while it increased the production of IL-6 at concentrations of 3 and 10 μM on all days and MCP-1 at concentrations of 3 and 10 μM on days 3 and 7 (Fig. 2b). IFN-γ production was dose-dependently suppressed on days 3 and 7. Nilotinib inhibited IL-17 production in a dose-dependent fashion, significantly at concentrations of 1 μM and higher on days 7and 14.

Imatinib and nilotinib inhibit antigen-specific proliferation of CD4+ T cells

GPI-immunized mice were killed on day 10 and inguinal lymph nodes were harvested. Single suspended cells were stained with CFSE-DA and cultured with 5 μg/ml rhGPI for 4 days in the presence of 1, 3, and 10 μM imatinib or nilotinib, and cell proliferation was analyzed by flow cytometry. Imatinib at 3 μM exerted a mild antiproliferative effect on CD4+ T cells, compared with GPI-antigen stimulation ($P < 0.05$), whereas imatinib at 10 μM and nilotinib at 3 and 10 μM demonstrated a marked antiproliferative effect on CD4+ T cells ($P < 0.01$) (Fig. 3).

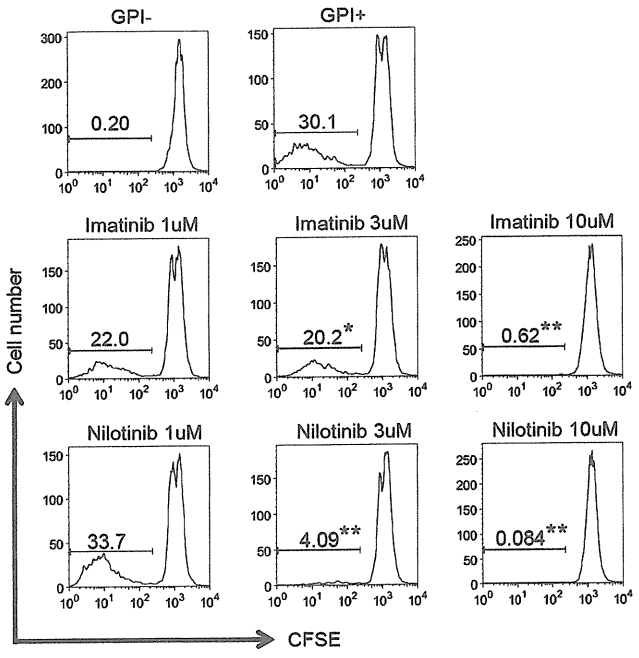

Fig. 3 Imatinib and nilotinib suppress CD4+ T-cell proliferation from arthritic lymph nodes. GPI-immunized mice were killed on day 10. Inguinal lymph nodes were harvested, and single-cell suspensions were prepared as described above. Cells stained with carboxyfluorescein diacetate succinimidyl ester (*CFSE-DA*) were cultured with 5 μg/ml of recombinant human (rh) GPI in 1, 3, and 10 μM imatinib or nilotinib for 4 days and then analyzed by flow cytometry. Imatinib at 3 μM exerted a mild antiproliferative effect on CD4+ T cells, whereas imatinib at 10 μM and nilotinib at 3 and 10 μM demonstrated a marked antiproliferative effect on CD4+ T cells. Data are representative of four independent experiments. *Numbers* represents the percentages of proliferating CD4+ T cells. $*P < 0.05$, $**P < 0.01$ compared with GPI-stimulated values

Analysis of IL-17 gene expression in joints

To determine serial changes of IL17 gene expression in the joints of GPI-induced arthritis, samples were taken on days 0, 7, and 14 and total RNA was extracted to synthesize cDNA. Real-time PCR was performed using GAPDH as an internal control. The IL-17 gene expression level on day 7 was significantly higher than that on day 0 ($P < 0.05$), while that on day 7 was higher than that on day 14 (Fig. 4). However, the expression levels of the IL-17 gene at any time point were significantly lower than the GAPDH mRNA expression level.

Discussion

In this study, we provide evidence to show that imatinib and nilotinib are effective against GPI-induced arthritis via different mechanisms. Imatinib and nilotinib inhibit Abl, Lck, KIT, PDGFR, DDR, and CSF-1R. Imatinib shows efficacy in murine models of arthritis, such as collagen-induced arthritis, anti-collagen antibody-induced arthritis, and K/BxN serum transfer-induced arthritis [13, 15–17]. Nilotinib has not previously been shown to have therapeutic effects in models of arthritis. In GPI-induced arthritis, administration of anti-CD4 mAb or anti IL-17 mAb markedly ameliorated the progress of arthritis [3]. Therefore, we considered that CD4+ T cells, especially Th17 cells, played a central role in the pathogenesis of GPI-induced arthritis. Although imatinib and nilotinib attenuate T-cell activation via inhibition of the T-cell

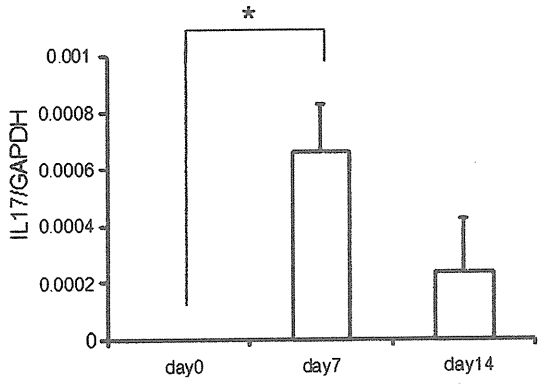

Fig. 4 Serial changes in expression levels of IL-17 in ankle joints of glucose-6-phosphate isomerase (*GPI*)-induced arthritis. To determine the time course of IL-17 expression in ankle joints of GPI-induced arthritis, samples on days 0, 7, and 14 were analyzed by real-time polymerase chain reaction (PCR). The IL-17 gene expression level on day 7 was significantly higher than that on day 0 ($P < 0.05$), while that on day 7 was higher than that on day 14. However, the expression levels of the IL-17 gene at any time point were significantly lower than the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression level. Data are means ± SEM of five mice per group. $*P < 0.05$ versus day 0 by Mann–Whitney *U*-test

receptor (TCR)-associated tyrosine kinase Lck, nilotinib inhibits Lck at approximately half the imatinib median inhibitory concentration (IC_{50}) [10]. Our in vitro study also showed that nilotinib suppressed IL-17 production and CD4+ T-cell proliferation more intensively than imatinib in antigen-stimulated splenocytes. Moreover, there is the possibility that nilotinib directly modulates T-cell differentiation to Th17, although the mechanism of such an effect remains to be elucidated. These factors may account for why nilotinib displayed more effectiveness than imatinib in the induction phase of GPI-induced arthritis. However, the administration of nilotinib from day 14 in the effector phase did not show any effect. This is probably because IL-17 does not have a crucial role in arthritic joints after day 14. We have already confirmed that IL-17 neutralization on day 14 was not effective in ameliorating GPI-induced arthritis [3]. In the present study, IL-17 gene expression in joints on day 14 tended to be lower than that on day 7. On the other hand, TNF-α gene expression in joints was similar on days 7, 14, and 28 [18]. Serum concentrations of TNF-α and IL-6 levels were higher than IFN-γ concentrations and IL-1β levels at the disease onset, although the data reflected systemic inflammation [4]. These cytokines might be key players in GPI-induced arthritis, especially later than 14 days after GPI-induction [3, 4].

Our data indicated that the severity of GPI-induced arthritis was suppressed by the administration of imatinib from day 0. TNF-α and IL-6 production in antigen-stimulated splenocytes on days 3, 7, and 14 was significantly suppressed by imatinib. In contrast, the production of TNF-α was not altered by nilotinib, suggesting a different mechanism from that of imatinib in affecting the cells. From the data in other models of arthritis, it seems that the cytokines TNF-α and IL-6 might be derived from macrophages and mast cells [13, 15]. Unexpectedly, we found in the present study that the production of IL-6 and MCP-1 in splenocytes in vitro was induced by nilotinib. The cells that dominantly produce IL-6 and MCP-1 are suspected to be macrophages, mast cells, and monocytes which can be modulated by the kinase activity associated with growth factor receptors, and so further investigation is needed. Imatinib significantly suppressed autoantibody production irrespective of the arthritis phase when the treatment started. Imatinib might directly inhibit B-cell proliferation and autoantibody production by blocking abelson kinase 1 (c-Abl) activity; otherwise, imatinib might suppress IL-6 expression, which stimulates B-cell activation and antibody production [19, 20]. However, it has not been shown whether nilotinib can affect Bcr-Abl-negative primary B cells. From our data showing that the administration of nilotinib from any time point did not influence autoantibody production, it seems that nilotinib may not work on B cells in GPI-induced arthritis.

The different profiles of cytokine production in splenocytes from mice treated with imatinib and nilotinib were confirmed (see Table 1 for a summary of results). Nilotinib has been exploited as a new Bcr-Abl inhibitor but it possesses a different kinase selectivity profile to that of imatinib [21]. Salih et al. [22] demonstrated that even a little difference in the kinase inhibition profile affected the signal transduction involved with cytokine secretion in immune cells. Imatinib showed no relevant effect on phosphatidylinositol 3-kinase (PI3K) or extracellular-signal-regulated kinase (ERK) activity and IFN-γ production in natural killer cells, while dasatinib, whose primary target is Bcr-Abl, the same as that of imatinib, decreased the phosphorylation of PI3K and ERK, causing lower IFN-γ production. Similar factors could explain the different mechanisms of imatinib and nilotinib action.

For the exploitation of kinase inhibitors, the selectivity of a compound is often determined by screening it against a small number of tyrosine kinases [23]. However, signal transduction pathways in cells interact, so that the efficacy of some drugs is likely to be related to the inhibition of multiple targets. The highest affinity off-target kinases were often kinases in the

Table 1 Suppression of cytokine production by imatinib and nilotinib in splenocytes of arthritic mice

Sampling day	Imatinib	Nilotinib
TNF-α		
Day 3	+++	0
Day 7	+++	0
Day 14	+++	0
IL-6		
Day 3	+++	0
Day 7	++	0
Day 14	++	0
MCP-1		
Day 3	+	0
Day 7	0	0
Day 14	0	0
IFN-γ		
Day 3	++	++
Day 7	++	+++
Day 14	++	0
IL-17		
Day 3	0	0
Day 7	++	+++
Day 14	+	+++

Glucose-6-phosphate isomerase (GPI)-immunized mice were killed on days 3, 7, and 14. The harvested splenocytes (2×10^6 cells/ml) were cocultured with 5 μg/ml of recombinant human (rh) GPI with 1, 3, and 10 μM imatinib or nilotinib for 72 h. The supernatants were assayed for tumor necrosis factor (TNF)-α, interleukin (IL)-6, monocyte chemotactic protein-1 (MCP-1), interferon (IFN)-γ, and IL-17 with a cytometric beads array or enzyme-linked immunosorbent assay (ELISA)

0, no change or increase; +, significant decrease at 10 μM; ++, significant decrease at 3 μM and more; +++, significant decrease at 1 μM and more

same group or family as the primary target, and some were kinases that were not closely related to their primary targets. To identify potential off-target kinases would be helpful for more accurately understanding the efficacy and safety of kinase inhibitors. Moreover, the identification of such off-target kinases could lead to unanticipated clinical applications for existing compounds. For example, the finding that imatinib inhibited KIT suggested its use for the treatment of gastrointestinal stromal tumors. Studying the effects of tyrosine kinase inhibitors such as nilotinib in disease models can also have the potential to discover new indications, related to either known or unknown kinase activities.

From a clinical perspective, as well as patients with chronic myeloid leukemia (CML) showing remission with imatinib, there are several case reports of patients with longstanding RA who have entered remission following the initiation of therapy with imatinib. An open-label study of imatinib in three patients with RA showed improvement following the initiation of treatment with imatinib 200–400 mg/day. Although it has not been published that there are any patients with RA who have experienced amelioration with nilotinib treatment since its approval in 2007, and it is expected that the clinical evidence will be reported [24, 25]. Our data in the present study are not sufficient to allow us to consider how to use these two compounds properly in clinical practice. But, according to its characteristic effectiveness on targeted cells and disease onset, nilotinib may be more appropriate than imatinib for treating inflammatory diseases relevant to T-cell cytokines, especially IL-17, in future applications. As imatinib has already been shown to have anti-inflammatory effects on mast cells, macrophages, and fibroblasts, as well as T cells and B cells, it could be employed for the treatment of immune-mediated and fibrotic diseases [26].

In conclusion, imatinib and nilotinib efficiently prevented GPI-induced arthritis via different mechanisms. Imatinib inhibited inflammatory and T-cell-derived cytokine production by splenocytes in vitro and autoantibody production in vivo, while, on the other hand, nilotinib suppressed T-cell-derived cytokine production, especially that of IL-17, in splenocytes. Further investigation to unravel these different mechanisms shown by similar compounds should shed light on new molecular targets underlying RA pathology.

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Conflict of interest None.

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ORIGINAL ARTICLE

Clinical features of reversible posterior leukoencephalopathy syndrome in patients with systemic lupus erythematosus

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Abstract To characterize reversible posterior leukoencephalopathy syndrome (RPLS) in systemic lupus erythematosus (SLE) in terms of treatments for resolution and its clinical course, we reviewed 28 cases of RPLS in SLE including our cases in view of the treatment. Of these, 15 cases improved with blood pressure control and 13 required immunosuppressive therapy for activity of SLE presenting neurological manifestations. Patients without immunosuppressants at onset of RPLS more frequently required immunosuppressive therapy to recover it than those precedingly using these agents [31% (4/13) versus 87% (13/15), $p = 0.008$, chi-square test]. Brain magnetic resonance imaging (MRI) is important for diagnosis of RPLS-SLE in the patient with SLE who develops neurological disturbance and rapidly increasing blood pressure. When 7-day therapy for hypertension and convulsion does not reverse the manifestations, immunosuppressive treatments would be recommended to reverse RPLS.

Keywords Reversible posterior leukoencephalopathy syndrome (RPLS) · Systemic lupus erythematosus (SLE) · Neuropsychiatric systemic lupus erythematosus (NPSLE) · Magnetic resonance imaging (MRI)

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Introduction

Systemic lupus erythematosus (SLE) involves the nervous system and presents with a number of different neurologic and psychiatric syndromes.

The American College of Rheumatology (ACR) has formulated case definitions, reporting standards, and diagnostic testing recommendations for 19 neuropsychiatric SLE (NPSLE) syndromes [1]. The most common neurologic manifestations of SLE include cognitive dysfunction, stroke, seizure, headaches, and peripheral neuropathy. Uncommon neurologic symptoms of SLE are movement disorders, chorea, cranial neuropathies, eye movement, and transverse myelitis and meningitis.

Reversible posterior leukoencephalopathy syndrome (RPLS) is a clinicoradiological syndrome that is associated with several conditions including hypertensive encephalopathy, chronic renal insufficiency, blood transfusion, and puerperal eclampsia [2]. RPLS produces clinical manifestations such as headache, confusion with conscious disorder, visual changes, and seizures with characteristic neuroimaging findings of posterior cerebral white matter edema. Imaging indicates presence of hyperpermeability of small vessels, one of the clinicopathological features of NPSLE.

RPLS also develops on the flare of SLE and is presumably induced by immunosuppressive therapy with cyclosporine or tacrolimus [3, 4].

Most cases of RPLS complicated with SLE (RPLS-SLE) are associated with triggers such as hypertension or use of immunosuppressive agents. Therefore, some cases of RPLS-SLE are recognized as a secondary complication of SLE rather than a primary effect of the disease [5].

We reviewed 28 cases of RPLS-SLE including our two cases in detail and propose management of RPLS-SLE that may be clinically useful.

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Association of a Functional Polymorphism in the 3′-Untranslated Region of *SPI1* With Systemic Lupus Erythematosus

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Objective. **SPI1, also referred to as PU.1, is an Ets family transcription factor that interacts with IRF2, IRF4, and IRF8. In view of the significance of the type I interferon pathway in systemic lupus erythematosus (SLE), this study was undertaken to investigate a possible association between *SPI1* polymorphisms and SLE.**

Methods. **A case–control association study was performed using 6 tag single-nucleotide polymorphisms (SNPs), as well as a SNP located upstream of *SPI1* previously found to be associated with acute myelogenous leukemia, in 400 Japanese patients with SLE and 450 healthy controls. Resequencing of all exons and known regulatory regions was performed to identify functional polymorphisms. Association of genotype and *SPI1* expression was examined using the GENEVAR database and reporter assays.**

Results. **A significant association was detected in 2 SNPs in intron 2 (rs10769258 and rs4752829) (P = 0.005 and P = 0.008, respectively, under the dominant model). The association was stronger in patients with nephropathy. Resequencing identified a potentially functional polymorphism in the 3′-untranslated region (3′-UTR), rs1057233, which was in strong linkage disequilibrium with the SNPs in intron 2. The number of risk alleles at rs1057233 was strongly correlated with *SPI1* messenger RNA (mRNA) level in the database analysis (P = 0.0002), and was confirmed by a reporter assay. Interestingly, rs1057233 alters a target sequence for microRNA hsa-miR-569 (miR-569). Transfection experiments demonstrated that miR-569 inhibits expression of a reporter construct with the 3′-UTR sequence containing the nonrisk allele but not the risk allele.**

Conclusion. **Our findings indicate that a SNP in the 3′-UTR of *SPI1* is associated with elevated *SPI1* mRNA level and with susceptibility to SLE.**

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by immunologic abnormalities and multiorgan injury involving the kidneys and the central nervous system. Although treatment and outcome have improved, prognosis is determined by the extent of such organ damage. The etiology of SLE is not yet fully understood; however, it is now apparent that multiple genetic and environmental factors are associated with its development.

Analysis of genetic factors in SLE has been greatly facilitated in the past few years by the progress in association studies. Both candidate-gene and genome-wide approaches have become much more efficient owing to the International HapMap Project and conve-

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nient and quick genotyping technologies. *IRF5*, *STAT4*, *ITGAM*, and *BLK* are among the genes that have been shown to be associated with SLE using these approaches (1–3).

Another important advance in understanding the pathogenesis of SLE in the past 10 years is the discovery of the role of the type I interferon (IFN) pathway (4). Elevated serum levels of IFN in SLE have been reported, and gene expression analyses have revealed upregulation of IFN-inducible genes in peripheral blood cells from SLE patients (5–7). *IRF5*, an IFN regulatory factor (IRF), is one of the genes outside the major histocompatibility complex (MHC) that is most consistently associated with SLE (8–11).

SPI1, also referred to as PU.1, is an Ets family transcription factor that is essential for lymphoid and myeloid development (12–14). The level of SPI1 expression is critical for determining cell fate, and decreased expression of SPI1 can lead to occurrence of leukemia and lymphoma (15–17). The *SPI1* gene is located on human chromosome 11p11. Its expression is regulated through the proximal promoter and upstream regulatory element located 17 kb upstream of the transcription start site (18,19). Antisense RNA transcribed from intron 3 of *SPI1* was also shown to regulate SPI1 expression by modulating messenger RNA (mRNA) translation (20).

Of interest, SPI1 has been shown to regulate expression of various genes in coordination with IRF2, IRF4, and IRF8 (21,22). Furthermore, in a recent study of identical twins discordant for SLE, the *SPI1* gene was identified as one of the significantly hypomethylated genes in affected twin members compared with unaffected twin members (23), indicating that SPI1 overexpression may play a role in the pathogenesis of SLE. These observations led us to test a possible association of *SPI1* polymorphisms with SLE, and to study the functional significance of the associated polymorphism.

PATIENTS AND METHODS

Patients and controls. Four hundred patients with SLE (28 men and 372 women with a mean ± SD age of 41.7 ± 13.7 years), and 450 healthy controls (227 men and 223 women with a mean ± SD age of 31.8 ± 8.8 years) were studied. Patients with SLE and healthy controls were recruited at the University of Tsukuba, Juntendo University, and the University of Tokyo. All patients fulfilled the American College of Rheumatology (ACR) classification criteria for SLE (24). Patients were categorized as those with and those without nephropathy, based on the ACR criteria. All patients and healthy controls were unrelated Japanese subjects living in the central part of Japan. This study was reviewed and approved by the Research Ethics Committees of University of Tsukuba, Juntendo University, and the University of Tokyo.

For the single-nucleotide polymorphisms (SNPs) rs4752829 (risk allele frequency in the population 0.43) and rs10769258 (risk allele frequency 0.60), this study had a power of 80% to detect an association when the genotype relative risk was ≥1.32 or ≥1.33, respectively, at the significance level 0.05 (25).

Preparation of genomic DNA. Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp blood kit (Qiagen) or a QuickGene-800 kit (Fujifilm). Whole-genome amplification was performed using a GenomiPhi DNA Amplification kit according to the recommendations of the manufacturer (Amersham Biosciences).

Genotyping. Tag SNPs were selected based on the HapMap Phase II data set for the Japanese in Tokyo, Japan (JPT) (http://hapmap.ncbi.nlm.nih.gov/index.html.ja). SNPs with an r^2 threshold of 0.85 and minor allele frequency of 0.05 were selected. A TaqMan SNP genotyping assay (Applied Biosystems) was used to determine the genotypes of all tag SNPs.

Genotyping of rs10769263, located ~17 kb upstream of the transcription start site of *SPI1*, was done by direct sequencing. The polymerase chain reaction (PCR) primers used were 5′-GGGGTACCTAGGCCTGAAGA-3′ and 5′-CTGTTGCCACGTCCACAC-3′. PCR products were subjected to sequencing reaction using a BigDye Terminator Cycle Sequencing kit, version 3.1, and sequenced with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Resequencing. All exons, the proximal promoter, upstream regulatory element (18,19), and antisense RNA transcription regulation site (20) in the *SPI1* gene were resequenced using genomic DNA from 15 patients with SLE and 16 healthy controls. Direct sequencing was conducted using a BigDye Terminator Cycle Sequencing kit, version 3.1 on an ABI Prism 3100 Genetic Analyzer. The genomic structure of the *SPI1* gene is shown in Figure 1A. The resequenced regions are indicated by boxes. Primers used for resequencing are shown in Supplementary Table 1, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131. The genotypes of 3′-untranslated region (3′-UTR) SNP rs1057233 were subsequently determined in all patients and controls by TaqMan SNP genotyping assay.

Association of *SPI1* 3′-UTR rs1057233 genotype with *SPI1* mRNA levels. For the association analysis of *SPI1* mRNA levels and the rs1057233 genotype, genotype data from HapMap samples from 45 Japanese subjects in Tokyo, Japan (JPT), 45 Han Chinese subjects in Beijing, China (CHB), 60 Utah residents with ancestry from northern and western Europe (CEU), and 60 Yoruban subjects in Ibadan, Nigeria (YRI) were obtained (http://hapmap.ncbi.nlm.nih.gov/index.html.ja).

Normalized mRNA data from Epstein-Barr virus–transformed lymphoblastoid cell lines derived from HapMap donors were obtained from the Gene Expression Variation (GENEVAR) project database at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/humgen/genevar/). The probe used for *SPI1* expression analysis was GI_4507174-S on the Illumina human whole-genome expression array (WG-6 version 1). Next, the correlation between the number of risk

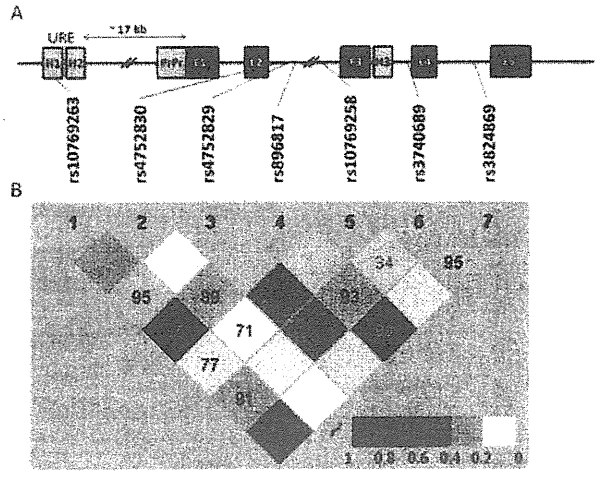

Figure 1. Structure of the *SPI1* gene and linkage disequilibrium plot of the single-nucleotide polymorphisms (SNPs) examined in this study. **A,** Genomic structure of the *SPI1* gene and SNPs typed in the present study. Boxes indicate resequenced regions. Gray boxes show regulatory regions, including the upstream regulatory elements (UREs) H1 and H2, the antisense RNA transcription regulation site H3, and the proximal promoter (PrPr) region. Black boxes show exon 1 (E1) through exon 5. The size of each box is not to scale. **B,** Linkage disequilibrium plot of the genotyped SNPs. Calculation of linkage disequilibrium parameters (r^2 and D′) based on genotype data from 450 healthy controls was performed using Haploview software. Values in each square are D′. Empty squares indicate that D′ is 1. Darker shading indicates higher r^2 values.

alleles, rs1057233 T, and *SPI1* mRNA levels was examined by simple regression analysis, as previously described (8,26).

Reporter assay. The *Renilla* luciferase constructs that contain 3′-UTR of *SPI1* were used for the reporter assay. The *SPI1* 3′-UTR carrying each C or T allele at rs1057233 was amplified with a forward primer (5′-CTCCCCTCGAGCGCTGGCCATAGCATTAAG-3′) and a reverse primer (5′-AAGAGGCGGCCGCGATGGATTGAGAATAACTTTACTTGTTT-3′) from a heterozygous genomic DNA sample. The PCR products were inserted into the 3′-UTR of the *Renilla* luciferase gene in the reporter vector psiCHECK-2 (Promega) by digestion using the restriction enzymes *Not* I and *Xho* I. The inserts containing rs1057233 C and T were confirmed by sequencing.

A human chronic myelogenous leukemia cell line, K562, and a human T cell lymphoma cell line, Jurkat, were maintained in RPMI 1640 with 10% fetal bovine serum (FBS) in a 37°C incubator supplemented with 5% CO_2. HEK 293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. MicroRNA hsa-miR-569 (miR-569) and the nontarget control were purchased from B-Bridge. Transfections of reporter vectors and miR-569 were performed in 96-well culture plates using Lipofectamine 2000, according to the recommendations of the manufacturer (Invitrogen). After 24 hours of transfection, luciferase activities were detected using a Dual-Glo Luciferase Assay System (Promega). Luminescence was measured with a TD-20/20 luminometer (Turner Biosystems). *Renilla* luciferase activities were normalized to firefly luciferase activities. Each assay was performed in 5 replicates. Student's *t*-test was used for statistical analysis of reporter assays.

Statistical analysis. Association analyses were conducted by chi-square test using 2 × 2 or 2 × 3 contingency tables. Deviation from Hardy-Weinberg equilibrium was tested by chi-square test. Linkage disequilibrium parameters (r^2 and D′) were calculated based on genotype data from healthy controls, using Haploview software, version 4.2 (available at http://www.broad.mit.edu/mpg/haploview/index.php). Sex differences between cases and controls were adjusted for using logistic regression analysis.

RESULTS

Association of *SPI1* tag SNPs with SLE. The *SPI1* gene is located on human chromosome 11p11 with a length of 24 kb. Among the 29 SNPs in the HapMap phase II data set in this gene, 14 were monomorphic in JPT samples. An additional 15 SNPs had a minor allele frequency of >0.05, and all of them were tagged by 6 tag SNPs based on the criteria of the r^2 threshold of 0.85. According to the HapMap JPT data, the *SPI1* region is covered by 2 linkage disequilibrium blocks which extend 30 kb and 4 kb, respectively. Together, these 2 blocks contain 28 SNPs with a minor allele frequency of >0.05, and all SNPs except for rs12422023 are tagged by the 6 tag SNPs genotyped in the present study. In addition, rs10769258, a SNP previously reported to be associated with susceptibility to acute myelogenous leukemia (27), was genotyped because of potential functional relevance (Figure 1).

The genotyping success rate was 100% for all SNPs. No SNPs showed significant deviation from Hardy-Weinberg equilibrium (Table 1). Two SNPs in significant linkage disequilibrium with each other (D′ = 0.977, r^2 = 0.484), rs4752829 and rs10769258, were found to be associated with SLE at a significance level of 0.05 (Table 1).

Nephropathy represents a severe manifestation of SLE, and some genes have been shown to be associated more strongly with SLE with nephropathy (28,29). Clinical information regarding the presence or absence of nephropathy was available in all cases in the present study. Therefore, we examined whether the association of *SPI1* was stronger in the patients with nephropathy. Both SNPs showed significant association with SLE with nephropathy, but not with SLE without nephropathy (Table 2).

Because the sex ratio was different between the case group and the control group, logistic regression

Table 1. Association study of *SPI1* SNPs in Japanese patients with SLE*

SNP, allele 1/2	Genotype count (%)			Frequency of allele 1	HWE *P*	1 vs. 2		(1/1 + 1/2) vs. 2/2	
	1/1	1/2	2/2			*P*	OR (95% CI)	*P*	OR (95% CI)
rs10769263 (enhancer), T/G									
SLE	272 (68)	110 (28)	18 (5)	0.82	0.12	0.96	1.01 (0.79–1.29)	0.38	0.73 (0.36–1.47)
Control	300 (67)	135 (30)	15 (3)	0.82	0.97				
rs4752830 (intron 2), C/T									
SLE	362 (91)	37 (9)	1 (0)	0.95	0.96	0.47	1.17 (0.76–1.80)	0.63	1.78 (0.17–19.11)
Control	401 (89)	47 (10)	2 (0)	0.94	0.62				
rs4752829 (intron 2), T/C									
SLE	83 (21)	215 (54)	102 (26)	0.48	0.12	0.05	1.21 (1.00–1.47)	0.008	1.49 (1.11–2.01)
Control	88 (20)	210 (47)	152 (34)	0.43	0.31				
rs896817 (intron 2), T/C									
SLE	18 (5)	107 (27)	275 (69)	0.18	0.08	0.68	1.05 (0.82–1.35)	0.87	0.98 (0.73–1.30)
Control	11 (2)	132 (29)	307 (68)	0.17	0.47				
rs10769258 (intron 2), A/G									
SLE	164 (41)	197 (49)	39 (10)	0.66	0.07	0.01	1.29 (1.06–1.57)	0.005	1.79 (1.19–2.70)
Control	160 (36)	217 (48)	73 (16)	0.60	0.97				
rs3740689 (intron 3), C/T									
SLE	146 (37)	197 (49)	57 (14)	0.61	0.47	0.09	1.18 (0.97–1.43)	0.049	1.44 (1.00–2.08)
Control	151 (34)	212 (47)	87 (19)	0.57	0.42				
rs3824869 (intron 4), A/G									
SLE	10 (3)	85 (21)	305 (76)	0.13	0.17	0.40	1.13 (0.85–1.51)	0.65	1.08 (0.78–1.48)
Control	5 (1)	96 (21)	349 (78)	0.12	0.57				
rs1057233 (3′-UTR), T/C									
SLE	167 (42)	194 (49)	39 (10)	0.66	0.11	0.03	1.24 (1.02–1.51)	0.03	1.59 (1.05–2.42)
Control	165 (37)	219 (49)	66 (15)	0.61	0.63				

* SNPs = single-nucleotide polymorphisms; SLE = systemic lupus erythematosus; HWE = Hardy-Weinberg equilibrium; OR = odds ratio; 95% CI = 95% confidence interval; 3′-UTR = 3′-untranslated region.

analysis was performed. The association essentially remained significant after adjustment for sex for all SLE patients versus controls (for rs10769258, additive model $P = 0.043$, dominant model $P = 0.048$ and for rs4752829, additive model $P = 0.067$, dominant model $P = 0.021$) and for SLE patients with nephropathy versus controls (for rs10769258, additive model $P = 0.016$, dominant model $P = 0.047$ and for rs4752829, additive model $P = 0.024$, dominant model $P = 0.006$).

Resequencing of *SPI1* to search for functional polymorphisms. Both of the associated SNPs were located in intron 2, and their functional significance was not easily determined. Therefore, we next searched for functional polymorphisms in *SPI1* by resequencing the upstream regulatory element, proximal promoter, antisense RNA transcription regulation site, and all exons of the *SPI1* gene (Figure 1A) in 15 patients with SLE and 16 healthy controls. Resequencing revealed 3 previously

Table 2. Preferential association of *SPI1* intron 2 SNPs with SLE with nephropathy*

SNP, allele 1/2	Genotype count (%)			Frequency of allele 1	1 vs. 2		(1/1+1/2) vs. 2/2	
	1/1	1/2	2/2		*P*	OR (95% CI)	*P*	OR (95% CI)
rs4752829, T/C								
SLE with nephropathy	43 (22)	111 (56)	43 (22)	0.50	0.02	1.33 (1.05–1.69)	0.002	1.83 (1.24–2.69)
SLE without nephropathy	40 (20)	104 (51)	59 (29)	0.45	0.41	1.10 (0.87–1.40)	0.23	1.24 (0.87–1.79)
Control	88 (20)	210 (47)	152 (34)	0.43		Reference		Reference
rs10769258, A/G								
SLE with nephropathy	86 (44)	94 (48)	17 (9)	0.68	0.007	1.40 (1.10–1.80)	0.01	2.05 (1.18–3.55)
SLE without nephropathy	78 (38)	103 (51)	22 (11)	0.64	0.16	1.19 (0.93–1.52)	0.07	1.59 (0.96–2.64)
Control	160 (36)	217 (48)	73 (16)	0.60		Reference		Reference
rs1057233, T/C								
SLE with nephropathy	87 (44)	93 (47)	17 (9)	0.68	0.02	1.34 (1.05–1.73)	0.03	1.82 (1.04–3.17)
SLE without nephropathy	80 (39)	101 (50)	22 (11)	0.64	0.26	1.15 (0.90–1.47)	0.18	1.41 (0.85–2.36)
Control	165 (37)	219 (49)	66 (15)	0.61		Reference		Reference

* SNPs = single-nucleotide polymorphisms; SLE = systemic lupus erythematosus; OR = odds ratio; 95% CI = 95% confidence interval.

Table 3. SNPs detected by resequencing and their linkage disequilibrium with *SPI1* SNPs associated with SLE*

SNP	Position	rs4752829		rs10769258	
		D′	r^2	D′	r^2
rs3740688	Intron 4	0.75	0.30	1	0.88
rs3882728	Intron 4	1	0.77	1	0.36
rs1057233	3′-UTR	0.97	0.45	0.99	0.92

* D′ and r^2 were calculated based on genotype data from 450 healthy Japanese individuals (for rs1057233) or 16 healthy Japanese individuals (for rs3740688 and rs3882728). SNPs = single-nucleotide polymorphisms; SLE = systemic lupus erythematosus; 3′-UTR = 3′-untranslated region.

described SNPs, all of which were in significant linkage disequilibrium with rs4752829 and rs10769258 in healthy individuals (Table 3).

The results of the association study of rs1057233 are shown in Tables 1 and 2. The T allele of rs1057233 was in strong linkage disequilibrium with the risk alleles of rs4752829 and rs10769258. As expected, rs1057233 showed an association with SLE comparable to that of rs4752829 and rs10769258. Among these 3 SNPs, rs1057233 was located in the 3′-UTR and was considered most likely to be functionally relevant.

Correlation of the number of rs1057233 risk alleles and *SPI1* expression. We next assessed the functional significance of the 3′-UTR SNP rs1057233. First, association between the rs1057233 genotype and *SPI1* mRNA level was analyzed using the GENEVAR database (http://www.sanger.ac.uk/humgen/genevar/), which displays mRNA expression profiles of B lymphoblastoid cell lines established from HapMap donors. The number of rs1057233 T alleles was significantly correlated with an increased level of *SPI1* mRNA in all 3 populations, JPT + CHB, CEU, and YRI, represented in the HapMap project (Figure 2).

Functional analysis of rs1057233 by reporter assay. To confirm the functional significance of rs1057233, we next performed a reporter assay using reporter constructs containing the *SPI1* 3′-UTR. We inserted *SPI1* 3′-UTR that included either the rs1057233 C allele or the rs1057233 T allele into the 3′-UTR region of *Renilla* luciferase of the psiCHECK-2 vector, which also carries firefly luciferase, for the purpose of normalization.

When the reporter vectors were transfected into the K562 cell line, the construct that contains the risk allele (rs1057233 T) showed significantly higher *Renilla* luciferase activity than did the nonrisk C allele (Figure 3A). Similar results were observed when HEK 293T cells were used, although such a difference was not observed when HeLa and Jurkat cells were used. These results indicate that, consistent with the results of the analysis using the GENEVAR database, rs1057233 indeed influences the expression of *SPI1*, possibly in a cell type–specific manner.

Alteration of the target sequence of miR-569 by the SNP rs1057233. MicroRNA are small noncoding transcripts of ~22 nucleotides that inhibit the translation or promote the degradation of complementary mRNA, usually by binding to the 3′-UTR. We therefore examined whether *SPI1* rs1057233 could be a target for microRNA. Analysis using TargetScan (http://www.targetscan.org/) indicated that rs1057233 is located within a predicted binding site of miR-569 (Figure 3B). The *SPI1* 3′-UTR that contains the nonrisk allele

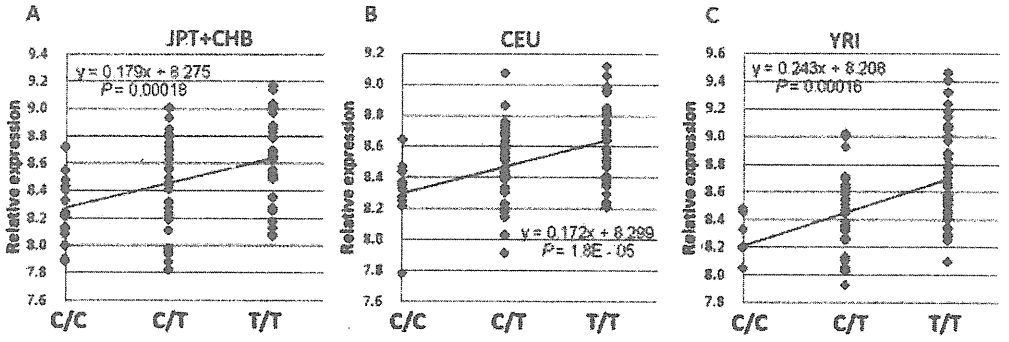

Figure 2. Association of rs1057233 genotype and *SPI1* mRNA levels. *SPI1* mRNA levels in the B lymphoblastoid cell lines established from donors in the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/index.html.en) were retrieved from the GENEVAR database (http://www.sanger.ac.uk/humgen/genevar/), and the correlation between the number of T alleles and mRNA levels was examined using simple regression analysis. A, Donors of Asian ancestry (JPT + CHB). B, Donors of European ancestry (CEU). C, Donors of African ancestry (YRI).

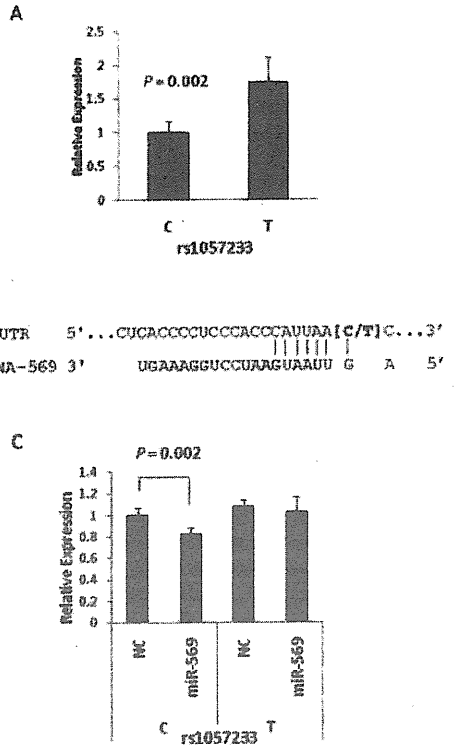

Figure 3. The risk allele at the *SPI1* 3′-untranslated region (3′-UTR) single-nucleotide polymorphism (SNP) rs1057233 alters the target sequence of microRNA hsa-miR-569 (miR-569) and is associated with elevated expression. **A**, Luciferase activity in constructs containing the rs1057233 C and T alleles, determined by reporter assay. K562 cells transfected with a reporter vector containing the risk allele (rs1057233 T) showed increased luciferase activity. Bars show the mean ± SD of 5 replicates. The experiment was repeated 3 times with similar results. **B**, Results of TargetScan (http://www.targetscan.org/) analysis. The analysis indicated a perfect match of the *SPI1* rs1057233 C allele, but not T allele, with the target sequence of miR-569. **C**, Inhibition of luciferase activity in constructs containing the rs1057233 C allele, determined by reporter assay. The reporter assay was performed by cotransfection of reporter vectors containing each allele of *SPI1* 3′-UTR and miR-569 into HeLa cells. Inhibition of luciferase activity was observed only in the reporter construct containing the nonrisk allele (C), and not in the construct containing the risk allele (T). Bars show the mean ± SD of 5 replicates. The experiment was repeated 3 times with similar results. NC = nontarget control.

(rs1057233 C) shows an exact match with positions 2–8 of the miR-569, whereas the risk allele (rs1057233 T) contains 1 mismatch.

We hypothesized that this mismatch might reduce the inhibitory effect of miR-569–mediated suppression of *SPI1*, resulting in higher *SPI1* expression. To test this, we performed a reporter assay by cotransfection of the reporter vectors and miR-569. Reporter constructs that contained the nonrisk allele (rs1057233 C) showed reduced luciferase activity in the presence of miR-569, whereas reporter activity of the constructs with the risk allele (rs1057233 T) was not affected by miR-569 (Figure 3C). This experiment was repeated 3 times with similar results.

DISCUSSION

In this study, we demonstrated that *SPI1* polymorphisms are associated with susceptibility to SLE in a Japanese population. The association was preferentially observed in patients with nephropathy. Resequencing of the *SPI1* gene identified a possible functional SNP, rs1057233, in the 3′-UTR. Expression of *SPI1* mRNA was correlated with the number of risk alleles (rs1057233 T), and a reporter assay confirmed the higher expression of the 3′-UTR sequence carrying rs1057233 T. Of particular interest, the rs1057233 SNP alters the target sequence of microRNA miR-569. Indeed, transfection experiments demonstrated that miR-569 can inhibit the expression of a reporter construct containing the nonrisk allele (rs1057233 C), but not the risk allele (T), suggesting that the loss of microRNA-mediated regulation of expression could be a potential molecular mechanism of overexpression of *SPI1* that might contribute to the occurrence of SLE.

SPI1 belongs to the Ets family, and is one of the key regulators in the differentiation of lymphoid and myeloid cells (12–14). Notably, another member of the Ets family, *ETS1*, has been shown to be associated with SLE (3,30). At an early stage of hematopoietic differentiation, SPI1 is highly expressed in hematopoietic stem cells, common lymphoid progenitors, granulocyte/macrophage progenitors, and early thymic progenitors (31,32). *SPI1* plays an important role in hematopoietic differentiation by regulating the expression of genes such as macrophage colony-stimulating factor receptor (33), granulocyte–macrophage colony-stimulating factor receptor (34), and interleukin-7 receptor (35). Moderate expression of SPI1 favors the B cell commitment and higher expression promotes myeloid differentiation (36). Thus, the SNP rs1057233 could possibly influence the hematopoietic differentiation profile.

In more differentiated cells, SPI1 expression is the highest in granulocytes and macrophages, modest in B cells, and absent in T cells. Although this remains a subject of controversy, several studies have indicated that SPI1 regulates the expression of genes that are required for terminal differentiation of B cells. Expres-

sion of Ig light and heavy chains is reported to be controlled by SPI1 (37), and higher expression of SPI1 might promote autoantibody production. The class II MHC transcription activator that induces class II MHC genes is also up-regulated by SPI1 (38,39). In macrophages, SPI1 expression induces the expression of cyclooxygenase 2 (40,41). Changes in the expression of these genes could also play a role in the pathogenesis of SLE.

Another important factor that links SPI1 and SLE is the IFN pathway. SPI1 has been shown to interact with IRF2, IRF4, and IRF8 in the induction of various genes including proinflammatory cytokines (21,22,42). IRF8 has also been shown to play a role in the induction of type I IFN (43,44). Thus, SPI1 may contribute to the production of inflammatory cytokines and "IFN signature" through interaction with IRF family transcription factors.

Of particular interest, a recent report suggested that epigenetic regulation of *SPI1* may play a role in SLE. Javierre et al (23) carried out a genome-wide DNA methylation analysis in monozygotic twins discordant for SLE, in which they compared methylation profiles of white blood cells from affected twin members with those from respective unaffected members. *SPI1* was listed as one of the 49 hypomethylated genes in the affected twin members (23), suggesting that it is epigenetically up-regulated in SLE. Taken together with the results of the present study, these findings indicate that *SPI1* may be involved in the pathogenesis of SLE through both genetic and epigenetic mechanisms.

Another new finding of this study was that rs1057233 alters the target sequence of miR-569. We present experimental evidence that expression of the nonrisk allele, but not the risk allele, was down-regulated by miR-569. These results may indicate a potential mechanism of overexpression of *SPI1* in the carriers of the risk allele. Since the expression profile of miR-569 remains unknown, investigation of the miR-569 expression pattern might provide new insight into the role of microRNA in SLE.

SPI1 has not been reported to be associated with SLE at the genome-wide significance level in any of the genome-wide association studies (GWAS) performed in Caucasian or Chinese populations (1–3,28,45,46). Among the *SPI1* SNPs, rs10769258 in intron 2 and rs3740689 in intron 3 have been examined in GWAS in Caucasians (1,2) (data available from dbGaP, http://www.ncbi.nlm.nih.gov/gap). Although rs1057233 in the 3′-UTR was not represented in the GWAS platform, this SNP appears to be in absolute linkage disequilibrium ($r^2 = 1$) with rs10769258 in CEU samples, according to the HapMap phase II database. Whether *SPI1* SNPs were represented in the platform used in the Chinese GWAS is unclear.

Although the reason for the difference in the association of *SPI1* with SLE between the GWAS and this study is unclear, it may be due to ethnic differences, for example, in the genes or environmental factors that interact with *SPI1*. Population difference in the genetic background was observed even between the closely related Chinese and Japanese populations (47,48).

It should be noted that the resequencing performed on 31 individuals in this study was aimed at detecting a functionally relevant common polymorphism which could account for the association of common SNPs (rs4752829 and rs10769258), and not at testing the multiple rare variant model. The latter requires resequencing of the entire *SPI1* gene in a large number of cases and controls, which should be performed in a different study.

In this study, 3 models (allelic, dominant, and recessive) were tested for 8 SNPs. In addition, for the 3 SNPs that showed an association with SLE, patients with and those without nephropathy were separately compared with controls under allelic and dominant models for the risk alleles; thus, the total number of comparisons was 36. When Bonferroni correction was applied, none of the comparisons remained significant. However, because of linkage disequilibrium, the genotype frequencies of the 8 SNPs are not independent, and the 3 models (allelic, dominant, and recessive) are obviously not independent. Therefore, Bonferroni correction is considered to be too conservative. Because there is no established method to correct for the number of tested SNPs and genetic models that are not independent, we have presented the uncorrected *P* values in this report. The sample size in this study was not sufficiently large to obtain conclusive results, and independent confirmation will be necessary to validate the association.

In conclusion, we detected an association of *SPI1* polymorphisms with SLE. A potentially causative allele was identified to be rs1057233 T in the 3′-UTR, which abolishes the miR-569 binding motif and is associated with elevated *SPI1* mRNA levels. Taken together with recent findings from other investigators (3,23,30), our findings indicate that Ets family genes including *SPI1* and *ETS1* may emerge as new and attractive molecular targets in SLE. Association of *SPI1* with SLE should be tested for independent replication to validate its role in SLE occurrence.

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. Tsuchiya 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. Hikami, Kawasaki, Tsuchiya.

Acquisition of data. Hikami, Kawasaki, I. Ito, Koga, S. Ito, Hayashi, Matsumoto, Tsutsumi, Kusaoi, Takasaki, Hashimoto, Arinami, Sumida, Tsuchiya.

Analysis and interpretation of data. Hikami, Kawasaki, I. Ito, Tsuchiya.

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ORIGINAL ARTICLE

Importance of serine727 phosphorylated STAT1 in IFNγ-induced signaling and apoptosis of human salivary gland cells

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Abstract

Aim: It is reported that in salivary glands of Sjögren's syndrome (SS), interferon gamma (IFNγ) and IFNγ-inducible genes containing signal transducers and activators of transcription 1 (STAT1) are upregulated and play a crucial role in the pathogenesis of SS. The aim of this study is to clarify which phosphorylation of STAT1, serine727 (Ser^{727}) or tyrosine701 (Tyr^{701}) of STAT1, is important for IFNγ signaling and IFNγ-induced apoptosis in salivary gland cells.

Methods: We established STAT1 Tyr^{701} variant (tyrosine to phenylalanine; Y701F) and STAT1 Ser^{727} variant (serine to alanine; S727A), which were transfected into human salivary gland (HSG) cells. HSG cells transfected with these mutant-STAT1 were analyzed on the expression of IFNγ-inducible genes and apoptosis after stimulation with IFNγ.

Results: In Y701F mutant-STAT1 transfected HSG cells (Ser^{727}-dominant HSG cells), IFNγ-inducible genes such as IP10, IRF1, and Fas expression were increased after stimulation with IFNγ. In Ser^{727}-dominant HSG cells, the induction of apoptosis after stimulation with IFNγ was also increased compared with S727A mutant-STAT1 transfected HSG cells (Tyr^{701}-dominant HSG cells).

Conclusion: Phosphorylation of Ser^{727} in STAT1 might be more important in IFNγ signaling and the induction of apoptosis in HSG cells than phosphorylation of Tyr^{701}. Accordingly, we propose that phosphorylation of Ser^{727} in STAT1 could be a potentially suitable new therapeutic target for SS patients to prevent the destruction of salivary glands.

Key words: apoptosis, human salivary gland cells, Interferon gamma, signal transducers and activators of transcription 1, Sjögren's syndrome.

INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized pathologically by focal lymphocytic infiltration of exocrine glands, especially lachrymal and salivary glands, and clinically by dry eyes and dry mouth.[1] Inflammatory cytokines, especially Interferon

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gamma (IFNγ), are considered to play an important role in the destruction of the exocrine glands, based on the detection of IFNγ messenger RNA (mRNA) expression in the salivary glands of SS patients.[2] Increased concentrations of IFNγ contribute to the upregulation of human leukocyte antigen (HLA) class II and co-stimulatory molecules on epithelial cells and antigen-presenting cells. IFNγ also upregulates Fas and caspase-8 expression and therefore fosters apoptosis.[3] Moreover, we also reported the upregulation of several IFNγ-inducible genes containing signal transducers and activators of transcription 1 (STAT1) in the salivary glands of SS patients.[4] Thus, the IFNγ signaling seems to play a crucial role in the pathogenesis of SS.

STAT1 is known as the mediator of IFNγ signaling. Maximal activation by STAT1 of the IFNγ signaling requires phosphorylation of both tyrosine701 (Tyr^{701}) and serine727 (Ser^{727}).[5,6] We reported previously the induction of STAT1-α phosphorylation and the different localization of Tyr^{701}-phosphorylated STAT1-α and Ser^{727}-phosphorylated STAT1-α in the labial salivary glands of patients with SS.[7]

Stephanou *et al.*[8] reported that induction of apoptosis and Fas expression by ischemia/reperfusion in cardiac myocytes required Ser^{727} of STAT1 but not Tyr^{701}, suggesting that Ser^{727}-phosphorylated STAT1-α is essential for the induction of apoptosis. However, it is not clear which phosphorylation of STAT1 induces apoptosis by IFNγ in salivary gland cells. To clarify this question, we established a STAT1 Tyr^{701} variant (tyrosine to phenylalanine; Y701F) and STAT1 Ser^{727} variant (serine to alanine; S727A), which were transfected into human salivary gland (HSG) cells. HSG cells transfected with these mutant-STAT1 were analyzed on the expression of IFNγ-inducible genes and apoptosis after stimulation with IFNγ.

MATERIALS AND METHODS

Production of mutant-STAT1 and transformation of mutant-STAT1 into HSG cells

We made the Tyr^{701} mutant (tyrosine to phenylalanine; Y701F) and Ser^{727} mutant (serine to alanine; S727A) STAT1 DNA fragments (Fig. 1a). These two mutant-STAT1 DNA fragments were inserted into myc-His vectors by double digestion with BamH I and Nod I (Fig. 1b). 1.0×10^5/mL of HSG cells were pre-cultured overnight, and then culture medium was changed to medium without sera. Mutant-STAT1 Y701F and S727A were transformed into HSG cells using FuGENE6 (Roche Applied Science, Mannheim, Germany). HSG cells transfected with mutant-STAT1 were selected by zeosin. HSG cells with Y701F mutant-STAT1 were Ser^{727}-dominant HSG cells, and HSG cells with S727A mutant-STAT1 were Tyr^{701}-dominant HSG cells.

IFNγ-inducible gene expression in mutant-STAT1-transfected HSG cells after stimulation with IFNγ

Human salivary gland cells transfected with Y701F, S727A mutant-STAT1 and empty vector were stimulated with IFNγ (2000 U/mL) for 24 h. These HSG cells were trypsinized and total RNA and cell lysate were extracted from HSG cells. Complementary DNA (cDNA) was synthesized by cDNA synthesis kit (Fermentas International, Burlington, ON, Canada). Reverse transcription–polymerase chain reaction (RT-PCR) and quantitative PCR were performed with cDNA using the human IFNγ-inducible protein 10 (IP10), interferon regulatory factor 1 (IRF1), Fas and CD40 specific primers. Human-glyceralaldehyde-3-phosphate dehydrogenase (GAPDH) was amplified to assess the cDNA yield and to analyze as the internal control.

IFNγ-inducible gene expression was examined at protein levels by Western blot analysis using the cell lysate of HSG cells. Total proteins were fractionated on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in 100% Block-Ace (Dainippon, Osaka, Japan) for 1 h and then incubated with rabbit anti-Fas antibody (1 : 500 dilution; Cell Signaling Technologies, Beverly, MA, USA) or mouse anti-β-actin antibody (2 μg/mL; BioVision, Mountain View, CA, USA) at 4°C overnight. Secondary antibodies, anti-rabbit IgG horseradish peroxidase (HRP) linked antibody (1 : 2000 dilution; Cell Signaling Technologies) or anti-mouse IgG HRP linked antibody (1 : 2000 dilution; Dako, Tokyo, Japan), were applied at room temperature for 1 h, and then proteins were detected by enhanced chemiluminescence using an ECL Western blot detection kit (Amersham, Little Chalfont, UK).

Apoptosis of mutant-STAT1-transfected HSG cells after stimulation with IFNγ

Human salivary gland cells transfected with Y701F, S727A mutant-STAT1 and empty vector were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and peni-

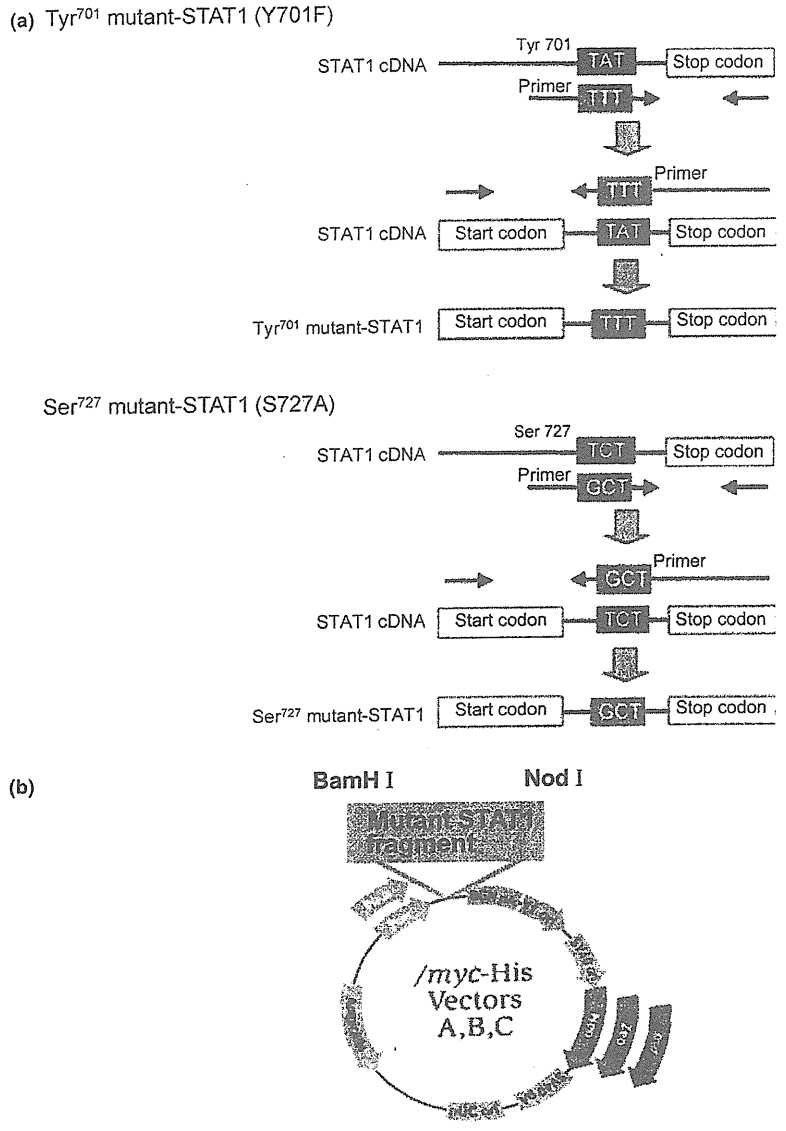

Figure 1 Production of mutant-STAT1 and transformation of mutant-STAT1 into HSG cells. (a) Production of tyrosine701 (Tyr^{701}) mutant (tyrosine to phenylalanine; Y701F) and serine727 (Ser^{727}) mutant (serine to alanine; S727A) STAT1 DNA fragments. (b) Y701F and S727A mutant-STAT1 DNA fragments were inserted into myc-His vectors by double digestion with BamH I and Nod I.

cillin/streptomycin. After stimulation with IFNγ (2000 U/mL) for 24 h, cells were trypsinized and harvested. Harvested HSG cells were washed in phosphate-buffered saline (PBS) and resuspended in binding buffer containing annexin-V-fluorescein isothiocyanate to monitor apoptosis-associated plasma membrane alteration for 20 min at room temperature. The samples were analyzed with a FACS Calibur flow cytometer (BD-Biosciences, Mountain View, CA, USA), and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

RESULTS

IFNγ-inducible gene expression was increased in Ser^{727}-dominant HSG cells after stimulation with IFNγ

Figure 2 shows the expression of IFNγ-inducible gene mRNA in mutant-STAT1 and empty vector-transfected HSG cells after stimulation with IFNγ. In Y701F mutant-STAT1-transfected HSG cells (Ser^{727}-dominant HSG cells), IP10 and IRF1 mRNA expression were increased after stimulation with IFNγ, whereas Fas

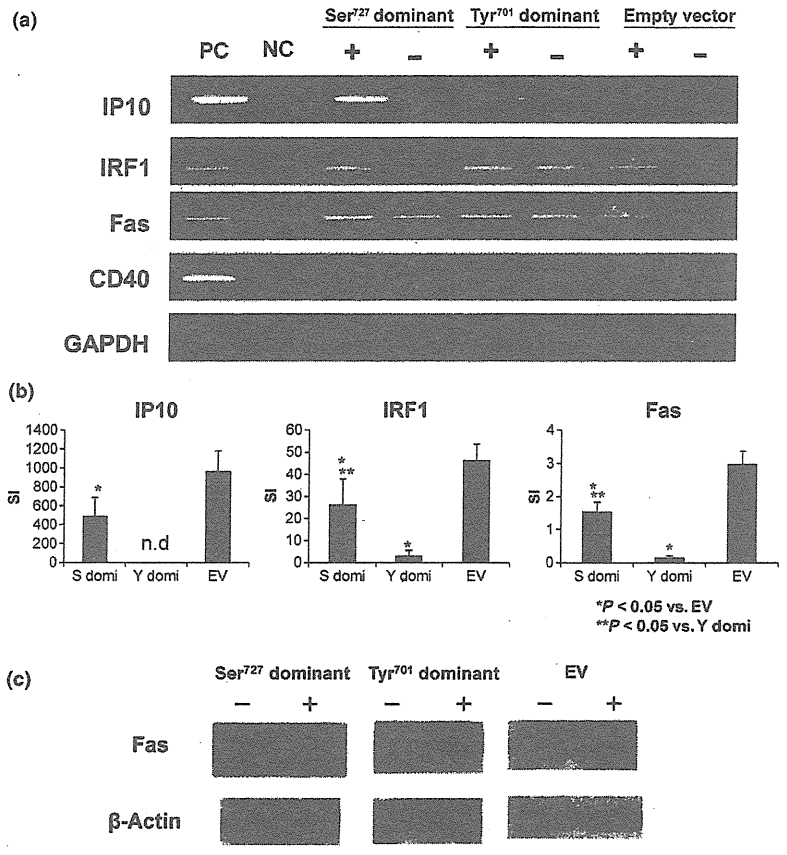

Figure 2 IFNγ-inducible gene expression in mutant-STAT1-transfected human salivary gland (HSG) cells after stimulation with IFNγ. (a) HSG cells transfected with Y701F, S727A mutant-STAT1, and empty vector were stimulated with IFNγ (2000 U/mL) for 24 h. mRNA expression of IFNγ-inducible genes (IP10, IRF1 and Fas) were analyzed by reverse transcription – polymerase chain reaction (RT-PCR). The expression of IP10, IRF1 and Fas were increased in Ser^{727}-dominant HSG cells after stimulation with IFNγ. (b) The results of quantitative PCR are shown as stimulation indexes (S.I), which were calculated by expression levels after stimulation with IFNγ/expression levels before stimulation. In Ser^{727}-dominant HSG cells, IRF1 and Fas mRNA expression levels were significantly increased after stimulation with IFNγ compared with Tyr^{701}-dominant HSG cells ($P < 0.05$, Mann–Whitney *U*-test). (c) Western blot analysis demonstrated that Fas protein expression in Ser^{727}-dominant HSG cells was increased after stimulation with IFNγ, although in Tyr^{701}-dominant HSG cells this was not increased. IP10, IFNγ-inducible protein 10; IRF1, interferon regulatory factor 1; GAPDH, human-glyceralaldehyde-3-phosphate dehydrogenase; PC, positive control; NC, negative control; +, after stimulation with IFNγ; –, before stimulation with IFNγ; EV, empty vector; S domi, Ser^{727}-dominant HSG cells; Y domi, Tyr^{701}-dominant HSG cells; n.d, not determined.

mRNA expression was increased slightly in RT-PCR analysis (Fig. 2a). On the other hand, in S727A mutant-STAT1-transfected HSG cells (Tyr^{701}-dominant HSG cells), only IP10 mRNA expression was increased slightly, whereas IRF1 and Fas mRNA expression were not increased after stimulation with IFNγ in RT-PCR analysis (Fig. 2a). The expression of CD40 mRNA was not detected in any type of HSG cell, neither before nor after stimulation with IFNγ. The results of quantitative PCR on IP10, IRF1 and Fas mRNA expression are shown as stimulation indexes (S.I.), which were calculated by expression levels after stimulation with IFNγ/expression levels before stimulation. In Ser^{727}-dominant HSG cells, IRF1 and Fas mRNA expression levels were significantly increased after stimulation with IFNγ compared with Tyr^{701}-dominant HSG cells ($P < 0.05$, Mann–Whitney *U*-test). S.I of IP10 in Tyr^{701}-dominant HSG cells was not determined, because the expression of IP10 before stimulation was not detected by quantitative PCR analysis (Fig. 2b).

Western blot analysis showed that in Ser^{727}-dominant HSG cells, Fas protein expression was also increased after stimulation with IFNγ, whereas in Tyr^{701}-dominant HSG cells, Fas protein expression was not increased (Fig. 2c).

Induction of apoptosis after stimulation with IFNγ was increased in Ser^{727}-dominant HSG cells

Figure 3 shows the induction of apoptosis after stimulation with IFNγ detected by annexin-V in mutant-STAT1 and empty vector-transfected HSG cells. The population of apoptotic cells before stimulation with IFNγ was 26.8%, 24.5% and 11.9%, and after stimulation with IFNγ, the population of apoptotic cells was changed to 34.9%, 22.8% and 12.9%, in Ser^{727}-dominant HSG cells, Tyr^{701}-dominant HSG cells, and empty vector transfected-HSG cells, respectively. Stimulation index (S.I.) with IFNγ was 1.30, 0.93 and 1.08 in Ser^{727}-dominant HSG cells, Tyr^{701}-dominant HSG cells, and empty vector transfected-HSG cells, respectively. The induction of apoptosis after stimulation with IFNγ was increased in Ser^{727}-dominant HSG cells compared with Tyr^{701}-dominant HSG cells.

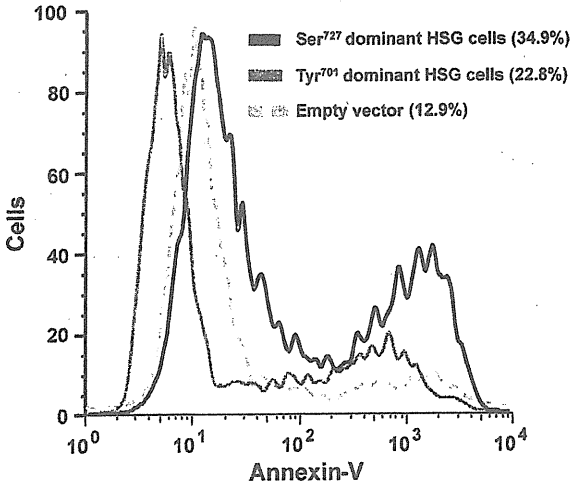

Figure 3 Apoptosis of mutant-STAT1-transfected human salivary gland (HSG) cells after stimulation with IFNγ. HSG cells transfected with Y701F, S727A mutant-STAT1, and empty vector were stimulated with IFNγ (2000 U/mL) for 24 h and then apoptotic cells were detected by annexin-V. The histogram of FLOWJO software (Tree Star, Ashland, OR, USA) showing the population of apoptotic cells after stimulation with IFNγ was 34.9%, 22.8% and 12.9%, in Ser^{727}-dominant HSG cells, Tyr^{701}-dominant HSG cells, and empty vector transfected-HSG cells, respectively. The induction of apoptosis after stimulation with IFNγ was increased in Ser^{727}-dominant HSG cells compared with Tyr^{701}-dominant HSG cells.

DISCUSSION

In Y701F mutant-STAT1-transfected HSG cells (Ser^{727}-dominant HSG cells), the expression of IFNγ-inducible genes such as IP10, IRF1 and Fas, were increased after stimulation with IFNγ. Moreover, in Ser^{727}-dominant HSG cells, the induction of apoptosis after stimulation with IFNγ was also increased compared with S727A mutant-STAT1-transfected HSG cells (Tyr^{701}-dominant HSG cells). These findings indicated that phosphorylation of Ser^{727} in STAT1 might be more important in IFNγ signaling and induction of apoptosis in HSG cells than phosphorylation of Tyr^{701}.

Previously, we reported that Tyr^{701}-phosphorylated STAT1 was localized in infiltrating lymphocytes and the adjacent ductal epithelium, while Ser^{727}-phosphorylated STAT1 was localized only in the ductal epithelium of labial salivary glands from SS patients (SS LSGs).[7] We also revealed that IP10, IRF1 and Fas genes were highly expressed in SS LSGs and colocalized with Ser^{727}-phosphorylated STAT1 but not with Tyr^{701}-phosphorylated STAT1.[7] We proposed that STAT1, especially Ser^{727}-phosphorylated STAT1, might function as a key molecule in the pathogenesis of SS, including the destruction of salivary glands.[7] Interestingly, these previous findings accord with the results in the present study.

In the present study, we showed the correlation of Ser^{727}-phosphorylated STAT1 with IFNγ signaling and the induction of apoptosis in HSG cells *in vitro*. In the pathogenesis of SS, IFNγ might induce phosphorylation of STAT1, especially Ser^{727}, in HSG cells, which cause IFNγ signaling and apoptosis. Thus, Ser^{727}-phosphorylated STAT1 might have essential roles in the destruction of salivary glands in patients with SS. On the other hand, Tyr^{701}-phosphorylated STAT1 might suppress IFNγ signaling and apoptosis. We previously showed that Tyr^{701}-phosphorylated STAT1 was localized in infiltrating lymphocytes in SS LSGs.[7] Thus, IFNγ might induce phosphorylation of Tyr^{701} of STAT1 in infiltrating lymphocytes, resulting in resistance to apoptosis.

In conclusion, phosphorylation of Ser^{727} in STAT1 might be important in IFNγ signaling and the induction of apoptosis in HSG cells. Accordingly, we propose that phosphorylation of Ser^{727} in STAT1 could be a potentially suitable new therapeutic target for SS patients to prevent the destruction of salivary glands.

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