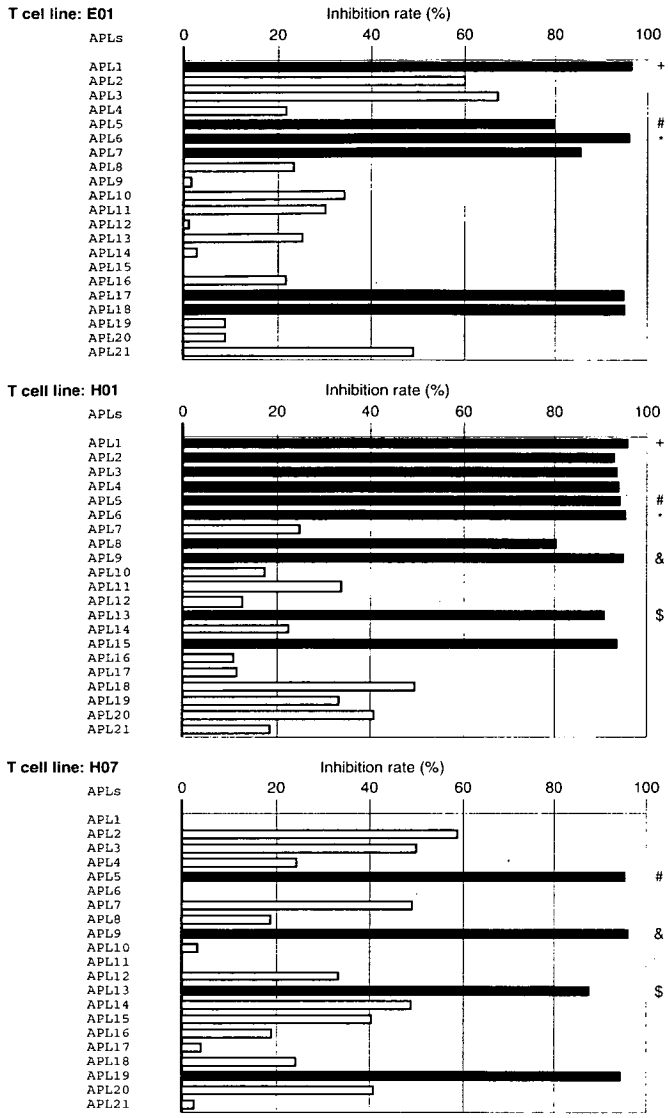

Fig. 2. Antagonistic activity of altered peptide ligands (*APL*) to T-cell lines. Three T-cell lines were established from two rheumatoid arthritis patients with DRB1*0101 and antagonistic activity of each APL was evaluated. The antagonistic activity was expressed as percentage of inhibition of the CII 256–271 peptide response. It was judged as positive when the percentage of inhibition was more than 80%. The *symbols* *, +, &, and $ designate APL inducing antagonistic activity in two T-cell lines; # designates APL inducing antagonistic activity in three T-cell lines

Results and discussion

The results demonstrated that seven APLs suppressed CII-specific T-cell response in E01 T-cell line, 10 APLs in H01, and four APLs in H07. Especially, APL5 (CII 262; G→A) inhibited the CII response in all three T-cell lines. Four other analog peptides, APL1 (CII 261; A→S), APL6 (CII 263; F→D), APL9 (CII263; F→S), and APL13 (CII 265; G→D), decreased T-cell proliferation in two T-cell lines. Although CII 263 (F) was considered an anchor residue, which is a binding site to DR1 molecule, some APLs induced T-cell suppression by antagonism when the residue was substituted. This might imply that there was another anchor residue on the peptide. Actually, CII 260 (I) was predicted as an anchor residue using the systems of prediction of MHC binding peptide (MHC Pred: http://www.jenner.ac.uk/MHCPred, RANKPEP: http://www.mifoundation.org/Tools/rankpep). CII 263 (F) might compose a TCR contact site when the peptide is bound to HLA molecule at CII 260 (I) or CII 264 (K).

To date, there have been some trials to change the T-cell response to CII. Fridkis-Hareli et al. reported that analog peptides based on CII 261–273 could block the binding of CII 261–273 peptide to DR1 molecule and suppress T-cell response to CII 261–273 peptide.[7] Myers et al. showed that CII 256–276 peptide substituted CII 263 (F→N) and CII 266 (E→D) induced lower IFNγ production and higher IL-4 and IL-10 production from splenocytes of DR1 transgenic mice, and collagen-induced arthritis was suppressed using this peptide.[8] On the other hand, we used antagonistic activity to change the T-cell response. This strategy is characterized by controlling the activation of antigen-specific T cells directly. Since RA patients do not necessarily have CII-reactive T cells, the antigen-specific therapy targeted to several autoantigens is expected to be an efficient method to control RA. This examination was probably the first trial using T cells of RA patients.

In conclusion, we observed evidence that at least four APLs of CII (256–271) were able to suppress the CII-specific T-cell proliferation. Further trials using more T-cell lines established from RA patients are required; however, these findings should shed light on a new therapy for RA in the antigen-specific fashion.

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Altered peptide ligands regulate muscarinic acetylcholine receptor reactive T cells of patients with Sjögren's syndrome

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In the generation of Sjögren's syndrome (SS), CD4 positive αβ T cells have a crucial role. Previous studies have provided evidence about the T cell receptor (TCR) Vβ and Vα genes on these T cells, and sequence analysis of the CDR3 region indicates the presence of some conserved amino acid motifs, supporting the notion that infiltrating T cells recognise relatively few epitopes on autoantigens.[1]

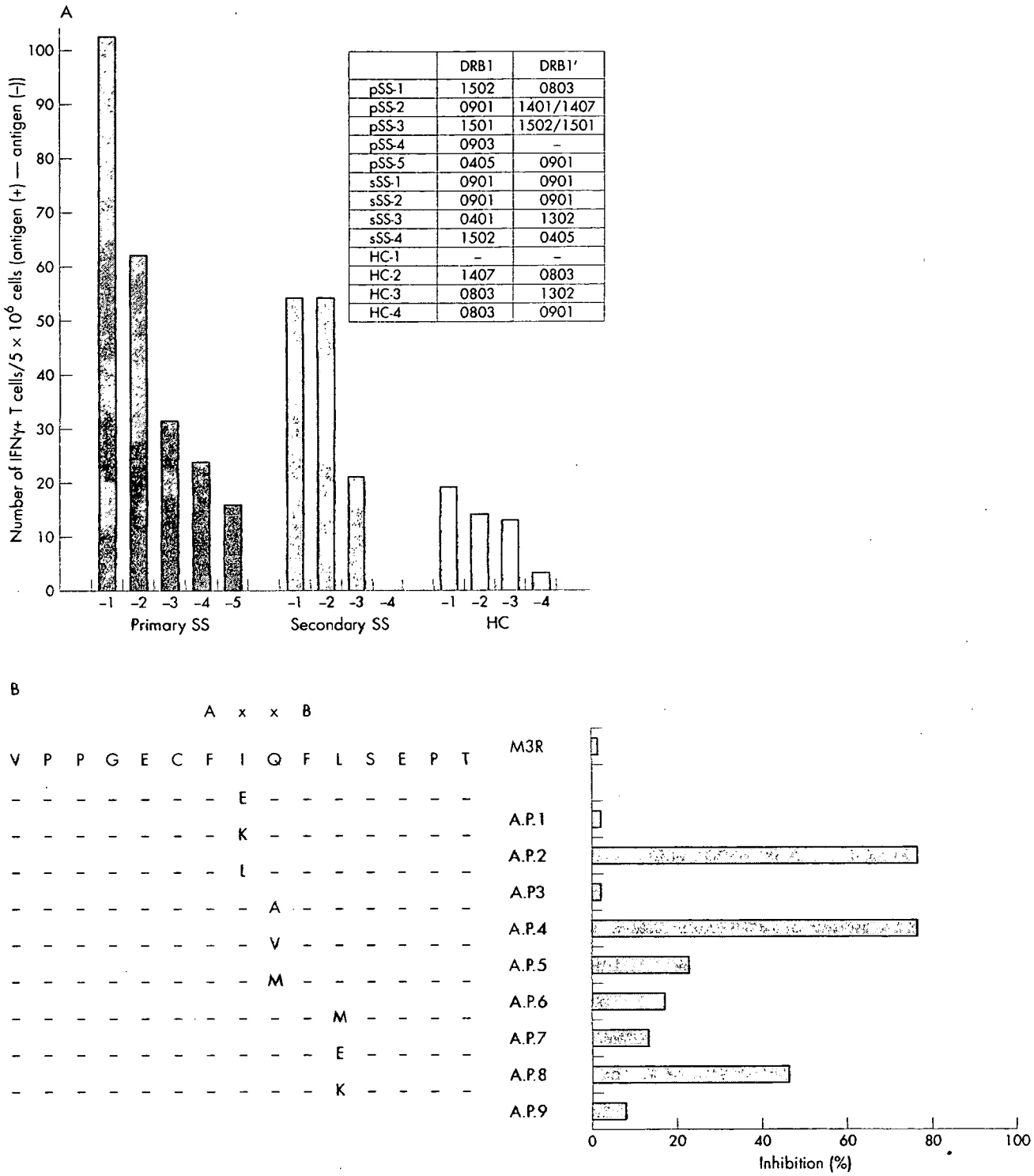
Candidate autoantigens recognised by T cells that infiltrate the labial salivary glands of patients with SS have been analysed, and Ro/SSA 52 kDa,[2] α-amylase, heat shock protein, and TCR BV6 have been identified, although Ro/SSA 52 kDa reactive T cells were not increased in peripheral blood.[3] Gordon *et al* indicated that anti-M3R autoantibodies occurred in SS and were associated with the sicca symptoms.[4] Recently, we provided evidence for the presence of autoantibodies against the second extracellular domain of muscarinic acetylcholine receptor (M3R) in a subgroup of patients with SS.[5] The M3R is an interesting molecule, because this portion has an important role in intracellular signalling,[5] although the function of anti-M3R autoantibodies remains unknown.

The mechanism through which a peptide is recognised by a TCR is flexible. If the amino acid residue of the peptide ligands for TCR is substituted by a different amino acid and can still bind to major histocompatibility complex molecules (altered peptide ligand), such an altered peptide ligand could regulate the activation of T cells. Several studies have shown that an altered peptide ligand could induce differential cytokine secretion, anergy, and antagonism of the response to the wild-type antigens.[6 7] The altered peptide ligand has the potential of being used therapeutically against T cell mediated diseases such as autoimmune diseases and allergic disorders.

As an extension to our previous study,[5] we focused in the present study on M3R reactive T cells and analysed T cell epitopes and their altered peptide ligands with the aim of regulating T cell proliferation and autoantibody production. The 25mer synthetic amino acids encoding the second extracellular domain of M3R (KRTVPPGECFIQFLSEPTITF GTAI, AA213-237) were used as the antigen for T cells, and the number of interferon (IFN) γ producing T cells was counted by flow cytometry using a magnetic activated cell sorting (MACS) secretion assay. The proportion of IFNγ-producing T cells among peripheral blood mononuclear cells (PBMCs) was high in two of five patients with primary SS (pSS) and two of four patients with secondary SS (sSS), compared with the level in four healthy control subjects (HC) (fig 1A). Three patients with SS and M3R reactive T cells (pSS-2, and sSS-1, 2) had the HLA-DR B1*0901 allele and the other patient (pSS-1) had HLA-DR B1*1502 and *0803 alleles. The 25mer amino acids contain the anchored motifs that bind to HLA-DR B1*0901. Thus, IFNγ production by T cells should be due to the recognition of antigen on the HLA molecule by the TCR on T cells.

The results shown in fig 1 were obtained as follows. Blood samples were collected from five Japanese patients with pSS and four Japanese patients with sSS followed up at the University of Tsukuba Hospital. All patients with SS satisfied both the Japanese Ministry of Health criteria for the classification of SS[8] and the revised EU-US criteria[9]. We also recruited four HC from our university. Approval for this study was granted from the local ethics committee, and written informed consent was obtained from all patients and HC who participated in this study.

Their HLA-DR allele was examined by the SSOP-PCR method, as described elsewhere. A 15mer peptide (VPPGECFIQFLSEPT) (M3R AA216–230) corresponding to the sequence of the second extracellular loop domain was also synthesised (Kurabo Industries, Osaka, Japan). PBMCs were purified with Ficoll-Paque and 5×10^6 cells were cocultured with 10 μg of M3R peptide (25mer) in 1 ml of RPMI-1640 with 10% of human AB serum (Sigma, St Louis, MO) for 12 hours at 37°C. As a positive control, 1 μg of staphylococcal enterotoxin B (Toxin Technology Inc, USA) was used. IFNγ-producing cells were identified by the MACS cytokine secretion assay (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, the cells were incubated with 20 μg of IFNγ detection antibody (Ab; Miltenyi Biotec), 20 μg of



	DRB1	DRB1′
pSS-1	1502	0803
pSS-2	0901	1401/1407
pSS-3	1501	1502/1501
pSS-4	0903	–
pSS-5	0405	0901
sSS-1	0901	0901
sSS-2	0901	0901
sSS-3	0401	1302
sSS-4	1502	0405
HC-1	–	–
HC-2	1407	0803
HC-3	0803	1302
HC-4	0803	0901

Figure 1 (A) M3R reactive T cells. (B) Selection of altered peptide ligands.

anti-CD4-FITC Ab (Becton Dickinson, Franklin Lakes, NJ, USA), and 5 μg of anti-CD3-APC Ab (Becton Dickinson) for 10 minutes at 4°C. After double washing with a cold buffer (phosphate buffered saline/0.5% bovine serum albumin with 2 mM EDTA), the cells were incubated with 20 μg of anti-phycoerythrin microbeads (Miltenyi Biotec) for 15 minutes at 4°C. After double washing, the cells were resuspended with 500 μl buffer and then passed through an MS column (Miltenyi Biotec), which was set to mini-magnet (Miltenyi Biotec). The column was set on the Falcon tube (Becton Dickinson), bead-binding cells were eluted by 1 ml of cold buffer, and IFNγ-producing cells were analysed by FACSCalibur (Becton Dickinson).

The 15mer peptide (M3R 216–230) and its nine altered peptide ligand candidates were synthesised (Sigma) (fig 1B). The purity of each peptide was >90%. The anchor positions binding to HLA-DR B1*0901 are AA222 and AA225, which are indicated as A and B in fig 1B. PBMCs from patient pSS-2 were used in this experiment; 1×10^6 cells were cultured with 10 μg of each peptide in 1 ml of RPMI-1640 with 10% human

AB serum. IFNγ-producing T cells were identified using MACS secretion assay as described in fig 1A.

To determine the altered peptide ligands of M3R in patients with SS, we synthesised nine 15mer peptides (VPPGECFI→E/K/LQFLSEPT, VPPGECFIQ→A/V/MFLSEPT, VPPGECFIQFL→M/E/KSEPT, M3R216–230), in which the anchored motif binding to the HLA-DR B1*0901 molecule is conserved, although one amino acid to TCR was different. Altered peptide ligands were selected based on inhibition of IFNγ production by M3R reactive T cells. Figure 1B shows that M3R 223I→K and M3R 224Q→A significantly suppressed the number of IFNγ-producing T cells, suggesting that they are candidates for selection as altered peptide ligands. The inhibition of IFNγ by other cytokines may not be likely, because interleukin 4 producing T cells were not increased (data not shown).

In conclusion, we have provided evidence for the presence of M3R reactive T cells in the serum of patients with SS and shown that VPPGECFKQFLSEPT (M3R 223I→K) and VPPGECFIAFLSEPT (M3R 224Q→A) are candidate altered peptide ligands of the second extracellular domain of M3R. Our findings may provide the basis of a potentially useful antigen-specific treatment for SS using altered peptide ligands of autoantigens recognised by autoreactive T cells.

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on timely referral should also include variables as discussed above. This is also suggested in gender studies in other health domains [9]. Multivariate analyses in large prospective studies will allow firm conclusions to be reached.

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Characterisation of Th1/Th2 type, glucose-6-phosphate isomerase reactive T cells in the generation of rheumatoid arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterised by an unknown inflammatory process in multiple joints. The K/BxN T cell receptor transgenic mouse model is a striking model of inflammatory arthritis characterised by arthritic manifestations similar to those of RA.[1] Matsumoto *et al* reported that arthritis could be provoked by linked T and B cell recognition of a ubiquitously expressed self antigen glucose-6-phosphate isomerase (GPI).[2] Recently, immunisation with recombinant human GPI was reported to induce T cell dependent arthritis in DBA/1 mice,[3] supporting the notion that GPI reactive T cells have a crucial role in the induction of arthritis.

In our previous study we reported the presence of high titres of anti-GPI antibodies (Abs) in some patients with RA, although a few control subjects were also positive.[4] To examine the role of GPI-specific T cells in patients with RA, we investigated the spontaneous Th1/Th2 response to GPI in patients with RA, systemic lupus erythematosus (SLE), and in healthy subjects with anti-GPI Abs.

To select anti-GPI Ab positive patients, an enzyme linked immunosorbent assay (ELISA) was performed using two different sources of GPI: a recombinant human GPI (huGPI), and a rabbit muscle GPI (raGPI; Sigma Chemical Co, St Louis, MO, USA), which have been described in detail previously.[4] Fifteen anti-GPI Ab positive patients with RA (from 185 with RA), four patients with SLE (from 135 with SLE), and four healthy subjects (from 145 controls) were studied (table 1). To analyse the possible relationship between HLA-DRB1 and anti-GPI Ab positivity, HLA-DRB1 alleles were screened. As shown in Table 1, 10 (67%) patients with RA and anti-GPI Abs shared the HLA-DRB1*0405 allele, which is one of the genes for susceptibility to RA in Japanese people, and five (33%) patients were DRB1*0901. In a recent report, the DRB1*0405 and *0901 alleles showed the most significant associations with RA in Korean people.[5] However, none of the four patients with SLE or four control subjects positive for anti-GPI Abs retained these alleles, suggesting a strong linkage between anti-GPI positive patients with RA with anti-GPI Abs and HLA DRB1*0405 and *0901 alleles (table 1).

To investigate the pathogenic relevance of GPI reactive T cells in subjects with anti-GPI Abs, a magnetic activated cell sorting cytokine secretion assay was performed using peripheral blood mononuclear cells plus GPI (in the presence of 10 μg purified human GPI protein digested by thrombin or 13.5 ng thrombin as a control). As a positive control, we used staphylococcal enterotoxin B (1 μg/ml). Cells (2×10^6) were harvested Ab-Ab directed against CD45 and either interferon (IFN) γ or interleukin (IL) 4 conjugates, and stained with phycoerythrin (PE)-conjugated anti-IFNγ or anti-IL4. Cells were magnetically labelled by anti-PE Ab microbeads, and were analysed on a FACSCalibur flow cytometer (Becton Dickinson). IFNγ secreting T cells were detected in seven (47%) patients with RA (RA3, 6, 7, 9, 10, 11, 15). IFNγ may be produced by GPI reactive T cells (table 1). IL4 secreting T cells were detected in four (27%) patients (RA1, 3, 7, 10), although they were less frequent than IFNγ+ T cells. Three patients (RA3, 7, 10) had both IFNγ and IL4 secreting T cells. In contrast, only one healthy subject (control 2) showed weak response to GPI (IFNγ and IL4). Interestingly, all seven patients with RA bearing GPI reactive IFNγ+ T cells shared either DRB1*0405 or *0901 (table 1). Our results demonstrated that GPI-specific Th1 and Th2-type cells (especially

Table 1 Anti-GPI Abs and DRB1 genotype in patients with RA, SLE, and in healthy subjects

Subject	huGPI	raGPI	DRB1 genotype	IFNγ+ T cells	IL4+ T cells
RA1	1.32	2087	0405 0901	4	9
RA2	2.73	3.02	0409 0803	0	0
RA3	1.33	1.15	0405 1501	190	12
RA4	2.43	2.55	0101 0803	0	0
RA5	1.79	3.14	0405 0401	6	3
RA6	1.65	2.67	0802 0901	15	3
RA7	1.88	1.15	1402 0901	55	10
RA8	2.60	3.47	0405 0901	0	0
RA9	2.46	1.70	1502 0405	8	1
RA10	1.93	2.65	0405 0803	16	24
RA11	1.72	0.95	0405 0803	49	3
RA12	1.40	1.61	0405 1502	7	0
RA13	1.49	0.94	0405 0803	0	0
RA14	1.39	0.99	0405 1502	0	0
RA15	2.48	3.32	0901 0901	20	2
SLE1	3.68	1.86	0803 1404	0	0
SLE2	1.32	1.38	0427 0427	0	0
SLE3	1.91	2.41	0101 0428	2	0
SLE4	2.89	2.97	0803 0803	0	0
Control1	3.75	3.54	1501 1403	2	0
Control2	3.05	3.19	1302 0803	9	4
Control3	2.19	3.28	1501 0803	3	0
Control4	2.5	2.59	1329 1406	1	1

*The cut off optical density was calculated from an ELISA of 145 healthy subjects; the mean value + two standard deviation was 1.32 to human recombinant GPI and 0.94 to rabbit native GPI. Double positive populations were considered anti-GPI Ab positive. For MACS cytokine secretion assay, positive cell numbers were determined after subtracting control cells, either reacted on thrombin or spontaneously secreting cytokines, from GPI reactive IFNγ+ and IL4+ T cells. The cut off cell numbers were calculated from the reaction of four patients with SLE and four control subjects who were all anti-GPI Ab positive. The mean value + two standard deviation was 7.6 to IFNγ and 3.6 to IL4. Bold numbers indicate a positive reaction to GPI.
Abs, antibodies; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; IFN, interferon; IL, interleukin.

Th1-type cells) were frequently detected in patients with RA with anti-GPI Abs, suggesting that these cytokines may be associated with the production of arthritogenic Abs, especially when associated with HLA-DRB1*0405 or *0901.

In conclusion, our findings suggest that GPI reactive IFNγ+/IL4+ T cells may have a crucial role in the generation of arthritis in HLA-DRB1*0405 or *0901 positive patients with RA and anti-GPI Abs.

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Muscarinic acetylcholine receptor autoantibodies in patients with Sjögren's syndrome

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Sjögren's syndrome (SS) is an autoimmune disease characterised by lymphocytic infiltration into the lachrymal and salivary glands, leading to dry eyes and mouth. Infiltration is also found in the kidneys, lungs, thyroid, and liver. Immunohistochemical studies have shown that most infiltrating lymphocytes around the labial salivary and lachrymal glands, and kidneys are CD4 positive αβ T cells. Previous studies with polymerase chain reaction provide evidence about the T cell receptor Vβ and Vα genes on these T cells, and sequence analysis of the CDR3 region indicates some conserved amino acid motifs, supporting the notion that infiltrating T cells recognise relatively few epitopes on autoantigens.[1]

Candidate autoantigens recognised by T cells infiltrating the labial salivary glands of patients with SS have been analysed, and Ro/SSA 52 kDa, α-amylase, heat shock protein, and T cell receptor BV6[2] have been identified. However, there is no direct evidence that these reactive T cells really attack and destroy the salivary glands. In contrast, the presence of autoantibodies (Abs) against M3 muscarinic acetylcholine receptor (M3R) has been reported, and it is suggested that an immune reaction to M3R plays a crucial part in the generation of SS.[3–5] Robinson, *et al* demonstrated that human anti-M3R Abs reduce the secretory function in NOD.Igμnull mice.[3] Moreover, Bacman *et al* clearly showed that human Abs against the second extracellular loop of M3R could

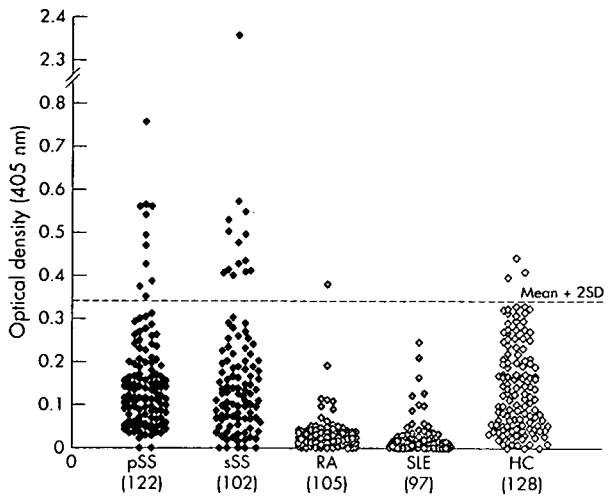

Figure 1 Optical density. pSS, primary Sjögren's syndrome; sSS, secondary Sjögren's syndrome, RA, rheumatoid arthritis, SLE, systemic lupus erythematosus, HC, healthy controls. Numbers in parentheses represent the number of patients in each group.

activate nitric oxide synthase coupled to the lachrymal gland M3R, suggesting that anti-M3R Abs are a new marker of dry eye SS.[4] The M3Rs are expressed on salivary and lachrymal glands, and thus they should be key receptors involved in the production of saliva and tears after stimulation of acetylcholine. Thence, autoantibodies against M3R could interfere with the production of saliva and tears. To test this hypothesis we analysed the prevalence of anti-M3R Abs in patients with SS.

Approval for this study was obtained from the local ethics committee and written informed consent was obtained from all patients and volunteers who participated in this study.

METHODS

Serum samples were collected from 122 Japanese patients with primary SS and 102 Japanese patients with secondary SS followed up at the Department of Internal Medicine, University of Tsukuba Hospital, Japanese Red Cross Mito Hospital, and Shimosizu National Hospital. All patients with SS satisfied the Japanese Ministry of Health criteria for the classification of SS. We also recruited 105 patients with rheumatoid arthritis, 97 with systemic lupus erythematosus, and 128 healthy subjects from our University.

A 25mer peptide (KRTVPPGECFIQFLSEPTITFGTAI) corresponding to the sequence of the second extracellular loop domain of the human M3R was synthesised (Kurabo Industries, Osaka, Japan). As a negative peptide, a 25mer peptide (SGSGSGSGSGSGSGSGSGSGSGS) was also synthesised (Kurabo Industries). Peptide solution (100 µl/well at 10 µg/ml) in 0.1 M Na_2CO_3 buffer, pH 9.6, was adsorbed to a Nunc-Immuno plate (Nalge Nunc International, Rochester, NY) at 4°C overnight, and blocked with 5% bovine serum albumin (Wako Pure Chemical Industries, Osaka) in phosphate buffered saline (PBS) for 1 hour at 37°C. Serum at 1:50 dilution in blocking buffer was incubated for 2 hours at 37°C. The plates were then washed three times with 0.05% Tween 20 in PBS, and 1 µl of alkaline phosphatase conjugated goat antihuman IgG (Fc; American Qualex, San Clemente, CA) diluted 1:1000 in PBS was added for 1 hour at room temperature. After extensive washing, 100 µl of *p*-nitrophenyl phosphate (Sigma, St Louis, MO) solution (final concentration 1 mg/ml) was added as alkaline phosphatase substrate. Plates were incubated for 1 hour at room temperature and the optical density at 405 nm was measured by plate spectrophotometry (Bio-Rad Laboratories, Hercules, CA; fig 1). Determinations were performed in triplicate and standardised between experiments.

RESULTS AND DISCUSSION

The 25mer synthetic amino acid encoding the second extracellular domain of M3R was used as the antigen, because this portion has an important role in intracellular signalling.[6] The binding activity of Abs to the second extracellular domain of M3R is dependent on the concentration of Abs using serial-diluted quantitative assay (data not shown). Figure 1 shows that Abs against M3R were more commonly detected in the serum of patients with primary (11/122 (9%), $p<0.05$) and secondary SS (14/102 (14%), $p<0.05$) than in those with other autoimmune diseases such as rheumatoid arthritis (1/105 (1%)) and systemic lupus erythematosus (0/97 (0%)), or healthy subjects (3/128 (2%)). These results clearly showed that autoantibodies against M3R are specifically present in SS, suggesting that anti-M3R Abs could be used as a diagnostic marker in a subgroup of patients with SS (9–14%). The proportions of patients positive for anti-M3R Ab and anti-SSA Ab, anti-SSB Ab, rheumatoid factor, and antinuclear factor were 68%, 29%, 57%, and 83%. In contrast, the proportions of patients negative for anti-M3R Ab with these autoantibodies were 65%, 6%, 59%, and 76%, respectively. Thus, anti-SSB Ab is strongly associated with anti-M3R Ab ($p<0.05$), although the homology between SSB and the M3R molecule is very low and the detailed mechanism remain unclear. The clinical feature is not significantly different between in patients with SS positive for anti-M3R Ab and negative patients.

In conclusion, we detected autoantibodies against M3R in a subgroup of patients with SS, suggesting that anti-M3R Ab could be used as a new diagnostic marker for SS. Further experiments on the functional analysis of anti-M3R Abs in SS using M3R transfectant cell lines should shed light on the relationship between the presence of anti-M3R autoantibodies and the pathogenesis of SS.

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CONCISE REPORT

Association of mannose binding lectin (MBL) gene polymorphism and serum MBL concentration with characteristics and progression of systemic lupus erythematosus

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Objective: To determine whether occurrence, characteristics, and progression of systemic lupus erythematosus (SLE) are associated with polymorphism of the mannose binding lectin (MBL) gene and with serum MBL concentration.

Methods: Codon 54 MBL gene polymorphism of 147 patients with SLE and 160 healthy controls was determined by polymerase chain reaction-restriction fragment length polymorphism. Serum concentration of MBL was measured by enzyme immunoassay. Fluctuations of serum MBL were analysed with respect to disease characteristics and activity.

Results: Frequency of homozygosity for codon 54 minority allele was 6% (9/147) in patients with SLE, and significantly higher than in controls ($p=0.0294$, Fisher's exact test). MBL polymorphism in patients with SLE was not significantly associated with disease characteristics or immunological phenotypes. Patients homozygous for the B allele tended to have a higher risk of infection during treatment. Levels of C3 and CH_{50} were slightly, but significantly, associated with serum MBL concentration in patients with SLE homozygous for the majority allele. During the course of SLE, serum MBL concentration increased in 6/14 patients, and decreased in 7 after initiation of immunosuppressive treatment.

Conclusions: MBL gene polymorphism influences susceptibility to SLE, but has no direct effect on disease characteristics. Serum MBL levels fluctuate during the course of SLE in individual patients. MBL genotyping may be useful in assessing the risk of infection during treatment of SLE.

Mannose binding lectin (MBL) is a molecule that shares many features with C1q. MBL comprises a trimer of three identical polypeptides, and several trimers further combine to form a bouquet-like structure.[1] MBL mediates lectin dependent activation of the complement pathway,[1] and has an important role in host defence against micro-organisms. People lacking this protein could develop severe episodes of bacterial infections from early life.[2] Several polymorphisms have been reported for the MBL gene, and a large interindividual difference in serum MBL concentration is caused by the possession of variant alleles. Codon 52, 54, and 57 polymorphisms are all on exon 1 of the MBL gene, and the presence of any of the minority alleles significantly reduces serum MBL concentration. Furthermore, homozygosity for minority alleles results in almost complete deficiency of serum MBL.[3] This has been attributed to increased degradation of the mutated protein.[4]

Recently, several studies have suggested that possession of MBL minority alleles may be associated with occurrence of systemic lupus erythematosus (SLE).[5 6] It is known that C1q deficiency is associated with severe symptoms of SLE.[7] Two possible explanations for associations between MBL or C1q deficiency and occurrence of SLE can be proposed: (*a*) MBL and C1q can bind to and initiate uptake of apoptotic cells into macrophages,[8 9] and abnormal clearance of apoptotic cells caused by MBL or C1q deficiency may result in overexpression of autoantigens; (*b*) viral infection is believed to be one of the causes of SLE,[10] and MBL or C1q deficiency may lead to more frequent infections.

This study was conducted on the premise that occurrence, characteristics, and progression of SLE are associated with polymorphism of the MBL gene and with serum MBL concentration. To our knowledge, this is the first study that has measured serum MBL concentration before and after immunosuppressive treatment in patients with newly diagnosed SLE.

PATIENTS AND METHODS

Samples from 147 Japanese patients with SLE followed up at our hospital, were used for the study. All patients fulfilled the 1997 American College of Rheumatology Classification Criteria for SLE. Samples from 160 Japanese healthy volunteers served as controls.

Genomic DNA was purified from peripheral blood leucocytes using the DNAQuick DNA purification kit (Dainippon Pharmaceuticals, Osaka, Japan), and stored at −30°C. Typing of the MBL gene allele was performed by polymerase chain reaction-restriction fragment length polymorphism, according to the method of Madsen *et al*.[3] The wild-type allele was designated allele A, and codon 54 substitution (glycine to aspartic acid) was designated allele B. Previous studies have shown that codon 52 and 57 polymorphisms are not present or extremely rare in the Japanese population.[11] Serum concentration of MBL was measured by a specific enzyme immunoassay using two rabbit polyclonal anti-MBL antibodies as described previously.[12]

Table 1 Codon 54 genotypes of the MBL gene in patients with SLE and healthy controls

	SLE	Healthy controls	p Value
AA + AB	138 (AA; 84, AB; 54)	158 (AA; 101, AB; 57)	0.0294
BB	9	2	
Total	147	160	

Allele A, codon 54 wild type majority allele; allele B, codon 54 variant minority allele.
p Value by Fisher's exact test.

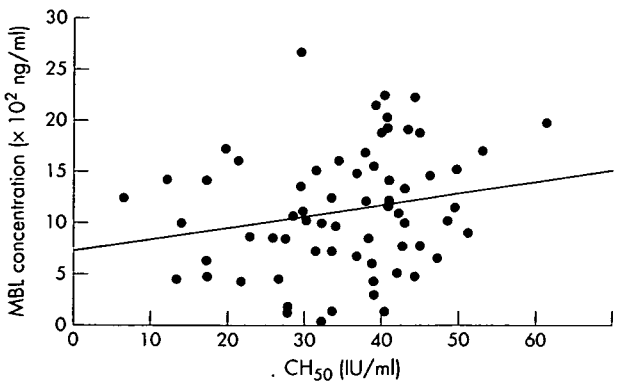

Figure 1 Relationship between serum MBL concentration and CH_{50} in genotype AA patients with SLE. $r_s = 0.253$, $p = 0.0412$ by Spearman's rank correlation test.

Fisher's exact test was used to compare the frequencies of genotypes AA/AB and BB, between disease and control groups, and to compare clinical characteristics between patients with genotypes AA/AB and those with BB. Mann-Whitney's U test was used to compare ages at diagnosis of SLE between patients with genotypes AA/AB and those with BB, and to compare serum MBL concentration between patients and controls of the same genotype. Spearman's rank correlation test was used to compare serum MBL concentration and the levels of anti-DNA antibody, C3, C4, and CH_{50}. Values of $p \leqslant 0.05$ were considered significant.

RESULTS

MBL gene genotypes were studied in patients with SLE and healthy controls (table 1). Among 147 patients with SLE, 9 were homozygous for allele B, which was significantly increased compared with controls ($p = 0.0294$).

We analysed the difference in disease characteristics among patients with SLE categorised by MBL genotypes. Ages (mean (SD)) at diagnosis of SLE tended to be younger in patients with allele B (AA: 32.5 (14.8); AB: 30.7 (15.2); BB: 23.4 (13.3)), but no significant differences were seen ($p = 0.0681$). Clinical characteristics, serological, and immunological measures did not significantly differ between genotype BB patients and other patients with SLE. This is

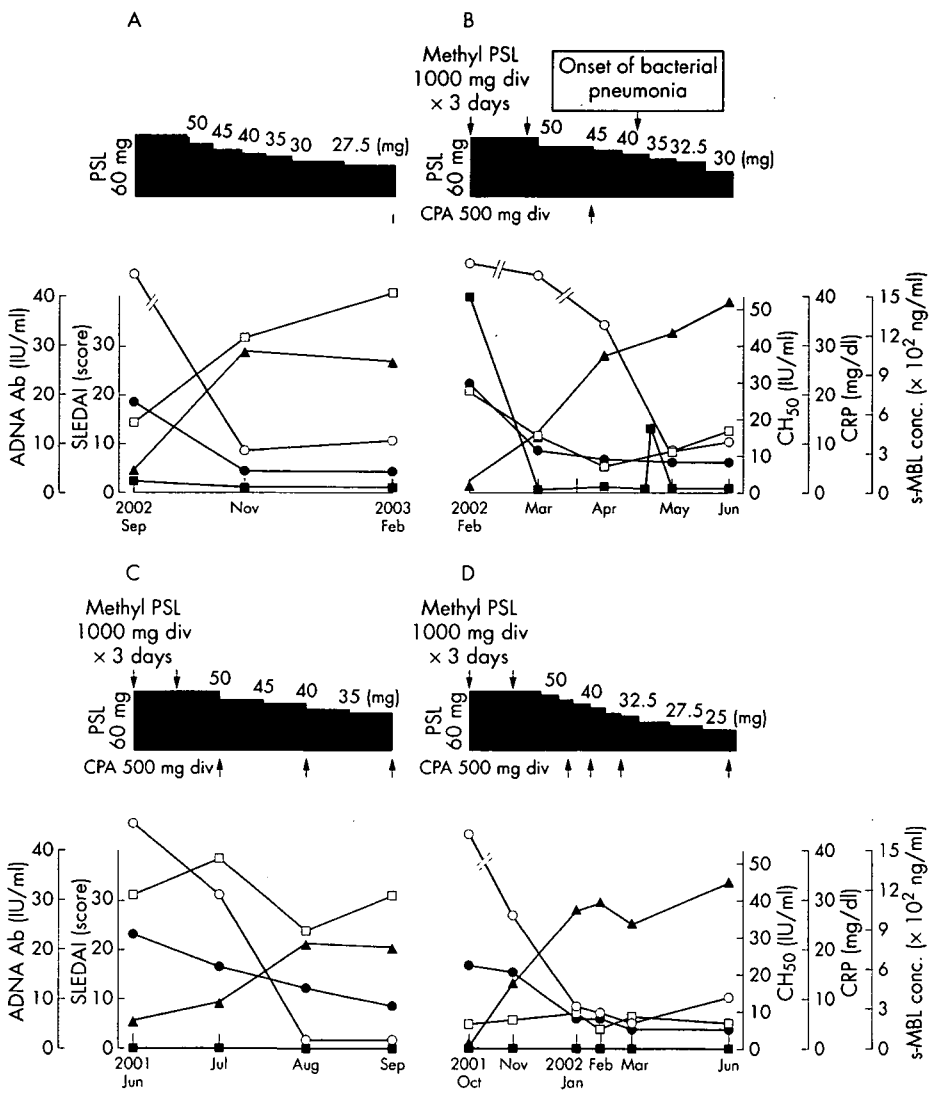

Figure 2 Fluctuation of serum MBL concentration and clinical variables during immunosuppressive treatment in patients with newly diagnosed SLE. Open squares, serum MBL concentrations (s-MBL); closed squares, C reactive protein (CRP); open circles, anti-DNA antibody (ADNA Ab); closed circles, SLE Disease Activity Index (SLEDAI); closed triangles: CH_{50}. PSL, prednisolone; CPA, cyclophosphamide.

most probably because of the small size of the BB cohort. However, incidence of infections requiring admission to hospital was significantly higher in patients with genotype BB than in other patients (genotype AA +AB; 35/132 patients, BB; 5/8 patients, $p = 0.0287$).

Serum MBL concentration reflected the MBL genotype of the individual subject, in accordance with previous reports[3] (data not shown). Among subjects with the same genotype, patients with SLE tended to have a higher MBL concentration than controls, but without statistical significance. The level of CH_{50} was weakly but significantly associated with serum MBL concentration in patients with SLE with genotype AA ($p = 0.0412$) (fig 1). In genotype AA patients, C3 was also associated with serum MBL concentration, although C4 was not (C3; $p = 0.0494$, C4; $p = 0.4265$). No significant relationship between anti-DNA antibody titre and serum MBL was found. In patients with other genotypes, no significant association was seen between serum MBL concentration and levels of anti-DNA antibody or complement components (data not shown).

We studied fluctuation of serum MBL concentration during immunosuppressive treatment in patients with newly diagnosed SLE (fig 2). In patient 1 with genotype AA (fig 2A), serum MBL increased in parallel with CH_{50} after initiation of methylprednisolone treatment, while the SLE Disease Activity Index (SLEDAI) and anti-DNA antibody decreased. In patient 2 with genotype AA (fig 2B), serum MBL concentration decreased after initiation of methylprednisolone pulse therapy, while CH_{50} increased. After CRP decreased to normal levels, MBL gradually increased in parallel with CH_{50}. In patient 3 with genotype AA (fig 2C), serum MBL did not show a clear trend, although disease activity steadily decreased. In patient 4 with genotype AB (fig 2D), serum MBL was low throughout, reflecting the MBL genotype. When the serum MBL concentration before and after immunosuppressive treatment was compared in 14 newly diagnosed patients, it increased in 6/14 patients (genotype AA: 2, AB: 4), and decreased in 7 patients (genotype AA: 5, AB: 2). There was no significant association between increase or decrease of serum MBL concentration and genotypes and clinical phenotypes in patients with SLE (data not shown).

DISCUSSION

Several studies have indicated that MBL gene polymorphism influences susceptibility to SLE.[5 6] When the components of the classical pathway of complement (C1q, C1r, C1s, C4, or C2) are deficient, it has been suggested that abnormal clearance of not only immune complexes[13] but also apoptotic cells[8] contributes to the occurrence of SLE. It has been indicated that inappropriate levels of apoptotic nuclei may be a major source of autoantigens in SLE.[14] Recently, it was reported that MBL can bind to apoptotic cells and initiate their uptake by macrophages,[9] and thus, abnormal clearance of apoptotic cells due to MBL deficiency may provide a source of autoantigens in SLE. However, deficiency of MBL is not an extremely high risk factor, in contrast with deficiencies of other complement molecules such as C1q.[7] The precise consequences of MBL deficiency for the onset and progression of SLE remain unclear. The lag time between occurrence of the first symptom attributable to SLE and diagnosis of definite SLE was reported to be significantly shorter for variant allele carriers than in those with genotype AA.[6] Therefore, the MBL gene may be a disease modifier locus rather than a true SLE susceptibility locus. Although no significant correlation between disease characteristics and MBL genotypes was seen, genotype BB was significantly associated with occurrence of infection in our patients, in accord with a previous report.[6] MBL genotyping may help in assessment of the risk of opportunistic infections in patients with SLE.

The balance of MBL production and consumption determines serum MBL levels. As the presence of MBL deposits in tissues of autoimmune patients has been demonstrated,[15 16] we expected that MBL would be consumed during active disease, and that serum MBL concentration might reflect disease activity and pathological features of SLE in individual patients. To test this hypothesis, we measured serum MBL concentration during immunosuppressive treatment in patients with newly diagnosed disease. As shown in fig 2, serum MBL concentration did fluctuate during the course of immunosuppressive treatment in patients with SLE, especially in genotype AA patients. In patients 1 and 2, the increasing phase of serum MBL concentration may reflect the decreased consumption of MBL while SLE activity gradually decreased, and the decreasing phase may reflect reduced production of MBL because MBL is an acute phase inflammatory protein.[17] Thus, MBL levels appear to reflect disease activity in some patients. The weak but significant association between serum MBL concentration and serum C3 or CH_{50} levels supports this view.

In conclusion, frequency of homozygosity for a minority allele of the MBL gene was increased in patients with SLE compared with controls, confirming previous studies. MBL gene polymorphism may have no direct effect on disease characteristics, but patients homozygous for the minority allele had significantly more frequent episodes of infections. Serum MBL levels did fluctuate during the course of SLE in individual patients, although the mechanism of their fluctuation and their consequences in SLE are unclear. The value of serum MBL monitoring in clinical practice should be determined in future studies.

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TCR Vα14$^+$ natural killer T cells function as effector T cells in mice with collagen-induced arthritis

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Summary

Natural killer (NK) T cells are a unique, recently identified cell population and are suggested to act as regulatory cells in autoimmune disorders. In the present study, designed to investigate the role of NKT cells in arthritis development, we attempted to induce arthritis by immunization of type II collagen (CIA) in Jα281 knock out (NKT-KO) and CD1d knock out (CD1d-KO) mice, which are depleted of NKT cells. From the results, the incidence of arthritis (40%) and the arthritis score (1·5 ± 2·2 and 2·0 ± 2·7) were reduced in NKT-KO and CD1d-KO mice compared to those in respective wild type mice (90%, 5·4 ± 3·2 and 2·0 ± 2·7, $P < 0·01$). Anti-CII antibody levels in the sera of NKT-KO and CD1d-KO mice were significantly decreased compared to the controls (OD values; 0·32 ± 0·16 and 0·29 ± 0·06 *versus* 0·58 ± 0·08 and 0·38 ± 0·08, $P < 0·01$). These results suggest that NKT cells play a role as effector T cells in CIA. Although the cell proliferative response and cytokine production in NKT-KO mice after the primary immunization were comparable to those in wild type mice, the ratios of both activated T or B cells were lower in NKT-KO mice than wild type mice after secondary immunization (T cells: 9·9 ± 1·8% *versus* 16·0 ± 3·4%, $P < 0·01$, B cells: 4·1 ± 0·5% *versus* 5·1 ± 0·7%, $P < 0·05$), suggesting that inv-NKT cells contribute to the pathogenicity in the development phase of arthritis. In addition, IL-4 and IL-1β mRNA expression levels in the spleen during the arthritis development phase were lower in NKT-KO mice, while the IFN-γ mRNA expression level was temporarily higher. These results suggest that inv-NKT cells influence cytokine production in arthritis development. In conclusion, inv-NKT cells may promote the generation of arthritis, especially during the development rather than the initiation phase.

Keywords: arthritis, natural killer T cell, T cells, TCR Vα14$^+$

Introduction

Natural killer (NK) T cells represent a novel lymphoid lineage distinct from conventional T cells, B cells, and NK cells. As invariant NKT cells (inv-NKT), whose TCR is a single invariant Vα14Jα281 chain, can secrete both Th1 and Th2 cytokines, it was suggested that inv-NKT cells play a role in immunomodulative function. Deficient or defective NKT cells are associated with certain autoimmune diseases [1–3], as well as with several animal models of autoimmune disease [4,5]. Recent studies showed that inv-NKT cell activation protected against type 1 diabetes in NOD mice [6], EAE [7], and MRL lpr/lpr mice [8]. Chiba *et al.* [9] reported that collagen-induced arthritis (CIA) was suppressed by NKT cell activation with OCH, an analogue of α-GalCer. However, the natural function of inv-NKT cells on the development of arthritis remains unclear.

To investigate the role of inv-NKT cells on arthritis in the CIA mouse model, NKT-cell-deficient mice were analysed. Arthritis was suppressed and anti-CII antibody levels were reduced in these mice. In addition, the ratios of both activated T and B cells were lower, and IL-4 and IL-1β mRNA expression was lower in the deficient mice during the development phase rather than induction phase of arthritis, while IFN-γ mRNA expression was temporarily higher. Therefore, we concluded that inv-NKT cells could promote the generation of arthritis and that they affected arthritis development rather than immunological initiation.

Materials and methods

Mice

Male C57BL/6 mice aged 10–12 weeks old were purchased from Charles River Japan Inc. (Yokohama, Japan). Male NKT cell (TCR Jα281) knock out mice (NKT-KO) [10] and male CD1d knock out mice (CD1d-KO) [11], in which the genetic background was C57BL/6, were used in this study. NKT-KO and CD1d-KO mice were established after backcrossing 10 generations or more to B6 mice, respectively, and were kindly provided by Prof M.Taniguchi (RIKEN Research Centre for allergy and Immunology, Yokohama, Japan). The study design was approved by the Ethical Committee of the University of Tsukuba.

Reagents

Chicken type II collagen (CII) was purchased from Sigma-Aldrich Corp. (St. Louis, USA). CII was dissolved in 0·1 M acetic acid and diluted with 0·01 M PBS (pH 7·4). Incomplete Freund's Adjuvant (IFA) and heat-inactivated *M. tuberculosis* (H37Ra) were purchased from Difco Laboratories (Detroit, USA).

Induction of collagen-induced arthritis (CIA) and evaluation of clinical severity

C57BL/6, NKT-KO and CD1d-KO mice were immunized intradermally at the base of the tail with 100 μg chicken CII emulsified in IFA containing 250 μg of inactivated *M. Tuberclosis* (H37Ra). On Day 21, the animals were boosted with an intradermal injection [12]. The animals were observed at 2- or 4-day intervals and evaluated for the severity of arthritis by scoring each paw. The observation period was set for 28 days after the booster immunization, because the clinical scores did not worsen after that in our preliminary experiment using a small number of animals. The scores ranged from 0 to 3 (0, no swelling or redness; 1, swelling or redness in one joint; 2, two joints or more involved; 3, severe arthritis of the entire paw and joints). The score for each animal was the sum of the score for all four paws.

Measurement of anti-CII antibody

C57BL/6, NKT-KO and CD1d-KO mice were sacrificed 28 days after the booster injection and sera were collected. Anti-CII IgG antibodies (anti-CII IgG Abs) were measured by ELISA [13].

Briefly, each mouse serum was diluted 100 000 times with blocking buffer (Block Ace, Dainippon Pharmaceuticals Co., Osaka, Japan) and was incubated in a type II collagen-coated well for 1 h at 37°C. The wells were washed 3 times with washing buffer (0·01 M Tris-HCl containing 0·05% Tween 20) and treated with biotinylated goat antimouse IgG antibody (Zymed Laboratories, Inc., South San Francisco, USA, diluted 4000 times with the blocking buffer) for 2 h at room temperature. After washing, avidin-alkaline phosphatase (EY laboratories, Inc., San Mateo, USA) diluted 4000 times with the blocking buffer was added to each well and incubated for 1 h at room temperature. After washing, colour development was carried out by an ELISA amplification system (Invitrogen Co., Carlsbad, USA) and the optical density was determined at 490 nm.

Measurement of the primary CII-specific response *ex vivo*

C57BL/6 and NKT-KO mice were immunized intradermally with 100 μg chicken CII emulsified in IFA containing 250 μg of inactivated *M. tuberclosis* (H37Ra). Nine days after immunization, spleens were removed. The red blood cells were removed from the splenocytes by treatment with 0·16 M Tris-NH_4Cl solution, and 2×10^5 cells were restimulated in triplicates with several concentrations of chicken CII (6·25, 12·5, 25, and 50 μg/ml) for 72 h and the proliferative response was estimated using the BrdU ELISA system (Cell Proliferation ELISA kit, Roche Diagnostics GmbH, Mannheim, Germany). IFN-γ and IL-4 concentrations in the culture supernatants were measured by ELISA using an immunoassay kit (Biosource International Inc., Camarillo, USA).

Flow cytometric analysis

Fluorescein isothiocyanate (FITC)-labelled anti-TCRβ mAb (clone H57-597), anti-CD45R(B220) mAb (clone RA3–6B2), and PE-labelled anti-CD69 mAb (clone H1·2F3) were purchased from eBioscience, Inc. (San Diego, USA). Rat antimouse FcγR II/III mAb (clone 2·4G2, BD Biosciences, San Diego, USA) was used as the Fc block. Splenocytes were collected from eight C57BL/6 male mice and eight NKT-KO male mice 5 days after the booster immunization and were treated with 0·16 M Tris-NH_4Cl solution. The cells were stained with mAbs and propidium iodide (PI, BD Biosciences, San Diego, USA), and were analysed by flow cytometry using EPICS XL-MCL (Beckman Coulter, Inc., Fullerton, USA).

Quantitative RT-PCR

The spleen was removed from three of the C57BL/6 male mice and three of the NKT-KO male mice 5, 10, 15 and 30 days after the booster immunization and they were preserved in RNA*later* (QIAGEN GmbH, Hilden, Germany). Total RNA was extracted by TriZol reagent (Invitrogen Co., Carlsbad, USA) and no genomic DNA contamination was confirmed using the GAPDH primer. First strand cDNA was synthesized using SuperScript III First Strand System (Invitrogen Co., Carlsbad, USA). The relative expression levels of IFN-γ, IL-1β, and IL-4 mRNA were determined by Taqman

RT-PCR technology (ABI PRISM 7700, Applied Biosystems, Foster city, USA). The target gene copy number of each sample was standardized by GAPDH gene expression. The primer-probe set for each cytokine and GAPDH was purchased from Applied Biosystems (Assay-on demand system).

Statistical analysis

Statistical analysis was carried out using the Fisher's exact test for the incidence of arthritis, and the Student's or Welch *t*-test for arthritis score, anti-CII antibody titre, percentage of CD69-positive cells, and relative cytokine mRNA expression.

Results

CIA in NKT-KO and CD1d-KO mice

To evaluate the association of Vα14-Jα281 NKT cells with the development of CIA, two different KO mice, Jα281-KO (NKT-KO) and CD1d-KO mice, were used and the incidence and severity of arthritis in each were compared to genetically matched C57BL/6 mice. In NKT-KO mice, the incidence (40%) and arthritis score (1·5 ± 2·2) were significantly reduced compared with the control mice (90%, 5·4 ± 3·2, $P < 0·01$) (Fig. 1a,b). In CD1d-KO mice, the incidence (40%) and arthritis score (2·0 ± 2·7) were also significantly reduced compared with control mice (90%, 6·4 ± 4·2, $P < 0·01$) (Fig. 1c,d). Judging from these findings, we hypothesize that inv-NKT cells function as effector T cells.

Anti-CII antibody in sera from NKT-KO and CD1d-KO mice

Serum anti-CII IgG Abs were also significantly decreased in NKT-KO and CD1d-KO mice (OD value: 0·32 ± 0·16 and 0·29 ± 0·06, respectively) compared with their controls (OD value: 0·58 ± 0·08 and 0·38 ± 0·08) ($P < 0·01$) (Fig. 2). The decrease in pathogenic anti-CII Ab levels is one reason why arthritis is suppressed in the NKT-cell-deficient mice.

Primary anti-CII response in NKT-KO mice

The immune system of mice was stimulated with adjuvant including microbial antigens as well as CII for the induction of CIA. NKT cells were stimulated with microbial antigens in the context of CD1d, an MHC class-I like molecule on APC [14]. Therefore, it is possible that the suppression of the incidence and severity of arthritis in NKT-KO mice is attributable to the reduction of the immune response to microbial antigens, leading to a reduced response to CII. To address this possibility, the degree of cell proliferation in NKT-KO mice was compared with those in C57BL/6 mice when stimulated with chicken CII after *in vivo* immunization with CII and inactivated *M. tuberculosis*. The results of the cell proliferation assay, mean OD values and S.D. at 6·25, 12·5, 25, and 50 μg/ml of CII, were 0·11 ± 0·06, 0·16 ± 0·04, 0·24 ± 0·04, and 0·42 ± 0·08 in C57BL/6 mice, and 0·08 ± 0·09, 0·13 ± 0·10, 0·20 ± 0·08, and 0·32 ± 0·14 in NKT-KO mice, respectively (Fig. 3a). This indicates that the cell response to CII is not significantly different between NKT-

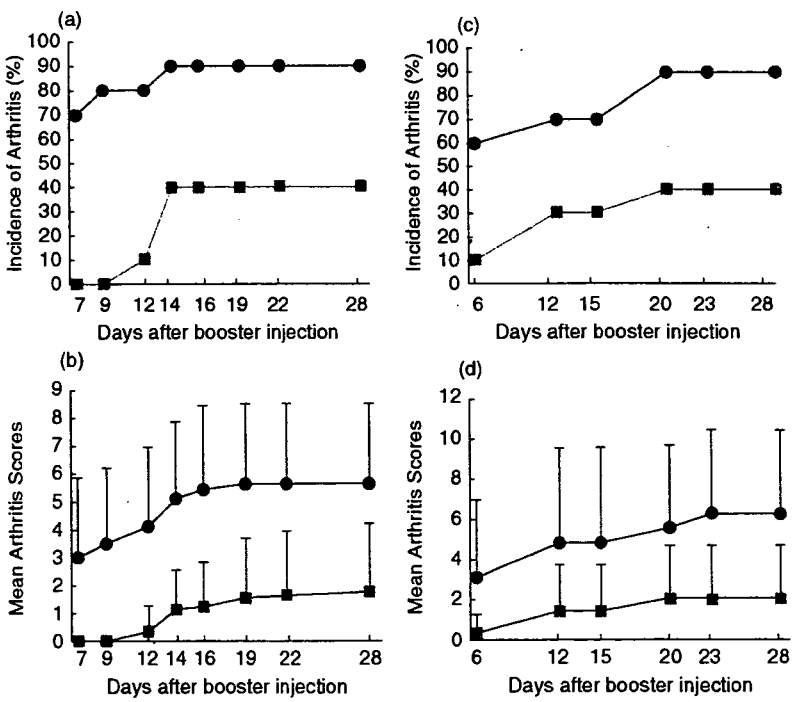

Fig. 1. Suppression of arthritis in NKT-KO mice and CD1d-KO mice. Ten NKT-KO (a,b) and 10 CD1d-KO mice (c,d) were immunized and boosted with chicken CII emulsified in IFA plus inactivated *M. tuberculosis* H37Ra. Ten C57BL/6 mice were used as the control in each examination. The incidence of CIA (a,c) and the severity of arthritis (b,d) were investigated. ■ represents each KO mice; ● represents C57BL/6 mice.

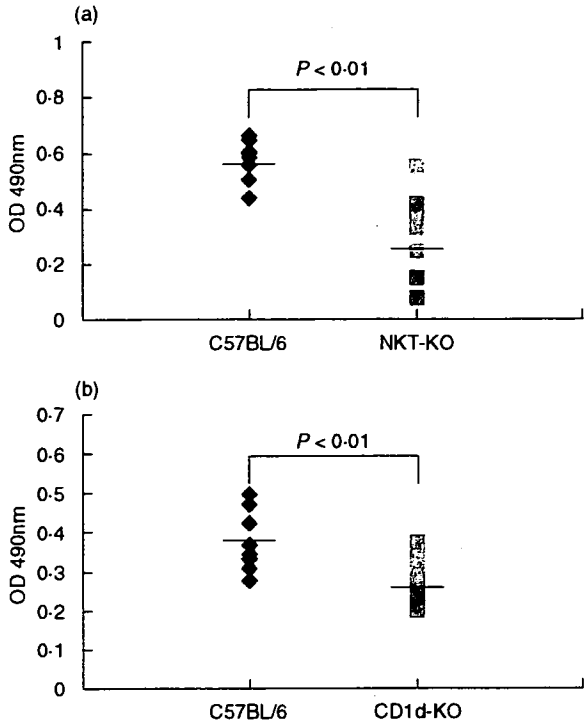

Fig. 2. Reduction of anti-CII Abs in NKT-KO and CD1d-KO mice. Twenty-eight days after the booster injection, the amount of anti-CII IgG antibody in the serum from NKT-KO (a) or CD1d-KO (b) mice and C57BL/6 mice was measured by ELISA.

KO and C57BL/6 mice at any antigen concentration tested, suggesting that the suppression of CIA in NKT-KO mice could not be ascribed to the reduced response to microbial antigen.

Inv-NKT cells have the potential to secrete several cytokines including TNF-α, IFN-γ and IL-4, which are effective or suppressive in the development of arthritis. To investigate whether the cytokine balance changed in NKT-cell-depleted mice during the induction phase of CIA, IFN-γ and IL-4 production was examined after a single immunization *in vivo* and stimulation *in vitro* with CII. The results demonstrate that there is no difference in IFN-γ or IL-4 production between inv-NKT cell positive and negative mice (Fig. 3b,c). Namely, the IFN-γ and IL-4 concentrations in supernatants stimulated with 50 μg/ml of CII were 84·3 ± 50·3 pg/ml and 10·6 ± 1·9 pg/ml in C57BL/6 mice, and 131·2 ± 64·6 pg/ml and 9·1 ± 1·3 pg/ml in NKT-KO mice, respectively. Therefore, the absence of inv-NKT cells did not have an effect on the cytokine balance after primary immunization with antigen and was not considered to have had an influence on the deviation towards a Th1 type response.

T and B cell activity in NKT-KO mice after the booster immunization with CII

To investigate the activation levels of T and B cells *in vivo* during the development phase of CIA (after the booster immunization), the ratio of CD69 expression, an early

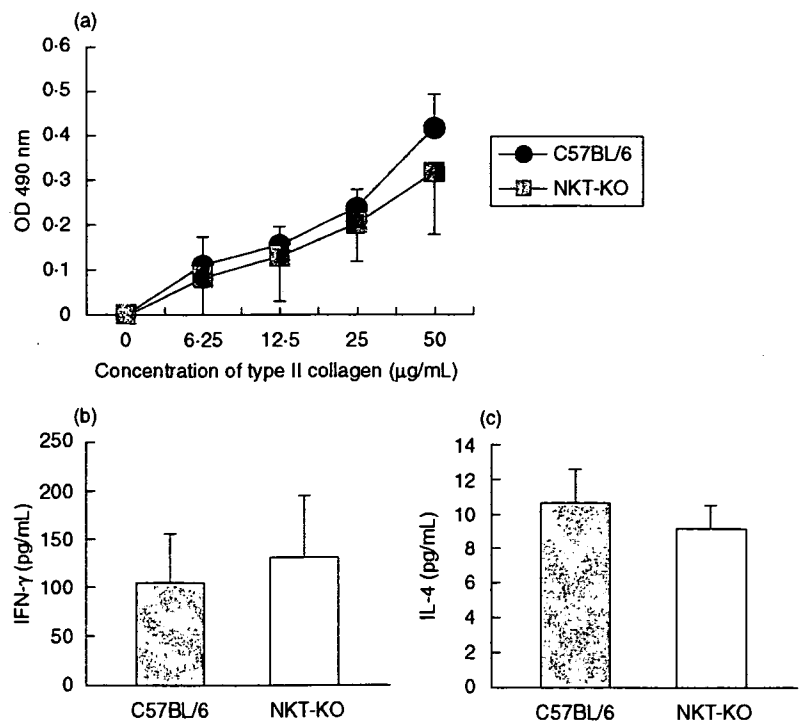

Fig. 3. Cell proliferation and cytokine production stimulated by CII in NKT-KO mice compared with those in C57BL/6 mice. Five NKT-KO and five C57BL/6 mice were immunized with chicken CII emulsified in IFA plus *M. tuberculosis* H37Ra. Nine days after immunization, splenocytes were stimulated with CII. The degree of cell proliferation was evaluated by a BrdU ELISA method (a). The concentrations of IFN-γ (b) and L-4 (c) in the culture supernatants were measured by ELISA.

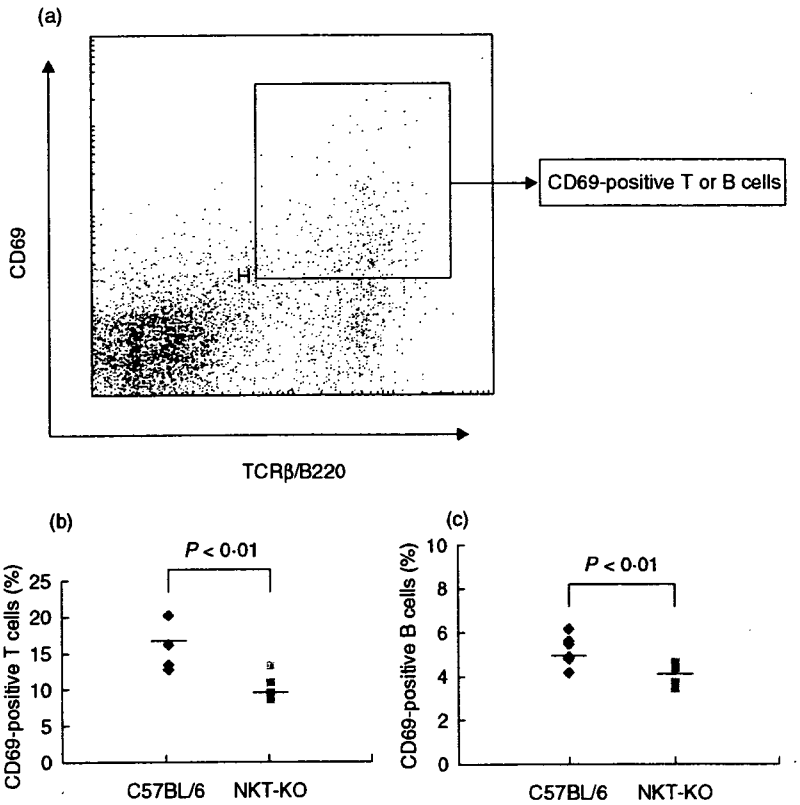

Fig. 4. Activation level of T and B cells after the booster immunization in NKT-KO mice compared with that in C57BL/6 mice. Eight NKT-KO and eight C57BL/6 mice were immunized and boosted with chicken CII emulsified in IFA plus inactivated *M. tuberculosis* H37Ra. Five days after the booster immunization, splenocytes were collected and stained with FITC-labelled anti-TCRβ or anti-B220, and PE-labelled anti-CD69 antibody. PI-negative cells were gated and FITC-PE double positive cells were counted (a). The proportions of CD69-positive T cells (b) or CD69-positive B cells (c) compared to the total number of T cells ($TCR\beta^+$ cells) or B cells ($B220^+$ cells) were calculated.

activation marker, on T and B cells was determined by flow cytometry. The results showed that the percentage of CD69-positive T cells in the spleen was lower in NKT-KO mice than in wild type mice (9·9 ± 1·8 in NKT-KO *versus* 16·0 ± 3·4 in C57BL/6, $P < 0·01$, Fig. 4b). The percentage of CD69-positive B cells was also lower in the spleen from NKT-KO mice compared with that of wild type mice (4·1 ± 0·5 *versus* 5·1 ± 0·7, $P < 0·05$, Fig. 4c). Therefore, the low incidence of arthritis in NKT-depleted mice was attributable to lower response of T and B cells after the booster immunization.

Cytokine mRNA expression in the spleen from NKT-KO mice after the booster immunization with CII

Further, to examine cytokine production *in vivo* in NKT-KO mice during the development of arthritis, IFN-γ and IL-4 mRNA expression in the spleen was measured by quantitative RT-PCR after the booster immunization. The results showed that relative IFN-γ mRNA expression in NKT-KO mice was higher than that in wild type mice around 10 or 15 days after the booster injection (1·38 ± 0·58 *versus* 0·64 ± 0·23 on day 10, $P < 0·05$, 1·94 ± 0·06 *versus* 1·05 ± 0·14 on day 15, $P < 0·01$, Fig. 5a), while relative IL-4 mRNA expression in NKT-KO mice was lower during the course of arthritis development (on days 5, 10, 15 and 30: 1·02 ± 0·02, 1·17 ± 0·56, 1·64 ± 0·17, and 4·02 ± 2·56 in NKT-KO *versus* 4·08 ± 1·03, 3·04 ± 0·07, 4·19 ± 0·21, and 9·06 ± 2·07 in C57BL/6, $P < 0·01$ or 0·05, Fig. 5b).

IL-1β plays a prominent role in the inflammation in CIA [15] and it is controlled by various cytokines. Since IFN-γ and IL-4 secretion was suggested to be changing in NKT-KO mice, the expression level of IL-1β mRNA in the spleen was also measured. The IL-β mRNA level was found to be lower in NKT-KO mice than in wild type mice after day 15 of the booster injection (on days 15 and 30: 0·84 ± 0·14, 0·73 ± 0·05 in NKT-KO *versus* 1·27 ± 0·07, 1·10 ± 0·24 in C57BL/6 mice, $P < 0·01$, Fig. 5c). Therefore, the low incidence of arthritis and alleviation of the symptoms in NKT-depleted mice was probably related to the suppression of IL-1β secretion.

Discussion

In this study, we revealed that a deficiency of inv-NKT cells induced a lower incidence of arthritis, and the results suggest that inv-NKT cells play a considerable role in arthritis development. Similar results have been shown in a different system by Chiba *et al.* [9]. The repeated administration of antigen for NKT cells, α-GalCer, exacerbated the arthritis of CIA (Ohnishi, Y. *et al.* unpublished observation), supporting this hypothesis.

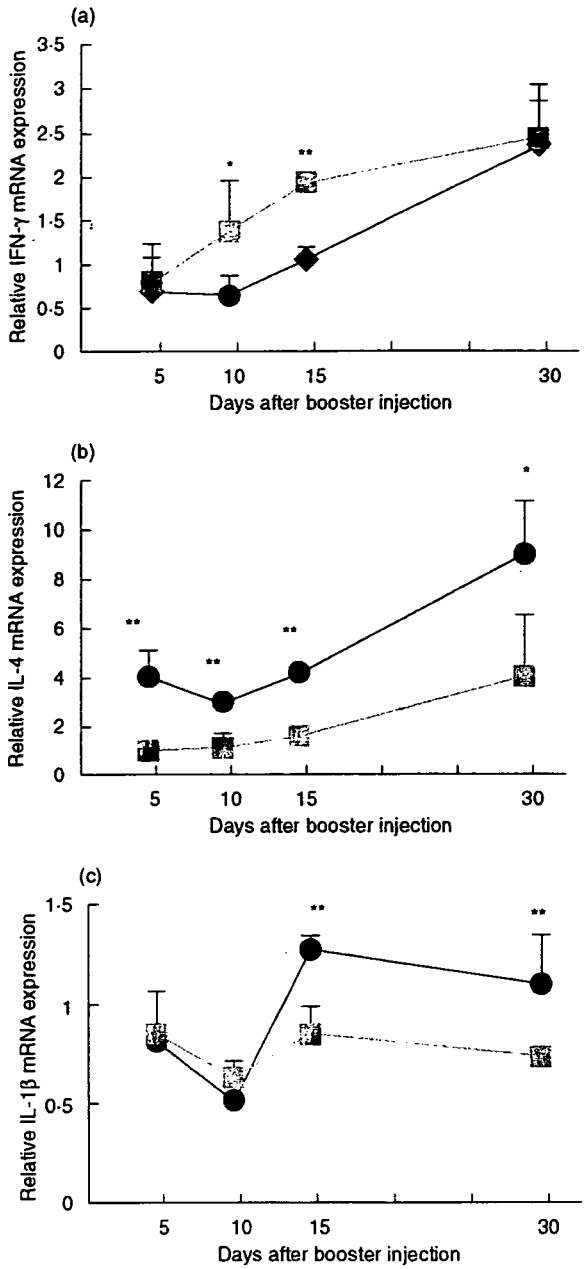

Fig. 5. Cytokine mRNA expression in the spleen after the booster immunization. Total splenic RNA was collected from three male C57BL/6 mice (●) and three male NKT-KO mice (■) 5, 10, 15, and 30 days after the booster immunization, and the relative expression levels of (a) IFN-γ, (b) IL-4 or (c) IL-1β mRNA were measured by the Taqman quantitative PCR method. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

Levels of the cell activation marker CD69 on T and B cells decreased in NKT-cell-deficient mice after secondary (booster) immunization, and serum anti-CII antibody levels were lower in the KO compared with wild type mice. Therefore, the low incidence of arthritis in NKT-deficient mice was due to the low activity of T and B cells during the development phase of arthritis. Some reports have shown that NKT cells activated by α-GalCer induce maturation of DC, and thereby, enhance the antigen-specific T cell response [16,17]. In addition, inv-NKT cells directly induce B cell proliferation and help antibody production [18]. Therefore, although the level of DC maturation was not investigated in this study, the lower incidence of arthritis was probably attributable to depletion of inv-NKT cells that effectively influences T and B cell activation. Further experiments, such as adaptive transfer of inv-NKT cells, are required to clarify inv-NKT cell function. Cell proliferative responses and the levels of cytokine secretion in KO mice were comparable to those of wild type mice after primary immunization (Fig. 3a–c). This suggests that inv-NKT cells are associated with the observed increase in T and B cell activation rather than with the initiation of CIA, including Th1/Th2 polarization, following antigen stimulation. Eberl *et al.* [19] reported that NKT cells contribute to the maintenance and persistent stimulation of memory T cells through cytokine secretion. We speculated that NKT cells are associated with the maintenance of T cells activated by antigen stimulation in CIA.

From the results of the measurement of cytokine mRNA expression levels, lower IL-4 and IL-1β secretion and temporarily higher IFN-γ secretion were observed in NKT-cell-depleted mice. Examination in IFN-γ KO mice showed that CIA was enhanced by genetic ablation of IFN-γ through up-regulation of IL-1β production, and therefore, IFN-γ plays a role in the regulation of IL-1β in CIA [20]. IL-4 has been reported to be an up-regulator of both type I and type II IL-1 receptors on monocytes [21–22]. Considering that IL-1β is a key mediator in the pathology of the CIA model, higher IFN-γ and lower IL-4 levels in NKT-KO mice might contribute to the alleviation of arthritis. In addition, endogeneous IL-4 not only acts directly on B cell activation, but also plays a crucial role in arthritis induced by the injection of anti-CII antibodies. [24]. There is the possibility that IL-4 is a key mediator for the suppression of arthritis in NKT-cell-deficient mice. Since IL-4 was reduced throughout the course of arthritis development, inv-NKT cells might act as the source of IL-4 for arthritis development. Further experiments, such as using anti-cytokine antibodies or cytokine augmentation, are required for confirmation of the involvement of these cytokines on the suppression of CIA.

Although inv-NKT cells have the potential to promote CIA development, arthritis developed at a low incidence with slight symptoms in NKT-deficient mice (40% of incidence and 1·5–2·0 of mean arthritis score, Fig. 1a–d). Therefore, inv-NKT cells are not essential for CIA establishment.

Chiba *et al.* [9] also showed that inv-NKT cells stimulated with OCH, an analogue of α-GalCer, could suppress the development of arthritis, and suggested that inv-NKT cells could play a role as suppressor cells. OCH is an artificially synthesized ligand and probably differs from the natural

ligands of inv-NKT cells, which are still unknown. Based on the results of this study, inv-NKT cells are considered to have the ability to enhance CIA in a natural state. Recent studies showed that NKT cells could be classified into several subsets based on their capability to secrete cytokines and their phenotype [25–27]. Subsets activated by OCH might be different from main subsets activated by the as yet unknown natural ligands in CIA.

In conclusion, two KO mouse models clearly show that inv-NKT cells can promote the generation of arthritis, especially during the development phase. Further experiments on the function of inv-NKT cells should shed light on the development and regulation of arthritis.

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Research article

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A functional variant of Fcγ receptor IIIA is associated with rheumatoid arthritis in individuals who are positive for anti-glucose-6-phosphate isomerase antibodies

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Abstract

Anti-glucose-6-phosphate isomerase (GPI) antibodies are known to be arthritogenic autoantibodies in K/B×N mice, although some groups have reported that few healthy humans retain these antibodies. The expression of Fcγ receptors (FcγRs) is genetically regulated and has strong implications for the development of experimental arthritis. The interaction between immune complexes and FcγRs might therefore be involved in the pathogenesis of some arthritic conditions. To explore the relationship between functional polymorphisms in FcγRs (*FCGR3A*-158V/F and *FCGR2A*-131H/R) and arthritis in individuals positive for anti-GPI antibodies, we evaluated these individuals with respect to *FCGR* genotype. Genotyping for *FCGR3A*-158V/F and *FCGR2A*-131H/R was performed by PCR amplification of the polymorphic site, followed by site specific restriction digestion using the genome of 187 Japanese patients with rheumatoid arthritis (including 23 who were anti-GPI antibody positive) and 158 Japanese healthy individuals (including nine who were anti-GPI antibody positive). We report here on the association of *FCGR3A*-158V/F functional polymorphism with anti-GPI antibody positive status. Eight out of nine healthy individuals who were positive for anti-GPI antibodies possessed the homozygous, low affinity genotype *FCGR3A*-158F (odds ratio = 0.09, 95% confidence interval 0.01–0.89; $P = 0.0199$), and probably were 'protected' from arthritogenic antibodies. Moreover, among those who were homozygous for the high affinity genotype FCGR3A-158V/V, there were clear differences in anti-human and anti-rabbit GPI titres between patients with rheumatoid arthritis and healthy subjects ($P = 0.0027$ and $P = 0.0015$, respectively). Our findings provide a molecular model of the genetic regulation of autoantibody-induced arthritis by allele-specific affinity of the FcγRs.

Introduction

Rheumatoid arthritis (RA) is a heterogeneous autoimmune disease that is characterized by chronic inflammatory polyarthritis [1]. One of the characteristic features of RA is the expression of several autoantibodies. The presence of such autoantibodies (e.g. rheumatoid factor [RF]), identified by screening, is commonly used as a diagnostic marker, although the pathogenic role played by autoantibodies in RA remains a mystery.

Fcγ receptors (FcγRs) play a pivotal role in the reaction between immune complex and myeloid cells. Three FcγR types have been identified in mice and humans (FcγRI, FcγRII and FcγRIII). In mouse arthritis models, FcγRIII deficient hosts exhibit resistance to collagen type II induced arthritis and anti-glucose-6-phosphate isomerase (GPI) antibody induced arthritis [2,3], suggesting that FcγRIII is indispensible in autoantibody dependent arthritis. In humans FcγRs are encoded by eight genes, and the genes encoding the low affinity FcγRs (*FCGR2A*, *FCGR3A*, *FCGR2C*, *FCGR3B* and

AP = alkaline phosphatase; bp = base pairs; ELISA = enzyme-linked immunosorbent assay; FcγR = Fcγ receptor; GPI = glucose-6-phosphate isomerase; GST = gluthathione-S-transferase; OD = optical density; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RA = rheumatoid arthritis; RF = rheumatoid factor.