CD4⁺CXCR4⁺ T cells as a novel prognostic biomarker in patients with idiopathic inflammatory myopathy-associated interstitial lung disease

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Abstract

Background. There is an unmet need for the development of new biomarkers for idiopathic inflammatory myopathy-associated interstitial lung disease (IIM-ILD).

Methods. Peripheral CD4⁺CXCR4⁺ T cells, stromal cell-derived factor-1 and Krebs von den Lungen-6 were measured in patients with IIM-ILD (n = 85) and controls. The relation to pulmonary functions, high-resolution CT scores, specific clinical phenotypes and survival was analysed. Cytokine-expression profiling of these CD4⁺CXCR4⁺ T cells and their co-culture with pulmonary fibroblasts were conducted.

Results. The peripheral percentages of CD4⁺CXCR4⁺ T cells were significantly elevated in IIM-ILD patients, and correlated with high-resolution CT score (r = 0.7136, P < 0.0001) and pulmonary function impairments, such as percentage of forced volume vital capacity (r = −0.4734, P = 0.0005). They were associated with anti-melanoma differentiation-associated gene 5 autoantibodies and the amyopathic DM phenotype. In IIM-ILD, peripheral percentages of CD4⁺CXCR4⁺ T cells ≥30% revealed a 6-month mortality as high as 47%. These CD4⁺CXCR4⁺ T cells express high levels of IL-21 and IL-6. In vitro blockade of IL-21 signalling by neutralization of IL-21 or Janus kinase inhibitor could abolish the fibroblast proliferation.

Conclusion. Overall, peripheral CD4⁺CXCR4⁺ T cells appear to be a potentially valuable novel biomarker associated with the severity and prognosis of IIM-ILD. They promote pulmonary fibroblast proliferation via IL-21, which may herald future targeted treatments for this severe disease.

Key words: idiopathic inflammatory myopathy, amyopathic dermatomyositis, CD4⁺CXCR4⁺ T cells, interleukin 21, interstitial lung disease

Introduction

Idiopathic inflammatory myopathies (IIMs) are a group of connective tissue diseases affecting primarily the skeletal muscles, with or without skin and extramuscular organ involvement. The typical adult onset-IIM phenotypes encompass PM, DM and amyopathic DM (ADM) [1]. Interstitial lung disease (ILD) is a common and severe complication of IIMs with poor prognosis [2]. Studies have shown that 5-year mortality among IIM-ILD ranges from 27% to 55% [3–5]. More importantly, ADM-ILD as a unique phenotype of IIM-ILD, which is prone to rapid
progression with a 6-month mortality as high as 50%, has been incrementally reported especially in East Asian regions in the past two decades [6–9].

Identifying key biomarkers in order to guide clinical judgement and treatment remains a substantial challenge in IIM-ILD research. Notably, myositis-specific autoantibodies, such as anti-melanoma differentiation-associated gene 5 (MDA5), have been identified and are associated with the rapid progressive form of ADM-ILD [10, 11], whereas antimyosynthetic-synthetase antibodies have been linked to chronic IIM-ILD [12]. Other biomarkers, such as Krebs von den Lungen-6 (KL-6) and surfactant protein D, are relatively non-specific lung injury markers that are not correlated well with ILD severity or the treatment response [13–15]. There are still unmet needs in terms of discovering possible mechanistically relevant biomarkers for IIM-ILD.

Currently, knowledge regarding the aetiology and pathogenesis of IIM-ILD is lacking; however, autoimmune responses mediated by abnormal T lymphocytes have been implicated. This is evidenced by the infiltration of predominant T cells in the muscle, skin, lung tissues and alveolar lavage fluid of such patients [16–18]. It is hypothesized that T cells in IIM-ILD patients may play a central role in the pro-fibrotic inflammatory process along with other molecular players such as cytokines, chemokines and growth factors [19].

In the current study, which started with phenotypic profiling of peripheral blood mononuclear cells (PBMCs) in IIM-ILD patients (Supplementary Fig. S1, available at Rheumatology online), we eventually identified a CD4+/CXCR4+ T cell subset that was strikingly elevated, and this elevation was associated with disease severity and patient survival rates. Indeed, CXCR4 and its ligand, stromal cell derived factor-1 (SDF-1), have been found to be a key axis in bleomycin-induced pulmonary fibrosis in mice [20] and in human ILD [21], as well as in RA- and SSc-associated ILD [22, 23]. Therefore, peripheral CD4+/CXCR4+ T cells may serve as a possible biomarker for IIM-ILD patients. Furthermore, the development of novel treatments with improved survival is the ultimate goal of the IIM-ILD research. The efficacies of current immunosuppressive treatments, such as glucocorticoids, cyclophosphamide and calcineurin inhibitors, are suboptimal. Our previous study also indicated that the efficacy of pirfenidone, a recent anti-fibrotic agent, is only moderate for this rapid progressive disease [24]. The identification of CD4+/CXCR4+ T cells may help in developing new targeted therapeutic strategies that deserve further investigation.

Materials and methods

Patients and ethical approval

In the current study, adult patients with IIM-ILD (n = 85, 17 PM-ILD, 25 DM-ILD and 43 ADM-ILD); IIMs without ILD (n = 19, four PM, eight DM and seven ADM); patients with RA-ILD (n = 14), patients with SSC-ILD (n = 7), patients with idiopathic pulmonary fibrosis (n = 6), patients with community acquired pneumonia (n = 7), and healthy controls (n = 13) were recruited from Ren Ji Hospital South Campus. The definitions of PM, DM, ADM and ILD [9, 24] were in accordance to previous reports. Malignancy-associated IIMs were excluded. The study was approved by the Institutional Review Board of Ren Ji Hospital (2015048k and 2016075). Informed consents was obtained from all participants. Patient clinical characteristics are listed in Table 1.

Flow cytometry

Bronchoalveolar lavage fluid (BALF) sample collection was conducted as previously described [24]. PBMCs were isolated from anti-coagulated peripheral blood using Ficoll reagent (Sigma-Aldrich, St Louis, MO, USA). PBMCs and BALF cells were evaluated by staining with the following antibodies: CD45RO-BV510, CD45RA-APC-Cy7, CD4/CD14-FITC, CD8/CD19-PE, CXCR4-APC, CXCR5-BV421, CCR7-PE-CY7, PD-1-PE, or ICOS-PerCP-Cy5.5, or matched isotype controls. Stained cells were assessed using the FACS Canto II platform (BD Biosciences, San Jose, CA, USA).

KL-6, SDF-1 and myositis-specific autoantibody analyses

The KL-6 concentrations in the serum were measured using the Lumipulse® G KL-6 Chemiluminescent Enzyme Immunoassay (CLEIA) Kit (Fujirebio, Tokyo, Japan) on a fully automated immunoassay system (LUMIPULSE G1200). SDF-1 concentrations were determined using the Bio-Plex Pro™ Human Chemokine SDF-1α + β Kit (Bio-Rad, Hercules, CA, USA). Anti-MDA5 and other myositis-specific autoantibodies were analysed using EUROLINE Autoimmune Inflammatory Myopathies 16 Ag(IgG) (Euroimmun, Lübeck, Germany).

Lung high-resolution CT scoring and pulmonary function evaluation

Lung high-resolution CT (HRCT) scoring was acquired as previously described [24, 25] in a single-blinded fashion. Pulmonary function test (predicted percentage of forced volume vital capacity (FVC) and single-breath diffusing capacity of the lung for carbon monoxide (DLCO/SB)) was performed on a JAEGGER platform (CareFusion, BD Biosciences).

CD4+/CXCR4+ T cell isolation and in vitro culture

Peripheral CD4+/CXCR4+ T cells were isolated from five ADM-ILD patients (all anti-MDA5 positive) and five ADM patients without ILD (all anti-MDA5 negative) using a CD4+ T cell isolation kit followed by an Anti-APC MultiSort Kit (BD Biosciences). For measurement of both the mRNA and protein levels of cytokines, CD4+/CXCR4+ T cells were seeded at 1 × 10⁶/well in 24-well plates in complete RPMI-1640 medium and cultured in a 37°C, 5% CO₂ incubator for 48 h. CD4+/CXCR4+ T cells and fibroblast co-culture were assessed in a 0.4 μm 12-well Transwell system (Corning, Corning, NY, USA). The upper chamber was filled with phychoamagglutin-stimulated CD4+/CXCR4+ T cells (5 × 10⁴ in 100 μL medium) while the lower chamber was filled with human fetal lung
**Table 1** Clinical characteristics of IIM patients and controls

<table>
<thead>
<tr>
<th></th>
<th>CAP (n = 7)</th>
<th>IPF (n = 6)</th>
<th>SSc-ILD (n = 7)</th>
<th>RA-ILD (n = 14)</th>
<th>IM (n = 19)</th>
<th>PM (n = 4)</th>
<th>DM (n = 8)</th>
<th>ADM (n = 7)</th>
<th>IM-ILD (n = 85)</th>
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<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>55.86 (15.00)</td>
<td>65.33 (7.89)</td>
<td>57.00 (6.81)</td>
<td>64.14 (13.48)</td>
<td>58.25 (11.59)</td>
<td>44.75 (22.80)</td>
<td>34.14 (17.59)</td>
<td>53.71 (11.33)</td>
<td>53.72 (8.63)</td>
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<td><strong>Female (%)</strong></td>
<td>0.00</td>
<td>33.33</td>
<td>100</td>
<td>71.43</td>
<td>75.00</td>
<td>50.00</td>
<td>85.71</td>
<td>1.42 (1.83)</td>
<td>1.22 (1.51)</td>
</tr>
<tr>
<td><strong>Disease duration</strong></td>
<td>0.03 (0.01)</td>
<td>0.97 (0.21)</td>
<td>7.93 (2.83)</td>
<td>14.24 (11.86)</td>
<td>3.03 (2.00)</td>
<td>0.91 (0.89)</td>
<td>0.97 (1.06)</td>
<td>0.88 (1.14)</td>
<td>1.03 (1.18)</td>
</tr>
<tr>
<td><strong>Disease duration for ILD (years)</strong></td>
<td>–</td>
<td>0.97 (0.21)</td>
<td>4.86 (2.72)</td>
<td>1.58 (2.95)</td>
<td>–</td>
<td>–</td>
<td>1.35 (1.21)</td>
<td>6.73 (7.17)</td>
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<tr>
<td><strong>CRP (&lt;8 mg/l)</strong></td>
<td>49.19 (65.18)</td>
<td>33.65 (54.62)</td>
<td>13.93 (17.97)</td>
<td>14.53 (19.04)</td>
<td>2.92 (0.63)</td>
<td>2.74 (3.23)</td>
<td>3.20 (1.43)</td>
<td>16.41 (13.77)</td>
<td>18.01 (40.74)</td>
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<tr>
<td><strong>ESR (0–20 mm/h)</strong></td>
<td>31.43 (35.49)</td>
<td>34.75 (42.36)</td>
<td>18.29 (11.41)</td>
<td>47.38 (24.81)</td>
<td>14.33 (11.68)</td>
<td>30.20 (29.18)</td>
<td>17.83 (10.13)</td>
<td>306.20 (29.18)</td>
<td>27.71 (29.36)</td>
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<tr>
<td><strong>CK (30–135 U/ml)</strong></td>
<td>70.00 (41.77)</td>
<td>64.67 (49.92)</td>
<td>228.17 (261.32)</td>
<td>43.25 (27.21)</td>
<td>755.33 (835.45)</td>
<td>306.20 (230.38)</td>
<td>55.50 (62.29)</td>
<td>592.22 (1244.29)</td>
<td>28.59 (19.94)</td>
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<tr>
<td><strong>Ferritin (11–306.6 ng/ml)</strong></td>
<td>49.59 (16.24)</td>
<td>24.74 (18.29)</td>
<td>240.68 (327.81)</td>
<td>282.30 (211.81)</td>
<td>358.03 (398.49)</td>
<td>487.34 (450.09)</td>
<td>189.68 (198.49)</td>
<td>938.51 (1108.23)</td>
<td>1238.72 (1430.43)</td>
</tr>
<tr>
<td><strong>Anti-MDA5 antibody positive (%)</strong></td>
<td>0.00</td>
<td>0.00</td>
<td>–</td>
<td>0.00</td>
<td>0.00</td>
<td>12.50</td>
<td>28.57</td>
<td>11.76</td>
<td>44.00</td>
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<tr>
<td><strong>Anti-ARS antibody positive (%)</strong></td>
<td>0.00</td>
<td>0.00</td>
<td>–</td>
<td>0.00</td>
<td>0.00</td>
<td>12.50</td>
<td>28.57</td>
<td>11.76</td>
<td>44.00</td>
</tr>
<tr>
<td><strong>KL-6 (105–435 U/ml)</strong></td>
<td>203.00 (52.06)</td>
<td>865.95 (531.16)</td>
<td>1061.57 (835.21)</td>
<td>1013.14 (816.02)</td>
<td>741.00 (672.02)</td>
<td>1105.70 (2019.28)</td>
<td>358.14 (193.72)</td>
<td>1107.59 (2329.15)</td>
<td>1146.86 (975.08)</td>
</tr>
<tr>
<td><strong>FVC %</strong></td>
<td>84.67 (4.71)</td>
<td>89.49 (4.81)</td>
<td>57.73 (8.05)</td>
<td>90.77 (12.23)</td>
<td>92.37 (10.68)</td>
<td>99.23 (13.97)</td>
<td>89.40 (12.80)</td>
<td>68.53 (16.05)</td>
<td>71.86 (24.91)</td>
</tr>
<tr>
<td><strong>DLCO/SB %</strong></td>
<td>77.77 (2.50)</td>
<td>57.82 (12.43)</td>
<td>31.87 (24.98)</td>
<td>68.25 (9.38)</td>
<td>72.17 (24.22)</td>
<td>75.23 (21.99)</td>
<td>70.10 (13.08)</td>
<td>43.74 (17.18)</td>
<td>46.39 (17.20)</td>
</tr>
<tr>
<td><strong>HRCT score</strong></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</table>

Data are mean (S.D.). ADM: amyopathic DM; ARS: aminoacyl-tRNA-synthetase, including either Jo-1, PL-7, PL-12, OJ or EJ; CAP: community acquired pneumonia; CK: Creatine kinase; DLCO/SB: single-breath diffusing capacity of the lung for carbon monoxide; FVC: forced volume vital capacity; HRCT: high-resolution CT; IIM: idiopathic inflammatory myopathy; ILD: interstitial lung disease; KL-6: Krebs von den Lungen-6; MDA5: melanoma differentiation-associated gene 5; IPF: idiopathic pulmonary fibrosis.
fi

Results

The peripheral percentage of CD4±CXCR4± T cells is increased among PBMCs and BALF in IIM-ILD patients

The peripheral percentage of CD4±CXCR4± T cells was significantly elevated in IIM-ILD patients compared with disease controls (community acquired pneumonia, idiopathic pulmonary fibrosis, RA-ILD, SSc-ILD and IIM alone) and healthy controls. In contrast, no differences existed among the different disease groups regarding the percentage of CD8±CXCR4± T cells (Fig. 1A) or the percentages of CD19±CXCR4± and CD14±CXCR4± cells among PBMCs (data not shown). Serum KL-6 concentrations were also elevated in IIM-ILD patients compared with healthy controls. However, elevated KL-6 levels are not specific to IIM-ILD and were also observed in idiopathic pulmonary fibrosis, RA-ILD, SSc-ILD and IIM patients without ILD. The CXCR4 ligand, SDF-1, was elevated in the serum of IIM-ILD patients compared with other controls (Fig. 1B). Accordingly, receiver operating characteristic analyses revealed a better predictive performance of the peripheral percentage of CD4±CXCR4± T cells for IIM-ILD than for SDF-1 or KL-6 levels (Fig. 1C). Furthermore, the percentage of CD4±CXCR4± T cells in BALF was also more pronounced among a few of the IIM-ILD patients than among patients with IIM alone and RA-ILD and community-acquired pneumonia patients (Fig. 1D).

The peripheral percentage of CD4±CXCR4± T cells is correlated with IIM-ILD lung structural changes and pulmonary function impairments

The peripheral percentage of CD4±CXCR4± T cells was strongly correlated with the extent of structural changes, as identified by HRCT score (r = 0.7136, P < 0.0001), in IIM-ILD patients; however, these changes were not correlated with KL-6 level (Fig. 2A). Furthermore, the peripheral percentage of CD4±CXCR4± T cells was also correlated with pulmonary function impairment as measured by the predicted FVC (r = -0.4734, P = 0.0005) and DLCO/SB (r = -0.3530, P = 0.0047) percentages (Fig. 2B and C).

The peripheral percentage of CD4±CXCR4± T cells predicts IIM-ILD prognosis

In IIM-ILD patients, high peripheral percentage of CD4±CXCR4± T cells identifies patients with acute progressive ILD with significant poor short-term survival rates. Peripheral percentages of CD4±CXCR4± T cells $\geq$30% or $<$30% revealed a 6-month mortality of 47.06% or 13.24%, respectively (Fig. 3A). The comparison data between these two groups are provided in Supplementary Table S2, available at Rheumatology online. Briefly, patients with high CD4±CXCR4± (≥30%) tend to be anti-MDA5 positive (82% vs 35%) and have baseline high HRCT score, low FVC% and higher ferritin levels. Indeed, the known poor prognostic factors of

Statistical analysis

All data analyses were performed with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance, Kruskal–Wallis non-parametric analysis or a paired Student’s t test was used as appropriate. IIM-ILD patients’ 6-month survival curves were generated using the Kaplan–Meier method. Receiver operating characteristic curves of the peripheral percentage of CD4±CXCR4± T cells, KL-6 and SDF-1 concentrations for IIM-ILD determination were generated, and the area under the curve was calculated. A Spearman correlation analysis was adopted when indicated. Multivariate analysis was applied using proportional hazards model. A P values <0.05 was considered statistically significant.

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fibroblast cells (1 x 10⁶ in 1000 µL medium) (Chinese Academy of Sciences, Shanghai, China). Anti-human IL-6 antibody (0.5 µg/ml, R&D Systems, Minneapolis, MN, USA), anti-human IL-21 antibody (0.5 µg/ml, Abcam, Cambridge, MA, USA), control IgG and the Janus kinase (JAK) inhibitor, tofacitinib (500 nmol/ml, Cell Signaling Technology, Danvers, MA, USA), were added into the lower chambers prior to co-incubation, when indicated. The proliferation of fibroblasts was analysed with the Cell Counting Kit-8 Cell Proliferation Kit (Dojindo Molecular Technologies, Kumamoto, Japan), as previously described [26].

Quantitative-PCR and cytometric bead array assay

Total RNA from PBMCs or cultured CD4±CXCR4± T cells was extracted using Trizol reagent (Thermo Fisher Scientific, Hilden, Germany). SYBR-Green-based real-time quantitative-PCR was then performed to determine the levels of expression of a series of genes using glyceraldehyde 3-phosphate dehydrogenase as an internal control. The relative expression levels of the target genes (IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-21, IFN-γ, TNF-α, TGF-β1, PD-1, ICOS, IL-12β, IL-23, Bcl6, T-bet, FoxP3, GATA3, RORγ, CXCR7, CXCR5, CXCR4, CXCR3, CXCR2, CCR7, CCR9, CCR5, CCR4, CCR3, CCR1, CCR3, and CCR2) were calculated using the 2-ΔΔCT method. Primers used for quantitative-PCR are listed in Supplementary Table S1, available at Rheumatology online. The expression profile of cytokines in CD4±CXCR4± T cell culture supernatants was measured with the cytometric bead array Human Soluble Protein Flex Set (BD Biosciences).

Western blot analysis

Total protein from co-cultured pulmonary fibroblasts was extracted by using protein lysis buffer (Beyotime Tech, Shanghai, China). Western blot was then performed with the primary antibodies of rabbit monoclonal anti-TGF-β (Abcam, Cambridge, UK, 1:1000 dilution), rabbit monoclonal anti-α-smooth muscle actin (SMA) (Cell Signaling Technology, 1: 1000 dilution), rabbit monoclonal anti-collagen I (Abcam, UK, 1:500 dilution), and secondary horse-radish peroxidase-conjugated antibody. The membrane was developed using enhanced chemiluminescence reagent, and densitometric analyses conducted using ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

All data analyses were performed with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance, Kruskal–Wallis non-parametric analysis or a paired Student’s t test was used as appropriate. IIM-ILD patients’ 6-month survival curves were generated using the Kaplan–Meier method. Receiver operating characteristic curves of the peripheral percentage of CD4±CXCR4± T cells, KL-6 and SDF-1 concentrations for IIM-ILD determination were generated, and the area under the curve was calculated. A Spearman correlation analysis was adopted when indicated. Multivariate analysis was applied using proportional hazards model. A P values <0.05 was considered statistically significant.
IIM-ILD, such as the presence of anti-MDA5 and the ADM phenotype, are inter-related to the peripheral percentage of CD4+CXCR4+ T cells (Fig. 3A and B). To further evaluate the relevant clinical and laboratory parameters in terms of survival, uni- and multivariate analysis was performed (Tables 2 and 3). The peripheral percentage of CD4+CXCR4+ T cells (>30%) and ferritin level (>1500 ng/ml), rather than the presence of anti-MDA5, were independent risk factors for mortality. In addition, the peripheral percentage of CD4+CXCR4+ T cells tended to decrease among survivors within the first month after treatments (Fig. 3C, \( P = 0.031, n = 19 \)).

ADM-ILD derived peripheral CD4+CXCR4+ T cells mediate pulmonary fibroblast proliferation in an IL-21-dependent manner

Peripheral CD4+CXCR4+ T cells, along with CD4+CXCR4+ and CD8+CXCR4+ T cells, were isolated from anti-MDA5 positive ADM-ILD patients and from anti-MDA5 negative ADM patients without ILD. Peripheral CD4+CXCR4+ T cells from ADM-ILD
patients express high mRNA levels and protein levels of IL-6 and IL-21, but not IL-1β, IL-4, IL-10, IL-17A, IFN-γ, or TNF-α (Fig. 4A). Notably, the immune phenotype of these CD4+CXCR4+ T cells was CXCR5−CCR7−ICOS−PD1−CD45RA+, which indicated that these cells were not circulating follicular helper T cells (Tfh) (Supplementary Fig S2, available at Rheumatology online). Nevertheless, ADM-ILD-derived peripheral CD4+CXCR4+ T cells promote in vitro pulmonary fibroblast proliferation and TGF-β, α-SMA and collagen I production (Fig. 4B). The neutralization of IL-21, but not IL-6, with a monoclonal antibody or the blockade of IL-21 signalling using a JAK inhibitor abolished the pro-fibrotic effect of these CD4+CXCR4+ T cells. There was also an additive suppressive effect when combined anti-IL21 and JAK inhibitor was used (Fig. 4C and D).

**Discussion**

IIM-ILD, and ADM-ILD in particular, continues to be a huge challenge for its high mortality and resistance to treatments [8, 24, 27]. T cell activation seems to play a key role in the pathogenesis of IIM-ILD [16–19]. Despite the fact that the level of evidence is not that strong, clinical practice has been switched to more T cell targeted treatments, for instance ciclosporin and tacrolimus, for IIM-ILD patients [28–31]. As an example, our colleagues have suggested the possible efficacy of basiliximab (anti-CD25),...
which is a biologic agent removing activated T cells, for a small case series of the patients exhibiting rapid progressive ADM-ILD [32].

As regards the underlying research work, we have pinpointed that the CD4+CXCR4+ T cell subset has an association with IIM-ILD. The percentage of circulating CD4+CXCR4+ T cells has a strong correlation with ILD structural damage (HRCT score), together with a moderate correlation with pulmonary function impairment (FVC and DLCO/SB). The peripheral CD4+CXCR4+ T cells have an association with the clinically severest IIM disease phenotype, i.e. ADM-ILD, together with the presence of anti-MDA5 antibody. More importantly, the peripheral proportion of CD4+CXCR4+ T cells parallels the short-term survival rate. The overall performance of CD4+CXCR4+ T cells in terms of association with ILD and forecasting of survival is apparently better than the currently available biomarkers, for instance KL-6 and anti-MDA5 antibody. The good predictive capacity and specificity of the peripheral CD4+CXCR4+ T cells suggest a role not only as a new biomarker for IIM-ILD, but also as a potential pathogenic driver in IIM-ILD.

It is hypothesized that the expression of CXCR4 facilitates the deployment of CD4+ T cells to the target organs, which is supported by the recovery of more abundant CD4+CXCR4+ T cells in the bronchoalveolar lavage from the IIM-ILD patients. As a matter of fact, it is known that the CXCR4 is a pro-fibrotic molecule; moreover, its ligand, SDF-1, is primarily released by macrophages as well as airway epithelial cells in fibrotic lungs. Furthermore, a CXCR4 antagonist (ADM3100) is capable of ameliorating bleomycin-induced pulmonary fibrosis in mice through a
reduction of fibroblast migration [20]. As further revealed by our data, these CD4+CXCR4+ T cells produce high levels of IL-21, together with promoting pulmonary fibroblast proliferation in vitro. Interestingly, the production of IL-21 by these cells is of great significance since it has been implicated in pulmonary fibrosis [33]. JAK is known to be required for IL-21/IL-21 receptor signalling [34]. As demonstrated by our data, in vitro neutralization of IL-21 or using a JAK inhibitor could inhibit the pro-fibrotic effect of these CD4+CXCR4+ T cells. It can also be noted that anti-IL-21 mAb is currently in clinical trial for RA [35], which will provide important safety data; on the other hand, case reports have shed light on the therapeutic effect of the JAK inhibitor on DM [36, 37]. It would be quite interesting to determine whether these two treatments could provide promising options for IIM-ILD.

In a bid to avoid ‘tunnel vision’ when considering this intricate disease, other aspects should also be considered. For instance, high levels of IL-6 are produced by these CD4+CXCR4+ T cells. Despite not directly mediating fibroblast proliferation in vitro, IL-6 is still likely to be required in the local milieu of inflammation [38]. This is suggested by the possible therapeutic effect of anti-IL-6 therapy (tocilizumab) in scleroderma-associated ILD [39].

As an example, the over-simplification of our in vitro experiment is likely to be primarily overcome through the application of the lately developed ex vivo human lung model [40]. It will be crucial to understand the crosstalk between those CD4+CXCR4+ T cells and other lung-resident cells, such as alveolar macrophages, in the inflammatory and fibrotic context of IIM-ILD.

A key limitation of our study is the relatively limited patient cohort for this rare disease, and samples were predominantly from ADM-ILD patients, which may hamper its generalizability for all IIM-ILD. Larger scale investigations, which include more IIM-ILD and control diseases, are considered to be essential.

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**Disclosure statement:** The authors have declared that no conflict of interest exists.

**Supplementary data**

Supplementary data are available at *Rheumatology* online.
Fig. 4 ADM-ILD-derived peripheral CD4+CXCR4+ T cells mediate pulmonary fibroblast proliferation in an IL-21-dependent manner

(A) mRNA and protein levels of IL-6 and IL-21 are increased in CD4+CXCR4+ T cells from ADM-ILD vs those from ADM alone (n=5 for each). (B) The peripheral CD4+CXCR4+ T cells or CD4+CXCR4+ T cells from patients, as indicated, were isolated. (C, D) They were cultured together with fibroblast cells in vitro in a Transwell system in the presence or absence of (C) an anti-IL-6 antibody, an anti-IL-21 antibody, or (D) the JAK inhibitor tofacitinib. After incubation, fibroblast proliferation was measured using the CCK-8 assay. TGF-β, a-SMA and collagen-I expression of fibroblasts were analysed by western blot (*P<0.05; **P<0.01; ***P<0.001). ADM: amyopathic DM; CCK-8: Cell Counting Kit-8; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; JAK: Janus kinase; a-SMA: a-smooth muscle actin.

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