Lung CD4+ T-cells in patients with lung fibrosis produce pro-fibrotic interleukin-13 together with interferon-γ

To the Editor:

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 probably involved through 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 characterised. 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.

This cross-sectional study included a cohort of: 1) 44 consecutive patients with PF-ILD (mean±SD age 67±6 years) referred for multidisciplinary diagnostic evaluation; and 2) 14 control patients, aged 50±13 years without ILD (data collection: years 2015–2019). The controls underwent bronchoscopy with BAL >6 months after resection of carcinoid tumour, and were considered healthy with no lung diseases. Seven patients with PF-ILD also had signs of emphysema. Contraindications for bronchoscopy were forced vital capacity (FVC) <50% predicted and/or diffusing capacity of the lung for carbon monoxide (D_{LCO}) <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 two control patients were ex-smokers.

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: 48 μm), and processed [4]. Peripheral blood mononuclear cells (PBMCs) were prepared using Lymphoprep (STEMCELL Technologies).

Cells from BAL and PBMC were treated as previously described [5]. Briefly, cells were stimulated with PMA/ionomycin for 3.5 h, stained for surface and intracellular antigens, acquired on a BD LSRFortessa (BD Bioscience), and FlowJo (LLC, Oregon) was used 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-IFN-γ 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).

MACS Cytokine secretion assay (MiltenyiBiotec) was used to identify viable cells producing IFN-γ and IL-13 for T-cell receptor (TCR) single-cell clonality analysis (anti-IFN-γ APC and anti-IL-13 PE (MiltenyiBiotec), anti-CD3 PECy7 and anti-CD8 PerCP/Cy5.5), and sorted on a BD FACSAriaIII. TCR sequencing was carried out as previously described [7]. The median (range) TCRαβ sequencing efficiency was 77% (59–78), of which half were paired TCRαβ sequences.
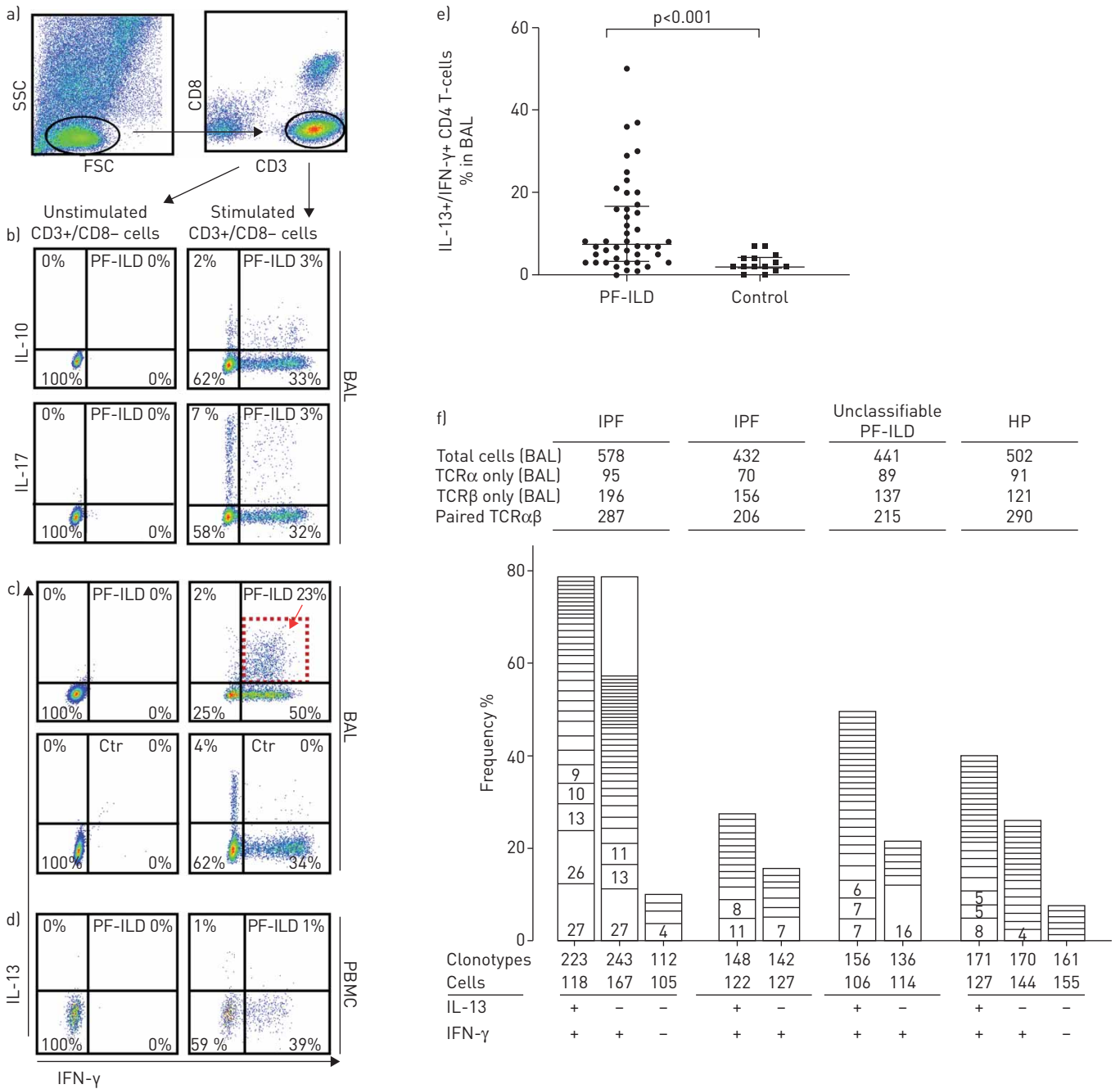
FIGURE 1 a–c) Cytokine production of bronchoalveolar lavage (BAL) CD4+ T-cells. Representative plots showing the gating strategy of BAL T-cells, pre-gated with viability dye, following 3.5 h stimulation with PMA/ionomycin in the presence of secretion blockade (stimulated). Cells cultured without PMA/ionomycin are shown as unstimulated. Progressive fibrosing interstitial lung disease (PF-ILD) and non-fibrotic controls. a) CD4+ T-cells were gated CD3+ and CD8– cells, and stained for b) IL-10, IL-17 and interferon (IFN)-γ or c and d) IL-13 and IFNγ. d) Identically treated peripheral blood mononuclear cells (PBMCs) from a representative ILD-patient are shown. e) Compiled percentages of T-cells (BAL) expressing IL-13 and IFN-γ are shown (scatter, median (interquartile range) between PF-ILD and controls. f) Distribution of TCR (T-cell receptor)-αβ clonotypes obtained by single-cell TCR sequencing of T-cells in BAL from four patients, as well as number of clonotypes and cells for IFN-γ+/IL-13+, IFN-γ+/IL-13– and IFN-γ–/IL-13– CD4+ T-cells. Expanded clonotypes observed in two cells or more are plotted as stacked boxes in the percentage of the total number of cells. IPF: idiopathic pulmonary fibrosis; HP: hypersensitivity pneumonitis.

Ethics approval was provided by the Regional Committee for Medical Research Ethics (2013/2358). Written informed consent was obtained.

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].

Following multidisciplinary evaluation, 44 patients were diagnosed with idiopathic pulmonary fibrosis (IPF) (n=32), hypersensitivity pneumonitis (HP) (n=8) or unclassifiable PF-ILD (n=4). Patients with PF-ILD had reduced lung function compared to controls (mean±SD FVC 78±15% versus 106±17% pred and D_{LCO} 50±8% versus 87±14% pred; p<0.001)].

Many T-cell cytokines may drive tissue fibrosis, including IL-10, IL-13, IL-17 and IFN-γ [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: 5% (4–6%) and 4% (3–9%) for IL-10 and 5% (3–7%) and 9% (4–12%) for IL-17, respectively (median and interquartile range (IQR) values). 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 IFN-γ (figure 1b). T-cells co-expressing IL-13 and IFN-γ were not detectable in PBMCs from patients or controls. Compiled analyses of BAL CD4+ IL-13+/IFN-γ+ cells showed a median of 8% (IQR 3–17%) in PF-ILD. This was more than four-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+/IFN-γ+ cells expressed identical TCR sequences indicating clonal expansion (figure 1c). Identical TCR sequences were observed in IL-13−/IFN-γ+ cells, but the degree of clonal expansion was lower (17–59%) than in IL-13+/IFN-γ+ cells. The IL-13+/IFN-γ+ T-cells had the smallest diversity with D50 of 0.29 (average), compared to IL-13−/IFN-γ+ and IL-13−/IFN-γ− T-cells with D50 of 0.39 and 0.48, respectively.

We describe a highly unusual phenotype of CD4+ T-cells in BAL of PF-ILD patients, which co-express both the Th1-associated cytokine IFN-γ, and the 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 IFN-γ [9, 10]. Importantly, in mice, such Th1/Th2-cells drive a pathogenic cascade characterised by airway hyperresponsiveness, 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+/IFN-γ+ CD4+ T-cells that we have identified may be important in the pathogenesis. In the present study, the IL-13+/IFN-γ+ 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+/IFN-γ+ 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 analysing bulk preparations of BAL T-cells with a panel of riboprobes corresponding to common TCRβ 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+/IFN-γ+ 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 CD28null T-cells in the blood may predict poor IPF prognosis [16]. Although analysis of CD4+CD28null T-cells from lung explants revealed no increase of such cells in IPF compared to controls [17], further analysis should assess whether IL-13+/IFN-γ+ 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.

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