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# Breg Cells Are Numerically Decreased and Functionally Impaired in Patients With Systemic Sclerosis

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***Objective.*** **Breg cells, a regulatory cell subset that produces interleukin-10 (IL-10), play a significant role in suppressing autoimmune responses and preventing autoimmunity. This study was undertaken to examine the number and function of Breg cells in patients with systemic sclerosis (SSc), a disease with many autoantibodies.**

***Methods.*** **Forty-five patients with SSc (12 with early SSc, 33 with established disease including 16 with SSc-associated pulmonary fibrosis [PF]), 12 healthy control subjects, and 10 patients with rheumatoid arthritis (RA)–associated PF were studied. The phenotypes of immature/transitional Breg cells (CD19+CD24$^{high}$ CD38$^{high}$) and memory Breg cells (CD19+CD27+ CD24$^{high}$) were evaluated by flow cytometry. The function of Breg cells was assessed by measuring the production of IL-10 after B cell activation. In addition, activation of p38 MAPK and STAT-3 was measured following stimulation of the cells with B cell receptor (BCR) and Toll-like receptor 9 (TLR-9).**

***Results.*** **Percentages of memory Breg cells were decreased in patients with early SSc (mean ± SEM 1.85 ± 0.38%), those with established SSc (1.6 ± 0.88%), those with SSc-associated PF (1.52 ± 0.17%), and those with RA-associated PF (1.58 ± 0.26%), compared to healthy controls (6.3 ± 0.49%; each $P < 0.001$). Percentages of transitional Breg cells were also decreased. Expression of IL-10 by Breg cells after stimulation with TLR-9 was impaired in patients with SSc, particularly those with SSc-associated PF. Activation of STAT-3 and p38 MAPK was impaired in naive and memory B cells from patients with SSc after stimulation with BCR and TLR-9. Expression of the stimulatory CD19 receptor was increased in B cells and also increased, to a lesser extent, in Breg cells from patients with SSc compared to healthy controls. Percentages of memory B cells were decreased in patients with SSc, particularly in those with SSc-associated PF.**

***Conclusion.*** **This is the first study to demonstrate that Breg cells are phenotypically and functionally impaired in patients with SSc. Furthermore, in SSc, B cells exhibit impaired p38 MAPK and STAT-3 activation upon stimulation with BCR and TLR-9. The findings of decreased numbers of Breg cells along with increased expression of CD19 support the idea of B cell autoaggression acting as an immunopathogenic mediator in SSc.**

Systemic sclerosis (SSc) is a chronic disease characterized by microvasculopathy (Raynaud's phenomenon and fibrointima proliferation), production of autoantibodies, and excessive collagen deposition in the skin and internal organs. The pathogenesis of SSc is incompletely understood, but immune cells are likely to be involved. There is evidence to suggest that T cells are implicated in the pathogenesis of the disease (1). Although frequencies of B cell infiltrates in SSc lesions vary (2–4), emerging evidence implicates B cells in the pathogenesis of SSc.

B cells from patients with SSc overexpress CD19, a stimulatory B cell receptor (BCR), and similar overexpression of CD19 in transgenic mice leads to spontaneous autoantibody production (5). In contrast, CD19

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deficiency in tight skin mice, a model of SSc, completely inhibits autoantibody production and significantly decreases skin fibrosis (6). Similarly, CD19-deficient mice exhibit reduced fibrosis in the bleomycin-induced lung fibrosis model of SSc (7). Many autoantibodies that promote inflammation, fibrosis, and autoimmunity are detected in patients with SSc. Anti–topoisomerase I antibodies bind to fibroblasts and promote inflammation (8), and autoantibodies to platelet-derived growth factor receptor (9) and matrix metalloproteinase 1 (10) promote fibrosis. Antibodies to CD22, an inhibitory BCR, are functional and associated with reduced activation of CD22 (11). Finally, coculture of B cells with SSc dermal fibroblasts induces collagen secretion in a cell interaction–dependent manner (12).

In recent years, Breg cells have been found to play a significant role in suppressing inflammatory immune responses and preventing autoimmunity through the production of interleukin-10 (IL-10), as demonstrated in studies of B10 cells in mice and humans (13–16). For instance, development and expansion of (IL-10–producing) B10 cells in mice inhibits symptoms when these cells are transferred into mice with established experimental autoimmune encephalomyelitis (EAE) (17). Peripheral blood Breg cells producing IL-10 in humans have been described mainly within the subsets of CD19+CD24$^{high}$CD38$^{high}$ B cells, also known as immature/transitional B cells (transitional Breg cells) (18–21), and CD19+CD24$^{high}$CD27+ memory B cells (memory Breg cells) (15,16). IL-10–producing Breg cells develop and expand in response to adaptive stimuli, such as CD40-mediated and IL-21–mediated signals from CD4+ T cells, and also in response to innate stimuli, such as lipopolysaccharide and CpG-containing oligonucleotide (ODN) (17,22).

The significance of Breg cells in the loss of immune tolerance in SSc is unclear. Therefore, in the present study, we evaluated the phenotypes of transitional and memory Breg cells in patients with SSc. In addition, we assessed the functional capacity of these Breg cells to produce IL-10. Finally, we investigated the ability of naive and memory B cells to activate p38 MAPK and/or STAT-3 following stimulation with BCR and Toll-like receptor 9 (TLR-9).

## PATIENTS AND METHODS

**Patients.** Forty-five patients with SSc were included in this study. Of these, 12 patients (10 female, 2 male; mean age 54.7 years, range 25–72 years) had disease of recent onset (<1 year) and had not received any immunomodulatory treatment prior to enrollment (designated as patients with early SSc). Thirty-three patients had established disease, of whom 18 had diffuse cutaneous SSc (dcSSc) (14 female, 4 male; mean age 56.2 years, range 28–85 years) and 15 had limited cutaneous SSc (lcSSc) (12 female, 3 male; mean age 61.8 years, range 44–81 years). Of the 33 patients with established disease, 16 had SSc-associated pulmonary fibrosis (PF) and 17 had SSc without PF. Among the 16 patients with SSc-associated PF (11 with dcSSc and 5 with lcSSc), 12 were taking low-dose steroids (<7.5 mg/day) in combination with azathioprine (5 patients) or methotrexate (9 patients). Among the 17 patients with SSc without PF (7 with dcSSc and 10 with lcSSc), 3 were taking low-dose steroids, 6 were taking methotrexate, and 3 were taking azathioprine. All of the patients with SSc were found to have antinuclear antibodies by indirect immunofluorescence, 17 were positive for anti–topoisomerase I antibodies, 13 for anticentromere antibodies, 7 for anti–RNA polymerase III antibodies, 1 for anti–topoisomerase I and anticentromere antibodies, and 1 for anti–topoisomerase I and anti–RNA polymerase II antibodies.

Ten patients with rheumatoid arthritis (RA)–associated PF (6 female, 4 male; mean age 63.7 years, range 49–74 years) and 12 healthy blood donors (9 female, 3 male; mean age 49.3 years, range 31–65 years) were included as disease controls and healthy controls, respectively. Among the patients with RA, all 10 were taking low-dose glucocorticoids, 7 were taking methotrexate, and 3 were taking leflunomide.

All patients fulfilled the American College of Rheumatology criteria for the classification of SSc or RA (23,24), and all study patients were attending the outpatient clinic of the Rheumatology Department at University General Hospital of Larissa (Larissa, Greece). Written informed consent was obtained from all patients and healthy blood donors. The protocol was approved by the local ethics committee of the University General Hospital of Larissa.

**Preparation of mononuclear cells.** Heparinized peripheral blood samples (~20–30 ml) were collected from patients and healthy controls after venipuncture. Peripheral blood mononuclear cells (PBMCs) were obtained by conventional centrifugation and separation over a discontinuous LymphoPrep gradient (Axis-Shield). PBMCs were collected and washed twice with serum-free RPMI 1640 medium (Invitrogen Life Technologies). Cell viability, determined by trypan blue exclusion, exceeded 95%. Relative lymphocyte percentages among viable cells (based on light-scatter properties) were determined by flow cytometry.

**Multiparameter flow cytometry analysis of phenotypes.** The following anti-human monoclonal antibodies (mAb) were used: fluorescein isothiocyanate (FITC)–conjugated anti-CD27 (clone MT271), phycoerythrin (PE)–conjugated anti-CD19 (clone HIB19), Alexa Fluor 488–conjugated anti-CD20 (clone FB1), PC5-conjugated anti-CD22 (clone HIB22), PC5-conjugated anti-CD24 (clone ML5), and allophycocyanin (APC)–conjugated anti-CD38 (clone HIT2) (all from BD Biosciences). PBMCs ($0.5–1 \times 10^6$ cells) were incubated with fluorochrome-conjugated mAb for 30 minutes on ice, washed twice in phosphate buffered saline containing 2% fetal calf serum (FCS), and fixed with 2% paraformaldehyde.

Flow cytometry analyses were performed using a FACSCalibur cytometer (Becton Dickinson) with logarithmic amplification and a forward and side light scatter–based gate for sorting of total lymphocyte populations. CaliBRITE beads (BD Biosciences) were used to adjust the instrument settings, set fluorescence compensation, and check the instrument

sensitivity. At least $3 \times 10^5$ cells within the lymphocyte gate were collected for each sample. Experimental testing was performed at least in duplicate in most of the significant analyses, and the results were reproducible. BD Bioscience CellQuest software was used for data acquisition and off-line analysis. For each of the gated populations, the percentage of cells and the geometric mean fluorescence intensity (MFI) were analyzed.

We typically titrated all selected surface and intracellular antibody clones for optimal staining of $0.5–1 \times 10^6$ cells per point. An aliquot of cells was used as an autofluorescence control, and a second aliquot was stained with the appropriate isotype control IgG antibody. Insignificant background staining was observed using appropriate control FITC-conjugated, PE-conjugated, PC5-conjugated, and APC-conjugated antibodies.

**Detection of intracellular IL-10 expression.** In order to measure the intracellular expression of IL-10, PBMCs were either left untreated or stimulated with 10 $\mu$g/ml CpG ODN (ODN2006, a TLR-9 ligand; InvivoGen) in fresh RPMI medium supplemented with 10% FCS for 24 hours. During the last 6 hours of culture, the cells were treated with brefeldin A (GolgiPlug; BD Biosciences) to block cytokine secretion and were then restimulated with 20 ng/ml phorbol myristate acetate (PMA) along with 1 $\mu$g/ml ionomycin (Sigma-Aldrich). Activated cells were stained for surface markers and then permeabilized with Perm Buffer (BioLegend) in accordance with the manufacturer's instructions. IL-10 was detected using PE-conjugated or unconjugated anti–IL-10 mAb (JES3–19F1 or JES3–9D7, respectively; BioLegend). Flow cytometry analyses were performed using a FACSCalibur cytometer (15).

**Flow cytometry analysis of phosphospecific p38 MAPK and STAT-3.** Phoshorylation of p38 MAPK and STAT-3 was measured using Phosflow technology, as previously described (25). Briefly, cells were first allowed to rest for 2 hours in low serum (1% FCS) and then activated either nonspecifically, using 20 ng/ml PMA together with 1 $\mu$g/ml ionomycin or sodium arsenite (500 $\mu M$), or specifically, using stimuli engaging the BCR (10 $\mu$g/ml polyclonal IgM) or TLR-9 (10 $\mu$g/ml ODN2006), for the indicated times to allow signal transduction and determine kinase activity. A parallel culture with unstimulated cells was used to determine basal levels of phosphorylation. Another culture was set up using the same stimuli in the presence of 3 m$M$ $H_2O_2$. This was done in order to deactivate phosphatases and enhance the strength of B cell signaling, as previously described (26).

Paraformaldehyde, prewarmed at 37°C, was added to the culture medium to give a final concentration of 2%, and the cells were fixed at 37°C for 15 minutes. Thereafter, the cells were harvested, washed, and permeabilized by slowly adding 1 ml of 75% (volume/volume) methanol (Fisher Scientific) in Tris buffered saline (TBS), while thoroughly vortexing at medium speed. Paraformaldehyde-fixed, methanol-permeabilized cells were rehydrated for 30–60 minutes by the addition of 1 ml of TBS-based wash buffer, followed by gentle resuspension and centrifugation. After washing, cells were treated with an Fc receptor–blocking reagent (Miltenyi Biotec), followed by 15 minutes of incubation at room temperature to allow blocking of Fc receptors. The cell pellet was washed twice, resuspended in 50 $\mu$l 2% bovine serum albumin–TBS (weight/volume), and incubated with fluorochrome-conjugated antibodies for 1 hour. The following reagents and mAb were used for Phosflow analysis: Alexa Fluor 647 dye (Molecular Probes, Invitrogen) and phosphospecific mAb to p38 MAPK (T180/Y182) and PE-conjugated STAT-3 (pS727) (both from BD Biosciences). According to the manufacturer's specifications, STAT-3 is phosphorylated at $Ser^{727}$ via the MAPK pathway. Activation through the $Ser^{727}$ residue leads to initiation of transcription.

**Magnetic cell isolation.** Peripheral blood B cells were purified by either positive or negative magnetic cell selection using CD19 mAb–coated microbeads and a B cell–negative isolation kit, respectively (Miltenyi Biotec), following the manufacturer's instructions. Sorting of total B cells in the naive and memory B cell subsets was carried out magnetically using a stepwise separation. First, B cells were negatively enriched using a B cell–negative isolation kit (Miltenyi Biotec), and then CD27 microbeads (Miltenyi Biotec) were used for the positive selection of memory B cells and negative selection of naive B cells. All procedures typically yielded B cell preparations that were >95% pure CD19+ cells and/or CD27+ cells, routinely assessed by fluorescence-activated cell sorter (FACS) staining.

**Statistical analysis.** Percentages of cells expressing cell surface markers and the MFI values are presented as the median ± SEM for each patient group. Differences between patients and healthy controls and between patient groups were tested using 2-tailed *t*-tests, one-way analysis of variance, and the nonparametric Mann-Whitney test. *P* values less than or equal to 0.05 were considered significant. The statistical calculations were performed with GraphPad Prism software.

## RESULTS

**Decreased frequency of memory and transitional Breg cells in SSc.** Peripheral blood lymphocytes from 12 patients with early SSc (6 with lcSSc and 6 with dcSSc), 33 patients with established SSc (18 with dcSSc and 15 with lcSSc), 10 patients with RA-associated PF, and 12 healthy controls were phenotypically analyzed by flow cytometry for the expression of CD19, CD24, CD38, and CD27 surface markers. This analysis enables us to reliably separate the subsets of $CD19+CD24^{high}CD38^{high}$ transitional Breg cells and $CD19+CD24^{high}CD27+$ memory Breg cells (27,28). Moreover, this combination of markers also identifies naive and memory B lymphocytes, as well as plasma cells, among CD19+ B cells (19). A representative staining of transitional and memory Breg cells from the peripheral blood of a healthy control subject is shown in Figure 1A. Cumulative data obtained from patients with early SSc, patients with established SSc, and healthy controls are shown in Figure 1B. Data are presented as the percentage of total lymphocytes, percentage of gated CD19+ B cells, and absolute counts of B cells per $\mu$l of blood. All cell subset frequencies were calculated as the mean ± SEM percentage of sorted positive cells in reference to negative staining with control antibodies.

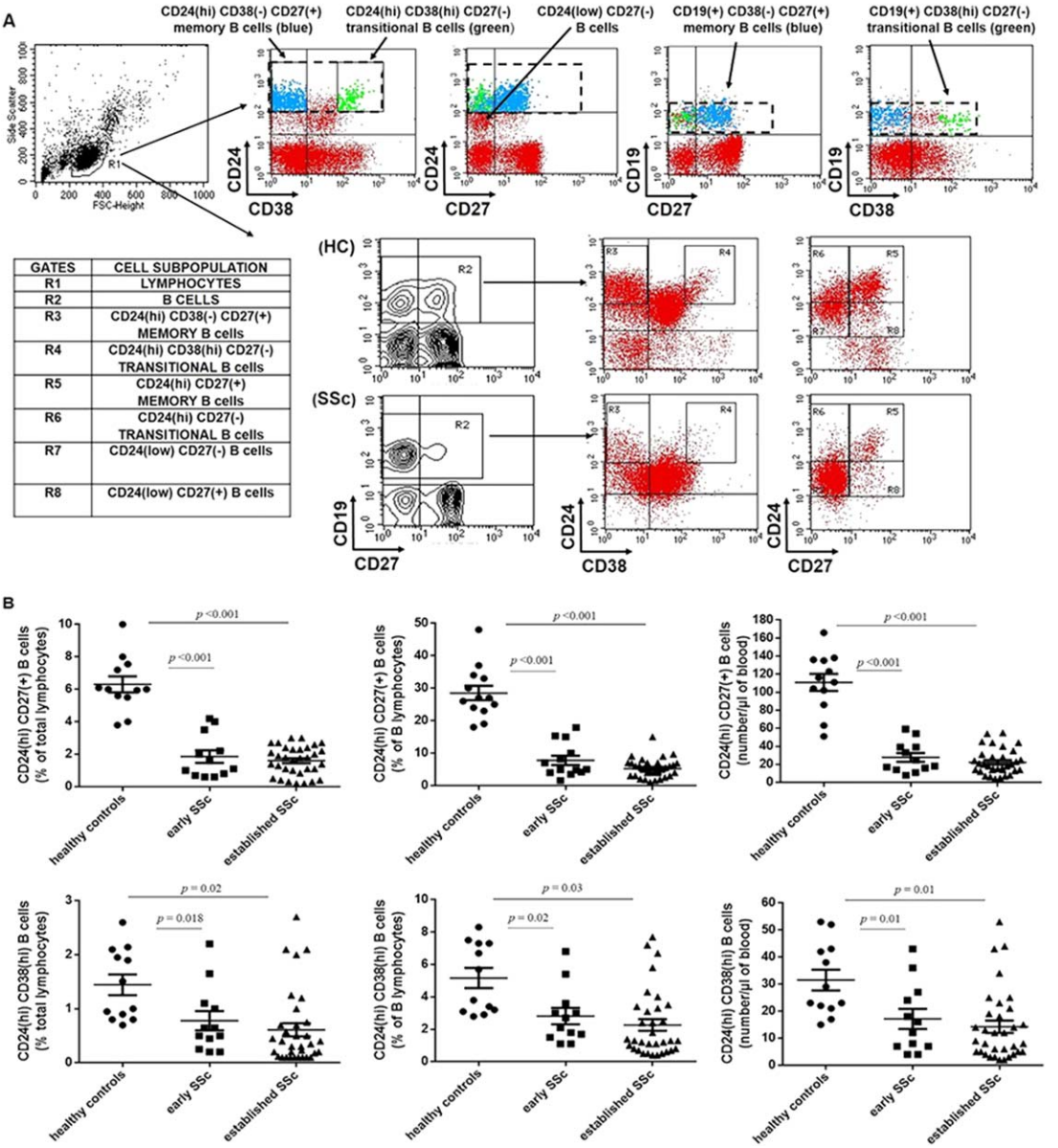


**Figure 1.** Typical flow cytometry analysis of memory and transitional Breg cells. **A,** Top, Peripheral blood mononuclear cells from a representative healthy control subject (HC) were stained with monoclonal antibodies to CD19, CD24, CD27, and CD38 and analyzed by flow cytometry. Total lymphocytes were gated based on forward and side light-scatter characteristics (gate R1), excluding dead cells and debris. Transitional Breg cells were identified based on high expression of the CD38 and CD24 markers (green). Memory Breg cells were identified based on high expression of CD24, positivity for CD27, and lack of CD38 expression (blue). Bottom, Subgating and phenotypes of each cell subset were analyzed in a representative healthy control subject and patient with systemic sclerosis (SSc). The mean percentages were calculated within both total lymphocytes (gate R1) and B lymphocytes (gates R1 and R2). **B,** Percentages of memory Breg cells (top) and transitional Breg cells (bottom) within total lymphocytes and total B cells, as well as the counts per $\mu$l of peripheral blood, were determined in patients with early SSc (n = 12), those with established SSc (n = 33), and healthy controls (n = 12). Results are the mean ± SEM.

Percentages of memory Breg cells within total lymphocytes were decreased in patients with early SSc and those with established SSc compared to healthy controls, with no significant difference between patients with early SSc and those with established SSc. Percentages of memory Breg cells in patients with early SSc (n = 12), patients with established SSc (n = 33), and healthy controls (n = 12) were 1.85 ± 0.38%, 1.6 ± 0.88%, and 6.3 ± 0.49%, respectively ($P < 0.001$ for early SSc or established SSc versus healthy controls; $P > 0.05$ for early SSc versus

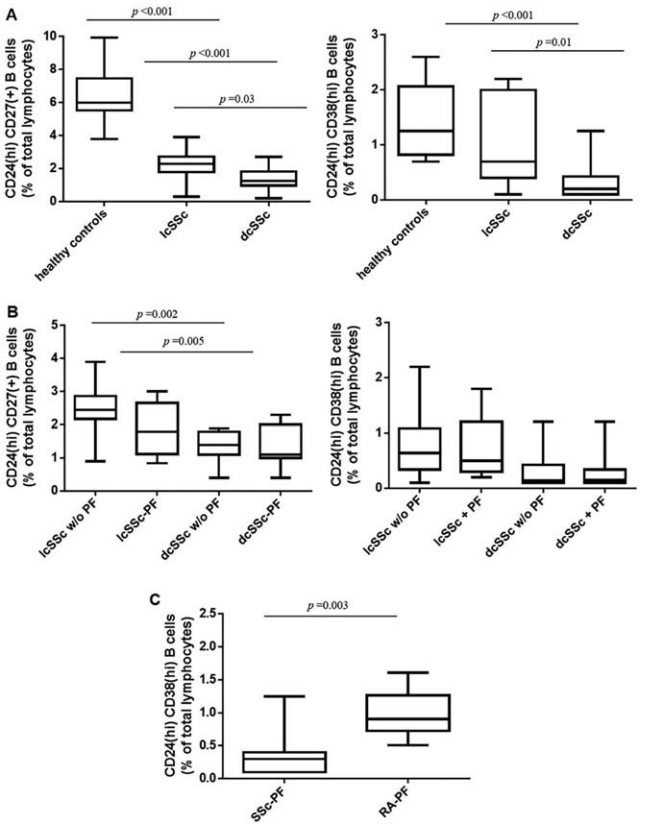


**Figure 2.** **A** and **B,** Distribution of memory Breg cells (left) and transitional Breg cells (right) within total lymphocytes in patients with limited cutaneous systemic sclerosis (lcSSc) (n = 15) and those with diffuse cutaneous SSc (dcSSc) (n = 18) compared to healthy controls (n = 12) **(A)** and between patients with lcSSc and those with dcSSc in the presence of pulmonary fibrosis (PF) (n = 5 and n = 11, respectively) or absence of PF (n = 10 and n = 7, respectively) **(B)**. **C,** Distribution of transitional Breg cells within total lymphocytes in SSc patients with PF (n = 16) compared to rheumatoid arthritis (RA) patients with PF (n = 10). Results are shown as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the whiskers outside the boxes represent the 5th and 95th percentiles.

established SSc) (Figure 1B). Percentages of transitional Breg cells in patients with early SSc, patients with established SSc, and healthy controls were $0.77 \pm 0.17\%$, $0.61 \pm 0.12\%$, and $1.44 \pm 0.19\%$, respectively ($P = 0.02$ for early SSc or established SSc versus healthy controls; $P > 0.05$ for early SSc versus established SSc) (Figure 1B).

**Frequency of memory and transitional Breg cells in lcSSc, dcSSc, and SSc-associated PF.** We next assessed the distribution of Breg cells (mean ± SEM percentages) in patients with established disease according to cutaneous involvement (lcSSc [n = 15] and dcSSc [n = 18]) (Figure 2A). Percentages of memory and transitional Breg cells within total lymphocytes were decreased to a greater extent in patients with dcSSc than in those with lcSSc. Percentages of memory Breg cells were $2.24 \pm 0.23\%$ in patients with lcSSc and $1.36 \pm 0.16\%$ in those with dcSSc ($P < 0.001$ for lcSSc or dcSSc versus healthy controls; $P = 0.03$ for lcSSc versus dcSSc). Percentages of transitional Breg cells were $0.99 \pm 0.19\%$ in patients with lcSSc and $0.34 \pm 0.08\%$ in those with dcSSc ($P > 0.05$ for lcSSc versus healthy controls; $P < 0.001$ for dcSSc versus healthy controls; $P = 0.01$ for lcSSc versus dcSSc).

We further examined memory and transitional Breg cells according to the presence of PF in patients with lcSSc and those with dcSSc. The percentage of memory Breg cells in patients with SSc-associated PF ($1.52 \pm 0.17\%$; n = 16) was significantly decreased compared to that in patients with SSc without PF ($2.07 \pm 0.19\%$; n = 17) ($P = 0.04$ between groups). Among SSc patients without PF, the percentages of memory Breg cells were $2.48 \pm 0.24\%$ in those with lcSSc (n = 10) ($P < 0.001$ versus healthy controls) and $1.39 \pm 0.19\%$ in those with dcSSc (n = 7) ($P < 0.001$ versus healthy controls). Among patients with SSc-associated PF, the percentages of memory Breg cells were $1.86 \pm 0.37\%$ in those with lcSSc (n = 5) and $1.36 \pm 0.18\%$ in those with dcSSc (n = 11) (both $P < 0.001$ versus healthy controls). Comparisons between the groups revealed that there were statistically significant differences only between patients with lcSSc without PF and those with dcSSc without PF and between patients with lcSSc without PF and those with dcSSc-associated PF ($P = 0.002$ and $P = 0.005$, respectively) (Figure 2B).

Percentages of transitional Breg cells were $0.59 \pm 0.15\%$ in SSc patients without PF (n = 17) and $0.39 \pm 0.11\%$ in those with SSc-associated PF (n = 16) ($P > 0.05$ between groups). Among SSc patients without PF, the percentages of transitional Breg cells were $0.81 \pm 0.23\%$ in those with lcSSc (n = 10) ($P = 0.02$ versus healthy controls) and $0.31 \pm 0.13\%$ in those with dcSSc (n = 7) ($P = 0.001$ versus healthy controls). Among patients with SSc-associated PF, the percentages of transitional Breg cells were $0.7 \pm 0.28\%$ in those with lcSSc (n = 5) ($P = 0.04$ versus healthy controls) and $0.27 \pm 0.09\%$ in those with dcSSc (n = 11) ($P = 0.001$ versus healthy controls). Comparisons between the groups revealed no statistically significant differences (all $P > 0.05$) (Figure 2B) (details on representative patients with lcSSc or dcSSc without PF compared to lcSSc or dcSSc patients with PF are available upon request from the corresponding author).

Memory and transitional Breg cells were also analyzed in patients with RA-associated PF. Similar to that observed in patients with SSc-associated PF, the

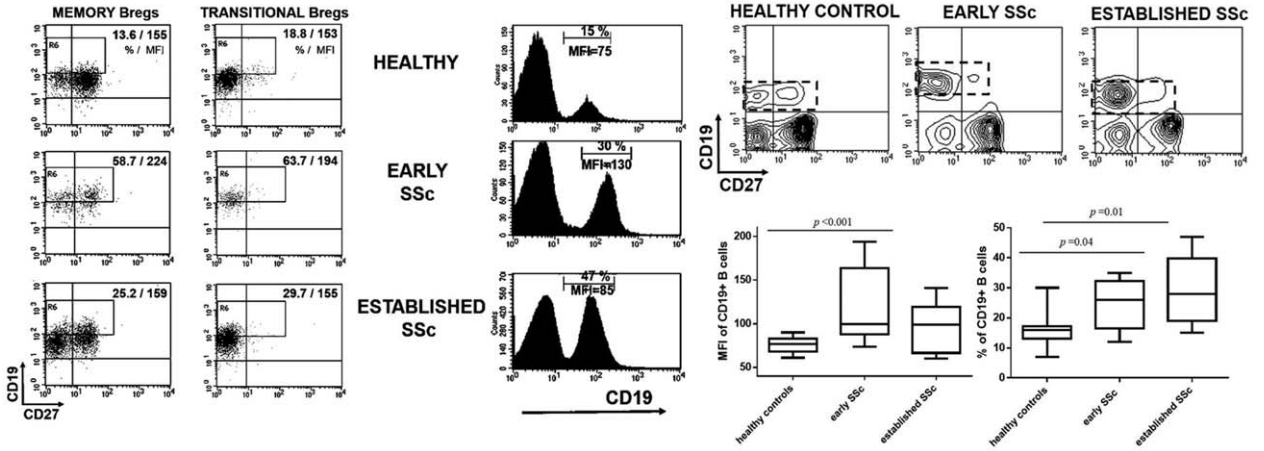


**Figure 3.** CD19 expression in Breg cells. Peripheral blood mononuclear cells from a representative healthy control subject, a patient with early systemic sclerosis (SSc), and a patient with established SSc were stained with monoclonal antibodies to CD19, CD24, CD27, and CD38. Left, Total lymphocytes were gated based on forward and side light-scatter characteristics (gate R1), excluding dead cells and debris. Memory Breg cells and transitional Breg cells were subgated as described in Figure 1 and plotted as CD19 versus CD27 dot plots. Gate R6 indicates $CD19^{high}$ expression, specified as a log-scale difference of $>10^2$. The percentage of cells within the $CD19^{high}$ gate (gate R6) and mean fluorescence intensity (MFI) values for CD19 expression within the $CD19^{high}$ gate are shown. Center and right, CD19 expression in total B cells was assessed in patients with early SSc (n = 12), those with established SSc (n = 33), and healthy controls (n = 12). Representative histograms show CD19 expression (left panels), and contour plots (top right panels) and cumulative data (bottom right panels) compare the MFIs of CD19 expression and the percentages of CD19+ cells among total B cells from each group. Results are shown as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the whiskers outside the boxes represent the 5th and 95th percentiles.

percentages of memory Breg cells were decreased in patients with RA-associated PF, but the difference between these 2 groups did not reach statistical significance (results not shown). However, the percentages of transitional Breg cells were decreased markedly in patients with SSc-associated PF, but not in those with RA-associated PF (Figure 2C). The percentage of memory Breg cells in patients with RA-associated PF was $1.58 \pm 0.26\%$ ($P < 0.001$ versus healthy controls; $P > 0.05$ versus SSc-associated PF). The percentage of transitional Breg cells in patients with RA-associated PF was $0.98 \pm 0.15\%$ ($P > 0.05$ versus healthy controls; $P = 0.003$ versus SSc-associated PF) (details available upon request from the corresponding author).

**Increased CD19 expression in Breg cells and B cells in early SSc and established SSc.** Since low expression of CD19 in Breg cells was observed in one study (29) and given that CD19 is overexpressed in SSc B cells (5), we assessed CD19 expression in Breg cells and B cells in patients with early SSc and those with established SSc. Increased expression of CD19 was detected in both phenotypic memory Breg cells and transitional Breg cells from patients with early SSc compared to healthy controls, whereas in patients with established SSc, expression of CD19 was also increased, but to a much lower extent, in both memory and transitional Breg cells (Figure 3).

Expression of CD19 was also elevated in total B cells. In patients with early SSc, CD19 expression in total B cells was 53% higher than that in healthy controls (mean ± SEM MFI $116.7 \pm 12.2$ versus $76.40 \pm 2.59$; $P < 0.001$) (Figure 3). CD19 expression in total B cells was also increased in patients with established SSc compared to healthy controls ($97 \pm 8.5$ versus $76.40 \pm 2.59$; $P > 0.05$) but to a lesser extent than that in patients with early SSc. The overall percentages of CD19+ B cells were also increased in patients with early SSc and those with established SSc compared to healthy controls (mean ± SEM $24.7 \pm 2.6\%$ and $29.3 \pm 3.2\%$ in early SSc and established SSc, respectively, versus $16.00 \pm 1.9\%$ in healthy controls) ($P = 0.04$ for both SSc groups versus healthy controls; $P = 0.01$ for established SSc versus healthy controls; $P > 0.05$ for early SSc versus established SSc) (Figure 3).

The mean ± SEM percentage of CD19+ CD27+ memory B cells was $4.10 \pm 0.43\%$ in patients with early SSc, as compared to $8.15 \pm 1.155\%$ in healthy controls ($P = 0.004$). Conversely, the percentage of CD19+CD27− naive B cells was significantly increased in patients with early SSc compared to healthy controls ($20.60 \pm 2.26\%$ versus $8.10 \pm 1.13\%$; $P = 0.001$) (results available upon request from the corresponding author). The ratio of naive B cells to memory B cells was increased in patients

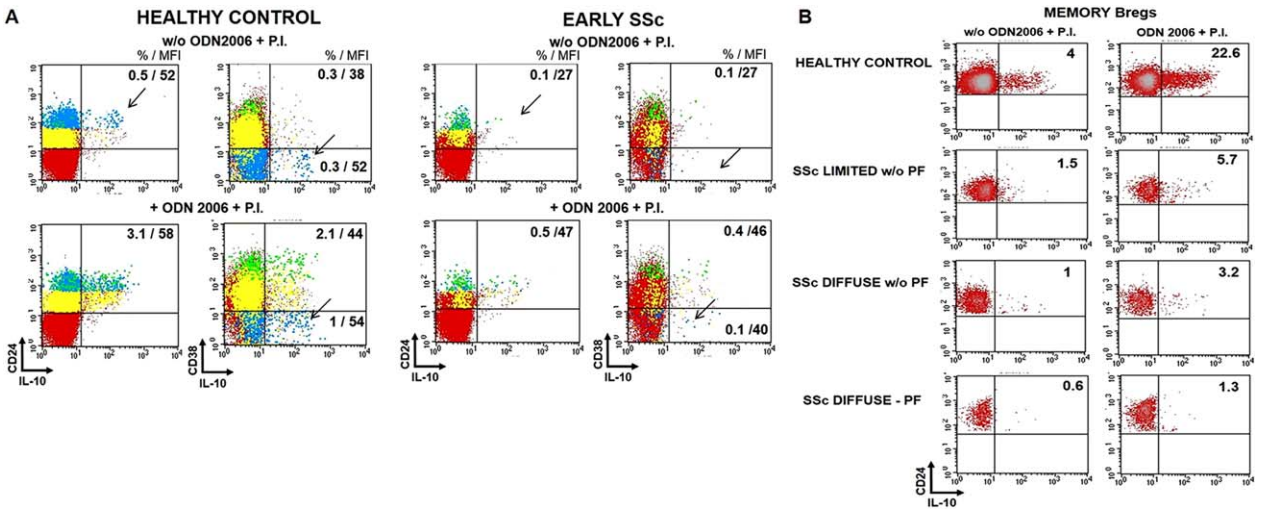


**Figure 4.** Flow cytometry analysis of intracellular expression of interleukin-10 (IL-10) by Breg cells. **A,** Peripheral blood mononuclear cells from a healthy control subject and a patient with early systemic sclerosis (SSc) were analyzed for IL-10 expression by flow cytometry. Dot plots show representative results from 1 of 5 experiments, denoting the percentage of IL-10–expressing cells and mean fluorescence intensity (MFI) of IL-10 expression among $CD24^{high}$ memory Breg cells (blue), $CD38^{high}$ transitional Breg cells (green), and $CD24^{low}$ B cells (yellow) in cultures stimulated with or without a CpG-containing oligonucleotide (ODN2006) and phorbol myristate acetate plus ionomycin (PI). **B,** IL-10 expression was assessed in memory Breg cells from patients with limited SSc without pulmonary fibrosis (PF) or patients with diffuse SSc with or without PF, as compared to healthy controls. Memory Breg cells were gated based on CD27 and CD24 expression in cultures stimulated with or without ODN2006 and propidium iodide. Values indicate the percentage of IL-10–expressing cells. Representative results from 1 of 5 independent experiments are shown.

with early SSc compared to healthy controls (mean ± SEM ratio 5.0 ± 0.62 versus 1.07 ± 0.14; $P = 0.001$). In patients with SSc-associated PF, memory B cells were nearly absent and naive B cells predominated, the latter constituting 30–50% of the total lymphocyte population. Decreased percentages of memory B cells were also detected, although to a lesser extent, in patients with RA-associated PF (results not shown).

**Defective IL-10 expression in phenotypic Breg cells in SSc.** Since production of IL-10 has long been considered the defining hallmark of Breg cells (15), we analyzed IL-10 intracellular staining in memory and transitional Breg cells from patients with early SSc and healthy controls. A representative experiment examining IL-10 expression by $CD19+CD27+CD24^{high}$ Breg cells and $CD19+CD24^{high}CD38^{high}$ Breg cells is illustrated in Figure 4A. In healthy controls, basal IL-10 expression derived mainly from $CD19+CD27+CD24^{high}$ memory Breg cells. After stimulation of the cells with ODN2006 for 24 hours, both memory and transitional Breg cells expressed high levels of IL-10. In contrast, in patients with early SSc, no basal IL-10 expression in memory and transitional Breg cells was detected, and very low IL-10 expression was detected after stimulation with ODN2006 (details with regard to IL-10 expression by each Breg cell subset, subgated based on their characteristic phenotypes, are available upon request from the corresponding author).

Memory and transitional Breg cells expressed comparable amounts of IL-10 in terms of the percentage of IL-10–positive cells. However, the MFI of IL-10 expression by memory Breg cells was higher compared to that of transitional Breg cells. A significant downregulation of IL-10 expression was also detected in memory Breg cells from patients with SSc-associated PF. Results of a representative experiment showing IL-10 expression by memory Breg cells in each SSc patient group with or without PF, as compared to healthy controls, are shown in Figure 4B.

**CD24 expression by Breg cells in early SSc.** In view of our findings demonstrating IL-10 production by $CD24^{high}$ cells but also considerable production of IL-10 by $CD24^{low}$ cells in the peripheral blood of healthy controls, we further characterized the expression of CD24 by B cells from patients with SSc. As illustrated in Figure 5A, $CD24^{high}$ Breg cells were detected in healthy controls but not in patients with early SSc. In healthy controls, the mean ± SEM percentage of $CD24^{high}$ cells was 6.4 ± 0.65%, compared to 1.4 ± 0.25% in patients with early SSc ($P < 0.001$). Percentages of $CD24^{low}$ cells were significantly increased in patients with early SSc compared to healthy controls (15 ± 1.4% versus 7.4 ± 0.9%;

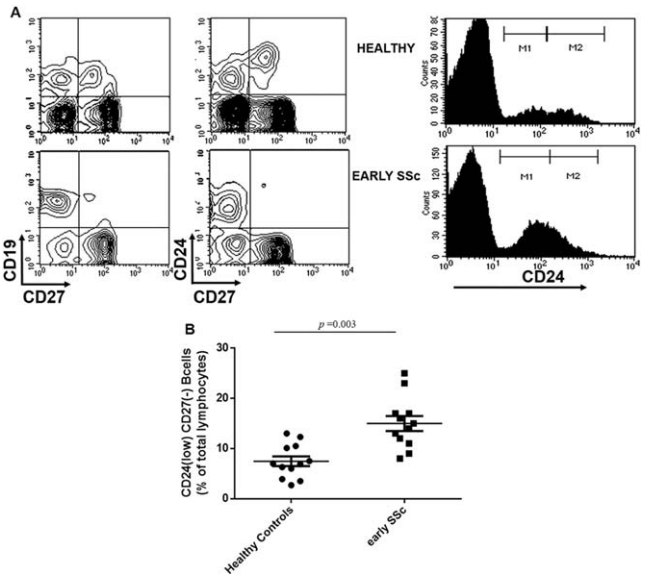


**Figure 5.** Low CD24 expression in CD27− lymphocytes in patients with early systemic sclerosis (SSc). **A,** Representative flow cytometry contour plots (left) and histograms (right) show expression of CD19, CD24, and CD27 in total lymphocytes from a representative patient with early SSc and a healthy control subject. **B,** Percentages of $CD24^{low}CD27-$ cells in total lymphocytes were determined in patients with early SSc and healthy controls. Each symbol represents an individual patient; bars show the mean ± SEM.

$P = 0.004$) (Figure 5B). Despite the high percentage of $CD24^{low}$ B cells in the peripheral blood of patients with early SSc, these B cells were unable to produce IL-10 upon stimulation with ODN2006 (results available upon request from the corresponding author).

**Impairment of p38 MAPK and STAT-3 activation in naive and memory B cells in early and established SSc.** In view of the reported data suggesting that IL-10 production by B cells stimulated through the TLRs is dependent on the activation of p38 MAPK (30) and STAT-3 (31), we investigated the effects of stimulation with ODN2006 (CpG ligand for TLR-9) on the phosphorylation of these signaling molecules in naive and memory B cells from SSc patients. We also examined the effects of BCR-specific stimulation using polyclonal IgM. $H_2O_2$ was also used to enhance B cell signaling (26).

P38 MAPK was phosphorylated in approximately one-third of the naive and memory B cells from healthy controls following stimulation with BCR or TLR-9. In the presence of $H_2O_2$, a synergistic increase in the percentages of phosphorylated p38 MAPK–positive B cells was noted. In contrast, the phosphorylation of p38 MAPK was impaired in naive and memory B cells from patients with early SSc compared to healthy controls (Figures 6A and B). This impairment was evident irrespective of whether naive or memory B cells were stimulated with either BCR, TLR-9, or $H_2O_2$ (Figures 6A and B). Phosphorylation of p38 MAPK and STAT-3 was also defective in total B cells from patients with early SSc and those with established SSc (results available upon request from the corresponding author).

## DISCUSSION

Our study demonstrated that the percentages of transitional Breg cells and memory Breg cells, as defined by the phenotypes $CD19+CD24^{high}CD38^{high}$ and $CD19+CD27+CD24^{high}$, respectively, were decreased in patients with SSc, particularly in those with SSc-associated PF. Furthermore, Breg cells were functionally impaired in patients with SSc, as they exhibited markedly decreased expression of IL-10 upon stimulation with TLR-9. Functional impairment of $CD19+CD24^{high}CD38^{high}$ Breg cells was also noted in patients with systemic lupus erythematosus (20). In antineutrophil cytoplasmic antibody–associated vasculitis, IL-10–producing Breg cells were reduced in one study (32), but other studies demonstrated that $CD19+CD24^{high}CD38^{high}$ Breg cells were numerically decreased but also able to express IL-10 upon stimulation (33,34). Furthermore, defective memory Breg cells have been detected in patients with Graves' disease (35).

In our study, we found that IL-10–expressing Breg cells were restricted to the $CD24^{high}$ phenotype. However, it should be mentioned that the precise phenotype of Breg cells (suppressive B cells) is still evolving. Thus, IL-10–expressing Breg cells with the $CD25^{high}CD71^{high}CD73-$ phenotype (36), $CD19+CD1d^{high}CD5+$ phenotype (37,38), or $CD25^{high}CD27^{high}CD86^{high}CD1d^{high}$transforming growth factor $\beta$ $(TGF\beta)^{high}$ phenotype (39) have been described. One study showed that IL-35 also expanded B10 cells (IL-35+ Breg cells), with the majority of them also expressing IL-35 receptor (40). In patients with SSc, Breg cell deficiency was characterized by impaired activation of p38 MAPK and STAT-3 in naive and memory B cells, irrespective of the stimulus. This reinforces the findings of previous studies showing that IL-10 production by B cells is dependent on the activation of p38 MAPK (30) and STAT-3 (31).

Breg cells, although they are found in small percentages in humans and mice, have an important immunoregulatory role in autoimmunity. B10 cells (IL-10–producing Breg cells) in humans and mice are potent regulators of macrophage and dendritic cell function (15). They negatively regulate inflammation and autoimmunity in animal models, as they have been found to suppress Th1 and Th17 immune responses (41). In mice, IL-10–producing B10 cells ameliorate collagen-

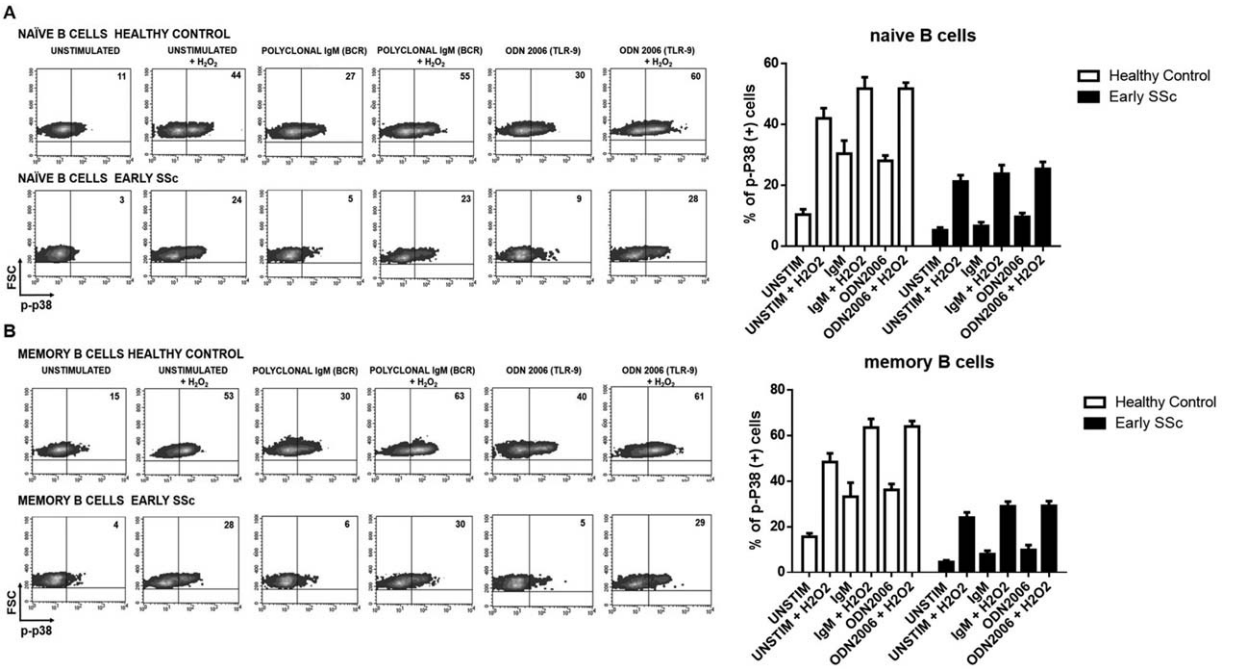


**Figure 6.** Impaired phosphorylation of p38 MAPK in naive and memory B cells from patients with early SSc. Phosphorylation of p38 MAPK was determined by phosphospecific flow cytometry in naive B cells (**A**) and memory B cells (**B**) from healthy control subjects and patients with early SSc. Cells were left unstimulated (unstim) or stimulated with polyclonal IgM (B cell receptor [BCR]) or ODN2006 (Toll-like receptor 9 [TLR-9]), in the presence or absence of $H_2O_2$ (which deactivates phosphatases). Left, Representative density plots illustrate the percentages of phosphorylated p38 MAPK–positive B cells under each condition. Right, Cumulative data from 5 independent experiments are shown. Bars show the mean ± SEM. See Figure 4 for other definitions.

induced arthritis by suppressing the generation of Th17 cells (42). In the chronic graft-versus-host disease (GVHD) mouse model of SSc, the early reconstitution of B10 cells suppresses chronic GVHD (43). IL-35+ Breg cells suppress experimental autoimmune uveitis (EAU), whereas adoptive transfer of IL-35+ Breg cells inhibits established EAU. In contrast, knockout mice lacking expression of IL-35 develop severe EAU (40). Glatiramar acetate (copaxone), used for the treatment of multiple sclerosis, may act via the stimulation of Breg cells (44). An mAb against TIM-1 expressed on Breg cells prevented the rejection of pancreas allografts (45). It should be mentioned that Breg cells can have suppressive action via IL-10–independent mechanisms, such as via TGF$\beta$ (46). The question still remains whether specialized B cell subsets with distinct regulatory capacities exist in a manner similar to that of Treg cells, or whether Breg cells exhibit plasticity. There is some evidence to suggest that the same B cells can promote either inflammation or tolerance through production of IL-6 or IL-10, depending on the specific stimulatory conditions of the microenvironment (47).

Two other findings in our study are worth mentioning: the increased expression of CD19 in B cells and the decreased percentages of memory B cells in patients with SSc. Our observations of increased expression of the stimulatory BCR CD19, increased percentages of naive B cells (CD19+CD27−), and reduced numbers of memory B cells (CD19+CD27+) are consistent with those in a previous study (48). Our study extended those observations to show a substantial increase in the expression of CD19 in patients with early SSc and in Breg cells. CD19 expression was less pronounced in B cells from patients with established SSc, and this might be related to treatment. Of note, IL-10–producing and FoxP3-expressing Breg cells are found to be predominantly within the $CD19^{low}CD5+$ B cell subset (29). CD19 is an important receptor, since its overexpression is associated with spontaneous autoantibody production, whereas deficiency in CD19 is associated with decreased fibrosis in animal models of SSc (6,7).

In our study, the decreased percentage of memory Breg cells in patients with SSc paralleled the decreased percentage of memory B cells. A near-complete absence of memory B cells (less than 5% of the total lymphocyte population) and aberrantly high percentages of naive B cells, typically in the magnitude of 30–50% of the total lymphocyte population, were main features in patients

with SSc-associated PF. Decreased percentages of memory B cells were also detected, although to a lesser extent, in patients with RA-associated PF. Deficiency in peripheral memory B cells has also been reported in other autoimmune conditions, such as Sjögren's syndrome, and autoimmune thrombocytopenia purpura (49,50). A decrease in memory B cells in SSc patients could be attributable to increased apoptosis. Spontaneous apoptosis was observed more frequently in memory B cells from SSc patients compared to healthy controls (48). It should be mentioned that memory B cells can migrate to the bone marrow in humans (51).

Our study convincingly demonstrates that Breg cells are decreased and functionally impaired in patients with SSc. The decreased number of memory B cells and their inability to produce IL-10 after the addition of an innate stimulus (TLR-9) may have important implications. Memory Breg cells suppress the monocyte innate immune response by suppressing tumor necrosis factor production (15,20). This finding, along with the increased expression of stimulatory CD19 receptor in B cells and the presence of functional anti-CD22 autoantibodies in patients with SSc (11), all support the notion of B cell autoaggression acting as an immunopathogenic mediator in this disease. In recent years, treatment with rituximab, an mAb that depletes B cells, decreased fibrosis in tight skin mice and in human SSc (4,52).

Our study provides a potential new treatment strategy for SSc. For instance, IL-35 may expand Breg cells in vivo in patients with SSc, as observed in a mouse model of uveitis, thus providing a potential treatment for SSc (40). Moreover, as has been observed in the EAE mouse model of multiple sclerosis (17), ex vivo expansion of B10 cells and reinfusion into patients with SSc may ameliorate disease.

## AUTHOR CONTRIBUTIONS

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. Dr. Sakkas had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Bogdanos, Sakkas.

**Acquisition of data.** Mavropoulos, Simopoulou, Varna, Liaskos, Katsiari, Sakkas.

**Analysis and interpretation of data.** Mavropoulos, Katsiari, Bogdanos, Sakkas.

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