eigenvectors 1 and 2 obtained from the PCA using EIGENSTRAT version 2.0 [42], along with European (CEU), African (YRI), Japanese (JPT), and Chinese (CHB) individuals obtained from the Phase II HapMap database (release 22) [29]. Subjects who were estimated to be outliers in terms of ancestry from East-Asian (JPT+CHB) clusters and excluded from the study are indicated by black arrows.
(TIF)

Figure S2 Quantile-Quantile plot (QQ-plot) of *P*-values in the GWAS for SLE. The horizontal axis indicates the expected $-\log_{10}$ (*P*-values). The vertical axis indicates the observed $-\log_{10}$ (*P*-values). The QQ-plot for the *P*-values of all SNPs that passed the quality control criteria is indicated in blue. The QQ-plot for the *P*-values after the removal of SNPs included in the previously reported SLE susceptibility loci is indicated in black. The gray line represents $y = x$. The SNPs for which the *P*-value was smaller than 1.0×10^{-15} are indicated at the upper limit of the plot.
(TIF)

Table S1 Basal characteristics of cohorts.
(DOC)

Table S2 Frequency of clinical characteristics of SLE in this GWAS.
(DOC)

Table S3 Distributions of eQTL positivity rates of the SNPs.
(DOC)

Table S4 Results of replication study 1 for Japanese patients with SLE.
(DOC)

Table S5 Results of replication studies 1 and 2 for Japanese patients with SLE.
(DOC)

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Author Contributions

Conceived and designed the experiments: Y. Okada, K. Shimane, Y. Kochi, A. Suzuki, M. Kubo, N. Kamatani, R. Yamada, Y. Nakamura, K. Yamamoto. Performed the experiments: Y. Kochi, N. Hosono, M. Kubo, K. Myouzen, T. Horiuchi, T. Tahira, K. Hayashi. Analyzed the data: Y. Okada, A. Takahashi, T. Tsunoda, K. Higasa, R. Yamada. Contributed reagents/materials/analysis tools: T. Horita, T. Atsumi, T. Koike, T. Ishii, A. Okamoto, K. Fujio, M. Hirakata, H. Amano, Y. Takasaki, Y. Kondo, S. Ito, T. Sumida, K. Takada, A. Mimori, K. Saito, Y. Tanaka, M. Kamachi, N. Nishimoto, Y. Kawaguchi, C. Terao, K. Ohmura, T. Mimori, F. Matsuda, O.W. Mohammed, K. Matsuda, K. Shimane, K. Ikari, H. Yamanaka. Wrote the paper: Y. Okada, K. Shimane, Y. Kochi, A. Suzuki, R. Yamada, K. Yamamoto.

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ORIGINAL ARTICLE

Cumulative association of eight susceptibility genes with systemic lupus erythematosus in a Japanese female population

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Although large-scale studies established many susceptibility genes to systemic lupus erythematosus (SLE), effect of each gene is not sufficiently large to be used alone to identify individuals with strong genetic predisposition. In this study, we analyzed the cumulative number of risk alleles at eight established susceptibility loci, *HLA-DRB1*, *IRF5*, *STAT4*, *BLK*, *TNFAIP3*, *TNIP1*, *FCGR2B* and *TNFSF13*, in 282 Japanese female SLE and 222 healthy female controls. The average number of risk alleles was significantly increased in SLE (8.07 ± 1.60) than healthy controls (7.02 ± 1.64) ($P=1.63\times10^{-12}$). Significant gene–gene interaction was not detected. When the subjects carrying seven risk alleles were used as a reference, the odds ratio (OR) for individuals carrying 10 and 11–13 risk alleles were 4.17 (95% confidence interval (CI) 1.89–9.19, $P=0.0002$) and 8.77 (95% CI 1.92–40.0, $P=0.0016$), respectively. In contrast, subjects with ⩽4 risk alleles were significantly decreased in SLE (OR 0.15, CI 0.03–0.67, $P=0.007$). The proportion of the patients with neurologic disorder was significantly increased in those carrying ⩾10 risk alleles than those with <10 (OR 2.30, CI 1.09–4.83, $P=0.025$). This study suggested that the cumulative number of risk alleles may efficiently distinguish groups with high and low genetic predisposition to SLE and its severe manifestation.
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Keywords: genetic risk score; polymorphism; susceptibility; systemic lupus erythematosus

INTRODUCTION

Recent large-scale association studies identified a number of novel susceptibility genes to systemic lupus erythematosus (SLE),[1–5] among which many have been replicated in independent studies. However, genetic effect of each susceptibility allele is generally modest; with the exception of *HLA-DRB1*, none reached the allelic odds ratio (OR) of 2. Thus, the effect of each susceptibility allele is not sufficient to identify the high-risk individuals when used alone.

One possible approach to solve this problem is to combine multiple risk allele genotypes of each individual into a genetic risk score. Such an approach has been employed in type 2 diabetes,[6,7] cholesterol levels and cardiovascular diseases,[8,9] prostate cancer[10] and rheumatic diseases such as gout,[11] rheumatoid arthritis[12] and systemic sclerosis.[13]

Our group has been intensively working on the susceptibility genes to SLE in a Japanese population. Thus far, we replicated association of *HLA-DRB1*15:01*, *STAT4* rs7574865T, *BLK* rs13277113A, *TNFAIP3* rs2230926G and *TNIP1* rs7708392C in Japanese, which had previously been reported in the Caucasian or Chinese populations.[14–18] In the process of these studies, we noted that the genetic contribution of *STAT4*, *BLK* and *TNIP1* was greater in the Japanese compared with the Caucasian populations, due to population differences in the risk allele frequencies.[15,16,18]

With respect to *IRF5*, we found that the initially reported Caucasian risk haplotype carrying rs2004640T, exon 6 insertion and rs10954213A[19] was nearly absent in Japanese[20] Moreover, although the association of rs2004640 was also observed in Japanese, another single nucleotide polymorphism (SNP) rs41298401, which is monomorphic in Caucasians, was more strongly associated with SLE.[20]

In *FCGR2B*, we identified association of a SNP rs1050501, which alters Ile at position 232 to Thr in the transmembrane region.[21] This association was replicated in multiple Asian populations.[22–24] This amino acid substitution was associated with reduced localization of

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inhibitory Fcγ receptor IIb molecule in the lipid raft, thereby resulting in hyperactivation of B cells[25] and monocytes.[26]

In the case of *TNFSF13* (APRIL), Koyama *et al.*[27] and our group[28] independently demonstrated association of rs11552708 (Gly67Arg) with SLE in Japanese. This association was also replicated in a study on mixed European-American, African-American and Hispanic populations.[29] Thus, *TNFSF13* is also an established susceptibility gene to SLE.

All of the above mentioned eight susceptibility genes have been independently replicated, and ORs of the risk genotypes are greater than 1.3 in Japanese.[14–18,20,21,28] In the case of SLE, a recent study from the United States reported association of cumulative risk allele number at 19 susceptibility loci with the age of onset among the patients;[30] however, association with genetic risk for the occurrence of SLE as compared with healthy controls was not analyzed.

In this study, we combined the genotype data at the eight susceptibility loci established in the Japanese, and tested the association of the cumulative risk allele number with susceptibility to SLE. When the subjects carrying seven risk alleles were used as a reference, the OR for individuals carrying 10 and 11–13 risk alleles were 4.17 and 8.77, respectively. In contrast, subjects with four or less risk alleles were significantly decreased in SLE (OR 0.15). In addition, the proportion of the patients with neurologic disorder was significantly increased in those carrying $\geqslant 10$ risk alleles than those with <10. Thus, this study suggested that the cumulative number of risk alleles could potentially be useful for distinguishing groups with high and low genetic predisposition to SLE and its severe manifestation.

MATERIALS AND METHODS

Patients and controls

A total of 282 Japanese female patients with SLE and 222 healthy female controls were recruited at University of Tsukuba, Juntendo University and the University of Tokyo. To exclude potential bias caused by gender-associated difference in the genetic factors for SLE, male patients and controls were excluded from this study. All patients and healthy individuals were native Japanese living in the central part of Japan. All patients fulfilled the American College of Rheumatology criteria for SLE.[31] Presence or absence of renal and neurologic disorders was also classified based on the American College of Rheumatology criteria.[31] This study was reviewed and approved by the research ethics committees of University of Tsukuba, Juntendo University and the University of Tokyo. Informed consent was obtained from all study participants. This study was conducted in accordance with the principles expressed in the Declaration of Helsinki.

Genotyping

Genotyping of *HLA-DRB1* was performed by PCR-microtiter plate hybridization (MPH) technique or by Luminex Multi-Analyte Profiling system (xMAP) with WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan) as described elsewhere.[32] *IRF5* rs41298401 genotype was determined by direct sequencing.[20] *FCGR2B* rs1050501 was genotyped using nested PCR and oligonucleotide hybridization as previously described,[21] or direct sequencing using the sequencing primer annealing at intron 5 (5′-AATACGGGCCTAGATCTGAATGTG-3′) described by Li *et al.*[22] after PCR using primers placed at exon 4 (5′-AAGGACA AGCCTCTGGTCAA-3′) and exon 7 (5′-CCCAACTTTGTCAGCCTCAT-3′).[21] *TNFSF13* rs11552708 was genotyped using PCR-single strand conformation polymorphism or direct sequencing, as previously described.[28] Other SNPs were genotyped using the TaqMan genotyping assay (Applied Biosystems, Foster City, CA, USA), as previously described.[15–18] Identity of the genotyping results were confirmed when two genotyping systems were used for a locus.

The genotyping data of the cases and controls were derived from our previous studies.[14–18,20,21,28] In addition, when necessary, additional genotyping was performed for individuals in whom any of the eight loci had not been genotyped.

Statistical analysis

Association of each susceptibility allele with SLE in the cases and controls analyzed this study was performed by Fisher's exact test using 3×2 contingency table. Gene–gene interaction was examined between each pair of genes using logistic regression analysis under the co-dominant model, where risk allele homozygotes $x_i=2$, heterozygotes $x_i=1$ and non-risk allele homozygotes $x_i=0$ for gene i. The logistic regression model for interaction between gene i and gene j was given by

$$\text{logit}(P) = \beta_0+\beta_i x_i + \beta_j x_j + \beta_{ij} x_i x_j.$$

The deviation from 0 was evaluated for β_{ij} by the Wald test.

The increase in the risk by carrying an additional risk allele was calculated using logistic regression analysis. The risk allele count and the disease status were used as independent and dependent variables, respectively.

The statistical significance of the difference in the average number of the risk alleles in cases and controls was examined using Student's t-test (two-tailed). When the OR and 95% confidence interval (CI) was calculated for individuals carrying different number of risk alleles, subjects carrying seven alleles, the median of the risk allele number in healthy controls, were used as a reference. The statistical significance was analyzed using Fisher's exact test.

The proportion of the patients with renal disorder, neurologic disorder or early onset (<20 years) was compared between those with $\geqslant 10$ and <10 risk alleles using χ^2-test.

RESULTS

Association between cumulative risk allele number and susceptibility to SLE

We selected eight established susceptibility alleles, *HLA-DRB1*15:01*, *IRF5* rs41298401C, *STAT4* rs7574865T, *BLK* rs13277113A, *TNFAIP3* rs2230926G, *TNIP1* rs7708392C, *FCGR2B* rs1050501C and *TNFSF13* rs11552708G, based on the following criteria: (1) at least one report of significant association in Japanese, (2) OR of the risk genotype >1.3 and (3) at least one independent replication.

These alleles were genotyped in 282 Japanese female patients with SLE and 222 healthy female controls. Table 1 shows the genotype frequencies of the examined loci in the patients and healthy controls. To exclude potential influence from gender-associated difference in the genetic background, we focused on female patients and controls. As a result, the sample size of this study was smaller than our previous studies where each locus was examined individually.[14–18,20,21,28] This resulted in lack of statistically significant association of *TNFAIP3*, *TNIP1* and *TNFSF13* in the subjects analyzed here.

The gene–gene interaction was examined between each pair of two loci (28 pairs) using logistic regression analysis under a co-dominant

Table 1 Genotype frequencies of each susceptibility gene with systemic lupus erythematosus in the Japanese female patients and controls examined in this study

		SLE (%)			*Healthy controls (%)*			
Locus	*Risk allele*	*11*	*12*	*22*	*11*	*12*	*22*	P
HLA-DRB1	*15:01	3.2	22.0	74.8	1.4	8.1	90.5	1.37×10^{-5}
IRF5	rs41298401C	73.8	25.9	0.4	60.8	35.1	4.1	3.71×10^{-4}
STAT4	rs7574865T	18.4	55.7	25.9	11.7	45.9	42.3	3.20×10^{-4}
BLK	rs13277113A	62.8	29.1	8.2	46.8	42.8	10.4	1.55×10^{-3}
TNFAIP3	rs2230926G	1.8	17.4	80.9	0.5	16.2	83.3	0.41
TNIP1	rs7708392C	58.9	34.4	6.7	51.4	40.5	8.1	0.24
FCGR2B	rs1050501C	10.3	36.5	53.2	4.1	38.3	57.7	0.03
TNFSF13	rs11552708G	39.7	48.6	11.7	36.9	47.7	15.3	0.48

Genotype frequencies of the tested eight susceptibility genes in Japanese female systemic lupus erythematosus patients (n=282) and healthy controls (n=222) are examined in this study. P values were calculated by Fisher's exact test using 2×3 contingency tables.
1=risk allele, 2=non-risk allele.

model. Statistically significant gene–gene interaction was not observed, except for a marginal interaction between *BLK* and *FCGR2B* (uncorrected P=0.04).

Table 2 shows the distribution of the cumulative risk allele number of each subject in the patients and healthy controls. Risk allele number was distributed between 4–13 in the patients and 3–11 in the controls. The average number of the risk alleles was significantly increased in the patients (8.07 ± 1.60 alleles) as compared with controls (7.02 ± 1.64 alleles) (P=1.63×10^{-12}, Student's t-test).

Figure 1 shows the OR and 95% CI for each group with increasing number of risk alleles, as compared with the individuals carrying seven risk alleles, the median risk allele count in the control group. In this analysis, groups with 11–13 and 3–4 risk alleles were combined due to the small number of the subjects in each group (Table 2). Because apparent gene–gene interaction was not observed, the increase in the risk by carrying an additional risk allele was estimated using logistic regression analysis, where the risk allele count and the disease status were used as independent and dependent variables, respectively. This analysis indicated that each additional risk allele increased the OR by 1.50 fold (95% CI: 1.33–1.69, P=4.5×10^{-11}).

When compared with individuals carrying seven risk alleles, those with eight or more risk alleles was associated with significantly elevated OR (P<0.05, Fisher's exact test) (Figure 1). Specifically, the ORs of individuals with 10 and 11–13 risk alleles were 4.17 (CI 1.89–9.19, P=0.0002) and 8.77 (CI 1.92–40.0, P=0.0016), respectively.

On the other hand, the OR in individuals having four or less risk alleles was significantly decreased in SLE (OR 0.15, CI 0.03–0.67, P=0.0070). When individuals carrying 11 or more risk alleles were compared with those carrying four or less alleles, the OR was 60 (CI 7.48–481.6, P=5.2×10^{-6}).

Power calculation was performed using computer simulation (1000 runs). This sample size provided 95.7% (seven versus eight or more alleles), 98.3% (seven versus 10 alleles), 94.9% (seven versus 11–13 alleles), 87.4% (seven versus 3–4 alleles) and 99.8% (3–4 versus 11–13 alleles) power to detect statistical difference at the significance level of 0.05.

Table 2 Numbers and percentages of the patients and healthy controls carrying each cumulative risk allele number

Number of risk alleles	*SLE (n=282)*	*Control (n=222)*
3	0 (0)	6 (2.7)
4	2 (0.7)	9 (4.1)
5	14 (5.0)	24 (10.8)
6	30 (10.6)	39 (17.6)
7	52 (18.4)	57 (25.7)
8	78 (27.7)	46 (20.7)
9	52 (18.4)	29 (13.1)
10	38 (13.5)	10 (4.5)
11	11 (3.9)	2 (0.9)
12	4 (1.4)	0 (0)
13	1 (0.4)	0 (0)

Abbreviation: SLE, systemic lupus erythematosus.
Percentages are shown in parentheses.

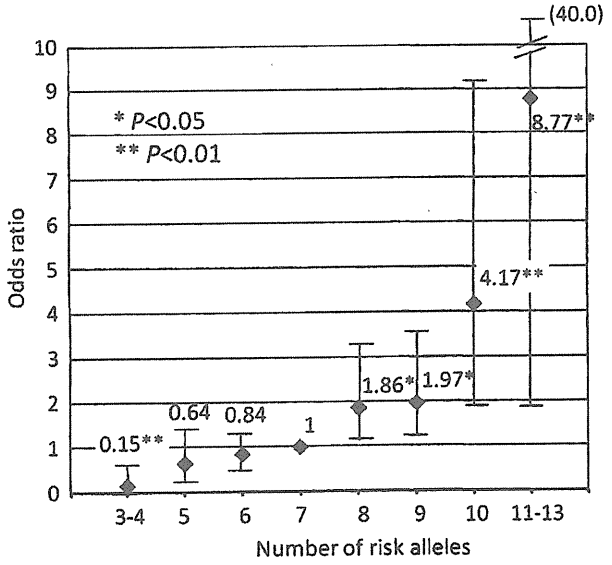

Figure 1 Odds ratios and 95% confidence intervals of the individuals carrying increasing numbers of risk alleles in eight SLE susceptibility loci. The reference group was the individuals with seven risk alleles, which was the median of the healthy controls. Statistical significance was tested using Fisher's exact test. Power calculation using computer simulation (1000 runs) demonstrated that the power to detect statistical significance at the significance level of 0.05 was 87.4% (3–4 alleles), 26.8% (5 alleles), 13.0% (6 alleles), 69.3% (8 alleles), 67.9% (9 alleles), 98.3% (10 alleles) and 94.9% (11–13 alleles), respectively.

Association between cumulative risk allele number and clinical subsets

We next examined whether the cumulative risk allele number is associated with severe clinical phenotypes such as renal disorder and neurologic disorder, as well as with early onset of SLE. First, we compared the mean risk allele number between the patients with and without each clinical feature. The mean number of risk alleles showed a tendency towards increase in the patients with renal disorder (8.21 ± 1.57 alleles, n=156) as compared with those without (7.90 ± 1.64 alleles, n=125), in those with neurologic disorder (8.39 ± 1.96, n=41) than those without (8.02 ± 1.53, n=232) and in the patients with early onset (<20 years) (8.10 ± 1.42, n=72) than those with later onset (≥20 years) (8.07 ± 1.66, n=208), although none of the differences reached statistical significance (P>0.05, Student's t-test).

We then compared the proportion of each clinical characteristics between the patients carrying ≥10 risk alleles and those with <10. As shown in Table 3, significant increase of neurologic disorder was observed in the patients carrying ≥10 risk alleles.

Table 3 Comparison of clinical phenotypes between SLE patients with ≥10 and <10 risk alleles

	≥10 risk alleles n *(%)*	*<10 risk alleles* n *(%)*	P	*OR (95% CI)*
Age of onset				
<20	11 (20.4)	61 (27.0)	0.317	0.69 (0.34–1.43)
≥20	43 (79.6)	165 (73.0)		
Renal disorder				
+	35 (64.8)	121 (53.3)	0.126	1.61 (0.87–2.99)
−	19 (35.2)	106 (46.7)		
Neurologic disorder				
+	13 (25.0)	28 (12.7)	0.025	2.30 (1.09–4.83)
−	39 (75.0)	193 (87.3)		

Abbreviations: CI, confidence interval; OR, odds ratio; SLE, systemic lupus erythematosus.
P values were calculated by χ^2-test. Power calculation using computer simulation (1000 runs) revealed that this sample size provided 20.9% (age of onset), 36.8% (renal disorder) and 61.4% (neurologic disorder) power to detect statistical difference at the significance level of 0.05.

DISCUSSION

Attempts to develop a marker for estimating the risk of an individual by using cumulative risk allele number at multiple loci have been reported in various complex diseases.[6–13] In this study, by combining the number of risk alleles at eight susceptibility loci, each having rather small allelic OR, it became possible to distinguish high and low risk groups with up to 60-fold difference in the genetic predisposition to SLE. However, it should be noted that the model proposed in this study is, at this point, not sufficient for the purpose of predicting disease onset or identifying a high-risk group that requires preventive intervention. This is because of the low incidence of SLE, lack of measures for prevention, as well as the limitations of the study approach as described later.

In this study, we simply counted the number of risk alleles of multiple susceptibility loci. Our model could potentially be improved by weighting each risk allele according to the size of genetic effects (ORs) as described in the study of RA,[12] or taking the appropriate genetic model (for example dominant, co-dominant or recessive) into account. However, the genetic effect of each allele and the model that provides the best fit sometimes vary considerably among studies. In addition, in most of the susceptibility genes, the causative allele, which accounts for the molecular mechanism of SLE has yet to be identified. The ORs of the unidentified causative alleles may be different from those observed for the 'proxy' SNPs. Therefore, we employed a model-free approach to simply count the number of risk alleles. Our data suggest that even this simple approach could potentially provide a useful parameter to estimate genetic predisposition to SLE of each individual. With establishment of many more susceptibility genes, this model is expected to improve its power to identify individuals with elevated genetic risk to develop SLE.

There are several limitations in this study. Because of the low prevalence of SLE, the sample size of this study is small, which resulted in the wide range of CIs in the subjects carrying 10 or more risk alleles. Although significant gene–gene interaction was not detected in every possible combination of two genes, it would be intriguing to examine the combined effects of specific risk genotypes at three or more susceptibility loci. However, such a study requires much larger number of subjects than this study. For these reasons, we admit that this study is somewhat preliminary and the results should be validated in a larger sample set.

One approach to increase the sample size in the study of rare diseases is to organize a large-scale international collaboration. However, we focused on the Japanese population, because we wanted to find a biomarker useful in clinical practice or preventive medicine in Japanese. The genetic background has been shown to be slightly different even among the closely-related East Asian populations.[33,34] Therefore, the next step will be to validate our results in a larger sample size through collaboration with multiple SLE centers in Japan.

Another possibility is the use of GWAS data of Japanese SLE. Although GWAS of Japanese SLE has not been reported yet, once the data become available, it might be possible to impute the genotypes at the susceptibility genes analyzed in this study to compare cumulative risk allele numbers between a large number of patients and controls.

It should be noted that the set of eight genes in this study was based on the Japanese data, and is not directly applicable to other populations. For example, the risk alleles of *PTPN22*[35] and *ITGAM*[1,2] as well as *HLA-DRB1*03:01*,[36] established risk factors for SLE in the Caucasian populations, are absent or very rare in the Japanese population. Thus, similar analysis should be carried out in each population, using the set of established susceptibility genes in the target population.

It should also be emphasized that this study considered only the SLE susceptibility genes that have already been replicated in the Japanese population. Recently reported SLE susceptibility genes, which have yet to be replicated in Japanese such as *TNFSF4*[4,5,37] were not included. For the same reason, we did not include the multiple SNPs in the HLA region, which were shown to be independently associated with SLE in other populations.[38] Therefore, our current model represents only a proportion of the whole genetic contribution to SLE, and should be improved in the future by incorporating additional susceptibility genes when their association was replicated in the Japanese.

The cumulative risk allele number might potentially be useful for the prediction of disease phenotype among the patients. In a recent study in the United States, the risk allele number was associated with early onset of SLE, which in turn was associated with severe disease.[30] In our data, the association between the risk allele number and early onset was not detected. However, of particular interest, the patients carrying $\geqslant$10 risk alleles exhibited more than twofold risk for developing neurologic disorder, one of the severe manifestations of the disease, than those with 10 risk alleles. Such information would be useful for personalized medicine; for example, more aggressive treatment may be considered for the patients with increased number of risk alleles.

The cumulative risk allele number may also be useful for research purpose. For example, longitudinal studies of healthy individuals carrying an increased number of risk alleles may efficiently identify environmental triggers or epigenetic modifiers that lead to the development of SLE. It would also be of interest to test association with the presence of antinuclear antibodies among healthy individuals.[39]

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RESEARCH ARTICLE Open Access

TLR7 single-nucleotide polymorphisms in the 3' untranslated region and intron 2 independently contribute to systemic lupus erythematosus in Japanese women: a case-control association study

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Abstract

Introduction: The Toll-like receptor 7 (*TLR7*) gene, encoded on human chromosome Xp22.3, is crucial for type I interferon production. A recent multicenter study in East Asian populations, comprising Chinese, Korean and Japanese participants, identified an association of a *TLR7* single-nucleotide polymorphism (SNP) located in the 3' untranslated region (3' UTR), rs3853839, with systemic lupus erythematosus (SLE), especially in males, although some difference was observed among the tested populations. To test whether additional polymorphisms contribute to SLE in Japanese, we systematically analyzed the association of *TLR7* with SLE in a Japanese female population.

Methods: A case-control association study was conducted on eight tag SNPs in the *TLR7* region, including rs3853839, in 344 Japanese females with SLE and 274 healthy female controls.

Results: In addition to rs3853839, two SNPs in intron 2, rs179019 and rs179010, which were in moderate linkage disequilibrium with each other (r^2 = 0.53), showed an association with SLE (rs179019: P = 0.016, odds ratio (OR) 2.02, 95% confidence interval (95% CI) 1.15 to 3.54; rs179010: P = 0.018, OR 1.75, 95% CI 1.10 to 2.80 (both under the recessive model)). Conditional logistic regression analysis revealed that the association of the intronic SNPs and the 3' UTR SNP remained significant after we adjusted them for each other. When only the patients and controls carrying the risk genotypes at the 3' UTR SNPpositionwere analyzed, the risk of SLE was significantly increased when the individuals also carried the risk genotypes at both of the intronic SNPs (P = 0.0043, OR 2.45, 95% CI 1.31 to 4.60). Furthermore, the haplotype containing the intronic risk alleles in addition to the 3' UTR risk allele was associated with SLE under the recessive model (P = 0.016, OR 2.37, 95% CI 1.17 to 4.80), but other haplotypes were not associated with SLE.

Conclusions: The *TLR7* intronic SNPs rs179019 and rs179010 are associated with SLE independently of the 3' UTR SNP rs3853839 in Japanese women. Our findings support a role of *TLR7* in predisposition for SLE in Asian populations.

Introduction

Toll-like receptors (TLRs) play a central role in detecting microbial pathogens. TLRs initiate innate immune responses and also induce adaptive immune responses [1]. Recently, TLRs have been strongly implicated in autoimmune diseases [2]. The *TLR7* and *TLR9* genes, which are expressed intracellularly in plasmacytoid dendritic cells (pDCs) and B cells, recognize single-stranded RNA and DNA containing cytidine-phosphate-guanosine motifs, respectively. Activation of pDCs by TLR7 and TLR9 induces a large amount of type I interferon (IFN). It has become evident that RNA- and DNA-containing immune complexes, which often exist in sera of patients with systemic lupus erythematosus (SLE), can activate TLR7 and TLR9 signaling [2].

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Several lines of evidence support a role of TLR7 in SLE pathogenesis [2]. Male BXSB mice bearing the Y chromosome-linked autoimmune accelerator (*Yaa*) gene develop severe SLE. It has been revealed that *Yaa* mutation is caused by a translocation of a portion of the X chromosome containing *TLR7* onto the Y chromosome [3,4]. *Yaa*-bearing mice have been demonstrated to have twofold overexpression of TLR7 protein and mRNA [3,4]. In contrast, lupus-prone MRL/Mp$^{lpr/lpr}$ mice lacking *TLR7* showed impaired production of antibodies to RNA-containing antigens, such as anti-Smith (anti-Sm) antibodies, and developed less severe disease [5]. Furthermore, upregulated expression of *TLR7* mRNA in peripheral blood mononuclear cells (PBMNCs) was observed in human SLE [6].

Recently, a multicenter collaborative study including our group reported an association of *TLR7*, located in Xp22.3, with SLE in combined East Asian populations [7]. In a discovery panel consisting mainly of Chinese and Korean populations, the association of 27 single-nucleotide polymorphisms (SNPs) in the *TLR7-TLR8* region with SLE was examined, and a significant association of the *TLR7* 3' untranslated region (3' UTR) SNP, rs3853839, was identified. Subsequently, the association of rs3853839 was replicated in two independent Chinese and Japanese case-control sets. The association was prominent in males with SLE. In addition, rs3853839 was associated with elevated expression of *TLR7*. The study also revealed some differences in the association of rs3853839 and other SNPs among Chinese, Korean and Japanese populations [7], indicating that systematic SNP screening should be performed in each population.

In this study, we examined the association of eight *TLR7* tag SNPs with SLE in Japanese women and discovered a newly identified association of two intronic SNPs, rs179019 and rs179010, with SLE. These SNPs and the 3'UTR rs3853839 were found to independently contribute to the genetic risk for SLE.

Materials and methods

Patients and controls

Three hundred forty-four Japanese female patients with SLE (mean age ± SD, 42.9 ± 13.8 years) and 274 healthy female controls (mean age ± SD, 31.3 ± 8.9 years) were recruited at University of Tsukuba, Juntendo University, Sagamihara National Hospital, and at the University of Tokyo. Among them, 296 SLE patients and 250 healthy controls were also examined in a previous study to replicate the association of rs3853839 with SLE in Japanese, but other SNPs were not investigated in that study [7]. All patients and healthy individuals were native Japanese living in the central part of Japan. All patients with SLE fulfilled the American College of Rheumatology criteria for SLE [8].

This study was carried out in compliance with the Declaration of Helsinki. The study was reviewed and approved by the research ethics committees of University of Tsukuba, Sagamihara National Hospital, the University of Tokyo and Juntendo University. Informed consent was obtained from all study participants.

Genotyping

Eight tag SNPs in the *TLR7* region were selected on the basis of the HapMap Phase II JPT (Japanese in Tokyo) data obtained from the HapMap database [9] with the criteria of minor allele frequency >0.1 and an r^2 threshold of 0.9. Genotyping of the tag SNPs was carried out using the TaqMan genotyping assay on the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Thermal cycling conditions consisted of initial denaturation at 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 seconds each and at 60°C for one minute each. TaqMan probes used in this study were as follows: Assay ID: C__15757400_10 (rs2302267), C___2259585_10 (rs179019), C___7625717_10 (rs1634322), C___2259582_10 (rs179016), C___2259578_10 (rs179012), C___2259576_10 (rs179010), C___2259575_10 (rs179009), and C___2259573_10 (rs3853839).

Expression analysis by real-time quantitative reverse transcription polymerase chain reaction assay

Total RNA was extracted from PBMNCs of 18 females with SLE using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), reverse transcribed into cDNA and used for real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) assay. Expression of *TLR7* was analyzed using the TaqMan Gene Expression Assay (Applied Biosystems), Hs00152971_m1. Amplification of cDNA was conducted using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems) under the following conditions: 50°C for 2 minutes and 95°C for 10 minutes, and 50 cycles at 95°C for 15 seconds and at 60°C for 1 minute, and then the cycle threshold (CT) value for each sample was obtained using Applied Biosystems 7300 System SDS version 1.4 software (Applied Biosystems). Relative quantitative levels were calculated on the basis of the CT value by a standard curve method and were normalized to β-actin (*ACTB*) expression (Hs99999903_m1). The experiments were done in triplicate for each sample.

Statistical analysis

Differences in allele and genotype frequencies between SLE patients and healthy controls were analyzed by using a χ^2 test with 2 × 2 contingency tables. When one or more of the variables in the contingency tables was 20 or less, Fisher's exact test was employed. Linkage

disequilibrium (LD) was analyzed using HaploView version 4.0 software (Broad Institute, Cambridge, MA, USA). Pairwise r^2 values were calculated on the basis of the genotypes of 274 healthy controls. Estimation of haplotype frequencies and association tests were performed using HaploView version 4.0 software.

To examine whether each SNP independently contributes to susceptibility to SLE, conditional logistic regression analysis was employed. Dominant, codominant and recessive models were tested for each SNP, and the model that provided the lowest *P* value was selected as the best fit model. As a result, the following were used as independent variables: rs3853839, C/C = 0, G/C = 1 and G/G = 2 under the codominant model for the G allele; rs179019, C/C = 0, C/A = 0, A/A = 1 under the recessive model for the A allele; rs179010, C/C = 0, C/T = 0, and T/T = 1 under the recessive model for the T allele.

The association of *TLR7* SNPs with *TLR7* mRNA expression was assessed by using the Kruskal-Wallis test.

Results

Association of *TLR7* SNPs with SLE in a Japanese female population

To systematically examine association of *TLR7* SNPs with SLE in Japanese, eight tag SNPs in the *TLR7* gene, including rs3853839 in the 3'UTR, which was recently shown to be associated with SLE in East Asian populations [7], were analyzed in 344 Japanese females with SLE and 274 healthy female controls. Because *TLR7* is located on an X chromosome, male and female individuals needed to be analyzed for the association separately. However, because of the female predominance of SLE (9:1 female to male ratio), the sample size of male SLE patients was too small to be analyzed statistically. Therefore, male patients and controls were excluded from this study. No deviation from the Hardy-Weinberg equilibrium was observed in the controls ($P > 0.05$).

In addition to the association of rs3853839 reported separately [7], the association of two SNPs in intron 2, rs179019 and rs179010, was newly detected (Figure 1 and Table 1). Significant association of rs179019 and rs179010 was observed under the recessive model for the A and T alleles, respectively (rs179019: $P = 0.016$, odds ratio (OR) 2.02, 95% confidence interval (95% CI) 1.15 to 3.54; rs179010: $P = 0.018$, OR 1.75, 95% CI 1.10 to 2.80). LD was present between rs179019 and rs179010 ($r^2 = 0.53$), while LD between rs3853839 and each of the intronic SNPs was modest ($r^2 = 0.02$ and 0.04) (Figure 1).

To examine the contribution of each SNP to susceptibility to SLE, conditional logistic regression analysis was conducted. As shown in Table 2, the association of rs3853839 remained significant after adjustment for the intronic SNP genotypes. Adjusted *P* values ($P_{adjusted}$) for rs3853839 under the codominant model were 0.040 and 0.047 after adjustment for rs179019 and rs179010, respectively. The association of rs179019 and rs179010 also remained significant after adjustment for rs3853839 (rs179019: $P_{adjusted} = 0.026$; rs179010: $P_{adjusted} = 0.042$). These results suggest that rs3853839 and the intronic SNPs are independently associated with SLE. In contrast, the association of rs179019 and rs179010 was eliminated when they were adjusted for each other as expected on the basis of LD between the two (Table 2 and Figure 1).

In agreement with these findings, when only the patients and controls carrying the risk genotypes of the 3' UTR SNP were analyzed, possession of both of the intronic SNP risk genotypes was significantly associated with SLE ($P = 0.0043$, OR 2.45, 95% CI 1.31 to 4.60) (Table 3).

SLE-associated SNPs rs179019, rs179010 and rs3853839 were estimated to form five major haplotypes (Table 4). When haplotype frequencies were compared between female SLE patients and healthy controls, tendencies for an increase of haplotype 3 containing all of the SLE risk alleles and a decrease of haplotype 2 containing none of them were observed, although the differences did not reach statistical significance (permutation *P*, haplotype 3 = 0.081; permutation *P*, haplotype 2 = 0.068). We next examined the haplotype association under the recessive model. Individuals homozygous for all three SNPs were considered to be homozygous for the haplotype. A significant association of haplotype 3 was detected under the recessive model (haplotype 3/3 versus others: $P = 0.016$, OR 2.37, 95% CI 1.17 to 4.80), but haplotype 1 ($P = 0.21$, OR 1.32, 95% CI 0.86 to 2.05) and haplotype 4 ($P = 1.0$, OR 0.80, 95% CI 0.11 to 5.68), which also contained the 3'UTR risk allele but not both of the intronic SNPs, were not associated. These results suggest that the combination of the intronic and 3'UTR risk alleles may be associated with higher SLE risk.

Association of *TLR7* SNPs with clinical subsets of SLE

We examined whether *TLR7* SNPs were associated with clinical phenotypes such as the presence of anti-Sm antibodies, anti-double-stranded DNA antibodies and renal disorder. Association was tested between SLE patients with each phenotype and healthy controls. The OR of rs179019 was slightly higher in the subset with renal disorder ($P = 0.011$, OR 2.25, 95% CI 1.21 to 4.18) than in all SLE patients ($P = 0.016$, OR 2.02, 95% CI 1.15 to 3.54) (Table 5), although no statistically significant association was observed in case-only analysis (SLE patients with renal disorder versus those without). The

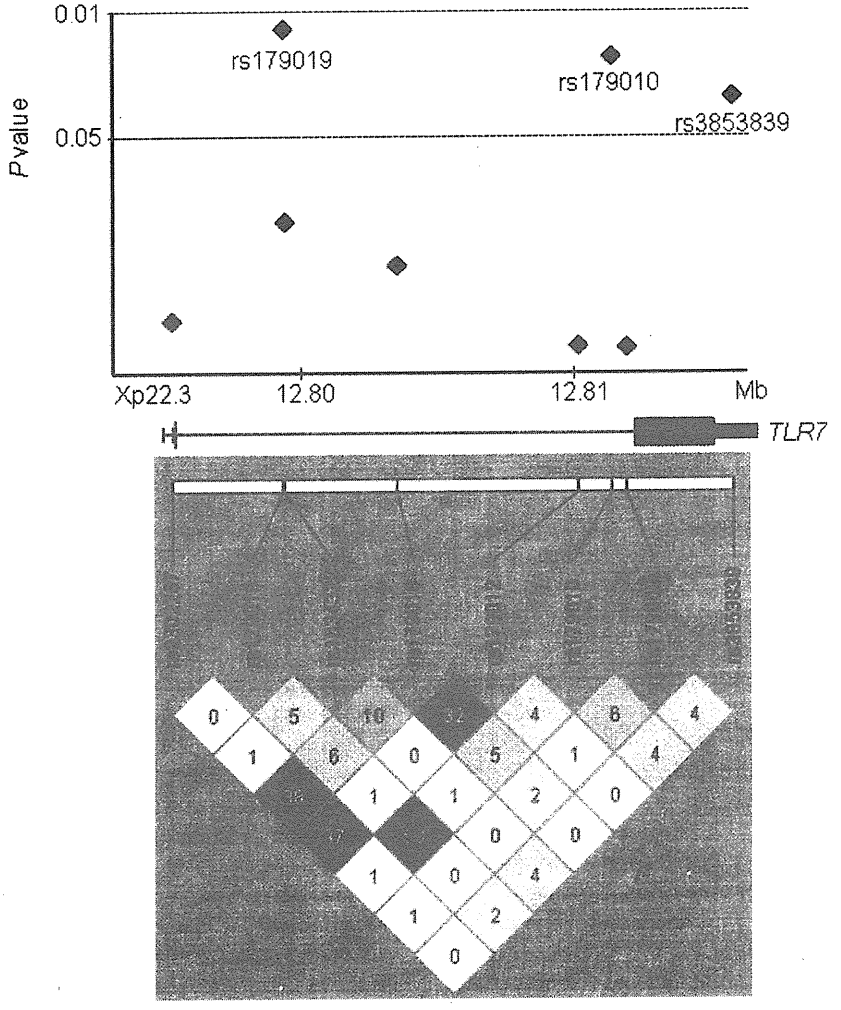

Figure 1 Association of tag single-nucleotide polymorphisms in the Toll-like receptor 7gene with systemic lupus erythematosus. Top: *P* values under the recessive model for minor alleles are indicated. Association was tested by χ^2 analysis using 2 × 2 contingency tables. Bottom: r^2 values based on data from 274 healthy Japanese women are shown.

association of rs179019 with renal disorder remained significant after adjustment for rs3853839 on the basis of logistic regression analysis ($P_{adjusted}$ = 0.019, OR 2.10, 95% CI 1.13 to 3.93 under the recessive model).

Analysis of association between *TLR7* SNPs and*TLR7* mRNA levels

To investigate the functional significance of the *TLR7* SNPs, we analyzed the association between*TLR7* SNPs and *TLR7* mRNA levels (Figure 2). The *TLR7* mRNA levels in PBMNCs from Japanese female SLE patients were measured using RT-PCR assay and were compared among individuals carrying each genotype. Although not statistically significant because of the limited sample size, a tendency toward an association of rs3853839G with elevated *TLR7* mRNA levels was observed (P = 0.20 by Kruskal-Wallis test). This tendency was consistent with the observations in the Chinese population [7], which demonstrated increased *TLR7* transcripts in individuals carrying rs3853839G. On the other hand, evidence for an association of the intronic SNPs with mRNA levels was not observed.

Discussion

In the recently reported multicenter study, an association of rs3853839 was originally found by screening the *TLR7-TLR8* region in Chinese and Korean populations and was subsequently replicated in Chinese and Japanese populations [7]. In the process of the study, some population difference was noted for rs3853839 and other SNPs,

Table 1 Association of *TLR7* SNPs with SLE in a Japanese population[a]

					Allelic association		Dominant model		Recessive model	
Study population	Genotype, *n* (%)			Risk allele, *n* (%)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)
rs3853839	G/G	G/C	C/C	G						
SLE	197 (57.3)	125 (36.3)	22 (6.4)	519 (75.4)	0.017	1.36 (1.06 to 1.75)	0.030	1.87 (1.05 to 3.31)	0.072	1.34 (0.97 to 1.84)
Controls	137 (50.0)	106 (38.7)	31 (11.3)	380 (69.3)						
rs179019	A/A	C/A	C/C	A						
SLE	45 (13.1)	131 (38.1)	168 (48.8)	221 (32.1)	0.17	1.19 (0.93 to 1.52)	0.77	1.05 (0.76 to 1.44)	0.016[b]	2.02 (1.15 to 3.54)
Controls	19 (6.9)	118 (43.1)	137 (50.0)	156 (28.5)						
rs179010	T/T	C/T	C/C	T						
SLE	61 (17.7)	156 (45.3)	127 (36.9)	278 (40.4)	0.062	1.25 (0.99 to 1.57)	0.36	1.16 (0.84 to 1.61)	0.018	1.75 (1.10 to 2.80)
Controls	30 (10.9)	133 (48.5)	111 (40.5)	193 (35.2)						

[a]*TLR7*, Toll-like receptor 7 gene; SNP, single-nucleotide polymorphism; 95% CI, confidence interval; OR, odds ratio; SLE, systemic lupus erythematosus. Genotype and allele frequencies are shown in parentheses (%). Association was tested by χ^2 analysis or Fisher's exact test using 2 × 2 contingency tables under the indicated models for rs3853839G, rs179019A and rs179010 T alleles. [b]Fisher's exact test was used.

even among these East Asian populations. Because association between *TLR7* and SLE had not been examined in a systematic manner in a Japanese population, we thought that *TLR7* SNPs other than rs3853839 might also contribute to SLE.

To explore such a possibility, we analyzed the association of eight tag SNPs in *TLR7* and the newly detected association of two SNPs in intron 2, rs179019 and rs179010. Conditional logistic regression analysis indicated that the association of the intronic SNPs cannot be explained by LD with rs3853839. In agreement with these results, the association of the intronic SNPs remained significant after excluding the effect of the 3'UTR SNP by testing the association only among individuals carrying the 3'UTR risk allele. Furthermore, haplotype analysis showed significant association of the haplotype containing all of the three SLE risk alleles, but not of the other haplotypes. All of these results support the possibility that the possession of both the 3'UTR and intronic risk alleles may confer further risk for SLE.

Although rs179019 and rs179010 were also investigated in the Discovery Panel in the previous study, the majority of whom were Chinese and Korean participants, no significant association was detected [7]. The Japanese patients and controls analyzed in this study were not included in the Discovery Panel. Population difference was also observed for rs3853839 between the Chinese and Korean populations, as this SNP was strongly associated with SLE in Chinese, but not in Koreans [7], suggesting that the genetic background with respect to *TLR7* association with SLE might be somewhat different, even among the closely related East Asian populations. Minor allele frequencies of rs179019 and rs179010 in the HapMap CHB (Han Chinese in Beijing) samples (rs179019: 30.9%, rs179010: 37.3%) available in the International HapMap database [9] are similar to those in the Japanese observed in this study (rs179019: 28.5%, rs179010: 35.2%). Thus, the difference in the association cannot be explained by differences in the minor allele frequencies. We cannot rule out the possibility that another SNP tagged by rs179019 and rs179010 in Japanese, but not in Chinese or Koreans because of difference in the LD status, might play a causative role. Such a possibility would be addressed by resequencing the entire *TLR7* region.

There is growing evidence to support involvement of type I IFN in the development of SLE. TLR7 is crucial for the production of type I IFN. Thus, the most plausible role of *TLR7* SNPs in SLE pathogenesis is likely to

Table 2 Conditional logistic regression analysis of *TLR7* SNPs[a]

				$P_{adjusted}$[b]		
SNP	Risk allele	Model	P^c	rs3853839	rs179019	rs179010
rs3853839	G	Codominant	0.021	NA	0.040	0.047
rs179019	A	Recessive	0.014	0.026	NA	0.24
rs179010	T	Recessive	0.019	0.042	0.42	NA

[a]*TLR7*, Toll-like receptor 7 gene; SNP, single-nucleotide polymorphism; NA, not applicable; [b]*P* value adjusted for each SNP by conditional logistic regression analysis using the indicated model; [c]*P* value for each SNP calculated by logistic regression analysis. The indicated model showed the lowest *P* value for each SNP.

Table 3 Independent effect of intron 2 SNPs in the carriers of the 3' UTR risk genotypes[a]

Risk genotype			Study group, *n* (%)				
rs3853839	rs179019	rs179010	SLE (*N* = 322)	Controls (*N* = 243)	*P*	OR	95% CI
G/G or G/C	A/A	T/T	42 (13.0)	14 (5.8)			
+	+	+	280 (87.0)	229 (94.2)	0.0043	2.45	1.31 to 4.60
+	Others					Reference	

[a]SNP, single-nucleotide polymorphism; 3' UTR, 3' untranslated region; OR, odds ratio; 95% CI, 95% confidence interval. Genotype frequencies are shown in parentheses (%). *P* value was calculated using Fisher's exact test.

Table 4 Estimated haplotype frequencies in SLE and controls[a]

Haplotype	rs179019	rs179010	rs3853839	SLE	Controls	Permutation *P* value
1	C	C	G	40.6%	38.0%	0.94
2	C	C	C	18.2%	24.1%	0.068
3[b]	A	T	G	26.1%	20.3%	0.081
4	C	T	G	8.5%	8.8%	1.0
5	A	T	C	5.2%	5.6%	1.0

[a]SLE, systemic lupus erythematosus; *P* values were calculated by permutation test (100,000 permutations) using HaploView version 4.0 software; [b]each haplotype was also tested for association under the recessive model. Individuals homozygous at all three SNPs were considered homozygous for the haplotype. Only haplotype 3 was significantly associated with SLE under the recessive model (SLE, 31 (9.0%) of 344; control, 11 (4.0%) of 274; $P = 0.016$ by Fisher's exact test; odds ratio 2.37, 95% confidence interval 1.17 to 4.80).

Table 5 Association study of *TLR7* SNPs with clinical characteristics of SLE[a]

		SLE total		Anti-Sm antibodies		Anti-dsDNA antibodies		Renal disorder	
SNP	Model	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)
rs3853839	Allele	0.017	1.36 (1.06 to 1.75)	0.032	1.65 (1.04 to 2.62)	0.014	1.40 (1.07 to 1.84)	0.025	1.40 (1.04 to 1.89)
rs179019	Recessive	0.016[b]	2.02 (1.15 to 3.54)	1.0[b]	0.89 (0.29 to 2.73)	0.029[b]	1.93 (1.07 to 3.48)	0.011[b]	2.25 (1.21 to 4.18)
rs179010	Recessive	0.018	1.75 (1.10 to 2.80)	0.67[b]	1.16 (0.51 to 2.67)	0.030	1.72 (1.05 to 2.83)	0.042	1.73 (1.02 to 2.95)

[a]*TLR7*, Toll-like receptor 7 gene; SNP, single-nucleotide polymorphism; SLE, systemic lupus erythematosus; anti-Sm, anti-Smith; dsDNA, double-stranded DNA; OR, odds ratio, 95% CI, confidence interval; [b]Fisher's exact test was used. Association was tested by χ^2 analysis or Fisher's exact test using 2 × 2 contingency tables under the indicated model for rs3853839G, rs179019A and rs179010 T allele. All SLE as well as each SLE subset were compared with healthy controls.

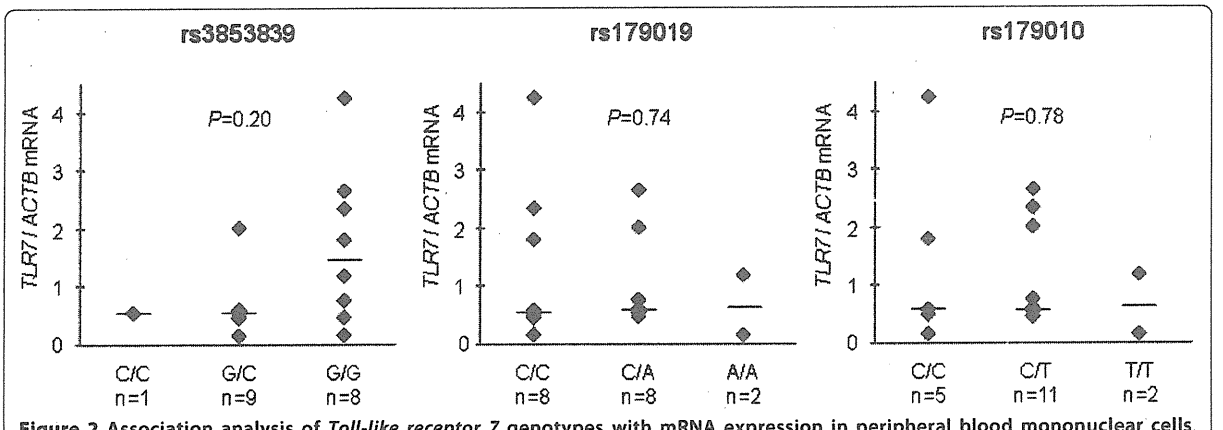

Figure 2 Association analysis of *Toll-like receptor 7* genotypes with mRNA expression in peripheral blood mononuclear cells. Association between *Toll-like receptor 7* (*TLR7*) single-nucleotide polymorphisms (SNPs) and *TLR7* mRNA levels was examined by using the Kruskal-Wallis test. Relative quantitative levels of *TLR7* mRNA were normalized to β-actin (*ACTB*) mRNA levels. Bars indicate median values in each group. The experiments were performed in triplicate.

be explained by elevated type I IFN production. The sera of SLE patients displayed elevated levels of type I IFN, and expression of IFN-inducible genes in PBMNCs was also upregulated in SLE [10]. Occasional occurrence of SLE symptoms following treatment with IFNα in patients with cancer or hepatitis underscored the relevance of type I IFN [10]. Type I IFN is thought to be a potential therapeutic target for SLE, and clinical trials of anti-IFNα antibodies in SLE are currently underway [11].

Recent genetic studies have identified an association of type I IFN pathway-related genes, IFN regulatory factor 5 (*IRF5*) and *STAT4*, with SLE in various populations [10,12-16]. An *IRF5* SLE risk haplotype has been shown to be associated with high serum IFNα activity in SLE patients [17], whereas the *STAT4* SLE risk variant was associated with increased sensitivity to IFNα *in vivo* [18]. These observations, as well as the previous study on *TLR7* showing upregulation of TLR7 in the risk genotype [7], suggest that SLE-associated alleles in the type I IFN pathway are gain-of-function alleles in nature.

Another potential role of *TLR7* polymorphisms may be related to the induction of proinflammatory cytokines. IRF5 is activated by TLR7 signaling and regulates the expression of many genes, including type I IFN and proinflammatory cytokines [19]. STAT4 is activated by type I IFN as well as interleukin 12 and plays a role in Th1 differentiation [20]. In view of these observations, the association between *TLR7* SNPs and SLE might also be explained by overproduction of proinflammatory cytokines in addition to type I IFN.

There are conflicting reports about copy number variation (CNV) of *TLR7*. Initially, the existence of CNV was reported by Kelley *et al.* [21]. They showed that, although common CNV was observed in Caucasians and African-Americans, no association with SLE was detected [21]. Recently, García-Ortiz *et al.* [22] reported an association of CNV with childhood-onset SLE in a Mexican population. In contrast to these observations, Shen *et al.* [7] did not find common *TLR7* CNV in multiple populations, including Asians. The latter observation is consistent with the fact that no CNV was registered in the Database of Genomic Variants [23], which includes results derived from the HapMap JPT (Japanese in Tokyo) samples.

Although our observation in the expression analysis supported the previous report that indicated the association between the risk allele of the 3'UTR SNP and elevated expression of *TLR7* [7], evidence for the association of the intronic SNPs with levels of *TLR7* mRNA was not observed, and therefore the molecular mechanism of the intronic SNPs requires further study. TLR7 is mainly expressed in pDCs and B cells. pDCs represent the major source of type I IFN, but constitute less than 1% of PBMNCs. If the intronic SNPs have a regulatory role in a cell type-specific fashion and influence the expression level of *TLR7* in pDCs but not in other white blood cells, such an effect may not have been detected in the analysis of total PBMNCs. In addition, the sample size of this study may not have been large enough for us to conclude that the intronic SNPs have no effect on the expression of *TLR7*.

Because we focused only on the Japanese population, the sample size of this study was limited and the observed statistical association was modest. Therefore, the association of the intronic SNPs should be confirmed in future independent studies.

Conclusions

TLR7 intronic SNPs rs179019 and rs179010 are associated with SLE independently of 3'UTR SNP rs3853839 in Japanese women. Our findings support the genetic role of *TLR7* SNPs in Asian populations with SLE.

Abbreviations

95% CI: 95% confidence interval; CNV: copy number variation; CpG: cytidine-phosphate-guanosine; IFN: interferon; LD: linkage disequilibrium; OR: odds ratio; PBMNCs: peripheral blood mononuclear cells; pDCs: plasmacytoid dendritic cells; RT-PCR: reverse transcription polymerase chain reaction; SLE: systemic lupus erythematosus; SNP: single-nucleotide polymorphism; ssRNA: single-stranded RNA; TLR: Toll-like receptor; UTR: untranslated region; Yaa: Y chromosome-linked autoimmune accelerator.

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Authors' contributions

AK participated in the study design; carried out all genotyping, expression analysis and statistical analyses; and wrote the manuscript. HF, YK, SI, TH, MK, IM, ST, YT, HH and TS recruited the patients and controls and collected clinical information. NT designed and coordinated the study and helped in the manuscript preparation. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Activation of natural killer T cells by α-carba-GalCer (RCAI-56), a novel synthetic glycolipid ligand, suppresses murine collagen-induced arthritis

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Summary

Alpha-carba-GalCer (RCAI-56), a novel synthetic analogue of α-galactosylceramide (α-GalCer), stimulates invariant natural killer T (NK T) cells to produce interferon (IFN)-γ. IFN-γ exhibits immunoregulatory properties in autoimmune diseases by suppressing T helper (Th)-17 cell differentiation and inducing regulatory T cells and apoptosis of autoreactive T cells. Here, we investigated the protective effects of α-carba-GalCer on collagen-induced arthritis (CIA) in mice. First, we confirmed that α-carba-GalCer selectively induced IFN-γ in CIA-susceptible DBA/1 mice *in vivo*. Then, DBA/1 mice were immunized with bovine type II collagen (CII) and α-carba-GalCer. The incidence and clinical score of CIA were significantly lower in α-carba-GalCer-treated mice. Anti-IFN-γ antibodies abolished the beneficial effects of α-carba-GalCer, suggesting that α-carba-GalCer ameliorated CIA in an IFN-γ-dependent manner. Treatment with α-carba-GalCer reduced anti-CII antibody production [immunoglobulin (Ig)G and IgG2a] and CII-reactive interleukin (IL)-17 production by draining lymph node (DLN) cells, did not induce apoptosis or regulatory T cells, and significantly increased the ratio of the percentage of IFN-γ-producing T cells to IL-17-producing T cells (Th1/Th17 ratio). Moreover, the gene expression levels of IL-6 and IL-23p19, Th17-related cytokines, were reduced significantly in mice treated with α-carba-GalCer. In addition, we observed higher IFN-γ production by NK T cells in α-carba-GalCer-treated mice in the initial phase of CIA. These findings indicate that α-carba-GalCer polarizes the T cell response toward Th1 and suppresses Th17 differentiation or activation, suggesting that α-carba-GalCer, a novel NK T cell ligand, can potentially provide protection against Th17-mediated autoimmune arthritis by enhancing the Th1 response.

Keywords: collagen-induced arthritis, glycolipid ligand, natural killer T cells, Th1, Th17

Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by chronic inflammation of the synovial tissues and subsequent destruction of multiple joints [1]. Although the pathogenesis of RA remains unclear, proinflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, play a central role in this process [2]. There is general agreement that interferon (IFN)-γ-producing T helper type 1 (Th1) cells play a pathogenic role in the development of RA. However, several recent studies on animal models of autoimmune diseases suggested that IL-17-producing Th17 cells, but not IFN-γ-producing Th1 cells, play a crucial role in the development of RA. For example, mice deficient in Th1 cytokines, such as IFN-γ- and IL-12-deficient mice, exhibited severe symptoms in collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) [3–5], whereas those deficient in Th17 cytokines, such as IL-17- and IL-23-deficient mice, were resistant to these diseases [6–9]. Harrington *et al.* [10] suggested that IFN-γ suppresses the differentiation of naive CD4 T cells to Th17 cells. Furthermore, Chu *et al.* [11] showed that IFN-γ also suppressed IL-17 production by differentiated Th17 cells. In addition, IFN-γ plays a suppressor role by inducing myeloid suppressor cells that induce apoptosis of activated T cells in the chronic immune response,

such as the late phase of mycobacterial infection or the autoimmune response [12,13]. It has also been reported that IFN-γ is necessary for the conversion of $CD4^+$ $CD25^-$ T cells to $CD4^+$ regulatory T cells (T_{regs}) during EAE [14]. Thus, IFN-γ is thought to be a suppressive cytokine in several animal models of autoimmune diseases.

Natural killer T (NK T) cells are a subset of T lymphocytes that express NK cell markers, such as NK1·1, in mice. In mice, the majority of NK T cells express an invariant T cell receptor (TCR) encoded by Vα14Jα18, which is associated with highly skewed sets of Vβs, mainly Vβ8·2. The receptor recognizes glycolipid antigen presented by CD1d, a non-classical antigen-presenting molecule [15,16]. Stimulation of TCR induces NK T cells to rapidly secrete large amounts of proinflammatory and anti-inflammatory cytokines, such as IL-4 and IFN-γ [16]. Because of this property, NK T cells are known as immune regulators. Functional defects within NK T cells and reduced numbers of these cells are associated with various human autoimmune diseases [17–20]. In animal models, NK T cells suppress the development and progression of diabetes mellitus [21], EAE [22] and systemic lupus erythematosus (SLE) [23]. However, NK T cells also act as effector cells in some murine models of RA by promoting Th17 responses, producing IL-17 and suppressing the production of transforming growth factor (TGF)-β [24–28]. These evidences suggest a dual function for NK T cells in autoimmunity.

Alpha-galactosylceramide (α-GalCer) is a potent NK T cell ligand. The synthetic ligand induces NK T cell activation and secretion of various cytokines, such as IFN-γ, IL-4, and IL-17 [29]. To control NK T cell activation and cytokine secretion, several analogues of α-GalCer have been synthesized. OCH, which is an α-GalCer analogue with a shorter sphingosine chain, stimulates IL-4 production selectively by NK T cells [30]. The α-GalCer analogue suppressed the EAE-inducing antigen-specific Th2 response. Conversely, α-C-GalCer, a C-glycoside (carbon glycoside) analogue of α-GalCer, activated iNK T cells at very low concentrations and promoted Th1 responses *in vivo* [31]. More recently, Tashiro *et al.* [32] synthesized α-carba-GalCer, which strongly induced NK T cell-mediated Th1 cytokines in a fashion similar to α-C-GalCer [32].

In the present study, we found that α-carba-GalCer inhibited the development of CIA. This suppressive effect was dependent on IFN-γ induced by NK T cells. The results also showed that α-carba-GalCer suppressed the production of both anti-type II collagen (CII) antibodies in serum and IL-17 in draining lymph nodes (DLNs) in response to CII. This lower pathogenic Th17 response resulted from enhancement of the Th1 response via α-carba-GalCer-dependent IFN-γ. Thus, α-carba-GalCer could be a potentially useful therapeutic agent for Th17-mediated autoimmune diseases.

Materials and methods

Mice

Male DBA/1J mice were purchased from Charles River Japan (Tokyo, Japan). The animals were kept under specific pathogen-free conditions and studied at 6–9 weeks of age. The Institutional Animal Care and Use Committee of the University of Tsukuba approved all experimental plans.

Reagents

α-GalCer was purchased from Funakoshi (Tokyo, Japan) and α-carba-GalCer (RCAI-56) was kindly provided by Dr Masaru Taniguchi (Riken Research Center for Allergy and Immunology, Yokohama, Japan). The structures of these two reagents are shown in Fig. 1a. The stock solutions of these glycolipids were dissolved originally in 100% dimethyl sulphoxide (DMSO) at 1 mg/ml and diluted in phosphate-buffered saline (PBS) just before injection into the mice. The

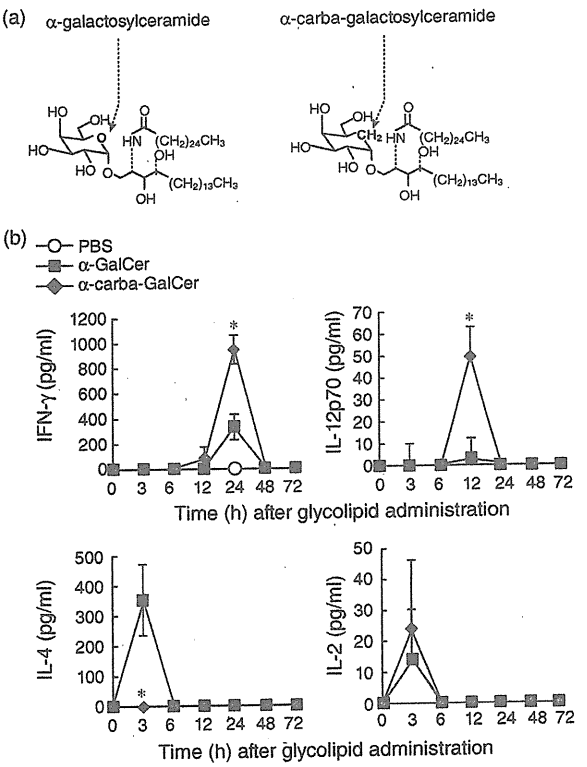

Fig. 1. Natural killer (NK) T cell response to α-carba-GalCer in CIA-susceptible mice. (a) Structure of α-galactosylceramide (α-GalCer) and α-carba-GalCer. (b) DBA/1 mice were injected intravenously (i.v.) with 2 μg of α-GalCer, α-carba-GalCer or vehicle (n = 3/group). The levels of interleukin (IL)-2, IL-4, IL-12 and interferon (IFN)-γ were measured by enzyme-linked immunosorbent assay (ELISA). Data are representative of three experiments. Values represent mean ± standard deviation (*$P < 0·05$ *versus* α-GalCer).

following monoclonal antibodies (mAbs) were used for flow cytometric analysis: allophycocyanin (APC)-conjugated anti-mouse CD4 (clone: GK1·5; eBioscience, San Diego, CA, USA), peridinin chlorophyll (PerCP)-conjugated anti-CD3, fluorescein isothiocyanate (FITC)- and PerCP-conjugated anti-CD19, FITC- or APC-conjugated anti-IFN-γ (clone XMG1·2; BioLegend, San Diego, CA, USA); and FITC- or phycoerythin (PE)-conjugated anti-IL-17 (clone TC11-18H10·1; BD Pharmingen, Franklin Lakes, NJ, USA), and PE-conjugated CD1d-tetramer (MBL International, Woburn, MA, USA). The following mAbs were used for anti-CII specific IgGs enzyme-linked immunosorbent assay (ELISA): polyclonal rabbit anti-mouse immunoglobulins/HRP (Dako, Glostrup, Denmark), rabbit anti-mouse IgG1-horseradish peroxidase (HRP) (Zymed Laboratories, San Francisco, CA, USA) and rat anti-mouse IgG2a-HRP (Zymed). Bovine type II collagen was purchased from Collagen Research Center (Tokyo, Japan) and dissolved under constant stirring overnight at 4°C in 0·05 M acetic acid in phosphate-buffered saline (PBS) to be used for immunization, or in 0·05 mM Tris-HCl, 0·2 M NaCl, pH 7·4 for ELISA.

Cell preparation

Lymphocytes were isolated from the liver, spleen or DLN, as described previously [33].

Induction of CIA and glycolipid administration

Mice were immunized subcutaneously (s.c.) at the base of their tails with 100 μg of bovine CII emulsified with complete Freund's adjuvant (CFA) (Difco, Detroit, MI, USA). An emulsion was formed by 2 mg/ml of CII with an equal volume of CFA. Two micrograms of either α-GalCer or α-carba-GalCer was added to and emulsified with CII/CFA. The emulsion was injected s.c. into the tail base. A booster dose of 100 μg of CII solution was injected intraperitoneally (i.p.) on day 21. For intracellular cytokine staining, 50 μg was injected into each footpad of the hind paw. Joint swelling was monitored and scored as follows: 0, no swelling or redness; 1, swelling or redness in one joint; 2, involvement of > 2 joints; and 3, severe arthritis affecting all paws and joints. The score for each animal represented the sum of the score for all four paws. The clinical score was calculated using the results of all mice in the group.

Antibody treatment

Systemic IFN-γ neutralization was carried out by treatment with anti-IFN-γ mAb, at 150 μg/mouse injected i.p. on day 0.

Enzyme-linked immunosorbent assay

To determine the CII-specific IgG subtype, bovine CII (10 μg/ml) was coated onto ELISA plates and incubated at 4°C overnight. After two washes with washing buffer (0·05% Tween 20 in PBS), the blocking solution [2% bovine serum albumin (BSA) in PBS] was applied for 1 h at room temperature. After two washes, serially diluted serum samples were added to the CII-coated wells for 1 h. After three washes, HRP-conjugated anti-mouse IgG, IgG1, or IgG2a was added at a final dilution of 1:4000 and incubated for 1 h. After three washes, colour was developed using peroxidase substrate (KPL). The plates were incubated for 15 min at room temperature, and the optical density was read at 450 nm using a microplate reader.

The concentrations of IL-2, IL-4, IL-10, IL-12, IL-17 and IFN-γ in the serum and in the culture supernatants were measured using an ELISA kit (Duoset; R&D Systems, Abingdon, UK), according to the protocols supplied by the manufacturer.

NK T cell response to glycolipid ligand

For the *in vivo* assay, naive DBA/1 mice ($n = 3$) were injected intravenously (i.v.) with 2 μg/mouse of glycolipid ligands and serum was collected at various time points. The concentration of cytokines in serum was determined by ELISA.

CII-reactive T cell response

Twelve days after CII/glycolipid injection, the DLN (inguinal) cells were collected and restimulated with 100 μg/ml of denatured bovine CII (60°C, 10 min) for 72 h. The cells were cultured in complete RPMI-1640 medium containing antibiotics and 5% fetal calf serum (FCS) and incubated at 37°C. The concentration of cytokines in the culture supernatants was determined by ELISA.

Flow cytometry

Cells were stained at 4°C in PBS containing 2% heat-inactivated FCS, incubated for 5 min with anti-CD16/32 to block Fcr receptors, and then incubated for 30 min with various mAbs at appropriate dilutions. A mouse T_{reg} cell staining kit (eBioscience) was used to stain T_{reg} cells following the protocol provided by the manufacturer. Apoptosis was examined by the annexin V/propidium iodide (PI) assay (eBioscience) using the protocol supplied by the manufacturer. Intracellular cytokines were stained using an intracellular staining kit (BD Pharmingen). Lymphocytes from CII-immunized mice were stimulated with phorbol myristate acetate (PMA) (50 μg/ml) and ionomycin (1 g/ml) in the presence of GolgiStop solution (BD Pharmingen) for 4 to 6 h. Flow cytometry was performed on a four-colour fluorescence activated cell sorter (FACS)Calibur. Dead cells were excluded based on the forward- and side-scatter characteristics. The results were analysed using Mac CellQuest software (BD Biosciences, San Jose, CA, USA).

Quantification of cytokine transcripts

Total RNA was extracted with an RNA extraction kit (Isogen; Nippon Gene, Tokyo, Japan) in accordance with the instructions provided by the manufacturer. cDNA was obtained by reverse transcription with a commercially available kit (Fermentas, Glen Burnie, MD, USA). We used a *Taq*Man assay-on-demand gene expression product (Applied Biosystems, Foster City, CA, USA). The expression levels of IL-6, IL-23p19, TGF-β and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (assay ID IL-6: Mm00446191; IL-23p19: D1160011; TGF- β: 01178819; GAPDH: 99999915, respectively; Applied Biosystems) were normalized relative to the expression of GAPDH. Analysis was performed with an ABI Prism 7500 apparatus (Applied Biosystems).

Statistical analysis

Values were expressed as the mean ± standard error of the mean (s.e.m.). Differences between groups were examined for statistical significance using the *t*-test. Probability values less than 0·05 were considered significant.

Results

NK T cell response to α-carba-GalCer in CIA-susceptible DBA/1 mice

First, we examined whether α-carba-GalCer causes a differential simulation of Th1 cytokine production in DBA/1 mice (known as the CIA-susceptible strain). Mice were injected with 2 μg of either α-carba-GalCer or α-GalCer and their blood cytokine levels were then measured at various time-points by ELISA. α-GalCer, but not α-carba-GalCer, increased IL-4 concentrations at 3 h after injection (Fig. 1b). Conversely, IFN-γ and IL-12 production was induced by both glycolipids, but the levels in α-carba-GalCer-treated mice were higher than those in mice treated with α-GalCer (Fig. 1b). IL-2-production was observed in both α-GalCer- and α-carba-GalCer-treated mice and the concentration of IL-2 in α-carba-GalCer-treated mice was comparable to that in α-GalCer-treated mice (Fig. 1b).

These data support the findings of a previous study [32], and suggest that α-carba-GalCer is a potent ligand for NK T cells and can selectively induce a Th1-type response.

α-carba-GalCer suppresses CIA in an IFN-γ-dependent manner

To examine the effects of α-carba-GalCer on the onset and severity of CIA, male *DBA/1* mice with type II collagen (CII) were injected s.c. with α-carba-GalCer, α-GalCer or vehicle on day 0. As shown in Fig. 2a, α-carba-GalCer treatment tended to reduce the incidence of CIA compared with the vehicle treatment, although the difference was not significant. In contrast, α-GalCer-treatment did not affect the incidence of the disease. The clinical score of arthritis of the α-carba-GalCer-treated group was significantly lower than that of the vehicle-treated groups ($P < 0·05$, Fig. 2b). To

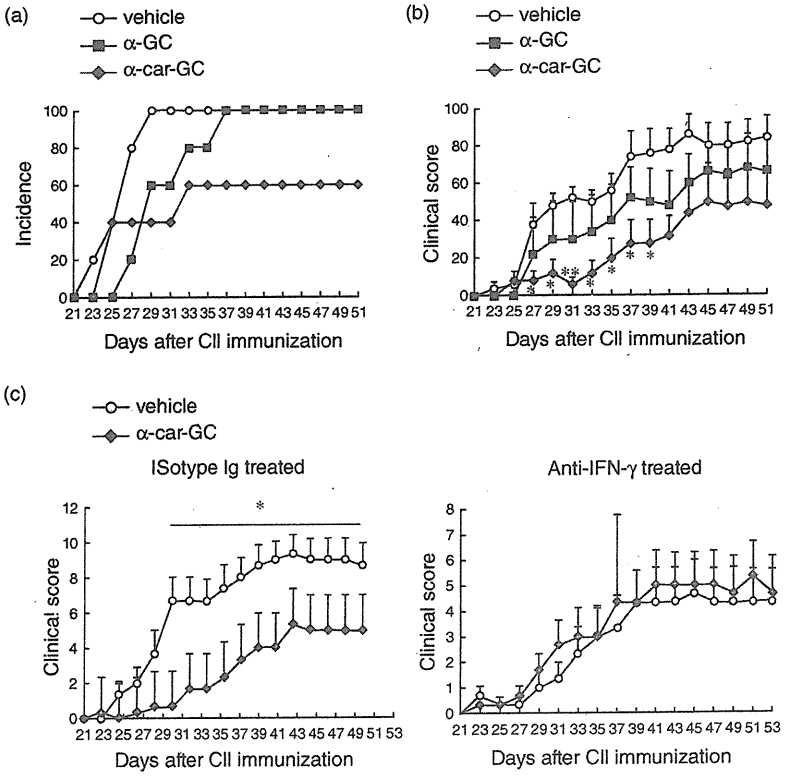

Fig. 2. Effects of α-carba-GalCer on CIA. DBA/1 mice were immunized with CII in CFA and 2 μg of α-galactosylceramide (α-GalCer) ($n = 5$), α-carba-GalCer ($n = 5$) or vehicle ($n = 5$). (a) Incidence and (b) clinical score of arthritis. Mice were immunized with CII/glycolipids as described above and injected intraperitoneally (i.p.) with anti-interferon (IFN)-γ (160 μg/mouse) or isotype on day 0. Subsequently (c) the clinical score of arthritis was monitored serially from day 21. Data are representative of two experiments. Values represent mean ± standard error of the mean of three mice (c) or five mice (a, b) per group (*$P < 0·05$ *versus* vehicle-treated mice).

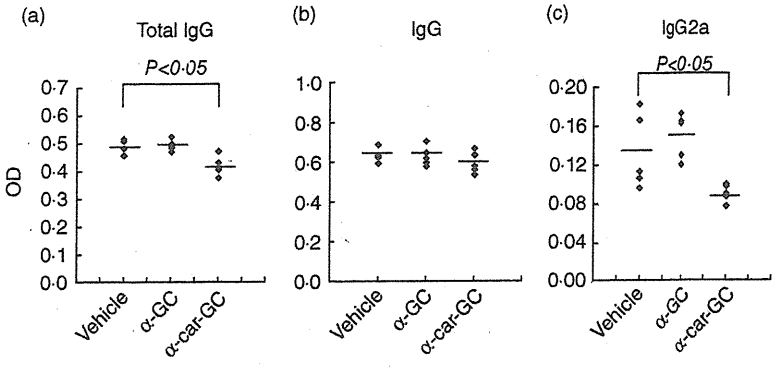

Fig. 3. Production of anti-type II collagen (CII) antibodies in α-carba-GalCer-treated mice. DBA/1 mice were immunized with CII in complete Freund's adjuvant (CFA) and 2 μg of α-galactosylceramide (α-GalCer) ($n = 5$), α-carba-GalCer ($n = 5$) or vehicle ($n = 5$). Sera were obtained on day 35, and (a) the titres of anti-CII-specific immunoglobulins (IgGs) (b) IgG1 and (c) IgG2a were analysed by enzyme-linked immunosorbent assay. Data are representative of three experiments. Values represent mean ± standard error of the mean of five mice per group.

determine whether this therapeutic effect was dependent on IFN-γ, IFN-γ was neutralized in the α-carba-GalCer-treated mice. IFN-γ neutralization at the time of CII immunization abolished the beneficial effect of α-carba-GalCer on CIA, but had no effect in the vehicle-treated mice (Fig. 2c). These data indicate that α-carba-GalCer ameliorates CIA and that this action is mediated through IFN-γ.

α-carba-GalCer suppresses anti-CII antibodies and CII-reactive IL-17 production

In general terms, CIA is thought to be an autoreactive T and B cell-dependent arthritis [34]. Therefore, we determined the anti-CII antibody titre in α-carba-GalCer-treated mice. As shown in Fig. 3a, the anti-CII IgG titre was significantly lower in the α-carba-GalCer-treated mice than in the control mice. Specifically, the anti-CII IgG2a titre was lower in α-carba-GalCer-treated mice, but there were no differences in the anti-CII IgG1 subclass titres among the groups (Fig. 3b,c). Twelve days after injection of CII/α-carba-GalCer, cells were collected from the DLN and restimulated with CII *in vitro*. IL-17 production by DLN cells was lower in the α-carba-GalCer-treated mice than in the control mice (Fig. 4). In contrast, IFN-γ production in α-carba-GalCer-treated mice was comparable to that in α-GalCer- and vehicle-treated mice. None of the cultures showed production of IL-4 and IL-10 (data not shown). These results suggest that α-carba-GalCer treatment suppresses antigen-specific Th17 cell and B cell responses in the development of CIA.

α-carba-GalCer does not alter the number of forkhead box P3 (FoxP3$^+$) T_{regs} or apoptotic T cells

IFN-γ is reported to play an important role in the induction of apoptosis and T_{regs} in autoimmune disease [13,14]. Therefore, we examined whether the beneficial effects of α-carba-GalCer were mediated by induction of apoptosis of T cells or T r_{egs}. As shown in Fig. 5a, treatment with α-carba-GalCer did not increase apoptosis, as assessed by the annexin/PI assay. Further analysis indicated that the proportion of FoxP3$^+$ T_{regs} was not significantly different between the α-carba-GalCer-treated mice and the control mice (Fig. 5b). These results suggest that the beneficial effects of α-carba-GalCer on CIA are unlikely to be mediated by induction of apoptosis or T_{regs}.

Alternation of the Th1/Th17 cytokine balance in α-carba-GalCer-treated mice

Because recent studies have shown that IFN-γ suppresses IL-17 production in CIA [10,11], we examined the hypothesis that the beneficial effect of α-carba-GalCer was due to the suppression of IL-17 production by IFN-γ. For this purpose, we determined the proportion of IFN-γ- and IL-17-producing T cells in α-carba-GalCer-treated mice. The proportion of IL-17-producing T cells in α-carba-GalCer-treated mice was significantly smaller than in vehicle-treated mice when analysed immediately *ex vivo* 10 days after α-carba-GalCer immunization with CII (Fig. 6a,b,

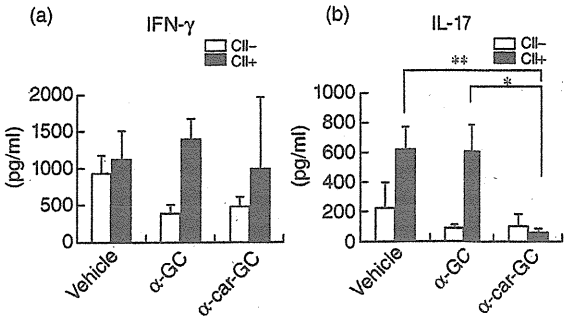

Fig. 4. CII-reactive T cell response in α-carba-GalCer-treated mice. DBA/1 mice were immunized with type II collagen (CII) in complete Freund's adjuvant (CFA) and 2 μg of α-galactosylceramide (α-GalCer) ($n = 3$), α-carba-GalCer ($n = 3$) or vehicle ($n = 3$). Twelve days after CII/glycolipid immunization, draining lymph node (DLN) cells were collected and then stimulated with CII for 72 h. Interferon (IFN)-γ and interleukin (IL)-17 levels in culture supernatant were determined by enzyme-linked immunosorbent assay. Data are representative of three experiments. Values represent mean ± standard error of the mean of three mice per group (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$).