

development of pathologic lesions by EC cytotoxicity (complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC)), activation of EC (proinflammatory cytokine secretion and expression of adhesion molecules), induction of coagulation, and induction of apoptosis [6-9].

Although new biologic drugs have been applied to the treatment of SLE, currently available therapies often introduce the additional risk of immunosuppression [10]. Bloom *et al.* [11] proposed a model for customized and specific therapeutic approaches against a highly pathogenic subset of lupus antibodies by using small molecules that neutralize them. AECAs may be good targets for such interventions, and identification of cell-surface targets of AECAs is required.

Target antigens of AECAs had been investigated intensively, but they are heterogeneous and classified into the following three groups: membrane component, ligand-receptor complex, and molecule adhering to the plasma membrane [12]. The cellular localization of the target antigen is considered to be a critical factor in the pathogenesis of autoantibodies [13], and it is generally accepted that autoantibodies against integral membrane proteins are usually pathogenic [14]. Although AECAs must be directed against the cell surface, most of the molecules reported to date as targets for AECAs are intracellular proteins [2,4,6,15]. Several groups have recently identified targets of AECAs by proteomics analysis [16,17]. However, extraction of some membrane proteins is difficult in proteomics analysis, and this may be one of the reasons that such proteins were not identified as AECA targets [2].

We constructed a retroviral vector system [18] to identify autoantigens expressed on the EC surface by using flow cytometry and identified the membrane protein fibronectin leucine-rich transmembrane protein 2 (FLRT2) as a novel autoantigen of AECAs in patients with SLE.

Materials and methods

Sources of human sera

Two hundred thirty-three patients with collagen diseases (196 female and 37 male patients) were enrolled in the study. The mean age was 42.5 years, with a range of 18 to 72 years. The patients comprised 95 with SLE and 138 with other collagen diseases. All of the patients were diagnosed according to the respective classification criteria [19-32]. Thirty-five age- and sex-matched healthy donors were enrolled as a control group. Sera were collected and stored at -20°C until use. All subjects gave written consent after the purpose and potential risks involved in the study were explained. The study protocol complied with the principles of the Declaration of Helsinki and was approved by the Ethical Committee of Tohoku University Graduate School of Medicine.

Cell culture

Human umbilical vein endothelial cells (HUVECs), human aortic endothelial cells (HAECs), human lung microvascular endothelial cells (HMVEC-Ls), and EGM-2 medium were purchased from Lonza (Basel, Switzerland). Human renal glomerular endothelial cells (HRGECs) and endothelial cell medium were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). The cells were grown in 5% CO₂ at 37°C on polystyrene flasks (BD Biosciences, Bedford, MA, USA). These ECs were used at sooner than the fifth passage. HEK293T cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA), Plat-E and Plat-GP packaging cells were purchased from Cell Biolabs (San Diego, CA, USA) and cultured in Dulbecco modified Eagle medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA). Rat myeloma cells, YB2/0, were purchased from ATCC and cultured in RPMI1640 medium (Sigma) containing 10% FBS.

IgG purification

IgG fractions were purified from sera by using HiTRAP Protein G HP columns (Amersham Biosciences, Roosendaal, The Netherlands). The concentration of purified IgG was determined by measuring the OD at 280 nm (OD₂₈₀). Purified IgG was stored at -20°C until use.

Flow cytometry

Binding activities of antibodies to the surface of ECs and FLRT2 molecules were measured by using FACSCalibur and FACSCanto II (Becton Dickinson, Franklin Lakes, NJ, USA) [17], and the data were analyzed with FlowJo Software (Tree Star, Ashland, OR, USA). In brief, attached cells were dissociated from plates by using Cell Dissociation Solution (Sigma) and washed with phosphate-buffered saline (PBS). Aliquots of 1×10^5 cells/tube were incubated in blocking buffer (PBS containing 1% bovine serum albumin and 50 mg/ml goat gamma globulin fraction (Sigma)) with primary antibodies at 4°C for 30 minutes. After washing, cells were incubated with secondary antibodies and 7-amino-actinomycin D (7-AAD) (BD Biosciences) at 4°C for 30 minutes and analyzed with flow cytometry.

Primary antibodies included 1:10 diluted human serum, 0.5 mg/ml of purified human IgG, and 10 µg/ml goat anti-human FLRT1/FLRT2/FLRT3 antibody (R&D Systems, Minneapolis, MN, USA). Secondary antibodies included 1:50 diluted fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated goat anti-human IgG (Abcam, Cambridge, UK), PE-conjugated donkey anti-goat IgG (Abcam), PE-conjugated mouse anti-human IgG1/IgG2/IgG3/IgG4 antibody (Beckman Coulter, Fullerton, CA, USA), and DyLight 650-conjugated anti human IgM antibody (Abcam). For staining of the intracellular FLRT2

domain, IntraStain (Dako, Glostrup, Denmark) and anti-human FLRT2 antibody (K-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used.

For measurement of AECA activity, the relative mean fluorescence intensity (MFI) ratio was calculated as follows: (sample MFI - control MFI)/control MFI \times 100 [33]. Relative MFI ratio of mean + 3 standard deviations (SD) among the control group was defined as the cutoff value for AECAs. For measurement of anti-FLRT2 activity against the cell-surface domain, the relative MFI ratio was calculated as follows: (MFI against FLRT2-expressing cells - MFI against non-FLRT2-expressing cells)/MFI against non-FLRT2-expressing cells \times 100. In each set of experiments, relative MFI ratios of titrated reference serum with high anti-FLRT2 activity were calculated, and a standard curve was generated. The relative MFI ratio was converted to arbitrary units (AUs) according to the standard curve. AU of mean + 3 SD in the control group was defined as the cutoff value for the anti-FLRT2 antibody. Recombinant human FLRT2 (R&D Systems) was added at the indicated dose in inhibition tests. The percentage inhibition was calculated as follows: % inhibition = (AECA titer of sample serum - AECA titer of sample serum with inhibitor)/AECA titer of sample serum \times 100.

HUVEC cDNA library

Total RNA was generated from HUVECs by using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and poly(A) + RNA was purified with an mRNA Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Double-stranded cDNA was synthesized by using a cDNA library construction kit (Takara Bio, Shiga, Japan). DNA fragments > 1,000 bp in length were ligated into the pMX vector (kindly donated by Toshio Kitamura, University of Tokyo, Tokyo, Japan).

Screening of cDNA library

The HUVEC cDNA library in pMX was retrovirally transfected into the YB2/0 rat myeloma cell line [34]. Aliquots of 1×10^7 YB2/0 cells expressing the HUVEC cDNA library were incubated with 0.5 mg/ml of IgG with high AECA activity at 4°C for 30 minutes. After washing, cells were incubated with FITC-conjugated goat anti-human IgG and 7-AAD at 4°C for 30 minutes. The cells showing a high level of FITC fluorescence signal were sorted with FACS Vantage (Becton Dickinson). Sorted cells were kept in culture until the cell number increased sufficiently for the next round of sorting. Subcloning of cells bound to IgG with AECA activity was performed by the limiting dilution method.

Genomic DNAs of clones were purified by using the Wizard SV Genomic DNA Purification system (Promega Corporation, Madison, WI, USA). DNA fragments from the HUVEC cDNA library were amplified by polymerase

chain reaction (PCR) by using TaKaRa LA Taq (Takara Bio) with primers corresponding to the 5' and 3' ends of the multiple cloning site of pMX (5'-GGTGGAC-CATCCTCTAGACTG, 3'-CCTTTTTCTGGAGAC-TAAAT, respectively). The PCR products were cloned into the pCR-TOPO vector (Invitrogen), and DNA sequences were analyzed with the BLAST program.

Expression of FLRT2 in HEK293T cells

The full-length FLRT2 fragment was amplified by PCR from genomic DNA of FLRT2-expressing YB2/0 clone sorted as described earlier, by using Phusion High-Fidelity DNA Polymerase (Finnzymes, Keilaranta, Espoo, Finland). Primer sequences were as follows: 5'-CCCACCACATTG-TATTTTATTTCC, 3'-CTTGATAACGCTGGGCCTCT. The FLRT2 fragment was inserted into the pMX-IRES-GFP vector (Cell Biolabs). An FLRT2 expression vector with deletion of the unique region was made by using an In-Fusion HD Cloning Kit (Clontech Laboratories, Madison, WI, USA) with two PCR segments constructed to omit the unique region (363 to 419 amino acids) and inserted into the pMX-IRES-GFP vector. pMX-FLRT2-IRES-GFP was transfected directly into HEK293T cells with FuGENE HD (Roche Diagnostics, Basel, Switzerland) or retrovirally transfected into HEK293T cells. Full-length FLRT1 and FLRT3 fragments were amplified as described earlier and inserted into the pMX-IRES-GFP vector.

Western blotting

Cells were lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA). The lysate was mixed with 5 \times sodium dodecyl sulfate (SDS) sample buffer and separated by electrophoresis on an 8% polyacrylamide gel. The proteins were then transferred onto Immobilon Transfer Membranes (Millipore, Billerica, MA, USA). The membranes were treated with 0.1 μ g/ml of goat anti-FLRT2 antibody and IRDye680-conjugated donkey anti-goat IgG (LI-COR Biosciences, Lincoln, NE, USA), and fluorescence intensity was determined with the Odyssey Infrared Imaging System (LI-COR).

CDC

CDC was assessed by the tetrazolium salt reduction method by using WST-1 (Roche Diagnostics) [35-37]. In brief, cells were seeded in 96-well culture plates at a concentration of 4×10^4 cells per well and cultured overnight. Cells were incubated with 100 μ l of diluted IgG for 30 minutes followed by addition of 50 μ l of rabbit complement (Cedarlane Laboratories, Burlington, ON, Canada) at the indicated concentrations for 2 hours at 37°C. Then 15 μ l of WST-1 was added, and cells were incubated for an additional 4 hours. Absorbance at 450 nm (A_{450}) was measured and expressed as relative fluorescence units (RFUs), reflecting the number of viable cells. Triton X-100 (1%)

and heat-inactivated complement were added to the wells to measure background or maximal absorbance of WST-1, respectively. Recombinant FLRT2 was added in the inhibition tests. The percentage cytotoxicity for each sample was calculated by using the formula:

$$\% \text{ cytotoxicity} = (\text{maximal RFU} - \text{sample RFU}) / (\text{maximal RFU} - \text{background RFU}) \times 100.$$

ADCC

ADCC was determined by using the LDH Cytotoxicity Detection Kit (Takara Bio) and the manufacturer's protocol [36].

The percentage cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = (\text{experimental} - \text{effector spontaneous} - \text{target spontaneous}) / (\text{target maximum} - \text{target spontaneous}) \times 100.$$

Detection of adhesion molecule expression

HUVECs were cultured overnight in 96-well culture plates and incubated with IgG (640 µg/ml) for 6 hours at 37°C. Harvested cells were stained with PE-conjugated anti-CD62E antibody (BioLegend, San Diego, CA, USA), allophycocyanin (APC)-conjugated anti-CD106 antibody (BioLegend), and Pacific blue-conjugated anti-CD54 antibody (BioLegend), and were analyzed with flow cytometry.

Detection of EC apoptosis

HUVECs were seeded in 48-well culture plates and incubated with test IgG (640 µg/ml) for 24 hours, and apoptosis in the harvested cells was measured with annexin V and 7-AAD (Apoptosis Detection Kit; BD Biosciences) with flow cytometry. Annexin V-positive/7-AAD-negative cells were measured as apoptotic cells.

Statistical analysis

Data were analyzed by using the two-tailed Student *t* test or Mann-Whitney *U* test for continuous variables. Pairwise comparisons were assessed by using the Wilcoxon signed-rank test. Spearman rank correlation was used to explore the associations between anti-FLRT2 titer and clinical parameters. All analyses were performed by using Prism software (GraphPad Software, La Jolla, CA, USA). In all analyses, *P* < 0.05 was taken to indicate statistical significance.

Results

Detection of AECA activity with flow cytometry

We first examined AECA activity in the sera from patients with collagen diseases by measuring binding activity of IgG to nonpermeabilized 7-AAD-negative HUVECs by using flow cytometry. The prevalence of AECAs was significantly higher in patients with SLE (50.5%) and other collagen diseases compared with normal controls (2.9%)

(Figure 1). As these data indicated the presence of autoantigens on the EC surface, we constructed a retroviral vector system to identify cell-surface target molecules of AECAs with flow cytometry.

Sorting of cells expressing cell-surface autoantigens with retroviral vector system

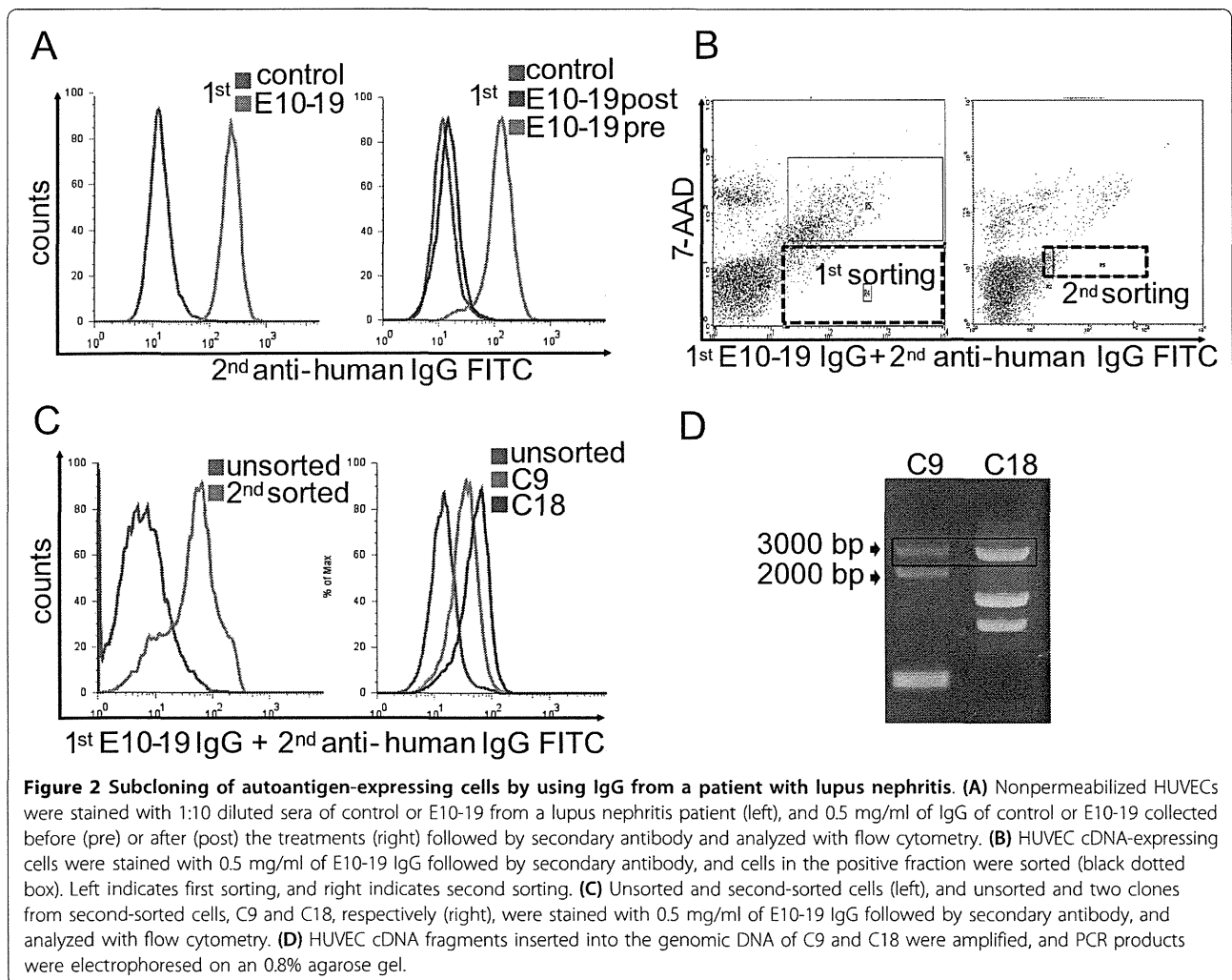
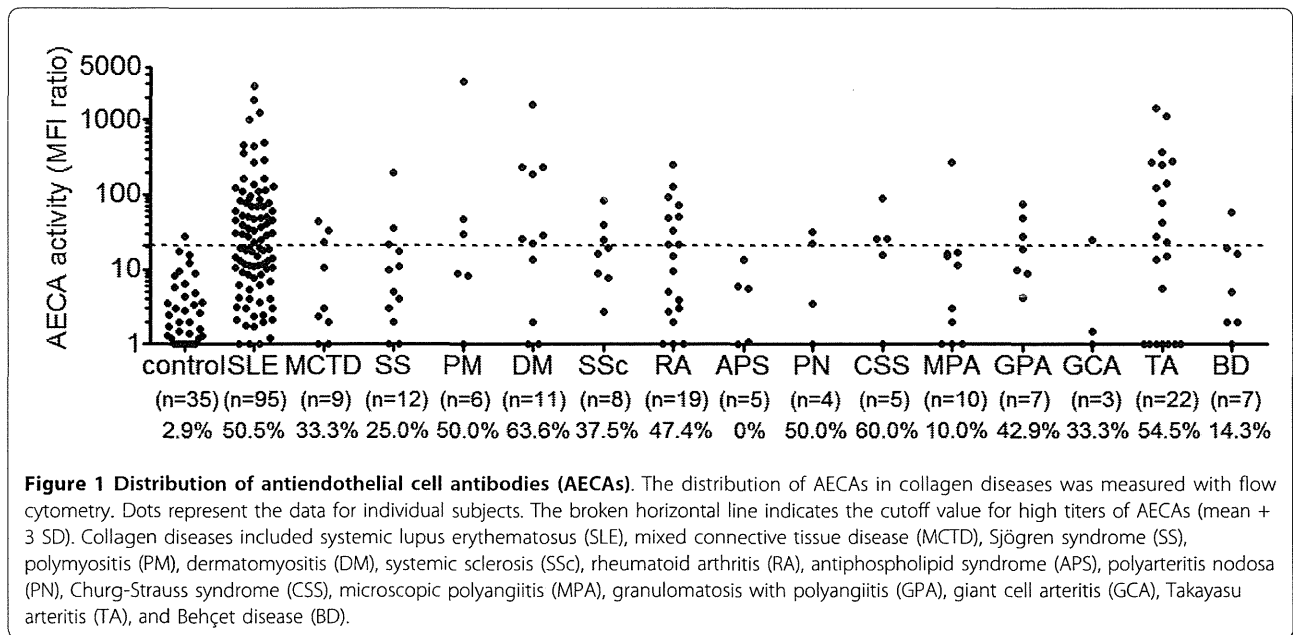
Among sera with AECA activity, one sample (E10-19) from an SLE patient with active lupus nephritis (WHO IV) showed strong AECA activity (Figure 2A). We selected this serum sample as the prototype of AECAs for subsequent cell sorting. Purified IgG from E10-19 serum also showed strong binding to the surface of HUVECs, and IgG from the same patient collected after the treatment with 1 mg/kg prednisolone and intravenous cyclophosphamide showed remarkably reduced AECA activity (Figure 2A).

The YB2/0 cell line expressing HUVEC cDNA was generated by stable transfection of the HUVEC cDNA library with the retroviral vector system. After staining of this cell line with E10-19 IgG and FITC-conjugated secondary antibody, cells with strong FITC signals were sorted with flow cytometry. After cell expansion, we repeated one more round of cell sorting to concentrate E10-19 IgG-binding cells (Figure 2B). After the second sorting, cells bound to E10-19 IgG were markedly increased, and several cell clones were established from the E10-19 IgG-binding cell population by the limiting dilution method. Two distinct clones with different E10-19 IgG-binding activities and gene profiles of transfected HUVEC cDNA were established, C9 and C18 (Figure 2C and 2D).

Identification of FLRT2 as a novel cell-surface autoantigen

After PCR amplification and cloning of HUVEC cDNA inserted into the genomic DNA of C9 and C18, DNA sequencing was performed followed by BLAST analysis. PCR bands of around 3,000 bp in C9 and C18 (Figure 2D, black box) were found to be an identical gene, that is, fibronectin leucine-rich transmembrane protein 2 (*FLRT2*) cDNA (GenBank accession number NM_013231.4). Real-time quantitative PCR and microarray analysis of YB2/0, C9, and C18 also supported the conclusion that only the *FLRT2* mRNA was overexpressed in both C9 and C18 (data not shown). Flow cytometry and Western blotting showed that FLRT2 protein was expressed on the cell surfaces of C9 and C18 (Figure 3A). Next, we generated an expression vector of FLRT2, which was transfected into HEK293T cells. E10-19 IgG showed significant binding activity to 7-AAD-negative FLRT2-expressing HEK293T cells (Figure 3B), indicating that E10-19 IgG has high anti-FLRT2 activity. Thus, the membrane protein FLRT2 was identified as a novel autoantigen.

Flow cytometry and Western blotting indicated that HUVECs and other ECs also expressed significant levels



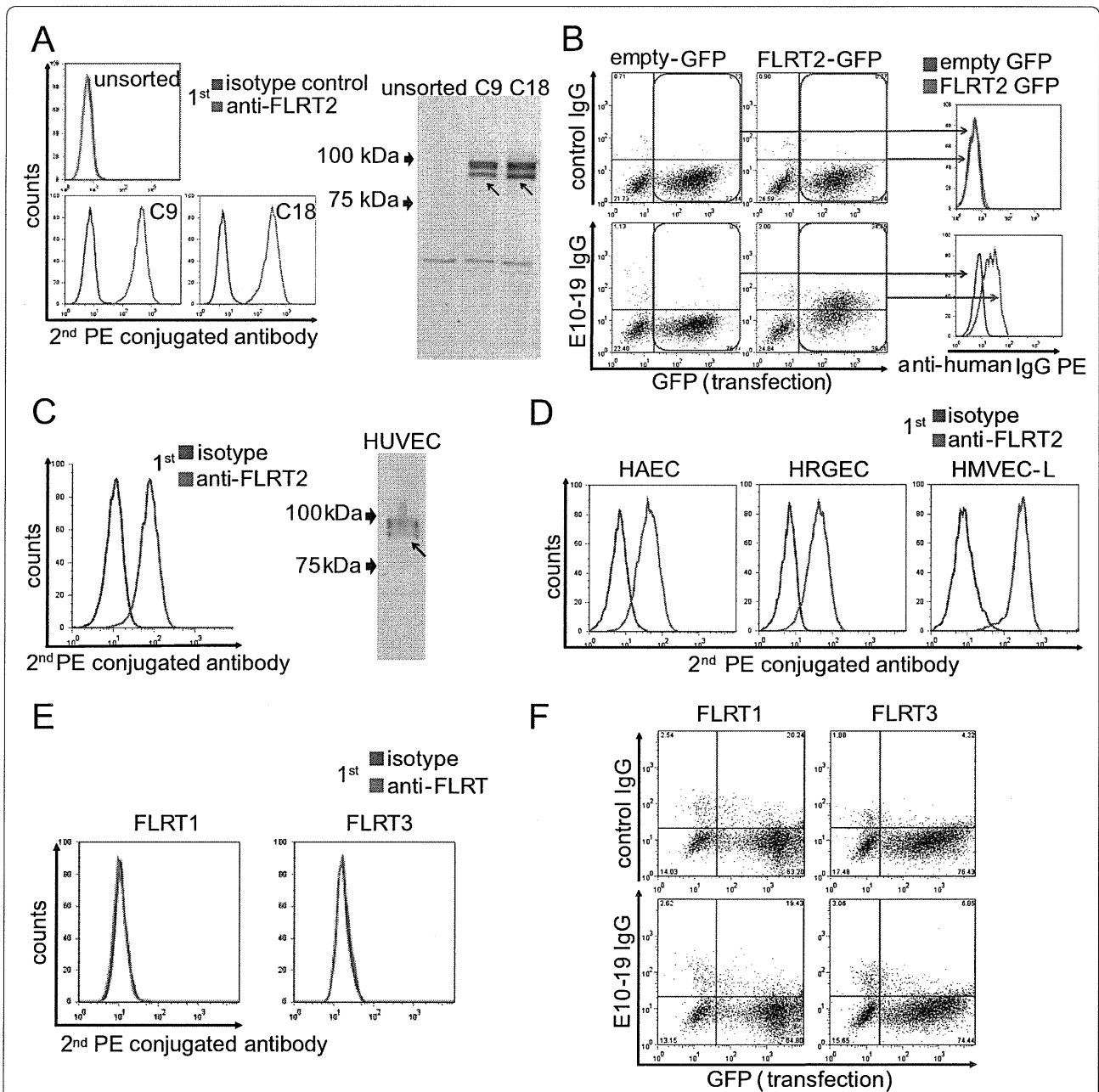


Figure 3 Identification of FLRT2 as a novel autoantigen of AECAs. (A) Unsorted, C9, and C18 were stained with isotype control or anti-FLRT2 antibody, followed by secondary antibody, and analyzed with flow cytometry (left). Western blotting of proteins from unsorted, C9, and C18 was performed, and they were stained with anti-FLRT2 antibody followed by secondary antibody (right). Arrows indicate the bands of FLRT2. Both of the two bands are FLRT2 because some of FLRT2 proteins were glycosylated. **(B)** Expression vector, empty-IRES-GFP, or FLRT2-IRES-GFP was transfected into HEK293T cells, and these cells were stained with 0.5 mg/ml of control IgG or E10-19 IgG, followed by secondary antibody, and analyzed with flow cytometry. Binding activities of IgG to cell-surface FLRT2 were analyzed in histograms (right) by gating for the GFP-positive transfected population (left). **(C)** HUVECs were stained with isotype control or anti-FLRT2 antibody followed by secondary antibody, and analyzed with flow cytometry (left). Western blotting of proteins from HUVECs was performed, and they were stained with anti-FLRT2 antibody followed by secondary antibody (right). The arrows indicate the bands of FLRT2. **(D)** HAECs, HRGECs, and HMVEC-Ls were stained with isotype control or anti-FLRT2 antibody followed by secondary antibody, and analyzed with flow cytometry. **(E)** HUVECs were stained with isotype control, anti-FLRT1 antibody, or anti-FLRT3 antibody followed by secondary antibody, and analyzed with flow cytometry. **(F)** Expression vector, FLRT1-IRES-GFP (left), or FLRT3-IRES-GFP (right) was transfected into HEK293T cells, and these cells were stained with 0.5 mg/ml of control IgG or E10-19 IgG, followed by secondary antibody, and analyzed with flow cytometry.

of FLRT2 on their cell surfaces (Figure 3C, D). FLRT2 is a member of the FLRT family, which includes FLRT1, FLRT2, and FLRT3 [38]. We examined whether other FLRTs were expressed on HUVECs with flow cytometry. Neither FLRT1 nor FLRT3 was expressed on the surface of these ECs, and E10-19 IgG showed no binding activity to either FLRT1 or FLRT3 (Figure 3E, F). These data indicated that among the FLRT family, FLRT2 was the only target molecule of AECAs.

Inhibition test and epitope mapping

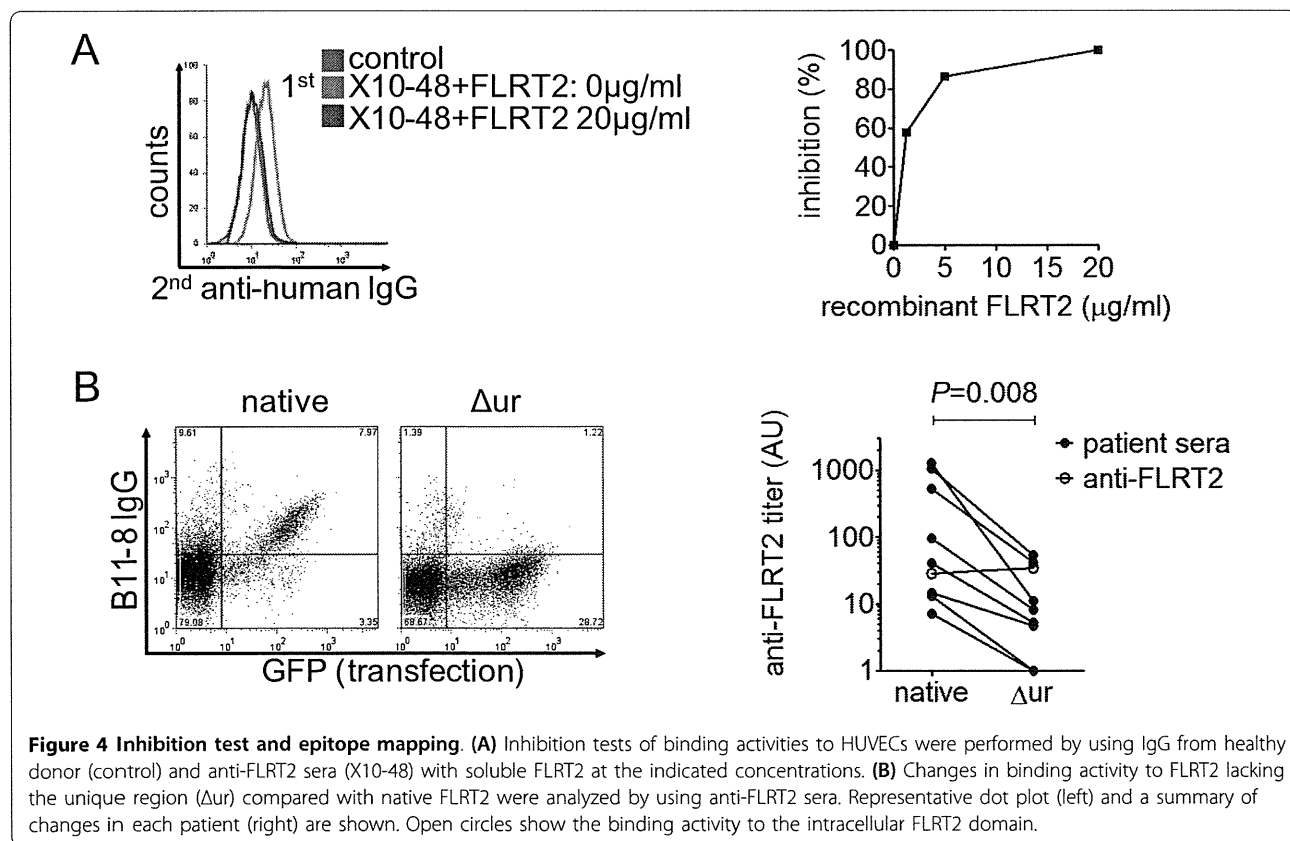
We conducted inhibition tests to determine whether the AECA activities of anti-FLRT2-positive SLE patients were due to anti-FLRT2 activity. Incubation with soluble recombinant FLRT2 inhibited the binding of patient IgG to HUVECs (Figure 4A). We further investigated the epitope of anti-FLRT2 antibody. FLRT2 contains extracellular leucine-rich repeats, unique region, fibronectin type III domain, and a cytoplasmic tail. As mentioned earlier, FLRT2 was the only member of the FLRT family that was bound by SLE IgG, so we hypothesized that the unique region of FLRT2 may be the major epitope for anti-FLRT2 antibody. To investigate this hypothesis, an expression vector of FLRT2 lacking the unique region (FLRT2 Δ ur) was generated. As shown in Figure 4B, the

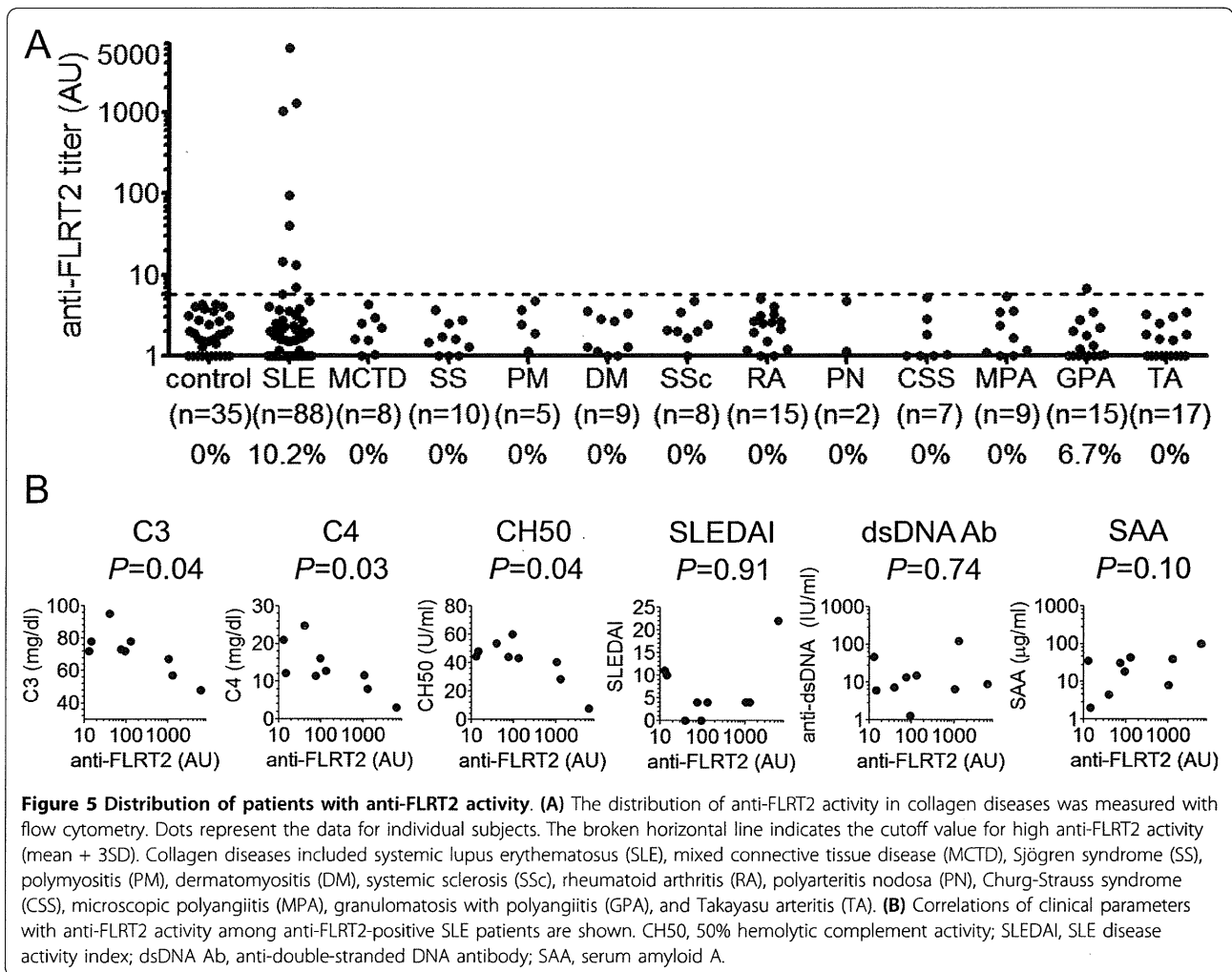
binding activity of the anti-FLRT2 antibody was significantly reduced when FLRT2 lacked its unique region ($P = 0.008$) compared with the equal binding activity of anti-FLRT2 antibody to the intracellular domain. These observations indicated that the major epitope was localized within the unique region of FLRT2.

Distribution of patients with anti-FLRT2 activity

Anti-FLRT2 activities were detected in nine (10.2%) of 88 patients with SLE and one (6.7%) of 15 patients with granulomatosis with polyangiitis (Wegener's). Healthy controls and other patients with collagen diseases, including diseases that showed a high prevalence of AECA activity, did not show anti-FLRT2 activity (Figure 5A). Strong anti-FLRT2 activities were detected in only SLE patients, indicating that anti-FLRT2 antibody is specific to SLE patients. Among 48 SLE patients with AECA positivity (Figure 1), 42 were examined for anti-FLRT2 activity, and nine patients (21.4%) were positive.

Among SLE patients with anti-FLRT2 positivity, anti-FLRT2 activity was significantly correlated with low levels of complement C3, C4, and CH50 (Figure 5B). No interrelations were found between anti-FLRT2 activity and the SLE disease activity index (SLEDAI), anti-dsDNA antibody titer, or serum amyloid A (SAA) level (Figure 5B).





Induction of endothelial cell killing by CDC

We next assessed the functional significance of anti-FLRT2 antibody by using IgG from the sera of two SLE patients with high FLRT2 activity (B11-8 and X10-48). IgG with anti-FLRT2 activity showed significant CDC activity against HUVECs compared with IgG from normal controls (Figure 6A). This CDC activity was inhibited by incubation with soluble recombinant FLRT2, and increased with a higher concentration of IgG (Figure 6B, C). Strong CDC activity was induced against FLRT2-expressing HEK293T cells, but not against mock-transfected HEK293T cells (Figure 6D). These observations confirmed the ability of the anti-FLRT2 antibody to induce CDC activity by binding to cell-surface FLRT2.

We also analyzed the IgG subclasses of anti-FLRT2 antibody with flow cytometry. In all anti-FLRT2 active IgG subclasses, IgG1 and IgG2 activities were strong, and IgG3 was weak. The presence of IgG4 varied between patients (Figure 6E). Compared with IgG, weak

IgM activity was detected (Figure 6F). None of these IgGs showed ADCC (Figure 6G).

Other pathogenic roles as AECAs

We examined further potentials for pathogenicity against EC activation and induction of apoptosis. The levels of expression of adhesion molecules (intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin) on HUVECs were not increased by incubation with IgG purified from B11-8 and X10-48 compared with control IgG (Figure 7A). Incubation of HUVECs with anti-FLRT2-positive IgG did not induce apoptosis (Figure 7B).

Discussion

Although the existence of AECAs in patients with SLE and other collagen diseases has been reported, its pathogenic significance remains unknown. The localization of the AECA target molecule on the cell surface should be

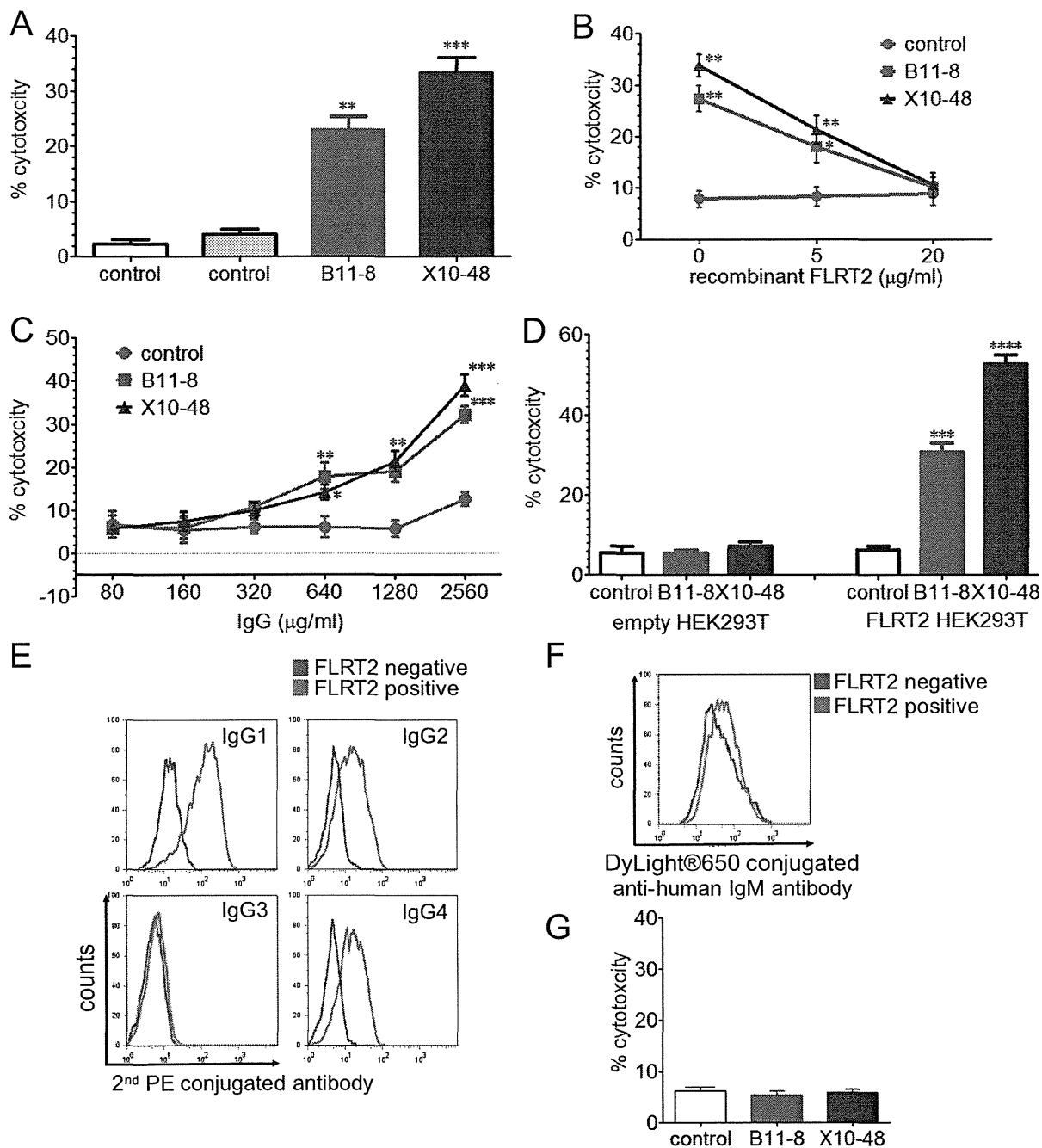


Figure 6 Complement-dependent cytotoxicity (CDC) of anti-FLRT2 antibody. CDC activities using two healthy control IgG and two anti-FLRT2 positive IgG, B11-8 and X10-48, at a concentration of 1.28 mg/ml with 1:3 diluted complement (A), 1.28 mg/ml of IgG, and 1:3 diluted complement with recombinant FLRT2 at the indicated concentrations (B), and various IgG concentrations with 1:6 diluted complement (C) against HUVECs were measured with the WST-1 assay. (D) CDC activities against mock transfected HEK293T cells (empty, left) and FLRT2-expressing HEK293T cells (FLRT2, right) by using 1.28 mg/ml of IgG and 1:3 diluted complement were measured with the WST-1 assay. HEK293T cells negative or positive for FLRT2 expression were stained with anti-FLRT2 antibody followed by secondary antibody against human IgG1, IgG2, IgG3, IgG4 (E), and IgM (F), and analyzed with flow cytometry. (G) ADCC activities using control IgG, B11-8, and X10-48, at a concentration of 1.28 mg/ml with an E:T ratio of 25:1 were determined with the lactate dehydrogenase detection method. Error bars indicate SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

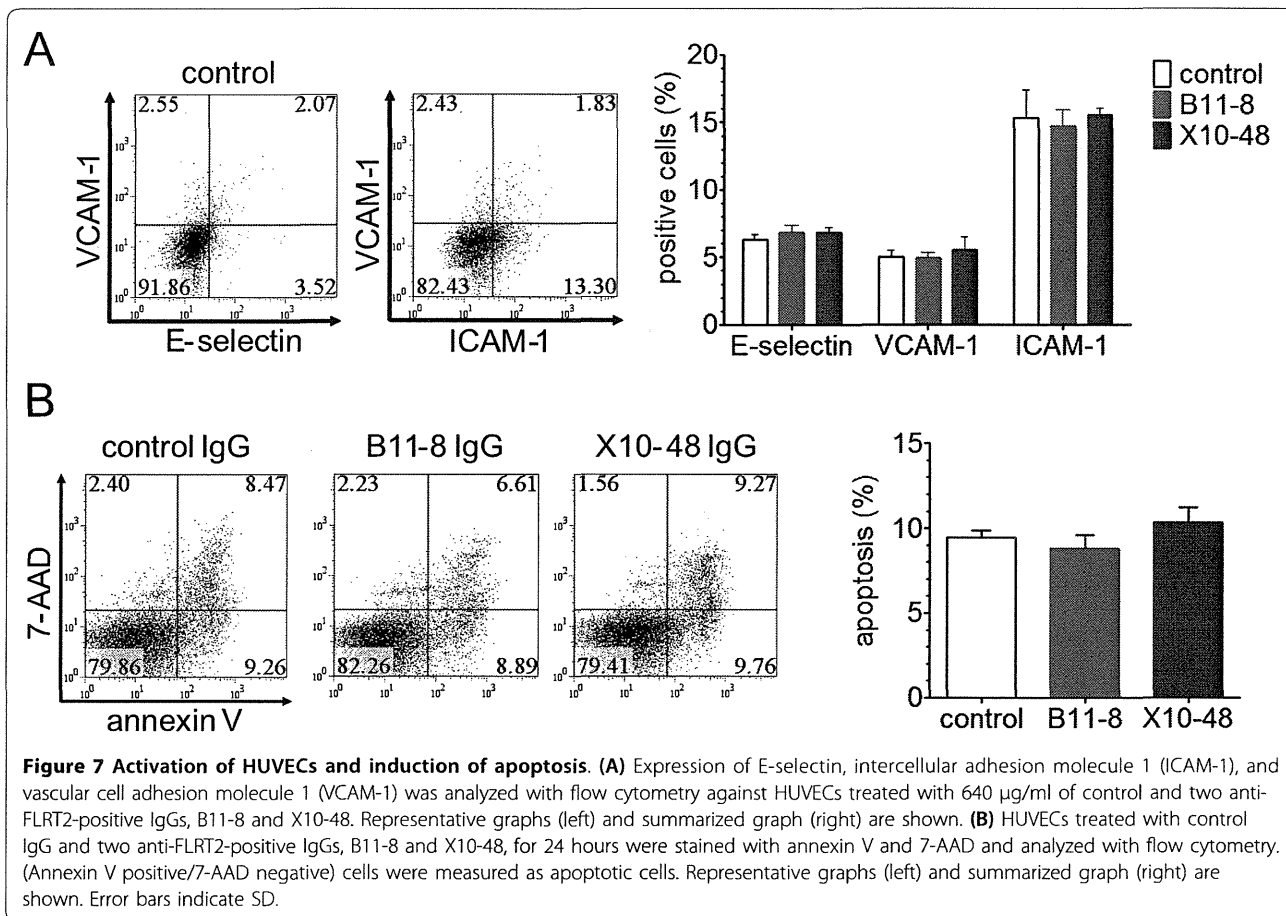


Figure 7 Activation of HUVECs and induction of apoptosis. (A) Expression of E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) was analyzed with flow cytometry against HUVECs treated with 640 µg/ml of control and two anti-FLRT2-positive IgGs, B11-8 and X10-48. Representative graphs (left) and summarized graph (right) are shown. **(B)** HUVECs treated with control IgG and two anti-FLRT2-positive IgGs, B11-8 and X10-48, for 24 hours were stained with annexin V and 7-AAD and analyzed with flow cytometry. (Annexin V positive/7-AAD negative) cells were measured as apoptotic cells. Representative graphs (left) and summarized graph (right) are shown. Error bars indicate SD.

an important factor for its pathogenicity *in vivo*, in terms of accessibility of the target molecule to AECAs.

Our strategy to identify AECA target molecules is to use a retroviral vector system and flow cytometry. As the localization of cellular molecules depends on their structures, only cell-surface molecules are expressed on the surface of YB2/0 cells transfected with the HUVEC cDNA library. AECAs can bind only to cell-surface molecules in flow cytometry. Therefore, sorting of IgG-binding cells can concentrate and isolate cells expressing autoantigens (target molecules for AECAs) on the cell surface. Although this system may present difficulties in sorting cells at very low frequency, we isolated and cloned autoantigen-expressing cells by repeated sorting, and this system was shown to be useful to identify cell-surface autoantigens. Whereas some cell-surface molecules were identified with this system previously [39], this is the first report of autoantigen identification.

With purified IgG from one SLE patient with high AECA activity (E10-19), two distinct clones were isolated and established, both of which were shown to have an identical gene, *FLRT2*. As we confirmed that E10-19 IgG bound specifically to cell-surface FLRT2 and FLRT2 was expressed on the cell surface of ECs, we concluded that

FLRT2 is a novel cell-surface autoantigen as a target molecule for AECAs in SLE patients.

Analysis of anti-FLRT2 activity among patients with various collagen diseases indicated that anti-FLRT2 antibody was specifically detected in SLE, and it accounted for 21.4% of cell-surface target molecules of AECAs in SLE. AECA activity of IgG from SLE patients with anti-FLRT2 activity was significantly inhibited by soluble recombinant FLRT2, indicating that FLRT2 is the major target on ECs for AECAs in these patients. Although heat-shock protein 60 (Hsp60) has been described as the target antigen of AECAs in SLE and has a proapoptotic effect [40,41], Hsp60 was not detected on freshly isolated unstressed HUVECs [40,41]. The remaining 78.6% of SLE patients with AECA activity in the present study may have other as-yet-unidentified target antigens.

FLRT2 is transmembrane protein and was identified as a novel gene family in the screening for extracellular matrix proteins expressed in muscle [38]. Although FLRT2 was shown to be expressed in the pancreas, skeletal muscle, brain, and heart with Northern blotting [38], we confirmed the expression of FLRT2 on HUVECs and other ECs (HAECs, HRGECs, and HMVEC-Ls), and treatment with neither tumor necrosis factor-α (TNF-α)

nor lipopolysaccharide (LPS) induced the expression of FLRT2 (data not shown). E10-19 IgG did not bind to FLRT1 and FLRT3, and these two molecules were not expressed on ECs. Consistent with these findings, the major epitope for anti-FLRT2 antibody was localized in the unique region within the extracellular domain of FLRT2.

FLRT2 has been reported to modulate signaling, interact with fibroblast growth factor receptor, promote cell proliferation, participate in craniofacial development, and promote heart morphogenesis [42-46]. Although we hypothesized that anti-FLRT2 antibody may affect some cellular behavior and induce expression of adhesion molecules, cell proliferation, and apoptotic cell death without complement in ECs, we did not find these activities in the present study.

Among SLE patients with anti-FLRT2 activity, complement levels were correlated significantly with the anti-FLRT2 antibody titer. Moreover, anti-FLRT2 antibody induced cell damage in a complement-dependent manner, suggesting that it has pathogenic roles in immune-mediated vascular damage. CDC activity of AECAs was reported in patients with SLE, Takayasu arteritis, hemolytic-uremic syndrome, and Kawasaki disease [2,4,35,47,48]. Although ADCC activity was not proven in our study, similar observations of AECAs producing CDC but not ADCC were reported previously [35,48].

As demonstrated in this study, FLRT2 is widely distributed in various types of ECs. Therefore, it is possible that anti-FLRT2 antibody is linked to systemic vascular injury. These observations indicate that it is necessary to evaluate the contributions of anti-FLRT2 antibody to atherosclerotic lesions because chronic inflammation is atherogenic in SLE [49,50]. Administration of gammaglobulin was reported to reduce CDC of AECAs against ECs [35], and this may apply to anti-FLRT2 antibody-induced damage. Furthermore, incubation with soluble recombinant FLRT2 inhibited the AECA activity and CDC activity in patients with anti-FLRT2 positivity, which suggests that neutralizing anti-FLRT2 antibodies might be the specific therapeutic approach.

Conclusions

We identified the membrane protein FLRT2 as a novel autoantigen of AECAs in SLE patients. Our retroviral vector system is useful for identification of cell-surface autoantigens. In addition to further investigations of the biologic significance of anti-FLRT2 antibody and its therapeutic applications, other cell-surface autoantigens of AECAs should be determined to achieve a comprehensive understanding of AECA-mediated vascular injury and the development of more-specific intervention strategies.

Abbreviations

ADCC: antibody-dependent cell-mediated cytotoxicity; AECAs: anti-endothelial cell antibodies; APC: allophycocyanin; AUs: arbitrary units; CDC: complement-dependent cytotoxicity; DMEM: Dulbecco modified Eagle medium; ECs: endothelial cells; FBS: fetal bovine serum; FITC: fluorescein isothiocyanate; FLRT: fibronectin leucine-rich transmembrane protein; HAECs: human aortic endothelial cells; HMVEC-Ls: human lung microvascular endothelial cells; HRGECs: human renal glomerular endothelial cells; Hsp60: heat shock protein 60; HUVECs: human umbilical vein endothelial cells; ICAM-1: intercellular adhesion molecule 1; LPS: lipopolysaccharide; MFI: mean fluorescence intensity; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; PE: phycoerythrin; RFUs: relative fluorescence units; SAA: serum amyloid A; SD: standard deviations; SDS: sodium dodecyl sulfate; SLE: systemic lupus erythematosus; SLEDAI: SLE disease activity index; TNF- α : tumor necrosis factor α ; VCAM-1: vascular cell adhesion molecule 1; 7-AAD: 7-amino-actinomycin D.

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Authors' contributions

TS and HF carried out the molecular biologic studies, flow cytometry, clinical evaluation, and functional assays and drafted the manuscript. MO participated in the design of the study, performed the molecular biologic studies, and helped to draft the manuscript. KN, RW, YT, NT, and TI participated in its design and helped to draft the manuscript. HH conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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A Genome-Wide Association Study Identified *AFF1* as a Susceptibility Locus for Systemic Lupus Erythematosus in Japanese

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease that causes multiple organ damage. Although recent genome-wide association studies (GWAS) have contributed to discovery of SLE susceptibility genes, few studies have been performed in Asian populations. Here, we report a GWAS for SLE examining 891 SLE cases and 3,384 controls and multi-stage replication studies examining 1,387 SLE cases and 28,564 controls in Japanese subjects. Considering that expression quantitative trait loci (eQTLs) have been implicated in genetic risks for autoimmune diseases, we integrated an eQTL study into the results of the GWAS. We observed enrichments of cis-eQTL positive loci among the known SLE susceptibility loci (30.8%) compared to the genome-wide SNPs (6.9%). In addition, we identified a novel association of a variant in the AF4/FMR2 family, member 1 (*AFF1*) gene at 4q21 with SLE susceptibility (rs340630; $P=8.3 \times 10^{-9}$, odds ratio = 1.21). The risk A allele of rs340630 demonstrated a cis-eQTL effect on the *AFF1* transcript with enhanced expression levels ($P < 0.05$). As *AFF1* transcripts were prominently expressed in CD4⁺ and CD19⁺ peripheral blood lymphocytes, up-regulation of *AFF1* may cause the abnormality in these lymphocytes, leading to disease onset.

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Author Summary

Although recent genome-wide association study (GWAS) approaches have successfully contributed to disease gene discovery, many susceptibility loci are known to be still uncaptured due to strict significance threshold for multiple hypothesis testing. Therefore, prioritization of GWAS results by incorporating additional information is recommended. Systemic lupus erythematosus (SLE) is an autoimmune disease that causes multiple organ damage. Considering that abnormalities in B cell activity play essential roles in SLE, prioritization based on an expression quantitative trait loci (eQTLs) study for B cells would be a promising approach. In this study, we report a GWAS and multi-stage replication studies for SLE examining 2,278 SLE cases and 31,948 controls in Japanese subjects. We integrated eQTL study into the results of the GWAS and identified *AFF1* as a novel SLE susceptibility loci. We also confirmed cis-regulatory effect of the locus on the *AFF1* transcript. Our study would be one of the initial successes for detecting novel genetic locus using the eQTL study, and it should contribute to our understanding of the genetic loci being uncaptured by standard GWAS approaches.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibody production, complement activation, and multi-organ damage [1]. Familial aggregation demonstrates that both genetic and environmental factors play a role in pathogenesis of SLE [2]. Genetic studies using candidate gene approaches, and recently, genome-wide association studies (GWAS), have uncovered more than 25 SLE susceptibility genes, including *HLA-DRB1*, *IRF5*, *STAT4*, *ITGAM*, *BLK*, *TNFAIP3*, and others [3–18]. However, most of these studies were conducted in European populations [3–13,15,17], and few studies have been conducted in Asian populations [14,16,18]. Since the epidemiology of SLE has demonstrated that the prevalence of disease substantially differs among populations, genetic backgrounds of SLE should be also heterogeneous across populations [19,20]. Therefore, additional studies in Asians might provide novel insights. It is of note that GWAS for SLE in Chinese populations identified novel loci that had not been detected in Europeans, such as *ETS1*, *IKZF1*, and *WDFY4* [14,16].

Another issue raised by the previous GWASs for complex diseases is that many susceptibility loci still remained uncaptured, owing to its strict significance threshold for multiple hypothesis testing [21]. In SLE, for example, the 26 risk loci identified by the previous GWAS explained only an estimated 8% of the total genetic susceptibility to the disease [15]. Therefore, it is still important to examine the sub-loci of GWAS, in order to reveal the entire picture of genetic etiology. To effectively explore these uncaptured loci, prioritization of GWAS results by incorporating additional information implicated in the disease pathophysiology is recommended [22,23]. Considering that abnormalities in B cell activity play essential roles in SLE [1] and that expression quantitative trait loci (eQTL) have been implicated to comprise approximately a half of genetic risks for autoimmune diseases [24], prioritization based on an eQTL study for B cells would be a promising approach for SLE [25]. Moreover, an eQTL itself assures the presence of functional variant(s) that regulate gene expression. Thus, eQTL increases the prior probability of the presence of disease-causal variant(s) in the locus more effectively

and unbiasedly, compared to other knowledge-based prioritizations such as gene pathway analysis [24].

Here, we report a GWAS and multi-stage replication studies for SLE examining 2,278 SLE cases and 31,948 controls in Japanese subjects. We integrated eQTL study into the results of the GWAS, which effectively enabled to detect a novel SLE susceptibility locus.

Results

GWAS for SLE

In the GWAS, 891 SLE cases and 3,384 controls in Japanese subjects were genotyped over 550,000 single nucleotide polymorphism (SNP) markers (Table S1, S2 and Figure 1). We applied stringent quality control (QC) criteria and evaluated associations of 430,797 autosomal SNPs, as previously described [26]. No substantial population stratification was demonstrated through principal component analysis (Figure S1) or a Quantile–Quantile plot of *P*-values (inflation factor, λ_{GC} = 1.088, Figure S2), suggesting homogenous ancestries of our study population [27].

We identified significant associations in six chromosomal loci that satisfied the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$ (Table 1 and Figure 2A). These loci have been reported to be associated with SLE susceptibility (*STAT4*, *TNFAIP3*, *HIP1*, *BLK*, *ETS1*, and the HLA region) [3–18]. We also observed significant replications in 17 of the previously reported SLE susceptibility loci [3–18] ($\alpha = 0.01$; Table 2). Of these, significant replications were enriched in the loci identified through the studies in Asian populations (80%; 8 of the 10 loci), including *RASGRP3*, *IKZF1*, *HIP1*, *WDFY4*, intergenic region at 11q23, *ETS1*, *SLC15A4*, *ELF1*, and *HIC2-UBE2L3* [14,16,18], compared to those in European populations (56.3%; 9 of the 16 loci) [3–13,15,17].

Incorporation of eQTL study into GWAS results

For the selection of SNPs incorporated in the replication studies of the potential association signals, we evaluated cis-eQTL effects of the SNPs using publically available gene expression data [28], and prioritized the results of the GWAS. After applying QC criteria, we evaluated the expression levels of 19,047 probes assayed in lymphoblastoid B cell lines from Phase II HapMap East-Asian individuals [29] using Illumina's human whole-genome expression array (WG-6 version 1) [28]. For each of the SNPs included in our GWAS, probes located within ± 300 kbp regions were focused on as cis-eQTLs (average 4.93 probes per SNP). We denoted the SNPs which exhibited significant associations with expression levels of any of the corresponding cis-eQTLs as eQTL positive (false discovery rate (FDR) *Q*-values < 0.2). We observed enrichments of eQTL positive loci among the SLE susceptibility loci (30.8%; 8 of the 26 evaluated loci) including a well-known eQTL gene of *BLK* [11,25] (Table 2), compared to the genome-wide SNPs (6.9%) and compared even to the SNPs in the vicinity of expressed loci (among the SNPs located within ± 10 kbp of probes used for the expression analysis, 13.1% were eQTL positive; Table S3).

By prioritizing the results of the GWAS using the eQTL study, we selected 57 SNPs from 1,207 SNPs that satisfied $P < 1.0 \times 10^{-3}$ in the GWAS. We subsequently referred the associations of the selected SNPs using the results of the concurrent genome-wide scan for SLE in an independent Japanese population (Tahira T et al. Presented at the 59th Annual Meeting of the American Society of Human Genetics, October 21, 2009). In the scan, 447 SLE cases and 680 controls of Japanese origin were evaluated using a pooled DNA approach [30]. We selected SNPs if any association signals were observed in the neighboring SNPs of the

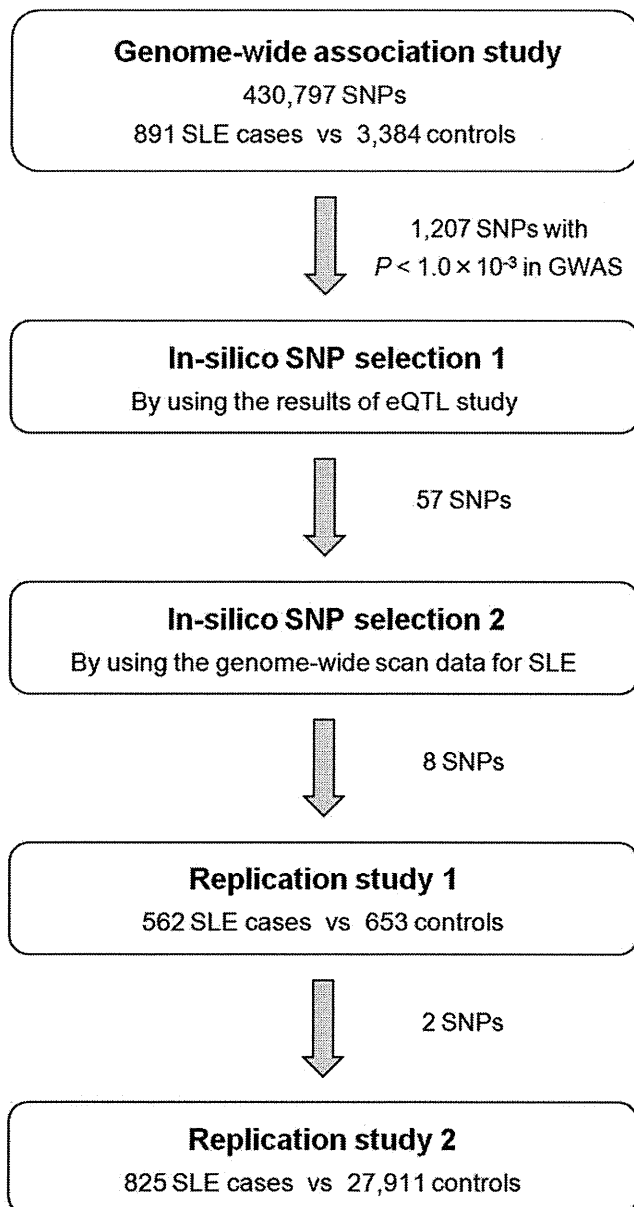


Figure 1. Design of the GWAS and multi-stage replication studies for SLE in Japanese subjects. A total of 2,278 SLE cases and 31,948 controls were enrolled. The clinical characteristics of the subjects are summarized in Table S1 and S2. Details of the genome-wide scan data for SLE referenced in the *in silico* SNP selection 2 are described elsewhere (Tahira T et al. Presented at the 59th Annual Meeting of the American Society of Human Genetics, October 21, 2009). doi:10.1371/journal.pgen.1002455.g001

pooled analysis. As a result, 8 SNPs remained for further investigation (Table S4).

Replication studies and identification of *AFF1*

Then, we performed two-stage replication studies using independent SLE cohorts for Japanese subjects (cohort 1 with 562 SLE cases and 653 controls, and cohort 2 with 825 SLE cases and 27,911 controls). First, we evaluated the selected 8 SNPs in the replication study 1. In the replication study 2, 2 SNPs that satisfied $P < 1.0 \times 10^{-6}$ in the combined study of GWAS and replication

study 1 were further evaluated (Figure 1). Among the evaluated SNPs, we observed significant replications in the SNP located in the genomic region of the *AF4/FMR2* family, member 1 gene (*AFF1*) at 4q21 (rs340630; $P = 4.6 \times 10^{-5}$ and $P = 0.0094$ in the two individual cohorts, respectively; Table 3, Table S5, and Figure 2B). The combined study for the GWAS ($P = 1.5 \times 10^{-4}$) and the replication studies demonstrated significant associations of rs340630 that satisfied the genome-wide significance threshold ($P = 8.3 \times 10^{-9}$, OR = 1.21, 95% CI 1.14–2.30).

Cis-eQTL effect of rs340630 on *AFF1* transcripts

Since the landmark SNP in the *AFF1* locus, rs340630, was prioritized through the eQTL study as an eQTL positive SNP (Table 3), we further validated its cis-eQTL effect using Epstein-Barr virus (EBV)-transfected B cell lines established from Japanese individuals (Pharma SNP Consortium (PSC) cells, $n = 62$). The correlation between rs340630 genotypes and the expression levels of *AFF1* was significant in the PSC cells stimulated with phorbol myristate acetate (PMA) ($R^2 = 0.074$, $P = 0.033$; Figure 3A). The expression levels increased with the number of SLE-risk (A) alleles. To further confirm this cis-regulatory effect, we performed allele-specific transcript quantification (ASTQ) of *AFF1*. The transcript levels of each allele were quantified by qPCR using an allele specific probe for a SNP in the 5'-untranslated region (rs340638), which was in absolute LD with rs340630 ($r^2 = 1.0$, $D' = 1.0$). We examined PSC-cells ($n = 17$) that were heterozygous for both rs340630 and rs340638. The mean ratio of each transcript (A over G allele; the A allele comprises a haplotype with the risk (A) allele of rs340630) were significantly increased to 1.07 compared to the ratio of the amount of DNA (1.00, $P = 0.012$) (Figure 3B). These results suggest that rs340630, or SNP(s) in LD with it, are a regulatory variant predisposing SLE susceptibility through increased expression levels of *AFF1*.

Expression of *AFF1* in CD4⁺ and CD19⁺ peripheral blood lymphocytes

AFF1 is known to be involved in cytogenetic translocations of acute lymphoblastic leukemia (ALL) [31]. Its fusion protein with the mixed-lineage leukemia gene (*MLL*) is implicated in the regulation of transcription and the cell cycle of lymphocytes [31]. To investigate the expression pattern of *AFF1* in normal tissues, we evaluated the transcript levels of *AFF1* in a panel of various tissues. We observed prominent expression of *AFF1* in CD4⁺ and CD19⁺ peripheral blood lymphocytes, implying an important role for *AFF1* in helper-T-cells and B-cells (Figure 3C).

Discussion

Through a GWAS and multi-staged replication studies consisting of 2,278 SLE cases and 31,948 controls in Japanese subjects, our study identified that the *AFF1* locus was significantly associated with SLE susceptibility.

As well as the identification of the novel SLE susceptibility locus, we observed significant replications of associations in the previously reported susceptibility loci. The replications were especially enriched in the loci identified through the studies in Asian populations, compared to those in European populations. Considering the ethnic heterogeneities in the epidemiology of SLE [19,20], these observations suggest the similarities in the genetic backgrounds of SLE shared within Asian populations, and also the existence of the both common and divergent genetic backgrounds encompassed between European and Asian populations.

Table 1. Results of a genome-wide association study for Japanese patients with SLE.

rsID ^a	Chr	Position (bp)	Cytoband	Gene	Allele ^b	No. subjects		Allele 1 freq.		OR (95%CI)	P
						Case	Control	Case	Control		
rs10168266	2	191,644,049	2q32	<i>STAT4</i>	T/C	891	3,384	0.37	0.27	1.59 (1.42–1.78)	2.7×10^{-16}
rs9501626	6	32,508,322	6p21	HLA region	A/C	891	3,381	0.20	0.12	1.86 (1.62–2.13)	1.0×10^{-18}
rs2230926	6	138,237,759	6q23	<i>TNFAIP3</i>	G/T	891	3,377	0.11	0.069	1.75 (1.47–2.08)	1.9×10^{-10}
rs6964720	7	75,018,280	7q11	<i>HIP1</i>	G/A	891	3,384	0.25	0.19	1.43 (1.27–1.63)	1.3×10^{-8}
rs2254546	8	11,381,089	8p23	<i>BLK</i>	G/A	891	3,384	0.78	0.72	1.42 (1.61–1.25)	4.1×10^{-8}
rs6590330	11	127,816,269	11q24	<i>ETS1</i>	A/G	891	3,368	0.48	0.39	1.44 (1.30–1.60)	1.3×10^{-11}

^aSNPs that satisfied the threshold of $P < 5.0 \times 10^{-8}$ were indicated.

^bBased on forward strand of NCBI Build 36.3.

SLE, systemic lupus erythematosus; OR, odds ratio.

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To effectively detect the novel SLE susceptibility locus, we integrated cis-eQTL effects of the SNPs and prioritized the results of the GWAS. In addition to identifying a novel locus for SLE-susceptibility, our study demonstrated approximately 30% of confirmed SLE-susceptibility loci were comprised of cis-eQTLs. We also confirmed cis-regulatory effect of the landmark SNP in the *AFF1* locus, rs340630, on *AFF1* transcripts, which had been prioritized through the eQTL study. These results would suggest that accumulation of quantitative changes in gene expression would accelerate the disease onset of SLE. It would also demonstrate the validity of applying eQTL study in the search of the susceptible genes for SLE or other autoimmune diseases, as previously suggested in the study for celiac disease [24]. To our knowledge, this is one of the initial studies to successfully discover a new locus by prioritizing GWAS results using eQTLs, and should contribute to the approaches assessing genetic loci still being uncaptured by recent large-scaled GWASs due to stringent significance threshold for multiple hypothesis testing [21].

We observed prominent expression levels of *AFF1* in CD4⁺ and CD19⁺ peripheral blood lymphocytes, which would imply an important role for *AFF1* in helper-T-cells and B-cells. In fact, *AFF1* is essential for normal lymphocyte development, as demonstrated in mice deficient for *AFF1*; severe reduction were observed in the thymic double positive CD4/CD8 population and the bone marrow pre-B and mature B-cell numbers [32]. The risk A allele of rs340630 demonstrated a cis-eQTL effect on the *AFF1* transcript with enhanced expression levels. As the *AFF1* locus was also demonstrated as an eQTL in primary liver cells [33], the cis-regulatory effect may hold in primary cells as well as lymphoblastoid cells used in the present study. However, because the mechanism of transcriptional regulation is substantially different among cell types [34], cell-type specific analyses including those for primary T-cells and B-cells are needed for understanding the precise role of *AFF1* variant in primary lymphocytes. Although further functional investigation is necessary, our observation suggested that *AFF1* is involved in the etiology of SLE through the regulation of development and activity of lymphocytes. It is of note that *AFF3*, which also belongs to the AF4/FMR2 family, is associated with susceptibility to autoimmune diseases [35].

One of our study's limitations is the selection of SNPs for the replication study using the results of the pooled DNA approach [30], which used a different genotyping platform from that of the present GWAS. Moreover, the association signals based on Silhouette scores in pooled analysis would be less reliable compared to those based on individual genotyping. Since direct comparisons of the association signals of the same single SNPs

between the studies would be difficult due to these issues, we adopted the complementary approach that referred the association signals of the multiple SNPs in the pooled analysis for each of the single SNPs in the GWAS, taking account of LD and physical distances between the SNPs. However, there would exist a possibility that the variant(s) truly associated with SLE was left not to be examined in the replication study. It should be noted that only 1 SNP among the 8 selected SNPs yielded the significant association with SLE, although further enrichments of the significant associations might be anticipated. To elucidate effectiveness and limitation of our approach, further assessments of the studies on the remaining loci would be desirable. It should also be noted that the control-case ratio of the subjects were relatively high in the replication study 2 (=33.8), and this disproportionate ratio could have induced potential bias on the results of the association analysis of the SNPs. However, considering the homogeneous ancestries of the Japanese population [27] and that principal component analysis did not demonstrate significant population stratification in the control subjects of the replication study 2 (data not shown), the bias owing to population stratification might not be substantial.

In summary, through a GWAS and multi-staged replication studies in a Japanese population integrating eQTL study, our study identified *AFF1* as a novel susceptibility locus for SLE.

Materials and Methods

Subjects

We enrolled 2,278 systemic lupus erythematosus (SLE) cases and 31,948 controls. SLE cases enrolled in the genome-wide association study (GWAS) ($n = 891$) or part of the 2nd replication study ($n = 83$) were collected from 12 medical institutes in Japan under the support of the autoimmune disease study group of Research in Intractable Diseases, Japanese Ministry of Health, Labor and Welfare: Hokkaido University Graduate School of Medicine, Tohoku University Graduate School of Medicine, the University of Tokyo, Keio University School of Medicine, Juntendo University School of Medicine, University of Occupational and Environmental Health, University of Tsukuba, Tokyo Medical and Dental University, National Center for Global Health and Medicine, Nagasaki University, Wakayama Medical University, and Jichi Medical University. SLE cases ($n = 562$) and controls ($n = 653$) enrolled in the 1st replication study were collected from Kyushu University. Some of the SLE cases ($n = 742$) and controls ($n = 27,911$) enrolled in the 2nd replication study were collected from Kyoto University, Tokyo Women's

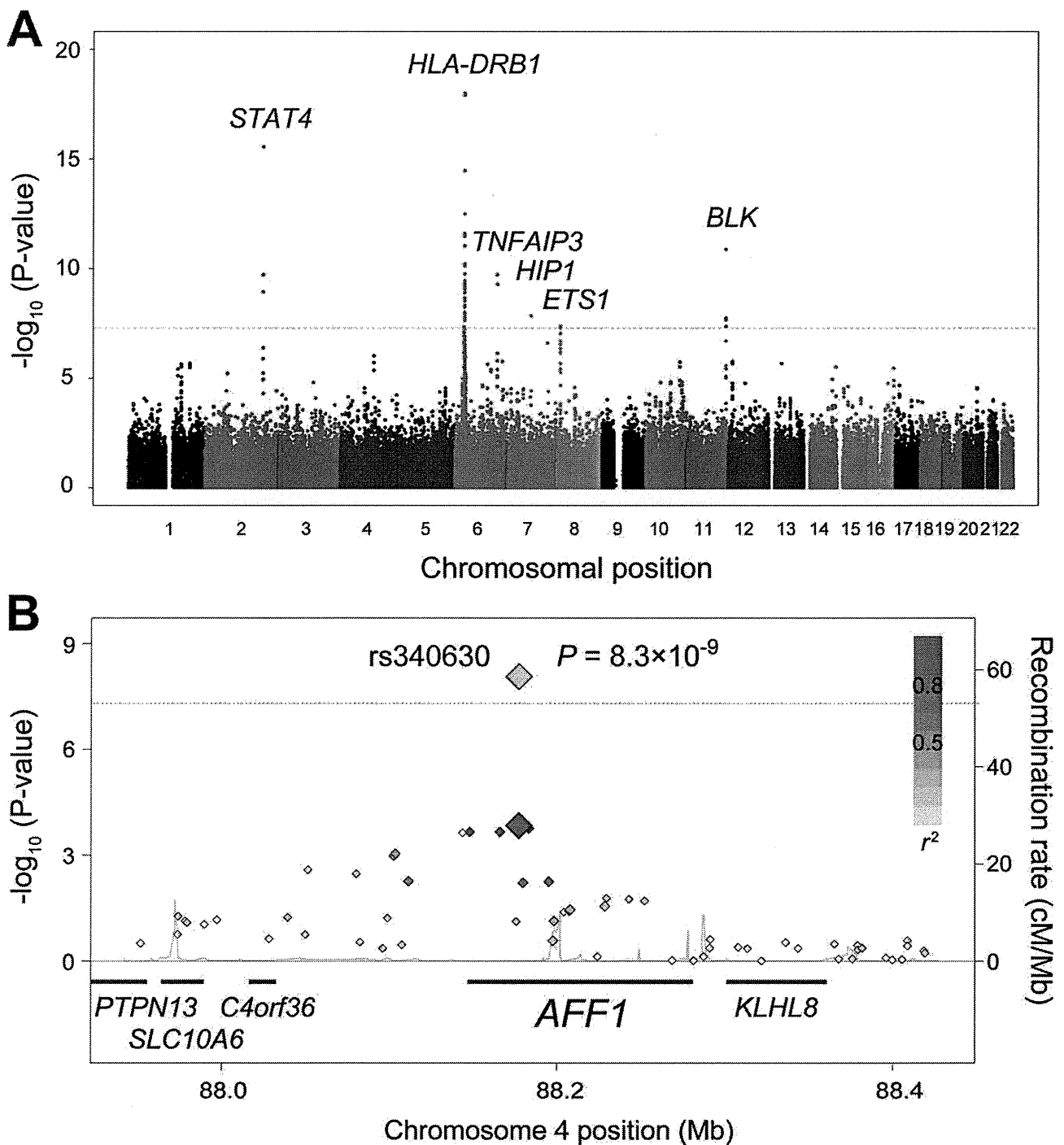


Figure 2. Associations of the *AFF1* locus with SLE. (A) A chromosomal plot of P -values in GWAS for SLE. (B) A regional plot in the *AFF1* locus. Diamond-shaped data points represent $-\log_{10}(\text{P-values})$ of the SNPs. Large-sized points indicate the P -values of the landmark SNP, rs340630 (green for the combined study and red for the GWAS). Density of red color represents r^2 values with rs340630. Blue line represents recombination rates. Lower part indicates RefSeq genes. Gray dashed horizontal lines represent the threshold of $P = 5.0 \times 10^{-8}$. The plots were drawn using SNAP, version 2.1 [47].

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Medical University, the University of Tokyo, and the BioBank Japan Project [36]. All subjects were of Japanese origin and provided written informed consent. SLE cases met the revised American College of Rheumatology (ACR) criteria for SLE [37]. Control subjects were confirmed to be free of autoimmune

disease. Some of the SLE cases were included in our previous studies [38–40]. Details of the subjects are summarized in Table S1 and S2. This research project was approved by the ethical committees of the University of Tokyo, RIKEN, and affiliated medical institutes.

Table 2. Associations among previously reported SLE-related loci.

rsID	Chr	Position (bp)	Cytoband	Gene	Allele ^a		Allele 1 freq.		OR (95%CI)	P	eQTL ^b	Identified by the studies in ^c	
					1/2	Case	Control	Case				Control	Caucasians
rs2205960	1	171,458,098	1q25	<i>TNFSF4</i>	T/G	0.23	0.18	1.35 (1.19–1.54)	3.0×10^{-6}		+		
rs3024505	1	205,006,527	1q32	<i>IL10</i>	A/G	0.019	0.014	1.34 (0.90–2.00)	0.15		+		
rs13385731	2	33,555,394	2p22	<i>RASGRP3</i>	C/T	0.90	0.87	1.37 (1.15–1.64)	6.0×10^{-4}	+			+
rs10168266	2	191,644,049	2q32	<i>STAT4</i>	T/C	0.37	0.27	1.59 (1.42–1.78)	2.7×10^{-16}		+		
rs6445975	3	58,345,217	3p14	<i>PXK</i>	G/T	0.25	0.23	1.09 (0.96–1.23)	0.18	+	+		
rs10516487	4	102,970,099	4q24	<i>BANK1</i>	G/A	0.91	0.89	1.28 (1.07–1.53)	0.0070		+		
rs10036748	5	150,438,339	5q33	<i>TNIP1</i>	T/C	0.75	0.72	1.16 (1.03–1.31)	0.014				+
rs9501626	6	32,508,322	6p21	<i>HLA-DRB1</i>	A/C	0.20	0.12	1.86 (1.62–2.13)	1.0×10^{-18}		+		
rs548234	6	106,674,727	6q21	<i>PRDM1</i>	C/T	0.40	0.34	1.30 (1.16–1.44)	2.3×10^{-6}	+	+		
rs2230926	6	138,237,759	6q23	<i>TNFAIP3</i>	G/T	0.11	0.069	1.75 (1.47–2.08)	1.9×10^{-10}	+	+		
rs849142	7	28,152,416	7p15	<i>JAZF1</i>	C/T	0.999	0.999	2.72 (0.25–29.8)	0.41				+
rs4917014	7	50,276,409	7p12	<i>IKZF1</i>	T/G	0.58	0.53	1.24 (1.11–1.38)	8.1×10^{-5}				+
rs6964720	7	75,018,280	7q11	<i>HIP1</i>	G/A	0.25	0.19	1.43 (1.27–1.62)	1.3×10^{-8}				+
rs4728142	7	128,361,203	7q32	<i>IRF5</i>	A/G	0.16	0.11	1.48 (1.28–1.72)	2.4×10^{-7}	+	+		
rs2254546	8	11,381,089	8p23	<i>BLK</i>	G/A	0.78	0.72	1.42 (1.25–1.61)	4.1×10^{-8}	+	+		
rs1913517	10	49,789,060	10q11	<i>WDFY4</i>	A/G	0.32	0.28	1.20 (1.07–1.35)	0.0013				+
rs4963128	11	579,564	11p15	<i>KIAA1542</i>	T/C	0.98	0.97	1.58 (1.03–2.44)	0.038	+	+		
rs2732552	11	35,041,168	11p13	<i>PDHX, CD44</i>	T/C	0.75	0.73	1.13 (1.00–1.27)	0.056		+		
rs4639966	11	118,078,729	11q23	Intergenic	T/C	0.32	0.28	1.22 (1.09–1.36)	7.3×10^{-4}				+
rs6590330	11	127,816,269	11q24	<i>ETS1</i>	A/G	0.48	0.39	1.44 (1.30–1.60)	1.3×10^{-11}				+
rs1385374	12	127,866,647	12q24	<i>SLC15A4</i>	T/C	0.19	0.16	1.21 (1.06–1.38)	0.0057				+
rs7329174	13	40,456,110	13q14	<i>ELF1</i>	G/A	0.30	0.25	1.32 (1.18–1.49)	2.2×10^{-6}				+
rs7197475	16	30,550,368	16p11	Intergenic	T/C	0.12	0.10	1.20 (1.02–0.41)	0.031				+
rs11150610	16	31,241,737	16p11	<i>ITGAM</i>	C/A	0.20	0.19	1.07 (0.94–1.22)	0.32	+	+		
rs12949531	17	13,674,531	17p12	Intergenic	T/C	0.28	0.27	1.02 (0.91–1.15)	0.73				+
rs463426	22	20,139,185	22q11	<i>HIC2, UBE2L3</i>	T/C	0.52	0.48	1.20 (1.08–1.33)	6.1×10^{-4}		+		

^aBased on forward strand of NCBI Build 36.3.^bDefined using gene expression data measured in lymphoblastoid B cell lines [28].^cBased on the previously reported studies for SLE susceptibility loci [3–18].

SLE, systemic lupus erythematosus; OR, odds ratio; eQTL, expression quantitative trait locus; GWAS, genome-wide association study.

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Genotyping and quality control

In GWAS, 946 SLE cases and 3,477 controls were genotyped using Illumina HumanHap610-Quad and Illumina Human-

Hap550v3 Genotyping BeadChips (Illumina, CA, USA), respectively. After the exclusion of 47 SLE cases and 92 controls with call rates <0.98, SNPs with call rates <0.99 in SLE cases or controls,

Table 3. Results of combined study for Japanese patients with SLE.

rsID	Chr	Position (bp)	Cytoband	Gene	Allele	Stage	No. subjects		Allele 1 freq.		OR (95%CI)	P	eQTL ^a
							Case	Control	Case	Control			
rs340630	4	88,177,419	4q21	<i>AFF1</i>	A/G	GWAS	891	3,383	0.56	0.51	1.22 (1.10–1.36)	1.5×10^{-4}	+
						Replication study 1	550	646	0.57	0.49	1.40 (1.19–1.64)	4.6×10^{-5}	
						Replication study 2	820	27,911	0.56	0.53	1.14 (1.03–1.26)	0.0094	
						Combined study	2,261	31,940	0.56	0.52	1.21 (1.14–1.30)	8.3×10^{-9}	

^aDefined using gene expression data measured in lymphoblastoid B cell lines [28].

doi:10.1371/journal.pgen.1002455.t003

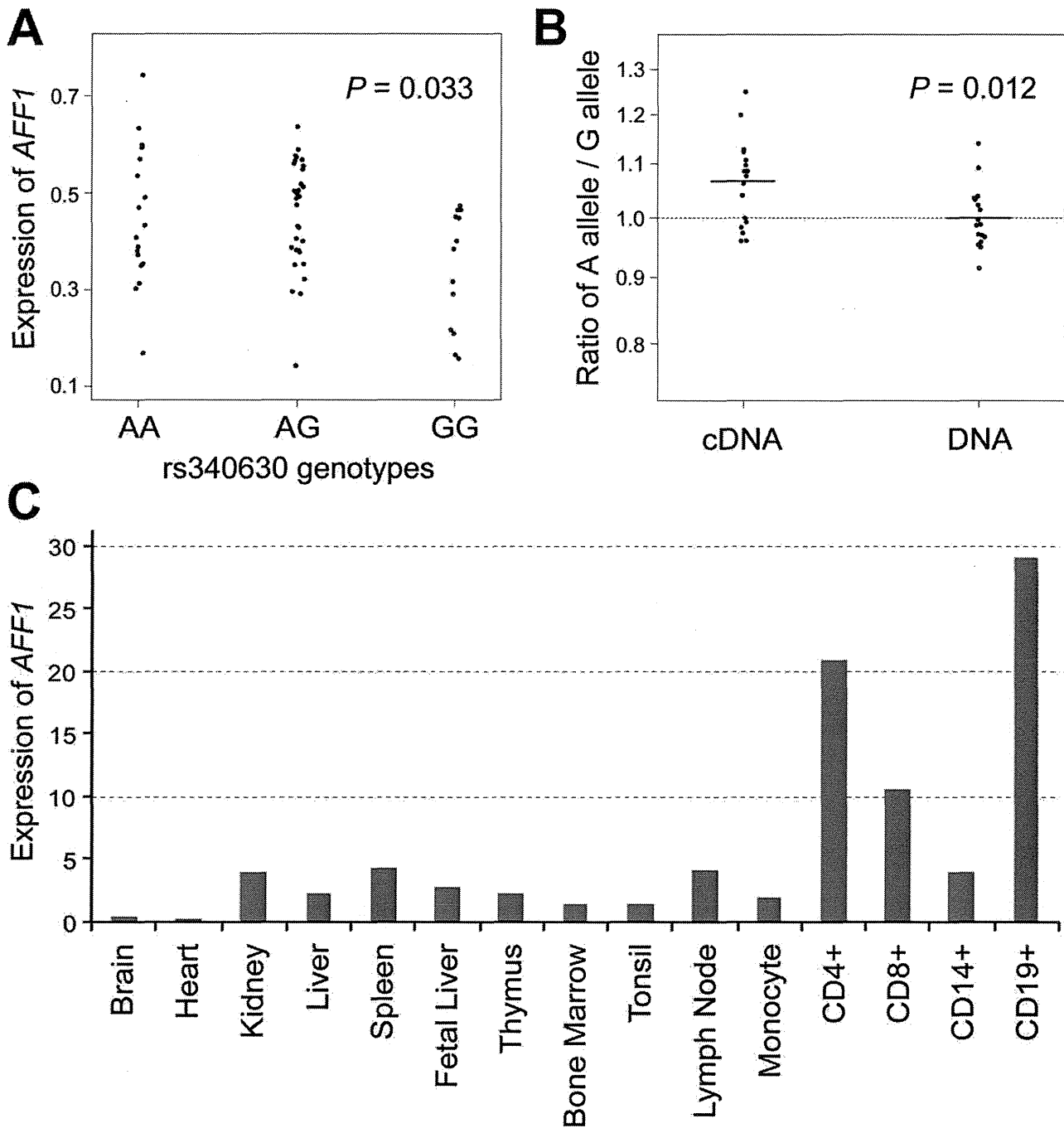


Figure 3. Association of rs340630 with *AFF1* expression. (A) Correlation between rs340630 genotypes and transcript levels of *AFF1* (NM_001166693) in EBV-transfected cell lines ($n = 62$) stimulated with PMA. (B) Allele-specific quantification (ASTQ) of *AFF1* transcripts. Allele specific-probes for rs340638 were used for quantification by qPCR. The ratios of A allele over G allele for the amounts of both cDNAs and DNAs were plotted in log scale for each cell line. (C) *AFF1* expression in various tissues. Transcripts levels of *AFF1* were quantified by qPCR and were normalized by *GAPDH* levels.
doi:10.1371/journal.pgen.1002455.g003

non-autosomal SNPs, and SNPs not shared between SLE cases and controls, were excluded. We excluded 7 closely related SLE cases in a 1st or 2nd degree of kinship based on identity-by-descent estimated using PLINK version 1.06 [41]. We then excluded 1 SLE cases and 1 controls whose ancestries were estimated to be distinct from East-Asian populations using PCA performed along with the genotype data of Phase II HapMap populations (release 24) [29] using EIGENSTRAT version 2.0 [42]. Subsequently,

SNPs with minor allele frequencies < 0.01 in SLE cases or controls, SNPs with exact P -values of Hardy-Weinberg equilibrium test $< 1.0 \times 10^{-6}$ in controls, or SNPs with ambiguous cluster plots were excluded. Finally, 430,797 SNPs for 891 SLE cases and 3,384 controls were obtained. Genotyping of SNPs in replication studies was performed using TaqMan Assay or Illumina HumanHap610-Quad Genotyping BeadChip (Illumina, CA, USA).

Association analysis of the SNPs

Association of SNPs in GWAS and replication studies were tested with Cochran-Armitage's trend test. Combined analysis was performed with Mantel-Haenszel method. Associations of previously reported SLE susceptibility loci [3–18] were evaluated using the results of the GWAS. Genotype imputation was performed for non-genotyped SNPs using MACH version 1.0 [43] with Phase II HapMap East-Asian individuals as references [29], as previously described [44]. All imputed SNPs demonstrated imputation scores, $R_{sq} > 0.70$.

eQTL study

We analyzed gene expression data previously measured in lymphoblastoid B cell lines from Phase II HapMap East-Asian individuals using Illumina's human whole-genome expression array (WG-6 version 1) (accession number; GSE6536) [28]. Expression data were normalized across the individuals. We used BLAST to map 47,294 Illumina array probes onto human autosomal reference genome sequences (Build 36). We discarded probes mapped with expectation values smaller than 0.01 to multiple loci, or for which there was polymorphic HapMap SNP(s) inside the probe. Then, 19,047 probes with exact matches to a unique locus with 100% identity and with a mean signal intensity greater than background were obtained. Genotype data of HapMap individuals were obtained for SNPs included in the GWAS. Associations of SNP genotypes (coded as 0, 1, and 2) with expression levels of each of the cis-eQTL probes (located within ± 300 kbp regions of the SNPs) were evaluated using linear regression assuming additive effects of the genotypes on the expression levels. Considering the significant overlap between eQTL and genetic loci responsible for autoimmune diseases [24], we applied relatively less stringent multiple testing threshold of FDR Q -values < 0.2 for the definition of eQTL. SNPs that exhibited this threshold with any of the corresponding cis-eQTL probes were denoted as eQTL positive.

Selection of SNPs enrolled in the replication studies

In order to select SNPs for further replication studies, we firstly integrated the results of GWAS and eQTL study. SNPs that satisfied $P < 1.0 \times 10^{-4}$ in GWAS, or the SNPs that satisfied $1.0 \times 10^{-4} \leq P < 1.0 \times 10^{-3}$ in GWAS and denoted as eQTL positive, were selected. Among these, SNPs most significantly associated in each of the genomic loci and not included in the previously reported SLE susceptibility loci [3–18] were further evaluated.

Then, the results of the concurrently proceeding genome-wide scan for SLE in the Japanese subjects using a pooled DNA approach were referred (Tahira T et al. Presented at the 59th Annual Meeting of the American Society of Human Genetics, October 21, 2009). In the scan, DNA collected from 447 SLE cases and 680 controls of Japanese origin were pooled respectively, and genotyped using GeneChip Human Mapping 500K Array Set (Affymetrix, CA, USA). SNPs were ranked according to the Silhouette scores estimated based on relative allele scores (RAS) between SLE cases and controls, and rank-based P -values were assigned [30]. By referring to association signals in multiple neighboring SNPs in the pooled analysis, we selected SNPs for replication study 1. Namely, if the SNP of interest was in LD ($r^2 > 0.5$) or was located within ± 100 kbp of SNPs showing association signals in the pooled analysis (rank-based $P < 0.01$), it would be selected. SNPs that satisfied $P < 1.0 \times 10^{-6}$ in the combined study of GWAS and replication study 1 were further evaluated in replication study 2 (Figure 1).

Quantification of *AFF1* expression

EBV-transformed lymphoblastoid cell lines ($n = 62$) were established by Pharma SNP Consortium (Tokyo, Japan) using peripheral blood lymphocytes of Japanese healthy individuals. Cells were incubated for 2 h in medium alone (RPMI 1640 medium containing 10% FBS, 1% penicillin, and 1% streptomycin) or with 100 ng/ml PMA. Conditions for cell stimulation were optimized before the experiment as previously described [45]. Cells were then harvested and total RNA was isolated using an RNeasy Mini Kit (Qiagen) with DNase treatment. Total RNA (1 μ g) was reverse transcribed using TaqMan Gold RT-PCR reagents with random hexamers (Applied Biosystems). Real-time quantitative PCR was performed in triplicate using an ABI PRISM 7900 and TaqMan gene expression assays (Applied Biosystems). Specific probes (Hs01089428_m1) for transcript of *AFF1* (NM_001166693) were used. Expression of *AFF1* in various tissues was also quantified using Premium Total RNA (Clontech). The data were normalized to *GAPDH* levels. *GUS* levels were also evaluated for internal control, and similar results were obtained. Correlation coefficient, R^2 , between rs340630 genotypes and transcript levels of *AFF1* was evaluated.

Allele-specific transcript quantification (ASTQ)

ASTQ of *AFF1* in PSC cells was performed as previously described [46]. DNAs were extracted by using a DNeasy Kit (QIAGEN). RNA extraction and cDNA preparation were performed as described above. For PSC cells ($n = 17$) that were heterozygous for both rs340630 (the landmark SNP of GWAS) and rs340638 (located in the 5'-untranslated region of *AFF1* and in absolute LD with rs340630), expression levels of *AFF1* were quantified by qPCR on an ABI Prism 7900 using a custom-made TaqMan MGB-probe set for rs340638. Primer sequences were 5'-CTAACTGTGGCCCGCGTTG-3' and 5'-CCCGGCGCA-GTTTCTGAG-3'. The probe sequences were 5'-VIC-CGAA-GACCGCCAGCGCCCAAC-TAMRA-3' and 5'-FAM-CGAA-GACCGCCGCGCCCAA-TAMRA-3'. Ct values of VIC and FAM were obtained for genomic DNA and cDNA samples after 40 cycles of real-time PCR. We also prepared genomic DNA of samples homozygous for each allele and mixed them at different ratios (2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2) to create a standard curve by plotting Ct values of VIC/FAM against the allelic ratio of VIC/FAM for each mixture. Using the standard curve, we calculated the allelic ratios for each genomic DNA and cDNA samples. We measured each sample in quadruplicate in one assay; tests were independently repeated twice.

Web resources

The URLs for data presented herein are as follows.
 NCBI GEO, <http://www.ncbi.nlm.nih.gov/geo>
 BioBank Japan Project, <http://biobankjp.org>
 PLINK software, <http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml>
 International HapMap Project, <http://www.hapmap.org>
 EIGENSTRAT software, <http://genepath.med.harvard.edu/~reich/Software.htm>
 MACH and mach2qtl software, <http://www.sph.umich.edu/csg/abecasis/MACH/index.html>
 SNAP, <http://www.broadinstitute.org/mpg/snap/index.php>

Supporting Information

Figure S1 Principal component analysis (PCA) plot of the subjects. PCA plot of subjects enrolled in the GWAS for SLE. SLE cases and the controls enrolled in the GWAS are plotted based on