Table III. Continued.

B, PM-1

	V					N-D-N									J				
clone	92					96									106				
BV9-2	C	A	S	S			Q	D	W	G	Q	A			N	E	K	L	BJ1S4
BV12-1	C	A	I			S	D	T	S	G	R	G			T	D	T	Q	BJ2S3
BV12-2	C	A	I			S	E	S	L	A						D	T	Q	BJ2S3
BV12-3	C	A	I			S	G	A	G					S	T	D	T	Q	BJ2S3
BV13-1	C	A	S	S			W	S	L	D	R	G	D			N	E	Q	BJ2S1
BV13-2	C	A	S	S			P	S	G	L	P	P			T	D	T	Q	BJ2S3
BV17-1	C	A	S	S			I	G	A	G	V				Y	N	E	Q	BJ2S1
BV17-2	C	A	S			N	A	R	P	F			S	G	A	N	V	L	BJ2S6
BV17-3	C	A	S	S			P	W	T	G	T				N	T	E	A	BJ1S1
BV17-4	C	A	S	S			I	Q	G	R						Q	P	Q	BJ1S5
BV20-1	C	A	W	S			V	G	G	S					S	Y	E	Q	BJ2S7
BV20-2	C	A	W	S			S	T	E								E	Q	BJ2S1
BV20-3	C	A	W	S			G	Q	A	A				S	T	D	T	Q	BJ2S3
BV20-4	C	A	W			G	P	D	I	P						E	T	Q	BJ2S7

C, PM-2

	V					N-D-N									J				
clone	92					96									106				
BV2-1	C	S	A			L	T	N	T							N	E	Q	BJ2S1
BV3-1	C	A	S	S			W	R							T	G	E	L	BJ2S2
BV4-1	C	S	V			V	G	T							T	D	T	Q	BJ2S3
BV5-1	C	A	S			A	R	D	S	G	R	L	R			E	K	L	BJ1S4
BV5-2	C	A	S	S			L	K	E	G	L	G		S	T	D	T	Q	BJ2S3
BV6-1	C	A	S	S			L	D	G	S				N	T	G	E	L	BJ2S2
BV6-2	C	A	S	S			L	G	P						N	Y	G	Y	BJ1S2
BV8-1	C	A	S	S			L	K	D								E	Q	BJ2S7
BV8-2	C	A	S			S	Y	G	T	G					S	Y	E	Q	BJ1S6
BV8-3	C	A	S			T	R	T	D	L					N	T	E	A	BJ1S1
BV11-1	C	A	S			S	L	G	T						N	Y	G	Y	BJ1S2
BV11-2	C	A	S			L	G	G	A	V	T				T	G	E	L	BJ2S2
BV13-1	C	A	S			S	H	D	G	P					N	Y	G	Y	BJ1S2
BV13-2	C	A	S			S	R	G	D	Y	A				N	E	K	L	BJ1S4
BV13-3	C	A	S			R	M	G	Q	G	F						E	Q	BJ2S7
BV16-1	C	A	S	S			P	G	G	N							E	Q	BJ2S7
BV16-2	C	A	S	S			Q	G	V	A	G	T	G	L		E	T	Q	BJ2S5
BV19-1	C	A	S			S	P	S	D	G	T				S	Y	E	Q	BJ2S7
BV23-1	C	A	S	S			S	P	R	T	W	A					Y	Q	BJ2S7
BV23-2	C	A	S	S			Y	N	Y	R	G	A	G	V		T	E	A	BJ1S1
BV25-1	C	A	S	S			Q	S	I	R	G	R					E	Q	BJ2S7

Table IV. CDR3 region of BALF T cells of healthy subjects (HS).

A, HS-1

Clone	V 92				N-D-N 96								J 106					
BV1-1	C	A	S	S			A	G	T			N	Q	E	T	Q	BJ2S5
BV1-2	C	A	S	S			V	T	G	G	S	L		N	E	Q	BJ2S1
BV2-1	C	S	A	K			G	E	R	G	G				E	Q	BJ2S1
BV2-2	C	S	A	R			I	G	T				Q	E	T	Q	BJ2S5
BV2-3	C	S	A			D	R	N					Q	E	T	Q	BJ2S5
BV2-4	C	S	A	S			K	T	G				T	G	E	L	BJ2S2
BV8-1	C	A	S	S			L	G						Y	E	Q	BJ2S7
BV20-1	C	A	W				K	R	E	S					E	Q	BJ2S1
BV20-2	C	A			F	T	G	Q	G	A			S	Y	E	Q	BJ1S5
BV20-3	C	A			S	R	D	R	G	L			N	Q	P	Q	BJ1S5

B, HS-2

Clone	V 92				N-D-N 96								J 106				
BV6-1	C	A	S	S		H	S	G	R	E	R		Y	N	E	Q	BJ2S1
BV8-1	C	A	S		Q	G						M	N	T	E	A	BJ1S1
BV8-2	C	A	S	S		F	G	A							E	Q	BJ2S7
BV8-3	C	A	S	S		F	S	G	T	S	G			N	E	Q	BJ2S1
BV11-1	C	A	S		V	M	M	T	G					T	E	A	BJ1S1
BV11-2	C	A	S		V	T	G	G					T	G	E	L	BJ2S2
BV13-1	C	A	S		S	R	G	D	Y	A			T	D	D	Q	BJ1S4
BV13-2	C	A	S		R	M	G	Q	G	F			T	G	E	L	BJ2S1
BV14-1	C	A	S	S		L	V	G	G	R					E	Q	BJ2S3
BV14-2	C	A	S	S		W	R	G						Y	E	Q	BJ2S2
BV15-1	C	A	T	S		G	P	A	D	E	N	G	S	S	Y	E	Q	BJ2S1
BV15-2	C	A	T	S		D	G								E	Q	BJ2S7
BV18-1	C	A	S	S		P	G	A	G				S	Y	N	E	Q	BJ2S7
BV18-2	C	A	S	S		P	Q	G	G					N	E	K	L	BJ2S1
BV18-3	C	A	S	S		P	P	G	P	L						E	Q	BJ2S1
BV21-1	C	A	S	S		S	Q	G	P	T	G				N	E	Q	BJ2S1
BV24-1	C	A	T	S		R	D	P	T	R						E	Q	BJ2S7

C, HS-3

Clone	V 92				N-D-N 96								J 106				
BV3-1	C	A	S	S		P	E	P	Q	G	V	R			T	Q	BJ2S5
BV7-1	A	A	S		Y	S	S	G						Y	E	Q	BJ2S7
BV7-2	A	A	S	Q		D	G	G	G	G			N	T	G	E	L	BJ2S2
BV10-1	C	A	S	S		S	P	L	G	A					P	Q	BJ1S5
BV10-2	C	A	S	S		K	T	E	R	E				Y	E	Q	BJ2S7
BV14-1	C	A	S	S		L	S	E	M	G				N	E	Q	BJ2S1
BV18-1	C	A	S	S		R	P	G	S	G				T	D	T	Q	BJ2S3
BV18-2	C	A	S	S		Q	T	E						N	I	Q	BJ2S4
BV20-1	C	A	W	S		P	L	A	L					E	T	Q	BJ2S5
BV20-2	C	A	W		A	L	G	I	A	S				N	E	Q	BJ2S1
BV24-1	C	A			S	S	N	T	P	K	A	S	L	V	G	E	T	Q	BJ2S5
BV25-1	C	A	S	S		T	S	G	T	G	D				T	E	A	BJ1S1
BV25-2	C	A	S	S		T	G	T	R	G					T	E	A	BJ1S1

Table V. Summary of conserved amino acid motifs in the TCR CDR3 region.

	RGS	BV2-1, 19-2, 20-3
	GLA	BV5-1, 10-1
	LQG	BV9-2, 13-3
DM-1	SGG	BV10-2, 24-2
	DRG	BV13-2, 20-3
	GTS	BV9-3, 24-2
	TSGR	BV4-2, 12-1
	GGS	BV8-2, 20-1
PM-1	GQA	BV9-2, 20-3
	GAG	BV12-3, 17-1
PM-2	GTG	BV8-2, 16-2

BV20-3, and GTS motif in TCR 9-3 and BV24-2 clones. In contrast, TSGR, GGS, GQA, GQA, GAG, and GTG motifs were found in TCR BV4-2 and BV12-1, BV8-2 and BV20-1, BV9-2 and BV20-3, BV12-3 and BV17-1, and BV8-2 and BV16-2 clones, respectively in patients with PM. These observations are specific for BALF T cells in PM and DM, since the same conserved amino acids motifs were not found in BALF T cells of IPF patients (13). Moreover, the different conserved amino acids in the CDR3 region between DM and PM patients suggest that antigens recognized by BALF T cells might be different from each other.

While some T cells clonally accumulate through antigen stimulation, many other T cells polyclonally expand by interleukin (IL)-2 stimulation. What are the antigens recognized by clonally expanded T cells? To our knowledge, there are no reports on antigens for BALF T cells in myositis, whereas heat-shock proteins or viral antigens are possible candidates for T cells in muscles of PM patients (15). The antigens might be different from those in patients with sarcoidosis, because the TCR BV gene repertoire is not skewed but heterogeneous (8-10). Moreover, antigens should not be the same as those in IPF patients (13), because of the distinct conserved amino acid motifs in the TCR CDR3 region. BALF-specific T cell clones suggest that the antigens might be pulmonary antigens, e.g., autoantigens in alveolar epithelial cells or extracellular proteins such as viral or bacterial particles. Further studies on the antigens recognized by BALF T cells in PM/DM patients are necessary to clarity the role of T cells in the pathogenesis of myositis.

The autoaggressive $CD8^+$ T cells seem to attack myocytes as cytotoxic T cells in a perforin-dependent manner. The majority of BALF T cells are CD8-positive similar to those infiltrating muscles, suggesting that BALF T cells may function as cytotoxic T cells against pulmonary epithelial cells and result in lung fibrosis. It is considered that PM and DM are pathogenetically different. In DM, there is no report that clonal expansion of T cells in the muscle is noted. Our results did not identify it clearly, but showed similar clonal expansion of T cells in BALF between PM and DM. This is considerable, therefore we need additional experimental data. However, the appropriate treatment for pulmonary fibrosis in patients with PM/DM is still non-specific therapy such as corticosteroid and immunosuppressant. Therefore, detection of clonally expanded T cells in BALF should be the first step in the development of specific regulators of cytotoxic T cells that induce interstitial pneumonitis in PM/DM patients.

In conclusion, we demonstrated clonal accumulation of BALF T cells in PM/DM patients with IP, suggesting that T cells in the lung expand via antigen-driven stimulation. Although the autoantigens recognized by pulmonary T cells have not yet been identified, we believe our results will be potentially useful for the design of new treatment strategies against IP in PM/DM patients that are specifically designed to target T cells and antigens.

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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]

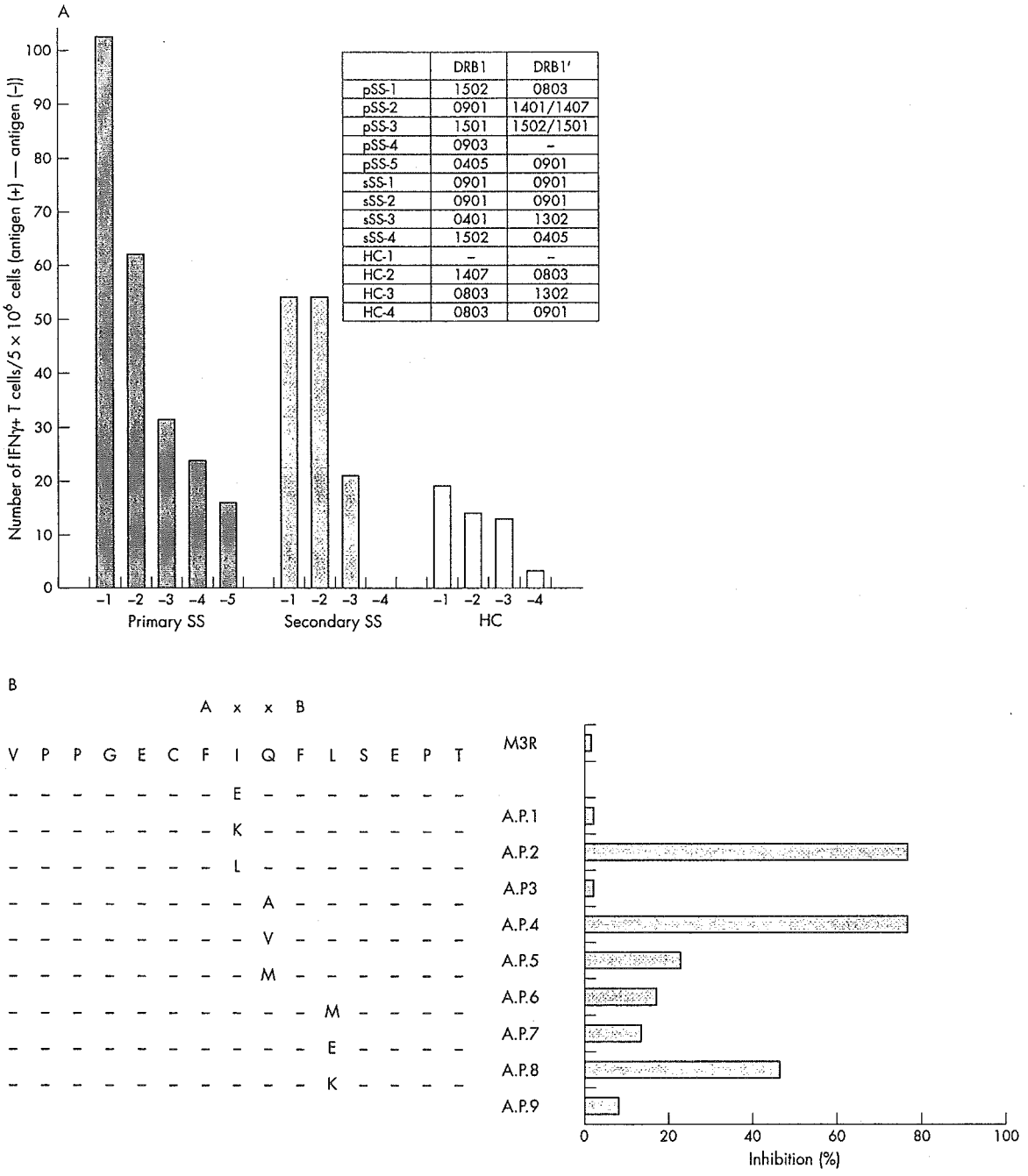
Candidate autoantigens recognised by T cells that infiltrate the labial salivary glands of patients with SS have been analysed, and Ro/SSA 52 kDa,[2] α-amylase, heat shock protein, and TCR BV6 have been identified, although Ro/SSA 52 kDa reactive T cells were not increased in peripheral blood.[3] Gordon *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℃. 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℃. 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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IL1RN genotype as a risk factor for joint pain in hereditary haemochromatosis?

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Hereditary haemochromatosis is a genetically determined disease of disrupted iron metabolism caused predominantly by the C282Y mutation in the HFE gene on chromosome 6.[1] If detected early, the symptoms of haemochromatosis can usually be prevented by venesection to remove excess iron and maintain normal iron stores.

One of the most common symptoms of haemochromatosis is joint pain (arthralgia) with or without associated joint disease (arthropathy),[2 3] and it has been generally assumed that arthritis in haemochromatosis is caused by iron deposition in the joints. Nevertheless, studies have failed to demonstrate any correlation between the level of iron overload and the presence or absence of arthritis or arthralgia,[4–6] nor does venesection ameliorate joint symptoms.[6] The interleukin cluster on chromosome 2 includes the interleukin (IL) 1α, IL1β, and IL1RN genes, and an association has been demonstrated between an IL1RN polymorphism and several inflammatory disorders, including osteoarthritis[7] but not rheumatoid arthritis.[8] This 86 bp polymorphism with five known alleles (a, b, c, d, and e) in intron 2 of the IL1RN gene has also been shown to be associated with expression levels.[9]

As far as we know, this study is the first to investigate genetic associations underlying joint pain in haemochromatosis. All participants gave informed written consent and the study was conducted with institutional ethics approval. In preliminary studies, we found that the frequency of the IL1IN polymorphism was significantly different ($p \leqslant 0.01$) between patients with haemochromatosis ($n = 313$) and controls ($n = 349$), and the IL1RN*a allele was more common in patients than in controls (data not shown).

We assessed 66 HFE C282Y homozygote patients (29 female, 37 male) and 52 healthy volunteers (25 women, 27 men) who were all wild type for the HFE mutation. All were available for clinical assessment of joint pain. Blood was collected for extraction of DNA and determination of serum ferritin (Immulite, 2000) and serum IL1RN levels (enzyme linked immunosorbent assay (ELISA), R&D, Minneapolis, USA). Clinical data, including the presence of joint pain, were collected through a questionnaire and by subsequent consultation with a physician (JR), who was unaware of their genetic status. Patient and control data were then divided according to whether they experienced joint pain or not. People who experienced joint pain as a result of injury

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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-

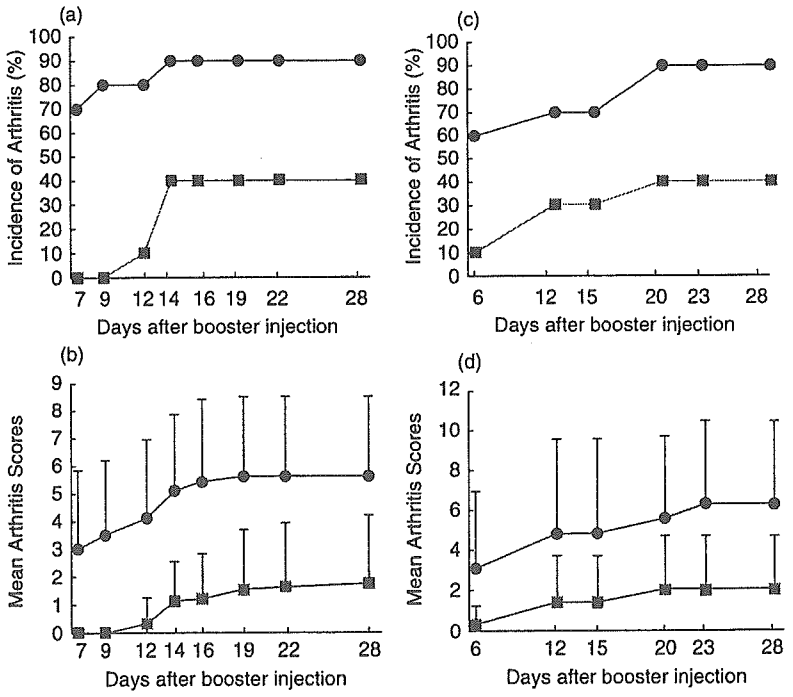

Fig. 1. Suppression of arthritis in NKT-KO mice and CD1d-KO mice. Ten NKT-KO (a,b) and 10 CD1d-KO mice (c,d) were immunized and boosted with chicken CII emulsified in IFA plus inactivated *M. tuberculosis* H37Ra. Ten C57BL/6 mice were used as the control in each examination. The incidence of CIA (a,c)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

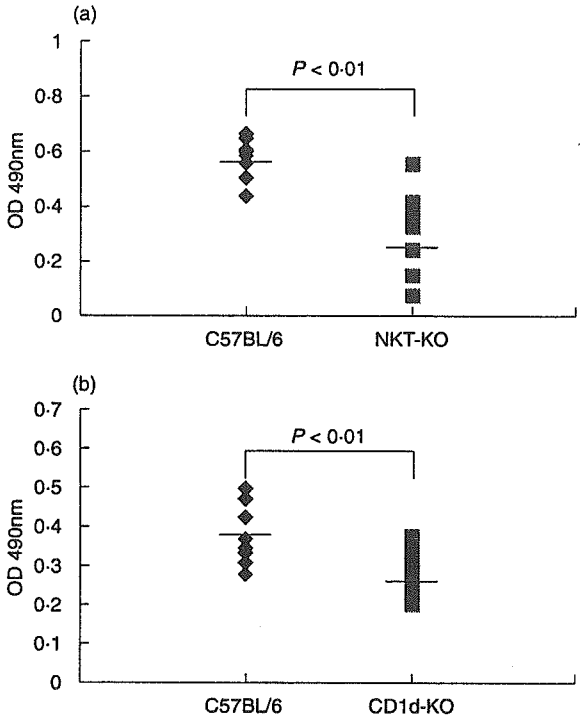

Fig. 2. Reduction of anti-CII Abs in NKT-KO and CD1d-KO mice. Twenty-eight days after the booster injection, the amount of anti-CII IgG antibody in the serum from NKT-KO (a) or CD1d-KO (b) mice and C57BL/6 mice was measured by ELISA.

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

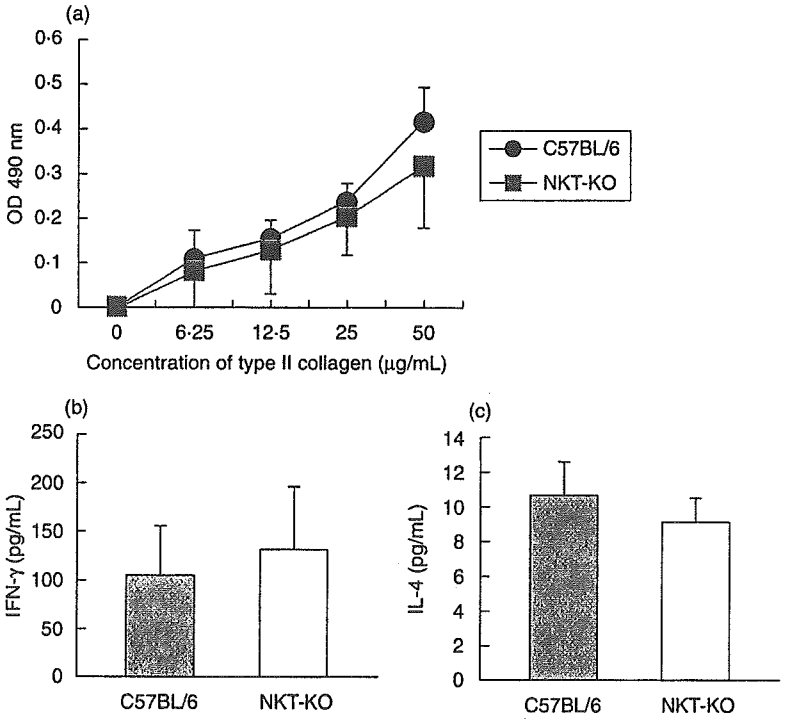

Fig. 3. Cell proliferation and cytokine production stimulated by CII in NKT-KO mice compared with those in C57BL/6 mice. Five NKT-KO and five C57BL/6 mice were immunized with chicken CII emulsified in IFA plus *M. tuberculosis* H37Ra. Nine days after immunization, splenocytes were stimulated with CII. 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.

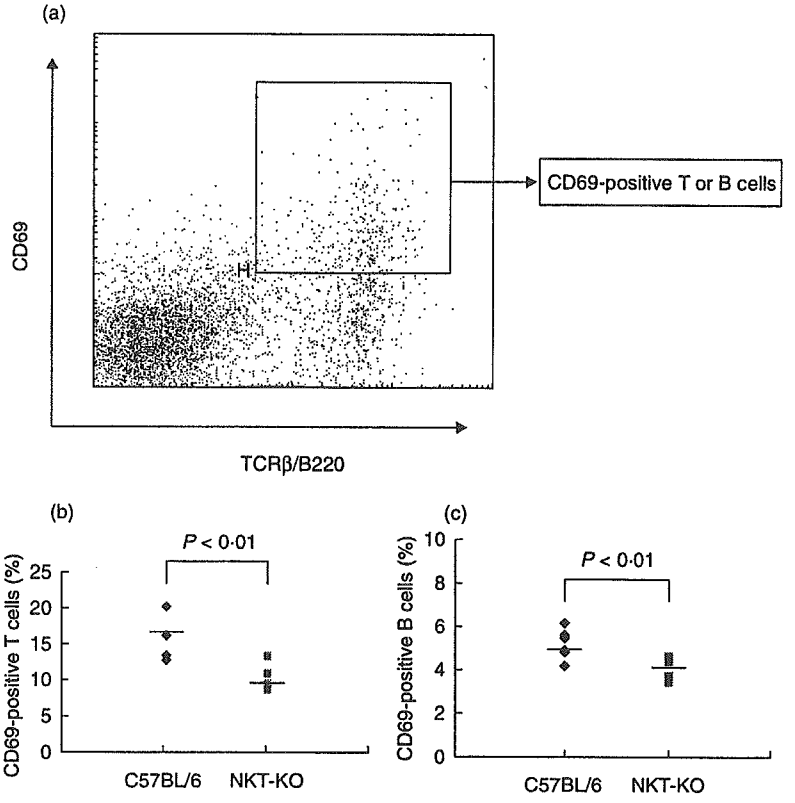

Fig. 4. Activation level of T and B cells after the booster immunization in NKT-KO mice compared with that in C57BL/6 mice. Eight NKT-KO and eight C57BL/6 mice were immunized and boosted with chicken CII emulsified in IFA plus inactivated *M. tuberculosis* H37Ra. Five days after the booster immunization, 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.

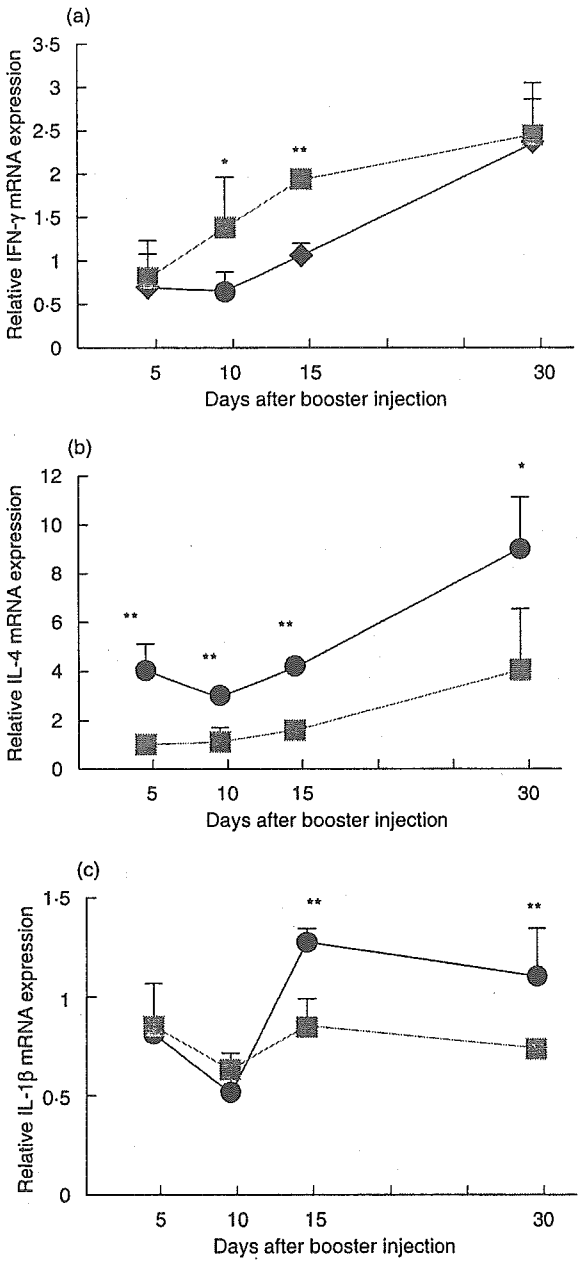

Fig. 5. Cytokine mRNA expression in the spleen after the booster immunization. Total splenic RNA was collected from three male C57BL/6 mice (●) and three male NKT-KO mice (■) 5, 10, 15, and 30 days after the booster immunization, and the relative expression levels of (a) IFN-γ, (b) IL-4 or (c) IL-1β 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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Analysis of abnormally expressed genes in synovium from patients with rheumatoid arthritis using a column gel electrophoresis-coupled subtractive hybridization technique

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Abstract. Rheumatoid arthritis (RA) is a chronic disease of unknown pathogenesis. To identify abnormally expressed genes in synovium from RA patients, we performed column gel electrophoresis-coupled subtractive hybridization (CGESH). CGESH is a newly developed subtractive hybridization technique to achieve sufficient enrichment of DNA sequences. CGESH was performed using restricted enzyme digested cDNA synthesized from mRNA of synovial tissues from one RA patient and one osteoarthritis (OA) patient. The obtained subtraction libraries (RA-OA) were screened by dot blot hybridization. The clones showing higher hybridization with the RA-OA probe were identified by sequence analysis and homology search. Their DNA sequencing revealed that the genes of HLA-DRB1, sequestosome 1, elongation factor 1α were included. Furthermore, a functionally unknown gene (FLJ00133) was also identified. It is reported that sequestosome 1 is a scaffold in the signal transduction of TNFα and interleukin 1, which are the important cytokines involved in the pathogenesis of RA. It is possible that other genes identified by the CGESH technique would be associated with the pathogenesis of RA, although there is no direct evidence yet. Our results imply that the CGESH technique is a useful tool to detect genes involved in the pathogenesis of RA. Further investigation of the functional roles of candidate genes should shed light on the pathogenesis of RA.

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Abbreviations: RA, rheumatoid arthritis; OA, osteoarthritis; CGESH, column gel electrophoresis-coupled subtractive hybridization; IGCR, in-gel competitive reassociation

Key words: rheumatoid arthritis, column gel electrophoresis-coupled subtractive hybridization

Introduction

Rheumatoid arthritis (RA) is a chronic disorder of unknown pathogenesis, that causes multiple joint destruction. Although new medications such as TNFα blocking agents are showing promising effects, deeper understanding of the disease is needed to overcome this possibly tragic condition. In order to understand the molecular mechanisms involved in the pathogenesis of RA, and to search for a possible target of RA-specific therapies, we wished to identify genes specifically expressed in the synovial tissues of patients with RA.

In the past, various strategies have been developed to examine tissue-specific differences in gene expression. We used column gel electrophoresis-coupled subtractive hybridization (CGESH) technique (1) for this purpose. The original technique which forms the basis of the CGESH, namely in-gel competitive reassociation (IGCR), was developed by Yokota *et al* (2,3). In this technique, electrophoresis size-separation of mixed restriction digested DNA samples to be compared and *in situ* denaturation-hybridization steps are carried out sequentially. These in-gel processes provide unique technical features that other batch subtractive hybridization techniques cannot. Firstly, size-dependent sequential fractionation of restriction digested DNA fragments results in an enormous reduction of sample complexity. This makes highly efficient hybridization possible. Secondly, closely related sequences will be physically separated and would not interfere with each other provided that they have different restriction fragment sizes. To overcome the drawbacks of this technique, such as its being time-consuming and too complex, Ozawa *et al* (1) made modifications to simplify the strategy of IGCR and developed the CGESH technique. While improving IGCR, this technique was originally used to detect differences between genome DNA samples (4,5), but if applied to cDNA samples, in addition to the advantages described above, it may

Table I. Oligonucleotides used for the CGESH experiment.

Name	Sequence	*HinP1* I site reconstitution
A20	5'-GACTGTCAAGGATCCCTTAG-3'	Yes
A10	3'-AGGGAATCGC-5'	
B20	5'-GATCGTGACAAGCTTCTGAC-3'	No
B10	3'-GAAGACTGGC-5'	
C20	5'-CAGACTCTGGAATTCGCATG-3'	Yes
C10	3'-AAGCGTACGC-5'	
Bio-C20	5'-biotin-CAGACTCTGGAATTCGCATG-3'	

CGESH, column gel electrophoresis-coupled subtractive hybridization. Underlining indicates *HinP1* I restriction enzyme site.

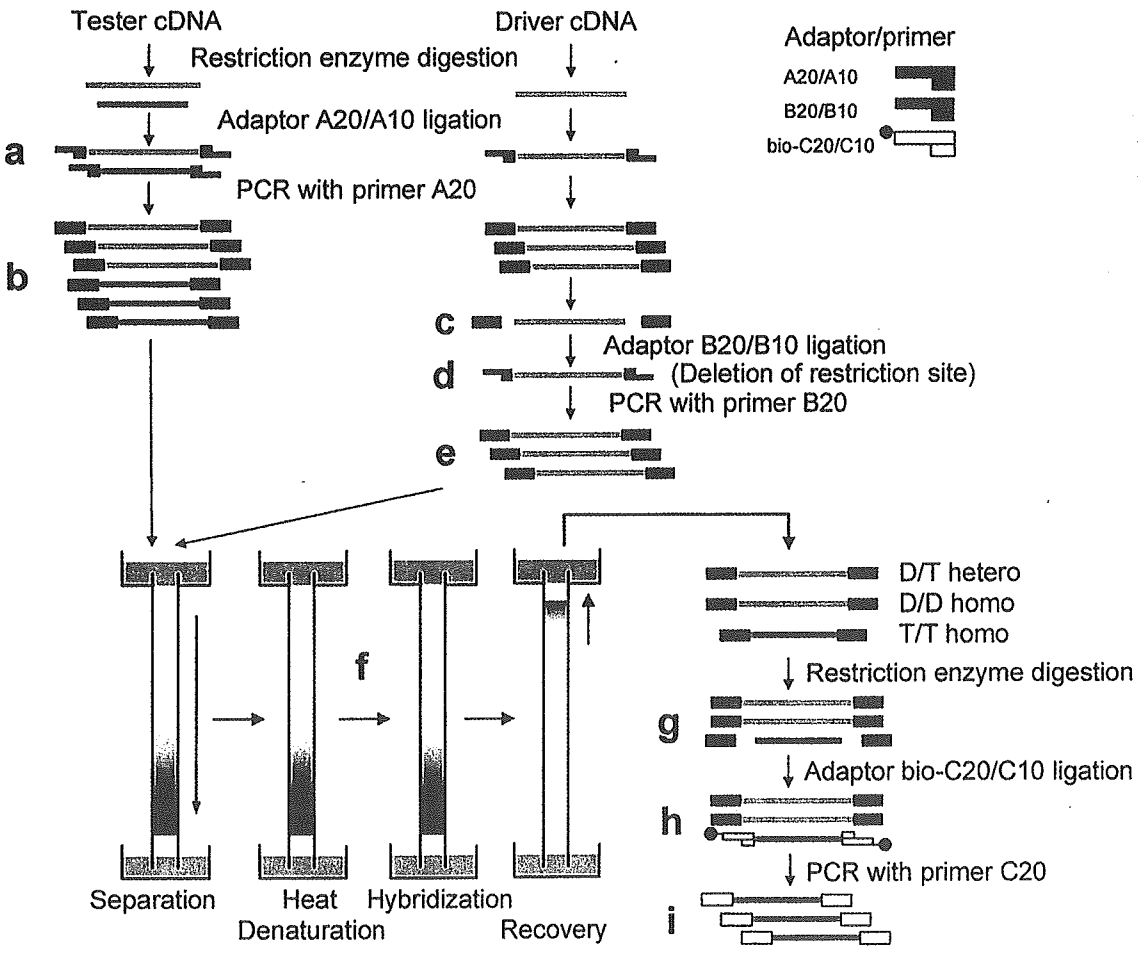

Figure 1. Summarized illustration of the steps of the column gel electrophoresis-coupled subtractive hybridization (CGESH) technique. *HinP1* I-digested driver or tester cDNA fragments were ligated with adaptor A20/A10, which preserves the *HinP1* I restriction enzyme site (a) and preamplified by PCR using primer A20 (b). Adaptor was removed from driver cDNA fragments by *HinP1* I digestion (c) and replaced with a new adaptor B20/B10, which does not reconstitute the *HinP1* I recognition site (d), and reamplified by PCR using primer B20 (e). Gel electrophoresis, denaturation, hybridization and recovery were carried out sequentially (f). Only perfectly reannealed tester-derived fragments can reconstitute the *HinP1* I site and the A20/A10 adaptor sequence are removed again by restriction digestion (g). A new adaptor, consisting of an oligonucleotide pair bio-C20/C10, is attached to both ends of the tester-derived fragments (h) and trapped by Streptavidin Sepharose beads (h). After removing unbound cDNA fragments by washing the beads, specifically captured cDNA fragments were amplified by PCR using primer C20 (i). See Table I for adaptor and primer sequences.

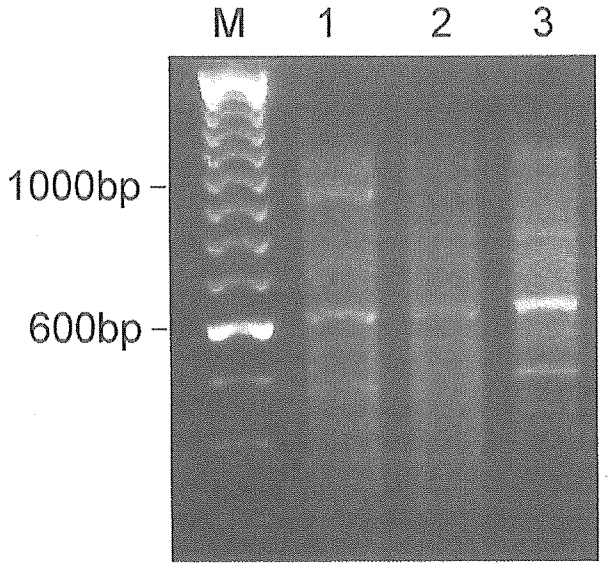

Figure 2. Electrophoresis pattern of the subtracted cDNA. Lane 1, driver cDNA amplified with primer A20, adaptor replaced and amplified with primer B20; Lane 2, tester cDNA amplified with primer A20; Lane 3, product of 1st round subtraction amplified with primer C20; Lane M, 100 bp DNA ladder.

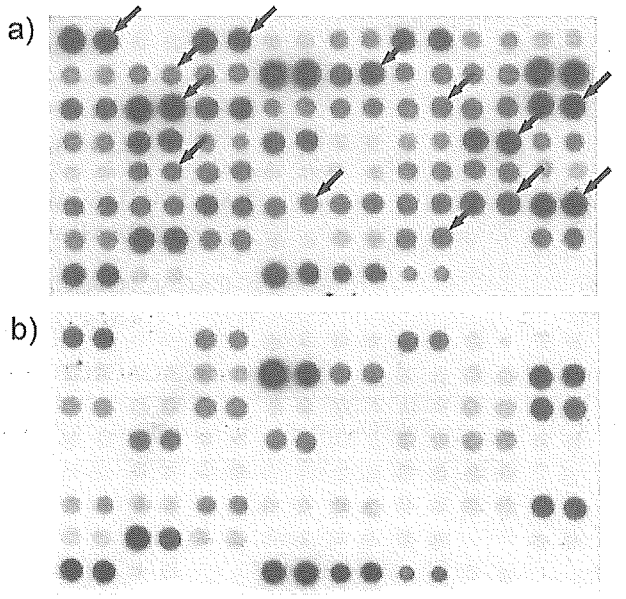

Figure 3. Screening of subtraction library by dot blot hybridization. Two identical cDNA blots of PCR-amplified inserts derived from the subtracted cDNA library were separately hybridized with DIG-labeled subtracted probe (a) and reverse-subtracted probe (b). Arrows indicate clones with a hybridization ratio of 2:1 or larger (RA-OA vs OA-RA) measured by a densitometer.

be possible to detect size variants such as those produced by alternative splicing, which are not detectable by conventional subtraction techniques. Thus, CGESH can be a powerful tool to identify known or unknown genes specifically expressed in a given tissue. Our objective was to detect genes specifically expressed in synovial tissues from RA patients using this newly developed technique.

Materials and methods

Synovial tissue was obtained under informed consent from patients with rheumatoid arthritis (RA) who met the criteria of the American Collage of Rheumatology (6), and patients with osteoarthritis (OA). Total RNA from synovial tissue was isolated using Isogen (Nippon Gene, Tokyo, Japan), and poly $(A)^+$ RNA was isolated from total RNA using a Oligotex-dt30 <Super> mRNA Purification Kit (Takara Bio, Shiga, Japan), following the protocols supplied by the manufacturers. The list of the oligonucleotide primers used in this study is provided in Table I.

CGESH technique. CGESH was performed essentially following the methods described by Ozawa *et al* (1), with some alterations (Fig. 1). Poly $(A)^+$ RNA (1 µg) from synovial tissues from RA or OA patients were subjected to cDNA synthesis. cDNA was synthesized using cDNA synthesis kit (Takara Bio) according to the manufacturer's instructions. cDNA from RA was designated as tester and that from OA as driver. Tester and driver cDNA were digested by a restriction enzyme *HinP1* I (New England Biolabs, Beverly, MA, USA), and ethanol-precipitated. Digested DNA fragments were ligated to oligonucleotide adaptor A20/A10 with T4 DNA ligase (New England Biolabs). The DNA was amplified using primer A20 by PCR using mini cycler PTC-150 (MJ Research, Waltham, MA, USA) under the following cycling conditions: 72°C for 1 min, 30 cycles of 94°C for 1 min and 68°C for 5 min, followed by 68°C for 20 min as a final extension step. Amplified driver DNA was digested with *HinP1* I, ligated with adaptor B20/B10, then amplified by PCR with primer B20 as described above. Tester DNA (10 ng) and driver DNA (1 µg) were mixed with formamide (10% final) to a final amount of 20 µl, and loaded on the top of the formamide-containing poly-acrylamide gel in a glass column (inner diameter: 5 mm; length: 130 mm), and electrophoresis was performed in the presence of a phosphate buffer. After gel separation, the column was incubated at 80°C for 10 min to denature dsDNA fragments in the gel. Then, the temperature was gradually lowered to hybridization temperature (37°C), and kept at 37°C overnight. After hybridization, DNA fragments were recovered by electrophoresis in the reverse direction, and collected by absorbing with Q-sepharose (Amersham Biosciences, Piscataway, CA, USA). Gel-recovered DNA was again digested with *HinP1* I, ligated with biotinylated adaptor bio-C20/C10. Biotinylated adaptor-ligated DNA fragments were absorbed with Streptavidin Sepharose (Amersham Bio-sciences). Amplification reaction was performed by adding the DNA-bound Streptavidin Sepharose slurry into a PCR reaction containing primer C20. The thermal cycling conditions were as described above.

Screening of differential expression. PCR products from the subtracted libraries were subcloned into pCR2.1-TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA). cDNA inserts of the cloned cDNA library were amplified by subjecting an aliquot of the bacterial culture directly to PCR.

Table II. Genes found to be up-regulated in RA compared to OA using the CGESH technique.

Clone No.	Accession No.	Identified gene
H237	NM_002124.1	Major histocompatibility complex, class II, DR ß 1 (HLA-DRB1)
H19, 180	NM_003900.2	Sequestosome 1 (SQSTM1) (p62)
H13, 167	NM_001402.4	Elongation factor 1 α 1 (EEF1A1)
H91, 129, 165, 190, 224, 252	NM_203339.1	Clusterin (CLU)
H95	NM_000852.2	Glutathione S-transferase pi (GSTP1)
H231	XM_059482.6	FLJ00133 protein (FLJ00133)

CGESH, column gel-electrophoresis subtractive hybridization.

Electrophoresis of the amplified product in agarose gels was always done to confirm that all clones were single. The amplified materials were then dot blotted onto nitrocellulose membranes in duplicates. Two identical membranes were prepared, and they were hybridized with either the subtracted probe (RA-OA) or the reverse-subtracted probe (OA-RA). Probes were labeled with a DIG DNA labeling kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's recommended protocol. Hybridization and detection via a chemiluminescence reaction were carried out employing a DIG luminescent detection kit (Roche diagnostics), according to the supplier's standard protocol. The amount of hybridization was semiquantified with a densitometer (LAS-3000; Fuji Photo Film, Tokyo, Japan). Clones with a hybridization ratio of 2:1 or larger (RA-OA vs OA-RA) were selected and plasmids were purified using a Qiagen plasmid mini kit (Qiagen, Hilden, Germany). Inserts were sequenced by chain termination reaction using an automated sequencer (ABI prism 310 genetic analyzer; Perkin Elmer, Wellesley, MA, USA). Nucleic acid homology searches were performed using the BLAST program at the National Center of Biotechnology Information (National Institutes of Health).

Results and Discussion

Using CGESH, we constructed one subtracted library using RA synovial tissue sample as tester and OA synovial sample as driver (RA-OA) (Fig. 2). In parallel, a reverse-subtracted library was prepared (OA-RA) to make a control probe for dot blot hybridization screening. This subtracted library was cloned, and a total of 273 clones were obtained. Thirteen clones with a hybridization ratio of 2:1 or larger (RA-OA vs OA-RA) measured by a densitometer in the dot blot hybridization were selected (Fig. 3). These clones were sequenced, and 6 genes (HLA-DRB1, sequestosome 1, elongation factor 1 α 1, clusterin, glutathione S-transferase pi and FLJ00133) were identified. Six clones (H91, 129, 165, 190, 224, 252) were derived from the clusterin gene, two clones (H19, 180) were sequestosome 1, and the other two clones (H13, 167) were elongation factor 1 α 1. This suggests that the dot blot hybridization worked well for selecting genes from the CGESH library, although the true frequency of this gene expression was not clarified. The sequences of the clones were identical to those submitted in the BLAST program (Table II).

HLA-DRB1, a class II MHC molecule, is important for antigen presentation, and would be important in the pathogenesis of RA (7,8). Increased expression of MHC class II molecules in RA synovial tissue is also reported (9). Sequestome 1, also known as p62, is reported to be a scaffold in the signal transduction of TNFα and interleukin-1 leading to the activation of NF-κB (10). These two important cytokines are well known to be involved in the pathogenesis of RA (11). Recently, it has been shown that p62 is an important mediator during osteoclastogenesis and induced bone remodeling (12). p62 may have a function in the joint destruction process in RA patients. Elongation factor 1 α 1 is reported to increase at the mRNA level in patients with RA synovial samples compared to OA synovial samples (13). Autoantibody against this protein has been reported to be present in patients with Felty's syndrome (14). The function of this protein is not fully understood, but may have a role in the pathogenesis of RA. Both clusterin and glutathione S-transferase pi genes are reported to be involved in the signal transduction of NF-κB (15,16). FLJ00133, which was identified in the NEDO human cDNA sequencing project at Kazusa DNA Research Institute (17), was also one of the genes identified in our study. The function of this gene product is yet to be clarified.

We used the dot hybridization technique to efficiently screen the clones obtained by CGESH. This method allows us to easily identify genes with higher expression in the tester sample than in the driver. However, it is true that dot blot hybridization cannot identify size variants such as alternatively spliced gene products. CGESH is theoretically potent for screening such variants because a size fractionation step is included in the procedure. A large number of clones that were considered non-specific in dot blot assays may contain such variants. By combining an efficient method to identify such clones, we would be able to make the most of the CGESH technique and be able to show that it is a powerful tool for identifying genes that are differently expressed between two tissues.

In summary, we have identified a number of genes possibly involved in the pathogenesis of RA by means of the CGESH technique, with RA samples as tester and OA samples as driver.

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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