implications for our understanding of the antiphospholipid syndrome. *Blood* 2010;**116**:1336–43.
51 Ioannou Y, Zhang JY, Passam FH, Rahgozar S, Qi JC, Giannakopoulos B *et al.* Naturally occurring free thiols within beta 2-glycoprotein I in vivo: nitrosylation, redox modification by endothelial cells, and regulation of oxidative stress-induced cell injury. *Blood* 2010;**116**:1961–70.
52 Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, Bauer JW *et al.* 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* 2006;**38**:550–5.
53 Abelson AK, Delgado-Vega AM, Kozyrev SV, Sanchez E, Velazquez-Cruz R, Eriksson N *et al.* STAT4 associates with systemic lupus erythematosus through two independent effects that correlate with gene expression and act additively with IRF5 to increase risk. *Ann Rheum Dis* 2009;**68**:1746–53.
54 Horita T, Atsumi T, Yoshida N, Nakagawa H, Kataoka H, Yasuda S *et al.* STAT4 single nucleotide polymorphism, rs7574865 G/T, as a risk for antiphospholipid syndrome. *Ann Rheum Dis* 2009;**68**:1366–7.
55 Ruiz-Irastorza G, Crowther M, Branch W, Khamashta MA. Antiphospholipid syndrome. *Lancet* 2010;**376**:1498–509.
56 Peaceman AM, Rehnberg KA. The immunoglobulin G fraction from plasma containing antiphospholipid antibodies causes increased placental thromboxane production. *Am J Obstet Gynecol* 1992;**167**:1543–7.
57 Rand JH, Wu XX, Guller S, Gil J, Guha A, Scher J *et al.* Reduction of annexin-V (placental anticoagulant protein-I) on placental villi of women with antiphospholipid antibodies and recurrent spontaneous abortion. *Am J Obstet Gynecol* 1994;**171**:1566–72.
58 Meroni PL, di Simone N, Testoni C, D'Asta M, Acaia B, Caruso A. Antiphospholipid antibodies as cause of pregnancy loss. *Lupus* 2004;**13**:649–52.
59 Holers VM, Girardi G, Mo L, Guthridge JM, Molina H, Pierangeli SS *et al.* Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. *J Exp Med* 2002;**195**:211–20.
60 Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D *et al.* Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest* 2003;**112**:1644–54.
61 Berman J, Girardi G, Salmon JE. TNF-alpha is a critical effector and a target for therapy in antiphospholipid antibody-induced pregnancy loss. *J Immunol* 2005;**174**:485–90.
62 Girardi G, Yarilin D, Thurman JM, Holers VM, Salmon JE. Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. *J Exp Med* 2006;**203**:2165–75.
63 Redecha P, Franzke CW, Ruf W, Mackman N, Girardi G. Neutrophil activation by the tissue factor/Factor VIIa/PAR2 axis mediates fetal death in a mouse model of antiphospholipid syndrome. *J Clin Invest* 2008;**118**:3453–61.
64 Seshan SV, Franzke CW, Redecha P, Monestier M, Mackman N, Girardi G. Role of tissue factor in a mouse model of thrombotic microangiopathy induced by antiphospholipid antibodies. *Blood* 2009;**114**:1675–83.
65 Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. *Nat Med* 2004;**10**:1222–6.
66 Shamonki JM, Salmon JE, Hyjek E, Baergen RN. Excessive complement activation is associated with placental injury in patients with antiphospholipid antibodies. *Am J Obstet Gynecol* 2007;**196**:167e1–5.
67 Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L *et al.* Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. *PLoS Med* 2011;**8**:e1001013.
68 Francis J, Rai R, Sebire NJ, El-Gaddai S, Fernandes MS, Jindai P *et al.* Impaired expression of endometrial differentiation markers and complement regulatory proteins in patients with recurrent pregnancy loss associated with antiphospholipid syndrome. *Mol Hum Reprod* 2006;**12**:435–42.
69 Girardi G. Role of tissue factor in the maternal immunological attack of the embryo in the antiphospholipid syndrome. *Clin Rev Allergy Immunol* 2010;**39**:160–5.
70 Pierangeli SS, Girardi G, Vega-Ostertag M, Liu X, Espinola RG, Salmon J. Requirement of activation of complement C3 and C5 for antiphospholipid antibody-mediated thrombophilia. *Arthritis Rheum* 2005;**52**:2120–4.
71 Romay-Penabad Z, Liu XX, Montiel-Manzano G, Papalardo De Martinez E, Pierangeli SS. C5a receptor-deficient mice are protected from thrombophilia and endothelial cell activation induced by some antiphospholipid antibodies. *Ann N Y Acad Sci* 2007;**1108**:554–66.
72 Oku K, Atsumi T, Bohgaki M, Amengual O, Kataoka H, Horita T *et al.* Complement activation in patients with primary antiphospholipid syndrome. *Ann Rheum Dis* 2009;**68**:1030–5.
73 Goldfarb RD, Parrillo JE. Complement. *Crit Care Med* 2005;**33**:S482–4.
74 Niculescu F, Niculescu T, Rus H. C5b-9 terminal complement complex assembly on apoptotic cells in human arterial wall with atherosclerosis. *Exp Mol Pathol* 2004;**76**:17–23.
75 Arumugam TV, Shiels IA, Woodruff TM, Granger DN, Taylor SM. The role of the complement system in ischemia-reperfusion injury. *Shock* 2004;**21**:401–9.
76 Marceau F, Hugli TE. Effect of C3a and C5a anaphylatoxins on guinea-pig isolated blood vessels. *J Pharmacol Exp Ther* 1984;**230**:749–54.
77 Speidl WS, Exner M, Amighi J, Kastl SP, Zorn G, Maurer G *et al.* Complement component C5a predicts future cardiovascular events in patients with advanced atherosclerosis. *Eur Heart J* 2005;**26**:2294–9.
78 Ghebrehiwet B, Silverberg M, Kaplan AP. Activation of the classical pathway of complement by Hageman factor fragment. *J Exp Med* 1981;**153**:665–76.
79 Huber-Lang M, Sarma JV, Zetpune FS, Rittirsch D, Neff TA, McGuire SR *et al.* Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med* 2006;**12**:682–7.
80 Ikeda K, Nagasawa K, Horiuchi T, Tsuru T, Nishizaka H, Niho Y. C5a induces tissue factor activity on endothelial cells. *Thromb Haemost* 1997;**77**:394–8.
81 Wiedmer T, Esmon CT, Sims PJ. Complement proteins C5b-9 stimulate procoagulant activity through platelet prothrombinase. *Blood* 1986;**68**:875–80.

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

Shigeru Iwata, MD, PhD,[a] Kunihiro Yamaoka, MD, PhD,[a] Hiroaki Niiro, MD, PhD,[b] Kazuhisa Nakano, MD, PhD,[a] Sheau-Pey Wang, MS,[a] Koichi Akashi, MD, PhD,[b] and Yoshiya Tanaka, MD, PhD[a] *Kitakyushu and Fukuoka, Japan*

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),

From [a]the First Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu, and [b]the Department of Medicine and Biosystemic Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka.
Supported in part by a Research Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare of Japan; the Ministry of Education, Culture, Sports, Science and Technology of Japan; and the University of Occupational and Environmental Health, Japan.
Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.
Received for publication September 25, 2011; revised February 3, 2012; accepted for publication March 7, 2012.
Available online April 25, 2012.
Corresponding author: Yoshiya Tanaka, MD, PhD, First Department of Internal Medicine, School of Medicine, University of Occupational & Environmental Health, Japan, 1-1 Iseigaoka, Yahata-nishi, Kitakyushu 807-8555, Japan. E-mail: tanaka@med.uoeh-u.ac.jp.
0091-6749/$36.00

doi:10.1016/j.jaci.2012.03.014

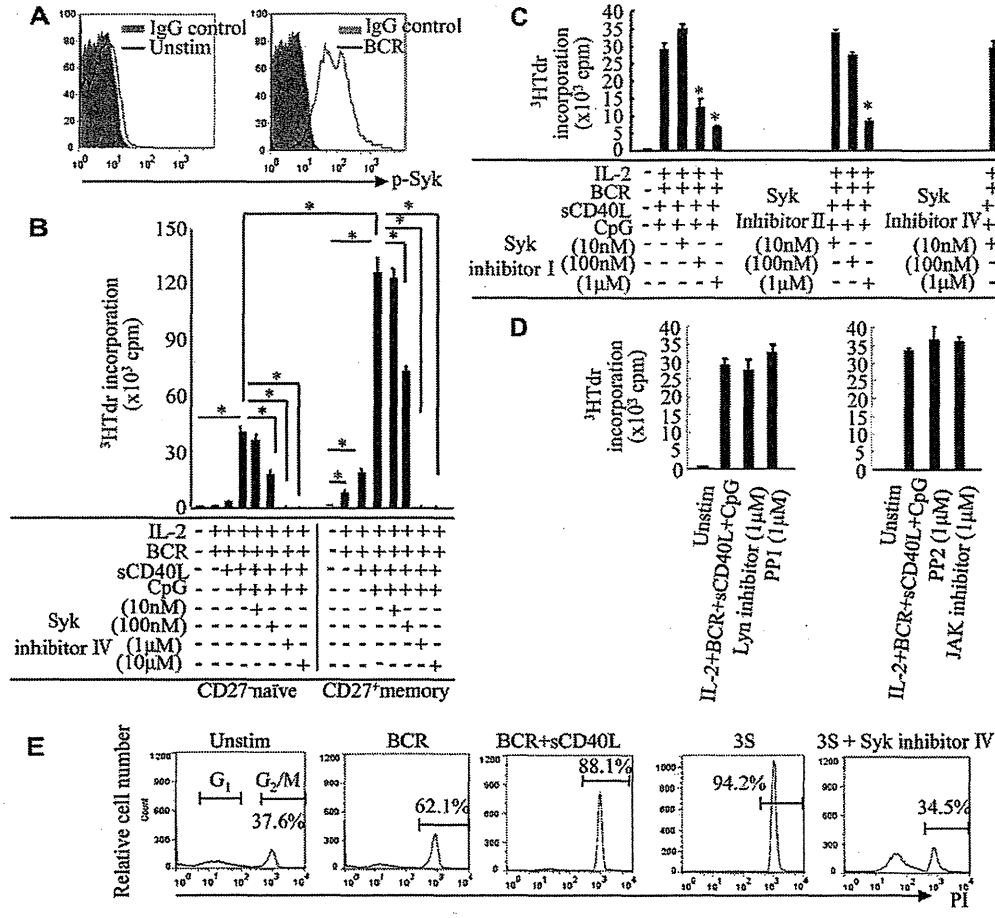

FIG 1. Syk regulates proliferation and cell-cycle progression in B-cell subsets on BCR, CD40, and TLR9 stimulation. **A**, BCR-induced phosphorylation of Syk (15 minutes). **B-D**, Tritiated thymidine *(³HTdr)* incorporation of human B cells was measured during the last 18 hours of the 72-hour culture. 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

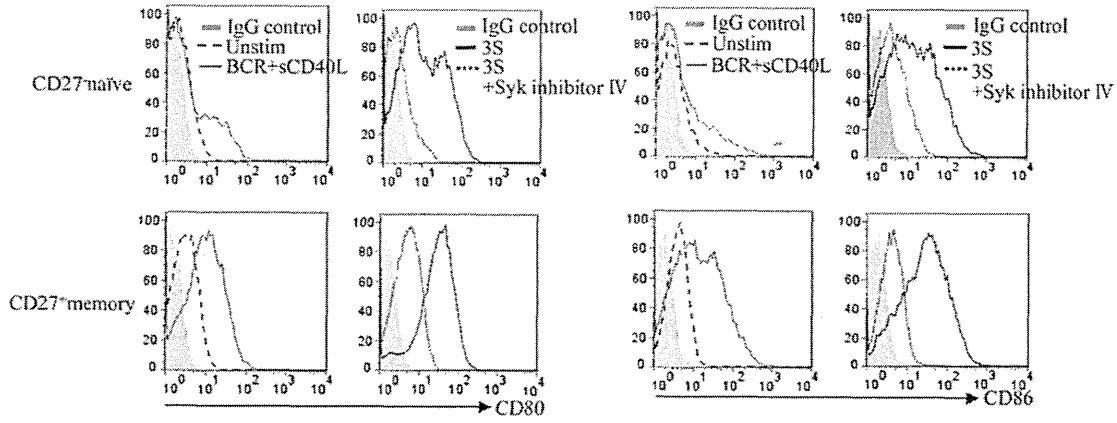

FIG 2. Syk regulates expression of CD80 and CD86 in B-cell subsets on stimulation. *Overlay histograms* depict relative fluorescence intensities of human naive and memory B cells cultured for 72 hours. *Unstim*, Before stimulation; *3S*, BCR, CD40, and TLR9 stimulation. Results are representative of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

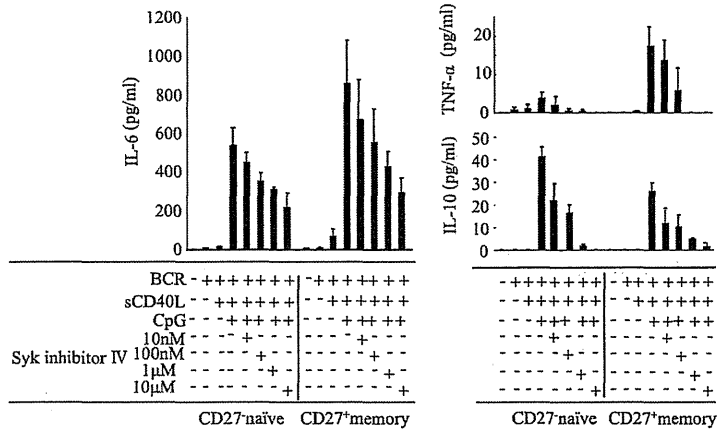

FIG 3. Syk regulates cytokine production in B-cell subsets on stimulation. Human peripheral blood naive and memory B cells were cultured for 72 hours, and supernatants were harvested and assayed by using the cytometric bead array for IL-6, TNF-α, and IL-10 content. Data are shown as means ± SDs and are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

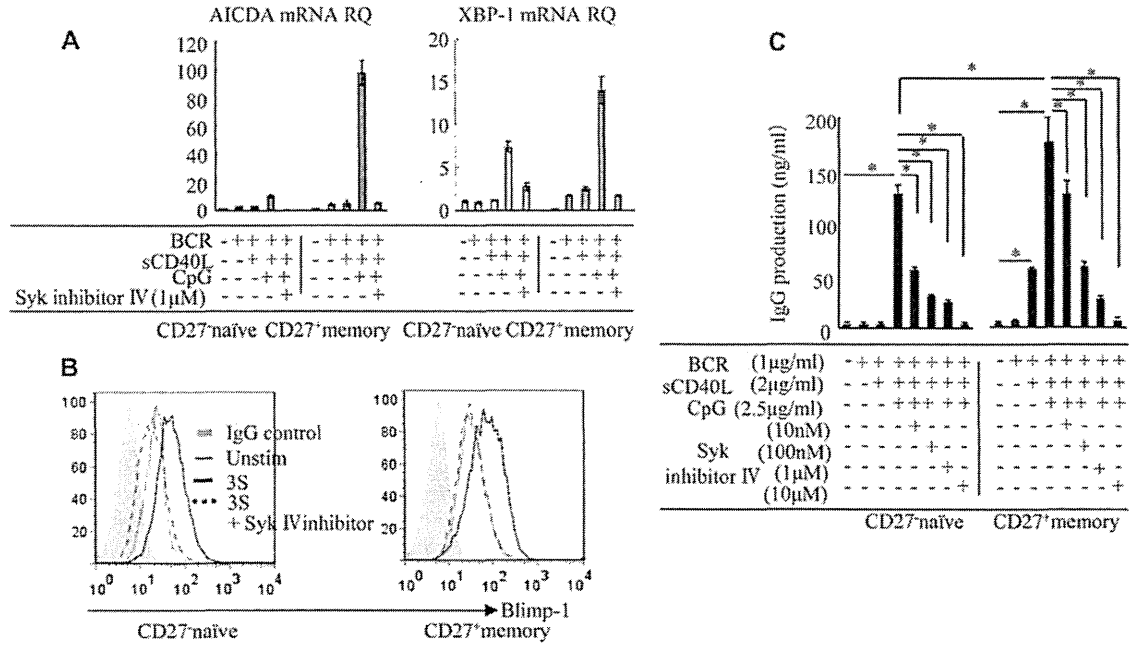

FIG 4. Syk regulates B-cell differentiation on BCR, CD40, and TLR9 stimulation. Naive and memory B cells were cultured for 48 hours (*AICDA* and *XBP-1* mRNA and Blimp-1) or for 5 days (IgG production). **A,** The level of *AICDA* and *XBP-1* mRNA was measured by using real-time PCR. *RQ,* Relative quantity. **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

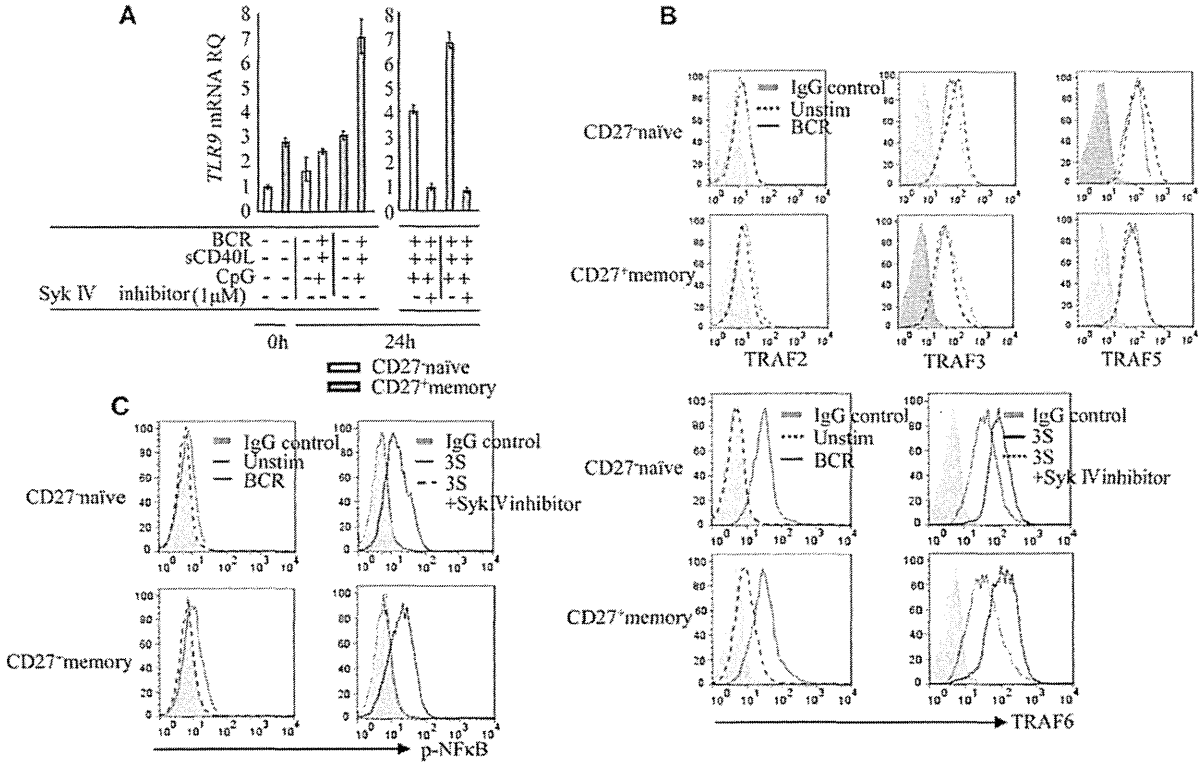

FIG 5. TLR9 and TRAF6 are key Syk-regulated molecules in B-cell subsets on stimulation. **A**, *TLR9* mRNA was quantified by using real-time PCR (TaqMan PCR kit) 24 hours later. *RQ*, Relative quantity; *sCD40L*, soluble CD40 ligand. **B** and **C**, TRAF2, TRAF3, TRAF5, and TRAF6 levels (48 hours later) 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.

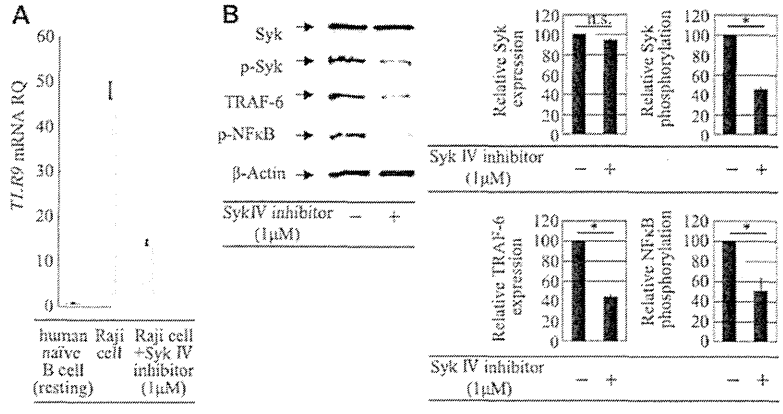

FIG 6. Syk inhibitor exerts marked inhibitory action, even at an activated state of B cells. Raji cells were cultured with RPMI containing 2% FCS for 48 hours. **A**, *TLR9* mRNA was quantified by means of real-time PCR. *RQ*, Relative quantity. **B**, Expression of Syk, phospho-Syk (Y348), TRAF6, and 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.

We thank Ms T. Adachi, Ms N. Sakaguchi, and Ms K. Noda for their excellent technical assistance.

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.

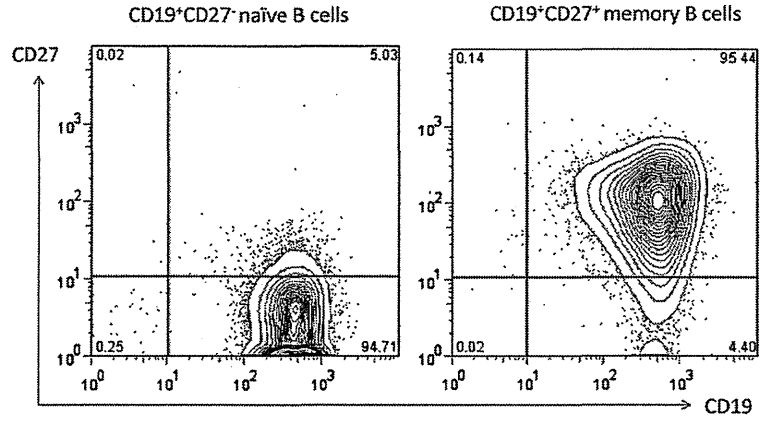

FIG E1. Phenotypic analysis of B-cell subsets in human peripheral blood. B cells were obtained by means of negative selection from PBMCs. $CD27^+$ memory B cells were then isolated by using positive selection from B cells with CD27 microbeads. The negative fraction of this isolation was assigned to $CD27^-$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

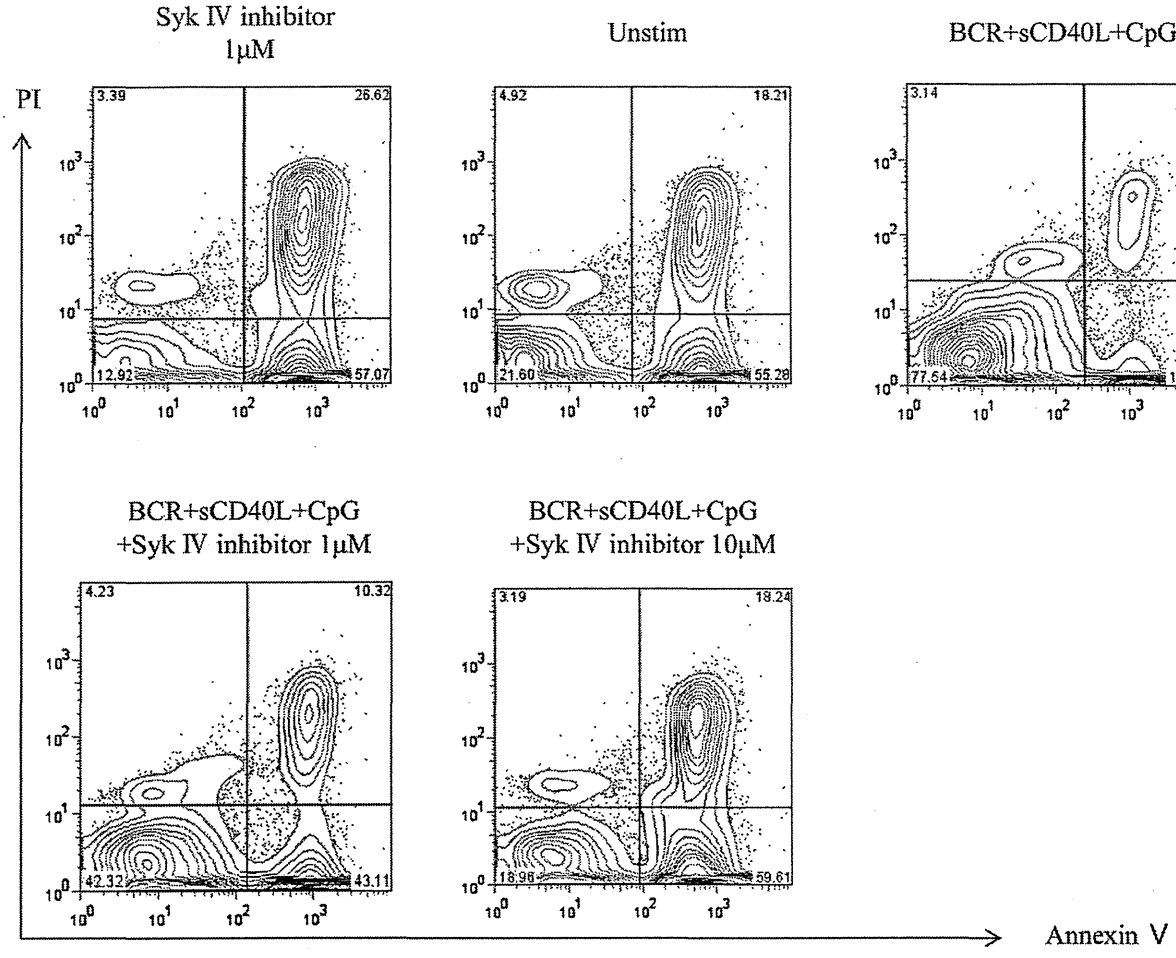

FIG E2. Syk provides survival signals for B cells after stimulation through all 3 receptors. B cells (2×10^5 per well) were cultured in triplicate in 96-well plates with anti-Igλ and anti-Igk antibodies (1 μg/mL), soluble CD40 ligand (*sCD40L*; 2 μg/mL), and CpG-ODN 2006 (2.5 μg/mL) with or without Syk 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).

B cell or T cell-dominant recurrence after rituximab therapy in patients with SLE

Systemic lupus erythematosus (SLE) is an autoimmune disease induced by autoreactive T cell activation and B cell autoantibody overproduction. The efficacy of rituximab in refractory SLE has been documented, although some patients show partial response only.[1–9] We report here two patients with SLE who showed T cell-dominant flare-up and two others who showed B cell-dependent flare-up, after long-term remission induced by rituximab administered at 375 mg/m^2 twice/week.

Rituximab rapidly depleted peripheral naive and memory B cells in patients with SLE. Patients with prolonged remission had persistent depletion of memory B cells for >2 years, whereas recovery of naive B cells occurred within 3–9 months. The expression levels of CD80 on B cells diminished rapidly and remained downregulated. Furthermore, CD69 and ICOS (inducible T-cell co-stimulator) expression levels on CD4+ T cells also decreased and remained at low levels.[10]

B cell-dominant recurrence occurred in two patients, who were concurrently positive for anti-ds-DNA antibodies and extractable nuclear antigen, with lupus nephritis (class II) before treatment (patients 2 and 3, table 1). Unlike patients with prolonged remission,[10] our patients had markedly high CD19+IgD memory B cells and overexpression of CD80 on CD19+ cells just before recurrence, with positive conversion of serum anti-ds-DNA antibodies and increased proteinuria (figure 1A and changes in Systemic Lupus Erythematosus Disease Activity Index and *British Isles Lupus Assessment Group Activity Index*, table 1). In contrast, no phenotypic changes were observed in T cells. Accordingly, the patients were re-treated with rituximab, which reduced CD19+IgD- B cells and anti-ds-DNA antibody to undetectable levels, and successfully controlled disease activity.

In contrast, a T cell-dominant recurrence was noted in two patients with negative anti-ds-DNA antibodies and extractable nuclear antigen. Patient 1 presented with fever, polyarthritis, lymphadenopathy and acute confusional state. Patient 4 had autoimmune haemolytic anaemia before treatment (table 1). In both patients, the signs and symptoms noted at initial presentation became evident again at recurrence after prolonged remission. Different from the previous two patients, there was neither an increase in memory B cells nor change in CD80 expression on B cells. Unlike patients with long-term remission [10], marked increases were noted in the number of CD4+CD45RO+ memory T cells and the expression levels of ICOS and CD69 on CD4+ T cells (figure 1B). Treatment commenced with intravenous cyclophosphamide pulse therapy and tacrolimus, which resulted in improvement of disease activity and peripheral CD4+ T cell abnormalities.

In conclusion, we experienced two patients with B cell-dominant recurrence and two patients with T cell-dominant one. SLE is known as a highly heterogeneous disease. Based on these results, rituximab-based B cell depletion therapy might expose the hidden B cell- or T cell-dependency during the SLE disease process. The phenotypic changes suggest that B cell- or T cell memory might be re-driven at recurrence after long-term remission. Thus, the phenotypic differences between B and

Table 1 Characteristics of patients with systemic lupus erythematosus who showed a flare-up after remission by RTX

Patient	Age/ sex	Disease duration (months)	Treatment prior to RTX	Initial major organ involvement	Anti-dsDNA antibody (IU/ml)	ENA	C3/ C4/ CH50	ANAs	Latency to relapse (years)	SLEDAI (day 0→6 months→ at relapse)	BILAG (day 0→6 months→ at relapse)	Phenotype of lymphocytes at flare
1	29/F	6	CS, IVCY	Lymphadenopathy CNS	5.4 (13.0)		114/ 18/31	320	2	9→0→17	12→0→21	Persistently low memory B cells, high number of memory T cells and expression levels of ICOS
2	32/F	108	CS, IVCY, CsA AZA, PE	Nephritis (IV)	52.3 (18.9)	Ro, Sm, RNP	90/ 15/39	640	3	16→0→20	17→0→9	High number of memory B cells and expression level of CD80. Persistently low expression levels of CD69 and ICOS
3	16/F	30	CS, IVCY MZ, CsA	Nephritis (IV)	610.7 (159.6)	- Ro -	54/8/ 25	320	1.5	13→0→10	23→0→13	High number of memory B cells and expression levels of CD80. Persistently low expression levels of CD69 and ICOS
4	19/F	74	CS, IVCY, PE AZA, TAC	AIHA	7.9 (5.0)		54/ <5/9	80	1	4→0→2	4→0→13	No recovery of B cells, high number of memory T cells and expression levels of CD69

AIHA, autoimmune haemolytic anaemia; ANAs, antinuclear antibodies; AZA, azathioprine; BILAG, British Isles Lupus Assessment Group Activity Index; CNS, central nervous system; CsA, cyclosporin; CS, prednisolone (or equivalent); ENA, extractable nuclear antigen; ICOS, inducible T-cell co-stimulator; IV, intravenous; IVCY, intravenous cyclophosphamide pulse therapy; MZ, mizoribine; PE, plasma exchange; RTX, rituximab; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; TAC, tacrolimus.

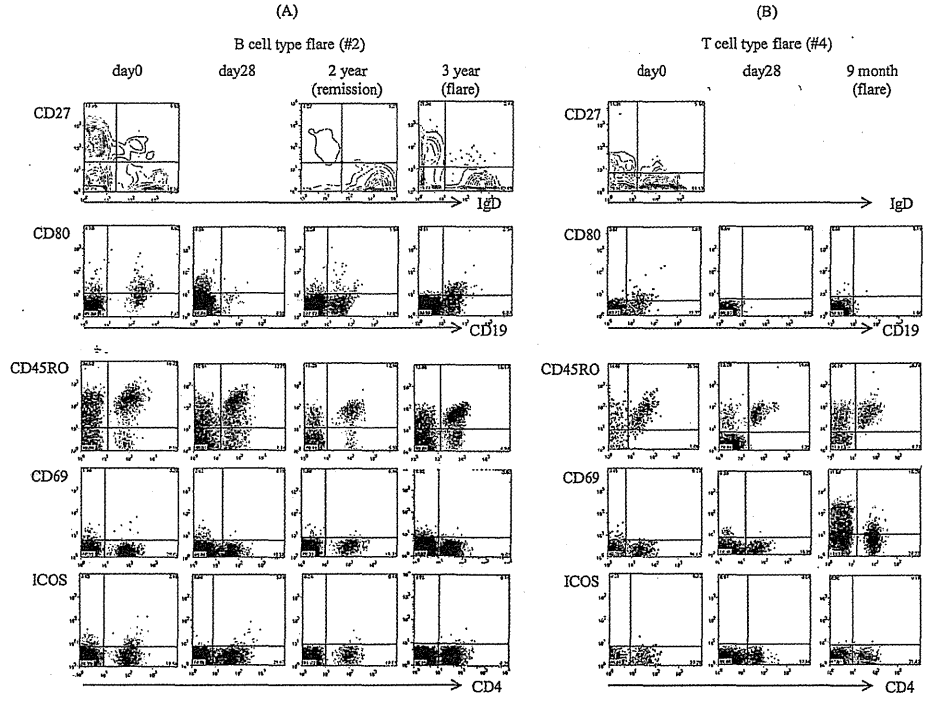

Figure 1 (A) B and T cell surface antigen expression levels before and 28 days after treatment, and at the time relapse of systemic lupus erythematosus, 3 years after treatment, in patient 2 with B cell-dominant relapse. (1) Changes in CD19+ cell subsets (abscissa: IgD, ordinate: CD27), in the numbers of naive B cells ($IgD^{+}CD27^{-}$), memory B cells ($IgD^{-}CD27^{+}$ class switched memory B cells and $IgD^{-}CD27^{-}$ double negative memory B cells) and plasma cells ($IgD^{-}CD27^{high}$). (2) Changes in the expression level of CD80 (costimulatory molecule expressed on $CD19^{+}$ cells). (3) Changes in $CD4^{+}$ cell subsets (*abscissa*: CD4; *ordinate*: CD45RO; changes in numbers of naive T cells and memory T cells). (4) Changes in the expression levels of CD69 and ICOS, which are costimulatory molecules expressed on CD4+ cells. (B) B and T cell surface antigen expression levels before and 28 days after rituximab treatment, and at the time of relapse, 9 months after treatment, in patient 4 who showed T cell-dominant relapse.

T cells after rituximab therapy could partly explain the heterogeneity of SLE. The results also indicate that differential targeting therapies should be considered according to such heterogeneity. However, further analysis of a large sample of patients is needed.

Shigeru Iwata, Kazuyoshi Saito, Mikiko Tokunaga, Yoshiya Tanaka

The First Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan

Correspondence to Yoshiya Tanaka, The First Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu 807-8555, Japan; tanaka@med.uoeh-u.ac.jp

Acknowledgements The authors thank T Adachi, N Sakaguchi and K Noda for the excellent technical assistance. This work was supported in part by a Research Grant-In-Aid for Scientific Research from the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the University of Occupational and Environmental Health, Japan.

Competing interests YT has received consulting fees, speaking fees and/or honoraria from Mitsubishi-Tanabe Pharma, Chugai Pharma, Eisai Pharma, Pfizer, Abbott Immunology Pharma, Daiichi-Sankyo, Janssen Pharma, Astra-Zeneca, Takeda Industrial Pharma, Astellas Pharma, Asahi-kasei Pharma and GlaxoSmithKline and has received research grant support from Mitsubishi-Tanabe Pharma, Bristol-Myers Squibb, Takeda Industrial Pharma, MSD, Astellas Pharma, Eisai Pharma, Chugai Pharma, Pfizer and Daiichi-Sankyo. The other authors declare no conflict of interest.

Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

Accepted 9 April 2011

Published Online First 14 May 2012

Ann Rheum Dis 2012;**71**:1749–1750.
doi:10.1136/annrheumdis-2012-201317

REFERENCES

1. **Looney RJ,** Anolik JH, Campbell D, *et al*. B cell depletion as a novel treatment for systemic lupus erythematosus: a phase I/II dose-escalation trial of rituximab. *Arthritis Rheum* 2004;**50**:2580–9.
2. **Leandro MJ,** Edwards JC, Cambridge G, *et al*. An open study of B lymphocyte depletion in systemic lupus erythematosus. *Arthritis Rheum* 2002;**46**:2673–7.
3. **Rastetter W,** Molina A, White CA. Rituximab: expanding role in therapy for lymphomas and autoimmune diseases. *Annu Rev Med* 2004;**55**:477–503.
4. **Anolik J,** Sanz I, Looney RJ. B cell depletion therapy in systemic lupus erythematosus. *Curr Rheumatol Rep* 2003;**5**:350–6.
5. **Tanaka Y,** Yamamoto K, Takeuchi T, *et al*. A multicenter phase I/II trial of rituximab for refractory systemic lupus erythematosus. *Mod Rheumatol* 2007;**17**:191–7.
6. **Tokunaga M,** Fujii K, Saito K, *et al*. Down-regulation of CD40 and CD80 on B cells in patients with life-threatening systemic lupus erythematosus after successful treatment with rituximab. *Rheumatology (Oxford)* 2005;**44**:176–82.
7. **Tokunaga M,** Saito K, Kawabata D, *et al*. Efficacy of rituximab (anti-CD20) for refractory systemic lupus erythematosus involving the central nervous system. *Ann Rheum Dis* 2007;**66**:470–5.
8. **Lu TY,** Ng KP, Cambridge G, *et al*. A retrospective seven-year analysis of the use of B cell depletion therapy in systemic lupus erythematosus at University College London Hospital: the first fifty patients. *Arthritis Rheum* 2009;**61**:482–7.
9. **Anolik JH,** Barnard J, Owen T, *et al*. Delayed memory B cell recovery in peripheral blood and lymphoid tissue in systemic lupus erythematosus after B cell depletion therapy. *Arthritis Rheum* 2007;**56**:3044–56.
10. **Iwata S,** Saito K, Tokunaga M, *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.

Mod Rheumatol
DOI 10.1007/s10165-012-0733-7

ORIGINAL ARTICLE

Age at onset and gender distribution of systemic lupus erythematosus, polymyositis/dermatomyositis, and systemic sclerosis in Japan

Akiko Ohta · Masaki Nagai · Motoko Nishina · Hiroyuki Tomimitsu · Hitoshi Kohsaka

Received: 17 May 2012 / Accepted: 27 July 2012

Abstract
Objectives The aim of this study was to describe age, gender distribution, and age at onset of systemic lupus erythematosus (SLE), polymyositis/dermatomyositis (PM/DM), and systemic sclerosis (SSc) in Japan.
Methods We used epidemiological information on 21,405, 6,327, and 10,058 patients with SLE, PM/DM, and SSc, respectively, in a Japanese nationwide registration database of patients with intractable diseases.
Results All three diseases occur predominantly in women, with the female-to-male ratio being 8.2:1, 2.6:1, and 7.7:1 for SLE, PM/DM, and SSc, respectively. The most susceptible age for SLE is 15–44 and 20–39 years for males and females, respectively. For PM/DM it is 45–64 and 40–64 years and for SSc, 50–69 and 40–59 for men and women, respectively.
Conclusions The basic descriptive epidemiological characteristics of SLE, PM/DM, and SSc in Japan, such as gender distribution, present age, and age at onset, were surveyed nationwide for fiscal 2007. It was found that these characteristics were similar to those in Western populations. Our finding provides new information on the natural history of disease development.

Keywords Age at onset · Epidemiology · Polymyositis/dermatomyositis (PM/DM) · Systemic lupus erythematosus (SLE) · Systemic sclerosis (SSc)

A. Ohta (✉) · M. Nagai · M. Nishina
Department of Public Health, Saitama Medical University Faculty of Medicine, 38 Morohongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan
e-mail: aohta@saitama-med.ac.jp

H. Tomimitsu
Department of Neurology and Neurological Science, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

H. Kohsaka
Department of Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

Introduction

Systemic lupus erythematosus (SLE), polymyositis/dermatomyositis (PM/DM), and systemic sclerosis (SSc) are systemic autoimmune diseases. Their chronic intractable nature has a significant impact on medical care utilization, activity of daily living, and quality of life. As these diseases are relatively rare, their epidemiological characteristics have not yet been described in detail in Japan. In such rare diseases, accumulation of large numbers of patients from the entire country is necessary for informative epidemiological studies.

The National Programme on Rare and Intractable Diseases was launched in Japan in 1972. Since then, the government has promoted research and expanded support for patients with a number of such diseases [1]. This programme established a nationwide registration system for patients with intractable diseases, including SLE, PM/DM, and SSc. Here, we describe age, gender distribution, and age at onset of these diseases using data from the registration system.

Materials and methods

Data sources

The Japanese government has established a nationwide registration system for patients with intractable diseases

Published online: 19 August 2012

under which registered patients are eligible for financial aid from the government for their treatment. Details of the registration system have been described elsewhere [1]. Most patients with SLE, PM/DM, or SSc are expected to be registered as having a designated intractable disease, although not all registered patients' data have been converted to electronic form. The electronic database has been effectively utilizable for epidemiological research since 2003. After obtaining permission from the Ministry of Health, Labour and Welfare (MHLW) of Japan, we used data of fiscal year 2007 consisting of patient sex, age, birth year, and disease-onset year.

Statistical analysis

We calculated the electronic data entry rate as the number of patients whose data was converted into electronic form divided by the total number of patients enrolled in the registration system. The latter information is contained in MHLW's Report on Public Health Administration and Services [2]. We ascertained age at disease onset as onset year minus birth year. Using onset age, we determined the most susceptible age as the minimum range that includes peak onset age and 50 % of onsets. All statistical analyses were performed with SAS version 9.1.3 software (SAS Institute Inc., Cary, NC, USA).

Ethical considerations

All data provided by the MHLW are anonymous, and researchers cannot access personal information about any patient.

Results

Table 1 shows the number of patients with SLE, PM/DM, and SSc whose data was converted into electronic form and the electronic data entry rate in fiscal 2007. We used electronic data for 21,405, 6,327, and 10,058 SLE, PM/DM, and SSc cases, respectively. The numbers of all patients registered with the database for the MHLW's Report on Public Health Administration and Services were 55,021 SLE and 37,975 PM/DM and SSc (PM/DM and SSc were not reported separately) [2]. The proportion of all patients with electronic data entered was therefore 39 % for SLE and 43 % for PM/DM and SSc. Estimating the number of all PM/DM- and SSc-registered patients separately, the number would be 14,714 (6,327/0.43) PM/DM and 23,391 (10,058/0.43) SSc. These can be considered to be the total number of registered patients in the entire Japanese (population 126 million).

Table 1 Number of patients with SLE, PM/DM, and SSc whose data was converted into electronic form, and the electronic data entry rate in fiscal 2007

Diseases	No. of electronic entries[a]	Total No. of patients[b]	Electronic data entry rate (a/b)
SLE	21,405	55,021	0.39
PM/DM	6,327	37,975	0.43
SSc	10,058		

SLE systemic lupus erythematosus, *PM/DM* polymyositis/dermatomyositis, *SSc* systemic sclerosis

[a] The number of patients whose data was converted into electronic form

[b] Total numbers of patients enrolled in the registration system was obtained from the 2007 Report on Public Health Administration and Services [2]

Table 2 Number of male and female patients with SLE, PM/DM, and SSc in fiscal 2007

Diseases	Total	Male	Female	Sex ratio
SLE	21,405	2,336	19,069	8.2
PM/DM	6,327	1,735	4,592	2.6
SSc	10,058	1,157	8,901	7.7

Sex ratio (female/male)

SLE systemic lupus erythematosus, *PM/DM* polymyositis/dermatomyositis, *SSc* systemic sclerosis

Table 2 shows the number of patients with SLE, PM/DM, and SSc stratified by sex. All three diseases predominantly affect women, with a female-to-male ratio of 8.2:1, 2.6:1, and 7.7:1 for SLE, PM/DM, and SSc, respectively.

Figure 1 shows the age distribution of male and female SLE, PM/DM, and SSc patients. The prevalence of SLE in women showed two peaks, at age 35–39 and 55–59 years, with a wide age distribution. The distribution of PM/DM was similar to SSc, with only a small number of patients <50 years, and peak prevalence at 55–59 years for PM/DM and 65–69 years for SSc. SLE distribution in men showed no significant age peak, and PM/DM and SSc were similar to that seen in women.

Figure 2 shows the distribution of age of onset. SLE onset peaked at 25–29 years in women, decreasing thereafter. Onset of both PM/DM and SSc in women also had one peak, but later, at 50–54 years of age, with PM/DM tending to have a younger onset than SSc. Age of SLE onset showed no peak in men, and again, PM/DM and SSc was similar in men and women.

These data on age at onset are summarized in Table 3. For SLE patients, age at the 50th percentile was 35 and

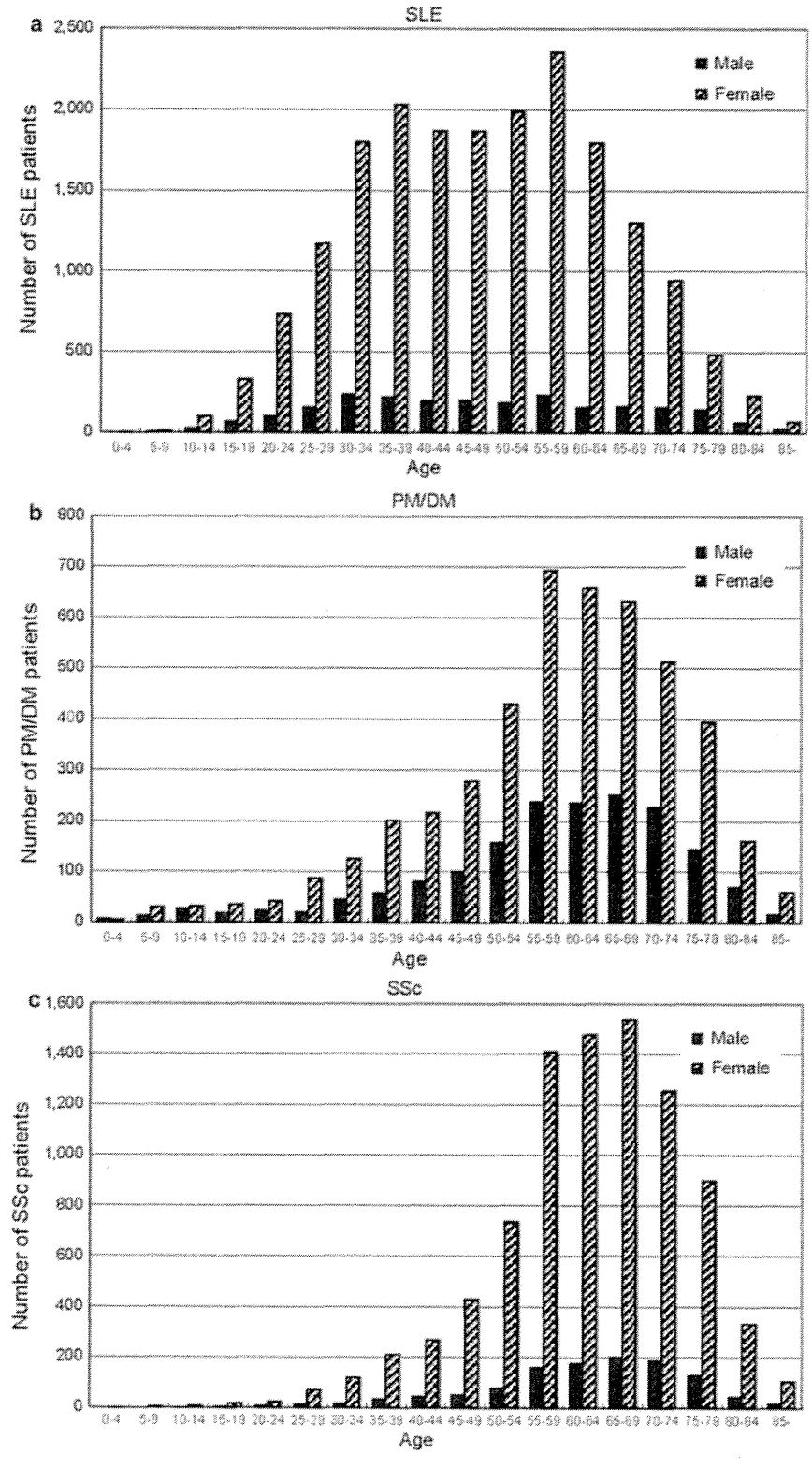

Fig. 1 Age distribution of SLE, PM/DM, and SSc in fiscal 2007. **a** *SLE* systemic lupus erythematosus, **b** *PM/DM* polymyositis/dermatomyositis, **c** *SSc* systemic sclerosis. *Solid bars* show the number of male patients, and *bars* with *right-slanting lines* show the number of female patients, by age

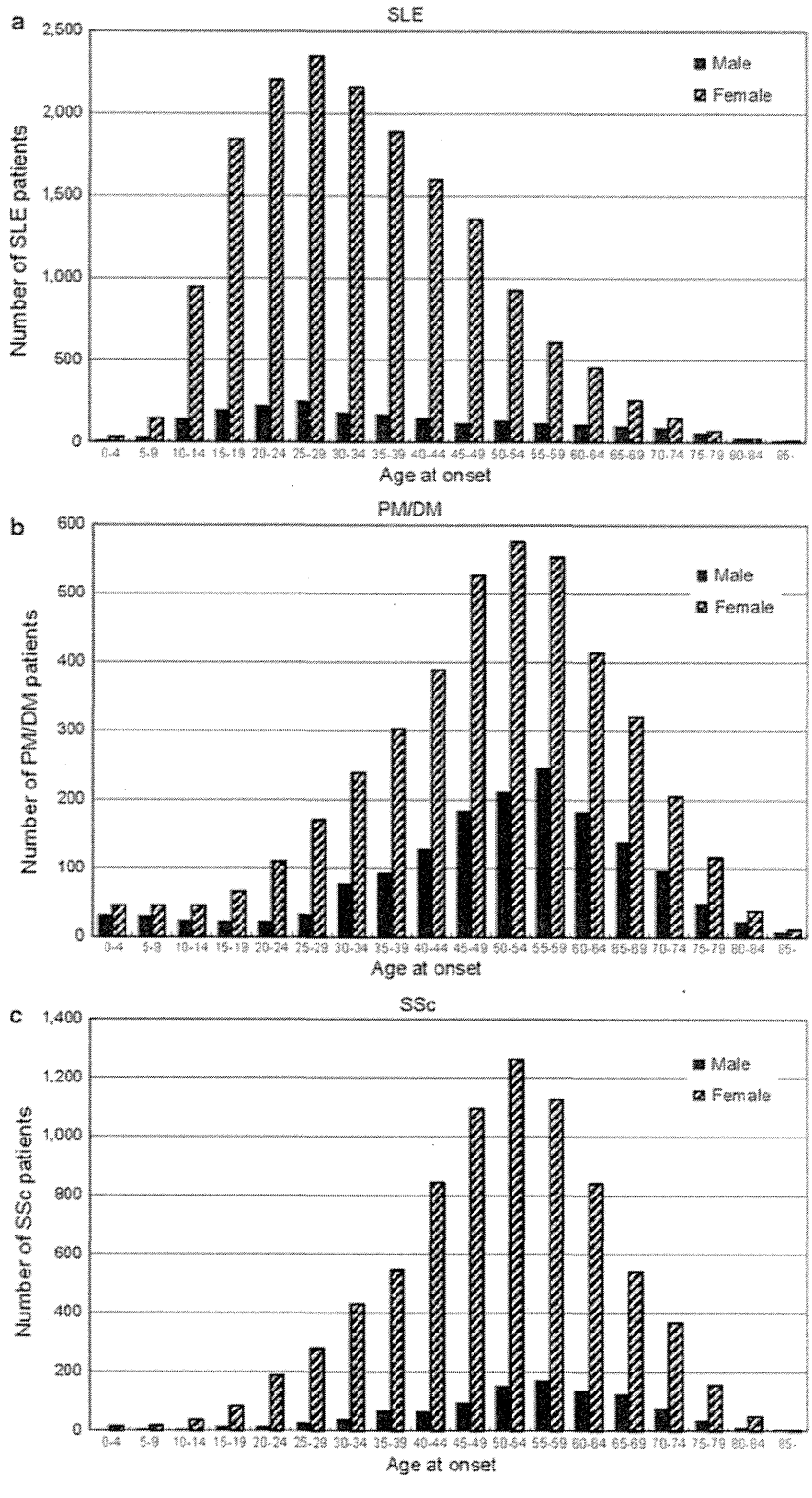

Fig. 2 Distribution of age at onset of SLE, PM/DM, and SSc in fiscal 2007. **a** *SLE* systemic lupus erythematosus, **b** *PM/DM* polymyositis/dermatomyositis, **c** *SSc* systemic sclerosis. *Solid bars* show the number of male patients, and *bars* with *right-slanting lines* show the number of female patients, by age at onset

Table 3 Distribution of age at onset of SLE, PM/DM, and SSc in fiscal 2007: percentile, mean, most susceptible age

Diseases	Sex	Number	Percentile (%)					Mean	SD	Most susceptible age[a] (years)
			10	25	50	75	90			
SLE	Male	2,042	15	23	35	54	68	38.5	19.3	15–44
	Female	16,995	16	22	32	43	54	33.7	14.5	20–39
PM/DM	Male	1,581	30	42	53	62	70	50.8	16.9	45–64
	Female	4,167	28	40	51	60	69	49.2	16.0	40–64
SSc	Male	1,018	35	46	55	64	70	54.0	14.1	50–69
	Female	7,875	32	42	51	59	67	50.2	13.7	40–59

Patients with unknown age at onset were excluded

SLE systemic lupus erythematosus, *PM/DM* polymyositis/dermatomyositis, *SSc* systemic sclerosis, *SD* standard deviation

[a] We determined the most susceptible age as the minimum range that includes the peak onset age and 50 % of onsets

32 years for men and women, respectively. For PM/DM and SSc, this was in the 50s for both sexes. Mean age at onset was similar to these 50th percentiles in all three diseases. We determined the most susceptible age as the minimum range that included peak occurrence and 50 % of onsets. This showed that men and women are most susceptible to SLE over the age range of 15–44 and 20–39 years, respectively. For PM/DM, this was 45–64 and 40–64, and for SSc, 50–69 and 40–59 years, for men and women, respectively.

Discussion

Here we report in detail present age and distribution of age at onset in patients with SLE, PM/DM, and SSc in Japan. Because these diseases are relatively rare, to produce an epidemiologically effective study, it is necessary to accumulate a large number of patients using nationwide surveys. We used data from a large number of such patients in Japan, which were especially informative regarding age of onset distribution. These data represent very important epidemiological information that has not been reported in any previous studies in Japan, with one exception that analyzed older data [3].

There are several reports describing age, occurrence frequency in men and women, and age of onset of SLE [4–6], PM/DM [7, 8], and SSc [9–11] in Western populations. In Caucasians, peak incidence of SLE occurs between ages 15 and 45 years, with a female-to-male ratio of 6–10:1 [4–6]. The pattern of occurrence of idiopathic inflammatory myopathy was bimodal, with a small childhood peak between 10 and 15 years and adult peak between 45 and 60 years [7, 8], with a female-to-male incidence ratio of 2.5:1 [7]. Age at onset of SSc is most commonly in the range of 45–65 years. As with the other two diseases, SSc is also predominant in women, with a female-to-male ratio of 4–6:1 [9–11]. The report presented here shows that the Japanese population seems similar to Western populations in the factors assessed here. However, age at PM/DM onset did not show a childhood peak. The reason for this may be that alternative financial support for medical treatment for children is provided by local government and therefore some of them are not registered in the national database. It means that a childhood peak may exist in Japanese, but our observation on the national database could not detect it.

There are some limitations to this study. One issue is data representativeness. The data we used, however, are derived from less than half of all registered patients, which depended on the electronic data entry rate. As the reason for the low entry rate is that some prefectures did not enter electronic data at a high enough rate due to financial or clerical problems, it is unlikely that the data entry rate differs between sexes and at different ages. In the observation of the characteristics of sex, age, and age of onset, we can expect that data is representative of the entire registered patient population. Other issues would be possible bias due to the use of data from the registration system, such as accuracy of disease diagnosis and coverage of all patients. Accuracy of data contained within this registration system is likely to be good, because specialist committees were organized in each prefectural government to check diagnoses according to standard criteria ordained by the government. Most patients are expected to be diagnosed and registered in this system, but clearly, we cannot be sure of the rate of omissions. Thus, calculations of prevalence rates from these data may be an underestimation. The prevalence of childhood patients may be also underestimated, as stated above. The last issue is the observation of incidence. We can distinguish newly registered cases, but as some of those may be recurrences or reregistration of patients who have moved (because when a patient changes address across prefectures, the registration may be renewed), not all the apparently newly registered cases are new incidence cases. Therefore, we did not show the incidence here.

In conclusion, gender distribution, present age, and age at onset of recent SLE, PM/DM, and SSc patients in Japan was surveyed nationwide for fiscal 2007. Our findings provide new information on the natural history of disease development in Japan, which, despite ethnic and other differences, appears similar to that familiar in Western populations.

Acknowledgments This study was supported by grants from Health and Labour Science Research of the Ministry of Health, Labour and Welfare, Japan: Research Committee on Epidemiology of Intractable Diseases and Research Committee on Autoimmune Diseases.

Conflict of interest None.

References

1. Hayashi S, Umeda T. 35 years of Japanese policy on rare diseases. Lancet. 2008;372:889–90.
2. Statistics and Information Department, Minister's Secretariat, Ministry of Health, Labour and Welfare. Report on Public Health Administration and Services 2007 (in Japanese). Tokyo: Statistics and Information Department, Ministry of Health, Labour and Welfare, Japan. 2009.
3. Ohta A, Nagai M, Nishina M, Shibazaki S, Ishijima H, Izumida M. Age at the onset of intractable disease: based on a clinical database for patients receiving financial aid for treatment (in Japanese). Nihon Koshu Eisei Zasshi. 2007;54:3–14.
4. Simard JF, Costenbader KH. Epidemiology and classification of systemic lupus erythematosus In: Hochberg MC, Silman AJ, Smolen JS, Weinblatt ME, Weisman MH, editors. Rheumatology, 5th ed. Philadelphia: Mosby/Elsevier; 2010. p. 1223–8.
5. Rus V, Maury EE, Hochberg MC. The epidemiology of systemic lupus erythematosus. In: Wallace DJ, Hahn BH, editors. Dubois' lupus erythematosus. 7th ed. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 34–44.
6. Boddaert J, Huong DL, Amoura Z, Wechsler B, Godeau P, Piette JC. Late-onset systemic lupus erythematosus: a personal series of 47 patients and pooled analysis of 714 cases in the literature. Medicine. 2004;83:348–59.
7. Oddis CV, Ascherman DP. Clinical features, classification, and epidemiology of inflammatory muscle disease. In: Hochberg MC, Silman AJ, Smolen JS, Weinblatt ME, Weisman MH, editors. Rheumatology. 5th ed. Philadelphia: Mosby/Elsevier; 2010. p. 1439–55.
8. Oddis CV, Conte CG, Steen VD, Medsger TA Jr. Incidence of polymyositis-dermatomyositis: a 20-year study of hospital diagnosed cases in Allegheny County, PA 1963–1982. J Rheumatol. 1990;17:1329–34.
9. Mayes M, Assassi S. Epidemiology and classification of scleroderma. In: Hochberg MC, Silman AJ, Smolen JS, Weinblatt ME, Weisman MH, editors. Rheumatology. 5th ed. Philadelphia: Mosby/Elsevier; 2010. p. 1361–6.
10. Steen VD, Oddis CV, Conte CG, Janoski J, Casterline GZ, Medsger TA Jr. Incidence of systemic sclerosis in Allegheny County, Pennsylvania. A twenty-year study of hospital-diagnosed cases, 1963–1982. Arthritis Rheum. 1997;40:441–5.
11. Chifflot H, Fautrel B, Sordet C, Chatelus E, Sibilia J. Incidence and prevalence of systemic sclerosis: a systematic literature review. Semin Arthritis Rheum. 2008;37:223–35.

Acta Derm Venereol 2012 Epub ahead of print

INVESTIGATIVE REPORT

Decreased miR-7 Expression in the Skin and Sera of Patients with Dermatomyositis

Yuka OSHIKAWA, Masatoshi JINNIN, Takamitsu MAKINO, Ikko KAJIHARA, Katsunari MAKINO, Noritoshi HONDA, Wakana NAKAYAMA, Kuniko INOUE, Satoshi FUKUSHIMA and Hironobu IHN
Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan

Expression of microRNA (miRNA) in the skin in dermatomyositis has not previously been studied in detail. In this study, we performed miRNA array analysis using miRNAs purified from dermatomyositis-involved skin and normal skin, and found that several miRNAs were up- or down-regulated in dermatomyositis skin. Among them, we focused on miR-7, one of the most down-regulated miRNAs in dermatomyositis skin. Total miRNAs were purified from serum, and hsa-miR-7 levels were measured with quantitative real-time PCR using the specific primer. Serum levels of miR-7 were significantly decreased in patients with dermatomyositis compared with normal subjects or patients with other autoimmune diseases. Thus, serum miR-7 levels might be a possible diagnostic marker for dermatomyositis. Clarifying the up- or down-stream events of down-regulated miR-7 in patients with dermatomyositis may lead to further understanding of the disease and a new therapeutic approach. ***Key words: autoimmune diseases; polymerase chain reaction; polymyositis.***

(Accepted June 19, 2012.)

Acta Derm Venereol 2012; 92: XX–XX.

Masatoshi Jinnin, Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan. E-mail: mjin@kumamoto-u.ac.jp

Polymyositis/dermatomyositis (PM/DM) is an inflammatory disease that affects multiple organs, including the muscles, skin and lungs. Cancer risk is increased in patients with PM/DM. This disease sometimes overlaps with other autoimmune disorders, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, systemic sclerosis (SSc), Sjögren's syndrome, and mixed connective tissue disease, thus autoimmunity may play a major role in the pathogenesis of PM/DM. However, the exact aetiology of PM/DM is unknown.

microRNAs (miRNAs) are a class of small non-coding RNAs, on average 22 nucleotides long, which usually inhibit the translation of target mRNA by binding to 3' UTR of the target (1). In humans, almost 1,700 miRNAs have been identified, and miRNAs have been implicated in various cellular events, such as immune response as well as cell development, cell differentiation, organogenesis, growth control or apoptosis. Accordingly, many publications have suggested the possibility that miRNAs are involved in the pathogenesis of various diseases. However, there are few reports about the relationships between miRNAs and PM/DM. Eisenberg et al. (2) reported that expression of several miRNAs are changed in the muscle tissues of PM/DM compared with those of normal control muscle. However, miRNA expression in the skin of DM patients has not been determined. In this study, we performed miRNA array using miRNAs purified from DM skin, and found that several miRNAs were up- or down-regulated in DM skin. Among them, we focused on miR-7, one of the most down-regulated miRNAs in DM skin. We evaluated the possibility that serum levels of miR-7 may be a useful marker for the diagnosis or evaluation of disease activity in DM patients.

MATERIALS AND METHODS

Patient material

Serum samples were obtained from 20 patients with DM. A summary of clinical/laboratory features of DM patients enrolled in this study are shown in Table I. All the patients fulfilled the criteria of Bohan & Peter (3, 4). Ten patients with clinically and histopathologically typical cutaneous lesions, but without myositis were diagnosed as clinically amyopathic DM (CADM) according to the previous criteria (5). Electromyographic examination and muscle biopsies were performed at the time of diagnosis. Lung involvement was diagnosed based on the findings of chest radiography, computed tomography of the chest, and lung function tests. Patients who had received treatments including steroids, azathioprine, and methotrexate were excluded. Control serum samples were also collected from 17 healthy volunteers, 5 patients with PM, 10 with SLE, and 10 with SSc. All serum samples were stored at –80°C prior to use.

Skin specimens were obtained from Gottron's eruption of 8 DM patients. Six SSc skin was used as disease control. Seven normal skin samples were obtained from routinely discarded limb skin of healthy human subjects undergoing skin grafts. Control and patient skin were collected and fixed in formaldehyde immediately after resections. The study design was approved by the Ethics Review Committee of Kumamoto University (number 177). Written informed consent was obtained before the patients and healthy volunteers were entered into this study according to the Declaration of Helsinki.