Genetic determinants and an epistasis of *LILRA3* and HLA-B*52 in Takayasu arteritis

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Takayasu arteritis (TAK) is a systemic vasculitis with severe complications that affects the aorta and its large branches. HLA-B*52 is an established susceptibility locus to TAK. To date, there are still only a limited number of reports concerning non-HLA susceptibility loci to TAK. We conducted a genome-wide association study (GWAS) and a follow-up study in a total of 633 TAK cases and 5,928 controls. A total of 510,879 SNPs were genotyped, and 5,875,450 SNPs were imputed together with HLA-B*52. Functional annotation of significant loci, enhancer enrichment, and pathway analyses were conducted. We identified four unreported significant loci, namely rs2322599, rs103294, rs17133698, and rs1713450, in *PTK2B*, *LILRA3*/*LILRB2*, *DUSP22*, and *KLHL33*, respectively. Two additional significant loci unreported in non-European GWAS were identified, namely *HSPA6*/*FCGR3A* and chr21q.22. We found that a single variant associated with the expression of *MICB*, a ligand for natural killer (NK) cell receptor, could explain the entire association with the *HLA-B* region. Rs2322599 is strongly associated with the expression of *PTK2B*. Rs103294 risk allele in *LILRA3*/*LILRB2* is known to be a tagging SNP for the deletion of *LILRA3*, a soluble receptor of HLA class I molecules. We found a significant epistasis effect between HLA-B*52 and rs103294 ($P = 1.2 \times 10^{-3}$). Enhancer enrichment analysis and pathway analysis suggested the involvement of NK cells ($P = 8.8 \times 10^{-5}$, enhancer enrichment). In conclusion, four unreported TAK susceptibility loci and an epistasis effect between *LILRA3* and HLA-B*52 were identified. HLA and non-HLA regions suggested a critical role for NK cells in TAK.

genome-wide association study | Takayasu arteritis | autoimmunity | HLA | epistasis

Takayasu arteritis (TAK) is a systemic vasculitis affecting the aorta and its large branches and is classified as one of the two major categories of large-vessel vasculitis (1). TAK mainly affects young women and was first reported in Japan (2), which is one of the countries with the highest prevalence of TAK. Currently, approximately 5,000 people in Japan are known to have the disease (~0.005% prevalence), but patients with TAK have been detected all over the world (2). Inflammation of aorta and its branches results in occlusion of arteries and organ damage (2). The resultant severe complications include aortic regurgitation, blindness, pulseless lower and upper limbs, pulmonary infarction, and renal failure. Although

Significance

Takayasu arteritis (TAK) is a systemic vasculitis with unknown etiology. We identified four unreported susceptibility genes to TAK through genome-wide association studies. We successfully fine-mapped HLA associations and showed that HLA-G is associated with TAK in addition to HLA-B*52. The association between PTK2B and TAK could be explained by expression regulation of PTK2B. We showed an epistasis effect of LILR3A, one of the four genes, with HLA-B52 on TAK susceptibility. Enhancer enrichment analysis of significant non-HLA markers showed natural killer cells as important cells in TAK. Not only the associations in the HLA region but also nonsignificant associations from GWAS suggest the involvement of NK cells. These findings would lead to a better understanding of TAK.

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corticosteroid and immunosuppressant agent are usually effective for patients, no gold standard exists for TAK treatment. Thus, innovative effective treatment and therapeutic targets are strongly desired.

Environmental and genetic factors are involved with TAK (2). The *HLA-B* locus, especially HLA-B*52 (3), is associated with its onset, and this association can be observed all over the world (2). In addition to HLA-B*52, HLA-B*67, another rare HLA-B allele, is also involved with TAK (4). Recently, two groups, including our own, simultaneously reported non-HLA susceptibility genes to TAK identified by genome-wide association study (GWAS), which included *IL12B* (5, 6). Rs6871626, the top SNP in *IL12B*, also showed associations with TAK severity (5). Based on the findings of the central role played by *IL12B* in TAK, we conducted a pilot clinical study of ustekinumab, a monoclonal antibody of IL12/23p40 encoded by *IL12B*, in patients with refractory TAK and achieved favorable response (7). Other non-HLA susceptibility genes reported in previous GWAS (5, 6, 8) include *FCGR2A/3A*, *IL-6*, *RPS9/LILRB3*, chr21q22, and *MLX*, as well as multiple other suggestive loci. The five significant loci except for *MLX* were reported in European studies. Thus, it is uncertain whether TAK shows similar genetic architecture aside from *HLA-B* and *IL12B* across different populations. Further analyses of TAK with more samples and denser markers would elucidate the genetic background and pathophysiology of TAK, as well as the critical cell types for potential therapeutic targets. Here, we conducted a GWAS to reveal genetic architectures and biological insights of TAK.

Results

GWAS of TAK Followed by Imputation. A total of 415 patients with TAK, of which 167 samples were from our previous GWAS, and 2,170 controls were genome-scanned with the use of Illumina Infinium arrays, and 510,879 SNPs were used for analyses (*SI Appendix*, Table S1). After quality control, 411 cases and 2,158 controls with 255,400 SNPs remained for subsequent analyses (*Methods* and *SI Appendix*, Fig. S1). Mean call rates for subjects and SNPs were both 99.9%. We next imputed genotypes across the whole genome by using the 1000 Genome p3v5 panel (9) as reference. We removed markers with r^2 less than 0.5 and allele frequency less than 5% in controls because this study is underpowered to detect rare alleles (*SI Appendix*, Table S2), and, as a result, 5,875,450 SNPs remained. We supplemented this with 2,625 SNPs in the arrays that satisfied quality control criteria and were not in the imputed 5.8 million SNPs to maximize the statistical power. We conducted logistic regression analysis to compare allele frequencies between cases and controls. Confounding bias to cause inflation of statistics was minimal [λ = 1.069, intercept after linkage disequilibrium (LD) score regression = 1.029; *SI Appendix*, Fig. S1]. We observed significant associations in *PTK2B* on chromosome 8 and *PTPN2* on chromosome 18, in addition to the two known loci, namely the HLA locus and *IL12B* region (5) (Fig. 1).

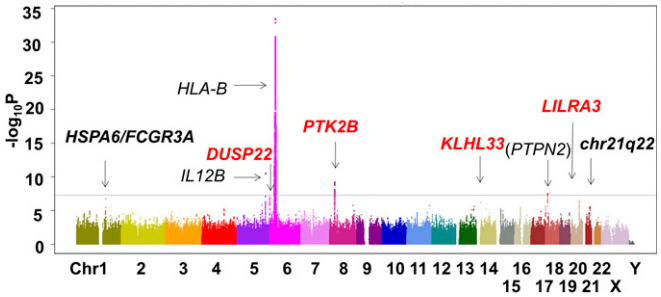

Fig. 1. GWAS reveals susceptibility loci to TAK. Manhattan plot of GWAS is indicated. Genes in bold red and black indicate unreported susceptibility loci across populations and in Asian GWAS, respectively. *PTPN2* reached GWAS significance, but this was not supported in replication.

Clarification of Associations Between the HLA and TAK. We obtained evidence that there is an additional independent locus apart from *HLA-B* in the HLA region, and that a single SNP associated with *MICB* expression explains the association of *HLA-B*. We imputed classical HLA alleles, amino acids, and SNPs in the HLA region by using SNP2HLA and combined them with the GWAS data. Rs117298325 in *HLA-B* demonstrated the smallest *P* value ($P = 3.6 \times 10^{-34}$; Fig. 2*A*). The C allele of rs117298325 was in strong LD ($r^2 = 0.93$) with the presence of HLA-B*52 ($P = 3.9 \times 10^{-30}$), which showed imputation accuracy of 99.7%. Despite the strong LD, the association of rs117298325 substantially remained after conditioning on HLA-B*52 ($P = 4.0 \times 10^{-7}$; *SI Appendix*, Fig. S2), but not vice versa ($P = 0.17$; Fig. 2*A*). We found that this was because the rs117298325 C allele also tagged the presence of HLA-B*67, another rare independent HLA-B allele (4) (Fig. 2*B*). The C allele was also associated with increased expression of *MICB*, MHC class I polypeptide-related sequence B, which encodes a ligand for NKG2D expressed on the surface of immune cells, especially natural killer (NK) cells (Fig. 2*C*). Because the strength of association of the top amino acid position (omnibus $P = 1.0 \times 10^{-24}$) was much weaker compared with those of rs117298325 or HLA-B*52 ($P \leq 3.9 \times 10^{-30}$), the association of rs117298325 or HLA-B*52 could not be explained by amino acid positions. Conditioning on rs117298325, we observed a substantial association in the intronic region of *HLA-G* (rs2523774; $P = 1.2 \times 10^{-7}$; Fig. 2*A* and *SI Appendix*, Table S3). Rs2523774 was strongly associated with the expression of *HLA-F* (*SI Appendix*, Fig. S3). HLA-G and HLA-F serve as ligands of NK cell receptors as well as classical HLA class I molecules (10).

Replication Study for 14 Regions. We conducted a replication study of non-HLA markers by using 219 cases and 3,770 controls, and a total of 14 regions were selected (detailed in *Methods*). All of the top markers in the suggestive regions ($P < 5.0 \times 10^{-5}$) not selected for replication are shown in *SI Appendix*, Table S4. All 14 regions showed call rates higher than 0.95 in cases and controls. Ten of the 14 regions showed the same risk alleles as the GWAS, and 4 of them demonstrated *P* values less than 0.01 (Table 1 and *SI Appendix*, Table S5). The *PTPN2* variant did not show the same risk allele as GWAS.

Combined Study. The overall associations revealed a total of six loci, namely *PTK2B* on chromosome 8, *LILR2B/3A* on chromosome 19, *DUSP22* on chromosome 6, *KLHL33* on chromosome 14, *HSPA6/FCGR3A* on chromosome 1, and a region on chr21q22, showing GWAS-significant associations (Table 1 and *SI Appendix*, Fig. S4). None of the significant markers showed deviation of Hardy–Weinberg equilibrium in GWAS or the replication study ($P \geq 0.10$). This study shows unreported GWAS-significant association of *PTK2B*, *LILR2B/3A*, *DUSP22*, and *KLHL33*, although the suggestive association of *PTK2B* and *LILR3A* were previously reported in a European population (we confirmed that the *LILR3A* association we found was different from that reported in the European population, as discussed later). Associations of *HSPA6/FCGR3A* and chr21q22 with TAK were reported in European populations (6, 8).

Functional Annotations of the Six Loci. All six loci showed evidence of associations with gene expression (*SI Appendix*, Table S6). Assessing the unreported susceptibility loci in detail, we found that none of the missense variants were in strong LD ($r^2 > 0.8$) with the top markers. The regional plots are shown in *SI Appendix*, Fig. S4. Rs2322599, which is located in an intronic region of *PTK2B*, showed the strongest expression quantitative trait loci (eQTL) association with *PTK2B* expression in the largest eQTL data set from whole blood (eQTL browser; *SI Appendix*, Fig. S5) (11). This could suggest that the association of TAK could be explained by *PTK2B* expression. The minor allele was protective against TAK and was associated with increased expression (*SI Appendix*, Table S7). Furthermore, we found a strong LD (r^2 = 0.81 in Japanese study) between rs2322599 and rs13260543, the top marker identified in the Turkish/US study (8) ($P = 9.0 \times 10^{-6}$ in the Turkish/US study), suggesting a common causative variant.

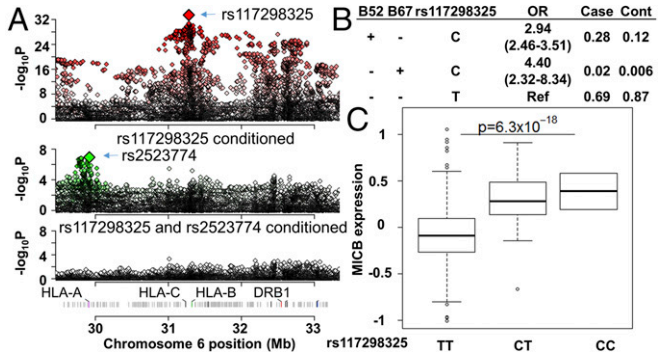

Fig. 2. Association between the HLA region and TAK susceptibility is explained by two variants in HLA class I, including HLA-B. (*A*) Sequential conditioning analyses in the HLA region. After condition on rs117298325, HLA-B associations disappeared and rs2523774 showed substantial evidence of association (*Middle*). After conditioning on the two SNPs, no markers showed association (*Bottom*). Color saturation indicates LD strength with the top SNPs measured by r^2. (*B*) Rs117298325 tags the two susceptibility HLA-B alleles, HLA-B*52 and B*67. OR, odds ratio with 95% CI. Haplotypic frequencies in cases and controls are indicated in the fifth and sixth columns. Ref, reference. (*C*) Rs117298325 risk allele (C allele) is associated with increased *MICB* expression in the Japanese eQTL data (35).

Rs103294 is sandwiched by *LILRB2* and *LILRA3* (*SI Appendix*, Fig. S4), which belong to LILR, leukocyte Ig-like receptor member genes of Ig superfamily. Regarding copy numbers, *LILRA3* is highly polymorphic among populations (12). Previous studies have shown that the risk allele T of rs103294 tags a 6.7-kb deletion of *LILRA3*, which is common in Asian populations (12–14). In fact, the T allele was strongly associated with decreased expression of *LILRA3* in the Japanese population ($P = 7.4 \times 10^{-13}$; Fig. 3*A*). The top SNP tagging a deletion indicates that the *LILRA3* gene is the responsible gene in this region. Although the Turkish/US study reported *LILRA3* as a suggestive locus, we found no evidence for LD between the top variants in the two studies ($r^2 < 0.040$).

Rs1713450 is located in the promoter region of *KLHL33*, Kelch-like family member 33 (*SI Appendix*, Fig. S4). This region is far from and not in LD with the HLA region. Genotype-Tissue Expression (GTEx), the biggest source of tissue-specific eQTL, revealed a significant association between expression of *KLHL33* across all cell types and rs1713450 (random-effect meta-$P = 2.5 \times 10^{-22}$). Rs67506652 is adjacent to rs1713450, and the two SNPs are almost in complete LD.

HSPA6/*FCGR3A* and chr21q22 regions were reported by the US/Turkish group, but were reported here in a non-European GWAS. The two top SNPs in both regions showed strong LD ($r^2 > 0.93$), so we have demonstrated that the two regions are susceptibility loci beyond populations.

Total Variance Explained by Significant Non-HLA Signals. A total of 6.1% variance in disease liability was explained by the eight non-HLA markers (the six SNPs identified here and the two loci we previously reported) and the top HLA SNP. The six loci explained 2.9% variance in disease liability.

Comparison of Genetic Associations Between Populations. Although we found involvement of the same loci with TAK beyond populations, we also found clear genetic differences between populations. The previous US/Turkish group reported *IL6* (rs2069837) and *LILRB3*/*RPS9* (rs11666543) loci (8) to be associated with TAK, but we did not find associations of these variants ($P \leq 0.25$; *SI Appendix*, Table S8) and other markers in these two loci (*SI Appendix*, Fig. S6).

Interaction Between *HLA-B* and a Variant in *LILRA3*. Based on the molecular binding of HLA class I and *LILRA3* (15), we hypothesized that rs109264 and HLA-B*52 might show an interaction. rs109264 and HLA-B*52 demonstrated a significant multiplicative interactive effect (interaction $P = 1.2 \times 10^{-3}$; Fig. 3*B*; detailed in *Materials and Methods*). The association of rs109264 was heavily dependent on the presence of HLA-B*52 (Fig. 3*B*). HLA-B*52 showed its association in a dominant manner (*SI Appendix*, Fig. S7 and Table S9).

Enrichment Analysis Using Enhancer Marks. We found that NK cells showed the strongest signal among immune-related cells when we assessed cell type-specific enrichment of enhancer marks in non-HLA markers ($P = 8.8 \times 10^{-5}$; Fig. 3*C* and *SI Appendix*, Table S10). When we added the two US/Turkish-specific associations, we obtained the same result ($P = 1.0 \times 10^{-5}$). We further analyzed our GWAS data with PASCAL, which uses *P* values of gene regions for its pathway analysis, controlling for LD structure in neighboring genes. NK cell pathway showed the most significant association ($P = 1.4 \times 10^{-4}$; *SI Appendix*, Table S11), and was the only pathway exceeding a significant level after Bonferroni's correction. Considering that the HLA region showed associations of classical/nonclassical class I molecules, which are strongly related to LILRA3, and that the top SNP is associated with *MICB* expression, HLA and non-HLA regions suggest the importance of NK cells in TAK. In fact, previous pathological analyses revealed that NK cells are one of the major tissue-infiltrating cells in patients with TAK (16).

Associations Between Unreported Signals and Clinical Phenotypes. No significant associations were found between the six SNPs and clinical phenotypes ($P > 0.06$; *SI Appendix*, Table S12). We did not find significant interactive effects on clinical manifestations.

Discussion

In the present study, we identified eight GWAS-significant loci to TAK, including two known regions, *IL12B* and the HLA region. This study contains more than 10% of all Japanese patients with TAK. Four loci were unreported, and two were unreported in the Japanese patients but in common with the Turkish/US populations. We showed clear genetic overlap of TAK beyond populations with some exceptions. We also found an epistatic multiplicative interactive effect between *LILRA3A* and HLA-B*52.

Table 1. Six loci showing significant associations in the 14 loci selected for replication study

				GWAS		Replication		Combined Study	
SNP	CH:BP	Region	A1/A2	A2 freq	*P* value	A2 freq	*P* value	OR (95% CI)	*P* value
rs2099684	1:161500130	*FCGR3A*	T/C	0.35/0.26	1.7×10^{-7}	0.36/0.26	7.8×10^{-6}	1.54 (1.36–1.75)	7.1×10^{-12}
rs17133698	6:255562	*DUSP22*	C/T	0.20/0.29	8.1×10^{-8}	0.28/0.32	0.099	0.66 (0.57–0.76)	2.8×10^{-9}
rs2322599	8:27211910	*PTK2B*	A/G	0.89/0.80	7.0×10^{-10}	0.87/0.79	2.5×10^{-4}	1.94 (1.62–2.31)	2.9×10^{-13}
rs1713450	14:20896245	*KLHL33*	T/C	0.22/0.15	6.5×10^{-7}	0.16/0.13	0.071	1.55 (1.34–1.80)	7.0×10^{-9}
rs103294	19:54797848	*LILR3A*	C/T	0.81/0.74	2.6×10^{-5}	0.83/0.75	1.4×10^{-4}	1.53 (1.31–1.77)	2.9×10^{-8}
rs4817988	21:40468838	21q.22	A/G	0.89/0.82	2.3×10^{-6}	0.86/0.80	0.001	1.77 (1.48–2.11)	2.8×10^{-10}

BP, base position of National center for Biotechnology Information build 37; CH, chromosome; A2 freq, A2 frequency in cases and controls, respectively.

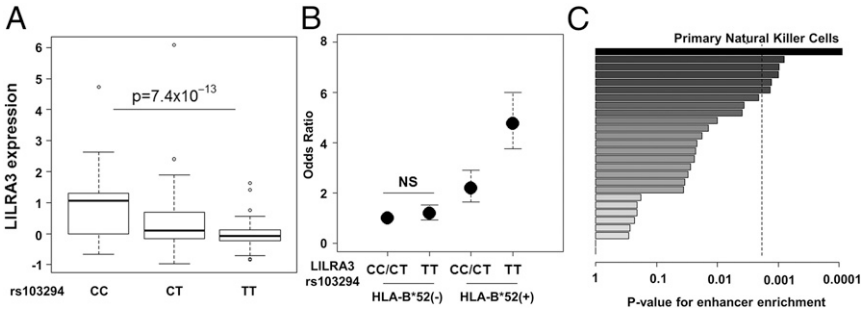

Fig. 3. Rs103294 is associated with *LILRA3* expression and shows a multiplicative interactive effect with HLA-B52. (*A*) Rs103294 risk allele (T allele) is associated with decreased expression of LILRA3 in the Japanese eQTL data (35). (*B*) An epistatic interactive effect is observed between TT genotype of rs103294 and HLA-B*52. TT genotype without HLA-B*52 did not show significant association with TAK susceptibility ($P = 0.13$; OR = 1.20; 95% CI = 0.95–1.53). Overall interactive and epistasis effects are evaluated by multiplicative interaction model in logistic regression (*Methods*). (*C*) Cell-specific enhancer enrichment analysis using non-HLA susceptibility SNPs to TAK revealed strongest signal in primary NK cells. We used the six loci in the present study and the two loci in our previous study. The vertical broken line indicates a significant level based on Bonferroni's correction.

Although we set the relatively nonstringent cutoff of Hardy–Wenberg equilibrium (HWE) before imputation to maximize the number of variants, we confirmed that none of the significant variants showed deviation from HWE, indicating that the cutoff did not lead to false-positive results. The present study is underpowered to detect low allele susceptibility variants and multiple signals in a single locus, but more samples in the future might clarify these points.

DUSP22 and *KLHL33* did not show strong associations in the replication study, but overall associations exceeded the level of significance. These suggest that the associations were mainly driven by the GWAS and that true effect sizes in the two loci might be smaller than those in the combined study. Further replication is essential to obtain convincing evidence of these associations.

The association of rs665268 in *MLX*, one of the two loci we found in the previous GWAS, was not strengthened in the present study [odds ratio (OR), 1.25; 95% CI, 1.08–1.45; $P = 0.0034$], suggesting that further increases in samples are necessary to achieve GWAS-significant association in this locus.

PTK2B encodes PYK2, a cytoplasmic tyrosine kinase activated by intracellular calcium influx and related with MAPK activation. PYK2 is expressed in a wide variety of cells, including lymphocytes and NK cells (17).

LILRA3 is the only member of the LILR family encoded as a soluble protein. LILRA3 binds to HLA class I molecules and is speculated to function as a regulator of other LILR family members (18). Its detailed functions and mechanisms are still not known. Previous studies reported associations between the risk allele of *LILRA3* in the present study and susceptibility to or clinical manifestations of rheumatic diseases in Chinese subjects (14).

KLHL33 encodes a member of a family of proteins with BTB/POZ domain, a BACK domain, and Kelch motifs. *KLHL33* shows a tissue-specific expression pattern, and the heart, which is commonly affected in TAK, is one of the organs in which *KLHL33* is strongly expressed according to GTEx. Based on histone modification and eQTL association, this region seems to be associated with TAK through alteration in the gene expression of *KLHL33*.

Rs17133698 is located 36 kb away from *DUSP22*, and its immune-related functions are not known. Further accumulation of functional analyses including trans-eQTL and interaction may clarify the association.

PTPN2 showed a significant association in GWAS, but the association was not replicated. A subset of GWAS publications report variants as significant if their result exceeds the significant level at some point in their analysis, even if the overall association does not show significance. However, we should carefully assess the association of *PTPN2*, and, because there is no concrete evidence for association, we should regard this as inconclusive.

This study identified unreported GWAS significance in *PTK2B* and *LILRA3* among various traits subjected to GWAS. The US/Turkish group reported suggestive associations in these two regions. There are clear genetic similarities beyond population observed for *PTK2B*, but this is not true for *LILRA3*. As LILR family genes display population-specific LD structures (12), it would be interesting to assess an association of deletion of *LILRA3* in the US/Turkish population. Associations were not observed for the *IL6* and *RPS9/LILRB3* regions in which the top two variants in the Turkish/US study were well-imputed ($r^2 = 0.87$) and genotyped, respectively. Although the two SNPs shared the risk directions with the US/Turkish data, the results suggest that these associations were population-specific or that the causative variants were not present in the Japanese population. Because it is obvious that there is a substantial overlap of susceptibility loci to TAK beyond populations, it would be feasible to conduct a transethnic meta-analysis of GWAS.

The present findings provide potential cellular candidates of therapeutic targets including NK cells. If animal models of TAK are developed, it would be interesting to test efficacy of depletion of NK cells on the phenotype.

The interactive effect between *LILRA3* and HLA-B*52 might suggest a strong direct or indirect binding of HLA-B*52 over other alleles. We did not find an interactive effect between rs103294 and HLA-B*67, with imputation accuracy of 91.7%. HLA-B*67 is specific to East Asian subjects and is relatively rare [1.2% incidence in the Japanese population (19)], and the present study is underpowered to detect significant effects. It would be interesting to assess interactive effects between *HLA-B* and *LILRA3* in patients with TAK in different populations. It will also be interesting to analyze the interactions between LILRA3 and HLA class I in various diseases in which HLA class I plays central effects.

Enhancer mark enrichment analysis suggested NK cells as the most promising cell type playing central roles in TAK. $CD8^+$ T cells, the second promising cell type, also seems compatible to TAK pathophysiology (16). It is not surprising that these cell types play critical roles in TAK based on pathological evidence, but it is important to identify critical cell types supported by genetic evidence. Although further experiments are necessary to confirm the critical cell types, together with the SNPs clustered in the enhancer marks, the list of the susceptibility regions also seems to support the involvement of NK cells.

Suppression of inhibitory mechanisms of NK cells and overactivation of NK cells may be involved with TAK. Inhibitory receptors in NK cells include KIR, CD94, and LIT-2 encoded by *LILRB1*. All recognize classical and/or nonclassical HLA class I molecules, including HLA-B and HLA-G. Binding between LILRA3 and HLA class I molecules, especially HLA-B*52, may be critical to preserve this inhibitory mechanism, and the deletion of *LILRA3* might result in failure of inhibition and lead to overactivation of NK cells and onset of TAK.

Activatory receptors of NK cells include NKG2D and CD16 encoded by *FCGR3A*. *HSP6A* encodes heat-shock protein 70, which is a stress-induced protein like MICB. Both are ligands of activating receptors in NK cells, and their increased expressions are associated with missing self recognition by NK cells (20) and activation of NK cells. PYK2 is a downstream molecule of activatory and inhibitory receptors in NK cells. As overexpression of PYK2 results in inhibition of cytolytic activity of NK cells (21), the protective association between TAK susceptibility and the A allele of rs2322599, which is associated with increased expression, seems compatible. IL-12 is important to regulate secretion of IFN-γ from NK cells (22).

The present study provides important clues in the understanding of TAK pathophysiology. Further analyses of TAK with more samples, together with extensive functional analyses, are necessary to expand our knowledge of TAK and obtain clues for effective management and treatment. It would also be interesting to analyze disease-specific, cell-specific gene expressions and epigenetic features to capture the entire genetic feature of TAK.

Methods

The Local Ethical Committee in Each Institution Approved the Study. This study was approved by ethical committees in Kyoto University Graduate School of Medicine, The University of Tokyo, Tokyo Medical and Dental University, Tohoku University School of Medicine, Osaka University Graduate School of Medicine, Yokohama City University Graduate School of Medicine, Aichi Cancer Center Research Institute, Nagasaki University Graduate School of Biomedical Sciences, Hokkaido University Graduate School of Medicine, Kawasaki Medical School, Ehime University Graduate School of Medicine, Sapporo Medical University School of Medicine, University of Occupational & Environmental Health, and Keio University. Written informed consent was obtained from each participant.

Study Subjects for GWAS. DNA obtained from 415 subjects collected by Kyoto University and 2,170 control subjects collected by Aichi Cancer Center Research Institute were used as case and control subjects for GWAS, respectively. All subjects were Japanese. The 415 subjects contained 167 case subjects from our previous study. TAK was diagnosed according to the criteria of the American College of Rheumatology (23) or the guideline provided by the Japanese Circulation Society (24). The control subjects were part of the hospital-based Epidemiologic Research Program at Aichi Cancer Center II who were originally recruited to serve as controls in association studies (25), and all participants were confirmed as not having any type of cancer.

Genotyping Method for GWAS. We performed GWAS for subjects by Illumina Infinium Human Core Exome Array or Human Core Array in combination with Human Exome Array (*SI Appendix*, Table S1). After the previous GWAS (5) using Human Exome Array, we expanded our samples, and they were genotyped by Human Core Exome Array. We additionally rescanned the case samples in the previous GWAS by Human Core Array. SNPs covered by the combination of the two arrays are almost identical to those by Human Core Exome Array.

Quality Control in GWAS. Quality control was performed on subjects and SNPs by using the following exclusion criteria in the GWAS. Subjects showing a call rate lower than 0.97 and relatedness with other subjects [PI_HAT calculated by plink >0.15; PI_HAT is the proportion of identity by descent (IBD) and defined as probability (IBD = 2) + 0.5 × probability (IBD = 1)] and outliers of the East Asian cluster based on principal component analysis were excluded from further analysis. SNPs with low call rate less than 0.95 and allele frequency less than 0.01 in cases or controls and showing deviation from Hardy–Weinberg equilibrium ($P < 1.0 \times 10^{-7}$ in control subjects) were excluded from imputation.

Imputation of Genotypes. Whole-genome imputation was performed to assess entire associations. GWAS data after the aforementioned quality control for SNPs in common among the three types of GWAS arrays (HumanCoreExome and the combination of HumanCore and HumanExome) was used for imputation. We imputed the GWAS data with use of the 1000 Genome Project p3v5 data as reference by using shapeit (26) and minimach3 (27) software for phasing and imputation, respectively.

Imputation of the HLA Region by Using SNP2HLA. HLA alleles and amino acid residues together with SNPs in the HLA region were imputed by SNP2HLA (19). We used the Japanese HLA reference panel (28) to obtain the best imputation accuracy. Imputation accuracy of HLA-B*52 and B*67 were calculated by comparing genotyping results previously described (4) and imputed results by means of correlation of dosage of the alleles. Because the association of B*67 with TAK was established, B*67 was included in the data set even though its frequency is less than 0.05 (as detailed later). When HLA-B*52 genotyping or imputation data were not available, rs9263739, in almost complete LD with HLA-B*52:01 (5), was used as a proxy of HLA-B*52 to assess synergistic effects of genetic determinants along with HLA-B*52.

Quality Control After Imputation. After imputation, cases of allele frequency less than 0.05 in control subjects were excluded. Markers with low imputation scores ($r^2 < 0.5$) were excluded from the analyses. We undertook this two-step filtering of allele frequency to ensure imputation accuracy of SNPs with allele frequency of approximately 0.05 and to avoid false-positive signals.

Evaluation of Confounding Bias. Confounding bias leading to inflation of statistics, including population structure, was assessed by a quantile-quantile plot first. Then, we conducted LD score regression (29) to assess an intercept of the present study. We used the LD score data of East Asian subjects originally contained in ldsc software (29).

Study Subjects for Replication. The replication study included 219 subjects collected by Tokyo Medical and Dental University or Tokyo University and 3,770 healthy subjects collected by Kyoto University. All subjects were Japanese. Again, TAK was diagnosed based on the criteria of American College of Rheumatology (23) or the guideline provided by the Japanese Circulation Society (24). The control subjects were obtained from the Nagahama study, a community-based prospective cohort study (30).

Selection of SNPs for Validation. After exclusion of the HLA region and the two loci identified in our previous GWAS, we obtained a list of SNPs showing *P* values less than 5.0×10^{-6} as candidate susceptibility markers. We regarded a set of variants as located in the same region if they were within 250 kb from the top SNPs. As a result, we identified 14 loci. We conducted conditioning analyses with the top variant as a covariate (discussed later) to assess potential multiple associations in a single locus and regarded other markers as independent only if they still satisfied *P* values less than 5.0×10^{-6}. None of the loci contained multiple independent signals. We further added one locus to the 14 loci that showed suggestive association ($5.0 \times 10^{-6} < P < 5.0 \times 10^{-5}$) in this study and was reported to show suggestive association in the US/Turkish study. TaqMan probe design for replication failed for a top SNP, rs7280413 ($P = 4.1 \times 10^{-6}$) on chromosome 21, so this SNP was excluded.

Genotyping Method for Replication. The TaqMan assay was adopted as the genotyping method for replication study. For the replication study, because it was difficult to amplify 80 DNA samples extracted many years ago with the use of TaqMan probes for rs564264, rs1569722, and rs72848097, we conducted direct sequencing for these SNPs. We did not observe allelic difference between different genotyping methods.

General Statistical Framework. Allele frequencies were compared between cases and controls by logistic regression analysis in GWAS, replication study, and the combined study. Overall significance for GWAS was evaluated in the results of the combined study in which we pooled the two studies in the same manner as in our first GWAS study of TAK (5). SNPs with *P* values less than 5.0×10^{-8} were regarded as significant. Cutoff significant levels of 0.05 with Bonferroni's correction were adopted for interactive effects, associations with clinical manifestations, and enhancer enrichment analysis. Statistical analyses were performed with R statistical software or PLINK version 1.90 (31).

Conditioning Analyses. We conducted conditioning analyses to find multiple susceptibility variants to TAK in a single locus. We added to covariates genotype dosages (0, 1, or 2) of variant(s) for which we wanted to perform adjustments.

Omnibus Test for the HLA Region. We conducted an omnibus test (*SI Appendix*) to assess associations of amino acid position based on imputed results by SNP2HLA. Detailed methods have been previously described (32).

Evaluation of LD and Haplotype. LD was assessed by Plink software version 1.9 (31) or Haploview software (33). Haplotype analysis was conducted by Haploview.

Functional Annotation. Functional annotation of top markers in the significant regions and other markers in strong LD ($r^2 > 0.8$) were evaluated by HaploReg (34). We selected enhancer and promoter histone marks and DNase and eQTL hits to show components related with gene expressions.

Evaluation of Associations Between SNPs and Gene Expression. We evaluated eQTL association of genetic determinants by using Japanese eQTL data from whole blood of 300 healthy individuals (35), GTEx (various kinds of cells) (36), and eQL browser (whole blood) (11). We took advantage of the Japanese data for gene expression of HLA and LILR regions because the HLA region is highly polymorphic and the LD structure of the two regions is diverse across different populations (13, 28). We reported the eQTL associations only if the association showed a *P* value less than 1.0×10^{-9}. We confirmed that the probes used to quantify gene expression did not hybridize sequences containing common variants (allele frequency > 1%) in *MICB* and *HLA-F*.

Pathway Analysis. We performed pathway analysis by using all GWAS signals, not just the significant loci, with PASCAL software (37). To avoid strong influence of GWAS-significant associations, we adopted the sum of *P* values of gene regions rather than the best *P* values to calculate gene scores used for pathway enrichment estimation. Because we conducted this analysis to find important cellular pathways, we focused on pathways in the BIOCARTA database.

Variance Explained. We calculated variance explained by the significant loci based on liability threshold model (38) assuming prevalence of TAK as 0.005%, in which we assume that subjects have a continuous risk score and that subjects who exceed a certain threshold develop TAK. Further details are described in *SI Appendix*.

Evaluation of Multiplicative Interactive Effects. An interactive effect was regarded as significant only if the interactive term showed a significant *P* value. We regarded multiplicative interaction as epistatic only when we found a significant multiplicative interaction and a genetic effect without the presence of the HLA-B allele with a *P* value more than 0.05. Further details are described in *SI Appendix*.

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1. Jennette JC, et al. (2013) 2012 revised International Chapel Hill consensus conference nomenclature of vasculitides. *Arthritis Rheum* 65:1–11.
2. Terao C, Yoshifuji H, Mimori T (2014) Recent advances in Takayasu arteritis. *Int J Rheum Dis* 17:238–247.
3. Yoshida M, Kimura A, Katsuragi K, Numano F, Sasazuki T (1993) DNA typing of HLA-B gene in Takayasu's arteritis. *Tissue Antigens* 42:87–90.
4. Terao C, et al. (2013) Association of Takayasu arteritis with HLA-B 67:01 and two amino acids in HLA-B protein. *Rheumatology (Oxford)* 52:1769–1774.
5. Terao C, et al. (2013) Two susceptibility loci to Takayasu arteritis reveal a synergistic role of the IL12B and HLA-B regions in a Japanese population. *Am J Hum Genet* 93: 289–297.
6. Saruhan-Direskeneli G, et al. (2013) Identification of multiple genetic susceptibility loci in Takayasu arteritis. *Am J Hum Genet* 93:298–305.
7. Terao C, et al. (2015) Ustekinumab as a therapeutic option for Takayasu arteritis: From genetic findings to clinical application. *Scand J Rheumatol* 45:80–82.
8. Renauer PA, et al. (2015) Identification of susceptibility loci in IL6, RPS9/LILRB3, and an intergenic locus on chromosome 21q22 in Takayasu arteritis in a genome-wide association study. *Arthritis Rheumatol* 67:1361–1368.
9. Abecasis GR, et al.; 1000 Genomes Project Consortium (2010) A map of human genome variation from population-scale sequencing. *Nature* 467:1061–1073, and erratum (2011) 473:544.
10. Goodridge JP, Burian A, Lee N, Geraghty DE (2013) HLA-F and MHC class I open conformers are ligands for NK cell Ig-like receptors. *J Immunol* 191:3553–3562.
11. Westra HJ, et al. (2013) Systematic identification of trans eQTLs as putative drivers of known disease associations. *Nat Genet* 45:1238–1243.
12. Hirayasu K, et al. (2008) Evidence for natural selection on leukocyte immunoglobulin-like receptors for HLA class I in Northeast Asians. *Am J Hum Genet* 82:1075–1083.
13. Hirayasu K, et al. (2006) Long-term persistence of both functional and non-functional alleles at the leukocyte immunoglobulin-like receptor A3 (LILRA3) locus suggests balancing selection. *Hum Genet* 119:436–443.
14. Du Y, et al. (2015) Impact of the leucocyte immunoglobulin-like receptor A3 (LILRA3) on susceptibility and subphenotypes of systemic lupus erythematosus and Sjögren's syndrome. *Ann Rheum Dis* 74:2070–2075.
15. Ryu M, et al. (2011) LILRA3 binds both classical and non-classical HLA class I molecules but with reduced affinities compared to LILRB1/LILRB2: Structural evidence. *PLoS One* 6:e19245.
16. Seko Y, et al. (1994) Perforin-secreting killer cell infiltration and expression of a 65-kD heat-shock protein in aortic tissue of patients with Takayasu's arteritis. *J Clin Invest* 93:750–758.
17. Gismondi A, et al. (1997) Proline-rich tyrosine kinase-2 activation by beta 1 integrin fibronectin receptor cross-linking and association with paxillin in human natural killer cells. *J Immunol* 159:4729–4736.
18. Hirayasu K, Arase H (2015) Functional and genetic diversity of leukocyte immunoglobulin-like receptor and implication for disease associations. *J Hum Genet* 60:703–708.
19. Jia X, et al. (2013) Imputing amino acid polymorphisms in human leukocyte antigens. *PLoS One* 8:e64683.
20. Schilling D, Tetzlaff F, Konrad S, Li W, Multhoff G (2015) A hypoxia-induced decrease of either MICA/B or Hsp70 on the membrane of tumor cells mediates immune escape from NK cells. *Cell Stress Chaperones* 20:139–147.
21. Sancho D, et al. (2000) The tyrosine kinase PYK-2/RAFTK regulates natural killer (NK) cell cytotoxic response, and is translocated and activated upon specific target cell recognition and killing. *J Cell Biol* 149:1249–1262.
22. Biron CA, Gazzinelli RT (1995) Effects of IL-12 on immune responses to microbial infections: A key mediator in regulating disease outcome. *Curr Opin Immunol* 7:485–496.
23. Arend WP, et al. (1990) The American College of Rheumatology 1990 criteria for the classification of Takayasu arteritis. *Arthritis Rheum* 33:1129–1134.
24. Anonymous; JCS Joint Working Group; Japanese Circulation Society (2011) Guideline for management of vasculitis syndrome (JCS 2008). *Circ J* 75:474–503.
25. Hamajima N, et al. (2001) Gene-environment interactions and polymorphism studies of cancer risk in the hospital-based epidemiologic research program at Aichi Cancer Center II (HERPACC-II). *Asian Pac J Cancer Prev* 2:99–107.
26. Delaneau O, Marchini J; 1000 Genomes Project Consortium; 1000 Genomes Project Consortium (2014) Integrating sequence and array data to create an improved 1000 Genomes Project haplotype reference panel. *Nat Commun* 5:3934.
27. Fuchsberger C, Abecasis GR, Hinds DA (2015) minimac2: Faster genotype imputation. *Bioinformatics* 31:782–784.
28. Okada Y, et al. (2015) Construction of a population-specific HLA imputation reference panel and its application to Graves' disease risk in Japanese. *Nat Genet* 47:798–802.
29. Bulik-Sullivan BK, et al.; Schizophrenia Working Group of the Psychiatric Genomics Consortium (2015) LD Score regression distinguishes confounding from polygenicity in genome-wide association studies. *Nat Genet* 47:291–295.
30. Yoshimura K, et al.; Nagahama Cohort Research Group (2013) Prevalence of post-micturition urinary incontinence in Japanese men: Comparison with other types of incontinence. *Int J Urol* 20:911–916.
31. Purcell S, et al. (2007) PLINK: A tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 81:559–575.
32. Raychaudhuri S, et al. (2012) Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. *Nat Genet* 44:291–296.
33. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: Analysis and visualization of LD and haplotype maps. *Bioinformatics* 21:263–265.
34. Ward LD, Kellis M (2012) HaploReg: A resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. *Nucleic Acids Res* 40:D930–D934.
35. Narahara M, et al. (2014) Large-scale East-Asian eQTL mapping reveals novel candidate genes for LD mapping and the genomic landscape of transcriptional effects of sequence variants. *PLoS One* 9:e100924.
36. GTEx Consortium (2013) The Genotype-Tissue Expression (GTEx) project. *Nat Genet* 45:580–585.
37. Lamparter D, Marbach D, Rueedi R, Kutalik Z, Bergmann S (2016) Fast and rigorous computation of gene and pathway scores from SNP-based summary statistics. *PLoS Comput Biol* 12:e1004714.
38. So HC, Gui AH, Cherny SS, Sham PC (2011) Evaluating the heritability explained by known susceptibility variants: A survey of ten complex diseases. *Genet Epidemiol* 35:310–317.