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PAPER

Increased Syk phosphorylation leads to overexpression of TRAF6 in peripheral B cells of patients with systemic lupus erythematosus

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Objective: Activation of B cells is a hallmark of systemic lupus erythematosus (SLE). Syk and TRAF6 are key signaling molecules in B-cell activation through BCR and CD40/TLR, respectively. Nevertheless, whether expression of Syk and TRAF6 is altered in SLE B cells remains unknown. **Methods:** Phosphorylation and/or expression of Syk and TRAF6 were analyzed by flow cytometry in peripheral blood mononuclear cells isolated from SLE patients. **Results:** Pronounced phosphorylation and expression of Syk were noted in B cells from SLE patients compared with healthy donors. Levels of Syk phosphorylation correlated with the disease activity score. TRAF6 was significantly over-expressed in B cells of SLE patients as compared with healthy donors, and significant correlation of levels of TRAF6 expression and Syk phosphorylation was observed in SLE patients. Levels of TRAF6 expression were more pronounced in CD27+ memory B cells than in CD27-naïve B cells. In vitro treatment of SLE B cells with a Syk inhibitor (BAY61-3606) reduced Syk phosphorylation as well as TRAF6 expression. **Conclusion:** Our results suggest that the activated Syk-mediated TRAF6 pathway leads to aberrant activation of B cells in SLE, and also highlight Syk as a potential target for B-cell-mediated processes in SLE. *Lupus* (2014) **0,** 1–10.

Key words: Systemic lupus erythematosus; renal lupus; neuropsychiatric lupus; Syk; TRAF6; B cell

Introduction

A hallmark feature of the pathogenesis of systemic lupus erythematosus (SLE) is the aberrant activation of autoreactive T cells and overproduction of autoantibodies by B cells. Recent evidence highlights that B cells not only produce pathogenic autoantibodies but also function as potent antigen-presenting cells and modulate immune responses via production of cytokines and chemokines.[1]

Spleen tyrosine kinase (Syk) is a 72 kDa non-receptor type protein tyrosine kinase (PTK)[2] that is activated via multichain immune receptors such as B-cell receptor (BCR), T-cell receptor (TCR) and Fc receptor (FcR), and widely expressed in immunocompetent cells such as mast cells, macrophages, neutrophils, B cells and T cells.[3,4] Syk inhibitors are effective for treating rheumatoid arthritis (RA), bronchial asthma, B-cell lymphoma and idiopathic thrombocytopenic purpura.[5–9] In rodent lupus models, Syk blockade prevents the development of skin and kidney lesions.[10,11]

The molecular mechanisms of BCR-mediated Syk activation have to date been investigated mainly in mouse B cells. Upon BCR ligation by antigens, PTKs such as Lyn and Syk are initially activated. Syk in turn propagates the signal by phosphorylating a wide array of downstream signaling molecules.[12] In general, BCR-triggered B cells require additional signals for efficient proliferation and differentiation. Recent evidence suggests that a combination of three stimuli, BCR triggering (1st signal), cognate T-cell help such as CD40 (2nd signal) and Toll-like receptor stimulation by endogenous nucleic acids and immunocomplexes (3rd signal), induces the most robust B-cell

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proliferation and differentiation, thus recapitulating the pathogenesis of SLE.[13–16]

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is a key molecule of CD40 and TLR9 signaling in B cells. CD40 is the most characterized member of the TNFR superfamily expressed in B cells, and its interactions with TRAFs have been thoroughly investigated. TRAF6 is involved in CD40-mediated expression of CD80[17] and IL-6[18] in B cells.

Recently we reported the close inter-relation of BCR and CD40/TLR9 pathways in human B cells by showing that BCR-induced Syk activation leads to optimal induction of TRAF6, allowing efficient activation of CD40/TLR9 signals required for proliferation and differentiation of memory B cells.[19] Whether an inter-relation between BCR and CD40/TLR9 signal, however, is also operational in SLE B cells remains unknown.

In the present study we demonstrate that Syk phosphorylation was significantly increased in B cells of patients with SLE. Levels of Syk phosphorylation correlated well with disease activity score. Moreover, TRAF6 was significantly overexpressed in B cells of patients with SLE compared with healthy donors, and a strong correlation of TRAF6 expression and Syk phosphorylation was observed. Notably, TRAF6 expression was indeed higher in $CD27^+$ memory B cells than that in $CD27^-$naïve B cells. Together, these results suggest that the Syk–TRAF6 loop plays a role in aberrant activation of B cells in patients with SLE.

Materials and methods

Isolation and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) from 25 healthy donors (HDs) and from 58 patients with SLE (all Japanese) who fulfilled the American College of Rheumatology revised criteria for SLE[20] were isolated from peripheral blood using lymphocyte separation medium (ICN/Cappel Pharmaceuticals, Aurora, Ohio, USA).

SLE clinical activity was assessed by the British Isles Lupus Assessment Group (BILAG) activity index[21] and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI).[22] Informed consent was obtained from each patient in accordance with the requirement of the study protocol approved by the Ethics Committee of Medicine and Medical Care, University of Occupational and Environmental Health, Japan. In the experiment shown in Figure 4, PBMCs were cultured at 37°C in RPMI with 10% FCS in a 5% CO_2 for 2 h with or without the Syk inhibitor (BAY61-3606).

Flow cytometric analysis

For intracellular staining of total Syk, phosphorylated Syk, and TRAF6 in $CD19^+$ B cells in HDs and SLE patients, PBMCs washed with PBS were fixed with PBS containing 1% formaldehyde and permeabilized with PBS containing 0.1% saponin (saponin-PBS). After washing, they were resuspended in saponin-PBS and stained with mouse anti-human Syk mAb (Abcam, Tokyo, Japan), mouse anti-human phospho-Syk (pY348) mAb (BD PharMingen) and mouse anti-human TRAF6 mAb (Santa Cruz Biotechnology), followed by washing with saponin-PBS. PE-labeled goat anti-mouse IgG pAb (BD PharMingen) was used as a secondary antibody. After washing with saponin-PBS, they were stained with fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD19 (BD Pharmingen, San Diego, California, USA) antibodies and allophycocyanin (APC)-conjugated mouse anti-human CD27 (BioLegend, San Diego, California, USA) antibodies. We thus determined the ratio of $CD19^+$ B cells in PBMCs; 10^5 cells of PBMCs were subjected for FACS analysis.

In this study, we used % positive ratio for analyzing Syk phosphorylation and TRAF6 expression in B cells, because $CD27^-$ (naïve) and $CD27^+$ (memory) B cells behaved differently. However, since almost all B cells of healthy controls and patients express Syk, albeit with different levels of expression, we used ΔMFI (mean fluorescence intensity) in analyzing Syk expression. We defined ΔMFI of Syk expression and % positive ratio of p-Syk and TRAF6 expression in B cells as follows:

(i) ΔMFI of Syk expression in B cells was calculated as MFI of Syk expression subtracted from that of IgG isotype control of untreated cells.
(ii) %positive ratio of p-Syk (or TRAF6) expression in B cells was calculated as percentage of p-Syk (or TRAF6)-positive $CD19^+$ B cells out of total $CD19^+$ B cells. We defined p-Syk (or TRAF6)-positive $CD19^+$ B cells as cells stained above background with the IgG control antibody.

Western blot analysis

These experiments were performed as previously described.[19]

Statistical analysis

Data are expressed as mean ± SD. Baseline and post-treatment values within each sample were compared using the Wilcoxon matched-pairs signed-rank test, and paired *t*-test. Correlation analysis was performed with the use of Spearman's correlation coefficients. *P*-values less than 0.05 were considered significantly different. All analyses were conducted using the PASW Statistics analysis software v18.0.

Results

The baseline characteristics of the patients with SLE are shown in Table 1. Twelve treatment-naïve patients were enrolled in the study. The majority of the patients were receiving oral prednisolone. Some patients were also receiving immunosuppressants. Although five patients had received rituximab treatment more than 2 years before entry, the number of $CD19^+$ B cells was completely recovered at study entry. The patients taking combination therapy were defined as those who were taking oral prednisolone in conjunction with immunosuppressants except for rituximab.

Syk expression was more pronounced, with statistically significant difference, in B cells from patients with active SLE compared with HDs (ΔMFI; HDs: 627.8 ± 437.1, inactive SLE: 824.5 ± 479.5, active SLE: 1018.0 ± 451.2, $p = 0.0076$, ANOVA) (Figure 1(a)). We next investigated Syk expression in gating on $CD19^+CD27^-$ naïve B cells and $CD19^+CD27^+$ memory B cells. Interestingly, Syk expression was more pronounced, with statistical difference, in $CD19^+CD27^+$ memory B cells compared with $CD19^+CD27^-$ naïve B cells from HDs (ΔMFI; HD; $CD19^+CD27^-$ naïve B cells: 536.9 ± 306.1, $CD19^+CD27^+$ memory B cells: 655.8 ± 458.6, $p = 0.0063$, paired *t*-test). On the other hand, Syk expression was at high levels and comparable between naïve and memory B cells from inactive/active SLE patients (ΔMFI; inactive SLE; $CD19^+CD27^-$ naïve B cells: 835.9 ± 468.6, $CD19^+CD27^+$ memory B cells: 805.1 ± 477.9, $p = 0.5312$, active SLE; $CD19^+CD27^-$ naïve B cells: 1171.5 ± 618.9, $CD19^+CD27^+$ memory B cells: 1115.4 ± 670.1, $p = 0.6232$) (Figure 1(b)). Moreover, we investigated ΔMFI of Syk expression in $CD27^+$ memory B cells/ΔMFI of $CD27^-$ naïve B cells in HDs and patients with inactive/active SLE. This ratio was significantly higher in HDs compared with inactive/active SLE patients (ΔMFI of Syk expression in $CD27^+$ memory B cells/ΔMFI of $CD27^-$ naïve B cells; HD: 1.22 ± 0.24, inactive SLE: 1.00 ± 0.27, active SLE, SLE: 1.00 ± 0.36, $p = 0.0033$; ANOVA) (Figure 1(c)). These results suggest that Syk expression is significantly higher in memory B cells than naïve B cells from HDs, while it is equally high on both subsets from patients with inactive/active SLE.

Even in the absence of stimuli, Syk phosphorylation was increased with statistically significant difference in SLE patients, especially active SLE patients who fulfilled a new BILAG A or two BILAG B flares, compared with HDs (p-Syk positive ratio among $CD19^+$ cells (%); HDs: 12.5 ± 8.5, inactive SLE: 32.6 ± 31.9, active SLE: 65.4 ± 21.4, $p < 0.001$, ANOVA). Levels of Syk phosphorylation significantly correlated with the disease activity score (SLEDAI, $p = 0.002$, $r = 0.400$, Spearman's test) (Figure 2(a)). CD86, an activation marker, was coexpressed with p-Syk in B cells from

Table 1 Characteristics of the study subjects

	healthy donors (n = 25)	*SLE patients (n = 58)*
Age, mean (range) years	36 (22–52)	41 (18–77)
Sex, no. of females / no. of males	23/2	52/6
Disease duration, mean (range) months		114 (1–324)
naïve to treatment		12
Prednisolone (or equivalent) treatment (mg)		10 (0.5–60)
Taking/No. taking		45/13
Immunosuppressant		
CY		8
CsA		2
AZA		12
MZ		3
MTX		1
TAC		5
History of treatment with rituximab		5
No. of patients taking combination therapy at study enrollment (%)		32/58 (55%)
Lymphocyte cell count (cells/μl), mean ± SD		1051 ± 577
Anti-ds-DNA antibody(average ± SD of positive)		114 ± 127
Serum complement (CH50)(average ± SD of positive)		12 ± 7
History of treatment with rituximab		5
SLEDAI score, mean (range)		6 (0–23)
BILAG score (one or more category A or two or more category B)		23/58

CY: cyclophosphamide; CsA: cyclosporine; AZA: azathioprine; MZ: mizoribine; MTX: methotrexate; TAC: taclorimus. Patients who have history of treatment with rituximab were enrolled more than 2 years after treatment with rituximab and the numbers of $CD19^+$ B cells have completely recovered. Taking combination therapy: treatment with prednisolone and/or immunosuppressive drug such as CsA, AZA, MZ, MTX, and TAC at that point. Patients with a history of rituximab were not included.

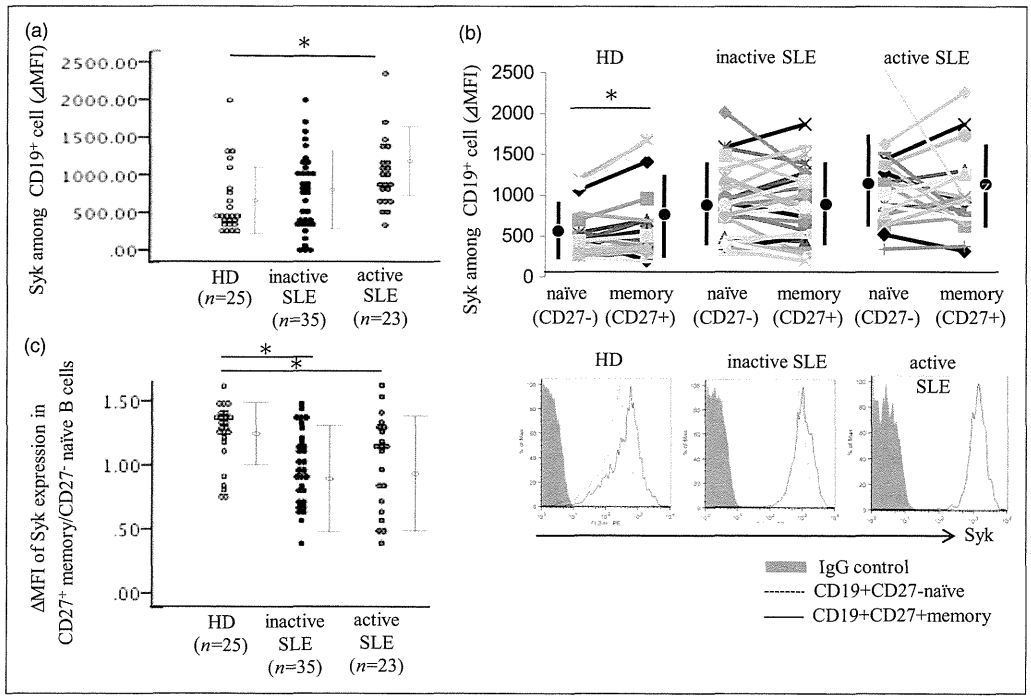

Figure 1 Expression of Syk in $CD19^+$ B cells from healthy donor (HD) and SLE patients. (a) Lymphocyte lesion on PBMC was gated. The geometric mean fluorescence intensity (MFI) of expression of Syk in $CD19^+$ B cells from inactive/active SLE patients was compared with that of HDs. ΔMFI of Syk expression on $CD19^+$ B cells = (MFI of Syk expression on $CD19^+$ B cells) – (MFI of IgG isotype control on $CD19^+$ B cells). (b) The geometric mean fluorescence intensity (MFI) of expression of Syk in $CD19^+CD27^-$ naïve and $CD19^+CD27^+$ memory B cells from inactive/active SLE patients compared with HDs. Representative data of Syk in $CD19^+CD27^-$ naïve and $CD19^+CD27^+$ memory B cells in HD #6, inactive SLE patient #33 and active SLE patient #17 (SLE) are shown. (c) ΔMFI of Syk expression in $CD27^+$ memory B cells/ΔMFI of $CD27^-$naïve B cells in HD and inactive/active SLE patients. $*p < 0.05$.

patients with active SLE (Figure 2(b)). Next, we also investigated Syk phosphorylation in $CD19^+CD27^-$ naïve and $CD19^+CD27^+$ memory B cells. Syk phosphorylation was at high levels and comparable between naïve and memory B cells from SLE patients (p-Syk positive ratio among $CD19^+$ cells (%); inactive SLE; $CD19^+CD27^-$ naïve B cells: 36.0±33.0, $CD19^+CD27^+$ memory B cells: 36.1 ± 29.3, $p = 0.9008$, active SLE; $CD19^+CD27^-$ naïve B cells: 59.0 ± 25.0, $CD19^+CD27^+$ memory B cells: 57.1 ± 22.1, $p = 0.5787$, paired t-test) (Figure 2(c)). In contrast to the correlation with SLEDAI, levels of anti-dsDNA antibodies and total hemolytic complement activity (CH50) in sera did not correlate with those of Syk phosphorylation (data not shown). These results suggest that Syk phosphorylation in B cells from SLE patients was increased, particularly in those with active disease.

To validate the evidence that Syk phosphorylation was increased in SLE B cells, we examined by Western blotting the phosphorylation of PLCγ, which is a downstream molecule of Syk and regulates calcium flux in B cells. Consistent with p-Syk expression levels, the levels of PLCγ phosphorylation in $CD19^+$ B cells from patients with SLE were clearly higher than those in HDs (Figure 3(a)). In addition, we tested the phosphorylation of Btk, which functions as key downstream molecule of Syk, in B cells. Again, the level of Btk phosphorylation in $CD19^+$ B cells from patients with SLE was significantly higher than that from HDs (B cell of p-Btk, HDs; 322.9 ± 28.1, SLE patients; $532.7 \pm 46.0\%$, $p = 0.008$, Wilcoxon's test) (Figure 3(b)).

We next tested the levels of TRAF6 expression in B-cell subsets from patients with SLE. Levels of TRAF6 expression were higher, with statistical difference, in SLE patients than those of HDs (TRAF6-positive ratio among $CD19^+$ cells (%); HDs: 2.7 ± 1.9, SLE: 16.9 ± 15.3, $p < 0.001$, Wilcoxon's test) (Figure 4(a)). Notably, TRAF6 expression was increased with statistical difference in patients with active SLE, compared with inactive

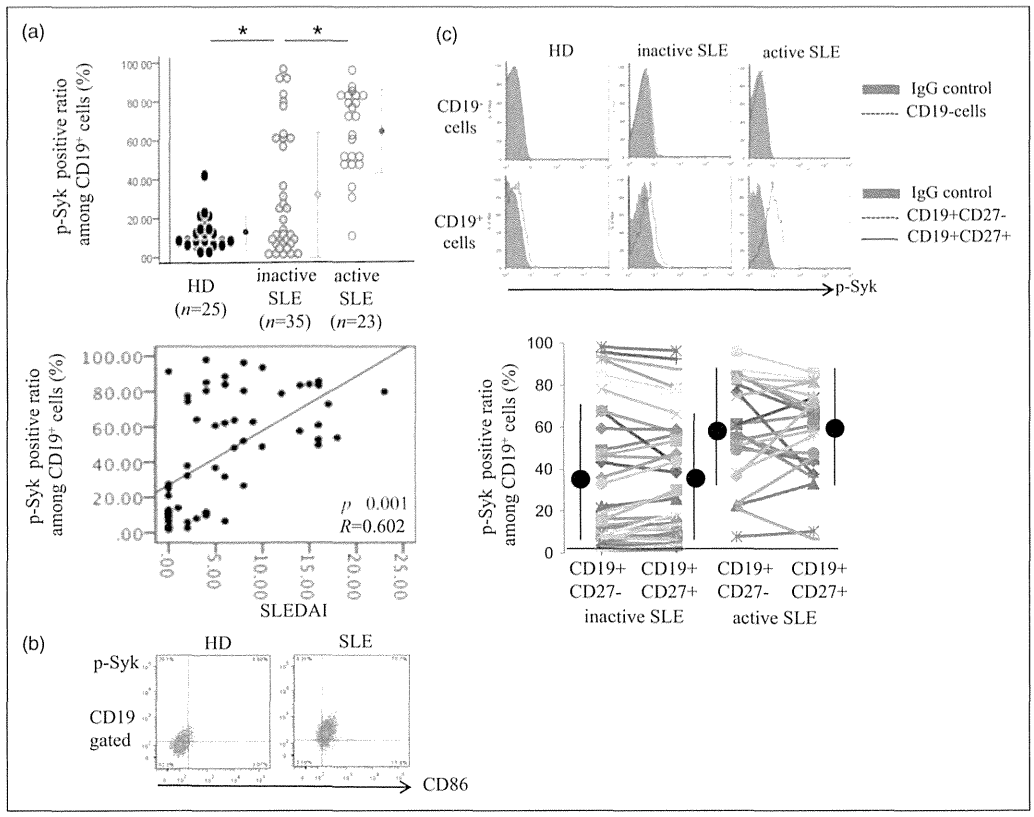

Figure 2 Phosphorylation of Syk in $CD19^+$ B cells from healthy donor (HD) and SLE patients. (a) Phospho-Syk positive ratio in $CD19^+$ B cells from inactive/active SLE patients compared with HDs. Patients with active SLE fulfilled a new BILAG A or two BILAG B flares. Lower panel depicts the correlation between phospho-Syk positive ratio in $CD19^+$ B cells from SLE patients and disease activity score (SLEDAI). (b) P-Syk phosphorylation and CD86 expression in $CD19^+$ cells. (c) Lymphocytes were separated to $CD19^-$ cells and $CD19^+$ cells. Representative data of phospho-Syk in $CD19^-$ cells and $CD19^+CD27^-$naïve and $CD19^+CD27^+$ memory B cells in HD #6, SLE patient #14 (inactive), and #1 (active) are shown. Phospho-Syk positive ratio in $CD27^-$naïve B cells and $CD27^+$ memory B cells in inactive/active SLE patients. $^*p < 0.05$.

SLE (TRAF6-positive ratio among $CD19^+$ cells (%); inactive SLE: 10.0 ± 10.9, active SLE: 27.4 ± 15.3, $p < 0.001$, Wilcoxon's test) and significantly correlated with disease activity assessed by SLEDAI ($p < 0.001$, $r = 0.460$, Spearman's test) (Figure 4(b)). We next tested the levels of TRAF6 expression in B-cell subsets from patients with SLE. Levels of TRAF6 expression were higher, with statistical difference, in memory ($CD19^+CD27^+$) B cells than those in naïve ($CD19^+CD27^-$) B cells of SLE patients (TRAF6-positive ratio among $CD19^+$ cells (%);$CD19^+CD27^-$ naïve B cells: 14.8 ± 12.4, $CD19^+CD27^+$ memory B cells: 24.0 ± 15.0, $p < 0.001$, paired t-test). In addition, we investigated TRAF6 expression on $CD27^-$ naïve and $CD27^+$ memory B cells in patients with active SLE. They exhibited a similar tendency which was, however, more remarkable in active disease (TRAF6-positive ratio among $CD19^+$ cells (%);$CD19^+CD27^-$ naïve B cells: 22.6 ± 13.7, $CD19^+CD27^+$ memory B cells: 34.0 ± 14.9, $p < 0.001$, paired t-test). (Figure 4(c)). These results suggest that TRAF6 expression, especially in memory B cells from SLE patients, was pronounced particularly in those with active disease.

We have very recently reported that BCR-induced Syk activation potently increases expression of TRAF6, a key molecule of CD40 and TLR9 signaling in human peripheral B cells.[19] This prompted us to test the correlation of TRAF6 expression and %positive ratio of p-Syk among $CD19^+$ B cells of patients with SLE. TRAF6 expression was induced by either BCR or CpG stimulation, but not CD40. The Syk inhibitor (BAY3616) selectively abrogated BCR-induced, but not CpG-induced TRAF6 expression (Figure 5(a)), suggesting that Syk is involved in TRAF6 expression downstream of the BCR, but

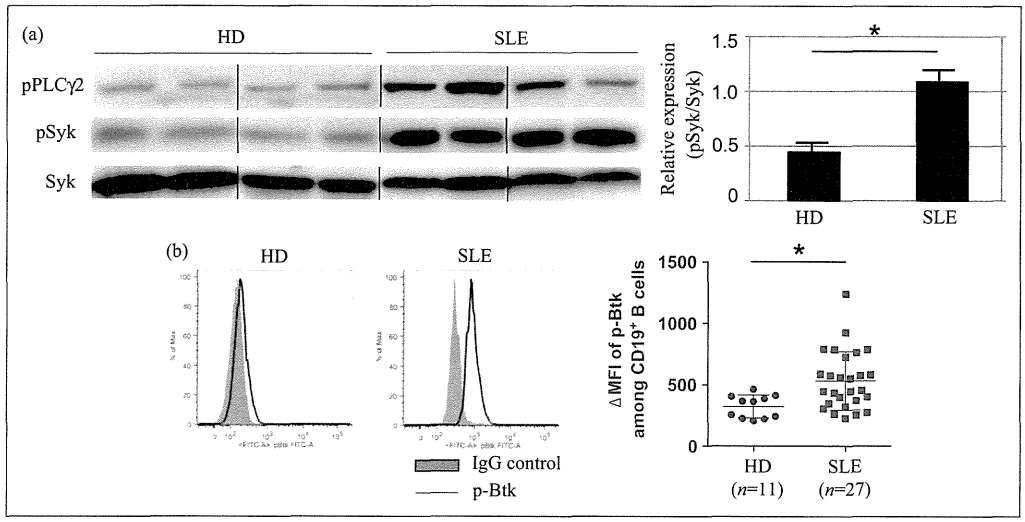

Figure 3 Activation of Syk and its downstream molecules in $CD19^+$ B cells from healthy donors (HDs) and SLE patients. (a) Expression of p-PLCγ along with Syk/p-Syk expression in $CD19^+$ B cells from HDs and SLE patients was assessed by Western blotting. (b) Phospho-Btk positive ratio in $CD19^+$ B cells from inactive/active SLE patients compared with HDs.

not TLR9. Interestingly, TRAF6 expression significantly correlated with %positive ratio of p-Syk among $CD19^+$ B cells ($p < 0.001$, $r = 0.776$, Spearman's test) (Figure 5(b)). To further test whether Syk blockade exerts inhibitory effects on SLE B cells, B cells were treated in vitro with or without the highly specific Syk inhibitor (BAY61-3606) for 2 h and expression of phospho-Syk and TRAF6 was analyzed. The Syk inhibitor significantly reduced Syk phosphorylation as well as TRAF6 expression (%positive ratio of p-Syk among $CD19^+$ B cells (%): Syk inhibitor (−) 45.6 ± 32.3, Syk inhibitor (+) 21.6 ± 17.5, $p < 0.001$; TRAF6-positive ratio among $CD19^+$ cells (%): Syk inhibitor (−) 17.7 ± 15.8, Syk inhibitor (+) 6.6 ± 6.1, $p < 0.001$, paired t-test) (Figure 5(c)). Moreover, we investigated the correlation between change ratio of % positive ratio of p-Syk and TRAF6 among $CD19^+$ B cells. They were significantly correlated ($p < 0.001$, $r = 0.517$, Spearman's test) (Figure 5(d)). These results suggest that the Syk–TRAF6 loop in B cells plays a role in SLE, which could be down-regulated by the Syk inhibitor.

Discussion

In this study we demonstrate that Syk phosphorylation was constitutively increased in SLE B cells. Levels of Syk phosphorylation correlated with the disease activity score. Moreover, TRAF6 expression was significantly up-regulated in SLE B cells and a strong correlation of TRAF6 expression and Syk phosphorylation was observed. Levels of TRAF6 expression were also higher in $CD27^+$ memory B cells, compared with $CD27^-$ naïve B cells. The Syk inhibitor significantly abrogated Syk phosphorylation and TRAF6 expression in SLE B cells in vitro.

We here show that in HDs the levels of Syk expression in memory B cells were significantly higher than those in naïve B cells. On the other hand, the levels of Syk expression were similarly high in both subsets from SLE patients irrespective of disease activity. This profile in B cells from SLE patients was not associated with altered cell viability (data not shown). It is thus feasible that a fundamental disease-associated process regulates the levels of Syk expression in lupus B cells. Further work is needed to address this issue.

BCR-induced calcium mobilization and protein tyrosine phosphorylation are both pronounced in SLE B cells,[23] suggesting that alterations in B-cell signaling occur at the proximity of the BCR. Intriguingly, a recent report showed that $CD19^{hi}$ memory B cells, which are enriched in autoreactivity, exhibit higher basal levels of Syk phosphorylation than $CD19^{lo}$ cells in patients with SLE.[24] CD19 is a positive regulator of BCR signaling in B cells; however, loss of CD19 does not affect Syk phosphorylation and activation.[25] This suggests that enhanced CD19 expression in SLE B cells

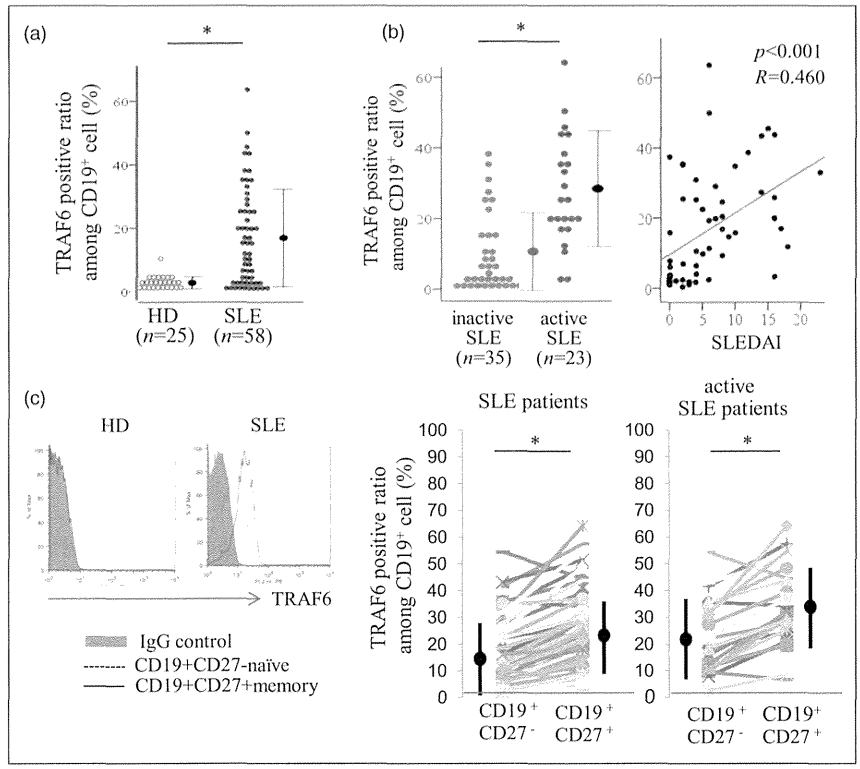

Figure 4 Expression of TRAF6 in $CD19^+$ B cells from healthy donor (HD) and SLE patients. (a) The percentage of positive expression of TRAF6 in $CD19^+$ B cells from HDs and SLE patients. (b) TRAF6 positive ratio in $CD19^+$ B cells from inactive/active SLE patients compared with HDs. Patients with active SLE fulfilled a new BILAG A or two BILAG B flares. Right panel depicts the correlation between TRAF6 positive ratio in $CD19^+$ B cells from SLE patients and disease activity score (SLEDAI). (c) Expression of TRAF6 in $CD19^+$ $CD27^-$ naïve and $CD19^+$ $CD27^+$ memory B cells from SLE patients. Representative data of TRAF6 expression in healthy donor (HD) #6 and active SLE patient #48 are shown. The right panel depicts the percentage of positive expression of TRAF6 in $CD19^+$ $CD27^-$naïve and $CD19^+$ $CD27^+$ memory B cells from SLE patients. $*p < 0.05$.

does not directly contribute to high basal levels of Syk phosphorylation. Consistent with this idea, our results show that B cells from patients with active SLE exhibit high basal levels of Syk phosphorylation regardless of CD19 levels, thus implying that Syk activation in SLE B cells is caused mainly by a CD19-independent mechanism.

We show here that basal TRAF6 expression is enhanced in B cells from patients with active SLE and significantly correlated with Syk phosphorylation (Figures 3 and 4). In SLE pathogenesis RNA- or DNA-containing autoantigens co-ligate BCRs and TLR-7/9, leading to robust activation, proliferation and differentiation of autoreactive B cells. In addition, the cognate interaction of T and B cells via the CD40L–CD40 system is crucial in the pathogenesis of autoimmune diseases including SLE.[13-16] TRAF6 is a key molecule downstream of CD40 and TLR9 signaling.[26] Recently we have shown that BCR stimulation alone strongly induces expression of TRAF6, but not TRAF2, 3 and 5, which is further enhanced by additional CD40 and TLR9 stimulation,[19] supporting the idea that Syk-mediated BCR signaling is a prerequisite for optimal induction of TRAF6, thereby allowing efficient activation of CD40 and TLR9 signaling. These results are further supported by a study of Kobayashi et al. showing that TRAF6 regulates activation of MAPK, NFκB and Akt.[27] Furthermore, single-nucleotide polymorphisms (SNPs) across the *TRAF6* gene have recently been evaluated in 7490 SLE and 6780 control subjects from different ancestries, and several SNPs (rs5030437, rs4755453 and rs540386) were considered statistically significant, although the impact of these SNPs on protein function remains to be clarified.[28] These results are consistent with our data showing the involvement of TRAF6 in the pathogenesis of SLE. Our current findings thus suggest that the novel Syk–TRAF6 loop is

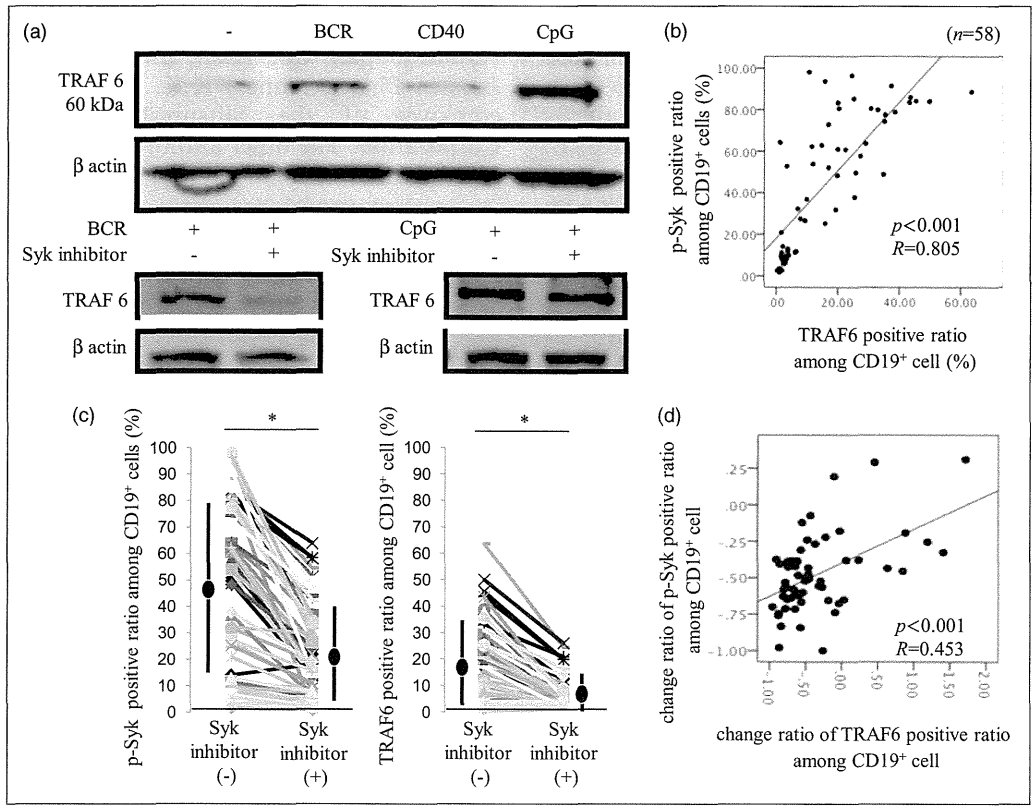

Figure 5 Effect of the Syk inhibitor on Syk phosphorylation and TRAF6 expression in $CD19^+$ B cells from SLE patients. (a) PB-B cells were stimulated with anti-BCR mAb (anti-Igλ and anti-Igk, 1 μg/ml each), soluble CD40L (2 μg/ml) or CpG-ODN2006 (2.5 μg/ml) during the last 24 h of total 72 h culture with or without Syk inhibitor 1 μM. Expression of TRAF-6 was assessed by Western blotting. β-actin was served as a loading control. (b) The correlation between phospho-Syk positive ratio in $CD19^+$ B cells (y-axis) and the percentage of positive expression of TRAF6 (x-axis) in $CD19^+$ B cells from active SLE patients. (c) The percentage of positive expression of phospho-Syk and TRAF6 in $CD19^+$ B cells from SLE. PBMCs were collected from blood of HD and patients with SLE. They were washed with PBS and cultured with 10% FCS RPMI with or without the Syk inhibitor (BAY61-3606) for 2 h in the absence of stimuli. (d) The correlation between change ratio of % positive ratio of p-Syk and TRAF6 among $CD19^+$ B cells. $*p < 0.05$.

constitutively operational and potentially generates robust CD40 and TLR9 signaling in SLE B cells.

It is established that memory B cells play more pathogenic roles than naïve B cells in SLE.[29-31] We found that TRAF6 expression was up-regulated with statistically significant difference in $CD27^+$ memory B cells in SLE patients compared with $CD27^-$naïve B cells (Figure 3). B-cell-specific disruption of TRAF6 in mice results in a lower number of mature B cells as well as inhibition of antibody class switching and impaired differentiation to plasma cells.[27] These results suggest a role of TRAF6 in the survival and differentiation of pathogenic memory B cells of SLE patients. It is important to note that in patients with SLE, TRAF6 is expressed at higher levels in memory than naïve B cells, while Syk expression and phosphorylation are comparable between the two subsets. This apparent discrepancy might be explained by the possibility that another stimulation cooperates with BCR–Syk activation to induce TRAF6 in memory B cells from patients with SLE. Notably, Bernasconi et al. reported that in the absence of stimuli, TLR9 is barely expressed in naïve B cells, while it is remarkably expressed in memory B cells.[32] Given that CpG (a TLR9 ligand) stimulation can induce TRAF6 expression in B cells (Figure 4(a)) and is implicated in SLE pathology, TRAF6 expression in memory B cells from patients with SLE might be triggered by synergy between BCR and TLR9 in vivo. Further work is required to substantiate this hypothesis.

Efficacy of Syk inhibitors was shown in the treatment of RA, bronchial asthma, B-cell lymphoma

and idiopathic thrombocytopenic purpura.[5-9] Syk blockade prevents the development of skin and kidney lesions in murine lupus;[10,11] however, it remains unknown whether this approach is therapeutically beneficial for treatment of human SLE. We show here that the Syk inhibitor profoundly suppressed Syk phosphorylation and TRAF6 expression in SLE B cells (Figure 4). A possible explanation for inhibitory effects of the Syk inhibitor on patients' B cells would be that it blocks a feed-forward loop of autophosphorylation of Syk by p-Syk. Collectively, these results raise the possibility that Syk blockade alleviates aberrant B-cell activation in human SLE.

Taken together, our current findings not only uncover a novel molecular explanation for aberrant B-cell signaling through the Syk–TRAF6 loop in patients with SLE, but also suggest the usefulness of phosphorylated Syk and TRAF6 in peripheral B cells as a biomarker for estimating disease activity and the efficacy of treatments. Moreover, our results stress that Syk inhibitors have considerable promise as new drugs in the treatment of autoimmune diseases including SLE.

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Conflict of interest Statement

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Activation of Syk in Peripheral Blood B Cells in Patients With Rheumatoid Arthritis

A Potential Target for Abatacept Therapy

Shigeru Iwata, Shingo Nakayamada, Shunsuke Fukuyo, Satoshi Kubo, Naoki Yunoue, Sheau-Pey Wang, Maiko Yoshikawa, Kazuyoshi Saito, and Yoshiya Tanaka

Objective. **B cells play a pivotal role in the pathogenesis of autoimmune diseases. Although Syk functions as a key molecule in B cell receptor signaling, the pathologic role of Syk in B cells in rheumatoid arthritis (RA) remains unclear. The purpose of this study was to assess the relevance of activation of Syk in B cells to the pathologic development of RA and to the responsiveness of RA patients to treatment with biologics.**

Methods. **Healthy subjects (n = 36) and patients with moderate or severe RA disease activity (n = 70) were studied. The phosphorylation of Syk (pSyk) in peripheral blood B cells was measured by flow cytometry, and its correlation with clinical characteristics and changes after administration of biologic agents was evaluated.**

Results. **Levels of pSyk in peripheral blood B cells were preferentially higher in patients with RA compared to healthy subjects. Patients with significantly higher pSyk levels were strongly positive for anti–citrullinated protein antibodies (ACPAs). High pSyk levels were not correlated with the severity of disease activity. Treatment with abatacept, but not tumor necrosis factor inhibitors, significantly reduced the levels of pSyk in RA peripheral blood B cells. Abatacept also significantly reduced the proportion of follicular helper T (Tfh) cells.**

Conclusion. **Levels of pSyk in peripheral blood B cells were significantly elevated in patients with RA, and these patients also exhibited strong positivity for ACPAs. These data suggest that abatacept seems to inhibit the phosphorylation of Syk in B cells, as well as the development of Tfh cells, thus highlighting the relevance of B cell–T cell interactions as a potential target of abatacept therapy in RA.**

Activated autoreactive B cells produce autoantibodies and inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor α (TNFα). The expression of costimulatory molecules, such as CD40 and CD80, is enhanced on B cells and is involved in the interactive activation with surrounding immunocompetent cells, including T cells. B cells have an antigen-presenting activity, particularly in autoimmune diseases, and are associated with the activation of autoreactive T cells. Therefore, B cells play an important role in the pathogenetic processes of rheumatoid arthritis (RA).

Rituximab, a chimeric anti-CD20 antibody, eliminates B cells through antibody- and complement-dependent cytotoxic activities. The efficacy of rituximab

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has been demonstrated in RA patients with high disease activity (in the Dose-Ranging Assessment: International Clinical Evaluation of Rituximab in Rheumatoid Arthritis [DANCER] trial [1]) and in RA patients resistant to TNF inhibitor therapy (in the Randomized Evaluation of Long-term Efficacy of Rituximab in Rheumatoid Arthritis [REFLEX] trial [2]). Rituximab was approved for the treatment of RA in the US in 2006 and is currently considered the second-line biologic agent, subsequent to TNF inhibitor therapy. In addition to these studies, some clinical studies have demonstrated the efficacy of a humanized anti-CD20 antibody, ocrelizumab, and a fully human anti-CD20 antibody, ofatumumab, in patients with RA resistant to TNF inhibitor therapy, indicating that B cells are an evident therapeutic target for RA.

Syk is a 72-kd nonreceptor tyrosine kinase discovered by Taniguchi et al (3) in 1991. Syk is involved in the signaling pathway through Fc receptors, which are broadly expressed on immunocompetent cells, such as B cells, dendritic cells, mast cells, macrophages, and neutrophils, and on molecules associated with cell adhesion, such as integrin (4,5).

Recently, the importance of Syk in the pathologic processes of RA has been reported. The results of a phase II clinical study of R406, a Syk inhibitor, in patients resistant to treatment with methotrexate (MTX) indicated that phosphorylation of Syk (measured as levels of pSyk) was increased in the synovial tissue of RA patients compared to healthy subjects and patients with osteoarthritis (6–8). Another experimental study using the synovial cells from these patients demonstrated that R406 inhibits TNFα-induced activation of mitogen-activated protein kinases and the expression of the matrix metalloproteinase 3 (MMP-3) gene, thus highlighting the significant role of Syk in synovial fibroblasts of RA patients (9).

In addition, previous studies elucidated the role of Syk in B cells. Syk has important roles in B cell maturation and survival (10,11). The Toll-like receptor 9 (TLR-9) signaling pathway is involved in the activation of B cells and autoantibody production by B cells (12,13). In this regard, we have recently demonstrated that signaling through Syk results in effective signal transduction of TLR-9 by inducing optimal expression of TNF receptor–associated factor 6 (TRAF6), and that this signaling is important for antibody production by B cells (14). Based on these results, we hypothesized that Syk phosphorylation in B cells is involved in the pathologic processes of RA through the production of autoantibodies, such as rheumatoid factor (RF) and anti–citrullinated protein antibodies (ACPAs).

T cells (especially Th1 and Th17 cells) also play a pivotal role in the pathogenesis of RA (15,16). Recently, follicular helper T (Tfh) cells, whose primary task is to drive the formation of B cell responses, have been recognized as a critical regulator of autoimmunity (17,18). We and other investigators have elucidated the mechanism of Tfh cell differentiation (19,20); however, the exact role of this T helper cell subset in RA remains elusive.

Abatacept, a fusion protein containing CTLA-4 and Ig, which is referred to as a T cell–selective costimulatory regulator, inhibits the activation of T cells. However, little is known about the T cell populations targeted by abatacept. The effect of abatacept on antigen-presenting cells has also been reported (21–23). The inhibitory effect of abatacept on T cell–dependent antibody production has been reported in mice and cynomolgus monkeys (24,25). Evidence suggests that abatacept also has an inhibitory effect on bone destruction, by suppressing the production of RF and ACPAs (26). However, the effect of abatacept on human B cells is unknown. Based on these observations, abatacept is predicted to regulate the activation of not only T cells but also B cells, directly and/or indirectly.

In this study, we observed significantly elevated Syk phosphorylation in the peripheral blood B cells of patients with RA compared to healthy subjects, and we demonstrated that the levels of pSyk were significantly high in patients who were strongly positive for ACPAs. Moreover, treatment with abatacept, but not with TNF inhibitors, significantly inhibited Syk phosphorylation in B cells. Interestingly, treatment with abatacept significantly reduced the proportion of Tfh cells, which could be a possible mechanism for the reduction in Syk phosphorylation in B cells. The results suggest that Syk plays an important role in ACPA production by B cells in patients with RA, and that abatacept inhibits both Syk phosphorylation in B cells and the development of Tfh cells.

PATIENTS AND METHODS

Patients. Table 1 summarizes the baseline characteristics of the 70 patients with RA. The healthy control subjects (n = 36) were either staff members of our hospital or healthy subjects who visited our hospital for medical examinations. Patients with RA who were resistant to treatment comprised those whose score of RA disease activity was >3.1 on the Disease Activity Score in 28 joints using erythrocyte sedimentation rate (DAS28-ESR) (27), despite having received treat-

Table 1. Characteristics of the study patients with rheumatoid arthritis (n = 70)*

Age, mean ± SD years	61.4 ± 15.1
Sex, no. female/no. male	60/10
Disease duration, mean ± SD months	91.5 ± 114.4
Prednisolone (or equivalent)	
No. not receiving treatment/total no.	11/70
Dosage, mean ± SD mg/day	3.4 ± 1.9
Methotrexate	
No. not receiving treatment/total no.	53/70
Dosage, mean ± SD mg/week	13.0 ± 3.6
Tender joint count, mean ± SD	8.5 ± 7.3
Swollen joint count, mean ± SD	7.3 ± 6.3
CRP, mean ± SD mg/dl	2.0 ± 3.0
ESR, mean ± SD mm/hour	53.2 ± 33.3
IgG, mean ± SD mg/dl	1,512.5 ± 452.5
RF	
Mean ± SD IU/ml	149.7 ± 407.7
No. negative/no. positive	21/49
ACPA status, no.	
Negative	22
Positive	6
Strongly positive	42
MMP-3, mean ± SD ng/ml	194.8 ± 246.7
DAS28-CRP, mean ± SD	4.7 ± 1.4
DAS28-ESR, mean ± SD	5.5 ± 1.4
CDAI, mean ± SD	26.3 ± 15.0
SDAI, mean ± SD	28.3 ± 16.8
HAQ score, mean ± SD	1.3 ± 0.9
No. not treated with biologics/total no.	57/70

* CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; RF = rheumatoid factor; ACPA = anti–citrullinated protein antibody; MMP-3 = matrix metalloproteinase 3; DAS28-CRP = Disease Activity Score in 28 joints using CRP level; CDAI = Clinical Disease Activity Index; SDAI = Simplified Disease Activity Index; HAQ = Health Assessment Questionnaire.

ment with adequate doses of antirheumatic drugs, mainly MTX, for a minimum of 3 months, and who showed no response or only a moderate response to treatment according to the European League Against Rheumatism (EULAR) improvement criteria (28). The Human Ethics Review Committee of the university reviewed and approved our study, including the collection of peripheral blood samples from healthy adults and patients with RA. Each subject provided a signed participation consent form.

Measurements. The background factors investigated were sex, age, duration of RA, and doses of corticosteroids and MTX. We also evaluated the severity of morning stiffness, number of swollen joints, number of tender joints, and patient's evaluations of pain and overall health by visual analog scales, in addition to global evaluations of health by the attending physician. The laboratory tests included measurements of the C-reactive protein (CRP) level, ESR, IgG, RF, ACPAs, and MMP-3. We consulted the American College of Rheumatology (ACR)/EULAR 2010 classification criteria for RA (29) to select the cutoff values for stratification of ACPA positivity. Low-positive ACPA refers to IU values that are higher than the upper limit of normal (ULN) but ≤3 times the ULN for the laboratory and assay, whereas high-positive ACPA refers to IU values that are >3 times the ULN for the laboratory and assay. The variables investigated included the DAS28 using CRP level (DAS28-CRP), DAS28-ESR, the Clinical Disease Activity Index (CDAI) (30), the Simplified Disease Activity Index (SDAI) (31), the Health Assessment Questionnaire (HAQ) (32), and history of biologics use.

Flow cytometry analysis. Peripheral blood mononuclear cells (PBMCs) from 36 normal healthy volunteers and from 70 patients with RA whose diagnosis met the ACR 1987 revised classification criteria for RA (33) were isolated from the peripheral blood using lymphocyte separation medium (ICN/Cappel Pharmaceuticals). For surface and intracellular staining, 2×10^5 PBMCs, which were acquired after strict deletion of dust by threshold adjustment, were subjected to fluorescence-activated cell sorting analysis. PBMCs were fixed with phosphate buffered saline (PBS) containing 1% formaldehyde and then permeabilized with PBS containing 0.1% saponin. After washing, the PBMCs were resuspended in saponin–PBS and stained with mouse anti-human Syk monoclonal antibodies (mAb) (Abcam) and mouse anti-human pSyk (pY348) mAb (BD PharMingen), followed by washing with saponin–PBS. Phycoerythrin-labeled goat anti-mouse IgG polyclonal antibody (BD PharMingen) was used as a secondary antibody. After washing with saponin–PBS, the PBMCs were stained with fluorescein isothiocyanate–labeled mouse anti-human CD19 antibodies (BD PharMingen).

The rate of pSyk expression in B cells was calculated as the percentage of pSyk-positive CD19+ B cells relative to total CD19+ B cells. We defined pSyk-positive CD19+ B cells as cells in which the intensity of staining was higher than the background staining with IgG control antibody. The proportion of CD19+ B cells (relative to total cells) in healthy donors and RA patients was a mean ± SD 15,199 ± 7,482 cells (7.6 ± 3.7%) and 12,844 ± 7,120 cells (6.6 ± 3.6%), respectively.

Tfh cells were stained with anti-CD4, anti-CXCR5, and anti–programmed death 1 (anti-PD-1) antibodies (BD PharMingen). The proportion of CD4+ cells (relative to total cells) was 20,364 ± 17,727 cells (8.2 ± 7.0%), while that of CD4+CXCR5+PD-1+ cells (relative to total cells) was 1,841 ± 3,940 cells (0.7 ± 1.5%). Stained cells were analyzed on a flow cytometer (FACSCalibur; BD PharMingen). The cells were collected and analyzed with FlowJo software (Tree Star).

In vitro B cell activation analysis. CD19+ B cells were purified from the peripheral blood of the healthy control subjects and RA patients. The cells were cultured in stimulation-free medium for 3 days to assess the production of IL-6 or for 5 days to assess the production of IgG. IL-6 production was determined using a BD Cytometric Bead Array human Flex set (BD PharMingen). Flow cytometry was carried out using a FACSCalibur and CellQuest software (Becton Dickinson). IgG levels in the culture medium were determined using a human IgG enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories).

Statistical analysis. Data are expressed as the mean ± SD. Differences between groups for variables with normal distribution and homoscedasticity were compared using Student's *t*-test. Differences between groups for variables with skewed distribution were compared using Wilcoxon's rank sum test. Analysis of variance followed by the Bonferroni/Dunn post hoc test was used to compare data from 3 groups with normal distribution. The Kruskal-Wallis test followed by the Bonferroni/Dunn post hoc test was used to compare data from

>3 groups with skewed distribution. Correlation analysis was performed using Spearman's correlation coefficients. Baseline and posttreatment values within each sample were compared using Wilcoxon's matched-pairs signed-rank test. *P* values less than 0.05 were considered significant. All analyses were conducted using PASW Statistics software version 18.0 (IBM).

RESULTS

Patient background. This study was conducted in 70 patients with RA who were receiving treatment in our hospital in Japan. The clinical features of the RA patients are described in Table 1. The washout period in patients who had previously received biologics (etanercept, golimumab, adalimumab, tocilizumab, abatacept) was more than 1 month. Infliximab required a 60-day washout.

High Syk phosphorylation in B cells of ACPA-positive RA patients. PBMCs were isolated from 36 healthy donors (as controls) and 70 patients with RA

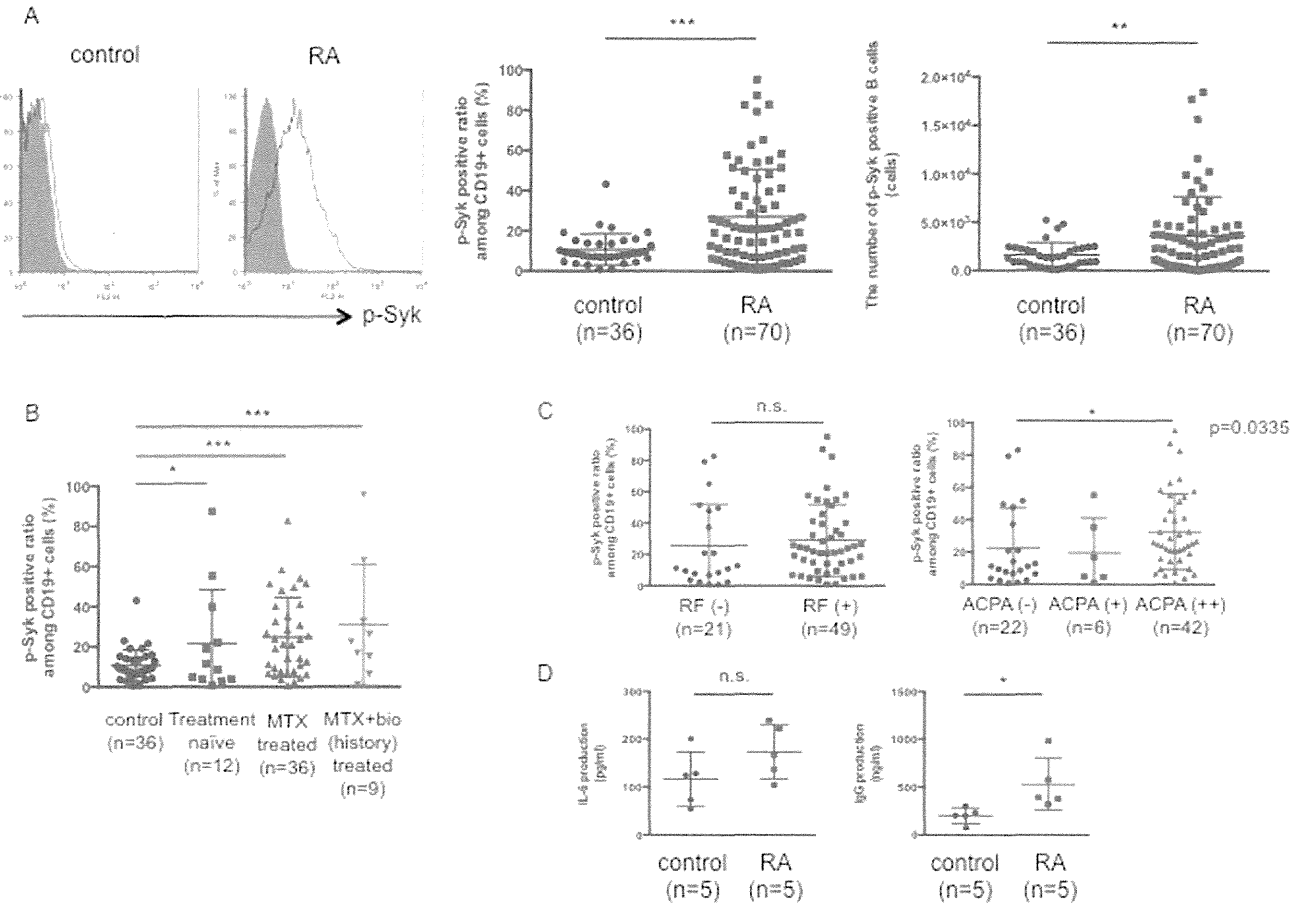

Figure 1. Phosphorylation of Syk in CD19+ B cells of healthy donors (controls) and patients with rheumatoid arthritis (RA). **A,** Representative histograms showing Syk phosphorylation in peripheral blood B cells from 70 RA patients and 36 healthy control subjects (left), and the ratio of pSyk-positive cells among CD19+ B cells (middle) and absolute number of pSyk-positive CD19+ B cells (right) in RA patients compared to healthy controls. **B,** Ratio of pSyk-positive cells among CD19+ B cells in 3 groups of RA patients: treatment-naive (n = 12), methotrexate (MTX)–treated (n = 36), and MTX + biologics (bio) (history)–treated (n = 9). RA patients treated with other disease-modifying antirheumatic drugs and/or corticosteroids were excluded. **C,** Ratio of pSyk-positive cells among CD19+ B cells in RA patients negative for rheumatoid factor (RF) (defined as <15 IU/ml, based on the normal limit at our hospital) or positive for RF (defined as ≥15 IU/ml), and RA patients negative (−), positive (+), or strongly positive (++) for anti–citrullinated protein antibodies (ACPAs) (defined as <4.5 units/ml, 4.5–13.5 units ml, and >13.5 units/ml, respectively, based on the normal limit at our hospital). **D,** Production of interleukin-6 (IL-6) (left) and IgG (right) by CD19+ B cells purified from the peripheral blood of healthy controls and RA patients. B cells were cultured in stimulus-free RPMI medium for 3 days (for IL-6) or 5 days (for IgG). Production of IL-6 in the supernatants was assayed by cytometric bead array, while IgG in the supernatants was quantified by enzyme-linked immunosorbent assay. Symbols represent individual subjects; bars show the mean ± SD. * = $P < 0.05$, ** = $P < 0.01$; *** = $P < 0.001$. NS = not significant.

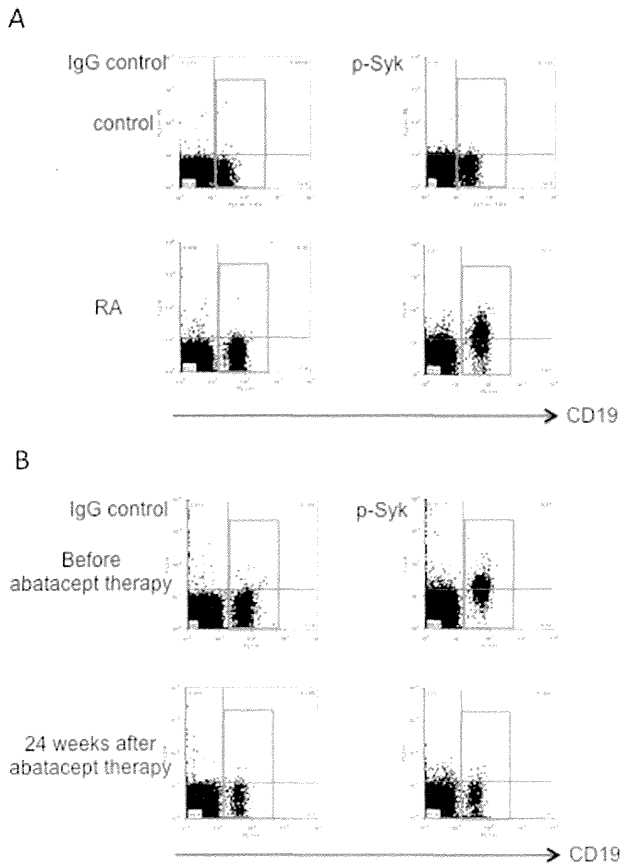

Figure 2. Phosphorylation of Syk in CD19+ B cells from the peripheral blood of healthy subjects (controls) and patients with rheumatoid arthritis (RA) and in RA patients before and after treatment with abatacept. **A,** Expression of pSyk on CD19+ B cells was assessed by flow cytometry in the peripheral blood mononuclear cells (PBMCs) of controls and RA patients. **B,** Levels of pSyk in CD19+ B cells were compared in RA patients before and 24 weeks after treatment with abatacept. In **A** and **B,** the lymphocyte region of PBMCs was gated for expression of pSyk on CD19+ B cells (indicated by gray-shaded boxed areas), in comparison to that in IgG control experiments.

and analyzed by flow cytometry to determine the levels of Syk phosphorylation in peripheral blood CD19+ B cells. The control subjects and RA patients were matched for sex but not age. Analysis of the effect of age on Syk phosphorylation showed that the level of pSyk in B cells was not correlated with age in either the control subjects or the RA patients (in controls [mean ± SD age 38.7 ± 9.6 years], Spearman's $r^2 = 0.0625$, $P = 0.23$; in RA patients, Spearman's $r^2 = 0.0008$, $P = 0.82$). Although the expression level of Syk in B cells was not different between the groups (results not shown), the level of Syk phosphorylation in B cells was significantly higher in RA patients compared to healthy controls (mean ± SD percentage of pSyk-positive B cells among CD19+ B cells, 27.7 ± 23.2% in RA patients versus 11.9 ± 8.2% in controls; $P = 0.0019$, by Student's *t*-test) (Figures 1A and 2A).

We estimated the absolute numbers of total B cells and pSyk-positive B cells in the RA patients and healthy controls. Although there was no significant difference in the percentage and absolute number of total B cells between RA patients and healthy controls (mean ± SD percentage of total B cells relative to number of lymphocytes, 11.2 ± 6.2% in RA patients versus 13.3 ± 6.6% in controls [$P = 0.08$, by Student's *t*-test]; absolute number of CD19+ cells, 12,844 ± 7,120 in RA patients versus 15,199 ± 7,482 in controls [$P = 0.09$, by Student's *t*-test]), the absolute number of pSyk-positive B cells was significantly higher in RA patients than in controls (mean ± SD 3,639 ± 4,021 versus 1,608 ± 1,285; $P = 0.0137$, by Student's *t*-test) (Figure 1A). In addition, the proportions of B cell subsets classified into CD19+CD27− naive B cells and CD19+CD27+ memory B cells were comparable between the RA patients and healthy controls (mean ± SD percentage of CD19+CD27− naive B cells, 83.0 ± 6.2% in RA patients versus 84.9 ± 5.0% in controls [$P = 0.24$, by Student's *t*-test]; percentage of CD19+

Table 2. Relationship between clinical characteristics and the ratio of pSyk-positive cells among CD19+ B cells in patients with rheumatoid arthritis*

	Spearman's rho†
Age	0.0961
Disease duration (mean months)	0.0320
Prednisolone (or equivalent) (mg/day)	0.0915
Methotrexate (mg/week)	−0.1516
Tender joint count (28 total)	0.0045
Swollen joint count (28 total)	0.1821
DAS28-CRP	0.0217
DAS28-ESR	0.1135
CDAI	0.0637
SDAI	0.0501
CRP level (mg/dl)	−0.0513
ESR (mm/hour)	0.1221
MMP-3 level (ng/ml)	0.2019
IgG level (mg/dl)	0.0311
Presence of ANAs	−0.0798

* DAS28-CRP = Disease Activity Score in 28 joints using C-reactive protein level; ESR = erythrocyte sedimentation rate; CDAI = Clinical Disease Activity Index; SDAI = Simplified Disease Activity Index; MMP-3 = matrix metalloproteinase 3; ANAs = antinuclear antibodies.
† Values are Spearman's rank correlation coefficients for each characteristic in relation to the percentage of pSyk-positive CD19+ B cells. None of the values were significant.

CD27+ memory B cells, 17.0 ± 6.0% in RA patients versus 15.1 ± 5.0% in controls [P = 0.20, by Student's t-test]). These results suggest that pSyk expression is up-regulated in RA patients compared to healthy control subjects irrespective of the proportions of B cell subsets.

We next investigated differences in pSyk levels among 3 groups of RA patients: treatment-naive RA patients (n = 12), MTX-treated RA patients (n = 36), and MTX + biologics (history)–treated RA patients (n = 9). RA patients who had been treated with other disease-modifying antirheumatic drugs and/or corticosteroids were excluded from the analysis. The expression levels of pSyk in all 3 groups of RA patients were significantly higher than those in the control group (mean ± SD percentage of pSyk-positive cells among CD19+ cells, 21.6 ± 7.7% in treatment-naive RA patients, 24.8 ± 3.3% in MTX-treated RA patients, and 30.8 ± 10.0% in MTX + biologics–treated RA patients versus 10.7 ± 1.3% in controls; P = 0.0036, by Student's t-test). There was no significant difference in the pSyk level among each of the 3 RA treatment groups (Figure 1B).

We then assessed the correlation between patient background characteristics and Syk phosphorylation in B cells (Table 2). Syk phosphorylation levels in B cells were not correlated with indices of RA disease activity, such as the tender joint count, swollen joint count, CRP level, ESR, MMP-3 level, DAS28-CRP, DAS28-ESR, CDAI, and SDAI. There was also no correlation with age, sex, duration of disease, use or dosage of steroids, or use or dosage of oral MTX. Interestingly, Syk phosphorylation was significantly higher in B cells of patients strongly positive for ACPAs (mean ± SD percentage of pSyk staining among CD19+ B cells, 22.2 ± 24.9% in RA patients negative for ACPAs and 19.5 ± 21.5% in RA patients positive for ACPAs versus 32.6 ± 23.5% in RA patients strongly positive for ACPAs; P = 0.0335, by Kruskal-Wallis test) (Figure 1C and Table 3).

We also investigated whether the up-regulation of pSyk expression in the B cells of RA patients was associated with enhanced B cell activation. CD19+ B cells were purified from the peripheral blood of the RA patients and healthy control subjects and then cultured in a stimulus-free medium for 3 or 5 days for assessment of IL-6 or IgG production, respectively. The levels of IL-6 tended to be more pronounced in the B cells of RA patients compared to healthy controls, but the difference was not statistically significant (mean ± SD IL-6 concentration, 174.0 ± 56.8 pg/ml in RA patients versus 116.6 ± 57.1 pg/ml in controls; P = 0.136, by Wilcoxon's rank sum test) (Figure 1D). IgG production by B cells was significantly higher in RA patients (mean ± SD 529.5 ± 270.7 ng/ml) compared to controls (204.1 ± 83.2 ng/ml; P = 0.033, by Wilcoxon's rank sum test) (Figure 1D). The results of these in vitro studies add support to the notion that pSyk expression in B cells is correlated with the production of autoantibodies in RA patients.

Table 3. Relationship between levels of Syk phosphorylation and subsets of clinical characteristics in patients with rheumatoid arthritis (RA)*

	pSyk, %†
Sex	
Male	27.4 ± 24.5
Female	32.6 ± 22.0
RA disease stage	
I	38.1 ± 28.6
II	24.6 ± 22.3
III	18.7 ± 11.4
IV	32.4 ± 27.8
RA functional class	
I	24.7 ± 19.9
II	28.4 ± 26.0
III	28.4 ± 16.1
RF	
Negative	25.4 ± 26.7
Positive	29.3 ± 23.0
ACPAs‡	
Negative	22.2 ± 24.9
Positive	19.5 ± 21.5
Strongly positive	32.6 ± 23.5

* RF = rheumatoid factor; ACPAs = anti–citrullinated protein antibodies.
† Values are the mean ± SD percentage of pSyk staining among CD19+ B cells.
‡ P = 0.0335 between groups.

Inhibition of Syk phosphorylation in B cells of RA patients following treatment with abatacept. The results presented thus far suggest that the phosphorylation of Syk in RA B cells is involved in the production of ACPAs. Autoantibody production by B cells requires the involvement of T cells, and treatment with abatacept can inhibit the activation of T cells. Based on this background, we hypothesized that abatacept inhibits Syk phosphorylation in B cells. For this purpose, we investigated the effect of abatacept on Syk phosphorylation by comparing it with the effect of TNF inhibitors (infliximab n = 10, golimumab n = 3, etanercept n = 1, adalimumab n = 1). Biologics-naive RA patients were selected for this analysis. Abatacept (n = 12) or a TNF inhibitor (n = 15) was administered to patients with MTX-resistant RA, and the change in B cell Syk phosphorylation after the treatment was investigated.

We found that the posttreatment clinical background was not significantly different between patients

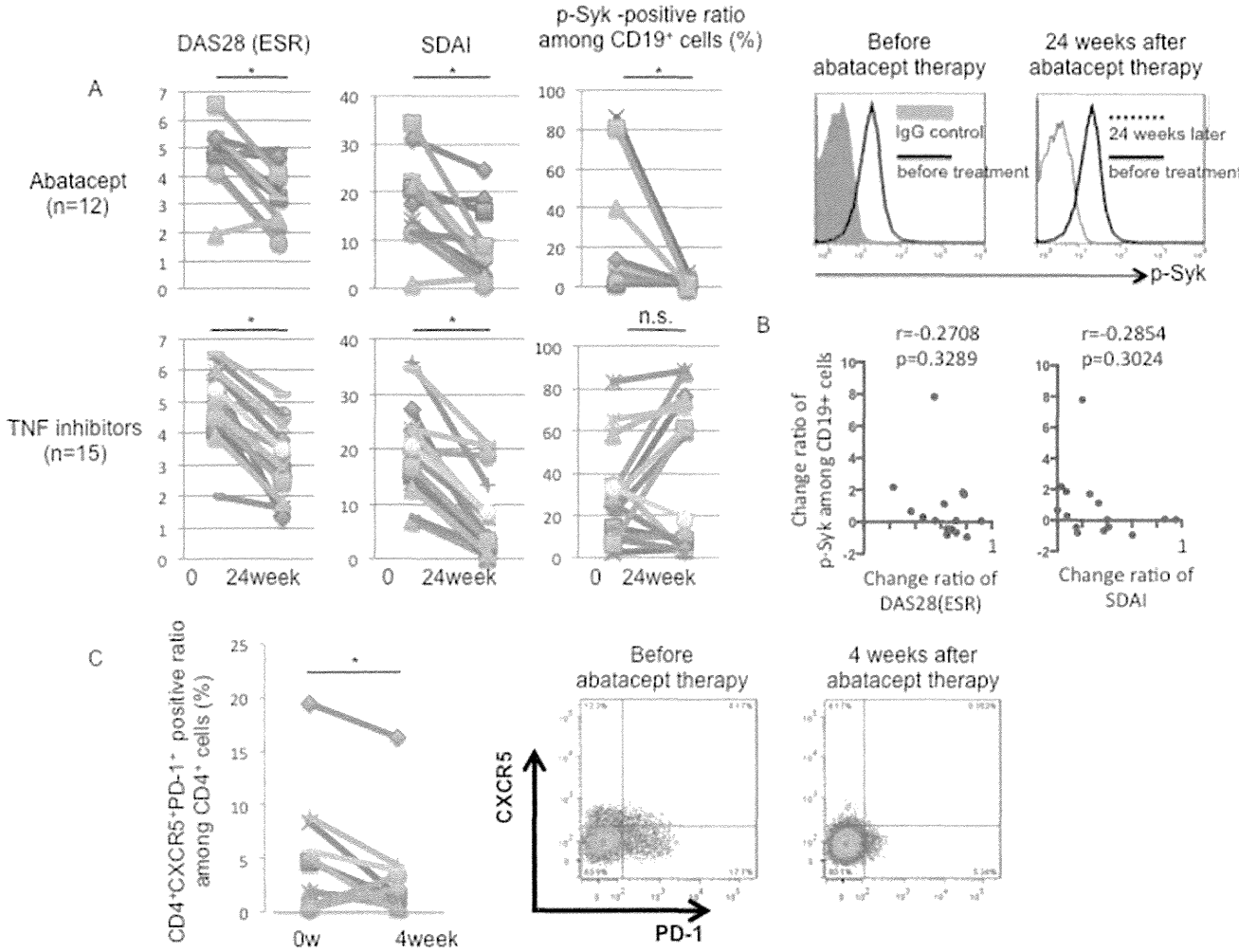

Figure 3. Changes in Syk phosphorylation in B cells and effects on follicular T helper (Tfh) cells among CD4+ T cells before and after treatment of rheumatoid arthritis (RA) patients with abatacept or tumor necrosis factor (TNF) inhibitors. **A,** Left, Changes in the Disease Activity Score in 28 joints using the erythrocyte sedimentation rate (DAS28-ESR), the Simplified Disease Activity Index (SDAI), and the ratio of pSyk-positive cells among CD19+ B cells were assessed in RA patients before and 24 weeks after treatment with abatacept or TNF inhibitors. Right, Changes in the levels of pSyk in CD19+ B cells were assessed in RA patients before and after treatment with abatacept. IgG served as control. **B,** The correlation between change in the ratio of pSyk-positive cells among CD19+ cells and change in the DAS28-ESR and SDAI was assessed in RA patients treated with TNF inhibitors. Change in the ratio was calculated as (value after treatment − value before treatment)/value before treatment. **C,** Left, Changes in the ratio of CD4+CXCR5+PD-1+ cells (Tfh cells) among CD4+ cells were assessed in RA patients before and 4 weeks after treatment with abatacept. Right, Representative flow cytometry data are shown. Colored bars and symbols in **A** and **C** represent individual patients. * = $P < 0.05$. NS = not significant; PD-1 = programmed death 1.

treated with abatacept and those treated with TNF inhibitors (results not shown), including the levels of ACPAs (mean ± SD 85.4 ± 91.9 units/ml in abatacept-treated RA patients versus 77.7 ± 89.6 units/ml in TNF inhibitor–treated RA patients). During the period from week 0 to week 24 after treatment, the DAS28 decreased from a mean ± SD 4.8 ± 1.1 to 3.4 ± 1.1 in the abatacept treatment group ($P = 0.002$, by Wilcoxon's matched-pairs signed-rank test) and from 4.9 ± 1.1 to 3.1 ± 1.2 in the patients treated with TNF inhibitors ($P < 0.001$, by Wilcoxon's matched-pairs signed-rank test). Furthermore, in patients treated with abatacept and those treated with TNF inhibitors, the SDAI decreased from 19.4 ± 10.1 to 9.6 ± 8.4 ($P = 0.0069$, by Wilcoxon's matched-pairs signed-rank test) and from 20.0 ± 8.5 to 7.4 ± 7.5 ($P < 0.001$, by Wilcoxon's matched-pairs

signed-rank test), respectively. These results indicate that a significant reduction occurred in both indices of disease activity (the DAS28-ESR and the SDAI) in both treatment groups after 24 weeks of administration.

Interestingly, in the abatacept treatment group, the percentage of pSyk-positive cells among CD19+ B cells diminished from week 0 to week 24 from a mean ± SD 21.4 ± 30.9% to 3.3 ± 3.8% (P = 0.0341, by Wilcoxon's matched-pairs signed-rank test), whereas in those treated with TNF inhibitors, this percentage increased from 30.0 ± 23.1% to 42.0 ± 34.8% from week 0 to week 24 (P = 0.1255, by Wilcoxon's matched-pairs signed-rank test). These results indicate that Syk phosphorylation in B cells was significantly decreased in the abatacept treatment group, whereas no change was observed in the TNF inhibitors group after 24 weeks of administration (Figures 2B and 3A). Although 2 different subsets of TNF inhibitor–treated patients were observed, one in which the pSyk levels increased and another in which the pSyk levels decreased after treatment with TNF inhibitors, the background features were similar in the 2 groups. However, the change in Syk phosphorylation in B cells after treatment was not correlated with the response to treatment (Figure 3B).

We next assessed the mechanism of abatacept-induced inhibition of Syk phosphorylation in B cells. For this purpose, we examined the proportion of Th1 cells (CD4+CXCR3+ cells) and Tfh cells (CD4+CXCR5+PD-1+ cells), which are CD4+ T cells that play important roles in the maturation and differentiation of B cells (17). Preliminary data (not shown) indicated that there was a significantly higher percentage of Tfh cells among CD4+ T cells in RA patients compared to healthy controls. However, there were no differences in the percentage of Th1 cells between the 2 groups (results not shown). Although the proportion of Th1 cells was not changed, treatment with abatacept significantly reduced the proportion of Tfh cells, from a mean ± SD 5.7 ± 5.7% at week 0 to 3.4 ± 4.7% at week 4 (P = 0.0206, by Wilcoxon's matched-pairs signed-rank test) (Figure 3C). In contrast, treatment with TNF inhibitors did not change the proportion of Tfh cells after 4 weeks of administration. Examination of the direct effect of abatacept on B cells showed that abatacept did not change the expression levels of the costimulatory molecules CD80 and CD86 on B cells (results not shown).

DISCUSSION

In this study, we revealed that Syk phosphorylation is enhanced in the peripheral blood B cells of patients with RA compared to healthy subjects, and we found that Syk phosphorylation was increased in RA patients strongly positive for ACPAs. We also found that treatment with abatacept resulted in inhibition of Syk phosphorylation in B cells, whereas treatment with TNF inhibitors did not produce the same effects. Treatment with abatacept also significantly reduced the proportion of Tfh cells.

Rituximab was approved for treatment of RA in 2006 in the US. The positioning of rituximab as the second-line biologic product that follows TNF inhibitor therapy has been established, and B cells are assumed to be the therapeutic target in RA. Whereas several studies have shown no abnormalities in peripheral blood B cells in patients with RA (34–37), others have identified B cell abnormalities in patients with RA, including a high proportion of IgD−CD27− double-negative memory B cells (38). In this regard, there is an increased likelihood of RA relapse in patients whose proportion of memory B cells increases after rituximab administration (39), and abnormalities of chemokine receptors in B cells are often detected in RA patients (40). In this study, we found a significant increase in Syk phosphorylation in peripheral blood B cells of RA patients compared to healthy subjects, suggesting that B cells are abnormal in RA patients.

How could this abnormality affect the pathologic processes of RA? Our results showed that the increased level of phosphorylation of Syk in B cells correlated with the production level of ACPAs, but not with the severity of disease activity, in patients with active RA (Figure 1 and Table 3). Consistent with these results, we have recently reported that signaling through Syk results in effective signal transduction of TLR-9 by induction of optimal expression of TRAF6, and that this signaling is important for the expression of various functions, such as antibody production, as well as for robust activation of B cells (14). These data suggest that the important role of Syk in B cells in the pathologic processes of RA is mediated, at least in part, through ACPA production.

These findings raise several important questions. How does Syk-related ACPA production affect the pathologic progression of RA? A high titer of ACPAs is an adverse prognostic factor for bone destruction (41), although the pathologic significance is still largely unknown. In a recent study, Amara et al (42) analyzed the immunoglobulin gene of IgG+ memory B cells collected from the synovial tissue of RA patients and found that patients who were positive for ACPAs had a gene sequence for an antibody that specifically binds to citrullinated antigen. In addition, Harre et al (43) re-

ported that ACPAs induce TNFα production by macrophages and indirectly induce the differentiation of osteoclasts. It has been demonstrated that the Syk inhibitor fostamatinib (R788) was ineffective in patients with active RA who did not respond to TNF inhibitors (44). Results of a recent study indicated that TNF inhibitors increase the levels of cytoplasmic Lyn, which phosphorylates Syk and plays a role in the initiation of the B cell receptor–mediated pathway (45). Our results (Figure 3A), however, showed 2 different subsets of TNF inhibitor–treated patients, one with an increase in pSyk levels and another with a decrease in pSyk levels after treatment with TNF inhibitors, despite improvement in disease activity at 24 weeks posttreatment. Further analysis is needed to explore this issue in more detail. Clarification of the relationship between Syk and TNFα and its role in RA pathologic processes may explain why Syk inhibitors are ineffective in patients with active RA who do not respond to TNF inhibitors.

In the treatment of RA, abatacept acts through a mechanism of action different from that of TNF inhibitors; it reduces T cell responses by limiting CD28-mediated signaling, which is required for T cell activation and differentiation. However, there is little or no information on the effect of abatacept on the pathologic development or progression of RA. The present findings showed that not only the phosphorylation of Syk but also the proportion of Tfh cells was significantly reduced by abatacept, while TNF inhibitors influenced neither Syk phosphorylation nor Tfh cell development. Platt and colleagues (46) demonstrated that treatment of mice with abatacept decreased the proportion of Tfh cells. Tfh cells are a critical T helper cell subset for the formation and function of B cells and play an important role in the pathogenesis of autoimmune diseases (17,18). Furthermore, B cells provide help for the survival of Tfh cells (47).

In a series of preliminary studies, the proportion of Tfh cells in RA patients was correlated significantly with the titers of various autoantibodies, such as RF and ACPAs, but was not correlated with the severity of disease activity or the levels of pSyk in B cells (results not shown). Previous studies showed that treatment with infliximab decreased the RF titer but did not change the ACPA level (48). In contrast, treatment with abatacept significantly reduced the levels of both RF and ACPAs (49). In the present study, treatment with TNF inhibitors did not reduce the proportion of Tfh cells, despite the fact that disease activity improved with the use of these drugs. However, the proportion of Tfh cells was significantly decreased by abatacept. Therefore, we argue that abatacept seems to selectively control Tfh cell activation, leading to the production of autoantibodies from pSyk-positive B cells. However, further studies are needed to determine whether this effect is selective for abatacept compared to other drugs such as MTX and tocilizumab.

Thus, the interaction between B cells and Tfh cells is required for autoantibody production. Our results suggest that abatacept inhibits Syk phosphorylation in B cells and inhibits the proliferation and differentiation of Tfh cells. Taken together, our findings highlight the importance of B cells in the pathogenesis of RA and describe the mode of action of abatacept, i.e., inhibition of B cell–T cell interactions. Further evaluation of Syk phosphorylation may help predict the response to abatacept therapy in patients with RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Tanaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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