Decreased levels of regulatory B cells in patients with systemic sclerosis: Association with autoantibody production and disease activity

著者	Matsushita Takashi, Hamaguchi Yasuhito, Hasegawa Minoru, Takehara Kazuhiko, Fujimoto Manabu
journal or publication title	Rheumatology (United Kingdom)
volume	55
number	2
page range	263-267
year	2015-10-05
URL	http://hdl.handle.net/2297/45453

doi: 10.1093/rheumatology/kev331

Decreased Levels of Regulatory B cells in Patients with Systemic Sclerosis: Association with autoantibody production and disease activity

Takashi Matsushita, [1] Yasuhito Hamaguchi, [1] Minoru Hasegawa, [2]

Kazuhiko Takehara, [1] and Manabu Fujimoto[3]

[1]Department of Dermatology, Faculty of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa 920-8641, Japan

[2] Department of Dermatology, University of Fukui, Fukui 910-1193, Japan

[3]Department of Dermatology, Faculty of Medicine, University of Tsukuba, Tennodai, Tsukuba 305-8575, Japan.

Running title: Regulatory B cells in SSc

Address correspondence and reprint requests to: Takashi Matsushita MD, PhD, Department of Dermatology, Faculty of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa 920-8641, Japan.

Phone: 81-76-265-2343 Fax: 81-76-234-4270

E-mail: t-matsushita@med.kanazawa-u.ac.jp

Abstract

Objective. B cell abnormalities characterized by autoantibody production and polyclonal B cell activation play an important role in the pathogenesis of systemic sclerosis (SSc). IL-10 producing regulatory B (Breg) cells play an important role in the negative immune response. Recently, we identified a human Breg cell subset and it was predominantly found within the $CD24^{hi}CD27^{+}$ B cell subpopulation. However, the role of Breg cells in SSc remains unknown. In this study, we investigated the clinical association of Breg cells in SSc patients.

Methods. Blood IL-10 producing Breg cell levels were determined by FACS in 35 SSc patients and 30 healthy subjects. In a follow-up study, we analyzed 6 individual dcSSc patients before and after treatment.

Results. The frequency of blood Breg cells was significantly lower in SSc patients than in healthy controls ($P<0.0001$). Similarly, the frequency of $CD24^{hi}CD27^{+}$ B cells was significantly lower in SSc patients with than in healthy controls ($P<0.0001$). SSc patients with decreased Breg cell levels more frequently had interstitial lung disease ($P<0.05$). Furthermore, Breg cell levels correlated negatively with the titer of anti-topoisomerase I Ab and anticentromere Ab in SSc patients. For a follow-up study, Breg cell levels in dcSSc patients after treatment were found to be significantly increased comparing with that before treatment ($P<0.05$), accompanied with decreased disease activity. Thus, Breg cell levels inversely correlated with disease activity of SSc.

Conclusion. These results suggest that decreased Breg cell levels may contribute to the development of SSc.

Keyowrds: systemic sclerosis, regulatory B cell, IL-10

Key messages

Regulatory B cells are decreased in patients with systemic sclerosis.

Decreased levels of regulatory B cells may acceralete autoantibody production in patients with systemic sclerosis .

Regulatory B cell levels inversely correlated with disease activity of SSc.

Systemic sclerosis (SSc) is a connective tissue disorder characterized by fibrosis in the skin and various internal organs, with autoimmune background. The two subsets were termed diffuse cutaneous (dcSSc) and limited cutaneous SSc (lcSSc) [1]. Autoantibodies are positive in over 90% of patients and these autoantibodies (Abs) react to various intracellular components. The autoantibodies associated with SSc include anti-DNA topoisomerase I and anticentromere Abs. It has been reported that SSc patients have distinct abnormalities of blood B lymphocyte compartments characterized by expanded naive B cells and activated memory B cells [2]. Furthermore, B cell depletion therapy suppresses the development of skin fibrosis in the tight-skin mouse [3, 4]. In addition, several recent studies have revealed a possible beneficial effect of antihuman CD20 antibody (rituximab) therapy for SSc patients [5].

Regulatory B (Breg) cells that produce IL-10 is now recognized as an important component of the immune system. Remarkably, Breg cells are potent negative regulators of inflammation and autoimmunity in mouse models of disease in vivo. Our recent study revealed that Breg cells play an inhibitory role in murine sclerodermatous chronic graft-versus-host disease (Scl-cGVHD), a mouse model of scleroderma [6]. Breg cells in humans normally represent <1% of peripheral blood B cells [7]. The phenotype of human Breg cells is reported to be $CD24^{hi}CD27^{+}$ B cell subset [7] or $CD19^{+}CD24^{hi}CD38^{hi}$ B cells [8]. It has been reported that pan-B cell depletion, including regulatory B cell depletion, worsens autoimmunity in humans [9]. Pan-B cell depletion is not always a beneficial effect in autoimmunity. Therefore, it is necessary to investigate Breg cells in systemic sclerosis.

Patients and Methods

Patients. Serum samples were obtained from 35 Japanese SSc patients (29 females and 6 males). All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology [10]. Their median (range) age was 57 (18-71) years and the median (range) disease duration was 2.0 (0.1-24) years. These patients were grouped according to the classification system proposed by LeRoy et al. [1]: 16 patients had dcSSc and 19 patients had lcSSc. Anti-topoisomerase I Abs were positive for 14 patients; anticentromere Abs for 11; and anti-RNA polymerases I/III Abs for 3. 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. Five patients were treated with a low-dose prednisolone (1-10 mg/day). Eight patients were treated with an intermediate-dose prednisolone (11-20 mg/day). In addition to an intermediate-dose of steroid treatment, 2 patients were treated with cyclosporine (2-3 mg/kg/day) and 2 patients were treated with intravenous cyclophosphamide pulse (500 mg/m^2). In a follow-up study, we analyzed 6 individual patients with dcSSc before and after immunosuppressant agents. At the first blood sampling, 1 patient was treated with 12 mg/day of prednisolone, while remaining 5 SSc patients did not received any immunosuppressant agents. Afterward, 4 patients were treated with 20-40 mg/day of prednisolone. Furthermore, 2 patients were treated with 6-cycle of intravenous cyclophosphamide pulse (500mg/m^2) in addition to 20 mg/day of prednisolone. Second blood sampling were taken after treatment. As disease control in this study, we also examined serum samples from 10 patients with dermatomyositis (DM) that fulfilled the American College of Rheumatology criteria [11] and Bohan and Peter criteria [12], respectively. Age- and sex-matched 30 healthy Japanese individuals were used as healthy controls. The whole study was approved by the ethics committee of the Institute of Medical,

Pharmaceutical and Health Sciences, Kanazawa University, and informed consent was obtained from all patients.

Clinical assessment. Complete medical histories, physical examinations, and laboratory tests were conducted for all patients at the time of blood sampling, 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) . Organ system involvement was defined as described previously [13].

Antibodies and flow cytometric analysis.

Anti-human mAbs included: CD19, CD24, CD27 from BD Biosciences (San Diego, CA, USA) and anti-IL-10 mAb from BioLegend (San Diego, CA, USA). Blood samples (50 μl) were stained at 4°C using mAbs for 20 minutes. Cells were analyzed on a BD FACSCant^{TM} II flow cytometer (BD Biosciences).

Analysis of IL-10 production. Heparinized blood samples were collected. Peripheral blood mononuclear cells (PBMCs) were resuspended (2 x 10^6 cells/ml) in medium and stimulated with CpG (ODN 2006, 10 μg/ml; Invivogen), CD40L (1 μg/ml; R&D Systems, Minneapolis, MN, USA), PMA (50 ng/ml; Sigma), ionomycin (1 μg/ml; Sigma), Brefeldin A (1X solution/ml; BioLegend). Stained cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and stained with anti-IL-10 mAb.

Statistical Analysis. Statistical analyses were performed using Student's *t*-test for comparison of sample means between two groups or one-way analysis of variance with Bonferroni *post hoc* tes for comparisons of more than two groups. The Pearson product–moment correlation coefficient was used to examine the relationship between two continuous variables. Wilcoxon matched paired rank test was used for matched paired samples. *P*-values <0.05 were

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

Results

Breg cell levels in SSc. The frequency of IL-10 producing Breg cells was significantly lower in SSc patients [5.43 (2.38-13.10) %] than in healthy controls [11.60 (4.29-18.80) %, $P<0.0001$; Fig. 1A-B]. The absolute number of Breg cells was also significantly lower in SSc patients [1.68 (0.08-15.30) x 10^6/L] than in healthy controls [4.31 (1.03-24.52) x 10^6/L, $P<0.0001$]. By contrast, the frequency of Breg cells in DM patients [9.57 (6.90-14.50) %] was comparable to that in healthy controls (Fig. 1B). Similarly, the frequency of $CD24^{hi}CD27^{+}$ B cells was significantly lower in SSc patients [16.2 (7.6-42.6) %] than in healthy controls [28.3 (17.1-62.7), $P<0.0001$]. The absolute number of $CD24^{hi}CD27^{+}$ B cells was also significantly lower in SSc patients [3.43 (0.22-65.98) x 10^6/L] than in healthy controls [12.99 (3.94-64.25) x 10^6/L, $P<0.01$].

Clinical correlation of Breg cell levels in SSc. Breg cell levels were decreased in 26% (9/35) of SSc patients in 26% (5/19) of dcSSc patients and in 25% (4/12) of lcSSc patients. There was no significant difference in disease duration between patients with decreased Breg cell levels and those with normal levels (Supplementary Table 1). In addition, there was no significant difference in modified Rodnan TSS and clinical features. SSc patients with decreased Breg cell levels more frequently had interstitial lung disease ($P<0.05$). In contrast, there was no significant difference in the prevalence of esophagus, heart, kidney, joint and muscle involvement. The frequency of elevated CRP levels was significantly increased in SSc patients with decreased Breg cell levels compared with normal levels ($P<0.05$). Furthermore, Breg cell levels correlated negatively with CRP in SSc patients ($P<0.05$, r=-0.343; Supplementary Fig. 1), although Breg cell levels did not correlated with CRP in healthy control and other diseases accompanied by elevated CRP levels, such as cellulitis and erysipelas (data not shown). Collectively, Breg cell levels may not correlate with disease severity.

Autoimmunity plays an important role in the pathogenesis of scleroderma. It was reported that the titer of a scleroderma specific autoantibody, such as anti-topoisomerase I Ab, reflects the severity of skin sclerosis in SSc patients [14]. Decreased levels of Breg cells might accelerate the autoimmunity of scleroderma. As a result, Breg cell levels were found to correlate negatively with the titer of anti-topoisomerase I and anticentromere Abs in SSc patients (P<0.05, r=-0.533; P<0.05, r=-0.684, respectively, Supplementary Fig. 1). Thus, decreased Breg cell levels were generally associated with the autoimmunity of SSc, rather than the severity of SSc.

Breg cell as a marker of disease activity.

To determine whether Breg cell levels are inversely correlated with disease activity of SSc, we analyzed 6 indivisual patient with dcSSc before and after treatment. Disease activity was defined as modified Rodnan TSS. After treatment, disease activity in 6 patients with dcSSc was declined, since modified Rodnan TSS in dsSSc patients were significantly decreased after treatment (P<0.01, Fig. 2). Breg and $CD24^{hi}CD27^{+}$ B cell levels in dcSSc patients after treatment were found to be significantly increased comparing with that before treatment (P<0.01 and P<0.05, respectively, Fig. 2), accompanied with decreased disease activity. To confirm whether immunosuppressant agents increase Breg cell levels, we compared Breg cell levels between the patients treated with immunosuppressant or not. Breg cell levels in SSc patients treated with immunosuppressant was tend to increased compared with that in SSc patients without treatment, while there was no significant difference (Supplementary Fig. 2). Taken together, Breg cell levels inversely correlated with disease activity of SSc.

Discussion

This is the first report to reveal decreased Breg cell levels in SSc. Decreased Breg cell levels were associated with the prevalence of interstitial lung disease. In addition, Breg cell levels correlated negatively with the titer of anti-topoisomerase I and anticentromere Ab in SSc patients. Although Breg cell levels did not correlate with the severity of skin sclerosis, Breg cell levels inversely correlated with disease activity of SSc. Therefore, decreased Breg cell levels were generally associated with pathogenesis of SSc.

It has been suggested that Th1 cytokines, such as interferon-γ and IL-2, generally decrease extracellular matrix deposition, whereas Th2 cytokines, such as IL-4, IL-5, IL-6, and IL-13, increase it [15]. To date, B cells have primarily been studied in the roles of effector B cells, which positively regulate the immune response via antibody production and IL-6 secretion. However, Breg cells have been identified and it was consequently revealed that B cells negatively control the immune response via IL-10 secretion [16]. Thus, B cells have two conflicting subsets. We elucidated the role of these two conflicting subsets in a mouse model of experimental autoimmune encephalomyelitis (EAE) [17]. EAE was exacerbated when B cells were depleted in the early phase, whereas it improved when B cells were depleted in the late phase. These results indicate that in the early phase, Breg cells play a major role and the lack of Breg cells exacerbates EAE. On the other hand, in the late phase, effector B cells are dominant and the lack of effector B cells improves EAE. These results reveal that both effector and regulatory B cells are involved in autoimmune disease and that the weights of their roles differ according to the disease stage. These results also indicate that Breg cells are involved in the onset of autoimmune disease because they are important in the early phase. In the current study, Breg cell levels were lower in SSc patients than in healthy individuals; however, we found no correlation with severity. On the other hand, a decreased level of Breg cells correlated with a

high autoantibody titer. Furthermore, Breg cell levels inversely correlated with disease activity of SSc. We recently reported that Breg cells are important in the suppression of Scl-cGVHD, a mouse model of systemic sclerosis [6]. The lack of Breg cells induced severe Scl-cGVHD, while replacement of Breg cells in the early phase restored the exacerbated Scl-cGVHD. However, replacement of Breg cells in the late phase had no effect on Scl-cGVHD. This suggests that Breg cells may play an important role in the onset of Scl-cGVHD. Thus, Breg cells are important for the pathogenesis of scleroderma.

Two large randomized controlled trials of Rituximab, which depletes human pan-B cells, were conducted with SLE patients with the expectation that it would be effective; however, it failed to achieve the primary endpoints [18, 19]. This ineffectiveness may have been due to the existence of Breg cells. If Breg cells are dominant in a patient with SLE, B cell depletion will worsen the condition of the patient with SLE. Thus, the effect of pan-B cell depletion depends on the balance of Breg cells versus effector B cells in the patient. A clinical trial is currently examining B cell depletion in systemic sclerosis, so it will be necessary to consider the existence of Breg cells in the future. Selective B cell depletion, which retains Breg cells, is the ideal treatment for patients with autoimmune disease.

Acknowledgements

This work was supported by grants-in-aid from the Ministry of Education, Science, and Culture of Japan, and Ministry of Health, Labour and Welfare of Japan.

We thank Ms M. Matsubara and Y. Yamada for their technical assistance.

The authors have declared no conflicts of interest.

References

1 LeRoy EC, Black C, Fleischmajer R, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988;15(2):202-5.

2 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(6):1918-27.

3 Matsushita T, Fujimoto M, Hasegawa M, et al. BAFF antagonist attenuates the development of skin fibrosis in tight-skin mice. The Journal of investigative dermatology 2007;127(12):2772-80.

4 Hasegawa M, Hamaguchi Y, Yanaba K, et al. B-lymphocyte depletion reduces skin fibrosis and autoimmunity in the tight-skin mouse model for systemic sclerosis. The American journal of pathology 2006;169(3):954-66.

5 McQueen FM, Solanki K. Rituximab in diffuse cutaneous systemic sclerosis: should we be using it today? Rheumatology 2015;54(5):757-67.

6 Le Huu D, Matsushita T, Jin G, et al. Donor-derived regulatory B cells are important for suppression of murine sclerodermatous chronic graft-versus-host disease. Blood 2013;121(16):3274-83.

7 Iwata Y, Matsushita T, Horikawa M, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood 2011;117(2):530-41.

8 Blair PA, Norena LY, Flores-Borja F, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. Immunity 2010;32(1):129-40.

9 Dass S, Vital EM, Emery P. Development of psoriasis after B cell depletion with rituximab. Arthritis Rheum 2007;56(8):2715-8.

10 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(5):581-90.

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

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

13 Sato S, Ihn H, Kikuchi K, Takehara K. Antihistone antibodies in systemic sclerosis. Association with pulmonary fibrosis. Arthritis Rheum 1994;37(3):391-4.

14 Sato S, Hamaguchi Y, Hasegawa M, Takehara K. Clinical significance of anti-topoisomerase I antibody levels determined by ELISA in systemic sclerosis. Rheumatology 2001;40(10):1135-40.

15 Chizzolini C. T lymphocyte and fibroblast interactions: the case of skin involvement in systemic sclerosis and other examples. Springer Semin. Immunopathol. 1999;21:431-50.

16 Fujimoto M. Regulatory B cells in skin and connective tissue diseases. J Dermatol Sci 2010;60(1):1-7.

17 Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J Clin Invest 2008;118(10):3420-30.

18 Merrill JT, Neuwelt CM, Wallace DJ, et al. Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. Arthritis Rheum

2010;62(1):222-33.
19 Rovin BH, Furie R, Latinis K, et al. Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. Arthritis Rheum 2012;64(4):1215-26.

Figure Legends

Figure 1. IL-10 producing regulatory B cell frequencies in SSc patients. **(A, left panel)** Representative B cell cytoplasmic IL-10 expression by control (Ctrl) individuals and SSc patients with after *in vitro* 48 h CD40L+CpG stimulation, with PMA, ionomycin, Brefeldin A added during the final 5 h of culture. **(A, right panel)** Representative $CD24^{hi}CD27^{+}$ B cells in Ctrl individuals and SSc patients. (**B**) IL-10 producing regulatory B cell and $CD24^{hi}CD27^{+}$ B cell frequencies of Ctrl individuals, SSc and dermatomyositis (DM) patients. Horizontal bars indicate group means. Blood IL-10 producing regulatory B cell and $CD24^{hi}CD27^{+}$ B cell frequencies were determined by FACS.*P<0.01, **P<0.0001.

Figure 2. Serial change of IL-10 producing regulatory B cell. The modified Rodnan TSS, IL-10 producing regulatory B cell and $CD24^{hi}CD27^{+}$ B cell levels were analysed in 6 indivisual patient with dcSSc before and after immunosuppressive treatment.*P<0.05, **P<0.01.

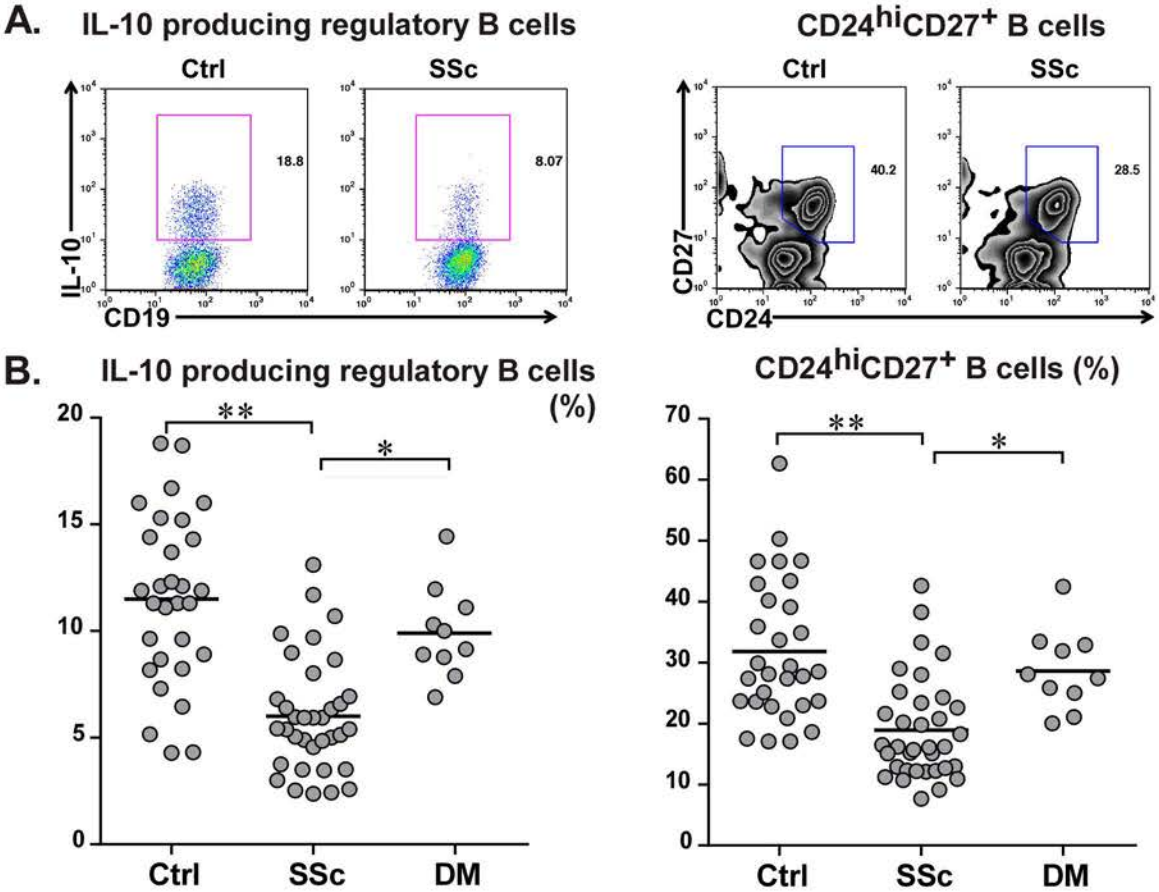


Figure 1
Matsushita et al.

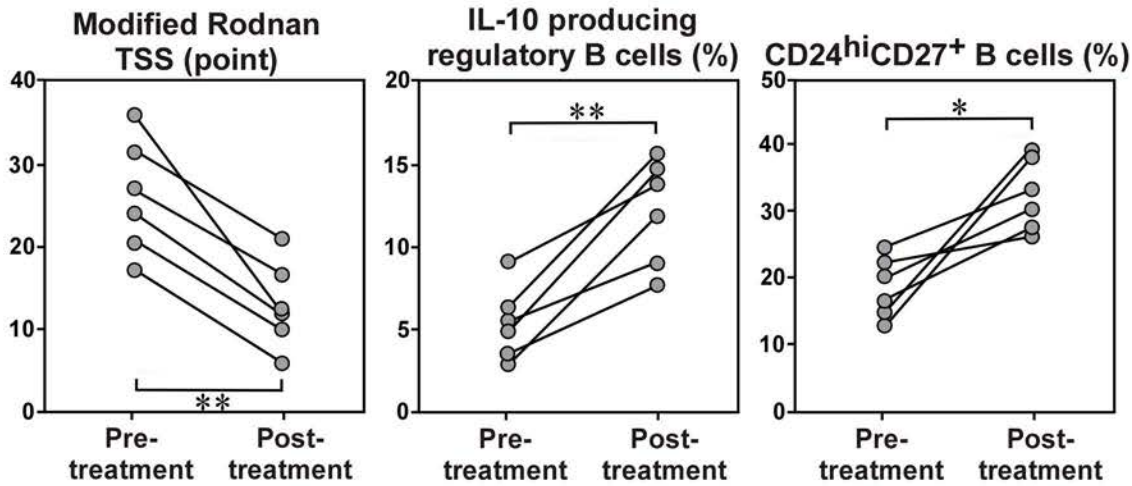
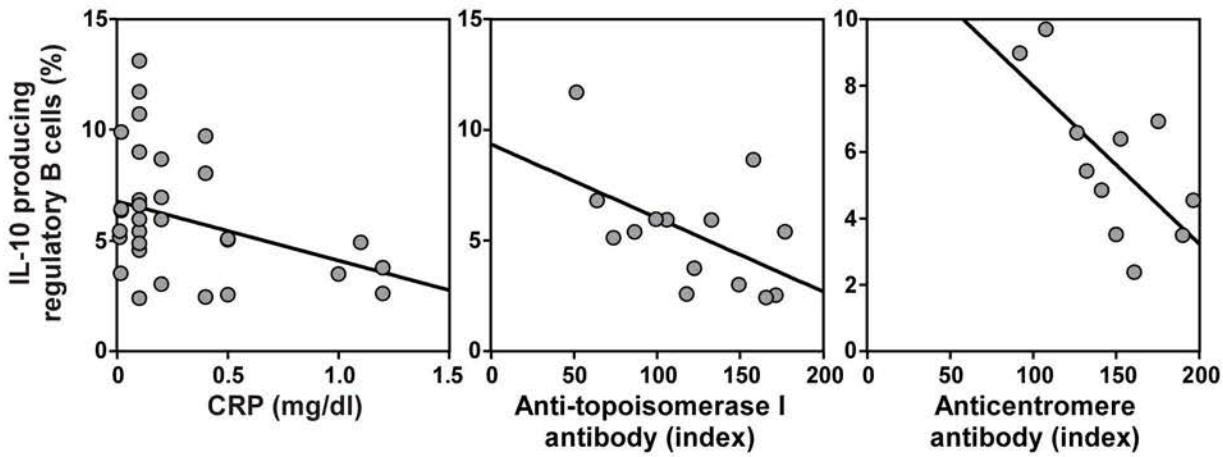
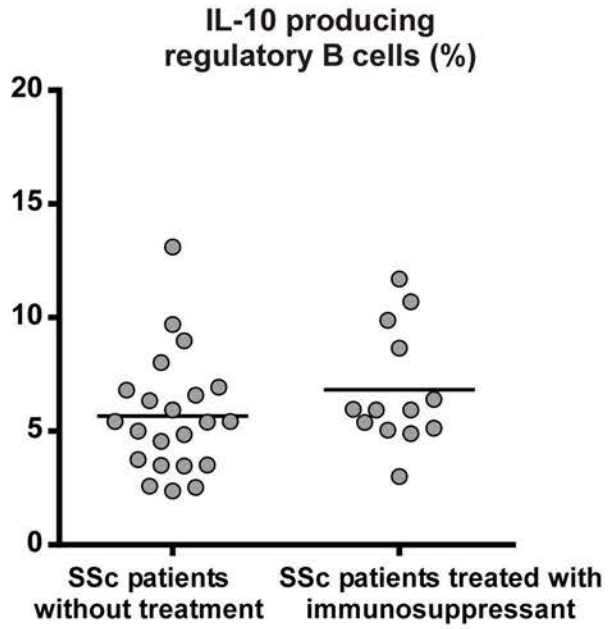


Figure 2
Matsushita et al.



Supplementary Figure 1
Matsushita et al.



Supplementary Figure 2
Matsushita et al.