Semaphorin 7a$^+$ Regulatory T Cells Are Associated with Progressive Idiopathic Pulmonary Fibrosis and Are Implicated in Transforming Growth Factor-β1–induced Pulmonary Fibrosis

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***Rationale*: Lymphocytes are increasingly associated with idiopathic pulmonary fibrosis (IPF). Semaphorin 7a (Sema 7a) participates in lymphocyte activation.**

***Objectives*: To define the relationship between Sema 7a and lymphocytes in IPF.**

***Methods*: We characterized the significance of Sema 7a$^+$ lymphocytes in humans with IPF and in a mouse model of lung fibrosis caused by lung-targeted, transgenic overexpression of TGF-β1. We determined the site of Sema 7a expression in human and murine lungs and circulation and used adoptive transfer approaches to define the relevance of lymphocytes coexpressing Sema7a and the markers CD19, CD4, or CD4$^+$CD25$^+$FoxP3$^+$ in TGF-β1–induced murine lung fibrosis.**

***Measurements and Main Results*: Subjects with IPF show expression of Sema 7a on lung CD4$^+$ cells and circulating CD4$^+$ or CD19$^+$ cells. Sema 7a expression is increased on CD4$^+$ cells and CD4$^+$CD25$^+$ FoxP3$^+$ regulatory T cells, but not CD19$^+$ cells, in subjects with progressive IPF. Sema 7a is expressed on lymphocytes expressing CD4 but not CD19 in the lungs and spleen of TGF-β1–transgenic mice. Sema 7a expressing bone marrow–derived cells induce lung fibrosis and alter the production of T-cell mediators, including IFN-γ, IL-4, IL-17A, and IL-10. These effects require CD4 but not CD19. In comparison to Sema 7a-CD4$^+$CD25$^+$FoxP3$^+$ cells, Sema7a$^+$CD4$^+$CD25$^+$ FoxP3$^+$ cells exhibit reduced expression of regulatory genes such as IL-10, and adoptive transfer of these cells induces fibrosis and remodeling in the TGF-β1–exposed murine lung.**

***Conclusions*: Sema 7a$^+$CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells are associated with disease progression in subjects with IPF and induce fibrosis in the TGF-β1–exposed murine lung.**

Keywords: Semaphorin; lung; fibrosis; TGF-β1; regulatory T cells

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The role of regulatory T cells (Tregs) in idiopathic pulmonary fibrosis (IPF) is unclear. Semaphorin 7a (Sema 7a) critically mediates experimentally induced lung fibrosis and lymphocyte activation; however, a relationship between Tregs and Sema 7a has not been assessed in IPF.

What This Study Adds to the Field

This study demonstrates that Sema 7a$^+$ expression on Tregs is increased in the circulation of subjects with rapidly progressive IPF. Sema 7a$^+$ Tregs are sufficient to induce fibrosis in the TGF-β1–exposed murine lung. These data suggest that strategies targeting Sema 7a and/or aberrant Treg responses might be beneficial in IPF.

Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease for which there is no cure and, currently, no approved therapies in the United States (1). Because treatments are limited, patients have few options beyond supplemental oxygen and lung transplantation (2). Thus, better understanding of the factors regulating this disease is critical. Current paradigms for the development of IPF include recurrent or prolonged epithelial injury followed by inflammation and an abnormal wound-healing response (3). The immunologic factors promoting these effects remain unclear but are important areas of investigation in both human disease and animal models (3).

Semaphorin 7A (Sema 7a) is a glycophosphatidylinositol-anchored membrane protein that is required for axon track formation during embryonic development (4, 5). Sema 7a also regulates inflammatory responses via stimulation of macrophage chemotaxis and cytokine production (6), dendritic cell migration and chemokine expression (7), modulation of T-cell function (6–8), and regulation of collagen production by fibrocytes (9). Hematopoietic expression of Sema 7a is sufficient to induce fibrosis in TGF-β1–induced murine lung fibrosis (9); however, the mechanism(s) mediating these effects remain unknown.

The role of lymphocytes in experimentally induced and human lung fibrosis is controversial (10). Strong experimental evidence demonstrates that lymphocytes are not required for bleomycin-induced collagen accumulation and remodeling in the murine lung (11). However, emerging data suggest that certain B- (12) and T-cell (13–15) populations participate in

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the immunopathogenesis of fibrosis. These reports are conflicting, with several studies suggesting that $CD4^+$ cells possessing suppressor or regulatory abilities either protect (14, 16) or promote (15, 17) fibrotic responses. Abnormalities in regulatory T cells (Tregs) are seen in the lungs and blood of patients with several forms of lung fibrosis (18), leading to the supposition that Tregs might impede fibrosis (16). Curiously, despite its importance as a regulator of lymphocyte biology, a role for Sema 7a in these processes has never been studied.

We characterized the significance of Semaphorin 7a in a cohort of subjects with IPF and in a mouse model of pulmonary fibrosis. Using a translational approach combining human studies with a model of experimentally induced murine lung fibrosis caused by transgenic TGF-β1 overexpression, we find that Sema $7a^+$ Tregs are increased in the blood of subjects with rapidly progressive IPF and that adoptive transfer of Sema $7a^+$ Tregs induces fibrosis in the TGF-β1–exposed murine lung.

METHODS

Detailed methods are available in the online supplement.

Animals

Mouse experiments were approved by Yale's Institutional Animal Care and Use Committee. The CC10-tTS-rtTA-TGF-β1 transgenic mice used in this study use the Clara cell 10-kD protein (CC10) promoter to specifically express bioactive human TGF-β1 to the lung (17). The Semaphorin 7a null mice (Sema $7a^{-/-}$) have been described previously (5) and were a gift from Dr. Alex Kolodkin, Johns Hopkins University. Mice with null mutations of CD4 ($CD4^{-/-}$) or CD19 ($CD19^{-/-}$) were purchased from Jackson Laboratories. All mice were backcrossed for more than 10 generations onto a C57BL/6 background.

Human Subjects

Studies were performed with approval from the Human Investigation Committee at Yale and the Institutional Review Board of the University of Pittsburgh. Subjects diagnosed with IPF based on current American

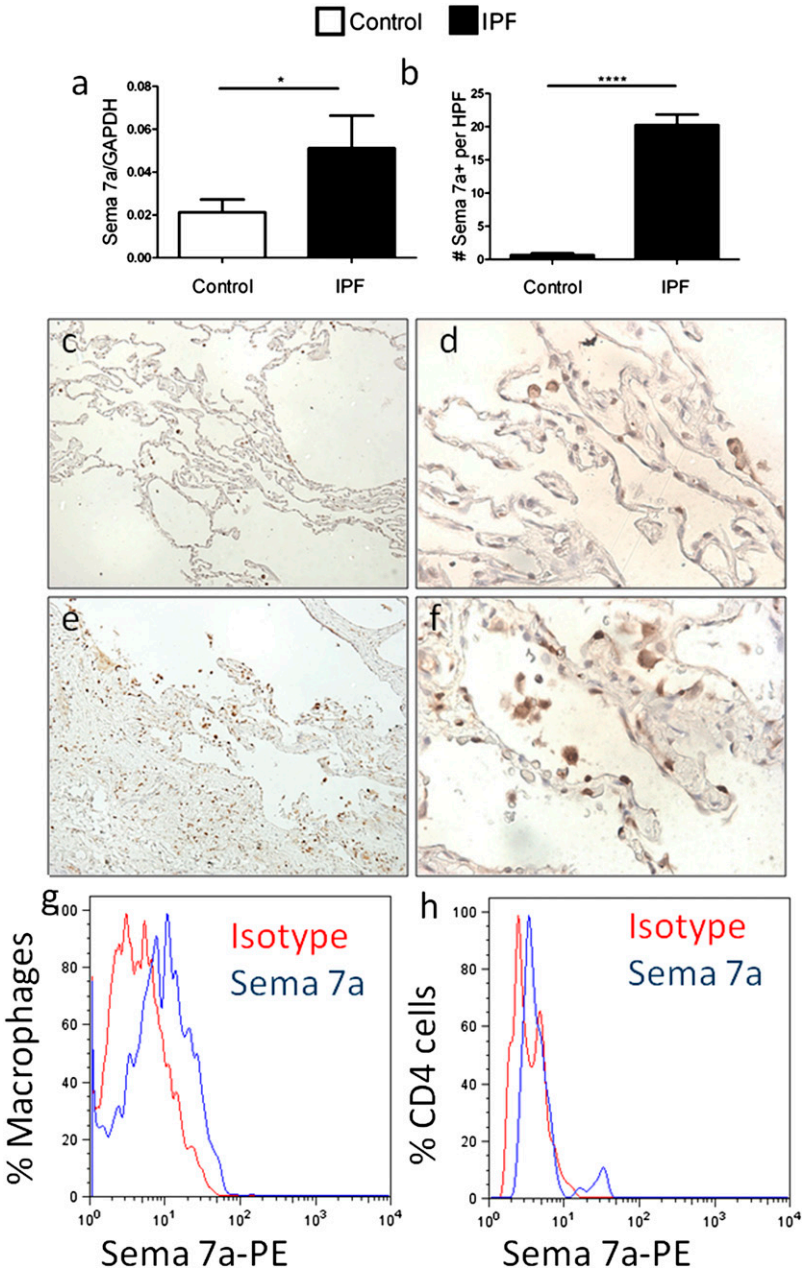

Figure 1. Detection of Semaphorin 7a (Sema 7a) in the human lung. (*a*) A 2.24-fold elevation in expression of Sema 7a, as measured by quantitative real-time polymerase chain reaction, was detected in the lungs of subjects with idiopathic pulmonary fibrosis (IPF) in comparison to control lung tissue (n = 5/group). (*b*) Quantification of Sema $7a^+$ cells after immunostaining in lung tissue sections from nonfibrotic control lung (n = 5) or subjects with IPF (n = 5) detected a nearly 20-fold increase in Sema $7a^+$ cells in IPF. (*c–f*) Representative sections from control (*c*, *d*) and IPF (*e*, *f*) patient lung tissue immunostained for Sema 7a cells and counterstained with hematoxylin. Sema $7a^+$ cells have the morphological appearance of inflammatory cells and appear brown. (*g*, *h*) Flow cytometric identification of Sema 7a on lung macrophages (*g*) and CD4 cells (*h*). Compared with isotype control (*red*) cells, the cells stained with PE-labeled Sema 7a show a significant shift to the right (*blue*). These images are representative of three separate samples. *c* and *e* are 4× original magnification; *d* and *f* are 40× original magnification. $*P \leq 0.05$, $****P \leq 0.0001$. Data are expressed as mean ± SE.

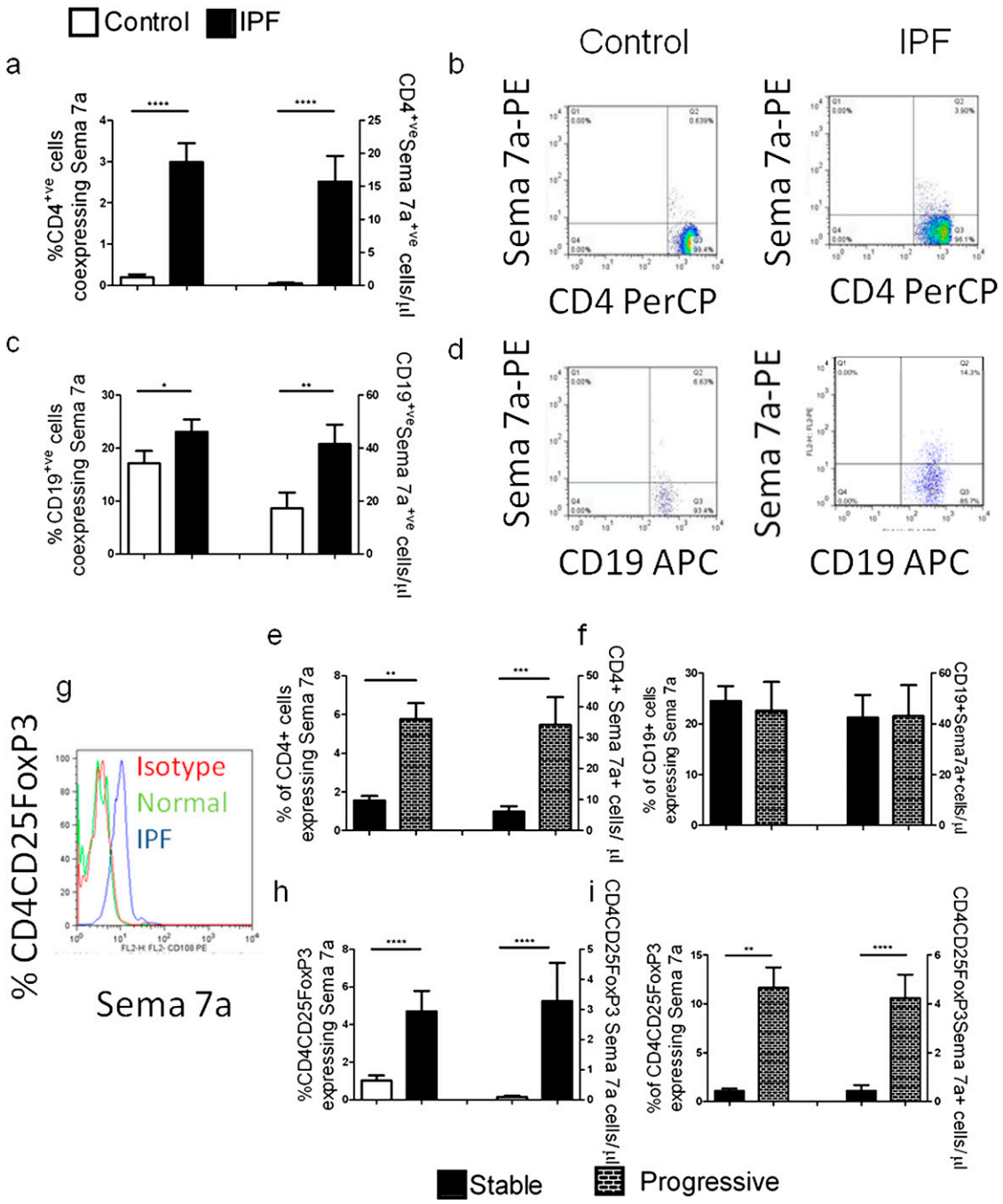

Figure 2. (*a*) A 15.04-fold increase in percentages (*left*) and a 50.7-fold increase in quantities (*right*) of $CD4^+$ cells that coexpress Semaphorin 7a (Sema 7a) are seen in the blood of subjects with idiopathic pulmonary fibrosis (IPF) (*solid bars*) compared with age-matched control subjects (*open bars*). (*b*) Flow cytometric detection of Sema 7a (*Y axis*) on CD4 cells (*X axis*) in (*left*) control subjects and (*right*) subjects with IPF. (*c*) A 1.35-fold increase in percentages (*left axis*) and a 2.41-fold increase in quantities (*right axis*) of $CD19^+$ cells coexpressing Sema 7a are seen in the blood of subjects with IPF (*solid bars*) compared with age-matched control subjects (*open bars*). (*d*) Flow cytometric detection of Sema 7a (*Y axis*) on and CD19 (*X axis*) in (*left*) normal and (*right*) IPF sample. (*e*) We found 3.72-fold increased percentages (*left axis*) and 5.55-fold increased quantities (*right axis*) of $CD4^+$ cells that coexpress Sema 7a in subjects with stable (*solid bars*) and progressive (*checkered bars*) IPF. (*f*) No increase in percentages (*left axis*) or quantities (*right axis*) of $CD19^+$ cells that coexpress Sema 7a in subjects with stable (*solid bars*) and progressive (*checkered bars*) IPF. (*g*) Histogram showing increased Sema $7a^+CD4^+CD25^+FoxP3^+$ Tregs in subjects with IPF (*blue line*) compared with normal control subjects (*green line*) and isotype control (*red line*). (*h*) Quantities (*left*) and percentages (*right*) of Sema $7a^+CD4^+CD25^+FoxP3^+$ Tregs are increased in IPF (*solid bars*) compared with control (*open bars*). (*i*) Compared with subjects with stable IPF, peripheral blood mononuclear cells obtained from subjects with rapidly progressive IPF demonstrate 10.77-fold increased percentages (*left*) and 9.51-fold increased quantities (*right*) of $CD4^+CD25^+FoxP3^+$ cells that coexpress Sema 7a. $^*P \leq 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P \leq 0.0001$. For comparisons *a–d*, IPF n = 38 and control n = 42. For comparisons *e–h*, stable n = 25 and progressive n = 13. Data are expressed as mean ± SE.

TABLE 1. CHARACTERISTICS OF SUBJECTS WITH PROGRESSIVE AND STABLE IDIOPATHIC PULMONARY FIBROSIS

	Progressive (*N* = *13*)	Stable (*N* = *25*)	*P* Value
Age, yr	72.71 (69.6813–75.7411)	68.22 (64.0894–72.3618)	0.1394
Sex			
Female	2 (15.38)	6 (24.00)	0.5366
Male	11 (84.62)	19 (76.00)	
Race			
White	12 (92.31)	22 (88.00)	0.6814
Not white	1 (7.69)	3 (12.00)	
FVC, % predicted	65.38 (57.89–72.86)	67.92(61.79–74.04)	0.5808
D_{LCO}, % predicted	42.38 (30.80–53.96)	44.84 (39.74–49.93)	0.6804
Outcome			
⩾10% Drop in % FVC	2		
AE (fatal)	5		
AE (nonfatal)	1		
Death	5		

Definition of abbreviation: AE = acute exacerbation; D_{LCO} = diffusing capacity of the lung for carbon monoxide.
Data are expressed as means with 95% confidence intervals or n (%).

Thoracic Society criteria (1, 19) were eligible. Exclusion criteria included: (*1*) inability to provide informed consent; (*2*) nonfibrotic lung disease; (*3*) unstable cardiac, vascular, or neurologic disease; (*4*) malignancy; (*5*) pregnancy; (*6*) chronic infection. Clinical data including age, sex, race/ethnicity, comorbidities, medications, and FVC % predicted and diffusion capacity of carbon monoxide (% D_{LCO}) were collected. Age-matched control subjects meeting the same exclusion criteria were recruited from the local community and from Yale's Program on Aging.

Doxycycline Administration

Eight- to ten-week-old TGF-β1 transgene-positive (Tg^+) mice or their wild-type littermate control mice (transgene-negative, Tg^-) were given doxycycline 0.5 mg/ml in their drinking water for up to 2 weeks.

Bone Marrow Transplantation

Transplantation of bone marrow–derived cells (BMDCs) was performed as previously described (9).

Adoptive Transfer of Tregs

Tregs were generated and injected via tail vein every 72 hours in a modification of previously described protocols (20, 21).

Animal Harvest

Bronchoalveolar lavage (BAL), tissue harvest, and Luminex determination of BAL cytokines were performed as described (9, 22).

Flow Cytometric Analysis

Processing of mouse or human lung and peripheral blood mononuclear cells (PBMCs) for fluorescence-activated cell sorter analysis was performed as previously described (9, 23). Tregs were identified using mouse or human Regulatory T Cell Staining Kit (EBioscience, San Diego, CA). Intracellular cytokine staining was performed using the Th1/Th2/Th17 Phenotyping Kit (BD Pharmingen, Franklin Lakes, NJ). Semaphorin 7a antibody was obtained from R&D Systems (Minneapolis, MN). Flow cytometry was performed using a BD FACSCalibur (San Jose, CA). Data were analyzed using Flow Jo v 7.5 software (Tree Star, Inc, Ashland, OR).

Sircol Analysis

Lung collagen was measured by Sircol Assay (Biocolor Ltd., Carrickfergus, UK) as previously described (9).

Histologic Analysis

Formalin-fixed and paraffin-embedded lung sections were stained with hematoxylin/eosin or Mallory's trichrome stains. Sema 7a immunohistochemistry was performed using anti-human Sema 7a antibody (Genetex, Irvine, CA).

Immunofluorescence

Immunofluorescence of mouse lung digests was performed using antibodies against CD19, CD4, F4/80 (BD Pharmingen), and Sema 7a (Genetex).

Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated and reverse transcribed (24). All primers were obtained from Superarray Bioscience (Frederick, MD).

Statistical Analysis

Parametric data were compared by Student *t* test or analysis of variance. Nonparametric data were compared using the Mann-Whitney *U*. Statistical analysis was performed using SAS (Research Triangle Park, NC). Graphs were generated using Graphpad (Graphpad Software Inc., La Jolla, CA).

RESULTS

Sema 7a Expression Is Increased in IPF Lungs

IPF is associated with TGF-β1 overexpression (24, 25). Because Sema 7a regulates TGF-β1–driven fibrosis, we believed that Sema 7a might be present in IPF lungs. Thus, we obtained IPF tissue from the Lung Tissue Research Consortium, or control tissue from the tumor-free margin of cancer resections performed at Yale, and assessed Sema 7a expression by quantitative real-time polymerase chain reaction and immunohistochemistry. Samples were assessed by a lung pathologist to confirm usual interstitial pneumonia pathology or the absence of cancer. Relative to control lungs, IPF lungs showed increased expression of Sema 7a ($P = 0.027$; Figure 1a) that was confirmed by immunohistochemistry in which, in contrast to control lungs, IPF lung demonstrated increased Sema 7a–expressing cells ($P < 0.0001$; Figure 1b). These findings were confirmed in a second cohort of patients with IPF and rejected organ donors obtained from the University of Pittsburgh Lung Transplant Program (Figures 1c–1f and data not shown).

Sema 7a Expression in the IPF Lung Localizes to $CD4^+$ Lymphocytes and Macrophages

We next determined the site(s) of Sema 7a expression in IPF. Because the autofluorescence of the IPF lungs rendered double

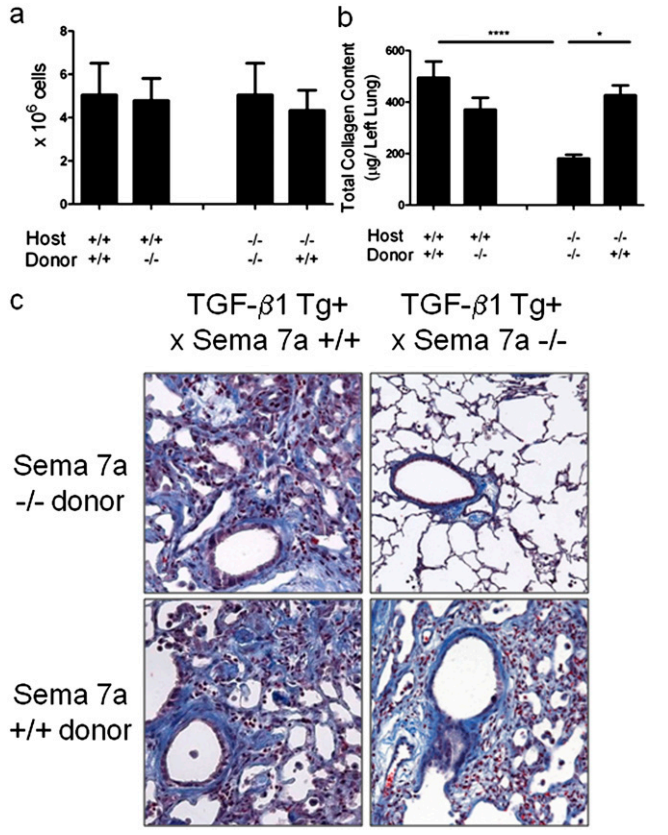

Figure 3. (*a–c*) Bone marrow–derived Semaphorin 7a (Sema 7a)$^+$ cells are sufficient for transforming growth factor (TGF)-β1–induced lung fibrosis. Reconstitution of TGF-β1 × Sema 7a$^{-/-}$ mice with bone marrow from Sema 7a$^{+/+}$ mice had no effect on bronchoalveolar lavage cell accumulation (*a*) but did restore the TGF-β1–induced lung fibrotic response as measured by total soluble collagen quantitated using the Sircol assay (*b*) and collagen deposition visualized using Masson trichrome staining (*c*). Removal of Sema 7a on BMDC had no effect on these outcomes. n ⩾ 5 mice/group, three repetitions. *$P \leqslant 0.05$, ****$P \leqslant 0.0001$. Data are expressed as mean ± SE.

labeling impossible, we performed flow cytometry on lung digests prepared from usual interstitial pneumonia/IPF lung biopsies performed at Yale. Here, Sema 7a was expressed on F4/80$^+$ macrophages and CD4$^+$ lymphocytes. Minimal expression was seen on CD8$^+$ or CD19$^+$ cells (Figures 1g and 1h and data not shown). Technical limitations prevented assessment of epithelial cells or fibroblasts in this manner, as well as assessment of normal lung tissue, and thus our analysis was restricted to immune cells in the IPF lung.

Sema 7a Expression Is Increased in the Blood of Subjects with IPF and Localizes to CD4$^+$ and CD19$^+$ Cells

Because Sema 7a was located on cells of presumed hematopoietic origin in IPF lungs, we believed that Sema 7a might be detected in the IPF circulation. Thus, we compared Sema 7a expression in archived mRNA obtained from normal and IPF PBMCs (n = 15/each group) and found that, similar to the lung, the IPF PBMCs demonstrated increased expression of Sema 7a mRNA ($P < 0.0001$; *see* Figure E1 in the online supplement).

We next prospectively enrolled a cohort of subjects with IPF (n = 38) and age-matched control subjects (n = 42) to prospectively define leukocyte expression of Sema 7a. Subject characteristics are shown in Table E1. Comparison of leukocyte subsets is shown in Figure E1. Because Sema 7a was expressed on CD4 cells and macrophages in IPF lungs, we expected to find augmented expression of Sema 7a on related populations in IPF blood. This prediction proved partially correct, as increased percentages and quantities of Sema 7a–expressing CD4$^+$ cells ($P < 0.0001$, both comparisons) but not monocytes were detected in IPF (Figures 2a and 2b; Figure E1). Examination of other populations found that percentages and quantities of Sema 7a$^+$ CD19$^+$ cells were increased in IPF ($P = 0.046$ and $P = 0.0028$, respectively; Figures 2c and 2d) and that neither quantities nor percentages of Sema 7a$^+$CD8$^+$ cells differed between control and IPF ($P > 0.05$, both comparisons; Figure E1).

We next sought clinical relevance for these increased Sema 7a$^+$ cells. Neither Sema 7a$^+$CD4+ nor Sema 7a$^+$CD19$^+$ cells were associated with % FVC or % D_{LCO} (Tables E2–E4); however, longitudinal follow-up revealed significantly increased percentages and levels of Sema 7a$^+$CD4$^+$ cells ($P = 0.044$ and $P < 0.0001$; Figure 2e) but not Sema 7a$^+$CD19$^+$ cells (Figure 2f) in subjects with IPF who within 1 year experienced progression defined as the composite outcome of either greater than or equal to 10% decline in % FVC, acute exacerbation as defined by Collard and colleagues (26), or death. Baseline characteristics of stable and progressive subjects were equivalent (Table 1). Thus, the increased detection of Sema 7a in the IPF circulation relates primarily to CD4$^+$ and/or CD19$^+$ cells, and Sema 7a$^+$ CD4$^+$ cells are most increased in those patients destined for short-term progression.

Circulating Sema 7a$^+$ Tregs Are Increased in Progressive IPF

These data indicate that circulating Sema 7a$^+$CD4$^+$ cells are increased in subjects with IPF with progressive disease, but they do not lend insight into the CD4$^+$ subpopulation(s) expressing Sema 7a. Because it has been previously shown that IPF Tregs are quantitatively reduced and demonstrate impaired suppressor function (27), and because a related semaphorin (Sema 3B) regulates Treg biology (28), we believed that the increased Sema 7a$^+$CD4$^+$ cells may be partially explained by Sema 7a$^+$ Tregs. This hypothesis was correct, as quantities and percentages of Sema 7a$^+$CD4$^+$CD25$^+$FoxP3$^+$ Tregs were increased in the blood of subjects with IPF compared with control subjects ($P < 0.0001$, both comparisons; Figures 2g and 2h). Sema 7a expression was also increased on presumed non-Treg populations (CD4$^+$CD25$^-$) cells in the subjects with IPF (Figure E2), but because we were primarily interested in the role of Tregs in these subjects we did not further characterize this population. Sema7a$^+$CD4$^+$CD25$^+$FoxP3$^+$ cells were not associated with % FVC or % D_{LCO} (Tables E2–E4), but, like the Sema 7a$^+$CD4$^+$ cells described above, longitudinal follow-up revealed that percentages and quantities of Sema7a$^+$CD4$^+$CD25$^+$FoxP3$^+$ cells were most increased in subjects with progressive IPF ($P = 0.0023$ and $P < 0.0001$, respectively; Figure 2h). No such association was detected for Sema 7a$^-$CD4$^+$CD25$^+$FoxP3$^+$ cells (Figure E2), suggesting that Sema 7a$^+$ Tregs might be unique to progressive IPF.

Hematopoietic Expression of Sema 7a Is Sufficient for Fibrosis and Remodeling in the TGF-β1–exposed Murine Lung

Our human data demonstrate that Sema 7a$^+$ cells are found in IPF lungs and blood. To determine if hematopoietic expression of Sema 7a reflects or mediates disease, we created bone marrow chimeras to assess whether Sema 7a$^+$ BMDCs are necessary, sufficient, or both, to induce fibrosis in the TGF-β1–exposed murine lung. Consistent with our previous results (9), chimeras

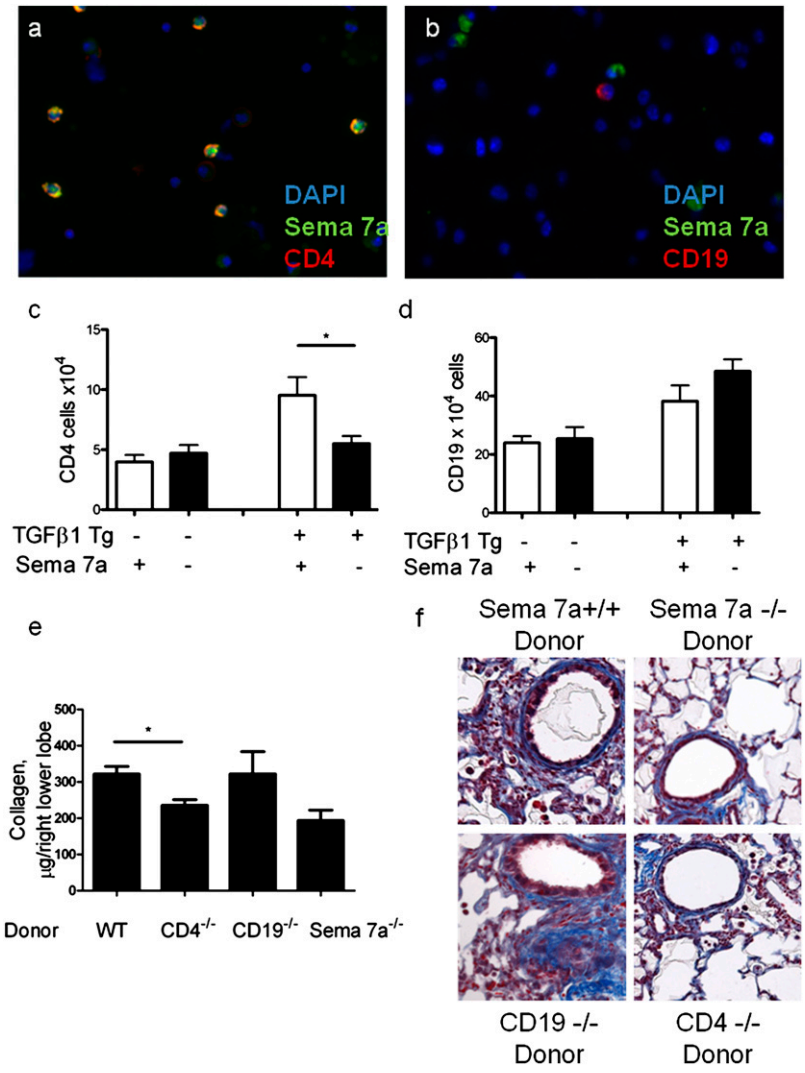

Figure 4. (*a*, *b*) Immunofluorescent colocalization of single cell suspensions from transforming growth factor (TGF)-β1 transgene-positive (Tg^+) mice showing Semaphorin 7a (Sema 7a) (*green*) and CD4 (*red*; *a*) and CD19 (*red*; *b*) indicates that in the TGF-β1–exposed murine lung, Sema $7a^+$ $CD19^+$ cells are very rare, whereas there is robust detection of Sema $7a^+$ $CD4^+$ cells. Nuclei are counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (*blue*). (*c*, *d*) Comparison of lung suspensions prepared the left lung of wild-type (WT) and TGF-β1 Tg^+ mice shows that Sema 7a deletion in the setting of TGF-β1 overexpression leads to reduced quantities of $CD4^+$ (*c*) but not $CD19^+$ cells (*d*). (*e*, *f*) TGF-β1 Tg^+ Sema $7a^{-/-}$ mice were reconstituted with donor bone marrow from wild-type, $CD4^{-/-}$, $CD19^{-/-}$, or Sema $7a^{-/-}$ mice. The effects of donor bone marrow on lung fibrosis after 14 days of transgene activation on (*e*) lung collagen as measured by Sircol assay and (*f*) lung collagen deposition as visualized using Masson's trichrome stain. n ⩾ 5 mice/group, two repetitions. *$P \leqslant 0.05$. Data are expressed as mean ± SE.

in which Sema 7a had been removed from the bone marrow showed unchanged BAL cell counts ($P = 0.88$; Figure 3a) and nonsignificant reductions in TGF-β1–induced lung fibrosis based on Sircol assay ($P = 0.13$; Figure 3b) and lung histology (Figure 3c). In contrast, the TGF-β1 × Sema $7a^{-/-}$ recipients of Sema $7a^{+/+}$ BMDCs also showed unchanged BAL inflammation ($P = 0.35$; Figure 3a), but collagen accumulation was increased ($P = 0.01$; Figure 3b) with remodeling of both periairway and parenchymal regions similar to that seen in the TGF-β1 × Sema $7a^{+/+}$ animals (Figure 3c). These data indicate that Sema $7a^{+/+}$ BMDCs are sufficient, but not necessary, for the induction of fibrosis and remodeling in the TGF-β1–exposed murine lung.

Sema 7a Is Highly Expressed on $CD4^+$ Cells, but Not $CD19^+$ Cells, in the TGF-β1 Model

These data suggested that the circulating Sema 7a–expressing $CD4^+$ or $CD19^+$ cells seen in our subjects with IPF might be mediating disease. To explore this idea, we performed immunofluorescent colocalization of Sema 7a and CD4 or CD19 on digested lungs and spleens of TGF-β1 mice. In these studies, nearly all $CD4^+$ cells coexpressed Sema 7a (Figure 4a and data not shown), but similar to the results seen in IPF lungs there was only minimal coexpression of Sema 7a and CD19 (Figure 4b and data not shown). Furthermore, Sema 7a deletion reduced accumulation of $CD4^+$, but not $CD19^+$ cells, in the TGF-β1–exposed murine lung (Figures 4c and 4d), further suggesting that certain population(s) of $CD4^+$ cell(s) might participate in Sema 7a–induced fibrosis.

Sema 7a–induced Lung Fibrosis Requires CD4 but Not CD19

Our combined human and mouse data suggested a role for CD4 cells in the pathogenesis of Sema 7a–induced fibrosis. A contribution of $CD19^+$ cells seemed less likely. To determine whether these predictions were correct, we again created bone marrow chimeras in which Sema 7a was restricted to the circulation, but this time we used donors with null mutations of CD4 or CD19. After transplantation and recovery, mice received doxycycline for 14 days, and lung fibrosis was assayed by Sircol and histology. As expected, TGF-β1 × Sema $7a^{-/-}$ recipients of Sema$7a^{+/+}CD4^{-/-}$ BMDCs failed to develop the increased total soluble lung collagen ($P = 0.04$; Figure 4e) and histologic appearance of fibrosis seen in the recipients of Sema $7a^{+/+}CD4^{+/+}$ marrow (Figure 4f). In contrast, TGF-β1 × Sema $7a^{-/-}$ mice transplanted with $CD19^{-/-}$ marrow demonstrated sustained lung collagen accumulation ($P > 0.05$ all comparisons; Figures 4e and 4f). These data indicate that CD4 expression is required for Sema 7a–expressing BMDCs to induce TGF-β1–induced murine lung fibrosis.

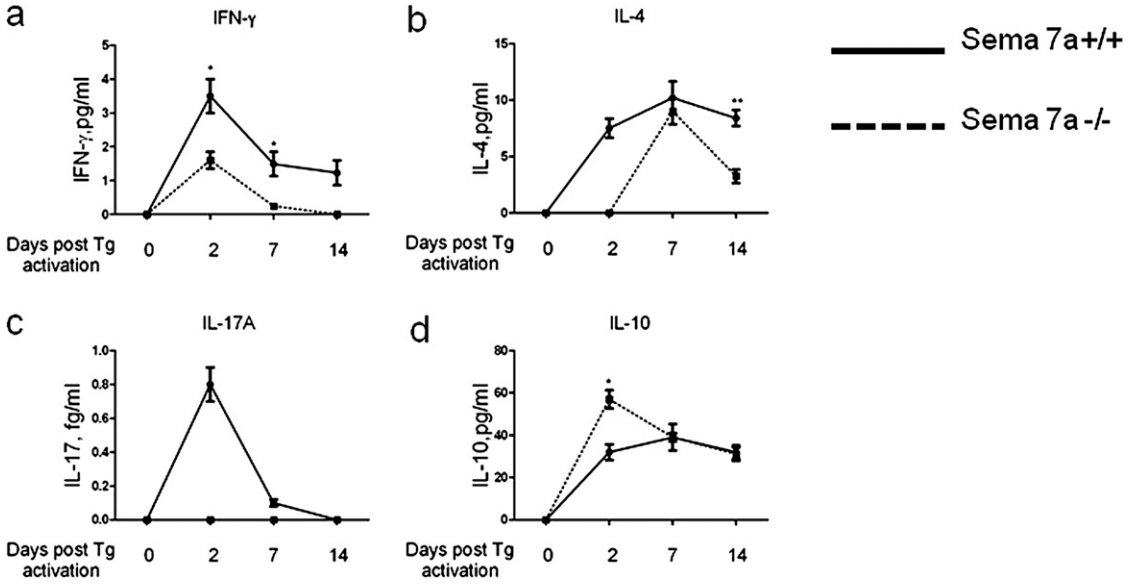

Figure 5. (*a–d*) Kinetic analysis of bronchoalveolar lavage levels of (*a*) IFN-γ, (*b*) IL-4, (*c*) IL-17A, (*d*) IL-10 in transforming growth factor-β1 transgene-positive × Semaphorin 7a (Sema 7a)$^{-/-}$ mice transplanted with bone marrow–derived cells from either Sema 7a$^{+/+}$ mice (*solid line*) or Sema 7a $^{-/-}$ mice (*dotted line*). n ⩾ 4 mice/group, two repetitions. *$P < 0.05$, **$P < 0.01$. Data are expressed as mean ± SE.

Transplantation of Sema 7a$^+$ BMDCs Increases Secretory Products of Effector but Not Tregs

Because the absence of CD4 influenced our model, it seemed likely that abnormalities in secretory products of specific effector and/or regulatory CD4$^+$ subpopulations might be seen. Thus, we repeated the bone marrow transplant studies, harvested mice at early (48 h), intermediate (Day 7), and late (Day 14) time points, and quantified BAL concentrations of mediators associated with Th1 cells (IFN-γ), Th2 cells (IL-4), Th17 cells (IL-17A), and Tregs (IL-10). Unlike TGF-β1 × Sema 7a$^{-/-}$ recipients of Sema 7a$^{-/-}$ BMDCs, recipients of Sema 7a$^{+/+}$ BMDCs demonstrated an early peak in IFN-γ that persisted (albeit at a lesser level) at the intermediate and late time points (Figure 5a). IL-4 levels increased at 48 hours and remained elevated at Days 7 and 14 (Figure 5b). IL-17A peaked at 48 hours and rapidly decreased thereafter (Figure 5c). IL-10 was reduced at 48 hours and then remained similar throughout (Figure 5d). Flow cytometry of lung digests performed at these same time points revealed increased production of IFN-γ, IL-4, and IL-17A by CD4$^+$ cells in the recipients of Sema 7a$^+$ BMDCs (Figure E3). Quantities of Tregs were unchanged in these mice.

Sema 7a$^+$ Tregs Display Reduced Expression of IL-10

Because Treg quantities were unchanged, but IL-10 detection was reduced in the recipients of Sema 7a$^+$ BMDCs, we believed it possible that Sema 7a$^+$ Tregs might exhibit reduced expression of the regulatory cytokine IL-10. To test this hypothesis, we generated Tregs from Sema 7a$^{+/+}$ and Sema 7a$^{-/-}$ mice and assessed these cells for IL-10 expression via quantitative real-time polymerase chain reaction. Consistent with the *in vivo* data shown above, when compared with Tregs that did not express Sema 7a, Tregs derived from Sema 7a$^{+/+}$ mice showed a significant reduction in the relative expression of IL-10 ($P = 0.043$; Figure 6a), suggesting that Sema 7a$^+$ Tregs suffer impaired regulatory function.

Adoptive Transfer of Sema 7a$^+$ Tregs Causes Fibrosis and Remodeling in the TGF-β1–exposed Lung

We last sought to define whether Sema 7a$^+$ Tregs might induce fibrosis in our model. Lacking a method to selectively deplete Sema 7a$^+$ Tregs, we again generated Tregs from Sema 7a$^{+/+}$ and null mutant mice and performed adoptive transfer of these cells into TGF-β1 × Sema 7a$^{-/-}$ mice. Compared with the recipients of Sema 7a$^{-/-}$ Tregs, lungs obtained from mice receiving Sema 7a$^{+/+}$ Tregs showed a 47.6% increase in collagen accumulation in the right upper lobe ($P = 0.015$; Figure 6b) and the histologic appearance of mild fibrosis (Figures 6c and 6d), thereby indicating that Sema 7a$^+$ Tregs are sufficient to induce remodeling and fibrosis in this model.

DISCUSSION

These studies show that Sema 7a is expressed in the lungs and blood of subjects with IPF. In the latter compartment, it localizes in part to CD4$^+$CD25$^+$FoxP3$^+$ Tregs and is associated with a more progressive clinical course. Murine modeling using lung-specific, doxycycline-inducible TGF-β1 overexpression finds that Sema 7a localizes to CD4$^+$ cells in the lungs and spleen and that CD4$^+$ cells are required for the profibrotic effects of Sema 7a$^+$ BMDCs. Sema 7a$^+$ Tregs exhibit reduced expression of the regulatory mediator IL-10 and are sufficient to cause mild fibrosis when adoptively transferred into TGF-β1 × Sema 7a$^{-/-}$ mice. When viewed in combination, these data implicate Sema 7a$^+$ Tregs in the immunopathogenesis of IPF and in experimentally induced lung fibrosis.

Our human studies show that Sema 7a$^+$CD4$^+$CD25$^+$FoxP3$^+$ Tregs are increased in the blood of subjects with rapidly progressive IPF. Our murine studies show that in contrast to Sema 7a$^-$CD4$^+$CD25$^+$FoxP3$^+$ cells, Sema 7a–expressing Tregs show reduced expression of regulatory mediators such as IL-10, and that adoptive transfer of Sema 7a$^+$ Tregs causes collagen accumulation and remodeling in the TGF-β1–exposed lung. Because adoptive transfer of Sema 7a$^-$ Tregs do not cause fibrosis in this model, and because Sema 7a$^-$ Tregs were not associated with progressive IPF, it is possible that Sema 7a expression identifies a population of Tregs that traffics to the lung and contributes to disease progression via impaired suppressor capabilities and permissive effects on profibrotic inflammatory responses, although this hypothesis will require further evaluation, and other mechanisms have not been ruled out (29). Because it has also

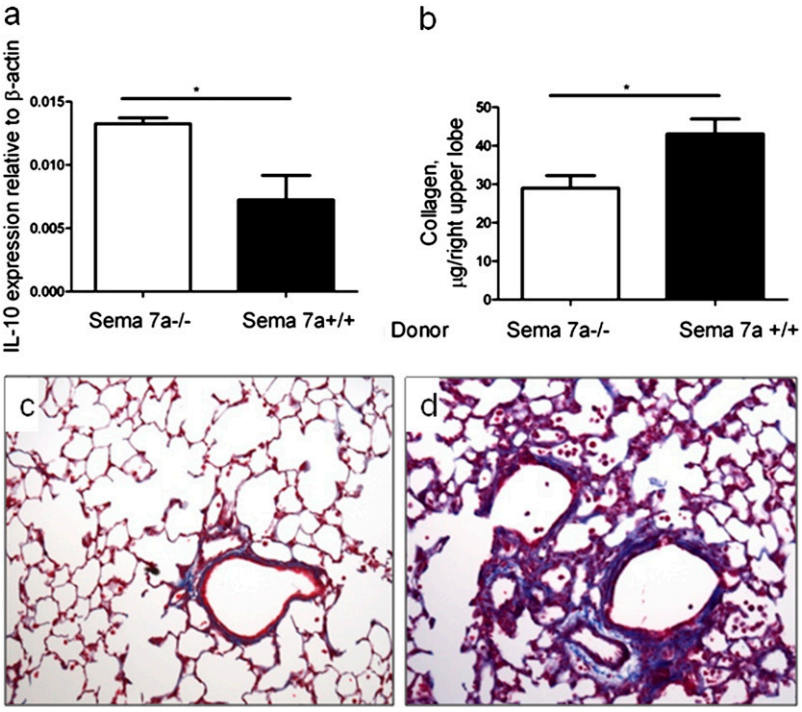

Figure 6. (*a*) Compared with *in vitro*–generated Tregs obtained from Semaphorin 7a (Sema 7a)$^{-/-}$ mice (*open bar*), a 1.82-fold decrease in the relative expression of IL-10 is seen in Sema 7a$^{+/+}$ Tregs (*solid bar*, n = 2 repetitions). (*b*) Compared with transforming growth factor (TGF)-β1 × Sema 7a$^{-/-}$ mice receiving Sema 7a–negative Tregs (*open bar*), adoptive transfer of Sema 7a^{+} CD4^{+}CD25^{+}FoxP3^{+} Tregs (*solid bar*) causes increased total soluble collagen in the right upper lobe as measured via Sircol assay. (*c*, *d*) Compared with TGF-β1 × Sema 7a$^{-/-}$ mice receiving Sema 7a–negative Tregs (*c*), adoptive transfer of Sema 7a^{+} CD4^{+}CD25^{+}FoxP3^{+} Tregs (*d*) causes lung fibrosis both in the periairway and septal regions as seen on these 10× images of Masson trichrome stain. n = 4–5 mice/group, two repetitions. *$P < 0.05$. Data are expressed as mean ± SE.

been shown that identification of CD4^{+}CD28^{-} cells in the blood of subjects with IPF predicts reduced event-free survival (30), it is intriguing to speculate that the Sema 7a^{+} Tregs we report are related to this cell population as well. Our human studies are limited by the relatively small number of subjects, enrollment at only one center, and the predominance of male subjects in our IPF cohort, which differs significantly from our enrolled control subjects. Nevertheless, our human findings demonstrate the important role that Sema 7a and dysregulated T-cell responses might play in the clinical monitoring and disease progression of IPF and thus warrant further study.

Our data also indicate that other CD4^{+} populations might participate in Sema 7a–induced lung fibrosis. For example, we detected increased Sema 7a^{+} CD4^{+}CD25^{-} cells in subjects with IPF, although because we did not further characterize these cells we cannot speculate on their identity. Transfer of Sema 7a^{+} BMDCs in the mouse model causes early peaks in Th1 and Th17-related mediators such as IFN-γ and IL17A, suggesting a role for these subsets in the early events in fibrosis, whereas the increase in IL-4 at later time points supports a role for Th2 cells in the establishment/maintenance of the fibrotic milieu. Sema 7a could influence one or all of these populations. Indeed, transfer of Sema 7a BMDCs and CD4^{+} cells causes a stronger fibrotic response than transfer of Sema 7a^{+} Tregs alone, supporting this hypothesis. Adoptive transfer of Sema 7a^{+} effector T-cell populations, as well quantification of these subsets in the IPF blood, would further clarify this question.

Although our study sheds light on the significance of Sema 7a^{+} Tregs in pulmonary fibrosis, several questions remain unanswered. We have not explored the contribution of Sema 7a expression on other cells, such as macrophages and epithelial cells, both of which are important drivers of Sema 7a–mediated T-cell activation (6, 8) and fibrosis (24, 31). Our study does not determine the receptor through which Sema 7a affects TGF-β1–induced lung fibrosis, although we have previously shown that Sema 7a's effects on human fibrocytes and murine lung fibrosis occur in a β1 integrin–dependent manner (9). We also have not defined expression of Sema 7a on murine Tregs, or a requirement for Sema 7a–expressing Tregs in our model, which will require the creation of reagents allowing site-specific Sema 7a deletion on Tregs. It should also be mentioned that we found increased Tregs in subjects with IPF compared with normal age-matched control subjects, which differs from one previously published report (27). Because our control values are consistent with previously published values for this age range (32, 33), this difference likely relates differences in the age and sex of our control subjects (both of which are known to influence quantities of circulating Tregs), unrecognized differences in the subjects with IPF studied related to recruitment under the updated 2010 American Thoracic Society criteria, and differences in processing technique. However, although certain details may differ, our manuscript supports the main conclusions of this prior study by highlighting the potential role of dysregulated Tregs in the immunopathogenesis of IPF, and our current studies demonstrating a novel role for Sema 7a^{+} Tregs in the induction of pulmonary fibrosis warrant further investigation.

Conclusions

Sema 7a^{+}CD4^{+}CD25^{+}FoxP3^{+} cells are elevated in subjects with rapidly progressive IPF and cause fibrosis in the TGF-β1–exposed murine lung. Further studies targeting Sema 7a and Tregs might lead to better understanding of the pathogenic immune responses driving IPF.

Author disclosures are available with the text of this article at www.atsjournals.org.

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