Reduced non-switched memory B cell subsets cause imbalance in B cell repertoire in systemic sclerosis

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ABSTRACT

Objective. *Analysis of peripheral blood B lymphocytes in patients with systemic sclerosis (SSc) has provided evidence for specific alterations in naive and memory B cell balance. However, memory B cell subsets in SSc have not been thoroughly investigated. This study sought to identify phenotypic abnormalities and activation markers in peripheral blood memory B cells in SSc subtypes.*

Methods. *Blood samples were obtained from 28 SSc patients with early form of disease (9 limited (lcSSc), 19 diffuse cutaneous SSc (dcSSc)) and 15 healthy controls. After magnetic bead separation of CD19+ B cells, multiparametric flow cytometry was performed and CD19+CD27-IgD+ naive, CD19+CD27+ memory, CD19+CD27+IgD+ non-switched memory CD19+CD27+IgD- switched memory, CD19+CD27-IgD- double negative (DN) memory, CD80+ or CD95+ activated cells were identified.*

Results. *The proportion of naive B cells was higher (p=0.046) in SSc than in controls, with a decreased percentage of memory (p=0.018), especially non-switched memory B cells (p=0.015). The dcSSc patients had a significantly higher frequency of switched memory and DN memory B cells compared to lcSSc patients (p=0.025 and p=0.031). Percentage of CD95+CD27+ memory and CD95+ DN memory B cells was also significantly elevated in dcSSc compared to lcSSc patients (p=0.038 and p=0.045).*

Conclusion. *We conclude that the decreased proportion of memory B cells in SSc is due to reduction of non-switched memory B cells, resulting in an imbalance between the tolerogenic and activated memory B cell types. Elevated switched and activated CD95+ DN memory B cells may serve as a biomarker for dcSSc and can have a pathogenic potential by cytokine and autoantibody production.*

Introduction

Systemic sclerosis (SSc) is a connective tissue disease with an autoimmune background characterised by excessive extracellular-matrix production, endothelial cell damage and vascular injury in the skin and visceral organs (1). Several immunological events may contribute to SSc development, including B cell functional abnormalities such as the production of certain autoantibodies and cytokines (2-4), modulation of T cell and dendritic cell functions, and also altered antigen presentation (5). Various autoantibodies recognysing different cellular components have been identified in SSc (6, 7), which closely correlate with the clinical manifestations of the disease (8-10). Of these the anti-topoisomerase-I (anti-Scl-70) and anti-RNA polymerase autoantibodies are highly associated with the diffuse cutaneous form of SSc (dcSSc) (7), while anti-centromere (ACA) antibodies are more frequently detected in the limited form of the disease (lcSSc). As another feature of abnormal B-cell functionality CD19 has been shown to be overexpressed in SSc patients and was related to polyclonal B cell activation leading to both multiple autoantibody production (11, 12), and infiltration into the lesions of the skin and lungs (13). Furthermore, by increased IL-6 and IL-10 production B cells in SSc patients may modulate the T helper cell response, activate macrophages and affect tissue fibrosis (14).

With regard to the B-cell distribution there are contradicting reports about the total B cell count in SSc, but the elevated naive and decreased, but activated memory B cell subsets were described by multiple research groups

(5, 15, 16). These findings closely correlate with the clinical efficacy of the anti-CD20 Rituximab (RTX) monoclonal antibody therapy in early stage of SSc (17) resulting in depletion of both circulating and dermal B cells (18), causing improvement of skin and pulmonary fibrosis (19-22).

CD19 is a widely used lineage marker of B cells (23), while CD27 is considered a universal memory B cell marker. Naive B cells can be characterised by their lack of CD27 expression and IgD display (24). The high frequency of mutations within productively rearranged VH genes in the CD27+ B cell populations supports the CD27-based distinction of naive and memory B cells (25). CD27+ memory B cells can be further divided into two major subpopulations: CD19+CD27+IgD- switched and CD19+CD27+IgM+IgD+ non-switched B cells. Switched memory B cells are generated during T-dependent responses, are able to recall rapidly their previous encounter with specific antigens, and produce large-scale, high-affinity, epitope-specific antibodies.

The origin and function of the non-switched memory cells is still not well defined, but they express Ig with mutated V-region without heavy chain class switch, thus are often referred to as "IgM memory" B cells (26). These cells may not require a T-B interaction and may be involved in T-independent immune responses. Changes in the distribution of non-switched and switched memory B cells were investigated in rheumatoid arthritis (RA), Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE), but it has not yet been examined in SSc (27-31).

Besides these B cells, a new CD19+CD27-IgD- double negative (DN) memory subset has also been described recently and has been implicated in the pathomechanism of autoimmune diseases (32-34). DN cells possess class-switched and mutated Ig genes through a mechanism similar to the germinal centre-derived CD27+IgD- switched memory cells (35). The increased frequency of the DN cells was described in SLE (32, 33) and in patients with mixed connective tissue disease (34); however it has not been investigated in SSc yet. In this study we performed an extensive phenotypic analysis of peripheral blood B cell subsets in SSc including non-switched, switched and DN memory B cells and their activation to evaluate whether their alterations could contribute to better understanding of the disease.

Table I. Clinical and laboratory characteristics of the SSc patients enrolled.

	SSc (n=28)	lcSSc (n=9)	dcSSc (n=19)
Age (years), mean (SD)	52.6 (12.7)	50.67 (9.98)	53.33 (13.7)
Gender (female), n (%)	23/28 (82.1%)	9/9 (100%)	14/19 (73.7%)
Disease duration (years), mean (SD)	3.0 (1.7)	3.0 (1.9)	2.21 (1.6)
Active disease[1], n (%)	14/28 (50%)	3/9 (33.3%)	11/19 (57.9%)
Organ involvement			
Pulmonary fibrosis[2], n (%)	18/28 (64.3%)	4/9 (44.4%)	14/19 (73.7%)
Pulmonary arterial hypertension[3], n (%)	1/28 (3.6%)	0/9 (0%)	1/19 (5.3%)
Renal crisis, n (%)	2/28 (7.1%)	0/9 (0%)	2/19 (10.5%)
MRSS, mean (SD)	8 (10.7)	2.0 (1.4)	10 (10.6)
Antibodies			
anti-centromere (ACA) +, n (%)	8/28 (28,6%)	6/9 (66,7%)	2/19 (10.5%)
anti-Scl-70+, n (%)	9/28 (32,1%)	2/9 (22.2%)	7/19 (36.8%)
anti-RNA-polymerase III+, n (%)	3/28 (10.7%)	0/9 (0%)	3/19 (15.8%)
Inflammatory laboratory parameters			
Elevated ESR[4], n (%)	4/28 (14.3%)	0/9 (0%)	4/19 (21.1%)
Hypocomplementaemia[5], n (%)	6/28 (21.4%)	4/9 (44.4%)	2/19 (10.5%)
Elevated CRP[6], n (%)	9/28 (32.1%)	3/9 (33.3%)	6/19 (31.6%)
Therapy			
immunosuppressive therapy	10/28 (35.7%)	0/9 (0%)	10/19 (52.6%)
-low-dose corticosteroid	5/28 (32.1%)	0/9 (0%)	5/19 (26.3
-cyclophosphamide+ corticosteroid	5/28 (17.9%)	0/9 (0%)	5/19 (26.3%)

[1]According to the European Scleroderma Study Group disease activity index (EScSG index >3); [2]Lung fibrosis detected by high resolution CT and/or forced vital capacity <80%; [3]Right ventricle pressure ≥40 mmHg measured by right heart catheterisation; [4]Erytrocyte sedimentation rate ≥30mmHg/h; [5]Complement 3 <0,9g/l and/or complement 4 <0,1g/l; [6]C-reactive protein ≥5 mg/l.

Materials and methods

Patients

We recruited 28 SSc patients (23 females and 5 males; 9 lcSSc and 19 dcSSc; mean (SD) age: 52.6 (12.7) years; mean (SD) disease duration: 3.0 (1.65) years) at the University of Pécs, Department of Rheumatology and Immunology, classified as dcSSc or lcSSc based on the criteria proposed by LeRoy *et al*. (36). All patients, except one fulfilled the 2013 ACR/EULAR SSc classification criteria (37). Onset of SSc was defined as the date of the first non-Raynaud's symptom. 15 age- and gender-matched healthy volunteers were also investigated. Blood samples were collected using standard procedure in vacuum tubes containing heparin. Our standard clinical investigation protocol (38, 39) included lung function tests, echocardiography, high-resolution computer tomography (HRCT) of the lungs and right heart catheterisation (RHC), if necessary. Pulmonary fibrosis was characterised by detection of fibrosis with high resolution CT and/or decreased forced vital capacity (FVC<80%). Disease activity was recorded according to the European Scleroderma Study Group (EScSG) disease activity index (40) and the disease was considered active with index values >3. The patients' basic characteristics are summarised in Table I.

All participants gave their informed consent to the study. The study was approved by the Hungarian National Ethics Committee (84-256/2008-1018EKU).

B cell enrichment

PBMCs were isolated using Ficoll density gradient centrifugation, followed by magnetic bead separation of CD19+ cells using EasySep™ Human CD19 Positive Selection Kit (Stemcell Technologies, USA) according to the manufacturer's instructions. achieving >95% purity.

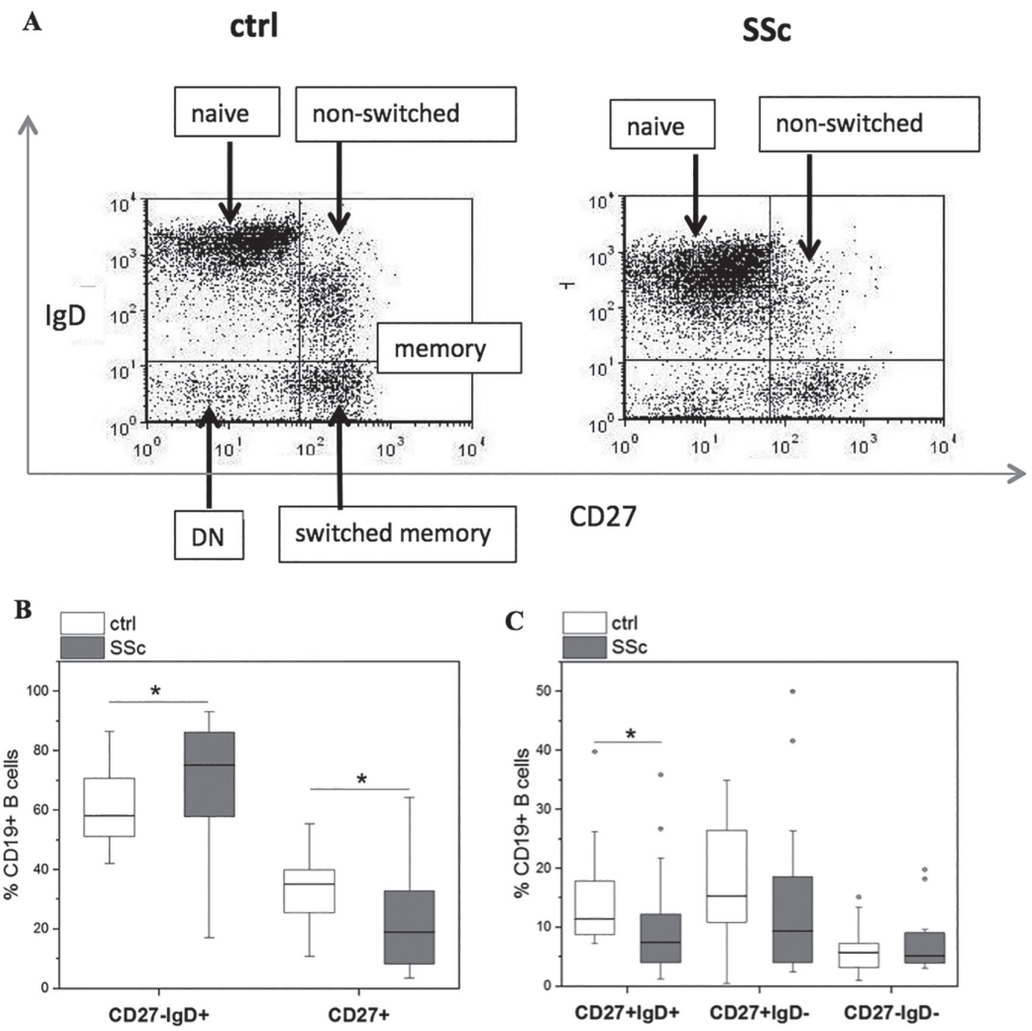

Fig. 1. Percentage of naive and memory B cell subsets in SSc patients and healthy controls (ctrl)
(**A**) Representative flow cytometry plots of MACS purified CD19+ B cells stained with CD27 and IgD. (**B**) Elevated naive (CD27-IgD+) and diminished memory (CD27+) B cells and (**C**) decreased non-switched (CD27+IgD+) memory B cells are shown as percentage of CD19+ total B cells. Boxes show interquartile ranges (IQR), whiskers indicate lowest and highest values, horizontal lines represent medians, circles indicate outliers of 1.5xIQR,
*represents significant *p*-values ($p<0.05$).

Flow cytometric detection of B cell subpopulations

Multiparametric flow cytometry was performed on the highly purified CD19+ cells with antibodies specific for CD20, CD27, IgD, CD80, and CD95 molecules. PE-conjugated anti-CD20 (2H7; eBioscience, USA) antibody was used to examine the purity of the separated B cells. To distinguish between naive and memory B cell subsets and to study the expression of activation markers, three-colour analysis was conducted using a combination of PE-conjugated anti-IgD (IA6-2; BD Pharmingen, USA), PE-Cy5 conjugated anti-CD27 (O323; eBioscience) and either FITC conjugated anti-CD80 (2010; eBioscience) or FITC conjugated anti-CD95 (DX2; eBioscience) antibodies. Stained cell images were acquired using a FACS Calibur flow cytometer (Becton Dickinson, USA) and analysed with FCS express 4 software (DeNovo software, USA). Using anti-CD27 and anti-IgD labelling CD27- naive, CD27+ memory, CD27+IgD+ non-switched, CD27+IgD- switched memory and CD27-IgD- DN B cell subsets were distinguished and were shown as percentage of total CD19+ B cells (Fig. 1A). The CD95 and CD80 expression of naive and memory cells were also analysed.

Statistical analysis

Statistical evaluation was performed with SPSS v. 22.0 statistics package (IBM, USA). Variables were expressed as medians and interquartile ranges. The non-parametric Kruskal-Wallis and Mann-Whitney U tests were used to compare data between the investigated groups. *p*-values <0.05 were considered significant.

Results

Memory and non-switched memory B cell ratios are decreased in SSc compared to healthy controls

To detect B cell abnormalities in SSc, we first compared naive and memory B cell ratios and then analysed memory B cell subsets as percentage of total CD19+ B cells isolated from PBMC. Similarly to previous findings (5) we found elevated CD27-IgD+ naive ($p=0.046$) and decreased CD27+ memory B cell ratios ($p=0.018$) in SSc patients compared to healthy controls (Fig. 1B). Upon comparison of the two SSc subgroups (dcSSc and lcSSc) to the healthy controls we observed a similar tendency, but significant alterations were found only in lcSSc cases (Table IIA).

Next we further analysed memory B cell subsets on the basis of CD27 and IgD expression. We distinguished CD27+IgD+ non-switched, CD27+IgD-

Table II. Statistical data for B-cell populations measured in the study*.

A

	A control	B SSc	C lcSSc	D dcSSc	A *vs.* B	A *vs.* C	A *vs.* D	C *vs.* D
					p-values			
CD27-IgD+	58.28 (51.16-70.85)	74.97 (57.76-88.45)	82.81 (74.92-90.32)	72.01 (44.94-83.23)	0.046	<0.001	0.493	0.016
CD27+	35.10 (25.53-40.04)	19.06 (7.74-32.95)	11.75 (5.02-20.94)	20.13 (10.93-45.23)	0.018	<0.001	0.228	0.037
CD27+IgD+	11.43 (8.78-17.8)	7.42 (3.76-12.43)	4.71 (1.42-12.29)	9.03 (6.39-14.52)	0.015	0.012	0.066	0.156
CD27+IgD-	15.27 (10.89-26.5)	9.36 (3.96-18.75)	6.55 (3.45-8.89)	12.72 (4.3-23.46)	0.063	0.002	0.430	0.025
CD27-IgD-	5.73 (3.2-7.31)	5.25 (3.88-9.12)	4.13 (3.32-6.05)	7.74 (4.1-9.24)	0.426	0.201	0.106	0.031
CD27+CD95+	7.47 (4.32-10.38)	5.44 (2.7-11.68)	3.1 (2.31-4.97)	9.5 (2.95-13.12)	0.559	0.024	0.706	0.038
CD95+CD27-IgD-	10.57 (8.71-12.1)	14.32 (9.06-16.33)	11.29 (8.06-14.71)	16.07 (10.01-17.05)	0.138	0.570	0.099	0.045

B

	Autoantibodies		*p*-values	pulmonary fibrosis		*p*-values
	ACA+	anti-Scl-70+		not present	present	
CD27-IgD+	89.27 (72.29-90.132)	72.02 (43.65-75.32)	0.027	89.27 (76.83-90.43)	72.02 (44.94-79.72)	0.099
CD27+	6.86 (4.73-19.76)	21.53 (18.14-44.43)	0.015	9.95 (5.34-21.41)	20.83 (11.55-37.62)	0.057
CD27+IgD+	3.18 (1.61-11.95)	10.27 (7.16-15.81)	0.074	4.67 (2.07-11.81)	9.33 (6.21-15.17)	0.072
CD27+IgD-	4.06 (2.84-8.16)	12.72 (9.36-22.28)	0.005	5.43 (3.01-9.04)	11.47 (6.48-23.46)	0.024
CD27-IgD-	5.41 (3.35-9.1)	7.74 (4.03-13.16)	0.370	4.1 (3.54-5.86)	7.74 (4.14-9.24)	0.036
CD27+CD95+	3.28 (2.23-5.44)	10.71 (4.4-13.45)	0.031	2.95 (1.82-6.42)	8.97 (3.39-12.85)	0.045
CD95+CD27-IgD-	9.41 (7.62-12.41)	16.17 (11.71-18.54)	0.036	10.09 (9.41-12.43)	16.11 (14.71-17.42)	0.009

*Data represent medians and IQRs.

switched and CD27-IgD- DN cells. Regarding memory B cell subsets, only the non-switched memory B cells percentage was significantly decreased in SSc samples compared to healthy controls (p=0.015). The frequency of switched memory and DN memory B cells was not significantly different in patients compared to healthy controls (p=0.063 and p=0.426) (Fig. 1C). Percentage of non-switched memory B cells diminished in both lcSSc and dcSSc patients, but significant difference was only in the lcSSc cases (p=0.012 and p=0.066) compared to the healthy controls (Table IIA).

Altered memory B cell subsets are associated with different clinical forms of the disease

Although both the total memory and non-switched memory B cell subsets in SSc patients were decreased compared to healthy controls, we asked whether the B memory cell reduction is coupled with further alterations in the memory B cell subpopulations in defined SSc subgroups, such as dcSSc or lcSSc and anti-Scl-70+ or ACA+ cases, respectively (Table I). Relative to total CD19+ B cells the proportion of CD27+IgD- switched memory B cells was significantly higher in dcSSc than in patients with lcSSc (p=0.025) (Fig. 2A and Table IIA). Correlation of memory B cell deviation with the presence of various autoantibodies revealed that this cell group was also significantly elevated in the anti-Scl-70 antibody positive group compared to ACA positive patients (p=0.005) (Fig. 2B and Table IIB). A similar tendency was found in the RNA polymerase III positive cases compared to ACA positive patients (data not shown). Furthermore SSc patients having pulmonary fibrosis also showed a significantly higher switched memory B cell proportion than patients without pulmonary fibrosis (p=0.024) (Fig. 2C and Table IIB).

To extend these findings on memory B cell alterations we also investigated a recently reported new population of memory B cells lacking the expression of CD27 and IgD surface markers called DN B cells (32) in SSc subgroups. In our analysis dcSSc patients had higher proportion of DN memory B cell subset than lcSSc cases (p=0.031) (Fig. 2A, Table IIA) and patients having pulmonary fibrosis also showed significantly higher DN memory B cell proportion than those without pulmonary fibrosis (p=0.036) (Fig. 2C and Table IIB). No significant associations were found with disease activity, duration of the disease, CRP or complement 3 and 4 serum levels (data not shown).

Fas receptor expressing memory B cells are increased in severe subset of SSc

Recent studies in phenotype analysis of SSc patients also suggested the contribution of B cell activation to the clinical features of SSc and their possible role in the pathogenesis of the disease, prompting us to determine the activation profile of B memory subsets in SSc patients. Frequencies of naive, memory B cells and memory B cell subsets expressing either CD80 or CD95 were also analysed in the defined groups of SSc patients. We found higher frequencies of CD27+CD95+ activated memory B cells (p=0.038) (Fig. 3A and Table II2A) and CD95+ DN memory B cells (p=0.045) were characteristic in dcSSc cases compared to lcSSc form (Fig. IIIC). The percentage of these memory B cell subsets was also elevated in patients with pulmonary fibrosis compared to patients not developing pulmonary fibrosis (p=0.045 and p=0.009 respectively) (Fig. 3B and 3D and Table IIB).

No significant associations were found with disease activity, duration of the disease, CRP or complement 3 and 4 serum levels (data not shown). Analysis of CD80+ B cell subsets revealed no significant alterations among the investigated SSc subgroups (data not shown).

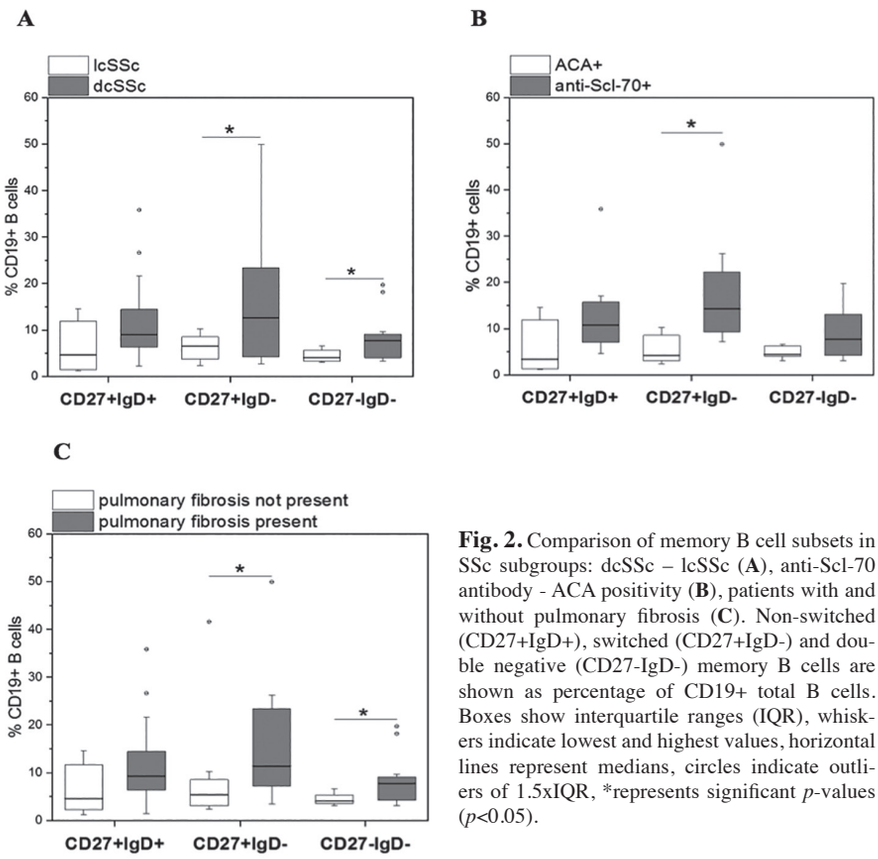

Fig. 2. Comparison of memory B cell subsets in SSc subgroups: dcSSc – lcSSc (**A**), anti-Scl-70 antibody - ACA positivity (**B**), patients with and without pulmonary fibrosis (**C**). Non-switched (CD27+IgD+), switched (CD27+IgD-) and double negative (CD27-IgD-) memory B cells are shown as percentage of CD19+ total B cells. Boxes show interquartile ranges (IQR), whiskers indicate lowest and highest values, horizontal lines represent medians, circles indicate outliers of 1.5xIQR, *represents significant *p*-values ($p<0.05$).

Effects of immunosuppressive therapy on the distribution of memory B cell subsets

Next we addressed the question, whether the memory B cell differences between lcSSc and dcSSc patients were influenced by the immunosuppressive therapy, as 10 patients with dcSSc with severe form of the disease had received low dose corticosteroid (n=5) or combined treatment (corticosteroid with cyclophosphamide (n=5) (Table I) during the period of investigation. Comparison of the untreated and treated dcSSc patients showed that low dose corticosteroid treatment alone did not change the B cell distribution. In case of combined treatment only the DN memory B cell ratio was higher in the treated dcSSc patients compared to the untreated dcSSc cases (9.67; 9.24-19.01 *vs*. 4.39; 3.62-7.99 *vs*. p=0.002).

Discussion

We studied the composition of memory B cell subpopulations in different groups of patients with diverse forms of SSc. B cells contribute to autoimmune disease development by their autoantibody and inflammatory cytokine production, modulation of antigen processing and presentation, and also by generating ectopic germinal centres (41). Flow cytometric analysis of peripheral blood lymphocytes has revealed changes in B cell subsets of patients with SLE, SS, RA and SSc (5, 15, 16, 27-31). Furthermore, B cell-depleting antibody (RTX) therapy is effective in the treatment of RA (42) and it shows some clinical efficacy in SSc (17-22), emphasising the importance of B cells in the development of the disease.

Studies focusing on changes in B cell homeostasis in SSc are limited. Since SSc is different in its pathogenic pathways from other systemic autoimmune diseases, the possible role of B cell subsets particularly in the development of fibrosis and vascular inflammation seems to be an important question. Expanded population of naive B cells accompanied by the reduction of memory B cells has been shown in patients with SSc when compared to healthy controls (5), which is in agreement with our findings. The sustained loss of memory B cells induces enhanced production of naive B cells in bone marrow to maintain B cell homeostasis. The decrease of memory B cells in peripheral blood could be explained by their enhanced apoptosis or by the migration of activated memory B cells to the inflamed tissues.

Our study is the first to address the relevance of memory B cell subpopulations and their abnormalities in the different subsets of SSc. We have found that the decreased memory B cell pool is due to the decreased frequency of non-switched memory B cells in patients with SSc compared to healthy controls. We have also shown that in dcSSc, in anti-Scl-70 antibody positive cases and in patients with clinically significant pulmonary fibrosis the memory B cell pool is decreased while the percentage of switched memory B cells is increased.

To exclude the modulatory effect of concomitant or previous immunosuppressant treatment, we also compared the B cell subsets in dcSSc patients with or without undergoing corticosteroid or steroid/cyclophosphamide combined treatment. Patients treated with steroid only did not show any difference to untreated patients; however, patients with higher disease index scores needing the more robust combined treatment showed an even more enhance increase of DN memory B-cell pool, which we attribute to the more severe conditions as primary factor, and not to the pharmacological effect of treatment. Thus in our view the elevated DN cell number is characteristic for the dcSSc if we compare it to lcSSC or controls, whereas further increase of DN memory B cell frequency within the dcSSc group is a sign of more severe onset of this form of the disease.

Non-switched memory B cells in peripheral blood may represent a mixture of germinal centre- and marginal zone-derived cells (43) resembling natural autoantibody producing B1 B cells. These cells are important in the protection against some infections and have a crucial role in maintaining self-tolerance, especially by the synthesis of IgM natural autoantibodies (43, 44). Reduction in the number

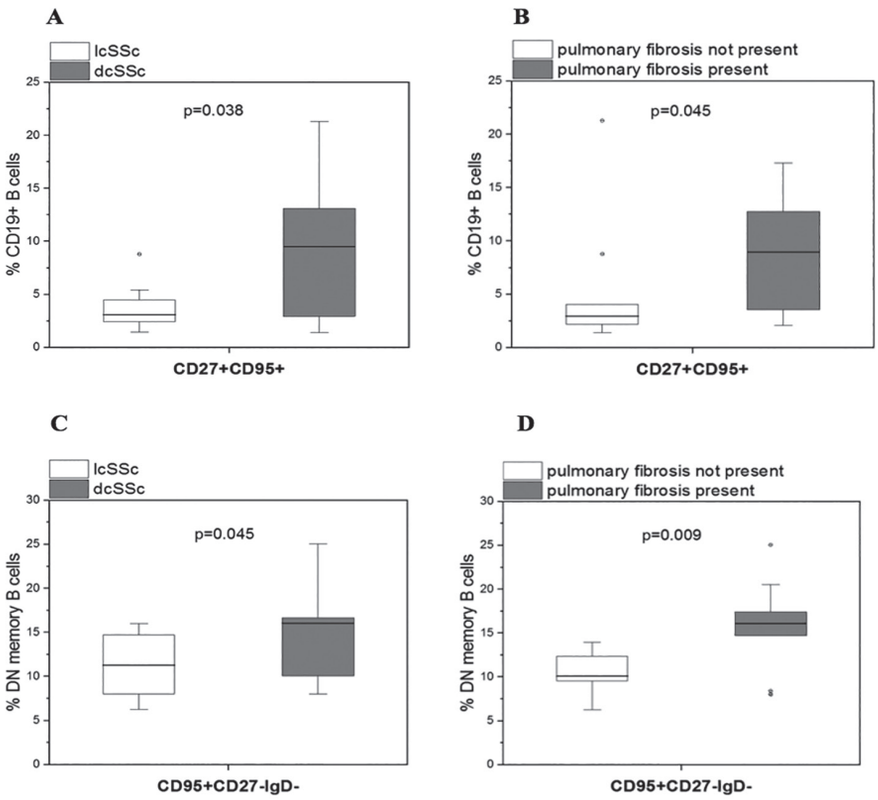

Fig. 3. Elevated CD95 expressing CD27+ memory B cells as percentage of CD19+ total B cells and increased CD95 expressing DN memory B cells are shown in dcSSc subset (**A**, **C**) and in patients having pulmonary fibrosis (**B**, **D**). Boxes show interquartile ranges (IQR), whiskers indicate lowest and highest values, horizontal lines represent medians, circles indicate outliers of 1.5xIQR.

of non-switched memory B cells was described in Sjögren's syndrome (SS) (31) and also in patients with SLE (44). These findings support the notion that the disturbed immunoregulation in SSc may, at least in part be due to the imbalance of tolerogenic T-independent (non-switched) and the T-dependent (switched) memory B cell responses.

The recently described new DN memory B cells lacking expression of both CD27 and IgD surface markers (32) have not been investigated in SSc subgroups. Of note, we found higher proportion of DN B cells in dcSSc and in patients with pulmonary fibrosis. Increased frequency of DN memory cells was also found in SLE and MCTD patients, and it was associated with higher disease activity index and the presence of autoantibodies (32, 34, 45). The DN memory cells phenotypically and morphologically resemble switched memory cells although the frequency of somatic mutations in DN cells is lower. There is a difference between the expression levels of IgG subclasses by these cell types suggesting that these cells could have different roles in the immune response (46).

In addition to the increased percentage of switched and DN memory B cells we have also found higher frequencies of CD95+CD27+ memory and CD95+ DN memory B cells in dcSSc and in patients having pulmonary fibrosis. The higher frequencies of these particular memory B cell subsets seem to be associated with the severe disease forms. The upregulation of CD95 was observed in SSc and was linked to the increased apoptosis of memory B cells (5). Although CD95 as Fas receptor is an important mediator of apoptosis (47), its signalling could be modulated by B cell receptor-derived signals e.g. as a receptor cross-talk and could result in the activation and survival of antigen specific B cells. Recent studies indicate that CD95 also plays a role in inflammation (48) since it can induce the production of pro-inflammatory cytokines in macrophages independent of caspase-mediated apoptotic signalling (49, 50). Consequently, CD95 expressing memory B cells may have a different cytokine expression profile and may also have a role in the maintenance of chronic inflammation present in SSc. Thus CD95 seems to be a useful marker for identifying atypical, activated DN memory B cells of pathogenic relevance and they represent an expanded population in patients with SLE and MCTD as well which correlate with disease activity (33, 34).

In conclusion, our results indicate that the frequency of non-switched memory B cells is lower in SSc than in healthy controls. Our results also showed that higher frequencies of switched memory and CD95+ DN memory B cells are associated with the presence of pulmonary fibrosis and the diffuse form of the disease, which may thus serve as potential biomarker for less favourable disease course. Further studies are necessary to identify those mechanisms that may connect the altered B-cell memory subset distribution and enhanced fibrosis in SSc patients.

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