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CD3 ζ defects in systemic lupus erythematosus

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ABSTRACT

The prototype autoimmune disease, systemic lupus erythematosus (SLE), has been known to be associated with deficiency of ζ chain, a component of the T-cell receptor–CD3 complex. Comprehensive analysis has shown that expression of the CD3 ζ chain is attenuated or absent in over half of SLE patients. Furthermore, aberrant transcripts of the CD3 ζ chain, including spliced variants lacking exon 7 or having a short 3′-untranslated region, have been detected in SLE T cells. Although attenuated expression of the CD3 ζ chain is also observed in cancer patients, infections and other autoimmune diseases, sustained attenuation of the CD3 ζ expression accompanied with aberrant transcripts are only observed in SLE. In this study, the authors review the unique features of CD3 ζ defects observed in SLE and discuss the molecular basis of the defects by recent findings in animal models, single-nucleotide polymorphisms and genome-wide association studies.

Systemic lupus erythematosus (SLE) is a prototype autoimmune disease with a wide spectrum of clinical manifestations.[1] While abundant production of autoantibodies and subsequent formation of immune complexes lead to tissue damage such as glomerulonephritis,[2 3] a particularly crucial role in SLE pathogenesis is played by SLE T cells.[2 4] In addition, comprehensive messenger RNA expression analysis has revealed that type I interferon (IFN)-related genes are upregulated in peripheral blood cells, partly through the increased production of IFN.[5–7] Although all of these events may interact with each other, the detailed molecular characterisation of SLE T cells is required. Here, we focus on the abnormal expression of the ζ chain of T-cell receptor (TCR)–CD3 complexes (CD3 ζ chain, TCR ζ chain, CD247) in patients with SLE, and discuss how the molecular basis of the defects affect developing autoimmune diseases.

DEFECTIVE EXPRESSION OF THE CD3 ζ CHAIN IN SLE PATIENTS

When SLE T cells are stimulated in vitro or in vivo through TCR–CD3 complexes, the response is usually attenuated in SLE T cells, compared with normal T cells.[8 9] However, the direct activation of protein kinase C with phorbol ester and ionomycin leads to normal or enhanced response in SLE T cells, raising a hypothesis that defects might reside in the proximal signal transduction molecules between the TCR–CD3 complexes and protein kinase C[9] (figure 1). Indeed, the tyrosine phosphorylation of cellular proteins, particularly the CD3 ζ chain, was diminished in SLE T cells in response to anti-CD3 and anti-CD4 treatment.[10–12] Surprisingly, the protein expression of the CD3 ζ chain was diminished in SLE patients.[10 11] While reduced protein expression of the CD3 ζ chain in cancer patients, infections and other autoimmune diseases such as rheumatoid arthritis has been reported to be transient,[13] the reduction was maintained throughout the course of the disease in more than half of the SLE patients.[12 14 15]

MECHANISM OF REDUCED PROTEIN EXPRESSION OF THE CD3 ζ CHAIN IN SLE PATIENTS

Several mechanisms are responsible for the decreased expression of the CD3 ζ chain, including low transcription activity,[11] generation of spliced variants,[10 16 17] increased ubiquitination,[18] increased caspase-3-dependent proteolysis,[19] redox status,[20] oxidative stress,[21] heat stress,[22] chronic exposure to pro-inflammatory cytokines[23] and direct contact with activated macrophages.[24] No mutations or deletions have been identified in the 5′-flanking region of the CD3 ζ gene in patients with SLE,[25] while other researchers have found those in the 5′-flanking region.[16] On the other hand, we and others have detected abnormal transcripts of the CD3 ζ chain, such as the splice variants including those lacking exon 7 and with the 3′-untranslated region (UTR).[10 16 26 27] The η (exon 1–7 plus exon 9) and the ι (exon 1–7 plus exon 10) variants have been shown to be generated by alternative splicing of the CD3 ζ chain on human and mouse chromosome 1q22–23[28–32] (figure 2); however, the functions of these splice variants are not fully understood. The CD3 ζ chain lacking exon 7 and short 3′-UTR variants, which are exclusively observed in SLE patients, are new class spliced variants[9] (figure 2).

ROLE OF UNIQUE SPLICE VARIANTS IN DEFECTIVE EXPRESSION IN THE CD3 ζ CHAIN

A 562-bp region containing the consensus sequence for mRNA stabilisation and a 31-nucleotide conserved sequence is missing from the short 3′-UTR splice variant.[17] As this conserved region is important for stabilisation, transportation and localisation of the CD3 ζ chain,[33] we speculated that this short 3′-UTR splice variant accounts for the downregulation of protein expression. To test this hypothesis, mRNA from the spliced variants was transfected into mouse T-cell hybridomas lacking CD3 ζ, resulting in instability of CD3 ζ mRNA and thereby leading to reduced protein expression.[27]

The 3′-UTR region of mRNA is known to control the turnover rate of presynthesised mRNA through interactions with trans-acting factors by altering mRNA stability and affecting its transportation and localisation.[34] mRNA 3′-UTR contains cis-acting, adenosine–uridine-rich elements that bind to trans-acting proteins and participate in either the stabilisation or destabilisation of transcripts. Adenosine–uridine-rich elements are located at positions +735, +803 and +1646 of the CD3 ζ mRNA,

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and the second element is found within the deleted sequence in the short 3′-UTR variant (figure 3). This notable absence may affect the stability of the short 3′-UTR variant mRNA. Using deletion mutants we found that the regions +871 to +950 and +1070 to +1136, which contain conserved regions one and two, respectively, are necessary to maintain the stability of CD3 ζ mRNA (figure 3). Similar transcript instability has been shown in another variant lacking exon 7,[35] suggesting that exon deletion and exon skipping also lead to the downregulation of protein expression through mRNA instability.

DEFECTS OF PROXIMAL SIGNAL TRANSDUCTION MOLECULES IN T CELLS IN MODEL ANIMALS

The SKG mouse, which models human autoimmune arthritis, exhibits a ζ-associated protein 70-kDa loss-of-function mutation, suggesting that one cause of the disease is defective proximal signalling molecules in T cells.[36] It is speculated that the thymic selection process has been altered by defective signal transduction, resulting in positive, but not negative, selection of autoreactive clones. More comprehensive data have been obtained by a study that tested whether the loss of tyrosine residues in each immunoreceptor tyrosine-based activation motif (ITAM) domain of a TCR–CD3 complex leads to autoimmunity.[37] That study demonstrated that scalable defects in signalling capability of the TCR–CD3 complex lead to multi-organ systemic autoimmune diseases such as interstitial pneumonitis, bowel inflammation and liver inflammation. Interestingly, a defect in two ITAM domains is sufficient to allow the development of autoimmune diseases and produces skewed cytokine production from interleukin (IL) 2 to IFNγ.

CONSEQUENCES OF EXPRESSING THE UNIQUE CD3 ζ CHAIN SPLICE VARIANTS

The evidence obtained from the model animal introduced an attractive hypothesis that defective signal transduction in T cells can be a cause of autoimmunity, partly through the altered thymic selection. One may raise the question as to whether other mechanisms may be responsible for developing skewed cytokine production and multi-organ disease. In this regard, it is interesting to know the common upregulated and downregulated genes after introducing two unique spliced variants of the CD3 ζ chain. After transfecting the short 3′-UTR and exon 7-lacking spliced variants from SLE patients into mouse T-cell hybridoma defective for CD3 ζ, DNA microarray analysis has been performed in two transfectants.[38] While only 16 common genes were upregulated in both the short 3′-UTR and exon 7-lacking variants, 36 shared genes were downregulated. We further supported that these results using real-time PCR, showing that expression levels of IL-2, IL-13, IL-15, IL-18 and transforming growth factor β-2 were significantly reduced in both spliced variants compared with those found after transfection with wild-type CD3 ζ.[38] In contrast, levels of Gsta4, Gzma, Lcn2, Mad3, Pmm1, Ptp4a3, Pvrl2, Sdc1, Selenbp1, Slc4a8, Tcf7 and Wasl were significantly increased. The possibility of whether these molecules may be involved in tissue inflammation or damage in SLE remains to be elucidated in the future.

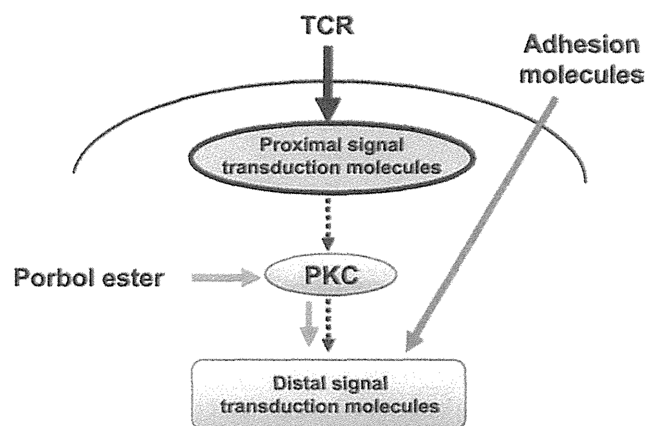

Figure 1 Functional defects of systemic lupus erythematosus (SLE) T cells. Phorbol ester can restore defects associated with SLE, indicating that proximal signal transduction molecules may be responsible for these defects. In contrast, adhesion molecules and their downstream signalling molecules are upregulated, raising the possibility that signals via adhesion molecules can bypass the proximal transduction molecules. PKC, protein kinase C; TCR, T-cell receptor.

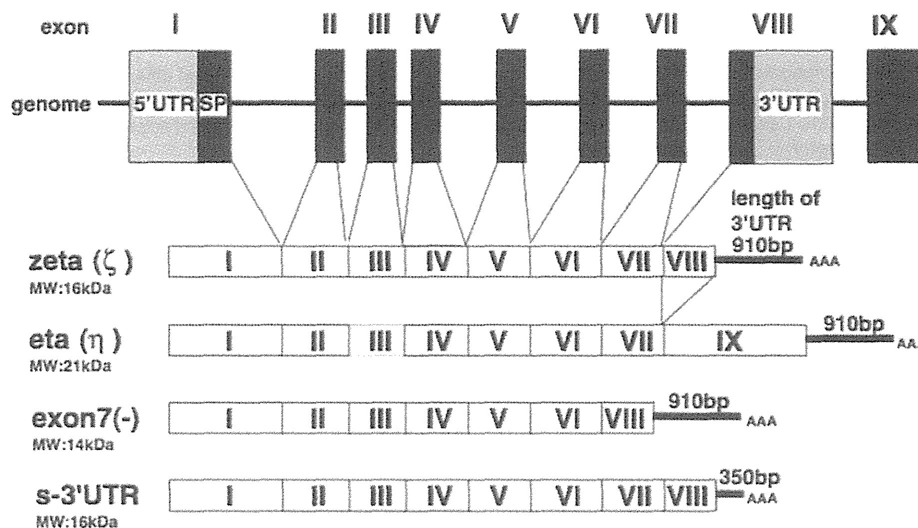

Figure 2 Structure of spliced variants of the human CD3 ζ chain. Exon–intron organisation of CD3 ζ chain genes and their transcripts for wild-type and spliced variants found in systemic lupus erythematosus (lacking exon 7 and short 3′-untranslated region; UTR).

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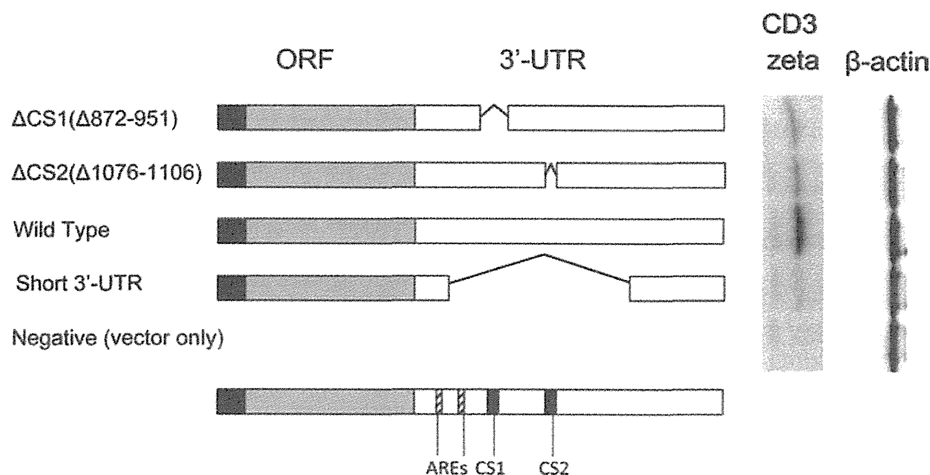

Figure 3 CD3 ζ protein expression by deleting conservative regions of the 3′-untranslated region (UTR). Structure of wild-type and deletion mutant constructs (left). CD3 ζ protein expression after transfection of mRNA from the spliced variants into mouse T-cell hybridomas lacking CD3 ζ (right). Deletion of conservative regions 1 (ΔCS1 (Δ position 872–951)), 2 (ΔCS2 (Δ position: 1076–1106)) and short 3′-UTR are indicated. ARE, adenosine–uridine-rich elements; CS, conservative sequence; ORF, open reading frame.

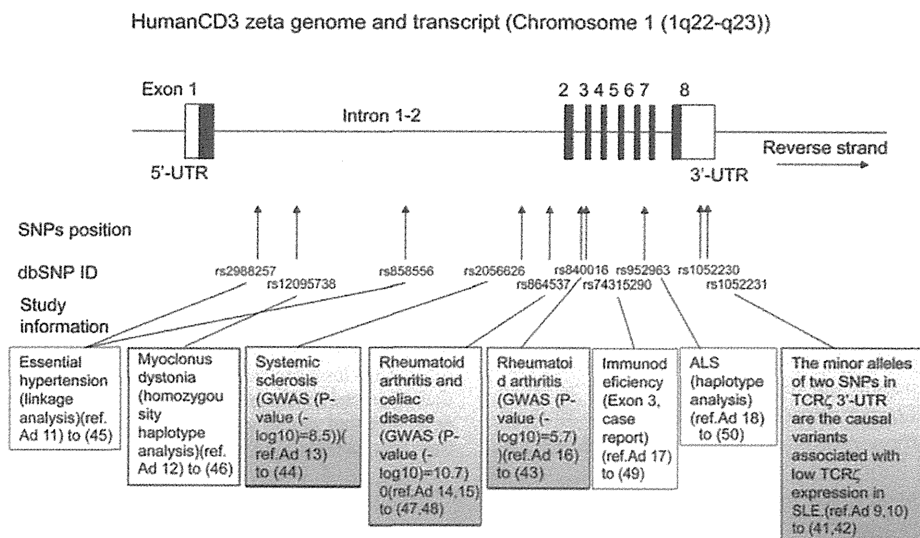

Figure 4 Single-nucleotide polymorphisms (SNP) of CD3 ζ related to systemic lupus erythematosus (SLE) and other diseases. Schemata of human CD3 ζ genome and transcripts are shown with summary information of diseases related to 10 reported SNP (position, dbSNP ID and study information) registered in the National Center for Biotechnology Information database. ALS, amyotrophic lateral sclerosis; GWAS, genome-wide association study; TCR, T-cell receptor; UTR, untranslated region.

mRNA EXPRESSION PROFILE OF PERIPHERAL BLOOD CELLS FROM PATIENTS WITH SLE

Large-scale microarray technology has been used to study global gene expression patterns of peripheral blood cells from lupus patients and control subjects in heterogeneous populations. These experiments demonstrated upregulated expression of genes in the IFN pathway in peripheral blood from SLE patients.[3 5–7] Furthermore, this signature gene expression served as an indicator for more severe diseases of the kidneys, haematopoietic cells and the central nervous system. IFN are known to have protean effects on the immune system and may therefore account for many of the immune system alterations that characterise SLE and contribute to autoimmunity.[3] As noted above, mice carrying reduced ITAM domains, such as mutated CD3 ζ, produced a substantial amount of cytokines, including IFNγ,[37] thereby prompting the attractive hypothesis that CD3 ζ defects are linked to IFN signature expression.

In fact, the level of CD3 ζ chain expression in SLE patients is inversely correlated with both in-vitro and serum levels of IFNγ,[39] whereas microarray analysis of transfectants with the spliced variant did not detect any IFN signature.[38] Recent proof-of-concept trials in SLE patients using monoclonal antibody against IFNα may provide a clue to understand the relationship between CD3 ζ and IFN in SLE patients.

SINGLE-NUCLEOTIDE POLYMORPHISMS AND GENOME-WIDE ASSOCIATION STUDIES OF THE TCR ζCD3 ζ CHAIN

The detailed mechanism of generating spliced variants of the CD3 ζ chain in SLE patients is potentially interesting, but is not fully

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understood. While no genomic mutations or deletions in either the splicing donor or acceptor sites have been reported in SLE,[40] two groups recently noted single-nucleotide polymorphisms (SNP) in the 3′-UTR region[41 42] (figure 4), reporting that the minor alleles of two SNP were causal variants associated with low TCR ζ expression and that one third of the mRNA was identical to that of the major alleles. It has been shown that the haplotype carrying the low-expression variants predisposes carriers to develop SLE.[41]

At present, 10 SNP found in the CD3 ζ gene are associated with autoimmune diseases, and have been registered in the National Center for Biotechnology Information database.[41–50] The genome-wide association study of SLE patients from several ethnic groups is spreading rapidly across the world (figure 4). Although no study of SLE has yet shown specific association for the CD3 ζ chain, a meta-analysis for rheumatoid arthritis[43] and a study for systemic sclerosis[44] have reported interesting findings with regard to the possible association between CD3 ζ SNP and rheumatoid arthritis and systemic sclerosis, both of which are located in the 78-kilo-base-long intron 1–2 region. Future analyses should focus on the functional consequences of these SNP on CD3 ζ expression.

Competing interests None.

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Efficacy of the Antiphospholipid Score for the Diagnosis of Antiphospholipid Syndrome and Its Predictive Value for Thrombotic Events

Kotaro Otomo, Tatsuya Atsumi, Olga Amengual, Yuichiro Fujieda, Masaru Kato, Kenji Oku, Tetsuya Horita, Shinsuke Yasuda, and Takao Koike

Objective. **To define the antiphospholipid score (aPL-S) by testing multiple antiphospholipid antibodies (aPL) and to evaluate its efficacy for the diagnosis of antiphospholipid syndrome (APS) and predictive value for thrombosis.**

Methods. **This study comprised 2 independent sets of patients with autoimmune diseases. In the first set of patients (n = 233), the aPL profiles were analyzed. Five clotting assays for testing lupus anticoagulant and 6 enzyme-linked immunosorbent assays (IgG/IgM anticardiolipin antibodies, IgG/IgM anti–β_2-glycoprotein I, and IgG/IgM phosphatidylserine-dependent antiprothrombin antibodies) were included. The association of the aPL-S with a history of thrombosis/pregnancy morbidity was assessed. In the second set of patients (n = 411), the predictive value of the aPL-S for thrombosis was evaluated retrospectively. Two hundred ninety-six of these patients were followed up for >2 years. The relationship between the aPL-S and the risk of developing thrombosis was analyzed.**

Results. **In the first set of patients, the aPL-S was higher in those with thrombosis/pregnancy morbidity than in those without manifestations of APS (P < 0.00001). For the aPL-S, the area under the receiver operating characteristic curve value was 0.752. In the second set of patients, new thromboses developed in 32 patients. The odds ratio (OR) for thrombosis in patients with an aPL-S of ≥30 was 5.27 (95% confidence interval [95% CI] 2.32–11.95, P < 0.0001). By multivariate analysis, an aPL-S of ≥30 appeared to be an independent risk factor for thrombosis (hazard ratio 3.144 [95% CI 1.383–7.150], P = 0.006).**

Conclusion. **The aPL-S is a useful quantitative index for diagnosing APS and may be a predictive marker for thrombosis in autoimmune diseases.**

Antiphospholipid antibodies (aPL) are a heterogeneous group of circulating immunoglobulins related to diverse clinical phenomena including arterial and venous thrombosis, pregnancy complications, livedo reticularis, valvular disease, nonthrombotic neurologic disorders, and thrombocytopenia. The term antiphospholipid syndrome (APS) is used to link thrombosis and/or pregnancy morbidity to the persistence of aPL as one of the most common causes of acquired thrombophilia (1).

In particular, anticardiolipin antibodies (aCL), anti–β_2-glycoprotein I (anti-β_2GPI), and lupus anticoagulant (LAC) are associated with APS. Assays for LAC are the most traditional laboratory method used to detect aPL. Lupus anticoagulants are immunoglobulins (IgG, IgM, IgA, or their combination) that interfere with in vitro phospholipid-dependent tests of coagulation (activated partial thromboplastin time [APTT], kaolin clotting time [KCT], dilute Russell's viper venom time [dRVVT]).

In the early 1980s, radioimmunoassays and enzyme-linked immunosorbent assays (ELISAs), which

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directly detected circulating aCL, were devised (2,3). Those aCL cross-reacted with negatively charged phospholipids, such as phosphatidylserine and phosphatidylglycerol (4). Thus, the term aCL was expanded to aPL. Further studies showed the requirement of a cofactor for the binding of autoimmune aCL to solid-phase phospholipids (5–7); β_2GPI was identified as that cofactor. Beta$_2$-glycoprotein I bears the epitopes for aCL binding that are exposed when β_2GPI binds to negatively charged phospholipids (8,9).

Prothrombin, another main phospholipid binding protein, has been reported to be a probable cofactor for LAC (10–13). An ELISA for the detection of antiprothrombin antibodies (APT) using prothrombin alone as the antigen coated onto irradiated plates (APT-alone assay) was described in 1995 (14). However, the association between APT alone and clinical manifestations of APS remains controversial (15). Our group (16) and other investigators (17,18) established an ELISA to detect antibodies against the phosphatidylserine/prothrombin complex (anti-PS/PT) and observed that IgG anti-PS/PT were highly prevalent in patients with APS compared with patients with other diseases (16). We also showed that the detection of anti-PS/PT strongly correlated with the clinical manifestations of APS and with the presence of LAC.

In consideration of this historical background and, moreover, the heterogeneity of the properties of aPL, we have performed multiple aPL assays, not only for research purposes but also as routine clinical practice in our autoimmune disease clinic. In the current study, we first tried to represent the aPL profile of each patient, using a quantitative score defined as the "antiphospholipid score" (aPL-S), and analyzed the value of the aPL-S for the diagnosis of APS. We then retrospectively analyzed the predictive value of the aPL-S for thrombotic events in patients with autoimmune diseases.

PATIENTS AND METHODS

Patients. This retrospective study included 2 sets of patients from our database. The first group comprised 233 consecutive patients with systemic autoimmune diseases who were examined at the Rheumatic and Connective Tissue Disease Clinic at Hokkaido University Hospital in 2006 (study 1).

Plasma and serum samples were obtained from the patients, and all testing for aPL was performed in our laboratory. The historical profiles, clinical manifestations, and diagnoses were carefully obtained by review of the medical records or by interviewing the patients (Table 1). Arterial thrombotic events comprised stroke, myocardial infarction, and iliac artery occlusion, as confirmed by computed tomography (CT) scanning, magnetic resonance imaging, or conventional angiography. Deep vein thrombosis and pulmonary thrombosis were defined as venous thrombosis and were confirmed by CT scanning, angiography, or scintigraphy. Pregnancy morbidity was defined by the revised Sapporo criteria for APS (1).

The second group comprised 411 consecutive patients who were examined at the Rheumatic and Connective Tissue Disease Clinic between January 1, 2002 and December 31, 2003 (study 2). Among these 411 patients, those who were followed up for <2 years were excluded from the study. The final population eligible for analysis of thrombosis risk comprised 296 patients. The median followup period for the eligible patients was 72 months. The clinical profiles of these patients are described in Table 2. The study was performed in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice.

Table 1. Characteristics of the 233 patients in study 1*

Diagnosis and manifestations	No. men/ no. women	Total
APS	5/32	37
Primary APS	1/12	13
APS with SLE	3/13	16
APS with other collagen disease	1/7	8
SLE	4/73	77
Rheumatoid arthritis	7/24	31
Sjögren's syndrome	0/18	18
Systemic sclerosis	5/9	14
Vasculitis syndrome	3/8	11
Polymyositis/dermatomyositis	0/8	8
Behçet's disease	1/5	6
Others	10/21	31
Clinical manifestations of APS	6/40	46
Thrombosis	6/32	38
Arterial thrombosis	3/24	27
Venous thrombosis	5/10	15
Pregnancy morbidity	0/14	14
Total	35/198	233

* APS = antiphospholipid syndrome; SLE = systemic lupus erythematosus.

Table 2. Characteristics of the 296 patients in study 2*

Diagnosis and manifestations	No. men/ no. women	Total
APS	3/40	43
Primary APS	2/17	19
APS with SLE	1/23	24
SLE without APS	10/79	89
Rheumatoid arthritis	8/42	50
Sjögren's syndrome	0/16	16
Systemic sclerosis	4/21	25
Vasculitis syndrome	3/2	5
Polymyositis/dermatomyositis	2/8	10
Behçet's disease	4/7	11
Others	9/39	48
Newly developed thrombosis	6/26	32
Arterial thrombosis	2/20	22
Venous thrombosis	4/10	14
Total	43/253	296

* APS = antiphospholipid syndrome; SLE = systemic lupus erythematosus.

Plasma samples. Venous blood was collected into tubes containing a one-tenth volume of 0.105*M* sodium citrate and was centrifuged immediately at 4°C. Plasma samples were depleted of platelets by filtration and then stored at –80°C until used.

Determination of LAC. Three clotting tests were performed for LAC determination, using a semiautomated hemostasis analyzer (STart 4; Diagnostica Stago) according to the guidelines recommended by the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis (19). For measurement of the APTT, a sensitive reagent with a low phospholipid concentration (PTT-LA test; Diagnostica Stago) was used for screening and mixing tests, and the results were confirmed with the use of a StaClot LA kit (Diagnostica Stago). The dRVVT was used to screen for the presence of LAC, and the results were confirmed with a Gradipore LAC test. The KCT was measured using a kaolin solution (Dade-Behring) according to a standard protocol. The cutoff level of positivity for the LAC tests was previously established as above the 99th percentile of levels in 40 healthy subjects, as used for our routine laboratory assays. For defining the aPL-S, the results of the 3 mixing procedures and the 2 confirming tests were used.

Anticardiolipin antibody–anti-β_2GPI ELISA. IgG and IgM aCL were assayed according to a standard aCL ELISA (20). Normal ranges for IgG aCL (>18.5 IgG phospholipid units) and IgM aCL (>7.0 IgM phospholipid units) were previously established, using the 99th percentile of the levels in 132 healthy controls as the cutoff level of positivity.

IgG and IgM anti-β_2GPI antibodies were determined by ELISA, as previously reported (21). Purified human β_2GPI was purchased from Yamasa. Irradiated microtiter plates (MaxiSorp; Nunc) were coated with 4 μg/ml of purified β_2GPI in phosphate buffered saline (PBS) at 4°C and washed twice with PBS. To avoid nonspecific binding of proteins, wells were blocked with 150 μl of 3% gelatin (BDH Chemicals). After 3 washes with PBS containing 0.05% Tween 20 (PBS–Tween 20; Sigma), 50 μl of serum diluted with PBS containing 1% bovine serum albumin (PBS–1% BSA; Sigma) in a 1:50 dilution was added in duplicate. Plates were incubated for 1 hour at room temperature and washed 3 times with PBS–Tween 20. Fifty microliters per well of the appropriate dilution of alkaline phosphatase–conjugated goat anti-human IgG and IgM (Sigma) in PBS–1% BSA was added. After 1 hour of incubation at room temperature and after 4 washes in PBS–Tween 20, 100 μl/well of 1 mg/ml of *p*-nitrophenyl phosphate disodium (Sigma) in 1*M* diethanolamine buffer (pH 9.8) was added. Following color development, optical density at 405 nm was measured by a Multiskan Ascent plate reader (ThermoElectron Corporation). Normal ranges for IgG (>2.2 units/ml) and IgM (>6.0 units/ml) anti-β_2GPI were established, using the 99th percentile of the levels in 132 nonpregnant healthy controls as the cutoff level of positivity.

ELISA for the detection of anti-PS/PT. Anti-PS/PT antibodies were detected by ELISA, as previously described (16). Briefly, nonirradiated microtiter plates (Sumilon Type S; Sumitomo Bakelite) were coated with 30 μl of a 50-μg/ml preparation of phosphatidylserine (Sigma) and dried overnight at 4°C. To avoid nonspecific binding of proteins, wells were blocked with 150 μl of Tris buffered saline (TBS) containing 1% fatty acid–free BSA (catalog no. A6003; Sigma) and 5 m*M* $CaCl_2$ (BSA–$CaCl_2$). After 3 washes in TBS containing 0.05% Tween 20 (Sigma) and 5 m*M* $CaCl_2$, 50 μl of a 10-μg/ml preparation of human prothrombin (Diagnostica Stago) in BSA–$CaCl_2$ was added to half of the wells in the plates, and the same volume of BSA–$CaCl_2$ alone (as sample blank) was added to the other half.

After 1 hour of incubation at 37°C, the plates were washed, and 50 μl of serum diluted 1:100 in BSA–$CaCl_2$ was added to duplicate wells. Plates were incubated for 1 hour at room temperature, followed by the addition of alkaline phosphatase–conjugated goat anti-human IgG or IgM and substrate. The anti-PS/PT antibody titer of each sample was derived from the standard curve according to dilutions of the positive control. Normal ranges for IgG (>2.0 units/ml) and IgM (>9.2 units/ml) anti-PS/PT antibodies were established, using the 99th percentile of the levels in 132 nonpregnant healthy controls as the cutoff level of positivity.

Statistical analysis. Statistical analysis was performed by Mann-Whitney U test, Fisher's exact test, or chi-square test, as appropriate. *P* values less than 0.05 were considered significant. The diagnostic accuracy of the aPL-S was assessed by receiver operating characteristic (ROC) curve analysis. The Kaplan-Meier approach was used to estimate the probability of thrombosis developing after aPL testing was performed. The risk of thrombosis was evaluated using multivariate Cox regression analysis. All statistical analyses were performed using SPSS software.

RESULTS

Definition of the aPL-S. To define the aPL-S, we used the first group of patients (n = 233) with autoimmune disease. In this population, the relative risks (approximated by odds ratios [ORs]) of having clinical manifestations of APS (thrombosis and/or pregnancy morbidity) were calculated for each aPL test. Furthermore, in each test, the specificity and sensitivity for the diagnosis of APS were calculated (Table 3). To define the aPL-S, we devised an original formula in which the aPL-S was determined by the OR, as follows: aPL-S = $5 \times \exp([OR] - 5)/4$. Consequently, an OR of 5 corresponds to an aPL-S of 5. The upper limit of the score for each aPL test was determined as 20.

In the aCL, anti-β_2GPI, and anti-PS/PT ELISAs, a second cutoff level was defined to separate patients with high antibody levels from those with medium or low levels of antibodies. The definition of high titers was established as more than the median levels of antibody-positive patients in each of the tests in the entire population studied. We observed that high levels of IgG aCL, anti-β_2GPI, and anti-PS/PT antibodies were closely related to the clinical manifestations of APS. In contrast, no relationship between clinical manifestations and titers of antibodies was observed in the IgM ELISAs.

Table 3. Relative risk of clinical manifestations of APS for each aPL test*

Test	Cutoff	Sensitivity, %	Specificity, %	OR (95% CI)	aPL score
APTT mixing	>49 sec.	39.1	89.3	5.36 (2.53–11.4)	5
Confirmation test, ratio	>1.3	19.6	95.2	4.81 (1.79–12.9)	2
	>1.1	30.4	90.9	4.38 (1.96–9.76)	1
KCT mixing	>29 sec.	45.6	88.8	6.64 (3.17–13.9)	8
dRVVT mixing	>45 sec.	28.2	90.9	3.93 (1.74–8.88)	4
Confirmation test, ratio	>1.3	17.4	94.7	3.72 (1.38–10.1)	2
	>1.1	28.3	90.4	3.7 (1.65–8.27)	1
IgG aCL, GPL					
High titers	>30	15.2	98.4	11 (2.72–44.5)	20
Medium/low titers	>18.5	19.5	94.6	4.31 (1.63–11.3)	4
IgM aCL, MPL	>7	6.52	96.3	1.79 (0.45–7.22)	2
IgG anti-β_2GPI, units					
High titers	>15	23.9	98.4	19.3 (5.11–72.7)	20
Medium/low titers	>2.2	30.4	92.5	5.4 (2.35–12.4)	6
IgM anti-β_2GPI, units	>6	8.7	91.4	1.02 (0.32–3.20)	1
IgG anti-PS/PT, units					
High titers	>10	19.6	97.8	11.1 (3.25–38.1)	20
Medium/low titers	>2	28.3	95.7	8.81 (3.39–22.9)	13
IgM anti-PS/PT, units	>9.2	6.52	98.9	6.45 (1.05–39.8)	8

* APS = antiphospholipid syndrome; aPL = antiphospholipid antibody; OR = odds ratio; 95% CI = 95% confidence interval; APTT = activated partial thromboplastin time; KCT = kaolin clotting time; dRVVT = dilute Russell's viper venom time; aCL = anticardiolipin antibody; GPL = IgG phospholipid units; MPL = IgM phospholipid units; anti-β_2GPI = anti-β_2-glycoprotein I; anti-PS/PT = anti-phosphatidylserine/prothrombin complex.

Therefore, the aPL scores for the IgG aCL, anti-β_2GPI, and anti-PS/PT antibody tests were separately defined.

For the determination of LAC, APTT, dRVVT, and KCT mixing tests were performed. In case of a positive APTT or dRVVT result, a complementary confirmation test was carried out, and an additional score was given. If the result of the confirmation test was >1.3, a score of 2 was added, and if the result was >1.1, a score of 1 was added. The aPL-S for each patient was calculated as the total scores for positive aPL tests and represents the complete aPL-S.

The partial aPL-S was defined using aPL tests that were included in the updated classification criteria for APS (1) and according to the guidelines recommended by the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis (19) and included tests for IgG/IgM aCL, IgG/IgM anti-β_2GPI, and LAC (only the APTT and dRVVT).

Correlation between the aPL-S and clinical manifestations. Among the first group of 233 patients, the aPL-S ranged from 0 to 86. Forty-six patients had experienced at least 1 of the clinical manifestations of APS (thrombosis and/or pregnancy morbidity), and the scores for these patients were higher than the scores for patients who did not have such manifestations (Figure 1A).

The prevalence of APS manifestations increased in accordance with increasing antiphospholipid scores. Patients were subdivided into 5 groups according to the aPL-S as follows: score of 0, scores of 1–9, scores of 10–29, scores of 30–59, and scores of ≥60. The prevalence of APS manifestations in the 5 groups was 10%, 26%, 29%, 56%, and 89%, respectively.

The partial aPL-S was also evaluated in the same population of patients and ranged from 0 to 56. When patients were subdivided into groups according to the partial aPL-S, the prevalence of APS manifestations was 13%, 23%, 36%, 44%, and 88% for a score of 0, scores of 1–9, scores of 10–19, scores of 20–39, and scores of ≥40, respectively.

Diagnostic value of the aPL-S for APS. The ROC curves for the aPL-S, the partial aPL-S, and the revised Sapporo criteria for APS showed a hyperbolic pattern, implying that the aPL-S is a potential quantitative marker for diagnosing APS (Figure 1B). The area under the curve (AUC) values were 0.752 for the aPL-S, 0.692 for the partial aPL-S, and 0.686 for the revised Sapporo criteria. ROC analysis was performed for each of the clinical manifestation of APS. ROC curves for either arterial thrombosis, venous thrombosis, or pregnancy

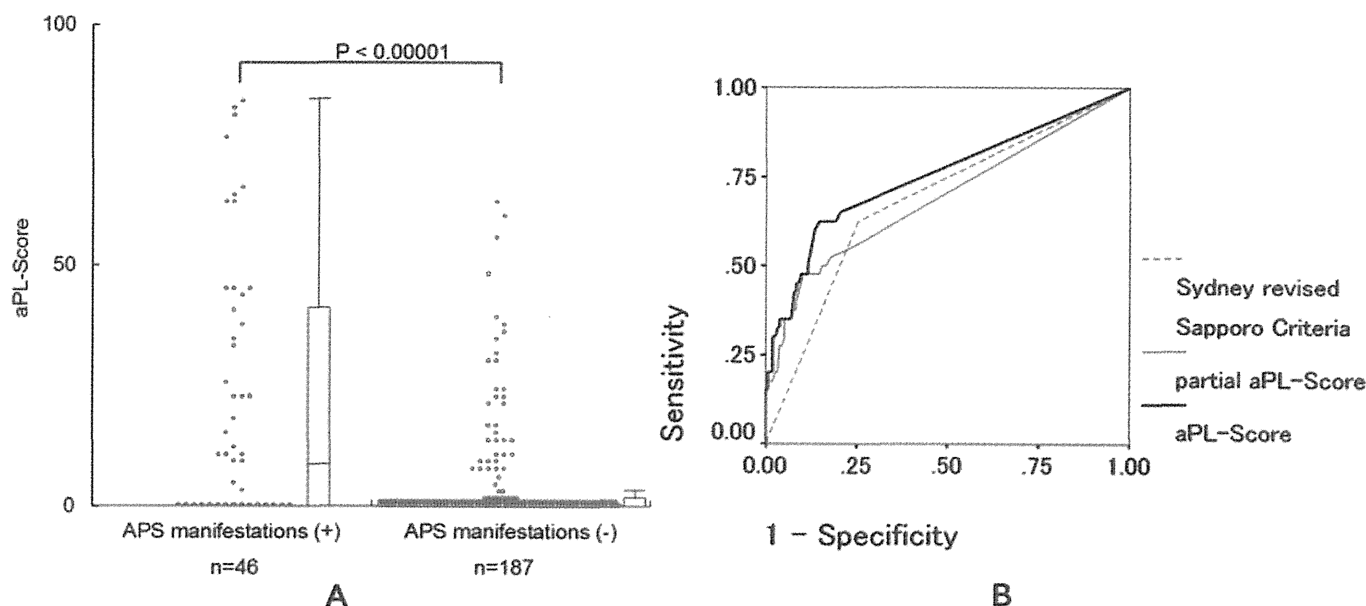

Figure 1. **A,** Distribution of the antiphospholipid scores (aPL-S) for patients in study 1 (n = 233). The aPL-S was defined as described in Patients and Methods. Data are shown as individual results as well as box plots, where each box represents the 25th to 75th percentiles; lines inside the box represent the median. The whisker represents the highest data still within 1.5 times the upper interquartile range. The scores for patients with antiphospholipid syndrome (APS) manifestations were significantly higher than those for patients without APS manifestations ($P < 0.00001$ by Mann-Whitney U test). **B,** Receiver operating characteristic (ROC) curves for the aPL-S, the partial aPL-S, and the revised Sapporo criteria for APS. Sensitivity and the specificity were calculated according to the presence of a history of clinical manifestations of APS. The area under the ROC curve values for the aPL-S and the partial aPL-S were 0.752 (95% confidence interval [95% CI] 0.656–0.849) and 0.692 (95% CI 0.588–0.795), respectively.

morbidity showed a hyperbolic pattern, and the AUC for each of them was larger than that for the revised Sapporo criteria (data not shown).

When the cutoff levels for the aPL-S and the partial aPL-S were defined as 30 and 20, respectively, the OR for the aPL-S (13.6 [95% confidence interval (95% CI) 4.81–38.7]) was higher than that for the revised Sapporo criteria (4.91 [95% CI 2.36–10.2]) and the partial aPL-S (7.85 [95% CI 2.99–20.7]). The sensitivity and specificity of an aPL-S of <30 were 35% and 96%, respectively, compared with 26% and 95%, respectively, for a partial aPL-S of <20 and 63% and 75%, respectively, for the revised Sapporo criteria.

Development of new thrombotic events. In the second group of patients, we retrospectively evaluated the relationship between the aPL-S and the risk of new thrombosis. This analysis included all thrombotic events that developed since the day the aPL-S was determined until the last followup in 2009.

During the followup period, new thromboses developed in 32 patients (22 arterial thrombotic events and 14 venous thrombotic events; some patients had both events). The aPL-S among patients in whom thromboses developed was significantly higher than that among those without thrombotic events during the followup (median score 5.5 versus 0; $P = 0.012$ by Mann-Whitney U test). This was also the case for the partial aPL-S (median score 5 versus 0; $P = 0.001$ by Mann-Whitney U test).

Predictive value of the aPL-S for APS manifestations. Patients with a higher aPL-S had a stronger risk of thrombosis compared with patients with lower scores. The ORs for newly developed thrombosis in patients with an aPL-S of ≥10, ≥30, and ≥50 were 2.86 (95% CI 1.33–6.6, $P = 0.006$), 5.27 (95% CI 2.32–11.95, $P < 0.0001$), and 5.31 (95% CI 1.81–15.53, $P = 0.0008$). The positive predictive values of an aPL-S of ≥10, ≥30, and ≥50 were 20%, 31%, and 35%, respectively, whereas the negative predictive values were 92%, 92%, and 91%, respectively. For the partial aPL-S, the positive predictive values of scores ≥10, ≥20, and ≥40 were 21%, 16%, and 25%, respectively, and the negative predictive values were 92%, 91%, and 91%, respectively (Figure 2A).

Effect of antithrombotic therapy. The effect of treatment on the aPL-S was evaluated in patients with an aPL-S of ≥30. This group included 39 patients (14 with primary APS, 15 with APS and SLE, and 10 with other autoimmune diseases), and 34 (87%) received

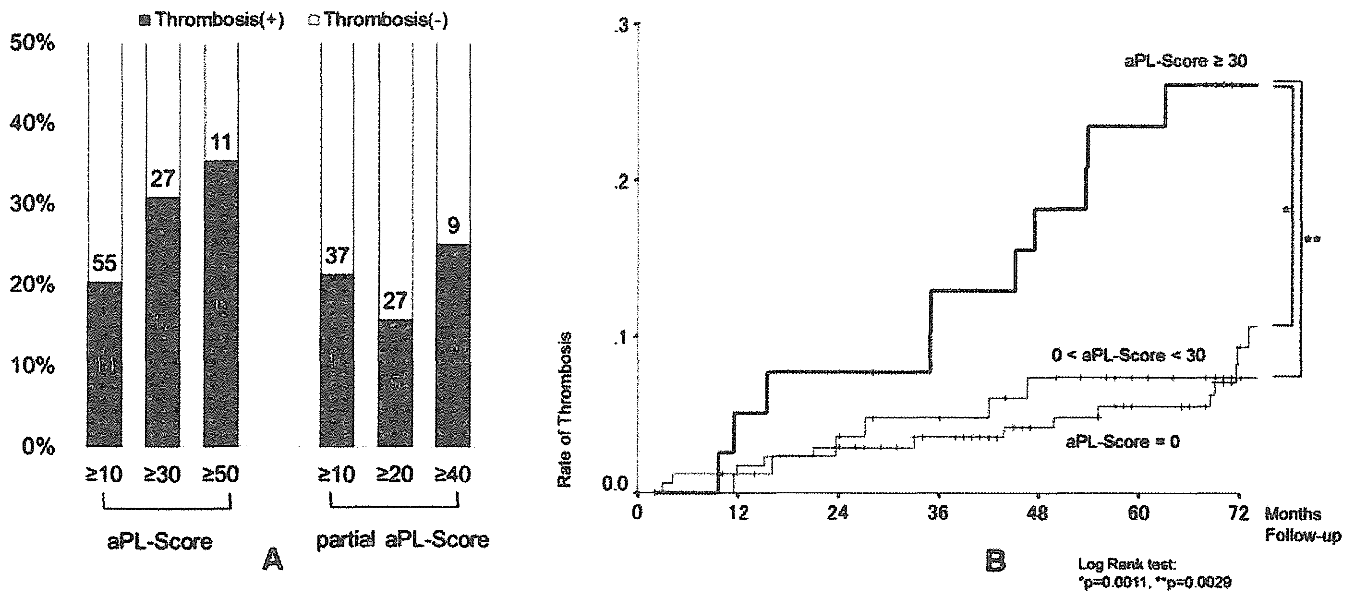

Figure 2. **A,** Positive predictive values (PPVs) of the antiphospholipid score (aPL-S) and the partial aPL-S for thrombosis in study 2. The numbers inside the bars represent the numbers of patients. The PPVs of an aPL-S of ≥10, ≥30, and ≥50 were 20.3%, 30.8%, and 35.3%, respectively. The PPVs of the aPL-S gradually increased in accordance with the cutoff value of the aPL-S, though the PPVs of the partial aPL-S did not. The negative predictive values of the aPL-S and the partial aPL-S were similar (90.5–92.2%). **B,** Kaplan-Meier analysis of the rate of thrombosis among patients in study 2, according to the aPL-S.

some antithrombotic medications. In 12 (31%) of these 39 patients, 15 new thromboses developed during the followup period despite antithrombotic therapy. The prevalence of thromboses among patients with an aPL-S of ≥30 was higher than that among those with a lower aPL-S (OR 5.40, 95% CI 2.38–12.23, $P = 0.00015$). The incidence rate of thrombosis among patients with an aPL-S of ≥30 was 5.144/100 person-years, whereas the rate among those with an aPL-S of 0 (no aPL) was 1.455/100 person-years. The rate of thrombosis among patients with an aPL-S of ≥30 was significantly higher than that among those with lower scores ($P = 0.0011$ for patients with an aPL-S of 0 and $P = 0.0029$ for patients with an aPL-S of 1–29, by log-rank test) (Figure 2B). In contrast, the partial aPL-S did not show significant correlation with the development of thrombosis.

To analyze the risk of thrombosis, multivariate Cox regression tests were conducted using the following data: aPL-S ≥30, age, sex, treatment with glucocorticoids, and the presence of hypertension, hyperlipidemia, diabetes, systemic lupus erythematosus, or rheumatoid arthritis at the time the aPL assays were performed. An aPL-S of ≥30 appeared to be an independent risk factor for thrombosis (hazard ratio [HR] 3.144, 95% CI 1.383–7.150, $P = 0.006$) (Table 4). A partial aPL-S of ≥20 was also analyzed using the same statistics but was not revealed to be an independent risk factor for thrombosis (HR 1.525, 95% CI 0.581–4.007, $P = 0.391$).

Table 4. Risk factors for thrombosis in autoimmune disease, as determined using multivariate analysis

Risk factor	Hazard ratio (95% CI)*
Glucocorticoid treatment	1.979 (0.809–4.842)
History of thrombosis	1.401 (0.640–3.068)
Hypertension	1.621 (0.750–3.504)
Hyperlipidemia	1.917 (0.927–3.966)
Diabetes	0.963 (0.394–2.355)
Age	1.017 (0.987–1.047)
Male sex	1.002 (0.385–2.606)
Systemic lupus erythematosus	1.052 (0.480–2.303)
Rheumatoid arthritis	0.470 (0.101–2.181)
Antiphospholipid score ≥30	3.144 (1.383–7.150)†

* 95% CI = 95% confidence interval.
† $P = 0.006$.

DISCUSSION

In this study, we demonstrated that the profile of aPL can be successfully quantitated as the aPL-S. The aPL-S level correlated with a history of thrombosis or pregnancy morbidity, suggesting that the aPL-S is a potential quantitative marker of APS. Therefore, the

current aPL-S can be unified and become a marker of the probability of having APS. Furthermore, we confirmed that the aPL-S had predictive value for recurrence and/or new onset of thrombotic events in the autoimmune disease setting. This fact suggests that treatment of APS can be modified considering the aPL-S.

Although aPL, as a group of autoantibodies sharing their properties in the phospholipid-associated molecules or reactions (22–27), have a strong link to thrombosis/pregnancy morbidity, the value of each aPL determination as a marker of APS is still not elucidated (28–32). Antiphospholipid antibodies are significantly prevalent in patients with infectious diseases, autoimmune diseases, malignant diseases, or hepatic diseases and even in healthy elderly individuals (33–37). One of the major issues involving the classification of APS has been avoiding overdiagnosis of APS by not accepting a positive result of a nonspecific aPL test as diagnostic (38). According to the APS criteria, aPL must be detected on 2 occasions not less than 12 weeks apart to determine that the presence of aPL is not transient. A low titer of aCL is not considered to be a marker of APS, although a "low positive" titer is a statistically abnormal laboratory phenomenon. However, efforts have not been successful enough, because aPL are found in many settings other than APS. In addition, updated diagnostic algorithms for catastrophic APS have been proposed, but no particular aPL has been proven to be associated with that syndrome (39).

In addition, standardization of each aPL assay has been extremely difficult. The presence of aPL defines the APS; thus, the greatest efforts have been made since the mid 1980s, when aCL were described (40,41). However, a number of variables in the assay, such as techniques, reagents, and standards, have hampered achievement of consensus (2), as described by de Groot et al in their article "Twenty-two years of failure to set up undisputed assays to detect patients with the antiphospholipid syndrome" (42). Considering the history of standardization, the establishment of a single aPL to define APS is unlikely in the near future.

In contrast, the premise that aPL represent the risk of thrombotic events and/or pregnancy morbidity either in the past or in the future would not be disputed (38,43–45). Accordingly, it would be more sensible to use aPL tests to establish an aPL profile as a marker of thrombotic risk rather than using these tests for diagnosis. Furthermore, combining multiple aPL tests would compensate for or reduce the disadvantage of each single aPL. From this point of view, our definition of the aPL-S has been proven to represent the "probability" or "likelihood" of having APS, depending on the level of the score; higher antiphospholipid scores were associated with higher risks of thrombotic events or pregnancy morbidity.

In the second part of the study, we retrospectively evaluated the value of the aPL-S for predicting the development of APS-related events in patients with autoimmune diseases. Despite receiving standard antithrombosis prophylaxis, many patients developed thrombosis during the followup period. In this cohort, the aPL-S showed a positive correlation with the risk of thrombotic events and had a significant predictive value. Those data would lead to a potential therapeutic strategy in which the intensity of antithrombotic treatment could be determined according to the aPL-S.

In clinical practice, all aPL tests are not available to all physicians. Therefore, we also defined a partial aPL-S that corresponds to the total score for the aPL tests included in the classification criteria for definite APS (1). For calculation of the partial aPL-S, the KCT mixing test and the anti-PS/PT IgG and IgM tests were excluded. The results for the complete aPL-S derived from the full battery of tests were compared with those for the partial aPL-S. A partial aPL-S seems to be a useful tool with which to evaluate the risk of thrombosis in patients with aPL (diagnostic value). However, although the aPL-S showed a positive predictive value for thrombosis that gradually increased in accordance with increasing scores, this increasing tendency was not observed with the partial aPL-S. None of the combinations of aPL tests used to define the aPL-S showed better relevance for the diagnosis of APS or for the prediction of thrombosis than the original complete aPL-S (data not shown). Inclusion of anti-PS/PT antibodies in the battery of aPL tests allows better quantification of the thrombosis risk.

Recently, Pengo et al (46) reported that in their cross-sectional study, patients with triple positivity for aCL, LAC, and anti-β_2GPI had a greater risk of thrombotic events than those who were positive for only 1 or 2 of these antibodies, which supports, in part, our findings. In the Pengo study, triple positivity was categorical (i.e., either present or absent), but our criteria were more quantitative, as proven by the ROC curves. Further, in the study of Pengo et al, anti-PS/PT antibodies were not considered. In their analysis, patients with prothrombin-dependent LAC and anti-PS/PT antibody positivity could be classified as single-positive for LAC, although this group of patients had a higher risk of APS than those with aPL positivity alone (47,48). In any case, the

combination of aPL tests should be considered when discussing the risk of thrombosis/pregnancy morbidity.

In the current study, we proved the efficacy of the aPL-S as a marker of the "probability" of APS and its value for predicting thrombosis in the setting of autoimmunity. This study is the first to attempt scoring the aPL profile, and the aPL-S successfully correlated with the risk of thrombotic events. However, the score could have other definitions, according to the population, and obviously the "true" predictive value should be validated in prospective studies. Higher accuracy of the aPL-S is obtained when all aPL tests are included. However, in clinical practice and trials, if all of the tests are not accessible, a partial aPL-S will provide important information regarding the thrombosis risk for each patient and consequently will help clinicians in making decisions about the therapeutic approach.

AUTHOR CONTRIBUTIONS

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

Study conception and design. Otomo, Atsumi, Amengual, Fujieda, Kato, Oku, Horita, Yasuda, Koike.

Acquisition of data. Otomo, Atsumi, Amengual, Fujieda, Kato, Oku, Horita, Yasuda, Koike.

Analysis and interpretation of data. Otomo, Atsumi, Amengual, Fujieda, Kato, Oku, Horita, Yasuda, Koike.

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

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

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

Abbreviations used

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

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

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

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

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

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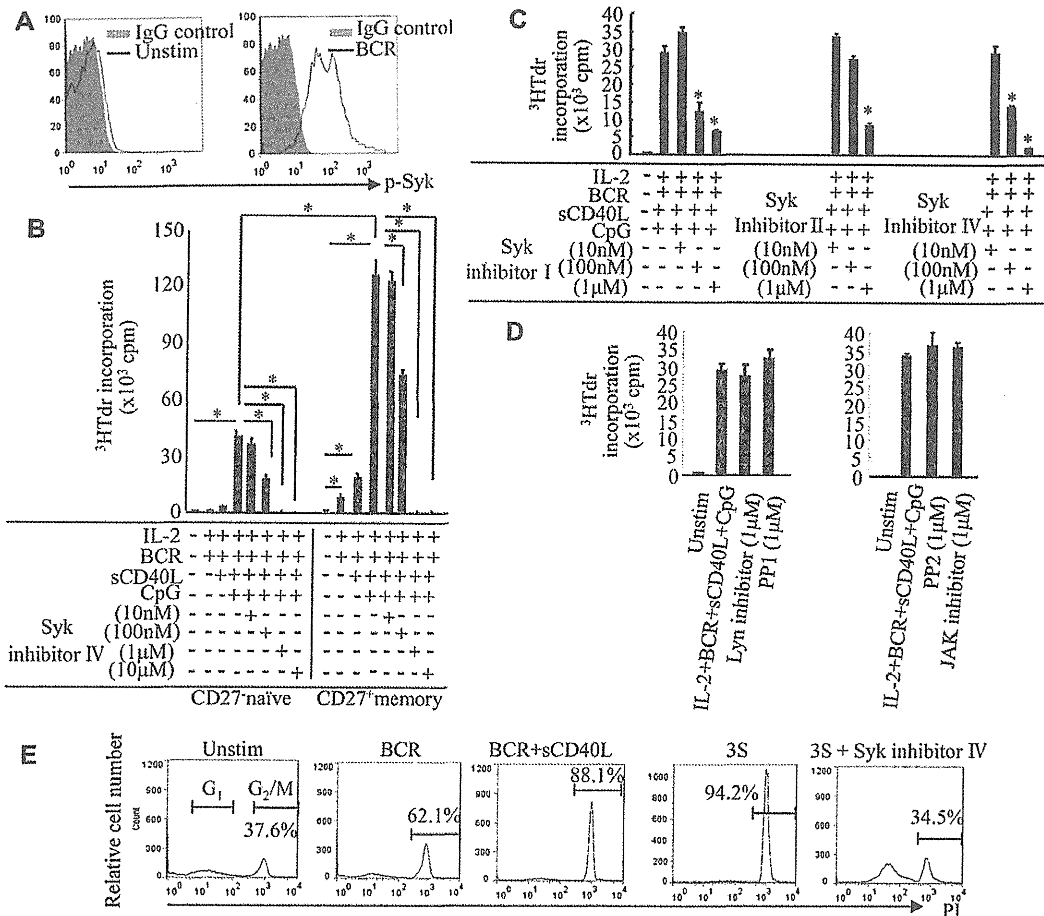

FIG 1. Syk regulates proliferation and cell-cycle progression in B-cell subsets on BCR, CD40, and TLR9 stimulation. **A**, BCR-induced phosphorylation of Syk (15 minutes). **B-D**, Tritiated thymidine (*^{3}HTdr)* incorporation of human B cells was measured during the last 18 hours of the 72-hour culture.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

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

METHODS

Reagents

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

Isolation, culture, and stimulation of B-cell subsets

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

Proliferation assay

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

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

Flow cytometric analysis

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

Apoptosis assay

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

Cell-cycle analysis

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

Cytokine production

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

IgG ELISA

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

Quantitative real-time PCR

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

Western blot analysis

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

Statistical analysis

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

RESULTS

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

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

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

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

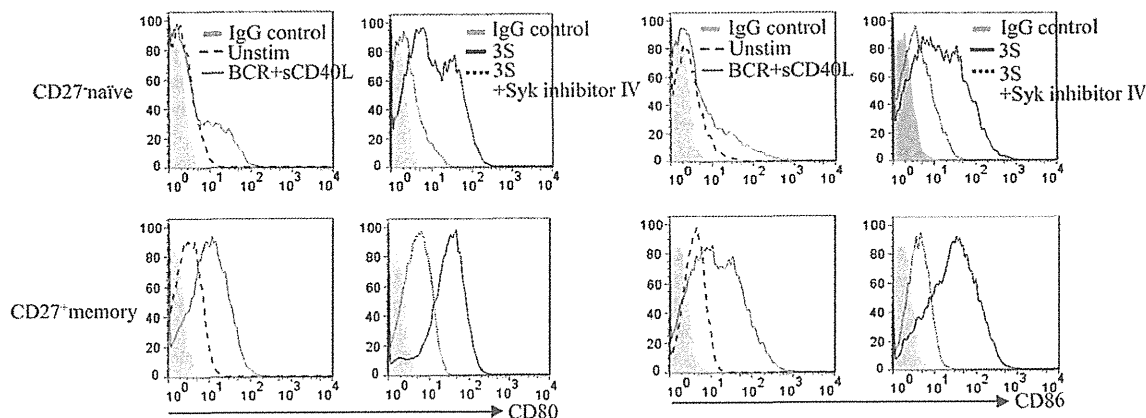

FIG 2. Syk regulates expression of CD80 and CD86 in B-cell subsets on stimulation. *Overlay histograms* depict relative fluorescence intensities of human naive and memory B cells cultured for 72 hours. *Unstim*, Before stimulation; *3S*, BCR, CD40, and TLR9 stimulation. Results are representative of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

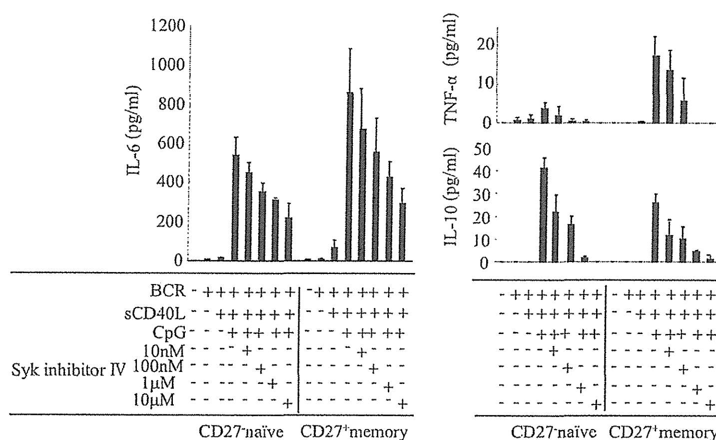

FIG 3. Syk regulates cytokine production in B-cell subsets on stimulation. Human peripheral blood naive and memory B cells were cultured for 72 hours, and supernatants were harvested and assayed by using the cytometric bead array for IL-6, TNF-α, and IL-10 content. Data are shown as means ± SDs and are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

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

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

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

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

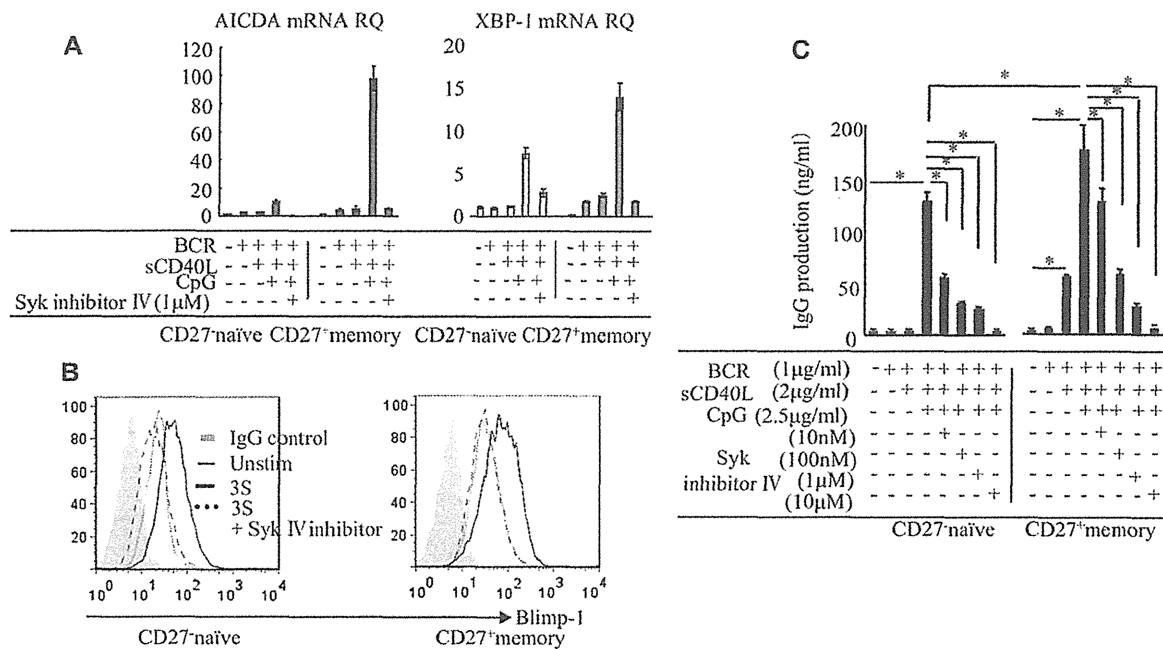

FIG 4. Syk regulates B-cell differentiation on BCR, CD40, and TLR9 stimulation. Naive and memory B cells were cultured for 48 hours (*AICDA* and *XBP-1* mRNA and Blimp-1) or for 5 days (IgG production). **A,** The level of *AICDA* and *XBP-1* mRNA was measured by using real-time PCR. *RQ,* Relative quantity.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

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

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

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

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

DISCUSSION

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

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

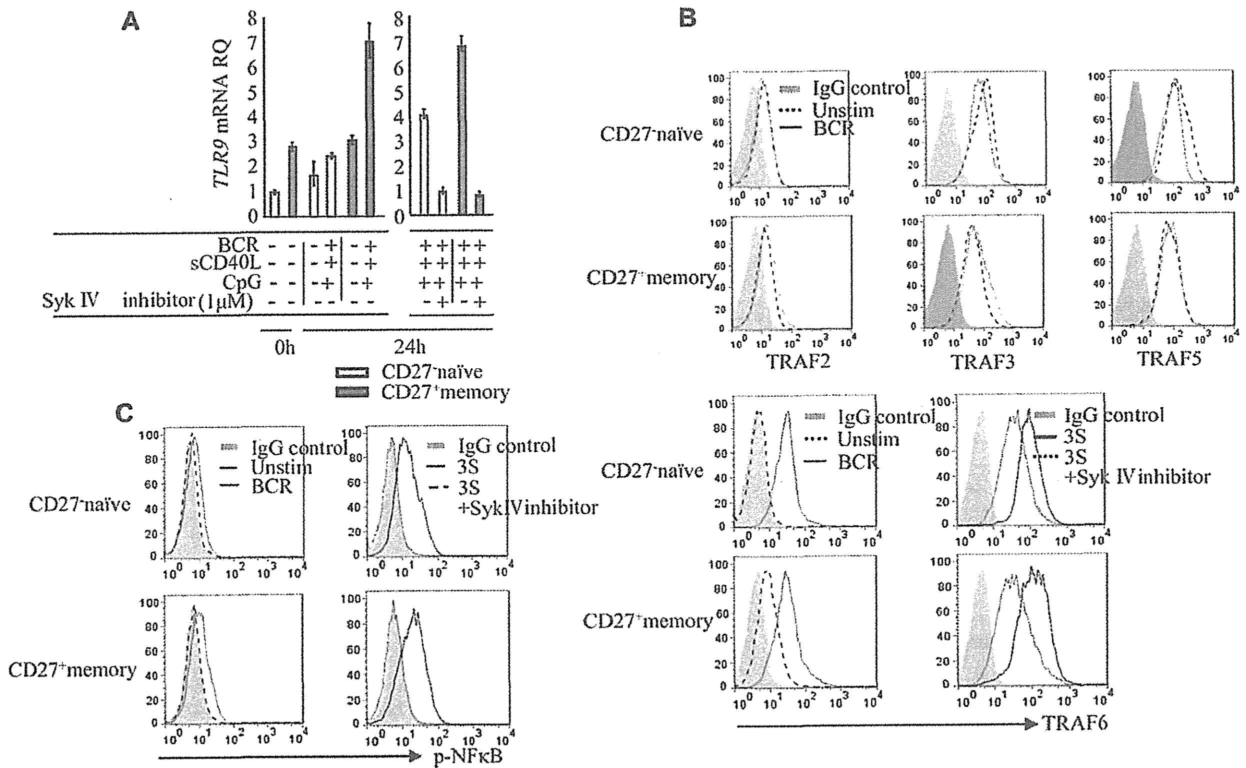

FIG 5. TLR9 and TRAF6 are key Syk-regulated molecules in B-cell subsets on stimulation. **A**, *TLR9* mRNA was quantified by using real-time PCR (TaqMan PCR kit) 24 hours later. *RQ*, Relative quantity; *sCD40L*, soluble CD40 ligand. **B** and **C**, TRAF2, TRAF3, TRAF5, and TRAF6 levels (48 hours later)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

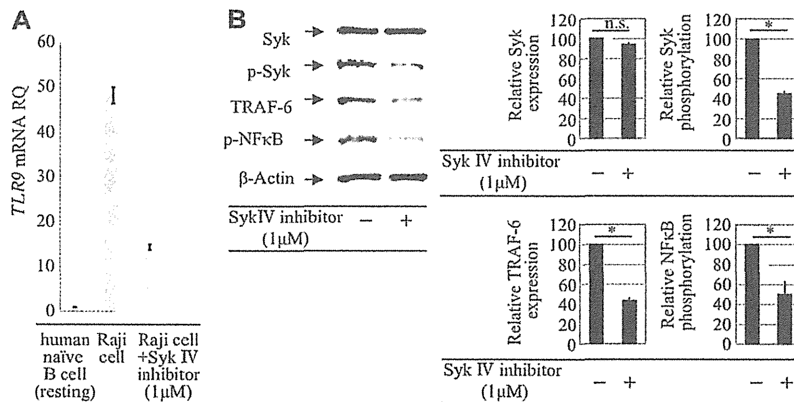

FIG 6. Syk inhibitor exerts marked inhibitory action, even at an activated state of B cells. Raji cells were cultured with RPMI containing 2% FCS for 48 hours. **A**, *TLR9* mRNA was quantified by means of real-time PCR. *RQ*, Relative quantity. **B**, Expression of Syk, phospho-Syk (Y348), TRAF6, and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

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

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

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

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

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

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

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

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

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