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Circulating follicular helper T cells are increased in systemic sclerosis and promote plasmablast differentiation through the IL-21 pathway which can be inhibited by ruxolitinib

Laure Ricard,1,2 Vincent Jachiet,1,2 Florent Malard,1,3 Yishan Ye,1 Nicolas Stocker,1 Sébastien Rivière,2 Patricia Senet,4 Jean-Benoit Monfort,4 Olivier Fain,2 Mohamad Mohty,1,3 Béatrice Gaugler,1,3 Arsène Mekinian1,2

ABSTRACT

Objectives Systemic sclerosis (SSc) is an autoimmune disease characterised by widespread fibrosis, microangiopathy and autoantibodies. Follicular helper T (Tfh) cells CD4+ CXCR5+PD-1+ cooperate with B lymphocytes to induce the differentiation of plasmocytes secreting immunoglobulins (Ig). Circulating Tfh (CTfh) cells are increased in several autoimmune diseases. However, there are no data about CTfh cells and their interaction with B cells in SSc. The aim of this study was to perform a quantitative and functional analysis of cTfh cells in SSc.

Methods Using flow cytometry, we analysed cTfh cells from 50 patients with SSc and 32 healthy controls (HC). In vitro coculture experiments of sorted cTfh and B cells were performed for functional analysis. IgG and IgM production were measured by ELISA.

Results We observed that cTfh cell numbers are increased in patients with SSc compared with HC. Furthermore, the increase in cTfh cells was more potent in patients with severe forms of SSc such as diffuse SSc and in the presence of arterial pulmonary hypertension. cTfh cells from patients with SSc present an activated Tfh phenotype, with high expression of BCL-6, increased capacity to produce IL-21 in comparison with healthy controls. In vitro, cTfh cells from patients with SSc had higher capacity to stimulate the differentiation of CD19+CD27+CD38hi B cells and their secretion of IgG and IgM through the IL-21 pathway than Tfh cells from healthy controls. Blocking IL-21R or using the JAK1/2 inhibitor ruxolitinib reduced the Tfh cells’ capacity to stimulate the plasmablasts and decreased the Ig production.

Conclusions Circulating Tfh cells are increased in SSc and correlate with SSc severity. The IL-21 pathway or JAK1/2 blockade by ruxolitinib could be a promising strategy in the treatment of SSc.

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease characterised by fibrosis of the skin and other organs, vascular impairment and deficient immune responses.1 Patients with SSc are classified into two groups depending on the extent of the skin involvement: the limited cutaneous SSc (lSSc) with skin involvement limited to face and hands, and the diffuse SSc (dSSc) with skin involvement proximal to elbows and knees. dSSc is a rapidly progressing disorder with more frequent visceral involvement and increased mortality rates.2 The exact pathophysiology of the disease is largely unknown and includes endothelial cell impairment, fibrosis and immune dysfunction. The CD4+ T cells are a major component of the infiltrate in the skin in the acute inflammatory stage of the disease.3 Peripheral T lymphocytes have increased expression of activation markers and secrete proinflammatory cytokines that activate fibroblasts and plasma cells.4 B lymphocytes are also reported to play a predominant role in SSc: autoantibodies are usually detected in the sera of patients with SSc5 and B-cell homeostasis is disturbed in SSc. Despite increased expression of activation markers (CD80, CD86 and CD95),3 SSc B lymphocytes secrete higher levels of the profibrotic cytokines IL-6 and TGF-β.6 The levels of the cytokine B-cell activating factor and a proliferation-inducing ligand implicated in B-cell survival are also increased in SSc serum and appear to be correlated with disease severity.8–10

What is already known on this topic?

► CD4+ T cells with a Tfh phenotype can infiltrate the skin of SSc patients, and induce in vitro myofibroblast differentiation.

What this study adds?

► Circulating Tfh cells are increased in SSc and correlate with SSc severity.

► Tfh cells from patients with SSc induce B-cell differentiation into plasmablasts secreting Ig via IL-21 secretion with increased capacity than healthy controls.

► IL-21 or JAK1/2 blockade by ruxolitinib could be a promising strategy in the treatment of SSc.

How might this impact on clinical practice or future developments?

► Clinical data with anti-JAK inhibition could be interesting in SSc patients
Follicular helper T (Tfh) cells are a subset of CD4+ T cells able to provide help to B cells to undergo proliferation, isotype switch and somatic hypermutation, resulting in long-lasting antibody responses.11,12 They are localised next to the germinal centre (GC) in secondary lymphoid organs and are characterised by their surface expression of C-X-C chemokine receptor type 5 (CXCR5), inducible costimulatory (ICOS) and programmed death cell protein 1 (PD-1), by the expression of the transcription factor B-cell lymphoma 6 (BCL-6) and the secretion of IL-21 and IL-4 cytokines.13-15 A circulating Tfh population (cTfh) has also been identified, which expresses CXCR5, PD-1 and ICOS and can help B-cell differentiation into plasma cells via IL-21 secretion.16 Increased frequencies of cTfh have been reported in several autoimmune diseases such as rheumatoid arthritis, juvenile dermatomyositis, Sjögren’s syndrome and systemic lupus erythematosus.16-19 Several subtypes of cTfh have been described whose frequencies could be disturbed in autoimmune diseases.16 Recently, a pathologically expanded population of CXCR5+PD-1+CD4+ T cells called T peripheral helper cells has been identified in the synovium of patients with rheumatoid arthritis which could also promote plasma cell differentiation.20 Furthermore, CD4+ T cells with a Tfh phenotype can infiltrate the skin of patients with SSc and induce in vitro myofibroblast differentiation.21 Thus, Tfh may play a predominant role in the pathophysiology of SSc disease and promote B-cell stimulation and activation favouring increased persistent inflammation. We performed a quantitative and functional analysis of the cTfh in patients with SSc in comparison with healthy controls. We demonstrate that cTfh are increased in SSc and correlated with disease severity. Moreover, cTfh from patients with SSc are more efficient than healthy controls to induce B-cell differentiation into plasma cells secreting IgG via the secretion of IL-21 cytokine. Ruxolitinib, a JAK1/2 inhibitor, significantly reduced in vitro cTfh capacity to induce plasma cells and their IL-21 secretion, suggesting that the JAK/STAT pathway blockade could be a potential therapeutic approach in SSc.

Patients and methods
Fifty-two consecutive patients with SSc (median age 61 years (range, 32–81), 80% women) and 38 healthy controls (median age 53 years (range, 23–64), 46% women) were prospectively included in the study. All patients with SSc enrolled in this study were followed at Saint-Antoine Hospital. All patients fulfilled the 2013 American College of Rheumatology/European League Against Rheumatism criteria for SSc.22 Patients with an associated autoimmune systemic disease, concomitant infectious disease or active neoplasm were excluded. None of the patients with SSc were receiving treatment with steroids or other immunosuppressive therapy at the time of the analysis. For each patient, the following data were analysed: age, gender, disease duration (from the date of the first non-Raynaud’s phenomenon), type of SSc (dSSc or lSSc), presence of active digital ulcers, presence of joint, heart, gastrointestinal and lung involvement, modified Rodnan skin score (mRss) and presence of pulmonary arterial hypertension. The lung involvement was defined as the presence of interstitial lung disease on high-resolution CT scan. Laboratory data included C reactive protein level, total lymphocyte count, plasma creatinine, urea, creatinine phosphokinase enzymes and antinuclear autoantibodies (anti-centromeres, anti-topoisomerase I, anti-PM-Scl, anti-RNA polymerase III autoantibodies). Characteristics of patients with SSc are depicted in table 1.

Table 1 Patients’ characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients with SSc (n=52)</th>
<th>Patients with dSSc (n=15)</th>
<th>Patients with lSSc (n=37)</th>
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<tr>
<td>Age (years)</td>
<td>61 (32–81)</td>
<td>59.5 (32–75)</td>
<td>61 (32–81)</td>
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<td>Age at first non-Raynaud symptom (years)</td>
<td>48 (26–72)</td>
<td>42 (27–67)</td>
<td>49 (26–72)</td>
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<td>Female sex n (%)</td>
<td>42 (81)</td>
<td>12 (73)</td>
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<td>Disease duration (years)</td>
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<td>European ethnicity n (%)</td>
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<td>8 (53)</td>
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<td>Skin involvement</td>
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<tr>
<td>Diffuse systemic sclerosis n (%)</td>
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<td>–</td>
<td>–</td>
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<td>Limited systemic sclerosis n (%)</td>
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<td>–</td>
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<td>Rodnan score</td>
<td>10 (0–36)</td>
<td>15 (10–36)</td>
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<td>Active digital ulcers n (%)</td>
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<td>4 (27)</td>
<td>4 (11)</td>
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<td>Pulmonary involvement</td>
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<td>Intestinal lung disease n (%)</td>
<td>21 (40)</td>
<td>11 (73)</td>
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<td>Pulmonary arterial hypertension n (%)</td>
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<td>3 (20)</td>
<td>2 (5)</td>
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<td>FVC (%) of the predicted value</td>
<td>103 (37–154)</td>
<td>75 (37–111)</td>
<td>106 (38–154)</td>
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<tr>
<td>DLCO (%) of the predicted value</td>
<td>61 (14–98)</td>
<td>55 (23–82)</td>
<td>62 (14–98)</td>
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<td>Other organ involvement</td>
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<tr>
<td>Joint involvement n (%)</td>
<td>6 (12%)</td>
<td>1 (7)</td>
<td>5 (14)</td>
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<tr>
<td>Kidney sclerosis n (%)</td>
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<td>1 (7)</td>
<td>0 (0)</td>
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<tr>
<td>Heart impairment n (%)</td>
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<td>1 (7)</td>
<td>0 (0)</td>
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<td>Laboratory data</td>
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<tr>
<td>BNP (mg/L)</td>
<td>34 (10–300)</td>
<td>39 (18–134)</td>
<td>34 (10–300)</td>
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<td>C reactive protein (mg/L)</td>
<td>1 (1–50)</td>
<td>8.9 (1–50)</td>
<td>1 (1–16)</td>
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<td>Autoantibodies</td>
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<td></td>
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<tr>
<td>Anti-centromeres n (%)</td>
<td>26 (50)</td>
<td>0 (0)</td>
<td>25 (71)</td>
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<tr>
<td>Anti-Scl70 n (%)</td>
<td>11 (22)</td>
<td>5 (33)</td>
<td>7 (20)</td>
</tr>
<tr>
<td>Anti-RNApol3 n (%)</td>
<td>5 (10)</td>
<td>5 (33)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Values are medians with ranges and frequencies with percentages.
Anti-RNApol3, anti-RNA polymerase 3 antibody; Anti-Scl70, anti-topoisomerase 1 antibody; BNP, Brain natriuretic peptide; DLCO, Diffusing capacity for carbon monoxide; FVC, Forced vital capacity.

Biological samples
Blood samples were collected in EDTA tubes (BD Biosciences, Le Pont de Claix, France). Healthy controls (HC) were obtained from healthy donors (‘Etablissement Français du Sang, Paris Saint-Antoine-Crozatier’). Peripheral blood mononuclear cells (PBMCs) were isolated with a standard gradient centrifugation procedure on a lymphocyte separation medium (Lymphosep separation media; Dutscher, Issy-les-Moulineaux, France).

Phenotype analysis by flow cytometry
After isolation, PBMCs were stained with the following fluorochrome-conjugated antibodies: CXCR3 (CD183) FITC, CCr6 PE-Vio770 (Miltenyi Biotec, Paris, France); CXCR5 (CD185) PE (eBioscience, ThermoFisher, Villebon, France); CD45RA ECD, PD-1 (CD279) PECY5.5, ICOS (CD278) APC, CD3 AA750 (Beckman Coulter, Villepinte, France). Patients with an associated autoimmune systemic disease, concomitant infectious disease or active neoplasm were excluded. None of the patients with SSc were receiving treatment with steroids or other immunosuppressive therapy at the time of the analysis. For each patient, the following data were analysed: age, gender, disease duration (from the date of the first non-Raynaud’s phenomenon), type of SSc (dSSc or lSSc), presence of active digital ulcers, presence of joint, heart, gastrointestinal and lung involvement, modified Rodnan skin score (mRss) and presence of pulmonary arterial hypertension. The lung involvement was defined as the presence of interstitial lung disease on high-resolution CT scan. Laboratory data included C reactive protein level, total lymphocyte count, plasma creatinine, urea, creatinine phosphokinase enzymes and antinuclear autoantibodies (anti-centromeres, anti-topoisomerase I, anti-PM-Scl, anti-RNA polymerase III autoantibodies). Characteristics of patients with SSc are depicted in table 1.
(Biolegend, Ozyme, Montigny-le-Bretonneux). T-lymphocyte and B-lymphocyte absolute numbers were determined using 50 µL of whole blood from patients and HC using Trucount tubes (BD Biosciences). Samples were stained with the following antibodies: CD19 FITC, CD45 ECD, CD3 AA750 (Beckman Coulter) for 15 min and then incubated for 15 min with 450 µL BD FACS Lysis Solution and subsequently analysed by flow cytometry. Compensation beads were used for compensation setting (VersaComp; Beckman Coulter). Cells were analysed on a Cytoflex flow cytometer (Beckman Coulter). Data were analysed using Kaluza V.5.1 software (Beckman Coulter).

Cell sorting
Total CD4+ T cells were isolated from PBMCs using a Magnisort Human CD4 T-cell negative selection kit (Invitrogen, ThermoFisher). T-cell purity was always more than 94%. CD4 T cells were then stained with the following fluorochrome-conjugated antibodies: CXCX5 (CD183) PE (eBioscience), CD4 Pacific Blue, CD-1 APC (Beckman Coulter). Tfh cells were defined as CD4+CXCR5+PD-1+ cells. Tfh cells, CD4+CXCR5+PD-1- and CD4+CXCR5+PD-1- were sorted using a flow cytometer (FACS Aria III; BD Biosciences). B lymphocytes were isolated from PBMCs by a magnetic beads CD19+ positive selection kit (eBioscience). All sorted cell populations exhibited high purity (>90%).

Stimulation of Tfh cells
Sorted Tfh cells or non-Tfh CD4+ T cells from nine patients with SSC were cultured at 50 000 cells per well in 200 µL of RPMI 1640 medium (Eurobio, Courtaboeuf, France) supplemented with 10% fetal bovine serum (Gibco, ThermoFisher) and with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and ionomycin (1 µg/mL) (Sigma-Aldrich, St. Louis, Missouri, USA). Supernatants were then collected after 36 hours of culture and IL-21 levels were measured by ELISA (IL-21 Human uncoated ELISA kit; ThermoFisher).

Coculture of T and B cells
Sorted Tfh cells, non-Tfh CD4+ T cells and purified CD19+ B cells were cocultured at 50 000 cells/well in 96-well plates (Starstedt) at a 1:1 ratio in 200 µL of TexMACS medium (Miltenyi Biotec) supplemented with gentamycine (Gibco, ThermoFisher) and with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and ionomycin (1 µg/mL) (Sigma-Aldrich, St. Louis, Missouri, USA). Supernatants were then collected after 6 days of culture. For blocking experiments, 10 µg/mL recombinant Human IL-21 Fc Chimera Protein (IL-21Fc) (R&D Systems, Lille, France), or 10 µg/mL anti-IL6R-IgG1 (Roactemra, tociluzimab; Roche), or 0.1 µg/mL ruxolitinib (InvivoGen, San Diego, California, USA) were added in the culture. The total IgG and IgM levels were measured in coculture supernatants by ELISA (IgG Total Human uncoated ELISA kit and IgM human uncoated ELISA kit; ThermoFisher) according to the manufacturers’ instructions.

RT-PCR analyses
RNA extractions from eight patients with SSC (n=3 dSSc and n=5 lSSc) and six HC were performed using RNeasy Mini kit (Qiagen, Cergy Pontoise, France). RNA was subjected to reverse transcription (High Capacity RNA-to-cDNA Master Mix; ThermoFisher) and quantified by real-time quantitative PCR using commercially available primer/probes sets (Assay-On-Demand; ThermoFisher Scientific): GAPDH (Hs00790910_m1), IL-21 (Hs00222327_m1), BLCL-6 (Hs00153368_m1), CXCL13 (Hs00757930_m1), BLIMP-1 (Hs00757930_m1). Real-Time PCR were performed on a 7500 Fast Dx Real-Time PCR Instrument (ThermoFisher Scientific). Relative expression for the mRNA transcripts was calculated using the ∆∆Ct method and GAPDH mRNA transcript as reference.

Statistical analysis
Data are expressed as means±SD, medians with ranges and numbers with their frequencies. The χ² test or Fisher test was used to compare qualitative values according to distribution and the Student t-test, Mann-Whitney U test or Wilcoxon test for continuous quantitative variables. The Pearson test was used to determine the correlation between variables. All analyses were performed using GraphPad Prism V.5.0 (GraphPad Software, San Diego, California, USA). A p value <0.05 was considered as statistically significant.

RESULTS
Circulating Tfh cells are increased in SSc and express high levels of PD-1 and activation markers
We analysed circulating Tfh (cTfh) cells in the blood of 50 patients with SSc and 32 healthy controls by flow cytometry. The cTfh cells were defined as CD4+CXCR5+PD-1+ cells among CD4+ T cells. The gating strategy used to identify cTfh cells is represented in figure 1A. The percentage and absolute numbers of cTfh cells among CD4+ T cells were significantly increased in patients with SSC as compared with HC (mean 7.2±3.6% vs 4.4±1.5% (p<0.0001) and 69.1±38.9 cells/µL vs 33.2±19.2 cells/µL (p<0.0001), respectively) (figure 1B,C).

We also evaluated the CXCR5+PD-1+CD4+ T cells and we observed that their number was also significantly increased in patients with SSc in comparison with HC: 128±79.4 cells/µL versus 77±60 cells/µL; p=0.001 (figure 1D). We then analysed the three major subsets of Tfh cells in human blood as previously described by Morita et al,16 namely Tfh1, Tfh2 and Tfh17 subsets which are characterised by their different expression of CXCR3 and CCR6 (figure 1A). As shown in figure 1E, the percentages of Tfh1 cells CXCR3+CCR6+, Tfh2 cells CXCR3+CCR6+ and Tfh17 CXCR3+CCR6+ cells did not differ in patients in SSc in comparison with healthy controls.

PD-1 signalling on Tfh cells promotes the selection and the survival of GC B cells, and regulates formation of long-lived plasma cells via interaction with the ligand PD-L1 on B cells. We evaluated the level of expression of PD-1 on Tfh cells from patients with SSc. High expression of PD-1 (PD-1high) or intermediate expression (PD-1int) by Tfh cells were defined according to the PD-1 intensity as shown in figure 1F. We observed that the frequencies of CD4+CXCR5+PD-1high cells were significantly increased in patients with SSc as compared with healthy controls (1.6±0.95% vs 1.1±0.6%; p=0.01) and for CD4+CXCR5+PD-1int cells (4.2±2.31% vs 3.2±0.96%; p=0.003) (figure 1G, H). The CD4+CXCR5+PD-1high Tfh cells have an increased expression (MFI) of ICOS (mean 4984±2716 vs 7845±3187; p<0.0001) and of HLA-DR (mean 10 590±5525 vs 14 560±8466; p=0.023) as compared with CD4+CXCR5+PD-1int cells (figure 1H, I). Thus, cTfh cells are increased in patients with SSc and express higher levels of PD-1 and other activation markers, such as ICOS and HLA-DR.

Circulating Tfh cells are increased in severe subtypes of SSc
We then analysed the proportions of cTfh according to SSc characteristics and subtypes. As expected, median Rodnan scale purity (>90%).

- 13 patients with SSc (3 dSSc and 10 lSSc) and 6 HC. Supernatants were then collected after 36 hours of culture and IL-21 levels were measured by ELISA (IL-21 Human uncoated ELISA kit; ThermoFisher).

- We then analysed the proportions of cTfh according to SSc characteristics and subtypes. As expected, median Rodnan scale
lSSc (p=0.0002) (table 1). We observed that cTfh cells were significantly expanded in the severe dSSc as compared with lSSc (9.3±4.9% vs 6.3±2.7%; p=0.04) (figure 2A). Only cTfh CXCR5+PD-1+ but not CXCR5−PD-1−CD4+ T cells were significantly increased in patients with dSSc (figure 2B). Circulating Tfh cell frequencies were also increased in patients with pulmonary arterial hypertension (13.4±5.8% vs 6.2±2.2%; p=0.003) (figure 2C). However, we did not observe differences in the proportions of cTfh in patients with active digital ulcers (p=0.053) or interstitial lung disease (figure 2D,E). Interestingly, cTfh frequencies correlated positively with mRSS scale (r=+0.33; p=0.023), but no correlation of Tfh cells and mRSS was observed when patients with dSSc and lSSc were analysed separately, probably because of the higher mRSS values in dSSc (figure 2F). The cTfh cell frequency did not correlate with C reactive protein levels, brain natriuretic peptide, diffusing capacity for carbon monoxide, forced vital capacity levels, the presence of scleroderma renal crisis and other organ involvements (data not
Figure 2  Circulating Tfh cells are increased according to disease severity. (A) Frequencies of cTfh among CD4+ T cells in limited cutaneous systemic sclerosis (lSSc) and in diffuse SSc (dSSc). (B) Frequencies of CXCR5−PD-1−CD4+ cells among CD4+ T cells in lSSc and in dSSc. (C) Frequencies of cTfh among CD4+ T cells in patients with SSc with or without pulmonary arterial hypertension (PAH). (D) Frequencies of cTfh among CD4+ T cells in patients with SSc with or without active digital ulcers. (E) Frequencies of cTfh among CD4+ T cells in patients with SSc with or without interstitial lung disease (ILD). (F) Correlation between frequencies of cTfh among CD4+ T cells and modified Rodnan skin score (mRSS) scale (A–E). Data represent the mean with SD; *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney U test. (F) Spearman test for patients with SSc (left panel), dSSc and lSSc (middle and right panel, respectively). ns, not significant.

shown). These data suggest that cTfh frequencies are increased in the severe dSSc subtype.

Circulating Tfh cells express IL-21 and induce plasma cell differentiation through IL-21
The cTfh cells are able to induce differentiation of naive B cells into plasma cells through IL-21 secretion. As cTfh are increased in patients with SSc, we evaluated the frequency of plasma cells in these patients, which were defined as CD19+CD27+CD38hiIgD− B cells in SSc and HC (figure 3A). The frequencies and absolute numbers of CD19+CD27+38hiIgD− plasma B cells were not statistically different in patients with SSc and in HC (figure 3B).

However, cTfh cell frequencies and numbers positively correlated with frequencies and numbers of CD19+CD27+38hiIgD− plasma B cells (r=+0.38; p=0.006) in patients with SSc, whereas no significant correlation was found in HC (figure 3C). Circulating CXCR5−PD-1−CD4+ T-cell numbers (r=+0.37; p=0.025) and frequencies (r=+0.514; p=0.0005) also positively correlated with CD19+CD27+38hiIgD− plasma B cells in patients with SSc, whereas no correlation was found for HC (figure 3D).
We further explored the functional capacity of cTfh from patients with SSC. We first characterised the expression of the transcription factor BCL-6 required for Tfh differentiation and B-cell help and Blimp-1 which antagonises BCL-6. We analysed by RT-PCR the expression of BCL-6 and Blimp-1 in sorted CXCR5⁺PD-1⁺CD4⁺ and CXCR5⁻PD-1⁻CD4⁺ T cells from patients with SSC and HC. As shown in figure 4A, for patients with SSC, BCL-6 expression was significantly higher in cTfh cells CXCR5⁺PD-1⁺CD4⁺ than in CXCR5⁻PD-1⁻CD4⁺ T cells (p=0.016), whereas Blimp-1 expression was not significantly different (p=0.55) (figure 4B). In addition, cTfh cells from patients with SSC also expressed higher BCL-6 than CXCR5⁻PD-1⁻CD4⁺ T cells (figure 4A and B). Then, we analysed the expression of IL-21 and CXCL-13 cytokines by cTfh and CXCR5⁻PD-1⁻CD4⁺ T cells. Sorted cTfh from patients with SSC expressed higher transcriptional levels of IL-21 than CXCR5⁻PD-1⁻CD4⁺ T cells (p=0.03) (figure 4D), and this increase was not observed for cTfh cells from HC (p=0.06) (figure 4D). In patients with SSC and HC, cTfh cells expressed significantly higher transcriptional levels of CXCL-13 than CXCR5⁻PD-1⁺CD4⁺ T cells (p=0.008 and p=0.02 for patients with SSC and HC, respectively) (figure 4C), whereas we did not detect any CXCL-13 expression by CXCR5⁻PD-1⁺CD4⁺ T cells (data not shown). cTfh cells from patients with SSC expressed significantly higher IL-21 than cTfh from HC (260±232 vs 100±0; p=0.02) (figure 4E).

In order to determine the capacity of cTfh cells from patients with SSC to induce plasmablast differentiation, sorted cTfh were cocultured with autologous CD19⁺ B cells. The proportion of CD19⁺CD27⁺CD38⁺Ig⁺⁺⁺ B cells cocultured with non-Tfh CXCR5⁻PD-1⁻CD4⁺ T cells (41.9±16.0% vs 7.1±5.6%; p<0.0001) (figure 5B) or B cells alone. The levels of total IgG and IgM were measured by ELISA in the supernatants of CD19⁺ B cells cocultured with cTfh and non-Tfh B cells.
BCL-6 and IL-21 are increased in cTfh from patients with systemic sclerosis (SSc). (A–B) Quantification of the expression of the transcription factor Bcl-6 (A) and Blimp-1 (B) in blood CD4+ T cells by reverse transcription PCR (RT-PCR) (n=8 patients with SSc, n=6 healthy controls (HC)). (C–D) RT-PCR analysis of chemokine CXCL13 (C) and cytokine IL-21 (D) and in blood CD4+ T cells from patients with SSc (n=7 for IL-21 experiment and n=8 for CXCL13) or HC (n=6). (E) RT-PCR analysis of cytokine IL-21 in cTfh or CXCR5−PD-1+ CD4+ T cells from patients with SSc (n=6) or HC (n=6). (A–E) Data represent mean with SD, *p<0.05, **p<0.01, ***p<0.001 by Wilcoxon test. ns, not significant.

Concentrations were significantly increased in cTfh and B-cell cocultures as compared with non-Tfh CXCR5−PD-1−CD4+ T-cell and B-cell cocultures (2384±2510 ng/mL vs 190±287 ng/mL; p<0.0001) and (4392±3191 ng/mL vs 1315±1436 ng/mL; p=0.005), respectively (figure 5C,D). Also, CXCR5−PD-1−CD4+ T cells induced higher plasmablast frequencies and IgG production than CXCR5−PD-1−CD4+ T cells (p=0.01 and p=0.0004, respectively) (figure 5B–C). Finally, CXCR5−PD-1−CD4+ T Th cells were more efficient than CXCR5−PD-1−CD4+ T cells in promoting IgM secretion (p=0.01) (figure 5D). In the cocultures of cells from HC, the same results were observed for plasmablast frequencies and Ig production (figure 5B–D). Interestingly, cTfh cells from patients with SSc were more efficient to induce plasmablasts and IgG or IgM production in comparison with healthy controls (figure 5B–D).

Altogether, these data suggest that cTfh from patients with SSc are fully functional in promoting plasmablast activation and Ig production.
**Figure 5**  Plasmablast (PB) cell frequencies and Ig secretion are increased in Tfh coculture with B cells. (A) Identification of PB by flow cytometry after 6 days of Tfh and B-cell cocultures, one representative example of patient. (B) Frequencies of PB after Tfh and B-cell cocultures or B-cell culture alone by flow cytometry (n=13 patients with systemic sclerosis (SSc), n=6 healthy controls (HC)). IgG (C) and IgM (D) in supernatants of coculture by ELISA (n=13 patients with SSc, n=6 HC). (B–D) Black bars represent patients with SSc, white bars represent HC. Data represent mean with SD, Mann-Whitney U test. ns, not significant.

**IL-21 blockade and JAK1/2 inhibition suppress the cTfh cells’ ability to induce plasma B-cell proliferation and Ig secretion**

Since IL-21 support the expansion of plasmablasts induced by cTfh, we performed blocking experiments of the IL-21 signaling pathway. Thus, cTfh sorted from the blood of patients with SSc were cocultured with autologous B cells in the presence of recombinant IL-21RFc or in the presence of ruxolitinib, a JAK1/2 inhibitor which can affect CD4+ T-cell proliferation and inflammatory cytokine production. After 6 days of cTfh and B-cell coculture with IL-21RFc from patients with SSc, plasmablast cell frequency significantly decreased (38.4±19.6% without IL-21RFc vs 13.2±13.8% with IL-21RFc; p=0.002) (figure 6A). In addition, a significant decrease of IgG (2821±3051 ng/mL vs 508±616 ng/mL; p=0.004) (figure 6B) and IgM secretion (4592±3191 ng/mL vs 962±978 ng/mL; p=0.0003) (figure 6C) was observed with IL-21RFc in cTfh and B-cell cocultures from patients with SSc. IL-21 blocking also reduced plasmablast cell frequencies...
Figure 6  cTfh cells promote plasmablast (PB) cell differentiation and Ig secretion through IL-21 that can be inhibited by ruxolitinib in patients with systemic sclerosis (SSc). (A) PB cell frequencies in Tfh-cell and B-cell cocultures in the presence of IL-21RFc (n=12 patients with SSc, n=6 healthy controls (HC)), or with anti-IL-6R (tocilizumab) (n=8 patients with SSc, n=6 HC). (B) IgG concentration in supernatants of Tfh and B-cell cocultures with or without IL-21RFc (n=11 patients with SSc, n=5 HC). (C) IgM concentration in supernatants of Tfh and B-cell cocultures with or without IL-21RFc (n=11 patients with SSc, n=5 HC) or anti-IL-6R (n=7 patients with SSc, n=5 HC). (D–F) PB frequencies, IgG and IgM levels in supernatants after Tfh-cell and B-cell cocultures with or without ruxolitinib (n=6 patients with SSc, n=6 HC). (G) Cytokine IL-21 production by CD4+ T cells after phorbol 12-myristate 13-acetate and ionomycin stimulation with or without ruxolitinib (n=9 patients with SSc). (A–G) Data represent mean with SD, *p<0.05, **p<0.01***p<0.001 by Mann-Whitney U test. ns, not significant.

We also assessed the role of IL-6 in plasmablast expansion induced by cTfh. IL-6 has been previously reported to be highly secreted by B cells in SSc7 and able to stimulate IL-21 production by CD4+ T cells.27 The addition of tocilizumab, an anti-IL-6 receptor monoclonal antibody, to CD19+ B and Tfh cell cocultures did not significantly reduce the frequency of plasmablasts (figure 6A) or their secretion of IgM (figure 6C) in patients with SSc and in HC.

The addition of ruxolitinib, a JAK1/2 inhibitor, to CD19+ B cells and cTfh cocultures from patients with SSc resulted in a significant decrease of plasmablast expansion (44.4±7.8% vs 4.7±2.9%; p=0.0007), of IgG (1568±937 ng/mL vs 56.8±75.7 ng/mL; p=0.02) and IgM secretions (4575±2447 ng/mL vs 105±132 ng/mL; p=0.002) (figure 6D–F). Similarly, in cTfh and B-cell cocultures from HC, the addition of ruxolitinib resulted in a significant reduction of plasmablast frequencies, IgG and IgM productions (figure 6D–F).
IL-21RFc or ruxolitinib to CXCR5−PD-1−CD4+ T-cell and CD19− B-cell cocultures from patients with SSc also reduced the plasmablast cell differentiation and IgG secretion (online supplementary figure 1).

Thus, these results indicated that ruxolitinib could affect plasmablast differentiation and their capacity to secrete immunoglobulins. In order to determine if ruxolitinib could directly impact the IL-21 production by cTfh, we stimulated in vitro cTfh from patients with SSc by PMA and ionomycin in the presence of ruxolitinib. We observed that IL-21 levels in the supernatants of activated cTfh were significantly reduced by ruxolitinib (3033±1419 pg/mL vs 1455±1101 pg/mL; p=0.013). Conversely, IL-21 secretion by CXCR5−PD-1−CD4+ was not affected by the presence of ruxolitinib (figure 6G). Taken together, these results demonstrate that cTfh from patients with SSc can drive the expansion of plasma cells and immunoglobulin production, which can be successfully inhibited by IL-21 neutralisation with ruxolitinib.

**DISCUSSION**

Autoimmune diseases are usually characterised by increased autoantibody production which can result in organ dysfunction through various mechanisms, such as increased apoptosis, recruitment of inflammatory cells or direct cytotoxicity. This increased production of autoantibodies can result from a tolerance breakdown during the B-cell development28 or be the consequence of somatic mutation and aberrant selection into the GCs.29 Tfh cells are crucial immune regulators of B-cell activation and differentiation and recently cTfh have been implicated in several autoimmune diseases such as lupus erythematosus, Sjogren’s syndrome and rheumatoid arthritis. Moreover, cTfh frequencies have been shown to correlate with disease severity and levels of autoantibodies.17–19 In SSc, B cells can secrete various autoantibodies that correlate with disease subtypes, disease severity and prognosis. Thus, several studies showed that B-cell homeostasis was altered in SSc; however, the precise mechanisms of B-cell excessive maturation remain understudied. In this study, we performed the functional analysis of Tfh cells from patients with SSc and analysed their interaction with B cells. First, we demonstrate that cTfh are increased in SSc and are correlated with disease severity. Indeed, the cTfh frequencies were significantly increased in patients with SSc with dSSc subtypes, which are characterised by more aggressive disease outcome and increased mortality. cTfh cells were also increased in patients with pulmonary arterial hypertension which represents a severe and life-threatening complication of SSc. The skin progression, as represented by the mRSS scale, is one of best predictors of disease activity in SSc, and the Tfh cell frequency is significantly correlated with the mRSS in our study. Further studies would be needed to analyse the cTfh cell frequencies and their functional capacity in patients under treatment who respond in particular with skin improvement. We did not analyse cTfh correlation with autoantibody levels in the serum of patients with SSc as ELISA tests used to detect autoantibodies provided only qualitative data, and more than 80% of patients with SSc were positive for autoantibodies in the serum. Another subset of CD4+ T cells, which can also stimulate plasma B-cell differentiation, has been recently described in rheumatoid arthritis and characterised as CXCR5−PD-1−CD4+ peripheral helper T cells. The T follicular helper-like cell frequencies were similar to HC, and these cells did not correlate with SSc severity. The CXCR5−PD-1−CD4+ peripheral helper T cells were expanded in the synovium of patients with rheumatoid arthritis30 and tissular Tfh cells have been shown to infiltrate the skin of patients with SSc.21 The increased frequencies of cTfh cells that we observed in patients with SSc could be related to overall increased Tfh cells in blood and tissues, or represent Tfh cell homeostasis disturbance with increased levels of circulating cells. Few studies have analysed circulating or tissue-infiltrating Tfh cell frequencies and homeostasis in SSc. Two studies described increased ICOS+ T cell in the skin of patients with SSc30 31 and one recent study showed that CD4+ ICOS−PD-1−CXCR5+ Tfh cells infiltrate the skin of patients with SSc. Moreover, the presence of Tfh cells in the skin was correlated with clinical disease and dermal fibrosis.32 These CD4+ ICOS−PD-1−CXCR5+ Tfh cells from SSc were able to induce dermal fibroblast differentiation into myofibroblasts in vitro.33 In a murine model of graft-versus-host disease, SSc ICOS+ Tfh cells were increased and neutralisation of IL-21 or the administration of an anti-ICOS antibody prevented clinical dermal fibrosis.34 In our study, we observed that cTfh cells expressing high levels of PD-1 also expressed high levels of ICOS.

We showed that cTfh cells from patients with SSc had an increased expression of the transcriptional factors BCL-6, and of CXCL13 and IL-21 cytokine. These factors are associated with B-cell interaction, suggesting that these cTfh are able to cooperate with B cells. BCL-6 is a master regulator of Tfh cells.35 This observation is in line with previous studies, showing that only Tfh cells from germinal centres (GC Tfh) expressed BCL-6, whereas BCL-6 expression is not detected in blood cTfh.36 32 Thus, our observation of the increased expression of BCL-6 in cTfh from patients with SSc together with the higher expression of IL-21 suggest that cTfh from patients with SSc may have a phenotype of effector cells, in contrast to the resting phenotype of cTfh from HC which do not express BCL-6.

Using cocultures with B cells, we show that Tfh from patients with SSc were fully functional and more efficient to induce plasmablast differentiation and their IgG and IgM secretion than cTfh from HC. This higher capacity could rely on the higher IL-21 expression. The IL-21 cytokine is known to be a potent plasma cell inducer33–35 and IL-21R is highly expressed in the skin of patients with SSc.36 37 In a murine model of bleomycin-induced fibrosis, increased IL-21 production from CD4+ T cells was correlated with dermal fibrosis and lung fibrosis.38 Moreover, IL-21 could induce fibrosis through activation of CD8+ T cells.39 The CD4+ T cells secreting IL-21 sorted from synovial fluid of patients with rheumatoid arthritis induce the production of MMP-1 and MMP-3 by a fibroblast-like synoviocyte that promotes joint inflammation, further supporting a role for IL-21 beyond the regulation of B-cell response.40 We demonstrated that IL-21 is highly secreted by cTfh from patients with SSc and blocking IL-21R significantly reduced Tfh cells’ capacity to induce plasmablast cells.

Blocking proinflammatory cytokines such as IL-17, TNFα or IL-6 with monoclonal antibodies has been successfully used to treat several immune diseases such as psoriasis,41 rheumatoid arthritis or ankylosing arthritis.42 However, the cytokine neutralisation strategy presents some limitations: some patients did not respond sufficiently or presented secondary treatment failure because of drug immunogenicity.43 The targeting of the Janus Kinase pathway has been recently experimented because the JAK signalling pathway is associated with various cytokine receptors.22 Ruxolitinib is a JAK1 and JAK2 antagonist, which inhibits intracellular signalling of multiple proinflammatory cytokines including IL-21.44 IL-21 expression has been significantly reduced by ruxolitinib in a rodent model of rheumatoid arthritis.45 In graft-versus-host disease, ruxolitinib showed...
promising results in patients with steroid-refractory disease and is currently being tested in prospective studies in rheumatoid arthritis, psoriasis, myositis and graft-versus-host disease. Treatment with ruxolitinib reduced skin and pulmonary fibrosis in bleomycin-induced pulmonary fibrosis and in adTBR-induced dermal fibrosis. We report that ruxolitinib inhibits the cTfh capacity to secrete IL-21 and decrease the proportions of plasmablasts induced by cTfh cells in vitro, similarly to IL-21 blockade.

The molecular events leading to the expansion of cTfh in patients with SSc have not been explored. Human cTfh cell differentiation and expansion pathways are not actually well established. IL-12 produced by activated dendritic cells could promote human Tfh cell differentiation. Other cytokines such as IL-6 and IL-21 could contribute to Tfh cell differentiation, inducing BCL-6 expression by CD4+ T cells. Recently, plasmacytoid dendritic cells have been involved in SSc pathogenesis via the aberrant expression of TLR-8. One can speculate that plasmacytoid dendritic cells could contribute to Tfh cell expansion by increasing proinflammatory cytokine production in the context of SSc. STAT-3 is the major transducer of IL-6 and IL-21 on T and B cells. In addition, IL-21 activates B cells via the JAK–STAT signalling pathway. STAT3 regulates IL-21 expression by CD4+ T cells and is important for Tfh differentiation. Furthermore, an increased expression of phosphorylated STAT3 has been reported in skin biopsies from patients with SSc. The mechanism of Tfh cell inhibition by ruxolitinib could be the inhibition of STAT-3 phosphorylation, which will modulate the proinflammatory cytokine receptor expression on T and B cells.

In conclusion, circulating Tfh cells are increased in SSc and correlate with severe SSc, notably in dSS subtypes. cTfh from patients with SSc present an activated phenotype and induce B-cell differentiation into plasmablasts secreting Ig via IL-21 secretion. IL-21 or JAK1/2 blockade by ruxolitinib could be a promising strategy in the treatment of SSc.

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REFERENCES

mice may be associated with dermal and pulmonary inflammation and fibrosis. *Int J Rheum Dis* 2016;19:392–404.


Berglund L, Avery DT, Ma CS, et al. IL-21 signalling via STAT3 primes human naive B cells to respond to IL-2 to enhance their differentiation into plasmablasts. *Blood* 2013;122:3940–50.

