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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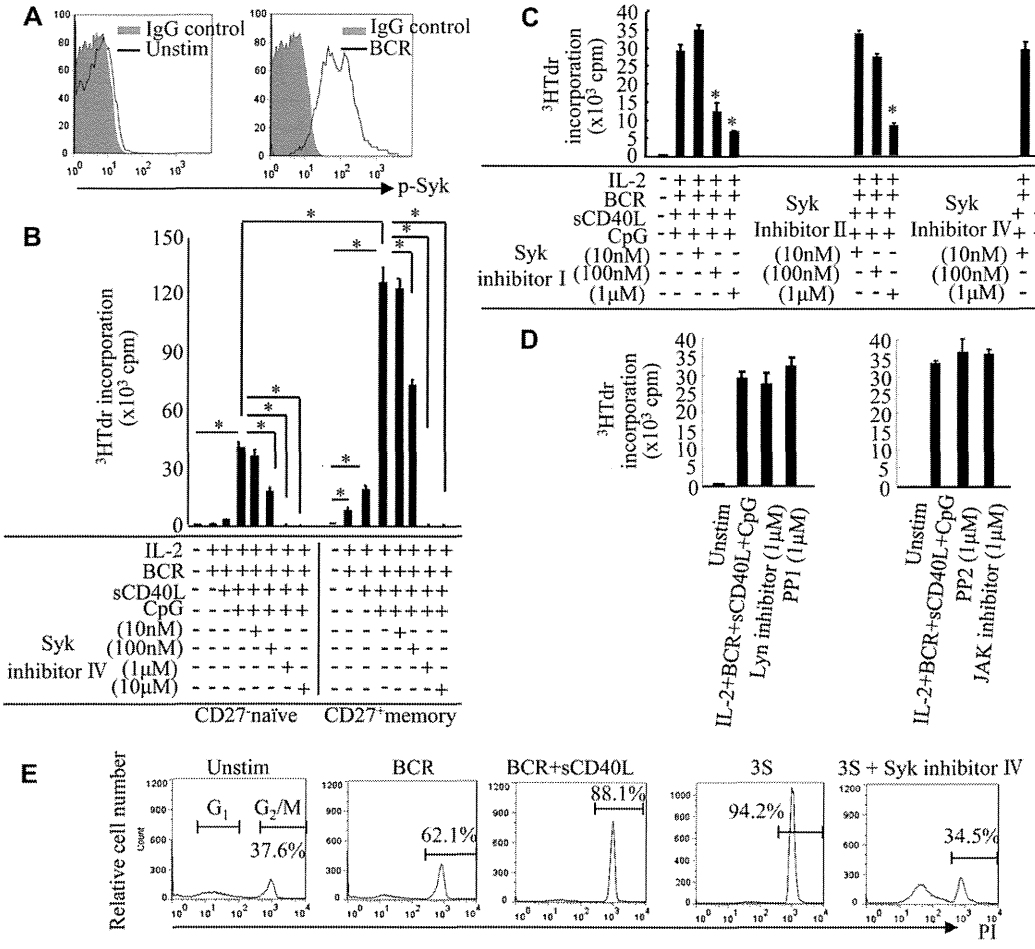

FIG 1. Syk regulates proliferation and cell-cycle progression in B-cell subsets on BCR, CD40, and TLR9 stimulation. **A**, BCR-induced phosphorylation of Syk (15 minutes). **B-D**, Tritiated thymidine *(^{3}HTdr)* incorporation of human B cells was measured during the last 18 hours of the 72-hour culture.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

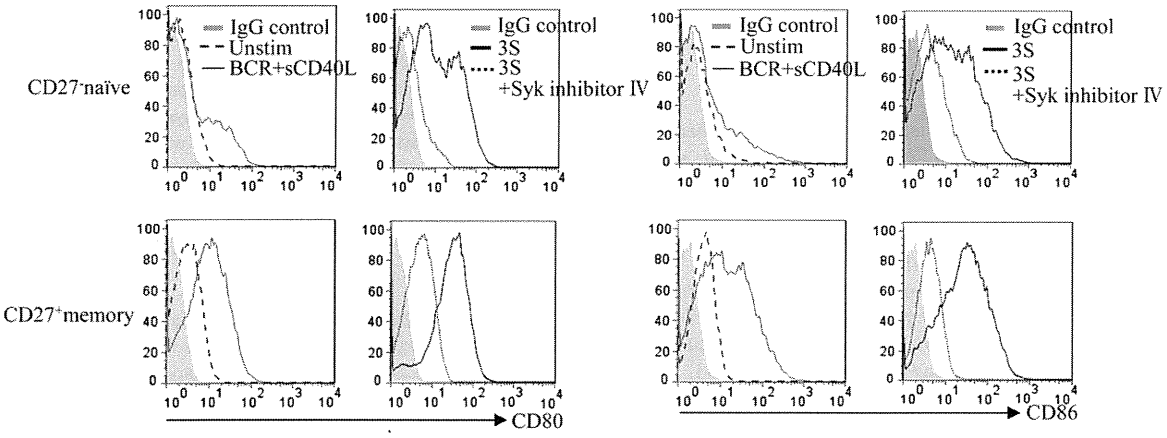

FIG 2. Syk regulates expression of CD80 and CD86 in B-cell subsets on stimulation. *Overlay histograms* depict relative fluorescence intensities of human naive and memory B cells cultured for 72 hours. *Unstim*, Before stimulation; *3S*, BCR, CD40, and TLR9 stimulation. Results are representative of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

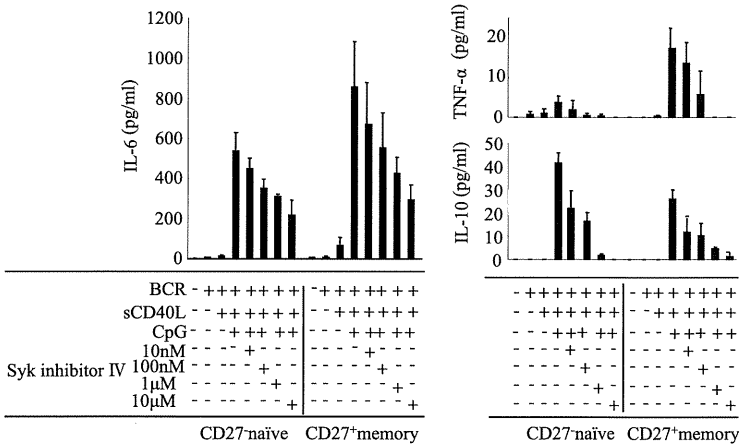

FIG 3. Syk regulates cytokine production in B-cell subsets on stimulation. Human peripheral blood naive and memory B cells were cultured for 72 hours, and supernatants were harvested and assayed by using the cytometric bead array for IL-6, TNF-α, and IL-10 content. Data are shown as means ± SDs and are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

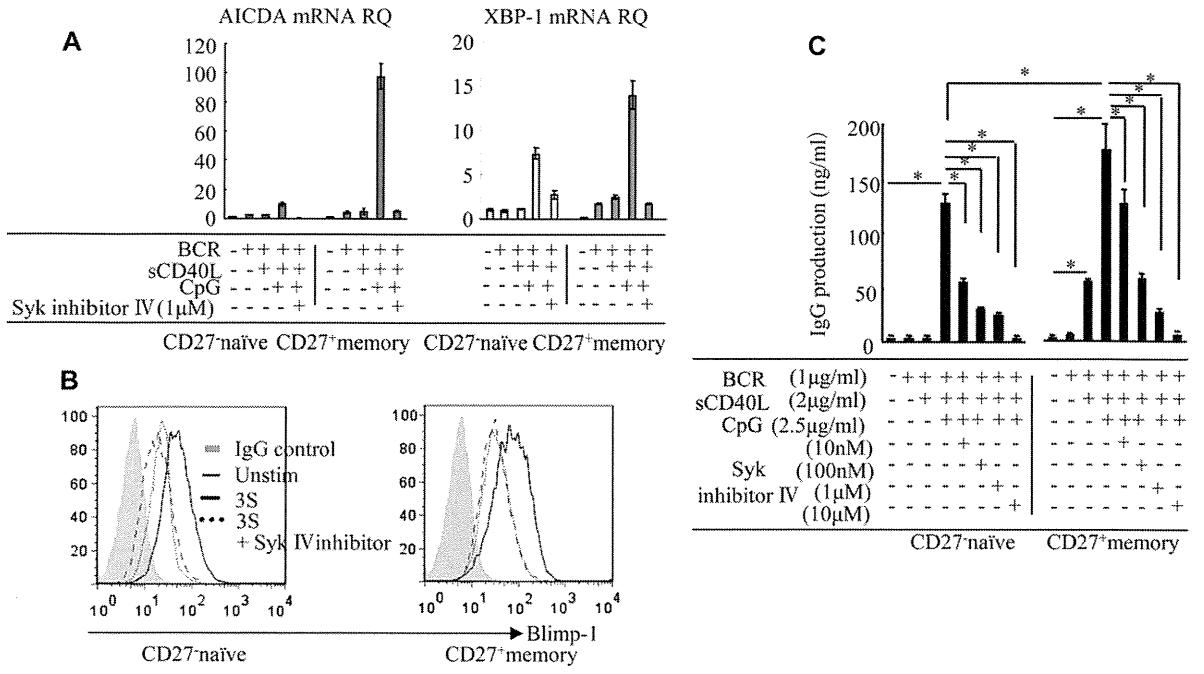

FIG 4. Syk regulates B-cell differentiation on BCR, CD40, and TLR9 stimulation. Naive and memory B cells were cultured for 48 hours (*AICDA* and *XBP-1* mRNA and Blimp-1) or for 5 days (IgG production). **A**, The level of *AICDA* and *XBP-1* mRNA was measured by using real-time PCR. *RQ*, Relative quantity.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

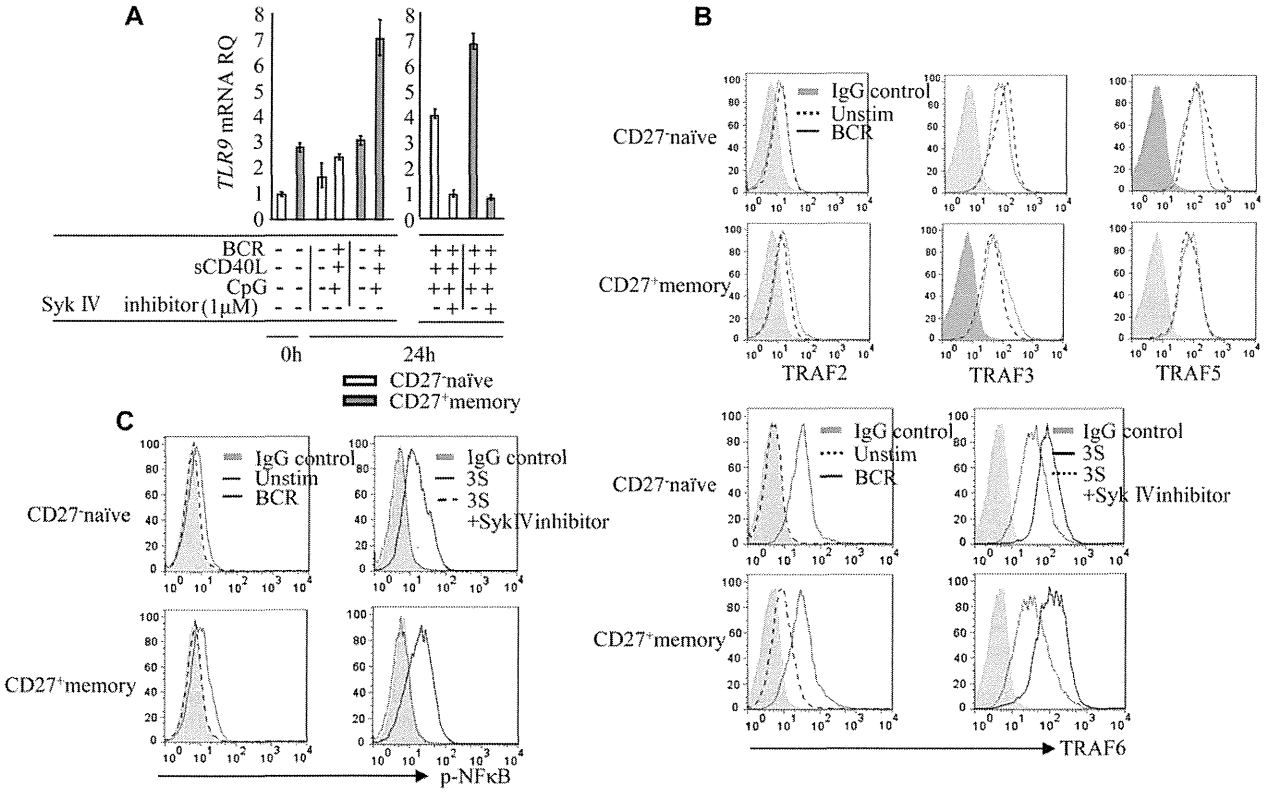

FIG 5. TLR9 and TRAF6 are key Syk-regulated molecules in B-cell subsets on stimulation. **A**, *TLR9* mRNA was quantified by using real-time PCR (TaqMan PCR kit) 24 hours later. *RQ*, Relative quantity; *sCD40L*, soluble CD40 ligand. **B** and **C**, TRAF2, TRAF3, TRAF5, and TRAF6 levels (48 hours later)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

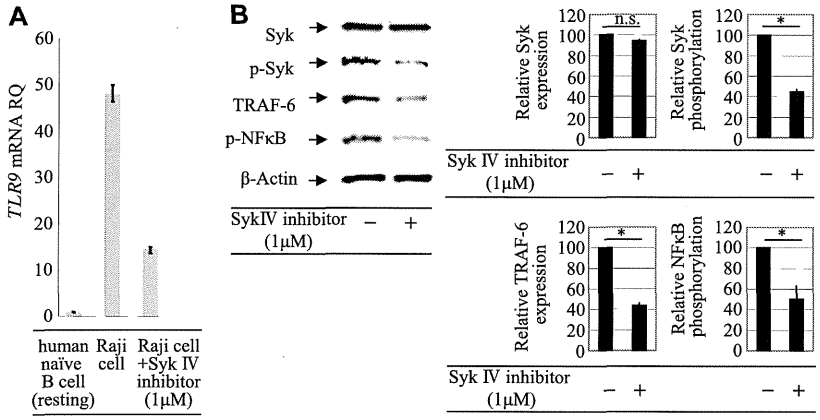

FIG 6. Syk inhibitor exerts marked inhibitory action, even at an activated state of B cells. Raji cells were cultured with RPMI containing 2% FCS for 48 hours. **A**, *TLR9* mRNA was quantified by means of real-time PCR. *RQ*, Relative quantity. **B**, Expression of Syk, phospho-Syk (Y348), TRAF6, and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

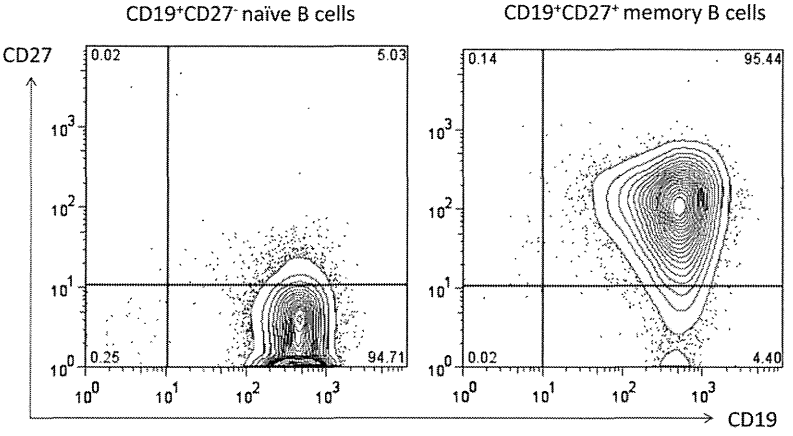

FIG E1. Phenotypic analysis of B-cell subsets in human peripheral blood. B cells were obtained by means of negative selection from PBMCs. $CD27^+$ memory B cells were then isolated by using positive selection from B cells with CD27 microbeads. The negative fraction of this isolation was assigned to $CD27^-$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

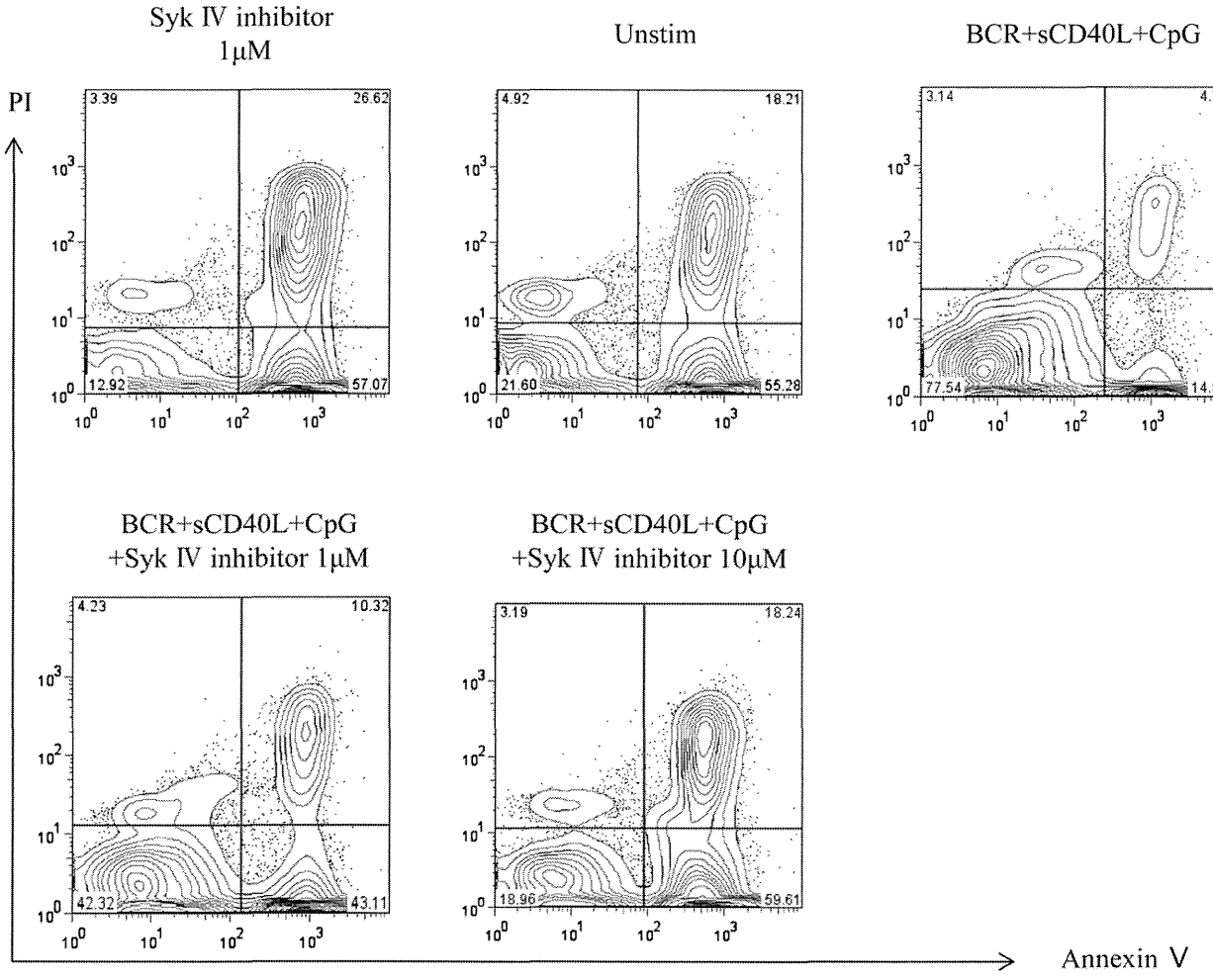

FIG E2. Syk provides survival signals for B cells after stimulation through all 3 receptors. B cells (2×10^5 per well) were cultured in triplicate in 96-well plates with anti-Igλ and anti-Igk antibodies (1 μg/mL), soluble CD40 ligand (*sCD40L*; 2 μg/mL), and CpG-ODN 2006 (2.5 μg/mL) with or without Syk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

Relapse patterns in IgG4-related disease

Immunoglobulin (Ig)G4-related disease (IgG4-RD) is a chronic inflammatory disorder characterised by elevated levels of serum IgG4 and swollen organs with fibrosis and infiltration by abundant IgG4-positive plasmacytes.[1 2] Glucocorticoid treatment is effective for achieving clinical remission in the short term,[3] but about 20%–30% of cases present with relapse after steroid doses are reduced.[4] Serial changes of serum IgG4 levels are often considered to reflect the disease activity in routine clinical practice,[5] but how disease relapse occurs is not well understood. We therefore analysed relapse patterns in IgG4-RD on the basis of data from the Sapporo Medical university And Related institutes database for investigation and best Treatments of IgG4-RD (SMART).

Subjects were 24 patients with IgG4-related dacryoadenitis and sialadenitis (IgG4-DS), the so-called IgG4-related Mikulicz's disease. Diagnoses were made according to the criteria of IgG4-DS,[6 7] and cases were registered to the SMART database. For patients with failure of organs other than the lacrimal and salivary glands (LSG), we initially prescribed 0.8 mg/kg/day of prednisolone (PSL) for 1 month, then reduced the dose by 10% every 2 weeks. For those patients without organ failure, we initially prescribed 0.6 mg/kg/day of PSL. We did not use immunosuppressants to spare steroids at the initial treatment. All patients were led to clinical remission once, but then they presented with recurrence during glucocorticoid treatment. Clinical patterns of the 24 cases at first visit and at relapse were analysed. 'Relapse' was defined as the condition in which the involved organs presented with swelling or dysfunction after previously achieving clinical remission.

The median time of relapse was 38 months (range, 8–105) since the initiation of glucocorticoid treatment. We found the possible prognostic factor was serial elevation of serum IgG4 levels, but it was not a complete marker. There were two patients who had a relapse without the elevation of serum IgG4 levels. The median dose of steroids at the time of relapse was 5 mg/day (range, 0–15). Nine cases showed only LSG involvement (Mikulicz's pattern), and 15 cases showed both LSG involvement and extraglandular (ExG) lesions at the first visit. At relapse, the group with only LSG involvement at first visit included four cases (44.4%) displaying Mikulicz's pattern, three cases (33.3%) with LSG involvement and ExG lesions, and two cases showing only ExG lesions. ExG lesions consisted of two cases with retroperitoneal fibrosis (RF), and one case each of autoimmune pancreatitis (AIP), tubulointerstitial nephritis (TIN) and lung involvement. Conversely, the group with LSG involvement and ExG lesions at the first visit showed four cases (26.7%) with Mikulicz's pattern, six cases (40.0%) with LSG involvement and ExG lesions, and five cases (33.3%) with only ExG lesions at relapse. Seven cases showed new organ failure at flare-up in this group. ExG lesions comprised two cases each with pulmonary lesions and TIN, and one case each with AIP, RF and pericarditis. Overall, 12 cases (representing half of the relapsed cases) showed new organ involvements (table 1).

IgG4-RD shows aspects of a systemic disease.[8] Relapse patterns are thus not always the same as the clinical form at the first visit. One reason may be that these organs possess inflammation at the first visit that goes undiagnosed clinically or on imaging. At the moment, it is important to follow patients with IgG4-RD regularly and systemically in daily clinical practice.

Table 1 Relapse patterns in IgG4-related dacryoadenitis and sialadenitis

		Relapse patterns		
Types of first visit	**Number of cases**	**Only LSG involvement**	**LSG involvement and ExG lesions**	**Only ExG lesions**
Only LSG involvement	9 (37.5%)	4 (44.4%)	3 (33.3%)	2 (22.2%)
LSG involvement and ExG lesions	15 (62.5%)	4 (26.7%)	6 (40.0%)	5 (33.3%)
Total	24	8	9	7

ExG, extraglandular; Ig, immunoglobulin; LSG, lacrimal and salivary glands.

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Competing interests None.

Patient consent Obtained.

Ethics approval Written consent to use the information from these cases was obtained from the patients in accordance with the Declaration of Helsinki. This study proceeded under the approval of our institutional IRB (SMU 22-57).

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REFERENCES

1. **Takahashi H,** Yamamoto M, Tabeya T, *et al.* The immunobiology and clinical characteristics of IgG4-related diseases. *J Autoimmun* 2012;**39**:93–6.
2. **Takahashi H,** Yamamoto M, Suzuki C, *et al.* The birthday of a new syndrome: IgG4-related diseases constitute a clinical entity. *Autoimmun Rev* 2010;**9**:591–4.
3. **Yamamoto M,** Harada S, Ohara M, *et al.* Beneficial effects of steroid therapy for Mikulicz's disease. *Rheumatology (Oxford)* 2005;**44**:1322–3.
4. **Khosroshahi A,** Stone JH. Treatment approaches to IgG4-related systemic disease. *Curr Opin Rheumatol* 2011;**23**:67–71.
5. **Tabata T,** Kamisawa T, Takuma K, *et al.* Serial changes of elevated serum IgG4 levels in IgG4-related systemic disease. *Intern Med* 2011;**50**:69–75.
6. **Yamamoto M,** Takahashi H, Shinomura Y. Mikulicz's disease and the extraglandular lesions. *Curr Immunol Rev* 2011;**7**:162–71.
7. **Umehara H,** Okazaki K, Masaki Y, *et al.* Comprehensive diagnostic criteria for IgG4-related disease (IgG4-RD), 2011. *Mod Rheumatol* 2012; **22**:21–30.
8. **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.

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The immunobiology and clinical characteristics of IgG4 related diseases

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ABSTRACT

Having the characteristic features of elevated serum IgG4 levels and prominent infiltration of IgG4-positive plasma cells with fibrosis in lesions, Mikulicz's disease (MD) has been recognized as an IgG4-related disease (IgG4-RD). Although incidence of autoimmune pancreatitis (AIP), one of the organ characteristics of IgG4-RD, has been internationally reported, there are only a few such reports of IgG4-related MD. The limited number of reports might be attributable to the low recognition of IgG4-related MD as a clinical entity as well as its misdiagnosis as Sjögren's syndrome (SS). Thus, we compared several clinical features of MD with SS to improve proper clinical diagnosis of MD in the clinical setting. A total of 70 SS and 70 MD cases evaluated at Sapporo Medical University Hospital were retrospectively analyzed. In SS patients, sicca symptoms were the most frequent (87%), followed by articular symptoms (23%), while lacrimal and salivary gland swelling were a rare (10%) and transient manifestation. In contrast, lacrimal or salivary gland swelling was observed in all patients with MD. Although nearly 60% of MD patients complained of sicca syndrome, skin rash and arthralgia were rare symptoms. Hypergammaglobulinemia was recognized in both SS and MD patients, but the occurrence of autoantibodies in patients with IgG4-related MD was low. Extraglandular organ involvement, often involving the retroperitoneum, pancreas, kidney and lung, was often discovered at the time of IgG4-related MD diagnosis. Although corticosteroid therapy tended to delay the hypofunction of salivary gland in SS patients, recovery of decreased function of salivary glands were observed in IgG4-related MD patients. These results suggest the beneficial effect of aggressive corticosteroid intervention in patients with IgG4-related MD. Although SS and MD are both chronic inflammatory diseases affecting the lacrimal and salivary glands, their clinical features and corticosteroid responsiveness are different. Thus, differential diagnosis of these conditions is warranted.

1. Introduction

As originally reported by Johann von Mikulicz-Radecki in 1892, Mikulicz's disease (MD) is a disorder characterized with symmetrical and persistent swelling of the lacrimal and salivary glands due to an unknown cause [1]. Until recently, MD has been categorized as a subtype of Sjögren's syndrome (SS), which is a chronic form of dacryoadenitis and sialoadenitis of autoimmune origin. However, in the early 2000s, Japanese researchers discovered that "Mikulicz's type" dacryoadenitis and sialoadenitis is associated with an elevated serum IgG4 level and significant infiltration of IgG4-positive plasma with fibrosis [2]. In addition, MD was also characterized by good responsiveness to corticosteroids and prompt recovery of glandular, in contrast to the irreversible tissue damage that accompanies SS [3]. MD is frequently complicated by autoimmune pancreatitis (AIP), retroperitoneal fibrosis, and tubulointerstitial nephritis (TIN) with extraglandular involvement. Indeed, the elevated serum IgG4 levels and fibrotic infiltration of IgG4-positive plasma cells are also observed in these extraglandular lesions. Thus, rather than a subtype of SS, MD is more appropriately regarded as a component of IgG4-related disease (IgG4-RD); a systemic disease which shares an etiology and clinical condition with AIP [4].

The Japanese Society of Pancreatology proposed diagnostic criteria for AIP in 2006 and attempted to encourage international adoption of their definition [5]. Gradually, AIP received international recognition that culminated with new international consensus diagnostic criteria developed by an international expert panel at the 14th Congress of the International Association of Pancreatology in 2010 [6]. Unfortunately, the number of international MD reports remains very low in contrast to those of AIP

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reports. Although few patients had been diagnosed with MD in Japan until several years ago, an increased number of reported cases would be predicted in response to the growing recognition of MD. Considering that IgG4-related AIP is frequently reported on an international level, it could not be assumed that IgG4-related MD occurs only in Japan. Rather, IgG4-related MD may continue to be misdiagnosed as SS in countries outside of Japan. Therefore, based on the experience at a single medical institution (Sapporo Medical University Hospital), we compared several clinical features of MD with SS with an aim to improve proper clinical diagnosis of MD.

2. Materials and methods

A retrospective survey of SS and MD was conducted in Sapporo Medical University Hospital. A total of 70 SS cases and 70 MD cases were analyzed. The diagnosis of SS was made according to the revised Japanese criteria with positivity of anti–SS–A antibody as a minimum requirement [7] and all cases were corresponding to primary SS. The diagnosis of MD was made according to the diagnostic criteria for IgG4-related MD determined by the Japanese Society for SS [8]. All patients with IgG4-related MD exhibited an increased level of serum IgG4 (840 ± 740 mg/dl) and a high ratio of serum IgG4/IgG (25.1 ± 9.4%). The average follow-up period among SS and MD cases was 117.3 and 46.8 months, respectively (Table 1). Antinuclear antibodies were measured using indirect fluorescence and a serum dilution reference value of ≤1:80. Rheumatoid factor levels were measured via a latex immunoassay (turbidimetric) with a reference value of <15 IU/ml. The Ouchterlony method with a serum dilution reference value of <1:1 was used to assess the presence of anti–SS–A and anti–SS–B antibodies. Salivary gland function was evaluated using the Saxon test [9].

3. Results

3.1. Clinical features

As outlined in Table 1, the average age at diagnosis was higher among IgG4-related MD cases than in SS cases (59.0 vs. 46.5 years, respectively). The youngest age at diagnosis of SS and IgG4-related MD was 17 and 25 years, respectively. The male-to female ratio of reported SS and MD cases was 2:68 and 29:41, respectively, indicating relative greater male risk for IgG4-related MD in contrast to SS. Initial sicca symptoms were present in nearly all SS patients (87.1%) and 58.6% of IgG4-related MD patients. Skin rash, arthralgia, and Raynaud's symptom were present as initial symptoms in numerous SS patients, including 3 young patients diagnosed with a fever of unknown origin. However, these symptoms were rarely found in IgG4-related MD patients. Although most (98.6%) IgG4-related MD patients revealed swelling of lacrimal and submandibular glands at diagnosis, only 10% of patients with SS had swelling of salivary glands and a transient enlargement of the parotid glands.

Table 1
Patient demographic characteristics at inclusion.

	Sjögren's syndrome	IgG4-related Mikulicz disease
No. Patients	70	70
Average age at diagnosis (yr)	46.5 (17–74)	59.0 (25–88)
(Male/female)	2/68	29/41
Follow-up period (month)	117.3 (15–394)	46.8 (1–206)
Initial symptom		
Sicca syndrome	61 (87.1%)	41 (58.6%)
Arthralgia	16 (22.9%)	0 (0.0%)
Skin rash	9 (12.9%)	1 (1.4%)
Lacrimal/salivary gland swellig	7 (10.0%)	70 (100.0%)
Nasal obstruction	0 (0.0%)	36 (51.4%)
Fever	7 (10.0%)	0 (0.0%)
Raynaud's phenomenon	3 (4.3%)	0.(0.0%)

3.2. Laboratory findings

As noted in Table 2, hypergammaglobulinemia was a characteristic feature of both diseases. Average levels of serum IgG at diagnosis in SS and IgG4-related MD patients were 1830 and 2533 mg/dl, respectively. Although hypocomplementemia (<30 U/ml of CH50) was rarely recognized in SS patients, approximately 20% of patients with IgG4-related MD demonstrated this finding. With the exception of a single patient, all others with IgG4-related MD showed negativity of the anti–SS–A antibody. Additionally, only a few positive cases for antinuclear antibody and rheumatoid factor were observed. Hematological tests were predominantly normal. Elevated serum CRP levels (>0.3 mg/dl) were observed in 3 patients (4.3%) with SS and 9 patients (12.9%) with IgG4-related MD.

3.3. Extraglandular lesions

As noted in Table 3, the most frequent extraglandular lesions in SS patients during the follow-up period were arthralgias (20.0%), followed by skin rashes (14.3%), cholestasis including primary biliary cirrhosis (8.6%), liver dysfunction (4.3%), and renal tubular acidosis (4.3%). The pattern of organ involvement was similar to those conventionally reported with SS [10]. Conversely, IgG4-related MD was most frequently accompanied by retroperitoneal fibrosis (20.0%), followed by AIP (18.6%), TIN (15.7%), and lung involvement (15.7%). Prostatitis and hypophysitis were also observed in IgG4-related MD. Although 9 patients (12.9%) with IgG4-related MD had superficial lymph node swelling, which required differential diagnosis from malignant lymphoma, systemic lymphadenopathy including the involvement of mediastinal lymph nodes was observed in 44 patients (62.9%) with IgG4-related MD. The introduction of the F-18 fludeoxyglucose positron emission tomography (an FDG-PET) scan has enabled early and highly sensitive detection of extraglandular lesions in IgG4-related MD. Interestingly, multiple organ involvement (with the exception of mediastinal lymph nodes) existed at diagnosis of IgG4-related MD in 41 (93.2%) out of 44 patients. While the effect of corticosteroid therapy after the diagnosis of IgG4-related MD should be considered, only 3 patients developed new extraglandular lesions during follow-up.

3.4. Prognosis in salivary gland function

As estimated using Saxon's test, the SS patients' salivary gland function at diagnosis and end of follow-up was 1.82 ± 1.45 g/2 min

Table 2
Laboratory findings at diagnosis.

	Sjögren's syndrome ($n = 70$)	IgG4-related Mikulicz disease ($n = 70$)
IgG (mg/d)	1830.4 ± 499.8	2533.9 ± 1633.2
IgA (mg/dl)	292.4 ± 138.6	196.0 ± 87.1
IgM (mg/dl)	131.7 ± 68.8	83.8 ± 68.2
CH50 (U/L)	43.5 ± 8.6	38.2 ± 12.8
Hypocomplimentemia	3/63 (4.8%)	15/70 (21.4%)
Antinuclear antibody + (≥160x)	34/70 (48.6%)	11/70 (15.7%)
Rheumatoid factor +	28/70 (40.0%)	14/70 (20.0%)
Anti–SS–A antibody +	70/70 (100%)	1/70 (1.4%)
Anti–SS–B antibody +	10/70 (14.3%)	0/70 (0.0%)
WBC (/mm^3)	4964 ± 1608	6006 ± 1567
PLT (× 10^4/mm^3)	21.6 ± 4.5	23.6 ± 6.0
Elevation of CRP (≥0.3 mg/dl)	3/70 (4.3%)	9/70 (12.9%)

Table 3
Organ involvement in Sjögren's syndrome and IgG4-related Mikulicz's disease.

	Sjögren's syndrome ($n = 70$)	IgG4-related Mikulicz disease ($n = 70$)
Joint	14 (20.0%)	0 (0.0%)
Skin	10 (14.3%)	1 (1.4%)
Eye	2 (2.9%)	0 (0.0%)
Pancreas	0 (0.0%)	13 (18.6%)
Biliary	6 (8.6%)	2 (2.8%)
Liver	3 (4.3%)	1 (1.4%)
Kidney	3 (4.3%)	11 (15.7%)
Retroperitoneum	0 (0.0%)	14 (20.0%)
Prostata	0 (0.0%)	5 (7.1%)
Lung	1 (1.4%)	11 (15.7%)
Heart	0 (0.0%)	2 (2.8%)
Pituitary	0 (0.0%)	1 (1.4%)
Parotid	7 (10.0%)	19 (27.1%)
Submandibular	2 (2.9%)	68 (97.1%)
Lacrimal	1 (1.4%)	69 (98.6%)

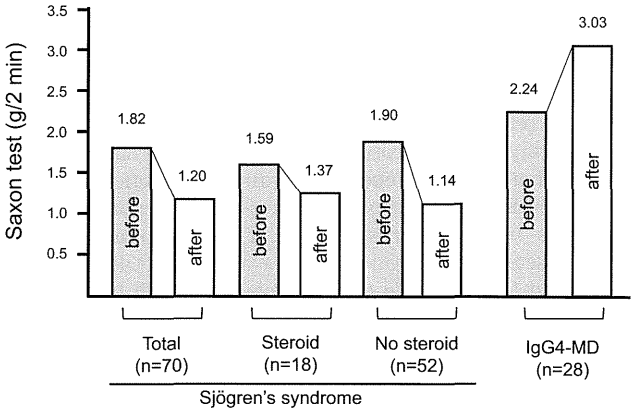

Fig. 1. Salivary gland function before and after follow-up in Sjögren's syndrome and IgG4-related Mikulicz's disease patients.

and 1.20 ± 0.98 g/2 min, respectively (Table 4, Fig. 1). The monthly and yearly rate of salivary gland function decrease in SS patients was −0.009 g/2 min and −0.1 g/2 min, respectively. The monthly rate of salivary gland function decrease was −0.0105 g/2 min in SS patients without corticosteroid therapy and −0.0048 g/2 min in SS patients with corticosteroid therapy. In contrast, salivary gland function at diagnosis and end of follow-up among 28 patients with IgG4-related MD was 2.24 ± 1.87 g/2 min and 3.02 ± 1.51 g/2 min, respectively (Table 5).

4. Discussion

Chronic dacryoadenitis and sialoadenitis, which showed symmetrical, persistent swelling of the lacrimal and salivary glands (so called MD), has been considered a subtype of SS [11]. However, recent research from Japan reported that an increased level of serum IgG4 and significant infiltration of IgG4-positive plasma cells with fibrosis in the lacrimal and salivary glands were observed in MD, and similarly in AIP [2]. In addition, MD has been found to be accompanied by a wide variety of extraglandular lesions. Thus, MD is now considered to comprise IgG4-RD with AIP, rather than belonging to SS [4]. In particular, AIP cases have been reported all over the world and international consensus diagnostic criteria were recently developed [5]. Although the diagnostic criteria for IgG4-related MD were created by the Japanese Society for SS [8], international consensus on clinical criteria for MD has yet to be established [12]. There are few reports regarding MD from countries other than Japan. However, many cases of IgG4-RD with AIP have been already reported internationally. Considering that about one-third of idiopathic retroperitoneal fibroses are assumed to be IgG4-related [13], MD is unlikely to be endemic to Japan. Rather, given that MD had previously been considered to be a subtype of SS and that the recognition for IgG4-RD is quite low in western countries, patients with IgG4-related MD might have been misdiagnosed with SS. Therefore, by comparing SS to IgG4-related MD patients evaluated at our institution, the purpose of this study was to find characteristic clinical features of IgG4-RD in order to help make a differential diagnosis.

This study found that the typical IgG4-related MD patient is a male or female around 60 years of age, presenting with swelling of the submandibular region and upper eyelids. Articular symptoms, pyrexia, and skin rash suggestive of systemic rheumatic diseases were rarely observed among IgG4-related MD patients. Sicca symptoms were initially recognized in approximately 60% of IgG4-related MD patients, lower than in SS patients. Thus, a diagnosis of SS and also IgG4-related MD should be considered when patients present with sicca symptoms such as oral thirst and dry eyes. Although IgG4-related MD patients showed a slightly higher level of serum IgG compared with SS patients, the positivity of disease-specific or -nonspecific autoantibodies was lower in IgG4-related MD patients. Hypocomplementemia was observed in approximately 20% of IgG4-related MD patients, particularly among cases with multiple organ involvement containing renal lesions [14]. When IgG4-related MD is suspected based on these symptoms, physical findings, and laboratory data, it is essential to perform serum IgG4 level measurement as well as immunostaining with anti-IgG4 antibody for tissue specimens from lacrimal and salivary glands. If the serum level of IgG4 exceeds 135 mg/dl and IgG4-positive plasma cells prominently infiltrate tissues with fibrosis, IgG4-related MD should be highly suspected. Finally, malignant diseases should be excluded prior to achieving a diagnosis.

In general, the most frequent extraglandular lesions in SS patients were arthralgias, followed by organ involvement such as kidney and lung according to literature report (Table 4) [15–17]. On

Table 4
Frequency of extraorgan involvement in Sjögren's syndrome on literature report.

	Fauchais AL [15] ($n = 445$)	Baimpa E [16] ($n = 536$)	Ramos-Casals [17] ($n = 1010$)
Articular	50%	30%	48%
Cutaneous	16%	11%	–
Renal	8%	8%	5%
Pulmonary	12%	19%	11%
Hepatobiliary	–	7%	–
Neuropathies	16%	19%	11%
Muscular	17%	0.4%	–
Thyroid	–	14%	–
Pancreas	–	–	0.5%

Table 5
Salivary gland function before and after follow-up.

	Follow-up period (month) mean (±SD)	Saxon's test (g/2 min)		
		Before	After	Monthly change mean (±SD)
Sjögren's syndrome ($n = 70$)	117.3 ± 83.2	1.82	1.20	−0.0090 ± 0.0166
steroid therapy ($n = 18$)	137.6 ± 98.3	1.59	1.37	−0.0048 ± 0.0187
no steroid therapy ($n = 52$)	110.3 ± 160.3	1.90	1.14	−0.0105 ± 0.0158
Mikulicz's disease ($n = 28$)	66.9 ± 41.1	2.24	3.03	0.0151 ± 0.0286

the other hand, the extraglandular organs involved in IgG4-related MD are clearly different from those involved in SS. Thus, IgG4-related MD should be suspected when various organ involvement, characterized by various indicators such as pancreatic swelling or retroperitoneal fibrosis, is recognized. In particular, the pancreatic involvement corresponding to AIP is characteristic of IgG4-RD. There are few reports regarding pancreatic lesion associated with SS [18]. Although Ramos-Casals reported 0.5% of pancreatitis in SS patients as one of extraglandular features, detailed disease description was not available [17]. In the case of IgG4-related MD, multiple organ involvement will be present at initial diagnosis. Therefore, systemic examination, including an FDG-PET scan, should be performed [19].

There have been only a few reports to assess the effectiveness of corticosteroid for the improvement of salivary gland function in SS patients. One earlier controlled trial compared oral prednisolone (30 mg, alternate days for 6 months) and placebo in SS [20]. There was no difference between groups in salivary flow rate, suggesting no beneficial effect of corticosteroid. However, a prospective study in 20 patients with SS found that low dose prednisolone maintenance (initial dose 15 mg daily, maintenance dose 5–7.5 mg daily) led to an increase of saliva production during 26.3 months of mean follow-up period [21]. This study confirmed that corticosteroid treatment did not cause significant improvement for salivary gland function in SS patients, and prognosis of salivary gland function was remarkably different between SS and IgG4-related MD (Fig. 1). Although conservative treatment without corticosteroid might be chosen for IgG4-related MD patients not presenting with subjective symptoms, the introduction of corticosteroid appeared to have a beneficial effect on glandular function.

5. Conclusion

IgG4-related MD comprises an important component of IgG4-RD, which is associated with AIP. Although there are few reports of MD from countries other than Japan, the number of reported IgG4-related MD cases will likely increase internationally over coming years, just as the number of AIP cases is also increasing. Most IgG4-related MD patients might be misdiagnosed with SS. Patients presented with a swelling of the lacrimal and salivary glands and sicca symptoms should be suspected for a diagnosis of IgG4-related MD. Physical examination for lacrimal and salivary glands and measurement of serum IgG4 are essential for proper diagnosis. In addition, negativity of anti–SS–A antibody and characteristic extraglandular lesions common to AIP might also suggest IgG4-related MD. Corticosteroid therapy is effective in improving salivary function among IgG4-related MD patients.

References

[1] Mikulicz J. Uber eine eigenartige symmetrishe Erkrankung der Tranen und Mundspeicheldrusen. Stuttgart: Beitr. z. Chir. Fesrschr. f. Theodor Billroth; 1892. pp. 610–30.
[2] Yamamoto M, Harada S, Ohara M, Suzuki C, Naishiro Y, Yamamoto H, et al. Clinical and pathological differences between Mikulicz's disease and Sjögren's Syndrome. Rheumatology (Oxford) 2005;44:227–34.
[3] Yamamoto M, Harada S, Ohara M, Suzuki C, Naishiro Y, Yamamoto H, et al. Beneficial effects of steroid therapy for Mikulicz's disease. Rheumatology 2005;44:1322–3.
[4] Takahashi H, Yamamoto M, Suzuki C, Naishiro Y, Shinomura Y, Imai K. Autoimmun Rev 2010;9:591–4.
[5] Okazaki K, Kawa S, Kamisawa T, Naruse S, Tanaka S, Nishimori I, et al. Clinical diagnostic criteria of autoimmune pancreatitis: revised proposal. J Gastroenterol 2006;41:626–31.
[6] Shimosegawa T, Chari ST, Frulloni L, Kamisawa T, Kawa S, Mino-Kenudson M, et al. International consensus diagnostic criteria for autoimmune pancreatitis: guidelines of the International Association of Pancreatology. Pancreas 2010; 40:352–8.
[7] Fujibayashi T, Sugai S, Miyasaka N, Hayashi Y, Tsubota K. Revised Japanese criteria for Sjögren's syndrome (1999): availability and validity. Mod Rheumatol 2004;14:425–34.
[8] Yamamoto M, Takahashi H, Shinomura Y. Mikulicz's disease and its extraglandular lesions. Curr Immunol Rev 2011;7:162–71.
[9] Kohler PF, Winter ME. A quantitative test for xerostomia. Arthritis Rheum 1985;28:1128–32.
[10] Anaya J-M, Talal N. Sjögren's syndrome and connective tissue diseases associated with other immunologic disorders. In: Koopman WJ, editor. Arthritis and allied condition. 13th ed. Pennsylvania: Williams & Wilkins; 1997. p. 1561–80.
[11] Morgan WS, Castleman B. A clinicopathologic study of "Mikulicz's disease.". Amer J Pathol 1953;29:471–503.
[12] Harrison JD, Rodriguez-Justo M. Commentary on IgG4-related sialadenitis: Mikulicz's disease, Kuttner's tumour, and eponymy. Histopathology 2011;58: 1164–6.
[13] Vaglio A, Palmisano A, Alberici F, Maggiore U, Ferretti S, Cobelli R, et al. Prednisone versus tamoxifen in patients with idiopathic retroperitoneal fibrosis: an open-label randomised controlled trial. Lancet 2011;378:338–46.
[14] Saeki T, Nishi S, Imai N, Ito T, Yamazaki H, Kawano M, et al. Clinicopathological characteristics of patients with IgG4-related tubulointerstitial nephritis. Kidney Int 2010;78:1016–23.
[15] Fauchais AL, Martel C, Gondran G, Lambert M, Launay D, Jauberteau MO, et al. Immunologcal profile in primary Sjögren's syndrome. Clinical significance, prognosis and long-term evolution to other auto-immune disease. Autoimmun Rev 2010;9:595–9.
[16] Baimpa E, Dahabreh IJ, Voulgarelis M, Moutsopoulous HM. Hematologic manifestations and predictors of lymphoma development in primary Sjögren's syndrome. Medicine 2009;88:284–93.
[17] Ramos-Casals M, Solans R, Rosas J, Teresa M, Gil A, Pino-Montes J, et al. Primary Sjögren's syndrome in Spain. Clinical and immunologic expression in 1010 patients. Medicine 2008;87:210–9.
[18] Afzelius P, Fallentin EM, Larsen S, Moller S, Schiodt M. Pancreatic function and morphology in Sjögren's syndrome. Scand J Rheumatol 2010;45:752–8.
[19] Suga K, Kawakami Y, Hiyama A, Hori K, Takeuchi M. F-18 FDG-PET finding in Mikulicz's disease and systemic involvement of IgG4-related lesions. Clin Nucl Med 2009;34:164–7.
[20] Fox PC, Datiles M, Atkinson JC, Macynski AA, Scott J, Fletcher D, et al. Prednisone and piroxicam for treatment of primary Sjögren's syndrome. Clin Exp Rheumatol 1993;11:149–56.
[21] Miyawaki S, Nishiyama S, Matoba K. Efficacy of low-dose prednisone maintenance for saliva production and serological abnormalities in patients with primary Sjögren's syndrome. Intern Med 1999;38:938–43.