SUPPLEMENTAL METHODS

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 employ the Clara cell 10-kDa protein (CC10) promoter to specifically express bioactive human TGF-β1 to the lung (17). The Semaphorin 7a null mice have been described previously (1) and were a gift from Dr. Alex Kolodkin, Johns Hopkins University. Mice will null mutations of CD4 or CD19 were purchased from Jackson Laboratories. All mice were backcrossed for >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 ATS criteria (2, 3) were eligible and were excluded based on: (1) Inability to provide informed consent; (2) Non-fibrotic lung disease; (3) Unstable cardiac, vascular, or neurologic disease; (4) Malignancy; (5) Pregnancy; (6) chronic infection. Clinical data including age, sex, race/ethnicity, co-morbidities, medications and percent-predicted Forced Vital Capacity (FVC) and Diffusion Capacity of Carbon Monoxide (DLCO) were collected. Age-matched controls were free of inflammatory conditions and were recruited from the local community and from Yale's Program on Aging. Subjects with IPF were followed prospectively for one year and categorized as "progressive" if they met one of the following three composite criteria: >10% decline in percent-predicted FVC, acute exacerbation according to the criteria of Collard et al (4), or death.

Doxycycline Administration

8-10 week old TGF-β1 Tg+ or their wild-type littermate controls (transgene negative, Tg-) mice were given doxycycline 0.5 mg/ml in their drinking water for up to 2 weeks.

Bone Marrow Transplantation

Bone marrow chimeras of the TGF-β1 and Sema $7a^{-/-}$ mice were created as previously described (5). Briefly, recipient mice were lethally irradiated with 800 cGy using a Seimens stabilipan Cesium irradiator. Donor mice were sacrificed and bone marrow was obtained by flushing the femurs with DMEM supplemented with 5% fetal bovine serum. Approximately eight hours after irradiation, recipient mice received 1 x 10^6 whole bone marrow derived cells (BMDCs) via tail vein injection. Following marrow transfer, recipient mice received antibiotics in their drinking water until the time of transgene activation.

Adoptive Transfer of Tregs

Regulatory T cells were generated and injected via tail vein every 72 hours in a modification of previously described protocols (6, 7). All procedures were performed under sterile conditions. Briefly, CD4+CD25- cells were isolated from murine spleens using magnetic labeled beads (Miltenyi, Auburn, CA). Purity of CD4+CD25- population was assessed by flow cytometry. Cells were cultured in antibiotic free RPMI and stimulated with monoclonal antibodies against CD3 and CD28 (kind gift of Dr. Lauren Cohn, Yale University) in the presence of recombinant murine TGF-β1 (R&D Systems) for 5 days. Treg phenotype was confirmed by FACS. Sema 7a expression was assessed using immunofluorescence as above. 5 x 10^5 cells were adoptively transferred into TG-Fβ1 x Sema 7a-/- mice via tail vein injection starting from the night before doxycycline administration.

Animal Sacrifice

Bronchoalveolar lavage, tissue harvest, and Luminex determination of BAL cytokines were performed as described (5, 8).

Flow cytometric analysis

Processing of mouse or human lung and PBMCs for FACS was performed as previously described (5, 9). 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). PE-labeled anti-human Semaphorin 7a antibody was obtained from R&D Systems (Minneapolis, MN). Flow cytometry was performed using a BD FACSCalibur. 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., UK) as previously described (5).

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

Immunofluoresence of mouse lung digests was performed using antibodies against CD19, CD4, F4/80 (BD Pharmingen) and Sema 7a (Genetex, Irvine CA). Fluorochrome-conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, CA). Slides were counterstained with DAPI and mounted with vectashield. Microscopy was performed using a Nikon Eclipse Ti-S microscope equipped with a Andor Technology camera

Quantitative Real Time PCR

RNA was isolated and reverse transcribed as we have previously described (10). Primers against human and/or murine Sema 7a, β-actin, TGF-β1, IL-10, and GAPDH were obtained from Superarray Bioscience (Frederick, MD). Quantitative PCR was performed using an ABI 7500.

Statistical Analysis

Data were assessed for normalcy using the D'Agostino and Pearson Omnibus test. Parametric data were compared by Student's t-test or ANOVA .Nonparametric data were compared using the Mann-Whitney U test. Categorical data were compared using Fischer's exact test. Statistical analysis was performed using SAS (Research Triangle Park, NC). Graphs were generated using Graphpad (Graphpad Software Inc., La Jolla, CA).

Table S1. Baseline characteristics of subjects

	IPF N=38	Control N=42	P value
Age, years	69.76 (59.97– 71.30)	65.60 (59.9 - 71.3)	0.2088
Sex Female Male	 8 (21.05%) 30(78.95%)	 22 (52.4%) 20 (47.6%)	0.0038
Race Caucasian Not Caucasian	 34 (89.47%) 4 (10.53%)	 38 (90.5%) 4 (9.5%)	0.2850
FVC, Percent Predicted	67.05 (62.47– 71.63)	NA	NA
DLCO, Percent Predicted	44.00 (39.13- 48.86)	NA	NA

Data are expressed as mean with 95% confidence intervals.

Table S2. P values of CD19+ Sema 7a+ cells compared by disease severity.

	% CD19+ cells also expressing Sema 7a	Number of CD19 cells expressing Sema 7a
Comparison of %FVC equal to or above vs. below the median value (66%)	0.38	0.61
Comparison of %DLCO equal to or above vs. below the median value (50%)	0.29	0.30

Table S3. P values of CD4+ Sema 7a+ cells compared by disease severity.

	%CD4+ cells also expressing Sema 7a	Number of CD4 cells expressing Sema 7a
Comparison of %FVC equal to or above vs. below the median value (66%)	0.22	0.14
Comparison of %DLCO equal to or above vs. below the median value (50%)	0.49	0.54

Table S4. P values of Sema 7a+ Tregs compared by disease severity.

	% of Tregs expressing Sema 7a	Number of Tregs expressing Sema 7a
Comparison of %Pred FVC equal to or above vs. below the median value (66%)	0.31	0.97
Comparison of %Pred DLCO equal to or above vs. below the median value (50%)	0.30	0.69

%FVC = percent predicted forced vital capacity

%DLCO = percent predicted diffusion capacity for carbon monoxide

Tables S2 – S4. Objective parameters of disease severity were divided at the median value. Percentages and quantities of Sema 7a+ lymphocyte populations of interest were compared in each group. P values are shown above.

Figure E1.

A.Expression of Sema 7a relative to β-actin in PBMCs obtained from control (white, n=15) and IPF (black, n=15) subjects. B-C. Comparison of percentages (right) and quantities (left) of CD4+ cells (B) and CD19+ cells (C) in PBMCs obtained from control (white bar, n=42) and IPF (black bar, n=38). Because gating for CD4 and CD19 cells were performed using different staining techniques (with and without permeabilization, respectively), the CD4 and C19 data are not directly comparable. Data are expressed as mean +/- standard error.

Figure E2.

A. Comparison of percentages (right) and quantities (left) of Sema 7a+ monocytes in control (white bar) vs IPF (black bar). B. Percentages (left) and quantities (right) of Sema 7a+ CD8+ cells do not differ between control (white) and IPF (black) PBMCS. C) Percentages and quantities of Tregs in control vs IPF. D) Percentages (right) and quantities (left) of Sema 7a+CD4+CD25- cells in control (white) vs IPF (black) PBMCs. E.) Percentages and quantities of Sema 7a-CD4+CD25+FoxP3+ cells in stable (black) vs. progressive (checkered) IPF. F.) Percentage and quantities of CD4+CD25+FoxP3+ cells in stable (black) vs. progressive (checkered) IPF. ****$p<0.0001$. For all comparisons, IPF n = 38 and control n = 42.

Figure E3.

Flow cytometric detection of CD4 cells co-expressing A) IFN-γ B) IL-4 C) IL-17A, along with D) CD4+CD25+FoxP3+ Tregs in lung cells obtained from TGF-β1 Tg+ x Sema 7a -/- mice transplanted with WT BMDCs (black line) or Sema 7a-/- bone marrow (dotted line). Analysis was performed at 48 hours, 7 days, and 14 days post transplant. $N \geq 4$ mice per group, one iteration. *$p<0.05$.

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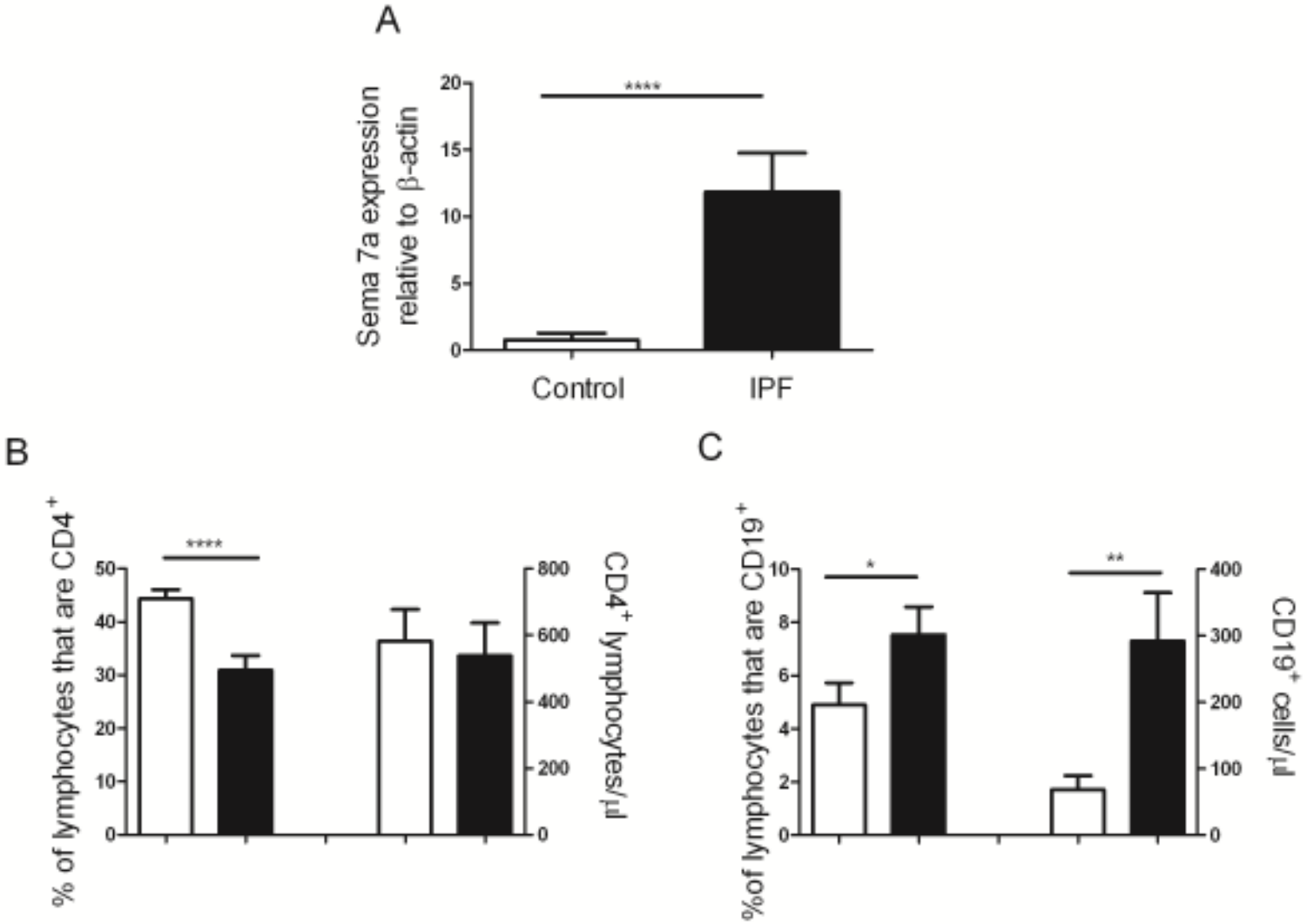
A
Sema 7a expression relative to β-actin
20
15
10
5
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****
Control
IPF
B
% of lymphocytes that are CD4+
50
40
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800
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400
200
0
CD4+ lymphocytes/μl
C
%of lymphocytes that are CD19+
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400
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100
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CD19+ cells/μl

a
Control
IPF
% of monocytes that are Sema 7a+
Sema 7a+ monocytes/μl
b
%of CD8+ cells that coexpress Sema 7a
CD8+Sema 7a+ cells/μl
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c
% of CD4+ cells that are Tregs
CD4+CD25+FoxP3+ cells/μl
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d
% of CD4+CD25- cells that co-express Sema 7a
CD4+CD25-Sema 7a+ cells/μl
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e
% CD4+CD25+FoxP3+ Tregs not expressing Sema 7a
Sema 7a-veCD4+CD25+FoxP3+/μl
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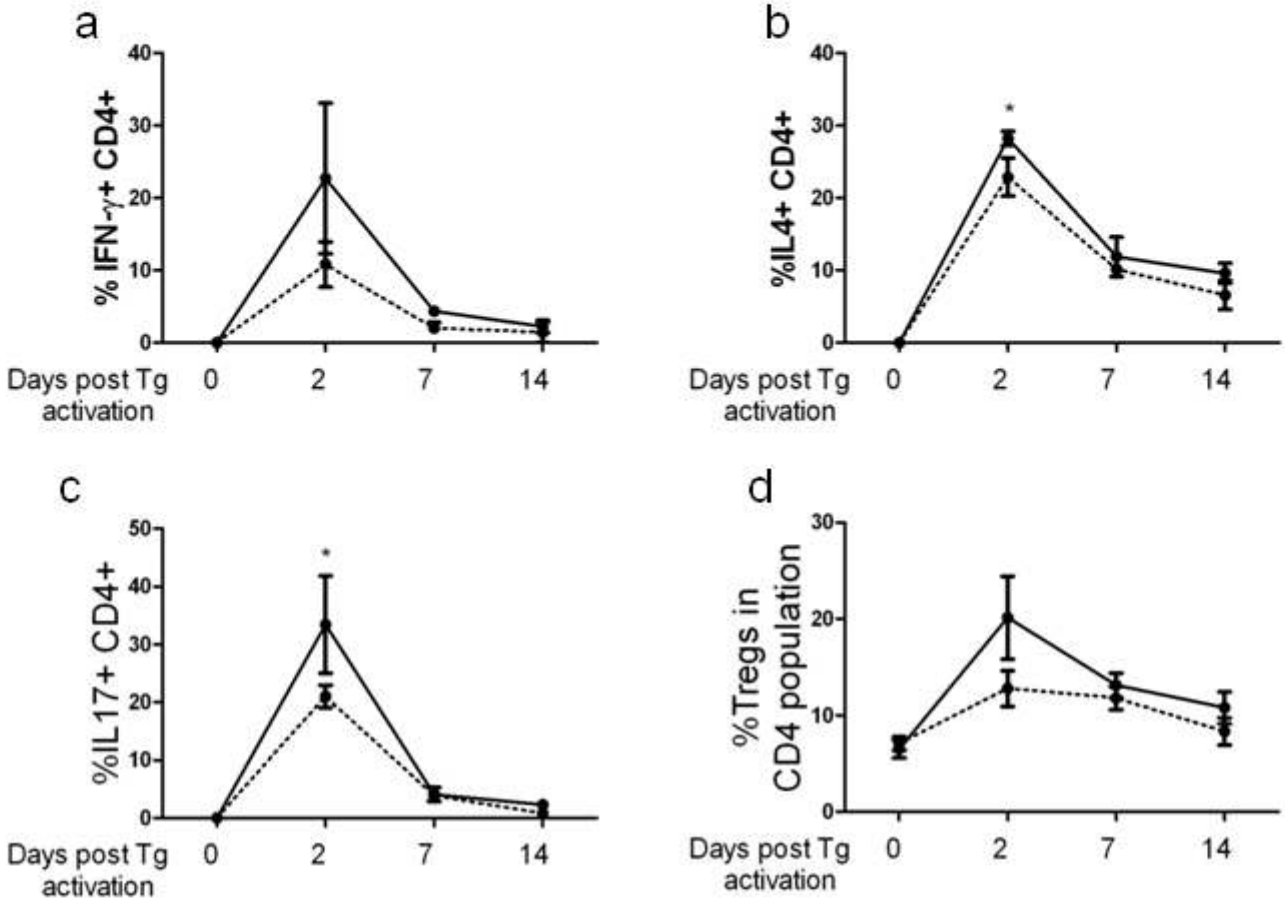
n.s.
f
% of CD4+ cells that are Tregs
Tregs/μl
n.s.
Stable
Progressive


a
% IFN-γ+ CD4+
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30
20
10
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Days post Tg activation
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7
14
b
%IL4+ CD4+
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c
%IL17+ CD4+
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d
%Tregs in CD4 population