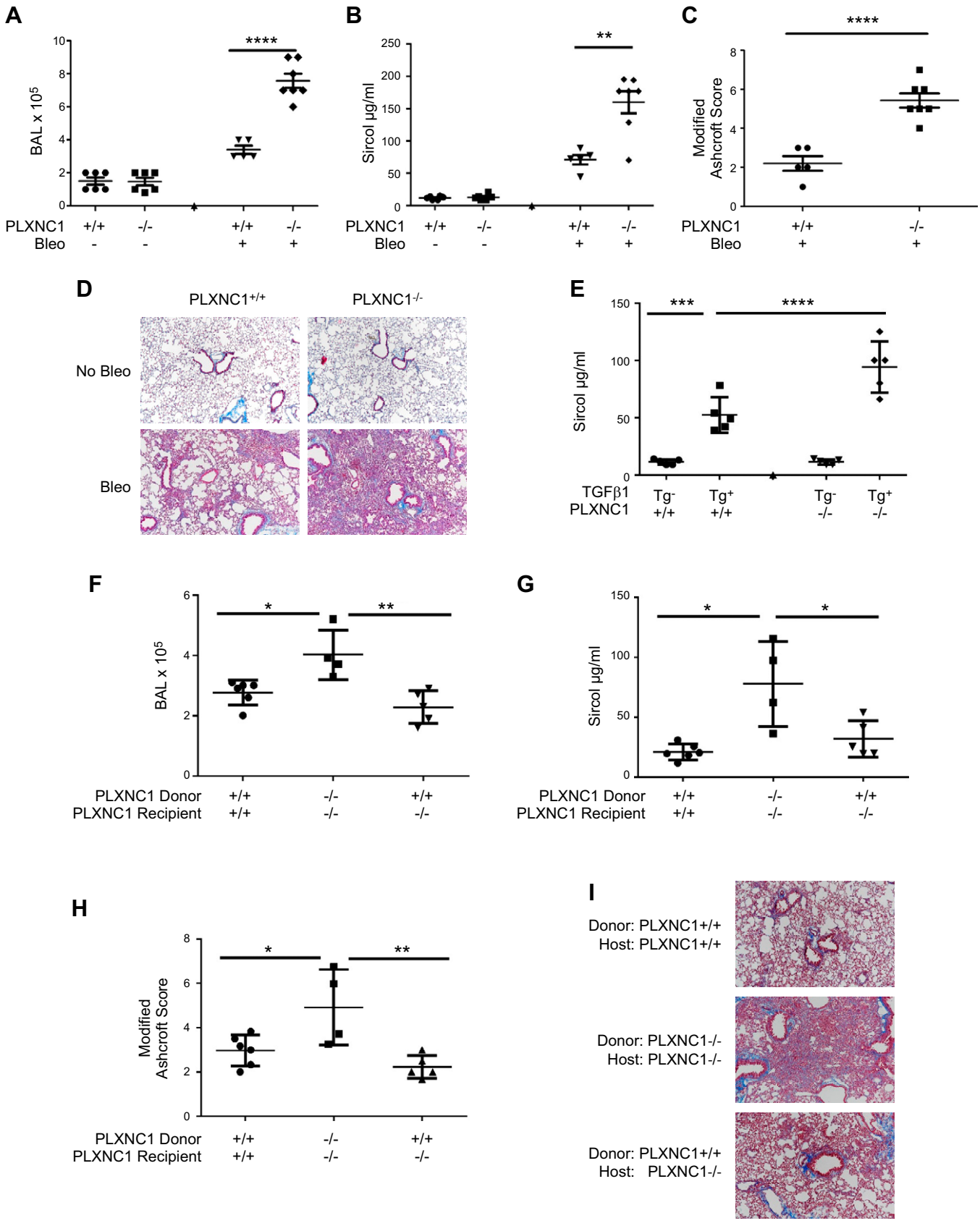

Figure 3. PLXNC1-null mice show exacerbated fibrosis in 2 mouse models of lung fibrosis, and this effect is abrogated by restoration of PLXNC1 expression on BMDCs. *A–D*) Relative to wild-type mice that were treated with a single dose of 0.8 U/kg inhaled bleomycin (Bleo), PLXNC1$^{-/-}$ mice demonstrate significantly increased lung inflammation as measured by BAL cell counts (*A*), collagen accumulation as measured by Sircol assay (*B*), and histologic evidence of remodeling (*C*, *D*). *E*) Relative to

(*continued on next page*)

which demonstrated restoration of PLXNC1 on macrophages in the lungs of PLXNC1$^{-/-}$ mice that underwent transplantation with PLXNC1$^{+/+}$ donor cells (Supplemental Fig. S3). One month after transplantation, mice were treated with inhaled bleomycin and humanely killed after 14 d. Our results showed replacement of PLXNC1 expression on BMDCs was sufficient to reduce both lung inflammation and fibrosis. Specifically, relative to PLXNC1$^{-/-}$ recipients of PLXNC1$^{-/-}$ BMDCs, replacement of PLXNC1 on BMDCs resulted in a 43.2% reduction in BAL cell counts ($P < 0.01$; Fig. 3*F*) that were in all groups composed primarily of macrophages (Supplemental Fig. S3*A*), a 57.5% reduction in lung collagen content ($P < 0.05$; Fig. 3*G*), and a 54.4% reduction in modified Ashcroft scores ($P < 0.01$; Fig. 3*H*, *I*). These data indicate that replacement of PLXNC1 on BMDCs, presumably including macrophages, is sufficient to counteract the effects of total body PLXNC1 deficiency in the bleomycin model.

Syt7 mediates excessive migration of PLXNC1$^{-/-}$ macrophages

Synthesis of the above data indicated that PLXNC1 deficiency on macrophages exacerbates existing fibrotic responses. Therefore, we thought it likely that PLXNC1 may be induced to protect against a gene with opposing functions. Here, we were led to examine a role for Syt7, a calcium binding protein that mediates fusion pore formation to allow cellular locomotion and vesicle formation. Syt7 has been implicated in the development of a wide range of diseases, including autoimmune myositis (34), diabetes (39), and inflammatory arthritis (40), but to date has not been evaluated in the context of macrophage-driven fibrotic processes affecting the lung. To determine whether a relationship exists between PLXNC1 and Syt7, we first repeated the scratch test studies and found that the migration of macrophages from Syt7-deficient mice (Syt7$^{-/-}$) did not differ significantly from macrophages that were obtained from wild-type mice. However, deletion of Syt7 in addition to PLXNC1 (PLXNC1$^{-/-}$ × Syt7$^{-/-}$) normalized the excessive migration of PLXNC1$^{-/-}$ cells ($P < 0.0001$; **Fig. 4*A***). Because Syt7 is dispensable for cellular migration when PLXNC1 is present, but is required in its absence, these data suggest that PLXNC1 opposes the migratory functions of Syt7 in adult mouse macrophages.

Syt7 is overexpressed in 2 separate models of pulmonary fibrosis and in 2 forms of fibrotic human lung disease

We next evaluated expression of Syt7 in the setting of experimentally induced lung fibrosis. Of interest, in both the bleomycin and TGF-β1 models, immunohistochemistry-based detection of Syt7 revealed a strong baseline expression in epithelial cells that was accompanied by a time-dependent increase in expression on alveolar macrophages that reached its height after 14 d ($P < 0.01$ in the bleomycin model, and $P < 0.05$ in the TGFβ1 model; Fig. 4*B*, *C*). Consistent with these findings, lung tissues from the LGRC data set showed that microarray levels of Syt7 in IPF lungs exceed that of controls ($U = 4099$; $Z = 3.879$; $P = 0.0001$; Fig. 4*D*), which displayed a moderate negative association with PLXNC1 expression (Spearman $R = -0.49$; $P < 0.0001$; Fig. 4*E*). Quantitative real-time PCR–based analysis of explanted lung tissues revealed similarly enhanced expression in both IPF and SSc-ILD relative to control ($P < 0.05$ for both comparisons; Fig. 4*F*). When viewed in conjunction with the underexpression of PLXNC1 described above, these data suggest that reductions in the PLXNC1/Syt ratio might contribute to pulmonary fibrotic responses.

Enhanced fibrosis caused by PLXNC1 deficiency is ameliorated by ubiquitous deletion of Syt7

To determine whether this increased expression of Syt7 facilitates the disrupted tissue homeostasis and collagen accumulation caused by PLXNC1 deficiency, we generated double knockout PLXNC1$^{-/-}$ and Syt7$^{-/-}$ mice and exposed them to low-dose inhaled bleomycin. Here, we reasoned that since macrophages are believed to function as amplifiers rather than initiators or effectors of fibrosis, deletion of genes that affect macrophage biology would result in reduction, but not normalization, of fibrotic end points at 14-d in the bleomycin model. Consistent with this prediction, relative to PLXNC1$^{-/-}$ animals with the Syt7 locus intact, bleomycin-challenged PLXNC1$^{-/-}$ mice that lacked Syt7 showed a 44.7% reduction in lung inflammation on the basis of BAL cell counts ($P < 0.05$; **Fig. 5*A***) that were predominantly macrophages (Supplemental Fig. S4), a 35.7% reduction in collagen accumulation on the basis of Sircol assay ($P < 0.01$; Fig. 5*B*), and a 60.0% reduction in indices of lung remodeling on the basis of modified Ashcroft scores and trichrome staining ($P < 0.0001$; Fig. 5*C*, *D*). Curiously, ubiquitous deletion of Syt7 had no effect on any of these end points when PLXNC1 was present (Fig. 5*A*–*D*). Similar reductions in collagen accumulation were seen in TGF-β1 Tg^{+} mice that were crossed with Syt7-deficient animals (TGF-β1 × Syt7$^{-/-}$) when they were treated with a PLXNC1-neutralizing antibody ($P = 0.049$; Fig. 5*E*). These data indicate that Syt7 is unable to contribute to fibrotic responses in the presence of PLXNC1,

TGF-β1 Tg^{+} mice with the PLXNC1 locus intact, TGF-β1 Tg^{+} animals that lacked PLXNC1 manifest increased collagen accumulation on the basis of Sircol assay after 28 d of doxycycline. *F*–*I*) Relative to total body PLXNC1-null mice (PLXNC1$^{-/-}$ recipients of PLXNC1$^{-/-}$ BMDCs), bleomycin-treated mice in which PLXNC1 has been restored on BMDC demonstrate attenuated lung inflammation on the basis of BAL cell counts (*F*), collagen accumulation on the basis of Sircol assay (*G*), and histologic evidence of fibrosis and remodeling on the basis of modified Ashcroft scores (*H*) and trichrome stains (*I*). Experiments were performed in triplicate. Histology images shown are at ×10 magnification. Data are shown as means ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$ after correction for multiple comparisons.

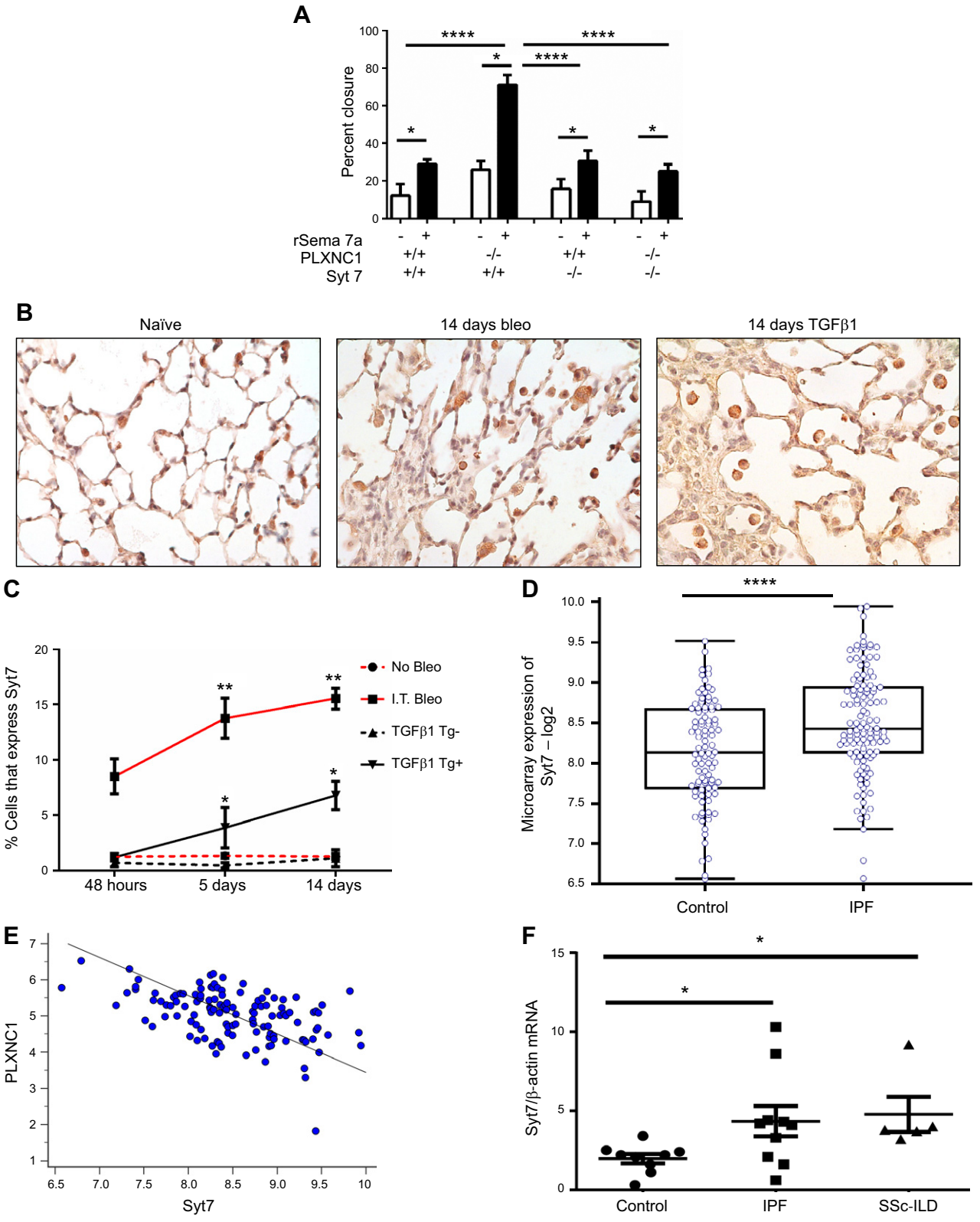

Figure 4. Syt7 is overexpressed in human pulmonary fibrosis and opposes PLXNC1's effects on macrophage migration. *A*) Syt7 deficiency ($Syt7^{-/-}$) significantly reduces migration of $PLXNC1^{-/-}$, but not $PLXNC1^{+/+}$, macrophages (n = 3 independent experiments). *B*) Immunodetection reveals that baseline Syt7 expression observed in cells with the morphology of type II pneumocytes (left) is accompanied by increased expression in cells with the morphology of alveolar macrophages in both bleomycin (bleo)-treated (middle) and TGF-β1 Tg^{+} (right) mouse lungs. Images are at ×40 magnification. *C*) $Syt7^{+}$ cells increase over time after bleomycin administration (red solid line) and TGF-β1 overexpression (black solid line). *D, E*) Microarray-based detection of Syt7 in the LGRC data set reveals significantly increased expression in IPF relative to controls (*D*), which display a moderate negative correlation with PLXNC1 (*E*) [Spearman $R = -0.49$; $P < 0.0001$]. *F*) Quantitative real-time PCR of total lung mRNA reveals increased Syt7 detection in both IPF and SSc-ILD relative to donor lung tissue. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$ after correction for multiple comparisons.

and that its absence ameliorates the increased fibrosis caused by PLXNC1 deficiency. When viewed in combination, these data suggest that PLXNC1 functions to oppose or neutralize Syt7.

DISCUSSION

These data provide new insight into the role that neuronally active proteins, such as PLXNC1 and Syt7, might play in the regulation of macrophage migration and mammalian lung fibrosis. Experiments performed in 2 *in vivo* modeling systems demonstrate an imbalance in the expression of PLXNC1 and Syt7 on macrophages in the fibrotic lung, and that attenuated levels of PLXNC1 results in enhanced Syt7-mediated macrophage migration. Constitutive deletion of PLXNC1 exacerbates fibrosis in a manner that is ameliorated either by replacement of PLXNC1 on BMDCs or by ubiquitous deletion of Syt7. These findings are also observed in patients with pulmonary fibrosis, as monocytes obtained from patients with IPF demonstrate both excessive migration and reduced expression of PLXNC1, and in lung tissues from both IPF and SSc-ILD, which display a relative reduction of PLXNC1 and an increase in Syt7. When viewed in combination, these findings demonstrate a previously unrecognized role for PLXNC1 in the suppression of Syt7-driven macrophage migration that contributes to fibrotic responses. The proposed mechanism of this effect is shown

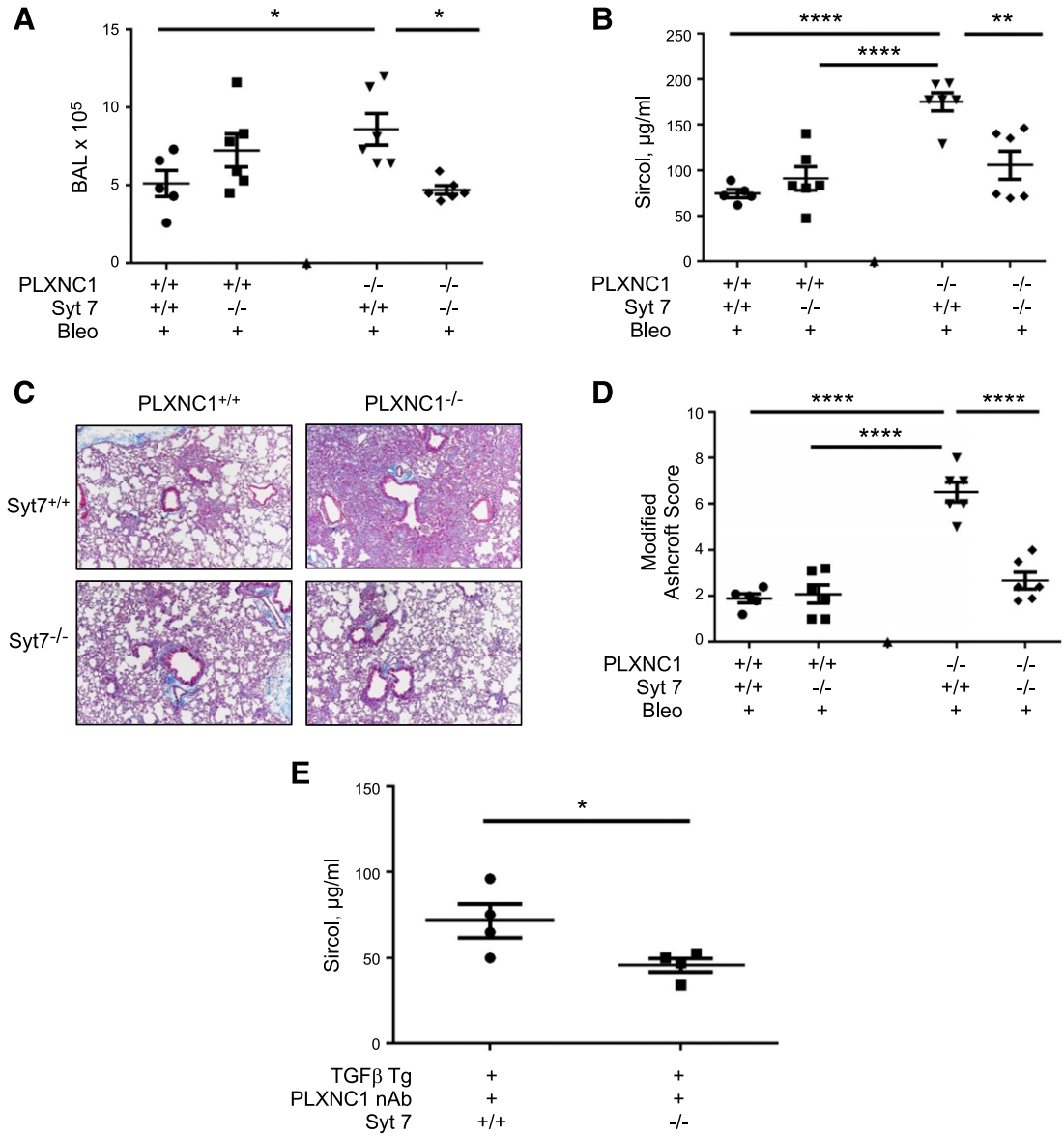

Figure 5. Deletion of Syt7 reverses excessive pulmonary fibrosis in mice that lack PLXNC1. *A–D*) Relative to PLXNC1$^{-/-}$Syt7$^{+/+}$ mice, PLXNC1$^{-/-}$Syt7$^{-/-}$ animals show significantly reduced BAL cell counts (*A*), Sircol levels (*B*), and Modified Ashcroft scores (*C*, *D*) in response to inhaled bleomycin. Images are at ×10 magnification. *E*) TGF-β1 Tg^{+} mice that were treated with PLXNC1-neutralizing antibody (PLXNC1 nAb) show reduced collagen accumulation measured by Sircol assay in the setting of Syt7 deficiency. Experiments were performed in triplicate. Data are shown as means ± SEM. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$ after correction for multiple testing.

in **Fig. 6**. Specifically, because Syt7 mediates cellular migration by driving lysosomal fusion with the plasma membrane, PLXNC1 may function to oppose this movement by halting cytoskeletal rearrangement. These findings may have great relevance for the broad array of fibrotic processes that have been linked to neuroimmune molecules, including but not limited to IPF, scleroderma, asthma, liver fibrosis, inflammatory bowel disease, and multiple sclerosis. In addition, these findings might have far-reaching implications that extend beyond fibrosis to other biologic processes that are mediated by these neuronally active proteins, such as brain development, neurotransmitter secretion and recycling, and lysosomal trafficking.

Since its initial discovery nearly 2 decades ago, PLXNC1 has been characterized as the inhibitory receptor of Sema 7a, in part, because of its inhibitory phosphorylation of Cofilin 1 (23–25) and its inactivation of the Ras homolog Rras (26). However, because PLXNC1 has never been studied in the context of fibrotic responses, our novel finding that ubiquitous PLXNC1 deletion dramatically worsens macrophage migration and collagen accumulation in 2 mouse models suggests that reduced or absent expression of this gene might contribute to human lung disease. Because bone marrow chimera data show that replacement of PLXNC1 on BMDCs is partially fibroprotective, these data suggest that PLXNC1 might act to dampen macrophage recruitment either into or within the lung during periods that precede development of established fibrosis, though it is possible that other cells and mechanisms may be involved. Of interest, it is known that human monocytes downregulate PLXNC1 as they mature (37) and that migratory macrophages in the CNS express markers of alternative activation (41). Thus, it is possible that the reduced expression of PLXNC1 by monocytes and macrophages represents accelerated differentiation, which would be consistent with published observations by our group and others that IPF and SSc-ILD monocytes seem to have undergone at least partial cell fate specification before entering the lung (28, 31).

Our data also present a novel role for the evolutionarily conserved, calcium-sensing protein, Syt7, in the regulation of macrophage migration and experimentally induced pulmonary fibrosis. Whereas Syt7 is well described as a regulator of neurotransmitter release (42), lysosome function (43), insulin secretion (39), and the fusion pore formation that drives cellular migration (40), a role for this protein in the setting of fibroproliferative lung disease, to date, has not been described. Because Syt7 is involved in surfactant recycling (44) and phagocytosis (45), and because studies of Syt7$^{-/-}$ mice revealed the development of spontaneous skin and muscle fibrosis resulting from impaired membrane resealing after injury (34), we were surprised to find that Syt7 is dispensable for experimentally induced macrophage migration, inflammation, and fibrosis in the lung. Only in the setting of PLXNC1 deficiency was there a discernable effect of Syt7 deletion, which indicates that PLXNC1 suppresses the migratory and fibrosis-promoting properties of Syt7. Whether PLXNC1 directly opposes Syt7-induced processes *via* cell autonomous effects on cytoskeletal rearrangement mediated *via* cofilin-1 or Rras remains to be determined. In addition, because Syt7 deficiency in the presynaptic terminus is phenocopied by calmodulin inhibition (46), these data suggest that pharmacologic interventions that target Syt7 or its substrates may be of benefit in pathologic processes that are characterized by insufficient expression of PLXNC1.

In a direct clinical extension of these studies, we also find evidence of altered PLXNC1 and Syt7 in 2 forms of human interstitial lung disease. These entities differ substantially in terms of patients affected, clinical presentation, and response to therapy (47). They are also believed to result from disparate pathogeneses, with IPF being an epithelial-driven process that might be subject to amplification by aberrant immune responses (16), whereas scleroderma features a vascular pathology and a significant primary autoimmune component (11). When viewed in combination with the animal modeling that showed that PLXNC1 deficiency worsens fibrogenic processes in 2

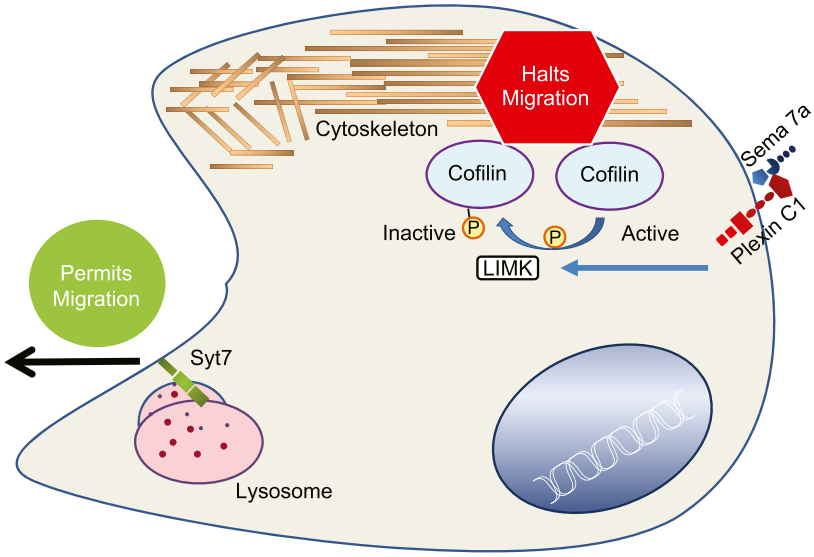

Figure 6. Schematic of the proposed interaction between PLXNC1 and Syt7. Migration of fibrosis-promoting cells, such as monocytes and macrophages, is mediated by a balance between PLXNC1 and Syt 7. Engagement of PLXNC1 by Sema 7a inhibits the cytoskeletal organization required for cell migration *via* the inhibitory phosphorylation of Cofilin in a LIMK-dependent pathway, thereby halting migration. In contrast, Syt7 promotes migration, perhaps by lysosomal fusion with the plasma membrane. A reduction in PLXNC1 relative to Syt7 seems to permit macrophage migration and to facilitate fibrosis in experimental modeling of pulmonary fibrosis. LIMK, LIM kinase.

distinct animal models, the findings of altered PLXNC1 and Syt7 expression in both disease states supports the idea that fibrosis might represent a final common pathway for a variety of different insults and that fibrosis-targeted interventions that show benefit in one disease might be efficacious in others. Finally, these data also suggest that rather than being specific to one relatively rare disease state, the PLXNC1–Syt7 relationship might have far-reaching ramifications as a convergent molecular mechanism that links divergent clinical entities.

Although important, our study has some limitations. Relevance of the bleomycin and TGF-β1 overexpression models to human lung disease is not firmly established, as human fibrosis is believed to result from a mismatch between simultaneously occurring injury and repair responses rather than one single insult. This may account for the finding that the fibrotic human lung, which is known to demonstrate the ongoing injury and fibrosis, displays reduced expression of PLXNC1, whereas the mouse models, in which injury and fibrosis occur sequentially, shows increased expression during the fibrotic phase. Regardless, the finding of alterations in PLXNC1 and Syt7 detection in separate cohorts of patients with distinct forms of pulmonary fibrosis suggests that our results have real human impact that warrants further investigation. We have not defined the precise mechanisms through which PLXNC1 deficiency worsens fibroproliferation, though our *ex vivo* modeling suggests that macrophages may be involved. We have not evaluated the effect of PLXNC1 expression on other cells, nor have we determined whether pharmacologic interventions that target PLXNC1 or Syt7 are beneficial. Nevertheless, despite these minor shortcomings, our work firmly frames PLXNC1 as a regulator of core fibrotic processes in mammalian lung fibrosis. Further investigation of this pathway might lead to widespread advances in the study of repair and remodeling. Fj

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AUTHOR CONTRIBUTIONS

X. Peng, M. Moore, A. Mathur, Y. Zhou, H. Sun, Y. Gan, J. D. Herazo-Maya, X. Hu, H. Pan, and C. Ryu performed experiments and analyzed data; N. Kaminski, A. Osafo-Addo, and R. J. Homer analyzed data; C. Feghali-Bostwick procured lung tissues; W. H. Fares and M. Gulati recruited participants and procured biospecimens; B. Hu, C.-G. Lee, and J. A. Elias performed data analysis; E. L. Herzog conceived of the experimental design and performed data analysis; and all authors participated in manuscript preparation and provided final approval of the submitted work.

REFERENCES

1. Raghu, G., Collard, H. R., Egan, J. J., Martinez, F. J., Behr, J., Brown, K. K., Colby, T. V., Cordier, J. F., Flaherty, K. R., Lasky, J. A., Lynch, D. A., Ryu, J. H., Swigris, J. J., Wells, A. U., Ancochea, J., Bouros, D., Carvalho, C., Costabel, U., Ebina, M., Hansell, D. M., Johkoh, T., Kim, D. S., King, T. E., Jr., Kondoh, Y., Myers, J., Müller, N. L., Nicholson, A. G., Richeldi, L., Selman, M., Dudden, R. F., Griss, B. S., Protzko, S. L., and Schünemann, H. J.; ATS/ERS/JRS/ALAT Committee on Idiopathic Pulmonary Fibrosis. (2011) An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *Am. J. Respir. Crit. Care Med.* **183**, 788–824
2. Wagner, G. R. (1997) Asbestosis and silicosis. *Lancet* **349**, 1311–1315
3. Vanhee, D., Gosset, P., Wallaert, B., Voisin, C., and Tonnel, A. B. (1994) Mechanisms of fibrosis in coal workers' pneumoconiosis. Increased production of platelet-derived growth factor, insulin-like growth factor type I, and transforming growth factor beta and relationship to disease severity. *Am. J. Respir. Crit. Care Med.* **150**, 1049–1055
4. Abid, S. H., Malhotra, V., and Perry, M. C. (2001) Radiation-induced and chemotherapy-induced pulmonary injury. *Curr. Opin. Oncol.* **13**, 242–248
5. Thannickal, V. J., Toews, G. B., White, E. S., Lynch III, J. P., and Martinez, F. J. (2004) Mechanisms of pulmonary fibrosis. *Annu. Rev. Med.* **55**, 395–417
6. Steen, V. D., Owens, G. R., Fino, G. J., Rodnan, G. P., and Medsger, T. A., Jr. (1985) Pulmonary involvement in systemic sclerosis (scleroderma). *Arthritis Rheum.* **28**, 759–767
7. Majumdar, S., Li, D., Ansari, T., Pantelidis, P., Black, C. M., Gizycki, M., du Bois, R. M., and Jeffery, P. K. (1999) Different cytokine profiles in cryptogenic fibrosing alveolitis and fibrosing alveolitis associated with systemic sclerosis: a quantitative study of open lung biopsies. *Eur. Respir. J.* **14**, 251–257
8. Lewis, M. J., Lewis III, E. H., Amos, J. A., and Tsongalis, G. J. (2003) Cystic fibrosis. *Am. J. Clin. Pathol.* **120**, S3–S13
9. King, T. E., Jr., Bradford, W. Z., Castro-Bernardini, S., Fagan, E. A., Glaspole, I., Glassberg, M. K., Gorina, E., Hopkins, P. M., Kardatzke, D., Lancaster, L., Lederer, D. J., Nathan, S. D., Pereira, C. A., Sahn, S. A., Sussman, R., Swigris, J. J., and Noble, P. W.; ASCEND Study Group. (2014) A phase 3 trial of pirfenidone in patients with idiopathic pulmonary fibrosis. *N. Engl. J. Med.* **370**, 2083–2092
10. Richeldi, L., du Bois, R. M., Raghu, G., Azuma, A., Brown, K. K., Costabel, U., Cottin, V., Flaherty, K. R., Hansell, D. M., Inoue, Y., Kim, D. S., Kolb, M., Nicholson, A. G., Noble, P. W., Selman, M., Taniguchi, H., Brun, M., Le Maulf, F., Girard, M., Stowasser, S., Schlenker-Herceg, R., Disse, B., and Collard, H. R.; INPULSIS Trial Investigators. (2014) Efficacy and safety of nintedanib in idiopathic pulmonary fibrosis. *N. Engl. J. Med.* **370**, 2071–2082
11. Tashkin, D. P., Elashoff, R., Clements, P. J., Goldin, J., Roth, M. D., Furst, D. E., Arriola, E., Silver, R., Strange, C., Bolster, M., Seibold, J. R., Riley, D. J., Hsu, V. M., Varga, J., Schraufnagel, D. E., Theodore, A., Simms, R., Wise, R., Wigley, F., White, B., Steen, V., Read, C., Mayes, M., Parsley, E., Mubarak, K., Connolly, M. K., Golden, J., Olman, M., Fessler, B., Rothfield, N., and Metersky, M.; Scleroderma Lung Study Research Group. (2006) Cyclophosphamide versus placebo in scleroderma lung disease. *N. Engl. J. Med.* **354**, 2655–2666
12. Raghu, G., Anstrom, K. J., King, T. E., Jr., Lasky, J. A., and Martinez, F. J.; Idiopathic Pulmonary Fibrosis Clinical Research Network. (2012) Prednisone, azathioprine, and N-acetylcysteine for pulmonary fibrosis. *N. Engl. J. Med.* **366**, 1968–1977
13. Murray, L. A., Chen, Q., Kramer, M. S., Hesson, D. P., Argentieri, R. L., Peng, X., Gulati, M., Homer, R. J., Russell, T., van Rooijen, N., Elias, J. A., Hogaboam, C. M., and Herzog, E. L. (2011) TGF-beta driven lung fibrosis is macrophage dependent and blocked by serum amyloid P. *Int. J. Biochem. Cell Biol.* **43**, 154–162
14. Gibbons, M. A., MacKinnon, A. C., Ramachandran, P., Dhaliwal, K., Duffin, R., Phythian-Adams, A. T., van Rooijen, N., Haslett, C., Howie, S. E., Simpson, A. J., Hirani, N., Gauldie, J., Iredale, J. P., Sethi, T., and Forbes, S. J. (2011) Ly6Chi monocytes direct alternatively activated profibrotic macrophage regulation of lung fibrosis. *Am. J. Respir. Crit. Care Med.* **184**, 569–581
15. Karmouty-Quintana, H., Philip, K., Acero, L. F., Chen, N. Y., Weng, T., Molina, J. G., Luo, F., Davies, J., Le, N. B., Bunge, I., Volcik, K. A., Le, T. T., Johnston, R. A., Xia, Y., Eltzschig, H. K., and Blackburn, M. R. (2015) Deletion of ADORA2B from myeloid cells dampens lung fibrosis and pulmonary hypertension. *FASEB J.* **29**, 50–60

16. Blackwell, T. S., Tager, A. M., Borok, Z., Moore, B. B., Schwartz, D. A., Anstrom, K. J., Bar-Joseph, Z., Bitterman, P., Blackburn, M. R., Bradford, W., Brown, K. K., Chapman, H. A., Collard, H. R., Cosgrove, G. P., Deterding, R., Doyle, R., Flaherty, K. R., Garcia, C. K., Hagood, J. S., Henke, C. A., Herzog, E., Hogaboam, C. M., Horowitz, J. C., King, T. E., Jr., Loyd, J. E., Lawson, W. E., Marsh, C. B., Noble, P. W., Noth, I., Sheppard, D., Olsson, J., Ortiz, L. A., O'Riordan, T. G., Oury, T. D., Raghu, G., Roman, J., Sime, P. J., Sisson, T. H., Tschumperlin, D., Violette, S. M., Weaver, T. E., Wells, R. G., White, E. S., Kaminski, N., Martinez, F. J., Wynn, T. A., Thannickal, V. J., and Eu, J. P. (2014) Future directions in idiopathic pulmonary fibrosis research. An NHLBI workshop report. *Am. J. Respir. Crit. Care Med.* **189**, 214–222
17. Zhu, S., Gladson, C. L., White, K. E., Ding, Q., Stewart, J., Jr., Jin, T. H., Chapman, H. A., Jr., and Olman, M. A. (2009) Urokinase receptor mediates lung fibroblast attachment and migration toward provisional matrix proteins through interaction with multiple integrins. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **297**, L97–L108
18. Ma, B., Herzog, E. L., Lee, C. G., Peng, X., Lee, C. M., Chen, X., Rockwell, S., Koo, J. S., Kluger, H., Herbst, R. S., Sznol, M., and Elias, J. A. (2015) Role of chitinase 3-like-1 and semaphorin 7a in pulmonary melanoma metastasis. *Cancer Res.* **75**, 487–496
19. Gan, Y., Reilkoff, R., Peng, X., Russell, T., Chen, Q., Mathai, S. K., Homer, R., Gulati, M., Siner, J., Elias, J., Bucala, R., and Herzog, E. (2011) Role of semaphorin 7a signaling in transforming growth factor β1-induced lung fibrosis and scleroderma-related interstitial lung disease. *Arthritis Rheum.* **63**, 2484–2494
20. Suzuki, K., Okuno, T., Yamamoto, M., Pasterkamp, R. J., Takegahara, N., Takamatsu, H., Kitao, T., Takagi, J., Rennert, P. D., Kolodkin, A. L., Kumanogoh, A., and Kikutani, H. (2007) Semaphorin 7A initiates T-cell-mediated inflammatory responses through alpha1beta1 integrin. *Nature* **446**, 680–684
21. Reilkoff, R. A., Peng, H., Murray, L. A., Peng, X., Russell, T., Montgomery, R., Feghali-Bostwick, C., Shaw, A., Homer, R. J., Gulati, M., Mathur, A., Elias, J. A., and Herzog, E. L. (2013) Semaphorin $7a^+$ regulatory T cells are associated with progressive idiopathic pulmonary fibrosis and are implicated in transforming growth factor-β1-induced pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* **187**, 180–188
22. Sultana, H., Neelakanta, G., Foellmer, H. G., Montgomery, R. R., Anderson, J. F., Koski, R. A., Medzhitov, R. M., and Fikrig, E. (2012) Semaphorin 7A contributes to West Nile virus pathogenesis through TGF-β1/Smad6 signaling. *J. Immunol.* **189**, 3150–3158
23. Czopik, A. K., Bynoe, M. S., Palm, N., Raine, C. S., and Medzhitov, R. (2006) Semaphorin 7A is a negative regulator of T cell responses. *Immunity* **24**, 591–600
24. Lazova, R., Gould Rothberg, B. E., Rimm, D., and Scott, G. (2009) The semaphorin 7A receptor Plexin C1 is lost during melanoma metastasis. *Am. J. Dermatopathol.* **31**, 177–181
25. Scott, G. A., McClelland, L. A., and Fricke, A. F. (2008) Semaphorin 7a promotes spreading and dendricity in human melanocytes through beta1-integrins. *J. Invest. Dermatol.* **128**, 151–161
26. Scott, G. A., McClelland, L. A., Fricke, A. F., and Fender, A. (2009) Plexin C1, a receptor for semaphorin 7a, inactivates cofilin and is a potential tumor suppressor for melanoma progression. *J. Invest. Dermatol.* **129**, 954–963
27. Granja, T., Köhler, D., Mirakaj, V., Nelson, E., König, K., and Rosenberger, P. (2014) Crucial role of Plexin C1 for pulmonary inflammation and survival during lung injury. *Mucosal Immunol.* **7**, 879–891
28. Mathai, S. K., Gulati, M., Peng, X., Russell, T. R., Shaw, A. C., Rubinowitz, A. N., Murray, L. A., Siner, J. M., Antin-Ozerkis, D. E., Montgomery, R. R., Reilkoff, R. A., Bucala, R. J., and Herzog, E. L. (2010) Circulating monocytes from systemic sclerosis patients with interstitial lung disease show an enhanced profibrotic phenotype. *Lab. Invest.* **90**, 812–823
29. Bauer, Y., Tedrow, J., de Bernard, S., Birker-Robaczewska, M., Gibson, K. F., Guardela, B. J., Hess, P., Klenk, A., Lindell, K. O., Poirey, S., Renault, B., Rey, M., Weber, E., Nayler, O., and Kaminski, N. (2015) A novel genomic signature with translational significance for human idiopathic pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol.* **52**, 217–231
30. Tourkina, E., Bonner, M., Oates, J., Hofbauer, A., Richard, M., Znoyko, S., Visconti, R. P., Zhang, J., Hatfield, C. M., Silver, R. M., and Hoffman, S. (2011) Altered monocyte and fibrocyte phenotype and function in scleroderma interstitial lung disease: reversal by caveolin-1 scaffolding domain peptide. *Fibrogenesis Tissue Repair* **4**, 15
31. Zhou, Y., Peng, H., Sun, H., Peng, X., Tang, C., Gan, Y., Chen, X., Mathur, A., Hu, B., Slade, M. D., Montgomery, R. R., Shaw, A. C., Homer, R. J., White, E. S., Lee, C. M., Moore, M. W., Gulati, M., Lee, C. G., Elias, J. A., and Herzog, E. L. (2014) Chitinase 3-like 1 suppresses injury and promotes fibroproliferative responses in mammalian lung fibrosis. *Sci. Transl. Med.* **6**, 240ra76
32. Davies, J. Q., and Gordon, S. (2005) Isolation and culture of human macrophages. *Methods Mol. Biol.* **290**, 105–116
33. Lee, C. G., Cho, S. J., Kang, M. J., Chapoval, S. P., Lee, P. J., Noble, P. W., Yehualaeshet, T., Lu, B., Flavell, R. A., Milbrandt, J., Homer, R. J., and Elias, J. A. (2004) Early growth response gene 1-mediated apoptosis is essential for transforming growth factor beta1-induced pulmonary fibrosis. *J. Exp. Med.* **200**, 377–389
34. Chakrabarti, S., Kobayashi, K. S., Flavell, R. A., Marks, C. B., Miyake, K., Liston, D. R., Fowler, K. T., Gorelick, F. S., and Andrews, N. W. (2003) Impaired membrane resealing and autoimmune myositis in synaptotagmin VII-deficient mice. *J. Cell Biol.* **162**, 543–549
35. Kang, S., Okuno, T., Takegahara, N., Takamatsu, H., Nojima, S., Kimura, T., Yoshida, Y., Ito, D., Ohmae, S., You, D. J., Toyofuku, T., Jang, M. H., and Kumanogoh, A. (2012) Intestinal epithelial cell-derived semaphorin 7A negatively regulates development of colitis *via* αvβ1 integrin. *J. Immunol.* **188**, 1108–1116
36. Hübner, R. H., Gitter, W., El Mokhtari, N. E., Mathiak, M., Both, M., Bolte, H., Freitag-Wolf, S., and Bewig, B. (2008) Standardized quantification of pulmonary fibrosis in histological samples. *Biotechniques* **44**, 507–511, 514–517
37. Chabbert-de Ponnat, I., Marie-Cardine, A., Pasterkamp, R. J., Schiavon, V., Tamagnone, L., Thomasset, N., Bensussan, A., and Boumsell, L. (2005) Soluble CD100 functions on human monocytes and immature dendritic cells require plexin C1 and plexin B1, respectively. *Int. Immunol.* **17**, 439–447
38. Landsman, L., and Jung, S. (2007) Lung macrophages serve as obligatory intermediate between blood monocytes and alveolar macrophages. *J. Immunol.* **179**, 3488–3494
39. Li, Y., Wang, P., Xu, J., Gorelick, F., Yamazaki, H., Andrews, N., and Desir, G. V. (2007) Regulation of insulin secretion and GLUT4 trafficking by the calcium sensor synaptotagmin VII. *Biochem. Biophys. Res. Commun.* **362**, 658–664
40. Colvin, R. A., Means, T. K., Diefenbach, T. J., Moita, L. F., Friday, R. P., Sever, S., Campanella, G. S., Abrazinski, T., Manice, L. A., Moita, C., Andrews, N. W., Wu, D., Hacohen, N., and Luster, A. D. (2010) Synaptotagmin-mediated vesicle fusion regulates cell migration. *Nat. Immunol.* **11**, 495–502
41. Vogel, D. Y., Heijnen, P. D., Breur, M., de Vries, H. E., Tool, A. T., Amor, S., and Dijkstra, C. D. (2014) Macrophages migrate in an activation-dependent manner to chemokines involved in neuro-inflammation. *J. Neuroinflammation* **11**, 23
42. Bacaj, T., Wu, D., Yang, X., Morishita, W., Zhou, P., Xu, W., Malenka, R. C., and Südhof, T. C. (2013) Synaptotagmin-1 and synaptotagmin-7 trigger synchronous and asynchronous phases of neurotransmitter release. *Neuron* **80**, 947–959
43. Czibener, C., Sherer, N. M., Becker, S. M., Pypaert, M., Hui, E., Chapman, E. R., Mothes, W., and Andrews, N. W. (2006) Ca^{2+} and synaptotagmin VII-dependent delivery of lysosomal membrane to nascent phagosomes. *J. Cell Biol.* **174**, 997–1007
44. Neuland, K., Sharma, N., and Frick, M. (2014) Synaptotagmin-7 links fusion-activated Ca^{2+} entry and fusion pore dilation. *J. Cell Sci.* **127**, 5218–5227
45. Becker, S. M., Delamarre, L., Mellman, I., and Andrews, N. W. (2009) Differential role of the Ca^{2+} sensor synaptotagmin VII in macrophages and dendritic cells. *Immunobiology* **214**, 495–505
46. Liu, H., Bai, H., Hui, E., Yang, L., Evans, C. S., Wang, Z., Kwon, S. E., and Chapman, E. R. (2014) Synaptotagmin 7 functions as a Ca^{2+}-sensor for synaptic vesicle replenishment. *eLife* **3**, e01524
47. Herzog, E. L., Mathur, A., Tager, A. M., Feghali-Bostwick, C., Schneider, F., and Varga, J. (2014) Review: interstitial lung disease associated with systemic sclerosis and idiopathic pulmonary fibrosis: how similar and distinct? *Arthritis Rheumatol.* **66**, 1967–1978

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