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Elevated Serum BAFF Levels in Patients with Systemic Sclerosis (SSc): Enhanced BAFF Signaling in SSc B Lymphocytes

Takashi Matsushita, [1] Minoru Hasegawa, [1] Koichi Yanaba, [1] Masanari Kodera, [1]

Kazuhiko Takehara, [1] and Shinichi Sato[2]

[1]Takashi Matsushita, MD, Minoru Hasegawa, MD, PhD, Koichi Yanaba, MD, PhD, Masanari Kodera, MD, Kazuhiko Takehara, MD, PhD: Department of Dermatology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan; [2]Shinichi Sato, MD, PhD: Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

Running title: Serum BAFF levels in SSc

Address correspondence and reprint requests to: Shinichi Sato, MD, PhD, Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.

Phone: 81-95-849-7331

Fax: 81-95-849-7335

E-mail: s-sato@net.nagasaki-u.ac.jp

Objective. To determine serum levels of B cell activating factor belonging to the tumor necrosis factor family (BAFF), a potent B cell survival factor, and clinical association in patients with systemic sclerosis (SSc).

Methods. Serum BAFF levels from 83 SSc patients were examined by ELISA. For a longitudinal study, 131 sera from 21 SSc patients were analyzed. BAFF mRNA expression in the skin was quantified by real-time RT-PCR. BAFF receptor (BAFF-R) expression on $CD19^+$ B cells was assessed by flow cytometry. IgG and IL-6 production by isolated B cells was examined by ELISA.

Results. Serum BAFF levels were elevated in SSc patients compared to healthy controls and correlated positively with the extent of skin fibrosis. In a longitudinal study, 21 SSc patients were classified as follows: 7 patients with decreasing BAFF levels; 11 with unchanged levels; and 3 with increasing levels. Decreasing BAFF levels were accompanied by regression of skin sclerosis, whereas increasing levels were associated with new onset or worsening of organ involvement. BAFF mRNA expression was up-regulated in the affected skin from early patients with diffuse cutaneous SSc. BAFF-R expression on B cells was increased in SSc patients relative to healthy controls. Furthermore, SSc B cells exhibited an enhanced ability to produce IgG and IL-6 by BAFF stimulation.

Conclusion. These results suggest that BAFF and its signaling in B cells contribute to B cell abnormalities and disease development in SSc.

Keywords: systemic sclerosis, BAFF, B cell, skin fibrosis, IL-6

Systemic sclerosis (SSc) is a connective tissue disorder characterized by excessive fibrosis in the skin and various internal organs, with autoimmune background. A variety of immunological abnormalities of T and B lymphocytes have been detected in SSc (1). Autoantibodies are positive in over 90% of patients and these autoantibodies react to various intracellular components. The autoantibodies associated with SSc include anti-DNA topoisomerase I, anticentromere, anti-RNA polymerases, anti-U3RNP, and anti-Th/To antibodies (Abs) (2). Furthermore, hyper-γ–globulinemia and B-cell hyperactivity are detected in SSc patients (3, 4). A recent study demonstrated that SSc patients have distinct abnormalities of blood B lymphocyte compartments characterized by expanded naive B cells and activated memory B cells (5). In a tight-skin mouse, a genetic model of SSc, chronic B cell activation is critical not only for induction of autoantibodies, but also for the development of skin fibrosis (6). Although the pathogenesis of SSc remains unknown, B cell abnormalities characterized by autoantibody production and polyclonal B cell activation play an important role.

B cell activating factor belonging to the tumor necrosis factor family (BAFF), also known as BLyS, TALL-1, and THANK, is a tumor necrosis factor (TNF) superfamily member (TNFSF13B) best known for its role in the survival and maturation of B cells (7). The BAFF gene encodes a putative type II transmembrane protein of 285 amino acids (8). A 152 amino acid form can also be shed from the membrane and is detectable in human serum (8-11). BAFF is produced by several cell types, including monocytes, macrophages, neutrophils, dendritic cells, and T lymphocytes (8, 12), and is a ligand for at least three TNF receptor superfamily (TNFRSF) members: B-cell maturation antigen (BCMA/TNFRSF17), transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI/TNFRSF13B), and BAFF receptor (BAFF-R/BR3/TNFRSF13C) (13, 14). All three receptors are primarily expressed by B cells (7). Among them, BAFF-R plays the central role in the BAFF system (13). BAFF/BAFF receptor

family appears to span nearly all stages of B-lineage differentiation, ranging from the development, selection, and homeostasis of naive primary B cells to the maintenance of long-lived bone marrow plasma cells (15, 16). BAFF also exhibits a strong costimulatory function for B cell activation *in vitro* (8, 12). Furthermore, excess BAFF rescues self-reactive B cells from anergy, which may play a crucial role of autoimmune induction and development (17). Mice overexpressing BAFF exhibit elevated B cell numbers in spleen and lymph node and characteristics of autoimmune diseases, including spontaneous autoantibody production, immunoglobulin (Ig) deposits in the kidneys, and glomerulonephritis (18-20). Thus, mice overexpressing BAFF appear to show autoimmune phenotype similar to patients with systemic lupus erythematosus (SLE) (14, 20).

In humans, previous reports have shown elevated serum BAFF levels in SLE, rheumatoid arthritis (RA), and Sjögren's syndrome patients (9-11, 21). Furthermore, elevated serum BAFF levels correlated with titer of autoantibodies, such as anti-double-stranded DNA Ab, rheumatoid factor (RF), and anti-Ro/La Ab in these autoimmune disorders, respectively (9, 10, 22). Despite distinct B cell abnormalities in SSc, serum BAFF levels were not previously investigated in SSc. In this study, we examined serum BAFF levels and related these results to clinical features in SSc patients. In addition, we assessed relevance of BAFF to altered B cell function in SSc. The current study showed elevated serum BAFF levels, their correlation with skin fibrosis, increased BAFF-R expression on B cells, and enhanced B cell function by BAFF stimulation in SSc. The results of this study suggest that BAFF and its signaling in B cells contribute to B cell abnormalities and disease development in SSc.

Patients and Methods

Patients. Serum samples were obtained from 83 Japanese patients with SSc (70 females and 13 males). All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology (23). Their median (range) age was 53 (20-80) years and the median (range) disease duration was 2.3 (0.2-30) years. These patients were grouped according to the classification system proposed by LeRoy et al. (24): 45 patients (35 females and 10 males) had diffuse cutaneous SSc (dcSSc) and 38 patients (35 females and 3 males) had limited cutaneous SSc (lcSSc). Anti-topoisomerase I Abs were positive for 43 patients; anticentromere Abs for 32; and anti-RNA polymerases I/III Abs for 8. The disease duration was calculated from the time of the first clinical event (other than Raynaud's phenomenon) that was a clear manifestation of SSc. The disease duration of patients with dcSSc and lcSSc was 2.0 (0.2-18) and 3.0 (0.3-30) years, respectively. At the first visit, 5 patients had been treated with low-dose steroids (prednisolone, 5-20 mg/day) and 8 patients with low-dose D-penicillamine (100-300 mg/day). None of the SSc patients had received other immunosuppressive therapy, and had a recent history of infection or other inflammatory diseases. As disease control in this study, we also examined serum samples from 25 patients with SLE and dermatomyositis (DM) that fulfilled the American College of Rheumatology criteria (25) and Bohan and Peter criteria (26, 27), respectively. Age- and sex-matched 25 healthy Japanese individuals [21 females and 4 males; age, 52 (26-72) years] were used as healthy controls. In addition, to assess the variability of serum BAFF levels over the course of a day, we obtained sera sampled from 6 healthy Japanese individuals [2 females and 4 males; age, 35 (31-42) years] in the morning, the daytime, and the night.

For a retrospective longitudinal study, we analyzed 131 serum samples from 21 patients with SSc (16 females and 5 males; 14 with dcSSc and 7 with lcSSc). Their median (range) age was 54 (20-68) years and the median (range) disease duration was 3.0 (0.2-10) years at the first

visit. The median (range) follow-up period was 6 (2-6) years after the first visit. At the first visit, 2 patients were treated with low-dose steroids (prednisolone, 5-20 mg/day). During the follow-up, 11 additional patients received low-dose steroids (prednisolone, 5-20 mg/day), while the remaining 8 patients did not receive oral steroids. In addition to steroid treatment, 2 patients were treated with low-dose D-penicillamine (100-300 mg/day) at the first visit. During the follow up, one additional received low-dose D-penicillamine (100-300 mg/day). One patient received steroid pulse therapy, followed by 40 mg/day of oral prednisolone for subacute deterioration of interstitial pneumonitis during the follow-up period. Fresh venous blood samples were centrifuged shortly after clot formation and all samples were stored at –70°C before use. The protocol was approved by the Kanazawa University Graduate School of Medical Science, and informed consent was obtained from all patients.

Clinical assessment. Complete medical histories, physical examinations, and laboratory tests were conducted for all patients at their first visit, with limited evaluations during follow-up visits. Skin score was measured by scoring technique of the modified Rodnan total skin thickness score (modified Rodnan TSS) (28). Organ system involvement was defined as described previously (29, 30): lung = bibasilar fibrosis on chest radiography and high-resolution computed tomography; esophagus = hypomotility shown by barium radiography; joint = inflammatory polyarthralgias or arthritis; heart = pericarditis, congestive heart failure, or arrhythmias requiring treatment; kidney = malignant hypertension and rapidly progressive renal failure without any other explanation; and muscle = proximal muscle weakness and elevated serum creatine kinase. Pulmonary function test, including vital capacity (VC) and diffusion capacity for carbon monoxide (DLco), was also tested. When the DLco and VC were <75% and <80%, respectively, of the predicted normal values, they were considered to be abnormal.

ELISA. Specific enzyme-linked immunosorbent assay (ELISA) kits were used for

measuring serum BAFF levels (R&D systems, Minneapolis, MN), according to the manufacturer's protocol. Each sample was tested in duplicate. The detection limit of this assay was 3.38 pg/ml. To assess the interference of RF in this ELISA system, RF was removed by incubation with an IgG-coated latex suspension. This absorption of the RF activity did not affect BAFF concentrations by ELISA (data not shown). Furthermore, serum BAFF levels did not correlate with RF levels in serum samples from SSc patients (data not shown). Therefore, there was no interference of RF in this ELISA system.

RNA isolation and real-time RT-PCR. Skin biopsy specimens were obtained from the forearm of 18 dcSSc patients [15 females and 3 males; median (range) age, 56 (26-71) years]. Ten of these patients were grouped into early dcSSc with disease duration of less than 3 years, while 8 patients were grouped into late dcSSc with disease duration of more than 6 years. None of the early dcSSc patients received therapy. All late dcSSc patients received low-dose steroids (prednisolone, 5-20 mg/day). Ten age- and sex-matched healthy Japanese individuals [7 females and 3 males; age, 48 (25-60) years] were used as healthy controls. In addition, we examined the skin from patients with hypertrophic scar [4 females and 2 males; age, 31 (23-40) years] as disease control of fibrotic skin disorders. All skin samples were snap-frozen in liquid nitrogen and stored at –80°C before use. Total RNA from skin was extracted using QIAGEN RNeasy spin columns (QIAGEN Ltd., Crawley, UK) and digested DNaseI (QIAGEN Ltd.) to remove chromosomal DNA in accordance with manufacturer's protocols. Total RNA was reverse transcribed to cDNA using Reverse Transcription System with random hexamers (Promega, Madison, WI). We performed real-time quantitative RT-PCR using the TaqMan® system (Applied Biosystems, Foster City, CA). We obtained BAFF probe and primers from TaqMan® Gene Expression Assays (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize mRNA, and probe and primers were from Pre-Developed

TaqMan® Assay Reagents (Applied Biosystems). Real-time PCR was performed on an ABI Prism 7000 Sequence Detector (Applied Biosystems) according to the manufacture's instructions. Relative expression of real-time PCR products was determined using the $\Delta\Delta C_T$ technique (Applied Biosystems User Bulletin #2) as previously described (31). A comparative threshold cycle (C_T) was used to determine gene expression relative to normal control (calibrator). We normalized each set of samples by housekeeping gene (GAPDH) using the formula of $\Delta C_T = C_{T\ BAFF} - C_{T\ GAPDH}$. One of the control samples was then chosen as a calibrator and relative mRNA levels were calculated by the expression $2^{-\Delta\Delta CT}$ where $\Delta\Delta C_T = \Delta C_{T\ sample} - \Delta C_{T\ calibrator}$. Hence, BAFF mRNA levels were expressed as an n-fold difference relative to the calibrator. Each reaction was done in, at least, triplicate.

Flow cytometric analysis. Blood samples were obtained from 15 SSc patients (12 females and 3 males; 10 with dcSSc and 5 with lcSSc). Their median (range) age was 35 (21-70) years and the median (range) disease duration was 2.1 (1-5) years at the first visit. Four of these SSc patients were treated with low-dose steroids (prednisolone, 5-20 mg/day). None of these SSc patients was treated with D-penicillamine or other immunosuppressive therapy. Ten age- and sex-matched healthy Japanese individuals [7 females and 3 males; age, 39 (25-52) years] were used as healthy controls. Two-color analysis was performed using phycoerythrin-conjugated anti-BAFF-R (BR3; eBioscience, San Diego, CA) and fluorescein isothiocyanate-conjugated anti-CD19 (B4; Beckman Coulter, Miami, FL) monoclonal antibodies (mAbs). For immunofluorescence staining, fresh heparinized whole blood samples were placed on ice immediately after collection. Blood samples (50 μl) were stained at 4°C using predetermined saturating concentrations of the test mAb for 20 minutes, as previously described (32). Blood erythrocytes were lysed after staining using the Whole Blood Immuno-Lyse kit as detailed by the

manufacturer (Beckman Coulter). Cells were analyzed on a FACScan flow cytometer (BD PharMingen).

Production of cytokines and IgG by purified B cells. Peripheral blood B cells were obtained from 15 SSc patients and 10 healthy controls, the same subjects examined for flow cytometric analysis described above. Peripheral blood mononuclear cells (PBMCs) from whole blood were isolated by VACUTAINER® CPT (Becton Dickinson, Franklin Lakes, NJ). Then, B cells were isolated from PBMCs by B cell Isolation Kit II, human (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, PBMCs (1×10^7) were incubated for 10 minutes with biotin-cojugated mAb mixtures containing mAb to CD2, CD14, CD16, CD36, CD43, and CD235a. B cells were then isolated by incubating anti-biotin magnetic beads. After the isolation, >95% of cells were $CD19^+$ by flow cytometric analysis.

Purified B cells (1×10^5) were cultured in RPMI1640 containing 10% heat-inactivated fetal calf serum (Gibco, Life Technologies, Paisley, UK) in 96-well flat-bottom plates (Becton Dickinson) with stimuli, at 37°C in a 5% CO_2 humidified atmosphere. The cells were stimulated with 0.01% *Staphylococcus aureus* Cowan strain (SAC; Sigma St. Louis, MO) plus 1 μg/ml of recombinant human BAFF (Peprotech, London, UK) as previously described (8). B cells were cultured for 72 hours, and IL-6 and IL-10 concentrations in the culture medium were measured by ELISA, according to the manufacturer's protocols (PharMingen, San Diego, CA). In addition, B cells were cultured for 8 days, and IgG concentrations in the culture medium were measured by ELISA (Bethyl Laboratories, Montgomery, TX). Each sample was done in triplicate.

Statistical Analysis. Statistical analysis was performed using Mann-Whitney U test for comparison of values, Fisher's exact probability test for comparison of frequencies, and Bonferroni's test for multiple comparisons. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. *P* values less than 0.05 were

considered statistically significant. The data were shown as the median (range) unless otherwise indicated.

Results

Serum BAFF levels in SSc. Serum BAFF levels were significantly higher in SSc patients with [1.26 (0.32-5.67) ng/ml] than healthy controls [0.78 (0.39-1.37), $P<0.001$; Figure 1]. Similarly, serum BAFF levels were significantly higher in patients with SLE [1.63 (0.56-5.52), $P<0.0001$] or DM [1.00 (0.21-4.38), $P<0.01$] than healthy controls. SLE patients had the highest serum BAFF levels. Concerning SSc subgroups, both dcSSc [1.30 (0.32-5.67)] and lcSSc [1.16 (0.60-2.34)] patients exhibited increased BAFF levels compared to healthy controls ($P<0.0005$ and $P<0.0001$, respectively). Furthermore, serum BAFF levels were significantly elevated in dcSSc patients relative to lcSSc patients ($P<0.05$). Since serum BAFF level may vary over short time periods, we assessed the variability of serum BAFF levels by measuring sera sampled several times over the course of a day in 6 healthy individuals. Serum BAFF levels were similar between samples obtained in the night [0.68 (0.52-0.82) ng/ml], those in the morning [0.69 (0.49-0.85) ng/ml], and those in the daytime [0.69 (0.52-0.82) ng/ml]. Furthermore, all blood samples examined in this study were obtained in the morning or the daytime. Thus, the variability of serum BAFF levels over a course of a day appeared to have no significant effect on our results.

Clinical correlation of serum BAFF levels in SSc. Clinical and laboratory parameters obtained at the first evaluation were compared between SSc patients with elevated serum BAFF levels and those with normal levels. Values higher than the mean + 2SD (1.245 ng/ml) of the control serum samples were considered to be elevated in this study. BAFF levels were elevated in 52% (43/83) of SSc patients with 56% (25/45) of dcSSc patients and 47% (18/38) of lcSSc patients. There was no significant difference in disease duration between patients with elevated BAFF levels and those with normal levels (Table 1). In addition, BAFF levels did not correlate with disease duration in dcSSc, lcSSc, and total SSc, respectively (data not shown). SSc Patients

with elevated BAFF levels exhibited significantly higher modified Rodnan TSS than those with normal levels (P<0.01; Table 1). BAFF levels correlated positively with modified Rodnan TSS in SSc patients (P<0.005, r=0.415; Figure 2). SSc patients with elevated serum BAFF levels more frequently had decreased %VC (P<0.05), arthralgia/arthritis (P<0.05), myositis (P<0.05), and elevated erythrocyte sedimentation rates (ESR; P<0.05). Furthermore, BAFF levels correlated positively with ESR in SSc patients (P<0.05, r=0.434; Figure 2). The level of IgG was significantly increased in SSc patients with elevated BAFF levels compared with healthy controls (P<0.05; Table 1). The level of IgG was higher in SSc patients with elevated BAFF than in SSc patients without elevated BAFF, but the difference was not statistically significant. BAFF levels did not significantly correlate with anti-topoisomerase I or anticentromere Ab levels determined by ELISA (data not shown). To determine whether BAFF is simply serving as a marker for dcSSc or serving as a marker for the skin score in dcSSc (and other manifestations, such as pulmonary fibrosis and renal disease), clinical features were compared between dcSSc patients with serum elevated BAFF levels and those with normal levels. As a result, modified Rodnan TSS was significantly higher in dcSSc patients with elevated BAFF levels [22 (8-42)] than those with normal levels [16 (5-24), P<0.005]. By contrast, the frequency of lung involvement and renal disease in dcSSc patients with elevated BAFF levels were similar to that in dcSSc patients with normal levels (72% versus 60% and 4% versus 5%, respectively). Thus, elevated BAFF levels were generally associated with the severity of skin sclerosis and inflammation in SSc, rather than dcSSc.

Longitudinal study of serum BAFF levels in SSc. To assess the changes in serum BAFF levels during the follow-up in 21 SSc patients (14 with dcSSc and 7 with lcSSc), the levels were studied for 6 years after the first visit (Figure 3). The changes in skin sclerosis during the follow-up period were assessed by modified Rodnan TSS (Figure 3). When the changes in BAFF

level showed more than a 50% increase or more than a 50% decrease at one or more time-points during the follow-up period compared with BAFF level at the first visit, the changes were defined as “increased” and “decreased”, respectively. Otherwise, the BAFF level was defined as “unchanged”. According to the criteria, 21 SSc patients were classified as follows: 7 (33%) patients with decreased BAFF levels; 11 (52%) with unchanged levels; and 3 (14%) with increased levels (Figure 3).

All patients in the “decreased” group had dcSSc with the disease duration of 2.0 (0.2-3.0) years (Figure 3A). At the first visit, none of the patients was treated with oral steroids and one patient with 200 mg/day of D-penicillamine. During the follow-up, all patients received low-dose steroids (prednisolone, 5-20 mg/day) and one additional patient received 300 mg/day of D-penicillamine. High BAFF levels (over 2.0 ng/ml) at their first visit were significantly decreased after 1 year and remained decreased thereafter. Furthermore, in comparison with modified Rodnan TSS at the first visit, it was significantly decreased after 2 years (36% decrease, $P<0.05$), 4 years (45%, $P<0.05$), and 6 years (54%, $P<0.05$). Thus, the decreased change in serum BAFF levels was associated with the improvement of skin sclerosis in early dcSSc patients with high BAFF levels at their first visit.

The “unchanged” group consisted of 4 patients with dcSSc and 7 with lcSSc (Figure 3B). The disease duration was 5.0 (1.0-10) years. At the first visit, one dcSSc patient was treated with 10 mg/day of oral prednisolone. After the first visit, 3 additional dcSSc patients received low-dose steroids (prednisolone, 5-20 mg/day). None of the patients received low-dose D-penicillamine. In general, serum BAFF levels are moderate (range 1.0-2.0 ng/ml) at the first visit and remained stable throughout the follow-up period. Modified Rodnan TSS tended to be decreased as the disease progressed; however, there was no significant difference.

Serum BAFF levels increased during the observation period in 3 dcSSc patients with

moderate BAFF levels (range 1.0-2.0 ng/ml) at their first visit (Figure 3C). The disease duration was 1.5 (0.3-8) years. One patient exhibited subacute deterioration of interstitial pneumonitis after serum BAFF levels increased and then treatment with steroid pulse therapy was started, followed by 40 mg/day of oral prednisolone. One patient who had not been treated with steroids developed scleroderma renal crisis with cardiac tamponade, which resulted in death at the time point of the highest BAFF level. One patient who had been treated with 20 mg/day of oral prednisolone due to interstitial pneumonitis exhibited deterioration of secondary pulmonary hypertension, leading to death at the time point of the highest BAFF level. In general, decreased change in BAFF levels correlated with the improvement of skin sclerosis, while increased change in BAFF levels was associated with new onset or worsening of major organ involvement.

BAFF mRNA expression in the dcSSc affected skin. To assess local BAFF expression in the affected skin from dcSSc patients, BAFF mRNA expression was quantified by real-time RT-PCR. The affected skin from early dcSSc patients showed fibrosis upon histological examination, while that from late dcSSc patients exhibited modest or absent skin fibrosis (data not shown). BAFF mRNA expression was significantly up-regulated in the affected skin from early dcSSc patients with disease duration of less than 3 years compared to that from late dcSSc patients with disease duration of more than 6 years, patients with hypertrophic scar, and normal controls ($P<0.005$, $P<0.05$, and $P<0.001$, respectively; Figure 4). BAFF mRNA expression in the skin from late dcSSc patients and patients with hypertrophic scar was similar to that from normal skin. Thus, local BAFF expression was up-regulated in fibrotic skin from early dcSSc patients.

BAFF-R expression on SSc B cells. Recently, BAFF activity has been shown to correlate with BAFF-R expression patterns *in vivo* (33). Therefore, BAFF-R expression on blood B cells from SSc patients was assessed with flow cytometric analysis. BAFF-R expression levels on SSc

B cells [mean fluorescence intensity 81 ± 40 (mean ± SD)] were significantly higher than those found in healthy controls (43 ± 7, $P<0.05$; Figure 5A).

Overproduction of IL-6 and IgG by SSc B cells. We investigated the role of BAFF in function of SSc B cells. B cells from SSc patients and healthy controls were stimulated with BAFF plus SAC, and culture supernatants were analyzed by ELISA to determine the amount of IL-6, IL-10, and IgG. SSc B cells stimulated with BAFF plus SAC produced 38% more IL-6 than those from healthy controls ($P<0.05$; Figure 5B), while IL-10 production by stimulated SSc B cells was similar to that by B cells from healthy controls (Figure 5C). Furthermore, stimulated SSc B cells exhibited significantly enhanced IgG production by 35% relative to B cells from healthy controls ($P<0.05$; Figure 5D). Thus, SSc B cells in SSc patients had a significantly enhanced ability to produce IL-6 and IgG by BAFF stimulation.

Discussion

This is the first report to reveal elevated serum BAFF levels in SSc. Elevated BAFF levels were associated with the severity of skin sclerosis. Our longitudinal study revealed that as serum BAFF levels decreased, skin sclerosis generally improved in early dcSSc patients with median disease duration of 2 years. A recent study using a tight-skin mouse has revealed that B cell activation status closely correlates with the development of skin sclerosis (6). Therefore, BAFF may be related to the development of skin sclerosis in SSc. In addition, 3 patients exhibited onset or worsening of major organ involvement parallel with an increase in serum BAFF levels. The results of this study suggest that serum BAFF levels reflect the disease severity and activity of SSc. However, we cannot exclude the possibility that changes in serum BAFF levels may be just reflecting responses to alterations in systemic inflammation, since various proinflammatory cytokines are known as potent inducers of BAFF (34).

Since all SSc patients whose BAFF levels were decreasing during the follow-up received treatment with low-dose steroids of 5-20 mg/day, reduction in BAFF levels might be caused by steroid treatment. Although serum BAFF levels were reduced with high-dose steroids in SLE, steroid dose of less than 30 mg/day did not decrease serum BAFF levels in SLE (21). Furthermore, 2 of 3 SSc patients whose BAFF levels were increasing throughout the follow-up period also received treatment with low-dose steroids. These results suggest that low-dose steroids are not the only factor for decreasing BAFF levels in SSc.

BAFF is an essential component of B cell homeostasis and a potent B cell survival factor associated with systemic autoimmune disease in animals (14, 18, 20). Previous studies have provided strong evidence that constitutive BAFF overproduction in mice leads not only to polyclonal hyper-γ–globulinemia, but also to spontaneous production of multiple antoantibodies, circulating immune complexes, and renal Ig deposits (14, 18, 20). In humans, elevated serum

BAFF levels have been found in SLE, RA, and Sjögren's syndrome (9-11, 21). Furthermore, elevated serum BAFF levels correlated with titer of autoantibodies and hyper-γ–globulinemia in these autoimmune disorders (9, 10, 22). Similar to SLE, RA, and Sjögren's syndrome, our results demonstrated that elevated BAFF levels were found in SSc and were associated with the increase in serum total IgG levels. Furthermore, elevated BAFF levels were associated with the severity of skin sclerosis. IL-6 and IgG by SSc B cells simulated with BAFF were enhanced. IL-6 induces concentration-dependent increases in the production of collagen and glycosaminoglycans from human dermal fibroblasts *in vitro* (35). Remarkably, blocking the IL-6 response by anti-IL-6 Ab results in a significant reduction in procollagen type I by culture dcSSc fibroblasts (36). Skin fibrosis in the tight-skin mice is improved with a parallel decrease in IL-6 production (6). In addition, serum IL-6 levels are elevated in patients with early dcSSc (37) and correlate with the extent of skin fibrosis (38). Therefore, augmented IL-6 production by SSc B cells stimulated with BAFF may be related to the development of fibrosis.

Previous studies have suggested that SSc B cells are chronically activated since the frequency of B cells expressing activation markers, including HLA-DR and CD25, is increased in SSc (3, 39, 40). In addition, we have shown that B cells from SSc patients overexpress CD19 by ~20% (32). CD 19 is a critical cell-surface signal transduction molecule on B cells that regulates basal signaling thresholds and accelerates signaling through B cell antigen receptor (41). Remarkably, transgenic mice with a similar increase in CD19 expression exhibit hyper-γ–globulinemia and produce characteristic autoantibodies with specificities similar to autoantibodies in human SSc (32). Furthermore, B cells from tight-skin mice display enhanced CD19 signaling with a chronically activated phenotype, hyper-γ–globulinemia, and spontaneous autoantibody production; these phenomena are completely eliminated by the loss of CD19 expression on B cells (6). Furthermore, BAFF stimulation enhances CD19 expression and

increases the ability of B cell antigen receptor to phosphorylate CD19 (42). Thus, excess BAFF may accelerate B cell hyperactivity via overexpression and phosphorylation of CD19 and thereby contribute to B cell abnormalities in SSc.

In RA patients, BAFF level in the synovial fluid greatly exceeds that in the blood (9). BAFF is also highly expressed in inflamed RA synovium and salivary glands of Sjögren's syndrome (11). Similar to RA and Sjögren's syndrome, our results showed that BAFF mRNA expression was up-regulated in the affected skin from early dcSSc patients. A recent study using DNA microarrays has revealed that genes characteristically expressed in B cells show differential expression between dcSSc and normal skin biopsies (43). Furthermore, analysis of lymphocyte population in dcSSc skin biopsies has demonstrated that B cell signature observed on the DNA microarrays is from $CD20^+$ B cells (43). Therefore, our results suggest that the dysregulation of BAFF expression is related to the development of skin fibrosis via activation of infiltrating B cells into the affected skin.

Since there are few established basic therapies for skin sclerosis and lung fibrosis in SSc, new therapeutic agents have been researched. Recently, BAFF have been shown to be a therapeutic target in SLE (44). Serum BAFF levels are increased and are correlated with autoantibody production in human SLE (21). Furthermore, inhibition of BAFF by TACI-Ig and BAFFR-Ig is successful in treating a murine model of SLE (14, 45). Moreover, treatment with BAFF antagonists, such as human anti-BAFF mAb, were already started in SLE patients and showed safety (46, 47). Our finding that elevated serum BAFF levels were associated with the disease severity and activity in SSc suggests that BAFF inhibition could be potential therapeutic targets of SSc as well as SLE.

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References

1. White B. Immunopathogenesis of systemic sclerosis. Rheum Dis Clin North Am 1996;22:695-708.
2. Okano Y. Antinuclear antibody in systemic sclerosis (scleroderma). Rheum. Dis. Clin. North. Am. 1996;22:709-35.
3. Famularo G, Giacomelli R, Alesse E, Cifone MG, Morrone S, Boirivant M, et al. Polyclonal B lymphocyte activation in progressive systemic sclerosis. J. Clin. Lab. Immunol. 1989;29:59-63.
4. Fleischmajer R, Perlish JS, Reeves JRT. Cellular infiltrates in scleroderma skin. Arthritis Rheum. 1977;20:975-84.
5. Sato S, Fujimoto M, Hasegawa M, Takehara K. Altered blood B lymphocyte homeostasis in systemic sclerosis: expanded naive B cells and diminished but activated memory B cells. Arthritis Rheum 2004;50:1918-27.
6. Saito E, Fujimoto M, Hasegawa M, Komura K, Hamaguchi Y, Kaburagi Y, et al. CD19-dependent B lymphocyte signaling thresholds influence skin fibrosis and autoimmunity in the tight-skin mouse. J Clin Invest 2002;109:1453-62.
7. Mackay F, Browning JL. BAFF: a fundamental survival factor for B cells. Nat Rev Immunol 2002;2:465-75.
8. Moore PA, Belvedere O, Orr A, Pieri K, LaFleur DW, Feng P, et al. BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. Science 1999;285:260-3.
9. Cheema GS, Roschke V, Hilbert DM, Stohl W. Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases. Arthritis Rheum 2001;44:1313-9.

10. Zhang J, Roschke V, Baker KP, Wang Z, Alarcon GS, Fessler BJ, et al. Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus. J Immunol 2001;166:6-10.

11. Groom J, Kalled SL, Cutler AH, Olson C, Woodcock SA, Schneider P, et al. Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome. J Clin Invest 2002;109:59-68.

12. Schneider P, MacKay F, Steiner V, Hofmann K, Bodmer JL, Holler N, et al. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. J Exp Med 1999;189:1747-56.

13. Thompson JS, Bixler SA, Qian F, Vora K, Scott ML, Cachero TG, et al. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. Science 2001;293:2108-11.

14. Gross JA, Johnston J, Mudri S, Enselman R, Dillon SR, Madden K, et al. TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. Nature 2000;404:995-9.

15. Harless SM, Lentz VM, Sah AP, Hsu BL, Clise-Dwyer K, Hilbert DM, et al. Competition for BLyS-mediated signaling through Bcmd/BR3 regulates peripheral B lymphocyte numbers. Curr Biol 2001;11:1986-9.

16. O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, et al. BCMA is essential for the survival of long-lived bone marrow plasma cells. J Exp Med 2004;199:91-8.

17. Thien M, Phan TG, Gardam S, Amesbury M, Basten A, Mackay F, et al. Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. Immunity 2004;20:785-98.

18. Mackay F, Woodcock SA, Lawton P, Ambrose C, Baetscher M, Schneider P, et al. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. J Exp Med 1999;190:1697-710.

19. Batten M, Groom J, Cachero TG, Qian F, Schneider P, Tschopp J, et al. BAFF mediates survival of peripheral immature B lymphocytes. J Exp Med 2000;192:1453-66.

20. Khare SD, Sarosi I, Xia XZ, McCabe S, Miner K, Solovyev I, et al. Severe B cell hyperplasia and autoimmune disease in TALL-1 transgenic mice. Proc Natl Acad Sci U S A 2000;97:3370-5.

21. Stohl W, Metyas S, Tan SM, Cheema GS, Oamar B, Xu D, et al. B lymphocyte stimulator overexpression in patients with systemic lupus erythematosus: longitudinal observations. Arthritis Rheum 2003;48:3475-86.

22. Mariette X, Roux S, Zhang J, Bengoufa D, Lavie F, Zhou T, et al. The level of BLyS (BAFF) correlates with the titre of autoantibodies in human Sjogren's syndrome. Ann Rheum Dis 2003;62:168-71.

23. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum 1980;23:581-90.

24. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr., et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988;15:202-5.

25. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1982;25:1271-7.

26. Bohan A, Peter JB. Polymyositis and dermatomyositis (first of two parts). N. Eng. J. Med. 1975;292:344-8.

27. Bohan A, Peter JB. Polymyositis and dermatomyositis (second of two parts). N. Eng. J. Med. 1975;292:403-7.

28. Clements PJ, Lachenbruch PA, Seibold JR, Zee B, Steen VD, Brennan P, et al. Skin thickness score in systemic sclerosis: an assessment of interobeservar variability in 3 independent studies. J. Rheumatol. 1993;20:1892-6.

29. Steen VD, Powell DL, Medsger TA, Jr. Clinical correlations and prognosis based on serum autoantibodies in patients with systemic sclerosis. Arthritis Rheum 1988;31:196-203.

30. Sato S, Ihn H, Kikuchi K, Takehara K. Antihistone antibodies in systemic sclerosis. Association with pulmonary fibrosis. Arthritis Rheum 1994;37:391-4.

31. Bloch G, Toma DP, Robinson GE. Behavioral rhythmicity, age, division of labor and period expression in the honey bee brain. J Biol Rhythms 2001;16:444-56.

32. Sato S, Hasegawa M, Fujimoto M, Tedder TF, Takehara K. Quantitative genetic variation in CD19 expression correlates with autoimmunity. J Immunol 2000;165:6635-43.

33. Hsu BL, Harless SM, Lindsley RC, Hilbert DM, Cancro MP. Cutting edge: BLyS enables survival of transitional and mature B cells through distinct mediators. J Immunol 2002;168:5993-6.

34. Nardelli B, Belvedere O, Roschke V, Moore PA, Olsen HS, Migone TS, et al. Synthesis and release of B-lymphocyte stimulator from myeloid cells. Blood 2001;97:198-204.

35. Duncan MR, Berman B. Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6. J Invest Dermatol 1991;97:686-92.

36. Kawaguchi Y, Hara M, Wright TM. Endogenous IL-1α from systemic sclerosis fibroblasts induces IL-6 and PDGF-A. J Clin Invest 1999;103:1253-60.

37. Hasegawa M, Sato S, Fujimoto M, Ihn H, Kikuchi K, Takehara K. Serum levels of interleukin 6 (IL-6), oncostatin M, soluble IL-6 receptor, and soluble gp130 in patients with systemic sclerosis. J. Rheumatol. 1998;25:308-13.

38. Sato S, Hasegawa M, Takehara K. Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. J. Dermatol. Sci. 2001;27:140-6.

39. Frieri M, Angadi C, Paolano A, Oster N, Blau SP, Yang S, et al. Altered T cell subpopulations and lymphocytes expressing natural killer cell phenotypes in patients with progressive systemic sclerosis. J Allergy Clin. Immunol. 1991;87:773-9.

40. Freundlich B, Jimenez SA. Phenotype of peripheral blood lymphocytes in patients with progressive systemic sclerosis: activated T lymphocytes and the effect of D-penicillamine therapy. Clin. Exp. Immunol. 1987;69:375-84.

41. Fujimoto M, Fujimoto Y, Poe JC, Jansen PJ, Lowell CA, DeFranco AL, et al. CD19 regulates Src family protein tyrosine kinase activation in B lymphocytes through processive amplification. Immunity 2000;13:47-57.

42. Hase H, Kanno Y, Kojima M, Hasegawa K, Sakurai D, Kojima H, et al. BAFF/BLyS can potentiate B-cell selection with the B-cell coreceptor complex. Blood 2004;103:2257-65.

43. Whitfield ML, Finlay DR, Murray JI, Troyanskaya OG, Chi JT, Pergamenschikov A, et al. Systemic and cell type-specific gene expression patterns in scleroderma skin. Proc Natl Acad Sci U S A 2003;100:12319-24.

44. Stohl W. A therapeutic role for BLyS antagonists. Lupus 2004;13:317-22.

45. Kayagaki N, Yan M, Seshasayee D, Wang H, Lee W, French DM, et al. BAFF/BLyS receptor 3 binds the B cell survival factor BAFF ligand through a discrete surface loop and promotes processing of NF-kappaB2. Immunity 2002;17:515-24.

46. Baker KP, Edwards BM, Main SH, Choi GH, Wager RE, Halpern WG, et al. Generation and characterization of LymphoStat-B, a human monoclonal antibody that antagonizes the bioactivities of B lymphocyte stimulator. Arthritis Rheum 2003;48:3253-65.

47. Furie R, Stohl W, Ginzler E, Becker M, Mishra N, Chatham W, et al. Safety, pharmacokinetic and pharmacodynamic results of a phase 1 single and double dose-escalation study of LymphoStat-B (human monoclonal antibody to BLyS) in SLE patients. Arthritis Rheum 2003;48 (Suppl.):S377.

Table 1. Clinical and laboratory data of patients with SSc showing elevated serum BAFF level.

	SSc	
	Elevated BAFF (n = 43)	Normal BAFF (n = 40)
Median (range) age at onset (yr)	52 (20-77)	55 (24-80)
Sex (female/male)	34/9	36/4
Median (range) disease duration (yr)	2.3 (0.2-27)	2.2 (0.5-30)
Subtype of SSc		
Diffuse cutaneous SSc	60%	50%
Limited cutaneous SSc	40%	50%
Median (range) modified Rodnan TSS	18 (2-42)**	9 (2-24)
Clinical features		
Pitting scars	45%	39%
Contracture of phalanges	68%	39%
Diffuse pigmentation	60%	45%
Telangiectasia	43%	47%
Organ involvement		
Lung	48%	47%
Decreased %VC	40%*	18%
Decreased %DLco	78%	63%
Esophagus	50%	47%
Heart	13%	11%
Kidney	5%	3%
Joint	38%*	16%
Muscle	23%*	5%
Laboratory findings		
Anti-topoisomerase I Ab	53%	50%
Anticentromere Ab	30%	48%
Anti-RNA polymerases I/III Ab	16%	3%
Elevated ESR	48%*	29%
Elevated CRP	23%	16%
Median (range) IgG (μg/ml)	1543 (958-3320) ***	1493 (722-2890)
Median (range) IgA (μg/ml)	301 (98-769)	289 (128-645)
Median (range) IgM (μg/ml)	140 (59-829)	150 (59-445)

All the clinical and laboratory parameters and serum BAFF levels were obtained at the first evaluation.

*P<0.05 or **P<0.01 vs. SSc patients with normal BAFF levels

***P<0.05 vs. serum total IgG levels [1320 (855-2250)] of healthy controls (n = 25).

ESR = erythrocyte sedimentation rates and CRP = C-reactive protein.

Figure Legends

Figure 1. Serum BAFF levels in patients with autoimmune diseases at the first evaluation. BAFF levels were determined by a specific ELISA in serum samples from patients with diffuse cutaneous SSc (dcSSc), limited cutaneous SSc (lcSSc), systemic lupus erythematosus (SLE), or dermatomyositis (DM) and from healthy controls (Control). The dashed lines indicate the cut-off value (mean + 2SD of the control samples). The lines inside the boxes indicate the median; the outer borders of the boxes indicate 25th and 75th percentiles; the bars extending from the boxes indicate the 10th and 90th percentiles.

Figure 2. The correlation of serum BAFF levels against modified Rodnan TSS and erythrocyte sedimentation rates (ESR) in patients with SSc at the first evaluation. Serum BAFF levels were determined by a specific ELISA.

Figure 3. Serial changes in serum BAFF levels and modified Rodnan TSS during the follow-up period. In a longitudinal study, 131 serum samples from 21 patients with SSc (14 with dcSSc and 7 with lcSSc) were analysed. SSc patients were classified into 3 groups: (**A**) patients whose BAFF levels decreased by more than 50% at one or more time-points, (**B**) patients whose BAFF levels changed by less than 50%, and (**C**) patients whose BAFF levels increased by more than 50%. **Top**, serum BAFF levels were determined by a specific ELISA. The dashed lines indicate the cut-off value (mean + 2SD of the control samples). *P<0.005 vs. serum BAFF levels at the first visit. **Bottom**, the extent of skin sclerosis was measured by modified Rodnan TSS at the first visit and after 2, 4, and 6 years. Data are shown as the median (range). **P<0.05 vs. modified Rodnan TSS at the first visit.

Figure 4. BAFF mRNA expression in the affected skin of dcSSc. BAFF mRNA expression was quantified by real-time RT-PCR in the skin from early dcSSc patients with disease duration of less than 3 years (early dcSSc), late dcSSc patients with disease duration of more than 6 years (late dcSSc), patients with hypertrophic scar, and healthy controls (Control). The affected skin from early dcSSc patients showed fibrosis by histological examination, while that from late dcSSc patients exhibited modest or absent skin fibrosis (data not shown). BAFF mRNA expression relative to a calibrator, one of the control samples, was expressed as $2^{-\Delta\Delta CT}$ (fold) and shown as a dot. The lines inside the boxes indicate the median; the outer borders of the boxes indicate 25th and 75th percentiles; the bars extending from the boxes indicate the 10th and 90th percentiles.

Figure 5. Representative expression of BAFF-R on $CD19^+$ B cells, and production of cytokines and IgG by peripheral blood B cells. Blood samples were obtained from 15 SSc patients and 5 healthy controls (Control). BAFF-R expression was assessed by 2-color immunofluorescence with flow cytometric analysis (**A**). The horizontal dashed lines are provided for reference. For production of cytokines and IgG, purified B cells (1×10^5) from blood samples were stimulated with either medium alone or recombinant human BAFF plus *Staphylococcus aureus* Cowan strain (SAC). Culture supernatants were analyzed by ELISA to determine the amount of IL-6 (**B**), IL-10 (**C**), and IgG (**D**).

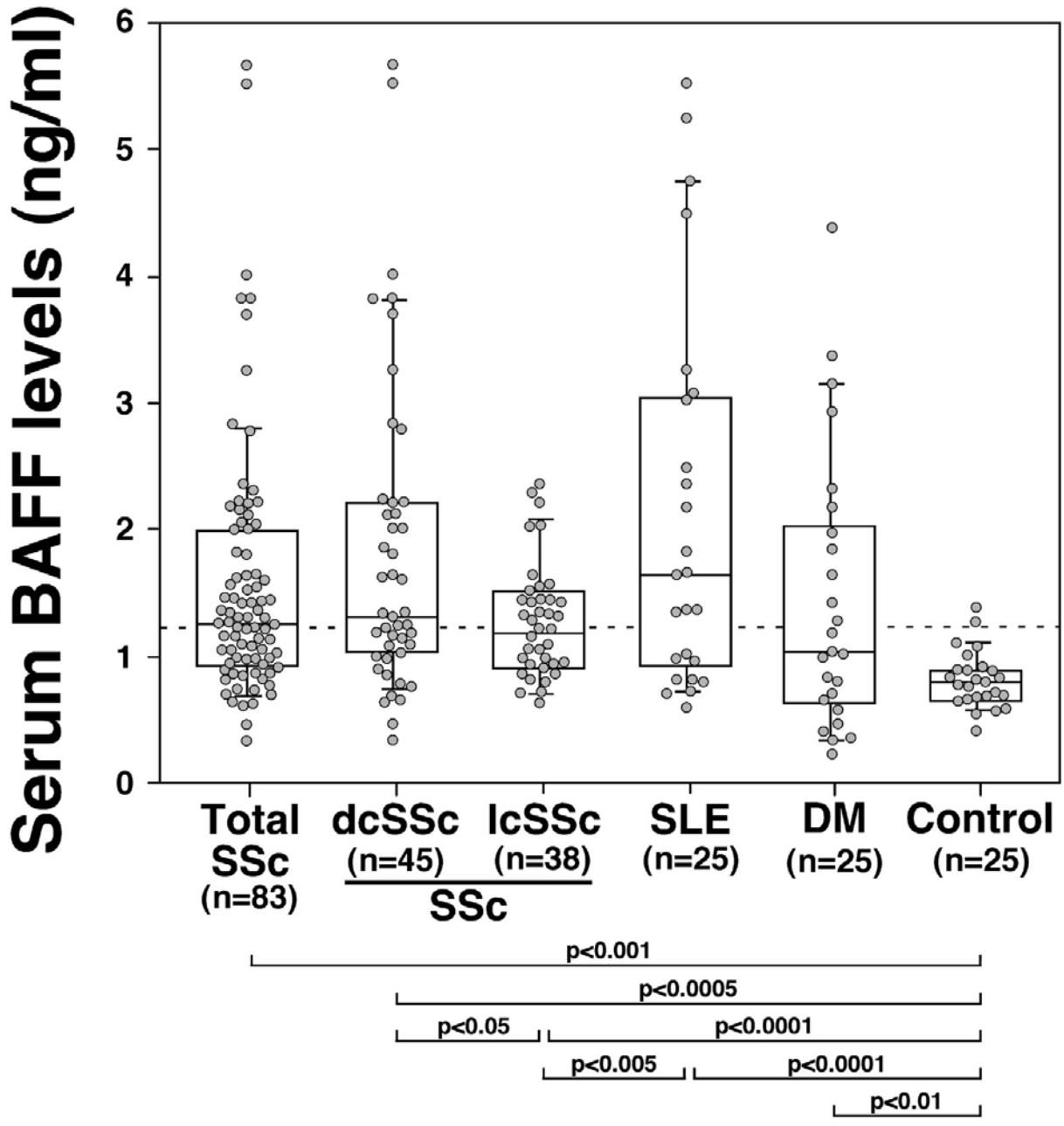

Figure 1
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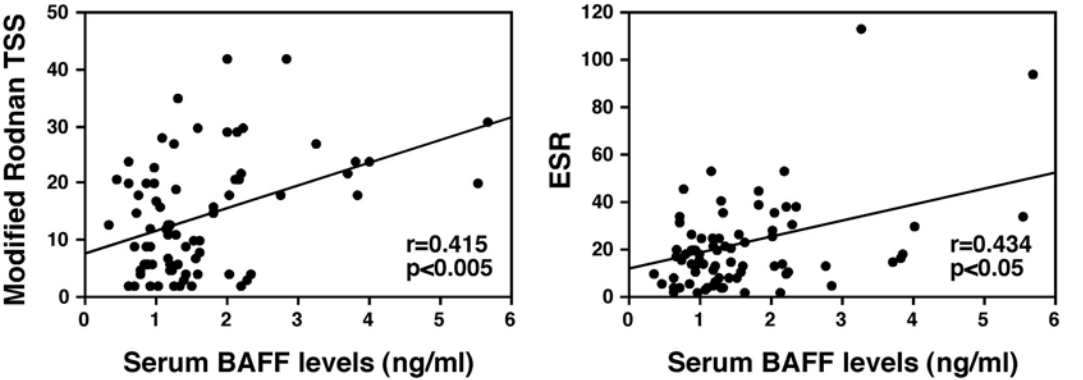

Figure 2
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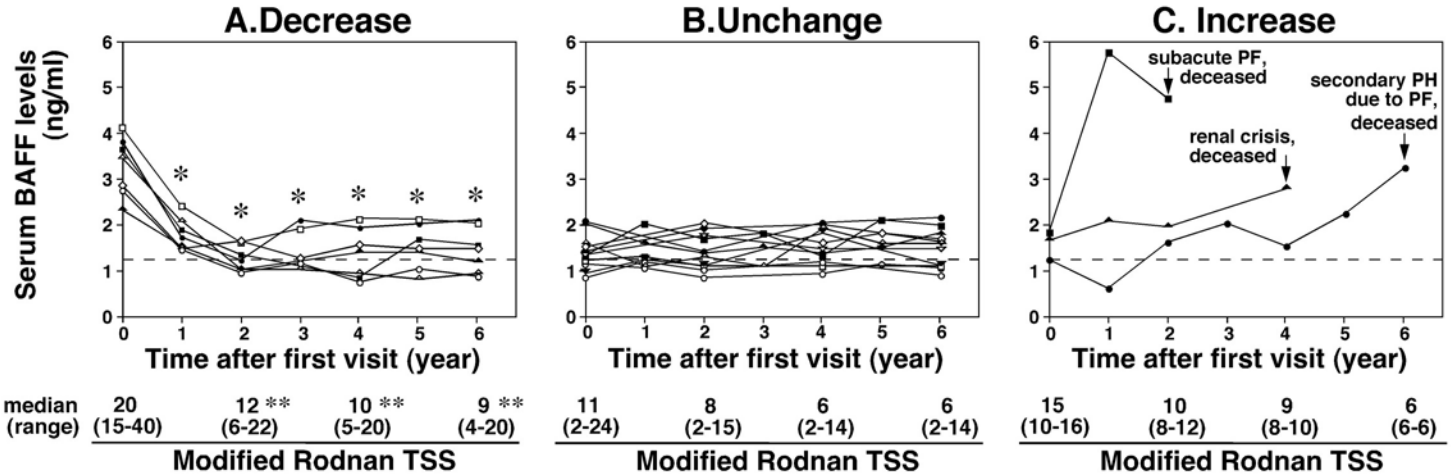

Figure 3
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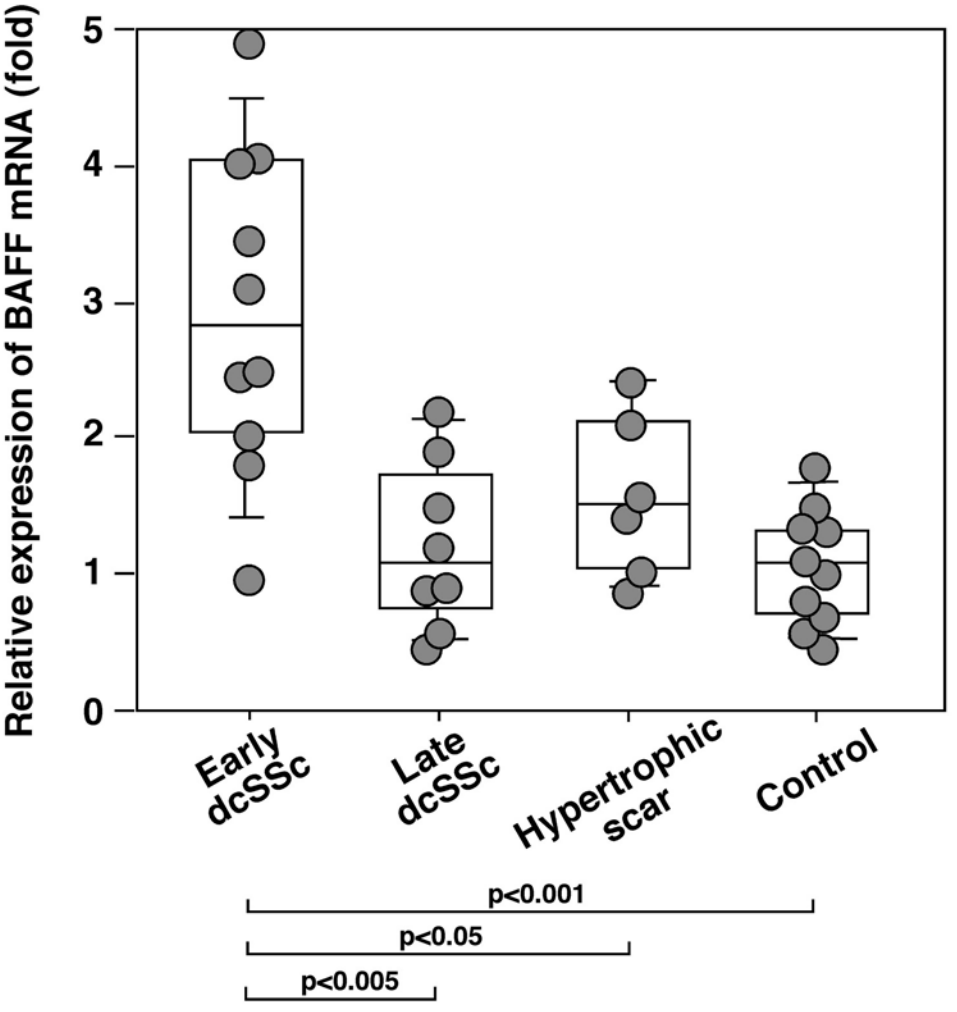

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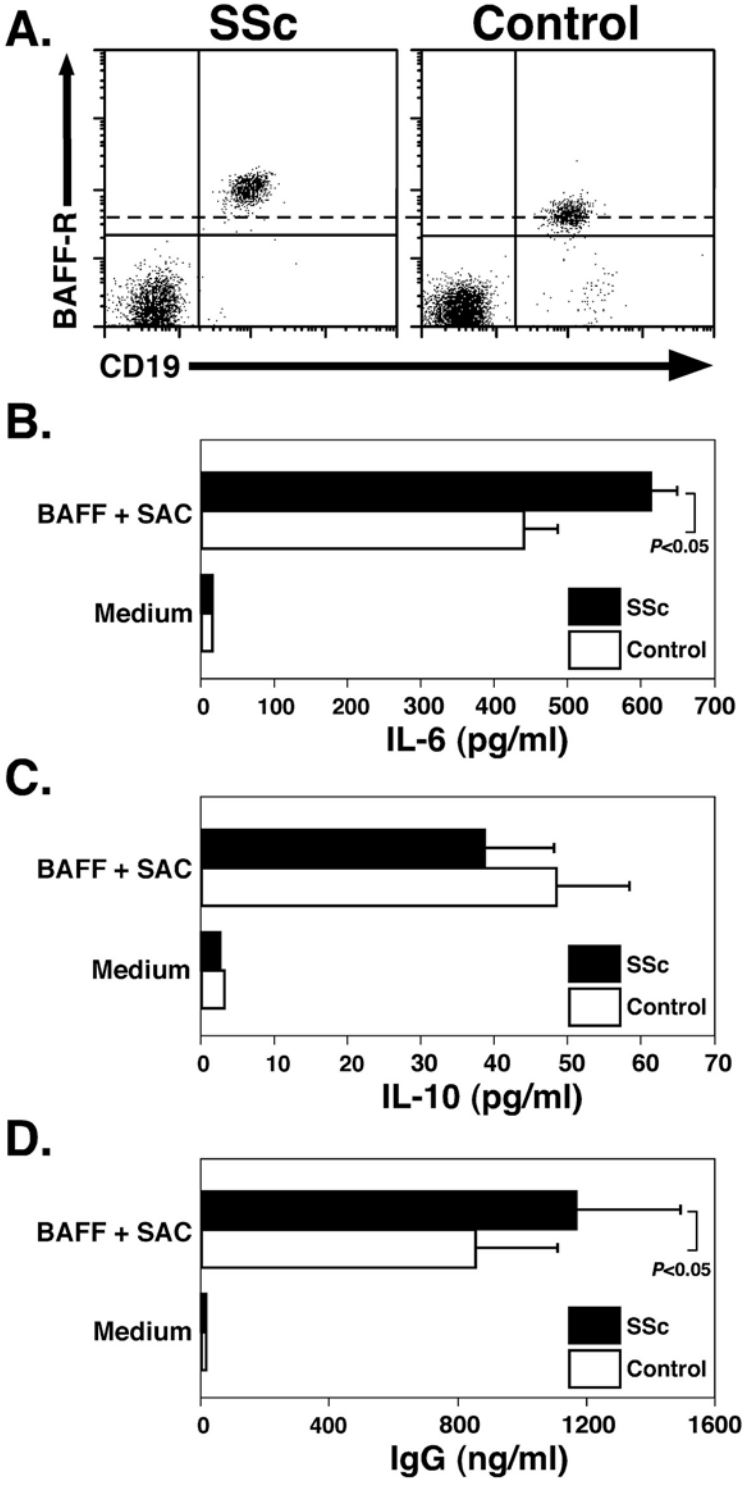

Figure 5
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