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. Cytofluorometry 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.[1] 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.[2] Recently, circulating antibodies to platelet-derived growth factor receptors, which activate fibroblasts and collagen synthesis, have been identified in SSc.[3] 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,[4–9] 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 induced in conventional T cells via activation without conferring suppressive activity.[10–14] Based on our observations,[14] FoxP3$^+$CD4 Tregs can be divided into CD4 CD45RA$^-$FoxP3^{bright}CD25^{bright} and CD4 CD45RA$^+$FoxP3$^+$CD25$^+$ fractions, while the remaining CD4 FoxP3$^+$ (ie, CD4 CD45RA$^-$FoxP3^{low}CD25$^+$) include a notable amount of non-regulatory, cytokine-secreting, activated T cells (=*non-regulatory FoxP3$^+$ T cells*). CD4 CD45RA$^-$FoxP3^{bright}CD25^{bright} Tregs are activated, highly suppressive and in vivo proliferating cells defined as activated Tregs (aTregs).[14] CD4 CD45RA$^+$FoxP3$^+$CD25$^+$ Tregs are fully functional, antigen-experienced cells and will therefore be hereafter referred to as resting Tregs (rTregs),[14] rather than naive Tregs. CD45RA$^+$Treg cells represent the main peripheral reservoir of CD45RA$^-$Treg cells.[14]

Recently, a new subset of T helper cells was described and named Th17 based on its production of interleukin 17A (IL-17A),-17F and -22.[15] Th17 cells play an important role in tissue damage. TGF-β, in association with inflammatory cytokines, facilitates Th17 differentiation (reviewed in reference[15]). TGF-β also plays a key role in the development of Tregs, as it induces differentiation and expansion of CD4 CD25 T regulatory cells from CD4 CD25$^-$ precursors.[16] It has been suggested that the transcription factor aryl hydrocarbon receptor and its ligands might regulate the Treg/Th17 balance by modulating TGF-β signalling within the nucleus.[17]

Because it was recently proposed that IL-17 together with TGF-β and IL-6 could be, at least in part, responsible for most prominent features of SSc such as fibroblast proliferation and endothelium activation,[18] we hypothesised that a Treg/Th17 imbalance could be a central component of SSc pathophysiology. On the one hand, normal[19] or increased[20–22] frequencies of circulating FoxP3-expressing T cells have been

observed in SSc. However, no clear distinction was made in these studies between activated and rTregs and non-regulatory FoxP3+ cells[14] and, as a result, no clear conclusion can be drawn regarding Treg cell status. On the other hand, IL-17 was reported elevated in the serum of SSc patients.[23 24] This finding was not confirmed in a more recent study, which nevertheless reported drastically increased Th17 frequencies in SSc.[25] 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.[26] 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).[27] We recorded at the time of blood collection the Modified Rodnan Skin Score (MRSS),[28] 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).[29] We recorded their change after 12 months (delta=Δ). Disease stages were defined as suggested by Steen and Medsger: for early lSSc, disease duration <5 years; for late lSSc, disease duration ≥5 years; for early dSSc, disease duration <3 years; and for late dSSc, disease duration ≥3 years.[30] The control group consists of 24 healthy volunteers matched for age and sex with the SSc group.

Skin biopsies were obtained from nine early SSc patients (eight dSSc and one lSSc) and seven healthy donors undergoing surgery. Thus, 5 mm punch biopsies in clinically affected and unaffected skin were obtained from all nine SSc patients and, in five cases, from transition areas. Treatments are detailed in the supplementary informations. Five patients (9.4%) had previously received cyclophosphamide but at least 1 year elapsed between immunosuppressive treatment and inclusion in the study. Seventeen (32.1%) were on low dose steroids (below 10 mg of prednisone a day) at the time of inclusion in the study.

In vitro culture assays, flow cytometry, cytokine detection procedures and statistical analysis. See supplementary informations.

Table 1 Characteristics of SSc patients and healthy controls

	SSc n=53	Controls n=24
Female sex (%)	45 (85)	20 (83)
Mean age upon inclusion in the study, years (±SD)	53.5±14.1	51.3±10
Mean age at SSc diagnosis, years (±SD)	46.2±13.6	
Median time between SSc diagnosis and analysis, months (range)	47 (0–317)	
Early SSc/late SSc	27/26	
dSSc/lSSc	35/18	
Median MRSS (range)	10 (0–37)	
ANA (%)	53 (100)	
Anti-Scl70 (%)	23 (43.4)	
ACA (%)	16 (30.2)	
Mean DLCO, % of predicted value (±SD)	58.8±18.4	
Mean serum creatinine level, μmol/l (±SD)	73.6±22.9	
IS during the past 6 months	None	
ST during the past 6 months (%)*	17 (32.1)	

*Steroid treatment consists of 3–10 mg of prednisone a day.
ACA, 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; lSSc, limited systemic sclerosis; MRSS, Modified Rodnan Skin Score; SSc, systemic sclerosis; ST, steroids.

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 $CD25^{bright}$ T cells,[14 31] circulating aTregs were purified as CD4 $CD45RA^{-}CD25^{bright}$ 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,[14] $FoxP3^{+}CD4$ T cells were divided into CD4 $CD45RA^{-}FoxP3^{bright}$ aTregs (gate c, figure 2B), CD4 $CD45RA^{+}FoxP3^{+}$ rTregs (gate a, figure 2B) and non-regulatory CD4 $CD45RA^{-}FoxP3^{low}$ 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 $5^{(1to17)}$ cells/mm^3 vs 1.51% (0.79 to 3.03) and $13^{(6–37)}$ cells/mm^3; p<0.0001 and p<0.0001, respectively) (figure 2C). Percentage of aTregs were correlated with the EScSG activity index (p=0.003, 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). lSSc patients had significantly less aTregs than dSSc patients (p=0.002) (figure 2C). aTregs were independent of disease duration, age, DLCO, ΔDLCO, 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).

rTregs decrease in late SSc stages

Proportions and absolute counts of rTregs (gate a, figure 2B) were significantly reduced in SSc compared with controls (0.72%

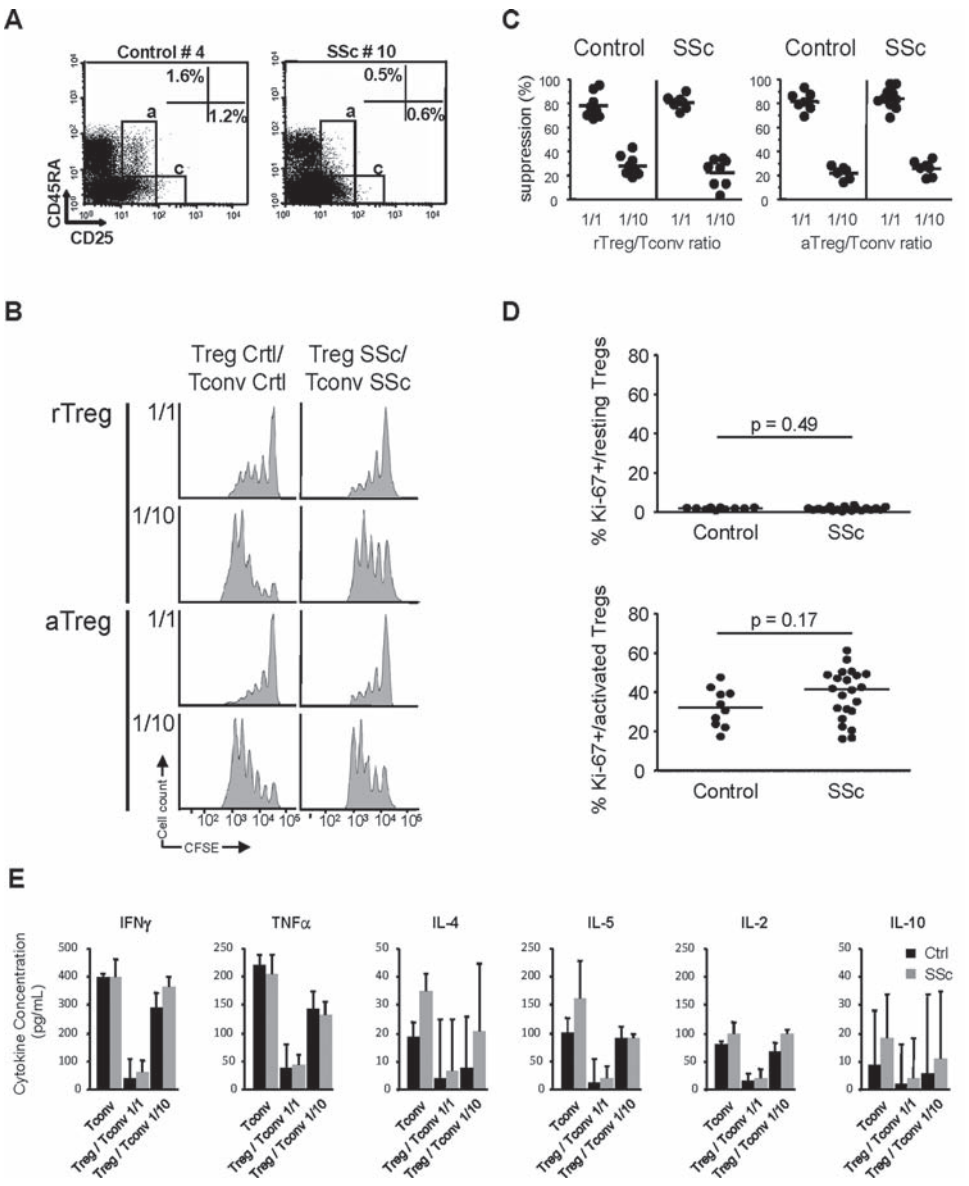

Figure 1 Phenotypic and functional characterisation of resting and activated regulatory T cells (Tregs) in systemic sclerosis (SSc) patients. (A) Flow cytometry analysis gated on CD4 peripheral lymphocytes. Resting Tregs (rTreg, CD4 CD45RA$^+$CD25$^+$) and activated Tregs (aTreg, CD4 CD45RA$^-$CD25^{bright}) were defined as indicated (gates a and c, respectively). Analysis of CD4 T cells from a representative control (ctrl) (out of 24 tested) and from a representative SSc patient (out of 53 tested) are presented. (B) SSc rTregs and aTregs are both suppressive. CFSE-labelled CD4 CD25$^-$T cells (Tconv) were flow-sorted from blood, stimulated with plate-bound anti-CD3 in the presence of irradiated autologous APCs and cocultured (1:1 or 1:10 ratios) with flow-sorted unlabeled rTreg and aTreg. Data from one representative SSc patient and control are presented. (C) Calculated percentages of rTreg and aTreg suppression for SSc patients (n=7–10) and controls (n=6–8). (D) Same proportions of proliferating Tregs in patients and controls. Proportions of circulating Ki-67$^+$ rTregs and aTregs were determined by flow cytometry in 10 controls and 22 SSc patients. (E) SSc Tregs block both autologous (grey bars, $n=4$) and healthy control (black bars, $n=2$) cytokine secretion. Treg to Tconv coculture ratios are indicated. Indicated cytokines were measured in supernatants after 84–90 h of coculture using a cytometric bead array kit. Mean values and SDs are indicated.

(0.10 to 5.29) and 4 (0 to 39) cells/mm^3 vs 1.63% (0.57 to 4.94) and 14 (7 to 59) cells/mm^3; p<0.0001 and p<0.0001, respectively) (figure 2C). Similar results were obtained when CD4 CD45RA$^+$CD25$^+$ T cells,[32] rather than CD4 CD45RA$^+$FoxP3$^+$ T cells, were monitored (data not shown). rTregs in SSc were negatively correlated with disease duration (p=0.004, r=−0.39) (supplemental figure S4) but not with any of the other parameters cited above. Late SSc patients had significantly less circulating rTregs than early SSc patients (0.60% (0.10 to 2.30) vs 1.02% (0.23 to 5.29) of CD4 T cells, respectively, p=0.007) (figure 2C).

A FoxP3 signature associated with active and diffuse disease

In multivariate analysis, the proportions of rTregs (p=0.02), aTregs (p=0.0003) 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=0.023), significantly higher proportions of rTregs (p=0.03), aTregs (p<0.0001), non-regulatory FoxP3$^+$ T cells (p<0.0001),

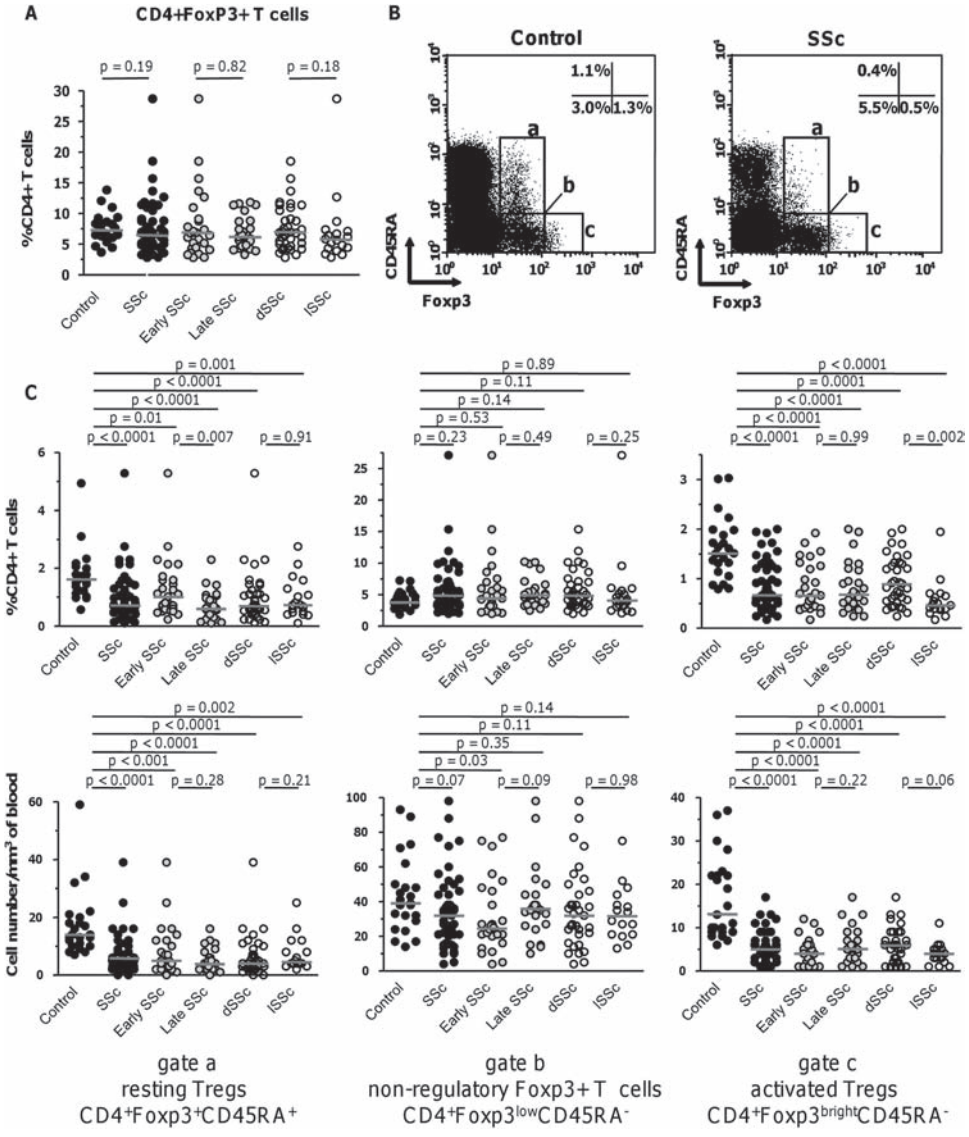

Figure 2 Resting Tregs and aTregs in systemic sclerosis (SSc) patients. PBMC analysis gated on CD4 cells. (A) Percentages of circulating CD4 FoxP3$^+$ T cells in patients and controls. (B) Gate definition for flow cytometry analysis according to FoxP3 and CD45RA fluorescence. Data from a representative healthy control (out of 24 tested) and from a representative SSc patient (out of 53 tested) are presented. (C) Percentages and numbers of circulating resting Tregs (gate a), non-regulatory FoxP3+ T cells (gate b) and activated Tregs (gate c) among CD4 T cells in patients and controls. Each dot represents an individual, and lines show median values. dSSC, diffuse systemic sclerosis; lSSc, limited systemic sclerosis.

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 (κ 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, immunohistological analyses, using anti-CD4 and anti-FoxP3 antibodies[31] 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 lSSc 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.

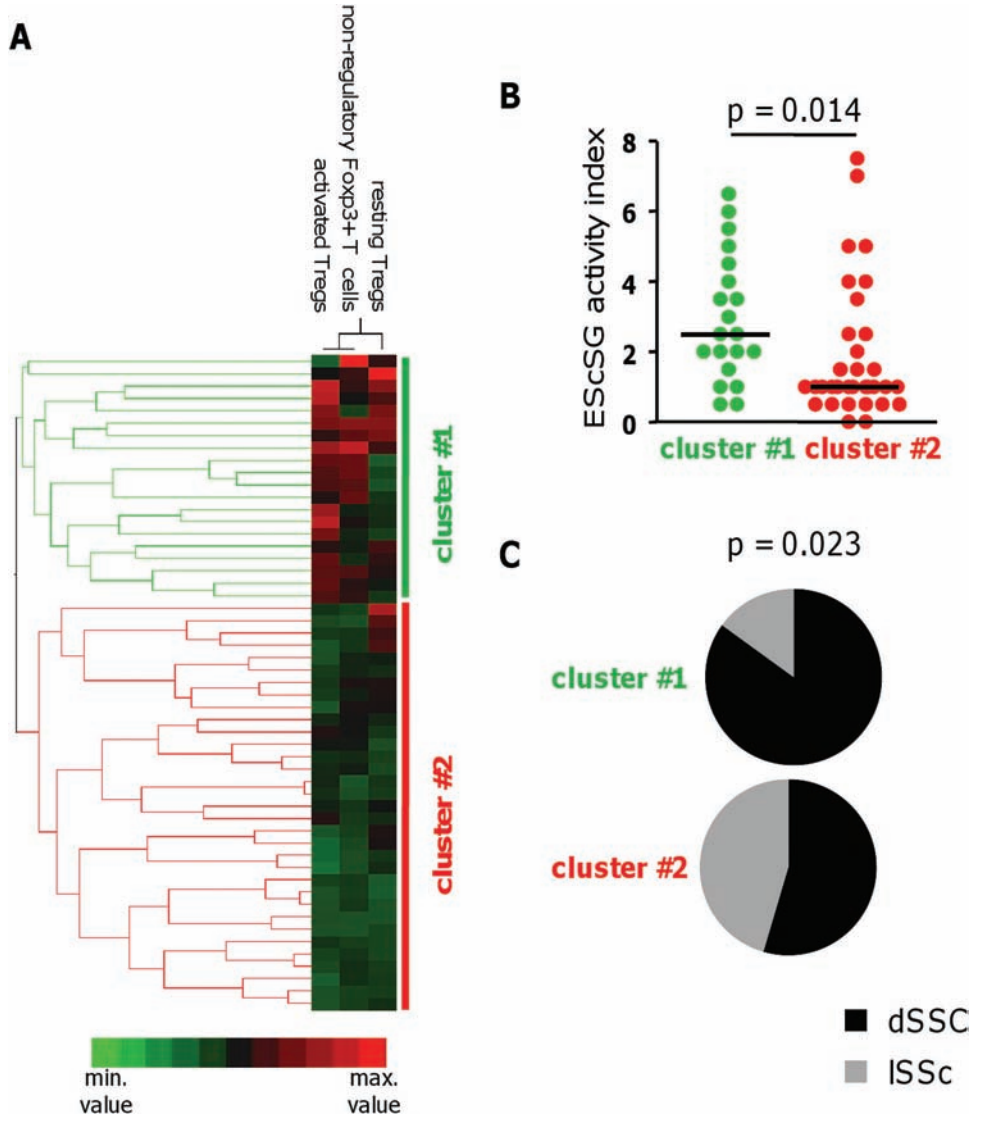

Figure 3 Systemic sclerosis (SSc) patients segregate in two different groups defined by their FoxP3$^+$ T cell status. (A) The dendrogram shows how the 53 SSc patients analysed cluster according to measured FoxP3$^+$ subsets proportions. The scale extends from minimum (green) to maximum (red) values. (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 (lSSc) among each cluster.

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 expansion[20–22] nor qualitative deficiency[20 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

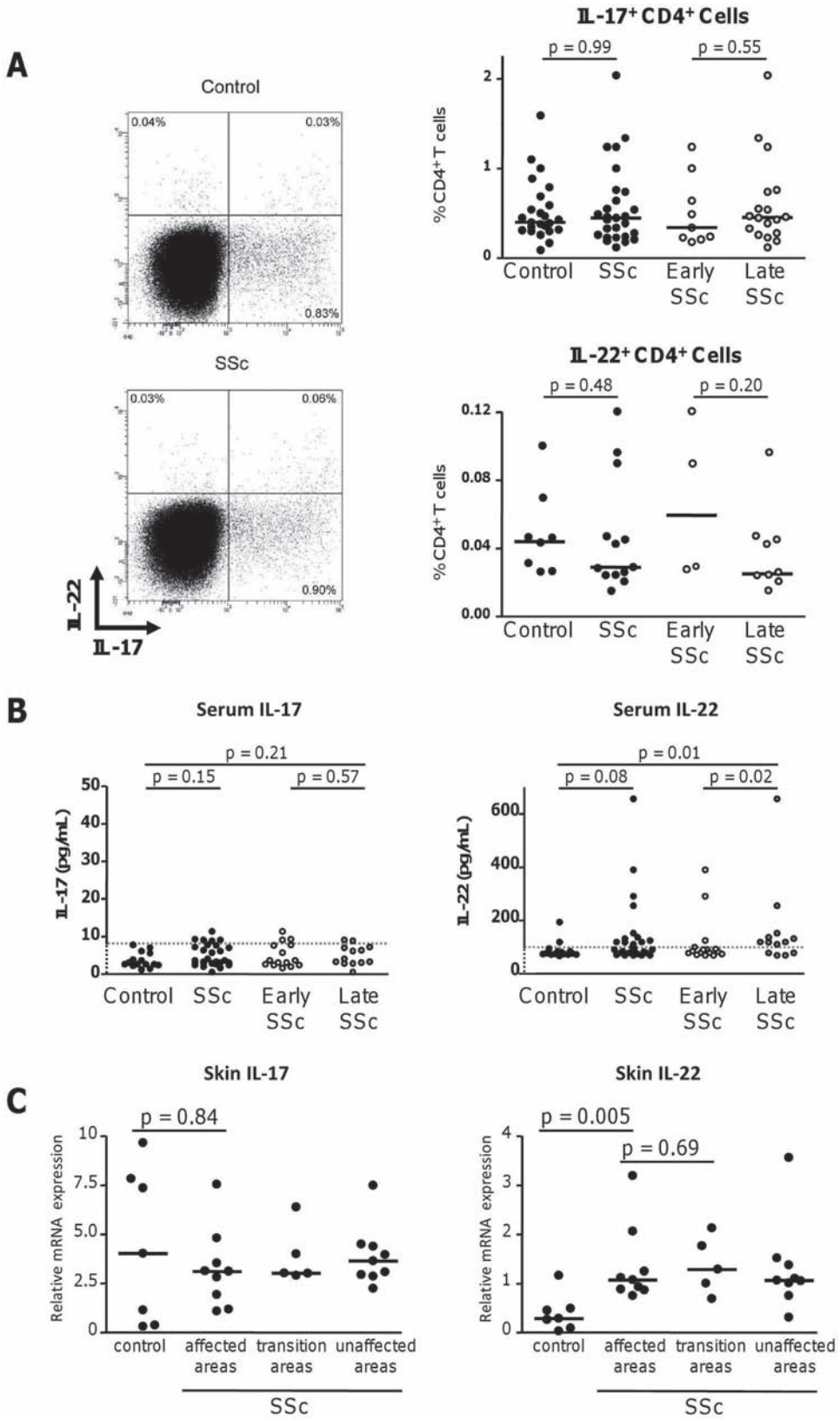

Figure 4 Interleukin 17 (IL-17) and IL-22 expression in systemic sclerosis (SSc) patients. (A) Flow cytometry of intracellular IL-17 and IL-22 in PBMCs assessed after 16 h of stimulation with anti-CD3 and anti-CD28. Analyses, gated on CD4 T cells, from a representative control and a representative SSc 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 skins areas were analysed as indicated.

of Treg,[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.[4–9] The reasons of this decline are still speculative. Numerous studies have demonstrated the central role of TGF-β in SSc.[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 T regulatory cells from CD4 CD25− precursors.[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,[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.[37] 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.[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.[25] This result could be due to a different analysis strategy as the study was done on purified CD3 cells and not with unfractioned 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).[24] The reasons for the discrepancy between our results and the one by Kurasawa *et al* are less clear.[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,[38] its expression differs from that of IL-17[39] 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.[40] 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.[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.[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.[43 44] 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 3C) 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.[45] 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.

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Patient consent Obtained.

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