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Overexpression of T-bet Gene Regulates Murine Autoimmune Arthritis

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Objective. **To clarify the role of T-bet in the pathogenesis of collagen-induced arthritis (CIA).**

Methods. **T-bet–transgenic (Tg) mice under the control of the CD2 promoter were generated. CIA was induced in T-bet–Tg mice and wild-type C57BL/6 (B6) mice. Levels of type II collagen (CII)–reactive T-bet and retinoic acid receptor–related orphan nuclear receptor γt (RORγt) messenger RNA expression were analyzed by real-time polymerase chain reaction. Criss-cross experiments using CD4+ T cells from B6 and T-bet–Tg mice, as well as CD11c+ splenic dendritic cells (DCs) from B6 and T-bet–Tg mice with CII were performed, and interleukin-17 (IL-17) and interferon-γ (IFNγ) in the supernatants were measured by enzyme-linked immunosorbent assay. CD4+ T cells from B6, T-bet–Tg, or T-bet–Tg/IFNγ$^{-/-}$ mice were cultured for Th17 cell differentiation, then the proportions of cells producing IFNγ and IL-17 were analyzed by fluorescence-activated cell sorting.**

Results. **Unlike the B6 mice, the T-bet–Tg mice did not develop CIA. T-bet–Tg mice showed overexpression of *Tbx21* and down-regulation of *Rorc* in CII-reactive T cells. Criss-cross experiments with CD4+ T cells and splenic DCs showed a significant reduction in IL-17 production by CII-reactive CD4+ T cells in T-bet–Tg mice, even upon coculture with DCs from B6 mice, indicating dysfunction of IL-17–producing CD4+ T cells. Inhibition of Th17 cell differentiation under an in vitro condition favoring Th17 cell differentiation was observed in both T-bet–Tg mice and T-bet–Tg/IFNγ$^{-/-}$ mice.**

Conclusion. **Overexpression of T-bet in T cells suppressed the development of autoimmune arthritis. The regulatory mechanism of arthritis might involve dysfunction of CII-reactive Th17 cell differentiation by overexpression of T-bet via IFNγ-independent pathways.**

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by autoimmunity, infiltration of the joint synovium by activated inflammatory cells, and progressive destruction of cartilage and bone. Although the exact cause of RA is not clear, T cells seem to play a crucial role in the initiation and perpetuation of the chronic inflammation in RA.

The Th1 cell subset has long been considered to play a predominant role in inflammatory arthritis, because T cell clones from RA synovium were found to produce large amounts of interferon-γ (IFNγ) (1). Recently, interleukin-17 (IL-17)–producing Th17 cells have been identified, and this newly discovered T cell population appears to play a critical role in the development of various forms of autoimmune arthritis in experimental animals, such as those with glucose-6-phosphate isomerase–induced arthritis (2) and collagen-induced arthritis (CIA) (3). Conversely, IFNγ has antiinflammatory effects on the development of experimental arthritis (4,5). IL-17 is spontaneously produced by RA synovium (6), and the percentage of IL-17–positive CD4+ T cells

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was increased in the peripheral blood mononuclear cells of patients with RA compared with healthy control subjects (7). It is therefore necessary to determine if autoimmune arthritis is a Th1- or a Th17-associated disorder.

The lineage commitment of each Th cell subset from naive CD4+ T cells is dependent on the expression of specific transcription factors induced under the particular cytokine environment. Differentiation of Th1 cells is dependent on the expression of the transcription factor T-bet, which is induced by IFNγ/STAT-1 signaling pathways and directly activates the production of IFNγ (8,9). Similarly, Th17 cell differentiation in mice is dependent on the transcription factor retinoic acid receptor–related orphan nuclear receptor γt (RORγt) induced by transforming growth factor β (TGFβ) and IL-6 (10). Previous studies showed that these transcription factors negatively regulate the differentiation of other T cell subsets by direct co-interaction and/or indirect effects of cytokines produced from each T cell subset (11,12). How the predominant differentiation of CD4+ T cells affects the development of autoimmune arthritis remains unclear, however.

In the present study, CIA was induced in C57BL/6 (B6) mice and T-bet–transgenic (Tg) mice under the control of the CD2 promoter. The results showed that CIA was significantly suppressed in T-bet–Tg mice as compared with B6 mice. IL-17 production was not detected in type II collagen (CII)–reactive T cells from T-bet–Tg mice, and a significant reduction in IL-17 production by CII-reactive CD4+ T cells from T-bet–Tg mice was observed even when they were cocultured with splenic dendritic cells (DCs) from B6 mice. IFNγ production was also reduced in T-bet–Tg mice as compared with B6 mice, and levels of IFNγ in CII-reactive CD4+ T cells from T-bet–Tg mice were not different from those in B6 mice. Inhibition of Th17 cell differentiation and predominant differentiation of Th1 cells under an in vitro condition favoring Th17 cell differentiation was observed in T-bet–Tg mice, and surprisingly, this inhibition was also observed in T-bet–Tg/IFNγ$^{-/-}$ mice. These results indicate suppression of Th17 cell differentiation by overexpression of T-bet, but not IFNγ. Our findings support the notion that the suppression of autoimmune arthritis in T-bet–Tg mice might be due to the direct inhibition of Th17 cell differentiation by T-bet overexpression in T cells.

MATERIALS AND METHODS

Mice. CD2 T-bet–Tg mice (12) were prepared by backcrossing mice on a C57BL/6 background. IFNγ$^{-/-}$ mice were obtained from The Jackson Laboratory. Littermates of T-bet–Tg mice were used as controls in all experiments. All mice were maintained under specific pathogen–free conditions, and the experiments were conducted in accordance with the institutional ethics guidelines.

Induction of CIA and assessment of arthritis. Native chicken CII (Sigma-Aldrich) was dissolved in 0.01*M* acetic acid and emulsified in Freund's complete adjuvant (CFA). CFA was prepared by mixing 5 mg of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco) and 1 ml of Freund's incomplete adjuvant (Sigma-Aldrich). Mice ages 8–10 weeks were injected intradermally at the base of the tail with 200 μg of CII in CFA on days 0 and 21. Arthritis was evaluated visually, and changes in each paw were scored on a scale of 0–3, where 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced swelling, and 3 = ankylosis. The scores in the 4 limbs were then summed (maximum score 12).

Histopathologic scoring. For histologic assessment, mice were killed on day 42 after the first immunization, and both rear limbs were removed. After fixation and decalcification, joint sections were cut and stained with hematoxylin and eosin. Histologic features of arthritis were quantified by 2 independent observers (YK and IM) who were blinded with regard to the study group, and a histologic score was assigned to each joint based on the degree of inflammation and erosion, as described previously (13). The severity of inflammation was scored on a scale of 0–5, where 0 = normal, 1 = minimal inflammatory infiltration, 2 = mild infiltration with no soft tissue edema or synovial lining cell hyperplasia, 3 = moderate infiltration with surrounding soft tissue edema and some synovial lining cell hyperplasia, 4 = marked infiltration, edema, and synovial lining cell hyperplasia, and 5 = severe infiltration with extended soft tissue edema and marked synovial lining cell hyperplasia. The severity of bone erosion was also scored on a scale of 0–5, where 0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe erosion with full-thickness defects in the cortical bone.

Analysis of cytokine profiles and cytokine and transcriptional factor gene expression. Inguinal and popliteal lymph nodes were harvested from each mouse on day 10 after the first immunization with CII. Single-cell suspensions were prepared, and lymph node cells (2×10^5/well on a 96-well round-bottomed plate) were cultured for 72 hours in RPMI 1640 medium (Sigma-Aldrich) containing 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 50 μ*M* 2-mercaptoethanol in the presence of 100 μg/ml of denatured chicken CII. The supernatants were analyzed for IFNγ, IL-4, IL-10, and IL-17 by enzyme-linked immunosorbent assay (ELISA) using specific Quantikine ELISA kits (R&D Systems).

Lymphocytes harvested on day 10 after immunization were used to obtain complementary DNA (cDNA) by reverse transcription, using a commercially available kit. A TaqMan Assay-on-Demand gene expression product was used for real-time polymerase chain reaction (PCR; Applied Biosystems). The expression levels of *Ifng*, *Il17a*, *Tbx21*, *Rorc*, *Il12a*, and *Il23a* were normalized relative to the expression of *gapdh*. Analyses were performed with an ABI Prism 7500 apparatus (Applied Biosystems).

Criss-cross coculture with CD4+ T cells and CD11c+ splenic dendritic cells. Ten days after the first CII immunization, CD4+ cells in draining lymph nodes were isolated by

positive selection, using a magnetic-activated cell sorter (MACS) system with anti-CD4 monoclonal antibody (mAb; Miltenyi Biotec). After treatment with mitomycin C, CD11c+ cells were isolated from the spleen by positive selection, using a MACS system with anti-CD11c mAb (Miltenyi Biotec). Criss-cross coculture for 72 hours was performed with 1×10^5 CD4+ cells and 2×10^4 CD11c+ cells in 100 μg/ml of denatured CII-containing medium. Cytokine production and transcription factor expression were then analyzed.

Measurement of collagen-specific immunoglobulin titers. Serum was collected from the mice on day 56 after the first immunization. A total of 10 μg/ml of CII in phosphate buffered saline (PBS) was coated overnight at 4°C onto 96-well plates (Nunc MaxiSorp; Nalge Nunc). After washes with washing buffer (0.05% Tween 20 in PBS), the blocking solution, including 1% bovine serum albumin in PBS, was applied for 1 hour. After washing, 100 μl of diluted serum was added, and the plates were incubated for 1 hour at room temperature. After further washing, horseradish peroxidase–conjugated anti-mouse IgG, IgG1, IgG2a, or IgG2b (1:5,000 dilution) in blocking solution was added, and the plates were incubated for 1 hour at room temperature. After washing, tetramethylbenzidine was added, and the optical density was read at 450 nm using a microplate reader.

Purification of CD4+ cells and in vitro T cell cultures. CD4+ cells (1×10^6/well) were cultured in medium with 1 μg/ml of soluble anti-CD3ε mAb (eBioscience), 1 μg/ml of soluble anti-CD28 mAb (BioLegend), 10 μg/ml of anti-IFNγ mAb (BioLegend), and 10 μg/ml of anti–IL-4 mAb (BioLegend) for a neutral condition. For Th17 cell differentiation, CD4+ cells (1×10^6/well) were cultured in medium with 1 μg/ml of soluble anti-CD3ε mAb, 1 μg/ml of soluble anti-CD28 mAb, 3 ng/ml of human TGFβ (R&D Systems), 20 ng/ml of mouse IL-6 (eBioscience), 10 μg/ml of anti-IFNγ mAb, and 10 μg/ml of anti–IL-4 mAb. On day 4, cells were restimulated for 4 hours with 50 ng/ml of phorbol myristate acetate and 500 ng/ml of ionomycin and used in the experiments.

Surface and intracellular staining and fluorescence-activated cell sorter (FACS) analysis. GolgiStop (BD PharMingen) was added during the last 6 hours of each culture. Cells were stained extracellularly, fixed, and permeabilized with Cytofix/Cytoperm solution (BD PharMingen). Then, intracellular cytokine staining was performed according to the manufacturer's protocol, using fluorescein isothiocyanate (FITC)–conjugated anti-IFNγ (BD PharMingen) and phycoerythrin (PE)–conjugated anti–IL-17 (BD PharMingen) or FITC-conjugated anti–IL-17 (BioLegend). A Treg cell staining kit (eBioscience) was used to stain T-bet, RORγt, and FoxP3 in cultured cells according to the manufacturer's protocol, using PE-conjugated anti–T-bet (eBioscience), allophycocyanin-conjugated anti-RORγt (eBioscience), and PE-conjugated anti-FoxP3 (eBioscience). Samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson), and data were analyzed with FlowJo software (Tree Star).

Statistical analysis. Data are expressed as the mean ± SEM or the mean ± SD. Differences between groups were examined for statistical significance using Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Construction of the T-bet transgene and tissue distribution of transcription factors and cytokine production in naive mice. To generate transgenic mouse lines that express high levels of T-bet specifically in T cells, mouse T-bet cDNA was inserted into a VA vector containing a human CD2 transgene cassette (14). To confirm the expression of the transgene, reverse transcription–PCR (RT-PCR) was performed to monitor the expression of *Tbx21* (coding for T-bet) in organs from the T-bet–Tg mice. *Tbx21* messenger RNA (mRNA) expression was detected in the lymphatic system and in nonlymphatic organs in T-bet–Tg mice, and the expression levels were higher than those in B6 mice (data available upon request from the author). Analysis by semiquantitative RT-PCR and quantitative PCR (data not shown) revealed that the expression levels of other transcription factors (*Gata3*, *Rorc*, and *Foxp3*) in T-bet–Tg mice were not different from those in B6 mice. As previously reported by Ishizaki et al (14), high production of IFNγ was observed even when CD4+ T cells isolated from the spleen of T-bet–Tg mice were cultured under neutral conditions (data available upon request from the author).

Failure to induce CIA and low CII-specific IgG production in T-bet–Tg mice. To assess whether T cell–specific T-bet expression affects the development of arthritis, we induced CIA in T-bet–Tg mice and in wild-type B6 mice. The incidence and severity of arthritis in T-bet–Tg mice were markedly suppressed compared with those in B6 mice (Figure 1A). Surprisingly, the majority of T-bet–Tg mice were essentially free of arthritis, and even when arthritis was present, it was of the mild type. Consistent with these findings, histologic analyses of the joints obtained from each mouse 42 days after immunization revealed that joint inflammation and destruction were significantly suppressed in T-bet–Tg mice compared with B6 mice (Figures 1B and C). These results indicated that enforced expression of T-bet in T cells suppressed the development of CIA.

Because the levels of CII-specific IgG correlate well with the development of arthritis (15), we examined CII-specific IgG production in T-bet–Tg mice. CII-specific IgG, IgG1, IgG2a, and IgG2b levels were significantly lower in T-bet–Tg mice than in B6 mice, as determined by ELISA (Figure 1D). Thus, enforced expression of T-bet in T cells suppresses the development of CIA and CII-specific IgG production.

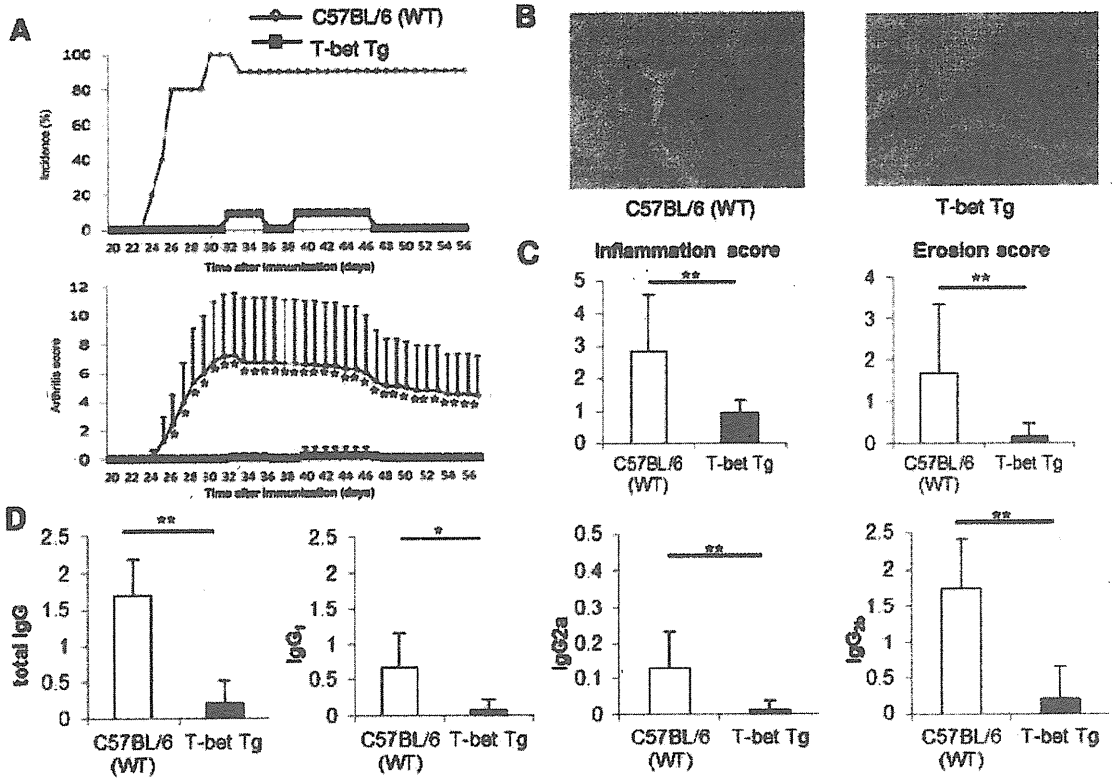

Figure 1. Significant suppression of collagen-induced arthritis (CIA) and type II collagen (CII)–specific IgG production in T-bet–transgenic (Tg) mice. On days 0 and 21, mice were immunized intradermally at several sites at the base of the tail with chicken CII emulsified with Freund's complete adjuvant. **A**, Incidence and severity of CIA. The arthritis score was determined as described in Materials and Methods. Data were obtained from 2 independent experiments involving 10 C57BL/6 (wild-type [WT]) mice and 11 T-bet–Tg mice. **B**, Hematoxylin and eosin–stained sections of the hind paws of mice obtained 6 weeks after the first immunization. Original magnification × 40. **C**, Inflammation and bone erosion scores in 7 C57BL/6 mice and 5 T-bet–Tg mice 6 weeks after the first immunization. Scores were determined as described in Materials and Methods. **D**, Serum levels of CII-specific IgG, IgG1, IgG2a, and IgG2b levels in 10 C57BL/6 mice and 11 T-bet–Tg mice 8 weeks after the first immunization, as measured by enzyme-linked immunosorbent assay. Values in **A**, **C**, and **D** are the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$ by Student's *t*-test.

Suppression of CII-reactive IL-17 production and IL-17 mRNA expression in T-bet–Tg mice. Because enforced T-bet expression in T cells suppressed the development CIA, we examined antigen-specific cytokine production and transcription factor expression in mice with CIA. CD4+ T cells harvested from draining lymph nodes were stimulated with CII in vitro, and then various cytokine levels in the supernatants were measured by ELISA. IL-17 production by CII-reactive T cells was significantly reduced in T-bet–Tg mice as compared with B6 mice (Figure 2A). IFNγ production by CII-reactive T cells also tended to be decreased in T-bet–Tg mice.

We analyzed CII-reactive cytokine and transcription factor mRNA expression levels by real-time PCR (Figure 2B). Similar to the ELISA results, *Il17a* expression tended to be lower in T-bet–Tg mice than in B6 mice. No difference in *Ifng* expression was observed between B6 and T-bet–Tg mice (Figure 2B). *Tbx21* expression tended to be higher in T-bet–Tg mice, whereas *Rorc* expression was lower in T-bet–Tg mice than in B6 mice ($P < 0.05$). The level of expression of *Il12a* (coding for IL-12p35) was also higher in T-bet–Tg mice than in B6 mice ($P < 0.05$). However, there was no difference in the expression levels of *Il23a* (coding for IL-23p19) between B6 mice and T-bet–Tg mice. These

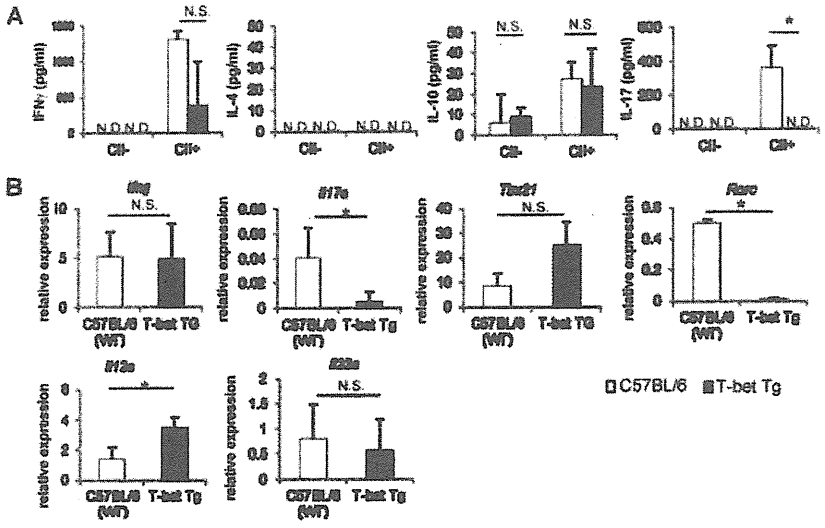

Figure 2. No production of interleukin-17 (IL-17) and low production of interferon-γ (IFNγ) in type II collagen (CII)–reactive CD4+ T cells. **A,** Ten days after the first CII immunization, lymphocytes derived from the draining lymph nodes of C57BL/6 (wild-type [WT]) mice and T-bet–transgenic (Tg) mice were cultured for 72 hours in the presence or absence of 100 μg/ml of denatured CII. Levels of IL-17, IFNγ, IL-4, and IL-10 in the supernatants were measured by enzyme-linked immunosorbent assay. **B,** After culture of lymphocytes with CII, cDNA was obtained, and levels of *Ifng*, *Il17a*, *Tbx21*, *Rorc*, *Il12a*, and *Il23a* expression were analyzed by real-time polymerase chain reaction. Values are the mean ± SD of 3 mice. * = $P < 0.05$ by Student's *t*-test. ND = not detected; NS = not significant.

results suggest that overexpression of T-bet on CD4+ T cells suppressed the expression of RORγt and IL-17.

No reduction of RORγt expression on CII-reactive CD4+ T cells in T-bet–Tg mice. CD4+ T cells from T-bet–Tg and B6 mice were cultured in vitro with CII, and analyses of T-bet and RORγt expression on CD4+ T cells were carried out by the intracellular staining method. T-bet expression on CII-reactive CD4+ T cells was significantly higher in T-bet–Tg mice than in B6 mice (Figure 3A). Surprisingly, the majority of T-bet+ CII-reactive T cells expressed RORγt in both the B6 mice and the T-bet–Tg mice (Figure 3A). Although there was no significant difference in the mean fluorescence intensity of RORγt between B6 mice and T-bet–Tg mice, the number of RORγt+ cells tended to be lower in T-bet–Tg mice (data available upon request from the author).

Moreover, in the case of CD4+ T cells examined under conditions favoring Th17 differentiation, RORγt expression on CD4+ T cells from T-bet–Tg mice was lower than that on cells from B6 mice (Figure 3B). Interestingly, most of the RORγt+ cells also expressed T-bet in the T-bet–Tg mice, and the proportion of IL-17–producing RORγt+ CD4+ T cells was lower in the T-bet–Tg mice than in the B6 mice. These findings support the notion that overexpression of T-bet not only suppresses RORγt expression on CD4+ T cells but also inhibits the production of IL-17 from RORγt+ T cells.

To investigate whether the suppression of arthritis and low antigen-specific cytokine production observed in T-bet–Tg mice was related to Treg cells, the next experiment analyzed FoxP3 expression on CD4+ T cells harvested from draining lymph nodes 10 days after immunization. There was no significant difference in the percentage of FoxP3+ cells among the CD4+ T cells between B6 mice and T-bet–Tg mice (data available upon request from the author). Thus, Treg cells do not seem to be involved in the suppression of CIA in T-bet–Tg mice.

Decreased numbers of T cells in the lymph nodes, spleen, and thymus of T-bet–Tg mice. To evaluate the low cytokine response and the low population of CII-reactive RORγt+CD4+ T cells in T-bet–Tg mice with CIA, we analyzed the lymphocyte subsets in the draining lymph nodes and spleen after immunization. The percentage and absolute number of CD3+ T cells were lower in both the draining lymph nodes and the

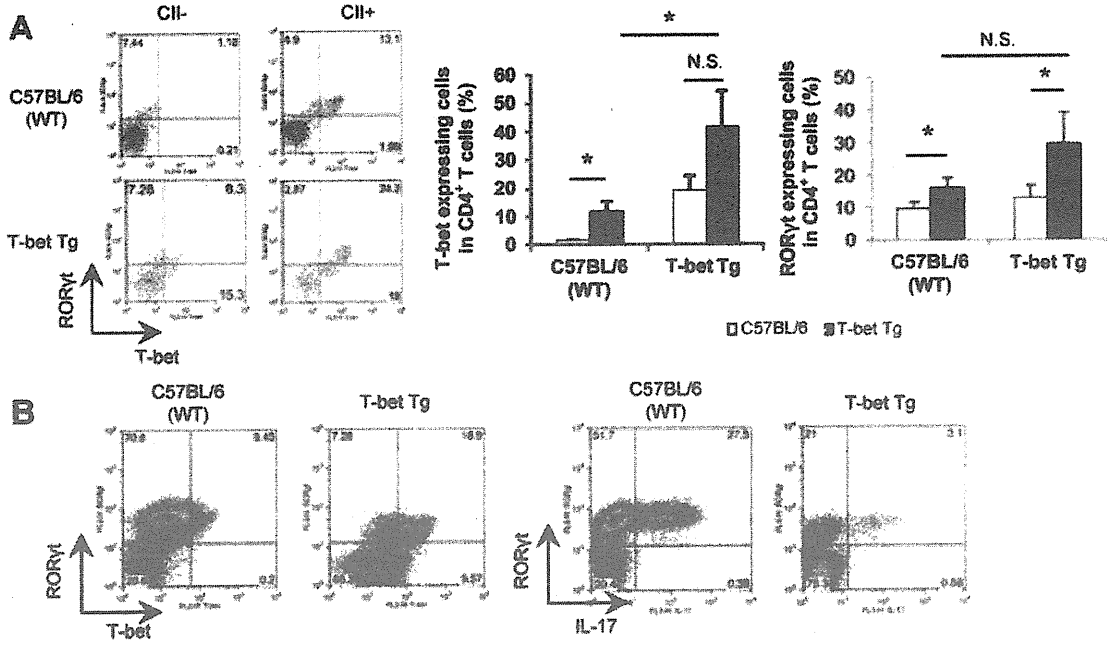

Figure 3. Suppression of Th17 cell differentiation by enforced expression of T-bet in T cells despite expression of retinoic acid receptor–related orphan nuclear receptor γt (RORγt). A, Ten days after the first type II collagen (CII) immunization, lymphocytes derived from the draining lymph nodes of C57BL/6 (wild-type [WT]) and T-bet–transgenic (Tg) mice were cultured for 72 hours in the presence or absence of 100 μg/ml of denatured CII. Levels of T-bet and RORγt expression on CD4+ T cells were analyzed by intracellular staining. Numbers in each compartment of the histograms are the percentage of transcription factor–expressing cells gated on CD4+ T cells. Values in the bar graphs are the mean ± SD of 3 mice per group. * = $P < 0.05$ by Student's *t*-test. NS = not significant. B, CD4+ T cells were isolated from the spleen of C57BL/6 and T-bet–Tg mice by magnetic-activated cell sorting and were then cultured for 96 hours with soluble anti-CD3 antibody, soluble anti-CD28 antibody, interleukin-6 (IL-6), and transforming growth factor β. Cytokine production and transcription factor expression on CD4+ T cells were analyzed by intracellular staining. Representative histograms from flow cytometric analysis of T-bet and RORγt expression with IL-17 production are shown. Numbers in each compartment are the percentage of positive cells gated on CD4+ T cells.

spleen of T-bet–Tg mice as compared with B6 mice (Figures 4A and B). The absolute number of CD4+ and CD8+ T cells also tended to be lower in T-bet–Tg mice (Figure 4B). Moreover, analysis of the thymus showed a significantly low number of total thymocytes in T-bet–Tg mice and the presence of an abnormal proportion of T precursor cells, such as a low number of double-positive T cells and CD4 single-positive T cells in T-bet–Tg mice (Figure 4C). These results suggest abnormal T cell development in the thymus of T-bet–Tg mice.

Inhibition of IL-17 production by CII-reactive CD4+ T cells in T-bet–Tg mice. To clarify whether T-bet overexpression on CD4+ T cells directly affects cytokine production, we performed criss-cross experiments using CD4+ T cells from B6 and T-bet–Tg mice, as well as DCs from B6 and T-bet–Tg mice in CII–containing medium, and measured IL-17 and IFNγ levels in the supernatants by ELISA. IL-17 production was detected in CII-reactive CD4+ T cells from B6 mice and in DCs from T-bet–Tg mice. Interestingly, IL-17 production was significantly reduced, even when CD4+ T cells from T-bet–Tg mice were cocultured with DCs from B6 mice (Figure 5A). These observations suggest that T-bet overexpression on CD4+ T cells is responsible for the inhibition of CII-reactive IL-17 production. No difference in IFNγ production was noted among the experimental conditions (Figure 5A), suggesting that reduced IFNγ production by CII-reactive CD4+ T cells from T-bet–Tg mice (Figure 2) was probably related to the reduced numbers of CD4+ T cells in draining lymph nodes. Moreover, intracellular staining revealed that RORγt expression was suppressed and T-bet expression was increased, even when CD4+ T cells from T-bet–Tg

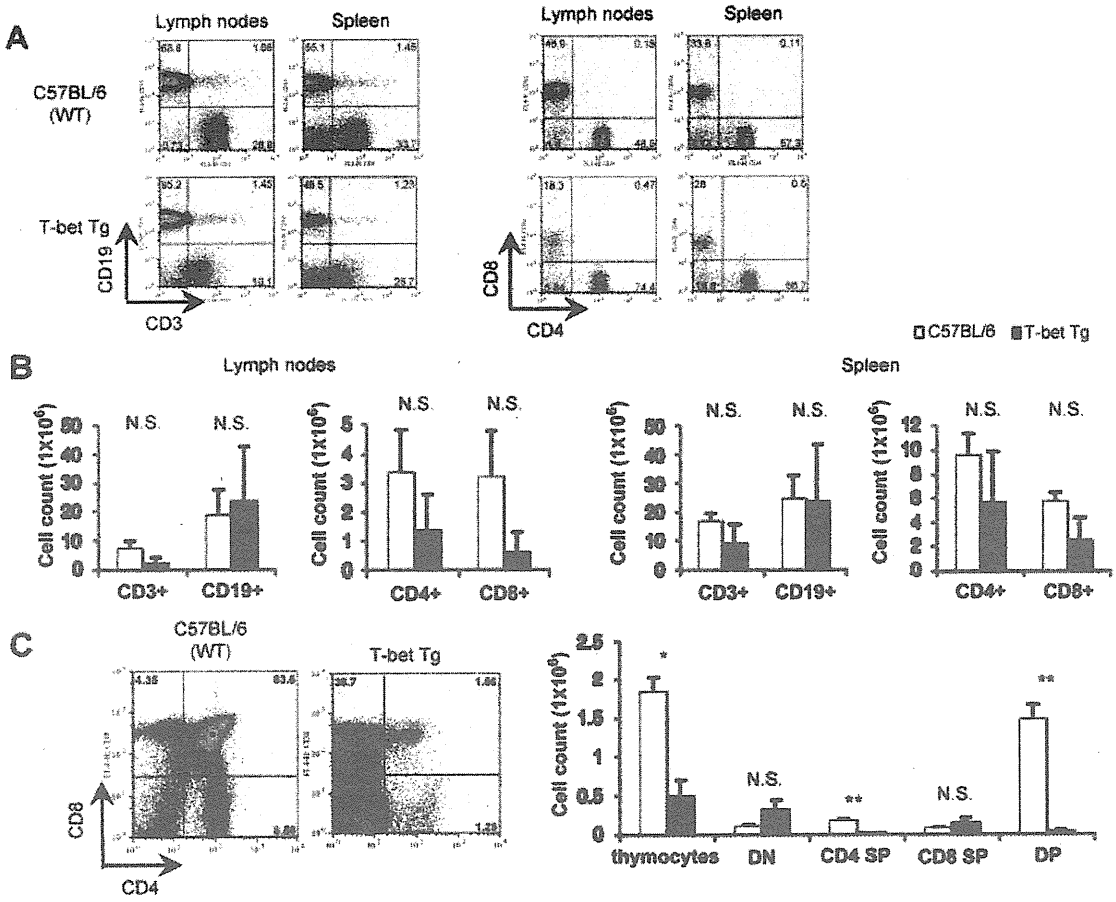

Figure 4. Decreased number of CD3+ T cells in spleen and lymph nodes and abnormal development of T precursor cells in the thymus in T-bet–transgenic (Tg) mice. **A,** Ten days after first immunization, the proportion of lymphocytes in draining lymph nodes and spleen were analyzed by fluorescence-activated cell sorting (FACS), and the absolute numbers of cells were calculated. Numbers in each compartment are the percentage of the parent population. **B,** The absolute numbers of CD3+, CD19+, CD4+, and CD8+ T cells in the lymph nodes and spleen of C57BL/6 (wild-type [WT]) and T-bet–Tg mice were determined. Values are the mean ± SD of 3 mice per group. NS = not significant. **C,** The proportion of T precursor cells in the thymus of nonimmunized mice was analyzed by FACS, and the absolute numbers of thymocytes, double-negative (DN) T cells, CD4 and CD8 single-positive (SP) T cells, and double-positive (DP) T cells were determined. Values in the bar graphs are the mean ± SD of 3 mice per group. * = $P < 0.05$; ** = $P < 0.01$ by Student's *t*-test.

mice were cocultured with DCs from B6 mice (Figure 5B). These results indicate that T-bet overexpression on CD4+ T cells suppressed CII-reactive IL-17 production by inhibition of the expression of RORγt.

Overexpression of T-bet directly suppresses Th17 cell differentiation via IFNγ-independent mechanisms. To clarify whether IFNγ production influences Th17 cell differentiation, we generated T-bet–Tg/IFNγ$^{-/-}$ mice. CD4+ T cells were isolated from the spleen of T-bet–Tg, T-bet–Tg/IFNγ$^{-/-}$, and B6 mice and were then cultured for Th17 cell differentiation. FACS analysis demonstrated that the proportion of IL-17–producing CD4+ T cells was lower in T-bet–Tg mice than in B6 mice, whereas the proportion of IFNγ-producing CD4+ T cells was higher in T-bet–Tg mice. Similarly, the proportion of IL-17–producing CD4+ T cells was also lower in T-bet–Tg/IFNγ$^{-/-}$ mice, although no IFNγ-producing CD4+ T cells were detected in

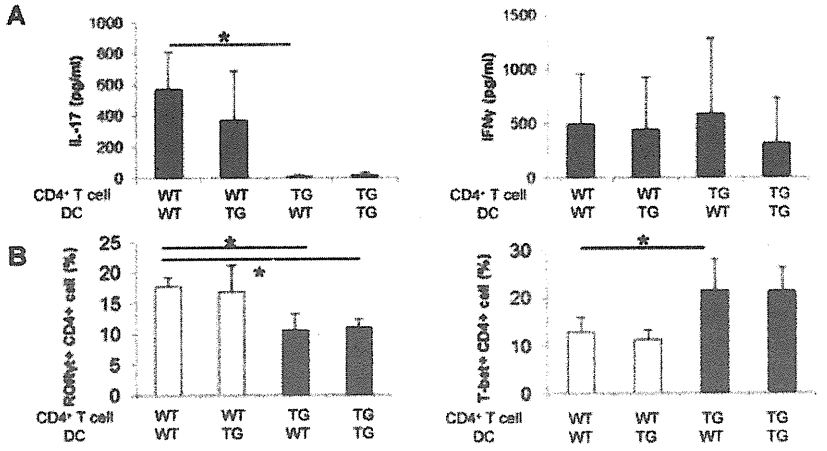

Figure 5. Impaired antigen-specific Th17 cell responses in T-bet–transgenic (Tg) mice with collagen-induced arthritis (CIA). Ten days after the first type II collagen (CII) immunization, CD4+ cells were isolated from draining lymph nodes of C57BL/6 (wild-type [WT]) mice and T-bet–Tg (TG) mice by positive selection using magnetic-activated cell sorting (MACS) with anti-CD4 monoclonal antibody (mAb). After treatment with mitomycin C, CD11c+ cells were isolated from the spleen by positive selection using a MACS system with anti-CD11c mAb. Criss-cross coculture for 72 hours was performed with 1×10^5 CD4+ cells and 2×10^4 CD11c+ cells in 100 μg/ml of denatured CII–containing medium. **A**, Levels of interleukin-17 (IL-17) and interferon-γ (IFNγ) in culture supernatants were measured by enzyme-linked immunosorbent assay. **B**, Expression of retinoic acid receptor–related orphan nuclear receptor γt (RORγt) and T-bet expression on CD4+ T cells were analyzed by intracellular staining. Representative data from flow cytometric analysis of the percentage of RORγt+ or T-bet+ cells in the CD4+ T cell subset are shown. Values are the mean ± SD of 3 mice per group. * = $P < 0.05$ by Student's *t*-test. DC = dendritic cells.

T-bet–Tg/IFNγ$^{-/-}$ mice (Figure 6). These results strongly support the view that inhibition of Th17 cell differentiation in T-bet–Tg mice cannot be due to overproduction of IFNγ, indicating that overexpression of T-bet directly suppresses Th17 cell differentiation in T-bet–Tg mice.

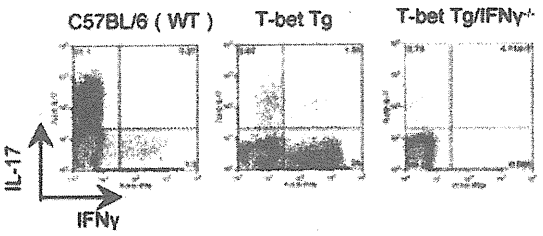

Figure 6. Suppressed expression of interleukin-17 (IL-17) by T-bet overexpression independently of interferon-γ (IFNγ) in T-bet–transgenic (Tg) mice. CD4+ T cells were isolated from the spleen of C57BL/6 (wild-type [WT]), T-bet–Tg, and T-bet–Tg/IFNγ$^{-/-}$ mice by magnetic-activated cell sorting and then cultured for 96 hours with soluble anti-CD3 monoclonal antibody (mAb), soluble anti-CD28 mAb, IL-6, and transforming growth factor β. IFNγ and IL-17 production by CD4+ cells was analyzed by intracellular cytokine staining. Numbers in each compartment are the percentage of cells secreting cytokines.

DISCUSSION

Recent studies showed that IL-17 plays a crucial role in the development of CIA (3) and other types of experimental arthritis (2). In contrast, it has been reported that IFNγ can suppress IL-17 production in vitro (16) and has antiinflammatory effects on the development of experimental arthritis (4,5). T-bet is a transcription factor known to induce the differentiation of naive CD4+ T cells to Th1 cells (8). Although the absence of T-bet can result in severe IL-17–mediated experimental autoimmune myocarditis via dysregulation of IFNγ (17), several studies have shown that T-bet is essential for the development of several models of autoimmunity, such as experimental autoimmune encephalitis (18,19), colitis (20), and diabetes mellitus (21). Nevertheless, the effect of T-bet expression on Th17 cell differentiation and function during arthritis remains unclear.

T-bet–Tg mice overexpress T-bet and mainly produce IFNγ in their T cells (14). Previous studies in T-bet–Tg mice suggested that overexpression of T-bet and a predominant Th1 response affect the pathogenesis of various diseases (14,22,23). To examine whether T-bet overexpression on T cells affects the regulation of

autoimmune arthritis, we induced CIA in T-bet–Tg mice and found marked suppression of CIA in T-bet–Tg mice.

To determine the reason for the low incidence of CIA in T-bet–Tg mice, we measured CII-reactive cytokine production and expression in vitro. IL-17 production from CII-reactive CD4+ T cells and *Il17a* expression were reduced in T-bet–Tg mice as compared with B6 mice. Although a predominant Th1 cell response was reported by Ishizuka et al (14), CII-specific IFNγ production was reduced in T-bet–Tg mice, and no significant difference was observed in *Ifng* expression between B6 mice and T-bet–Tg mice. Furthermore, *Il12a* expression was significantly higher in T-bet–Tg mice than in B6 mice, suggesting that overexpression of T-bet on T cells seems to affect innate immune cells, because the main producers of IL-12 are DCs and macrophages, not CD4+ T cells.

In criss-cross coculture experiments with CD4+ T cells and splenic DCs from B6 mice and T-bet–Tg mice, CII-reactive IL-17 production was also reduced even when CD4+ T cells from T-bet–Tg mice were cocultured with DCs from B6 mice, although there was no significant difference in IL-17 production by CD4+ T cells from B6 mice cocultured with DCs from either B6 mice or T-bet–Tg mice. In contrast, no difference in IFNγ production was observed under all coculture conditions examined. Moreover, suppression of RORγt expression and high expression of T-bet on CD4+ T cells were observed even when CD4+ T cells from T-bet–Tg mice were cocultured with DCs from B6 mice. These findings indicate that T-bet overexpression on CD4+ T cells might suppress CII-reactive IL-17 production resulting from suppression of RORγt expression in an IFNγ-independent manner, and that overexpression of T-bet has no direct effect on DC function.

CII-specific IgG levels correlate well with the development of arthritis (15). We observed significant suppression of CII-specific IgG production in the T-bet–Tg mice as compared with the B6 mice. A previous study showed that IL-17 is required for anti-CII antibody production (3). Therefore, the suppression of anti-CII antibody formation might be due to lower CII-reactive IL-17 production in T-bet–Tg mice.

To evaluate the low cytokine response to CII in T-bet–Tg mice, we analyzed lymphocytes obtained after immunization from draining lymph nodes and spleen. The percentage and absolute number of T cells tended to be lower in both the draining lymph nodes and spleen of T-bet–Tg mice compared with B6 mice. Moreover, significantly lower numbers of total thymocytes and an abnormal proportion of T precursor cells were observed in T-bet–Tg mice. The latter phenomenon could be due to T-bet transgene expression on double-negative thymic cells in T-bet–Tg mice. Because previous observations showed that T-bet interferes with GATA-3 function (11) and that GATA-3 was required for the development of early thymic T cells (24), one of the reasons for abnormal T cell development in the thymus might be the dysfunction of GATA-3 by overexpression of T-bet. These results suggest that overexpression of T-bet in thymic T cells affects T cell development, is responsible for the low number of T cells in spleen and lymph nodes, and is related to the low cytokine production against CII in T-bet–Tg mice.

To assess the effect of T-bet on CD4+ T cell differentiation in T-bet–Tg mice, we performed in vitro induction of Th17 cells. Analysis of T-bet–Tg mice showed a reduction in IL-17–producing CD4+ T cells and an increase in IFNγ-producing CD4+ T cells in spite of the condition favoring Th17 differentiation, which indicates suppression of Th17 cell differentiation and predominance of Th1 cell differentiation in vitro in T-bet–Tg mice. These results did not contradict the previous findings that the phenotype of polarized Th1 cells was not affected by Th cell–polarizing conditions (25). It is possible that suppression of CII-reactive IL-17 production in T-bet–Tg mice was not associated with IFNγ. For this reason, we generated T-bet–Tg/IFNγ$^{-/-}$ mice and performed in vitro induction of Th17 cells in these mice. Surprisingly, in T-bet–Tg/IFNγ$^{-/-}$ mice, the levels of IL-17–producing CD4+ T cells were also markedly reduced under Th17 cell differentiation–favoring conditions, indicating an IFNγ-independent suppressive pathway against Th17 cell differentiation. Although previous studies showed that suppression of Th17 cell differentiation was mediated through IFNγ signal transduction (16), our findings allow us to propose a new hypothesis: Th17 cell differentiation is regulated by a pathway that is distinct from the IFNγ signaling pathway. Therefore, we suggest that T-bet expression either directly or indirectly suppresses Th17 cell differentiation via an IFNγ-independent mechanism.

Tbx21 expression was significantly higher in T-bet–Tg mice as compared with B6 mice, and FACS analysis of CII-reactive CD4+ T cells revealed a significantly higher percentage of T-bet+ cells among the CD4+ T cell subset in T-bet–Tg mice. While there was no significant difference in the percentage of RORγt+ cells among the CD4+ T cell subset in T-bet–Tg mice as compared with B6 mice, *Rorc* expression was down-regulated on CII-reactive CD4+ T cells in T-bet–Tg mice. In the case of CD4+ T cells under

conditions favoring Th17 cell differentiation, RORγt expression on CD4+ T cells from T-bet–Tg mice was lower than that on cells from B6 mice. Interestingly, most of the RORγt+ cells also expressed T-bet in T-bet–Tg mice, and the proportion of IL-17–producing RORγt+ T cells in the CD4+ cell subset was lower in T-bet–Tg mice than in B6 mice. These findings support the notion that overexpression of T-bet not only suppresses RORγt expression on CD4+ T cells, but also inhibits the production of IL-17 from RORγt+ T cells.

Previous studies showed that RORγt expression is positively regulated by several transcription factors, such as runt-related transcription factor 1 (RUNX-1), interferon regulatory factor 4, and STAT-3 (26–28). Lazarevic et al (29) recently reported that T-bet prevented RUNX-1–mediated activation of the gene encoding RORγt, followed by the suppression of Th17 cell differentiation. In addition to direct promotion of RORγt expression, RUNX-1 also acts as a coactivator, together with RORγt, and induces the expression of *Il17a* and *Il17f* (26); therefore, T-bet inhibits IL-17 production by RORγt+ cells induced by RUNX-1 (29). Although further studies will be required to identify the effect of T-bet overexpression on the function of RUNX-1, it might be associated with the suppression of Th17 cell differentiation that was observed in the T-bet–Tg mice.

In conclusion, our results demonstrated that overexpression of T-bet in T cells suppressed the development of autoimmune arthritis. The regulatory mechanism of CIA might involve dysfunction of CII-reactive Th17 cell differentiation by overexpression of T-bet via IFNγ-independent pathways. These findings should enhance our understanding of the pathogenesis of autoimmune arthritis and help in the development of new therapies for RA.

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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. Sumida 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. Sugihara, Hayashi, Yoh, Takahashi, Matsumoto, Sumida.

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A Genome-Wide Association Study Identified *AFF1* as a Susceptibility Locus for Systemic Lupus Eyrthematosus 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 has 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, $\lambda_{GC} = 1.088$, 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 $P<5.0\times10^{-8}$ (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*, *IIIP1*, *WDFY4*, intergenic region at 11q23, *ETS1*, *SLC15A4*, *ELF1*, and *IIIC2-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 $P<1.0\times10^{-3}$ 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

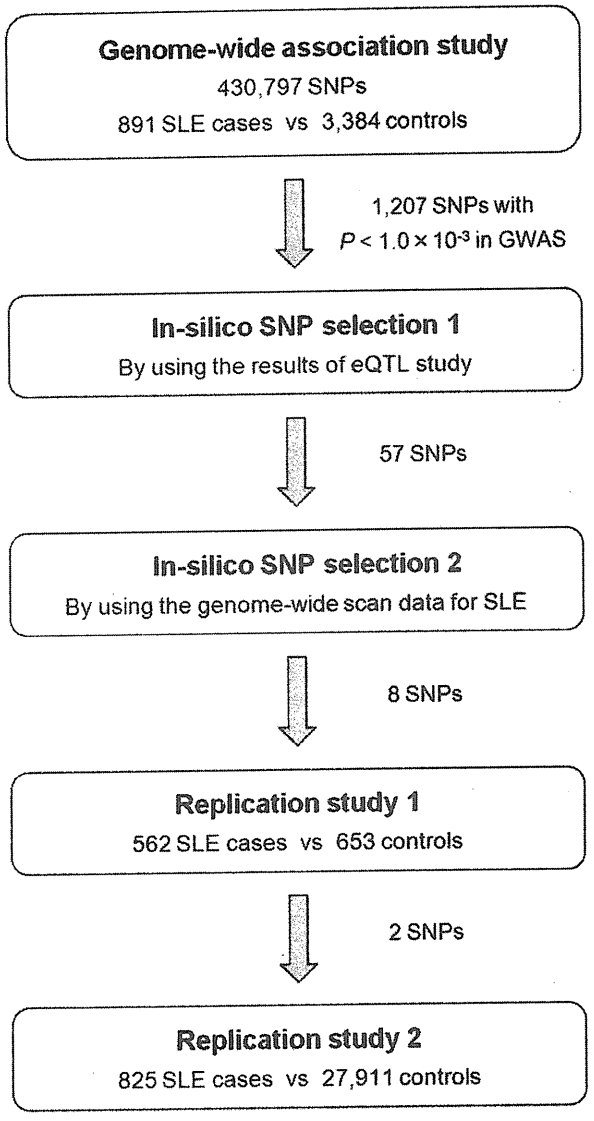

Figure 1. Design of the GWAS and multi-stage replication studies for SLE in Japanese subjects. A total of 2,278 SLE cases and 31,948 controls were enrolled. The clinical characteristics of the subjects are summarized in Table S1 and S2. Details of the genome-wide scan data for SLE referenced in the *in 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 $P<1.0\times10^{-6}$ 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; $P=4.6\times10^{-5}$ and $P=0.0094$ in the two individual cohorts, respectively; Table 3, Table S5, and Figure 2B). The combined study for the GWAS ($P=1.5\times10^{-4}$) and the replication studies demonstrated significant associations of rs340630 that satisfied the genome-wide significance threshold ($P=8.3\times10^{-9}$, 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 ethnical 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*
					1/2	Case	Control	Case	Control		
rs10168266	2	191,644,049	2q32	*STAT4*	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	*TNFAIP3*	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	*HIP1*	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	*BLK*	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	*ETS1*	A/G	891	3,368	0.48	0.39	1.44 (1.30–1.60)	1.3×10^{-11}

[a]SNPs that satisfied the threshold of $P<5.0\times10^{-8}$ were indicated.
[b]Based on forward strand of NCBI Build 36.3.
SLE, systemic lupus erythematosus; OR, odds ratio.
doi:10.1371/journal.pgen.1002455.t001

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

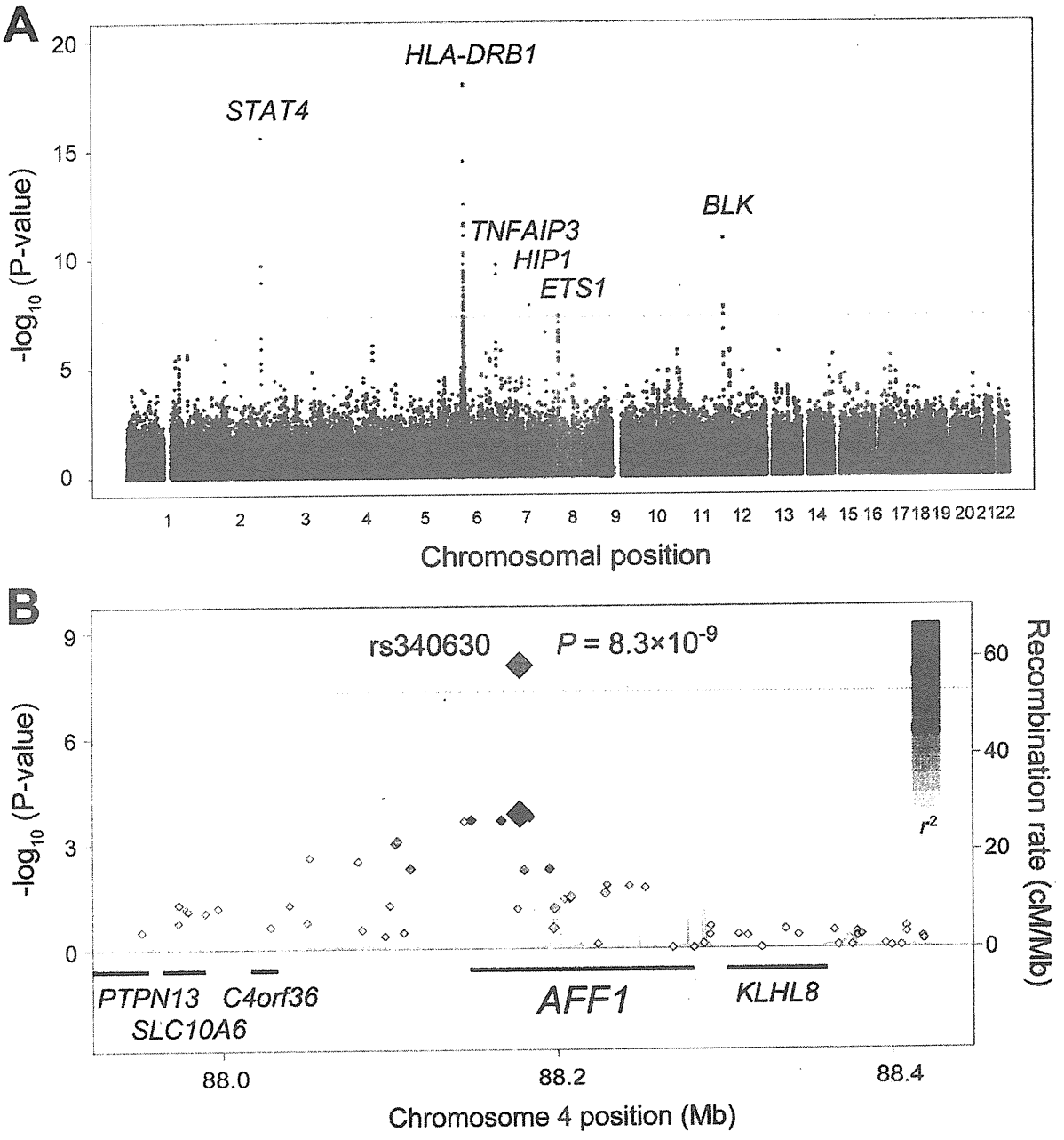

Figure 2. Associations of the *AFF1* locus with SLE. (A) A chromosomal plot of *P*-values in GWAS for SLE. (B) A regional plot in the *AFF1* locus. Diamond-shaped data points represent $-\log_{10}$ (*P*-values) of the SNPs. Large-sized points indicate the *P*-values of the landmark SNP, rs340630 (green 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].
doi:10.1371/journal.pgen.1002455.g002

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				Caucasians	Asians
rs2205960	1	171,458,098	1q25	*TNFSF4*	T/G	0.23	0.18	1.35 (1.19–1.54)	3.0×10^{-6}		+	
rs3024505	1	205,006,527	1q32	*IL10*	A/G	0.019	0.014	1.34 (0.90–2.00)	0.15		+	
rs13385731	2	33,555,394	2p22	*RASGRP3*	C/T	0.90	0.87	1.37 (1.15–1.64)	6.0×10^{-4}	+		+
rs10168266	2	191,644,049	2q32	*STAT4*	T/C	0.37	0.27	1.59 (1.42–1.78)	2.7×10^{-16}		+	
rs6445975	3	58,345,217	3p14	*PXK*	G/T	0.25	0.23	1.09 (0.96–1.23)	0.18	+	+	
rs10516487	4	102,970,099	4q24	*BANK1*	G/A	0.91	0.89	1.28 (1.07–1.53)	0.0070		+	
rs10036748	5	150,438,339	5q33	*TNIP1*	T/C	0.75	0.72	1.16 (1.03–1.31)	0.014			+
rs9501626	6	32,508,322	6p21	*HLA-DRB1*	A/C	0.20	0.12	1.86 (1.62–2.13)	1.0×10^{-18}		+	
rs548234	6	106,674,727	6q21	*PRDM1*	C/T	0.40	0.34	1.30 (1.16–1.44)	2.3×10^{-6}	+	+	
rs2230926	6	138,237,759	6q23	*TNFAIP3*	G/T	0.11	0.069	1.75 (1.47–2.08)	1.9×10^{-10}	+	+	
rs849142	7	28,152,416	7p15	*JAZF1*	C/T	0.999	0.999	2.72 (0.25–29.8)	0.41		+	
rs4917014	7	50,276,469	7p12	*IKZF1*	T/G	0.58	0.53	1.24 (1.11–1.38)	8.1×10^{-5}			+
rs6964720	7	75,018,280	7q11	*HIP1*	G/A	0.25	0.19	1.43 (1.27–1.62)	1.3×10^{-8}			+
rs4728142	7	128,361,203	7q32	*IRF5*	A/G	0.16	0.11	1.48 (1.28–1.72)	2.4×10^{-7}	+	+	
rs2254546	8	11,381,089	8p23	*BLK*	G/A	0.78	0.72	1.42 (1.25–1.61)	4.1×10^{-8}	+	+	
rs1913517	10	49,789,060	10q11	*WDFY4*	A/G	0.32	0.28	1.20 (1.07–1.35)	0.0013			+
rs4963128	11	579,564	11p15	*KIAA1542*	T/C	0.98	0.97	1.58 (1.03–2.44)	0.038	+	+	
rs2736552	11	35,041,168	11p13	*PDHDC,CD44*	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	*ETS1*	A/G	0.48	0.39	1.44 (1.30–1.60)	1.3×10^{-11}			+
rs1385374	12	127,866,647	12q24	*SLC15A4*	T/C	0.19	0.16	1.21 (1.06–1.38)	0.0057			+
rs7329174	13	40,456,110	13q14	*ELF1*	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	*ITGAM*	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		+	
rs4820426	22	20,139,185	22q11	*HIC2,UBE2L3*	T/C	0.52	0.48	1.20 (1.08–1.33)	6.1×10^{-4}		+	

[a]Based on forward strand of NCBI Build 36.3.
[b]Defined using gene expression data measured in lymphoblastoid B cell lines [28].
[c]Based 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.
doi:10.1371/journal.pgen.1002455.t002

Genotyping and quality control

In GWAS, 946 SLE cases and 3,477 controls were genotyped using Illumina HumanHap610-Quad and Illumina HumanHap550v3 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]
					1/2		Case	Control	Case	Control			
rs340630	4	88,177,419	4q21	*AFF1*	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}	

[a]Defined using gene expression data measured in lymphoblastoid B cell lines [28].
doi:10.1371/journal.pgen.1002455.t003

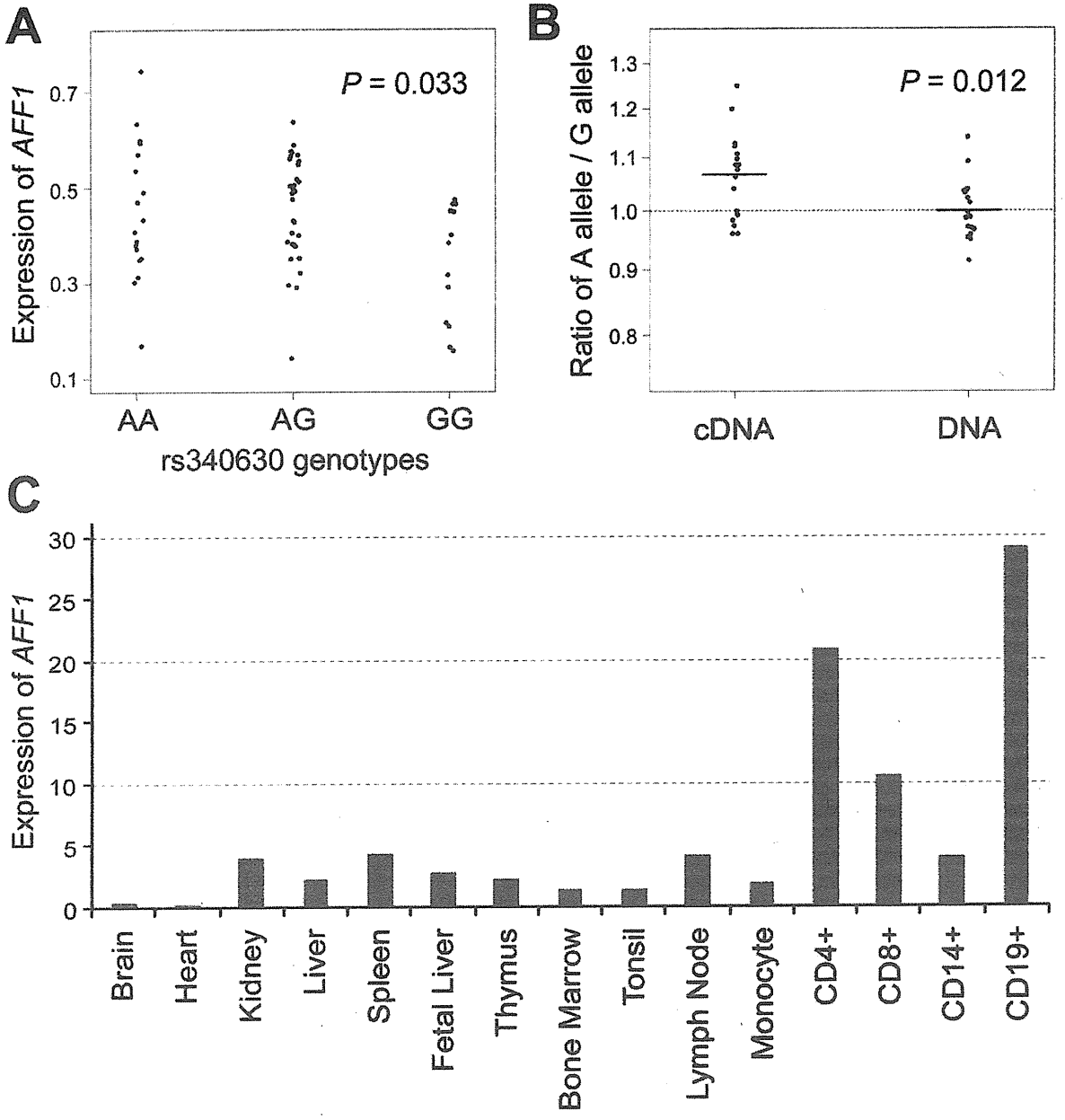

Figure 3. Association of rs340630 with *AFF1* expression. (A) Correlation between rs340630 genotypes and transcript levels of *AFF1* (NM_001166693) in EBV-transfected cell lines ($n = 62$) stimulated with PMA. (B) Allele-specific quantification (ASTQ) of *AFF1* transcripts. Allele specific-probes for 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 $<1.0\times10^{-6}$ 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-Haenzel 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, *Rsq*, >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 $P<1.0\times10^{-4}$ in GWAS, or the SNPs that satisfied $1.0\times10^{-4}\leq P<1.0\times10^{-3}$ 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 $P<1.0\times10^{-6}$ 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 μg) 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′-CCCGGCGCAGTTTCTGAG-3′. The probe sequences were 5′-VIC-CGAAGACCGCCAGCGCCCAAC-TAMRA-3′ and 5′-FAM-CGAAGACCGCCGGCGCCCAA-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