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Treatment with imatinib results in reduced IL-4–producing T cells, but increased CD4⁺ T cells in the broncho-alveolar lavage of patients with systemic sclerosis

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Abstract

T cells, particularly those producing IL-4, are implicated in inflammation-mediated fibrosis. In our phase I/IIa open-label pilot study in 15 patients with scleroderma-interstitial lung disease (SSc-ILD), high-dose imatinib treatment showed modest improvement in lung function and skin score, but with several adverse events. Here, we investigated T cell phenotype and cytokine production in bronchoalveolar lavage (BAL) from patients enrolled in this trial. We found that IL-4⁺ T cells showed a stronger correlation with ground glass opacity (GGO) than fibrosis scores on lung high-resolution computer tomography scans. Frequencies of IL-4⁺ T cells also discriminated patients with high (≥ 20) versus low (< 20) GGO scores. Functional annotation clustering of proteins that correlated with T cells identified two major clusters that belonged to immune/inflammatory and wounding response. Repeat analyses after one year of treatment in 10 BAL samples, one each from the right middle and lower lobes of lung from 5 patients, showed that post-imatinib, IL-4⁺ T cells were profoundly reduced but CD4⁺ T cells increased, except in one patient who showed worsening of SSc-ILD. Post-imatinib increase in CD4⁺ T cells correlated with soluble ICAM-3 and PECAM-1 levels in BAL, which associated with the lack of worsening in SSc-ILD. Thus,

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imatinib might confer its therapeutic effect in fibrosis via re-directing T cell responses from type 2 to other, non-type 2 cytokine producing CD4⁺ T cells.

Keywords

IL-4; T cells; imatinib; scleroderma; fibrosis

1. Introduction

Uncontrolled tissue repair or fibrosis is a common end-result of tissue injury, including inflammation. Chronic inflammation and fibrosis are hallmarks of systemic sclerosis (SSc, scleroderma) that involves multiple organs. SSc-associated interstitial lung disease (SSc-ILD) is a major cause of morbidity and mortality, but its pathogenesis is poorly understood. Studies in patients with SSc-ILD and animal models suggest a role for T cells that produce type-2 cytokines such as IL-4 [1, 2]. It is unclear whether any of the treatments currently used in patients with SSc modulate the production of these cytokines.

Currently, no treatment reverses fibrosis and no drug has been approved by the Food and Drug Administration for SSc. However, lack of worsening of lung fibrosis in SSc-ILD patients treated with cyclophosphamide [3] provides hope that fibrosis might be amenable to therapy. Imatinib, a tyrosine kinase inhibitor approved for the treatment of leukemia and gastrointestinal tumors [4], inhibits tyrosine kinase activity of c-abl, c-kit and c-fms [5]. Interestingly, c-abl deficiency attenuates the induction of extracellular matrix proteins by transforming growth factor β (TGF β), a pro-fibrotic cytokine [6]. Imatinib also blocks the tyrosine kinase activity of platelet-derived growth factor (PDGF) receptor that is implicated in fibrosis [7]. Thus, imatinib may target two pro-fibrotic pathways activated in SSc. Indeed, several case reports in patients with SSc, nephrogenic systemic fibrosis, and graft-versus-host-disease report regression of fibrosis following imatinib treatment [8–10].

Thus we and others have evaluated the effect of imatinib in SSc-ILD in a phase I/IIa one-year open-label trial [11, 12]. Treatment with imatinib led to an estimated improvement of forced vital capacity (FVC) % of 1.74% and modified Rodnan skin score (MRSS) of 3.9 units [11]. To assess the impact of T cell alterations in relation to disease and imatinib treatment, we correlated T cell subsets with proteins in BAL and disease parameters including high-resolution computerized tomography (HRCT) findings and pulmonary function tests (PFT). We report that IL-4⁺ T cells in BAL that correlate with baseline HRCT findings of SSc-ILD decrease significantly post-imatinib. However, CD4⁺ T cells increased in all except one patient who had worsening of SSc-ILD post-imatinib.

2. Materials and Methods

2.1 Patients

Patients are participants in a single center, open-label study of imatinib for treatment of active SSc-ILD with disease duration ≤ 7 years, FVC $< 85\%$ predicted and $\geq 50\%$ predicted, dyspnea on exertion, and presence of ground glass opacity (GGO) on HRCT (Table 1). The study “Pilot Study to Examine the Use of Imatinib (Gleevec) for the Treatment of Active Alveolitis in Systemic Sclerosis” was approved by the University of California at Los Angeles Institutional Review Board and informed consent was obtained from all patients. The clinical trial was conducted under IND#55,666 and registered with clinicaltrials.gov (NCT00512902) [11]. Major exclusion criteria included: FVC $< 50\%$, FEV1/FVC ratio $< 65\%$, clinically significant abnormalities on HRCT not attributable to SSc-ILD, and clinically significant pulmonary hypertension requiring treatment. Seven patients completed

one-year treatment with imatinib and eight discontinued the study. Seven of the eight patients had SSc-related or imatinib-related adverse events, and one patient was lost to follow up before completing the 12-month study. BAL samples were analyzed in 11 patients at baseline and in five of these 11 patients after 12-month treatment with imatinib. Patient 3 did not have a post imatinib HRCT and Patient 4 did not have baseline HRCT. Thus Patient 4 and 3 were not included in the baseline and post-imatinib HRCT correlations respectively.

2.2. Treatment

Imatinib (Novartis Pharmaceuticals, Inc) was initiated at a dose of 100 mg per day. Safety laboratory studies were performed every 2 weeks (CBC with differential counts, renal panel, hepatic panel, and urinalysis) till a stable dose was achieved and then were performed every 2 months. Imatinib was increased by 100 mg every 2 weeks to a maximum dose of 600 mg per day. Patients were allowed low dose prednisone (< 10 mg per day) or equivalent during the trial. Patients were required to discontinue putative “disease-modifying” treatments for scleroderma (including immunosuppressives, potassium aminobenzoate, photopheresis, colchicine, or any other experimental therapy) at least 4 weeks before the study.

2.3. High-resolution CT scans

Volumetric CT scans were obtained in full inspiration and analyzed by an expert thoracic radiologist blinded to clinical and hemodynamic information. A linear scoring system was employed that accounted for the percentage of area affected in each lobe separately for extent of parenchymal abnormality in three categories: GGO, fibrosis, and honeycombing. This scoring system is based on that reported [13, 14] but differs by scoring to the closest percentage and not the quartiles. The following radiographic definitions were employed (Fig. S1): GGO represents hazy parenchymal opacity in the absence of reticular opacity, or architectural distortion; lung fibrosis represents reticular opacification, traction bronchiectasis, and bronchiolectasis; and honeycombing represents clustered air-filled cysts with dense walls. Each lung was scored separately and then each of five lobes were scored separately (upper, middle and lower lobes) for each category of parenchymal abnormality: fibrosis, GGO, and honeycombing.

2.4. Pulmonary Function Tests

Baseline measures of FVC, total lung capacity (TLC), and single-breath diffusing lung capacity for carbon monoxide (DLCO) were obtained on study-certified equipment in accordance with recommended standards. The quality of the tests was carefully monitored by centralized over-reading. Serial pulmonary function tests were performed at the start of the study and every 3 months for the duration of the study.

2.5. Collection of BAL

150 to 180 cc of sterile normal saline was flushed in the middle and lower lobes of the right lung and BAL fluid was collected. 30-ml was sent for routine lab analysis and the remainder was sent to our laboratory within 30 minutes of collection. Cells and fluid were separated by centrifugation. Separated fluid was stored in 2–5 ml aliquots at –80°C.

2.6. Flow cytometry

BAL cells were stained for cell surface markers of T cells using anti-CD3-APC, clone SK7 (BDBiosciences, CA), anti-CD4-FITC, clone RPA-T4 (BDBiosciences, CA), and anti-CD8-PEcy5, clone RPA-T8 (BD Biosciences, CA). For intracellular staining, cells were incubated for 30 minutes at 4°C with the cell surface stains followed by permeabilization and incubation with anti-IL-4-PE, clone MP4-25D2 (eBioscience, San Diego, CA), anti-IFN γ -PEcy5, clone 4S.B3 (eBioscience, San Diego, CA), and anti-IL-13-FITC, clone PVM13-1

(eBioscience, San Diego, CA) or with appropriate isotype controls, as per manufacturer's recommendations. Cells (2×10^5) were acquired on a FACSCalibur at UCLA Translational Immunology Core and data analyzed using FlowJo (Tree Star Inc, WA) software. Cell frequencies are reported as a percent of total cells to compensate for inter-sample differences in fluid volume procured for analysis.

2.7. Multiplex protein analysis

BAL samples were analyzed for cytokines and inflammatory markers using SearchLight technology, a multiplex ELISA based technology (Aushon Biosystems, MA). A preliminary analysis of 165 proteins was conducted in BAL samples from three randomly selected patients. Proteins that were detected in these samples were chosen for analysis in this study (Table S2). Results are expressed as the mean of duplicates for each cytokine.

2.8. Bioinformatics

Proteins that showed a correlation with T cell subsets were chosen and gene ontology (GO) annotation and clustering of proteins, was analyzed using web based tools, Database Annotation, Visualization and Integrated Discovery (DAVID) and Gostat [15–17]. DAVID provides integrated solutions for the annotation and analysis of genome-scale datasets derived from high-throughput technologies such as microarray and proteomic platforms. Gostat determines statistically significant over- or under-represented GO categories within lists of genes.

2.9. Statistics

Graph Pad prism version 5 and STATA 10.2 were used for analysis. Spearman non-parametric correlation analysis was performed to determine correlations between cell frequencies and HRCT scores or PFT values. Groups were compared using Wilcoxon rank sum test. Wilcoxon signed rank test was used to compare baseline and post-imatinib values. Correlation values were interpreted as follows: 0.00–0.20, no correlation; 0.21–0.40, low; 0.41–0.60, moderate; 0.61–0.80, marked; and 0.81–1.00, high correlations [18]. As this is a hypothesis-generating analysis, we did not account for multiple comparisons.

3. Results

Demographic and clinical characteristics of SSc patients enrolled in this study are described in Table I. HRCT scans of lung were performed at baseline and scored for GGO, fibrosis, and honeycombing (Fig. S1). BAL samples were collected from the right middle lobe (RML) and right lower lobe (RLL) and pooled for analysis in the first two patients. However, their HRCT scores were substantially different between RML and RLL. Hence, in subsequent patients we analyzed the RML and RLL samples separately. As shown in Fig. S2, fibrosis and GGO scores were higher in RLL compared to RML in patients in this study ($p < 0.001$).

3.1. T cell subsets in the BAL of SSc-ILD patients at baseline

BAL samples collected at baseline were analyzed for T cell subsets and their cytokine production. *Ex vivo* intracellular cytokine analysis revealed a large population of T cells that produced IL-4, but not IFN- γ , in the BAL of SSc-ILD patients (Fig. 1B, C). Both CD4⁺ and CD4⁺ T cells produced IL-4 without any further *in vitro* stimulation (Fig. 1C). In some patients, >90% of the BAL T cells produced IL-4 *ex vivo* (Fig. 1C, **right panel**), whereas other patients (Fig. 1C, **left panel**) had a few IL-4⁺ T cells in their BAL. Data from all patients are summarized in Fig. 1D. As shown in Fig. 1E, there were substantial interlobar differences in the frequency of T cell subsets ($p < 0.05$ for CD4⁺CD3⁺IL-4⁺ and CD8⁺ T

cells). We found 15–1000-fold differences in the frequencies of CD4⁺CD3⁺IL-4⁺ and CD4⁺CD3⁺IL-4⁺ cells and 7-1000-fold differences in the frequencies of CD4⁺/CD8⁺ T cells between the two lobes in about half of patients, although the differences between RML and RLL were not statistically significant.

3.2. CD4⁺, but not CD8⁺, T cells correlate with GGO at baseline

To understand the implications of BAL T cells in SSc-ILD, T cell subsets in BAL samples collected at baseline were correlated with HRCT scores and PFTs. CD4⁺ T cells moderately correlated with GGO, weakly with fibrosis scores (Fig. 2A), and inversely with DLCO (Table S1). CD8⁺ T cells, on the other hand, did not correlate with GGO, and weakly correlated with fibrosis (Fig. 2A).

3.3. IL-4-producing T cells correlate more strongly with GGO than with fibrosis at baseline

IL-4⁺ T cells, both CD4⁺ and CD4⁺ subsets, showed a marked correlation with GGO and a low correlation with fibrosis (Fig. 2A). In previous studies, ≥20% fibrosis was found to be an independent predictor of disease progression as assessed by FVC and mortality [19, 20]. We thus divided the BAL samples in two groups based on 20% cut-off for GGO and fibrosis scores, and found that the frequencies of CD4⁺IL-4⁺ and CD4⁺CD3⁺IL-4⁺ cells discriminated patients based on 20% GGO, but not based on 20% fibrosis cut-off (Fig. 2B, C). There was no difference in the frequency of CD4⁺ or CD8⁺ T cells between the two groups. Thus, IL-4⁺ T cells in BAL correlate with GGO that is believed to represent early stages of SSc-ILD [21].

3.4. Bioinformatic analysis for GO annotations and GO clusters for proteins that correlate with T cell subsets in BAL

To begin to understand probable mechanisms and potential roles of T cell abnormalities in BAL of SSc patients, we sought correlation between the frequency of T cells in BAL and 96 proteins that were measured using a multiplex assay, as shown in Table S2. CD4⁺ T cells correlated with a number of chemokines and proteins including CCL8, CCL24, CXCL10, G-CSF, IL-1RA, PAI-1 active, sex hormone binding thyroglobulin (SHBG), and visfatin (Nampt). Analysis of GO annotations for these proteins revealed enrichment of GOs namely cell proliferation and extracellular proteins; immune response, inflammatory response, chemokine activity, and signaling proteins for CD4⁺ T cells (Fig. 3A).

As shown in Table S2, the frequencies of both CD4⁺IL-4⁺ and CD4⁺CD3⁺IL-4⁺ cells correlated with a similar set of proteins. The GO annotations for these CD4⁺/CD4⁺ IL-4⁺ T cell associated proteins, as determined using Gostat [17], include extracellular region, signaling, immune and inflammatory response, chemotaxis and cell movement, cytokine activity, cell proliferation, and apoptosis (Fig. 3B). Clustering of these proteins using Gostat and DAVID identified two major clusters (Fig. 3C). A cluster including CCL5, CXCL10, IL-1a, IL-15 and MIF associated with GO terms “response to wounding” and “defense response”. Proteins Areg, CCL5, CXCL10, IL-1a, IL-15, TRAIL/TNFSF10 and MIF associate with GO terms inflammatory and immune response.

3.6. Imatinib-treated patients had reduced IL-4⁺ T cells but increased CD4⁺ T cells in the BAL

Of 15 patients enrolled in the trial, seven completed one-year treatment with imatinib. IL-4⁺ T cells, both CD4⁺ and CD4⁺, that were abundant in BAL at baseline (Fig. 1C) became undetectable in 7 of 11 post-imatinib BAL samples (Fig. 4A). However, CD4⁺ T cells were increased or unchanged in all but two BAL samples, both of which were from patient #9 who had worsened SSc-ILD as shown by increased lung fibrosis and GGO and reduced

FVC post-imatinib (Fig. 4B, C). HRCT scores and FVC values were stable or slightly improved in all patients who had increased CD4⁺ T cells post-imatinib (Fig. 4B, C). This observation is further illustrated in Table 2 and Fig. S3, showing that an increase in CD4⁺ T cells post-imatinib markedly correlated with FVC and DLCO, and negatively with fibrosis. Thus, IL-4⁺ T cells were significantly reduced in the BAL of imatinib-treated patients. Additionally, increased CD4⁺ T cells in BAL correlated with improved/stable SSc-ILD after treatment.

To begin to understand possible mechanisms whereby imatinib might elicit above cellular changes, we asked if proteins that correlated with BAL cells at baseline were modulated by imatinib treatment. Table S3 describes correlation of changes in protein levels with changes in T cell subsets following imatinib treatment. We found that increased CD4⁺ T cells post-imatinib correlated with changes in BAL levels of CCL5, CCL20, CXCL9, CXCL10, intracellular adhesion molecule-3 (ICAM-3), and platelet endothelial cell adhesion molecule-1 (PECAM-1) ($r=0.4-0.82$) (Table S3 and Fig. 5A). Soluble ICAM-3 and PECAM-1, which have been reported to inversely correlate with lung fibrosis [22, 23], increased in all samples, except patient #9 who had worsened fibrosis (Fig. 5B) and FVC, and reduced CD4⁺ T cells.

4. Discussion

Many reports have described T cell subsets in relation to lung function in patients with SSc [1, 24, 25]. A few studies have also investigated T cells in relation to lung pathology as defined by histology or HRCT findings of SSc-ILD [26]. In this article, we extend these observations and report a relation between T cell subsets at the affected site (BAL) and HRCT-defined GGO and fibrotic lesions of SSc-ILD. CD4⁺ T cells and CD4⁺/CD4⁻ IL-4⁺ T cells showed a stronger correlation with GGO than with fibrosis scores. Importantly, we found a marked reduction in CD4⁺/CD4⁻ IL-4⁺ T cells in the lung of patients post imatinib. However, total CD4⁺ T cells increased after imatinib treatment. The increases in BAL CD4⁺ T cells as well in BAL levels of ICAM-3 and PECAM-1 appear to identify patients with stable / improved SSc-ILD after imatinib treatment. These data, albeit pilot, have implications for the development of new, targeted therapies for SSc-ILD.

We found substantial inter-lobar differences in HRCT scores and T cell subsets in BAL from patients with SSc-ILD. These data along with a previous study showing substantial inter-lobar variability in the percentage of neutrophils in BAL from patients with connective tissue disease associated ILD [27] emphasize the need for obtaining lavage from more than one lobe. Interlobar differences in lymphocyte and T cell infiltration have also been reported in patients with interstitial lung disease and HIV infection [28, 29].

Several studies have ascribed a role for type 2 cytokines in reduced lung function in SSc [24, 30–32]. Cytokines in most studies, however, were detected after *in vitro* polyclonal stimulation that reflects the ability of T cells to make a cytokine, but may not reflect the *in vivo* production of a cytokine. Surprisingly, we detected a large population (>90% in some patients, Fig. 1C, **right panel**) of BAL T cells producing IL-4 by direct *ex vivo* cytokine analysis, suggesting a profound *in vivo* type-2 cytokine deviation in the lungs of patients with SSc. On the other hand in patients with very little or no HRCT abnormalities few to no IL-4⁺ T cells were detected (Fig. 1C, **left panel**). We found that the frequencies of CD4⁺/CD4⁻ IL-4⁺ T cells in BAL markedly correlated with GGO and discriminated patients with high versus low GGO scores, but did not distinguish patients based on high versus low fibrosis scores. The implication of such differential association of T cell subsets with GGO that is believed to represent early events in SSc-ILD [21] versus more advanced fibrosis remains to be determined.

The GO annotations and clusters of proteins that correlated with T cell subsets in BAL (Table S2, Fig. 3) suggest new pathways whereby T cells might contribute to the pathogenesis of SSc-ILD. CD4⁺ T cells correlated with PAI-1 active and G-CSF, which are involved in migration of lymphocytes and collagen producing cells and in Th2 differentiation, respectively [33]. CD4⁺ T cells also correlated with CD14, IL-1RA, G-CSF, PAI-1 active, SHBG and Nampt, which are known to be involved in regulation of immune response, leukocyte migration, and Th2 differentiation [33–37]. A similar set of proteins correlated with both CD4⁺CD3⁺IL-4⁺ and CD4⁺CD3⁺IL-4⁺ cells, suggesting a common mechanism of induction and/or function of these cells in lung. The CD4⁺/CD4⁺ IL-4⁺ T cell associated proteins were enriched into two GO clusters that were associated with GO terms of inflammatory and immune responses, and response to wounding. These data warrant further studies to examine whether and how CD4⁺/CD4⁺ IL-4⁺ T cells influence immune, inflammatory and wound healing responses, leading to the development of GGO in patients with SSc.

Recent open-label trials and case reports have shown a potential efficacy of imatinib against SSc-ILD, SSc-associated pulmonary hypertension and skin fibrosis [11, 12, 38, 39]. Imatinib also caused many adverse effects at the dose regimen used [11]. These effects may be related to many immune effects elicited by this drug, including inhibition of T cell proliferation and production of cytokines IL-2, TNF α and IFN γ *in vitro* [40–42]. To the best of our knowledge, our data represent the first demonstration of a marked reduction in IL-4 producing T cells *in vivo* at the effected site following treatment with imatinib in humans. It remains to be determined how this finding is related to imatinib's effect on tyrosine kinases PDGFR β and Abl that were reported to be reduced in the skin of two patients treated with imatinib [43]. The latter study also identified an imatinib-responsive signature with genes involved in multiple functional pathways, including cell proliferation, matrix and vascular remodeling, immune signaling, and growth factor signaling [43]. Several proteins involved in these cellular functions correlated with frequencies of IL-4⁺ T cells in SSc patients' BAL at baseline in our study. Changes in many of these proteins also correlated with changes in T cell subsets elicited by imatinib. Taken together, these observations suggest a link between cellular and molecular changes elicited by imatinib in skin and lung of patients with SSc.

Our results show that while CD4⁺IL-4⁺ cells that comprised a substantial fraction of CD4⁺ T cells at baseline in our patients were significantly reduced, IL-4⁺CD4⁺ T cells were significantly increased post-imatinib. The increase in CD4⁺ T cells in BAL (Fig. 4) and peripheral blood (our manuscript in preparation) also appeared to correlate with reduced or stable SSc-ILD (Fig. 4). These findings in BAL are likely to have implications for disease in other organs, as one patient who exhibited worsening of ILD post-imatinib also showed no reduction in MRSS whereas the other four imatinib-treated patients had improved skin disease. We further found that the increase in CD4⁺ T cells in BAL post-imatinib correlated with increases in BAL levels of ICAM-3 and PECAM-1 (CD31), which in turn also correlated with the lack of worsening of SSc-ILD (Fig. 4 and 5). Soluble ICAM-3 may inhibit interaction between cell surface ICAM-3 and LFA-1 thereby preventing co-stimulation of T cells, whereas soluble PECAM-1 levels can inhibit transmigration of cells into affected tissues [23, 44]. Thus, we speculate that treatment with imatinib may induce the migration or generation of CD4⁺ T cells that might be protective in SSc-ILD. Further studies are warranted to identify the phenotype and function of these potentially protective CD4⁺ T cells in the lungs of patients with SSc-ILD.

In summary, we report that the long-term treatment with imatinib halts the worsening of lung fibrosis in some patients who completed the trial, reduces IL-4⁺ CD4⁺/CD4⁺ T cells, but increases certain CD4⁺ T cells in the lungs of patients with SSc-ILD. IL-4⁺CD4⁺/CD4⁺ T cells in BAL from these patients at baseline associated with a number of proteins that

were enriched in GO clusters associated with immune, inflammatory, and wound healing response. Further elucidation of these cell-protein associations and their potential interactions will identify new targets for manipulation of fibrotic diseases.

Highlights

- In patients with systemic sclerosis-associated interstitial lung disease (SSc-ILD), IL-4⁺ T cells in lung fluid (bronchoalveolar lavage, BAL) correlate with HRCT lesions of ground glass opacity and with proteins involved in multiple cellular functions including signaling, immune and inflammatory response, chemotaxis and cell movement, cytokine activity, cell proliferation, and apoptosis.
- IL-4⁺ T cells in BAL are significantly reduced after 12-month treatment with imatinib. However, IL-4⁻ CD4⁺ T cells, along with ICAM-3 and PECAM-1 levels, increase in BAL after treatment.
- There is a substantial variability in the BAL frequency of T cell subsets between the right middle and lower lobes of lung

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. T cell subsets in the BAL of patients with SSc-ILD at baseline: interlobar differences
BAL cells were stained for surface markers (A) and for IL-4 and IFN- γ or respective isotype controls (B, C) by intracellular staining. (A) Representative dot plots show CD4⁺ and CD8⁺ T cells. Numbers in quadrants indicate frequencies of CD4⁺ and CD8⁺ as percent of lymphocyte gate (small cells). (B) Representative dot plots depicting very few IFN γ producing cells (top panel, isotype control; bottom panel, IFN γ staining). Numbers in quadrants indicate frequencies of IFN γ ⁺ cells as percent of CD3⁺ cells. (C) Representative dot plots from two SSc patients depicting the abundance (right panel) or paucity (left panel) of IL-4 producing cells. Top panel, isotype control; bottom panel, IL-4 staining. Numbers in quadrants indicate frequencies of IL-4⁺ cells as percent of CD3⁺ cells. (D) Summarizes

frequencies of IL-4 producing cells as the proportion of T cells (23.5 ± 7.1) and 19.3 ± 6.6 ($CD4^+CD3^+IL-4^+$) mean $\pm$ SE; n = 16). (E) Frequencies of T cell subsets ($CD4^+$, $CD8^+$, $CD4^+CD3^+IL-4^+$ and $CD4^+CD3^+IL-4^-$), expressed as the % of total BAL cells, were compared between the two lobes (n=8 patients, excluding patients 1 and 2 where BAL was pooled). Values <0.01 are plotted at 0.01. There were substantial interlobar differences that were statistically significant for $CD8^+$ and $CD4^+CD3^+IL-4^+$ cells ($p < 0.05$, low versus high values regardless of the lobe, paired t-test).

Fig. 2. Relation of T cell subsets in BAL with HRCT scores of SSc-ILD at baseline

(A) Frequencies of T cell subsets were correlated with GGO (top panels) and fibrosis scores (lower panels). Colors indicate values from individual patients. Closed and open symbols indicate RML and RLL, respectively. Arrows exemplify interlobar differences in T cell frequencies and HRCT scores. T cell subset frequencies are expressed as the % of total BAL cells. The frequencies are plotted at 0.01 if no positive cells were undetected in the respective BAL. HRCT values that were <0.5 are plotted at 0.5. R-values from non-parametric Spearman correlations are indicated on plots. ** $p < 0.001$ ($n = 19$ lobes from 12 patients; BAL from patients 1 and 2 were pooled). (B) Frequencies of T cell subsets were compared between patients with severe ($n = 6$ lobes, 5 patients) versus mild ($n = 13$ lobes, 8

patients) disease based on 20% cut-off for GGO scores. Patients 3 and 9 had more severe in disease in lower lobe (>20% GGO) compared to middle lobe. (C) Frequencies of T cell subsets were compared between patients with severe (n=5 lobes, 4 patients) versus mild (n=14 lobes, 10 patients) disease based on 20% cut-off for fibrosis scores. Patients 3, 5 and 9 had more severe in disease in lower lobe (>20% fibrosis) compared to middle lobe. * indicates significant difference between the two groups.

Fig. 3. T cell subset associated proteins in BAL and their GO annotations

BAL fluid was processed and cells were separated. Cells were analyzed for T cell subsets and supernatant was tested for 96 cytokines and inflammatory mediators. Proteins that correlated with T cell subsets are listed in Table S2. We used web-based tools, namely Gostat and DAVID, to identify GO annotations and clustering for these proteins that correlated with T cell subsets. (A, B) Most abundant GOs among proteins that correlated with individual T cell subsets. GOs that were identified only once are not shown. $CD4^+CD3^+IL-4^+$ and $CD4^-CD3^+IL-4^+$ cells were analyzed together, as these T cell subsets correlated with a similar set of proteins. (C) Clustering of proteins based on cellular processes they are known to be involved in. $IL-4^+$ $CD4^+/CD4^-$ T cell associated proteins were highly enriched in two clusters. Proteins that are included in these two clusters are shown along with their enrichment scores.

Fig. 4. Effect of imatinib treatment on T cell subsets in BAL

(A) Frequencies of T cell subsets, expressed as % of gated lymphocytes (upper panels) or as % of total BAL cells (lower panels), at baseline (*pre*) and post-imatinib (*post*) were compared using Wilcoxon signed rank test. Each line represents *pre* and *post* values in BAL from the same lobe of a patient. Patients 3 and 4 were included in this analysis. (B, C) Frequencies of CD4⁺ T cells (red symbols) were associated with fibrosis scores or % predicted FVC (% FVCp) shown in blue symbols at baseline (filled symbols) and post-imatinib (empty symbols). Results of patients who completed the trial and where both *pre* and *post* values were available are shown. Results from individual lobes (RML, right middle lobe; RLL, right lower lobe) are shown in (B) and excluded patients 3 and 4. In (C), BAL

CD4⁺ T cell frequencies from the two lobes were averaged and associated with % FVCp. Patient #9 (arrows) who showed worsened SSc-ILD (increased HRCT scores and reduced FVC) exhibited reduced CD4⁺ T cells. Patients 3 and 4 were excluded from this analysis. CD4⁺ T cells increased post-treatment in all other subjects who also had stable SSc-ILD.

Fig. 5. Association between changes in ICAM-3 and PECAM-1 levels, CD4⁺ T cell frequencies, and fibrosis post-imatinib

(A) Changes (post-imatinib–baseline [*post–pre*]) in CD4⁺ T cell frequencies (shown as % of total BAL cells) and in ICAM-3 and PECAM-1 levels (pg/ml) were calculated (n=8 BAL samples from 5 patients; pooled samples from patients #1 and 2). Correlation between these changes are shown (** $p<0.01$). (B) BAL levels of ICAM-3 and PECAM-1 (shown in red) and fibrosis scores on HRCT (blue) are shown at baseline (*filled symbols*) and post-imatinib (*empty symbols*). Levels of these two proteins increased post-imatinib, except in the lobes that had worsened HRCT scores post-treatment, indicated by arrow (RML, right middle lobe; RLL, right lower lobe).

Table I

Demographic and clinical characteristics of patients enrolled in the study

N=15	Mean $\pm$ SD / N (%)
Age, years	44.0 $\pm$ 12.4
Disease duration, years	4.2 $\pm$ 2.9
Gender: N (%)	
Female	11 (73.3%)
Race: N (%)	
White	10 (66.7%)
Asian	3 (20.0%)
Hispanic	2 (13.3%)
MRSS (0–51), Mean (SD)	19.5 $\pm$ 10.2
FVC% predicted (%), Mean (SD)	66.2 $\pm$ 15.4
DLCO% predicted (%), Mean (SD)	51.4 $\pm$ 11.2
TLC% predicted (%), Mean (SD)	74.5 $\pm$ 11.2

Of 15 patients enrolled in the trial, one patient (#4) did not have a baseline HRCT scan and one patient (#3) did not have a post-imatinib HRCT. Thus Patients 4 and 3 were not included in the baseline and post-imatinib HRCT correlations respectively. The mean FVC in patients in this study was lower than the mean TLC, which is likely due to a normal or increased residual volume in patients with SSc-ILD [45, 46]. MRSS – Mean Rodnan Skin Score, FVC – forced vital capacity, DLCO – diffusing capacity of lung for carbon mono-oxide, TLC – total lung capacity.

Table II

Correlation between changes in cellular versus clinical parameters post-imatinib

	Fibrosis	GGO	FVC	DLCO
CD4 ⁺	-0.70	-0.10	0.67	0.61
CD8 ⁺	-0.10	0.40	-0.03	0.14
CD4 ⁺ CD3 ⁺ IL-4 ⁺	-0.48	-0.10	-0.03	-0.26
CD4 ⁻ CD3 ⁺ IL-4 ⁺	0.39	0.51	-0.67	-0.42

Changes in cellular and clinical parameters between baseline and post-imatinib were calculated, and correlated with each other. PFTs were available at both baseline and post-imatinib in 5 patients. HRCT values Spearman values $r > 0.4$ are shown in bold.