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PD-1 up-regulation on CD4⁺ T cells promotes pulmonary fibrosis through STAT3-mediated IL-17A and TGF- β 1 production

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Competing interests: N.K. and J.D.H.-M. are inventors on a patent application (number 20180101642) held by Yale University that covers a blood 52-gene expression signature useful to improve outcome prediction in IPF. N.K. is also an inventor on a patent application (number 9913819) held by Yale University that covers a method of preventing or treating a fibrotic lung disease. W.P.D. is an inventor on a patent application (number 62613600) submitted by Vanderbilt University that covers checkpoint inhibition in organ fibrosis.

Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials.

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Abstract

Pulmonary fibrosis is a progressive inflammatory disease with high mortality and limited therapeutic options. Previous genetic and immunologic investigations suggest common intersections between idiopathic pulmonary fibrosis (IPF), sarcoidosis, and murine models of pulmonary fibrosis. To identify immune responses that precede collagen deposition, we conducted molecular, immunohistochemical, and flow cytometric analysis of human and murine specimens. Immunohistochemistry revealed programmed cell death-1 (PD-1) up-regulation on IPF lymphocytes. PD-1⁺CD4⁺ T cells with reduced proliferative capacity and increased transforming growth factor- β (TGF- β)/interleukin-17A (IL-17A) expression were detected in IPF, sarcoidosis, and bleomycin CD4⁺T Cells. PD-1⁺ T helper 17 cells are the predominant CD4⁺T cell subset expressing TGF- β . Coculture of PD-1⁺CD4⁺ T cells with human lung fibroblasts induced collagen-1 production. Strikingly, ex vivo PD-1 pathway blockade resulted in reductions in TGF- β and IL-17A expression from CD4⁺ T cells, with concomitant declines in collagen-1 production from fibroblasts. Molecular analysis demonstrated PD-1 regulation of the transcription factor STAT3 (signal transducer and activator of transcription 3). Chemical blockade of STAT3, using the inhibitor STATTIC, inhibited collagen-1 production. Both bleomycin administration to PD-1 null mice or use of antibody against programmed cell death ligand 1 (PD-L1) demonstrated significantly reduced fibrosis compared to controls. This work identifies a critical, previously unrecognized role for PD-1⁺CD4⁺ T cells in pulmonary fibrosis, supporting the use of readily available therapeutics that directly address interstitial lung disease pathophysiology.

INTRODUCTION

Pulmonary fibrosis represents progressive remodeling of lung architecture by deposition of connective tissue elements after persistent stimulation from antigenic (for example, *Schistosoma* eggs) and nonantigenic sources (for example, bleomycin in a mouse model) (1–3). Although tissue repair in its early stages is beneficial, continued connective tissue deposition results in scarring that eventually leads to organ fibrosis and death without therapeutic interventions (4). While new therapies continue to emerge, enhancing our understanding of the immunologic foundation responsible for pulmonary fibrosis reveals innovative therapeutics that directly target pathophysiology. Numerous cytokines have been implicated in pulmonary fibrosis (5–7). The importance of interleukin-17A (IL-17A)–producing CD4⁺ T cells has been demonstrated in pulmonary fibrosis (8). Increased IL-17A in the bronchoalveolar lavage of idiopathic pulmonary fibrosis (IPF) patients has also been

reported (9). Longitudinal increases in programmed cell death-1 (PD-1)⁺CD4⁺ T cells are present during sarcoidosis pulmonary progression, with T helper 17 (T_H17) cells as the T cell subset expressing the greatest percentage of PD-1 (10). Furthermore, genome-wide association studies implicate the T_H17 signaling pathway in sarcoidosis pulmonary progression (11); transcriptomic analysis implicates adaptive immune dysfunction in IPF (12, 13).

To date, a link establishing PD-1 and IL-17A interactions in pulmonary fibrosis has not been established. The purpose of the current study is to use two disease systems, IPF and sarcoidosis, to test the hypothesis that PD-1 up-regulation on T_H17 cells induces pulmonary fibrosis. Here, we report that PD-1⁺CD4⁺ T cells produce both transforming growth factor- β (TGF- β) and IL-17A through increased signal transducer and activator of transcription 3 (STAT3) transcription. Coculture of CD4⁺ T cells with human lung fibroblasts (HLFs) induces collagen-1 production; while blockade of the PD-1 pathway significantly decreases both STAT3, TGF- β and IL-17A expression, with concurrent reductions in collagen-1. These findings establish the biological significance of the PD-1 pathway, suggesting STAT3, IL-17A, or PD-1 as potential therapeutic targets for patients suffering from pulmonary fibrosis.

RESULTS

PD-1⁺CD4⁺ T cells with diminished proliferative capacity are present in IPF subjects

To delineate the role of co-inhibitory molecules in interstitial lung disease, we first examined PD-1 expression on systemic IPF CD4⁺ T cells. PD-1 cell surface expression was significantly higher on IPF CD4⁺ T cells compared to healthy controls (HC) ($P < 0.0001$, unpaired two-tailed t test) (Fig. 1A; gating strategy is shown in fig. S1A). Increased PD-1 expression on IPF CD4⁺ T cells was still apparent after restricting analysis to age-matched HC subjects ($P = 0.02$, unpaired two-tailed t test; fig. S1B and Table 1). We then defined the IPF CD4⁺ T cell subset demonstrating the highest percentages of PD-1⁺ T cells. Intriguingly, PD-1⁺T_H17 cells were the highest [$P < 0.001$, one-way analysis of variance (ANOVA) with Tukey's post hoc test], as was seen in sarcoidosis subjects (Fig. 1B and fig. S1C). The functional significance of PD-1 up-regulation was demonstrated by significant reductions in proliferative capacity in IPF PD-1⁺CD4⁺ T cell CD3/CD28 T cell receptor (TCR) stimulation ($P < 0.0001$, unpaired two-tailed t test; Fig. 1C).

To examine whether PD-1 pathway expression is also present in IPF lungs, immunohistochemical analysis for PD-1 expression in IPF human lung biopsies was performed. Increased PD-1 expression was detected in 10 IPF lungs compared to 5 HC lungs. Representative sections are shown in Fig. 1D. PD-L1 expression was also noted within IPF biopsies (Fig. 1D). Grading the prevalence of PD-1 in IPF and HC samples revealed that IPF specimens had significantly higher PD-1⁺ lymphocytes than HC specimens (Fig. 1E).

PD-1⁺T_H17 cells display the highest expression of TGF-β1

TGF-β is synthesized and processed within the cell and then secreted as a large latent TGF-β complex in which it is bound to latency-associated peptide (LAP) (14). Excessive TGF-β production results in organ fibrosis and, eventually, compromised organ function (15, 16). The presence of PD-1⁺CD4⁺ T cells in fibrosing conditions, such as IPF and sarcoidosis, led us to examine whether CD4⁺ T cells from these subjects secrete TGF-β and whether distinctions in TGF-β expression by T cell subsets are present. Active TGF-β1 expression was in ~70% of sarcoidosis and IPF CD4⁺ T cell cultures compared to ~30% in HC (HC/sarc, $P = 0.03$; HC/IPF, $P = 0.01$; one-way ANOVA with Tukey's post hoc test). No significant differences were observed in active TGF-β1 between sarcoidosis and IPF CD4⁺ T cells (Fig. 2A). A greater percentage of total CD4⁺ T cells producing membrane-bound TGF-β1 (LAP/TGF-β1) in sarcoidosis and IPF patients compared to HC was also ($P < 0.0001$, one-way ANOVA with Tukey's post hoc test). LAP/TGF-β1 mean fluorescence intensity (MFI) was also significantly increased in sarcoidosis and IPF CD4⁺ T cells compared to HC ($P < 0.01$, one-way ANOVA with Tukey's post hoc test; Fig. 2B) (representative histograms are shown in fig. S2, A to C). PD-1 up-regulation was seen on sarcoidosis and IPF CD4⁺ T cells expressing TGF-β1 (Fig. 2C). These findings demonstrate the presence of TGF-β-secreting CD4⁺ T cells with PD-1 up-regulation in patients with fibrotic lung disease.

TGF-β promotes the development of regulatory T cells (T_{regs}) through the transcription factor FOXP3 (17). Hence, we investigated whether increased TGF-β1 expression observed in CD4⁺ T cells was derived from T_{regs}. We first examined the overall percentages of T_{regs} in sarcoidosis and IPF patients. The percentage of T_{regs} was higher in sarcoidosis subjects only compared to HC (HC/sarc, $P < 0.001$; HC/IPF, $P = 0.08$, one-way ANOVA with Tukey's post hoc test; fig. S3A). No significant differences in LAP/TGF-β1 production by sarcoidosis or IPF T_{regs} were apparent (HC/sarc, $P = 0.45$; HC/IPF, $P = 0.38$; $P = 0.08$, one-way ANOVA with Tukey's post hoc test; Fig. 2D). No distinctions in PD-1 expression on sarcoidosis or IPF T_{regs} producing TGF-β1 were seen ($P = 0.30$, one-way ANOVA with Tukey's post hoc test; Fig. 2E) (representative histogram is shown in fig. S3B).

The proinflammatory cytokine IL-6 modulates TGF-β promotion of induced T_{reg} development in favor of T_H17 development (18–20). We detected increased IL-6 production in systemic sarcoidosis CD4⁺ T cells compared to HC CD4⁺ T cells ($P < 0.0001$, unpaired two-tailed t test; fig. S4). We therefore examined LAP/TGF-β1 secretion from T_H17 cells. A significant difference in LAP/TGF-β1 secreted by sarcoidosis and IPF T_H17 cells compared to HC was noted (HC/sarc, $P = 0.03$; HC/IPF, $P = 0.0092$, one-way ANOVA with Tukey's post hoc test; Fig. 2F). The sarcoidosis T_H17 cells that demonstrated increased frequency and intensity of LAP/TGF-β1 were derived from patients with high baseline PD-1 MFI ($P = 0.04$, one-way ANOVA with Tukey's post hoc test) but not those with baseline PD-1 MFI consistent with HC ($P = 0.9534$; high/low, $P = 0.03$; one-way ANOVA with Tukey's post hoc test; Fig. 2F). We observed significantly higher PD-1 on sarcoidosis and IPF T_H17 cells expressing TGF-β1 compared to HC ($P < 0.0001$, one-way ANOVA with Tukey's post hoc test; Fig. 2G). T_H1 cells, not producing IL-17A, from sarcoidosis and IPF patients produced higher amounts of LAP/TGF-β1 compared to HC (HC/sarc, $P = 0.02$; HC/IPF, $P < 0.01$;

one-way ANOVA with Tukey's post hoc test; fig. S5A). IPF CD4⁺ T cells that were dual producers of IL-17A and interferon- γ (IFN- γ) had significantly higher LAP/TGF- β 1 expression compared to HC ($P < 0.001$), but LAP/TGF- β 1 expression by sarcoidosis subjects was not distinct ($P = 0.68$, one-way ANOVA with Tukey's post hoc test; fig. S5B). The percentage of T follicular helper cells producing LAP/ TGF- β 1 was higher in IPF patients ($P < 0.01$, one-way ANOVA with Tukey's post hoc test), but not in sarcoidosis subjects ($P = 0.89$, one-way ANOVA with Tukey's post hoc test; fig. S5C). Evaluation of TGF- β 1 expression by PD-1 in CD4⁺ T cells showed significantly more TGF- β 1 production by PD-1–positive cells than PD-1–negative cells (sarc, $P < 0.0001$; IPF, $P = 0.03$; unpaired two-tailed t test; fig. S5D). Collectively, T_H17 cells had the highest percentage of TGF- β 1 secretion in sarcoidosis and IPF cohorts (Fig. 2H). Assessment of intracellular TGF- β 1 expression by flow cytometry revealed significantly higher expression in sarcoidosis and IPF CD4⁺ T cells producing IL-17A compared to HC ($P = 0.02$ and $P = 0.0032$, respectively, unpaired two-tailed t test; fig. S6, A to C).

PD-1 pathway blockade reverses the capacity of sarcoidosis and IPF CD4⁺ T cells to induce HLF-derived collagen-1 production in vitro

To determine the biological and physiological significance of increased TGF- β 1 secretion by sarcoidosis and IPF T_H17 cells, we assessed collagen-1 production after coculture of HLF with patient CD4⁺ T cells. We isolated HC, sarcoidosis, and IPF CD4⁺ T cells to 90 to 98% purity and cocultured each with an HLF cell line. Figure S7A depicts the gating strategy using flow cytometry. During analysis, we gated out CD3⁺CD4⁺ T cells and used the absence of CD45 expression to identify the fibroblast population as established previously (21, 22).

We observed a significant increase in percentage of collagen-1 produced by fibroblasts cocultured with sarcoidosis or IPF CD4⁺ T cells compared to HLF cultured alone or in combination with HC CD4⁺ T cells (HC and HLF, $P = 0.76$; sarcoidosis and HLF, $P < 0.0001$; IPF and HLF, $P < 0.01$; overall, $P = 0.01$, one-way ANOVA with Tukey's post hoc test; Fig. 3, A and B). Elevated collagen-1 was also observed in coculture supernatants by enzyme-linked immunosorbent assay (ELISA), confirming the aforementioned results (sarcoidosis, $P = 0.0031$; IPF, $P < 0.0001$, overall one-way ANOVA with Tukey's post hoc test; Fig. 3, A and B). We repeated the coculture experiments with four other distinct HLF lines and noted increased collagen production in those as well (table S1). Notably, only sarcoidosis CD4⁺ T cells with high PD-1 induced collagen-1 production, whereas CD4⁺ T cells with PD-1 expression akin to HC did not induce collagen-1 production after coculture experiments with HLF ($P = 0.14$, one-way ANOVA; fig. S7, B and C).

Increased collagen-1 production after coculture of TCR-stimulated PD-1⁺CD4⁺ T cells with HLF led us to examine the effects of PD-1 pathway blockade on HLF-derived collagen-1 production. Purified sarcoidosis CD4⁺ T cells were cultured overnight with or without the addition of α PD-1, α PD-L1, and α PD-L2 blocking antibodies, followed by CD3/CD28 TCR stimulation. HLFs were added the following day at a 1:10 ratio (HLF/T cell). The percentage of collagen-1 produced by HLF (CD3⁺CD4⁺CD45⁺) cultured in the presence of sarcoidosis CD4⁺ T cells after PD-1 pathway blockade dropped from 13.5% (mean)

preblockade to 7.5% (mean) postblockade analogous to the control group [HLF alone (mean, 7.5%)]. This decline reflects a 44% reduction in collagen-1 production from HLF (Fig. 3C). Fibroblast collagen-1 expression was only reduced in the cells that had been cultured with sarcoidosis CD4⁺ T cells expressing high baseline PD-1 ($P < 0.001$, paired two-tailed t test; Fig. 3C), but not in those that had been cultured with sarcoidosis T cells from patients who expressed PD-1 levels akin to HC ($P = 0.25$, paired two-tailed t test; Fig. 3C) (representative histograms are shown in fig. S7D). Reductions in collagen-1 production from HLF after PD-1 pathway blockade of IPF CD4⁺ T cells were also apparent ($P < 0.01$, one-way ANOVA with Tukey's post hoc test; Fig. 3D) (representative histograms are shown in fig. S7E). We conducted confirmatory ELISA for collagen-1 production in coculture supernatants after 24 hours of HLF coculture with TCR-stimulated sarcoidosis PD-1⁺CD4⁺ T cells and noted a significant increase in collagen production between baseline (HLF only) and preblockade conditions ($P = 0.03$, paired two-tailed t test), as well as a significant reduction in collagen after PD-1 pathway blockade ($P = 0.03$, paired two-tailed t test; Fig. 3E). These results were confirmed by Western blot using antibodies against COL1A1, α -smooth muscle actin (α -SMA), and fibronectin (Fig. 3F). Significant differences in COL1A and α -SMA expression were noted ($P = 0.01$ and $P < 0.001$, respectively; overall, one-way ANOVA with Tukey's post hoc test); reduced fibronectin expression was also noted ($P = 0.07$; overall, one-way ANOVA with Tukey's post hoc test; Fig. 3F). No significant differences were noted in the culture supernatant by Western blot assays (fig. S8). These results indicate that PD-1⁺CD4⁺ T cells have the capacity to induce pulmonary fibrosis through stimulation of HLF collagen-1 production.

PD-1 regulates IL-17A and TGF- β 1 expression in sarcoidosis and IPF CD4⁺ T cells

To understand how PD-1 regulates collagen-1 production by HLF, we assessed for two key mediators of organ fibrosis: IL-17A and TGF- β 1. After PD-1 pathway blockade, the percentage of sarcoidosis IL-17A-producing CD4⁺ T cells in the coculture experiments significantly declined from a mean of 15% to a mean of 3%, reflecting an 80% reduction ($P < 0.01$, paired two-tailed t test; Fig. 4A). The detection of PD-1⁺T_H17 cells in sarcoidosis and IPF subjects supported further inquiry into the effects of PD-1 pathway blockade on TGF- β 1, a key mediator of T_H17 development. PD-1 pathway blockade significantly reduced TGF- β 1 secretion in sarcoidosis and IPF CD4⁺ T cells ($P < 0.01$ and $P = 0.04$, respectively; paired two-tailed t test; Fig. 4, B and C). Representative histograms are depicted in fig. S9 (A to C). Using ELISA analysis, we confirmed increased active TGF- β 1 expression after HLF coculture with sarcoidosis CD4⁺ T cells, accompanied by significantly reduced expression after PD-1 pathway blockade ($P = 0.01$, one-way ANOVA with Tukey's post hoc test; Fig. 4D). To confirm the source of TGF- β 1 in coculture experiments, we assessed for TGF- β 1 expression by T_H17 cells using flow cytometry. PD-1 pathway blockade effectively reduced TGF- β 1 secretion from T_H17 cells in sarcoidosis subjects with high baseline PD-1 ($P = 0.04$, paired two-tailed t test; Fig. 4E), but not in subjects with PD-1 levels akin to HC ($P = 0.49$, paired two-tailed t test; Fig. 4F). TGF- β 1 expression was significantly reduced in IPF T_H17 cells after blockade of PD-1 pathway ($P = 0.02$, paired two-tailed t test; Fig. 4G).

PD-1 blockade reduces expression of the transcription factors STAT3 and RORC in CD4⁺ T cells

STAT3 is an essential transcription factor for T_H17 cell development (23). To identify the molecular mechanism by which PD-1 regulates IL-17A production, we first focused on STAT3 expression. Flow cytometric analysis revealed significantly higher STAT3 expression in sarcoidosis subjects compared to HC after CD3/CD28 TCR stimulation (percentage, $P=0.03$; MFI, $P=0.01$, unpaired two-tailed t test; Fig. 5A). We also conducted quantitative PCR analysis for *STAT3* mRNA expression in HC, sarcoidosis, and IPF CD4⁺ T cells. A significant increase in *STAT3* mRNA expression in sarcoidosis and IPF subjects was apparent compared to HC (HC/sarc, $P=0.01$; HC/IPF, $P=0.01$; $P<0.01$, one-way ANOVA with Tukey's post hoc test) (Fig. 5B). Significant differences between sarcoidosis and IPF subjects were not detected ($P=0.14$, one-way ANOVA with Tukey's post hoc test; Fig. 5B). We then assessed *STAT3* mRNA expression after PD-1 pathway blockade in sarcoidosis CD4⁺ T cells. Significant reductions in *STAT3* mRNA expression were detected after PD-1 blockade ($P=0.04$, paired two-tailed t test; Fig. 5C). To confirm that the PD-1 pathway regulates collagen-1 production through phospho-STAT3 (pSTAT3)-mediated IL-17A production, we used the small-molecule inhibitor of pSTAT3, STATTIC (24). After STATTIC administration to sarcoidosis CD4⁺ T cells overnight, coculturing of these cells with HLF resulted in significant reductions in collagen-1 production ($P=0.0032$, one-way ANOVA with Tukey's post hoc test) to baseline levels (that is, HLF cultured alone) ($P=0.82$, one-way ANOVA with Tukey's post hoc test; Fig. 5D). Significant reductions in IL-17A-producing CD4⁺ T cells after STATTIC inhibition were also noted ($P=0.0032$, paired two-tailed t test; Fig. 5E), thus underlying the physiologic significance of STAT3-mediated T_H17 cell differentiation in pulmonary fibrosis. Although diminution of IL-17A expression is one mechanism relevant to STATTIC inhibition of collagen production, the capacity for STATTIC to inhibit STAT1 in T cells may also have a role (fig. S10).

Because our results demonstrated that both these factors are increased in sarcoidosis patients, we examined RAR-related orphan receptor C (RORC) expression in CD4⁺ T cells. A significant increase in *RORC* mRNA was apparent between HC and sarcoidosis patients ($P=0.02$, one-way ANOVA with Tukey's post hoc test; fig. S11A). *RORC* expression was significantly reduced after PD-1 blockade ($P=0.03$, one-way ANOVA with Tukey's post hoc test) to control levels ($P=0.97$, one-way ANOVA with Tukey's post hoc test; fig. S11A). Comparison of T_{regs} and T_H17 cells from sarcoidosis and IPF patients based on PD-1 expression demonstrated an imbalance in the ratios of these PD-1⁺ subsets that was not apparent in PD-1-negative cells ($P<0.0001$, one-way ANOVA with Tukey's post hoc test; fig. S11, B and C). Furthermore, IL-6 has also been reported to induce the conversion of FOXP3⁺ T_{regs} into T_H17 cells in autoimmune disorders (25, 26). We therefore examined RORC expression using flow cytometry in PD-1-positive and PD-1-negative sarcoidosis and IPF T_{regs}. Our results demonstrated a significant increase in *RORC* expression in PD-1⁺FOXP3⁺ cells from both sarcoidosis and IPF patients compared to PD-1-FOXP3⁺ cells (sarc, $P<0.0001$; IPF, $P=0.0041$; unpaired two-tailed t test; fig. S11, D and E).

Elevated expression of T_H17-related pathway genes is associated with reduced IPF life expectancy

To further assess the relevancy of T_H17 cells in interstitial lung diseases, we screened PBMCs from IPF patients for differences in gene expression profiles and clinical outcomes. Two distinct T_H17 gene profiles were evident with significant differences in transplant-free survival (TFS) in the discovery cohort [hazard ratio (HR), 2.77; 95% confidence interval (CI), 0.96 to 8.01; $P = 0.042$; Fig. 6A]. Similar to the cellular investigation, enhanced TFS was present among IPF subjects having genetic signatures of reduced *TGF- β* and *IL17A* and increased *IFN- γ* expression (Fig. 6A). Similar results were observed in the validation cohort where two clusters of patients were found to have significant differences in overall survival (HR, 5.24; 95% CI, 2.09 to 13.08; $P = 0.0001$; Fig. 6B). The HR for mortality and TFS ranged from 2.77 to 5.24, indicating an increased risk of dying or requiring a lung transplant for survival in patients with a genetic profile consistent with increased T_H17 cell signatures (Fig. 6, A and B). High STAT3 expression was also found to be a predictor of worse TFS in IPF patients ($P = 0.005$), with an HR of 3.82 (Fig. 6C). These results were confirmed in the validation cohort with an HR of 3.5 (Fig. 6D), which suggest that dysregulation of T_H17 genes is associated with IPF progression and poor disease outcome, providing further evidence of T_H17 cell involvement in human interstitial lung disease.

The cohort with better TFS also demonstrated increased STAT4 expression, which could be secondary to increased IL-12 production. IL-12 in combination with other cytokines, such as IL-7, promotes the differentiation of IFN- γ -producing CD4⁺ T cells. However, in vitro human studies have shown that increased IL-12 can drive the expansion of IL-17A-producing CD4⁺ T cells (27). Microarray analysis of the discovery cohort demonstrated that increased IL-12A and IL-12B expression was associated with reduced TFS in IPF patients ($P = 0.03$ and $P = 0.002$, respectively; fig. S12).

Bleomycin murine model reveals reduced fibrosis in PD-1 null mice

To evaluate the presence of PD-1⁺CD4⁺ T cells in pulmonary fibrosis induced by other mechanisms, we assessed for their presence in murine lungs after intranasal bleomycin administration, a model of pulmonary fibrosis due to acute lung injury. PD-1 up-regulation and diminished effector function were noted in CD4⁺ T cells from single-cell lung suspensions of bleomycin-treated C57BL/6 mice (Fig. 7). The percentage of unstimulated CD4⁺ T cells expressing PD-1 and PD-1 MFI was significantly higher at day 21 after treatment in the bleomycin-treated group than that in the phosphate-buffered saline (PBS)-treated group (percentage, $P = 0.02$; MFI, $P < 0.01$; unpaired two-tailed t test; Fig. 7A). After TCR stimulation ex vivo, both groups demonstrated increased PD-1 expression, with a significantly higher percentage of CD4⁺ T cells expressing PD-1 in the bleomycin-treated group ($P < 0.01$, unpaired two-tailed t test; Fig. 7B). Notably, PD-1 up-regulation was accompanied by reduced CD4⁺ T cell proliferation in the bleomycin-treated mice compared to cells obtained from control mice ($P < 0.001$, unpaired two-tailed t test; Fig. 7C), thus confirming the functional significance of PD-1 up-regulation on pulmonary CD4⁺ T cells after bleomycin administration.

T_H17 cells from single-cell lung suspensions of bleomycin-treated mice also displayed increased LAP/TGF- β 1 secretion in comparison to cells from PBS-treated mice (Fig. 7D). Furthermore, no significant differences in TGF- β 1 expression were apparent between T_{regs} from the control and bleomycin-treated mice ($P = 0.92$, unpaired two-tailed t test; Fig. 7E). When comparing TGF- β 1 secretion from $CD4^+$ T cells after bleomycin administration, we observed that T_H17 cells were responsible for most of the LAP/TGF- β production ($P < 0.0001$, one-way ANOVA with Tukey's post hoc test) among the $CD4^+$ T cell subsets (Fig. 7F). The detection of PD-1 $^+$ CD4 $^+$ T cells in a model of pulmonary fibrosis due to acute lung injury indicates that PD-1 pathway may be a common point of immunologic convergence in pulmonary fibrosis despite the etiology.

To determine whether interrupting the PD-1 pathway can dampen fibrosis, wild-type (WT) and PD-1 null C57BL/6 mice were challenged with bleomycin via intranasal administration and were then euthanized at day 14. In separate experiments, WT mice were either treated with anti-PD-L1 or an isotype control before or after bleomycin. To analyze fibrosis parameters, we measured pulmonary trichrome staining and hydroxyproline content by high-performance liquid chromatography (HPLC) or the Sircol assay. Representative hematoxylin and eosin (H&E) and trichrome sections are shown in Fig. 7G. Fibrosis degree and severity were significantly higher by Ashcroft scoring in WT C57BL/6 compared to PD-1 null mice (Fig. 7G). Assessment of hydroxyproline content by HPLC confirmed these distinctions (Fig. 7G). Significantly lower levels of hydroxyproline were noted in PD-1 null mice compared to WT C57BL/6 (41.7 ± 17.0 versus 111.3 ± 8.7 , $P = 0.003$, unpaired two-tailed t test; Fig. 7G). We also assessed the role of anti-PD-L1 therapy administered 4 days after intranasal bleomycin on murine pulmonary fibrosis. We noted significantly reduced weight loss in mice treated with anti-PD-L1 compared to those treated with isotype antibody, as well as significant reductions in pulmonary collagen content after administration of antibody against PD-L1 compared to isotype before or after bleomycin administration (Fig. 7H). With the Sircol assay, pulmonary collagen content was higher in mice treated with the isotype control (156.2 ± 23.59) than that in mice treated with anti-PD-L1 antibody (85.45 ± 14.63) ($P = 0.03$, unpaired two-tailed t test; Fig. 7H). Furthermore, significant differences in PD-1 $^+$ CD4 $^+$ T cells and pSTAT3 (Y705) were also detected between isotype and anti-PD-L1-treated cohorts ($P < 0.0001$ and $P = 0.01$, respectively, unpaired two-tailed t test; Fig. 7, I and J). One mechanism by which PD-1 reduces pSTAT3 expression is through decreased IL-23R expression (fig. S13). A significant correlation between PD-1 expression and pSTAT3 (Y705) was also noted in bleomycin-treated mice ($P = 0.0028$; $R^2 = 0.5398$; Fig. 7K). These findings provide in vivo confirmation of the relevance of the PD-1 pathway in interstitial lung disease.

DISCUSSION

These investigations identify PD-1 up-regulation as a critical mediator of human interstitial lung disease through increased IL-17A production via the STAT3 signaling pathway. Key investigations have implicated aberrant STAT3 signaling and T_H17 cells in murine models of pulmonary fibrosis pathogenesis (9, 28, 29). Here, we report that PD-1 up-regulation on human T_H17 cells creates not only an immunosuppressive microenvironment but also a profibrotic one. We demonstrate that IL-17A and TGF- β 1 produced by PD-1 $^+$ CD4 $^+$ T cells

induce collagen-1 production from HLF, a process that can be impeded by PD-1 pathway blockade. Mechanistically, PD-1 pathway blockade or small-molecule inhibition of STAT3 results in reduced collagen production from HLF.

Immune dysfunction in CD4⁺ T cells secondary to PD-1 upregulation in sarcoidosis subjects experiencing loss of pulmonary function has been noted (10). However, the observation of a profibrotic component to PD-1 up-regulation has not been previously described; even more striking is the observation of PD-1 regulation of IL-17 and TGF- β expression. TGF- β expression by T_{regs} has been reported (30). We detected T_H17 cells that secrete intracellular and membrane-bound TGF- β . T_{regs} have plasticity, allowing them to differentiate into T_H17 cells in conditions of increased IL-6/pSTAT3 expression (31). We noted increased IL-6 production from sarcoidosis CD4⁺ T cells of subjects experiencing disease progression; these same T cells exhibit PD-1 up-regulation. It is feasible that the TGF- β -secreting T_H17 cells began as T_{regs}, but increased IL-6 production from sarcoidosis CD4⁺ T cells tilted the balance in favor of developing a T_H17 phenotype. We observed PD-1⁺ T_{regs} that also contain the transcription factor RORC in sarcoidosis and IPF subjects. Strikingly, PD-1⁺T_H17 cells are present in pulmonary fibrosis of distinct etiologies [chronic antigenic stimulation from microbial antigens (for example, sarcoidosis) (32), genetic alterations in the Src homology 2 domain-containing phosphotyrosine phosphatase-2 (Shp2) signaling pathway (for example, IPF) (33), or bleomycin-induced pulmonary fibrosis], suggesting that the PD-1/STAT3/T_H17 pathway may represent a point of immunologic convergence resulting in organ fibrosis. The presence of PD-1⁺CD4⁺ and CD8⁺ T cell up-regulation in liver and skin fibrosis has also been reported (34).

The observation that PD-1 can regulate STAT3 is particularly provocative. In cancers, such as melanoma and chronic lymphocytic leukemia (CLL), STAT3 regulation of PD-1 on CD4⁺ and CD8⁺ T cells has been reported (35). Reduced PD-1 expression with STAT3 inhibition was noted on these cells, as well as reduced PD-L1 expression on CLL cells, after treatment with the STAT3 inhibitor ibrutinib. It was also notable that ibrutinib only affected the PD-1 pathway and not other checkpoint inhibitors (35). We noted PD-1 regulation of STAT3 in sarcoidosis CD4⁺ T cells. These observations in malignancies suggest that PD-1 and STAT3 may directly or indirectly regulate each other. In melanoma cells, STAT3 transcription has been shown to be negatively regulated by phosphatidylinositol 3-kinase (PI3K) (36). This pathway may have particular relevance to PD-1 manipulation of STAT3 in sarcoidosis subjects due a recent report demonstrating the capacity of PD-1 to inhibit PI3K expression in sarcoidosis CD4⁺ T cells (37). It is possible that PD-1 indirectly regulates STAT3 transcription through inhibition of PI3K, resulting in increased STAT3. There is also a possible role for exomes derived from T_H17 cells to regulate the PD-1 pathway.

Administration of bleomycin to PD-1 null mice resulted in significant reductions in fibrosis. Despite the model's predilection to pulmonary fibrosis after bleomycin administration, we observed significant reductions in pulmonary fibrosis in the PD-1 null mice compared to WT using multiple complementary assays. Administration of antibody against PD-L1 also demonstrated significant reductions in pulmonary collagen content. Mechanistically, direct targeting of the PD-1 pathway demonstrated reductions in PD-1⁺CD4⁺ T cells, as well as pSTAT3 in these cells. Significant reductions in collagen production were noted by

immunoblot analysis of the cell lysates, primarily by reductions in collagen-1 and fibronectin expression. We did not observe distinctions in α -SMA between baseline and stimulated coculture conditions, most likely due to assessment at the 24-hour time point. Previous reports have indicated a delay in the detection of myofibroblast differentiation at 24-hour assessments (38). These data demonstrate the capacity of PD-1 pathway blockade to reduce pulmonary fibrosis due to acute lung injury (bleomycin).

This investigation is distinct from the previous publication reporting increased immunosuppressive PD-1⁺CD4⁺ T cells in sarcoidosis subjects experiencing disease progression (10). This work demonstrates that PD-1 up-regulation is also profibrotic through its capacity to enhance STAT3 expression, leading to production of profibrotic cytokines, such as TGF- β and IL-17A. STAT3 inhibition of STAT3 has been shown to reduce collagen-1 content and connective tissue growth factor using the bleomycin murine model of pulmonary fibrosis (39–41). Furthermore, it has been previously demonstrated that genetic enhancement and ablation of STAT3 in the murine bleomycin model increase and reduce lung fibrosis, respectively (42).

A limitation of this study is that other immunologic and nonimmunologic contributors to lung fibrosis were not examined. For example, mammalian sterile 20-like kinase 1 signaling from dendritic cells negatively regulates IL-17-producing CD4⁺ T helper cell (T_H17) differentiation (43). In addition, semaphorin 7A⁺ T_{regs} with reduced IL-10 expression have also been implicated in IPF progression (44). An investigation into the effects of PD-1 on IL-10 inhibition of IL-17A production by CD4⁺ T cells is warranted to identify the aberrancies that facilitate progression of fibrotic lung disease in humans and murine models. In addition, whereas gene expression profiles of both IPF cohorts cluster around STAT1 and STAT4 expression, the T_H17 STAT3 bias is more apparent in the validation cohort. There are several factors that affect the distinctions in expression results between the two cohorts, including cell compartment differences (PBMCs in discovery cohort versus whole blood in validation cohort), different RNA extraction methods (TRIzol in discovery cohort versus PAXgene in validation cohort), and differences in clinical practices across centers in two different continents (University of Chicago versus Imperial College London). STAT3 assessment in the discovery cohort revealed increased STAT3 expression among IPF patients with reduced TFS, thus validating the role of STAT3 in disease progression among IPF patients. Despite the reproducibility of the results, further validation using standardized gene expression methods may be required in additional patient cohorts to confirm our findings.

In conclusion, these data identify relevant mechanisms for PD-1⁺CD4⁺ T cell induction of pulmonary fibrosis. Blockade of the PD-1 pathway significantly altered IL-17A expression in CD4⁺ T cells via decreased pSTAT3 expression, resulting in significant declines in collagen-1 production. In vivo investigations reveal significant decreases in bleomycin-induced pulmonary fibrosis with the absence of *PDCDI* in their genetic background or after administration of anti-PD-L1 antibody. This work identifies readily available, U.S. Food and Drug Administration–approved therapeutics such as inhibitors of STAT3 signaling (for example, metformin and ibrutinib), which have been shown to alter epithelial-mesenchymal transition (EMT) (45), or antibodies directed against IL-17A (46) as viable therapeutic options for patients suffering from interstitial lung disease.

MATERIALS AND METHODS

Study design

This is a study using ex vivo human sample experiments and in vivo mouse experiments to demonstrate the ability of PD-1 to induce pulmonary fibrosis through STAT3 regulation of IL-17A and TGF- β 1 production. Primary data are located in table S2, and the details are described in Supplementary Materials and Methods. For study participation, clinical and radiographic criteria were used to define sarcoidosis as has been previously described (47, 48). PBMCs of patients from the University of Cincinnati (UC), the Cleveland Clinic (CC), and the Vanderbilt University Medical Center (VUMC) were used. All subjects provided written informed consent that was approved by the appropriate institutional review boards (CC 14-004, UC 2013-8269, and VUMC 040187). All investigations with human subjects were conducted according to the principles expressed in the Helsinki Declaration. There were four subject cohorts: HC, subjects with sarcoidosis that have active disease, sarcoidosis subjects with resolved disease, and IPF patients. The sarcoidosis patients with active disease were characterized by reductions in forced vital capacity (FVC), radiographic progression, or pulmonary symptom acceleration. Sarcoidosis subjects who had resolved disease were distinguished by normalized FVC or chest radiograph and resolution of pulmonary symptoms despite not being on immunosuppressive therapy. Patients with high PD-1 expression were distinguished from patients with low PD-1 according to percentage of positive cells and MFI. Subjects expressing low PD-1 were defined as having PD-1 percentages and MFIs similar to HC. Study subject demographics are provided in Tables 1 and 2.

Microarray data were analyzed on two previously published IPF patient cohorts evaluated at the University of Chicago ($N = 45$) (GSE27957) (13) and the Imperial College London ($N = 55$) (GSE93606) (49), using cluster 3 for hierarchical clustering (50) and the survival package of MedCalc software. Immunohistochemistry for PD-1 and PD-L1 was conducted as previously described (10). Coculture experiments were conducted as previously described (51). Studies involving large and independent experimental cohorts of mice were performed once. All bleomycin (52) and PD-1 pathway blockade experiments (53) were performed as previously described. Mouse cohorts received antibody injections at the dosage regimens indicated in Supplementary Materials and Methods. Although the investigators were not blinded when they administered therapeutic antibodies, they were blinded while assessing the results. All murine procedures were performed according to the protocol approved by the Institutional Animal Care and Use Committee (protocol M1700043-00). Collagen quantification was conducted by hydroxyproline (54) or Sircol assay as previously described (55). Primary data are located in table S2.

Statistical analysis

Comparisons between cohorts were performed using an unpaired two-tailed Student's *t* test. Preblockade and PD-1 pathway blockade cohort comparisons were analyzed using a paired Student's *t* test. Multiple group comparisons were performed using a one-way ANOVA with Tukey's post hoc test. Proliferation data were analyzed using the Mann-Whitney *U* test. Statistical analysis for all figures was carried out using Prism version 6.0 (GraphPad

Software). A $P < 0.05$ was considered statistically significant. Fold change and false discovery rate values were calculated using the Significance Analysis of Microarrays (SAM) tools. All data were used in analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. PD-1 up-regulation in fibrotic lung disease.

(A) Baseline PD-1 on total CD4⁺ T cells (HC, $n = 24$; IPF = 25). (B) PD-1 expressed on CD4⁺ T cell subsets (HC, $n = 10$; T_H1, T_{reg}, and T_H17, $n = 7$). (C) Percentage of proliferating CD4⁺ T cells from the peripheral blood of HC ($n = 14$) and IPF subjects ($n = 20$) after TCR stimulation with plate-bound CD3/CD28 antibodies. Corresponding histograms illustrating proliferative capacity for a HC and IPF patient. (D and E) Immunohistochemistry (IHC) assessment for PD-1 and PD-L1 (brown) in healthy and IPF pulmonary biopsies. Increased PD-1 expression on lymphocytes (upper right quadrant, arrows). Increased PD-L1 expression (lower right quadrant, arrows). $N = 12$. Comparisons between cohorts were performed using an unpaired two-tailed Student's t test. Multiple group comparisons were performed using a one-way ANOVA with Tukey's post hoc test. Proliferation data were analyzed using the Mann-Whitney U test. Bars indicate SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. NS, no significance; CFSE, carboxyfluorescein diacetate succinimidyl ester; FMO, fluorescence minus one.

Fig. 2. T_H17 cells are most likely to secrete TGF- β 1 in association with PD-1 up-regulation after ex vivo stimulation.

Purified CD4⁺ T cells from the peripheral blood of HC, sarcoidosis, and IPF patients were TCR-stimulated and cultured for 24 hours. (A) Increased active TGF- β 1 in the cell culture supernatants of sarcoidosis and IPF CD4⁺ T cells (HC, $n = 9$; sarc, $n = 6$; IPF, $n = 8$). (B) Frequency (left) and MFI (right) for total CD4⁺ T cells secreting TGF- β 1 (HC, $n = 12$; sarc, $n = 14$; IPF, $n = 7$). (C) PD-1 percentages for total CD4⁺ T cells expressing TGF- β 1 (HC, $n = 6$; sarc, $n = 10$; IPF, $n = 7$) and corresponding histograms. (D) Percentage of T_{regs}

expressing TGF- β 1 (HC, $n = 14$; sarc, $n = 13$; IPF, $n = 7$). **(E)** PD-1 expression on T_{regs} that are positive for TGF- β 1 (HC, $n = 8$; sarc, $n = 10$; IPF, $n = 7$). **(F)** T_H17 cells secreting TGF- β 1 (frequency and MFI) in HC ($n = 6$), sarcoidosis patients according to baseline PD-1 expression (high, $n = 8$; low, $n = 5$) and IPF subjects ($n = 7$). **(G)** Percentage of T_H17 cells that are secreting TGF- β 1 and also expressing PD-1 (HC, $n = 6$; sarc, $n = 13$; IPF, $n = 7$). Histograms depict PD-1 expression on TGF- β 1⁺ T_H17 cells. **(H)** Percentage of TGF- β 1 secreted by CD4⁺ T cell subsets. Comparisons between cohorts were performed using an unpaired two-tailed Student's t test. Multiple group comparisons were performed using a one-way ANOVA with Tukey's post hoc test. Bars indicate SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. NS, no significance for (A) to (G).

Fig. 3. PD-1 blockade normalizes HLF collagen-1 production after coculture with patient T cells. Purified CD4⁺ T cells were cultured overnight with or without the presence of anti-PD-1, anti-PD-L1, and anti-PD-L2. The following day, cells were TCR-stimulated and cocultured with purified HLF. **(A)** Percentage of fibroblasts (CD3⁺CD4⁺CD45⁺) expressing collagen-1 when cultured alone ($n = 14$) and when cultured with HC ($n = 8$) or sarcoidosis ($n = 12$) CD4⁺ T cells. Collagen-1 assessment in cell culture supernatants from an independent experiment (HLF, $n = 10$; HC, $n = 8$; sarc, $n = 8$). **(B)** Collagen-1 expression by HLFs when cultured in the presence of purified CD4⁺ T cells isolated from IPF patients ($n = 4$). Collagen-1 in cell culture supernatants from an independent experiment (HLF, $n = 10$; HC, $n = 8$; IPF, $n = 5$). **(C)** Purified sarcoidosis CD4⁺ T cells from the same patients were cultured with (postblockade) or without (preblockade) the addition of PD-1-blocking antibodies. HLFs were added the following day, and the cells were cocultured at 37°C in 5% CO₂ atmosphere. Percentage of fibroblasts expressing collagen-1 at baseline and in cocultures with sarcoidosis CD4⁺ T cells subsequent to PD-1 blockade [HLF (baseline), $n = 14$; preblockade, $n = 13$; postblockade, $n = 13$]. Collagen-1 expressed by fibroblasts cocultured

with CD4⁺ T cells expressing high or low baseline PD-1 levels, before and after PD-1 blockade (high, $n = 9$; low, $n = 4$). (D) Percentage of collagen-1 expressed by HLFs cocultured with IPF CD4⁺ T cells pre- and postblockade ($n = 4$). (E) Collagen-1 assessment in coculture supernatants by HLF at baseline (HLF only) and with sarcoidosis CD4⁺ T cells before and after PD-1 pathway blockade and 24-hour TCR stimulation ($n = 8$). (F) Coculture lysates were immunoblotted with antibodies against COL1A1, α -SMA, and fibronectin after 24-hour TCR stimulation with and without the addition of PD-1–blocking antibodies. Representative Western blots for three sarcoidosis patients and quantification of results are depicted (baseline, $n = 2$; preblockade and postblockade, $n = 5$). Comparisons between cohorts were performed using an unpaired two-tailed Student's t test. Preblockade and PD-1 pathway blockade cohort comparisons were analyzed using a paired Student's t test. Multiple group comparisons were performed using a one-way ANOVA with Tukey's post hoc test. Bars indicate SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. NS, no significance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 4. PD-1 blockade reduces IL-17A and TGF- β 1 expression.

Purified CD4⁺ T cells were cultured overnight with (postblockade) or without (preblockade) the presence of anti-PD-1, PD-L1, and PD-L2. The following day, cells were TCR-stimulated and cocultured with HLF. (A) CD4⁺IL-17A⁺ T cells with and without PD-1 blockade ($n = 6$). Representative FACS plots illustrating CD4⁺IL-17A⁺ T cell percentages pre- and postblockade. (B) Percentage of CD4⁺ T cells from sarcoidosis and (C) IPF patients secreting TGF- β 1 pre- and postblockade (sarc, $n = 11$; IPF, $n = 7$). (D) Free active TGF- β 1 in sarcoidosis coculture supernatants subsequent to PD-1 blockade. (E and F) Sarcoidosis TH17 cells expressing TGF- β 1 after PD-1 pathway blockade according to CD4⁺ T cell baseline PD-1 levels (high PD-1, $n = 8$; low PD-1, $n = 5$). (G) Percentage of TH17 cells that are TGF- β 1⁺ before and after PD-1 blockade in IPF patients ($n = 7$). Comparisons between cohorts were performed using an unpaired two-tailed Student's t test. Preblockade and PD-1 pathway blockade cohort comparisons were analyzed using a paired Student's t test. Multiple group comparisons were performed using a one-way ANOVA with Tukey's post hoc test. Bars indicate SEM. * $P < 0.05$, ** $P < 0.01$. NS, no significance.

Fig. 5. PD-1 blockade reduces STAT3 mRNA and protein.

Purified CD4⁺ T cells from HC and sarcoidosis peripheral blood mononuclear cells (PBMCs) were TCR-stimulated for 24 hours at 37°C in 5% CO₂. STAT3 protein expression was assessed using flow cytometry. (A) Percentage and MFI for CD4⁺ T cells expressing STAT3 (HC, $n = 4$; sarc, $n = 8$). (B) *STAT3* gene expression was assessed using quantitative reverse transcription polymerase chain reaction (PCR). (HC, $n = 6$; high PD-1, $n = 6$; low PD-1, $n = 4$; IPF, $n = 4$). (C) *STAT3* mRNA expression from sarcoidosis and IPF CD4⁺ T cells after PD-1 pathway blockade ($n = 8$). (D) CD4⁺ T cells were cultured overnight with or

without the pSTAT3 inhibitor STATTIC. The following day, cells were TCR-stimulated in the presence of HLFs and cultured at 37°C in 5% CO₂. Percentage of collagen-1 produced by fibroblasts that have been cultured in the presence of sarcoidosis CD4⁺ T cells without STATTIC (gray bar) and with STATTIC (green bar) (HLF, $n = 14$; sarcoidosis without STATTIC, $n = 5$; STATTIC, $n = 5$). Histograms illustrate percentage of collagen produced by fibroblasts before and after STAT3 inhibition. (E) Percentage of CD4⁺ T cells producing IL-17A before and after STATTIC inhibition and representative histograms. Comparisons between cohorts were performed using an unpaired two-tailed Student's t test. Preblockade and PD-1 pathway blockade cohort comparisons were analyzed using a paired Student's t test. Multiple group comparisons were performed using a one-way ANOVA with Tukey's post hoc test. Bars indicate SEM. * $P < 0.05$, ** $P < 0.01$. NS, no significance.

Fig. 6. T_H17 gene expression profiles in IPF.

(A) Hierarchical clustering of IPF discovery [University of Chicago ($N=45$) (GSE27957)] and (B) validation cohort [Imperial College London ($N=55$) (GSE93606)] based on a T_H17 signature in PBMCs (discovery cohort) and whole blood (validation cohort) by GeneChip microarrays. Two major clusters of IPF patients were identified. Distinctions are present in transplant-free survival (TFS) over the course of 3.5 years post-diagnosis and overall survival between clusters in both the discovery and validation cohorts, respectively. (C) TFS for clusters 1 and 2 from the discovery cohort and (D) validation cohort based on the expression of STAT3.

Fig. 7. Bleomycin-induced lung fibrotic murine model reveals reduced lung fibrosis in PD-1 null mice or after PD-L1 blockade.

(A) PD-1 percentages and MFI on CD4⁺ T cells from single-cell lung suspensions 21 days after bleomycin treatment. Matching histograms depicting PD-1 on CD4⁺ T cells. (B) Percentage and MFI for lung-derived CD4⁺PD-1⁺ T cells after TCR stimulation with plate-bound anti-CD3/CD28 antibodies after bleomycin treatment (PBS, $n = 4$; bleomycin, $n = 8$). Representative histograms showing percentage of PD-1 on CD4⁺ T cells after TCR stimulation. (C) CD4⁺ T cell proliferation subsequent to 5-day TCR stimulation and corresponding histograms (PBS, $n = 3$; bleomycin, $n = 5$). (D) Percentage of TH17 cells

secreting TGF- β 1 (PBS, $n = 4$; bleomycin, $n = 8$). Representative FACS plots illustrating percentage of TGF- β 1 in a PBS- and bleomycin-treated mouse. (E) TGF- β 1 percentages expressed by T_{regs} in PBS- treated ($n = 4$) and bleomycin- treated ($n = 8$) mice. Flow cytometry plots demonstrating percentage of TGF- β 1 expressed by T_{regs}. (F) Percentage of TGF- β 1 secreted by CD4⁺ T cell subsets. (G) Representative images for H&E- and trichrome-stained lungs with Ashcroft scoring 14 days after bleomycin injury from WT and PD-1 null mice. Scale bar, 100 μ m. $n = 4$ in each cohort. Lungs were processed for measuring hydroxyproline levels by HPLC ($n = 4$ in each bleomycin cohort; $n = 2$ in NS cohort). (H) Trichrome-stained lungs and quantification of collagen content after isotype or anti-PD-L1 treatment 4 days after bleomycin administration to mice, as well as weight differences for isotype and anti-PD-L1 bleomycin-treated mice ($n = 7$). (I) Percentage of CD4⁺PD-1⁺ T cells by flow cytometry after anti-PD-L1 antibody administration to bleomycin-treated mice ($n = 5$). (J) Percentage of phosphorylated STAT3 at Tyr⁷⁰⁵ in CD4⁺ T cells from single-cell lung suspensions of bleomycin-treated mice after isotype and anti-PD-L1 antibody treatment ($n = 10$ and $n = 12$, respectively). (K) Linear correlation between PD-1 and pSTAT3 (Y705) after bleomycin administration. Comparisons between cohorts were performed using an unpaired two-tailed Student's t test. Preblockade and PD-1 pathway blockade cohort comparisons were analyzed using a paired Student's t test. Multiple group comparisons were performed using a one-way ANOVA with Tukey's post hoc test. Proliferation data were analyzed using the Mann-Whitney U test. PD-1/pSTAT3 correlation analyzed using Spearman correlation. Bars indicate SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. NS, no significance.

Table 1.

Demographics of IPF and age-matched control populations.

	Healthy control	IPF
Number	5	33
Sex (female, male)	0, 5	7, 26
Age (year), median (minimum, maximum)	61 (44, 68)	66 (49, 80)
Race	5 C	33 C

C, Caucasian.

Table 2.

Demographics of peripheral blood sarcoidosis, IPF, and control populations.

	Healthy control	Sarcoidosis	IPF
Number	32	46	33
Sex (female, male)	19, 13	32, 14	7, 26
Age (year), median (minimum, maximum)	44 (25, 68)	54 (30, 75)	66 (49, 80)
Race	4 AA; 28 C	17 AA; 28 C; 1 H	33 C

AA, African-American; H, Hispanic.