Association of Defective Regulation of Autoreactive Interleukin-6–Producing Transitional B Lymphocytes With Disease in Patients With Systemic Sclerosis

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Objective. Systemic sclerosis (SSc) has the highest case-specific mortality of any rheumatic disease, and no effective therapy is available. A clear manifestation of SSc is the presence of autoantibodies. However, the origin of autoantibody-producing B lymphocytes, their mechanisms of activation and autoantibody production, and their role remain unclear. This study was undertaken to identify mechanisms that contribute to pathogenic B cell generation and involvement in SSc and to assess the altered distribution and function of B cells in SSc patients.

Methods. Multicolor flow cytometry was performed to determine B cell subset distribution, cytokine production, and tolerance induction in SSc patients and healthy controls. Cytokine production following stimulation of the cells ex vivo was determined by multiplex assay.

Results. A range of defects in B lymphocyte tolerance and cytokine production in SSc were noted. There was evidence of altered distribution of transitional B cell subsets, increased production of interleukin-6 (IL-6) and IL-8, and defective tolerance induction in SSc B cells. In addition, B cells from SSc patients had a reduced ability to produce IL-10 when stimulated through innate immune pathways. In contrast to healthy individuals, tolerance checkpoints in SSc patients failed to suppress the emergence of B cells that produce autoantibodies with specificity to the Scl-70 antigen, which is strongly associated with SSc. These defects were paralleled by altered intracellular signaling and apoptosis following B cell receptor engagement.

Conclusion. Our findings provide new insights into mechanisms underlying defective B lymphocyte responses in patients with SSc and their contribution to disease.

Systemic sclerosis (SSc) is a severe inflammatory disease characterized by excessive extracellular matrix deposition in the skin and visceral organs (1). It has a complex pathogenesis with 2 major hallmarks: autoimmunity and inflammation leading to widespread damage to blood vessels and progressive interstitial and perivascular fibrosis (2).

A key feature of autoimmunity in SSc is the presence of high levels of autoantibodies to nuclear proteins, including topoisomerase I enzyme, centromere, RNA polymerase, endothelial cells, and platelet-derived growth factor receptor (1,3). Evidence of the pathogenic roles of these autoantibodies comes from studies showing the association between their specificity and which tissues and organs are involved. Importantly, these autoantibodies appear prior to disease onset. Furthermore, B lymphocytes accumulate at sites of disease, such as around small vessels in the skin (4) and in the alveolar interstitium in patients with lung involvement (5). In addition to producing autoantibodies, B lymphocytes contribute to fibrosis by producing interleukin-6 (IL-6) (6).

The notion of a role played by B lymphocytes in SSc is supported by studies of disease pathogenesis in

mouse models (7–9) and the success of treating patients with rituximab, a chimeric monoclonal antibody (mAb) against human CD20 (10,11). In the tight skin mouse model of SSc, which is associated with constitutive CD19-mediated signaling (7,12), inhibition of CD19 expression abrogates autoantibody production and IL-6 production by B cells and ameliorates skin fibrosis (7,8). Similar effects were noted when CD19 expression was suppressed in the mouse model of bleomycin-induced SSc (9). Furthermore, in a previous study, patients with diffuse cutaneous SSc (dcSSc) with interstitial lung disease who were positive for anti–Scl-70 autoantibodies were shown to benefit significantly from treatment with rituximab (10). In that study, no patient receiving rituximab exhibited lung function deterioration, whereas 5 of 6 placebo-treated patients experienced worsening disease (10). Additionally, skin thickening, collagen deposition in the skin, and health quality improved in rituximab-treated patients but not in placebo-treated patients (10–14). Furthermore, rituximab reduced the level of plasma IL-6, decreased the activity index, depleted skin B lymphocytes, and reduced dermal myofibroblasts and hyalinized collagen in the skin (15).

The cause of defective B cell responses in SSc remains unclear. However, defective tolerance and deregulation of autoreactive B cell responses are potential causes of B cell–mediated pathology. High-affinity self-reactive B cells are normally deleted in the bone marrow, but some that recognize self with low affinity or those that do not encounter self antigens in the bone marrow escape censure and migrate to the periphery as transitional B cells. Migrant transitional B cells go through a series of tolerance checkpoints and maturational phases to become mature B cells. This pathway was initially described in mice as immature B cells transiting to the spleen to mature into B cell receptor (BCR) cells that are responsive to antigen engagement (16). Murine transitional B cells are distinguishable from mature cells based on the expression level of CD23, CD21, and the developmental marker CD24 (17). In humans, recent studies from a number of laboratories including ours have revealed that transitional human B cells (CD24^{high}CD38^{high}) do not constitute a single population but can be divided into 4 subsets (18). These subsets have subtle differences in maturational and tolerance status and capacity to produce IL-10 and IL-6 (18,19).

The present study was carried out to explore if defects in the maturation of, or tolerance induction in, B cells at the transitional stage are related to the emergence of high-affinity pathogenic B cells in SSc patients. Transitional B cells were studied for their ability to produce IL-6 since its production is a key mechanism by which B cells could promote fibrosis (6–8). In addition, the potential of transitional B cells in SSc to produce IL-10 was studied to assess their ability to be involved in immune regulation (19,20). We used multicolor flow cytometry and a secretome profiling approach to characterize the distribution and functional characteristics of B cells in SSc to identify potential pathways through which pathogenic B cells could emerge and contribute to pathogenesis.

PATIENTS AND METHODS

Patients. All patients fulfilled the 2013 American College of Rheumatology/European League Against Rheumatism criteria for SSc (21). Patients were classified as having limited cutaneous SSc (lcSSc) when skin thickening was present distal to elbows and knees and as having dcSSc when skin thickening affected both distal and proximal areas. Blood samples were obtained from 88 patients with SSc attending the clinics after obtaining their informed consent. Interstitial lung disease was diagnosed based on characteristic changes visualized by high-resolution computed tomography. Patients were diagnosed as having pulmonary arterial hypertension if they had mean pulmonary artery pressure of ≥25 mm Hg and normal pulmonary capillary wedge pressure on right-sided heart catheterization. Scleroderma renal crisis (SRC) was defined as new-onset systemic hypertension (>150/85 mm Hg) and a documented decrease in estimated glomerular filtration rate of ≥30% or confirmed features on renal biopsy (22). Blood samples from 17 healthy controls (mean ± SD age 40.2 ± 11.3 years [range 25–60 years]) were included for comparison. Some of the samples from both patients and controls were studied on multiple occasions. The study was approved by the London-Hampstead National Research Ethics Service Committee (REC reference 6398) and was conducted in accordance with the 2013 Declaration of Helsinki.

B lymphocyte enrichment. B lymphocytes were enriched from whole blood by negative selection using the EasySep human B cell enrichment kit (StemCell Technologies). All experiments presented in this study were carried out using freshly enriched B lymphocytes from blood samples obtained on the same day. The protocol of B cell enrichment involves using a cocktail of mAb with dual specificity for non–B cell components of blood mononuclear cells and red blood cells. The cocktail aggregates all non–B cells with red blood cells, and these are then separated on Ficoll-Paque. The protocol is fast, and the purity of enriched B cells is >95%.

Cell staining and flow cytometry. Enriched B cells were stained with combinations of fluorochrome-conjugated antibodies (18). Monoclonal antibodies were purchased from the following suppliers. Allophycocyanin (APC)–eFluor 780–conjugated anti-human CD10 (clone SN5c), phycoerythrin (PE)–Cy5.5–conjugated anti-human CD19 (clone HIB19), PE–Cy7–conjugated anti-human CD32 (clone 6C4), fluorescein isothiocyanate (FITC)–conjugated anti-human IgD (clone IA6-2), eFluor 450–conjugated anti-human CD21 (clone HB5), and PE-conjugated anti-human CD24 (clone eBioSN3) mAb were all from eBioscience. Brilliant Violet 605–conjugated anti-human IgM (clone MHM-88), Brilliant Violet 785–conjugated anti-human CD19 (clone HIB19), and Brilliant Violet 711–conjugated anti-human CD27 (clone O323) were from

BioLegend. PerCP–Cy5.5–conjugated anti-CD38 mAb (clone HIT2) was from BD Biosciences. To determine intracellular cytokine levels, cells were fixed, permeabilized, and stained with either APC-conjugated anti-human IL-6 mAb (clone MQ2-13A5) or APC-conjugated anti-human IL-10 mAb (clone JES3-9D7) (both from BD Biosciences) and assessed using an LSRFortessa fluorescence-activated cell sorter (FACS) and FACSDiva software.

For measuring the frequency of IL-6– or IL-10– producing B lymphocytes, enriched cells were stimulated with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (250 ng/ml) in the presence of GolgiPlug for 6 hours at 37°C (Bio-Rad) and then stained with different combinations of fluorochrome-conjugated mAb and assessed using an LSRFortessa analyzer. For measuring IL-10 production in the supernatant of ex vivo stimulated B cells, enriched cells were cultured with mitomycin C–treated mouse L fibroblasts transfected with complementary DNA for human CD40L. In some experiments, the cells were stimulated with the soluble Toll-like receptor 9 (TLR-9) agonist CpG-containing oligonucleotide (CpG ODN) either alone or in the presence of 10 μg/ml of mouse mAb to human CD40 (clone G28-5) precoated onto culture plates. The cells were cultured for 72 hours at 37°C. For coculture experiments involving the CD40L-transfected L cell fibroblasts, these were first incubated with 10 μg/ml mitomycin C for 2 hours, and then enriched B cells were added at a ratio of 1:5 mouse L fibroblasts:B cells. Culture supernatants were collected, centrifuged, and tested for cytokine levels using Meso Scale Discovery multiplex kits as previously described (23).

Apoptosis, either spontaneous or following engaging the BCR with 25 μg/ml goat $F(ab')_2$ anti-human IgM (Jackson ImmunoResearch) for 8 hours at 37°C, was detected by staining the cells with FITC-conjugated annexin V (BioLegend).

For measuring phospho–STAT-3 and phospho–NF-κB p65, enriched B cells were rested at 37°C for 3 hours and then stimulated with either anti-CD40 mAb (10 μg/ml), CpG ODN (1 μM), or the combination of both for 10 minutes. The cells were fixed, permeabilized, and stained with either Alexa 488–conjugated anti–phospho–STAT-3 mAb (clone D3A7) or Alexa 488–conjugated anti–phospho–NF-κB p65 mAb (clone 93H1) (Cell Signaling Technology) and then with different combinations of conjugated mAb for surface proteins. The results were analyzed using LSRFortessa.

Detection and quantification of autoantibody-producing B cells. Enzyme-linked immunospot (ELISpot) assay kits were obtained from Mabtech. The assay was performed according to the manufacturer's instructions and used to detect the frequency of anti–Scl-70 autoantibody–secreting B cells in FACS-sorted B cell subsets from patients with SSc. Membranes were coated overnight at 4°C with 10 μg/ml of Scl-70, and FACS-sorted B cell subsets were left to settle on the coated membranes for 20 hours at 37°C. The membranes were washed, and biotinylated antibody specific for human IgM was added followed by streptavidin–horseradish peroxidase (HRP) conjugate. Spots identified were for IgM antibodies specific for Scl-70 antigen secreted by B cells revealed with the HRP substrate 3,3′,5,5′-tetramethylbenzidine. The number of spots on each membrane was counted and analyzed using an automated AID ELISpot Reader System.

Statistical analysis. All data are presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism version 6.05. Student's *t*-test was used for comparing patients and healthy controls. *P* values less than 0.05 were considered significant.

RESULTS

Patient cohort. The SSc patient cohort included 88 patients; 74 (84.1%) were women. Forty patients (45.5%) had dcSSc, and 48 (54.5%) had lcSSc. The dcSSc group had a mean ± SD age of 49 ± 11.9 years (range 25–75 years) and disease duration of 7.7 ± 7 years (range 1–31 years). The lcSSc group had a mean ± SD age of 61 ± 10.9 years (range 31–83 years) and disease duration of 13.6 ± 9.4 years (range 2–49 years). Twenty-four patients (27.3%) had anticentromere antibodies, and 15 (17.0%) had anti–RNA polymerase antibodies. Six patients (6.8%) had anti–U3 RNP autoantibodies, 5 (5.7%) had anti–U1 RNP autoantibodies, 10 (11.4%) had unidentified antinuclear autoantibodies (ANAs), 4 (4.5%) were ANA negative, 1 (1.1%) had anti–Jo-1 autoantibodies, and 2 (2.3%) had anti-PM/Scl autoantibodies. Twenty-three patients (26.1%) had interstitial lung disease, and the majority were seropositive for anti–Scl-70 autoantibodies (25%; n = 22). Two patients (2.3%) had SRC, and 4 (4.5%) had pulmonary arterial hypertension. Forty-seven of the patients (53.4%) were treated with immunosuppressive agents, including mycophenolate mofetil (n = 30) and methotrexate (n = 7) (24). A majority of the patients who were not receiving immunosuppressive agents had lcSSc (n = 33).

Altered distribution of B cell subsets in patients with SSc. To explore the role of B cells in SSc pathogenesis and mechanisms that could explain the expansion of autoreactive B cells, we first determined the total numbers of B cells and distribution of subsets in the patients and healthy controls. In general, patients with SSc had higher numbers of B cells than healthy controls (mean ± SEM 153.7 ± 32.9 versus 99.2 ± 20.3 cells/μl blood, respectively; *P* < 0.01). When B cell subsets were categorized based on CD24 and CD38 expression, the SSc patients had significantly more transitional B cells (CD24^high^CD38^high^) (mean ± SEM 19.85 ± 1.87 cells/μl blood corresponding to 13.6 ± 0.93% of total B cells) than the healthy controls (mean ± SEM 7.045 ± 1.09 cells/μl blood corresponding to 6.81 ± 0.62% of total B cells; *P* < 0.001) (Figure 1A). However, the percentages of mature naive (CD24+CD38+) B cells and memory (CD24^high^CD38+) B cells were lower in the patients (mean ± SEM 85.57 ± 6.21 cells/μl blood and 21.66 ± 2.15 cells/μl blood corresponding to 58.7 ± 2.4% and 14.81 ± 1.18% of total B cells, respectively) than the healthy controls (68 ± 4.85 cells/μl blood and
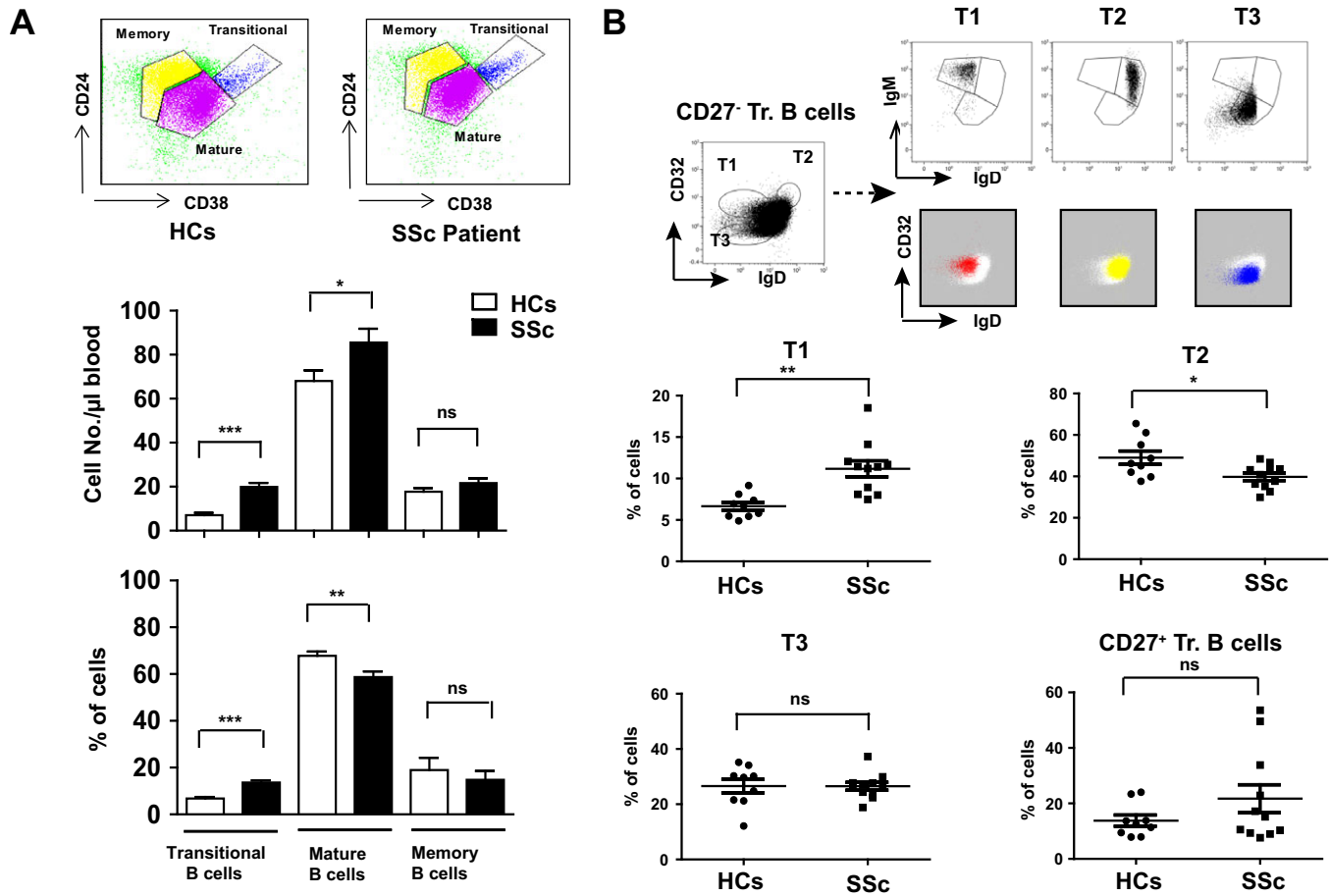
Figure 1. Distribution of B cell subsets in healthy controls (HCs) and patients with systemic sclerosis (SSc). **A**, Top, Gating strategy for identifying transitional (Tr), mature, and memory B lymphocytes in peripheral blood based on expression levels of CD24 and CD38. Middle, Numbers of transitional, naive/mature, and memory B cells per microliter of blood in healthy controls and patients with SSc. Bottom, Frequency of transitional, naive/mature, and memory B cells in the blood of healthy controls and SSc patients. Bars show the mean ± SEM (n = 10 healthy controls and 10 SSc patients). **B**, Top, Staining and gating strategies used for identifying CD27+ and transitional T1, T2, and T3 subsets of CD24^{high}CD38^{high} B cells. Middle and bottom, Frequencies of T1, T2, T3, and CD27+ transitional B cell subsets. Symbols represent individual samples; horizontal lines and error bars show the mean ± SEM (n = 9 healthy controls and 11 SSc patients). * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, by Student's *t*-test. NS = not significant.

17.72 ± 1.66 cells/μl blood corresponding to 67.76 ± 1.87% and 18.98 ± 1.66% of total B cells, respectively) ($P < 0.01$ for mature B cells and $P = 0.056$ for memory B cells). The increase in transitional B cells in SSc patients is consistent with other autoimmune diseases, such as systemic lupus erythematosus and Sjögren's syndrome (18).

We next carried out experiments to determine numbers, responses, and regulation of transitional B cell subsets to determine why they are increased in SSc patients. Transitional B cells go through a series of developmental stages and tolerance checkpoints and, therefore, identifying defects in any of these stages could provide insights into specific defects in regulation and maturation. We recently categorized transitional CD24^{high}CD38^{high} B cells into 4 subsets using 10-color

flow cytometry and the FLOCK (Flow clustering without K) algorithm (18). The 4 subsets are distinguishable based on the expression of CD27, CD10, CD21, IgM, IgD, and CD32 within CD24^{high}CD38^{high} B cells. Transitional B cells in subset 1 (T1) express high levels of IgM, CD10, and CD32 and low levels of IgD and CD21. Cells in subset 2 (T2) express medium levels of IgM, IgD, CD10, and CD32 and high levels of CD21. Cells in subset 3 (T3) express low levels of IgM, IgD, CD10, CD21, and CD32 (Figure 1B). Finally, one subset of transitional B cells expresses high levels of CD27, CD24, and CD38, and was designated CD27+ transitional B cells (18).

In the present study, SSc patients had significantly more T1 B cells than healthy controls (mean ±
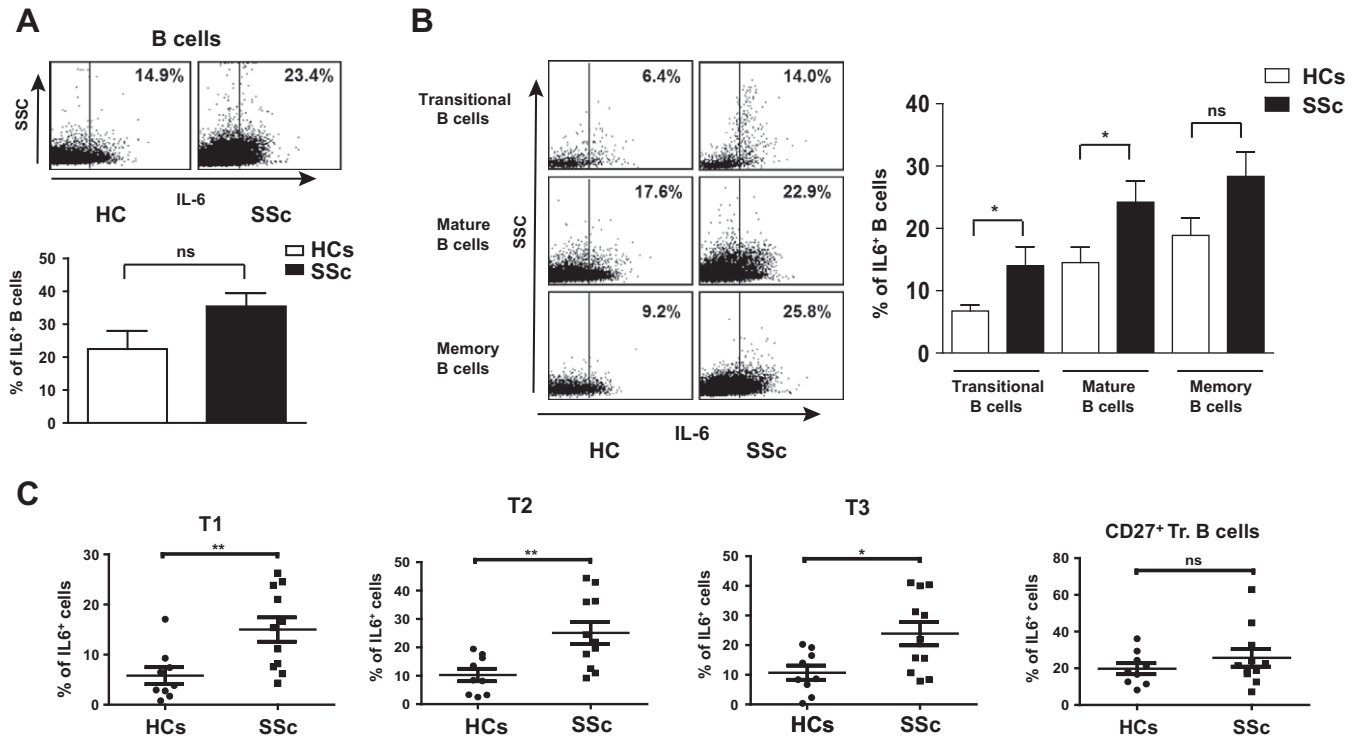
Figure 2. Interleukin-6 (IL-6) expression in B cells and B cell subsets in healthy controls (HCs) and patients with systemic sclerosis (SSc). **A**, Top, Fluorescence-activated cell sorting (FACS) quadrants from a healthy control and an SSc patient, showing the gating strategy for identifying IL-6–positive ex vivo B cells stimulated with phorbol myristate acetate and ionomycin. Bottom, Frequency of IL-6–positive ex vivo B cells in the blood of healthy controls and patients with SSc. Bars show the mean ± SEM (n = 10 healthy controls and 11 SSc patients). **B**, Left, FACS quadrant profiles of B cells from a healthy control and a patient with SSc, depicting the strategy for quantifying IL-6–positive transitional (Tr), naive/mature, and memory B cell subsets. Right, Frequency of IL-6–positive transitional, naive/mature, and memory B cells. Bars show the mean ± SEM (n = 4 healthy controls and 10 SSc patients). **C**, Percentage of IL-6–positive T1, T2, T3, and CD27+ transitional B cells. Symbols represent individual samples; horizontal lines and error bars show the mean ± SEM (n = 9 healthy controls and 11 SSc patients). * = $P < 0.05$; ** = $P < 0.01$, by Student's t-test. NS = not significant.

SEM 1.32 ± 0.21 versus 0.33 ± 0.06 cells/μl blood corresponding to $11.2 \pm 1.0\%$ versus $6.7 \pm 0.5\%$ of transitional B cells; $P < 0.01$) (Figure 1B). In contrast, there was an increase in the total number but a decrease in the frequency of T2 cells in patients (mean ± SEM 4.87 ± 0.78 versus 2.52 ± 0.57 cells/μl blood corresponding to $39.8 \pm 1.8\%$ versus $49.0 \pm 3.2\%$ of transitional B cells; $P < 0.05$). There was no difference in the frequency of T3 cells. There was, however, a trend toward an increased number and frequency of CD27+ transitional B cells in the patients but this was not statistically significant (mean ± SEM 1.82 ± 0.42 versus 0.70 ± 0.16 cells/μl blood corresponding to $21.7 \pm 5.0\%$ versus $13.8 \pm 2.0\%$ of transitional B cells; $P = 0.054$).

Increased IL-6–producing transitional B cells in patients with SSc. To assess whether the response of B cells in patients with SSc is different from that in healthy controls, we studied cytokine production by the cells. Enriched B cells were activated with PMA and ionomycin in the presence of GolgiPlug and stained for intracellular IL-6. FACS analyses showed that the number of IL-6–positive B cells was higher in patients compared with healthy controls (mean ± SEM 51.8 ± 7 versus 22.8 ± 6.2 cells/μl blood corresponding to $35.4 \pm 4\%$ versus $22.5 \pm 5.5\%$ of total B cells) (Figure 2A). The numbers of IL-6–positive transitional, mature naive, and memory B cells were also higher in the patients (Figure 2B). The mean ± SEM numbers and frequencies of IL-6–positive transitional B cells were 2.8 ± 0.7 cells/μl blood corresponding to $14.0 \pm 3.1\%$ of transitional B cells in the patients compared with 0.5 ± 0.13 cells/μl blood corresponding to $6.7 \pm 1.0\%$ of transitional B cells in the healthy controls ($P < 0.01$ and $P < 0.05$, respectively). The mean ± SEM frequencies of IL-6–positive T1, T2, and T3 B cells in the SSc patients were $15.0 \pm 2.4\%$, $25.1 \pm 3.9\%$, and $23.9 \pm 4.0\%$, respectively. These were significantly higher than

the frequencies in the healthy controls (5.8 ± 1.7% of T1 B cells, 10.3 ± 2.2% of T2 B cells [both $P < 0.01$ versus SSc patients], and 10.7 ± 2.4% of T3 B cells [$P < 0.05$ versus SSc patients]) (Figure 2C).

The SSc B cell profiles and frequencies revealed associations with disease severity and symptoms. For example, SSc patients with severe lung fibrosis had more IL-6–positive B cells in all subsets compared with patients with mild disease. Similarly, patients with dcSSc had more IL-6–positive B cells than patients with lcSSC (data available upon request from the corresponding author). Furthermore, serum from anti–Scl-70–positive patients had more IL-6–positive B cells than patients who were negative for this autoantibody. However, these differences were not statistically significant.

Altered IL-10 production by B cells from SSc patients in response to stimulation. Transitional B cells are normally purged from most self-reactive cells by passing through a series of tolerance checkpoints after migrating from the bone marrow. Some self-reactive transitional B cells, however, survive this censorship and mature as polyreactive B cells. These cells are believed to participate in innate immunity as a first line of defense against infections and become polyreactive IgM-producing B cells or mature into follicular/marginal zone B cells. These "natural" polyreactive B cells are, therefore, proposed to be important for immunity, and some have been shown to develop into Breg cells that produce IL-10 (25). However, some transitional B cells can also become high-affinity autoreactive B cells and contribute to pathology in patients with autoimmune diseases (20).

To determine whether the increase in the number of IL-6–positive B cells reflects a reduction in IL-10 production in SSc, we determined the frequency of IL-10–positive B cells. Patients with SSc had more ex vivo–activated IL-10–positive B cells than healthy controls (results available upon request from the corresponding author). Analyses of IL-10–positive B cell subsets showed more IL-10–positive transitional, mature, and memory B cells in the patients compared with the healthy controls, although the differences were not statistically significant (results available upon request from
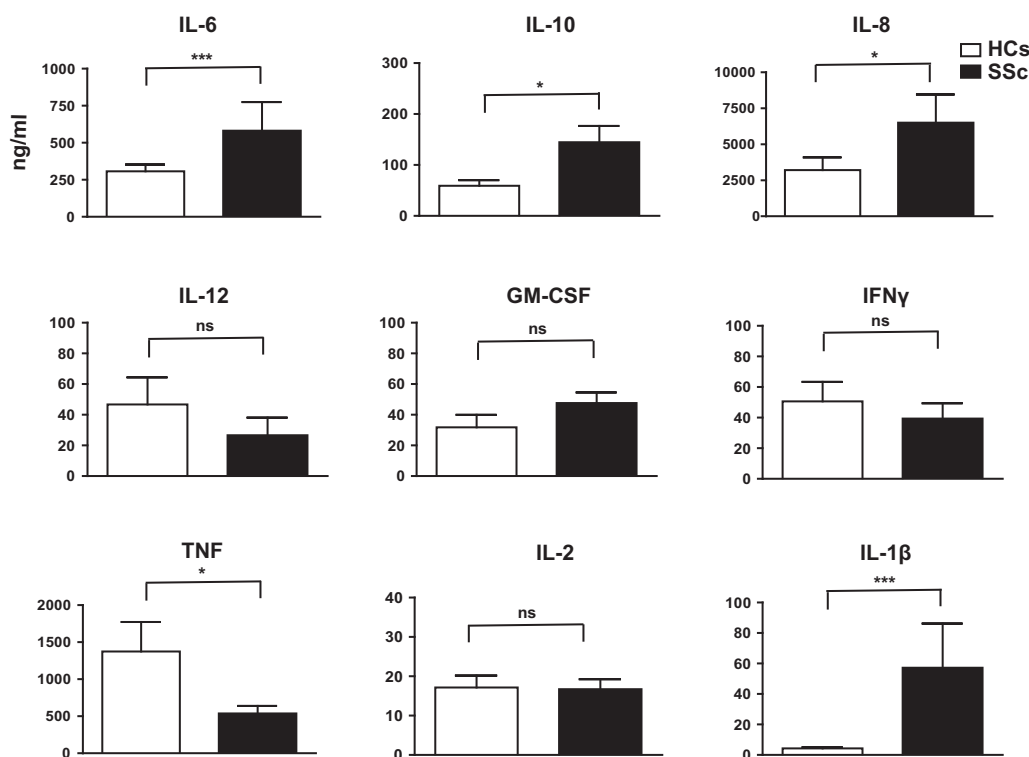

Figure 3. Cytokine production by B lymphocytes from healthy controls (HCs) and patients with systemic sclerosis (SSc). Enriched B lymphocytes were cultured for 72 hours in plates precoated with mitomycin C–treated mouse L fibroblasts transfected with cDNA for human CD40L. Culture supernatants were collected, centrifuged, and tested for the indicated cytokines using MSD Multiplex kits. Bars show the mean ± SEM (n = 5 healthy controls and 10 SSc patients). * = $P < 0.05$; *** = $P < 0.001$, by Student's t-test. IL-6 = interleukin-6; GM-CSF = granulocyte–macrophage colony-stimulating factor; IFNγ = interferon-γ; TNF = tumor necrosis factor; NS = not significant.

the corresponding author). There was a trend toward increased IL-10–positive T1 and T3 B cells in the patients compared with the healthy controls, but the differences were not significant (results available upon request from the corresponding author).

We next assessed whether the functional capacity of B cells to produce IL-10 could be impaired, as has been reported in patients with SLE (25). For this purpose, enriched B cells were stimulated with either CD40 alone, TLR-9 alone (with its agonist CpG ODN), or through both pathways simultaneously, and the level of IL-10 in culture supernatants was determined. When B cells were stimulated through CD40 alone, B cells from the SSc patients produced more IL-10 (mean $\pm$ SEM 144.7 $\pm$ 31.8 pg/ml) than those from the healthy controls (59.1 $\pm$ 17.1 pg/ml) ($P <$ 0.05) (results available upon request from the corresponding author). However, when B cells were stimulated by engaging TLR-9 alone, or through both TLR-9 and CD40, B cells from the patients produced significantly less IL-10 (mean $\pm$ SEM 82.0 $\pm$ 36.0 pg/ml and 44.2 $\pm$ 18.0 pg/ml, respectively) than B cells from the healthy controls (mean $\pm$ SEM 190.3 $\pm$ 35.7 pg/ml and 163.4 $\pm$ 37.9 pg/ml, respectively) ($P <$ 0.05).

To determine if these results reflect further functional differences between B cells from patients with SSc and healthy controls, we studied the ability of B cells to produce cytokines thought to be relevant to SSc pathogenesis. B cells were cocultured for 72 hours with mouse L fibroblasts transfected with human CD40L, and the level of cytokines in the culture supernatants was measured. B cells from SSc patients produced significantly more IL-1β and IL-8 ($P <$ 0.001 and $P <$ 0.05, respectively) and more granulocyte–macrophage colony-stimulating factor (GM-CSF) than B cells from healthy controls. In contrast, B cells from the patients produced less IL-12, interferon-γ, and tumor necrosis factor than B cells from the healthy controls (Figure 3).

Altered responsiveness of transitional B cells in SSc to BCR engagement. Our previous studies of transitional B cell responses to BCR engagement, including apoptosis, revealed subtle differences between the subsets (18). For example, there was a steady increase in the level of Bcl-2 expression in T1 cells through to CD27+ transitional B cells. Analysis of spontaneous cell death (apoptosis/necrosis), however, showed no significant differences between the subsets. With BCR engagement, there was clear evidence of a higher rate of apoptosis in the T1 subset compared with the other 3 subsets and with mature B cells. On the basis of these findings, we proposed a pathway for an evolving

maturation and response to BCR engagement in transitional B cells (18). In the present study, there was a notable reduction in BCR-mediated apoptosis in all of the transitional B cell subsets in the SSc patients, particularly in the T1 subset (mean $\pm$ SEM 13.6 $\pm$ 4.1%) compared with the healthy controls (47.1 $\pm$ 12.9%) ($P <$ 0.05) (Figure 4A). This reduced ability of transitional B cells to undergo BCR-mediated apoptosis was consistent with the higher number of IL-6–producing B cells in the SSc patients.

Our previous studies also showed that differences between transitional and mature B cells in response to stimulation are associated with altered Ca^{2+}, phosphatidylinositol 3-kinase, and phospholipase Cγ2 signaling (18). Therefore, we examined signaling pathways that could be related to altered IL-6 and IL-10 production by transitional B cells in the SSc patients following TLR-9 engagement (26). For this purpose, we examined the phosphorylation of NF-κB and STAT-3 in transitional B cells. The phosphorylation of NF-κB p65 and STAT-3 are key in CD40-mediated as well as TLR-9–mediated cytokine production. In addition, NF-κB1 represses transcription of the IL-6 gene since its loss leads to uncontrolled RelA-driven transcription of IL-6 (27). No consistent significant differences were seen in the level of NF-κB p65 phosphorylation of unstimulated B cells from the patients and healthy controls (Figure 4B). However, when B cells were stimulated through TLR-9, there was a significant reduction in the phosphorylation of STAT-3 in T1 and CD27+ transitional B cells from the SSc patients (mean $\pm$ SEM 121.8 $\pm$ 10.4 and 177.0 $\pm$ 22.7 mean fluorescence intensity [MFI], respectively) compared with the healthy controls (155.6 $\pm$ 5.6 and 251.8 $\pm$ 10.6 MFI, respectively) ($P <$ 0.05 for both) (Figure 4C).

Anti–Scl-70 autoantibody–producing transitional B cells survive the T1–T2 tolerance checkpoint at high frequency in seropositive SSc patients. The available evidence indicates that the BCR repertoire differs between transitional and mature B cells (28,29). Thus, a major fraction of transitional B cells is polyreactive and suggested to give rise to natural antibody–producing mature B cells (30). For the purpose of the present study, we examined differences in the frequency of B cells capable of producing IgM antibodies with specificity for Scl-70, which is associated with SSc (31,32). To achieve this objective, the transitional B cell subsets were subjected to FACS and incubated for 4 days with CpG (to mimic T cell–independent stimulation), and the levels of autoantibody to Scl-70 produced by the different transitional B cell subsets were determined by ELISpot. Our previous studies established that the T1 and CD27+ subsets had the highest
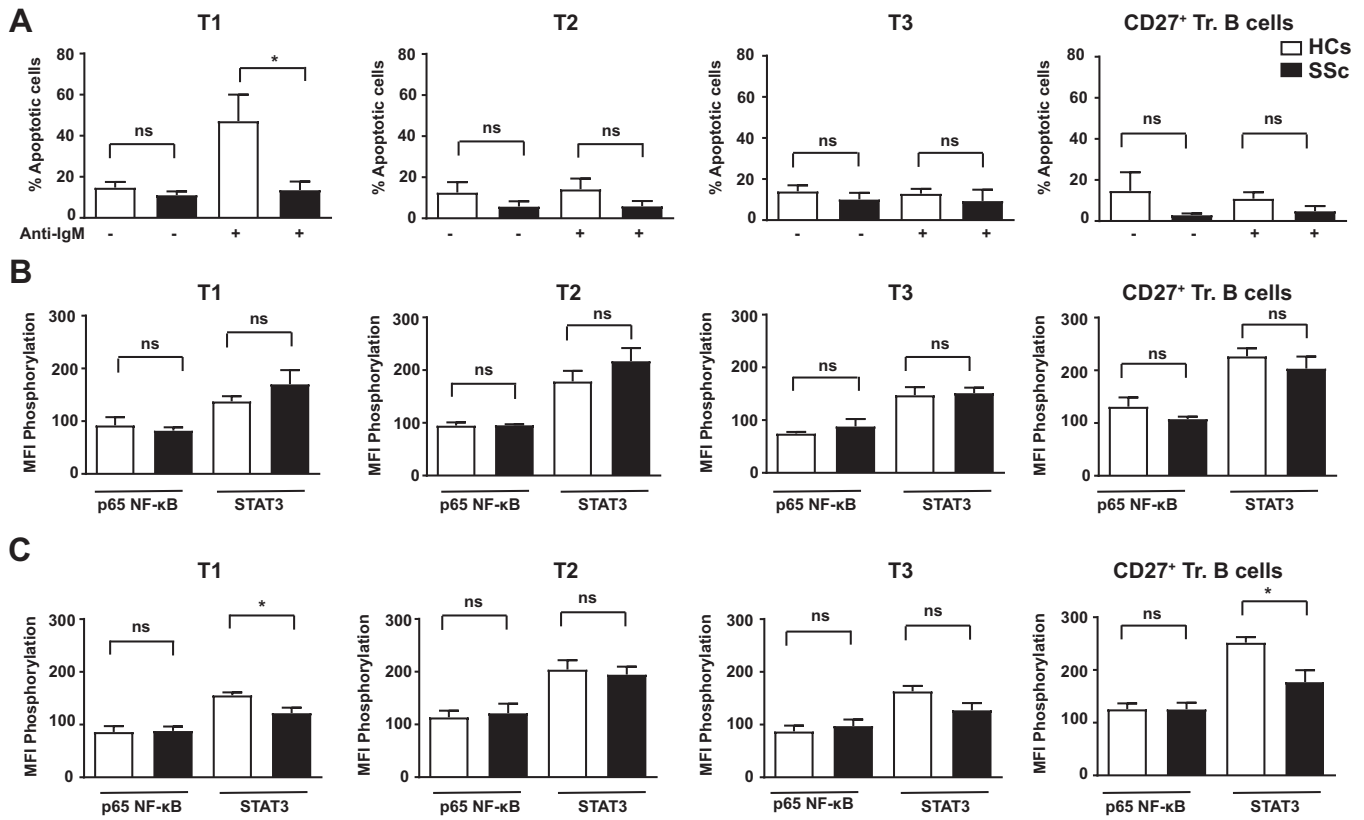
Figure 4. Altered responses of transitional (Tr) B cells from patients with systemic sclerosis (SSc) to B cell receptor (BCR) and Toll-like receptor 9 (TLR-9) engagement. **A,** Percentages of cells undergoing spontaneous apoptosis and BCR-induced apoptosis (+ anti-IgM) in transitional B cell T1, T2, T3, and CD27+ subsets in healthy controls (HCs) and SSc patients. Apoptotic cells were identified by staining with annexin V. Bars show the mean ± SEM (n = 8 healthy controls and 9 SSc patients). **B,** Mean fluorescence intensity (MFI) of basal phosphorylation levels of NF-κB p65 and STAT-3 (with no stimulation) in transitional B cell subsets in healthy controls and SSc patients. Bars show the mean ± SEM (n = 5 healthy controls and 4 SSc patients). **C,** MFI of phosphorylation of NF-κB p65 and STAT-3 in transitional B cell subsets stimulated by engaging TLR-9 with its ligand CpG-containing oligonucleotide. Bars show the mean ± SEM (n = 5 healthy controls and 4 SSc patients). * = $P < 0.05$ by Student's *t*-test. NS = not significant.

frequencies of transitional B cells capable of differentiating to IgM-secreting cells. Interestingly, however, in the present study the ELISpot results revealed that the T2 subset of transitional B cells in SSc patients who are seropositive for anti–Scl-70 contained most of the B cells that produced IgM autoantibodies that bound to the Scl-70 antigen (Figure 5A). Consistent with the detection of serum anti–Scl-70 autoantibodies in patients, the numbers of IgM anti–Scl-70–positive transitional B cells were significantly higher in seropositive patients than in seronegative patients.

DISCUSSION

The development of B lymphocytes in the bone marrow involves a delicate balance between generating a repertoire capable of combating pathogens and eliminating strongly self-reactive cells. Changes in this

balance result in defective immunity or, alternatively, the generation of pathogenic self-reactive B cells leading to autoimmune diseases (33). Despite the tight regulation of the B cell repertoire in the bone marrow, however, some self-reactive B cells escape tolerance and migrate to the periphery as transitional B cells. These cells undergo further development in the periphery, including passing through a number of tolerance checkpoints to mature to follicular, marginal zone, or Breg cells (16,17,34).

Results of studies from many laboratories, including ours, show that polyreactive/autoreactive B lymphocytes exist in significant numbers in transitional B cells in healthy individuals as well as in patients with autoimmune diseases (18). However, it appears that whereas the vast majority of self-reactive transitional B cells with the potential to become pathogenic cells are tolerized in healthy controls, patients with SSc show defects in the
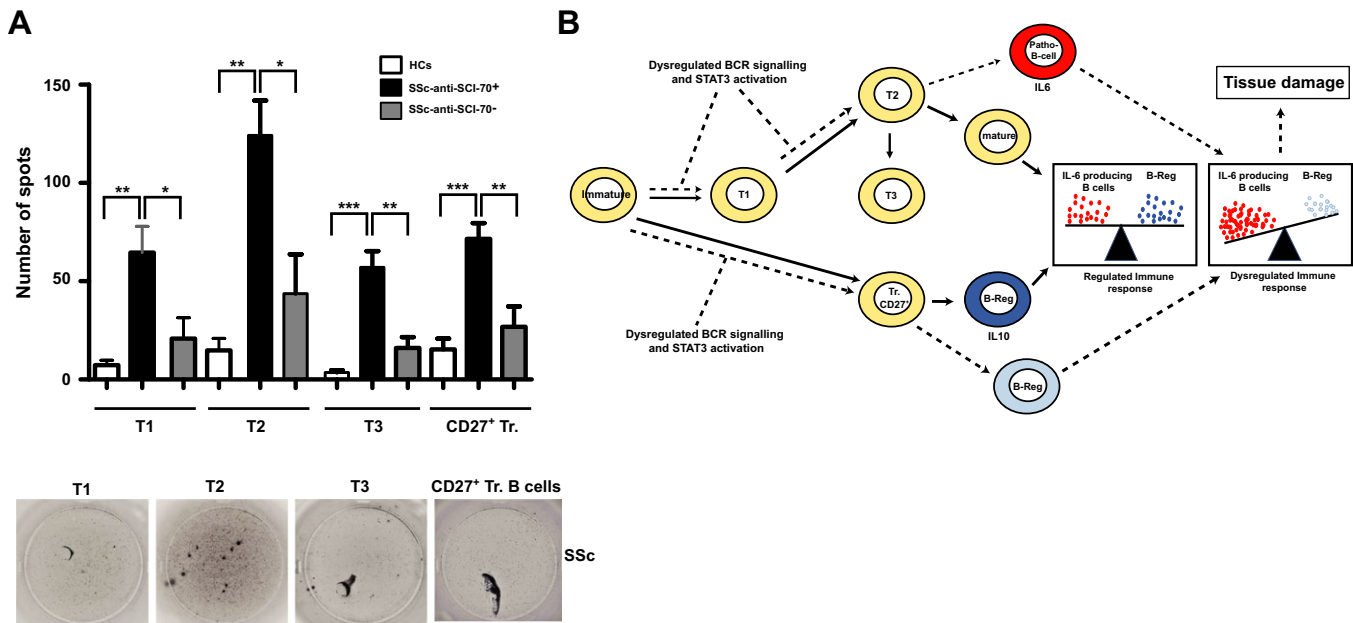
Figure 5. A, Top, Frequency of IgM anti–Scl-70 autoantibody–producing B cells in the T1, T2, T3, and CD27+ transitional (Tr) B cell subsets in healthy controls (HCs), anti–Scl-70–positive patients with systemic sclerosis (SSc), and anti–Scl-70–negative patients with SSc, as determined by enzyme-linked immunospot (ELISpot) assay. Bars show the mean $\pm$ SEM number of spots per 10^4 cells (n = 4 healthy controls, 6 SSc patients who were seropositive for anti–Scl-70 autoantibodies, and 6 SSc patients who were seronegative for anti–Scl-70 autoantibodies). * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, by Student's t-test. Bottom, ELISpot wells detecting anti–Scl-70 autoantibody–producing B cells. Sorted B cells were seeded at 10^4 cells/well. Each spot represents one IgM anti–Scl-70 autoantibody–producing B cell in the T1, T2, T3, and CD27+ transitional B cell subsets from an anti–Scl-70–positive patient with SSc. **B**, Schematic illustration depicting a proposed developmental program for transitional B cells in health and in SSc. Transitional B lymphocytes migrate from the bone marrow to become follicular, marginal zone, or Breg cells. Transitional B cells undergo a series of maturational events and tolerance checkpoints to prevent the emergence of high-affinity self-reactive B cells. The model envisions that during maturation in healthy individuals, some polyreactive/self-reactive transitional B cells survive tolerance checkpoints to become polyreactive Breg cells. In patients with SSc, however, tolerance at the T1–T2 stage is defective, leading to the eventual expansion of potentially pathogenic interleukin-6 (IL-6)–producing B cells, some with specificity for Scl-70. Broken arrows indicate where defective selection could occur. BCR = B cell receptor. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40390/abstract.

regulation of these cells at an early stage of their developmental program. Thus, in patients with SSc, IL-6–producing B cells and cells capable of producing autoantibodies with specificity for the hallmark SSc autoantigen Scl-70 are not eliminated or reduced. Furthermore, the results suggest that the failure to eliminate potentially pathogenic B cells in patients with SSc occurs at the T1–T2 maturational stage.

Early studies of the phenotype and functions of murine transitional B cells revealed that 3 major subsets of these cells exist (17,35,36). In humans, however, the phenotype and biology of transitional B cells have remained less well-defined. Our recent study using multicolor flow cytometry and functional assessment revealed that 4 different subsets of human transitional B cells exist (18). The criteria set used for the analysis of transitional B cells in the present study, established based on our earlier study, showed that, overall, the number of transitional B cells was higher in patients with SSc than in healthy

controls. There was a higher frequency of IL-6–producing B cells among transitional B lymphocytes in patients with SSc than in healthy controls.

The elevated frequency of IL-6–producing B cells in SSc patients could be relevant for further understanding of the regulation of B cells and how they could be involved in the pathogenesis of SSc, since IL-6 is implicated in key aspects of SSc. For example, IL-6 promotes the proliferation of T and B lymphocytes and monocytes and their resistance to apoptosis (37). In addition, IL-6 reduces the suppressive functions of Treg cells (38). Furthermore, IL-6 plays a significant role in the development of Th1 and Th17 cells (39,40). IL-6 production is, therefore, a key function through which B cells could contribute to SSc pathogenesis (10,11,19). This proposition is supported by observations that the therapeutic benefits of B cell depletion in murine models of autoimmune diseases and in human patients relates to reduced IL-6 production by B cells (8,10,11,20). Other studies

have also shown that IL-6 production by B cells is involved in promoting the development of pathogenic Th17 in chronic autoimmune diseases (39).

In addition to transitional B cells, the present study confirmed that the frequency of IL-6–producing B cells is increased in mature and memory B cells. This generalized increase in the number of IL-6–producing B cells could relate either to a genetic predisposition to higher IL-6 production or to defective censorship of transitional B cells that have the potential to mature to pathogenic autoantibody- and/or IL-6–producing B cells in SSc. Perhaps relevant to the latter possibility is the notable increase in the proportion of IL-6–producing transitional B cells as they progress from T1 to T2 cells. This may implicate a defect in the checkpoint mechanism(s) that regulate autoreactive T1–T2 B cell maturation. Clearly, however, further studies are needed to verify and explore the molecular mechanism(s) that lead to such an outcome.

Interestingly, whereas the frequency of T1 cells was significantly higher in patients with SSc compared with healthy controls, the frequency of T2 cells did not differ between the 2 groups. These 2 subsets have different capacities to undergo apoptosis following BCR engagement. Thus, whereas T1 cells in healthy controls undergo apoptosis following BCR engagement, T2 cells are more resistant. In contrast, T1 cells from patients with SSc appeared to be resistant to BCR-mediated apoptosis. This finding is consistent with our recent studies of transitional B cell subsets and implies that tolerance checkpoints acting at the T1–T2 stage of translational B cell maturation could be where defective tolerance mechanisms result in the survival of autoreactive IL-6–producing B cells in patients with SSc. Any defect(s) at this stage are likely to have notable effects on the repertoire of the T2 and T3 cells and possibly, of course, mature B cells. Indeed, our results show that the T2 subsets of transitional B cells in patients with SSc contained B cells that produced autoantibodies with specificity for the Scl-70 antigen. Based on our previous studies, this observation could reflect a specific failure to deplete or tolerize Scl-70–specific B cells at the T1–T2 maturational stage in SSc. Thus, the frequency of Scl-70–specific B cells at the T1 stage is too low to detect among the large number of polyreactive B cells. However, most polyreactive T1 B cells are depleted or tolerized as they mature to T2 cells, allowing for a relative increase in the frequency of Scl-70–specific B cells, rendering them detectable among the surviving T2 B cells (18).

The underlying molecular mechanisms in defective tolerization of Scl-70–specific B cells, and perhaps other self-reactive B cells, at the T1–T2 stage in SSc remain unclear, but they are likely to involve genetic factors that are manifested in defective intracellular signaling and/or enhanced IL-6 production. Interestingly, however, preliminary studies of intracellular signaling did not reveal differences in basal NF-κB p65 phosphorylation between transitional B cells from SSc patients and healthy controls. However, both T1 and CD27+ B cells from the patients showed a trend toward reduced TLR-9–induced phosphorylation of STAT-3. These findings are consistent with the observation that transitional B cells in patients with SSc produced lower levels of IL-10 when stimulated through TLR-9.

Transitional B cells are proposed to also give rise to Breg cells, which can suppress autoimmunity and chronic inflammation through IL-10–dependent and independent mechanisms (18,41). Our findings with regard to the reduced ability of B cells in patients with SSc to produce IL-10 following TLR-9 but not CD40 engagement could provide further clues as to the cause of defects in the regulation of B cell responses in this disease. TLR-9 signaling induces IL-10 production by naive B cells, whereas CD40/BCR engagement enhances IL-10 production by activated B cells (26). This is important since the available evidence suggests that the differentiation to Breg cells occurs through 2 stages. During the initial stage of differentiation, TLR-9 engagement promotes IL-10–producing B cells and then CD40/BCR engagement expands such Breg cells (42). Notably, our results showed that STAT-3 activation was defective in patients with SSc when the cells were stimulated with TLR-9 but not when the cells were stimulated through the CD40 coreceptor (results not shown).

This study also revealed that B cells from patients with SSc produce significantly higher levels of the proinflammatory cytokines GM-CSF, IL-1β, and IL-8 than those from healthy controls. It will be of interest to directly evaluate the pathogenic contribution of these cytokines to the disease process in SSc. Further investigation is also needed to define the mechanisms that lead to the imbalance in the maturation of transitional B cells to IL-6–producing, potentially pathogenic B cells rather than IL-10–producing Breg cells in SSc.

It is notable that the majority of the SSc patients included in the present study were receiving immunomodulating agents that possibly affected their B cell profiles. It is likely that these drugs influence the level of IL-6 production by B cells as well as the number and percentage of IL-6–producing cells. A detailed study of individual patients is needed to assess the effect of treatment with immunomodulating agents on B cell profiles in SSc.

Additionally, the SSc B cell profiles and frequencies observed in this study showed an association with disease severity and symptoms. For example, SSc patients with severe lung fibrosis had more IL-6–positive B cells within all subsets than patients with mild disease (data not shown). Similarly, patients with diffuse scleroderma had more IL-6–positive B cells than patients with limited scleroderma (data available upon request from the corresponding author). Further, anti–Scl-70–positive patients had more IL-6–positive B cells than patients who were seronegative for the autoantibody. However, these differences were not statistically significant. Therefore, a larger study is needed to address this issue conclusively.

In normal individuals, significant numbers of early immature B cells display varying degrees of self-reactivity or polyreactivity, but the level of autoreactivity progressively declines within the B cell repertoire with cell maturation (43). Consistent with previous studies in lupus and rheumatoid arthritis, our results highlight inadequate elimination of autoreactive B cells in patients with autoimmune disease (43–45). The fact that very few, if any, spots were detected in B cells from the healthy controls suggests that polyreactive autoantibodies are unlikely to have influenced our observations. However, the possibility that some of the Scl-70–bound IgM could also bind dsDNA, insulin, or, indeed, other self antigens cannot be excluded.

Studies in mice and humans have provided multiple lines of evidence to indicate that defects in central tolerance exist in patients with autoimmune diseases. However, further studies are required to determine which of these defects is primary and which are secondary to the development of autoimmunity as well as reveal the genetic basis for any such defects. Several studies have suggested that effective censoring of autoreactive cells relies on multiple factors including self-antigen availability, avidity, and the ability to crosslink the BCR and engage TLRs and the abundance of survival factors, particularly BAFF (46,47). Further investigations will also be needed to define the mechanisms that lead to the defect in central tolerance in SSc.

In conclusion, this study sheds new light on potential mechanisms underlying defective B lymphocyte selection and regulation in patients with SSc and, possibly, highlights functional imbalances between transitional B cells that differentiate to become protective rather than pathogenic B cells in SSc. Based on findings from this study, a framework summarizing predictions for pathways of transitional B cell development and possible defects in the regulation in patients with SSc can be suggested (Figure 5B).

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