eigenvectors 1 and 2 obtained from the PCA using EIGENSTRAT version 2.0 [42], along with European (CEU), African (YRI), Japanese (JPT), and Chinese (CHB) individuals obtained from the Phase II HapMap database (release 22) [29]. Subjects who were estimated to be outliers in terms of ancestry from East-Asian (JPT+CHB) clusters and excluded from the study are indicated by black arrows.
(TIF)

Figure S2 Quantile-Quantile plot (QQ-plot) of *P*-values in the GWAS for SLE. The horizontal axis indicates the expected $-\log_{10}$ (*P*-values). The vertical axis indicates the observed $-\log_{10}$ (*P*-values). The QQ-plot for the *P*-values of all SNPs that passed the quality control criteria is indicated in blue. The QQ-plot for the *P*-values after the removal of SNPs included in the previously reported SLE susceptibility loci is indicated in black. The gray line represents $y = x$. The SNPs for which the *P*-value was smaller than 1.0×10^{-15} are indicated at the upper limit of the plot.
(TIF)

Table S1 Basal characteristics of cohorts.
(DOC)

Table S2 Frequency of clinical characteristics of SLE in this GWAS.
(DOC)

Table S3 Distributions of eQTL positivity rates of the SNPs.
(DOC)

Table S4 Results of replication study 1 for Japanese patients with SLE.
(DOC)

Table S5 Results of replication studies 1 and 2 for Japanese patients with SLE.
(DOC)

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Conceived and designed the experiments: Y. Okada, K. Shimane, Y. Kochi, A. Suzuki, M. Kubo, N. Kamatani, R. Yamada, Y. Nakamura, K. Yamamoto. Performed the experiments: Y. Kochi, N. Hosono, M. Kubo, K. Myouzen, T. Horiuchi, T. Tahira, K. Hayashi. Analyzed the data: Y. Okada, A. Takahashi, T. Tsunoda, K. Higasa, R. Yamada. Contributed reagents/materials/analysis tools: T. Horita, T. Atsumi, T. Koike, T. Ishii, A. Okamoto, K. Fujio, M. Hirakata, H. Amano, Y. Takasaki, Y. Kondo, S. Ito, T. Sumida, K. Takada, A. Mimori, K. Saito, Y. Tanaka, M. Kamachi, N. Nishimoto, Y. Kawaguchi, C. Terao, K. Ohmura, T. Mimori, F. Matsuda, O.W. Mohammed, K. Matsuda, K. Shimane, K. Ikari, H. Yamanaka. Wrote the paper: Y. Okada, K. Shimane, Y. Kochi, A. Suzuki, R. Yamada, K. Yamamoto.

References

1. Lipsky PE (2001) Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity. Nat Immunol 2: 764–766.
2. Sestak AL, Shaver TS, Moser KL, Neas BR, Harley JB (1999) Familial aggregation of lupus and autoimmunity in an unusual multiplex pedigree. J Rheumatol 26: 1495–1499.
3. Sigurdsson S, Nordmark G, Goring HH, Lindroos K, Wiman AC, et al. (2005) Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus. Am J Hum Genet 76: 528–537.
4. Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, et al. (2006) A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. Nat Genet 38: 550–555.
5. Graham RR, Kyogoku C, Sigurdsson S, Vlasova IA, Davies LR, et al. (2007) Three functional variants of IFN regulatory factor 5 (IRF5) define risk and protective haplotypes for human lupus. Proc Natl Acad Sci U S A 104: 6758–6763.
6. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, et al. (2007) STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 357: 977–986.
7. Cunninghame Graham DS, Graham RR, Manku H, Wong AK, Whittaker JC, et al. (2008) Polymorphism at the TNF superfamily gene TNFSF4 confers susceptibility to systemic lupus erythematosus. Nat Genet 40: 83–89.
8. Nath SK, Han S, Kim-Howard X, Kelly JA, Viswanathan P, et al. (2008) A nonsynonymous functional variant in integrin-alpha(M) (encoded by ITGAM) is associated with systemic lupus erythematosus. Nat Genet 40: 152–154.
9. Harley JB, Alarcon-Riquelme ME, Criswell LA, Jacob CO, Kimberly RP, et al. (2008) Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. Nat Genet 40: 204–210.
10. Kozyrev SV, Abelson AK, Wojcik J, Zaghlool A, Linga Reddy MV, et al. (2008) Functional variants in the B-cell gene BANK1 are associated with systemic lupus erythematosus. Nat Genet 40: 211–216.
11. Hom G, Graham RR, Modrek B, Taylor KE, Ortmann W, et al. (2008) Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. N Engl J Med 358: 900–909.
12. Graham RR, Cotsapas C, Davies L, Hackett R, Lessard CJ, et al. (2008) Genetic variants near TNFAIP3 on 6q23 are associated with systemic lupus erythematosus. Nat Genet 40: 1059–1061.
13. Musone SL, Taylor KE, Lu TT, Nititham J, Ferreira RC, et al. (2008) Multiple polymorphisms in the TNFAIP3 region are independently associated with systemic lupus erythematosus. Nat Genet 40: 1062–1064.
14. Han JW, Zheng HF, Cui Y, Sun LD, Ye DQ, et al. (2009) Genome-wide association study in a Chinese Han population identifies nine new susceptibility loci for systemic lupus erythematosus. Nat Genet 41: 1234–1237.
15. Gateva V, Sandling JK, Hom G, Taylor KE, Chung SA, et al. (2009) A large-scale replication study identifies TNIP1, PRDM1, JAZF1, UHRF1BP1 and IL10 as risk loci for systemic lupus erythematosus. Nat Genet 41: 1228–1233.
16. Yang W, Shen N, Ye DQ, Liu Q, Zhang Y, et al. (2010) Genome-wide association study in Asian populations identifies variants in ETS1 and WDFY4 associated with systemic lupus erythematosus. PLoS Genet 6: e1000841. doi:10.1371/journal.pgen.1000841.
17. Lessard CJ, Adrianto I, Kelly JA, Kaufman KM, Grundahl KM, et al. (2011) Identification of a systemic lupus erythematosus susceptibility locus at 11p13 between PDHX and CD44 in a multiethnic study. Am J Hum Genet 88: 83–91.
18. Yang J, Yang W, Hirankarn N, Ye DQ, Zhang Y, et al. (2011) ELF1 is associated with systemic lupus erythematosus in Asian populations. Hum Mol Genet 20: 601–607.
19. Hopkinson ND, Doherty M, Powell RJ (1994) Clinical features and race-specific incidence/prevalence rates of systemic lupus erythematosus in a geographically complete cohort of patients. Ann Rheum Dis 53: 675–680.
20. Danchenko N, Satia JA, Anthony MS (2006) Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden. Lupus 15: 308–318.
21. Yang J, Benyamin B, McEvoy BP, Gordon S, Henders AK, et al. (2010) Common SNPs explain a large proportion of the heritability for human height. Nat Genet 42: 565–569.
22. Raychaudhuri S, Plenge RM, Rossin EJ, Ng AC, Purcell SM, et al. (2009) Identifying relationships among genomic disease regions: predicting genes at pathogenic SNP associations and rare deletions. PLoS Genet 5: e1000534. doi:10.1371/journal.pgen.1000534.
23. Cantor RM, Lange K, Sinsheimer JS (2010) Prioritizing GWAS results: A review of statistical methods and recommendations for their application. Am J Hum Genet 86: 6–22.
24. Dubois PC, Trynka G, Franke L, Hunt KA, Romanos J, et al. (2010) Multiple common variants for celiac disease influencing immune gene expression. Nat Genet 42: 295–302.
25. Cookson W, Liang L, Abecasis G, Moffatt M, Lathrop M (2009) Mapping complex disease traits with global gene expression. Nat Rev Genet 10: 184–194.
26. Kochi Y, Okada Y, Suzuki A, Ikari K, Terao C, et al. (2010) A regulatory variant in CCR6 is associated with rheumatoid arthritis susceptibility. Nat Genet 42: 515–519.
27. Yamaguchi-Kabata Y, Nakazono K, Takahashi A, Saito S, Hosono N, et al. (2008) Japanese population structure, based on SNP genotypes from 7003 individuals compared to other ethnic groups: effects on population-based association studies. Am J Hum Genet 83: 445–456.
28. Stranger BE, Nica AC, Forrest MS, Dimas A, Bird CP, et al. (2007) Population genomics of human gene expression. Nat Genet 39: 1217–1224.
29. The International HapMap Consortium (2003) The International HapMap Project. Nature 426: 789–796.

30. Pearson JV, Huentelman MJ, Halperin RF, Tembe WD, Melquist S, et al. (2007) Identification of the genetic basis for complex disorders by use of pooling-based genomewide single-nucleotide-polymorphism association studies. Am J Hum Genet 80: 126–139.
31. Xia ZB, Popovic R, Chen J, Theisler C, Stuart T, et al. (2005) The MLL fusion gene, MLL-AF4, regulates cyclin-dependent kinase inhibitor CDKN1B (p27kip1) expression. Proc Natl Acad Sci U S A 102: 14028–14033.
32. Isnard P, Core N, Naquet P, Djabali M (2000) Altered lymphoid development in mice deficient for the mAF4 proto-oncogene. Blood 96: 705–710.
33. Schadt EE, Molony C, Chudin E, Hao K, Yang X, et al. (2008) Mapping the genetic architecture of gene expression in human liver. PLoS Biol 6: e107. doi:10.1371/journal.pbio.0060107.
34. Ernst J, Kheradpour P, Mikkelsen TS, Shoresh N, Ward LD, et al. (2011) Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473: 43–49.
35. Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, et al. (2010) Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nat Genet 42: 508–514.
36. Nakamura Y (2007) The BioBank Japan Project. Clin Adv Hematol Oncol 5: 696–697.
37. Hochberg MC (1997) Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 40: 1725.
38. Suzuki A, Yamada R, Kochi Y, Sawada T, Okada Y, et al. (2008) Functional SNPs in CD244 increase the risk of rheumatoid arthritis in a Japanese population. Nat Genet 40: 1224–1229.
39. Shimane K, Kochi Y, Horita T, Ikari K, Amano H, et al. (2010) The association of a nonsynonymous single-nucleotide polymorphism in TNFAIP3 with systemic lupus erythematosus and rheumatoid arthritis in the Japanese population. Arthritis Rheum 62: 574–579.
40. Myouzen K, Kochi Y, Shimane K, Fujio K, Okamura T, et al. (2010) Regulatory polymorphisms in EGR2 are associated with susceptibility to systemic lupus erythematosus. Hum Mol Genet 19: 2313–2320.
41. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, et al. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 81: 559–575.
42. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, et al. (2006) Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet 38: 904–909.
43. Li Y, Willer C, Sanna S, Abecasis G (2009) Genotype imputation. Annu Rev Genomics Hum Genet 10: 387–406.
44. Okada Y, Takahashi A, Ohmiya H, Kumasaka N, Kamatani Y, et al. (2011) Genome-wide association study for C-reactive protein levels identified pleiotropic associations in the IL6 locus. Hum Mol Genet 20: 1224–1231.
45. Aikawa Y, Yamamoto M, Yamamoto T, Morimoto K, Tanaka K (2002) An anti-rheumatic agent T-614 inhibits NF-kappaB activation in LPS- and TNF-alpha-stimulated THP-1 cells without interfering with IkappaBalpha degradation. Inflamm Res 51: 188–194.
46. Akamatsu S, Takata R, Ashikawa K, Hosono N, Kamatani N, et al. (2010) A functional variant in NKX3.1 associated with prostate cancer susceptibility down-regulates NKX3.1 expression. Hum Mol Genet 19: 4265–4272.
47. Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, et al. (2008) SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. Bioinformatics 24: 2938–2939.

Phenotypic changes of lymphocyte in a patient with IgG4-related disease after corticosteroid therapy

Immunoglobulin G4-related disease (IgG4RD) is a novel clinical disease entity characterised by elevated serum IgG4 and tissue infiltration by IgG4-positive plasma cells.[1 2] Interleukin 4 (IL-4) and IL-10, which were detected with B cells in the salivary gland in this disease,[3] direct naive B cells to switch to IgG4 production.[4] B cells are therefore considered to be important for the pathogenesis of IgG4RD. However, the phenotype of B cells in patients with IgG4RD remains elusive. In this report we show the phenotypic changes of peripheral blood B cells in a patient with IgG4RD analysed by flow cytometry during treatment with a corticosteroid.

In January 2011 a 53-year-old man presented with symmetrical swelling of the lachrymal glands and a tumour located in the left junction of the renal pelvis and ureter, serum IgG4 >135 mg/dl and IgG4+/IgG+ cells >40% with significant invasion of lymphocytes and plasma cells, typical tissue fibrosis and sclerosis in the salivary gland. He was diagnosed with IgG4RD based on the guidelines for diagnosis.[5] Treatment with corticosteroid 40 mg (0.6 mg/kg) was started. One year later the bilateral renal pelvic tumour had disappeared and serum IgG (IgG4) decreased from 1692 (341) mg/dl to 969 (61.5) mg/dl despite tapering of the corticosteroid dose to 2 mg/day.

Meanwhile, before treatment, memory B cells (CD19 gated IgD-CD27 or CD27+ CD38-) and plasmablasts (CD19 gated $CD27^{high}CD38^{high}$ or IgD-$CD27^{high}$, $CD19^{low}CD38^{high}$) increased in a patient with IgG4RD compared with healthy donors in peripheral blood. Moreover, the expression levels of the costimulatory molecules CD80 were upregulated. Spleen tyrosine kinase (Syk) is a tyrosine kinase expressed in various immunocompetent cells including B cells. We have reported that the engagement of immune receptor phosphorylates Syk, resulting in proliferation and cytokine production on B cells.[6] It is noteworthy that Syk phosphorylation was also markedly increased in CD19 cells in the patient compared with those in healthy donors.

However, $CD27^{high}CD38^{high}$ plasmablasts in the patient decreased at 1 month and disappeared 6 months after treatment with corticosteroid, whereas the percentage of memory B cells almost did not change. Although he remained in low disease activity after tapering of the corticosteroid dose, the expression levels of CD80 and phospho-Syk on CD19 cells did not change until 1 year later (figure 1).

Taken together, although corticosteroid therapy effectively decreased peripheral plasmablasts, activated memory B cells were resistant to treatment in IgG4RD. These results are supported by those of Khosroshahi et al who demonstrated that B cell depletion therapy by rituximab was effective in some patients with IgG4RD refractory to corticosteroid.[7] The present data could therefore partly explain why IgG4RD is difficult to maintain in remission after reduction in the dose of corticosteroid. The results also indicate that the combined use of immunosuppressants, B cell-targeted therapies or Syk inhibitors may be considered for the treatment of IgG4RD, as shown in systemic lupus erythematosus, rheumatoid arthritis and idiopathic thrombocytopenic purpura.[8–10] However, further analysis of a large sample of patients is needed.

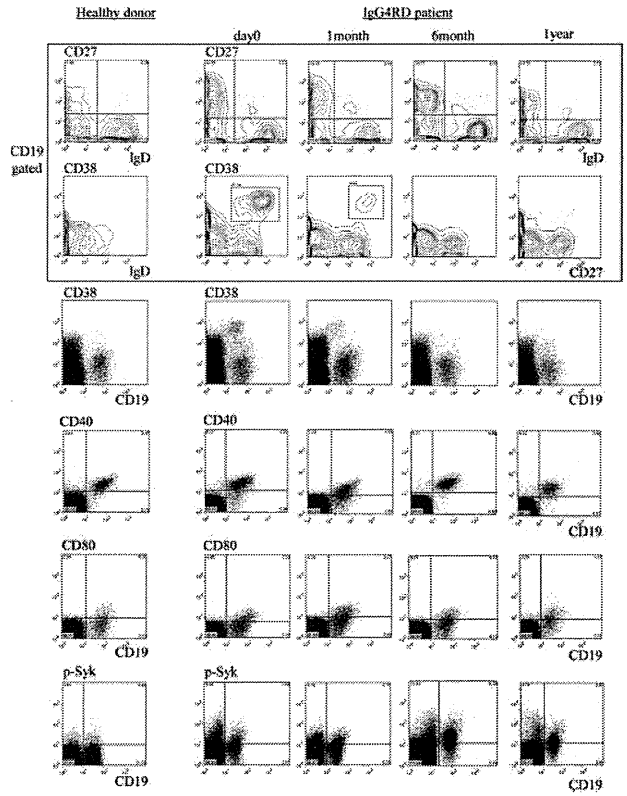

Figure 1 Phenotypic changes of B cells in a patient with IgG4-related disease before treatment, 1 month and 6 months after treatment with corticosteroid therapy. In the upper 10 contour line graphs, peripheral blood mononuclear cell (PBMCs) were gated on CD19-positive cells and further separated with CD27 and IgD, or CD38 and CD27. In the former, the left quadrant identified plasma cells (IgD-$CD27^{high}$) and class-switched memory B cells (IgD-CD27+). The right upper quadrant identified IgM memory B cells (IgD+CD27). The right lower quadrant identified naïve B cells (IgD+CD27+). In the latter, CD27−CD38 + identified naïve B cells, CD27+ CD38− identified memory B cells and $CD27^{high}CD38^{high}$ identified plasmablasts and plasma cells. In the lower four line graphs, PBMC were double-stained by CD19 (x-axis) and IgG isotype control, CD38, CD40, CD80 and Syk phosphorylation, respectively (y-axis). $CD19^{low}CD38^{high}$ identified plasmablasts and plasma cells.

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Patient consent Obtained.

Ethics approval Ethics approval was obtained from the committee in University of Occupational and Environmental Health, Japan.

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REFERENCES

1. **Hamano H,** Kawa S, Horiuchi A, *et al*. High serum IgG4 concentrations in patients with sclerosing pancreatitis. *N Engl J Med* 2001;**344**:732–8.
2. **Masaki Y,** Dong L, Kurose N, *et al*. Proposal for a new clinical entity, IgG4-positive multiorgan lymphoproliferative syndrome: analysis of 64 cases of IgG4-related disorders. *Ann Rheum Dis* 2009;**68**:1310–15.
3. **Tanaka A,** Moriyama M, Nakashima H, *et al*. Th2 and regulatory immune reactions contribute to IgG4 production and the initiation of Mikulicz disease. *Arthritis Rheum* 2012;**64**:254–63.
4. **Punnonen J,** Aversa G, Cocks BG, *et al*. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA* 1993;**90**:3730–4.
5. **Umehara H,** Okazaki K, Masaki Y, *et al*. Comprehensive diagnostic criteria for IgG4-related disease (IgG4-RD), 2011. *Mod Rheumatol* 2012;**22**:21–30.
6. **Iwata S,** Yamaoka K, Niiro H, *et al*. Amplification of Toll-like receptor-mediated signaling through spleen tyrosine kinase in human B-cell activation. *J Allergy Clin Immunol* 2012;**129**: 1594–601.
7. **Khosroshahi A,** Bloch DB, Deshpande V, *et al*. Rituximab therapy leads to rapid decline of serum IgG4 levels and prompt clinical improvement in IgG4-related systemic disease. *Arthritis Rheum* 2010;**62**:1755–62.
8. **Deng GM,** Liu L, Bahjat FR, *et al*. Suppression of skin and kidney disease by inhibition of spleen tyrosine kinase in lupus-prone mice. *Arthritis Rheum* 2010;**62**:2086–92.
9. **Weinblatt ME,** Kavanaugh A, Genovese MC, *et al*. An oral spleen tyrosine kinase (Syk) inhibitor for rheumatoid arthritis. *N Engl J Med* 2010;**363**:1303–12.
10. **Podolanczuk A,** Lazarus AH, Crow AR, *et al*. Of mice and men: an open-label pilot study for treatment of immune thrombocytopenic purpura by an inhibitor of Syk. *Blood* 2009;**113**:3154–60.

Phenotypic changes of lymphocyte in a patient with IgG4-related disease after corticosteroid therapy

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Amplification of Toll-like receptor–mediated signaling through spleen tyrosine kinase in human B-cell activation

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Background: B cells are activated by combined signals through the B-cell receptor (BCR) and CD40. However, the underlying mechanisms by which BCR signals synergize with Toll-like receptor (TLR) signaling in human B cells remain unclear.
Objective: We sought to elucidate a role of spleen tyrosine kinase (Syk), a key molecule of BCR signaling, in TLR-mediated activation of human B cells.
Methods: Human naive and memory B cells were stimulated with combinations of anti-BCR, soluble CD40 ligand, and CpG. Effects of the Syk inhibitors on several B-cell functions and expression of TLR9, TNF receptor–associated factors (TRAFs), and phospho–nuclear factor κB in B cells were assessed.
Results: Activation of BCR synergized with CD40- and TLR9-mediated signals in driving robust proliferation, cell-cycle progression, expression of costimulatory molecules, cytokine production, and immunoglobulin production of human B-cell subsets, especially memory B cells. However, the Syk inhibitors remarkably abrogated these B-cell functions. Notably, after stimulation through all 3 receptors, B-cell subsets induced marked expression of *TLR9*, TRAF6, and phospho–nuclear factor κB, which was again significantly abrogated by the Syk inhibitors.
Conclusion: Syk-mediated BCR signaling is a prerequisite for optimal induction of TLR9 and TRAF6, allowing efficient propagation of TLR9-mediated signaling in memory B cells. These results also underscore the role of Syk in aberrant B-cell activation in patients with autoimmune diseases. (J Allergy Clin Immunol 2012;129:1594-601.)

Key words: Syk, Toll-like receptor 9, TNF receptor–associated factor 6, B cells

Abbreviations used
AICDA: Activation-induced cytidine deaminase
BCR: B-cell receptor
FITC: Fluorescein isothiocyanate
NF-κB: Nuclear factor κB
PI: Propidium iodide
SLE: Systemic lupus erythematosus
Syk: Spleen tyrosine kinase
TLR: Toll-like receptor
TRAF: TNF receptor–associated factor
XBP-1: X-box binding protein 1

B cells play a pivotal role in initiation and perpetuation of autoimmune diseases, including systemic lupus erythematosus (SLE). Activated self-reactive B cells not only are a source of pathogenic autoantibodies but also exert effector functions, including antigen presentation, cytokine production, and modulation of the T-cell repertoire. We recently reported that B-cell depletion therapy with rituximab for refractory patients with SLE not only rapidly depleted both naive and memory B cells in peripheral blood but also rapidly downregulated the expression levels of CD69, CD40 ligand, and inducible costimulator on $CD4^+$ T cells.[1] Thus B cells can facilitate autoimmune processes in both antibody-dependent and antibody-independent manners.

B cells are effectively activated by combined signals through B-cell receptor (BCR) and CD40; however, they require additional signals for efficient proliferation and differentiation. Accordingly, when combined with BCR and CD40 stimulation, Toll-like receptor (TLR) signaling by nucleic acids[2] induces the most robust B-cell activation.[3] In patients with SLE, RNA- or DNA-containing self-antigens coligate BCRs and TLR7 or TLR9, causing activation, proliferation, and differentiation of self-reactive B cells. However, the underlying mechanisms by which BCR signals potentiate TLR signaling in human B cells remain unclear.

On BCR ligation by antigens, protein kinases, including Lyn, an Src family kinase Lyn, and spleen tyrosine kinase (Syk), are initially activated.[4] Activation of Syk is a key event for further propagation of downstream signaling molecules in B cells.[5] In addition to BCR, Syk is activated through T-cell receptor and Fc receptor.[6,7] Notably, Syk inhibitors exert potent therapeutic efficacy against rheumatoid arthritis, as well as bronchial asthma and idiopathic thrombocytopenic purpura.[8-10] Moreover, Syk blockade prevents the development of skin and kidney lesions in mice with lupus.[11,12] Our current understanding of BCR-mediated Syk activation, however, extrapolates mainly from rodent studies.

In this study we demonstrate that Syk-mediated BCR signaling is a prerequisite for optimal induction of TLR9, TNF receptor–associated factor (TRAF) 6, and nuclear factor κB (NF-κB),

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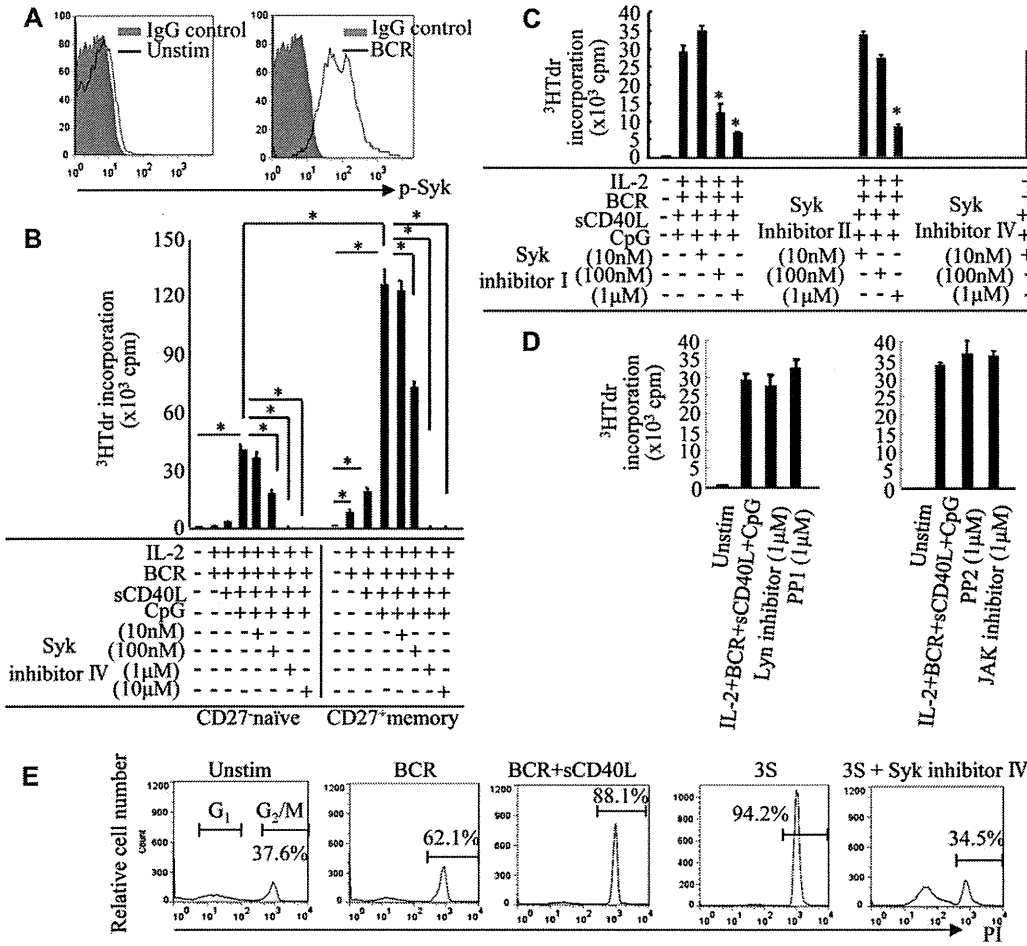

FIG 1. Syk regulates proliferation and cell-cycle progression in B-cell subsets on BCR, CD40, and TLR9 stimulation. **A**, BCR-induced phosphorylation of Syk (15 minutes). **B-D**, Tritiated thymidine *(^{3}HTdr)* incorporation of human B cells was measured during the last 18 hours of the 72-hour culture. The data are shown as means ± SDs. *$P < .05$. *sCD40L*, Soluble CD40 ligand. **E**, FACS histograms of nuclear DNA content in memory B cells 24 hours later. *Unstim*, Before stimulation; *3S*, BCR, CD40, and TLR9 stimulation. Results are representative of 3 independent experiments.

thereby driving efficient TLR9 signaling that is critical for the proliferation and differentiation of human memory B cells.

METHODS

Reagents

Syk inhibitor I, Syk inhibitor II, Syk inhibitor IV, BAY61-3606, PP1, and PP2 were purchased from Merck (Darmstadt, Germany). Lyn peptide inhibitor was purchased from Tocris Bioscience (Ellisville, Mo). PF-956980 (JAK3 kinase inhibitor) was provided from Pfizer, Inc (New York, NY). Anti-BCR mAbs (anti-Igλ and anti-Igk), recombinant human IL-2, recombinant human CD40 ligand, and phosphorothioate-protected CpG-oligonucleotide 2006 (CpG-ODN 2006; 5′-TCGTCGTTTTGTCGTTTTGTCG TT-3′) were from BD PharMingen (San Diego, Calif), R&D Systems (Minneapolis, Minn), PeproTec (Rocky Hill, NJ), and Greiner Bio-One (Tokyo, Japan), respectively.

Isolation, culture, and stimulation of B-cell subsets

This study protocol has been approved by the ethics committee of our university. PBMCs from 3 healthy donors were isolated with lymphocyte separation medium (ICN/Cappel Pharmaceuticals, Aurora, Ohio). B cells were obtained by means of negative selection from PBMCs by using the memory B-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD27$^+$ memory B cells were then isolated by means of positive selection from B cells with CD27 microbeads. The negative fraction of this isolation was assigned to CD27$^-$ naive B cells. Purity of naive and memory B cells was greater than 90% (see Fig E1 in this article's Online Repository at www.jacionline.org). B cells were cultured in RPMI 1640 (Wako Pure Clinical Industries, Osaka, Japan) supplemented with 10% FCS (Tissue Culture Biologicals, Tulare, Calif), 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen, Carlsbad, Calif). According to a previous study,[13] we used the combination of anti-Igκ and anti-Igλ mAbs for BCR stimulation and initially ensured strong induction of Syk phosphorylation by these antibodies (Fig 1, *A*). CD40 stimulation with recombinant human CD40 ligand is hereafter referred to simply as CD40 stimulation. CpG-ODN 2006 is a type B CpG-ODN specific for human TLR9 and mainly activates B cells but only weakly stimulates IFN-α secretion in plasmacytoid dendritic cells.[14]

Proliferation assay

Purified B cells were stimulated in 96-well plates (1×10^5 per well) with anti-BCR mAbs (anti-Igλ and anti-Igκ, 1 μg/mL each), soluble CD40 ligand (2 μg/mL), and CpG-ODN (2.5 μg/mL) with or without IL-2 (10 ng/mL). Cells were cultured for 72 hours and pulsed with 0.5 μCi (18.5 kBq) per well of tritiated thymidine during the last 18 hours of culture and then harvested with a semiautomatic cell harvester (Abe Kagaku, Chiba, Japan), and

their uptake of tritiated thymidine was determined with a scintillation counter (Aloka LSC-3500ETM, Tokyo, Japan).

Flow cytometric analysis

After washing, B-cell subsets were incubated in blocking buffer (0.25% human globulin, 0.5% human albumin [Yoshitomi, Osaka, Japan], and 0.1% NaN_3 in PBS) in a 96-well plate at 4°C for 15 minutes. Cells were then suspended in 100 μL of FACS solution (0.5% human albumin and 0.1% NaN_3 in PBS) and treated with fluorescein isothiocyanate (FITC)–labeled murine IgG1κ, anti-human CD80 (BD PharMingen, San Diego, Calif), or anti-human CD86 (Dako Japan, Kyoto, Japan) for 30 minutes at 4°C. Cells were washed 3 times with FACS solution and analyzed with a FACSCalibur (Becton–Dickinson, San Jose, Calif) and FlowJo software (Tomy Digital Biology, Tokyo, Japan). For intracellular staining of phosho-Syk, Blimp-1, TRAF2, TRAF3, TRAF5, TRAF6, and phospho–NF-κB, cells were fixed with PBS containing 1% formaldehyde and permeabilized with saponin-PBS (PBS containing 0.1% saponin, 0.1% BSA, 0.1% NaN3, and 0.01 mol/L HEPES). After washing, cells were resuspended in saponin-PBS and stained with mouse anti-human phospho-Syk (pY348) (BD PharMingen), goat anti-human Blimp-1 (N-20; Santa Cruz Biotechnology, Santa Cruz, Calif), rat anti-human TRAF2 (MBL), rabbit anti-human TRAF3 (Santa Cruz Biotechnology), rabbit anti-human TRAF5 (Santa Cruz Biotechnology), mouse anti-human TRAF6 (Santa Cruz Biotechnology), or rabbit anti-human phospho–NF-κB p65 (Ser 536, 93H1; Cell Signaling Technology, Tokyo, Japan), followed by washing with saponin-PBS. FITC-labeled donkey anti-goat IgG (Santa Cruz Biotechnology), phycoerythrin-labeled goat anti-rat (BD PharMingen), phycoerythrin-labeled goat anti-rabbit (CALTAG), FITC-labeled rat anti-mouse (BD PharMingen), and FITC-labeled goat anti-rabbit IgG (BD PharMingen) were used as secondary antibodies. Isotype-matched goat IgG, rat IgG, rabbit IgG, or mouse IgG controls (all from Sigma-Aldrich, St Louis, Mo) were used to evaluate the background.

Apoptosis assay

Purified B cells were stimulated for 72 hours in 96-well plates (2×10^5 per well) with anti-BCR mAbs (anti-Igλ and anti-Igκ, 1 μg/mL each), soluble CD40 ligand (2 μg/mL), and CpG-ODN (2.5 μg/mL) with or without Syk inhibitor IV. After culture, cells were double-stained with FITC–Annexin V and propidium iodide (PI) in Apoptosis Detection kit I (BD PharMingen). The percentage of apoptotic cells was measured by using flow cytometry.

Cell-cycle analysis

For cell-cycle analysis, cells were suspended in PI staining buffer (50 μg/mL PI, 5 mmol/L EDTA, 1 μg/mL DNase-free RNase, and 0.1% saponin in PBS). The samples were then incubated for 30 minutes at 37°C, and DNA content was analyzed by using flow cytometry.

Cytokine production

Levels of IL-6, IL-10, IL-12 p70, and TNF-α in culture were determined by using the BD Cytometric Bead Array human Flex set, according to the manufacturer's instructions (BD PharMingen).

IgG ELISA

For quantification of *in vitro* IgG secretion, B-cell subsets were cultured with anti-BCR mAbs, CD40 ligand, and CpG-ODN 2006 in 96-well plates (1×10^5 per well) for 5 days. IgG levels in culture were determined by using a human IgG ELISA Quantitation Kit (Bethyl Laboratories, Inc, Montgomery, Ala).

Quantitative real-time PCR

Total RNA was prepared by using the RNeasy Mini Kit (Qiagen, Chatsworth, Calif). First-strand cDNA was synthesized, and quantitative real-time PCR was performed in the Step One Plus instrument (Applied Biosystems, Foster City, Calif) in triplicate wells in 96-well plates. TaqMan target mixes for X-box binding protein 1 *(XBP-1)* (Hs00152973-m1), AICDA (Hs00757808-m1), and *TLR9* (Hs00964360-m1) were purchased from Applied Biosystems. *XBP-1*, activation-induced cytidine deaminase *(AICDA)* and *TLR9* mRNA expression levels were normalized to the levels of 18S ribosomal RNA (Hs99999901-m1, Applied Biosystems) as an endogenous control, and the relative quantity compared with the PBMC sample as a reference was calculated by using the quantification-comparative cycle threshold ($\Delta\Delta C_T$) formula. Relative quantity was calculated by using the $\Delta\Delta C_T$ formula–referenced sample of PBMCs.

Western blot analysis

Raji cells were lysed in an NP-40 buffer containing NaCl, Tris-HCL (pH 8.0), distilled water, and protease inhibitor. Lysates were then mixed with an equal volume of sample buffer solution (2-mercaptoethanol; Wako Pure Chemical Industries) and boiled for 5 minutes. Proteins were separated by means of SDS-PAGE, transferred onto nitrocellulose membranes (Whatman, Tokyo, Japan), blocked with 5% skim milk, and immunoblotted with anti-human Syk, anti-human phospho-Syk (pY348), anti-human TRAF6, anti-human phospho–NF-κB p65 (Ser 536, 93H1), and horseradish peroxidase–labeled anti-secondary (#NA931V and #NA934V; GE Healthcare, Osaka, Japan) by using immunoreaction enhancer solution (Can Get Signal; Toyobo, Osaka, Japan). Blots were developed with ECL Western Blotting Detection Reagents (GE Healthcare) and visualized with a light-capture instrument (ATTO, Tokyo, Japan).

Statistical analysis

All statistical analyses were performed with JMP version 8.0.2 statistical software (SAS Institute Inc, Cary, NC). Statistical significance of differences between the pretreatment and posttreatment values was tested by using the Wilcoxon test. *P* values of less than .05 were considered statistically significant.

RESULTS

Syk is critical for proliferation and cell-cycle progression in memory B cells

We investigated the effect of BCR, CD40, and TLR9 stimulation on the proliferation of B-cell subsets. BCR stimulation alone remarkably induced Syk phosphorylation; however, it had only marginal effects on DNA synthesis in B cells (Fig 1, *A* and *B*). Combined stimulation of BCR, CD40, and TLR9 strongly induced DNA synthesis in both naive and memory B cells, although significantly more so in the latter. This robust proliferation was inhibited by Syk inhibitor IV (BAY61-3606) in a dose-dependent manner (Fig 1, *B*). Similar data were obtained with another Syk inhibitor (Syk inhibitors I and II; Fig 1, *C*). In contrast to these Syk inhibitors, non-Syk inhibitors (PP1, PP2, and JAK inhibitor) were not effective, even at high concentrations (Fig 1, *D*). Syk inhibitor IV was hereinafter used for further experiments. We next tested cell-cycle progression in memory B cells after BCR, CD40, and TLR9 stimulation (Fig 1, *E*). The percentage of cells in the G_2/M phase without stimulation was 37.6%. This value increased further up to 94.2% with combined stimulation of BCR, CD40, and TLR9. Consistent with our results (Fig 1, *B* and *C*), Syk inhibitor IV significantly inhibited G_2/M phase progression in memory B cells. Together, these results suggest a critical role for Syk in BCR-, CD40-, and TLR-induced proliferation and cell-cycle progression in human memory B cells.

Syk regulates expression of costimulatory molecules and cytokine production in B-cell subsets

We tested expression of the costimulatory molecules CD80 and CD86 in B cells (Fig 2). Both were only marginally expressed in

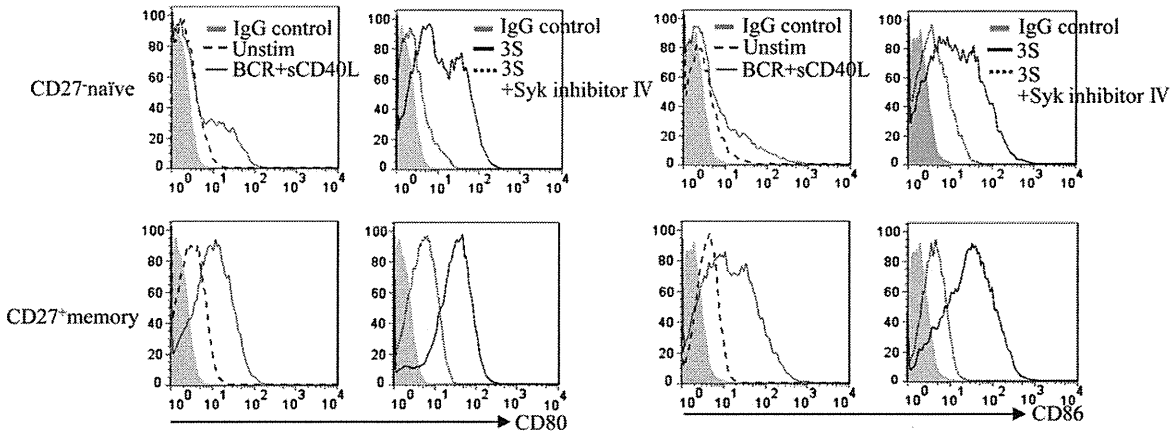

FIG 2. Syk regulates expression of CD80 and CD86 in B-cell subsets on stimulation. *Overlay histograms* depict relative fluorescence intensities of human naive and memory B cells cultured for 72 hours. *Unstim*, Before stimulation; *3S*, BCR, CD40, and TLR9 stimulation. Results are representative of 3 independent experiments.

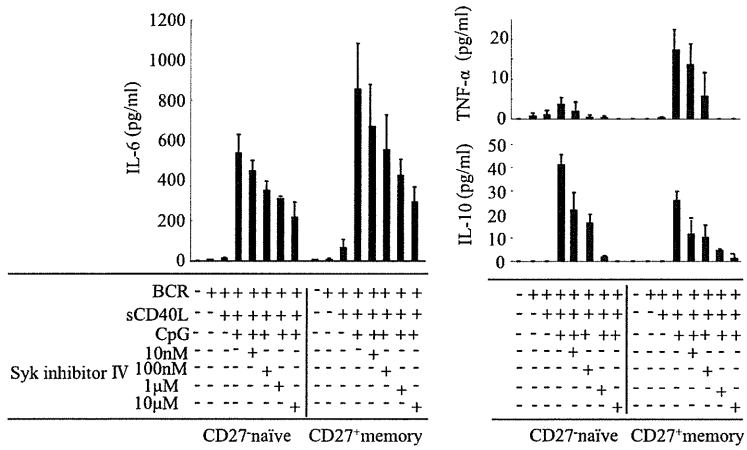

FIG 3. Syk regulates cytokine production in B-cell subsets on stimulation. Human peripheral blood naive and memory B cells were cultured for 72 hours, and supernatants were harvested and assayed by using the cytometric bead array for IL-6, TNF-α, and IL-10 content. Data are shown as means ± SDs and are representative of 3 independent experiments. *sCD40L*, Soluble CD40 ligand.

memory but not naive B cells without stimulation. Combined stimulation of BCR, CD40, and TLR9 induced significant expression of CD80/CD86 in memory B cells compared with that seen in naive cells. Syk inhibitor IV almost completely canceled CD80/CD86 expression in both subsets, suggesting a role of Syk in expression of costimulatory molecules in B cells.

We next analyzed cytokine production (IL-6, IL-10, and TNF-α) by B-cell subsets (Fig 3). Combined stimulation with BCR, CD40, and TLR9 induced production of the proinflammatory cytokines IL-6 and TNF-α in naive and memory cells, although more markedly in the latter. Syk inhibitor IV clearly inhibited production of these cytokines in both subsets in a dose-dependent fashion. In contrast to proinflammatory cytokines, anti-inflammatory IL-10 production was more pronounced in naive than memory B cells, which is consistent with a recent study that IL-10–producing B cells are enriched in human $CD27^{-}CD38^{hi}$ B cells.[15] Again, dose-dependent suppression of IL-10 production by Syk inhibitor IV was observed in both subsets. We failed to detect IL-12 p70, IL-2, IFN-α, and IFN-γ under any conditions (data not shown). These results suggest the critical role of Syk in BCR-, CD40-, and TLR-induced cytokine production in B-cell subsets and also underscore the therapeutic efficacy of Syk inhibitors in decreasing the inflammatory consequences of autoimmune diseases by modulating proinflammatory cytokines, such as TNF-α and IL-6.

Syk regulates B-cell differentiation on BCR, CD40, and TLR9 stimulation

On strong stimulation, B cells differentiate to plasma cells and undergo class-switching along with expression of critical molecules, such as *AICDA*, *XBP-1*, and Blimp-1. Both naive and memory B cells strongly induced expression of *AICDA*, *XBP-1*, and Blimp-1 after BCR, CD40, and TLR9 stimulation, which was inhibited by Syk inhibitor IV (Fig 4, *A* and *B*). In addition, IgG production induced by BCR, CD40, and TLR9 stimulation, which was particularly high in memory B cells, was again greatly reduced by Syk inhibitor IV in a dose-dependent manner (Fig 4, *C*).

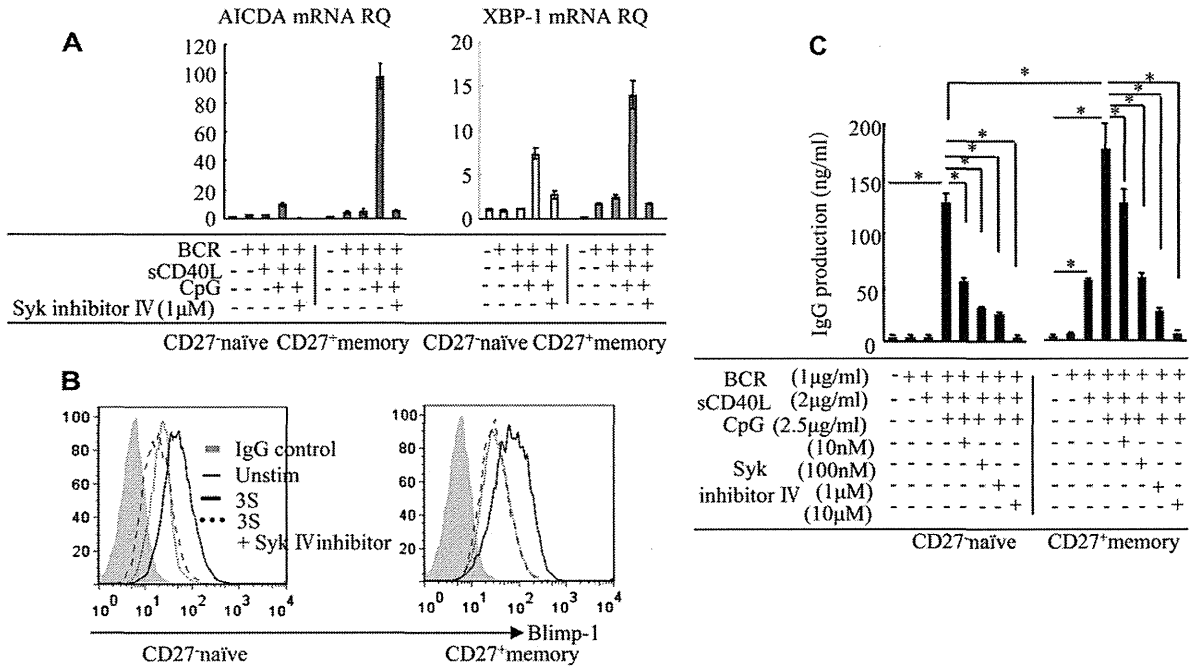

FIG 4. Syk regulates B-cell differentiation on BCR, CD40, and TLR9 stimulation. Naive and memory B cells were cultured for 48 hours (*AICDA* and *XBP-1* mRNA and Blimp-1) or for 5 days (IgG production). **A**, The level of *AICDA* and *XBP-1* mRNA was measured by using real-time PCR. *RQ*, Relative quantity. **B**, Blimp-1 expression was measured by means of flow cytometry. *Unstim*, Before stimulation; *3S*, BCR, CD40, and TLR stimulation. **C**, IgG in the supernatant was quantified by using ELISA. Data are shown as means ± SDs and are representative of 3 independent experiments. $*P < .05$. *sCD40L*, Soluble CD40 ligand.

These results suggest that Syk also regulates B-cell differentiation induced by BCR, CD40, and TLR9 stimulation.

TRAF6 is a key Syk-regulated molecule in B-cell subsets on stimulation

Syk is a key downstream signaling molecule of BCR, but not CD40 or TLR9, in B cells.[16,17] Considering that Syk blockade significantly abrogates proliferation, cytokine production, and differentiation after BCR, CD40, and TLR9 stimulation (Figs 1-4), we particularly sought to elucidate the mechanisms by which Syk regulates TLR9 signaling in human B-cell subsets. Given that TLR9 expression is significantly induced in BCR-stimulated B cells and that TRAFs are the critical downstream molecules in CD40 and TLR9 signaling in B cells,[18,19] we reasoned that TLR9 and TRAFs were possible candidates. Memory B cells constitutively expressed more *TLR9* mRNA than naive B cells (Fig 5, *A*). On BCR, CD40, and TLR9 stimulation, *TLR9* mRNA expression was more drastically induced in memory than naive B cells. Syk inhibitor IV inhibited expression of *TLR9* mRNA in memory B cells to the level seen in unstimulated naive B cells (Fig 5, *A*). Among TRAFs, expression of TRAF2, TRAF3, and TRAF5 was constitutively detected; however, their expression was not affected by BCR stimulation (Fig 5, *B*). In contrast, TRAF6 expression was only slightly detected in memory B cells without stimulation. BCR stimulation alone, however, potently increased TRAF6 expression in both subsets (Fig 5, *B*). TRAF6 expression was further pronounced by additional CD40 and TLR9 stimulation, and strong NF-κB phosphorylation was correlatively observed. Expression of these molecules was blocked by Syk inhibitor IV (Fig 5, *B* and *C*).

Without stimuli, Raji cells exhibit higher basal (tonic) signaling that supports proliferation and survival.[20] In these cells TLR9 mRNA was expressed at a much higher level than in unstimulated naive B cells, which was markedly reduced by Syk inhibitor IV (Fig 6, *A*). In addition, these cells constitutively exhibited pronounced expression and phosphorylation of Syk. Syk inhibitor IV clearly inhibited Syk phosphorylation without affecting its protein levels. Of note, TRAF6 expression and NF-κB phosphorylation were strongly reduced as well by Syk inhibitor IV (Fig 6, *B*). These suggest that Syk blockade exerts an inhibitory action on expression of TLR9, TRAF6, and NF-κB phosphorylation, even in B cells with high basal BCR signaling.

DISCUSSION

In this study we demonstrate that engagement of BCR in conjunction with ligation of CD40 and TLR9 induces remarkable proliferation, expression of costimulatory molecules, cytokine production, and immunoglobulin production in human B cells, especially the memory subset. Moreover, the Syk inhibitor suppresses all of these functions to background levels, at least in part through inhibition of expression of TLR9 and TRAF6, resulting in decreased phosphorylation of NF-κB.

We show that combined stimulation with BCR and CD40 was sufficient to activate memory B cells, whereas it had less effect on naive B cells. However, Additional CpG stimulation caused potent activation of both subsets, although always more strongly in the memory subset, suggesting that memory B cells exhibit a lower threshold for activation compared with naive B cells. Memory B cells can survive without antigenic stimulation, and they can be fully activated only by cognate T-cell help and

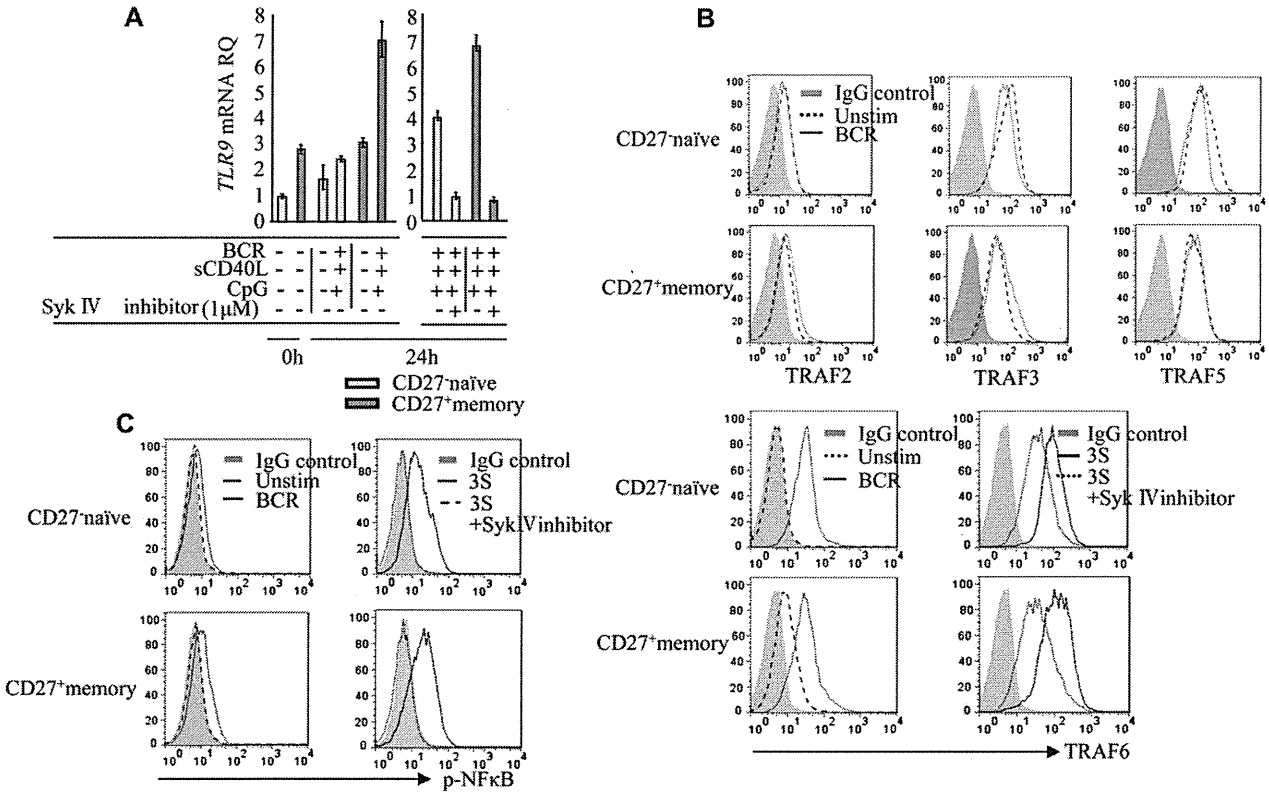

FIG 5. TLR9 and TRAF6 are key Syk-regulated molecules in B-cell subsets on stimulation. **A**, *TLR9* mRNA was quantified by using real-time PCR (TaqMan PCR kit) 24 hours later. *RQ*, Relative quantity; *sCD40L*, soluble CD40 ligand. **B** and **C**, TRAF2, TRAF3, TRAF5, and TRAF6 levels (48 hours later) and NF-κB phosphorylation (p65; 12 hours later) were measured by means of flow cytometry (intracellular staining). *Unstim*, Before stimulation; *3S*, combination of BCR, CD40, and TLR9 stimulation. Data are representative of 3 independent experiments.

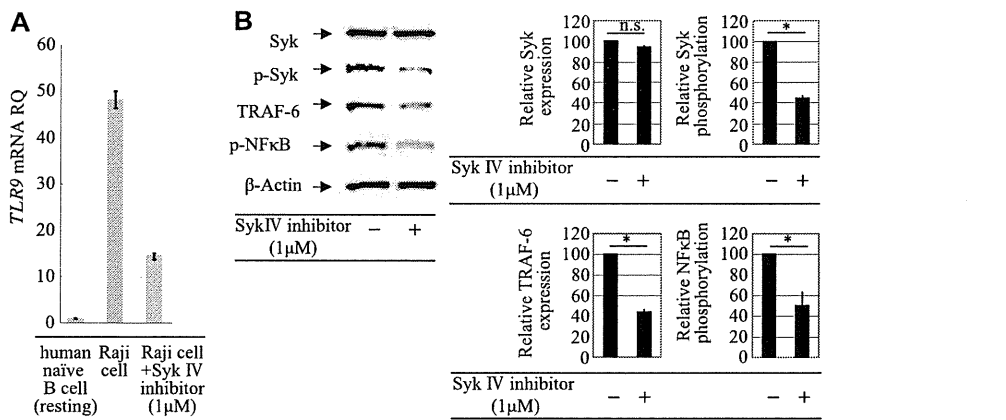

FIG 6. Syk inhibitor exerts marked inhibitory action, even at an activated state of B cells. Raji cells were cultured with RPMI containing 2% FCS for 48 hours. **A**, *TLR9* mRNA was quantified by means of real-time PCR. *RQ*, Relative quantity. **B**, Expression of Syk, phospho-Syk (Y348), TRAF6, and phospho-NF-κB (p65) was assessed by means of Western blotting. The intensity of bands was quantified and normalized with respect to those of corresponding β-actin. The resulting values were expressed as the percentage in reference to that of cells without Syk inhibitor IV. Data are shown as means ± SDs and are representative of 3 independent experiments. $*P < .05$. *n.s.*, Not significant.

cytokines.[21-23] In addition, the costimulatory molecules CD80 and CD86, as well as TLR9 and TRAF6, are weakly expressed in memory B cells in the nonstimulated (steady) state (Figs 2 and 5). These findings suggest that a basal BCR tonic signal in memory B cells is higher than in naive B cells, which might account for the maintenance of serologic memory.[24,25]

What signaling molecules are responsible for a basal BCR tonic signal in memory B cells? We recently showed that without

BCR stimulation, weak activation of Syk is constitutively observed in memory B cells.[26] Given that Syk activation is a key event for further propagation of the BCR signaling pathway,[4] these findings support our rationale that blockade of Syk activation regulates the functions of memory B cells. Surprisingly, the effects of the Syk inhibitor on B-cell functions were more dramatic than we had initially expected: it almost completely abrogated B-cell proliferation, activation, cytokine production, and differentiation induced by a combinatorial stimulation of BCR, CD40, and TLR9 (Figs 1-4). We also evaluated B-cell survival by determining the percentage of apoptotic cells with FITC–Annexin V and PI. Consistent with our previous study,[26] without stimuli, a considerable fraction of B cells spontaneously underwent apoptotic cell death *in vitro*, and such cell death was not affected by the Syk inhibitor, excluding nonspecific cytotoxic effects of this inhibitor on B-cell survival (see Fig E2 in this article's Online Repository at www.jacionline.org). On stimulation with BCR, CD40, and TLR9, apoptotic cell death (Annexin V^+PI^- and AnnexinV^+PI^+) was considerably protected. This protection was indeed abrogated by the Syk inhibitor in a dose-dependent manner, suggesting that Syk provides survival signals as well for B cells after stimulation through all 3 receptors (see Fig E2).

It remains somewhat unclear whether Syk is directly activated in CD40 and TLR9 signaling pathways in B cells.[16,17] Ying et al[27] showed that Syk is synergistically activated in B cells on BCR/CD40 costimulation, suggesting a role for Syk in CD40 signaling. Sanjuan et al[28] showed, using human monocytic cell lines, that tyrosine phosphorylation of TLR9 by the Src family kinases leads to the recruitment and activation of Syk, suggesting a role for Syk in TLR9 signaling. In contrast to these findings, we found that robust proliferation in memory B cells after CD40, TLR9, or both stimulation is not influenced by the Syk inhibitor (data not shown). Thus other regulatory mechanisms of B-cell activation by the Syk inhibitor are more likely to exist.

We show here that Syk is a regulator of expression of TLR9 and TRAF6, both of which are critical for TLR9-induced NF-κB activation. Consistent with our results, a previous study showed that *TLR9* mRNA is expressed at high levels in memory B cells and its expression is enhanced by BCR cross-linking,[18] although involvement of Syk in this process was not investigated. NF-κB activation regulates *TLR9* mRNA expression induced by BCR, CD40, and TLR9 stimulation,[29] suggesting that NF-κB–induced TLR9 expression forms a novel feed-forward loop in NF-κB activation in B cells. Blockade of Syk-mediated BCR signaling could thus shut off this loop, thereby inhibiting NF-κB activation and TLR9 expression. Indeed, we found that Syk inhibition reduces expression of TLR9 mRNA in memory B cells to the levels seen in unstimulated, steady-state naive B cells (Fig 5, *A*).

TRAF6 plays a pivotal role in TLR9-induced c-Jun N-terminal kinase activation, CD80 expression,[30] and IL-6 production.[31] B cell–specific disruption of TRAF6 results in a lower number of mature B cells, as well as inhibition of antibody class-switching and impaired differentiation to plasma cells.[32] We found that BCR stimulation alone strongly induces TRAF6 expression, which is further enhanced by additional CD40 and TLR9 stimulation (Fig 5, *B*). TRAF6 expression, as well as NF-κB phosphorylation, on B-cell activation is markedly inhibited by Syk blockade. These findings clearly suggest that Syk-mediated BCR signaling is a prerequisite for optimal induction of TRAF6, allowing efficient propagation of TLR9 signaling.

Our current findings provide a novel insight into B-cell aberrations in patients with SLE. The prevailing hypothesis of B cell–mediated autoimmunity is that both autoantigen-triggered BCR signals and costimulatory signals are required for activation of autoreactive (pathogenic) B cells, which are particularly enriched in the memory subset. However, recent studies showed that TLR7 and TLR9 can recognize self-derived RNA and DNA, respectively, and that TLR signaling is necessary for autoantibody production in mice with lupus.[33,34] BCR-induced calcium mobilization and protein tyrosine phosphorylation were both pronounced in B cells from mice with SLE,[35] indicating that alterations in B-cell signaling already occur at the proximity of the BCR. We here demonstrate that Syk-mediated BCR signaling is a prerequisite for optimal induction of TLR9 and TRAF6, thereby allowing efficient propagation of CD40 and TLR9 signaling, which are critical for the proliferation and differentiation of human memory B cells. Our current findings also underscore the potential role of Syk in B cell–mediated pathologic processes in patients with autoimmune diseases, namely Syk-mediated BCR signaling, could be already activated probably by autoantigens and that Syk inhibitors have potential as new drugs in the treatment of autoimmune diseases, including SLE and RA.

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Clinical implications: Syk inhibitors might be promising for controlling the aberrant TLR9 signaling that is related to the proliferation and differentiation of pathogenic memory B cells in patients with autoimmune diseases, including SLE and RA.

REFERENCES

1. Iwata S, Saito K, Tokunaga M, Yamaoka K, Nawata M, Yukawa S, et al. Phenotypic changes of lymphocytes in patients with systemic lupus erythematosus who are in longterm remission after B cell depletion therapy with rituximab. J Rheumatol 2011;38:633-41.
2. Krug A. Nucleic acid recognition receptors in autoimmunity. Handb Exp Pharmacol 2008;183:129-51.
3. Ruprecht CR, Lanzavecchia A. Toll-like receptor stimulation as a third signal required for activation of human naïve B cells. Eur J Immunol 2006;36:810-6.
4. Taniguchi T, Kobayashi T, Kondo J, Takahashi K, Nakamura H, Suzuki J, et al. Molecular cloning of a porcine gene syk that encodes a 72-kDa protein-tyrosine kinase showing high susceptibility to proteolysis. J Biol Chem 1991;266:15790-6.
5. Kulathu Y, Grothe G, Reth M. Autoinhibition and adapter function of Syk. Immunol Rev 2009;232:286-99.
6. Wong WS, Leong KP. Tyrosine kinase inhibitors: a new approach for asthma. Biochim Biophys Acta 2004;1697:53-69.
7. Beaven MA, Baumgartner RA. Downstream signals initiated in mast cells by Fc epsilon RI and other receptors. Curr Opin Immunol 1996;8:766-72.
8. Meltzer EO, Berkowitz RB, Grossbard EB. An intranasal Syk-kinase inhibitor (R112) improves the symptoms of seasonal allergic rhinitis in a park environment. J Allergy Clin Immunol 2005;115:791-6.
9. Podolanczuk A, Lazarus AH, Crow AR, Grossbard E, Bussel JB. Of mice and men: an open-label pilot study for treatment of immune thrombocytopenic purpura by an inhibitor of Syk. Blood 2009;113:3154-60.
10. Weinblatt ME, Kavanaugh A, Genovese MC, Musser TK, Grossbard EB, Magilvay DB. An oral spleen tyrosine kinase (Syk) inhibitor for rheumatoid arthritis. N Engl J Med 2010;363:1303-12.
11. Bahjat FR, Pine PR, Reitsma A, Cassafer G, Baluom M, Grillo S. An orally bioavailable spleen tyrosine kinase inhibitor delays disease progression and prolongs survival in murine lupus. Arthritis Rheum 2008;58:1433-44.
12. Deng GM, Liu L, Bahjat FR, Pine PR, Tsokos GC. Suppression of skin and kidney disease by inhibition of spleen tyrosine kinase in lupus-prone mice. Arthritis Rheum 2010;62:2086-92.
13. Sakurai D, Kanno Y, Hase H, Kojima H, Okumura K, Kobata T. TACI attenuates antibody production costimulated by BAFF-R and CD40. Eur J Immunol 2007;37:110-8.

14. Krug A, Rothenfusser S, Hornung V, Jahrsdörfer B, Blackwell S, Ballas ZK, et al. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur J Immunol 2001;31:2154-63.
15. Blair PA, Noreña LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, 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;32:129-40.
16. Graham JP, Arcipowski KM, Bishop GA. Differential B-lymphocyte regulation by CD40 and its viral mimic, latent membrane protein 1. Immunol Rev 2010;237:226-48.
17. Krieg AM. A role for Toll in autoimmunity. Nat Immunol 2002;3:423-4.
18. Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naïve B cells and constitutive expression in memory B cells. Blood 2003;101:4500-4.
19. Xie P, Kraus ZJ, Stunz LL, Bishop GA. Roles of TRAF molecules in B lymphocyte function. Cytokine Growth Factor Rev 2008;19:199-207.
20. Guo Q, Qian L, Guo L, Shi M, Chen C, Lv X, et al. Transactivators Zta and Rta of Epstein-Barr virus promote G0/G1 to S transition in Raji cells: a novel relationship between lytic virus and cell cycle. Mol Immunol 2010;47:1783-92.
21. Maruyama M, Lam KP, Rajewsky K. Memory B-cell persistence is independent of persisting immunizing antigen. Nature 2000;407:636-42.
22. Tangye SG, Avery DT, Deenick EK, Hodgkin PD. Intrinsic differences in the proliferation of naïve and memory human B cells as a mechanism for enhanced secondary immune responses. J Immunol 2003;170:686-94.
23. Kindler V, Zubler RH. Memory, but not naïve, peripheral blood B lymphocytes differentiate into Ig-secreting cells after CD40 ligation and costimulation with IL-4 and the differentiation factors IL-2, IL-10, and IL-3. J Immunol 1997;159:2085-90.
24. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science 2002;298:2199-202.
25. Lam KP, Kuhn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell 1997;90:1073-83.
26. Tabrizi SJ, Niiro H, Masui M, Yoshimoto G, Iino T, Kikushige Y, et al. T cell leukemia/lymphoma 1 and galectin-1 regulate survival/cell death pathways in human naive and IgM+ memory B cells through altering balances in Bcl-2 family proteins. J Immunol 2009;182:1490-9.
27. Lin YC, Huang DY, Chu CL, Lin WW. Anti-inflammatory actions of Syk inhibitors in macrophages involve non-specific inhibition of toll-like receptors-mediated JNK signaling pathway. Mol Immunol 2010;47:1569-78.
28. Sanjuan MA, Rao N, Lai KT, Gu Y, Sun S, Fuchs A, et al. CpG-induced tyrosine phosphorylation occurs via a TLR9-independent mechanism and is required for cytokine secretion. J Cell Biol 2006;172:1057-68.
29. An H, Yu Y, Zhang M, Xu H, Qi R, Yan X, et al. Involvement of ERK, p38 and NF-kappaB signal transduction in regulation of TLR2, TLR4 and TLR9 gene expression induced by lipopolysaccharide in mouse dendritic cells. Immunology 2002;106:38-45.
30. Rowland SL, Tremblay MM, Ellison JM, Stunz LL, Bishop GA, Hostager BS. A novel mechanism for TNFR-associated factor 6-dependent CD40 signaling. J Immunol 2007;179:4645-53.
31. Jalukar SV, Hostager BS, Bishop GA. Characterization of the roles of TNF receptor-associated factor 6 in CD40-mediated B lymphocyte effector functions. J Immunol 2000;164:623-30.
32. Kobayashi T, Kim TS, Jacob A, Walsh MC, Kadono Y, Fuentes-Pananá E, et al. TRAF6 is required for generation of the B-1a B cell compartment as well as T cell-dependent and -independent humoral immune responses. PLoS One 2009;4:e4736.
33. Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchick MJ. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. Immunity 2006;25:417-28.
34. Viglianti GA, Lau CM, Hanley TM, Miko BA, Shlomchik MJ, Marshak-Rothstein A. Activation of autoreactive B cells by CpG dsDNA. Immunity 2003;19:837-47.
35. Liossis SN, Kovacs B, Dennis G, Kammer GM, Tsokos GC. B cells from patients with systemic lupus erythematosus display abnormal antigen receptor-mediated early signal transduction events. J Clin Invest 1996;98:2549-57.

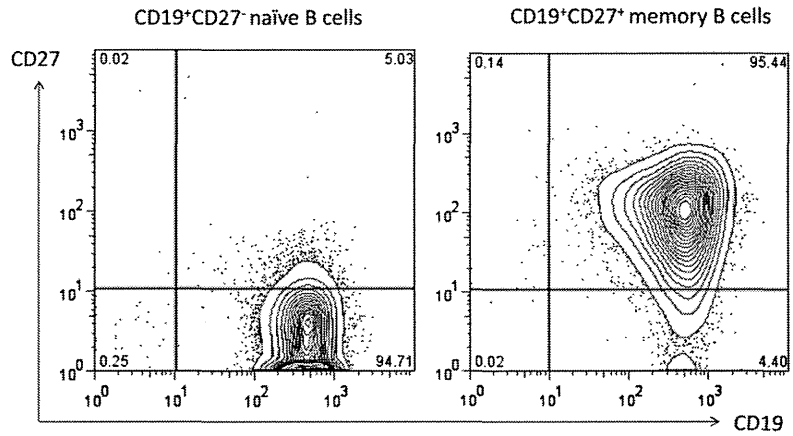

FIG E1. Phenotypic analysis of B-cell subsets in human peripheral blood. B cells were obtained by means of negative selection from PBMCs. $CD27^+$ memory B cells were then isolated by using positive selection from B cells with CD27 microbeads. The negative fraction of this isolation was assigned to $CD27^-$ naive B cells. The purity of naive and memory B cells was greater than 90% (*x-axis*, CD19; *y-axis*, CD27).

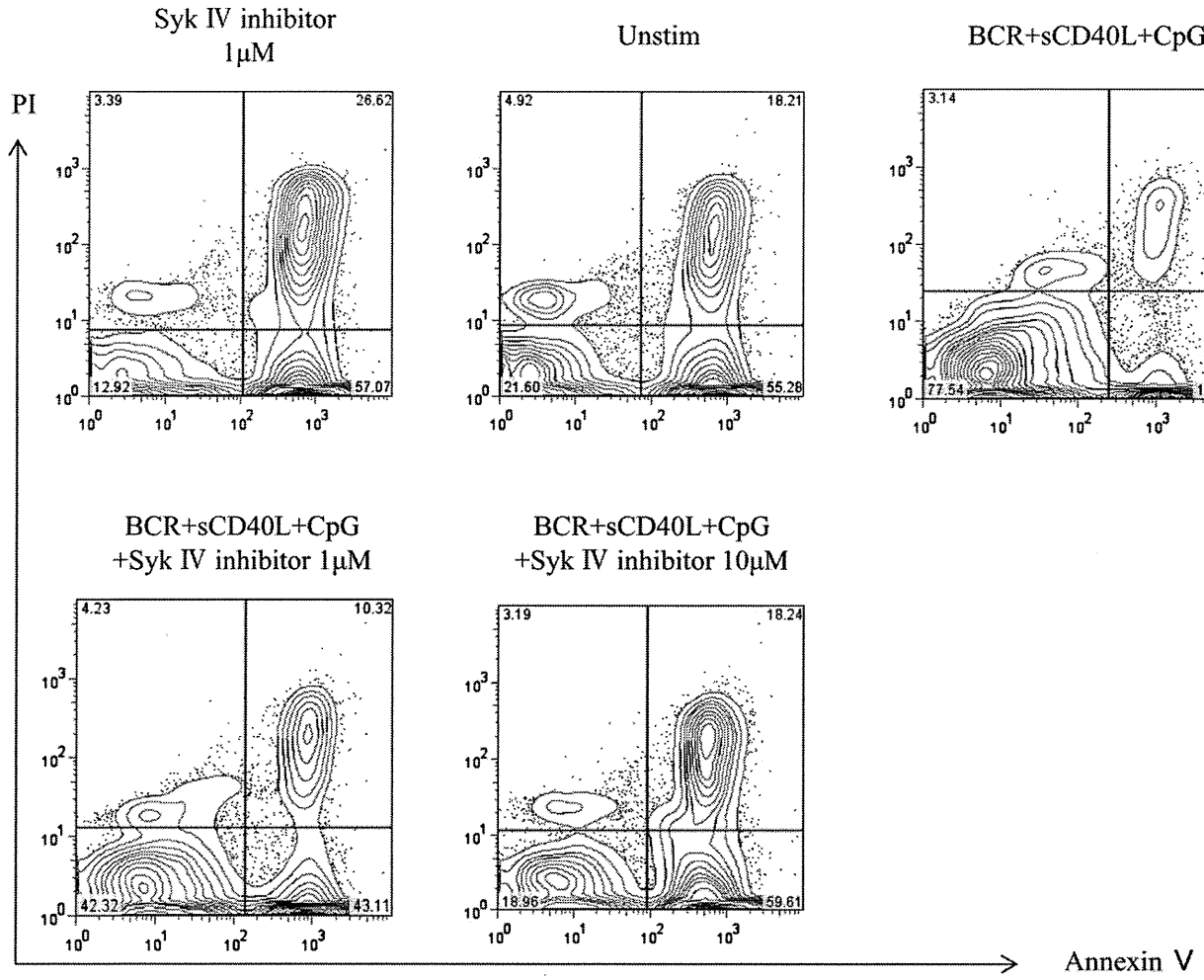

FIG E2. Syk provides survival signals for B cells after stimulation through all 3 receptors. B cells (2×10^5 per well) were cultured in triplicate in 96-well plates with anti-Igλ and anti-Igk antibodies (1 μg/mL), soluble CD40 ligand (*sCD40L*; 2 μg/mL), and CpG-ODN 2006 (2.5 μg/mL) with or without Syk inhibitor IV for 72 hours. The percentage of apoptotic B cells was assessed by means of double-staining with FITC–Annexin V and PI (*x-axis*, PI; *y-axis*, Annexin V).

Serum immune complex containing thrombospondin-1: a novel biomarker for early rheumatoid arthritis

The diagnosis of rheumatoid arthritis (RA) is based on classification criteria set by the 2010 RA classification criteria including serological assessment of rheumatoid factor (RF) and anticitrulline-containing protein/peptide (anti-CCP) antibody.[1 2] Anti-CCP antibody is specific (94–99%) for RA; however, 25% of patients with established RA and 40% of patients with early RA are negative for this marker.[3 4] Novel biomarkers, especially for early RA and/or for RA lacking RF and anti-CCP antibody markers (ie, seronegative RA) are therefore urgently required. Circulating immune complexes (CICs) present in the human

body are likely to contain many different antigens that may reflect underlying disease, so antigens incorporated into CICs are promising candidates for diagnostic biomarkers. We developed a novel proteomic strategy (immune complexome analysis) to identify and profile antigens in CICs and used this method to analyse CICs in patients with established RA and controls (healthy donors and patients with osteoarthritis).[5] CIC-associated thrombospondin-1 (TSP-1) was found in 81% and CIC-associated platelet factor 4 (PF4) in 52% of patients with established RA, but neither protein was found in CICs from any of the controls.[5] Both proteins are known as endogenous inhibitors of angiogenesis[6–8]; the formation of CICs may promote angiogenesis. We evaluated the diagnostic potential of CIC-associated TSP-1 and CIC-associated PF4 in patients with early RA divided into seropositive and seronegative groups.

Serum samples were collected from 25 disease-modifying antirheumatic drug (DMARD)-naïve seropositive patients with early RA (mean±SD age 52.8±18.4 years; 21 women; disease duration 0.25–12 months; CRP 0.01–8.55 mg/dl) and 15 seronegative patients with early RA (mean±SD age 60.5±17.9 years; 8 women; disease duration 1–6 months; CRP 0.02–14.4 mg/dl) at Nagasaki University Hospital. All the seropositive patients were positive for RF and 20 were positive for anti-CCP antibody, while all the seronegative patients were negative for both RF and anti-CCP antibody. The diagnosis of RA was made by the 2010 RA classification criteria as well as administration of DMARDs within the first 12 months.[1 2] Serum samples from 16 patients with Sjögren's syndrome (SS) (mean±SD age 60.9±13.0 years) and 14 patients with systemic lupus erythematosus (SLE) (mean±SD age 42.6±12.4 years) who fulfilled the international criteria for the diagnosis of SS[9] and SLE[10] and 11 healthy donors (mean±SD age 49.5±10.3 years) were used as controls. CICs purified by magnetic beads with immobilised protein G were reduced and alkylated, followed by tryptic digestion. The peptide mixture (1 μl) was subjected to nano-liquid chromatography/electrospray ionization/tandem mass spectrometry. More details of the analytical method can be found in our earlier report.[5]

As shown in table 1, CIC-associated TSP-1 was found only in patients with early RA and was not found in disease controls (patients with SS or SLE) or healthy donors (100% specific). Twenty-two (55%) of the total of 40 patients with early RA (56% (14/25) of the seropositive patients and 53% (8/15) of the seronegative patients) had CIC-associated TSP-1. PF4-containing CICs were found in only three patients (8%) with early RA compared with 52% of the patients with established RA.[5] These PF4-containing CICs may therefore promote disease progression.

In conclusion, we have shown that CIC-associated TSP-1 has high potential as a novel biomarker for diagnosing early and/or seronegative RA. Further analyses using a large number of patients are warranted to determine the clinical benefit of using this novel biomarker.

Table 1 Number of patients with early RA carrying CIC-associated TSP-1 or CIC-associated PF4

	Early RA patients (n=40)				
	Seropositive (n=25)	Seronegative (n=15)	SS patients (n=16)	SLE patients (n=14)	Healthy donors (n=11)
TSP-1	14	8	0	0	0
PF4	3	0	0	0	0

CIC, circulating immune complex; PF, platelet factor; RA, rheumatoid arthritis; SS, Sjögren's syndrome; SLE, systemic lupus erythematosus; TSP, thrombospondin.

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REFERENCES

1. **Aletaha D,** Neogi T, Silman AJ, *et al.* 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum* 2010;**62**:2569–81.
2. **Aletaha D,** Neogi T, Silman AJ, *et al.* 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Ann Rheum Dis* 2010;**69**:1580–8.
3. **van Venrooij WJ,** Zendman AJ. Anti-CCP2 antibodies: an overview and perspective of the diagnostic abilities of this serological marker for early rheumatoid arthritis. *Clin Rev Allergy Immunol* 2008;**34**:36–9.
4. **Somers K,** Geusens P, Elewaut D, *et al.* Novel autoantibody markers for early and seronegative rheumatoid arthritis. *J Autoimmun* 2011;**36**:33–46.
5. **Ohyama K,** Ueki Y, Kawakami A, *et al.* Immune complexome analysis of serum and its application in screening for immune complex antigens in rheumatoid arthritis. *Clin Chem* 2011;**57**:905–9.
6. **Jou IM,** Shiau AL, Chen SY, *et al.* Thrombospondin 1 as an effective gene therapeutic strategy in collagen-induced arthritis. *Arthritis Rheum* 2005;**52**:339–44.
7. **Lawler J.** Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth. *J Cell Mol Med* 2002;**6**:1–12.
8. **Maione TE,** Gray GS, Petro J, *et al.* Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. *Science* 1990;**247**:77–9.
9. **Vitali C,** Bombardieri S, Jonsson R, *et al.* Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002;**61**:554–8.
10. **Cohen AS,** Fries JF, Winchester RJ, *et al.* The 1982 revised criteriafor the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982;**25**:1271–7.

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Original article

The diagnostic utility of anti-melanoma differentiation-associated gene 5 antibody testing for predicting the prognosis of Japanese patients with DM

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Abstract

Objective. Interstitial lung disease (ILD), especially rapidly progressive ILD (RPILD), is a major poor prognostic factor in patients with DM. We investigated the association of anti-melanoma differentiation-associated gene 5 (MDA5) antibody (Ab) with clinical characteristics and mortality in Japanese patients with DM.

Methods. Seventy-nine DM patients, comprising 58 classic DM and 21 clinically amyopathic DM (CADM) patients, were enrolled. Serum Abs were screened by immunoprecipitation assays, and an immunosorbent assay (ELISA) was used for MDA5. The relationships of clinical characteristics and mortality with each Ab were investigated.

Results. Anti-MDA5 Ab was detected in 17 patients. Anti-clinically amyopathic DM 140 kDa polypeptide Abs (anti-CADM-140 Abs) were found in 16 of the 17 anti-MDA5 Ab^+ patients. Skin ulcers, palmar papules, CADM, RPILD and mediastinal emphysema were widely distributed in anti-MDA5 Ab^+ patients. Mortality at 6 months as well as 5 years was also significantly higher in anti-MDA5 Ab^+ patients than in anti-MDA5 Ab^- patients. In a multivariable Cox regression analysis, mortality was independently associated with anti-MDA5 Ab (relative hazard 6.33; 95% CI 1.43, 28.0). All of the deaths in anti-MDA5 Ab^+ patients were attributed to respiratory failure of RPILD; however, RPILD did not worsen in any of the anti-MDA5 Ab^+ patients who survived the first 6 months.

Conclusion. The presence of anti-MDA5 Ab identifies the characteristic skin, musculoskeletal, pulmonary and prognostic features in patients with DM. In addition, anti-MDA5 Ab seems to predict a group of patients with CADM-complicated fatal RPILD.

Key words: anti-MDA5 Ab, CADM, RPILD.

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Introduction

A number of autoantibodies can be detected in the sera of patients with DM, some of which are specific to DM and are known as myositis-specific autoantibodies (MSAs). Moreover, these autoantibodies are closely associated with clinical manifestations of DM, such as symptoms, complications, reactivity to therapy and prognosis [1].

In recent years, the autoantibodies found in patients with inflammatory myopathies have been mainly classified into several types by immunoprecipitation assays: anti-aminoacyl-tRNA synthetase antibodies (anti-ARS Abs), Abs to the signal recognition particle (anti-SRP Abs), anti-Mi2 Abs, PM/Scl-100 Abs and PM/Scl-75 polypeptides Abs (anti-PM-Scl Abs), anti-clinically amyopathic DM 140 kDa polypeptide Abs (anti-CADM-140 Abs), anti-155/140 kDa polypeptide Abs (anti-p155/140 Abs) and autoantibodies to a 142 kDa protein (anti-MJ Abs). These autoantibodies are strongly associated with the clinical presentation [2–6]. In this regard, we have reported a high frequency of rapidly progressive interstitial lung disease (RPILD) and clinically amyopathic DM (CADM) associated with anti-CADM-140 Abs [7, 8]. Recently RNA helicase encoded by melanoma differentiation-associated gene 5 (MDA5) was identified as a major autoantigen in patients with CADM, which is targeted by anti-CADM-140 Abs [9, 10].

Gono *et al.* [11] have also recently reported that anti-MDA5 Ab predicts a fatal outcome in patients with DM combined with RPILD; however, the long-term prognosis and other clinical characteristics of anti-MDA5 Ab$^+$ DM patients remain to be elucidated. In the present study we have tried to investigate the clinical value of anti-MDA5 Ab for DM patients in a single cohort.

Patients, materials and methods

Patients

Sera samples were obtained from 79 patients with DM who were undergoing medical treatment at the Graduate School of Biomedical Sciences, Nagasaki University, from September 1999 to August 2010, and were stored at −20°C until use. Most of the sera samples were obtained at the first visit so the interval from initiation of therapy was minimal. We collected the data from all of the DM patients examined in our department. Twenty-one patients did not fulfill Bohan and Peter's criteria [12, 13] but fulfilled Sontheimer's criteria (CADM) [14, 15] because of the absence of clinical skeletal muscle symptoms and the presence of persistent clinical DM skin features. Clinical manifestations, laboratory data, radiographic data and the presence of internal malignancies were extracted from medical records and verified by T.K., N.I. and K.F. The patients were diagnosed with ILD according to the results of chest X-ray and high-resolution chest CT, reported by Japanese board-certified radiologists. All of the subjects underwent routine examination of internal malignancies and chest radiography. A subset of patients with RPILD was defined as those presenting with progressive dyspnoea and progressive hypoxaemia, and a worsening of interstitial change on chest radiography within 1 month from the onset of respiratory symptoms, as described previously [2]. A signed consent form to participate in the study, which was approved by the Institutional Review Board of Nagasaki University, was obtained from each patient.

Immunoprecipitation and ELISA

MSAs, including anti-CADM-140 Abs, anti-ARS Abs and anti-155/140 Abs, were detected by immunoprecipitation assays using extracts of leukaemia cell line K562, as described previously [3]. Interpretation of the results of immunoprecipitation was undertaken without knowledge of patients' clinical status. An ELISA system using recombinant MDA5 as an antigen source was performed as described previously [10]. All samples were examined in duplicate, and the Ab units were calculated from the optical density at 450 nm, using a standard curve obtained from serial concentrations of a serum sample containing a high titre of anti-CADM-140 Abs. The cut-off level was set at 8.0 U, based on 10 S.D. above the mean value obtained from 32 healthy control sera. Interpretation of the results of ELISA was undertaken without knowledge of the clinical status of the patients and the results of immunoprecipitation assays.

Statistical analysis

Fisher's exact probability test and the Mann–Whitney U-test were used to compare the differences. We also examined the cumulative survival rates from the first visit to the hospital with DM-related symptoms up to 5 years by the multivariate Cox proportional hazard model adjusted for patient age at symptoms onset, gender, with or without CSs and with or without immunosuppresants. A $P < 0.05$ was considered significant.

Results

Clinical characteristics of anti-MDA5 Ab$^+$ patients

Table 1 summarizes the 17 DM patients with anti-MDA5 Ab and the 62 DM patients without anti-MDA5 Ab. There were 21 patients with CADM in the present study and we have found that anti-MDA5 Ab is detected in 14 of 21 patients. In this group, 11 of 14 (79%) patients had complicated RPILD and 7 (50%) patients died. Our present data confirm the recent publications regarding the characteristics of anti-MDA5 Ab$^+$ patients, including the CADM, RPILD, low CK, high ferritin and high mortality found in these patients [11]. Since anti-MDA5 Ab is mostly attributed to anti-CADM-140 Abs, a high prevalence of palmar papules and mediastinal emphysema, which has been reported as typical of anti-CADM-140 Abs$^+$ DM patients by our group [7], was also preferentially found in anti-MDA5 Ab$^+$ patients. The present finding that skin ulcers are highly prevalent in anti-MDA5 Ab$^+$ patients is new, however. Muscle biopsy or lung biopsy was not performed. Skin biopsies were taken from eight patients positive for anti-MDA5 Abs, and six patients were