EXTENDED REPORT

Activated and resting regulatory T cell exhaustion concurs with high levels of interleukin-22 expression in systemic sclerosis lesions

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ABSTRACT
Objective Transforming growth factor-β is considered to play a key role in the process of fibrosis in systemic sclerosis (SSc) and in the development of regulatory T cells (Treg) and pro-inflammatory Th17 T cells producing interleukin 17 (IL-17) and IL-22. The authors therefore postulated that SSc could be characterised by a marked Treg/Th17 imbalance. Previous works did not distinguish between the different subsets of Treg and the non-regulatory FoxP3+ cells leading to inconsistent results.

Methods Combined phenotypic and functional analysis of Th17 cells and FoxP3+ CD4 T cells, discriminating activated Tregs and resting Tregs from non-regulatory FoxP3+ T cells, in blood and skin of SSc patients.

Results In early disease stages, there is a decreased proportion of activated Tregs. A concomitant resting Treg deficit becomes more apparent with disease progression. Active and diffuse forms of the disease are characterised by a relatively higher proportion of all FoxP3+ subsets, including non-regulatory T cells. No peripheral or local IL-17 amplification was observed. However, the authors found significantly increased IL-22 transcription levels in SSc lesional skin, as compared with healthy skin. Cytotofluorometry confirmed the existence in SSc patients and controls of a distinct subset of T cells producing IL-22 in the absence of IL-17.

Conclusion SSc pathogenesis does not appear to be linked to IL-17, but rather to IL-22-producing cells with skin-homing potential and a concomitant quantitative Treg defect. Active and diffuse forms of the disease are associated with a FoxP3 signature. Altogether, our data depict a status of regulatory/pro-inflammatory T cell imbalance in SSc.

INTRODUCTION
Systemic sclerosis (SSc) is an incurable disease characterised by fibrosis of the skin and organs, damage to endothelial cells leading to widespread vasculopathy and immunological abnormalities. Numerous studies have demonstrated the crucial role of several fibrogenic cytokines/growth factors and mediators, such as transforming growth factor-β (TGF-β), in initiating the process of fibrosis and orchestrating interactions among lymphocytes, fibroblasts, endothelial cells and monocytes/macrophages. Recently, circulating antibodies to platelet-derived growth factor receptors, which activate fibroblasts and collagen synthesis, have been identified in SSc. However, the precise triggering event(s) initiating the pathogenic sequence leading to fibrosis remains unknown.

There is growing evidence that T cell proliferation and cytokine secretion play a major role in SSc suggesting that this condition could be associated with a generally defective control of T cell activation. Naturally occurring CD4 regulatory T cells (Tregs) can suppress the immune response of CD4 and CD8 T cells. FoxP3 is still considered to be the most specific marker for Tregs, although its expression can be postulated that SSc could be characterised by a marked Treg/Th17 imbalance. Previous works did not distinguish between the different subsets of Treg and the non-regulatory FoxP3+ cells leading to inconsistent results. Although several studies have suggested that IL-17 might play a key role in the development of fibrosis in SSc, the precise role of cytokine secretion in this context remains unknown.

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observed in SSc. However, no clear distinction was made in these studies between activated and rTregs and non-regulatory FoxP3+ cells and, as a result, no clear conclusion can be drawn regarding Treg cell status. On the other hand, IL-17 was reported elevated in SSc tissues. Here, we show that Tregs retain suppressive activity in SSc but are reduced in numbers. Furthermore, we document a decline of the circulating aTreg subset with disease progression. At the same time, no Th17 amplification was observed. However, IL-22 was found increased in lesional and non-lesional skin.

**PATIENTS, MATERIALS AND METHODS**

**Patients and controls**

Blood samples were obtained from 53 consecutive patients with SSc (table 1).

SSc was diffuse (dSSc) in 35 patients and limited in 18 patients (lSSc). We recorded at the time of blood collection the Modified Rodnan Skin Score (MRSS), the serum creatinine level, the diffusing capacity of the lung for carbon monoxide (DLCO) and the European Scleroderma Study Group (EScSG) activity index that evaluate disease activity using clinical and laboratory items resulting in a score ranging from 0 to 10 (0=no activity; 10=maximal activity). Finally, although circulating IL-22-secreting T cells were found associated with interstitial lung disease in SSc, expression of IL-22 has not been previously studied in SSc tissues. Here, we show that Tregs retain suppressive activity in SSc but are reduced in numbers. Furthermore, we document a decline of the circulating aTreg subset with disease progression. At the same time, no Th17 amplification was observed. However, IL-22 was found increased in lesional and non-lesional skin.

**RESULTS**

**SSc aTregs and rTregs suppress autologous T cell proliferation and cytokine secretion**

Live human Tregs cannot be purified using intracellular FoxP3 as a marker. Because there is a linear correlation between CD25 and FoxP3 levels expressed by CD4 CD25bright T cells, circulating aTregs were purified as CD4 CD45RA FoxP3low T cells (sorting gate c, figure 1A), SSc aTregs inhibited autologous, anti-CD3-induced, CD4 CD25+ T-cell proliferation as efficiently as control aTregs (figure 1B,C). SSc rTregs, defined as CD4 CD45RA CD25+ T cells (sorting gate a, figure 1A), also proved to be as immunosuppressive as control rTregs (figure 1B,C). The percentage of proliferating (ie, Ki-67+) cells aTregs was similar in SSc patients and in controls (figure 1D). Like in controls, rTregs remained non-proliferating in patients (figure 1D). SSc Tregs inhibited the production of IL-2, IL-4, IL-5, IL-10, interferon γ (IFNγ) and tumour necrosis factor-α by CD4 CD25+ autologous T cells (figure 1E).

Altogether, we conclude from these results that Treg immunosuppressive activity is preserved in SSc patients.

**Decreased circulating aTregs in SSc patients**

The proportion of FoxP3+CD4 is similar in SSc patients compared with controls (median (range) 6.42% (2.85 to 28.70) of CD4 T cells vs 7.15% (3.66 to 13.81); p=0.19) (figure 2A).

Based on our observations, FoxP3+CD4 T cells were divided into CD4 CD45RA FoxP3bright aTregs (gate c, figure 2B), CD4 CD45RA FoxP3+ rTregs (gate a, figure 2B) and non-regulatory CD4 CD45RA FoxP3low T cells (gate b, figure 2B). Proportions and absolute counts of in SSc patients with controls compared with (median (range) 0.66% (0.17 to 2.50) of CD4 T cells and SSSc patients (p=0.002, r=0.40) (supplemental figure S1), the MRSS (p=0.02, r=0.29) and the serum creatinine level (p=0.04, r=0.28). SSc patients had significantly less aTregs than dSSc patients (p=0.002) (figure 2C). aTregs were independent of disease duration, age, DLCO, ADLCO, ΔMRSS, Δserum creatinine, ΔEScSG activity index, the presence of interstitial lung disease and the steroid/immunosuppressive regimen.

The frequencies of non-regulatory FoxP3− T cells were similar between groups (figure 2C). Yet, among SSc patients, increased frequencies of non-regulatory FoxP3+ T cells correlated with increased serum creatinine levels (p=0.009, r=0.36) (supplemental figure S2) but not with any of the other parameters cited above. The proportions of IFNγ, IL-17, IL-22- and IL-2-producing cells among non-regulatory FoxP3+CD4 T cells were similar in controls and SSc (supplemental figure S3).

**Table 1** Characteristics of SSc patients and healthy controls

<table>
<thead>
<tr>
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<th>SSc n=53</th>
<th>Controls n=24</th>
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<tr>
<td>Female sex (%)</td>
<td>45 (85)</td>
<td>20 (83)</td>
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<tr>
<td>Mean age upon inclusion, years (±SD)</td>
<td>53.5±14.1</td>
<td>51.3±10</td>
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<tr>
<td>Mean age at SSc diagnosis, years (±SD)</td>
<td>46.2±13.6</td>
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<tr>
<td>Median time between SSc diagnosis and analysis, months (range)</td>
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<tr>
<td>Early SSc/late SSc</td>
<td>27/26</td>
<td></td>
</tr>
<tr>
<td>dSSc/SSc</td>
<td>35/18</td>
<td></td>
</tr>
<tr>
<td>Median MRSS (range)</td>
<td>10 (0–37)</td>
<td></td>
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<tr>
<td>ANA (%)</td>
<td>53 (100)</td>
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<tr>
<td>Anti-Scl70 (%)</td>
<td>23 (43.4)</td>
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<tr>
<td>ACA (%)</td>
<td>16 (30.2)</td>
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<tr>
<td>Mean DLCO, % of predicted value (±SD)</td>
<td>58.8±18.4</td>
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<tr>
<td>Mean serum creatinine level, μmol/l (±SD)</td>
<td>73.6±22.9</td>
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<tr>
<td>IS during the past 6 months</td>
<td>None</td>
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<tr>
<td>ST during the past 6 months (%)</td>
<td>17 (32.1)</td>
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*Steroid treatment consists of 3–10 mg of prednisone a day.

AC, anticentromere antibody; ANA, antinuclear antibody; Anti-Scl70, antitopoisomerase I antibody; DLCO, diffusing capacity of the lung for carbon monoxide; dSSc, diffuse systemic sclerosis; IS, immunosuppressive therapy; SSc, limited systemic sclerosis; MRSS, Modified Rodnan Skin Score; SSc, systemic sclerosis; ST, steroids.

**IS during the past 6 months None**

**DISCUSSION**

The proportion of FoxP3+CD4 is similar in SSc patients compared with controls (median (range) 6.42% (2.85 to 28.70) of CD4 T cells vs 7.15% (3.66 to 13.81); p=0.19) (figure 2A).

Based on our observations, FoxP3+CD4 T cells were divided into CD4 CD45RA FoxP3bright aTregs (gate c, figure 2B), CD4 CD45RA FoxP3+ rTregs (gate a, figure 2B) and non-regulatory CD4 CD45RA FoxP3low T cells (gate b, figure 2B). Proportions and absolute counts of aTregs are significantly decreased in SSc patients compared with controls (median (range) 0.66% (0.17 to 2.00) of CD4 T cells and SSSc patients (p=0.002, r=0.40) (supplemental figure S1), the MRSS (p=0.02, r=0.29) and the serum creatinine level (p=0.04, r=0.28). SSc patients had significantly less aTregs than dSSc patients (p=0.002) (figure 2C). aTregs were independent of disease duration, age, DLCO, ADLCO, ΔMRSS, Δserum creatinine, ΔEScSG activity index, the presence of interstitial lung disease and the steroid/immunosuppressive regimen.

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A FoxP3 signature associated with active and diffuse disease

In multivariate analysis, the proportions of rTregs (p=0.02), aTregs (p=0.003) and non-regulatory FoxP3+ T cells (p=0.002) among CD4 T cells were independently associated with the diagnosis of SSc, compared with controls. A higher proportion of aTregs (p=0.01), but not of other FoxP3+ subsets, was independently associated with the diagnosis of dSSc versus lSSc.

Hierarchical cluster analysis based on FoxP3+ subsets proportions resulted in the delineation of two clusters within SSc patients. Cluster #1 patients (n=20) have more likely dSSc (p=0.025), significantly higher proportions of rTregs (p=0.08), aTregs (p<0.0001), non-regulatory FoxP3+ T cells (p<0.0001), and a FoxP3 signature associated with active and diffuse disease.
MRSS (p=0.049) and EScSG activity scores (p=0.014) than cluster #2 patients (n=33) (figure 3). Validity and reproducibility of this partition in two clusters were assessed using non-hierarchical k-means cluster analysis ($\kappa$ agreement value=0.96).

**FoxP3+ T cells are not redistributed to the skin of SSC patients**

Histological analysis of affected areas of SSC skin showed small pericapillary lymphocytic infiltrates (data not shown). Real time quantitative PCR showed that CD3 was more abundant in SSC skin (supplemental figure S5). Yet, immunohistochemical analyses, using anti-CD4 and anti-FoxP3 antibodies did not reveal the presence of FoxP3+ cells in SSC-associated skin sclerosis (data not shown). We rather observed that FoxP3 transcripts were less abundant in SSC than in control skin, as evaluated by real time quantitative PCR (supplemental figure S6).

**IL-17 production is similar in SSC patients and controls**

IL-17 could only be detected in the serum of five out of 28 SSC patients and was not above detection level in any of the 15 controls (p=0.15). No differences in IL-17 serum levels were found between SSC patients at late and early disease stage (p=0.57) (figure 4B, left). In addition, IL-17 levels also did not differ between ISSc and dSSc (data not shown). Considering that IL-17 serum detection might not be sensitive enough to evaluate the Th17 axis in SSC, we measured the percentage of circulating CD4 T cells expressing IL-17 following CD3/CD28 stimulation. No significant differences between controls and SSC patients were observed (0.4% (0.1 to 1.6) and 0.5% (0.1 to 2.0) of CD4 T cells, respectively, p=0.99) (figure 4A). The frequencies of Th17 cells did not significantly differ between early and late SSC patients (p=0.55) (figure 4A). Finally, as shown in figure 4C, left, IL-17 transcripts were not more abundant in SSC patients than in controls.
Increased IL-22 expression in SSc

We investigated other putative Th17-associated markers. Global proportions of circulating IL-22-secreting cells were not found to be increased in SSc patients, as compared with controls (p=0.48). Twelve out of 29 SSc patients had serum IL-22 levels above detection threshold, as compared with two out of 15 controls (p=0.08). A significantly increased proportion of late SSc patients (nine out of 13 patients) had circulating IL-22 levels above detection threshold, as compared with early disease stage SSc patients (three out of 16 patients) (p=0.02, figure 4B, right). No difference was found between lSSc and dSSc (data not shown). Finally, IL-22 transcripts were significantly more abundant in SSc skin, as compared with healthy skin samples (p=0.005) (figure 4C, right). Of note, β-actin levels were similar in SSc and control skin (data not shown). Results were confirmed using RS9 (GenBank accession # NM_001013) as other endogenous gene reference (data not shown). IL-22 transcriptional levels were similar in clinically affected areas, in what was considered unaffected areas as well as in transition areas between the former two regions (figure 4C, right).

DISCUSSION

The assessment of Treg cell performed in the present study relies on a functional delineation of FoxP3-expressing CD4 T cells allowing at the same time to discriminate aTregs, rTregs and non-regulatory FoxP3+ T cells.14 These distinctions were not clearly made in previous studies addressing the same issue.19–22 We report that aTregs are decreased in SSc patients irrespective of disease stage whereas the decline in rTregs is mainly manifest in late stages. Nevertheless, SSc aTregs and rTregs retain their ability to block CD4 CD25− T cell proliferation and to inhibit cytokine secretion. Thus, contrary to what has been reported previously, there is neither quantitative expansion20–22 nor qualitative deficiency20 33 34 of bona fide Tregs in SSc. It cannot be concluded either that Treg counts are unchanged in SSc patients compared with controls.19 These discrepancies are most likely related to the observation that FoxP3 expression can be induced in conventional T cells via activation without conferring suppressive activity.10–14 Since previous studies in SSc were based on the analysis of global FoxP3 expression of CD4 T cells and/or did not discriminate between the different subsets

Figure 3  Systemic sclerosis (SSc) patients segregate in two different groups defined by their FoxP3+ T cell status. (A) The dendrogram shows how the 53 SSc patients analysed cluster according to measured FoxP3+ subsets proportions. The scale extends from minimum (green) to maximum (red) values. (B) European Scleroderma Study Group (EScSG) activity index in main patient clusters defined above. Each dot represents an individual, and lines show median values. (C) Proportions of diffuse systemic sclerosis (dSSc) and limited systemic sclerosis (ISSc) among each cluster.
Figure 4  Interleukin 17 (IL-17) and IL-22 expression in systemic sclerosis (SSc) patients. (A) Flow cytometry of intracellular IL-17 and IL-22 in PBMCs assessed after 16 h of stimulation with anti-CD3 and anti-CD28. Analyses, gated on CD4 T cells, from a representative control and a representative SSc patient are presented. Data summary for proportions of circulating IL-17 producing cells among CD4 T cells in SSc patients (n=27) and controls (n=24) (top right). Data summary for proportions of IL-22 producing cells in SSc patients (n=13) and controls (n=8) are presented below. (B) Left: serum IL-17 levels in controls (n=15) and SSc patients (n=28); right: serum IL-22 levels in controls (n=15) and SSc patients (n=29). Detection threshold of ELISA test was 8 pg/ml for IL-17 and 100 pg/ml for IL-22 (indicated by a dotted line). Statistical analysis was done using Fisher’s exact test. (C) Skin cDNA samples of 9 SSc patients and seven controls were subjected to real-time quantitative PCR analysis using primers specific for β-actin, IL-17 (left) and IL-22 (right). In patients, various skin areas were analysed as indicated.
of Treg,\textsuperscript{19–22} relatively abundant non-regulatory FoxP3+CD4 T cells could have masked the modifications of the more discrete Treg subsets.

The quantitative Treg defect observed could account for the T cell activation and expansion associated with SSc.\textsuperscript{4–9} The reasons of this decline are still speculative. Numerous studies have demonstrated the central role of TGF-β in SSc.\textsuperscript{2} This cytokine has also been found to play a key role in the development of Tregs, as it induces differentiation and expansion of CD4 CD25+ regulatory cells from CD4 CD25− precursors.\textsuperscript{16,35–36} Therefore, in the context of increased TGF-β levels, like in SSc, an increased pool of Tregs could have been expected at all stages. Interestingly, we indeed observed through hierarchical analysis of FoxP3+ subsets that patients with diffuse and/or active SSc have relatively higher proportions of both regulatory and non-regulatory FoxP3+ T cells, compared with patients with less active disease. However, long term and strong stimulation by TGF-β over Treg cells could result in a high turn-over and differentiation of the latter and, consequently, in an exhaustion of the Treg pool. This could explain why we also found a negative correlation between circulating rTregs numbers and the time elapsed since disease onset, while rTreg levels were not correlated with age. As it was recently shown that human rTreg can convert into aTreg in vivo,\textsuperscript{14} it is tempting to conclude that repetitive TCR-mediated stimulation could have resulted in the peripheral conversion of most natural rTreg cells into antigen-experienced aTregs.

Altogether, there is a quantitative Treg defect in SSc that is less pronounced in diffuse and/or active disease, this apparent paradox being likely the result of a compensatory, but inefficient, amplification of regulatory cells in the context of active inflammation.

It was recently confirmed that TGF-β, along with IL-23 and pro-inflammatory cytokines, is essential to the differentiation of the Th17 lineage.\textsuperscript{57} In a TGF-β-related condition like SSc, it could therefore have been expected to observe an amplification of Th17 cells. Indeed, increased IL-17 production by SSc T cells has been reported, especially in early stages of the disease.\textsuperscript{23,24} In a recent study, very high frequencies of circulating Th17 cells (up to 40% of CD4 CD45RO+ cells and up to 20% of CD4 CD45RA+ cells) were reported in SSc patients.\textsuperscript{25} This result could be due to a different analysis strategy as the study was done on purified CD3 cells and not with unfraccionated PBMC. In our own study, we did not find significantly elevated IL-17 levels neither by ELISA nor by intracellular flow cytometry. It should be noted that in the study reported by Murata et al, most of the IL-17 serum levels were below the minimum significant detection level of their ELISA assay (5 pg/ml).\textsuperscript{24} The reasons for the discrepancy between our results and the one by Kurasawa et al are less clear.\textsuperscript{23} In an effort to use a more sensitive and possibly more appropriate assay, we quantified IL-17 transcripts in SSc skin lesions. IL-17 mRNA levels were not found elevated. We can only speculate that Kurasawa et al had access to patients in their very early stage of the disease in order to explain the discrepancy between their results and ours. Nevertheless, it is fair to conclude that in SSc, Treg depletion is not associated with a concomitant amplification of Th17 cells.

Even if IL-22 is mostly expressed by Th17 cells,\textsuperscript{38,39} its expression differs from that of IL-17\textsuperscript{36} and recently, a novel subset of IL-22-, but not IL-17-producing T cells, with skin homing potential was described in healthy subjects and in inflamed tissues.\textsuperscript{40–42} However, the existence of a truly separate ‘Th22’ subset remains unclear. IL-22 is a key cytokine in the regulation of inflammatory responses, particularly in the skin where it mediates keratinocyte migration and differentiation and epithelial hyperplasia leading to the epidermal remodelling and the thickening of the epidermis with dermal infiltration of macrophages, some of the pathological features seen in SSc skin.\textsuperscript{39–42} In the present study, we confirm the existence in SSc patients and in healthy subjects of a CD4 IL-17-IL-22+ circulating subset.\textsuperscript{43,44} It was impossible for ethical reasons to obtain enough SSc skin material to perform cytofluorometric analysis on lesional T cells in order to directly confirm their expected skin tropism.\textsuperscript{45–46} We were also unable to set up immunohistochemistry staining to confirm the presence of Th22 cells in situ. However, the observation that IL-22, but not IL-17 transcripts, were found more likely elevated in SSc skin samples than in normal skin (figure S3C) is strongly suggestive of the presence of ‘IL-22-producing cells only’ associated with SSc.

Finally, it should be noted that we found elevated IL-22 transcripts in SSc lesions and in apparently unaffected areas. An SSc-associated gene expression pattern was also observed both in clinically affected and clinically unaffected skin using DNA microarrays.\textsuperscript{47} Both results further emphasise the systemic nature of this condition that has covert manifestations in early stages of the disease and in apparently unaffected organs.

Contributors All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Study conception and design: AM, CP, KD, ST, ML, MM, LA, MH, J-CP CF, HY, ZA and GG. Analysis and interpretation of data: AM, CP, KD, ST, ML, MM, LA, MH, J-CP, CF, HY, ZA and GG. AM, CP, KD, ST and LA contributed equally to the work.

Acknowledgements The authors thank the patients and controls who participated in this study; Cécile Badoual and Philippe Le Pelletier for technical support in skin biopsy analyses; and the staff of the Internal Medicine department who actively participated in this study.

Funding This study was supported by Assistance Publique-Hôpitaux de Paris (CIB Pitié-Salpêtrière) and by the Institut National de la Santé et de la Recherche Médicale. M Larsen was supported by the European FP6 ‘ATTACK’ programme (contract: LSHC-CT-2005-018914) and S Trad by the Association pour la Recherche sur le Cancer.

Competing interests None.

Patient consent Obtained.

Ethics approval Approval provided by the local ethics committee of the Pitié-Salpêtrière hospital.

Provenance and peer review Not commissioned; externally peer reviewed.

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Basic and translational research


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*Ann Rheum Dis* 2012 71: 1227-1234
doi: 10.1136/annrheumdis-2011-200709

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