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PADI4 and HLA-DRB1 Are Genetic Risks for Radiographic Progression in RA Patients, Independent of ACPA Status: Results from the IORRA Cohort Study

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Abstract

Introduction: Rheumatoid arthritis (RA) is a systemic, chronic inflammatory disease influenced by both genetic and environmental factors, leading to joint destruction and functional impairment. Recently, a large-scaled GWAS meta-analysis using more than 37,000 Japanese samples were conducted and 13 RA susceptibility loci were identified. However, it is not clear whether these loci have significant impact on joint destruction or not. This is the first study focused on the 13 loci to investigate independent genetic risk factors for radiographic progression in the first five years from onset of RA.

Methods: Sharp/van der Heijde score of hands at 5-year disease duration, which represents joint damage, were measured retrospectively and used as an outcome variable in 865 Japanese RA patients. Genetic factors regarded as putative risk factors were RA-susceptible polymorphisms identified by the Japanese GWAS meta-analysis, including HLA-DRB1 (shared epitope, SE), rs2240340 (*PADI4*), rs2230926 (*TNFAIP3*), rs3093024 (*CCR6*), rs11900673 (*B3GNT2*), rs2867461 (*ANXA3*), rs657075 (*CSF2*), rs12529514 (*CD83*), rs2233434 (*NFKBIE*), rs10821944 (*ARID5B*), rs3781913 (*PDE2A-ARAP1*), rs2841277 (*PLD4*) and rs2847297 (*PTPN2*). These putative genetic risk factors were assessed by a stepwise multiple regression analysis adjusted for possible non-genetic risk factors: autoantibody positivity (anti-citrullinated peptide antibody [ACPA] and rheumatoid factor), history of smoking, gender and age at disease onset.

Results: The number of SE alleles ($P = 0.002$) and risk alleles of peptidyl arginine deiminase type IV gene (*PADI4*, $P = 0.04$) had significant impact on progressive joint destruction, as well as following non-genetic factors: ACPA positive ($P = 0.0006$), female sex ($P = 0.006$) and younger age of onset ($P = 0.02$).

Conclusions: In the present study, we found that *PADI4* risk allele and HLA-DRB1 shared epitope are independent genetic risks for radiographic progression in Japanese rheumatoid arthritis patients. The results of this study give important knowledge of the risks on progressive joint damage in RA patients.

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Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by the chronic synovitis and the localized destruction of cartilage and bone resulting in deteriorated physical function and reduced quality of life. It has been recognized that early therapeutic intervention can prevent progress of joint damage and provide long-term benefits to the patients of RA. The therapeutic recommendations for the management of RA indicate patients may use non-biologic and/or biologic disease-modifying anti-rheumatic drugs (DMARDs) in consideration of the presence of poor prognostic factors.[1–3].

To date, prognostic markers of joint damage have been studied extensively and reported; anti-cyclic citrullinated peptides antibody (ACPA) positive,[4–7] rheumatoid factor (RF) positive, [6,7] the history of smoking, [8,9] the high level of disease activity measured using composite measures,[10–12] gender [4,13] and the age of disease onset.[13–15].

Since RA is a complex disease influenced by both genetic and environmental factors, susceptibility genes to the disease have been widely investigated and identified, especially in the era of genome-wide association studies (GWAS) and GWAS meta-analyses.[16–18] Recently, a large-scaled GWAS meta-analysis was conducted using samples from more than 9,000 Japanese RA patients and 38,000 controls. As a result, nine novel RA susceptibility loci were identified; *B3GNT2*, *ANXA3*, *CSF2*, *CD83*, *NFKBIE*, *ARID5B*, *PDE2A-ARAP1*, *PLD4* and *PTPN2*. [16] The study also showed that some previously reported RA susceptibility genes satisfied the genome-wide significance threshold ($P<5.0 \times 10^{-8}$); HLA-DRB1, *PADI4*, *TNFAIP3* and *CCR6*. [16] Of these 13 RA-susceptible loci, HLA-DRB1 shared epitope (SE) have been reported to have impact on disease severity.[19–21] However, the question remains whether if the other RA-susceptible genes have significant impact on joint destruction.

The purpose of this study is to explore genetic risk factors associated with radiographic progression in RA patients.

Methods

Patients and Evaluation of Radiographic Joint Damage

Tokyo Women's Medical University Genome Ethics Committee approved the present study and each individual signed an informed consent form after receiving a verbal explanation of the study. All the patients satisfied the American College of Rheumatology 1987 revised criteria for RA. [22] DNA samples from RA patients were obtained from the IORRA (Institute of Rheumatology Rheumatoid Arthritis cohort study) DNA collection. [16] IORRA is a project of observational RA cohort with an enrollment of over 5,000 Japanese RA patients, and DNA samples were collected from 2,068 patients. [23,24] All these DNA samples were included in the Japanese GWAS meta-analysis. [16].

Radiographic data at 5-year disease duration were collected retrospectively from the medical records of the patients. Of the patients who donated DNA samples, Sharp/van der Heijde score (SHS) of the hands representing radiographic joint damage (a higher score indicating more damage) was available in 865 patients who have not received biologic agents. [25] Proper anteroposterior radiographs of the hands were scored by a single experienced reader as described elsewhere. [26] Since it has been well known that the rate of radiologic progression develops rapidly in early disease course of RA, joint damage scores of the same disease duration, 5 years, were used. Interobserver and intraobserver agreements (0.85 and 0.95, respectively) indicated good reliability.

The reasons of the exclusion for the patients who treated with biologic agents were as follows: the apparent reported dissociation between clinical and radiologic outcomes in patients with RA who are treated with biologic agents, which could be a confounding factor for the study; [27] the year of RA onset for most patients in this study was before 2000 (70.2%), while the first biologic agent was not launched in the Japanese market until 2003, and the number of the patients who have ever used biologic agents in the first 5-year of disease duration was not sufficient for the sub-analysis targeted on biologic agents.

Assessment Measures, Non-genetic Factors

From the IORRA database and medical records of the patients, demographic, clinical, biological and therapeutic data during the first 5-year after onset of RA were collected, including ACPA status (ACPA titers were measured with second [MESACUP CCP test, Medical and biological laboratories] or third generation [QUANTA Lite CCP3 IgG ELISA, Inova Diagnostics] kit), [28] RF status (determined by a latex agglutination turbidimetric immunoassay method), history of smoking, gender and the age at onset. The age at onset was defined as the age at the onset of first symptoms, according to the patient's self-report, and it did not mean the age that satisfied the 1987 ACR criteria.

ACPA, RF, history of smoking and gender were categorized into two dichotomous variables: ACPA (positive [> = 4.5 IU/ml] = 1, negative = 0), RF (positive [> = 15.0 IU/ml] = 1, negative = 0; maximum value in the first 5 years was used), history of smoking (ever smoked = 1, never = 0) and gender (female = 1, male = 0). Data of age at onset was used as continuous variables.

Assessment Measures, Genetic Factors

HLA-DRB1 SE and twelve single nucleotide polymorphisms that have been reported as RA susceptibility polymorphisms using a large-scaled GWAS meta-analysis of Japanese were chosen for the study. [16] There were rs2240340 (*PADI4*, peptidyl arginine deiminase type IV), rs2230926 (*TNFAIP3*, tumor necrosis factor, alpha-induced protein 3), rs3093024 (*CCR6*, C-C chemokine receptor type 6), rs11900673 (*B3GNT2*, UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2), rs2867461 (*ANXA3*, annexin A3), rs657075 (*CSF2*, colony stimulating factor 2), rs12529514 (*CD83*, CD83 molecule), rs2233434 (*NFKBIE*, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon), rs10821944 (*ARID5B*, AT rich interactive domain 5B [MRF1-like]), rs3781913 (*PDE2A-ARAP1*, *PDE2A*; phosphodiesterase 2A, cGMP-stimulated, *ARAP1*; ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1), rs2841277 (*PLD4*, phospholipase D family, member 4) and rs2847297 (*PTPN2*, protein tyrosine phosphatase, non-receptor type 2). The risk alleles were defined as the allele that increases the risk of RA based on a prior report. [16].

Genotyping

Duplicate samples and negative controls were included to ensure accuracy of genotyping. High-resolution polymerase chain reaction (PCR) based DNA typing of HLA-DRB1 locus was performed using the sequence-based typing method with the AlleleSEQR DRB1 typing kit (Abbott Japan), according to the manufacturer's instructions. Assignment of HLA-DRB1 alleles was performed using Assign software. HLA-DRB1 SE were defined as alleles encoding amino acid sequences of QKRAA/QRRAA/RRRAA in positions 70–74 of HLA-DRB1. Genotyping of non-HLA RA susceptibility single-nucleotide polymorphisms (SNPs) were performed using the TaqMan fluorogenic 5′ nuclease assay according to the manufacturer's instructions (Applied Biosystems,

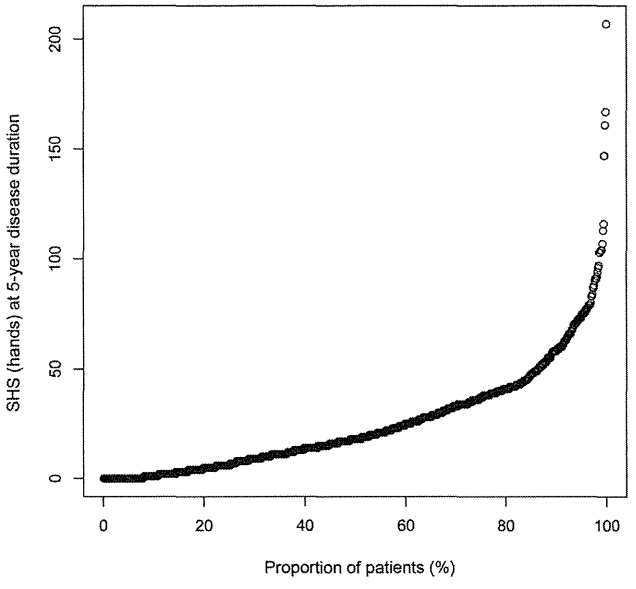

Figure 1. Probability plot of Sharp/van der Heijde score of the hands at the 5-year disease duration. Each point on the plot represents the Sharp/van der Heijde score (SHS) of the hands at the 5-year disease duration, which representing approximate value of the radiographic progression in the first 5 years after onset of RA, in an individual patient. A zero value represents a patient without any radiographic progression, and the right-side tail represents patients with the most progression.
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Tokyo, Japan) as described elsewhere. [16] All PCRs were performed using GeneAmp PCR System 9700 (Applied Biosystems), DNA sequencing for HLA typing on 3130×l Genetic Analyzer (Applied Biosystems) and endpoint fluorescent readings for TaqMan assays on ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems).

Statistical Analysis

First, the putative risk factors including non-genetic factors on joint damage were assessed using univariate linear regression analyses (univariate-based feature selection process). Any variable showing a significance level (alpha = 0.05) was selected as a candidate for a stepwise multiple regression analysis (backward elimination) to evaluate the putative risk factor as an independent risk of radiographic damage in RA patients. Number of reported risk alleles on disease susceptibility (0, 1 and 2) was used for the RA susceptible polymorphisms to test the additive effect of the alleles. [16] The dependent variable was the radiographic progression in the first 5 years after onset of RA, calculated as SHS of hands at the 5-year disease duration. Since some RA patients may show more rapid radiographic progression than others[29–31], the SHS (hands) were log-transformed to obtain a normal distribution for all statistical analyses. [32,33].

All valuables were standardized using "scale" command in R software to calculate standardized regression coefficients (ß) in the stepwise multiple regression analysis. Statistical analyses were performed using the R software package (http://www.r-project.org/).

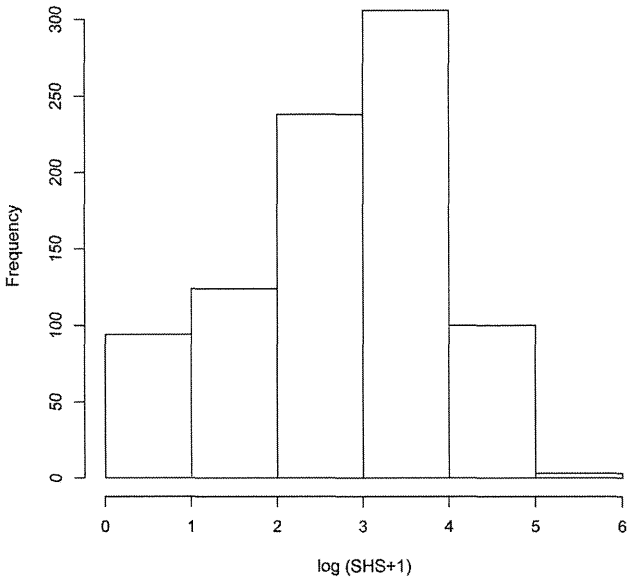

Figure 2. Histogram of distribution of the log-transformed SHS (hands).
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Results

Demographic, Clinical and Biological Characteristics of the Patients

Demographic, clinical, biological and therapeutic characteristics of the patients are shown in Table 1. Median age of the patients at

Table 1. Demographic and clinical characteristics of patients at 5 years from onset.

Age at 5-year disease duration, years	54 (46–62)
Sex, female	738 (85.3)
Year of disease onset	
<1990	141 (16.3)
1990<1995	195 (22.5)
1995<2000	271 (31.3)
2000<	258 (29.8)
SHS (hands)	18 (6–37)
ACPA, positive*	739 (87.8)
RF, positive†	781 (90.3)
History of smoking, ever	301 (35.2)
Medication in the first 5-year from the onset	
DMARDs use, ever	735 (92.3)
Methotrexate use, ever	399 (50.1)
Biologic agents use, ever	0 (0)
Corticosteroid use, ever	375 (47.4)

Data are presented as median (interquartile range) or n (%).
*Cut-off = 4.5 IU/ml.
†Maximum value in the first 5-year period of the disease was used, cut-off = 15.0 IU/ml.
SHS, Sharp/van der Heijde score; ACPA, anti-citrullinated peptide antibody; RF, rheumatoid factor; DMARDs, disease-modifying anti-rheumatic drugs.
doi:10.1371/journal.pone.0061045.t001

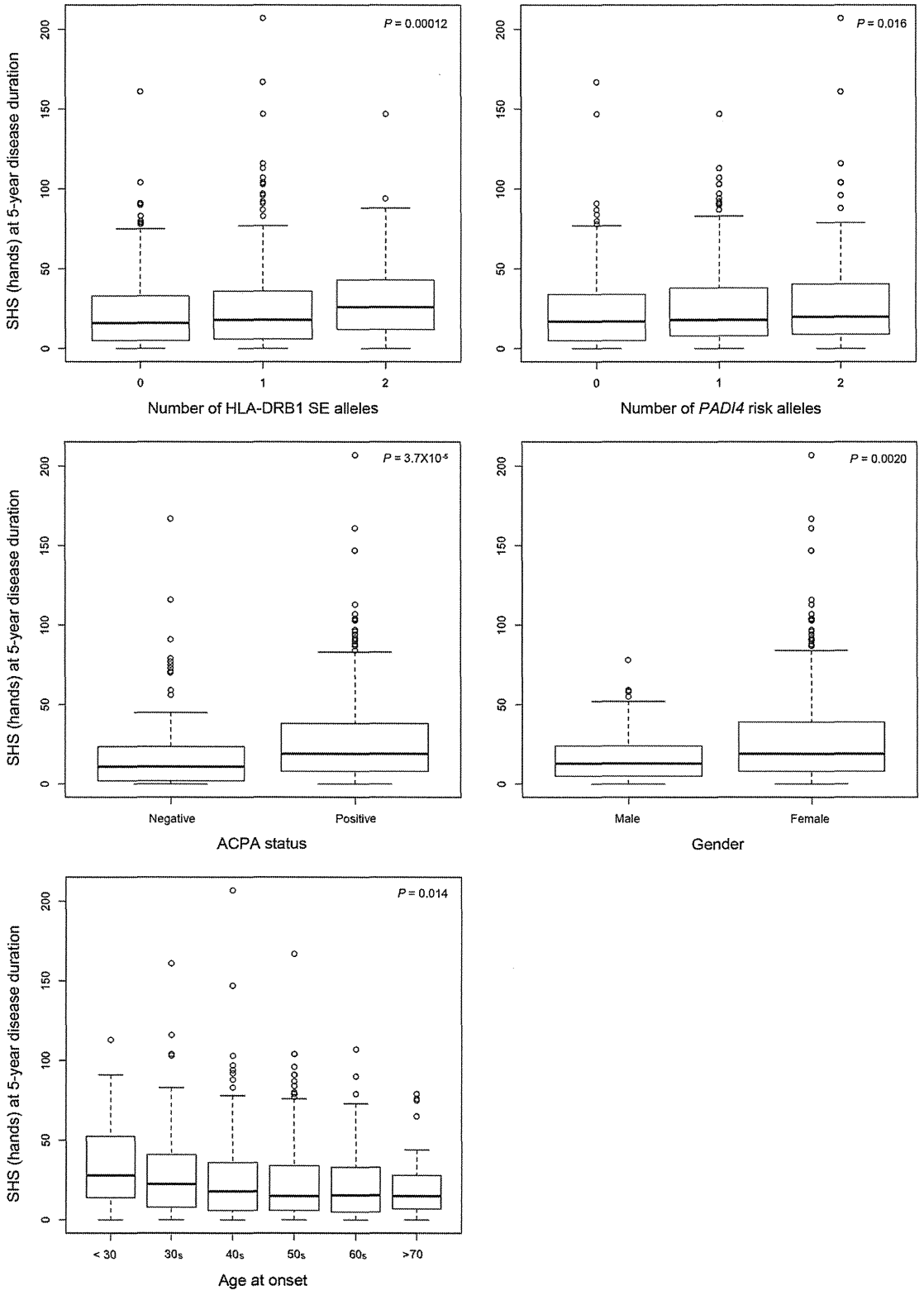

Figure 3. Boxplots representing the distribution of Sharp/van der Heijde score (SHS) of the hands in each category of independent risk factors for joint destruction. Risk factors; the number of HLA-DRB1 shared epitope, the number of PADI4 risk alleles, ACPA status (negative [<4.5 IU/ml] and positive), gender (female and male) and age at onset (categorized as "age under 30", "30 s", "40 s", "50 s", "60 s" and "age over 70"). Each box represents the interquartile range of values, with the bold line showing the median value. The vertical lines show maximum and minimum value that fall within 1.5 box lengths, the open circles show extreme values >1.5 box plot lengths. The P values were given by the univariate linear regression analyses (a log-transformed SHS was used as the dependent variable). PADI4, peptidyl arginine deiminase type IV ACPA, anti-citrullinated peptide antibody.
doi:10.1371/journal.pone.0061045.g003

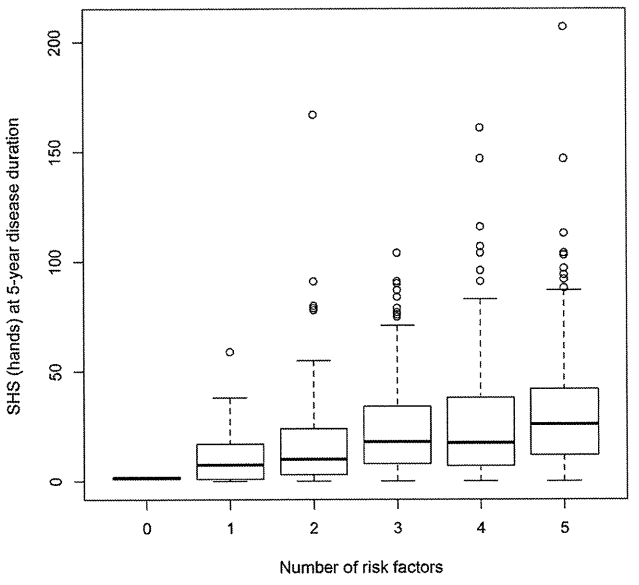

Figure 4. Boxplots representing the distribution of Sharp/van der Heijde score (SHS) of the hands according to the number of the risk factors. Risk factors; SE allele carrier, PADI4 risk allele carrier, ACPA positive, female and age at onset under 50. Each box represents the interquartile range of values, with the bold line showing the median value. The vertical lines show maximum and minimum value that fall within 1.5 box lengths, the open circles show extreme values >1.5 box plot lengths. PADI4, peptidyl arginine deiminase type IV ACPA, anti-citrullinated peptide antibody.
doi:10.1371/journal.pone.0061045.g004

5-year disease duration was 54 years, 85.3% of the patients were female, 87.8% were ACPA positive and 90.3% were RF positive. Median SHS (hands) at 5-year disease duration was 18 (interquartile range 6–37) and yearly progression rate (SHS/disease duration) was 3.6 (Figure 1 and 2). The distribution of SHS (hands) was similar to those in recent clinical studies in which some patients had extreme progressive joint destruction compared to others.[29–31] Half of the patients had prior use of MTX (50.1%) for their treatment of RA in the first 5 years of the disease. The patients who had used biologic agents in the first 5-year disease duration were excluded from the study. Since ACPA measurements started only in the early 2000 s in Japan, data of ACPA in the first 5-years from the onset could not be collected in most patients in this study, and they were substituted by recent data.

SNPs and HLA-DRB1 Genotyping

The overall genotyping success rate was 98.1% and the genotype concordance rate was 100% as assessed by duplicate samples. After the application of quality control criteria for genotyping (remove samples that consistently fail for ≥20% [3/13] SNPs, SNP call rate >95% overall after removing samples that consistently fail), 857 of 865 samples and all polymorphisms passed for the analyses. The following HLA-DRB1 alleles were classified as belonging to SE: DRB1*0101, DRB1*0401, DRB1*0404, DRB1*0405, DRB1*0410, DRB1*1001, DRB1*1402 and DRB1*1406. Frequency of SE carrier was 70.4% (n = 605) and 130 patients were homozygous for SE (15.1%).

Risk Factors for Radiographic Joint Damage

The univariate analysis identified 6 covariates initially as potential candidates; ACPA positive, RF positive, female sex, younger age at onset, HLA-DRB1 SE and *PADI4* risk allele (Table 2). The stepwise multiple regression analysis revealed all tested candidates except RF as independent risks for radiographic joint destruction (Table 3 and Figure 3). Patients with higher number of risk factors had more joint damage (Figure 4). Patients with extremely high joint damage score (SHS [hands] at 5-year disease duration more than 100, n = 13) were all females and had either SE or *PADI4* risk allele.

In the power calculation with a sample size of 830 (the number of samples used in the stepwise multivariate analysis), a 22% change of SHS of the hands with and without a risk by power 0.69 and an 11% change by power 0.23 could be detected.

Discussion

To date, a lot of studies focused on disease severity of RA have been conducted using various endpoints: radiographic progression, disease activity, functional impairment, presence of extra-articular features, complication or death.[34–36] Since a major symptom of RA is the chronic synovitis of multiple joints, which leads to highly damaged joints, restriction of activities of daily living and deterioration of quality of life, SHS that represent radiographic damage in joints has been thought to be a reliable index to assess the disease severity.

One of the difficulties in a study using joint damage score to evaluate RA severity is that the radiographic change is highly influenced by the disease duration. The patients with longer disease duration tend to have more accumulated damage; furthermore, rates of progression in joint damage are nonlinear, it is significantly faster in the early stage than the late phase of the disease. [37] Though the problem can be solved by using the radiographic joint damage score of the same disease duration, such data must be collected from a large number of patients. One of the strong points of this study was that we could obtain hundreds of SHS data from the same disease duration of 5 years, from a large RA cohort project, IORRA. As a result, we were able to perform powerful statistical analyses on joint destruction.

RA is caused by a combination of genetic and environmental factors, and to date, plenty of RA-susceptible polymorphisms have been identified, especially in the era of GWAS. However, genetic factors associated with joint destruction in RA patients have not been extensively studied. Although we had tested the association between joint destruction and some susceptible polymorphisms, no significant association was found thus far. [26,38,39] One of the reasons for the negative association may be due to the small sample size. By utilizing a larger size of DNA samples, we could find that HLA-DRB1 SE and *PADI4* risk allele were genetic risk factors for joint destruction in RA patients.

Hence, the genetic background of disease severity of RA is not yet fully known, although one thing may be for sure; there is little doubt that HLA-DRB1 SE, the strongest genetic factor to RA susceptibility, has impact on the disease severity, as was confirmed in this study.[19–21,40] HLA-DRB1 SE may play a central role for genetic component of RA, and the association between HLA-DRB1 SE and RA susceptibility or severity has been repeatedly reported across the different ethnic populations.

However, RA susceptible genes outside the HLA region have not been fully replicable across racial or ethnic groups. A representative example is *PADI4*, which was first reported in 2003 as RA susceptible gene in a Japanese population. [41] Since then, several reports using Caucasian samples showed negative association between RA susceptibility and *PADI4* polymorphisms, while the association was repeatedly confirmed in Asian populations.[42–47] Currently, based on amassing of research evidence,

Table 2. Univariate linear regression analysis on putative risk factors for radiographic progression: non-genetic and genetic factors.

Putative risk/gene(s)	Polymorphism	alleles *	MAF	risk allele	n	ß	*P* value
ACPA (positive)					834	0.14	3.7×10^{-5}†
RF (positive)					857	0.12	0.00043†
Smoking status (ever)					848	−0.056	0.10
Gender (female)					857	0.11	0.0020†
Age of onset					857	−0.084	0.014†
HLA-DRB1	SE	+/−	0.428	SE	853	0.13	0.00012†
PADI4	rs2240340	G/A	0.442	A	856	0.082	0.016†
TNFAIP3	rs2230926	T/C	0.089	C	847	−0.027	0.43
CCR6	rs3093024	C/T	0.487	T	852	−0.011	0.74
B3GNT2	rs11900673	C/T	0.320	T	852	0.015	0.66
ANXA3	rs2867461	A/G	0.454	G	822	−0.020	0.56
CSF2	rs657075	G/A	0.391	A	832	0.019	0.59
CD83	rs12529514	T/C	0.163	C	843	−0.030	0.39
NFKBIE	rs2233434	T/C	0.239	C	828	0.028	0.42
ARID5B	rs10821944	T/G	0.398	G	842	−0.032	0.35
PDE2A-ARAP1	rs3781913	A/C	0.278	A	848	0.062	0.073
PLD4	rs2841277	T/C	0.287	T	853	−0.013	0.70
PTPN2	rs2847297	A/G	0.360	G	854	−0.032	0.36

*Alleles shown as major allele/minor allele.
†P<0.05.
ACPA, anti-citrullinated peptide antibody; RF, rheumatoid factor. MAF; Minor allele frequency in the tested population, SE, shared epitope; *PADI4*, peptidyl arginine deiminase type IV; *TNFAIP3*, tumor necrosis factor, alpha-induced protein 3; *CCR6*, C-C chemokine receptor type 6; *B3GNT2*, UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2; *ANXA3*, annexin A3; *CSF2*, colony stimulating factor 2; *CD83*, CD83 molecule; *NFKBIE*, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon; *ARID5B*, AT rich interactive domain 5B [MRF1-like]; *PDE2A*, phosphodiesterase 2A, cGMP-stimulated; *ARAP1*, ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1; *PLD4*, phospholipase D family, member 4; *PTPN2*, protein tyrosine phosphatase, non-receptor type 2.
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PADI4 is considered as RA susceptibility gene even in Caucasian populations though its impact on disease susceptibility is lower than in Asian populations. [16].

It is interesting that *PADI4* risk allele had impact on joint damage independent of ACPA status, which is the most significant finding of this study. *PADI4* gene encodes one of PADI enzymes that catalyse the post-translational modification reaction generating citrulline residues from arginine, [41] and the serum titer of antibodies against citrullinated peptides, ACPA, which is an established prognostic marker for joint destruction in RA patients, is significantly correlated to *PADI4* risk alleles.[48–50] Thus, to date, the relationship between *PADI4* gene and disease severity of RA have been reported mainly in the context of association of *PADI4* haplotypes (or alleles) with serum titer (or positivity) of ACPA.[41,48–50] Recently, Bang et al. [51] indicated that *PADI4* gene contributed to the development of RA, regardless of ACPA status. Combined with our results, the *PADI4* gene is likely to play an additional role in the development and disease progression of RA along with its role in ACPA formation. Subsequent studies should elucidate the unidentified role of *PADI4* in the pathogenesis of RA.

Table 3. Stepwise multiple regression analysis on risk factors for radiographic progression (n = 830).

Risk factors	ß	95% CI for ß	*P* value
ACPA (positive)	0.12	0.05–0.17	0.00056
Gender (female)	0.09	0.03–0.16	0.0059
Age of onset	−0.07	−0.14– −0.01	0.024
HLA-DRB1 SE	0.11	0.04–0.17	0.0021
PADI4 risk allele	0.07	0.004–0.14	0.037

Multiple R squared value = 0.055.
95% CI, 95% confidence interval; ACPA, anti-citrullinated peptide antibody; SE, shared epitope; *PADI4*, peptidyl arginine deiminase type IV.
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Numerous clinical studies have indicated that severe, tight control with aggressive treatment in RA patients with remission as a target would help to lower the risk of progression of joint damage, which is especially critical in patients with uncontrollable risk factors. Although prediction of progressive joint damage in RA patients is still far from perfect, the use of identified risk factors (HLA-DRB1-SE positive, *PADI4* risk allele positive, ACPA positive, younger age of onset and female sex) should make it easier for rheumatologists to make their treatment decisions in the future.

Our cohort study has strong points, but also still has some limitations. Since the study was a retrospective cohort study, we were able to collect radiographic data from only 865 of 2,068 patients with DNA sample. Loss of patients could affect the results, although the baseline characteristics of the patients with radiographic data were similar to the whole DNA cohort of IORRA. As a result of the limited sample size, the study was underpowered to detect minor effect on joint destruction. Though we used the data of SHS (hands) at the same disease duration, because radiographs at baseline (onset of the disease) were not available in most

patients, they are only approximate values of delta-SHS in the first 5 years of the disease.

Conclusions

In conclusion, we have identified HLA-DRB1 SE and *PADI4* risk alleles as independent risk factors for progressive joint destruction in the first five years from onset of RA, as well as several non-genetic factors; ACPA positive, younger age of onset and female sex. Results of this study may help patients with these risk factors receive early aggressive intervention to change their natural disease course of RA.

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Author Contributions

Conceived and designed the experiments: KI. Performed the experiments: TS KI EI. Analyzed the data: TS KI KY EI. Contributed reagents/materials/analysis tools: KI KY YT AT HY SM. Wrote the paper: TS KI.

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Neutrophils Are Essential as a Source of IL-17 in the Effector Phase of Arthritis

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Abstract

Objective: Th17 has been shown to have a pivotal role in the development of arthritis. However, the role of IL-17 in the T cell-independent effector phase has not fully been examined. We investigated whether IL-17 is involved in the effector phase of arthritis by using K/BxN serum-induced arthritis model.

Methods: K/BxN serum was transferred into IL-17 knockout (KO) mice, SCID mice and their control mice, and arthritis was evaluated over time. In order to clarify the source of IL-17 in the effector phase, neutrophils or CD4+ T cells collected from IL-17 KO or control mice were injected into IL-17 KO recipient mice together with K/BxN serum. To examine if neutrophils secrete IL-17 upon stimulation, neutrophils were stimulated with immune complex in vitro and IL-17 in the supernatant was measured by ELISA.

Results: K/BxN serum-induced arthritis was much less severe in IL-17 KO mice than in WT mice. Since K/BxN serum-transferred SCID mice developed severe arthritis with high serum IL-17 concentration, we speculated neutrophils are the responsible player as an IL-17 source. When wild type (WT) but not IL-17 KO neutrophils were co-injected with K/BxN serum into IL-17 KO mice, arthritis was exacerbated, whereas co-injection of WT CD4+ T cells had no effect. In vitro, stimulation of neutrophils with immune complexcaused IL-17 secretion.

Conclusions: Neutrophils are essential as a source of IL-17 in the effector phase of arthritis. The trigger of secreting IL-17 from neutrophils may be immune complex.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovitis leading to destruction of articular cartilage and bone. The pathophysiology of RA is still unclear, but recently the important role of IL-17-producing T cells (Th17) has been highlighted in arthritis development in human and several mouse models. IL-17 (especially IL-17A) is a proinflammatory cytokine that is thought to contribute to the inflammation, cartilage destruction and bone erosion in RA. IL-17 is upregulated in the synovium and the synovial fluids of RA patients [1]. IL-17 induces fibroblasts, endothelial cells or macrophages to secrete IL-6, TNFα and IL-1 [2,3,4]. IL-17 can synergize with IL-1 and TNFα, although it may also have direct pathological effects. In experimental arthritis models, the critical role of IL-17 has been clearly demonstrated. Spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist was completely abrogated in the absence of IL-17 [5]. Collagen-induced arthritis [3] was also apparently suppressed in IL-17 knock out (KO) mice and was prevented by anti-IL-17 antibody treatment [6].

K/BxN mouse is a KRN T cell receptor transgenic mouse crossed with NOD mouse, and develops severe arthritis similar to RA [7]. KRN T cells recognize glycolytic enzyme glucose-6-phosphatase isomerase (GPI), and the autoantibodies against GPI cause arthritis. Transfer of K/BxN arthritic serum or purified anti-GPI antibodies into normal mice induces arthritis similar to K/BxN mice [8]. In the K/BxN model, T cells and B cells are required for inducing arthritis, but once the anti-GPI antibody is generated, arthritis can develop without lymphocytes [9]. K/BxN serum-transfer arthritis is thus a useful model to analyze the effector phase of arthritis. The innate immune system including neutrophils [10], mast cells [11], Fcγ receptor and C5a [12] has been shown to be essential in this arthritis development. Although IL-1 and TNFα, but not IL-6, were shown to be important in this arthritis model [13], it has not been clear whether IL-17 works in the effector phase of arthritis, regardless of the findings that non-T cells produce IL-17. In the present study, we tested whether IL-17

is involved in K/BxN serum-induced arthritis using IL-17 KO mice and we found that IL-17 derived from neutrophils affects arthritis severity in the effector phase.

Materials and Methods

Mice

C57BL/6JJcl (B6), FOX CHASE SCID C.B-17/Icr-scid/scidJcl (SCID), FOX CHASE SCID C.B-17/Icr-+/+Jcl (SCID WT), NOD/SciJcl (NOD) mice were purchased from Japan Clea Inc. (Tokyo, Japan). KRN TCR transgenic (B6 background: K/B) and Cα (TCRα chain) KO mice were kindly provided by Drs. D. Mathis and C. Benoist, Harvard Medical School, Boston, MA. Fcγ receptor KO (FcR KO) mice were kindly provided by Dr. T. Takai, Tohoku University, Sendai. All mice were maintained in our animal facility under specific pathogen–free conditions. K/BxN arthritic mice were obtained by crossing K/B with NOD mice, and the sera were pooled at eight weeks old. Arthritic adult K/BxN mice were bled, and the sera collected from eight-week-old K/BxN mice were pooled. All animal procedures were approved by the Ethics Committee of Kyoto University.

Induction of K/BxN Serum-induced Arthritis and Arthritis Scoring

Recipient mice were usually i.p. injected with 200 μl of K/BxN sera at days 0 and 2. In the experiments of neutrophil or $CD4^+$ T cell transfer, recipient mice were i.v. injected with 200 μl of K/BxN sera at days 0 and 2. Arthritis was evaluated visually, and the swelling of each paw was scored on a scale of 0–3, where 0 = no evidence of inflammation, 1 = subtle inflammation or localized edema, 2 = easily identified swelling localized to either the dorsal or ventral surface of the paw, and 3 = swelling of all aspects of the paw. Clinical indices for all four paws were added as a composite score. Ankle thickness was measured with a caliper.

Histological Examination

Dissected ankles were fixed in 4% neutral buffered paraformaldehyde, demineralized and stained with hematoxylin and eosin (H&E).

Isolation of Murine Neutrophils from Bone Marrow (BM) and $CD4^+$ T Cells from Spleen

BM neutrophils were isolated using a mouse Anti-Ly-6G MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of neutrophils was >95% as determined by May-Giemsa staining. Splenic $CD4^+$ T cells were isolated using a mouse Pan T Cell Isolation Kit II (Miltenyi Biotec).

Neutrophil Stimulation in Vitro

Soluble murine peroxidase-anti-peroxidase (mPAP) ICs consisting primarily of two horseradish peroxidase (HRP) molecules bound to three anti-peroxidase IgGs were obtained from Jackson ImmunoResearch Laboratories (Bar Harbor, HE, USA). Immunomagnetically purified neutrophils from mouse BM were incubated at a concentration of 8×10^6/ml in RPMI 1640 medium containing 10%FCS, penicillin, or streptomycin for 3 hours, followed by culture with mPAP IC (200, 20, and 2 μg/ml), HRP (Sigma Aldrich, Saint Luis, MO, USA: 200 μg/ml), anti-HRP antibody (Thermo Fisher Scientific, Waltham, MA, USA: 200 μg/ml) or PBS for 1 hour before collecting the culture supernatant.

IL-17 Measurements by ELISA

Mouse IL-17 Quantikine EILSA kit was purchased from R&D systems (Minneapolis, MN, USA). Cell culture supernatant and sera from arthritic mice were assayed in accordance with the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using the Student's *t*-test. Data are expressed as means ± SEM unless otherwise stated.

Results

IL-17 Exacerbates K/BxN Serum-induced Arthritis

To clarify whether IL-17 has any roles in the effector phase of arthritis, we induced K/BxN serum-induced arthritis in IL-17 KO and WT B6 mice. We assessed the clinical index and ankle thickness over time. Interestingly, the severity of arthritis in IL-17 KO mice was much milder than that in WT mice (Fig. 1). Histological examination of ankle joints also revealed that the inflammation, bone and cartilage destruction of serum induced arthritis were much less in IL-17 KO mice than in WT mice (Fig. 2). These results clearly indicate that IL-17 has pivotal roles in aggravating arthritis in the effector phase.

CD4+T Cells are not the Source of IL-17 in Arthritis Effector Phase

We hypothesized that T cells were not the source of IL-17 in arthritis effector phase since it was reported that T cells are not essential in this phase. To confirm this, we injected K/BxN arthritic sera or BxN control sera into SCID and control mice (C.B-17/Icr-+/+). We assessed their clinical index and ankle thickness over time, and measured their serum concentration of IL-17 at days 7 and 21. The arthritis induced in SCID mice was as severe as that in control mice (Fig. 3A), and the concentrations of serum IL-17 in the SCID mice were similar to those in the control mice (Fig. 3B). In contrast, in both SCID and control mice, the concentrations of serum IL-17 in BxN serum-injected non-arthritic mice were significantly lower than those in K/BxN serum-induced arthritic mice (Fig. 3B). Since the concentration of IL-17 in the injected K/BxN serum was ~50 pg/ml (data not shown) and that of K/BxN serum-induced SCID mice was ~140 pg/ml (Fig. 3B), it was evident that the serum IL-17 in the K/BxN serum-induced SCID mice was not derived from K/BxN serum itself but from non-T non-B inflammatory cells.

Neutrophils Exacerbate Arthritis via IL-17 in the Effector Phase

We were curious what types of cells secrete IL-17 in the effector phase of arthritis. We focused on neutrophils, because it was reported that they could secrete IL-17 [14] and that they are essential for the development of K/BxN serum transfer arthritis [10]. We injected neutrophils (2×10^6/body) collected from BM of IL-17 KO or WT mice into IL-17 KO mice together with K/BxN serum at days 0 and 2, and the disease parameters were followed over time. As we expected, arthritis became significantly more severe when IL-17 sufficient neutrophils were injected compared with when IL-17 KO neutrophils were injected (Fig. 4A). The results were consistent in the three independent experiments. In order to exclude IL-17 producing-T cells are contaminated, we injected neutrophils (2×10^6/body) collected from BM of IL-17 KO or αβT cell deficient (Cα KO) mice into IL-17 KO mice together with K/BxN serum at days 0 and 2. As shown in Fig. 4B, Cα KO neutrophils (which can produce IL-17) exacerbated

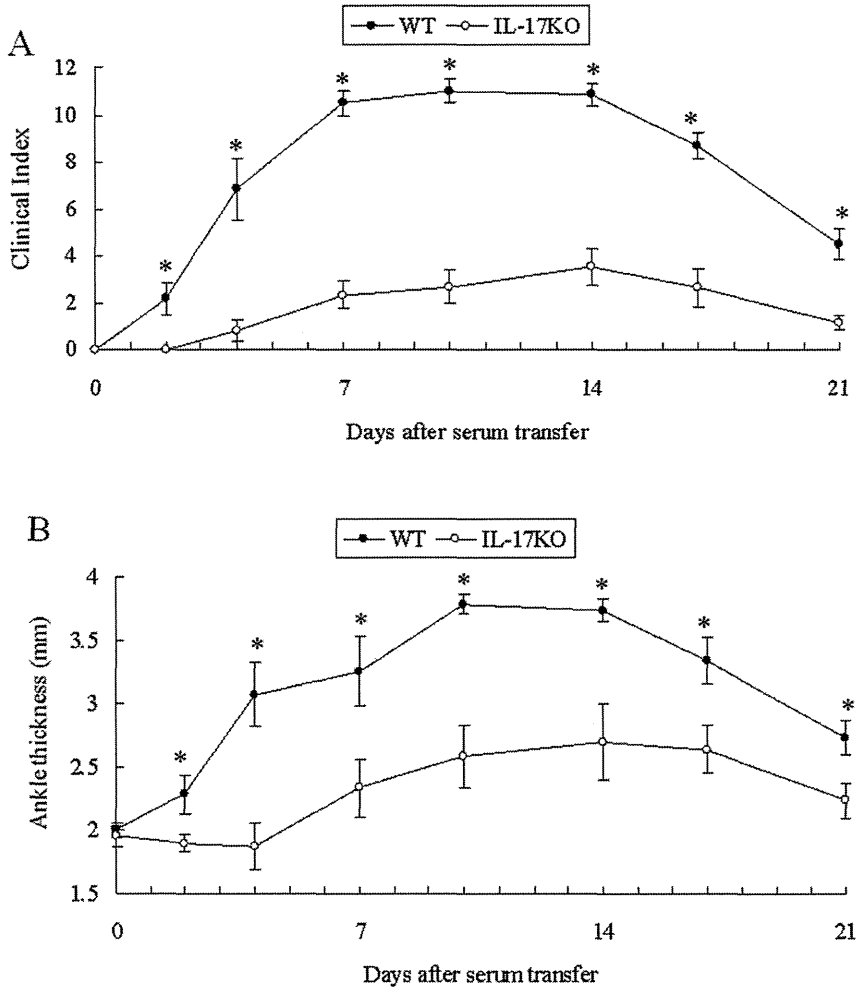

Figure 1. K/BxN serum-induced arthritis in IL-17 knockout mice. K/BxN sera (200 μl/body) were i.p. injected at days 0 and 2 into IL-17 KO (○) and WT mice (●) (n = 6 each). Clinical index (A) and ankle thickness (B) were monitored for 21 days. The results were from two independent experiments, both of which showed similar results. *$p<0.05$.
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arthritis of recipient mice more severely than the IL-17 KO neutrophils.

We next collected splenic CD4+ T cells from IL-17KO or WT mice and injected them (1×10^7/body) into IL-17 KO mice at day0, while K/BxN serum (200 μl/body) was injected at days 0 and 2. As shown in Fig. 5, there were no statistical differences in arthritis severity between the recipients of IL-17KO and WT CD4+ T cells. All these results clearly indicate that neutrophils but not $CD4^+$ T cells are the major source of IL-17 in the effector phase and affect the severity of arthritis. The results were consistent in the two independent experiments.

Neutrophils can Secrete IL-17

Wipke et al. showed that mouse peroxidase-anti-peroxidase (mPAP) immune complex (IC) activates neutrophils through Fcγ receptor to get autoantibodies into joints in this model [15]. We hypothesized that ICs trigger neutrophils to secrete IL-17. We collected neutrophils from BM of B6 mice and incubated them for 3 hours, followed by culture with mPAP-IC for additional 1 hour. We measured the IL-17 concentration in the culture supernatant by ELISA. IL-17 was detected in the supernatant of cultures in a dose-dependent manner of IC (Fig. S1).

To clarify whether ICs stimulate neutrophils through Fcγ receptor for IL-17 secretion, we used FcR KO neutrophils to stimulate with mPAP-IC. We found that they secrete significantly

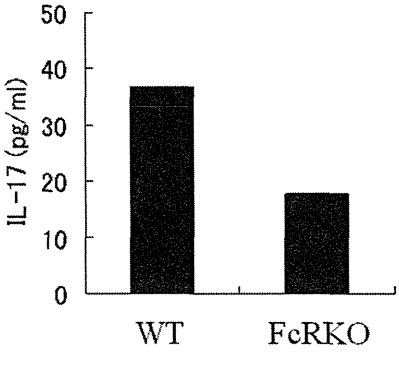

Figure 2. Histopathology of ankle joints from arthritic IL-17KO and WT mice. Arthritis was induced as described in the legend for figure 1 and the mice were sacrificed at days 7 and 21 after initial serum transfer. H&E stainings of ankle joints are shown (scale bar represents 100 μm).
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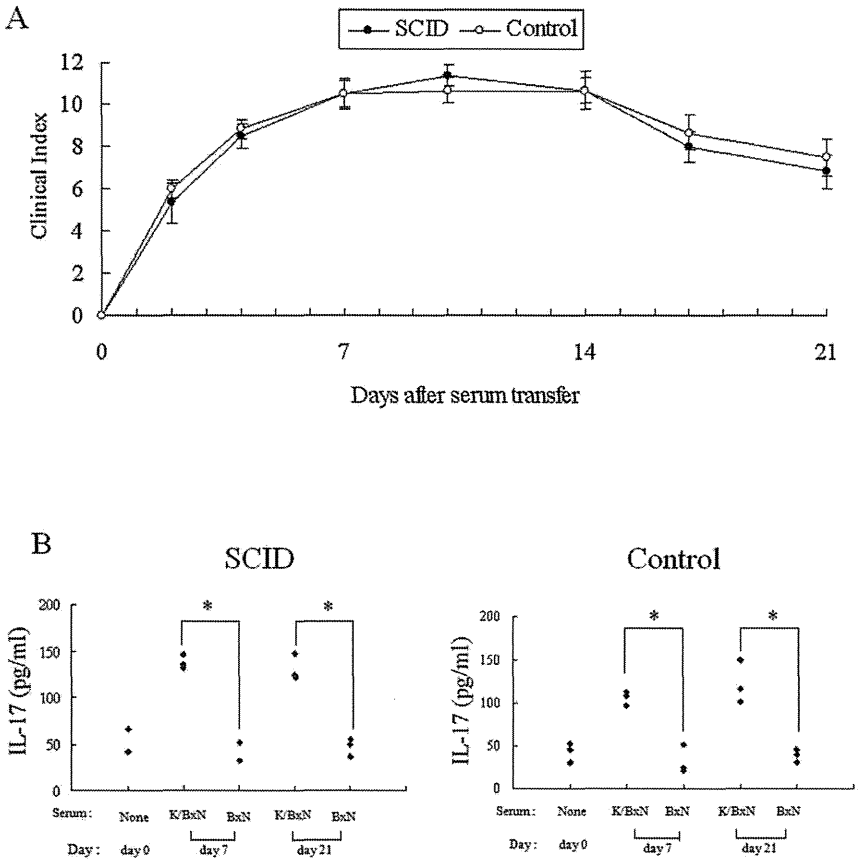

Figure 3. Arthritis severity and IL-17 in the sera of SCID or control mice which received K/BxN or BxN sera. K/BxN arthritic sera or BxN control sera were injected into SCID (C.B-17/Icr-scid/scid) and corresponding control mice (C.B-17/Icr-+/+) at days 0 and 2. (A) Clinical Index was monitored for 21 days. (B) Blood was sampled at days 0, 7 and 21 and IL-17 concentrations in the sera of each mouse were measured by EILSA. *$p<0.05$.
doi:10.1371/journal.pone.0062231.g003

lower amount of IL-17 than the WT neutrophils (Fig. S2). This result clearly shows that ICs stimulate neutrophils through FcR, although there may be other pathways as well.

Furthermore, we stimulated B6 neutrophils with HRP only, mouse anti-HRP antibody only, or PBS. IL-17 was detected in the supernatant of cultures stimulated by anti-HRP antibody and mPAP-IC, but not HRP or PBS (Fig. 6). On the other hand, IL-17 mRNA was not detected in any groups (data not shown). These results indicate that IL-17 is secreted from prestored pool in neutrophils by the stimulation of IC or anti-HRP antibodies themselves.

Discussion

In this report, we clearly showed that IL-17 operates as a proinflammatory cytokine in the effector phase of the K/BxN arthritis model. In this phase, neutrophils are the important source of IL-17 production.

It was rather surprising that IL-17 KO mice showed hyporesponsiveness to K/BxN serum transfer, since this means that IL-17 from cells other than $CD4^+$ T cells are important for arthritis development. Although the distinct $CD4^+$ helper T cell subset is famous for IL-17 producing cells (known as Th17), several innate immune cells have recently been reported to secrete IL-17 in inflammatory or autoimmune diseases. TCRγδ T cells secrete IL-17 in Mycobacterium Tuberculosis infection [16], in experimental antoimmune encephalomyelitis model mice [17], and in collagen-induced arthritis [18]. Invariant natural killer T cells also produce IL-17 [19]. Neutrophils are reported to secrete IL-17 in several mouse models of asthma [20], ANCA-associated vasculitis [21] and kidney ischemia-reperfusion injury [22]. Since IL-17 was detected in the sera of SCID mice which had been treated with K/BxN sera (Fig. 3), the source of IL-17 in the effector phase should not be TCRαβ, TCRγδ, or NKT cells. From the neutrophil transfer experiments (Fig. 4*A*), it was evident that neutrophils are the major source of IL-17 in this K/BxN serum-induced arthritis model. However, K/BxN serum-induced arthritis in IL-17KO mice which had been reconstituted with WT neutrophils was not as severe as that in the WT mice (Figs. 1A & 4A). Even when we injected 5 times more number (1×10^7/body) of WT neutrophils, the arthritis severity did not increase (data not shown). These results suggest that some innate immune cells other than neutrophils are also sources of IL-17 in K/BxN serum transfer arthritis. Mast cells are a possible source of IL-17 [23].

Jacobs et al. reported that IL-17-producing KRN T cells amplified the inflammatory process in the K/BxN serum-induced arthritis model [24]. In contrast, T cells did not augment the arthritis in our study (Fig. 5). The differences between these results are that Jacobs et al. transferred KRN T cells into recipients in which APC express $I\text{-}A^{g7}$ and lack B cells, whereas we transferred normal B6 T cells into IL-17 KO mice. Therefore, their T cells were activated but ours were not. If activated T cells are present, such T cells may augment the effector phase of arthritis. Jacobs

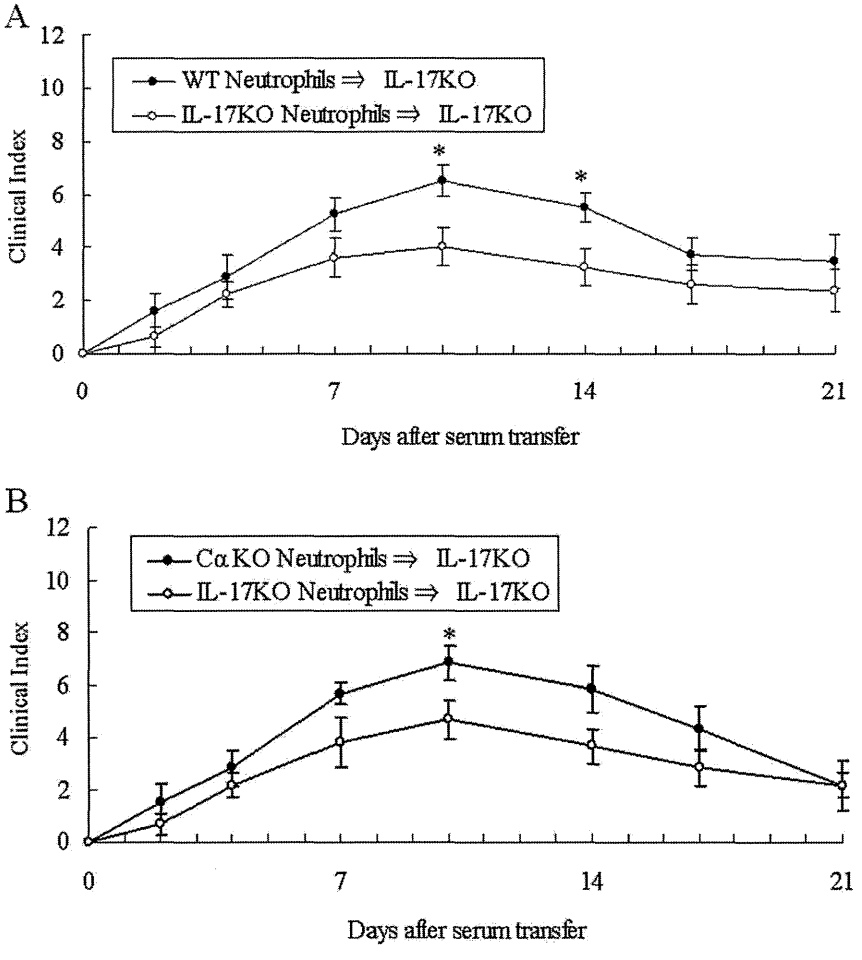

Figure 4. IL-17 from neutrophils aggravates K/BxN serum-induced arthritis. (A) Neutrophils (2×10^6/body) collected from bone marrow of IL-17 KO (○) or WT (●) mice were injected into IL-17 KO mice together with K/BxN sera (200 μl/body) at days 0 and 2. Clinical index of arthritis is shown. The results were from three independent experiments, both of which showed similar results. n = 8 mice in each group. (B) Neutrophils (2×10^6/body) collected from bone marrow of IL-17 KO (○) or Cα KO (●) mice were injected into IL-17 KO mice together with K/BxN sera (200 μl/body) at days 0 and 2. Clinical index of arthritis is shown. n = 6 mice in each group.*$p<0.05$.
doi:10.1371/journal.pone.0062231.g004

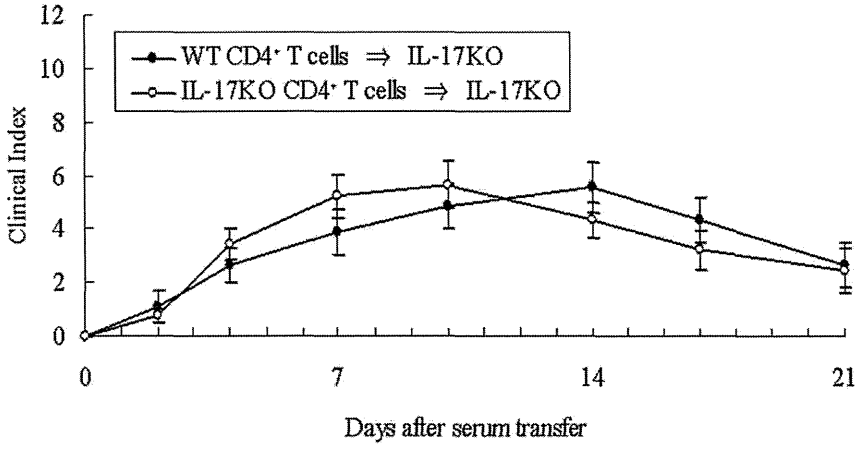

Figure 5. CD4+ T cells do not aggravate K/BxN serum-induced arthritis. $CD4^+$ T cells (1×10^7/body) collected from spleen of IL-17 KO (○) or WT (●) mice were injected into IL-17 KO mice at day 0. K/BxN sera (200 μl/body) were injected at days 0 and 2. Clinical index of arthritis is shown. The results were from two independent experiments, both of which showed similar results. n = 9 mice in each group.
doi:10.1371/journal.pone.0062231.g005

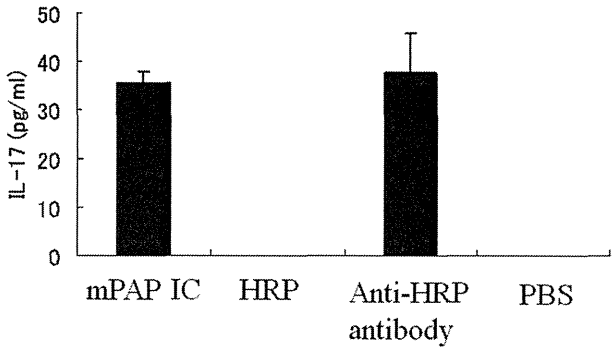

Figure 6. Neutrophils secrete IL-17 after stimulation in vitro. Neutrophils (8×10^6 cells/ml) collected from the bone marrow cells of B6 mice were cultured for three hours, followed by incubation with mPAP: murine peroxidase (HRP) -anti-peroxidase immune complex (IC), HRP only, anti-HRP antibody only or PBS for additional one hour. The supernatants were collected and the concentration of IL-17 was measured by ELISA. The results were reproducible in four independent experiments and the representative results are shown.
doi:10.1371/journal.pone.0062231.g006

et al. also showed that anti-IL-17 antibodies offer no protection against K/BxN serum-induced arthritis. Such results may be due to the shortage of antibody amount or incomplete blocking of IL-17 in the intimate interaction between effector cells.

Actually it was recently reported that the severity of arthritis in IL-17 receptor (IL-17R) deficient mice is milder than that of IL-17R WT mice using K/BxN serum-induced arthritis model [25]. Their results are consistent with our results, but they did not refer to the source of IL-17. Since the severity of K/BxN serum-induced arthritis in their IL-17R deficient mice is similar to that in our IL-17A KO mice, IL-17A may have the dominant role in the IL-17 family members for arthritis induction, although possible important role of IL-17F cannot be excluded.

The trigger causing neutrophils to secrete IL-17 is not yet clear. Wipke et al. demonstrated that the immune complex can stimulate neutrophils through FcR to increase local vascular permeability as the initiation of arthritis [15]. We found similar results, in that the same immune complex (HRP-anti-HRP antibodies) can trigger neutrophils to secrete IL-17 in vitro (Fig. 5). However, we found that not only HRP-anti-HRP antibody immune complex, but also anti-HRP antibody itself can stimulate neutrophils, which was not tested in Wipke's paper. There still remains a possibility that anti-HRP antibody may directly stimulate neutrophils. Another possibility is that anti-HRP Ab crossreacts with a certain molecule to form ICs that stimulate neutrophils. In order to test whether GPI-anti-GPI Ab IC can stimulate neutrophils, we cultured neutrophils in RPMI medium containing 10% K/BxN arthritic serum with or without GPI protein (200 μg/ml), but we could not detect IL-17 in the culture supernatant (data not shown). This implies that neither GPI-anti-GPI antibody immune complex nor soluble factors in K/BxN serum can trigger neutrophils to secrete IL-17. The stimulating factor of neutrophils to secrete IL-17 remains to be determined.

Pathological functions of IL-17 in human arthritides such as RA have not completely clarified, but IL-17 is thought to be working on accumulation of neutrophils in synovial space, activation of synovial cells and osteoclasts, which lead to joint inflammation, synovial cell proliferation, cartilage and bone destruction. In this paper, we focused on the effector phase of arthritis and did not analyze the initiation phase in which antigen recognition and T-B cell interaction occur. Since IL-17 is produced from various types of cells and works on multiple cell types, it is difficult to dissect the pathological mechanisms in arthritis. By dividing the phase of arthritis, we were able to find the major player of the arthritis effector phase, IL-17-producing neutrophils. However, we have to be careful when we apply our findings to human diseases, which sometimes behave differently from mouse model. Human studies are also needed to warrant our mouse results.

In summary, this is the first report to clearly show that IL-17 is critical in the effector phase of arthritis and that neutrophils are the major source of IL-17, at least in the effector phase. These results demonstrate a new pathogenic role of neutrophils in the arthritis development.

Supporting Information

Figure S1 Immune complex stimulate neutrophils to secrete IL-17 in a dose depend manner. We collected neutrophils from the bone marrow cells of B6 mice and cultured them (8×10^6 cells/well) for 3 hours, followed by incubation with 200, 20 and 2 μg/ml of mPAP: murine peroxidase (HRP) -anti-peroxidase immune complex (IC) for additional 1 hour. The supernatants were collected and the concentration of IL-17 was measured by ELISA.
(JPG)

Figure S2 Immune complex stimulate neutrophils through Fcγ receptor. We collected neutrophils from the bone marrow cells of Fcγ receptor (FcR) knockout (KO) mice or wild type (WT) B6 mice and cultured them (8×10^6 cells/well) for 3 hours, followed by incubation with 200 μg/ml of mPAP-IC for additional 1 hour. The supernatants were collected and the concentration of IL-17 was measured by ELISA.
(JPG)

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Author Contributions

Conceived and designed the experiments: MK KO NY. Performed the experiments: MK KO. Analyzed the data: MK KO NY CT MH HY DK TF TM. Contributed reagents/materials/analysis tools: MK KO CT YI. Wrote the paper: MK KO.

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Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population

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Rheumatoid arthritis is a common autoimmune disease characterized by chronic inflammation. We report a meta-analysis of genome-wide association studies (GWAS) in a Japanese population including 4,074 individuals with rheumatoid arthritis (cases) and 16,891 controls, followed by a replication in 5,277 rheumatoid arthritis cases and 21,684 controls. Our study identified nine loci newly associated with rheumatoid arthritis at a threshold of $P < 5.0 \times 10^{-8}$, including *B3GNT2*, *ANXA3*, *CSF2*, *CD83*, *NFKBIE*, *ARID5B*, *PDE2A-ARAP1*, *PLD4* and *PTPN2*. *ANXA3* was also associated with susceptibility to systemic lupus erythematosus ($P = 0.0040$), and *B3GNT2* and *ARID5B* were associated with Graves' disease ($P = 3.5 \times 10^{-4}$ and 2.9×10^{-4}, respectively). We conducted a multi-ancestry comparative analysis with a previous meta-analysis in individuals of European descent (5,539 rheumatoid arthritis cases and 20,169 controls). This provided evidence of shared genetic risks of rheumatoid arthritis between the populations.

Rheumatoid arthritis is a complex autoimmune disease characterized by inflammation and the destruction of synovial joints and affects up to 1% of the population worldwide. To date, more than 35 rheumatoid arthritis susceptibility loci, including *HLA-DRB1*, *PTPN22*, *PADI4*, *STAT4*, *TNFAIP3* and *CCR6*, among others, have been identified by GWAS in multiple populations[1–12] and by several meta-analyses of the original GWAS[13–16]. In particular, each meta-analysis of these GWAS uncovered a number of loci that were not identified in the single GWAS, leading to recognition of the enormous power of the meta-analysis approach for detecting causal genes in disease. However, these previous meta-analyses have been performed solely in European populations[13–16] and not in Asian ones. As multi-ancestry studies on validated rheumatoid arthritis susceptibility loci showed the existence of both population-specific and shared genetic components of rheumatoid arthritis[10,17], additional studies in Asian populations might provide useful insight into the underlying genetic architecture of rheumatoid arthritis, which would otherwise be difficult to capture using the studies in a single population. Here, we report a meta-analysis of GWAS and a replication study for rheumatoid arthritis in a Japanese population that was conducted by the Genetics and Allied research in Rheumatic diseases NETworking (GARNET) consortium[10,12]. We subsequently performed a multi-ancestry comparative analysis that incorporated results from a previously conducted meta-analysis of individuals of European ancestry[15].

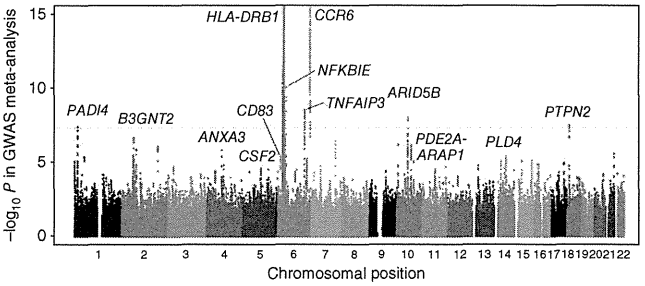

Figure 1 Manhattan plots of the GWAS meta-analysis for rheumatoid arthritis in the Japanese population. The genetic loci that satisfied the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$ (gray line) in the meta-analysis or in the combined study of the meta-analysis and the replication study are presented. The *y* axis shows the $-\log_{10} P$ values of the SNPs in the meta-analysis. The SNPs for which the *P* values were smaller than 1.0×10^{-15} are indicated at the upper limit of the plot.

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Table 1 Results of the GWAS meta-analysis and the replication studies for rheumatoid arthritis

					Associations in Japanese								Associations in Europeans[c]			
					GWAS meta-analysis			Replication study		Combined study			GWAS meta-analysis			
						Allele 1 freq.							Allele 1 Freq.			
rsID[a]	Chr.	Position (bp)	Cytoband	Gene(s)	Allele 1/2	RA	Control	OR (95% CI)[b]	*P*	OR (95% CI)[b]	*P*	OR (95% CI)[b]	*P*	RA	Control	OR (95% CI)[b]	*P*
SNPs with significant associations ($P < 5.0 \times 10^{-8}$ in the combined study)																	
rs11900673	2	62306165	2p15	*B3GNT2*	T/C	0.31	0.28	1.15 (1.08–1.21)	3.5×10^{-6}	1.09 (1.04–1.14)	6.0×10^{-4}	1.11 (1.07–1.15)	1.1×10^{-8}	0.13	0.13	1.05 (0.98–1.13)	0.17
rs2867461	4	79732239	4q21	*ANXA3*	A/G	0.46	0.44	1.13 (1.08–1.19)	4.7×10^{-6}	1.12 (1.08–1.17)	1.2×10^{-7}	1.13 (1.09–1.17)	1.2×10^{-12}	0.37	0.37	0.98 (0.92–1.04)	0.52
rs657075	5	131458017	5q31	*CSF2*	A/G	0.38	0.36	1.12 (1.06–1.18)	3.2×10^{-5}	1.11 (1.06–1.16)	3.8×10^{-6}	1.12 (1.08–1.15)	2.8×10^{-10}	0.10	0.10	1.04 (0.95–1.13)	0.37
rs12529514	6	14204637	6p23	*CD83*	C/T	0.16	0.14	1.19 (1.10–1.27)	6.8×10^{-6}	1.11 (1.05–1.18)	6.0×10^{-4}	1.14 (1.09–1.19)	2.0×10^{-8}	0.055	0.053	1.11 (0.99–1.24)	0.074
rs2233434	6	44340898	6p21.1	*NFKBIE*	G/A	0.24	0.21	1.23 (1.16–1.31)	9.2×10^{-11}	1.17 (1.11–1.23)	2.2×10^{-9}	1.19 (1.15–1.24)	5.8×10^{-19}	0.059	0.040	1.57 (1.11–2.21)	0.0099
rs10821944	10	63455095	10q21	*ARID5B*	G/T	0.39	0.36	1.17 (1.11–1.23)	1.0×10^{-8}	1.15 (1.10–1.20)	3.0×10^{-10}	1.16 (1.12–1.20)	5.5×10^{-18}	0.29	0.26	1.11 (1.05–1.17)	1.9×10^{-4}
rs3781913	11	72051144	11q13	*PDE2A-ARAP1*	T/G	0.71	0.69	1.11 (1.05–1.17)	3.2×10^{-4}	1.13 (1.08–1.18)	6.7×10^{-7}	1.12 (1.08–1.16)	5.8×10^{-10}	0.45	0.43	1.04 (0.99–1.09)	0.13
rs2841277	14	104462050	14q32	*PLD4*	T/C	0.72	0.69	1.11 (1.05–1.18)	2.8×10^{-4}	1.18 (1.13–1.24)	7.0×10^{-12}	1.15 (1.11–1.19)	1.9×10^{-14}	0.47	0.46	1.02 (0.96–1.09)	0.54
rs2847297	18	12787694	18p11	*PTPN2*	G/A	0.37	0.33	1.16 (1.11–1.23)	3.5×10^{-8}	1.06 (1.01–1.11)	0.013	1.10 (1.07–1.14)	2.2×10^{-8}	0.36	0.34	1.10 (1.05–1.15)	9.2×10^{-5}
SNPs with suggestive associations ($5.0 \times 10^{-8} \leq P < 5.0 \times 10^{-6}$ in the combined study)																	
rs4937362	11	127997949	11q24	*ETS1-FLI1*	T/C	0.71	0.68	1.13 (1.07–1.19)	2.0×10^{-5}	1.07 (1.02–1.12)	0.0061	1.09 (1.06–1.13)	7.5×10^{-7}	0.46	0.44	1.06 (1.01–1.11)	0.015
rs3783637	14	54417868	14q22	*GCH1*	C/T	0.76	0.74	1.13 (1.07–1.20)	6.5×10^{-5}	1.07 (1.02–1.13)	0.0062	1.10 (1.06–1.14)	2.0×10^{-6}	0.88	0.88	0.99 (0.88–1.11)	0.87
rs1957895	14	60978085	14q23	*PRKCH*	G/T	0.40	0.39	1.12 (1.06–1.18)	4.1×10^{-5}	1.07 (1.02–1.12)	0.0022	1.09 (1.05–1.13)	3.6×10^{-7}	0.093	0.089	1.01 (0.95–1.07)	0.73
rs6496667	15	88694672	15q26	*ZNF774*	A/C	0.38	0.35	1.13 (1.07–1.19)	4.7×10^{-5}	1.07 (1.02–1.11)	0.0050	1.09 (1.05–1.13)	1.4×10^{-6}	0.21	0.20	1.07 (1.01–1.13)	0.031
rs7404928	16	23796341	16p12	*PRKCB1*	T/C	0.65	0.62	1.13 (1.07–1.19)	1.5×10^{-5}	1.05 (1.01–1.10)	0.026	1.08 (1.05–1.12)	4.0×10^{-6}	0.75	0.75	1.01 (0.94–1.09)	0.79
rs2280381	16	84576134	16q24	*IRF8*	T/C	0.86	0.84	1.16 (1.08–1.25)	1.0×10^{-4}	1.09 (1.03–1.15)	0.0049	1.12 (1.07–1.17)	2.4×10^{-6}	0.62	0.60	1.05 (0.99–1.11)	0.081
SNPs in previously reported rheumatoid arthritis susceptibility loci ($P < 5.0 \times 10^{-8}$ in the GWAS)																	
rs766449	1	17547439	1p36	*PADI4*	T/C	0.44	0.40	1.17 (1.11–1.24)	4.6×10^{-8}	–	–	–	–	0.38	0.37	1.09 (1.03–1.05)	0.0022
rs2157337	6	32609122	6p21.3	*HLA-DRB1*	C/T	0.59	0.44	1.99 (1.88–2.11)	2.6×10^{-118}	–	–	–	–	0.69	0.46	2.50 (2.39–2.62)	$< 1.0 \times 10^{-300}$
rs6932056	6	138284130	6q23	*TNFAIP3*	C/T	0.092	0.073	1.35 (1.23–1.49)	3.2×10^{-9}	–	–	–	–	0.044	0.034	1.41 (1.24–1.60)	1.3×10^{-7}
rs1571878	6	167460832	6q27	*CCR6*	C/T	0.54	0.48	1.31 (1.24–1.39)	3.2×10^{-19}	–	–	–	–	0.47	0.43	1.13 (1.08–1.19)	5.9×10^{-7}

Chr., chromosome; Freq., frequency; RA, rheumatoid arthritis; OR, odds ratio; CI, confidence interval.
[a]SNPs with $P < 5.0 \times 10^{-6}$ in the combined study of the GWAS meta-analysis and the replication study or SNPs with $P < 5.0 \times 10^{-8}$ in the GWAS meta-analysis are annotated according to forward strand and NCBI Build 36.3. Full results of the replication study are provided in **Supplementary Table 3**. [b]Odds ratio of allele 1. [c]Associations in the previous meta-analysis in European populations[15].

The meta-analysis included 4,074 rheumatoid arthritis cases (with 81.4% and 80.4% of the subjects being positive for antibody to cyclic citrullinated peptide (anti-CCP) and rheumatoid factor, respectively) and 16,891 controls from three GWAS of Japanese subjects (from the BioBank Japan Project[10,18], Kyoto University[12] and the Institute of Rheumatology Rheumatoid Arthritis (IORRA)[19]; **Supplementary Table 1**). After the application of stringent quality control criteria, including principal-component analysis (PCA; **Supplementary Fig. 1**) for each GWAS, the meta-analysis was conducted by evaluating ~2.0 million autosomal SNPs with minor allele frequencies (MAFs) ≥0.01, which were obtained through whole-genome imputation of genotypes on the basis of the HapMap Phase 2 East Asian panels (Japanese in Tokyo (JPT) and Han Chinese in Beijing (CHB)). The inflation factor of the test statistics in the meta-analysis λ_{GC} was as low as 1.036, suggesting no substantial effects of population structure (**Supplementary Table 2**). The quantile-quantile plot of *P* values showed a marked discrepancy in the values in its tail from those anticipated under the null hypothesis that there is no association—even after removal of the SNPs located in the human leukocyte antigen (HLA) region, the major rheumatoid arthritis susceptibility locus—thereby showing the presence of significant associations in the meta-analysis (**Supplementary Fig. 2**).

We identified seven loci in the current meta-analysis that satisfied the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$. These included previously known rheumatoid arthritis susceptibility loci, such as *PADI4* at 1p36, *HLA-DRB1* at 6p21.3, *TNFAIP3* at 6q23 and *CCR6* at 6q27 (refs. 1,3,6,10,15) (the smallest $P = 2.6 \times 10^{-118}$ was found at the *HLA-DRB1* locus; **Fig. 1** and **Table 1**). To our knowledge, the other three loci identified, *NFKBIE* at 6p21.1, *ARID5B* at 10q21 and *PTPN2* at 18p11, are newly associated ($P = 9.2 \times 10^{-11}$, 1.0×10^{-8} and 3.5×10^{-8}, respectively).

To validate the associations identified in the meta-analysis, we conducted a replication study of two independent Japanese rheumatoid arthritis case-control cohorts (cohort 1: 3,830 rheumatoid arthritis cases and 17,920 controls, cohort 2: 1,447 rheumatoid arthritis cases and 3,764 controls; **Supplementary Table 1**). To increase the number of subjects and enhance statistical power, genotype data obtained from other GWAS projects conducted for non-autoimmune diseases in Japanese using Illumina platforms were used for the replication control panels. For each of the 46 loci that exhibited $P < 5.0 \times 10^{-4}$ in

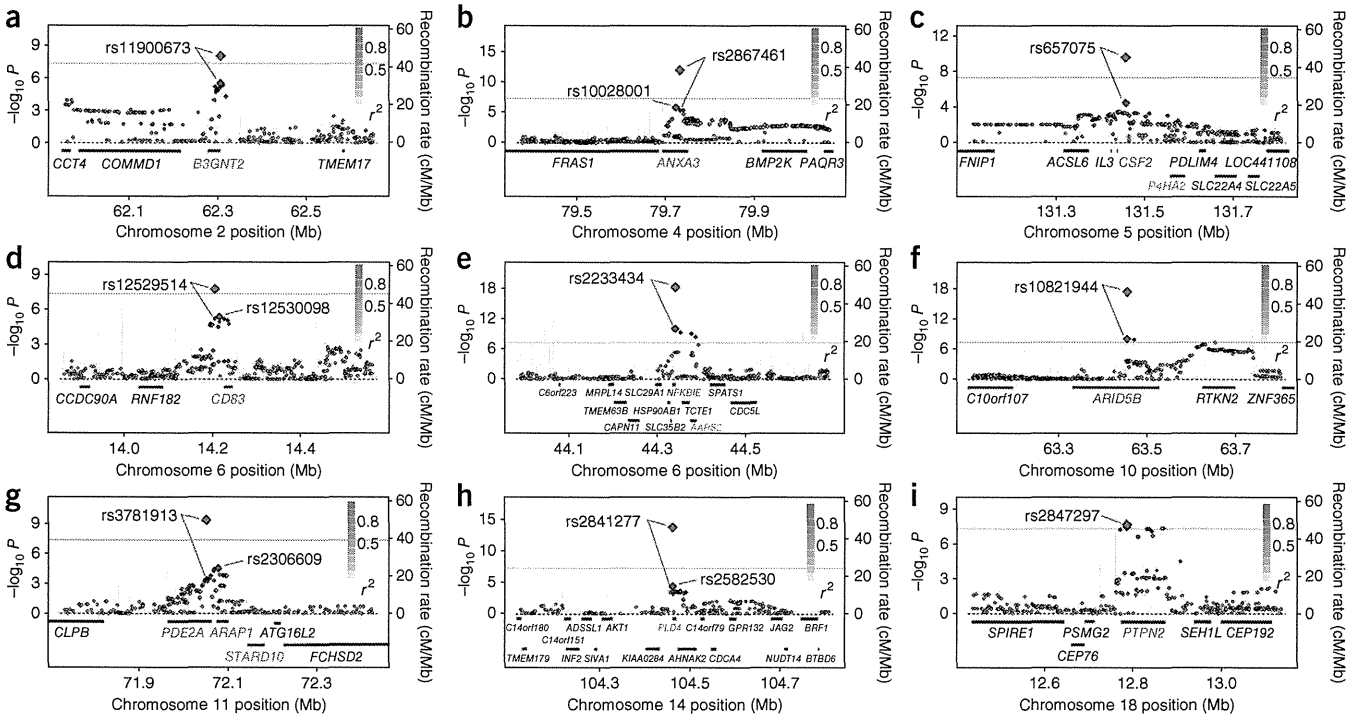

Figure 2 Regional plots of the loci newly associated with rheumatoid arthritis at the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$ in the combined study of the meta-analysis and the replication study. (**a**–**i**) Regional plots are shown at *B3GNT2* (**a**), *ANXA3* (**b**), *CSF2* (**c**), *CD83* (**d**), *NFKBIE* (**e**), *ARID5B* (**f**), *PDE2A-ARAP1* (**g**), *PLD4* (**h**) and *PTPN2* (**i**). Diamonds represent the $-\log_{10} P$ values of the SNPs, and the red diamonds represent the $-\log_{10} P$ values of the SNPs in the meta-analysis. Red color for the smaller circles represents the r^2 value with the most significantly associated SNP (larger red circle). The purple circle represents the *P* value in the combined study. The blue line shows the recombination rates given by the HapMap Phase 2 east Asian populations (release 22). RefSeq genes at the loci are indicated below. Genes nearest to the marker SNPs at the loci are colored blue (**Supplementary Note**), and genes implicated in eQTL analysis are colored red (**Supplementary Table 4**). At 11q13, two genes (*PDE2A* and *ARAP1*) that are nearest to the SNP selected for the replication study and the most significant SNP in the meta-analysis are highlighted. The plots were drawn using SNP Annotation and Proxy Search (SNAP) version 2.2.

the meta-analysis and had not been reported as rheumatoid arthritis susceptibility loci[1–16], we selected a marker SNP for the replication study (Online Methods and **Supplementary Table 3**).

In the combined analyses of the meta-analysis and the replication study, including a total of 9,351 rheumatoid arthritis cases and 38,575 controls, we identified six newly associated loci, in addition to the *NFKBIE*, *ARID5B* and *PTPN2* loci, that satisfied the significance threshold of $P < 5.0 \times 10^{-8}$, including *B3GNT2* at 2p15, *ANXA3* at 4q21, *CSF2* at 5q31, *CD83* at 6p23, *PDE2A-ARAP1* at 11q13 and *PLD4* at 14q32 (**Figs. 1** and **2** and **Table 1**). Of these loci, *NFKBIE* had the smallest *P* value (5.8×10^{-19}). Although association with rheumatoid arthritis has been described for the *CSF2* and *PTPN2* loci[11,15,16,20,21], ours is the first report to our knowledge validating these associations with a threshold of $P < 5.0 \times 10^{-8}$. Suggestive associations were also observed in *ETS1-FLI1* at 11q24, *GCH1* at 14q22, *PRKCH* at 14q23, *ZNF774* at 15q26, *PRKCB1* at 16p12 and *IRF8* at 16q24 ($5.0 \times 10^{-8} \leq P < 5.0 \times 10^{-6}$). A summary of the genes in the newly associated loci and the results of *cis* expression quantitative trait locus (*cis* eQTL) analysis of the marker SNPs are provided (**Supplementary Table 4** and **Supplementary Note**).

Previous studies have reported associations of rheumatoid arthritis susceptibility loci with other autoimmune diseases[4,10,15,16]. Therefore, we assessed the association of these newly identified susceptibility loci with systemic lupus erythematosus (SLE) by examining the results of an SLE GWAS in the Japanese population (891 cases and 3,384 controls)[22] and in Graves' disease by genotyping 1,783 cases[10] (the controls from the SLE analysis were used for testing for Graves' disease). We observed significant associations of the *ANXA3* locus with SLE and of the *B3GNT2* and *ARID5B* loci with Graves' disease, which showed the same directional effects of the alleles as in rheumatoid arthritis ($P < 0.05/9 = 0.0056$, Bonferroni correction of the number of loci; **Supplementary Table 5**). It should be noted that relatively small sample sizes in the SLE and Graves' disease cohorts might yield limited statistical power, and further evaluations enrolling larger numbers of subjects would be desirable.

To highlight genetic backgrounds of rheumatoid arthritis that are common and divergent in different ancestry groups, we conducted a multi-ancestry comparative analysis of the present study in Japanese and a previous GWAS meta-analysis in Europeans that included 5,539 rheumatoid arthritis cases and 20,169 controls[15] (**Fig. 3a–c**). First, we compared associations in the reported[1–16] or newly identified rheumatoid arthritis susceptibility loci (**Fig. 3a** and **Supplementary Table 6**). Of the 46 rheumatoid arthritis risk variants evaluated, 6 were monomorphic in Japanese, and all were polymorphic in Europeans. We observed significant associations at 22 loci in Japanese and at 36 loci in Europeans (false discovery rate (FDR) < 0.05, $P < 0.0030$), with 14 loci being shared between the populations. Of the newly associated rheumatoid arthritis susceptibility loci identified in our Japanese meta-analysis, significant associations were also observed in the European meta-analysis at the *ARID5B* and *PTPN2* loci ($P = 1.9 \times 10^{-4}$ and 9.2×10^{-5}, respectively; **Table 1**). Significant positive correlation of odds ratios was observed between the studies ($r = 0.822$, $P = 8.1 \times 10^{-11}$; **Fig. 3b**), suggesting that a substantial proportion of genetic factors are shared between

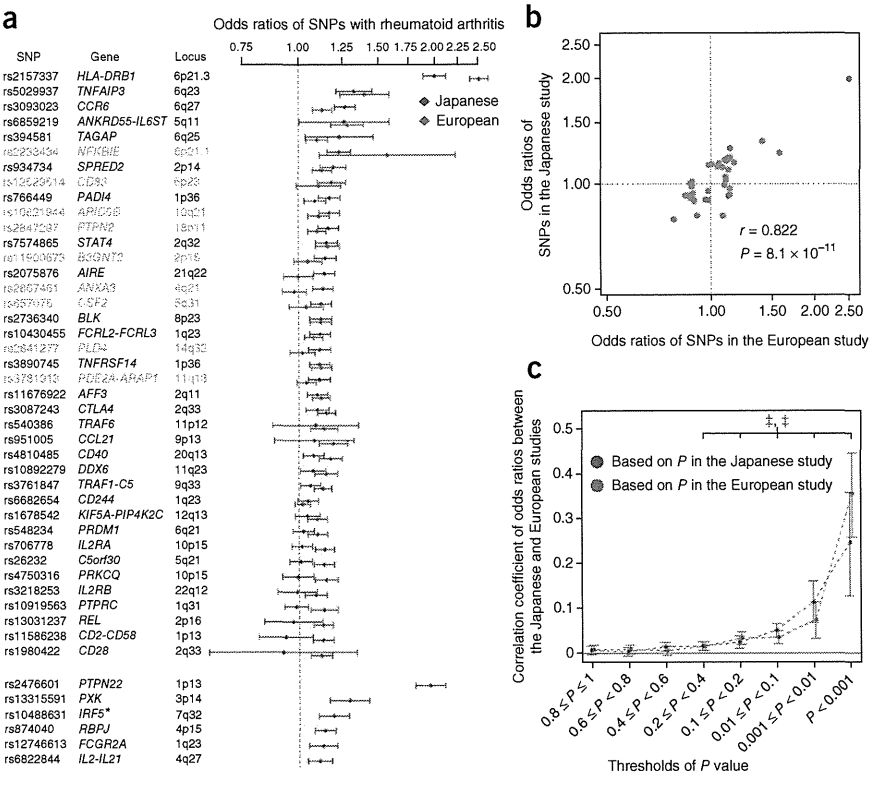

Figure 3 Overlap of the associations with rheumatoid arthritis between Japanese and European populations. (**a**) Forest plots of SNPs in the rheumatoid arthritis susceptibility loci (**Supplementary Table 6**). We selected the genetic loci that have been validated to be associated with rheumatoid arthritis susceptibility by showing associations in the reports of multiple cohorts or satisfying the genome-wide significant threshold ($P < 5.0 \times 10^{-8}$) in previous studies, including in the meta-analysis and replication phases[1–16]. For each of the loci, the most significant SNP among those reported in the previous or present study were selected[1–16]. SNPs in the newly identified rheumatoid arthritis susceptibility loci are colored green. Odds ratios and 95% confidence interval (CI) values are based on rheumatoid arthritis risk alleles, and the SNPs are ordered according to the odds ratios in the Japanese study. Several SNPs were monomorphic in the Japanese population. The odds ratios of these SNPs in the European study are presented below. The asterisk indicates that an association of another variant at the *IRF5* locus was reported in the Japanese population[24]. (**b**) Correlation of the odds ratios of the SNPs in the validated rheumatoid arthritis susceptibility loci between the two populations. SNPs that were polymorphic in both populations were used; odds ratios were based on the minor allele in the Japanese population. (**c**) Correlation of the odds ratios of the genome-wide SNPs, excluding the rheumatoid arthritis susceptibility loci. Correlations were evaluated for sets of SNPs stratified by the thresholds based on the meta-analysis *P* values in each population after pruning of the SNPs by LD ($r^2 < 0.3$). Correlation coefficient and 95% CI are indicated on the *y* axis. Significant correlation of the odds ratios was observed (‡, $P < 0.005$), even for the SNPs that showed moderate associations with rheumatoid arthritis (meta-analysis $P < 0.4$ in each population).

the two ancestry groups[17]. When the rheumatoid arthritis cases of the Japanese GWAS meta-analysis were stratified into anti-CCP–positive or rheumatoid factor–positive cases ($n = 3,209$) and controls ($n = 16,891$), similar results were observed (data not shown). Nevertheless, most of the SNPs assessed here are not necessarily causal variants, and further fine mapping of the loci is warranted to precisely evaluate the shared genetic predisposition between the populations.

Next, we compared regional associations within each of the loci and identified unique patterns in the *ARID5B* locus at 10q21 (**Supplementary Fig. 3**). In Japanese, three peaks of association were observed ($P = 1.0 \times 10^{-8}$ at rs10821944, $P = 5.7 \times 10^{-8}$ at rs10740069 and $P = 8.5 \times 10^{-6}$ at rs224311). These three variants were in weak linkage disequilibrium (LD) in Japanese ($r^2 < 0.10$), indicating independent associations with each of the other SNPs that satisfied a region-wide significance threshold of $P < 3.5 \times 10^{-5}$ (conditional $P = 4.3 \times 10^{-6}$, 1.7×10^{-5} and 1.8×10^{-5}, respectively) (**Supplementary Fig. 3**). In contrast, there was only one peak of association in Europeans ($P = 1.2 \times 10^{-6}$ at rs12764378; $r^2 = 0.59$ with rs10821944 in Europeans), and no additional association was observed in conditional analysis with rs12764378 (the smallest conditional $P = 2.2 \times 10^{-4}$), suggesting that the number of independent associations may be different at this locus in the two populations.

Finally, we conducted polygenic assessment for common variants showing modest associations to rheumatoid arthritis (those not meeting the genome-wide association threshold). This approach has been recognized to be a means to explain a substantial proportion of genetic risk[23]. For the SNPs that were shared between the two meta-analyses but not included in the validated rheumatoid arthritis susceptibility loci, we adopted LD pruning of the SNPs ($r^2 < 0.3$). We then evaluated the correlation of odds ratios of the SNPs between the two meta-analyses and observed a significant positive correlation ($r = 0.023$, $P < 1.0 \times 10^{-300}$). When the SNPs were stratified according to the *P* values in each meta-analysis, significant positive correlations of odds ratios were observed for the SNPs, even for those showing modest association ($P < 0.4$ in the meta-analysis of Japanese or Europeans; $r = 0.014$–0.36 for each *P* value range, $P < 0.005$ for each correlation test) (**Fig. 3c**). Correlations (r) of odds ratios observed herein suggest substantial overlap of the genetic risk of rheumatoid arthritis between the two populations, not only in the validated rheumatoid arthritis susceptibility loci but also at the loci showing nonsignificant associations. This suggests the usefulness of a meta-analysis approach involving multiple ancestry groups in identifying additional susceptibility loci.

In summary, we identified multiple new loci associated with rheumatoid arthritis through a large-scale meta-analysis of GWAS in Japanese. Multi-ancestry comparative analysis provided evidence of significant overlap in the genetic risks of rheumatoid arthritis between Japanese and Europeans. Thus, findings from the present study should contribute to the further understanding of the etiology of rheumatoid arthritis.

URLs. GARNET consortium, http://www.twmu.ac.jp/IOR/garnet/home.html; The BioBank Japan Project (in Japanese), http://biobankjp.org/; International HapMap Project, http://www.hapmap.org/; PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/; EIGENSTRAT, http://genepath.med.harvard.edu/~reich/Software.htm; MACH and mach2dat, http://www.sph.umich.edu/csg/abecasis/MACH/index.