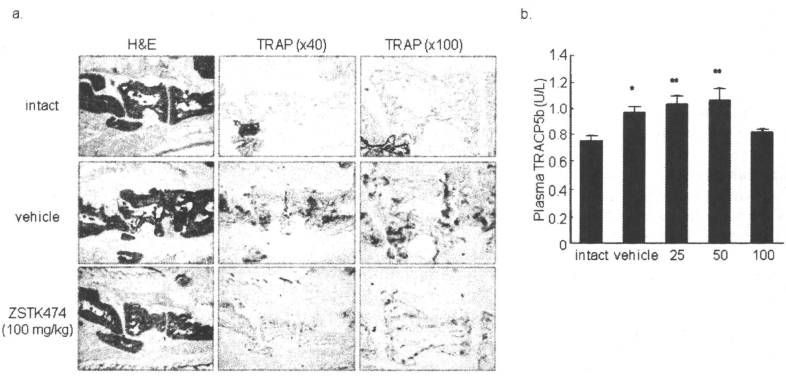

Figure 5 Administration of ZSTK474 inhibited *in vivo* OC formation and bone resorption in CIA mice. a) The synovial sections described above were stained with H&E and also with TRAP to examine *in vivo* OC formation. Representative results are shown. **b)** Plasma levels of TRACP5b were measured. The levels of TRACP5b in vehicle- 25 mg/kg, and 50 mg/kg ZSTK474-treated mice, but not 100 mg/kg ZSTK474-treated mice, were significantly raised in comparison with that of intact mice (*$P < 0.05$, **$P < 0.01$).

(PDGF) in murine embryonic fibroblasts [29]. In OCs, the SH3 domain of tyrosine kinase, *c-src*, interacts with the p85 regulatory domain of PI3-K, and this signaling pathway is crucial for colony-stimulating factor-1-induced OC spreading [22]. Therefore, ZSTK474 might suppress the cytoskeletal change of OCs, resulting in the reduced bone resorption observed in this study.

ZSTK474 suppressed inflammation and also protected against joint destruction in CIA in mice. Although it is difficult to ascertain the direct effect of ZSTK474 on OCs in this model, the TRAP-staining of the synovial tissue sections demonstrated marked reduction of OC formation. In addition, plasma levels of TRACP5b, that reported to correspond with systemic but not localized bone resorption [42], were not increased in 100 mg/kg ZSTK474-treated mice. This result implied that 100 mg/kg of ZSTK474 possibly prevented the systemic bone resorption.

Both the semi-therapeutic and therapeutic treatments of ZSTK474 ameliorated joint inflammation in a mouse model of RA. This anti-rheumatic effect might be explained by contribution of PI3-K to activation, proliferation and migration of inflammatory cells, such as lymphocytes, macrophages, neutrophils, mast cells and synovial fibroblasts [9]. However, the titers of antibody to type II collagen were not significantly different between vehicle- and ZSTK474-treated mice in this experiment (data not shown). Regarding migration, chemokine receptors, such as the MCP-1 receptor and the RANTES receptor, are GPCRs that associate with PI3-Kγ and induce signals for chemotaxis of the inflammatory cells [9]. It was reported that the PI3-Kγ-selective inhibitor suppressed joint inflammation in mouse CIA by inhibiting migration of neutrophils to the joints [43]. This inhibitory process might occur in the ZSTK474-treated mice. Additionally, synovial pannus tissues of patients with RA express phosphorylated Akt [44] and exhibit tumor-like behaviors, such as angiogenesis, proliferation and invasion. A recent report demonstrated potent antiangiogenic activity for ZSTK474, which could be attributed to both inhibition of VEGF secretion by cancer cells and inhibition of PI3-K in endothelial cells [45]. These findings also account for the effects of ZSTK474 on CIA mice. In addition, ZSTK474 did not affect the count of peripheral white blood cells and red blood cells (data not shown). Further studies are underway to evaluate how ZSTK474 exerts anti-inflammatory activity *in vivo*.

Clinical studies have demonstrated that the degree of inflammation and the progression of joint destruction do not always correspond with each other [46,47]. In current therapy for RA, anti-rheumatic drugs are required not only to control the inflammation but also to suppress the joint destruction. On the other hand, recent reports have shown convincing pathogenic evidence for the involvement of class I PI3-K and Akt signaling pathways in synovial fibroblasts [44,48-52] and other cells [43,53,54] in patients with RA. Synovial tissue from patients with RA expressed higher levels of phosphorylated Akt than that

from patients with osteoarthritis [44]. Moreover, blocking the PI3-K/Akt pathway by intracellular gene transfer of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which dephosphorylates phosphatidylinositol - 3,4,5 - tris - phosphate (PtdIns(3,4,5)P_3) and attenuates the downstream signals of PI3-K, CIA in rats [52]. Taken together, the present results indicate that PI3-K could be a potent target for RA therapy.

Conclusions

We have demonstrated inhibitory effects of ZSTK474 on *in vitro* OC formations and CIA in mice. Inhibition of PI3-K with ZSTK474 may potentially have an anti-rheumatic effect in patients with RA.

Abbreviations

CIA: collagen-induced arthritis; ERK: extracellular signal-regulated kinase; FBS: fetal bovine serum; GCPRs: G-protein-coupled receptors; MAPK: mitogen-activated protein kinase; M-CSF: macrophage-colony stimulating factor; NFATc1: nuclear factor of activated T cells c1; OCs: osteoclasts; PDGF: platelet-derived growth factor; PI3-K: phosphoinositide 3-kinase; PTEN: phosphatase and tensin homolog deleted chromosome 10; RA: rheumatoid arthritis; RANK: receptor activator of nuclear factor κB; RANKL: RANK ligand; SHIP: Src homology-2 (SH2)-containing inositol-5-phosphatase; TNF: tumor necrosis factor; TRAP: tartrate-resistant acid phosphatase; α-MEM: alpha-minimum essential medium

Competing interests

KH, SM and TW were employed by Zenyaku Kogyo Co., Ltd (Tokyo, Japan), which is the proprietary company of ZSTK474. TY has a research fund from Zenyaku Kogyo Co., Ltd. ST, NT, TK and YT declare that they have no competing interests.

Authors' contributions

ST performed data acquisition and was involved in drafting of the manuscript. NT contributed to the study design and did most of the drafting of the manuscript. KH designed the *in vivo* and part of the *in vitro* experiments, and carried out the analysis and interpretation of data; he was also involved in drafting of the manuscript. TK participated in the *in vitro* experiments and gave helpful advice. SM contributed essentially to the animal experiments. TW provided the synthesized PI3-K inhibitors used in this study. TY fundamentally participated in the concept of the study using ZSTK474. YT supervised conception and design of the study. All authors read and approved the final manuscript.

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RESEARCH ARTICLE Open Access

Association of TNFAIP3 interacting protein 1, TNIP1 with systemic lupus erythematosus in a Japanese population: a case-control association study

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Abstract

Introduction: *TNFAIP3* interacting protein 1, *TNIP1* (ABIN-1) is involved in inhibition of nuclear factor-κB (NF-κB) activation by interacting with TNF alpha-induced protein 3, A20 (*TNFAIP3*), an established susceptibility gene to systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Recent genome-wide association studies revealed association of *TNIP1* with SLE in the Caucasian and Chinese populations. In this study, we investigated whether the association of *TNIP1* with SLE was replicated in a Japanese population. In addition, association of *TNIP1* with RA was also examined.

Methods: A case-control association study was conducted on the *TNIP1* single nucleotide polymorphism (SNP) rs7708392 in 364 Japanese SLE patients, 553 RA patients and 513 healthy controls.

Results: Association of *TNIP1* rs7708392C was replicated in Japanese SLE (allele frequency in SLE: 76.5%, control: 69.9%, $P = 0.0022$, odds ratio [OR] 1.40, 95% confidence interval [CI] 1.13-1.74). Notably, the risk allele frequency in the healthy controls was considerably greater in Japanese (69.9%) than in Caucasians (24.3%). A tendency of stronger association was observed in the SLE patients with renal disorder ($P = 0.00065$, OR 1.60 [95%CI 1.22-2.10]) than in all SLE patients ($P = 0.0022$, OR 1.40 [95%CI 1.13-1.74]). Significant association with RA was not observed, regardless of the carriage of human leukocyte antigen DR β1 (*HLA-DRB1*) shared epitope. Significant gene-gene interaction between *TNIP1* and *TNFAIP3* was detected neither in SLE nor RA.

Conclusions: Association of *TNIP1* with SLE was confirmed in a Japanese population. *TNIP1* is a shared SLE susceptibility gene in the Caucasian and Asian populations, but the genetic contribution appeared to be greater in the Japanese and Chinese populations because of the higher risk allele frequency. Taken together with the association of *TNFAIP3*, these observations underscore the crucial role of NF-κB regulation in the pathogenesis of SLE.

Introduction

TNFAIP3 (tumor necrosis factor α-induced protein 3) encodes a ubiquitin-editing protein, A20, known as an inhibitor of nuclear factor-κB (NF-κB). Several adaptor molecules are thought to associate with A20 and be involved in inhibition of NF-κB [1]. TNIP1 (TNFAIP3 interacting protein 1), also known as ABIN (A20-binding inhibitor of NF-κB)-1, is one such adaptor molecule binding to A20. *TNIP1* mRNA is strongly expressed in peripheral blood lymphocytes, spleen and skeletal muscle, and the expression is also detected in kidney [2]. *TNIP1* expression is induced by NF-κB, and in turn, overexpression of *TNIP1* inhibits NF-κB activation by TNF [1], although deficiency of *TNIP1* has few effects on NF-κB inhibition [3]. Thus, TNIP1 appears to play a

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role in NF-κB inhibition, at least partly by interacting with A20. In addition, TNIP1 was shown to inhibit TNF-induced apoptosis independently of A20 [3].

TNFAIP3, located at 6q23, has been identified as a susceptibility gene for both systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) in Caucasian and Asian populations [4-8]. Recently, Shimane et al. [9] replicated association of *TNFAIP3* single nucleotide polymorphisms (SNPs) with SLE and RA in a Japanese population. We also detected association of *TNFAIP3* rs2230926 with Japanese SLE patients in an independent study [10].

Recently a genome-wide association study (GWAS) reported association of *TNIP1* (5q32-q33.1) as well as *TNFAIP3* SNPs with psoriasis in the Caucasian populations [11]. Subsequently, two recent GWAS revealed association of *TNIP1* intronic SNPs rs7708392 and rs10036748, which are in strong linkage disequilibrium (LD) with SLE in the Caucasian (European-American and Swedish) and Chinese Han populations, respectively [8,12]. These observations underscored the role of the pathway involving *TNFAIP3-TNIP1* in the genetic predisposition to SLE. The association of *TNIP1* with SLE needs to be further confirmed.

Recently, it has become increasingly clear that SLE and RA share a number of susceptibility genes. *TNFAIP3* [4-10], *STAT4* [13,14] and *BLK* [15,16] represent such shared susceptibility genes. *TNIP1* has been shown to be upregulated in synovial tissues from RA [17], raising a possibility that *TNIP1* may also play a role in the pathogenesis of RA. To date, association of RA with *TNIP1* has not been reported.

This study was conducted to examine whether *TNIP1* was associated with SLE and RA in a Japanese population.

Materials and methods

Patients and controls

Three hundred sixty-four Japanese patients with SLE (21 males and 343 females, mean ± SD age, 42.8 ± 13.9 years), 553 patients with RA (43 males and 510 females, mean ± SD age, 58.0 ± 11.3 years) and 513 healthy controls (238 males and 275 females, mean ± SD age, 34.1 ± 9.9 years) were recruited at University of Tsukuba, Juntendo University, Sagamihara National Hospital, Matsuta Clinic and the University of Tokyo (Table 1). All patients and healthy individuals were native Japanese living in the central part of Japan. All patients with SLE and RA fulfilled the American College of Rheumatology criteria for SLE [18] and RA [19], respectively. Consecutive patients ascertained in rheumatology specialty hospitals or clinics were recruited. The patients with SLE were classified into subgroups according to the presence or absence of renal disorder, neurologic disease and serositis based on the definition of ACR criteria [18], anti-dsDNA and anti-Sm antibodies, and age of onset (< 20 yr). The numbers of the missing data were 5 (renal disorder), 3 (neurologic disease), 21 (serositis), 19 (anti-dsDNA antibody), 22 (anti-Sm antibody) and 6 (age of onset). Patients with RA and healthy controls were stratified by the presence or absence of human leukocyte antigen DR β1 (*HLA-DRB1*) shared epitope. The numbers of the missing data were 6 (RA) and 15 (controls).

Table 1 Characteristics of the patients and healthy controls studied

	SLE	RA	Healthy controls
n	364	553	513
Male/female	21/343	43/510	238/275
Age*	42.8 ± 13.9	58.0 ± 11.3	34.1 ± 9.9

*Mean ± SD.

The control group consisted of healthy volunteers without any signs or symptoms of autoimmune diseases recruited at the same institutes.

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

Genotyping

Genotyping of *TNIP1* rs7708392 was carried out using the TaqMan genotyping assay (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions, using a TaqMan probe C__29349759_10. *HLA-DRB1* was genotyped at the sequence level using a polymerase chain reaction (PCR) microtiter plate hybridization assay as previously described [20].

Statistical analysis

A case control association study was conducted by χ^2 test using 2 × 2 contingency tables. The null hypotheses tested in this study were that there was no difference in the genotype or allele frequencies between all SLE patients and healthy controls, between SLE subsets and healthy controls, between all RA patients and all healthy controls, or between RA patients and healthy controls stratified by the presence or absence of *HLA-DRB1* shared epitope.

The power to detect association was calculated on the basis of the frequency of the rs7708392C allele in Japanese healthy controls (69.9%). The sample size of this study (364 SLE patients, 553 RA patients and 513 controls) had the power of 80% to detect association when the genotype relative risk was1.36 (SLE) and 1.32 (RA) or greater, respectively [21].

To adjust for the gender difference between patients and healthy controls (Table 1), multiple logistic

regression analyses were employed. The following were used as independent variables: for the genotypes of rs7708392, C/C = 1, C/G = 0, G/G = 0 under the recessive model for the C allele, C/C = 2, C/G = 1, G/G = 0 under the codominant model, and for gender, male = 0, female = 1.

The interaction between *TNIP1* rs7708392 and *TNFAIP3* rs2230926, which we recently replicated to be associated with SLE [10], was examined in 308 SLE, 372 RA and 449 healthy controls, using logistic regression analysis. Codominant (risk allele homozygotes $x_i = 2$, heterozygotes $x_i = 1$, nonrisk allele homozygotes $x_i = 0$), dominant (risk allele homozygotes $x_i = 1$, heterozygotes $x_i = 1$, nonrisk allele homozygotes $x_i = 0$) and recessive (risk allele homozygotes $x_i = 1$, heterozygotes $x_i = 0$, nonrisk allele homozygotes $x_i = 0$) models for gene i were tested. 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 b_{ij} by the Wald test. Population attributable risk percentage (PAR%) was estimated by the formula:

$$\text{PAR\%} = \text{Pe}\,(\text{RR}-1)/(\text{Pe}\,[\text{RR}-1]+1)\times 100,$$

where Pe represents the risk genotype frequency in the population and RR represents the relative risk of the risk genotype [22]. Although RR cannot be determined from the case-control study design, it can be approximated by odds ratio (OR) when the incidence of the disease is sufficiently low. Because the incidence of SLE has been reported to be 3.0 in Japan and 1.8-7.6 in the United States per 100,000 persons per year [23] and is sufficiently small, OR can be adequately used for an approximation for RR. The PAR% in the Caucasian populations were calculated using the raw genotype count data for the previously reported study (cases: C/C 293, C/G 1,389, G/G 1,632, controls: C/C 735, C/G 4,510, G/G 7,050) [12].

Results

Replication of *TNIP1* association with SLE in Japanese

The association of *TNIP1* rs7708392 with SLE, recently demonstrated in the Caucasian (European-American and Swedish) populations [12], was examined in a Japanese population. Departure from Hardy-Weinberg equilibrium was observed neither in the cases nor in the controls ($P > 0.3$). As shown in Table 2, rs7708392C allele was significantly increased in Japanese SLE patients (76.5%) compared with healthy controls (69.9%, $P = 0.0022$, OR 1.40, 95% confidence interval [95% CI] 1.13-1.74), confirming the association in the Caucasians. The association was also detected under the recessive model for the rs7708392C allele ($P = 0.0023$, OR 1.52, 95% CI 1.16-2.00). Notably, the risk allele frequency was considerably greater in the Japanese (69.9%) than in the Caucasian healthy controls (24.3%) [12]. In the Japanese, PAR% was estimated to be 20.4% under the recessive model for the C allele (OR 1.52, population frequency of C/C 48.9%) and 31.0% under the dominant model (OR 1.50, population frequency of C/C + C/G 90.8%). These estimates were substantially greater than in the Caucasian populations, where the PAR% was 3.0% under the recessive model (OR 1.53, population frequency of C/C 6.0%) and 14.1% under the dominant model (OR 1.39, population frequency of C/C + C/G 42.7%).

Because the female-to-male ratio was different between SLE patients and healthy controls (Table 1), we carried out multiple logistic regression analysis to examine the association after adjustment for gender. The association with SLE remained significant both under the recessive model for rs7708392C ($P = 0.030$, OR 1.40, 95% CI 1.03-1.89) and under the codominant model ($P = 0.033$, OR 1.30, 95% CI 1.02-1.65).

Association of *TNIP1* with Clinical Subsets of SLE

We next analyzed whether *TNIP1* was associated with clinical subsets such as presence or absence of renal disorder, neurological disease, serositis, anti-dsDNA antibody, anti-Sm antibody, as well as the age of onset (< 20 yr). When the association was tested between patients having each phenotype and healthy controls, a tendency of stronger association was observed in the subsets with renal disorder and anti-dsDNA antibody as compared with all SLE (Table 2). These associations remained significant after adjustment for gender using logistic regression analysis (nephropathy positive versus controls: $P = 0.0070$, OR 1.50, 95% CI 1.12-2.01 under the codominant model and $P = 0.011$, OR 1.59, 95% CI 1.11-2.26 under the recessive model; anti-dsDNA positive versus controls: $P = 0.033$, OR 1.32, 95% CI 1.02-1.71 under the codominant model and $P = 0.024$, OR 1.45, 95% CI 1.05-2.00 under the recessive model).

On the other hand, significant association was not observed in the patient subsets having neurologic disease, serositis, anti-Sm antibody, and the patients with the age of onset <20 yr.

Lack of Association with RA

We next tested association of *TNIP1* rs7708392 with RA. Although a slight tendency toward association was observed, significant association with RA was not detected (Table 3). Significant association was not detected after the adjustment for gender ($P = 0.847$, OR 1.02, 95% CI 0.83-1.26 under the codominant model, $P = 0.753$, OR 1.04, 95% CI 0.80-1.36 under the recessive model), nor after stratification according to the presence or absence of *HLA-DRB1* shared epitope (Table 3).

Table 2 Association study of *TNIP1* rs7708392 with SLE in a Japanese population

	Genotype			Allele	Allelic association		Recessive model*	
	C/C	C/G	G/G	C	*P*	OR (95%CI)	*P*	OR (95%CI)
All SLE (*n* = 364)	216 (59.3)	125 (34.3)	23 (6.3)	557 (76.5)	0.0022	1.40 (1.13-1.74)	0.0023	1.52 (1.16-2.00)
SLE subsets								
Renal disorder + (*n* = 203)	126 (62.1)	68 (33.5)	9 (4.4)	320 (78.8)	0.00065	1.60 (1.22-2.10)	0.0015	1.71 (1.23-2.38)
Neurologic disorder + (*n* = 53)	28 (52.8)	21 (39.6)	4 (7.5)	77 (72.6)	0.55	1.14 (0.73-1.79)	0.59	1.17 (0.66-2.05)
Serositis + (*n* = 55)	33 (60.0)	18 (32.7)	4 (7.3)	84 (76.4)	0.16	1.39 (0.88-2.20)	0.12	1.57 (0.89-2.75)
Anti-dsDNA Ab + (*n* = 280)	169 (60.4)	93 (33.2)	18 (6.4)	431 (77.0)	0.0026	1.44 (1.14-1.83)	0.0021	1.59 (1.18-2.13)
Anti-Sm Ab + (*n* = 67)	37 (55.2)	26 (38.8)	4 (6.0)	100 (74.6)	0.26	1.27 (0.84-1.91)	0.33	1.29 (0.77-2.15)
Onset <20 yr (*n* = 86)	46 (53.5)	34 (39.5)	6 (7.0)	126 (73.3)	0.37	1.18 (0.82-1.70)	0.43	1.20 (0.76-1.90)
Healthy controls (*n* = 513)	251 (48.9)	215 (41.9)	47 (9.2)	717 (69.9)		reference		reference

OR: odds ratio, 95% CI: confidence interval. Genotype and allele frequencies are shown in parentheses (%).

Association was tested by χ^2 analysis using 2 × 2 contingency tables. All SLE group as well as each SLE subset was compared with healthy controls.

*Association was tested under the recessive model for rs7708392C allele.

Lack of Evidence for Genetic Interaction between *TNFAIP3* and *TNIP1*

Finally, we examined whether genetic interaction exists between *TNFAIP3* and *TNIP1* SNPs, because molecular interaction is known between the protein products of these genes. Although all combinations of the codominant, dominant and recessive models for each gene were examined, statistically significant gene-gene interaction was not detected ($P > 0.05$).

Discussion

In the present study, we replicated the association of *TNIP1* rs7708392 with SLE in a Japanese population, which indicated that *TNIP1*, as well as *TNFAIP3*, is a susceptibility gene to SLE shared by the Caucasian and Asian populations. Because both TNIP1 and A20 are thought to be involved in the inhibition of NF-κB activation, genetic association of these genes implicates a causal role of NF-κB regulation pathway in the pathogenesis of SLE.

Kalergis et al. [24] demonstrated that expression of IκB-α, an inhibitor of NF-κB, was decreased in Fcγ receptor IIb-deficient mice which present lupus-like symptoms, and the symptoms were reduced by treatment with NF-κB inhibitors. Previous studies demonstrated that *TNFAIP3* risk allele rs2230926G (127Cys) leads to reduced inhibitory activity of NF-κB activation [6] or reduced mRNA level of *TNFAIP3* [10]. In view of these observations, it is speculated that the risk allele of *TNIP1* may also be associated with reduced inhibitory activity of *TNFAIP3-TNIP1* pathway.

TNIP1 rs7708392 is located in intron 1. Expression analysis using the GENEVAR [25] and the International HapMap databases [26] as previously described [27] did not show significant effect of rs7708392 genotypes on the mRNA level of *TNIP1* (data not shown). Although the direct molecular mechanism of the risk allele to cause SLE remains unclear, it is possible that the risk allele may be associated with the selection of splicing

Table 3 Association study of *TNIP1* rs7708392 with RA in a Japanese population

	Genotype			Allele	Allelic Association		Recessive Model*	
	C/C	C/G	G/G	C	*P*	OR (95%CI)	*P*	OR (95%CI)
All RA (*n* = 553)	292 (52.8)	215 (38.9)	46 (8.3)	799 (72.2)	0.23	1.12 (0.93-1.35)	0.21	1.17 (0.92-1.49)
All healthy controls (*n* = 513)	251 (48.9)	215 (41.9)	47 (9.2)	717 (69.9)		reference		reference
HLA-DRB1 shared epitope positive								
RA (*n* = 376)	203 (54.0)	142 (38.7)	31 (8.2)	548 (72.9)	0.76	1.04 (0.80-1.37)	0.57	1.11 (0.78-1.56)
Healthy controls (*n* = 202)	104 (51.5)	83 (41.1)	15 (7.4)	291 (72.0)		reference		reference
HLA-DRB1 shared epitope negative								
RA (*n* = 171)	86 (50.3)	70 (40.9)	15 (8.8)	242 (70.8)	0.67	1.07 (0.80-1.43)	0.68	1.08 (0.74-1.58)
Healthy controls (*n* = 296)	143 (48.3)	125 (42.2)	28 (9.5)	411 (69.4)		reference		reference

OR: odds ratio, 95% CI: confidence interval. Genotype and allele frequencies are shown in parentheses (%).

Association was tested by χ^2 analysis using 2 × 2 contingency tables. Comparisons were made between all RA and all healthy controls, HLA-DRB1 shared epitope positive RA and controls, and shared epitope negative RA and controls.

*Association was tested under the recessive model for rs7708392C allele.

variant. To date, at least 11 splice variants of *TNIP1* have been identified [1]. Presence of alternative exon 1A and 1B, as well as splice variants lacking exon 2, has been described. Because rs7708392 is located between exon 1B and exon 2, it is possible that this SNP may influence the usage of the splicing isoform. It is also possible that other causative SNPs in tight LD with rs7708392 may exist. Such a possibility would be addressed by resequencing the entire *TNIP1* gene.

Interestingly, in sharp contrast to the Caucasian populations, the risk rs7708392C constituted the major allele in the Japanese population. This resulted in substantially higher PAR% in the latter. We previously reported similar findings in *STAT4* and *BLK* SNPs [14,15]. In Chinese, a SNP rs10036748, which is in tight LD with rs7708392 in Japanese (r^2 = 0.81, HapMap database [26]), has been shown to be associated with SLE. The frequencies of rs10036748 risk allele in Chinese (cases 79.7%, controls 66.1%) [8] are similar to those of rs7708392 in Japanese (Table 2). It should be noted that, because the information used to estimate the PAR % was based on the data from a variant that has not been shown to be the causal variant in *TNIP1*, and the estimates of the allele frequency and OR (as an approximation for RR) were taken from a rather small case-control study, the PAR% values shown here represent rough estimates. Nevertheless, the data suggest that the significance of *TNIP1* in the genetic background of SLE may be substantially greater in the Asian than in the Caucasian populations.

In the association analysis with the clinical subsets, none of the case-only comparisons (cases with each clinical phenotype versus those without) reached statistical significance, partly because of the insufficient statistical power caused by the small sample size due to stratification. However, preferential association of *TNIP1* with renal disorder and anti-dsDNA antibody was suggested by comparison with healthy controls. In our subjects, preferential association with renal disorder was also observed for *TNFAIP3* [10].

On the other hand, association was not observed with the SLE subsets having neurological disease, serositis, anti-Sm antibody and age of onset <20. It is interesting to note that renal disorder and presence of anti-dsDNA are significantly correlated in SLE, while neurologic disorders are not, suggesting that these clinical features might represent different clinical subsets of SLE [28]. In view of this, our findings could be interpreted such that polymorphisms in *TNIP1-TNFAIP3* pathway might play a significant role in the subset of SLE characterized by renal disorder and anti-dsDNA antibody, but not in the subset with neurologic disease. Such a hypothesis should be validated in future large-scale studies.

No strong evidence for association of rs7708392 with RA was obtained in this study. The sample size in this study (553 RA patients and 513 controls) provides 80% power to detect associations with genotype relative risk of 1.32 or greater, but we cannot rule out a possibility of weak association. Recently published meta-analysis of GWAS in Caucasians also failed to demonstrate statistically significant association of *TNIP1* SNP with RA, although similarly to our observation, a tendency for association was detected [29]. Thus, while a role of *TNFAIP3* is observed both in SLE and RA genetics, *TNIP1* appears to play a major role in SLE, but not in RA. Such a difference might possibly imply that the molecular mechanism of *TNIP1* association might not be fully explained by A20 modification. In support of this possibility, TNIP1 has been shown to block TNF-induced programmed cell death in *TNFAIP3* deficient cells, indicating that TNIP1 does not always require A20 to perform its anti-apoptotic function [3]. Thus, further analysis on the molecular mechanisms involving these molecules is required.

Conclusions

Association of *TNIP1* with SLE was confirmed in a Japanese population. *TNIP1* is a shared SLE susceptibility gene in the Caucasian and Asian populations, but the genetic contribution appeared to be greater in the Asians because of the higher risk allele frequency in the population. Taken together with the association of *TNFAIP3*, these observations underscore the crucial role of NF-κB regulation in the pathogenesis of SLE.

Abbreviations

95%CI: 95% confidence interval; ABIN-1: A20-binding inhibitor of NF-κB -1; CI: confidence interval; GWAS: genome-wide association studies; HLA-DRB1: human leukocyte antigen DR β1; LD: linkage disequilibrium; NF-κB: nuclear factor-κB; OR: odds ratio; PAR%: population attributable risk percentage; PCR: polymerase chain reaction; RA: rheumatoid arthritis; RR: relative risk; SLE: systemic lupus erythematosus; SNP: single nucleotide polymorphism; TNFAIP3: tumor necrosis factor α-induced protein 3; TNIP1: TNFAIP3 interacting protein 1.

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Authors' contributions

AK participated in the study design, carried out all genotyping and statistical analyses, and wrote the manuscript. JO carried out statistical analysis with AK and helped in the manuscript preparation. SI, HF, TH, DG, IM, MK, KM, ST, YT, HH and TS recruited Japanese patients with SLE and collected clinical information. RRG and TWB provided Caucasian data. NT designed and coordinated the study and helped in the manuscript preparation. All authors read and approved the final manuscript.

Competing interests

RRG and TWB are employees of Genentech, Inc. (South San Francisco, CA, USA). The other authors declare that they have no competing interests.

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Pathogenic role of immune response to M3 muscarinic acetylcholine receptor in Sjögren's syndrome-like sialoadenitis

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ABSTRACT

The aim of this study was to clarify the role of the immune response to muscarinic type 3 receptor (M3R) in the pathogenesis of Sjögren's syndrome (SS). $M3R^{-/-}$ mice were immunized with murine M3R peptides and their splenocytes were inoculated into $Rag1^{-/-}$ ($M3R^{-/-} \rightarrow Rag1^{-/-}$) mice. $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice had high serum levels of anti-M3R antibodies and low saliva volume. Histological examination showed marked infiltration of mononuclear cells in the salivary glands and immunohistochemistry demonstrated that the majority of these cells were $CD4^+$ T cells with a few B cells and several IFN-γ- and IL-17-producing cells. Apoptotic cells were present in the salivary glands of $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Moreover, transfer of only $CD3^+$ T cells from $M3R^{-/-}$ immunized with M3R peptides into $Rag1^{-/-}$ mice resulted in cell infiltration and destruction of epithelial cells in the salivary glands, suggesting that M3R reactive $CD3^+$ T cells play a pathogenic role in the development of autoimmune sialoadenitis. Our findings support the notion that the immune response to M3R plays a crucial role in the pathogenesis of SS-like autoimmune sialoadenitis.

1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by infiltration of lymphocytes into lacrimal and salivary glands, and clinically by dry eyes and dry mouth [1]. Immunohistochemical studies have shown that most infiltrating lymphocytes around the labial salivary and lachrymal glands are CD4-positive αβ T cells [2]. B cells are also detected in inflammatory lesions and act as antigen-presenting cells (APC) and produce autoantibodies such as anti-Ro/SSA and anti-La/SSB antibodies. Previous studies analyzed autoantigens recognized by T cells infiltrating the labial salivary glands of patients with SS and several candidate autoantigens such as Ro/SSA 52 kDa, [3] α-amylase, [4] heat shock protein, T cell receptor BV6 [5] and M3 muscarinic acetylcholine receptor (M3R) [6,7] have been identified.

M3R is the major muscarinic acetylcholine receptor in the salivary glands. Studies in M3R knockout ($M3R^{-/-}$) mice showed the involvement of these receptors in saliva secretion [8], suggesting that dysfunction of the M3R signaling in salivary glands can lead to a decrease in salivary secretion. The presence and specificity of anti-M3R antibodies in SS have been investigated using enzyme-linked immunosorbent assay (ELISA) or flow cytometry assay [9,10]. Robinson et al. [11] demonstrated that human anti-M3R antibodies reduced salivary secretion in NOD $Ig\mu^{null}$ mice. We also reported previously the presence of M3R reactive T cells in some patients with SS [12]. At this stage, however, it is not clear whether the immune response to M3R plays a role in the onset and progression of SS.

Amagai et al. [13] described a mouse model of pemphigus vulgaris (PV) using the combination of desmoglein 3 knockout ($Dsg3^{-/-}$) mice without Dsg3 and adoptive splenocytes transfer to lymphocyte-free Dsg3-positive $Rag2^{-/-}$ mice ($Dsg3^{-/-} \rightarrow Rag2^{-/-}$). In the $Dsg3^{-/-} \rightarrow Rag2^{-/-}$ mice, the Dsg3 molecule induces a powerful immune response and the cells break self-tolerance and attack Dsg3 autoantigens. The same approach might be applicable to T cell-mediated autoimmune disease as well as antibody-mediated disease such as PV.

Our research interest is the pathogenic role of the immune response against M3R in patients with SS. In the present study, we

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immunized M3R$^{-/-}$ mice with murine M3R peptides and transferred their splenocytes into Rag1$^{-/-}$ mice (M3R$^{-/-}$ → Rag1$^{-/-}$). We succeeded in establishing M3R-induced SS-like sialoadenitis in M3R$^{-/-}$ → Rag1$^{-/-}$ mice as a new murine model of SS. The results also showed that CD3^{+} T cells play a crucial role in the generation of autoimmune sialoadenitis. We also discuss the possible molecular mechanism of M3R-induced autoimmunity in SS.

2. Materials and methods

2.1. Mice

C57BL/6j (B6) mice (M3R$^{+/+}$) were purchased from Charles River Laboratory (Yokohama, Japan). Rag1$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). M3R$^{-/-}$ mice, which were generated from B6 mice, were kindly supplied by Dr. Matsui (Tokyo-Nishi Tokushukai Hospital, Tokyo, Japan). Mice were maintained under specific pathogen-free conditions in the Laboratory Animal Resource Center. All experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals at Tsukuba University.

2.2. Synthesized peptides encoding M3R extracellular regions

Six types of peptides encoding murine M3R extracellular domains: N-terminus 1 (MTLHSNSTTSPLFPNISSSWVHSPSEAGLP, N1), N-teminus 2 (VHSPSEAGLPLGTVSQLDSYNISGTSGNFS, N2), N-terminus 3 (NISQTSGNFSSNDTSSDPLGGHTIWQV, N3), 1st extracellular loop (FTTYIIMNRWALGNLACDLW, 1st), 2nd extracellular loop (QYFVGK RTVPPGECFIQFLSEP, 2nd) and 3rd extracellular loop (VLVNTF CDSCIPKTYWNLGY, 3rd), were synthesized chemically by a solid-phase procedure and purified by high performance liquid chromatography (AnyGen, Korea).

2.3. Immunization of mice

M3R$^{+/+}$ and M3R$^{-/-}$ mice were immunized intradermally at the base of the tail with a mixture of free-form extracellular peptides (each 20 μg) in Incomplete Freund's Adjuvant (IFA, Difco, Detroit, MI) containing 250 μg of inactivated Mycobacterium tuberculosis (H37RA, Difco). Pertussis toxin (500 ng; Sigma–Aldrich, Tokyo, Japan) was injected intraperitoneally on the day of immunization. On day 10, each mouse received another intradermal injection of the same mixture of peptides (each 20 μg) emulsified with IFA containing 250 μg of H37RA (Fig. 1A).

2.4. Analysis of cytokine profiles

On day 20 after the first immunization, the spleen was isolated from each M3R$^{+/+}$ and M3R$^{-/-}$ mouse and homogenized. The red blood cells were removed from the splenocytes by treatment with 0.16 M NH_4Cl solution. Then, the homogenates were adjusted to 1×10^6 cells/ml and incubated with RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1000 U/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol, together with a mixture of extracellular peptides (each 5 μg/ml) for 72 h under 5% CO_2 at 37 °C. After collection of the supernatant, the levels of IFN-γ, IL-4 and IL-17 were measured by enzyme-linked immunosorbent assay (ELISA) (IFN-γ, IL-4, and IL-17; R&D Systems Minneapolis, MN).

2.5. Adoptive transfer and cell sorting

On day 20 after the first immunization, the spleens were isolated from M3R$^{+/+}$ and M3R$^{-/-}$ mice. The spleens from each group of mice (each $n = 3$) were pooled and homogenized. The red blood cells were removed from the splenocytes by treatment with 0.16 M NH_4Cl solution. After washing with phosphate buffered saline (PBS), the cells were resuspended in PBS.

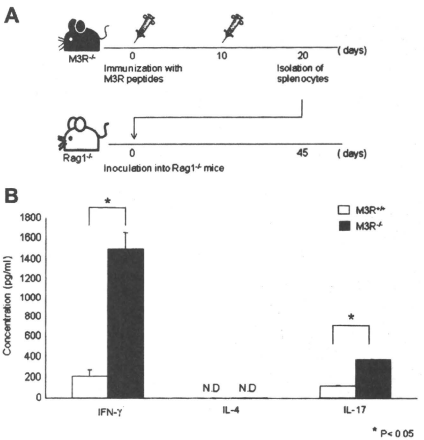

Fig. 1. Cytokine profile of M3R reactive T cells in M3R$^{-/-}$ mice immunized with M3R peptides. (A) M3R$^{-/-}$ and M3R$^{+/+}$ mice were immunized with a murine M3R peptide mixture on days 0 and 10. On the day of immunization, 500 ng of pertussis toxin was injected intraperitoneally. Ten days after booster immunization, the spleens were isolated and transferred into Rag1$^{-/-}$ mice. At day 45 after the transfer, the salivary glands of Rag1$^{-/-}$ recipient mice were analyzed histologically. (B) M3R$^{-/-}$ and M3R$^{+/+}$ mice were immunized followed by a booster dose of M3R peptides mixture emulsified in IFA plus M. tuberculosis. Ten days later, the spleens were isolated from M3R$^{+/+}$ and M3R$^{-/-}$ mice and stimulated with the M3R peptides mixture. The concentrations of IFN-γ, IL-4 and IL-17 in the culture supernatants were measured by enzyme-linked immunosorbent assay. N.D.: not detected. Values are mean ± SD of three independent experiments ($n = 3$ mice per experiment). *$P < 0.05$ (Mann–Whitney U test).

CD3^{+} T cells were sorted from splenocytes of M3R$^{+/+}$ and M3R$^{-/-}$ by using the Pan T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The high purity of CD3^{+} T cells (>93%) was confirmed by FACSCalibur (BD PharMingen, San Diego, CA). Cells were resuspended in PBS and 1×10^7 cells were inoculated intravenously into Rag1$^{-/-}$ mice (Fig. 1A).

2.6. Measurement of salivary volume

Mice were first anesthetized with intraperitoneal injection of pentobarbital (1.0 mg/kg), then injected subcutaneously with pilocarpine (25 mg/kg). This was followed 15 min later by collection of saliva from the oral cavity using a 200-μl micropipette. The volume of the sample was measured and expressed relative to body weight. Changes in saliva volume were calculated relative to the volume measured at baseline, using the formula [dayN saliva volume (ml)/weight (g)]/[day0 saliva volume (ml)/weight (g)].

2.7. Analysis of antibody levels by enzyme-linked immunosorbent assay

The peptide solution containing all types of extracellular peptides (each 5 μg/ml) was mixed with 0.1 M Na_2CO_3 buffer, pH 9.6, absorbed onto a Nunc-Immunoplate (Nalge Nunc Int, Rochester, NY) at 4 °C overnight, then blocked with 1% bovine serum

albumin (Wako Pure Chemical Industries, Osaka, Japan) in PBS for 1 h at room temperature. Serum at 1:50 dilution in blocking buffer was incubated for 2 h at room temperature. The plates were then washed five times with 0.05% Tween 20 in PBS, and HRP-conjugated anti-mouse IgG antibody diluted 1:1000 in blocking buffer was added for 1 h at room temperature. After washing, 100 μl of TMB solution (Kirkegaard & Perry Laboratories, Inc, Washington, DC) was added as a substrate and then the plates were incubated for 15 min at room temperature in the dark. After adding 50 μl of 1 M phosphate buffer to stop the enzymatic reaction, the optical density was measured at 450 nm by plate spectrophotometry (Bio-Rad Laboratories, Hercules, CA).

2.8. Histological score

The inflammatory lesions were graded histologically (histological sore) using the method proposed by Greenspan et al. [14] as follow: focus score was described as the number of a focus composed of >50 mononuclear cells per 4 mm^2 of tissue. Histological evaluation of the salivary glands was performed in a blind manner, and at least one tissue section from each salivary gland was examined.

2.9. Immunohistochemical analysis

Sections of the salivary glands were thawed, dried, and then fixed with acetone for 10 min. They were incubated with biotin-conjugated anti-mouse CD4, CD8, I-A^b, CD11c (Biolegend, San Diego, CA), rat anti-mouse Thy1 (Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-mouse B220, CD86, F4/80 (Biolegend) or HRP-conjugated rabbit anti-mouse IgG antibodies (DAKO, Japan) for 2 h. To detect primary antibodies, horseradish peroxidase (HRP)-conjugated streptavidin or HRP-conjugated anti-rat IgG antibodies were added for 30 min. HRP activity was detected using 3,3-diaminobenzidine (DAB; Nichirei, Tokyo) as a substrate. The stained sections were counterstained with Mayes's hematoxylin for 30 s, and mounted with aqueous medium. Control slides were incubated with a dilution buffer containing isotype-matched antibodies instead of the primary antibodies.

Double immunofluorescence staining was performed as follow: Sections of the saliva glands were thawed, dried, and then fixed with 4% paraformaldehyde PBS (Wako) for 10 min. The sections were incubated with rat anti-mouse IFN-γ (Biolegend) and rat anti-mouse IL-17 antibodies (Santa Cruz Biotechnology) for 30 min. To detect primary antibodies, Alexa Fluor 546 goat anti-rat IgG antibody was added for 30 min. The nuclear DNA was stained with DAPI (Sigma–Aldrich).

2.10. Terminal deoxynucleotidyl transferase-mediated nick and labeling staining

Apoptotic cells were detected by *in situ* apoptosis detection kit (Takara Biomedicals, Tokyo). Briefly, frozen sections were fixed with 4% paraformaldehyde for 30 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase/methanol. After washing in PBS, the sections were treated for 5 min on ice with the permeabilisation buffer and then incubated with TdT enzyme reaction for 90 min at 37 °C. An HRP-conjugated anti-FITC antibody was added for 30 min at 37 °C, and the HRP activity was detected using DAB as a substrate. Nuclei were counterstained with hematoxylin.

2.11. Statistical analysis

Data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Mann–Whitney *U* test. *P* values less than 0.05 were considered significant.

3. Results

3.1. High production of IL-17 and IFN-γ by splenocytes of $M3R^{-/-}$ mice

To compare the immune response to M3R between in $M3R^{-/-}$ and $M3R^{+/+}$ mice, both groups of mice were immunized with the M3R peptide mixture and their splenocytes were cultured with M3R *in vitro* at 20 days after first immunization (Fig. 1A). As shown in Fig. 1B, the production of IL-17 and IFN-γ by splenocytes of immunized $M3R^{-/-}$ mice far exceeded that of $M3R^{+/+}$ mice. On the other hand, IL-4 production was below the detection level in both groups. These results suggest a higher immune response to M3R in $M3R^{-/-}$ than $M3R^{+/+}$ mice.

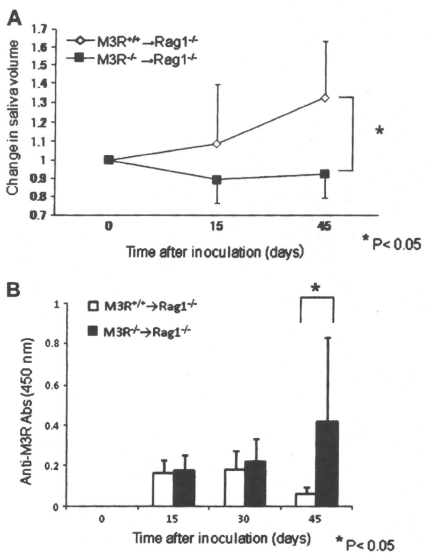

Fig. 2. Reduced salivary secretion and increased levels of anti-M3R antibodies in $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. $Rag1^{-/-}$ mice inoculated with splenocytes from immunized $M3R^{-/-}$ and $M3R^{+/+}$ mice by intravenous injection into the tail vein. (A) Saliva was collected from $Rag1^{-/-}$ mice at 0, 15, 45 days after the adoptive transfer. The saliva samples were collected from each mouse 15 min after stimulation with pilocarpine. The volume of each saliva sample was measured and adjusted for body weight, and calculated relative to the volume measured at baseline, using the formula [dayN saliva volume (μl)/weight (g)]/[day0 saliva volume (μl)/weight (g)]. (B) Titer of anti-M3R antibodies in sera of $Rag1^{-/-}$ mice obtained at days 0, 15, 30, and 45 after adoptive transfer, determined by ELISA. *$P < 0.05$ versus saliva volume and M3R antibodies derived from $Rag1^{-/-}$ mice inoculated with splenocytes of immunized $M3R^{+/+}$ mice at the same time point, by Mann–Whitney *U* test. Values are mean ± SD of two independent experiments ($n = 6$ mice per experiment).

3.2. Development of sialoadenitis in Rag1$^{-/-}$ mice inoculated with splenocytes from immunized M3R$^{-/-}$ mice

To expose M3R reactive cells to the antigen, we isolated splenocytes from the immunized M3R$^{-/-}$ and M3R$^{+/+}$ mice and inoculated them into M3R-positive Rag1$^{-/-}$ mice (Fig. 1A). First, we examined saliva volume at days 0, 15, and 45 to determine the effect of inoculation of splenocytes of immunized M3R$^{-/-}$ or M3R$^{+/+}$ mice in Rag1$^{-/-}$ mice. The saliva volume decreased gradually from day 15–45 in Rag1$^{-/-}$ mice inoculated with splenocytes of M3R$^{-/-}$ mice compared with those inoculated with splenocytes of M3R$^{+/+}$ mice (Fig. 2A). At day 45 after the cell transfer, serum anti-M3R antibodies were significantly higher in M3R$^{-/-}$ → Rag1$^{-/-}$ mice than M3R$^{+/+}$ → Rag1$^{-/-}$ mice (Fig. 2B).

To examine the influence of transferred splenocyte from M3R$^{-/-}$ or M3R$^{+/+}$ immunized with the M3R peptides on salivary glands of Rag1$^{-/-}$ mice, M3R$^{-/-}$ → Rag1$^{-/-}$ mice and M3R$^{+/+}$ → Rag1$^{-/-}$ mice were analyzed histologically. Histological examination of mice at day 45 after inoculation showed marked mononuclear infiltration in the salivary glands and lacrimal glands of M3R$^{-/-}$ → Rag1$^{-/-}$ mice but not in M3R$^{+/+}$ → Rag1$^{-/-}$ mice (Fig. 3A). However, no inflammatory lesions were observed in the intestine or liver (Fig. 3C). Quantitative analysis of the inflammatory lesions of the salivary glands indicated a higher histological sore for M3R$^{-/-}$ → Rag1$^{-/-}$ mice than M3R$^{+/+}$ → Rag1$^{-/-}$ mice (Fig. 3B). These results suggested that autoimmune response against M3R-induced infiltration of mononuclear cells in the salivary glands and salivary dysfunction.

To characterize the infiltrating cells in the inflammatory lesions of salivary glands, the frozen sections were analyzed immunohistochemically. The majority of infiltrating cells in M3R$^{-/-}$ → Rag1$^{-/-}$ mice were Thy1^{+} CD4^{+} T cells, with only few CD8^{+} T cells. Other cells such as B220^{+}, CD11c^{+} cells and macrophage formed a minor population and were localized around T cells (Fig. 4A). Furthermore, deposition of IgGs was sometimes noted in the salivary glands of M3R$^{-/-}$ → Rag1$^{-/-}$ mice (Fig. 4A). MHC class II and CD86 were expressed in ductal epithelial cells of the inflammatory lesions of salivary glands from M3R$^{-/-}$ → Rag1$^{-/-}$ mice, suggesting that activated epithelial cells acted as APC.

We also examined cytokine production in the salivary glands of M3R$^{-/-}$ → Rag1$^{-/-}$ by immunofluorescence staining. The salivary glands of M3R$^{-/-}$ → Rag1$^{-/-}$ mice, but not those of M3R$^{+/+}$ → Rag1$^{-/-}$ mice, showed differential expression of IFN-γ and IL-17 (Fig. 4B). A few apoptotic cells were noted in the lesions of salivary glands isolated from M3R$^{-/-}$ → Rag1$^{-/-}$ mice on TUNEL staining (Fig. 4D), which might explain the salivary destruction and dysfunction.

3.3. M3R reactive T cells and development of sialoadenitis

The above findings indicated that the infiltrated cells in the salivary glands of M3R$^{-/-}$ → Rag1$^{-/-}$ mice were mainly T cells. To confirm that M3R reactive T cells play a role in the development of sialoadenitis in M3R$^{-/-}$ → Rag1$^{-/-}$ mice, we transferred purified splenic CD3^{+} T cells or CD3^{+} T cells-free splenocytes (CD3^{-} cells) from M3R$^{-/-}$ mice immunized with M3R peptides mix into Rag1$^{-/-}$ mice (M3R$^{-/-}$ CD3+

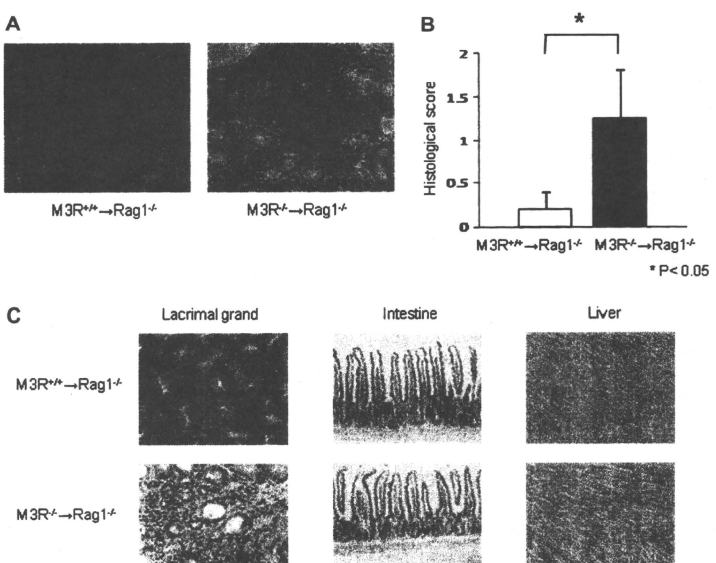

Fig. 3. Infiltration of salivary glands in M3R$^{-/-}$ → Rag1$^{-/-}$ mice. Salivary glands isolated from Rag1$^{-/-}$ mice at day 45 after inoculation of splenocytes from immunized M3R$^{-/-}$ and M3R$^{+/+}$ mice. (A) Salivary glands were prepared into 4-μm thick sections, and each section was stained with Mayer's hematoxylin and eosin (H&E). Representative images of five to seven mice in each group. (B) Mean grade (histological score) of inflammatory lesions in salivary glands of M3R$^{+/+}$ → Rag1$^{-/-}$ and M3R$^{-/-}$ → Rag1$^{-/-}$ mice. Values are mean ± SD of five mice. (C) H&E-stained sections of the lacrimal glands, intestine and liver isolated from M3R$^{+/+}$ → Rag1$^{-/-}$ mice and M3R$^{-/-}$ → Rag1$^{-/-}$ mice. (Original magnification 100×).

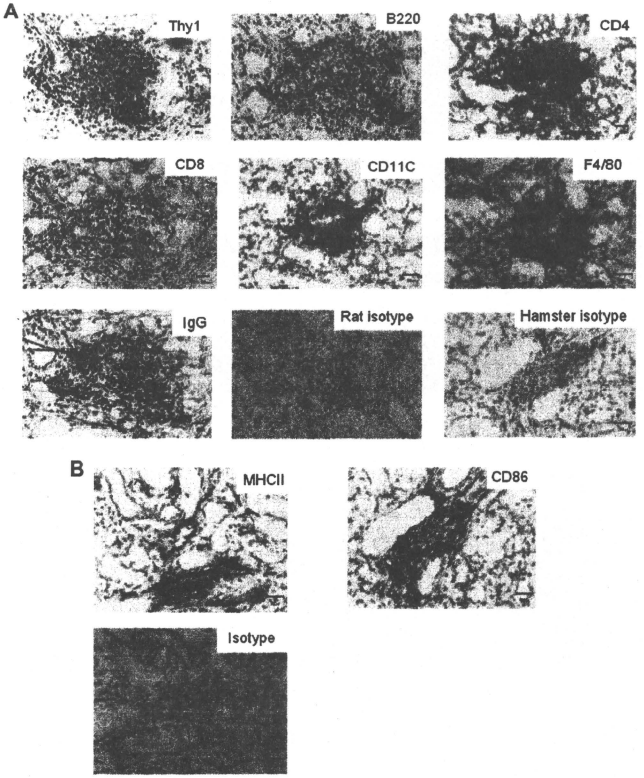

Fig. 4. Histological analysis of salivary glands isolated from M3R$^{-/-}$→Rag1$^{-/-}$ mice. (A) Inflammatory lesions in salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice stained with anti-Thy1, B220, CD4, CD8, CD11c, F4/80 as macrophage specific marker, IgG antibodies and control antibodies. Representative images of five to seven mice. (B) Expression of MHC class II and CD86 detected by immunohistochemistry using anti-I-A^{b}, CD86 and control antibodies. Representative images of three to five mice. (C) Immunofluorescence analysis of IFN-γ and IL-17 in salivary glands of M3R$^{+/+}$→Rag1$^{-/-}$ and M3R$^{-/-}$→Rag1$^{-/-}$ mice. The stained sections were counterstained with H&E, and mounted with aqueous medium. Stained sections were observed at 100× original magnification. Representative images of three to five mice from each group. (D) Apoptotic cells in sections of salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice detected by the TUNEL method. Note the presence of a few apoptotic cells in the salivary glands of M3R$^{+/+}$→Rag1$^{-/-}$ mice. Representative images of three to five mice from each group. Bars = 30 μm.

cells→Rag1$^{-/-}$ mice or M3R$^{-/-}$CD3^{-} cells→Rag1$^{-/-}$ mice). The purity of CD3^{+} T cells was 93.6% and CD3^{-} T cell included only 1.6% of CD3^{+} T cells (Fig. 5A). Histological analysis showed marked cell infiltration in the salivary glands of M3R$^{-/-}$ CD3+ cells→Rag1$^{-/-}$ mice, but not in M3R$^{-/-}$CD3^{-} cells→Rag1$^{-/-}$ mice (Fig. 5A). The majority of infiltrating cells in the salivary glands of M3R$^{-/-}$ CD3+ cells→Rag1$^{-/-}$ mice were Thy1+ CD4+ T cells, compared with only a few CD8^{+} T cells (Fig. 5B). These findings suggest that M3R reactive T cells play a crucial role in the pathogenesis of sialoadenitis in the M3R$^{-/-}$→Rag1$^{-/-}$ mice.

4. Discussion

Our previous results (6) demonstrated that 14% of patients with SS harbor autoantibodies against M3R in their sera. In another study, we also reported that peripheral lymphocytes of 4 out of 9 patients with SS included M3R-reactive T cells (12). These results suggested that the underlying pathomechanism of sialoadenitis in patients with SS could perhaps involve the immune response to M3R. The present study is an extension to our previous investigation and was designed to clarify the role of the M3R reactive immune response in the development of SS. For this purpose, M3R$^{-/-}$ mice were immunized with M3R peptides, their splenocytes were transferred into Rag1$^{-/-}$ mice, and the development of SS-like sialoadenitis was examined. Our results showed the development of mild to severe sialoadenitis in the salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice, indicating the involvement of the M3R reactive immune response in the development of sialoadenitis. The infiltrating cells in the salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice were mainly CD4^{+} T cells with a few surrounding B cells. The histological findings resemble those of infiltration of lymphocyte subset in the labial salivary glands of

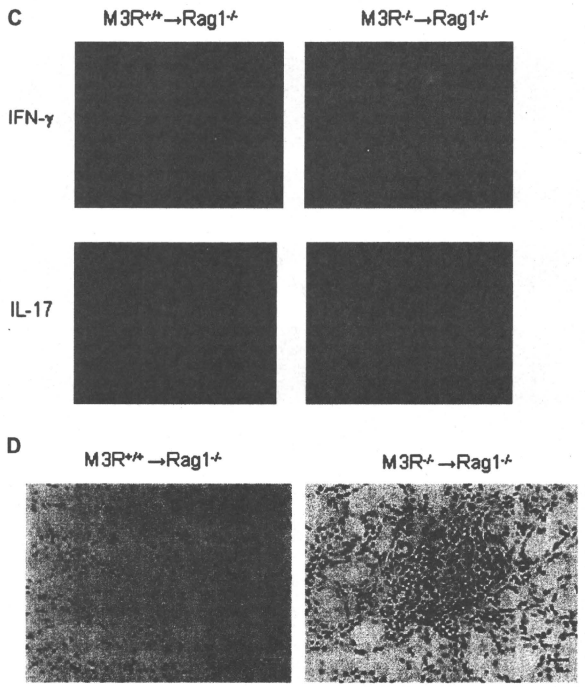

Fig. 4. *(continued).*

patients with SS [15]. Moreover, the $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice had high levels of anti-M3R antibodies and low saliva volume, similar to patients with SS. Previous studies analyzed the levels of anti-M3R antibodies in patients with SS by three methods including peptide-based ELISA, flow cytometry assay and western blot analysis [9,16,17]. Previously, we established peptide-based ELISA for detection of anti-M3R antibodies in SS patients [10,12] and thus applied this method in our mouse model in this study. The results suggest that the M3R molecule acts as an autoantigen to induce an autoimmune reaction in some patients with SS as well as experimental M3R-induced sialoadenitis.

What is the mechanism of M3R-induced sialoadenitis in our mouse model? In $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice, anti-M3R autoantibodies were detected in the serum. IFN-γ– and IL-17-producing cells were also detected in the salivary glands of $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Moreover, the adoptive $CD3^+$ T cell transfer experiments showed that $CD3^+$ T cells are essential for the development of sialoadenitis in $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Thus, M3R reactive T cells are important in the development of antigen-induced sialoadenitis in our mouse model. Although we have no direct evidence in support of a pathogenic role for IFN-γ-producing Th1 cells or IL-17-producing Th17 cells, the latter type of cells might be important in the pathogenesis of M3R-induced sialoadenitis, because none of the IFN-γ dominant T-bet transgenic mice immunized with M3R peptides developed sialoadenitis (data not shown). In this regard, recent studies reported the expression of IL-17 in the salivary glands of patients with SS [18,19]. Thus, further studies using IFN-$\gamma^{-/-}$ mice or IL-$17^{-/-}$ mice are necessary.

Why does the combination of $M3R^{-/-}$ mice immunized M3R peptides and $Rag1^{-/-}$ mice enhance the generation of antigen-driven sialoadenitis? It is possible that immunization using a mixture of M3R peptides that includes the N region, and the 1st, 2nd, and 3rd extracellular domains of the M3R molecule, could have induced the immune response against exogenous antigens such as M3R in $M3R^{-/-}$ mice. In fact, M3R reactive T cells and anti-M3R antibodies appeared in $M3R^{-/-}$ mice immunized with M3R peptides. It is possible that these M3R reactive lymphocytes proliferate in $Rag1^{-/-}$ mice and attack the M3R molecule expressed in their own salivary glands, resulting in sialoadenitis.

Why is tolerance to M3R autoantigens broken down in patients with SS? While there is no evidence for the expansion of M3R reactive T cells and B cells in the salivary glands of patients with SS, it is possible that overexpression of M3R autoantigen on APC present in the salivary glands plays a role in the induction of M3R lymphocytes in the peripheral blood with subsequent infiltration of these cells into the tissues [20,21]. The high expression of M3R in the salivary glands may be triggered by previous bacterial or viral infections in the local tissues [22–24].

Based on the results of the present study, we propose that the pathogenesis of sialoadenitis in patients with SS might involve

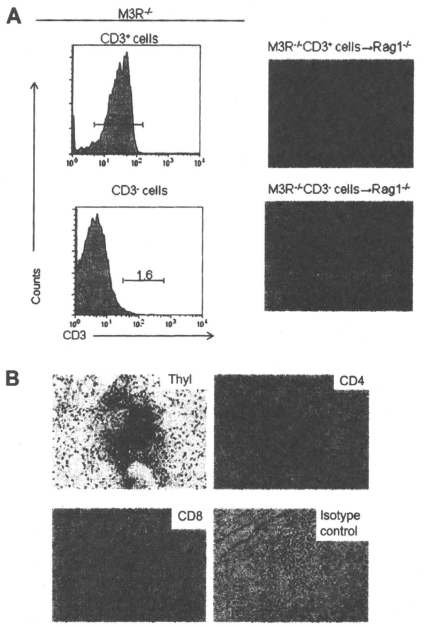

Fig. 5. Role of M3R reactive T cells in the development of sialoadenitis. $CD3^+$ or $CD3^-$ splenocytes of $M3R^{+/+}$ and $M3R^{-/-}$ mice immunized with M3R peptide mix obtained on day 20 after immunization were inoculated into $Rag1^{-/-}$ mice. (A) Salivary glands isolated from $Rag1^{-/-}$ mice at day 45 after transfer of $CD3^+$ or $CD3^-$ cells. Salivary glands were prepared into 4-μm thick sections, and each section was stained with H&E. Representative images of five to seven mice. (B) Sections of salivary glands of $M3R^{-/-}$ $CD3^+$ cells→$Rag1^{-/-}$ mice stained with anti-CD4, CD8, Thy1 and isotype control. Representative images of three to five mice from each group. Magnification 40×.

induction of M3R reactive T cells and B cells in the salivary glands. Importantly, the altered peptide ligands (APL) for M3R binding to the HLA-DR molecule are known to suppress the M3R reactive T cells *in vitro* [25,26]. Therefore, the antigen-induced sialoadenitis in patients with SS could be regulated by APL of M3R. Targeting this antigen-specific modification of autoimmunity in SS could be a potentially useful therapeutic strategy.

In conclusion, we established a new model mouse of sialoadenitis in SS using M3R immunized $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Experiments using the new model suggest that the M3R reactive immune response, especially M3R reactive T cells, play a crucial role in the pathogenesis of SS. Targeting this antigen-specific modification of autoimmunity could be a potentially suitable therapeutic strategy in autoimmune diseases such as SS.

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Sex-specific association of X-linked Toll-like receptor 7 (TLR7) with male systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is a multisystem, autoimmune disease that predominantly affects women. Previous findings that duplicated Toll-like receptor 7 (*Tlr7*) promotes lupus-like disease in male BXSB mice prompted us to evaluate *TLR7* in human SLE. By using a candidate gene approach, we identified and replicated association of a *TLR7* 3′UTR SNP, rs3853839 (G/C), with SLE in 9,274 Eastern Asians ($P_{combined} = 6.5 \times 10^{-10}$), with a stronger effect in male than female subjects [odds ratio, male vs. female = 2.33 (95% CI = 1.64–3.30) vs. 1.24 (95% CI = 1.14–1.34); $P = 4.1 \times 10^{-4}$]. G-allele carriers had increased *TLR7* transcripts and more pronounced IFN signature than C-allele carriers; heterozygotes had 2.7-fold higher transcripts of G-allele than C-allele. These data established a functional polymorphism in type I IFN pathway gene *TLR7* predisposing to SLE, especially in Chinese and Japanese male subjects.

functional polymorphism | disease susceptibility | autoimmunity | type I interferon

Systemic lupus erythematosus [SLE; Online Mendelian Inheritance in Man (OMIM) no. 152700] is a multisystem, autoimmune disease with strong genetic and environmental components (1). SLE predominantly affects women, with a female-to-male ratio of approximately 9:1. Male patients with SLE, although rare, tend to have more severe disease and poorer outcome (2), suggesting potential sex dimorphism in the disease development. Although the sex effect has often been attributed to sex hormones, the fact that XXY male subjects have approximately a 14-fold higher risk of developing SLE than 46 XY men indicates that X-linked genes may be risk factors for human SLE (3).

Located at Xp22.2, Toll-like receptor 7 (*TLR7*; OMIM no. 300365) and its functionally related gene *TLR8* (OMIM no. 300366) encode proteins that play critical roles in pathogen recognition and activation of innate immunity (4). They recognize endogenous RNA-containing autoantigens and induce the expression of type I IFN, a pivotal cytokine in the pathogenesis of SLE (5). In lupus-prone BXSB mice, the translocation of a segmental duplication of X chromosome to Y chromosome creates the Y-linked autoimmune accelerator (Yaa) locus, which was associated with autoreactive B cell responses to RNA-related antigens and exacerbation of glomerulonephritis in male mice (6). Although translocated X chromosome segment in Yaa may contain as many as 16 genes, the major gene for causation of the autoimmune phenotypes was identified to be *TLR7* (7), making it a potential susceptibility gene for SLE. By using a candidate gene approach, we report herein that a functional polymorphism in 3′UTR of *TLR7* is associated with SLE in Chinese and Japanese populations, with a stronger effect in male than female subjects.

Results

Discovery and Replication of the Association of a *TLR7* 3′UTR SNP with SLE in Eastern Asian Population. We genotyped 27 SNPs from the *TLR7–TLR8* region (12 in *TLR7* and 15 in *TLR8*) in 1,434 SLE cases and 1,591 control subjects of Eastern Asian ancestry using the Beadstation Infinium II platform (Illumina). Eleven SNPs in *TLR7* and 12 SNPs in *TLR8* that showed a minor allele frequency greater than 0.01 were included in association analysis (Fig. 1*B* and Table S1). We observed evidence of association with SLE in 2 *TLR7* SNPs (rs5935436 and rs3853839) and 2 *TLR8* SNPs (rs3764880 and rs4830805; Fig. 1*B*). Only rs5935436 and rs3853839 in *TLR7* remained significant after Bonferroni correction ($P = 0.041$ and $P = 0.016$, respectively). The strongest association signal was detected at rs3853839 among Chinese subjects [cases vs. controls, 563 vs. 522; $P = 6.3 \times 10^{-6}$; odds ratio (OR) = 1.67 (95% CI = 1.33–2.08)], but not in Korean subjects [$P = 0.32$; OR = 0.92 (95% CI = 0.79–1.08); Fig. 1*B*], suggesting potential genetic heterogeneity of SLE between the two populations.

We next performed a haplotype-based association test using Haploview 4.03 software. The "GAACAC" haplotype formed by rs2897827, rs5935436, rs2302267, rs179019, rs5743740, and rs179016 had a frequency of 3.1% in SLE cases and 4.8% in con-

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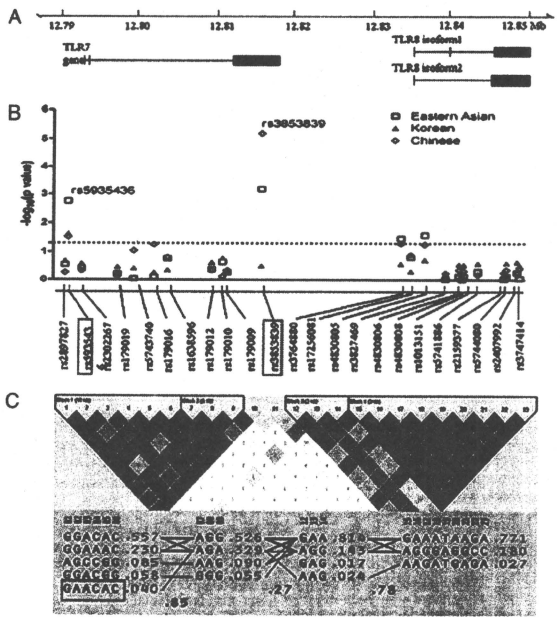

Fig. 1. A functional SNP rs3853839 in 3′UTR of *TLR7* is associated with SLE in a Chinese population. (*A*) *TLR7* and *TLR8* gene structure. (*B*) Eleven *TLR7* SNPs and 12 *TLR8* SNPs were genotyped in 1,434 SLE cases and 1,591 healthy controls of Eastern Asian descent. The two SNPs (rs5935436, rs3853839) that showed significant association after Bonferroni correction are highlighted. (*C*) Two haplotype blocks were constructed based on the strength of LD in each gene region. The R^2 values of each SNP pair are depicted. The protective haplotype GAACAC is highlighted.

trols ($P = 0.0017$; Fig. 1*C*). Given that only this *TLR7* protective haplotype carries the minor allele of rs5935436, we genotyped rs5935436 in replication studies to represent the SLE-associated haplotype. Rs3853839 was in low linkage disequilibrium (LD) with other SNPs in the region. To minimize missing any other common polymorphisms, we sequenced 5′ promoter region (2 kb upstream) as well as three exons (including 3′UTR) of *TLR7* in 48 Chinese female patients with SLE, which revealed no additional polymorphism and raised the possibility that rs3853839 might be causal.

To verify the association of rs3853839 and rs5935436 with SLE, we then conducted a replication study in two independent case-control Chinese and Japanese panels. Whereas rs5935436 was not replicated in these studies ($P = 0.97$ and $P = 0.25$, respectively), rs3853839 showed a consistent association with SLE in both replication panels [Chinese, 2,340 vs. 2,436; $P = 9.0 \times 10^{-4}$; OR = 1.21 (95% CI = 1.08–1.35); and Japanese, 560 vs. 913; $P = 0.007$; OR = 1.28 (95% CI = 1.07–1.53); Table 1]. A combined analysis of discovery and replication panels showed compelling evidence supporting that G allele of *TLR7* SNP rs3853839 conferred risk for SLE [$P_{combined} = 6.5 \times 10^{-10}$, $OR_{combined} = 1.27$ (95% CI = 1.17–1.36)]. Furthermore, G allele showed an additive effect in female subjects, as the OR for female homozygous GG versus CC was 1.45 (95% CI = 1.17–1.79) and OR for heterozygous GC versus CC was 1.15 (95% CI = 0.93–1.43), which reinforced a role of rs3853839 in developing SLE. Stratification by clinical subsets including age of disease onset, presence of mucocutaneous manifestations, lupus nephritis, dsDNA, or anti-RNA binding protein antibodies (presence of one or more autoantibodies to Ro/SSA, La/SSB, RNP, and Sm) (8) revealed a weak association of G allele with the presence of anti-RNA binding protein [$P = 0.049$, OR = 1.16 (95% CI = 1.00–1.35); missing data, 45%], suggesting that *TLR7* binding of RNA containing immune complexes might play a role in the initiation and perpetuation of autoimmunity.

Significant Male Effect of rs3853839 in the Risk of Developing SLE. Of interest, we observed a stronger association of rs3853839 with male SLE. In the discovery panel, G allele showed higher OR in male patients compared with female patients [OR = 1.79 (95% CI = 1.03–3.13) vs. 1.22 (95% CI = 1.06–1.39); Table 1], especially in Chinese subjects [OR = 5.56 (95% CI = 1.85–16.7) vs. 1.54 (95% CI = 1.22–1.96)]. This notable male effect was verified in both replication panels: Chinese male patients with SLE had a significantly higher frequency of G allele than controls [92% vs. 81%; OR = 2.73 (95% CI = 1.57–4.74)]; in contrast, female cases only showed a modest increase of G allele versus controls [83% vs. 80%; OR = 1.19 (95% CI = 1.06–1.35)]. A similar finding was also observed in the Japanese subject replication panel. In the combined analysis, G allele occurred in 89% of male cases ($n = 358$), but only 77% of male controls ($n = 1,550$),

Table 1. Association of rs3853839 with SLE in Eastern Asian populations

Ethnicity and panel	Case/control	G risk allele frequency		*P* value	OR (95% CI)
		Case	Control		
Eastern Asian*:					
Discovery					
Male	126/229	0.83	0.73	0.038	1.79 (1.03–3.13)
Female	1,308/1,362	0.79	0.76	3.00E-03	1.22 (1.06–1.39)
All	1,434/1,591	0.80	0.76	6.70E-04	1.24 (1.1–1.41)
Chinese: Replication 1					
Male	196/931	0.92	0.81	2.31E-04	2.73 (1.57–4.74)
Female	2,144/1,505	0.83	0.80	4.24E-03	1.19 (1.06–1.34)
All	2,340/2,436	0.83	0.80	9.02E-04	1.21 (1.08–1.35)
Japanese: Replication 2					
Male	36/390	0.89	0.69	0.014	3.51 (1.22–10.2)
Female	524/523	0.75	0.71	0.037	1.23 (1.01–1.49)
All	560/913	0.75	0.70	7.00E-03	1.28 (1.07–1.53)
Eastern Asian*: Combined					
Male	358/1,550	0.89	0.77	1.33E-06	2.33 (1.64–3.30)
Female	3,976/3,390	0.80	0.77	1.19E-07	1.24 (1.14–1.34)
All	4,334/4,940	0.81	0.77	6.50E-10	1.27 (1.17–1.36)

*Includes subjects of Chinese, Korean, or Japanese descent.

showing a strong association with SLE ($P = 1.33 \times 10^{-6}$) and significantly higher OR in male versus female subjects [2.33 (95% CI = 1.64–3.30) vs. 1.24 (95% CI = 1.14–1.34); $P = 4.1 \times 10^{-4}$]. The significant male sex effect of rs3853839 in SLE was confirmed by a higher G allele frequency in male cases than female cases ($P = 1.1 \times 10^{-4}$; OR = 1.90 (95% CI = 1.36–2.64)] and by a higher OR in male subjects than the OR calculated by using only female homozygotes [GG vs. CC, OR = 1.45 (95% CI = 1.17–1.79); $P = 0.02$].

***TLR7* SNP rs385839 Confers Elevated Expression of *TLR7* in Vivo.** The consistent association of rs3853839 with SLE led us to investigate the potential functional consequences of the G risk allele. We first examined whether the 3′UTR polymorphism affected the expression level of *TLR7* transcripts. We isolated peripheral blood mononuclear cells (PBMCs) from selected Chinese individuals carrying various genotypes and measured the mRNA expression levels of *TLR7* by using real-time PCR. As shown in Fig. 2*A*, male controls carrying G allele had significantly higher *TLR7* transcripts than those carrying C allele ($P = 0.02$). Similarly, female cases and controls homozygous for G allele showed notably higher *TLR7* mRNA expression than those carrying homozygous C allele ($P = 7.4 \times 10^{-6}$ and $P = 0.03$, respectively). No sex differences in *TLR7* mRNA expression levels were observed between normal controls carrying the same allelic genotype of rs3853839 ($P = 0.07$ or $P = 0.55$ for GG females vs. G males or CC females vs. C males, respectively; Fig. 2*A*). The sex comparison was not replicated in patients with SLE because of insufficient sample size of blood collected from male patients with SLE.

To exclude potential bias between individuals, we next assessed the allelic specific transcriptional expression of *TLR7* in Chinese female G/C heterozygotes by pyrosequencing. We extracted genomic DNA (gDNA) and cDNA from PBMCs of heterozygous SLE patients and controls and determined the exact ratios of G/C in gDNA and cDNA for each individual. As shown in Fig. 2*B*, the mean G/C ratio in gDNA of all individuals was 1.05, which was consistent with the theoretical value. However, SLE and control female heterozygotes showed a 2.7-fold higher G/C ratio in *TLR7* transcripts than that in gDNA ($P = 2 \times 10^{-4}$ and $P = 2 \times 10^{-3}$, respectively; $P_{overall} = 1.2 \times 10^{-6}$), meaning that there was significantly higher level of G allele–containing *TLR7* transcripts than C allele–containing transcripts in PBMCs, even though there was same amount of gDNA that contains either allele.

To investigate whether the disparity observed between transcript and gDNA was a result of imbalanced X chromosome inactivation (XCI) in female subjects, we analyzed the XCI pattern in these individuals by measuring the degree of methylated CAG alleles in androgen receptor (*AR*) gene as previously described (9). Fig. 2*C* showed no significant skewed XCI in either SLE or controls ($P = 0.8$), suggesting that skewed XCI or somatic selection might not account for the current finding. Taken together, these data suggested rs3853839 polymorphism could play a functional role in the regulation of *TLR7* transcript levels in vivo.

To investigate whether allelic differences of rs3853839 corresponded to TLR7 protein levels, we isolated proteins from PBMCs of healthy Chinese male subjects carrying the G or C allele for Western blot analysis. Fig. S1 showed that five of the six male G allele carriers had higher expression levels of TLR7 protein than the two C allele carriers after normalization of the amount of protein loading using β-actin. Although the result was not statistically significant ($P = 0.21$, probably because of the very small sample size), the finding of higher TLR7 protein expression of the G risk allele carriers was consistent with what we observed at mRNA levels of *TLR7*.

We next investigated that whether the functional SNP rs3853839 might influence the IFN reaction downstream of *TLR7*. A prominent feature of patients with SLE is an increased expression of type I IFN–regulated genes (i.e., IFN signature) in PBMCs compared with unaffected controls (10, 11). We measured expression levels of four type I IFN–regulated genes, including lymphocyte antigen 6 complex, locus E (*LY6E*), myxovirus resistance 1 (*MX1*), IFN-inducible protein with tetratricopeptide repeats 1 (*IFIT1*), and *IFIT3* by real-time PCR in PBMCs from clinically inactive female SLE patients carrying various rs3853839 genotypes and calculated an IFN score for each individual. As shown in Fig. 2*D*, female SLE patients carrying GG genotype showed a more pronounced IFN signature in PBMCs compared with those carrying GC and CC genotypes, indicating rs3853839 might modulate expression levels of *TLR7*, affecting IFN response in vivo.

Discussion

There is compelling evidence supporting the contribution of *TLR7* to the development of autoimmunity. Transgenic mice with a twofold overexpression of *TLR7* would have increased production of RNA-related autoantibodies and develop spontaneous