
Increased expression levels of FcγRIIB on naïve and double-negative memory B cells in patients with systemic sclerosis

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Received on July 30, 2018; accepted in revised form on December 7, 2018.

Clin Exp Rheumatol 2019; 37 (Suppl. 119): S23-S31.

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EXPERIMENTAL RHEUMATOLOGY 2019.

Key words: FcγRIIB, B cell, systemic sclerosis, interstitial lung disease

ABSTRACT

Objective. In systemic sclerosis (SSc), B cell hyperactivation and abnormality are considered to play an important role in the disease pathophysiology. We aimed to clarify if the abnormal activation of B cells involves inhibitory FcγRIIB on B cells in SSc patients.

Methods. Blood samples were collected from 76 SSc patients (38 limited cutaneous SSc and 38 diffuse cutaneous SSc) and 59 healthy controls. We evaluated the expression levels of FcγRIIB on different B cell subsets. B cells were classified into five subsets based on their surface phenotype as measured by flow cytometry: naïve B cells (CD19⁺IgD⁺CD27⁻), pre-switched memory B cells (CD19⁺IgD⁺CD27⁺), double-negative (DN) memory B cells (CD19⁺IgD⁻CD27⁻), switched memory B cells (CD19⁺IgD⁻CD27^{mid}), and plasmablasts (CD19⁺IgD⁻CD27^{high}). The expression levels of the activation markers CD80, CD86, and CD95 were also examined.

Results. The expression levels of FcγRIIB on SSc naïve and DN memory B cells were significantly increased compared to healthy controls ($p < 0.05$ and $p < 0.001$, respectively). CD80, CD86, and CD95 expression levels were significantly higher in all five B cell subsets, except for CD80 in switched memory B cells and plasmablasts. Increased FcγRIIB expression levels on DN memory B cells were associated with disease activity as assessed by the European Scleroderma Study Group Activity Index, presence of interstitial lung disease (ILD), and reduced lung function. Intravenous cyclophosphamide pulse therapy decreased FcγRIIB expression levels on memory B cell subsets.

Conclusion. SSc B cells may exhibit compensatory elevation in the expression levels of FcγRIIB in order to suppress the abnormal activation of B cells. In addition, FcγRIIB expression

levels may serve as a marker of severe complications, such as ILD, in SSc.

Introduction

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterised by excessive extracellular matrix deposition in the skin and internal organs with autoimmune involvement. A representative feature of autoimmunity in SSc is the existence of autoantibodies (autoAbs) that target antigens not only in the nucleus or cytoplasm (1), but also endothelial cell antigen (2), matrix metalloproteinase (3), or the platelet-derived growth factor receptor (4). Although the pathophysiology of SSc remains unclear, B cell abnormalities characterised by autoantibody (autoAb) production and polyclonal B cell activation are considered to play an important role (5). SSc patients have expanded naïve and activated memory B cells that may result in B cell hyperactivation in SSc. For instance, the expression levels of CD19, a B cell-specific member of the Ig superfamily and a positive regulator of B cell function, on blood B cells from SSc patients were significantly higher (~20%) compared to healthy individuals (6). In clinical application, B cell-targeted therapies, including anti-CD19 antibody (Ab) and anti-CD20 Ab, are currently under development for both skin thickening and lung fibrosis in SSc patients (7-9).

Memory B cells are divided into three distinct subsets depending upon relative expression levels of CD27 and IgD in humans: CD27⁺IgD⁺ pre-switched, CD27⁻IgD⁻ double-negative (DN), and CD27^{mid}IgD⁻ switched memory B cell subsets (10). Among these populations, the DN memory B cell compartment has attracted attention recently. DN memory B cells possess class-switched and mutated immunoglobulin (Ig) genes through a mechanism similar to the germinal centre-derived CD27^{mi-}

Funding: This work was supported by a grant on Research on Intractable Diseases from the Ministry of Health, Labor and Welfare of Japan (to Kazuhiko Takehara).

Competing interests: none declared.

dIgD- switched memory B cells (11). The increased frequency of DN memory B cells was described in patients with systemic lupus erythematosus (SLE) (12) and mixed connective tissue disease (13). In SSc, the frequency of memory B cells was decreased due to reduction of pre-switched memory B cells, whereas SSc patients with diffuse cutaneous type (dcSSc) had a higher frequency of DN and switched memory B cells compared to those with limited cutaneous type (lcSSc) (14).

FcγRIIB (CD32B) is a low-affinity receptor for the Fc fragment of IgG and the only FcγR expressed by B cells (15). When FcγRIIB is cross-linked to the B cell receptor (BCR), the B cell activation threshold is increased and autoAb production is decreased. FcγRIIB on dendritic cells, macrophages, activated neutrophils, mast cells, and basophils inhibits the functions of activating FcγRs, such as phagocytosis and pro-inflammatory cytokine release (16, 17). This accumulating evidences indicates that FcγRIIB expression and function underlies the importance in regulating susceptibility to autoimmune disease and defense against foreign pathogens (18). In fact, previous studies reported an association of FcγRIIB and autoimmune connective tissue diseases. Kyogoku *et al.* demonstrated the association of a polymorphism of FCGR2B (I232T) with susceptibility to Japanese SLE patients (19). Decreased FcγRIIB expression levels were observed in memory B cells, but not on naïve B cells from patients with SLE (20). In rheumatoid arthritis (RA), the association of the FcγRIIB (I232T) variant with increased radiological joint damage was reported, suggesting a critical determinant of the FcγRIIB polymorphism in disease severity (21). On the other hand, increased FcγRIIB expression levels were found on naïve and transitional B cells in SSc patients (22). However, FcγRIIB expression levels on each memory B cell subset and an association with clinical characteristics in SSc have not been elucidated.

In the present study, we evaluated FcγRIIB expression levels on B cell subsets in SSc patients, including naïve and pre-switched, DN, and switched

memory B cells and plasmablasts. In addition, we examined the correlation of FcγRIIB expression levels with cell surface activation markers and clinical characteristics.

Materials and methods

Patients

We analysed 76 Japanese patients with SSc (68 women and 8 men) who visited Kanazawa University Hospital. All patients fulfilled the criteria for classification of definite SSc proposed by the American College of Rheumatology (23). Table I summarises the profile of the 76 SSc patients enrolled in this study. The median (range) age was 51 (20-77) years and disease duration was 5.3 (0.2-34) years. SSc patients were classified into lcSSc or dcSSc according to the criteria proposed by LeRoy *et al.* (24): 38 patients (35 women and 3 men) had lcSSc and 38 patients (33 women and 5 men) had dcSSc. Indirect immunofluorescence tests were performed using slides of monolayer HEp-2 cells (Medical & Biological Laboratories [MBL], Nagoya, Japan) as a substrate. Anticentromere Abs (ACA) were considered to be positive if serum diluted 1:40 produced a characteristic staining pattern on HEp-2 cells. Anti-topoisomerase I (anti-topo I) Abs and anti-RNA polymerase III (anti-RNAP) Abs were screened by enzyme-linked immunosorbent assay (ELISA) kits (MBL) according to the manufacturer's instructions. ACA were positive in 24 patients, anti-topo I Abs in 25, and anti-RNAP Abs in 15. The remaining 14 patients had other autoAbs.

Patients with interstitial lung disease (ILD) that was defined as "active" based on findings including chest computed tomography, pulmonary function test, and levels of serum ILD markers including KL-6 and SP-D, were treated with intravenous cyclophosphamide (IVCY) pulse therapy (500 mg/m²). In this study cohort, 19 patients were diagnosed as having active ILD and received IVCY pulse therapy, whereas 29 patients with ILD were judged to have "inactive" disease and thus did not undergo IVCY pulse therapy. None of the patients without ILD received IVCY pulse therapy. Meanwhile, 25 patients

were treated with prednisolone for thickened skin. Cyclosporine A (2-3 mg/kg/day) was given to two patients. Among the 76 SSc patients enrolled in the present study, 32 patients received no immunosuppressive therapy, 24 received prednisolone only, 18 received prednisolone with IVCY pulse therapy, 1 had prednisolone with cyclosporine A, and 1 underwent prednisolone, cyclosporine A, and IVCY pulse therapy. In a follow-up study, we analysed six individual patients with ILD before and after IVCY pulse therapy. All six patients were treated with six cycles of IVCY pulse therapy in combination with 20 mg/day of prednisolone at the initial dosage. Prednisolone was then tapered and continued at a maintenance dosage. The mean (range) dosage of prednisolone at blood sampling was 10 (1-20) mg/day. The second set of blood samples were taken after IVCY pulse therapy. As healthy controls, 59 age- and sex-matched healthy Japanese individuals were used. Ethics committee approval for this study was obtained at Kanazawa University. All subjects provided informed written consent according to the Declaration of Helsinki.

Clinical assessments

The modified Rodnan total skin thickness score (mRSS) was used to semi-quantitatively assess the degree of skin sclerosis (25). Organ system involvement is defined principally as described elsewhere (26).

Disease activity was assessed according to the European Scleroderma Study Group Activity Index (EScSG-AI) (27) and the revised European Scleroderma Trials and Research group (EUSTAR) activity index (28). Disease severity was measured using the Scleroderma Disease Severity Scale (DSS) (29) and the summed DSS score was assessed (30). The EScSG-AI in dcSSc was significantly higher than that in lcSSc (2.42 vs. 1.18, $p < 0.0001$) (Table I). The revised EUSTAR activity index was also higher in dcSSc patients compared to lcSSc (2.38 vs. 0.78, $p < 0.0001$). The summed DSS scores of 7.89 and 4.13 for dcSSc and lcSSc, respectively, significantly differed ($p < 0.0001$).

Table I. Clinical profile of 76 systemic sclerosis patients.

	SSc (n = 76)	lcSSc (n = 38)	dcSSc (n = 38)
Age at onset, median (range) years	51 (20-77)	52 (20-75)	49 (23-77)
No. of females/no. of males	68/8	35/3	33/5
Disease duration, median (range) years	5.3 (0.2-34)	7.5 (0.3-34)	3.2 (0.2-13)
MRSS, median (range)	12 (0-42)	3 (0-15)	21 (5-42)
EScSG activity index, average (range)	1.80 (0-4.5)	1.18 (0-2.5)	2.42 (1-4.5)
Revised EUSTAR activity index, average (range)	1.58 (0-6.25)	0.78 (0-2.5)	2.38 (0-6.25)
Medsger disease severity index, average (range)	6.01 (1-15)	4.13 (1-8)	7.89 (3-15)
Clinical features			
Raynaud's phenomenon	95% (72/76)	97% (37/38)	92% (35/38)
Nailfold bleeding	54% (41/76)	50% (19/38)	58% (22/38)
Pitting scar	26% (20/76)	8% (3/38)	45% (17/38)
Digital ulcers	28% (21/76)	18% (7/38)	37% (14/38)
Contracture of phalanges	36% (27/76)	11% (4/38)	61% (23/38)
Diffuse pigmentation	42% (32/76)	21% (8/38)	63% (24/38)
Telangiectasia	22% (17/76)	18% (7/38)	26% (10/38)
Calcification	4% (3/76)	3% (1/38)	5% (2/38)
Organ involvement			
Interstitial lung disease	63% (48/76)	42% (16/38)	84% (32/38)
Oesophagus	37% (28/76)	26% (10/38)	47% (18/38)
Heart	3% (2/76)	0% (0/38)	5% (2/38)
Kidney	5% (4/76)	3% (1/38)	8% (3/38)
Arthralgia/arthritis	24% (18/76)	16% (6/38)	32% (12/38)
Muscle weakness	9% (7/76)	8% (3/38)	11% (4/38)
Autoantibodies			
Anti-topoisomerase I antibody	33% (25/76)	18% (7/38)	47% (18/38)
Anti-centromere antibody	32% (24/76)	55% (21/38)	8% (3/38)
Anti-RNA polymerase III antibody	20% (15/76)	8% (3/38)	32% (12/38)
Others	18% (14/76)	21% (8/38)	16% (6/38)
Treatment			
Corticosteroid	33% (25/76)	29% (11/38)	37% (14/38)
IVCY + Corticosteroid	25% (19/76)	8% (3/38)	42% (16/38)
No immunosuppressive therapy	42% (32/76)	63% (24/38)	21% (8/38)

dcSSc: diffuse cutaneous SSc; lcSSc: limited cutaneous SSc; mRSS: modified Rodnan total skin thickness score; EScSG: European Scleroderma Study Group.

Flow cytometric analysis

To distinguish between naïve B cells, memory B cell subsets, and plasma-blasts, and to examine the expression of FcyRIIB and activation markers, seven-color analysis was performed by staining the cells with the following antibodies: FITC-conjugated anti-CD19 (HIB19), PE-conjugated anti-CD32 (FUN-2), PerCP/Cy5.5-conjugated anti-CD27 (O323), BV510-conjugated anti-IgD (IA6-2), PE/Cy7-conjugated anti-CD80 (2D10), BV421-conjugated anti-CD86 (IT2.2), and APC-conjugated anti-CD95 (DX2) (all from BD Biosciences, NJ, USA). Heparinised blood samples (50 µl) were stained at 4°C with mAb for 20 minutes, as previously described (31). Blood erythrocytes were lysed after staining using the RBC Lysis Buffer as detailed by the

manufacturer (BD Biosciences). Cells were analysed on a BD FACSCant II flow cytometer (BD Biosciences). Positive and negative populations of cells were determined using nonreactive isotype-matched mAb (BD Biosciences) as controls for background staining.

Real-time RT-PCR

Heparinised blood samples were obtained from 68 SSc patients and 53 healthy controls, and peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll-Hypaque gradient centrifugation. CD19⁺ B cells were purified by magnetic labeling with CD19 microbeads (Miltenyi Biotec, Paris, France GmbH). Total RNA was isolated from CD19⁺ cells using RNeasy spin columns (Qiagen, Crawley, UK). Total RNA was reverse tran-

scribed to complementary DNA using a reverse transcription system with random hexamers (Promega, Madison, WI, USA). FcyRIIB messenger RNA (mRNA) was analysed using real-time reverse transcription-polymerase chain reaction (RT-PCR) on an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA, USA). Sequence-specific primers and probes were designed using Pre-Developed TaqMan Assay Reagents (Applied Biosystems). GAPDH was used to normalise mRNA. Relative expression of real-time PCR products was determined using the $\Delta\Delta C_t$ method (32) to compare target gene and GAPDH mRNA expression. One of the control samples was chosen as a calibrator sample.

Statistical analysis

Statistical analyses were performed using Student's *t*-test for comparison of sample means between two groups or the Bonferroni adjustment for multiple comparisons. Spearman's rank correlation was used to examine the relationship between two continuous variables. The data shown are the median (range) unless otherwise indicated. *p*-values <0.05 were considered statistically significant. Statistical analyses were performed using JMP version 10 statistical software (Cary, NC).

Results

Frequency of each B cell subset in SSc patients

Human blood memory B cells can be identified in CD19⁺ B cells depending on the relative expression levels of CD27 and IgD (10) (Fig. 1A). We first compared the percentage of total CD19⁺ B cells between SSc patients and healthy controls. Frequencies of CD19⁺ B cells in mononuclear cells were comparable between the two groups (Fig. 1B). We then analysed B cell subsets as the percentage of total CD19⁺ B cells. Similar to previous findings (14, 22), we found increased CD27-IgD⁺ naïve B cell (*p*<0.001), decreased CD27⁺IgD⁺ pre-switched memory B cell (*p*<0.001), and increased CD27-IgD⁻ DN memory B cell ratios (*p*<0.001) in SSc patients, compared to healthy controls (Fig. 1B).

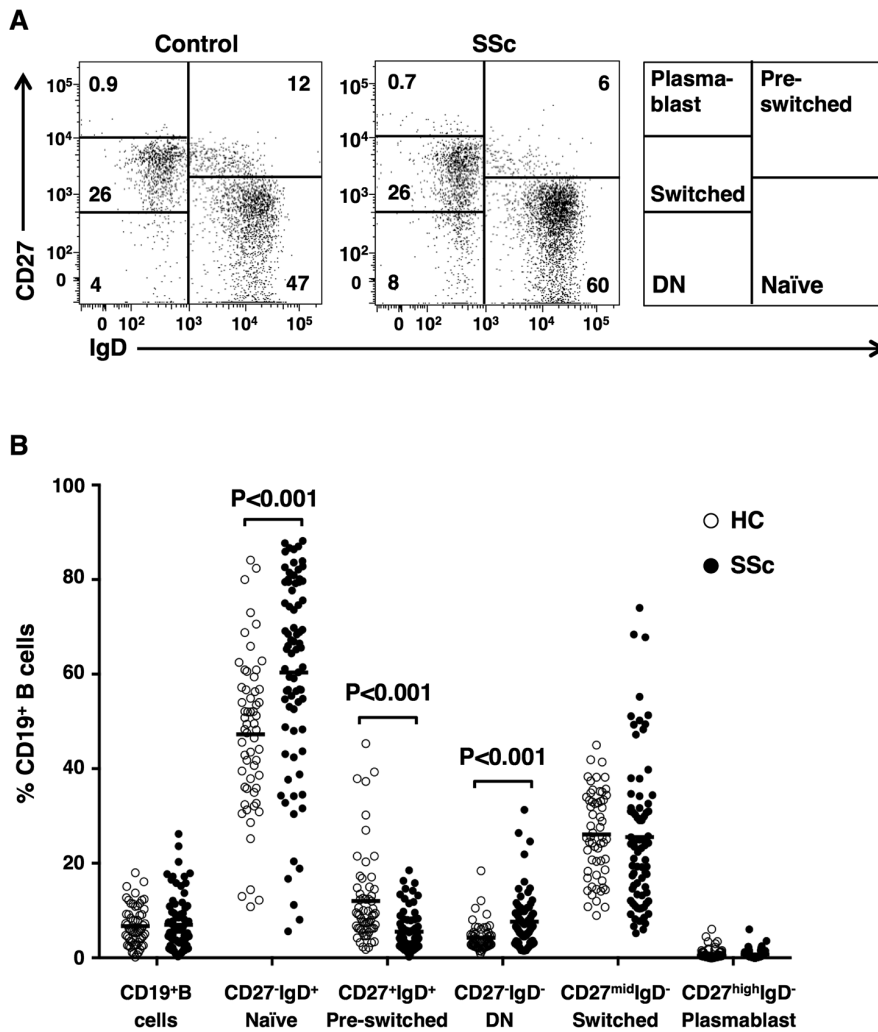


Fig. 1. Percentage of naive B cell, memory B cell, and plasmablast subsets in healthy controls and SSc patients. **A.** Representative flow cytometry dot plots of CD19⁺ B cells stained with CD27 and IgD. CD19⁺ B cells are divided into 5 subsets depending on the relative expression of CD27 and IgD: CD27⁻IgD⁺ naïve, CD27⁺IgD⁺ pre-switched memory, CD27⁻IgD⁻ double-negative (DN) memory, and CD27^{mid}IgD⁻ switched memory B cells and CD27^{high}IgD⁻ plasmablasts. **B.** Scatter plots represent the percentages of total B cell and B cell subsets in 76 healthy controls (HC; open circles) and 76 SSc patients (closed circles). Horizontal lines indicate the mean of each set of values.

FcγRIIB expression levels in SSc patients

We analysed FcγRIIB expression levels in each B cell subset (Fig. 2). In naïve B cells, FcγRIIB expression levels were significantly increased in SSc patients compared to healthy controls ($p < 0.05$) (Fig. 2A and 2B). Among memory B cell subsets, FcγRIIB expression levels on DN memory B cells in SSc patients were significantly increased compared to those in healthy controls ($p < 0.001$) (Fig. 2A and 2B). We then examined FcγRIIB mRNA expression on B cells and confirmed that FcγRIIB mRNA expression on B cells was significantly increased in SSc patients compared to healthy controls ($p < 0.01$) (Fig. 2C).

Therefore, FcγRIIB expression levels were increased in naïve and DN memory B cells in SSc patients.

Since immunosuppressive therapy may alter FcγRIIB expression levels on B cells, we compared FcγRIIB expression levels in SSc subsets based on the first blood sampling condition. The prednisolone dosage and the length of time from the initiation of prednisolone to blood sampling did not affect FcγRIIB expression levels (Supplemental Figure 1A and 1B). We divided SSc patients into four subgroups: SSc patients without ILD or any immunosuppressive therapy, including oral corticosteroid and IVCY at the first blood sampling (SSc w/o Tx, w/o ILD) (n=21), SSc

patients with ILD but without any immunosuppressive therapy (SSc w/o Tx, w/ ILD) (n=11), SSc patients with oral corticosteroid therapy (SSc w/ Steroid) (n=25), and SSc patients with a history of IVCY therapy (SSc w/ IVCY) (n=19). In naïve B cells, FcγRIIB expression levels were significantly increased in SSc patients with steroid treatment (SSc w/ Steroid) and IVCY treatment (SSc w/ IVCY) compared to those in healthy controls ($p < 0.05$ and $p < 0.05$, respectively) (Fig. 3). In DN memory B cells, SSc patients having ILD without treatment (SSc w/o Tx, w/ ILD), those with steroid (SSc w/ Steroid), and those with IVCY therapy, exhibited significantly increased FcγRIIB expression levels compared to healthy controls ($p < 0.05$, $p < 0.01$, and $p < 0.01$, respectively). Switched memory B cells from SSc patients that had ILD without treatment (SSc w/o Tx, w/ ILD) also showed significantly increased FcγRIIB expression levels compared to healthy controls as well ($p < 0.05$). On the other hand, the present study did not find a significant association of FcγRIIB expression levels with skin thickness (data not shown). Therefore, FcγRIIB expression levels on naïve and DN memory B cells were mainly increased in SSc patients with ILD.

FcγRIIB expression levels correlate with immune activation in SSc patients

Previous studies described that B cells from SSc patients exhibit an activated phenotype (22, 33). In order to evaluate whether FcγRIIB expression levels are associated with immune activation markers in SSc B cells, the surface expression of CD80, CD86, and CD95 was measured. The expression levels of CD80, CD86, and CD95 on B cells were significantly stronger in all B cell subsets, except for CD80 on switched memory B cells and plasmablasts (Suppl. Fig. 2A). We then examined the correlation of the expression levels of FcγRIIB and immune activation markers. CD95 expression on naïve B cells, switched memory B cells, and plasmablasts was positively, but moderately, associated with FcγRIIB expression levels ($r = 0.23$, $p < 0.05$, $r = 0.32$, $p < 0.01$, and $r = 0.29$, $p < 0.05$, respectively). Then, we

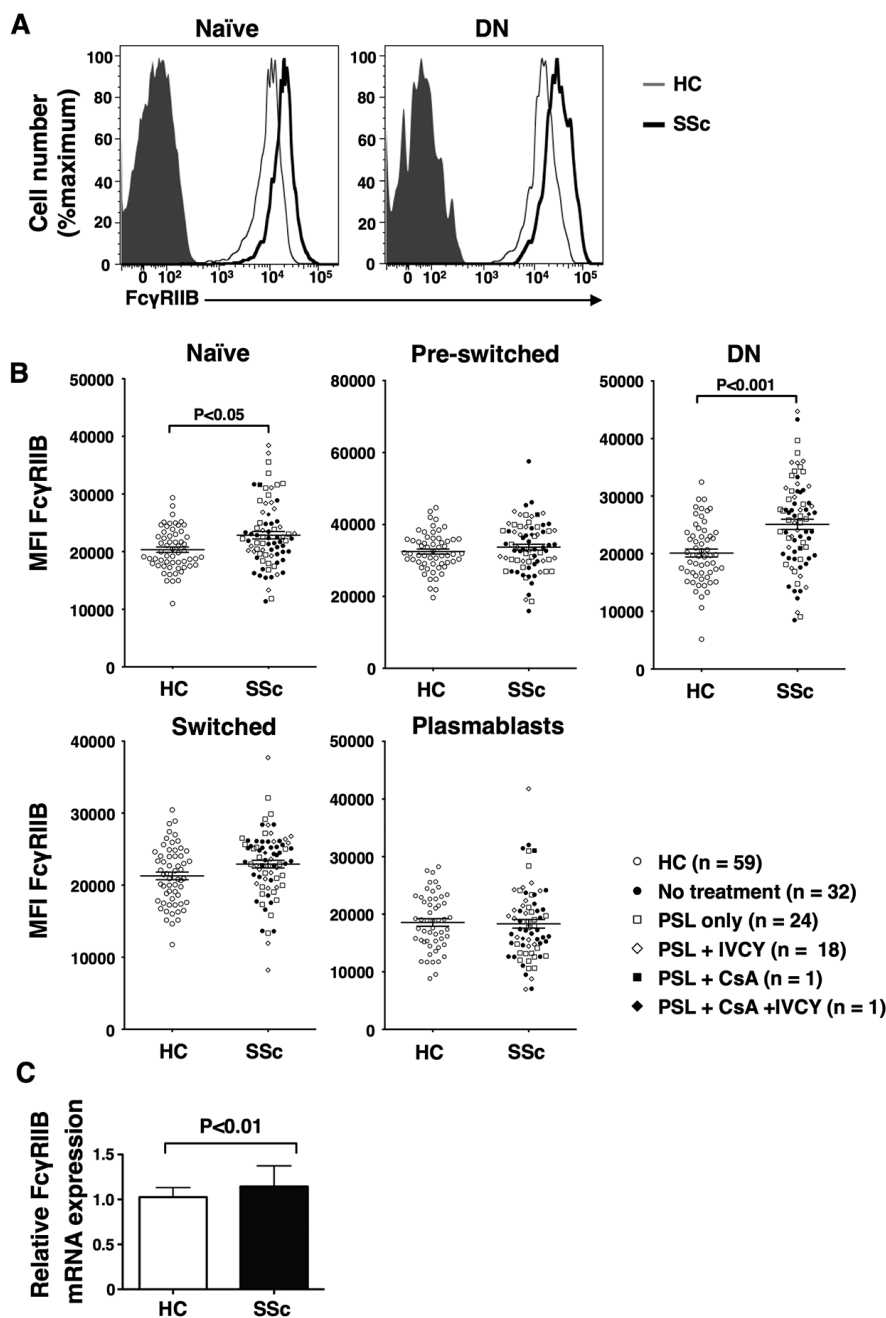


Fig. 2. FcγRIIB expression on B cells. **A**, Representative histograms of FcγRIIB expression on naïve and double-negative (DN) B cells from healthy controls (HC) and SSc patients. The shaded curve represents the control staining. **B**, Scatter plots represent FcγRIIB expression on each B cell subset in HC and SSc patients. Open and closed circles indicate data obtained from HC and SSc patients that received no immunosuppressives, respectively; open and closed squares indicate data from SSc patients that received only corticosteroid and those that received corticosteroid and cyclosporine A (CsA), respectively; open and closed diamonds indicate data from SSc patients that underwent corticosteroid and intravenous cyclophosphamide (IVCY) therapy and corticosteroid, cyclosporine A, and IVCY therapy, respectively. MFI means fluorescence intensity. **C**, FcγRIIB mRNA expressions on B cells. Bar histograms show the relative mRNA expression of FcγRIIB in B cells in HC (open bar) and SSc patients (closed bar).

evaluated the expression of activation markers with or without the presence of ILD, since ILD is a cause of increased mortality (34). SSc patients with ILD exhibited significantly increased levels of CD80 on pre-switched memory B

cell cells, CD86 on switched memory B cells, and CD95 on DN memory B cell cells (all comparisons with $p < 0.05$) (Supplemental Figure 2B). Thus, B cell subsets from SSc patients had an activated phenotype with increased expression

of CD80, CD86 and CD95, and were, in part, associated with FcγRIIB expression levels.

Clinical association of FcγRIIB expression levels in SSc patients

The 76 SSc patients were classified into two groups in which the early group was within 1 year of disease onset and the long group was more than 1 year past disease onset. The FcγRIIB expression levels were similar between the early group and long group for each B cell subset (data not shown).

It has been reported that an increased ratio of DN memory B cell cells correlated with ILD in SSc (14). Therefore, we examined the clinical and laboratory characteristics of patients with SSc, according to FcγRIIB expression levels on DN memory B cells (Table II). The EScSG-AI was significantly increased in patients with higher expression levels of FcγRIIB relative to those with normal FcγRIIB expression levels in DN memory B cells (1.64 vs. 2.47, $p < 0.05$). The revised EUSTAR activity index and summed DSS score tended to be higher for patients with higher expression levels of FcγRIIB, although the differences did not reach statistical significance. Patients with higher expression levels of FcγRIIB ($>$ healthy control mean + 2SD) on DN memory B cells had ILD more frequently (87% vs. 57%, $p < 0.05$) and IVCY therapy (47% vs. 20%, $p < 0.05$) than those with normal levels. The total lung capacity (% of predicted), forced vital capacity (% of predicted), and diffusing capacity of carbon monoxide (% of predicted) were all significantly decreased in patients with higher FcγRIIB expression levels compared to those with normal FcγRIIB expression levels ($p < 0.005$, $p < 0.01$, and $p < 0.05$, respectively). Heart involvement occurred significantly more often in patients with higher expression levels of FcγRIIB compared to those with normal levels (13% vs. 0%, $p < 0.005$). On the other hand, the frequency of telangiectasia was significantly lower in patients with higher expression levels of FcγRIIB (0% vs. 28%, $p < 0.05$). Collectively, these data suggest that the expression levels of FcγRIIB on DN memory B cells potentially correlate with disease

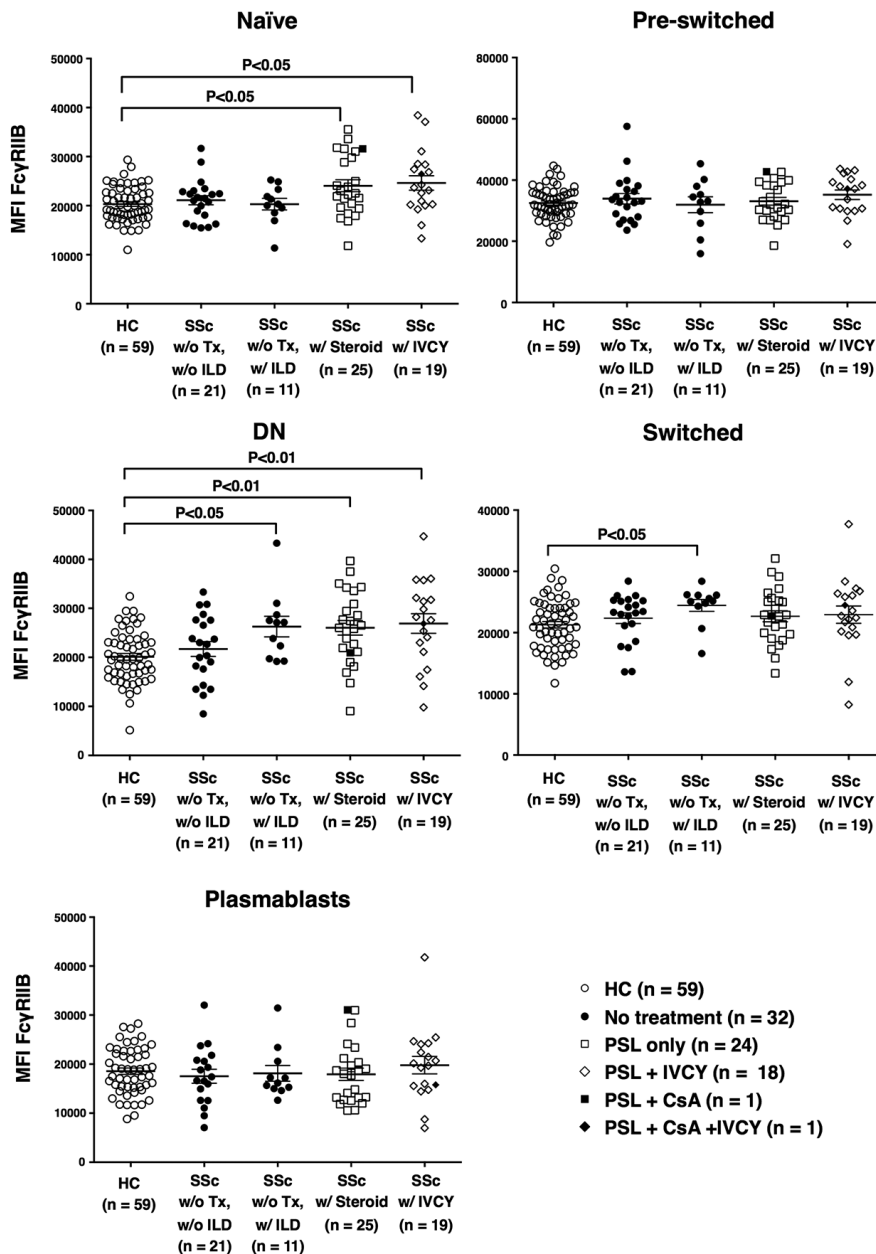


Fig. 3. Comparison of FcγRIIB expression in SSc subgroups that were classified at the time of the first blood sample collection. Open and closed circles indicate data obtained from HC and SSc patients that received no immunosuppressives, respectively; open and closed squares indicate data from SSc patients that received only corticosteroid and those that received corticosteroid and cyclosporine A (CsA), respectively; open and closed diamonds indicate data from SSc patients that underwent corticosteroid and intravenous cyclophosphamide (IVCY) therapy and corticosteroid, cyclosporine A, and IVCY therapy, respectively. The horizontal bars indicate the mean value in each group. MFI means fluorescence intensity.

severity. For other B cell subsets, there was no correlation between FcγRIIB expression levels and clinical characteristics (data not shown).

FcγRIIB expression levels as a marker of disease activity for ILD

In order to determine whether FcγRIIB expression levels are associated with ILD disease activity, we analysed the

six individual patients with ILD before and after IVCY therapy. FcγRIIB expression levels on pre-switched memory B cells, DN memory B cells, and switched memory B cells after IVCY therapy were significantly decreased compared to before IVCY therapy (all comparisons, $p < 0.05$) (Fig. 4). Therefore, FcγRIIB expression levels were decreased in memory B cell subsets ac-

companied with improvement of ILD, suggesting that FcγRIIB expression levels may serve as an activity marker for ILD in patients with SSc.

Discussion

In the present study, we confirmed important alterations in the frequency of different B cell subsets that exhibited an increased ratio of naïve and DN memory B cells, but decreased pre-switched memory B cells, in agreement with previous studies (14, 22, 33). In addition, this study revealed for the first time increased FcγRIIB expression levels on DN memory B cells in SSc patients, as well as an association of increased FcγRIIB expression levels with disease activity of SSc. Therefore, increased FcγRIIB expression levels may play an important role in the development of SSc and serve as a biomarker.

One of the novel findings of the present study is that naïve and DN memory B cells from SSc patients showed higher expression levels of the inhibitory receptor FcγRIIB. Previous studies have demonstrated that memory B cells exhibit reduced expression levels of FcγRIIB in patients with autoimmune diseases, including SLE and RA (20, 35-37). Mackay et al. reported that FcγRIIB expression was selectively decreased on memory B cells in patients with SLE and this alternation was correlated with decreased FcγRIIB-mediated suppression of a BCR-induced calcium response (20). RA patients exhibited reduced expression levels of FcγRIIB on memory B cells and plasmablasts as compared to healthy individuals. This shift on FcγRIIB expression was associated with high levels of anti-citrullinated vimentin autoAbs (36). In another study, patients with active RA have an increased frequency of FcγRIIB low/negative cells in the memory B cell subset, and CD40L expression on CD4 T cells correlated with the frequency of FcγRIIB low/negative cells (37). The authors included in their discussion that high levels of CD40L present on circulating T cells cause B cell activation and FcγRIIB downregulation, resulting in secondary protection of memory B cells from FcγRIIB-mediated cell death (37). Collectively, these results sug-

Table II. Clinical and laboratory characteristics of systemic sclerosis patients, according to FcγRIIB expression levels on double-negative memory B cells.

	FcγRIIB normal (n=61)	FcγRIIB increased (n=15)	p
Age at onset, median (range) years	49 (20-75)	57 (47-77)	0.290
No. of females/no. of males	56/6	13/2	0.182
Disease duration, median (range) years	5.9 (0.3-34)	3.0 (0.2-11)	0.379
MRSS, median (range)	12 (0-42)	10 (2-25)	0.865
EScSG activity index, average (range)	1.64 (0-3.5)	2.47 (1-4.5)	0.016
Revised EUSTAR activity index, average (range)	1.49 (0-6.25)	1.95 (0-4)	0.274
Medsger disease severity index, average (range)	5.77 (1-14)	7.00 (3-15)	0.247
Clinical features			
Raynaud's phenomenon	97% (59/61)	87% (13/15)	0.236
Nailfold bleeding	56% (34/61)	47% (7/15)	0.691
Pitting scar	25% (15/61)	33% (5/15)	0.491
Digital ulcers	25% (15/61)	40% (6/15)	0.232
Contracture of phalanges	34% (21/61)	40% (6/15)	0.314
Diffuse pigmentation	44% (27/61)	33% (5/15)	0.686
Telangiectasia	28% (17/61)	0% (0/15)	0.020
Calcification	3% (2/61)	7% (1/15)	0.546
Organ involvement			
Interstitial lung disease	57% (35/61)	87% (13/15)	0.035
Total lung capacity, % of predicted	93 (49-134)	74 (39-105)	0.002
Vital capacity, % of predicted	101 (55-159)	82 (44-110)	0.007
DLco, % of predicted	63 (36-100)	49 (24-76)	0.013
IVCY therapy	20% (12/61)	47% (7/15)	0.031
Oesophagus	41% (25/61)	20% (3/15)	0.137
Heart	0% (0/61)	13% (2/15)	0.004
Kidney	5% (3/61)	7% (1/15)	0.786
Arthralgia/arthritis	20% (12/61)	40% (6/15)	0.097
Muscle weakness	7% (4/61)	20% (3/15)	0.107
Autoantibodies			
Anti-topoisomerase I antibody	28% (17/61)	33% (8/15)	0.060
Anti-centromere antibody	36% (22/61)	13% (2/15)	0.090
Anti-RNA polymerase III antibody	21% (13/61)	13% (2/15)	0.487
Others	16% (10/61)	27% (4/15)	0.358

mRSS: modified Rodnan total skin thickness score; EScSG: European Scleroderma Study Group; DLco: diffusing capacity of carbon monoxide; IVCY: intravenous cyclophosphamide.

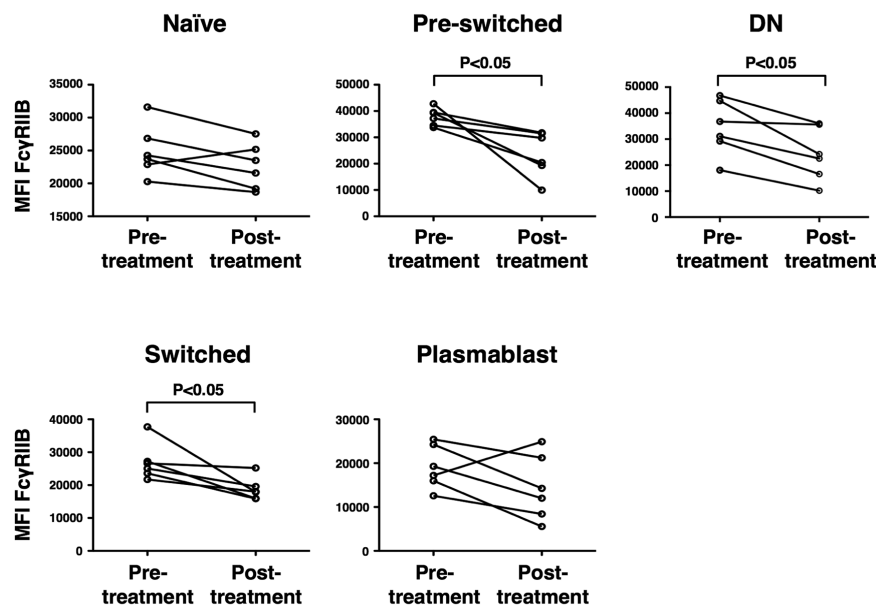


Fig 4. Changes of FcγRIIB expression levels on each B cell subset were analysed in six individual patients with SSc before and after intravenous cyclophosphamide therapy. MFI mean fluorescence intensity.

gest that decreased expression levels of FcγRIIB on memory B cells play, in part, a crucial role in the development of SLE and RA, resulting in exacerbated B cell function and autoAb production. On the other hand, the present study found that naïve B cells from SSc patients had increased expression levels of FcγRIIB, which is in accordance with a previous study (22). Although FcγRIIB expression levels on memory B cells were comparable between SSc patients and healthy controls in Soto's study, the present study revealed that FcγRIIB expression levels were increased on DN memory B cells in SSc by dividing the memory B cell subset into three distinct compartments. Since the clinical phenotype of SSc substantially differs from that of SLE and RA, the role of FcγRIIB on B cells in SSc may also constitutively differ from its role in SLE and RA.

With regard to the B cell distribution, there are contradicting reports about total B cell numbers in SSc, although increased naïve B cells and decreased, but activated memory B cells have been described by several studies (14, 22, 33). However, there is only one study that analyses the frequency and phenotypic abnormality in detail in memory B cell subsets in SSc. Simon *et al.* reported that SSc patients had a higher proportion of naïve B cells and a decreased percentage of memory B cells, especially pre-switched memory B cells (14). These findings correspond to the present study, whereas the frequency of DN memory B cells was different between the present study and Simon's study. The frequency of DN memory B cells was increased in our study, but not in their study. This discrepancy might be due to the different patient numbers enrolled. Our study included a relatively large number of patients (n=76) compared to their study (n=28). It is possible that the difference in the number of enrolled patients may contribute to the difference of the frequency in DN memory B cells.

To exclude an effect of concomitant or previous immunosuppressive therapy, we compared FcγRIIB expression levels in SSc patients with or without corticosteroid or corticosteroid/

IVCY combined therapy. In SSc patients without any immunosuppressive therapy at the time of blood sample collection, SSc patients with ILD had higher expression levels of FcγRIIB on DN memory B cells. Patients receiving corticosteroid or corticosteroid/IVCY combined therapy also showed higher expression levels of FcγRIIB on naïve and DN memory B cells. It appears that the pharmacological effect of immunosuppressive therapy does not influence FcγRIIB expression levels, since IVCY therapy decreased expression levels of FcγRIIB on the memory B cell subsets examined.

Recently, attention has been drawn to a role of DN memory B cells in the pathophysiology of autoimmune diseases. In SLE, an increased frequency of DN memory B cells is significantly associated with higher disease activity, a history of nephritis, and disease-specific autoAbs (12). TNF-inhibitors or tocilizumab treatment reduced DN memory B cells concomitant with improvement of disease activity in RA patients (38, 39). It has been reported that higher frequencies of DN memory B cells were observed in patients with dcSSc and in those with ILD (14). Although the present study did not find an association of FcγRIIB expression levels with skin thickness, SSc patients with increased expression levels of FcγRIIB on DN memory B cells had ILD, reduced lung function, and received IVCY therapy more frequently, suggesting that FcγRIIB expression levels on DN memory B cells appear to be associated with more severe disease. In addition, disease activity as assessed by EScSG-AI was correlated with FcγRIIB expression levels on DN memory B cells. Further study is needed to clarify a role of DN memory B cells in the pathophysiology of SSc.

The activated phenotype of B cells in SSc has been attributed to an increased expression of the activating molecules CD19 and CD21 (6). Expression of CD19 was significantly increased on memory B cells as well as on naïve B cells, suggesting that CD19 overexpression in SSc memory B cells is related to their hyperreactivity (33). In addition, activation markers on SSc B cells, such

as CD80, CD86, and CD95, were also increased (14, 22, 33). In another study, B cells from SSc patients present an activated phenotype, which leads to an overproduction of immunoglobulin, including autoantibodies, and active production of cytokines, such as profibrotic IL-6 (40). Our study confirmed that the levels of activation markers on B cell subsets were increased. Although it remains unclear how increased expression levels of FcγRIIB on naïve and DN memory B cells could be involved in the development of SSc, one possibility is that it may be a compensatory increase to suppress the exacerbated humoral response observed in SSc B cells.

In the present study, FcγRIIB expression levels were decreased by IVCY treatment but not corticosteroid. In RA, adalimumab normalised CD86 expression on memory B cells and reduced FcγRIIB expression levels, mainly on naïve B cells (36). In another study, infliximab treatment resulted in increased expression levels of FcγRIIB on neutrophils in some patients (41). Asahi *et al.* reported that eradication of *Helicobacter pylori* in immune thrombocytopenic purpura patients shifts the balance of Fcγ receptors on monocytes toward inhibitory FcγRIIB (42). In patients with chronic inflammatory demyelinating polyneuropathy, FcγRIIB expression levels were up-regulated on monocytes and B cells after intravenous immunoglobulin therapy (43). Taken together, these findings indicate that various treatments can have differing effects on FcγRIIB expression levels on particular cell subsets.

In conclusion, the present study analysed for the first time FcγRIIB expression in SSc B cell subsets. A molecular mechanism in which FcγRIIB expression levels are increased still remains unclear. Further studies are needed to clarify the mechanisms through which the changes of FcγRIIB expression in specific B cell subsets affect the function of B cells in SSc as well as the induction of fibrosis in skin and lung.

Acknowledgments

We thank Ms Masako Matsubara, Yuko Yamada, and Natsuho Yoshifuji for technical assistance.

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