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Defective regulation of autoreactive IL-6-producing transitional B lymphocytes is associated with disease in patients with systemic sclerosis

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Running Title: Defective transitional B-cell tolerance in SSc

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Abstract

Background: Systemic Sclerosis (SSc) has the highest case-specific mortality of any rheumatic disease and has no effective therapy. A clear manifestation of SSc is the presence of auto-antibodies. However, the origin of autoantibody-producing B lymphocytes and mechanisms of their activation, auto-antibody production and role remain unclear.

Objective: To identify mechanisms that contribute to pathogenic B cell generation, involvement in SSc and assess the altered distribution and function of B cells in patients.

Methods: Multi-colour flow cytometry was used 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 carried out by multiplex analysis.

Results: The study reveals a range of defects in B lymphocyte tolerance and cytokine production in SSc. Evidence is provided for altered distribution of transitional B cell subsets, increased production of IL-6 and IL-8 and defective tolerance induction in SSc B cells. In addition, the study reveals that B cells in SSc have a reduced ability to produce IL-10 when stimulated through innate immune pathways. In contrast to healthy individuals, tolerance checkpoints in SSc patients fail to suppress the emergence of B cells that produce autoantibodies with specificity to the Scl-70 antigen, a specificity strongly associated with SSc. These defects are paralleled by altered intracellular signalling and apoptosis following B cell receptor (BCR) engagement.

Conclusion: The study provides new insights into mechanisms underlying defective B lymphocyte responses in patients with SSc and their contribution to pathology.

INTRODUCTION

Systemic sclerosis (SSc) is a severe inflammatory disease characterized by excessive extracellular matrix (ECM) deposition in the skin and visceral organs (1). It has complex pathogenesis with two 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 high levels of auto-antibodies (auto-Abs) to nuclear proteins including topoisomerase I enzyme, centromere and RNA polymerase and endothelial cell and platelet derived growth factor receptor (1, 3). Evidence for pathogenic roles of these auto-Abs comes from studies revealing the association between their specificity and which tissues and organs are involved. Importantly also, these auto-Abs appear prior to disease onset. Furthermore, B lymphocytes accumulate at sites of disease, around small vessels in the skin (4) and in alveolar interstitium in patients with lung involvement (5). In addition to producing auto-Abs, B lymphocytes contribute to fibrosis through producing interleukin 6 (IL-6) (6).

The 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 against human CD20 (10, 11). In the tight-skin mouse model of SSc that is associated with constitutive CD19-mediated signaling (7, 12), inhibition of CD19 expression abrogates auto-Ab production, IL-6 production by B cells and ameliorate skin fibrosis (7, 8). Similar effects were noted when CD19 expression was suppressed in the bleomycin-treated mouse model of SSc (9). Furthermore, SSc patients with interstitial lung

and diffuse disease who are positive for anti-Scl70 auto-Abs benefit significantly from treatment with rituximab (10). Thus, no patient receiving rituximab exhibited lung function deterioration, whereas 5 out of 6 placebo-treated patients had a worsening of their disease. 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, activity index, depleted skin B lymphocytes and reduced dermal myofibroblasts and hyalinised 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 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 to B cell receptor- (BCR) responsive cells to antigen engagement (16). Murine transitional B cells are distinguishable from mature cells based on the level of CD23, CD21 and the developmental marker CD24 expression (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 current study was carried out to explore if defects in the maturation of, or tolerance induction in B cells at the transitional stage could relate to the emergence of high affinity pathogenic B cells in patients. Transitional B cells were studied for their ability to produce IL-6 as 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 a multi-colour 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.

METHODS

Patients

All patients fulfilled the 2013 EULAR/ACR criteria for SSc (21). Disease subsets were defined as patients with limited cutaneous SSc (lcSSc) when skin thickening was present distal to elbows and knees and as diffuse cutaneous SSc (dsSSc) when skin thickening affected both distal and proximal areas. Blood samples from 88 patients with SSc attending the clinics were obtained for the purpose of this study after their informed consents. Patients with interstitial lung disease were diagnosed based on characteristic changes visualised by high-resolution computed tomography (CT). Pulmonary arterial hypertension was diagnosed by right heart catheterization with mean pulmonary artery pressure of ≥25 mm Hg and normal pulmonary capillary wedge pressure. 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 (HCs) (mean age 40.2±11.3 years; 25-60 years) were also included for comparisons. Some of the tested 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 compliance with the Helsinki Declaration of 2013.

B lymphocyte enrichment

B lymphocytes were enriched from whole blood by negative selection using the EasySep Human B Cell Enrichment Kit (StemCell Technologies, Grenoble, France). All experiments presented in this study were carried out using freshly-enriched B lymphocytes from blood samples taken on the same day. The protocol of B cell enrichment involves using a cocktail of monoclonal antibodies (mAbs) with dual specificity for non-B cell components of blood mononuclear cells (MNCs) and red blood cells (RBCs). The cocktail aggregates all non-B cells with RBCs and these are then separated on Ficoll-Paque. The protocol is fast and purity of enriched B cells is >95%.

Cell staining and flow cytometry

Enriched B cells were stained with combinations of fluorochrome-conjugated antibodies (18). mAbs were purchased from the suppliers indicated as follows: APC-eFlour780-conjugated anti-human CD10 (Clone SN5c), PE-Cy5.5-anti-human CD19 (Clone: HIB19), PE-Cy7-anti-human CD32 (Clone 6C4), FITC-anti-human IgD (Clone: IA6-2), eFlour 450-anti-human CD21 (Clone: HB5) and PE-anti-human CD24 (Clone: eBioSN3) mAbs were all from eBioscience. Brilliant Violet 605-anti-human IgM (Clone: MHM-88), brilliant Violet 785-anti-human CD19 (Clone: HIB19) and brilliant Violet 711-anti-human CD27 (Clone: O323) were from BioLegend. PerCP-Cy5.5-anti-CD38 mAb (Clone: HIT2) was from BD Biosciences. For intracellular cytokines, stained cells were fixed, permeabilized and stained with either APC-anti-human IL-6 mAb (Clone: MQ2-13A5), APC-anti-human IL-10 mAb (Clone: JES3-9D7) (both from BD Bioscience) and were assessed by LSR-Fortessa FACS machine and FacsDiva software.

For measuring the frequency of IL-6- or IL-10-producing B lymphocytes, enriched cells were stimulated with PMA (50ng/mL) and Ionomycin (250ng/mL) in the presence of Golgi-Plug for 6 hours at 37°C (BIO-RAD) and then stained with different combinations of fluorochrome-conjugated mAbs and assessed by LSR-Fortessa. 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 cDNA for human CD40L. In some experiments, the cells were stimulated with the soluble TLR9 agonist CpG-ODN either alone or in the presence of 10µg/mL of mouse mAb to human CD40 (clone G28-5) pre-coated onto culture plates. The cells were cultured for 72 hours at 37°C. For co-culture experiments involving the CD40L-transfected L-fibroblasts, these were first incubated with 10µg/mL mitomycin C for 2 hours then enriched B cells 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 (MSD) multiplex kits as described (23).

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

For measuring phospho-STAT3 and phospho-NF-κB p65, enriched B cells were rested at 37°C for 3 hours and then stimulated either with anti-CD40 mAb (10ug/mL), CpG-ODN (1uM) or the combination of both for 10 minutes. The cells were fixed, permeabilized and stained with either Alexa 488-conjugated anti-phospho-STAT3 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 mAbs for surface proteins. The results were analysed using LSR-Fortessa.

Detection and quantification of autoantibody-producing B cells

ELISpot assay kits were obtained from Mabtech (Germany). 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^{o}C with 10µg/ml of scl-70 and FACS-sorted B cell subsets left to settle on the coated membranes for 20 hours at 37^{o}C. The membranes were washed and biotinylated antibody specific for human IgM added followed by streptavidin-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 (TMB). The number of spots on each membrane was counted and analysed using an automated AID ELISpot Reader System.

Statistical analyses

All data are presented as the mean±standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism version 6.05 (Graph Pad software, San Diego, CA). Student's *t*-test was used for comparing patients and the HCs and data considered significant when *P* values were <0.05.

RESULTS

Patient cohort

The demographic and clinical profiles of the SSc patient cohort studied are summarised in Supplementary Table 1**.** The cohort included 88 patients; 74 (84.1%) were females. Forty patients (45.5%) had diffuse cutaneous SSc (dcSSc) and 48 (54.5%) limited cutaneous SSc (lcSSc). The dcSSc group had a mean age of 49±11.9 years (range 25-75 years) and disease duration of 7.7±7 years (1-31 years). The mean age of patients in the lcSSc group was 61±10.9 years (31-83 years) and mean disease duration of 13.6± 9.4 (2-49 years). Twenty

four patients had anti-centromere antibodies (27.3%) and fifteen (17.0%) anti-RNA polymerase antibodies. Six patients (6.8%) had anti-U3RNP auto-antibodies (auto-Abs), five (5.7%) anti-U1RNP auto-Abs, ten (11.4%) had unidentified anti-nuclear auto-Abs (ANA), four (4.5%) were ANA negative, one (1.1%) had anti-Jo1 auto-Abs and one patient had antiPM-Scl auto-Abs. Twenty three patients (26.1%) had interstitial lung disease and the majority seropositive for anti-Scl70 auto-Abs (n= 22; 25%). Two patients (2.3%) had SRC and 4 (4.5%) pulmonary arterial hypertension. Forty seven of the patients (53.4%) were treated with immunosuppressive agents including mycophenolate mofetil, 30 patients, or methotrexate, 7 patients (24). A majority of the patients who were not on immunosuppressive drugs had lcSSc (n=33).

Altered distribution 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 total numbers of B cells and distribution of subsets in the patients and HCs. The results showed that patients with SSc, generally, had higher numbers of B cells compared with the HCs (153.7±32.9 vs. 99.2±20.3 cells/μL blood, respectively; $P<0.01$). When B cell subsets were categorised based on CD24 and CD38 expression, the data showed that the patients had significantly more transitional B cells ($CD24^{hi}CD38^{hi}$) (19.85±1.87 cells/μL blood corresponding to 13.6±0.93% of total B cells) compared with the HCs (7.04.5±1.09 cells/μL blood corresponding to 6.81±0.62% of total B cells; $P<0.001$) (Figure 1A). However, the percentage of mature naïve ($CD24^{+}CD38^{+}$) and memory B cell numbers ($CD24^{hi}CD38^{+}$) was lower in the patients (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% total B cells, respectively) compared with the HCs (68±4.85 cells/μL blood and 17.72±1.66 cells/μL blood corresponding to 67.76±1.87% and 18.98±1.66% of total B cells; $P<0.01$ and

P=0.056). 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 studies to determine numbers, responses and regulation of transitional B cell subsets to determine why they are increased in the SSc. 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 regulatory/maturation. We recently categorized transitional $CD24^{high}CD38^{high}$ B cells into 4 subsets using a 10-color flow cytometry with FLOCK (Flow clustering without K) (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 subset 1 (T1) of B cells, expresses high levels of IgM, CD10 and CD32 and low levels of IgD, and CD21. Subset 2 (T2), expresses medium levels of IgM, IgD, CD10 and CD32 but high levels of CD21. T3 expresses low levels of IgM, IgD, CD10, CD21 and CD32 (Fig. 1B). Finally, one subset of transitional B cells expresses high levels of CD27, CD24 and CD38, and was designated transitional $CD27^{+}$ B cells (18). The current study revealed that SSc patients have significantly more T1 B cells than HCs (1.32±0.21 vs. 0.33±0.06 cells/μL blood corresponding to 11.2±1.0% vs. 6.7±0.5% of transitional B cells; *P*<0.01) (Fig. 1B, the scatter graph panels). In contrast, there was an increase in the total number but a decrease in the frequency of T2 cells in patients (4.87±0.78 vs. 2.52±0.57 cells/μL blood corresponding to 39.8±1.8% vs. 49.0±3.2% of transitional B cells; *P*<0.05). There was no differences in the frequency of T3 cells. There was, however, a trend towards increased $CD27^{+}$ transitional B cells in the patients but this was not statistically significant (1.82±0.42 and 0.70±0.16 cells/μL blood corresponding to 21.7±5.0% and 13.8±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 cell in patients with SSc is different from HCs, we studied cytokine production by the cells. Enriched B cells were activated with PMA and Ionomycin in the presence of Golgi-Plug and stained for intracellular IL-6. FACS analyses showed that the number of IL-6^+ B cells was higher in patients compared with HCs (51.8±7 vs. 22.8±6.2 cells/μL blood corresponding to 35.4±4% vs. 22.5±5.5%, of total B cells) (Fig. 2A). The number of IL-6^+ cells was also higher in transitional, mature naïve and memory B cells in the patients (Fig. 2B). The number and frequency of IL-6^+ transitional B cells were 2.8±0.7 cells/μL blood corresponding to 14.0±3.1% of transitional B cells in the patients compared with 0.5±0.13 cells/μL blood corresponding to 6.7±1.0% of transitional B cells in the HCs (P<0.01 and <0.05, respectively). When the number of $IL6^+$ transitional B cell subsets was determined, the frequency for IL-6^+ T1, T2 and T3 B cells in the SSc patients were 15.0±2.4%, 25.1±3.9% and 23.9±4.0% respectively. These were significantly higher than the HCs at 5.8±1.7%, 10.3±2.2% (P<0.01) and 10.7±2.4% (P<0.05), respectively) (Fig. 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^+ B cells in all the subsets compared with patients with mild disease. Similarly, patients with dcSSc had more IL-6^+ B cells than patients with lcSSC (Supplementary Figure 2). Furthermore, serum from anti-Scl-70^+ patients had more IL-6^+ B cells than those without this auto-Ab. 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 defence against infections and become polyreactive IgM-producing B cells, or mature to 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 to regulatory B cells (Bregs) 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$^+$ B cells reflects a reduction in IL-10-producing in SSc, we determined the frequency of IL-10$^+$ B cells. These analyses revealed that patients with SSc had more *ex vivo*-activated IL-10$^+$ B cells than the HCs (Supplementary Figure 1A). Results of IL-10$^+$ B cell subset analyses showed that transitional, mature and memory B cells all contained more IL-10$^+$ cells in the patients compared with the HCs although the differences were not statistically significant (Supplementary Figure 1B). When the frequency of IL-10$^+$ B cells in transitional B cells were determined there was a trend towards increased IL-10$^+$ T1 and T3 B cells in the patients compared with the HCs but the differences were not significant (Supplementary Figure 1C).

We next assessed if 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 either through CD40 alone, TLR9 alone (with its agonist CpG-ODN) or stimulating the cells through both pathways simultaneously and the level of IL-10 in culture supernatants determined. When B cells were stimulated through CD40 alone, B cells from the SSc patients produced more IL-10 (144.7±31.8pg/mL) than the HCs (59.1±17.1pg/mL) (P<0.05) (Supplementary Figure 1D). However, when B cells were stimulated by engaging TLR9 alone, or through both TLR9 and CD40, B cells from the patients produced

significantly less IL-10 (82.0±36.0pg/mL and 44.2±18.0pg/mL, respectively) than B cells from the HCs (190.3±35.7pg/mL and 163.4±37.9pg/mL, respectively) (P<0.05).

To determine if this reflects further functional differences between B cells from patients with SSc and HCs, we studied the ability of B cells to produce cytokines thought to be relevant to SSc pathogenesis. B cells were co-cultured for 72 hours with the mouse L-fibroblasts transfected with human CD40L and the level of cytokines in the culture supernatants measured. Results of cytokine level measurements revealed that B cells from SSc patients produced significantly more IL-1β and IL-8 (P<0.001 and P<0.05, respectively) and also GM-CSF compared with B cells from the HCs. In contrast, B cells from the patients produced less IL-12, IFNγ and TNFα than B cells from the HCs (Fig. 3).

Altered responsiveness of transitional B cells in SSc to B cell receptor (BCR) engagement

Our 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, however, there was clear evidence for a higher rate of apoptosis in the T1 subset compared with the other 3 subsets and also 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). Results of studying BCR-mediated apoptosis in transitional B cell subsets in the current study showed notable reduction in apoptosis in all the subsets in the SSc patients particularly in the T1 subset (13.6±4.1) compared with the HCs (47.1±12.9) (P<0.05) (Fig. 4A). This reduced ability of transitional B

cells in the patients to undergo BCR-mediated apoptosis was consistent the higher number of IL-6-producing B cells.

Our previous studies also revealed that differences between transitional and mature B cells in response to stimulation is associated with altered Ca^{2+}, PI3K and PLCγ2 signalling (18). Therefore, we examined signaling pathways that could relate to altered IL-6 and IL-10 production by transitional B cells in the SSc patients following TLR9-engagement (26). For this purpose, we examined the phosphorylation of nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) in transitional B cells. The phosphorylation of NF-κB p65 and STAT3, are key in CD40 as well as TLR9-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 between unstimulated B cells from the patients and HCs in the level of NF-κB p65 phosphorylation (Fig. 4B). However, when the B cells were stimulated through the TLR9, there was a significant reduction in the phosphorylation of STAT3 in T1 and $CD27^+$ transitional B cells from the SSc patients (121.8±10.4 and 177.0±22.7 in MFI) compared with the HCs (155.6±5.6 and 251.8±10.6, respectively) (P<0.05 for both) (Fig. 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 is different between transitional and mature B cells (28, 29). Thus, a major fraction of transitional B cells is polyreactive and are suggested to give rise to natural antibody-producing mature B cells (30). For the purpose of the current study, we examined differences in the frequency of B cells capable of producing IgM antibodies with specificity for Scl-70, a specificity strongly associated with SSc patients

(31, 32). To achieve this objective, the transitional B cell subsets were FACS-sorted and incubated for 4 days with CpG (to mimic T cell-independent stimulation) and levels of auto-antibody to Scl-70 produced by the different transitional B cell subsets determined by ELIspot. Our previous studies established that the T1 and $CD27^+$ subsets had the highest frequency of transitional B cells capable of differentiating to IgM-secreting cells. Interestingly, however, the ELIspot results revealed that the T2 subset of transitional B cells in SSc patients who are seropositive for anti-Scl-70 was the subset containing most of the B cells that produced IgM autoantibodies that bound to the Scl-70 antigen (Fig. 5A). Consistent with the detection of serum anti-Scl-70 auto-antibodies in patients, the number of IgM anti-$Scl\text{-}70^+$ transitional B cells were significantly higher in seropositive compared with 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 results either 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 part of 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 regulatory B 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 HCs, patients with SSc show defects in the regulation of these cells at an early stage of their developmental program. Thus, patients with SSc fail to eliminate or reduce the number of IL-6-producing B cells and also cells capable of producing autoantibodies with specificity for the hallmark SSc auto-antigen Scl-70. Furthermore, the results suggest that the failure to eliminate potentially pathogenic B cells in patients with SSc occurs at the T1 to T2 cell maturational stage.

Early studies of the phenotype and functions of murine transitional B cells revealed that three major subsets of these cells exist (17, 35, 36). In humans, however, the phenotype and biology of transitional B cells remained less well-defined than in mice. A recent study in our laboratories using multi-colour flow cytometry and functional assessment revealed that 4 different subsets of human transitional B cells exist (18). The criteria set for the analysis of transitional B cells in the current study, established based on our earlier study, show that, overall, the number of transitional B cells is higher in patients with SSc than HCs. The results also reveal that transitional B lymphocytes in patients with SSc contain a higher frequency of IL-6-producing B cells than in HCs. This 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 disease. 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 from 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 study confirmed that the frequency of IL-6-producing B cells is also 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 regulates autoreactive T1 to T2 B cell maturation. Clearly, however, further studies are required 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 the HCs, the frequency of T2 cells was not different between the two groups. These two subsets have different capacities to undergo apoptosis following BCR engagement. Thus, whereas T1 cells in HCs undergo apoptosis following BCR engagement, T2 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 the stage 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 is 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/tolerize Scl-70-specific B cells at the T1 to T2

maturational stage in SSc. Thus, the frequency of Scl-70-specific B cells at the T1 stage is low to detect among the large number of polyreactive B cells. However, when most polyreactive T1 B cells are depleted/tolerized as they mature to T2 cells, this allows for a relative increase in the frequency of Scl-70-specific B cells rendering them detectable within 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 it is likely to involve genetic factors that are manifested in defective intracellular signaling and/or enhanced IL-6 production. Interestingly, however, the preliminary studies of intracellular signaling did not reveal differences in basal NF-κB p65 phosphorylation between transitional B cells from SSc patients and HCs. However, both T1 and $CD27^+$ B cells from the patients showed a trend towards 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 TLR9.

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 regards to the reduced ability of B cells in patients with SSc to produce IL-10 following TLR9 but not CD40 engagement could provide further clues as to the cause of defects in the regulation of B cell responses in this disease. TLR9-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 two stages. During the initial stage of differentiation, TLR9 engagements promotes IL-10-producing B cells and then CD40/BCR engagement expands such Breg cells (42). Noteworthy, our results showed

defective TLR-9 induced STAT3 activation in patients with SSc but not when the cells were stimulated through the CD40 co-receptor (not shown).

The study has also revealed that B cells from patients with SSc produce significantly higher levels of the pro-inflammatory cytokines GM-CSF, IL-1β and IL-8 than the HCs. It will be of interest to evaluate directly the pathogenic contribution of these cytokines to the disease process in SSc. Further investigation will also be required to define the mechanisms that lead to the imbalance in the maturation of transitional B cells to become IL-6-producing potentially-pathogenic B cells rather than IL-10-producing Breg cells in SSc.

It was notable in this study that the majority of the SSc patients studied were on immunomodulating drugs that possibly could affect the B cell profiles seen. It is likely that these drugs could influence the level of IL-6 production by B cells as well as the number/percentage of IL-6-producing cells. A detailed study of individual patients is required to assess the effect of treatment with immunomodulating drugs on the B cell profiles in SSc as reported in our study. Additionally, the SSc B cell profiles/frequencies reported showed an association with disease severity/symptoms. For example, SSc patients with severe lung fibrosis had more IL-6^+ B cells within all subsets compared with patients with mild disease (not shown). Similarly, patients with diffuse scleroderma had more IL-6^+ B cells than patients with limited scleroderma (Supplementary Figure 2). Further, anti-Scl-70 positive patients had more IL-6^+ B cells B cells than seronegative patients for the autoantibody. However, these differences were not statistically significant. Therefore, a larger study will be required to address this issue conclusively.

In normal individuals, significant numbers of early immature B cells display varying degrees of self-reactivity/polyreactivity but the level of autoreactivity progressively declines within the B cell repertoire with cell maturation (43). In agreement with previous studies in lupus

and rheumatoid arthritis patients, our results highlight inadequate elimination of autoreactive B-cells in patients with autoimmune disease (43-45). The fact that very few if any spots could be detected in B cells from the HCs suggests that polyreactive autoantibodies are unlikely to have influenced our observation. However, it is not possible to completely exclude the possibility that some of the Scl-70 bound IgM could also bind dsDNA, insulin or, indeed, other self-antigens.

Studies in mice and humans have provided multiple lines of evidence to indicate that defects in central tolerance exists 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 rely on multiple factors including self-antigen availability, avidity and the ability to cross-link the BCR and engage TLRs and the abundance of survival factors, particularly BAFF/B-cell activating factor (46, 47). Further investigations will also be required to define the mechanisms that lead to the defect in central tolerance in SSc.

In conclusion, this study sheds new lights 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 the disease. Based on findings from this study a framework summarising predictions for pathways of transitional B cell development and possible defects in the regulation in patients with SSc can be suggested (Fig. 5B).

AUTHOR CONTRIBUTIONS

T.E.T. performed the experiments, analysed data, had intellectual input and wrote the first draft of the manuscript. Q.S., J.B. and S.H. helped in the design and analysis of B-cell phenotype by FACS. V.H.O., C.P.D., P.J-O made intellectual contribution to the analyses, gathered and provided reagents and clinical samples and data on the patients. D.J.A. and R.A.M. designed the experimental strategy, contributed to analysing the data and writing, editing and revising the manuscript.

KEY WORDS

Systemic Sclerosis; Transitional B-cells; Defective Tolerance Checkpoints, Pro-inflammatory cytokines

ABBREVIATIONS

SSc: Systemic sclerosis

HC: Healthy control

MNC: Mononuclear cell

Breg: Regulatory B-cell

PMA: Phorbol 12-myristate 13-acetate

FACS: Fluorescence-activated cell sorting

mAb: Monoclonal antibody

BCR: B-cell receptor

HRP: Horse-radish-peroxidase (HRP)

TMB: Tetramethylbenzidine

TLR: Toll-like receptor

NF-κB: Nuclear factor kappa B

STAT: Signal transducer and activator of transcription

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FIGURE LEGENDS

Figure 1: B cell subset distribution in healthy controls (HCs) and patients with SSc. A) The upper panel shows the gating strategy for identifying transitional, mature and memory B lymphocytes in peripheral blood based on expression levels of CD24 and CD38**.** The middle panel provides the actual numbers of transitional, naïve/mature and memory B cell in each μL of blood in HCs and patients with SSc. The bottom histogram shows the frequency of transitional, naïve/mature and memory B cells in the blood of the two groups. The data is presented as the Mean + standard error of the mean (SEM) from experiments using blood from 10 HCs and 10 SSc patients. * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns: non-significant for statistical differences using Student's *t* test. **B**) The top panel depicts the staining and gating strategies used for identifying transitional T1, T2, T3 and The $CD27^+$ subsets within $CD24^{hi}CD38^{hi}$ B cells as described before (18). The low scatter graph panels show the frequency of T1, T2, T3 and $CD27^+$ B cells subsets within transitional B cells in the 9 HCs and 11 patients with SSc**.**

Figure 2: IL-6 expression in total B cells and B cell subsets in HCs and patients with SSc. A) The top panel depicts FACS quadrants from one HC and one SSc patient showing the gating strategy for identifying IL-6^+ *ex vivo* B cells stimulated with PMA and Ionomycin. The lower histogram summarises the frequency of IL-6^+ *ex vivo* B cells from the blood of HCs and patients with SSc. The data is presented as the Mean + SEM for 10 HCs and 11 SSc patients. **B)** The left panel is for FACS quadrant profiles of B cells from one HC and one patients with SSc depicting the strategy for quantifying IL-6^+ transitional, naïve/mature and memory B cell subsets. The histogram panel on the right summarises the frequency of IL-6^+ transitional, naïve/mature and memory B cells in 4 HCs and 10 patients with SSc. **C**) Percentage of IL-6^+ T1, T2, T3 and $CD27^+$ transitional B cell subsets in 9 HCs and 11

patients with SSc. * indicates $p<0.05$, ** indicates $p<0.01$, ns: non-significant for statistical differences using the Student's *t* test.

Figure 3: Cytokine production by B lymphocytes from HCs and patients with SSc. Enriched B lymphocytes were cultured for 72 hours in plates pre-coated with mitomycin C-treated mouse L-fibroblast transfected with cDNA for human CD40L. Culture supernatants were collected, centrifuged and tested for the cytokines shown in the figure using MSD Multi-Plex kits. Data represents the Mean + SEM for the indicated cytokines produced by B lymphocytes from 5 HCs and 10 SSC patients SSc. * indicates $p<0.05$, *** indicates $p<0.001$, ns: non-significant for statistical differences using Student's *t*-test.

Figure 4: Altered responses of transitional B cells from patients with SSc to BCR and TLR9 engagement. A) Bar charts representing percentages of spontaneous (two left columns) or BCR-induced apoptosis (two right columns, + anti-IgM) in each chart. Apoptotic cells were identified by staining with Annexin V^+. B cell subsets from 8 HCs and 9 patients with SSc were studied**.** The data are presented as the Mean + SEM. * indicates $p<0.05$, ns: non-significant statistical differences using the Student's *t* test. **B)** Summary of basal NF-κB p65 and STAT3 phosphorylation data (with no stimulation) in transitional B cell subsets in HCs and SSc patients. Mean fluorescence intensity (MFI) for basal phosphorylation levels of NF-κB p65 and STAT3 in transitional B cell subsets from 5 HCs and 4 patients with SSc. **C)** Bar charts summarising NF-κB p65 and STAT3 phosphorylation data in transitional B cell subsets stimulated by engaging TLR9 with its ligandin CpG-ODN. The results are presented as MFI for levels of TLR-induced phosphorylation of NF-κB p65 and STAT3 in transitional B cell subsets from the 5 HCs and 4 SSc patients.

Figure 5: The frequency of anti-Scl-70 autoantibody-producing B cells in transitional B cells. A) ELIspot of IgM anti-Scl-70-producing transitional B cells in HCs and SSc patients. The number of transitional B cell subsets that produced anti-Scl-70/10^4 cells is shown. The results are presented as the Mean+SEM obtained from experiments using blood from 4 HCs, 6 SSc patients who were seropositive and 6 SSc patients who were seronegative for anti-Scl-70 autoantibodies. * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns: non-significant for statistical differences using Student's *t* test. **B)** Cartoon 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 regulatory B 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 IL-6-producing B cells, some with specificity for Scl-70. The dotted arrows represent where defective selection could occur.

SUPPLENETARY FIGURES AND TABLE

Figure 1: The frequency of IL-10^+ B cells and IL-10 production in HCs and SSc patients. A) The left panel shows a FACS quadrants depicting IL-10^+ B cells in one HC and one SSc patient. IL-10^+ B cells were identified following activation through CD40 by co-culture of the cells for 72 hours on plates pre-coated with mitomycin C-treated L-fibroblasts transfected with human DC40L cDNA. The histogram on the right summarises the frequency of IL-10^+ B cells in 8 HCs and 10 SSc patients. **B**) FACS quadrants for identifying IL-10^+

transitional, mature and memory B cells. The histogram on the right summarises % of IL-10^+ B cells. **C**) Percentage of IL-10^+ T1, T2, T3 and $CD27^+$ transitional B cells in 8 HCs and 10 SSc patients. **D**) A histogram summarising results of quantifying IL-10 production by B cells. The B cells were from 4 HCs and 10 SSc patients. The cells were enriched and cultured for 72hr either with mitomycin C-treated mouse L-fibroblast expressing human CD40L alone, activated through TLR9 or activated with a combination of anti-CD40 mAb and CpG-ODN. The level of IL-10 was determined using MSD Multiplex kits. * indicates $p<0.05$, ** $p<0.01$, ns: non-significant using Student's *t*-test.

Figure 2: IL-6 expression in total B cells and B cell subsets in HCs and patients with limited and diffuse SSc. A) Bar chart summarising the frequency of IL-6^+ *ex vivo* B cells from the blood of HCs and patients with limited and diffuse SSc. **B)** Bar chart summarising the frequency of IL-6^+ T1, T2, T3 and $CD27^+$ transitional B cell subsets. The results are presented as the Mean+SEM IL-6^+ B cells in the blood of 9 HCs, 4 patients with limited SSc and 7 patients with diffuse SSc. * indicates $p<0.05$, ** $p<0.01$, ns: non-significant for statistical differences between the groups using the Student's *t* test.

Table 1: The epidemiological, serological profiles and the major internal organ involvement in the SSc patient cohort studied**.** Abbreviations: Anti-Scl-70: Anti-scleroderma-70 antibodies; Anti-U3RNP: Anti-U3 ribonucleoprotein antibodies; Anti-U1RNP: Anti-U1 ribonucleoprotein antibodies; Anti-Jo1 antibodies: Anti-aminoacyl tRNA synthetase antibodies; Anti-PM-Scl: Anti-polymyositis Scleroderma antibodies; ANA: Anti-nuclear antibodies.