gel (CEQ™ Separation Gel-LPA I; Beckman Coulter) to determine the size and fluorescence intensity by using an automated capillary DNA sequencer (CEQ™ 8000; Beckman Coulter). Data were analysed by using Genetic Analysis System software (Beckman Coulter).

Human leucocyte antigen D-related (HLA-DRB1) genotyping

HLA-DRB1 genotyping was performed using the Genoresearch HLA-DRB1 kit (Medical Biological Laboratories, Tokyo, Japan) according to the manufacturer's instructions.

Detection of antibodies to SMEZ-2 and TSST-1 in plasma samples

Levels of immunoglobulin antibodies against SMEZ-2 and TSST-1 in plasma samples were assayed by an ELISA method using rTSST-1 or rSMEZ-2 as antigens. Recombinant proteins were diluted to 1 μg/ml in 10 mM phosphate-buffered saline (PBS, pH 7·4), and a 100 μl diluted toxin was added to each well of a 96-well microplate (Nalge Nunc International, Rochester, NY, USA). The plates were incubated overnight at 4°C to allow binding of antigens to the wells. Unbounded antigens were removed by aspiration, and the wells were washed four times with washing buffer. After blocking with 1% bovine serum albumin (BSA)–PBS, the wells were washed four times with washing buffer and filled with dilution buffer (PBS containing 0·1% BSA). The toxin-coated plates were stored at 4°C until assay.

Plasma samples from 27 patients with active UC and seven healthy controls were diluted to 1 : 200 with dilution buffer and 100 μl diluted plasma was added to the toxin-coated wells. The plates were then incubated overnight at 4°C. At the end of the incubation time, the wells were washed four times with washing buffer. One hundred μl peroxidase-conjugated anti-human IgG antibody (Southern Biotechnology Associates, Birmingham, AL, USA) (diluted to 1 : 10 000 with dilution buffer) was added to each well; the plates were then incubated at 30°C for 2 h. The wells were again rinsed four times with washing buffer. The product was visualized by subsequent reaction with 100 μl 3,3′,5,5′-tetramethlbenzidine (TMB) solution (Wako, Osaka, Japan) for 5 min at room temperature. The reaction was terminated by addition of 50 μl of 1 M sulphuric acid, and the absorbance of each well was read at 450 nm with a plate spectrophotometer (Multiskan JX; Thermo Labsystems). The antibodies to SMEZ-2 and TSST-1 in plasma samples were corrected with the antibodies to BSA.

Detection of plasma anti-streptolysin-O antibody

Detection of plasma anti-streptolysin-O (ASO) antibody, a marker for group A streptococcal infections, was performed by SRL Inc. (Hachioji, Tokyo, Japan), a clinical diagnosis laboratory. Determination of anti-SMEZ-2, anti-TSST-1 (above) and ASO titres was to investigate the nature of the background SA and contribution to TCRBV4 skewing (see Results section).

Statistical analysis

For statistical analysis, a software package StatView 5·0 for Windows (SAS Institute, Cary, NC, USA) was used. For all comparisons except *in vitro* studies, non-parametric tests (Mann–Whitney *U*-test, Wilcoxon's signed-rank test, Kruskal–Wallis test and Spearman's rank correlation test) were applied. For three independent samples, it was confirmed that there were significant differences by Kruskal–Wallis test before the comparison between each two groups was performed. Paired *t*-test was used for comparisons *in vitro* studies. Accordingly, results of comparisons are given as mean or median values. $P < 0{\cdot}05$ was considered significant.

Results

Demography of patients with UC

Twenty-seven patients (19 males and eight females) with active UC, clinical activity index (CAI) ≥ 5 [32], were enrolled into the study (Table 1). The mean age at entry was 33 years (range 16–64 years); the mean disease duration was 5·5 years (range 1 month–32 years) and the mean CAI was 9·4, range 5–17. HLA-DRB1 typing revealed the presence of the allele (*1502) in 11 of 23 patients (48%); *1502 is known to be associated with UC in Asians [33,34]. HLA-DRB1 typing was not performed in four patients because DNA could not be collected.

Selective expansion of TCRBV subfamilies in PBMC from patients with UC

Initially, we performed TCR repertoire analysis on PBMC from 23 of 27 patients with active UC to determine whether T cell features were associated with UC. The expansion of TCR-bearing T cells in patients with UC was defined as significant when the percentage frequency of the relevant T cell subset was greater than 5%, and exceeded the mean percentage plus 3 standard deviations (s.d.) of the corresponding T cells bearing the relevant TCR in 20 healthy controls. Four patients, UC-05, UC-14, UC-18 and UC-20, were excluded from the analysis due to unsuccessful collection of RNA. Twenty-one of 23 (91%) patients with UC had skewed a TCR repertoire in any TCRBV subfamily. In particular, 14 of 23 (61%) patients had skewed TCR in the TCRBV4 (Fig. 1). There was no selective expansion of TCRAV subfamilies in patients with UC (data not presented).

The percentage frequency of TCRBV4-bearing T cells was increased markedly ($P < 0{\cdot}0001$) in patients with UC compared with healthy controls (Fig. 2a), but was not associated

Table 1. Baseline demography of the 27 patients who were included in this study.

Patient ID	Age (years)	Gender	UC duration (years)	CAI[a]	Location	Severity	Medication[b]
UC-01	41	Male	12	14	Left	Severe	5-ASA
UC-02	16	Male	2	11	Total	Severe	PSL, SASP
UC-03	21	Female	2	14	Total	Severe	PSL, SASP
UC-04	52	Male	12	6	Left	Moderate	5-ASA, SASP
UC-05	17	Female	2	9	Total	Severe	PSL, SASP
UC-06	45	Male	5	9	Left	Moderate	PSL, 5-ASA
UC-07	30	Male	2	9	Left	Moderate	PSL, SASP
UC-08	33	Male	16	8	Total	Severe	5-ASA
UC-09	37	Male	11	11	Total	Severe	PSL
UC-10	29	Male	10	7	Left	Moderate	PSL, 5-ASA
UC-11	19	Male	4	5	Total	Moderate	5-ASA
UC-12	19	Male	1·75	11	Total	Moderate	SASP
UC-13	42	Female	19	5	Total	Severe	5-ASA
UC-14	34	Female	2·7	9	Total	Severe	PSL, 5-ASA
UC-15	25	Male	6	5	Total	Moderate	PSL, 5-ASA
UC-16	20	Male	0·08	8	Total	Severe	SASP, 5-ASA
UC-17	47	Male	0·25	10	Total	Moderate	5-ASA, betamethasone
UC-18	20	Male	0·25	9	Total	Severe	PSL, 5-ASA, SASP
UC-19	18	Female	2	10	Total	Severe	PSL, SASP
UC-20	29	Male	4	9	Left	Moderate	PSL, 5-ASA
UC-21	64	Female	0·08	5	Total	Moderate	5-ASA
UC-22	38	Male	0·16	17	Left	Severe	PSL, 5-ASA
UC-101	57	Male	32	9	Total	Moderate	PSL, SASP
UC-102	48	Male	0·67	10	Total	Moderate	5-ASA
UC-103	32	Male	0·16	9	Total	Moderate	PSL, 5-ASA
UC-104	35	Male	0·08	12	Total	Severe	SASP
UC-105	24	Female	0·16	12	Total	Severe	PSL, 5-ASA

[a]CAI indicates the disease activity. The final score is the total of several score for symptoms and signs, and the maximum value is 21 [29]. [b]5-ASA; 5-aminosalicylic acid, SASP; salazosulfapyridine, PSL; prednisolone, UC: ulcerative colitis.

with HLA-DRB1 types. CDR3 size spectratyping was undertaken to determine whether the TCRBV4 T cells were expanded in a monoclonal or polyclonal manner. Figure 2b shows that there were multiple Gaussian-like patterns with three nucleotide intervals, indicating polyclonal expansion in all CDR3 size distributions [35].

TCRBV4-bearing T cells expansion by rSMEZ-2

It has been suggested that TSST-1 or SMEZ-2 might induce selective expansion of TCRBV4-bearing T cells [36,37]. We were interested to identify which SA stimulates the TCRBV4-bearing T cells *in vitro*. The percentage frequency of TCRBV2-bearing T cells increased in the PBMC from all four donors when stimulated with rTSST-1. In contrast, the percentage frequency of TCRBV4-bearing T cells did not increase by TSST-1 stimulation, but increased significantly in PBMC from all five donors when stimulated by rSMEZ-2. Similarly, the percentage frequency of TCRBV2-bearing T cells did not increase by rSMEZ-2 (Fig. 3). Further, rSMEZ-2 also stimulated TCRBV8-bearing T cells (data not presented).

Association of SMEZ-2 titre with TCRBV4 skewing and ASO level

We were convinced that our experimental method could detect the SMEZ-2-induced polyclonal expansion of TCRBV4- and TCRBV8-bearing T cells (without TCRBV2-bearing T cells) both qualitatively and quantitatively. We then examined antibody levels to SMEZ-2 in the plasma samples from 27 patients with UC and from seven healthy donors to investigate exposure of patients to SMEZ-2 (infection with *Streptococcus pyogenes*). The level of antibodies against SMEZ-2 in patients with skewed TCRBV4 was significantly higher compared with the level in healthy volunteers ($P = 0{\cdot}0305$, Fig. 4a). Additionally, there was a significant correlation between the percentage of TCRVB4 and the level of anti-SMEZ-2 titre ($\rho = 0{\cdot}606$, $P = 0{\cdot}0045$, Fig. 4b), and the levels of ASO antibody and anti-SMEZ-2 titre ($\rho = 0{\cdot}456$, $P = 0{\cdot}0227$) (Fig. 4c). In contrast, there was no significant correlation between the percentage TCRVB4 and the level of antibodies against TSST-1 (data not presented).

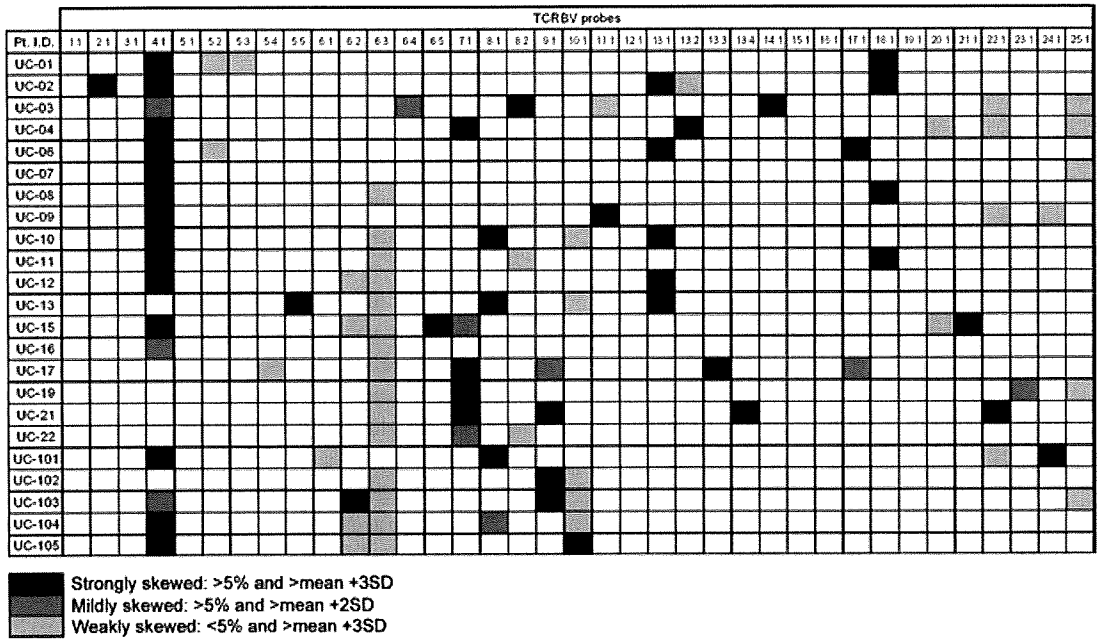

Fig. 1. T cell receptor β-chain (TCRBV) gene expression profile in peripheral blood mononuclear cells (PBMC) of patients with active ulcerative colitis (UC). Total RNA was extracted from PBMC and reverse-transcribed into cDNA and the adaptor was ligated. This adaptor-ligated cDNA was then used as a template for individual polymerase chain reaction (PCR) amplifications. The primer sets were then applied to the adaptor sequence and TCRBC gene elements. The PCR products were determined by semiquantitative PCR–enzyme-linked immunosorbent assay. Twenty-one of 23 (91%) patients had strongly skewed TCR repertoire in any TCRBV subfamilies and intense skew was observed in TCRBV4.

Expansion of intestinal TCRBV in patients with UC

The expansion of TCRBV in pairs of PBMC and intestinal mucosa from an additional five patients was investigated for the polyclonal expansion of TCRBV4-bearing T cells, as the phenomenon observed in PBMC. The percentage frequencies of TCRBV4, BV6·2, BV6·5 and BV8 in the intestinal biopsy samples from UC patients were significantly higher than the level in PBMC specimens from healthy donors (Fig. 5a). The mean percentage frequencies were 10·3%, 4·6%, 4·7% and 7·9%, respectively. There was no significant increase in any other subfamily.

CDR3 size spectratyping was performed in three patients, UC-101, UC-104 and UC-105, who showed skewed TCRBV4 in PBMC. Similar to the results in PBMC, TCRBV4 and BV8-bearing T cells within local mucosal lesions had multiple Gaussian-like patterns with three nucleotide intervals indicating polyclonal expansion in these patients, except on TCRBV8 within intestinal T cells in UC104 (Fig. 5b). These observations implied that intestinal T cells might also have been exposed to SMEZ-2. In contrast, a few peaks, indicating oligoclonal expansion of T cells, were detected in TCRBV6·2- and TCRBV6·5-bearing T cells within the intestinal mucosa from these three patients (data not presented).

Association of TCRBV4 skewing with UC duration

We wished to investigate any probable relationship between TCRBV4 skewing and the UC disease parameters. There was no association between CAI and the percentage TCRBV4. However, there was a significant ($P = 0{\cdot}0314$) association between UC disease duration and the TCRBV4 skewing (Fig. 6).

Discussion

T cells are believed to be involved intimately in the regulation of the immune function in patients with IBD [20,21,38,39], while injury to the mucosal tissue is caused mainly by granulocytes and monocytes/macrophages [1,40,41]. Hence dysregulated immune activity seen in patients with UC might reflect abnormal T cell behaviour and bacterial SA appear to be potential factors for dysregulated T cells. Accordingly, this study was to further understanding on T cell features associated with UC, whether the TCR repertoire of T cells is biased in patients with UC, and to what extent bacterial SA are involved. Certainly, a better understanding of T cell involvement in the immunopathogenesis of UC is desirable and should be valuable for designing effective therapeutic interventions.

Initially, we performed TCR repertoire analysis on PBMC from patients with active UC because this condition is characterized by multiple systemic clinical manifestations and activated T cells are known to increase not only in the inflamed intestinal mucosa but also in the peripheral blood [42], albeit the total peripheral blood lymphocyte count appears to be compromised in patients with UC [39,43,44]. We found an increase in the percentage of T cells expressing

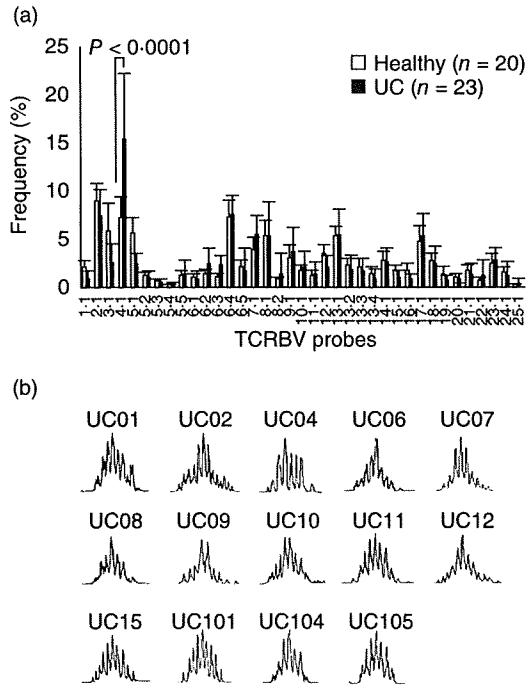

Fig. 2. The polyclonal expansion of T cell receptor β-chain 4 (TCRBV4)-bearing T cells in peripheral blood mononuclear cells (PBMC) of patients with active ulcerative colitis (UC). (a) The percentage of TCRBV4 in PBMC specimens of patients with UC was strikingly high compared with healthy individuals ($P < 0{\cdot}0001$). Mean ± s.d. values are presented; the *P*-value is by the Mann–Whitney *U*-test. (b) The CDR3 size spectratype profiles of the TCRBV4 gene rearrangement. The TCRBV4 gene was selected among the PBMC of 14 patients with skewed TCRBV4. Each of the 14 subjects had 7–10 peaks showing a Gaussian-like distribution.

TCRBV4, which appears to be a polyclonal expansion of PBMC in many patients with UC. This specific TCRBV and polyclonal expansion of T cells suggests involvement of bacterial SA in the immunopathogenesis of UC. In line with this thinking, it is widely known that bacterial SA can activate TCRBV-bearing T cells without any other specific antigen due to the cross-linking of HLA class II molecules and the specific interaction of SA with TCRBV. This can lead to systemic immune disorders [45,46]. It is also known that systemic immune disorders might trigger other immune-related episodes such as toxic shock syndrome, Kawasaki disease, psoriasis vulgaris and atopic dermatitis [29,47–49]. Accordingly, interaction of bacterial SA with TCRBV-bearing T cells should serve as an appropriate model to investigate mechanisms of immune disorders.

Staphylococci and *Streptococci* are common bacterial flora in the pharynx, the larynx, the paranasal sinus and the colon. SMEZ-2 produced by *S. pyogenes* and TSST-1 produced by *Staph. aureus* are known as SA, which activate preferentially the TCRBV4-bearing T cells [36,37]. Yang *et al.* reported that the clinical symptom score improved after the sinus was ablated in patients with both chronic rhinosinusitis and UC; these patients had been infected with *Staph. aureus* [50]. However, TSST-1 strongly stimulates TCRBV2-bearing T cells. By contrast, SMEZ-2 could strongly stimulate TCRBV4 and BV8-bearing T cells without the expansion of TCRBV2-bearing T cells [36]. Our *in vitro* study also showed an increase in the percentage frequency of TCRBV4 and BV8-bearing T cells without the expansion of TCRBV2-bearing T cells (by SMEZ-2 stimulation).

The level of antibody against SMEZ-2 in patients with the skewed TCRBV4 was significantly higher than the level in healthy volunteers. There was significant correlation between the ASO levels, the marker for the infection with *S. pyogenes*, and the level of antibodies against SMEZ-2 (although the ASO levels were mainly within the normal range). Moreover, the percentage frequency of TCRBV4-bearing T cells correlated with the level of antibodies against SMEZ-2, but did not correlate with the antibodies against TSST-1. These observations support a perception that many patients with UC are infected with *S. pyogenes*, which can specifically promote the expansion of TCRBV4-bearing T cells by its SA.

Interestingly, we found an increase in the percentage of TCTBV4-bearing T cells at the sites of inflamed intestinal mucosa, which were expanded polyclonally similar to the results in PBMC. Moreover, the expansion of TCRBV8-bearing T cells was also observed in the same patients. Previous reports have indicated that antigen-specific T cells exist within the intestinal mucosa not only in patients with IBD, but also in healthy adults [51–55]. The TCRBV4 repertoire is

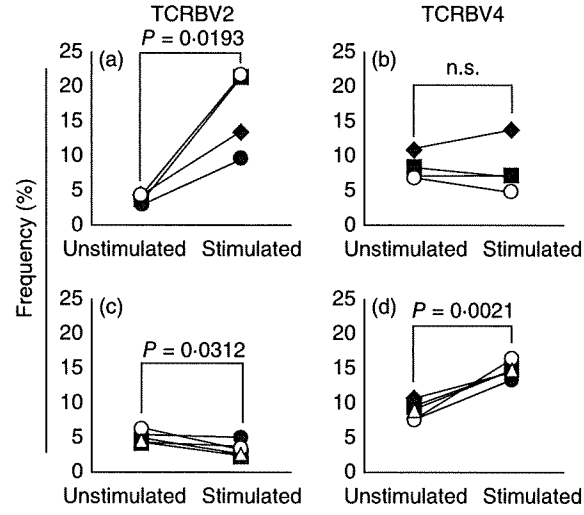

Fig. 3. Changes in the percentage frequency of T cell receptor β-chain (TCRBV) families following stimulation by two different bacterial superantigens (SA), toxic shock syndrome toxin 1 (TSST-1) and streptococcal mitogenic exotoxin Z-2 (SMEZ-2). TSST-1 stimulated TCRBV2-bearing T cells (a) and did not stimulate TCRBV4-bearing T cells (b). SMEZ-2 stimulated TCRBV4-bearing T cells (d) without the expansion of TCRBV2-bearing T cells (c). The *P*-values are by paired *t*-test.

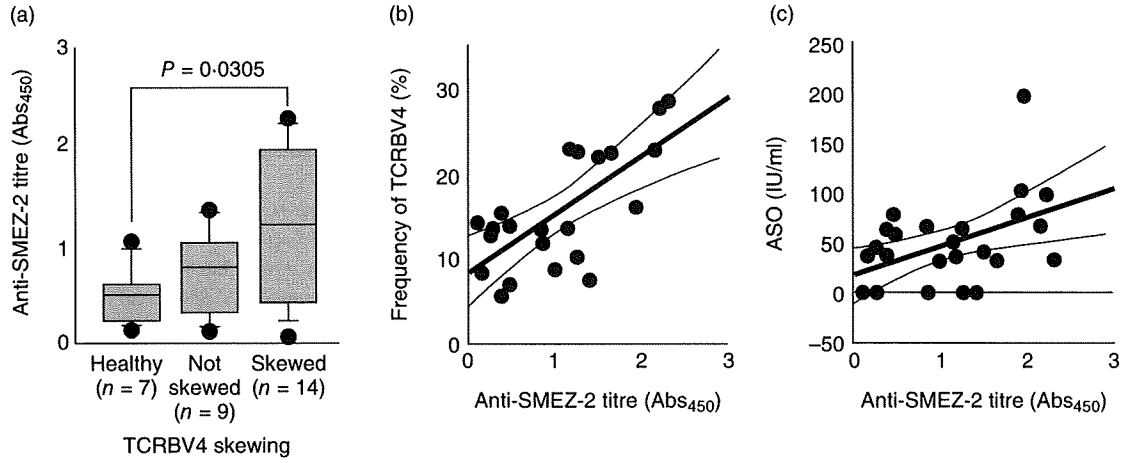

Fig. 4. Elevated anti-streptococcal mitogenic exotoxin Z-2 (SMEZ-2) antibody in patients with active ulcerative colitis (UC) who had skewed T cell receptor β-chain 4 (TCRBV4). (a) The anti-SMEZ-2 titre was significantly higher in patients with skewed TCRBV4 compared with healthy volunteers ($P = 0{\cdot}0305$, by Mann–Whitney *U*-test). (b) The correlation of the percentage of TCRBV4 with anti-SMEZ-2 titre ($n = 23$, $\rho = 0{\cdot}606$, $P = 0{\cdot}0045$, by Spearman's rank correlation). The anti-streptolysin-O (ASO) level (c) also showed significant correlation with the anti-SMEZ-2 titre ($n = 27$, $\rho = 0{\cdot}352$, $P = 0{\cdot}0149$, by Spearman's rank correlation test), but no correlation with anti-toxic shock syndrome toxin 1 (TSST-1) titre was seen.

monoclonal within the intestinal mucosa even during infancy, although other TCRBV subfamilies show polyclonal behaviour [56]. Indeed, with our semiquantitative TCR repertoire and sequence analyses, we found that TCRBV6·2- and TCRBV6·5-bearing T cells had expanded within the intestinal mucosa and that these T cells had some common TCRBV-BJ motifs like those reported previously [51] (data not shown). Small amounts of SA might specifically stimulate TCRBV subfamilies and this could lead to the production of inflammatory cytokines, causing disordered host immune function. Such immune disorders might, in turn, cause exaggerated reaction to food antigens and/or self-antigens. In fact, we detected monoclonal or oligoclonal expansions of TCRBV6·5-bearing T cells in the inflamed intestinal tissue, suggesting that selective antigenic pressures are prevalent among activated intestinal T cells. Chott *et al.* [51] have shown that there is a common TCRBV-BJ motif within $CD8^+$ mucosal T cells and these may recognize common foreign antigens. Essentially, the findings reported by Chott and colleagues are in line with the perception that T cell abnormality is a feature in immune pathology. We have also detected some common TCRBV-BJ motifs within the mucosal T cells, such as TCRBV6S7-BJ2S7, in addition to polyclonal expansion of TCRBV4-bearing T cells not only within the mucosa but also in PBMC.

Based on the knowledge that within the lymphocyte population certain T cell subsets are major factors in the immune pathology of intestinal mucosa, while other subsets are essential regulators, controlling IBD [57], we were looking for any relationship between factors associated with skewed TCR repertoire and the UC disease, together with a special interest in the role of bacterial SA in any prevailing T cell behaviour. There was no significant correlation between the percentage frequency of TCRBV4 and CAI. However,

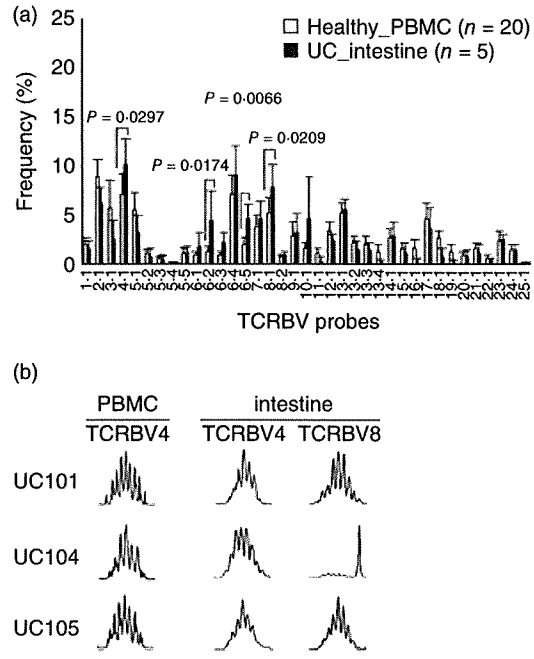

Fig. 5. Polyclonal expansion of T cell receptor β-chain 4 (TCRBV4) and TCRBV8-bearing T cells within the intestinal mucosa of patients with active ulcerative colitis (UC). (a) The percentage of TCRBV4, BV6·2, BV6·5 and BV8 in intestinal samples from patients with UC were significantly higher than the level in the peripheral blood mononuclear cells (PBMC) of healthy individuals ($P = 0{\cdot}0297$, $P = 0{\cdot}0174$, $P = 0{\cdot}0066$ and $P = 0{\cdot}0209$, respectively). Mean ± s.d. values are presented; the *P*-values are by Mann–Whitney *U*-test. (b), CDR3 size spectratype profiles of TCRBV gene rearrangements. Three patients who had skewed TCRBV4 within the intestinal mucosa were selected for spectratyping. On the TCRBV4 and TCRBV8 genes, these three subjects had 6–8 peaks with Gaussian-like distribution and spectratype profile except on TCRBV8 in UC104, showing polyclonal expansion.

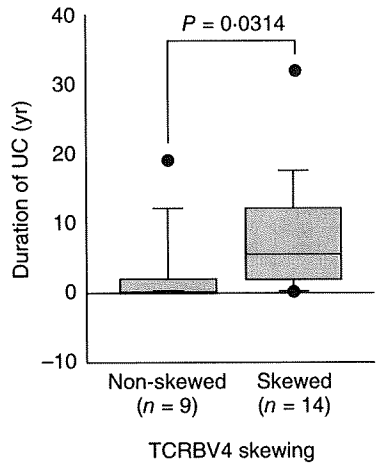

Fig. 6. Significant ($P = 0{\cdot}0314$, by Mann–Whitney U-test) association between duration of ulcerative colitis and T cell receptor β-chain 4 (TCRBV4) skewing. Twenty-three patients were classified into two subgroups based on the skewing of TCRBV4. The skewing was defined to be significant if the percentage frequency of the relevant T cells was greater than 5%, and exceeded the mean percentage plus 3 s.d. of the T cells bearing the corresponding TCR in 20 healthy controls.

when patients with skewed TCRBV4 and non-skewed TCRBV4 were compared with respect to the duration of UC, there was a very significant difference between the two subgroups. It was assumed that long-term exposure (even at a low dose) to bacterial SA such as SMEZ-2 promotes exacerbation, hypersensitivity reaction or exaggerated reaction to food antigens (or self-antigens) within the intestinal immune system and this might give rise to systemic immune disorders. Such exuberant immune activation might cause the remission–relapsing cycles seen in patients with UC (reflecting dysregulated T cell function). The long duration of disease, together with chronic immunosuppressive medication, might provide increased opportunity for infection. Future studies should strengthen our findings and increase understanding of the aetiology of IBD.

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Arthritis and pneumonitis produced by the same T cell clones from mice with spontaneous autoimmune arthritis

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Abstract

SKG mice, a newly established model of rheumatoid arthritis (RA), spontaneously develop autoimmune arthritis accompanying extra-articular manifestations, such as interstitial pneumonitis. To examine possible roles of T cells for mediating this systemic autoimmunity, we generated T cell clones from arthritic joints of SKG mice. Two distinct $CD8^+$ clones were established and both showed *in vitro* autoreactivity by killing syngeneic synovial cells and a variety of MHC-matched cell lines. Transfer of each clone to histocompatible athymic nude mice elicited joint swelling and histologically evident synovitis accompanying the destruction of adjacent cartilage and bone. Notably, the transfer also produced diffuse severe interstitial pneumonitis. Clone-specific TCR gene messages in the inflamed joints and lungs of the recipients gradually diminished, becoming hardly detectable in 6–11 months; yet, arthritis and pneumonitis continued to progress. Thus, the same $CD8^+$ T cell clones from arthritic lesions of SKG mice can elicit both synovitis and pneumonitis, which chronically progress and apparently become less T cell dependent in a later phase. The results provide clues to our understanding of how self-reactive T cells cause both articular and extra-articular lesions in RA as a systemic autoimmune disease.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology that primarily affects the synovial membranes of multiple joints (1, 2). A cardinal feature of joint inflammation in RA is proliferative inflammation of the synovium, i.e. synovitis, which leads to the destruction of adjacent cartilage and bone. In addition, RA frequently accompanies extra-articular manifestations, for example the development of rheumatoid factors, rheumatic nodules, vasculitis and interstitial lung disease (ILD). Recent studies with high-resolution imaging have indeed revealed a high prevalence of ILD in patients with RA (3–6). RA is thus a systemic disease; yet, the immunological basis of this systemic autoimmunity is poorly understood.

T cells appear to play a key role in the development of RA as suggested by the infiltration of T cells, especially $CD4^+$ T cells, into the synovial tissue of RA (7–9) and the association of genetic susceptibility to RA with particular alleles of HLA-DR (10, 11). On the other hand, there is evidence in humans and animal models that stimulated synoviocytes, composed of macrophage-like and fibroblast-like synovial cells, can

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themselves mediate joint destruction in a T cell-independent manner (12, 13). A key issue in elucidating the pathogenetic mechanism of RA is, therefore, to determine how self-reactive T cells contribute to the initiation and progression of synovitis and possibly extra-articular lesions such as ILD.

The SKG strain of mice spontaneously develops T cell-mediated chronic autoimmune arthritis (14–16). The strain possesses a mutation in the gene encoding a Src homology 2 domain of the ζ-associated protein of 70 kDa (ZAP-70), a key signal transduction molecule in T cells (17, 18). Impaired signal transduction through SKG ZAP-70 results in thymic positive selection and failure in negative selection of highly self-reactive T cells that include potentially arthritogenic T cells (14). The SKG arthritis progresses chronically, starting from small joints of the digits and symmetrically progressing to larger joints, such as the wrists and ankles. Histologically, affected joints show hyperplasia of synoviocytes, inflammatory cell infiltration, pannus formation and destruction of cartilage and bone, eventually leading to joint deformity. As extra-articular lesions, they develop interstitial pneumonitis, dermatitis, necrobiotic nodules akin to rheumatic nodules in RA and systemic vasculitides. Serologically, they spontaneously develop IgM-type rheumatoid factors, auto-antibodies against type II collagen and antibodies cross-reactive with *Mycobacterium tuberculosis* heat shock protein (hsp) 70. IL-1, tumor necrosis factor (TNF)-α, IL-6 or IL-17 deficiency inhibits the development of arthritis in SKG mice (15, 19), similar to the effects of anti-cytokine therapies in RA (20, 21). Thus, autoimmune disease in SKG mice closely resembles RA in clinical and immunopathological characteristics. In addition, considering recent findings that genetic polymorphism of a signaling molecule at a TCR proximal step involving ZAP-70 significantly contributes to the susceptibility to RA and other autoimmune diseases (22, 23), SKG mice can be a suitable model for elucidating how a T cell-intrinsic anomaly contributes to the development of RA as a systemic autoimmune disease.

In this study, we have attempted to determine the role of T cells in SKG autoimmune disease by establishing T cell clones from their arthritic lesions. We have established two distinct $CD8^+$ clones and show that both of them have the potential to induce not only arthritis but also pneumonitis. This indicates that inflammation in both the joints and the lung can be mediated, at least in part, by common autoreactive T cell clones in SKG mice. In addition, by adoptively transferring these T cell clones to normal mice, we show that autoreactive T cells are able to initiate arthritis; yet, the arthritis can progress apparently in a T cell-independent manner in a later phase. These findings contribute to our understanding of how T cells cause chronic arthritis and ILD in RA.

Materials and methods

Mice

SKG and (SKG × BALB/c)F_1 mice (14) were maintained in the animal facility of Kyoto University under a microbially conventional condition. Female C.B-17 SCID mice (Clea Japan, Tokyo, Japan), DBA/1J, BALB/c and BALB/c-nu/nu mice (Charles River Japan, Kanagawa, Japan) were maintained under specific pathogen-free conditions at Kyoto University or Discovery Research Laboratories of Shionogi & Co., Ltd. All experiments were approved by the Animal Care and Use Committee at Kyoto University and Shionogi & Co., Ltd.

Culture medium

The culture medium for SKG T cell lines and clones was AIM-V supplemented with 20% RPMI-1640, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol (ME), 2 mM L-glutamine, ×1 penicillin/streptomysin (Gibco BRL, Gaithersburg, MD, USA), 10% heat-inactivated FCS (Hyclone, Logan, UT, USA), 10% rat T-STIM™ with Con A (Becton Dickinson, Franklin Lakes, NJ, USA), 100 U/ml of recombinant mouse IL-2 (Genzyme, Cambridge, MA) and 5 μg/ml of Con A (Sigma, St Louis, MO, USA).

Establishment of T cell clones from arthritic joints of SKG mice

To establish T cell lines, severely swollen joints of SKG mice were aseptically excised, finely minced and cultured until clusters of mononuclear cells were confirmed in bulk culture. Outgrown T cells were cloned in 96-well microplates by using SKG synovial cells (1×10^3) as feeder cells. Synovial cells were prepared as previously described (16). Briefly, synovial tissues from wrist and ankle joints were digested with 400 Mandl U/ml of Liberase Brendzyme II (Roche) in RPMI-1640 medium for 1 h at 37°C; digested cells were filtrated through a nylon mesh to prepare single-cell suspensions. A typical composition of the synoviocyte preparation was ~10% $CD11b^+$ monocyte/macrophages, ~20% $Gr\text{-}1^+$ granulocytes, ~1% T cells and other cells. Several days later non-adherent cells were removed by washing the plates with culture medium. T cells that had outgrown from the bulk culture of synovial cells were dispensed at 1, 5, 20 or 50 cells per well and apparently single colonies were propagated in the culture medium described above. Clonality of each cell was confirmed by microplate hybridization assay (MHA) (24) and sequence analysis of TCR. Established T cell clones were maintained without feeder cells. Dengue 2F7 and 3F2 T cell clones, established by immunization of BALB/c mice with the NS3 peptide of dengue virus, were kindly provided by Dr H. Masaki (Kinki University). All cultures were performed in a humidified atmosphere of 7.5% CO_2 at 37°C.

Cytokine detection

Cytokine production by T cell clones were analyzed by ELISA. T cell clones were stimulated with 10 ng/ml of phorbol myristate acetate (PMA) (Wako Chemicals USA, Inc., Richmond, VA, USA) and 0.4 μg/ml of ionomycin (Calbiochem, Darmstadt, Germany) in culture medium at 1×10^6 cells/ml for 16 h. The supernatants were assayed for various cytokines using specific ELISA kits (Endogen, Woburn, MA, USA, and Axis-Shield, Oslo, Norway) according to the manufacturer's protocol. Cytokine mRNA levels in the joints and lungs of clone recipient mice were analyzed by quantitative PCR as described previously (25).

MHA for TCR AV and BV family and sequence analysis

MHA, cDNA synthesis and PCR amplifications of TCR of each T cell clone were performed as described previously (24). The

PCR products cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) were analyzed for TCR sequences using CEQ DTCS-Quick Start Kit according to the manufacturer's protocol (Beckman Coulter Inc., Fullerton, CA, USA).

^{51}Cr release cytotoxicity assay

BALB/3T3 fibroblast line (H-2^d), J774 macrophage line (H-2^d), p815 mastocytoma line (H-2^d), EL-4 lymphoma line (H-2^b), L929 fibroblast line (H-2^k) obtained from Dainippon Sumitomo Pharma (Osaka, Japan) and synovial cells of SKG mice (H-2^d) were used as target cells. Synovial cells (1×10^4) were seeded in 96-well flat-bottom plates with 40 U/well of IFN-γ for 2 days and radiolabeled with 2.5 μCi/well of $Na_2^{51}CrO_4$ (Daiichi Radioisotope Laboratories, Ltd, Tokyo, Japan) for 2 h. Other target cells (3×10^5) were radiolabeled with 20 μCi of $Na_2^{51}CrO_4$ for 2 h and seeded in 96-well round-bottom plates at 1×10^4 cells per well. Effector cells (4×10^5) were added in each well in triplicate and incubated for 8 h. Relative cytotoxicity was calculated as follows from the radioactivity released in the culture supernatant; percent specific lysis = 100(experimental − spontaneous)/(maximal − spontaneous) counts per minute. Maximal lysis and spontaneous release were determined from target cells incubated with surfactant ×7 (Flow Laboratories, ICN Biomedicals, Inc., Aurora, OH, USA) or without effector cells, respectively.

Adoptive transfer

Spleen T cells from SKG mice or (SKG × BALB/c)F_1 mice and each SKG T cell clones (1×10^7) were intravenously transferred to C.B-17 SCID mice (8 weeks) or BALB/c-nu/nu mice (6 weeks), respectively. Control dengue 2F7 and 3F2 clone were collected 10–14 days after *in vitro* stimulation with specific peptide-pulsed irradiated (33 Gray) BALB/c spleen cells and transferred as described above. Severity of arthritis was scored weekly as previously described (14).

Clinical assessment of arthritis

Joint swelling was monitored by inspection and scored as follows: 0, no joint swelling; 0.1, swelling of one finger joint; 0.5, mild swelling of wrist or ankle and 1.0, severe swelling of wrist or ankle. Scores for all fingers and toes, wrists and ankles were totalled for each mouse (14).

Histological assessment of interstitial pneumonitis

Interstitial pneumonitis was evaluated microscopically depending on diffusely affected area: –, normal histology; +, 10–30%; ++, 30–60%; +++, >60% of the sections of the lungs showed pneumonitis.

Histology and immunohistochemistry

Tissues were fixed in 10% neutral formalin, paraffin embedded and stained with Haematoxylin & Eosin (H&E). Joints were additionally decalcified for 3 weeks in 10% EDTA in PBS before staining. For immunohistochemistry of joints, deparaffinized sections were incubated with 20% normal rabbit serum (Dako, Hamburg, Germany) in PBS for 15 min to block non-specific binding, primary rat anti-Ly-6G mAb (Gr-1, RB6-8C5; BD PharMingen) with appropriate dilutions overnight at 4°C, biotinylated polyclonal rabbit anti-rat antibody (Dako) and HRP-conjugated streptavidin (Dako). The slides were developed using diaminobenzidene (Elite Kit; Vector, Burlingame, CA, USA) and counterstained with Mayer's hematoxylin.

For immunohistochemistry of lungs, tissues were fixed in 4% phosphate-buffered PFA (pH 7.4) and embedded in Tissue-Tek OCT compound (Ted Pella, Inc., Redding, CA, USA). Cryostat sections were stained with rat mAbs to mouse CD4 (H129.19), CD8a (53-6.7), CD45R/B220 (RA3-6B2), Ly-6G (RB6-8C5) (BD PharMingen) and F4/80 (Cl: A3-1) (CALTAG Laboratories, Burlingame, CA, USA) with appropriate dilutions followed by incubation with biotinylated secondary antibodies and HRP-conjugated streptavidin. The slides were developed as described above.

Southern blot analysis

The persistence of transferred clones in the recipients was assessed by Southern blot analysis. Two micrograms of total RNA of each tissue was treated with DNaseI and reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA, USA). Nested PCRs were performed as described previously (24) to amplify TCR β chain of 35S or dengue 2F7 with the primers specific for V, J and C region. Ten microliters of the PCR products were separated on 2% agarose gel, transferred onto Hybond-N+ membranes (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. The membranes were prehybridized overnight with PerfectHyb (TOYOBO CO., Ltd, Osaka, Japan) at 54°C and hybridized with the third complementarity-determining region (CDR3)-specific probes labeled with ^{32}P-deoxyadenosine triphosphate for 3 h at 54°C. The membranes were washed in ×2 standard saline citrate (SSC) and 0.1% SDS at room temperature and ×0.2 SSC and 0.1% SDS at 37°C. RNA extracts of 35S and dengue 2F7 clones, diluted to 1% of concentration with RNA of L9 cells, were used as positive controls. The detection limits of 35S and dengue 2F7 were compared using the serial dilution of positive controls and both systems detected the RNA extract corresponding to the amount of one cell.

The sequences of PCR primers and probes are as follows; 35S: first PCR (BV8S3-1: 5′-ATA TGG TGC TGG CAA CCT TC-3′ and MCB1: 5′-AGG ATT GTG CCA GAA GGT AG-3′), second PCR (BV8S3-2: 5′-ACC AGA ACA ACG CAA GAA GAC T-3′ and MCB2: 5′-TTG TAG GCC TGA GGG TCC-3′), third PCR (BV8S3-3: 5′-TTC CTC CTG CTG GAA TTG GC-3′ and BJ1.5: 5′-TAG AAC AGA GAT CGA GTC CC-3′) and probe (5′-AGT GGG ACA GGG GGC AAC CA-3′). Dengue 2F7: first PCR (BV8S1-1: 5′-CCC AAA GTC CAA GAA GCA AG-3′ and MCB1), second PCR (BV8S1-2: 5′-GTA CAA GGC CTC CAG ACC AA-3′ and MCB2), third PCR (BV8S1-3: 5′-TGG CTT CCC TTT CTC AGA CA-3′ and BJ2.7: 5′-AAG GAG ACC TTG GGT GGA GT-3′) and probe (5′-TGC CAC CAA CGA CAA CTC CT-3′).

Results

Induction of arthritis and interstitial pneumonitis in SCID mice by the transfer of SKG splenic T cells

In our conventional housing environment, SKG mice started to develop arthritis around 2 months of age and

histologically evident mild interstitial pneumonitis around 6 months of age (14). To determine the role of T cells in SKG mouse autoimmunity, we transferred splenic T cells from 3-month-old arthritic SKG mice (without histologically evident pneumonitis or colitis) to T/B-cell-deficient C.B-17 SCID mice, which are histocompatible with SKG mice on the BALB/c background (14). Within 2 months after transfer, the recipient developed arthritis (14) and mild but histologically evident interstitial pneumonitis (Table 1, Fig. 1); they also developed mild colitis (data not shown). Similar cell transfer from non-arthritic heterozygotes of the SKG mutation failed to induce such lesions in the recipients. Age-matched SCID mice similarly maintained in our facility did not develop these lesions histologically (data not shown). The results thus indicate that SKG T cells are able to adoptively transfer arthritis and also have a potential to induce interstitial pneumonitis and colitis when transferred to SCID mice.

Establishment of T cell clones from arthritic joints

To analyze the mechanism of such T cell-mediated inflammatory tissue damage in multiple organs, we attempted to establish T cell clones from arthritic joints of SKG mice, as described in Materials and methods. Two T cell clones, designated 35S and 73S, were established in separate experiments. The clones were maintained and expanded with culture medium containing IL-2 and Con A (see Materials and methods). $CD8^+$ CTL clones specific for dengue virus NS3 protein were used as control.

Cytofluorometric analyses revealed that the 35S and 73S clones were $CD8^+$. Both expressed α and β chains of the TCR, and the expression level of the TCR on 35S was slightly lower than normal (Fig. 2). In response to *in vitro* PMA and ionomycin stimulation, 35S and 73S produced IFN-γ but no detectable amount of TNF-α, IL-4, IL-5, IL-6, IL-10 or IL-17 by ELISA (Table 2).

Clonality of each T cell line was confirmed by MHA (24) (data not shown) and sequence analysis of the TCR α and β chains with determination of the amino acid sequences of the TCRs (Table 3). Interestingly, these T cell clones shared in common the BV8S3 TCR Vβ subfamily; yet, the CDR3 sequences of the TCR β chains were different (26–29).

Table 1. Induction of arthritis, interstitial pneumonitis and colitis in SCID mice by the transfer of SKG splenic T cells

Spleen cell donor	Recipients	Arthritis	Interstitial pneumonitis	Colitis
SKG	1	++ (4.6)	++	+
	2	++ (4.0)	++	+
	3	++ (4.0)	+	+
	4	++ (3.0)	+	–
(SKG × BALB/c)F_1	1	–	–	–
	2	–	–	–
	3	–	–	–
	4	–	–	–

Cells (1×10^7) of T cells prepared from spleens of indicated mice were intravenously transferred to 8-week-old SCID mice. The severity of arthritis, interstital pneumonitis and colitis in these mice was histologically assessed 2 months later.

Autoreactivity of T cell clones

In ^{51}Cr release cytotoxicity assay to determine cytotoxic activity of the SKG clones against syngeneic synovial cells, 35S and 73S lysed SKG synovial cells prepared by crude collagenase digestion of inflamed synovium (44.0 and 16.3% of specific lysis, respectively, at a high 40:1 ratio), while control dengue 2F7 clone did not (Fig. 3A). 35S lysed not only syngeneic synovial cells but also MHC-matched cell lines, such as BALB/c-derived 3T3 cells, macrophage-like J774 cells and DBA/2 ($H\text{-}2^d$)-derived P815 cells, whereas the clone failed to lyse allogenic EL-4 ($H\text{-}2^b$) lymphoid or L929 ($H\text{-}2^k$) fibroblast cell line (Fig. 3B). Thus, 35S appears to recognize a ubiquitous self-peptide in an MHC-restricted manner. These

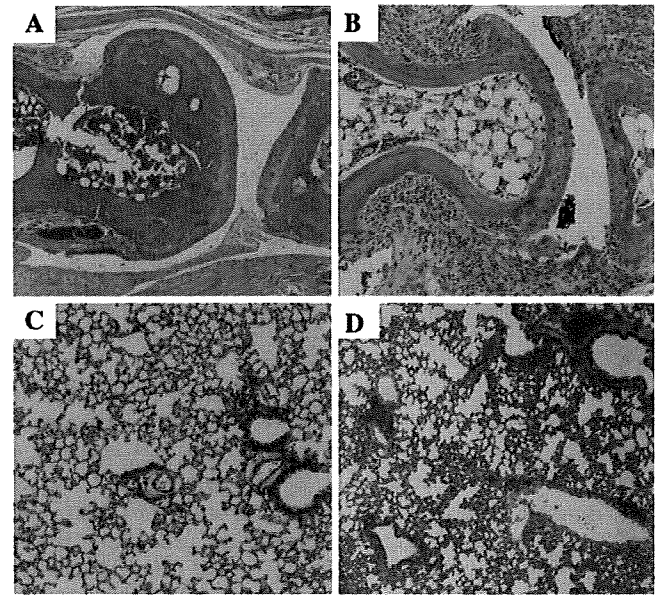

Fig. 1. Arthritis and pneumonitis in SCID mice transferred with T cells from SKG mice. Histology of a joint (A) and lung (C) of a SCID mouse T cell transferred from (SKG × BALB/c)F_1 mouse. Arthritis (B) and interstitial pneumonitis (D) in a SCID mouse T cell transferred from a SKG mouse. H&E staining (A and B, ×100; C and D, ×50).

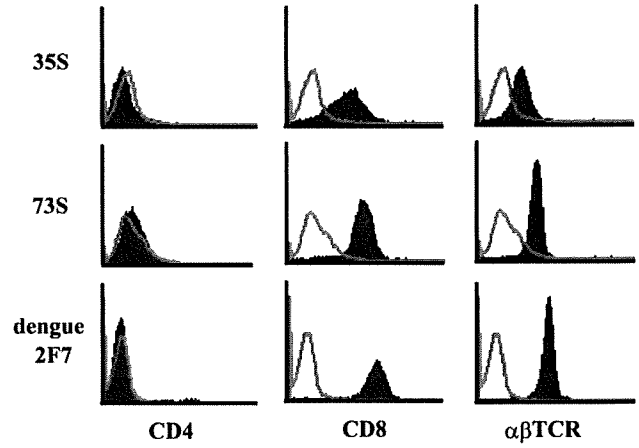

Fig. 2. Expression levels of CD4, CD8 and αβ TCR on 35S, 73S and dengue 2F7 clones.

functional characteristics, together with cell surface and cytokine-secreting profiles, indicate that 35S and 73S are CTL and that they bear self-reactive specificity.

Induction of synovitis in BALB/c nude mice by adoptive transfer of T cell clones

To examine possible arthritogenicity of the T cell clones, they were transferred to BALB/c nude mice once, and the degree of joint swelling of each recipient mouse was assessed once a week for 12 months (Fig. 4). Transfer of 35S and 73S clones induced joint swelling with incidences of 57.1% (4 out of 7 mice) and 42.9% (3 out of 7 mice), respectively, during the observation period; synovitis was histologically evident in 71.4% (5 out of 7 mice) in each transfer (Table 4, Fig. 5). Once joint swelling started in one joint following cell transfer, it slowly progressed with remissions and exacerbations, leading to swelling of other joints in a symmetrical fashion (Figs 4 and 5A–D). Two mice showed progressive debilitation to death without an apparent cause, although one of them showed dermatitis; with debilitation, joint swelling somehow remitted in these mice.

Table 2. Cytokine production (ng/ml) of T cell clones derived from SKG joints and control clones

	TNF-α	IFN-γ	IL-4	IL-5	IL-10	IL-6	IL-17
35S	0.02	180	0.03	<0.02	<0.04	0.2	<0.01
73S	0.02	80	0.03	<0.02	1.2	<0.05	<0.01
Dengue 2F7	0.2	10	ND	ND	<0.04	<0.05	<0.01
Dengue 3F2	0.02	20	ND	ND	<0.04	<0.05	<0.01

Culture supernatant of activated cells by PMA and ionomycin for 16 h were assayed by ELISA. ND, not done.

Table 3. CDR3 sequences of the TCR α and β chain used by the SKG T cell clones

TCR α chains	AV	V	N	J	AJ
35S	3S6	C A V T	S D	S G T Y Q R F	13
73S	3S1	C A A S M	R R	N S G T Y Q R F	13
Dengue 2F7	2S2/7	C A A		N Q G G R A L I F	15
Dengue 3F2	2S2/7	C A A	S G R D	Y A N K M I F	47
TCR β chains	**BV**	**V**	**N-D-N**	**J**	**BJ**
35S	8S3	C A S S G	T G G	N Q A P L F	1.5
73S	8S3	C A S S G	W G D	A E Q F F	2.1
Dengue 2F7	8S1	C A T	N D N	S Y E Q Y E	2.7
Dengue 3F2	8S2	C A S E	T R	E Q Y F	2.6

The amino acid sequences of the V, D and J regions of the TCR were determined according to the nucleotide sequences. AV and BV gene families were assigned according to Arden *et al.* (26). AJ genes were numbered according to Koop *et al.* (27). BJ genes were assigned according to Malissen *et al.* (28) and Gascoigne *et al.* (29).

Histologically, swollen joints showed marked synovial and peri-articular inflammation when examined 6–12 months after cell transfer (Fig. 5E and F). The inflammation accompanied a marked proliferation of synovial lining cells, infiltration of inflammatory cells into subsynovial tissue and joint cavity and active angiogenesis; pannus eroded the adjacent cartilage and bone (Fig. 5F). Gr-1-positive neutrophils were abundant among the infiltrating cells, as observed in the arthritic lesions of SKG mice (14, 15), whereas few T cells infiltrated into the inflammation sites (Fig. 5G and H).

In accordance with the appearance of multinuclear cells at the interface between proliferating synoviocytes and bone, many tartrate-resistant acid phosphatase-positive osteoclasts were observed in the inflamed joints (Fig. 6A–D). Safranin-O staining revealed a decrease in proteoglycan in the articular cartilage matrix of severely affected joints (Fig. 6E and F). Notably, Gr-1-positive cells, mainly neutrophils, also increased in the bone marrow (BM) of the affected recipients (Fig. 6G and H).

A high level of circulating rheumatoid factors was detected in one mouse out of seven recipients of the 35S clone and in none of the recipients of other clones (data not shown).

Some of the swollen joints following transfer of 35S $CD8^+$ clones exhibited higher expression levels of IL-17 mRNA assessed by quantitative reverse transcription (RT)–PCR than those from mice transferred with control $CD8^+$ clones (Supplementary Figure 1A, available at *International Immunology* Online), despite that 35S failed to produce IL-17 upon *in vitro* stimulation.

Taken together, the $CD8^+$ T cell clones prepared from arthritic lesions of SKG mice were able to induce arthritis in athymic nude recipients, leading to the destruction of the surrounding cartilage and the bone.

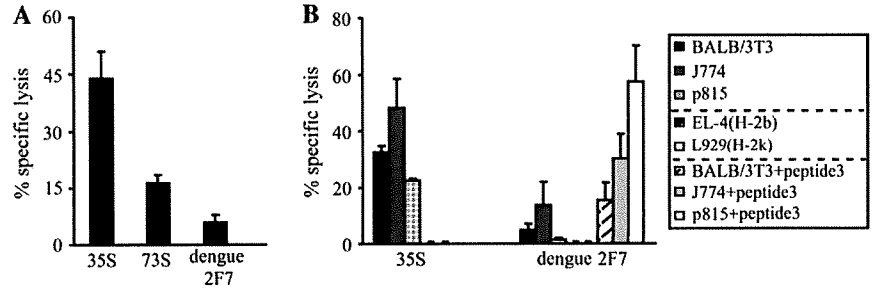

Fig. 3. *In vitro* self-reactivity of SKG T cell clones. (A) CTL activity of SKG T cell clones against SKG synovial cells. CTL clones specific for dengue virus NS3 protein, dengue 2F7, was used as control. IFN-γ-treated target cells were ^{51}Cr labeled in adherent condition and incubated with effector cells for 8 h (E:T ratio = 40). (B) CTL activity of SKG T cell clones against various types of cell lines (E:T ratio = 40). CTL activity of dengue 2F7 clone was also analyzed against $H\text{-}2^d$ cells pulsed with a specific peptide (E:T ratio = 10). All assays were conducted in triplicate with 8 h of incubation. The mean and standard deviation of three independent experiments are shown in each bar.

Induction of interstitial pneumonitis in BALB/c nude mice by the transfer of T cell clones

Notably, histologically evident severe alveolitis and diffuse interstitial pneumonitis also developed in all the recipients of 35S and 73S but not in those recipients of dengue 2F7 and 3F2 clones (Table 4 and Fig. 7A–D). Some recipients of 35S and 73S developed only pneumonitis without histologically evident synovitis. No histologically apparent inflammation was observed in other tissues/organs including the liver and the colon in any of these recipient mice (data not shown). The diffuse pulmonary lesions (Fig. 7A and B) comprised thickening of the alveolar walls, and perivascular and peribronchiolar infiltration by inflammatory cells (Fig. 7C and D). Immunohistochemical analysis of the 73S recipients 6 months after cell transfer revealed the infiltration of a large number of granulocytes as Gr-1$^+$ cells (Fig. 7E), macrophages as F4/80$^+$ cells (Fig. 7F) and B cells as B220$^+$ cells (Fig. 7G) into the alveolar walls and spaces and also the perivascular and peribronchiolar area where only a small number of CD8$^+$ T cells were detected, which might be transferred to CD8$^+$ clones or derived from nude mice (30) (Fig. 7H). CD4$^+$ T cells were occasionally found in the lesions and could be those derived from endogenous T cells that might develop extrathymically in aged nude mice (Fig. 7I) (30).

The pulmonary tissues with severe interstitial pneumonitis following CD8$^+$ clone transfer exhibited higher expression levels of IL-17 mRNA by quantitative RT–PCR compared with the mice transferred with control CD8$^+$ clones (Supplementary Figure 1B, available at *International Immunology* Online).

Thus, the SKG arthritogenic T cell clones are able to induce interstitial pneumonitis when transferred to athymic nude mice.

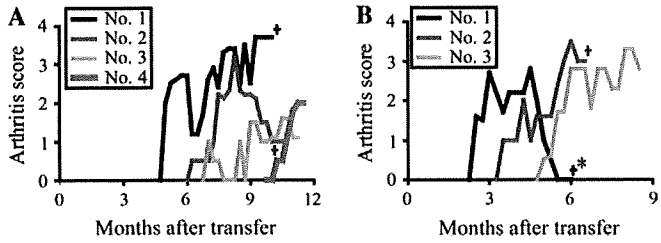

Fig. 4. Time course of joint swelling in the recipient mice of SKG T cell clones, 35S (A) and 73S (B). Score for all paws were totalized for each mouse. +, Sacrificed at the indicated time points; *, the mouse developed dermatitis at 5 months after transfer.

Detection of transferred clones in recipient mice

Since T cells were hardly detected by immunohistochemistry at the site of synovitis or pneumonitis 6 months after clone transfer (data not shown and see above), the persistence of transferred clones in the recipients was assessed by RT–PCR amplification of TCR β chain gene and Southern blot analysis of the products with a CDR3 sequence-specific probe. We adopted this method to avoid detecting nude mouse-derived oligoclonal endogenous T cells that may expand with aging (see above) (30–32). For example, a clone-specific TCR message of the 35S clone was detected in the majority of recipient spleens 1 month after transfer but not in the spleens examined 6 months later (Fig. 8). As shown in Fig. 9, the messages were

Table 4. Development of arthritis and interstitial pneumonitis in BALB/c nude mice transferred with T cell clones

Clone	Individual recipients	Macroscopically evident arthritis			Histological analysis	
		Onset (months)	Sacrifice (months)	Clinical score[a]	Synovitis[b]	Interstitial pneumonitis[c]
35S	1	5	10	3.7	++	+
	2	6	11	3.2	++	++
	3	7	11	1.6	++	+++
	4	10	12	2.0	++	+++
	5	–	9	0	+	±
	6	–	12	0	—	+++
	7	–	12	0	—	+++
73S	1	2.5	6	2.8	++	++
	2	3.5	6	3.5	++	+
	3	5	9	3.3	++	++
	4	–	8	0	+	++
	5	–	9	0	+	+++
	6	–	12	0	—	++
	7	–	12	0	—	++
Dengue 2F7	1	–	9	0	—	—
	2	–	9	0	—	—
	3	–	9	0	—	—
	4	–	9	0	—	—
	5	–	12	0	—	—
	6	–	12	0	—	—
	7	–	12	0	—	—
Dengue 3F2	1	–	12	0	—	—
	2	–	12	0	—	—

Six-week-old BALB/c nude mice were intravenously injected with 1×10^7 cells of each clone. The incidence of joint swelling of the recipient mice was examined weekly. Mice were sacrificed 6–12 months after cell transfer.

[a]Maximum clinical score of arthritis.

[b]–, Without change; +, microscopically observed synovitis without joint swelling; ++, macroscopically obvious joint swelling.

[c]–, Normal histology; +, 10–30%; ++, 30–60%; +++, >60% of the sections of the lungs showed pneumonitis (Fig. 7).

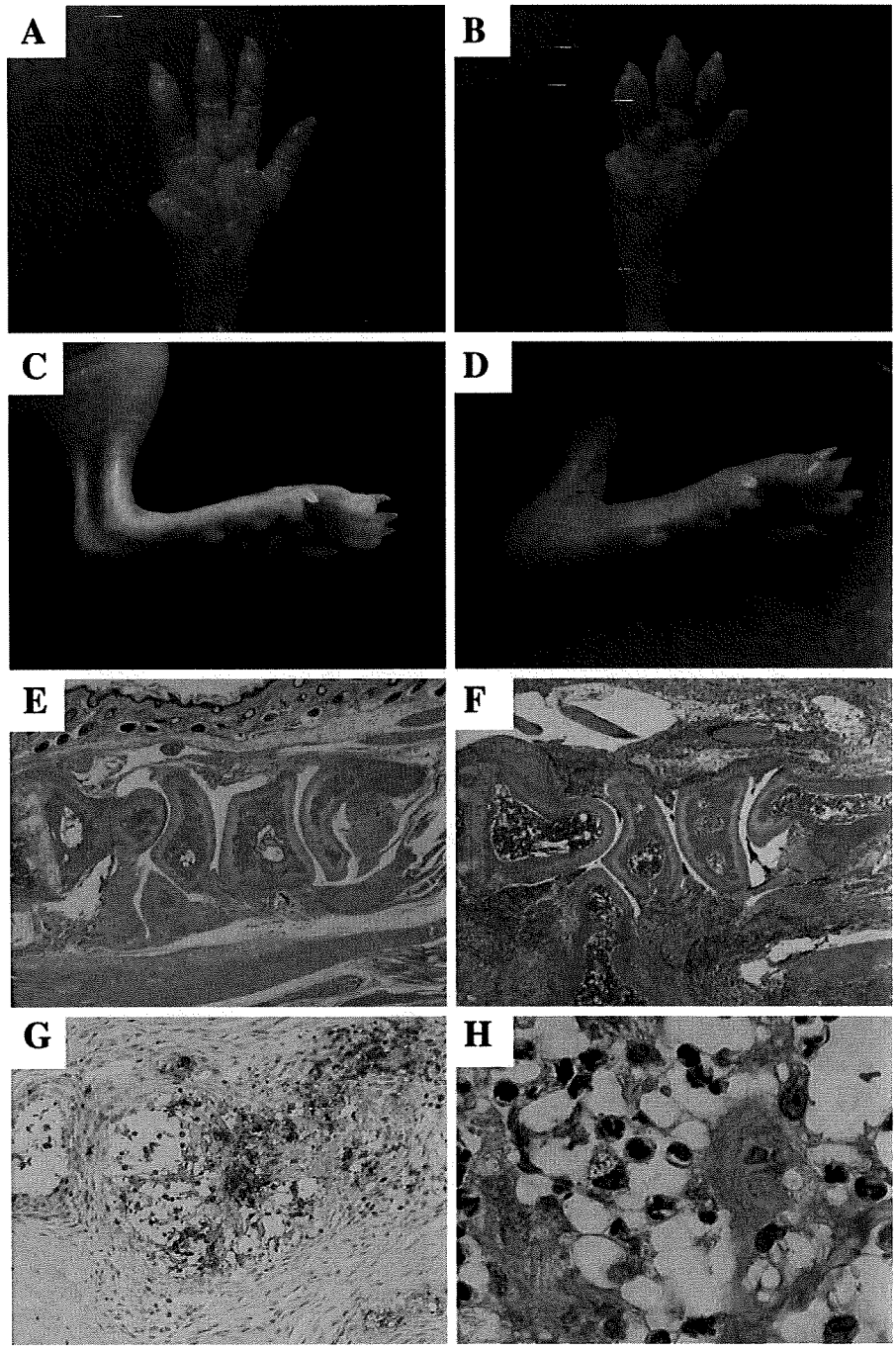

Fig. 5. Arthritis in athymic nude mice transferred with SKG T cell clones. (A–D) Macroscopic views of a forepaw (A) and a hind paw (C) of a recipient of control dengue 2F7 and a forepaw (B) and a hindpaw (D) of a recipient of 35S. (E–H) Histology of the joints of recipients of control dengue 2F7 (E) or 35S (F). Proliferation of the synovial lining cells, erosive destruction of cartilage and bone and infiltration of inflammatory cells is noted in a joint of a 35S recipient (F) (H&E staining, ×40). (G) Gr-1-positive cells were abundant among the infiltrating cells in a joint of 35S recipient mouse (×200). High-magnification view (×1000) of the synovial lesion in 35S transferred mouse, showing that most of the infiltrating cells are granulocytes or monocytes (H) (H&E staining). (A, C and E) 12 months after transfer. (B, D and F–H) 10 months after transfer.

detected in every tested tissue with high frequency for the first 3 months after cell transfer; the detection rate became lower with time; clone-specific TCR signals were not detected in most tissues examined at 6–11 months after transfer, irrespective of the swelling of the joints and the presence of interstitial pneumonitis by histological examination. These findings collectively indicate that the T cell clones initiate arthritis but the progression and persistence of the disease may not require the expansion of the clones even if a small number of them might persist in the joints and the lung.

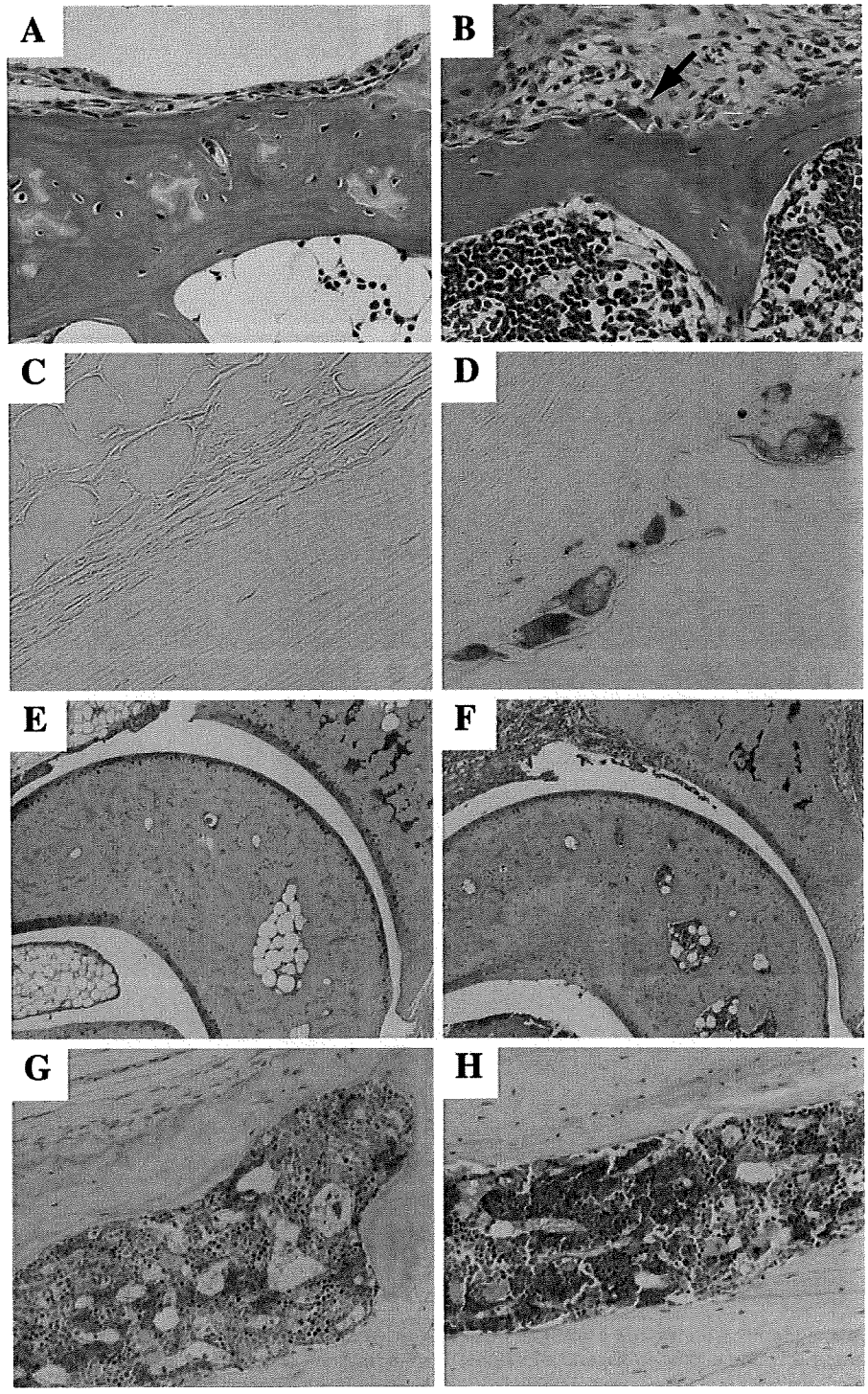

Fig. 6. Bone and cartilage destruction in athymic nude mice transferred with SKG T cell clones. High magnification of H&E-stained sections of a nude mouse recipient of dengue 2F7 (A) or 35S (B), showing bone erosion by pannus and BM activation (×400). Multinuclear cells (osteoclasts) (arrow) are also observed. Tartrate-resistant acid phosphatase-positive cells (osteoclasts) are detected in a 35S recipient (D) but not in a 2F7 recipient (C) (×400). By Safranin-O staining, proteoglycan stained red decreases in the articular cartilage matrix of a recipient of 35S (F) but not in a recipient of 2F7 (E) (×100). By immunohistochemistry, Gr-1-positive cells increase in the BM of a 35S recipient (H) but not in a 2F7 recipient (G) (×200). (A, C, E and G) 12 months after transfer; (B, D, F and H) 10 months after transfer.

Discussion

In this study, we have established two distinct $CD8^+$ T cell clones from arthritic lesions of SKG mice. Interestingly, both exhibited *in vitro* autoreactivity against not only synoviocytes but also a variety of MHC-matched cell lines and elicited both arthritis and interstitial pneumonitis when transferred to

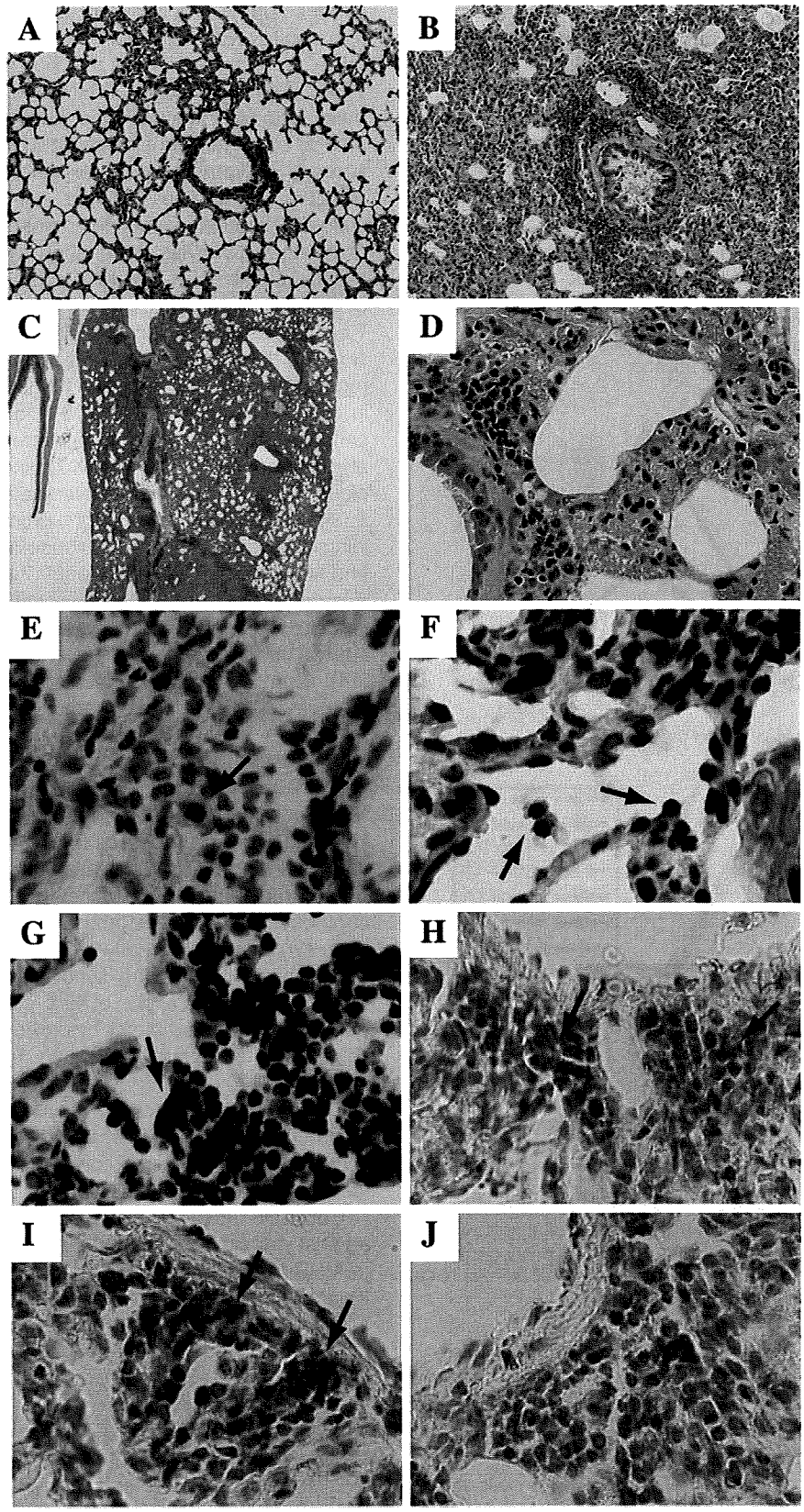

Fig. 7. Interstitial pneumonitis induced by the transfer of SKG T cell clones. (A–D) H&E-stained sections of the lungs of the recipients of control dengue 2F7 clone (A) or 73S clone (B–D) (A–B, ×100). Lower (C, ×10) and higher (D, ×400) magnification of the lung of 73S clone recipient show thickening of alveolar walls diffusely in the lung. (E–J) Serial sections of a lung of a 73S recipient mouse were stained for Ly-6G (Gr-1) (E), F4/80 (F), B220/CD45R (G), CD8a (H) or CD4 (I), with staining control (J) (×400). Typically positive cells in these stainings are arrowed. (A–J) 6 months after transfer.

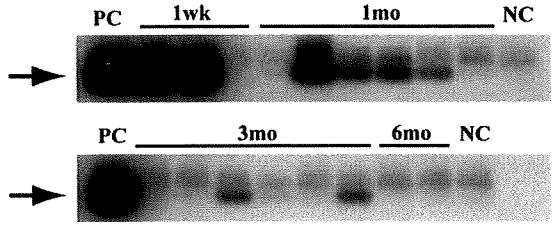

Fig. 8. Detection of a clone-specific TCR message of 35S clone in spleens by RT–PCR amplification and Southern blot analysis. After transfer of 1 × 10^7 clone cells to BALB/c nude mice, RNA was extracted from spleens at indicated days. PC, positive control (RNA from 35S clone diluted to 1%); NC, negative control (RNA from a 6-month-old non-treated BALB/c athymic nude mouse). The separate lanes represent individual mice.

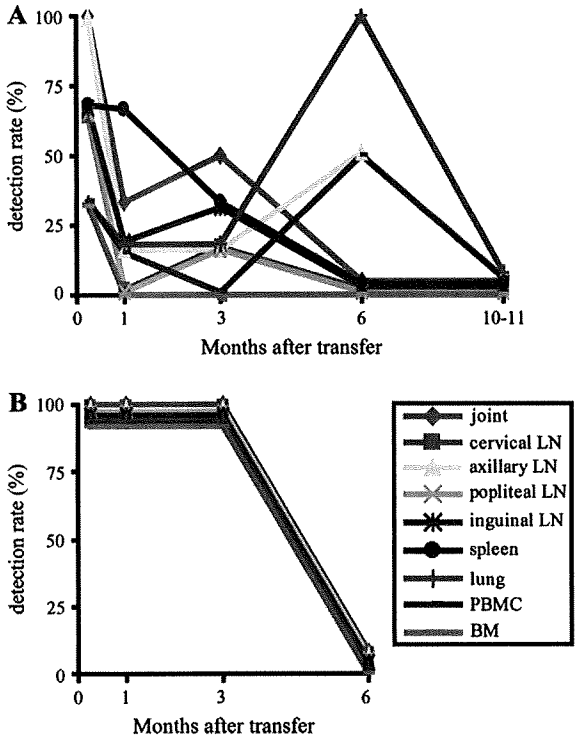

Fig. 9. Detection of TCR mRNA of the transferred clones in recipient BALB/c nude mice. 35S (A) or dengue 2F7 (B) in the recipients were detected by Southern blot analysis using primers and probes specific for TCR V and J region and CDR3 sequences of each clone. All mice with at least one positive signal out of four joints were considered to be positive. (A) $n = 3$ at 1 week; $n = 6$ at 1 month; $n = 6$ at 3 months; $n = 2$ at 6 months; $n = 2$ at 10–11 months. (B) $n = 2$ in every group. No signal was detected in control 6- or 11-month old BALB/c nude mice in each Southern blot analysis (data not shown).

histocompatible T cell-deficient mice. Furthermore, the arthritic and pulmonary lesions chronically progressed irrespective of the decline in the number of transferred T cell clones to hardly detectable levels in either lesion.

Our previous study showed that bulk $CD4^+$ T cells alone from arthritic SKG mice were able to transfer the disease to athymic nude mice, whereas bulk $CD8^+$ T cells alone were not and that abundant $CD4^+$ T cells and only a small number of $CD8^+$ T cells were found by immunohistochemistry in the arthritic subsynovial tissue of arthritic SKG mice (14). These apparently opposing results with $CD8^+$ T cell clones versus bulk $CD8^+$ T cells indicate that potentially arthritogenic $CD8^+$ T cells are present in SKG mice and may usually need $CD4^+$ T cell help for induction of arthritis; yet, they are potentially able to mediate arthritis without $CD4^+$ T cell help if they are strongly activated, clonally expanded to a large number or possibly selected for stronger self-reactivity during *in vitro* culture. It remains to be determined how $CD8^+$ clones elicit proliferative synovitis rather than cytotoxic killing of certain cellular elements in the joint. One possibility is that these $CD8^+$ clones, which exert *in vitro* killing activity at a high T cell/target cell ratio, might also be able to stimulate synoviocytes through secreting cytokines. It is of interest in this regard that the joints and the lungs with severe pneumonitis in some recipients of the $CD8^+$ clones showed active transcription of IL-17 mRNA (Supplementary Figure 1, available at *International Immunology* Online). Although the $CD8^+$ clones did not produce detectable amounts of IL-17 by *in vitro* stimulation, they might produce the cytokine in the joints or interact with nude mouse-derived α/β or γ/δ T cells and stimulate them to secrete IL-17 (33, 34). It is of note that a large number of $Gr\text{-}1^+$ mature neutrophils exuded into the joint fluid and infiltrated into the subsynovial tissue of the recipient nude mice, as in the arthritic lesions of SKG mice (14). BM of the clone recipients also showed an increase in the number of $Gr\text{-}1^+$ mature neutrophils. It remains to be determined how $CD8^+$ T cells mediate arthritis and pneumonitis in SKG mice by recruiting other cellular elements including neutrophils, how they increase neutrophils in the BM and whether IL-17, which is capable of recruiting neutrophils, is involved in these processes (35, 36).

It also needs further investigation whether IFN-γ secreted by the transferred $CD8^+$ clones or their killing activity could contribute to the development of synovitis. IFN-γ may activate synoviocytes directly or indirectly through activating macrophages, facilitating synoviocyte proliferation. It might up-regulate the expression of MHC class I in synovial cells, rendering them susceptible to cytotoxic activity of $CD8^+$ T cells. With these apparently opposing activities of arthritogenic $CD8^+$ T cells (i.e. killing versus proliferation of synoviocytes), they mediate proliferative synovitis rather than synoviocyte destruction presumably because synoviocytes might be more sensitive *in vivo* to the stimulatory effect than the cytotoxicity (see Discussion below).

The $CD8^+$ clones exhibited *in vitro* cytotoxic activity against not only syngeneic synovial cells but also a variety of MHC-matched lymphoid and non-lymphoid cell lines. Although their precise antigen specificities need to be determined, this finding suggests that these clones may recognize a ubiquitous self-antigen (for example, ubiquitous cellular protein such as hsp complexed with MHC or the MHC molecule itself) expressed in the joint and lung and other tissues, rather than a common self-antigen exclusively expressed in the joint and lung. If this is the case, how are the joint and the lung selectively affected by these T cell clones? For the following reasons, one could attribute this to unique characteristics of the synoviocytes, and possibly the alveolar macrophage, as the target of this autoimmunity. Compared with other tissue cells, the synoviocytes are

highly sensitive to pro-inflammatory cytokines, for example systemic overproduction of transgenic TNF-α or IL-1 almost exclusively produces chronic arthritis even in mice deficient of both T and B cells (37–39); similarly, systemic deficiency of the IL-1R antagonist, and resulting overproduction of IL-1, or systemic alteration of signal transduction via IL-6 receptor results in predominant development of arthritis with no inflammatory damage to other tissues (40, 41). These findings collectively indicate that synoviocytes are much more sensitive to the SKG self-reactive T cell clones (at least to those secreting pro-inflammatory cytokines) than other tissue cells, even if the common self-antigens recognized by the clones are ubiquitously expressed. In addition, synoviocytes are unique in that they are the target cells and also the mediators of autoimmunity, i.e. upon stimulation (e.g. by cytokines or via cell contact stimulation by self-reactive T cells), they proliferate and secrete pro-inflammatory cytokines (e.g. IL-1, IL-6 and TNF-α) and other inflammatory substances (matrix metalloproteinases and prostaglandins), mediating inflammation and tissue damage (42). It is likely that the cells composing the alveolar walls, in particular the alveolar macrophages, are sensitive and responsive to T cell self-reactivity in a similar manner as synoviocytes and that excessively and chronically activated macrophages might mediate alveolitis and interstitial inflammation. A similar mechanism might also be responsible for the development of colitis in SKG mice (Table 1).

We do not assert, however, that SKG arthritis and pneumonitis are solely mediated by T cells recognizing a ubiquitous common self-antigen. We have previously shown that SKG mice spontaneously produce IgG isotype auto-antibody specific for joint-rich type II collagen or IgG antibody cross-reactive with hsp-70 of Tuberculosis bacilli (14). This indicates that helper $CD4^+$ T cells that specifically react with these self-antigens may also be induced in SKG mice either primarily or secondarily to joint damage. Moreover, we have recently shown that some self-reactive T cells in SKG mice may not be arthritogenic but can polyclonally stimulate antigen-presenting cells in the spleen and lymph nodes to secrete IL-6 and other cytokines, which in turn facilitate differentiation of potentially arthritogenic self-reactive T cells to T_h17 effector T cells that mediate synovitis (19). In addition to our current approach to the characterization of antigen specificity of SKG autoimmune T cells by preparing T cell clones, efforts are being made to further characterize infiltrating T cells *in situ* at a single-cell level by amplifying their TCR message.

Tracing the fate of transferred T cell clones revealed that clone-specific TCR gene messages gradually diminished not only in the inflamed joints and the lungs but also in the regional lymph nodes and spleens of the recipients, becoming hardly detectable in 6–11 months; yet, inflammation in the joints and the lung continued to progress and severe arthritis and pneumonitis were apparent even 12 months after clone transfer. Thus, initial triggering of synovitis requires arthritogenic T cells; yet, synovitis apparently becomes less T cell dependent in a later phase, albeit it chronically progresses with the formation of pannus destroying adjacent cartilage and bone, as in human RA (2). This may correlate with the findings in humans that T cell-targeted mAb therapy is not much efficacious in the treatment of RA at a chronic stage (43). Further characterization of each stage of disease development in SKG mice will contribute to our understanding of the cellular and molecular basis of the T cell-dependent and -independent phases of disease progression in the joints and also in the lung in RA.

In conclusion, we have shown that $CD8^+$ T cell clones established from arthritogenic lesions of SKG mice are capable of mediating not only arthritis but also interstitial pneumonitis immunopathologically resembling ILD in RA. This provides a possible common pathogenetic basis between arthritis and ILD in RA. The etiology of RA is largely obscure at present (1, 2). Yet, there are recent findings that genetic polymorphism of the PTPN22-encoded lymphoid tyrosine phosphatase, which alters signal transduction at a TCR proximal step involving ZAP-70, contributes significantly (second only to MHC polymorphism) to the susceptibility to RA and other autoimmune diseases (22, 23, 44, 45). The polymorphism might be responsible for thymic generation of arthritogenic and other self-reactive T cells. Further elucidation of the mechanism by which such autoreactive T cells are generated and activated in SKG mice, and characterization of putative ubiquitous self-antigen recognized by self-reactive T cells capable of mediating arthritis and pneumonitis, would facilitate our understanding of the etiology and the pathogenetic mechanism of RA as a systemic autoimmune disease. This should help devising preventive or curative measures for the disease.

Supplementary data

Supplementary figure is available at *International Immunology* Online.

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Disclosures

The authors declare no conflicting interests.

Abbreviations

BM	bone marrow
CDR3	the third complementarity-determining region
H&E	haematoxylin & eosin
hsp	heat shock protein
ILD	interstitial lung disease
MHA	microplate hybridization assay
PMA	phorbol myristate acetate
RA	rheumatoid arthritis
RT	reverse transcription
SSC	standard saline citrate
TNF	tumor necrosis factor
ZAP-70	ζ-associated protein of 70 kDa

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Full Paper

Differential Coupling of Human Endothelin Type A Receptor to $G_{q/11}$ and G_{12} Proteins: the Functional Significance of Receptor Expression Level in Generating Multiple Receptor Signaling

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Abstract. This study examines the influence of receptor expression level on signaling pathways activated via endothelin type A receptor (ET_AR) expressed in Chinese hamster ovary cells at 32,100 (ET_AR-high-CHO) and 893 (ET_AR-low-CHO) fmol·mg protein^{-1}. Endothelin-1 (ET-1) elicited a sustained increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which was dependent on $G_{q/11}$ protein, phospholipase C (PLC), Na^+/H^+ exchanger (NHE), and p38 mitogen–activated protein kinase (p38MAPK) in ET_AR-high-CHO, whereas the sustained $[Ca^{2+}]_i$ increase was negligible in ET_AR-low-CHO. Functional study with Cytosensor™ microphysiometer showed that ET-1 evoked an NHE1-mediated increase in extracellular acidification rate (ECAR) in ET_AR-high-CHO and ET_AR-low-CHO. In ET_AR-high-CHO, the ECAR response at 30 min after ET-1 stimulation was insensitive to $G_{q/11}$ and PLC inhibitors, but sensitive to the p38MAPK inhibitor. In ET_AR-low-CHO, the ECAR response at 30 min was sensitive to these inhibitors. Western blot analysis demonstrated that ET-1–induced p38MAPK phosphorylation in ET_AR-low-CHO but not in ET_AR-high-CHO was mediated via $G_{q/11}$ and PLC. The $G_{q/11}$/PLC-independent p38MAPK phosphorylation in ET_AR-high-CHO was suppressed by expression of the C terminus of $G_{\alpha 12}$ protein to disrupt receptor-G_{12} protein coupling. These results provide evidence for multiple signaling pathways of ET_AR that were activated via at least the $G_{q/11}$/PLC/NHE, G_{12}/p38MAPK/NHE, and $G_{q/11}$/PLC/p38MAPK/NHE cascades in an expression level–dependent manner.

Keywords: endothelin type A receptor, receptor expression level, intracellular free Ca^{2+} concentration, Na^+/H^+ exchanger, p38 mitogen–activated protein kinase

Introduction

Endothelin type A receptor (ET_AR) belongs to the superfamily of G protein-coupled receptors (GPCRs) that transduce the binding of their agonists into activation of G protein–regulated effectors and elevation of the corresponding second messengers. It is generally accepted that human ET_AR can couple with several subfamilies of a heterotrimeric G protein family including G_q, G_s, and G_{12}. Typically, G_q protein activated by stimulation of ET_AR with its agonist, endothelin-1 (ET-1), induces formation of second messengers such as inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) via phospholipase Cβ (PLCβ); G_s induces formation of cyclic AMP (cAMP) via adenylyl cyclase (AC); G_{12} induces formation of actin stress fiber via a Rho/ROCK system (1, 2).

Ca^{2+} signal plays a key role in controlling diverse cellular functions such as contraction, proliferation, and transcription. Activation of endogenous ET_AR in vascular smooth muscle cells and recombinant ET_AR expressed in Chinese hamster ovary (CHO) cells evokes

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