Lung CD4+ T cells in patients with lung fibrosis produce pro-fibrotic IL-13 together with IFNγ

Short title: Distinct T cell phenotype in lung fibrosis

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Progressive fibrosing interstitial lung diseases (PF-ILD) have poor prognosis and survival, and their pathogenesis is not well understood[1]. Mechanistically, lung fibrosis is thought to result from distorted wound-healing following tissue insults and inflammation, leading to scar formation by excess deposition of extracellular matrix proteins and destruction of lung architecture[2]. The fibrotic process is complex, and CD4+ T cells are likely involved by their production of a wide range of cytokines and growth factors that promote fibroblast proliferation and differentiation, collagen production, and stimulate production of pro-fibrotic mediators by tissue macrophages[3]. However, CD4+ T cells in PF-ILD are poorly characterized. To this end, we performed a detailed analysis of phenotype, cytokine production and clonality of T cells from the lungs [bronchoalveolar lavage (BAL)] of PF-ILD patients. We found that BAL from PF-ILD lungs contained high numbers of clonally expanded CD4+ T cells that produced an unusual combination of interferon (IFN) γ and pro-fibrotic interleukin (IL)-13. Such cells were not found in patient blood or in control BAL samples.

Materials and Methods

Subjects

This cross-sectional study included a cohort of: (i)44 consecutive patients with PF-ILD [mean(SD) age 67(6)] referred for multidisciplinary diagnostic evaluation and (ii)14 control patients, aged 50(13) without ILD (data collection: year 2015-2019). The controls underwent bronchoscopy with BAL>6 months after resection of carcinoid tumor, and considered healthy with no lung diseases. 7 patients with PF-ILD had also signs of emphysema. Contraindications for bronchoscopy were FVC<50% predicted and/or DLCO<40% predicted. Exclusion criteria were age>75 years, anti-fibrotic treatment, infections and active smoking during the last year. 30 patients with PF-ILD and 2 control patients were ex-smokers.

BAL and Peripheral blood mononuclear cells (PBMC)

BAL was performed with the patient in supine position and the bronchoscopy wedged in a middle lobe segment (Instillation;3×40 mL Ringer solution, 37°C). Recoveries of the second and third aliquot were used for the cell analysis. BAL was filtered (pore size;48μm), and processed[4]. PBMC were prepared using Lymphoprep (STEMCELL Technologies).

Flowcytometric cytokine analysis

Cells from BAL and PBMC were treated as described[5]. Briefly, cells were stimulated with PMA/Ionomycin for 3.5h, stained for surface and intracellular antigens, acquired on a BD LSRFortessa (BD Bioscience), and FlowJo (LLC, Oregon) for analysis. CD4+ T cells were gated as CD3+/CD8− since surface CD4 expression is reduced by PMA/Ionomycin stimulation[6]. Analysis of untreated cells showed that >90% of the CD3+/CD8− T cells were CD4+. Cells were stained with anti-IFNγ Alexa488, anti-IL-10 BV421, anti-IL-17 BV421, anti-IL-13 PE, anti-CD3 PECy7, anti-CD4 BV421 anti-CD8 PerCP/Cy5.5 (Biolegend) and e780 live/dead discriminator dye (ThermoFisherScientific).

Clonality analysis

MACS Cytokine secretion assay (MiltenyiBiotec) was used to identify IFNγ and IL-13 producing viable cells for T cell receptor (TCR) single-cell clonality analysis [anti-IFNγ APC and anti-IL-13 PE (MiltenyiBiotec), anti-CD3 PECy7 and anti-CD8 PerCP/Cy5.5], and sorted on a BD FACSAriaIII. TCR sequencing were carried out as described[7]. The average TCRαβ sequencing efficiency was 77% (59-78) [median (range)] of which half were paired TCRαβ sequences.

Ethics

Approval: Regional Committee for Medical Research Ethics (2013/2358). Written informed consent was obtained.

Statistics

Statistical comparisons were performed using Mann Whitney or t-tests when appropriate (SPSS V26). Degree of clonal expansion was described by Diversity50 (D50) (Larger D50 shows larger diversity and less clonality)[7].

Results

Following multi-disciplinary evaluation, 44 patients were diagnosed with IPF (n=32), HP (n=8) or unclassifiable PF-ILD (n=4). Patients with PF-ILD had reduced lung function compared to controls [mean (SD) FVC % predicted 78(15) vs 106(17) and DLCO % predicted 50(8) vs 87(14), $p<0.001$]. Many T cell cytokines may drive tissue fibrosis, including IL-10, IL-13, IL-17 and IFNγ[8]. Thus, cytokine production of lung T cells from BAL was assessed after short-term stimulation with PMA/ionomycin followed by intracellular cytokine staining (Figure 1a). No significant differences of IL-10+ and IL-17+ CD4+ T cells between PF-ILD and controls were detected, [median (IQR)] 5% (4-6) and 4% (3-9) for IL-10 and 5% (3-7) and 9% (4-12) for IL-17, respectively. In contrast, BAL CD4+ T cells from PF-ILD patients contained a larger fraction of IL-13 producing T cells compared to controls, and the majority of these co-expressed IFNγ (Figure 1b). T cells co-expressing IL-13 and IFNγ were not detectable in PBMC from patients or controls. Compiled analyses of BAL CD4+ IL-13+/IFNγ+ cells showed a median of 8% (IQR 3-17) in PF-ILD. This was more than 4-fold higher than the controls [2% (2-4)].

To assess clonal expansion, single-cell TCRαβ sequencing was performed in BAL cells in four randomly selected patients with final diagnosis IPF (n=2), unclassifiable PF-ILD (n=1) and HP (n=1). The analysis showed that 28-80% IL-13+/IFNγ+ cells expressed identical TCR sequences indicating clonal expansion (Figure 1c). Identical TCR sequences were observed in IL-13–/IFNγ+ cells, but the degree of clonal expansion was lower (17-59%) than in IL-13+/IFNγ+ cells. The IL-13+/IFNγ+ T cells had the smallest diversity with D50 of 0.29 (average), compared to IL-13–/IFNγ+ and IL-13–/IFNγ– T cells with D50 of 0.39 and 0.48, respectively.

Discussion

We describe a highly unusual phenotype of CD4+ T cells in BAL of PF-ILD patients, which co-express both the Th1-associated cytokine, IFNγ, and pro-fibrotic Th2-associated cytokine, IL-13. Such cells were not present in patients' blood and were hardly detectable in BAL from non-fibrotic controls. These distinct T cells had undergone clonal expansion probably due to local antigenic stimulation, and may be specifically related to pathogenic processes in the lungs of PF-ILD patients. To our knowledge, T cells with such "dual" phenotype have not previously been described in humans. However, chronic IL-18 stimulation of T cells may induce production of IL-13 in combination with IFNγ[9, 10]. Importantly, in mice such Th1/Th2-cells drive a pathogenic cascade characterized by airway hyper-responsiveness, lung inflammation and lung fibrosis[10, 11]. Earlier studies have shown increased levels of IL-13 in the lungs in patients with PF-ILD[2], and it is thus tempting to speculate that the lung-associated IL-13+/IFNγ+ CD4+ T cells that we have identified may be important in the pathogenesis. In the present study the IL-13+/IFNγ+ CD4+ T cells display characteristics of tissue resident memory T cells (Trm) by being present in BAL-samples, but undetectable in peripheral blood of PF-ILD patients. However, markers to directly identify lung CD4+ Trm are lacking[12]. Moreover, it is unclear to what extent T cells obtained by BAL reflect the cellular profiles of the lung parenchyma [13]. Future efforts should therefore be directed at assessing if the IL-13+/IFNγ+ CD4+ T cells are *bona fide* Trm, their anatomical location, and whether they are amenable to depletion from the tissue.

In human diseases, clonally expanded tissue resident memory T cells may promote chronic inflammation[14]. Earlier studies analyzing bulk preparations of BAL T cells with a panel of riboprobes corresponding to common TCR□ genes indicated expanded T cell clones in patients with lung fibrosis[15]. Here we directly show by single cell analysis of paired TCR sequences that IL-13+/IFNγ+ CD4+ T cells exhibit a high degree of clonal expansion indicating that they have

expanded due to local antigenic stimulation. Repeated antigen stimulation may lead to downregulation of CD28 in T cells. Increased numbers of $CD28^{null}$ T cells in blood may predict poor IPF prognosis [16]. Although analysis of $CD4^{+}CD28^{null}$ T cells from lung explants revealed no increase of such cells in IPF compared to control [17], further analysis should assess whether IL-13+/IFNγ+ CD4+ T cells display this phenotype.

There is a need for better treatment options in PF-ILD, and our identification of a novel, clonally expanded population of lung-associated T cells with a distinct phenotype indicates a potential value of targeting these cells for therapeutic purposes. Future efforts to disentangle their antigenic specificities and the molecular mechanisms that support their maintenance in lung tissue are warranted.

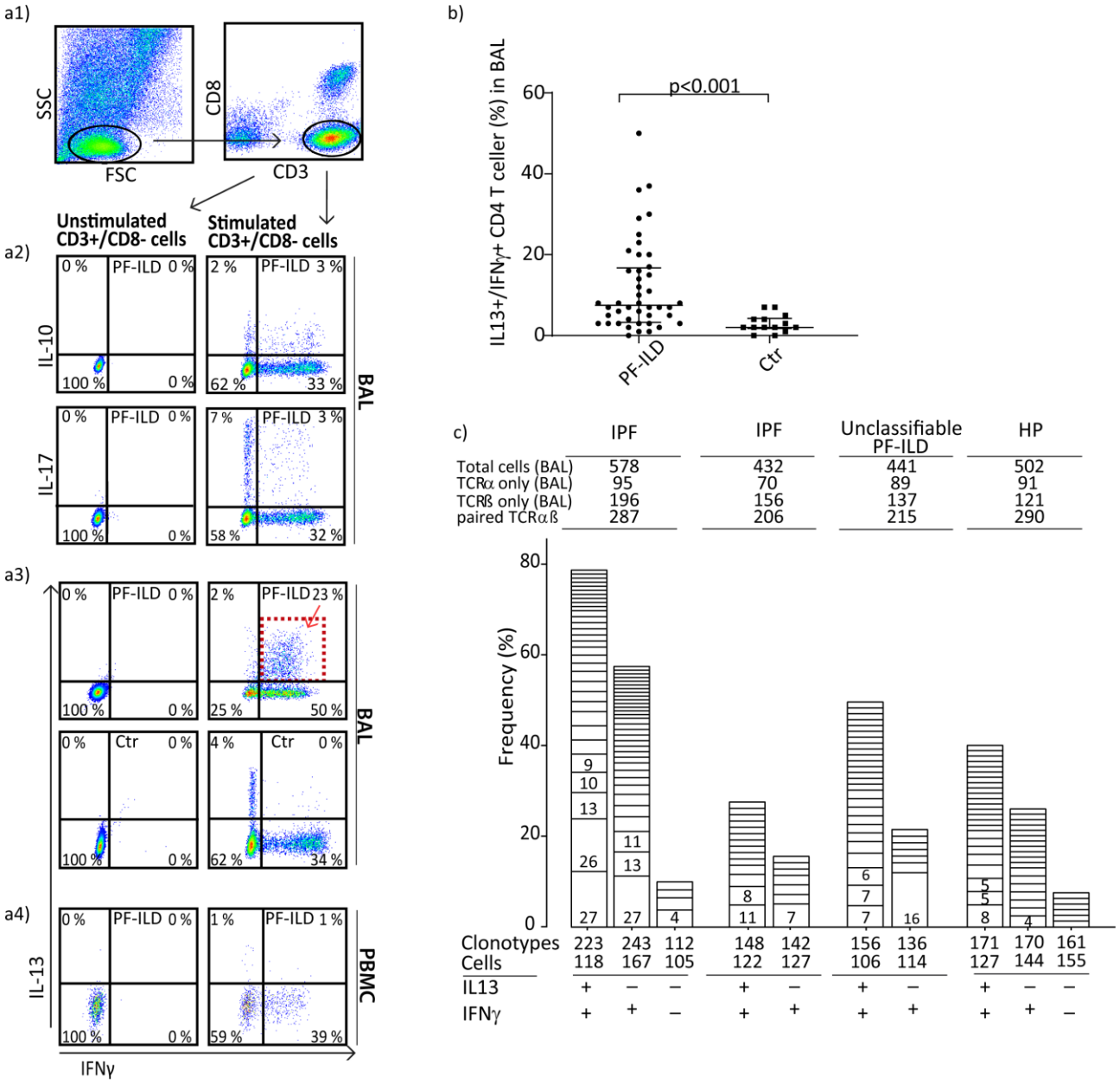
Author contributions: All authors contributed to the design of the study, to interpretation of the results, and in revising the manuscript. LIBS, SHW, OM, LW, EB participated in data collection and performed the laboratory work. LIBS, SHW, ØM, EB drafted the manuscript. The final version has been approved by all authors.

References

1. Cottin V, Hirani NA, Hotchkin DL, Nambiar AM, Ogura T, Otaola M, Skowasch D, Park JS, Poonyagariyagorn HK, Wuyts W, Wells AU. Presentation, diagnosis and clinical course of the spectrum of progressive-fibrosing interstitial lung diseases. *European Respiratory Review* 2018: 27(150): 180076.
2. Wynn TA. Integrating mechanisms of pulmonary fibrosis. *The Journal of experimental medicine* 2011: 208(7): 1339-1350.
3. Gieseck RL, 3rd, Wilson MS, Wynn TA. Type 2 immunity in tissue repair and fibrosis. *Nat Rev Immunol* 2018: 18(1): 62-76.
4. Gregersen S, Holm AM, Fevang B, Ueland T, Sikkeland LI, Aalokken TM, Mynarek G, Kongerud J, Aukrust P, Johansen B, Froland SS. Lung disease, T-cells and inflammation in common variable immunodeficiency disorders. *Scand J Clin Lab Invest* 2013: 73(6): 514-522.
5. Ballke C, Gran E, Baekkevold ES, Jahnsen FL. Characterization of Regulatory T-Cell Markers in CD4+ T Cells of the Upper Airway Mucosa. *PLoS One* 2016: 11(2): e0148826.

6. O'Neil-Andersen NJ, Lawrence DA. Differential modulation of surface and intracellular protein expression by T cells after stimulation in the presence of monensin or brefeldin A. *Clin Diagn Lab Immunol* 2002: 9(2): 243-250.
7. Bartolome-Casado R, Landsverk OJB, Chauhan SK, Richter L, Phung D, Greiff V, Risnes LF, Yao Y, Neumann RS, Yaqub S, Oyen O, Horneland R, Aandahl EM, Paulsen V, Sollid LM, Qiao SW, Baekkevold ES, Jahnsen FL. Resident memory CD8 T cells persist for years in human small intestine. *The Journal of experimental medicine* 2019: 216(10): 2412-2426.
8. Borthwick LA, Wynn TA, Fisher AJ. Cytokine mediated tissue fibrosis. *Biochim Biophys Acta* 2013: 1832(7): 1049-1060.
9. Hata H, Yoshimoto T, Hayashi N, Hada T, Nakanishi K. IL-18 together with anti-CD3 antibody induces human Th1 cells to produce Th1- and Th2-cytokines and IL-8. *International immunology* 2004: 16(12): 1733-1739.
10. Hayashi N, Yoshimoto T, Izuhara K, Matsui K, Tanaka T, Nakanishi K. T helper 1 cells stimulated with ovalbumin and IL-18 induce airway hyperresponsiveness and lung fibrosis by IFN-γ and IL-13 production. *Proceedings of the National Academy of Sciences* 2007: 104(37): 14765-14770.
11. Ramalingam TR, Gieseck RL, Acciani TH, K MH, Cheever AW, Mentink-Kane MM, Vannella KM, Wynn TA. Enhanced protection from fibrosis and inflammation in the combined absence of IL-13 and IFN-γ. *The Journal of pathology* 2016: 239(3): 344-354.
12. Szabo PA, Miron M, Farber DL. Location, location, location: Tissue resident memory T cells in mice and humans. *Sci Immunol* 2019: 4(34): eaas9673.
13. Fujimori F, Shimizu T, Takada T, Narita J, Suzuki E, Gejyo F. Differences in lymphocyte profile between BAL fluid and human lung tissue from patients with interstitial lung disease. *Br J Biomed Sci* 2008: 65(2): 63-67.
14. Clark RA. Resident memory T cells in human health and disease. *Science translational medicine* 2015: 7(269): 269rv261.
15. Feghali-Bostwick CA, Tsai CG, Valentine VG, Kantrow S, Stoner MW, Pilewski JM, Gadgil A, George MP, Gibson KF, Choi AM, Kaminski N, Zhang Y, Duncan SR. Cellular and humoral autoreactivity in idiopathic pulmonary fibrosis. *Journal of immunology (Baltimore, Md : 1950)* 2007: 179(4): 2592-2599.
16. Herazo-Maya JD, Noth I, Duncan SR, Kim S, Ma S-F, Tseng GC, Feingold E, Juan-Guardela BM, Richards TJ, Lussier Y, Huang Y, Vij R, Lindell KO, Xue J, Gibson KF, Shapiro SD, Garcia JGN, Kaminski N. Peripheral Blood Mononuclear Cell Gene Expression Profiles Predict Poor Outcome in Idiopathic Pulmonary Fibrosis. *Science translational medicine* 2013: 5(205): 205ra136-205ra136.
17. Habiel DM, Espindola MS, Kitson C, Azzara AV, Coelho AL, Stripp B, Hogaboam CM. Characterization of CD28null T cells in idiopathic pulmonary fibrosis. *Mucosal immunology* 2019: 12(1): 212-222.

Figure 1: a1-3) Cytokine production of alveolar (BAL) CD4+ T cells. Representative plots showing the gating strategy of BAL T cells, pre-gated with viability dye, following 3.5h stimulation with PMA/ionomycin in the presence of secretion blockade (stimulated). Cells cultured without PMA/ionomycin are shown as unstimulated. Progressive fibrosis interstitial lung disease (PF-ILD), non-fibrotic control (Ctr). (a1) CD4+ T cells were gated CD3+ and CD8− cells, and stained for **(a2)** IL-10, IL-17 and IFNγ or **(a3-4)** IL-13 and IFNγ. **a4)** Identically treated PBMCs from a representative ILD-patient are shown. **b)** Compiled percentages of T cells (BAL) expressing IL-13 and IFNγ are shown (scatter, median (IQR) between PF-ILD and Ctr. **c)** Distribution of TCR (T cell receptor)-αβ clonotypes obtained by single-cell TCR sequencing of T cells in BAL from 4 patients, as well as number of clonotypes and cells for IFNγ+/IL13+, IFNγ+/IL13− and IFNγ−/IL13− CD4+ T cells. Expanded clonotypes observed in 2 cells or more are plotted as stacked boxes in the percentage of the total number of cells. Idiopathic pulmonary fibrosis (IPF), hypersensitivity pneumonitis (HP).


a1)
SSC
FSC
CD8
CD3
Unstimulated CD3+/CD8- cells
Stimulated CD3+/CD8- cells
a2)
IL-10
IL-17
BAL
PF-ILD
a3)
Ctr
a4)
IL-13
IFNγ
PBMC
b)
IL13+/IFNγ+ CD4 T celler (%) in BAL
p<0.001
PF-ILD
Ctr
c)
IPF
IPF
Unclassifiable PF-ILD
HP
Total cells (BAL) 578 432 441 502
TCRα only (BAL) 95 70 89 91
TCRß only (BAL) 196 156 137 121
paired TCRαß 287 206 215 290
Frequency (%)
Clonotypes 223 243 112 148 142 156 136 171 170 161
Cells 118 167 105 122 127 106 114 127 144 155
IL13
IFNγ