Table 2 Haplotype frequency of rs3766379 and 6682654

|	rs3766379	rs6682654			n Case	Control	Frequency Case	Control
Haplotype_1	T	C	Susceptible[a]	Susceptible[a]	935	474	0.61	0.52
Haplotype_2	C	C	Nonsusceptible	Susceptible	86	56	0.06	0.06
Haplotype_3	C	T	Nonsusceptible	Nonsusceptible	519	388	0.34	0.42
				Sum	1,540	918	1.00	1.00

	P value	Odds ratio	95% c.i.
Haplotype_1 versus haplotype_3	0.000009	1.47	1.24–1.75
Haplotype_1 versus others	0.000011	1.45	1.23–1.71
Haplotype_3 versus others	0.000021	1.44	1.22–1.70

[a]"Susceptible" and "nonsusceptible" indicate the alleles more frequently and less frequently observed, respectively, for individual SNPs in affected individuals.

CD244 are associated with rheumatoid arthritis in a Japanese population. Because the association in the segment containing *CD244* was validated and the strongest association within the segment was detected at SNPs of *CD244* using independent sample sets, this association is likely to originate from genetic heterogeneity of *CD244*, although it does not negate the possibility that other nearby genes, including *LY9*, have risk variants for autoimmune diseases. Because the genetic factors in rheumatoid arthritis vary between East Asians and individuals of European origin, as clarified by a genome-wide association study of rheumatoid arthritis[28], we searched the Wellcome Trust Case Control Consortium study[29] for the association between the SNPs in *CD244* and *LY9* and rheumatoid arthritis but did not identify any positive signals. This might suggest that the association in the *CD244* locus is another example of an ethnicity-specific genetic factor. We have also demonstrated that the polymorphisms have an allele-specific effect on the expression of *CD244* with *in vitro* expression assays, consistent with the report on whole-genome gene expression profile of cell lines in HapMap individuals[22]. Although these data suggest that the rheumatoid arthritis-susceptible variants of rs3766379 and rs6682654 upregulate expression of *CD244*, their causative role in increasing the risk of autoimmune disease must be investigated further to determine its validity and precise molecular mechanism. Further investigations are needed to clarify the genetic roles of *CD244* and its variants on rheumatoid arthritis and autoimmunity.
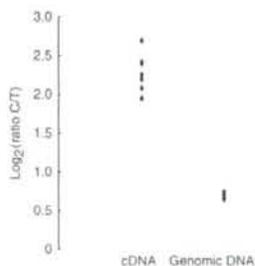
Figure 4 Allelic imbalance of gene expression of *CD244* in an EBV-transformed lymphoblastoid cell line with the heterozygous genotypes. To compare the relative abundance of RNA transcribed from each allele in the cell lines, we measured each sample in quadruplicate in one assay; tests were independently repeated twice. Genomic DNA was used as a control for equal biallelic representation.

METHODS

Affected and unaffected individuals. Two independent Japanese rheumatoid arthritis case-control cohorts, rheumatoid arthritis case-control cohort 1 (830 affected individuals and 658 controls) and rheumatoid arthritis case-control cohort 2 (1,112 affected individuals and 940 controls), and a case cohort of individuals with SLE ($n = 555$) were recruited for collection of genomic DNA through several medical institutes in Japan. All individuals with rheumatoid arthritis met the revised criteria of the American College of Rheumatology (ACR) for rheumatoid arthritis, and all individuals with SLE fulfilled the 1997 ACR criteria for SLE[30]. The mean age of the 830 individuals with rheumatoid arthritis was 64.3 years (range 28–92 years), 83.7% were female and 75.0% were positive for rheumatoid factor. The control samples consisted of 658 individuals from the general population; the mean age was 48.6 years (range 3–92 years), and 57.4% were female. The mean age of the second rheumatoid arthritis cohort was 60.5 years (range 16–88 years), 89.7% were female and 76.8% were positive for rheumatoid factor. The SLE cohort consisted of 555 individuals; the mean age was 44.4 years (range 17–86 years), and 89.7% were female. The mean age of the second control cohort from the general population was 52.6 years (range 18–93 years), and 25.0% were female. Informed consent was obtained from each subject or, in the case of minors, from an individual with parental authority, and the study was approved by the ethics committee at the Center for Genomic Medicine of the Institute of Physical and Chemical Research (RIKEN).

SNP analysis. DNA extraction, design of PCR primers, PCR experiments, DNA sequencing, SNP discovery and genotyping of 91 SNPs were performed as described previously[9].

Luciferase assay. We constructed luciferase reporter plasmids by cloning 31 nucleotides adjacent to each SNP into the pGL3-promoter vector (Promega) upstream of the SV40 promoter. We introduced a SacI site at the 3' end of antisense oligonucleotides (**Supplementary Table 2** online) and inserted them into a vector that was digested with SmaI and SacI restriction enzymes (TaKaRa)[10]. We also cloned a cDNA of USF-1 into pcDNA3.1/nV5-DEST for protein expression in mammalian cell lines. As a control vector, we used LacZ cDNA inserted into pcDNA3.1/nV5-DEST (Invitrogen). We sequenced the inserts of all constructs. Vector pRL-TK was used to normalize for variations in transfection efficiency. The luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega)[10].

Quantification of allelic variation in gene expression using real-time Taq-Man PCR. We obtained EBV-transformed lymphoblastoid cell lines from the Health Science Research Resources Bank of Japan. We prepared genomic DNA and total RNA from ten cell lines that were heterozygous with rs6682654 as a target SNP. We performed allelic expression analyses by TaqMan assay using SNP genotyping probes. Briefly, the $\log_2$ of the ratio of FAM intensity to VIC intensity for *CD244* was plotted against the $\log_2$ of (FAM allele/VIC allele) for mixtures of homozygous DNAs at six different ratios (3:1, 2:1, 3:2, 2:3, 1:2 and

1:3), with correction based on the signal intensity of heterozygote controls for a standard line. In this case, the VIC allele is the allele associated with susceptibility to rheumatoid arthritis, whereas the FAM allele is not associated with risk. We then measured the allelic ratio for each cDNA and genomic DNA from each individual by real-time TaqMan PCR. Based on the standard line, we calculated the allelic ratio of cDNA and genomic DNA. We measured each sample in quadruplicate in one assay; tests were independently repeated two times.

Electrophoretic mobility shift assays (EMSAs). After lysing the cytoplasm, we prepared nuclear extracts from Jurkat E6-1 cells using nuclear lysis buffer containing 20 mM HEPES, 20% (vol/vol) glycerol, 500 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 1 mM DTT, 0.1% (vol/vol) NP-40 and protease inhibitor cocktail (Nacalai Tesque). We stored the nuclear extracts in aliquots at −80 °C until use. We used constructed biotin-labeled double-stranded 31-nucleotide synthetic oligonucleotide probes for each SNP (**Supplementary Table 2**) using the Biotin 3′ End DNA Labeling Kit (Pierce). Biotin-labeled probes were incubated with nuclear extracts after preincubation with 1 μg poly(dI-dC) as a nonspecific competitor of DNA for 20 min at 25°C. The binding reaction mixtures were then separated by electrophoresis on 5% nondenaturing polyacrylamide gel with 0.5× TBE running buffer. For competition studies, we preincubated a 100-fold excess of unlabeled oligonucleotide probe with the nuclear extract before adding the biotin-labeled probe. In the supershift assay, nuclear extract was preincubated with antibody to USF-1 (Santa Cruz) or with nonimmunized rabbit serum in the reaction mixture, followed by the addition of biotin-labeled probe. DNA-protein complexes were detected using the Lightshift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's instructions.

Statistical analysis. The case-control association of each SNP was tested with the Cochran-Armitage trend test. We tested association between haplotypes and phenotype using the χ^2 test. The Mantel-Haenszel method was used to combine genotype data sets. We calculated pairwise LD indices r^2 and D', defined LD blocks, inferred haplotype frequency and identified tag SNPs as previously described[18] (minor allele frequency >5% and $r^2 > 0.9$) using Haploview 3.2. The contour plot of pairwise LD was drawn with Microsoft Excel. We used genomic control[21] to assess population stratification. We used THESIAS software for conditional logistic analysis[23]. Associations between s.e.m. and genotype were tested with the χ^2 test. The correlation between gene expression and the number of an allele of SNPs was evaluated with Pearson's correlation coefficient and tested with a linear regression model. Expression differences between genotypic groups were tested with Student's t-test.

Accession codes. GenBank: human chromosome 1 genomic DNA sequence, NT_004487.18; mRNA sequence for human CD244, NM_016382.2. NCBI GEO: microarray data, GSE6536.

URLs. HapMap project: http://www.hapmap.org/index.html; Haploview software: http://www.broad.mit.edu/mpg/haploview/; SNPHAP: http://www-gene.cimr.cam.ac.uk/clayton/software/; NCBI GEO: http://www.ncbi.nlm.nih.gov/geo/; The Wellcome Trust Case Control Consortium: http://www.wtccc.org.uk, TRANSFAC: http://helixweb.nih.gov/transfac/.

Note: Supplementary information is available on the Nature Genetics website.

AUTHOR CONTRIBUTIONS
A.S. performed most of the experiments and wrote the manuscript; R.Y. and Y.O. performed the data analysis and managed DNA samples and clinical information; Y. Kochi and M.M. summarized clinical data and performed SNP genotyping; T.S. and A.M. managed DNA samples and clinical information; K.S., K.M. and Y. Kamatani performed the SLE association study; Y.H. managed SLE samples; T.T., A.T. and N.K. performed the data analysis; M.K. contributed to SNP genotyping and managed DNA samples; Y.N. managed DNA samples and the project; and K.Y. planned and supervised the whole project.

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