
Regulatory B cell imbalance correlates with Tfh expansion in systemic sclerosis

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ABSTRACT

Objective. Systemic sclerosis (SSc) is an autoimmune disease with fibrosis, microangiopathy and immune dysfunction. B cell abnormalities characterised by autoantibody production and polyclonal B cell activation play an important role in the pathogenesis of SSc. We previously identified an expansion of functional and activated circulating T follicular helper (cTfh) cells in SSc patients. The aim of this study was to analyse the frequency of regulatory B (Breg) cell subsets and the correlation with Tfh in SSc patients.

Methods. Circulating Breg cells CD24^{hi}CD38^{hi} and CD27⁺CD24^{hi} levels and cTfh cells CD4⁺CXCR5⁺PD1⁺ were determined by cytometry in 50 SSc patients and 32 healthy subjects.

Results. The frequency of Breg cells CD24^{hi}CD38^{hi} and CD24^{hi}CD27⁺ was significantly reduced in patients with SSc as compared to controls ($p=0.02$ and $p<0.001$, respectively). In contrast, when examining the CD21^{low} B cell subset, the frequency was significantly increased in SSc patients compared to healthy controls, ($p<0.001$). There was no difference in Breg cell levels in patients with diffuse SSc and limited SSc. However, CD24^{hi}CD27⁺ Breg cell frequency was significantly decreased in SSc patients with pulmonary arterial hypertension ($p=0.014$), but not in patients with interstitial lung disease ($p=0.058$). Furthermore, we observed a negative correlation between cTfh and CD24^{hi}CD27⁺ Breg cell levels in SSc patients but not in healthy controls ($p=0.02$).

Conclusion. These results suggest that Breg cell subsets may participate in the regulation of cTfh and disease severity. Decreased CD24^{hi}CD27⁺ Breg cell frequency may contribute to the development of SSc.

Introduction

Systemic sclerosis (SSc) is an autoimmune disease characterised by fibrosis, microangiopathy and immune dysfunction. The exact pathophysiology of SSc is not well established, and mechanisms leading to dysimmunity remain to be established (1, 2). B lymphocytes are involved in the pathophysiology of SSc and several autoantibodies can be detected in the sera of SSc patients including anti-DNA topoisomerase I (anti-topo I) and anti-centromere antibodies (3). It has been previously reported that B cell homeostasis is disturbed in SSc patients, with increased naïve and reduced numbers of memory B lymphocytes (4, 5); as well as an increased level of the B cell activating factor (BAFF) cytokine in the blood of SSc patients (6, 7). In addition, B lymphocytes in patients with SSc secrete high levels of IL-6 and TGF- β and are able to promote skin fibrosis (8). The beneficial effect of B cell depletion with rituximab in SSc patients further supports the involvement of B cells in disease development and/or progression (9). The maintenance of immune tolerance and prevention of autoimmune induction is exerted by various regulatory B cell subpopulations which in humans, include CD24^{hi}CD38^{hi} transitional B cells and CD24^{hi}CD27⁺ B cells, the human equivalent of murine B10 cells (10, 11). Another subset of B lymphocytes, the CD21^{low} B cells (CD19⁺CD27⁻CD21^{low}) has been found to be expanded in several autoimmune diseases such as lupus erythematosus, Sjögren's syndrome and SSc, and was involved in the development of chronic graft-versus-host disease (5, 12-15). We recently reported that circulating T follicular helper (cTfh) cells were increased in SSc patients and they presented an activated phenotype with the

expression of BCL6 and HLA-DR and could also activate B cell plasmablasts to secrete immunoglobulins (5, 16). As human Breg cells could control Tfh development (17), we hypothesised that an imbalance in Breg cell subpopulations in patients could affect cTfh expansion and function. The aim of this study was to investigate frequencies of Breg cell subsets including CD21^{low} B cells, CD24^{hi}CD38^{hi} and CD24^{hi}CD27⁺ in the peripheral blood of SSc patients and to correlate them with cTfh expansion and clinical manifestations of the disease.

Patients and methods

Patients

In this prospective study we included 50 SSc patients and 32 healthy controls. All patients fulfilled the 2013 American College of Rheumatology (18) /European League Against Rheumatism (19) criteria for SSc (20). Patients with a concomitant infectious disease, active neoplasm or an associated autoimmune disease were excluded. SSc patients were free of any immunomodulatory treatment (steroids, hydroxychloroquine, DMARDs, biologics) at the time of the analysis since at least 3 months. All SSc patients enrolled in this study were followed at Saint-Antoine Hospital and had given written informed consent. This study was approved by the Ethic committee of Kremlin Bicêtre University, France (N° ID-RCB 2017-AO3380-53).

For each patient, the following clinical data were analysed: age, gender, disease duration (from the date of the first non-Raynaud's phenomenon), type of SSc (diffuse dSSc or limited ISSc), presence of active digital ulcers, presence of joint, heart, gastrointestinal and lung involvement, modified Rodnan skin score (mRSS), presence of pulmonary arterial hypertension (PAH). For pulmonary hypertension: confirmed by right heart catheterisation when mean pulmonary arterial pressure was found to be >20mmHg at rest. The lung involvement was defined as the presence of interstitial lung disease (ILD) on high resolution computed tomography (CT) scan. Laboratory data included C-reactive protein level, total lympho-

cyte count, plasma creatinine, urea, creatinine phosphokinase enzymes and antinuclear autoantibodies (anti-centromeres, anti-topoisomerase I, anti-PM-Scl, anti-RNA polymerase III autoantibodies). Healthy controls were obtained from "l'Etablissement Français du Sang" (median age 54 years [range, 23–64], 44% female).

Biological samples

Venous blood samples were collected in EDTA tubes (BD Biosciences, Le Pont de Claix, France). 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).

Flow cytometry analysis

For B lymphocyte analysis, PBMCs were stained with the following fluorochrome-conjugated antibodies: IgD FITC, CD45 BV510 (both from BD Biosciences), and CD19 PC7, CD27 PE, CD24 ECD, CD38 PECY5.5, CD21 Pacific Blue (all from Beckman Coulter, Villepinte, France). Cells were analysed on a Cytoflex flow cytometer (Beckman Coulter) using Kaluza 5.1 software (Beckman Coulter). Living lymphocytes were identified by the SSC/CD45 profiles.

For Tfh analysis, PBMCs were stained with the following fluorochrome-conjugated antibodies: CXCR5 (CD185) PE (eBioscience, ThermoFisher, Villebon, France); CD45RA ECD, PD-1 (CD279) PECY5.5, ICOS (CD278) APC, CD3 AA750 (Beckman Coulter); CD45 BV510, CD4 BV650, HLA-DR BV786 (BD Biosciences).

Statistical analysis

Data are expressed as means \pm SD, medians with ranges, and frequencies with percentages. Qualitative values were compared with the parametric chi-square test or Fisher's exact test according to distribution, and continuous quantitative variables with the Student's t-test or Mann-Whitney test. The Pearson test was used to determine the correlation between variables. The analyses were done using Graphpad

Prism 5.0 (GraphPad Software, San Diego, CA) and a $p < 0.05$ value was considered as significant.

Results

Increase of CD21^{low} B cells is balanced by decreased Breg CD24^{hi}CD38^{hi} and CD24^{hi}CD27⁺ subsets in patients with SSc

SSc patient characteristics are depicted in Supplementary Table S1. The median age was 61 years, 80% were female and 32% had dSSc.

The gating strategy to identify the various B cell subpopulations is shown in Figure 1A. Among B cell subsets, the frequencies and the absolute numbers of memory B cells (CD19⁺CD27⁺) (mean 27.4 \pm 15.0% vs. 34.2 \pm 9.9%; $p=0.008$) and (57.4 \pm 7.7 cells/ μ L vs. 68.9 \pm 8.1 cells/ μ L; $p=0.04$), respectively and of pre-germinal centre B cells (CD19⁺CD27⁺IgD⁺CD38⁺) (0.34 \pm 0.27% and 0.45 \pm 0.26%; $p=0.03$) were significantly decreased in SSc compared to healthy controls (Fig. 1B-D). The absolute numbers and frequencies of plasmablast B cells were similar in both groups (Fig. 1D).

When examining the CD21^{low} B cell subset, we observed that the frequency and absolute numbers were significantly increased in SSc patients compared to healthy controls: 5.3 \pm 4.3% versus 2.3 \pm 1.3% ($p < 0.001$) and 10.50 \pm 8.9 cells/ μ L versus 3.7 \pm 1.8 cells/ μ L ($p < 0.001$), respectively (Fig. 1E-F). By contrast, the frequency of Breg cells CD24^{hi}CD38^{hi} and CD24^{hi}CD27⁺ was significantly reduced in patients with SSc as compared to controls: 0.25 \pm 0.22% versus 0.34 \pm 0.23% ($p=0.02$); and 19.0 \pm 12.6% vs. 29.0 \pm 8.9% ($p < 0.001$), respectively (Fig. 1G-H).

Correlation of Breg cell levels with clinical severity

Among the 50 SSc patients, there were 16 with dSSc and 34 with ISSc. The median mRSS was significantly higher in patients with dSSc, as expected (data not shown). There was no difference in total B cells, CD21^{low}, CD24^{hi}CD38^{hi} and CD24^{hi}CD27⁺ Breg proportions in patients with dSSc and ISSc (data not shown).

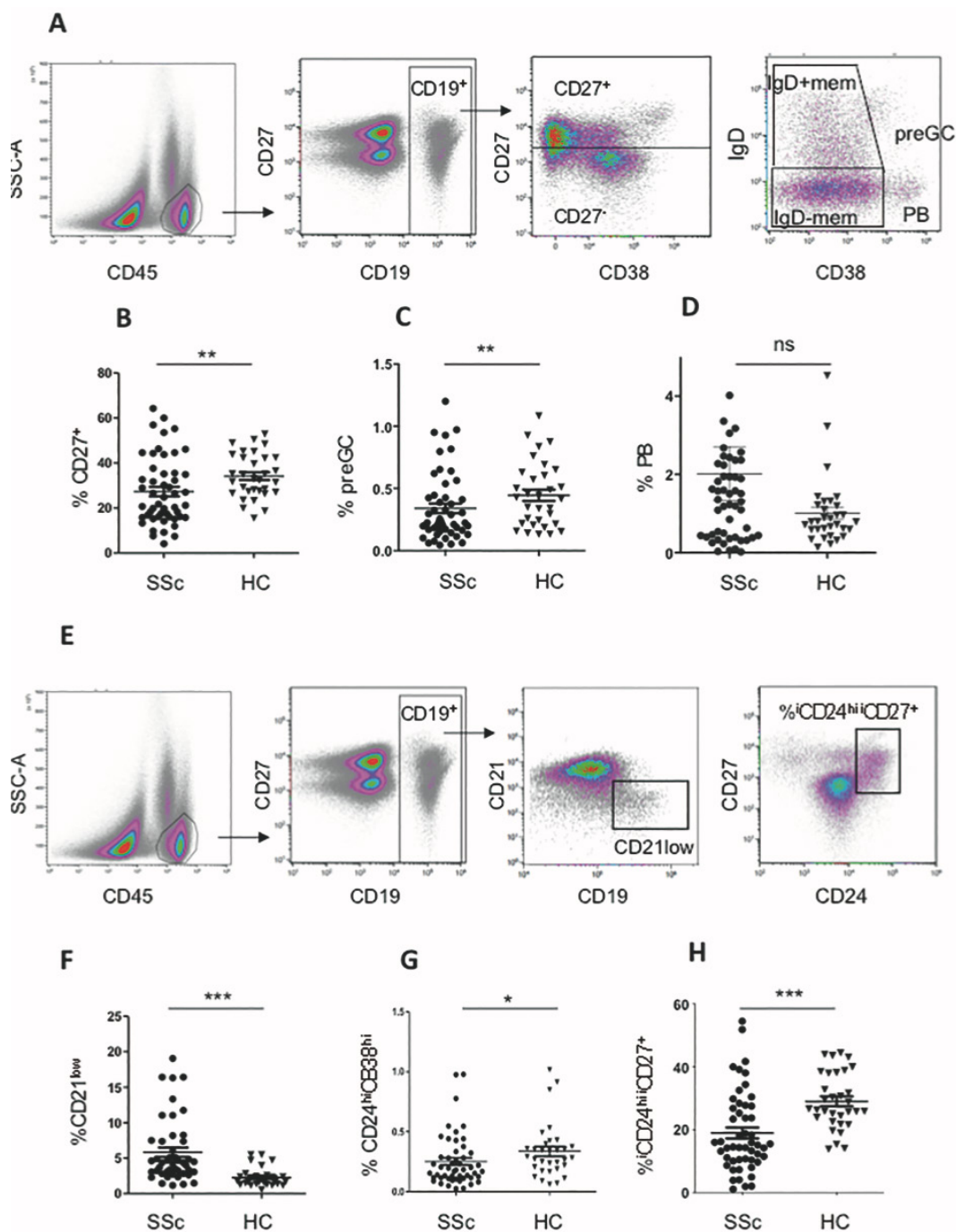


Fig. 1. B cell subpopulations frequencies in SSc patients.

(A) Gating strategy to identify the B lymphocyte subpopulations by flow cytometry; Frequencies of memory B CD27⁺ B cells; (B) pre-germinal centre B cells (preGC) and (C) plasmablast cells (PB) within total CD19⁺ B cells in SSc patients (n=50) and healthy controls (n=32); (E) Gating strategy to identify CD21^{low} and CD24^{hi}CD27⁺ B cells by flow cytometry; Frequencies of CD21^{low} B cells (F), CD27⁺CD24^{hi}CD38^{hi} (G) and CD24^{hi}CD27⁺ (H) Breg cells within CD19⁺ cells in SSc patients (n=50) and healthy controls (n=32).

Data expressed as means with standard deviation; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Mann-Whitney test.

We then analysed the frequencies of CD21^{low} B cells and CD24^{hi}CD38^{hi} and CD24^{hi}CD27⁺ subsets according to SSc severity. The CD24^{hi}CD27⁺ Breg cell frequency was significantly decreased in SSc patients with PAH ($8.6 \pm 4\%$ vs. $20.6 \pm 13\%$; $p = 0.014$), but not in patients with ILD ($15 \pm 10\%$ vs. $23 \pm 14\%$; $p = 0.058$) (Suppl. Fig. S1). Breg CD24^{hi}CD27⁺ frequency was not different in SSc patients with active

digital ulcers and did not correlate with the mRSS scale or BNP levels.

No difference was observed for CD24^{hi}CD38^{hi} and CD21^{low} B cell frequencies with respect to the presence of ILD, of active digital ulcers, of arterial hypertension, and no correlation was detected with mRSS or BNP levels (data not shown).

Furthermore SSc patients with high plasmablast cells (above 1%) had more

PAH than those with plasmablast cells above 1% (25% vs. 0% ; $p = 0.03$) (data not shown).

Decrease in Breg cells correlates with the expansion of cTfh in SSc patients

In a previous study analysing cTfh in SSc patients, we observed an expansion of these cells, notably in dSSc patients. As Breg cells could be involved in the regulation of Tfh, we evaluated

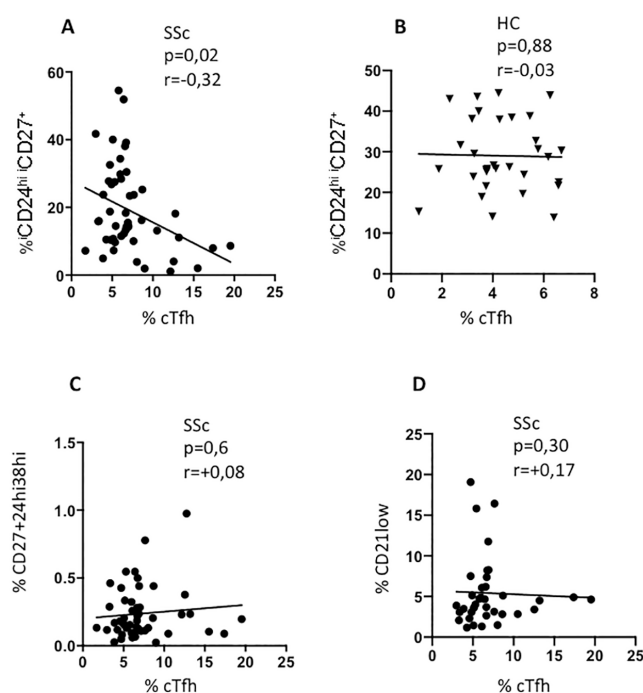


Fig. 2. Decrease of CD24^{hi}CD27⁺ inversely correlates with cTfh in SSc patients.

Inverse correlation between CD24^{hi}CD27⁺ B cells and CD4⁺CXCR5⁺PD1⁺cTfh in SSc patients (A) or healthy controls (B). No correlation between CD27⁺CD24^{hi}CD38^{hi} B cells and cTfh (C) or CD21^{low} B cells and cTfh (D).

A-D, Spearman test.

the correlation of cTfh frequency with Breg cell subsets. Interestingly, we observed a negative correlation between cTfh and CD24^{hi}CD27⁺ Breg cells in SSc patients but not in healthy controls (Fig. 2A-B). We did not observe any correlation between cTfh and CD24^{hi}CD38^{hi} B cells or between cTfh and CD21^{low} B cells (Fig. 2C-D).

Discussion

In this study, we report the decrease of Breg cell subpopulations CD24^{hi}CD38^{hi} and CD24^{hi}CD27⁺ in patients with SSc in comparison to healthy controls, with a more pronounced decrease of Breg lymphocytes in SSc with PAH impairments. In contrast, the CD21^{low} B cells were increased in SSc patients as compared to healthy controls. These CD21^{low} B cells have already been described in different autoimmune diseases such as rheumatoid arthritis (21), Sjögren's syndrome (12) and systemic lupus erythematosus (13, 22). This particular B cell population is predominantly composed of memory B cells (23) and expresses high levels of activation markers, inhibitory receptors and a peculiar pattern of homing receptors. These cells are usually considered as having features of anergic and exhausted cells, as characterised by increased apoptosis and decreased

proliferation after stimulation (12). These CD21^{low} B cells highly express autoreactive antibodies and thus can be enriched by autoreactive B cell clones that may have been selected by self-antigens.

In our study, we did not detect differences in CD21^{low} B cells according to clinical severity as previously reported (5). Marrapodi *et al.* reported that CD21^{low} B cells in SSc were increased in comparison to healthy controls with a higher prevalence of PAH in those with more than 10% of CD21^{low} B cells (15, 24).

Regulatory B cells is a relatively newly recognised subset of B cells which have an immunoregulatory role by suppressing excessive inflammatory responses through the inhibition of T CD4⁺ Th1 and Th17 cell proliferation and the capacity to express inflammatory cytokines and the induction of regulatory T lymphocytes (Tregs) (25). Several studies have demonstrated decreased Breg cells in various autoimmune diseases, arguing for the potential involvement of these B cell subsets in the regulation of autoimmune diseases. Data about Breg cell impairment in SSc are still scarce but show decreased frequencies of several Breg cell subpopulations in various subtypes of SSc. Thus, Breg cell numbers could be lower in patients with severe and ex-

tensive SSc, as found in dSSc, in patients with PAH and ILD impairments (26, 27). Few studies have analysed the functional impairment of regulatory B cells in SSc and have mainly showed decreased numbers of IL-10 positive B cells (26, 28). The TIM-1⁺ IL-10 expressing B cells are reduced in SSc patients and have reduced ability to suppress CD4⁺ T cell production of inflammatory cytokines (28). The results reported by Matsushita *et al.* showing that Breg cell levels correlated negatively with the titre of anti-topo I antibody (Ab) and anti-centromere Ab in SSc patients and are in line with our observations of a negative correlation between CD24^{hi}CD27⁺ Breg cells and cTfh (26). We did not detect a correlation between the CD24^{hi}CD38⁺ Breg cell subset and cTfh, suggesting that these two subsets display different functional characteristics as previously reported (29). It remains to be investigated whether these mechanisms are involved in the direct regulation of Tfh, which are likely IL-10 independent because both of these two subsets express IL-10 (29).

It is interesting to highlight that BAFF inhibition therapies have been found to promote Breg cell numbers, by selectively depleting B effector cells producing IL-6 while sparing Breg cells (24). In addition, immunosuppressive therapies such as autologous haematopoietic stem-cell transplantation increased regulatory T and CD24^{hi}CD38^{hi} B cells at 6- and 12 months at higher levels in responders than in non-responders (30). Moreover, BAFF has been shown to regulate Tfh cells and promote their accumulation (31). Thus, a selective B cell depletion sparing Breg cells in SSc could be a potent therapeutic strategy (24).

Taken together, increased numbers of CD21^{low} B cells in the blood of SSc patients instead of being eliminated, and impaired regulatory B cell function could be part of a favorable environment to break tolerance leading to the development and/or extension of SSc. Further studies are necessary to determine the functional profile of Breg cell subsets and their ability to regulate cTfh and disease severity.

Conclusions

These results suggest that Breg cell subsets may participate in the regulation of cTfh and disease severity. Decreased CD24^{hi}CD27⁺ Breg cell frequency may contribute to the development of SSc.

Take home messages

- The Breg cell subpopulations CD24^{hi}CD38^{hi} and CD24^{hi}CD27⁺ are decreased whereas the CD21^{low} B cells were increased in SSc patients.
- Regulatory B cells were negatively correlated with cTFH in systemic sclerosis.
- The regulatory B cells decrease is more pronounced in SSc with PAH.
- B reg cells seem to participate to cTFH expansion and to the disease severity in systemic sclerosis.

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