Table 1. Pt data.

Patient	Age	Sex	Allergy	Past medical history
1	75	M	None	None
2	69	F	None	HT, arrhythmia, hyperlipidaemia
3	37	F	Food (soymilk)	Hyperlipidaemia, osteoporosis
4	64	M	None	None
5	65	M	None	HT
6	59	M	None	Tinea
7	78	F	None	Emphysema
8	38	M	None	None
9	65	M	None	None
10	70	F	None	HT, hyperlipidaemia
11	30	F	None	None
12	60	F	Pollinosis	HT, DM, hyperlipidaemia

HT, hypertension; DM, diabetes mellitus.

to clonal expansion of T cells. Furthermore, superantigens (SAs) produced by bacteria or viruses are known to bind to the outside of the MHC class II α-chain (TCRAV) and V-region of the TCR β-chain (TCRBV) to form a cross-link, such that a given SA can stimulate all T cells that bear the appropriate TCRBV in polyclonal settings [8]. Because CDR3 has different sequences and lengths, it is possible to analyse the diversity of TCRs by using a CDR3 size spectratyping method that provides a rapid scan of all TCR V-region transcripts grouped according to the utilized V-region gene and the chain length [12,13].

The aim of the present study was to elucidate the characteristics of the TCR repertoires in lesions and PBMCs of OLP patients without HCV infection using the AL–PCR and MHA methods and CDR3 spectratyping. We characterized further the T cell phenotypes and cytokine expression levels.

Materials and methods

Patients and samples

The OLP tissue specimens and peripheral blood were obtained from 12 patients with reticular type OLP at Tsurumi University Hospital and Yokohama Rosai Hospital (Table 1). The patient group comprised seven women and six men with an age range of 30–78 years (mean age: 59·2 years). None of the subjects had amalgam restorations or HCV infection. Informed consent was obtained from all patients. The study was approved by the local Research Ethical Committees at Tsurumi University, Yokohama Rosai Hospital and National Sagamihara Hospital. Lesional tissue specimens were taken from the buccal mucosa after biopsy for diagnostic purposes. Tissue specimens of normal oral mucosa (NOM) were obtained from six healthy volunteers. Control healthy peripheral blood samples were obtained from nine healthy volunteers, and age-matched to the patient group. The tissue specimens were obtained immediately after biopsy and soaked in RNAlater™ (Qiagen, Hilden, Germany). PBMCs were isolated from peripheral blood by H-SMF (Jimro Co. Ltd., Gunma, Japan) gradient centrifugation. The specimens were cryopreserved until RNA extraction.

The TCR repertoire analysis

Crude cellular RNAs from PBMCs and tissue specimens were extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The AL–PCR and MHA methods were carried out as described previously [9]. Briefly, 1 μg of total RNA was reverse-transcribed to double-stranded cDNAs using a SuperScript II cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, except for priming with a BSL-18B primer adaptor containing a Not I site. The P20EA/P10EA universal adaptors were ligated at the 5′ ends of the BSL-18B-primed cDNAs. Three rounds of Cα- and Cβ-specific PCR were performed using Cα and Cβ sequence-specific oligonucleotide probes (SSOPs), respectively, to prepare amplified and biotinylated TCR cDNA pools. Hybridization was carried out between biotinylated PCR products and AV or BV SSOPs immobilized on carboxylate-modified enzyme-linked immunosorbent assay plates (Sumitomo Bakelite, Tokyo, Japan). Positive hybridization was detected using p-nitrophenylphosphate (Nacalai Tesque, Osaka, Japan), and the visualized signals were estimated at 405 nm using a Multiskan JX Microplate Reader (Thermo Labsystems, Helsinki, Finland). Relative expansion of the TCRAV or TCRBV repertoire was calculated by the following formula: frequency (%) = X100 (corresponding SSOP signal)/(ΣTCRV SSOP signals).

CDR3 size analysis of TCRs

The above-described second PCR products were labelled by 20 cycles of PCR amplification with fluorescent dye-labelled C region-SSOPs [14]. After mixing with size markers (CEQ™ DNA Size Standard Kit-600; Beckman Coulter, Inc., Fullerton, CA, USA), the labelled PCR products were loaded onto a polyacrylamide sequencing gel (CEQTM Separation Gel-LPA I; Beckman Coulter, Inc.) to determine their sizes and fluorescence intensities using an automated capillary DNA sequencer (CEQ™ 8000; Beckman Coulter, Inc.). Data were analysed using the Genetic Analysis System Software (Beckman Coulter, Inc.).

Histology and immunohistochemistry

For histopathology, 6-μm sections of paraffin-embedded tissue specimens were stained with haematoxylin and eosin (H&E). Immunohistochemical studies were performed for the expression of CD3 (Dako, Kyoto, Japan), CD4 (Nichirei, Tokyo, Japan), CD8 (Nichirei), CD14 (Dako),

CD20 (Dako) and human leucocyte antigen D-related (HLA-DR) (Dako) using commercially available monoclonal antibodies following the ENVISION technique with 3,3-diaminobenzidinetetrahydrochloride as the indicator agent.

Real-time PCR

The mRNA expression levels of CD markers and cytokines were examined in PBMCs and tissue specimens by real-time quantitative PCR using a LightCycler® (Roche Diagnostics, Tokyo, Japan). Interleukin (IL)-2, IL-4, IL-5 and tumour necrosis factor (TNF)-α were examined in PBMCs and tissue specimens by real-time quantitative PCR using the LightCycler®. Total RNA was reverse-transcribed into cDNAs using a PrimeScript® RT Reagent Kit (Takara Bio, Shiga, Japan). One-tenth of each cDNA sample was amplified with a SYBR® Premix Ex Taq™ RT–PCR Kit (Takara Bio) according to the manufacturer's instructions. Primer pairs were obtained from Takara Bio. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as an internal control. The results were expressed as the ratio of the amount of the respective cytokine-specific cDNA to the amount of GAPDH-specific cDNA.

Statistical analysis

Statistical analyses were carried out using StatView version 5·0 for Windows (SAS Institute, Cary, NC, USA). The non-parametric Mann–Whitney rank test was applied for all comparisons. Accordingly, the results of the comparisons are given as the mean or median. Values of $P < 0·05$ were considered to indicate statistical significance.

Results

The TCR repertoire analysis

First, we examined the TCR repertoires in NOM tissue specimens and found that they varied markedly among individuals, with no significant differences from healthy PBMCs (Figs 1 and 2c). On the other hand, the TCR repertoires in OLP tissue specimens were quite similar, with significant increases in TCRAV8-1, AV22-1, BV2-1, BV3-1 and BV5-1 compared with healthy PBMCs ($P < 0·05$) (Fig. 2a, b). Similarly, there were significant increases in TCRAV8-1, BV2-1, BV3-1 and BV5-1 in OLP PBMCs compared with healthy PBMCs ($P < 0·05$) (Fig. 3). However, there were no significant differences in the TCR repertoires between OLP and NOM tissue specimens (Fig. 2c). The TCR repertoires in NOM tissue specimens varied markedly among individuals. The averages of the individual subfamilies seemed to increase, because the standard deviation values were very high. Therefore, the statistical differences were not significant even when we compared the frequencies in PBMCs and NOM tissue specimens.

T cell clonality in increased TCR repertoires

Using CDR3 size spectratyping, we further studied whether T cell clonality was present in the increased TCR repertoires, including TCRAV8-1, AV22-1, BV2-1, BV3-1 and BV5-1. The results revealed that all these TCR repertoires were composed of polyclonal expansion in OLP tissue specimens, whereas those in NOM tissue specimens showed monoclonal or oligoclonal patterns (Fig. 4). T cell clonality was confirmed by CDR3 size spectratyping. Among the TCR repertoires of OLP PBMCs, T cells bearing TCRAV8-1, BV2-1, BV3-1 and BV5-1 were subjected to CDR3 size spectratyping. The expansion of TCRV-bearing T cells showed polyclonal patterns in OLP PBMCs and healthy PBMCs (data not shown).

Histopathological features of NOM and OLP tissue specimens

The H&E staining of OLP tissue specimens revealed band-like infiltrates of lymphocytes in the superficial lamina propria and basement membrane destruction (Fig. 5). $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes were detected among the infiltrating cells toward the basal membrane (Fig. 6a–c). NOM tissue specimens contained low numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes (Fig. 6a–c). Expression of CD20 was detected in both OLP and NOM tissue specimens (Fig. 6d). HLA-DR expression was increased significantly in OLP tissue specimens compared with NOM tissue specimens (Fig. 6e). The $HLA\text{-}DR^+$ cells were considered to be antigen-presenting cells, as they were large compared with the lymphocytes.

Quantification of CD markers and cytokine expression levels

The mRNA expression levels of CD4, CD8, IL-2, IL-4, IL-5 and TNF-α in PBMCs and tissue specimens were measured by real-time quantitative PCR. GAPDH gene expression was used as an internal control. The mRNA expression levels represented by the ratio of the amount of each cytokine-specific cDNA to the amount of GAPDH-specific cDNA were compared between tissue samples or PBMCs. CD4 and CD8 were expressed in all samples (Fig. 7). In NOM and OLP tissue specimens, CD8 was expressed more highly than CD4 (Fig. 7a) ($P < 0·005$). The CD4/CD8 ratio was higher in OLP tissue specimens than in NOM tissue specimens ($P < 0·001$). There was no difference between the CD4/CD8 ratios in healthy PBMCs and OLP PBMCs (Fig. 7b). The mRNA expression levels of IL-2, IL-4, IL-5 and TNF-α were measured in healthy and OLP PBMCs and NOM and OLP tissue specimens to determine the cytokine environment in OLP patients (Fig. 8). The IL-5 and TNF-α expression levels were higher in NOM tissue specimens than in OLP tissue specimens ($P < 0·001$). The IL-5

(a)

NOM-01
NOM-02
NOM-03
NOM-04
NOM-05
NOM-06
Healthy PBMCs
Freuency (%)
TCRAV probes
TCRBV probes

(b)

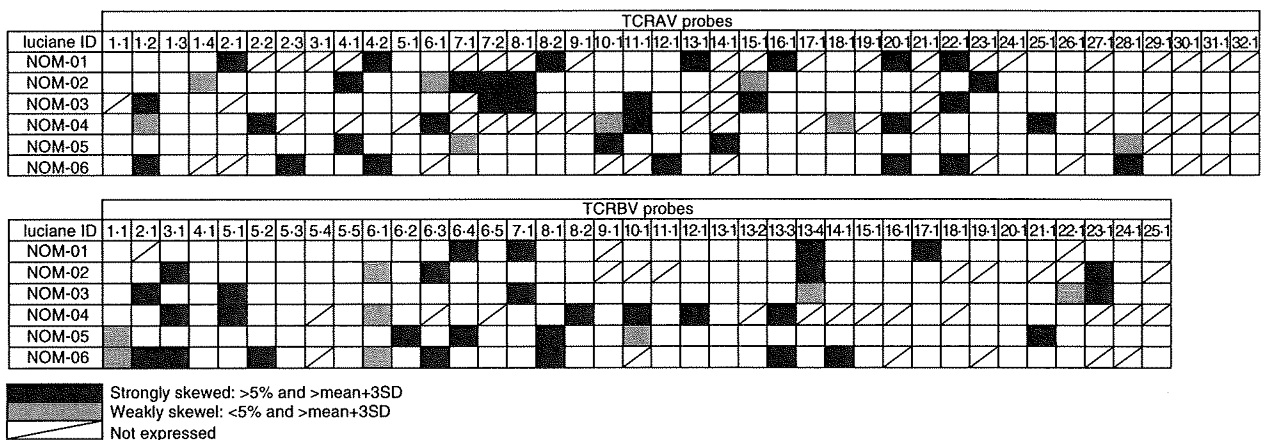

Fig. 1. Mean percentage frequencies of TCRVA and TCRVB in normal oral mucosa (NOM) tissue specimens from six healthy volunteers. The expressions of the different T cell receptor (TCR) repertoires in individual NOM tissue specimens are shown as mean ± standard deviation (a). TCRVA and TCRVB gene expression profiles in the NOM tissue specimens of healthy volunteers (b).

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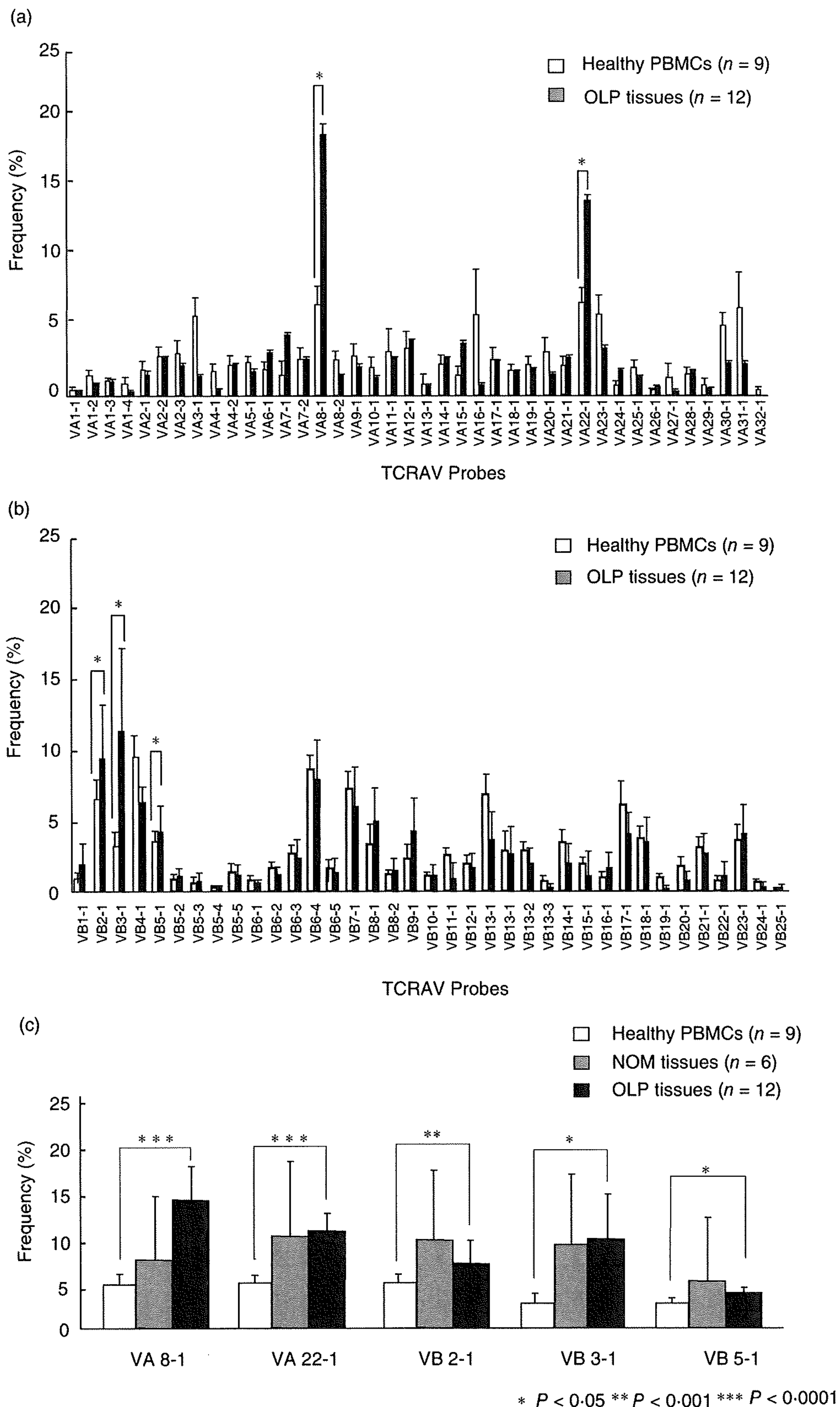

Fig. 2. The T cell receptor (TCR) repertoires in oral lichen planus (OLP) tissue specimens are skewed in comparison with those in healthy peripheral blood mononuclear cells (PBMCs). There are significant increases in TCRVA8-1 and VA22-1 (a) as well as VB2-1, VB3-1 and VB5-1 (b) in OLP tissue specimens compared with healthy PBMCs ($*P < 0{\cdot}05$). There are no significant increases in VA8-1, VA22-1, VB2-1, VB3-1 and VB5-1 in normal oral mucosa (NOM) tissue specimens compared with healthy PBMCs (c). All data are shown as mean ± standard deviation.

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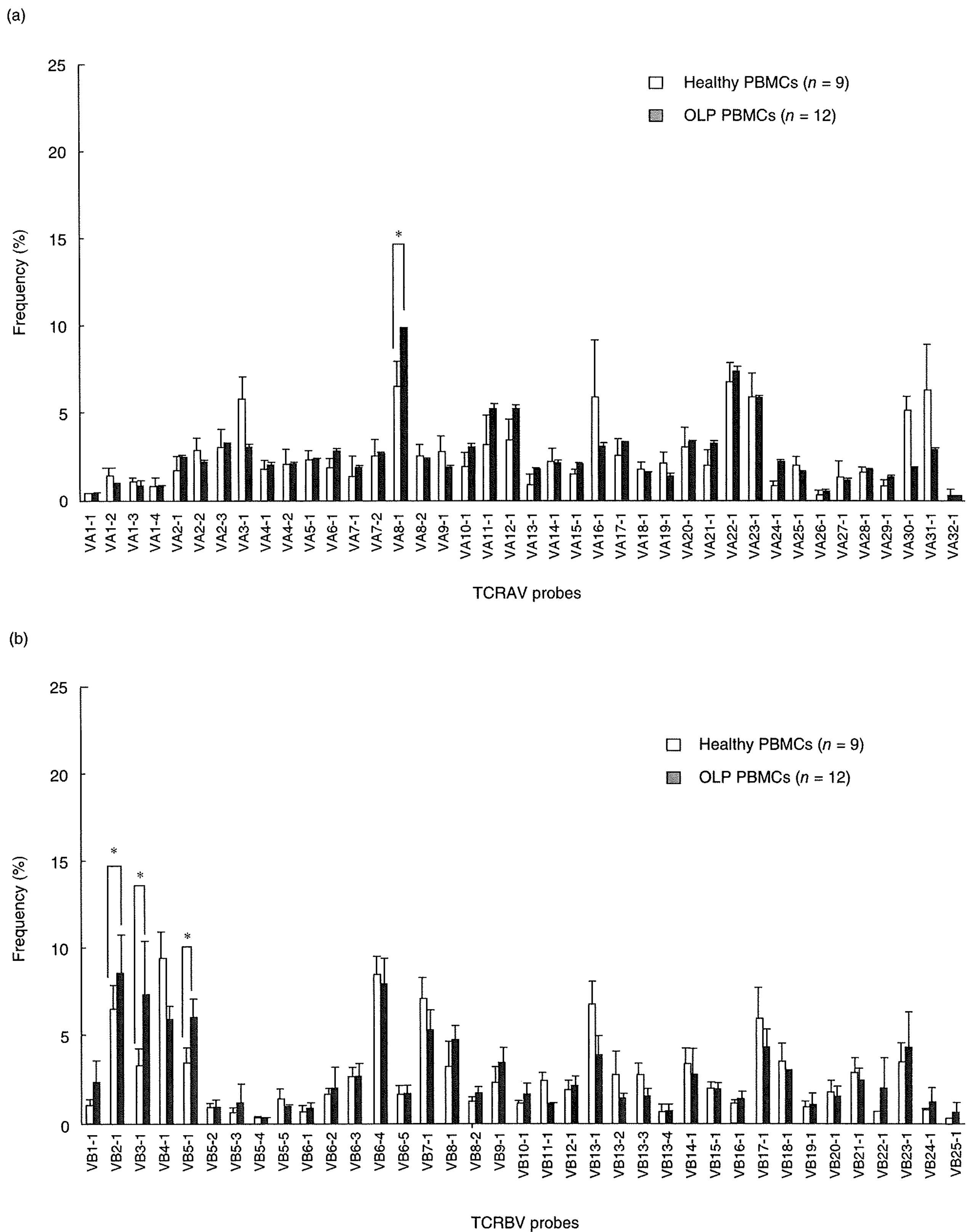

Fig. 3. The T cell receptor (TCR) repertoires in oral lichen planus (OLP) peripheral blood mononuclear cells (PBMCs) are skewed in comparison with those in healthy PBMCs. There are significant increases in T cells bearing TCRVA8 (a) as well as VB2, VB3 and VB5 (b) in OLP PBMCs compared with healthy PBMCs (* $P < 0{\cdot}05$).

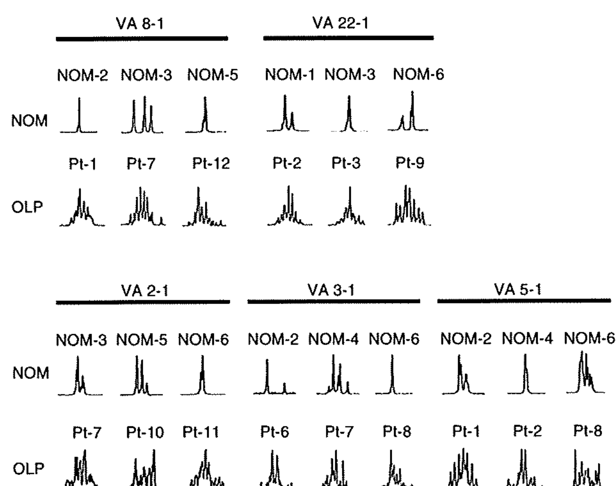

Fig. 4. Polyclonal expansion of T cell receptors (TCRs) within the oral mucosa of patients with oral lichen planus (OLP). Because the TCR repertoire analysis revealed skewed numbers of T cells bearing TCRVA8-1, VA22-1, VB2-1, VB3-1 and VB5-1, CDR3 size spectratyping was performed for all samples. In NOM tissue specimens, the CDR3 size distributions show monoclonal or oligoclonal patterns. In contrast, the CDR3 spectratyping patterns for OLP tissue specimens exhibit polyclonal or oligoclonal patterns.

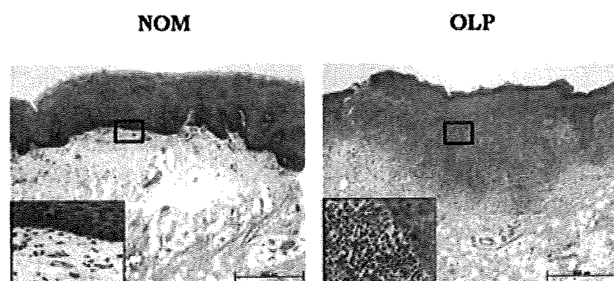

Fig. 5. Histopathological findings for normal oral mucosa (NOM) and oral lichen planus (OLP) tissue specimens. In NOM tissue specimens, lymphocytes are habitually present. In OLP tissue specimens, band-like infiltrates of lymphocytes in the superficial lamina propria and disruption of the basement membrane are detected. The insets show higher magnification images of the boxed regions. Magnifications: ×20 and ×200.

expression levels were higher in OLP PBMCs than in healthy PBMCs ($P < 0{\cdot}05$).

Discussion

Using the sensitive and reliable AL–PCR and MHA methods, we detected significant increases in TCR repertoires, namely TCRAV8-1, AV22-1, BV2-1, BV3-1 and BV5-1, in OLP tissue specimens compared with healthy PBMCs. In contrast, we detected variable TCRAV and TCRBV repertoires in NOM tissue specimens, with no particular specificity. Therefore, we consider that OLP lesions show a tendency towards

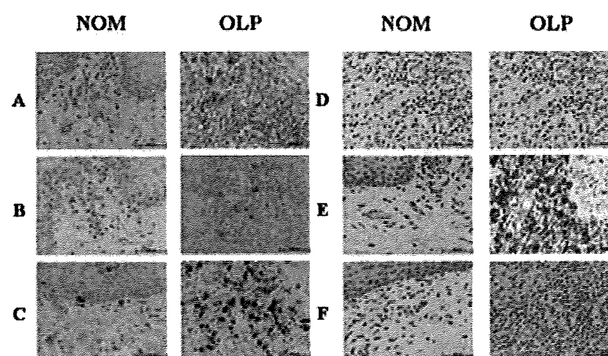

Fig. 6. Immunohistochemical staining for CD3 (a), CD4 (b), CD8 (c), CD20 (d) and human leucocyte antigen D-related (HLA-DR) (e) using commercially available monoclonal antibodies following the ENVISION technique with 3,3-diaminobenzidinetetrahydrochloride as the indicator agent. (f) Negative control. $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes are detected among the infiltrating cells toward the basal membrane (a–c). Normal oral mucosa (NOM) tissue specimens contain low numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes (a–c). Expression of CD20 is detected in both OLP and NOM tissue specimens (d). HLA-DR expression is increased significantly in OLP tissue specimens (e). Magnification: ×200.

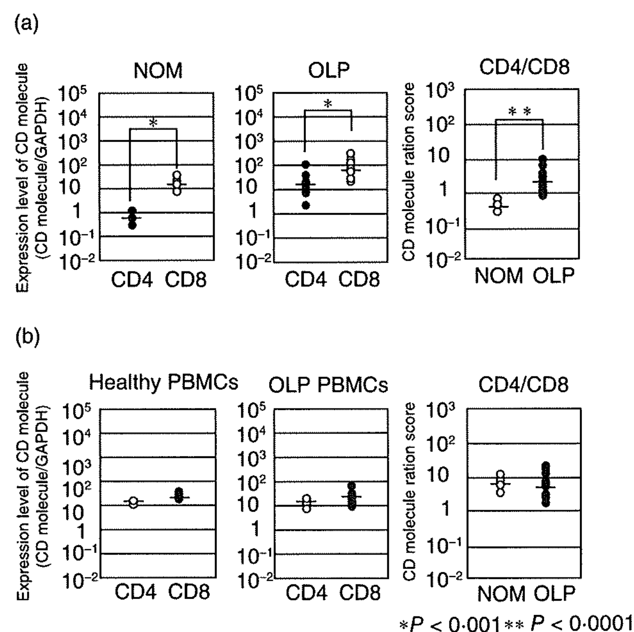

Fig. 7. Comparison of the expression levels of T cell phenotypes between normal oral mucosa (NOM) ($n = 6$) and oral lichen planus (OLP) ($n = 12$) tissue specimens (a) or healthy ($n = 9$) and OLP ($n = 12$) peripheral blood mononuclear cells (PBMCs) (b). The mRNA expression levels of $CD4^+$ and $CD8^+$ T cells were measured by real-time quantitative polymerase chain reaction (PCR). Each dot indicates a single sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as an internal control. The expression levels of $CD8^+$ T cells are higher than $CD4^+$ T cells in NOM and OLP tissue specimens ($^*P < 0{\cdot}001$). The CD4/CD8 ratio is higher in OLP tissue specimens than in NOM tissue specimens ($^{**}P < 0{\cdot}0001$).

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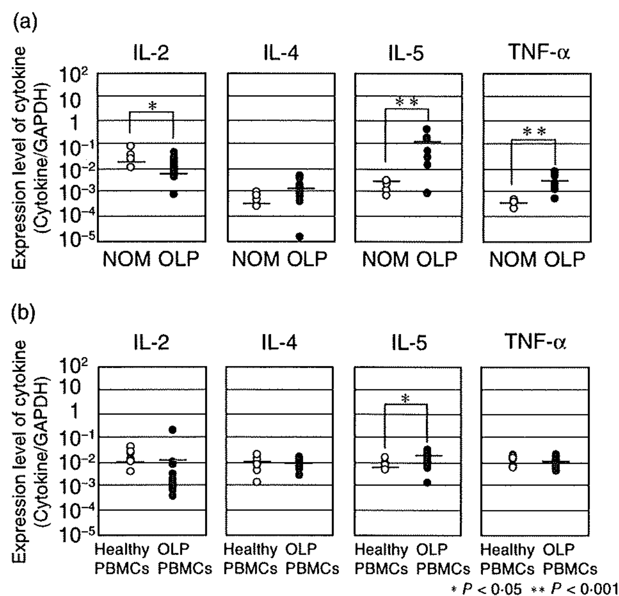

Fig. 8. Comparison of the expression levels of several cytokines between normal oral mucosa (NOM) ($n = 6$) and oral lichen planus (OLP) ($n = 12$) tissue specimens (a) or healthy ($n = 9$) and OLP ($n = 12$) peripheral blood mononuclear cells (PBMCs) (b). The mRNA expression levels of interleukin (IL)-2, IL-4, IL-5 and tumour necrosis factor (TNF)-α were measured by real-time quantitative polymerase chain reaction (PCR). Each dot indicates a single sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as an internal control. The mRNA levels expressed by the ratio of the amount of the each cytokine-specific cDNA to the amount of GAPDH-specific cDNA were compared between NOM and OLP tissue specimens or healthy and OLP PBMCs. (a) The expression levels of IL-5 and TNF-α are higher in OLP tissue specimens than in NOM tissue specimens. The IL-2 expression levels are lower in OLP tissue specimens than in NOM tissue specimens. (b) The IL-5 expression levels are higher in OLP PBMCs than in healthy PBMCs.

increased infiltration of T cells with these TCR repertoires. The observation that increased usage of TCRAV8-1, BV2-1, BV3-1 and BV5-1 was also detected in OLP PBMCs suggests that T cells with these TCR repertoires expand into the blood and infiltrate the lesional mucous membrane, where they may participate in the inflammatory process.

Previous studies have reported that usage of TCRAV2, BV2, BV3, BV6, BV13, BV14, BV15 and BV19 is increased in OLP lesions and that BV22 and BV23 are increased in *in vivo*-expanded cells [4–6]. Our observation that TCRBV3-1 was increased in OLP lesions is compatible with a previous observation, that T cells bearing TCRBV3 are increased in OLP lesions as evaluated by immunohistochemistry [4]. The increased usage of TCRBV2-1 in OLP lesions detected in the present study is compatible with a previous finding of increased usage of TCRBV2 detected by semiquantitative PCR [6]. In the present study, we did not find increased usage of TCRAV2, BV6, BV13, BV14, BV15 or BV19. The discrepancy between our results and previous findings may be attributed to differences in the detection methods or the series of examined patients. Because the AL–PCR and MHA methods are highly reliable for quantitative evaluation of TCR usage [9], we believe that the present findings are more reliable than the previously reported findings. We examined reticular-type OLP patients without HCV infection, whereas previous reports included erosive and atrophic types. It is unclear whether the patients in the previous reports were associated with HCV infection. Differences in the characteristics of the examined patients in the present and previous studies may represent another possible reason for the differing results.

In the present study, we have demonstrated a striking difference in the clonality of TCR repertoires between NOM and OLP tissue specimens by CDR3 size spectratyping. The TCR repertoires of TCRAV8-1, AV22-1, BV2-1, BV3-1 and BV5-1 appeared to be polyclonal in OLP tissue specimens, but oligoclonal in NOM tissue specimens. These findings suggest that the band-like infiltrates in OLP lesional mucosa are composed of polyclonally expanded T cells. It has been suggested that SAs are involved in the pathogenesis of OLP [6]. In fact, TCRBV2-1, BV3-1 and BV5-1 are known to be activated by various SAs, including TSST-1, SEB, SEC3, SED and SEE [5]. The pattern of TCR usage in T cells expanded by each SA is usually restricted. For instance, TCRBV2 and BV4 are expanded by TSST-1, while TCRBV1, BV5, BV6, BV7, BV9 and BV18 are expanded by SEA [15,16]. Andersen *et al.* [17] reported preferential expression of certain Vα regions among SA-reactive T cells and suggested that the TCR-α chain may modulate the level of activation through an interaction with MHC. In addition, Petersson *et al.* [18,19] reported that *Staphylococcal enterotoxin H* induces TCR Vα27-specific expansion of T cells. There are no previous reports of SAs that induce specific expansion of T cells bearing TCRAV8-1, AV22-1, BV2-1, BV3-1 or BV5-1, suggesting that the pattern of TCR usage expansion observed in the present study may not be related to a particular SA or that a currently unknown SA is involved in OLP pathogenesis. It has been suggested that T cell clones are deleted in peripheral blood by some SAs [20,21]. In the present study, there were significant decreases in T cells bearing VA3-1, VA16-1, VA31-1, VA32-1 and VB4-1 in the OLP PBMCs compared with healthy PBMCs. These observations support further the hypothesis that SAs may be contribute to OLP pathogenesis, and that T cells with limited TCR repertoires are involved in its inflammatory process.

Previous studies have indicated that autoimmunity may be involved in the pathogenesis of OLP. The lichenoid tissue reactions observed in OLP lesions resemble the histological patterns of graft-*versus*-host disease [1]. Lichen planus-like lesions in oral mucosa have been described in patients after autologous bone marrow transplantation [22,23]. Peripheral T lymphocytes from OLP patients have been shown to induce cell lysis of oral epithelial cells [24]. In addition, injection of Ia-reactive T cell clones into syngeneic mice

causes lichenoid epidermal changes in an experimental model [25]. It is thus possible that autoimmune mechanisms may be involved in the pathogenesis of OLP. Interestingly, a recent study revealed that TCRAV22 is expressed by T cell clones specific for desmoglein-3, a pemphigoid antigen [26]. Desmoglein-3 is a known surface molecule of basal cells in the oral epithelium, implying that T cells bearing TCRAV22 may cause inflammation of the mucous membrane by responding to this molecule on the basal keratinocytes. The issue of whether autoreactive T cells participate in the pathogenesis in OLP remains to be clarified in future studies. In the present study, expansion of TCRVA22-1-bearing T cells was observed distinctively in OLP lesions. This observation raises another possibility that T cells activated by SAs may recognize self-antigens, due probably to cross-reactivity.

While the majority of intraepithelial lymphocytes in OLP are $CD8^+$ cytotoxic T cells, most lymphocytes in the lamina propria are $CD4^+$ helper T cells [27,28]. In the present study, $CD8^+$ cytotoxic T cells were found significantly in the lamina propria and the mRNA expression levels in $CD4^+$ and $CD8^+$ T lymphocytes were increased in OLP. These findings suggest that $CD4^+$ T cells are increased in OLP lesions, as the CD4/CD8 ratio was higher in OLP tissue specimens than in NOM tissue specimens. Therefore, investigation of the TCR repertoires of $CD8^+$ T cells infiltrating OLP lesions may lead to a better understanding of OLP pathogenesis.

The finding that TNF-α expression was increased in OLP tissue specimens compared with NOM tissue specimens is compatible with a previous report [29]. Surprisingly, we found increased levels of IL-5 expression, but not IL-4 expression, in OLP tissue specimens and PBMCs. It has been suggested that mast cells and regulated upon activation normal T cell expressed and secreted (RANTES) may play roles in OLP pathogenesis [30]. IL-5 is known to be important for mast cell survival, and may therefore be involved in the mast cell-dependent inflammatory process.

In conclusion, we have observed skewed TCR repertoires in tissue specimens and PBMCs from OLP patients. Furthermore, the CDR3 distributions of the skewed TCR subfamilies exhibited polyclonal or oligoclonal patterns. Thus, polyclonal T cell expansion in the blood and mucous lesions may be induced in individuals with OLP. T cells responding to SAs, certain self-antigens or external antigens may be involved in the pathogenesis of OLP. Further analyses are required to understand fully the exact aetiology of OLP.

Acknowledgements

We thank Drs Motohiro Kobayakawa, Kazutoshi Kamei and Naoki Hasegawa from Yokohama Rosai Hospital for providing some of the biopsy specimens used in this study. This study was supported by Grants-in-Aid from the Ministry of Health, Labor and Welfare of Japan, and the Japan Society for the Promotion of Science.

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Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2007.03443.x

Bacterial superantigens and T cell receptor β-chain-bearing T cells in the immunopathogenesis of ulcerative colitis

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Accepted for publication 21 May 2007

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Summary

Ulcerative colitis (UC) is a chronic relapsing–remitting inflammatory bowel disease (IBD) that affects the colon and the rectum producing debilitating symptoms, which impair ability to function and quality of life. The aetiology of IBD is incompletely understood, but within the lymphocyte population, specific T cell subsets are known to be major factors in the development of intestinal immune pathology while different subsets are essential regulators, controlling IBD. Hence, IBD is thought to reflect dysregulated T cell behaviour. This study was to investigate if the normal molecular configuration of the T cell receptor (TCR) repertoire is compromised in patients with UC. The percentage of T cell-bearing β-chain 4 (TCRBV4) was high in patients with UC, and T cells showed polyclonal expansion in the presence of bacterial superantigens (SA) such as streptococcal mitogenic exotoxin Z-2 (SMEZ-2), indicating that bacterial SA promote specific TCRBV family expansion. Further, in patients with UC, the duration of UC was significantly longer in patients with skewed TCRBV4 compared with patients without TCRBV4 skewing, suggesting that long-term exposure to bacterial SA such as SMEZ-2 might promote systemic immune disorders like the remission-relapsing cycles seen in patients with UC. In conclusion, our observations in this study support the perception that the systemic activation of T cells by enteric bacterial SA might lead to a dysregulated, but exuberant immune activity causing the remission and flare-up cycle of mucosal inflammation in patients with UC. Future studies should strengthen our findings and increase understanding on the aetiology of IBD.

Keywords: bacterial superantigens, inflammatory bowel disease, T lymphocytes, TCR repertoire, ulcerative colitis

Introduction

Ulcerative colitis (UC) is a chronic relapsing–remitting inflammatory bowel disease (IBD) that affects the colon and the rectum and produces debilitating symptoms, which impair both ability to function and quality of life [1]. At present, factors which exacerbate and perpetuate UC are not well understood. Multiple factors, including environmental influences [2–4], enteric flora [1,5–7] and genetic susceptibility [8], are thought to contribute to the dysregulated immune function [9,10] seen in patients with IBD. However, active UC is associated frequently with infiltration of a vast number of leucocytes, mainly activated T cells, monocytes/macrophages and polymorphonuclear cells, into the intestinal mucosa [1,11–15]. In line with these observations, several studies have suggested that T cells are major players in the immunopathogenesis of UC [9,12–18]. Accordingly, certain immunosuppressants such as cyclosporin A, a T cell-specific immunosuppressive agent, has been used to induce remission of active UC [19]. Given that certain T cell subsets such as the $CD4^+CD25^+$ phenotype (known popularly as regulatory T cells) are involved intimately in the control of intestinal immune pathology [20,21], it is logical to look for factors which are associated with dysregulated T cell features in the peripheral blood and within the intestinal mucosa of patients with UC.

T cells are known to recognize the antigen presented by antigen-presenting cells through the T cell receptor (TCR) in the context of major histocompatibility complex (MHC) class I and class II molecules [22]. The fine specificity of T cells is determined by the TCR displayed on the cell surface, a heterodimer composed of an α-chain and a β-chain or a γ-chain and a δ-chain. The variable regions of these chains are responsible for antigen recognition and are encoded with variable (V), joining (J) and diversity (D) (for the β-chain) gene segments. Random insertion of non-germinal element (N) nucleotides or deletion of nucleotides has been observed in the VN(D)NJ junction region called CDR3, and is thought to be responsible for an antigenic peptide content [23,24]. Thus, any specific recognition of antigens by CDR3 can lead to the clonal expansion of T cells. Further, it has been known that superantigens (SA) such as staphylococcal enterotoxins and streptococcal pyrogenic exotoxins produced by bacteria bind to the outside of the MHC class II α-chain and V region of TCR β-chain (TCRBV) to form a cross-linking; a given SA can stimulate all T cells that bear the appropriate TCRBV in polyclonal settings [22]. As CDR3 has different sequences and lengths, it is possible to analyse the diversity of the TCRs by using the CDR3 size spectratyping method that provides a rapid scan of all TCRBV transcripts, grouped according to the utilized Vβ gene and the lengths of the chains [25,26]. Using this technique with TCR repertoire analysis, it is possible to investigate the diversity of TCRs [27–29]. By using TCR repertoire analysis and CDR3 spectratyping, this study aimed to gain further understanding on T cell profiles associated with UC, to investigate if the normal molecular configuration of the TCR repertoire of T cells is compromised in patients with UC.

Materials and methods

Test samples

Peripheral blood samples were obtained from 22 patients with active UC and 20 healthy controls after obtaining informed consent at the Sakura Medical Center of Toho University (Japan). The study protocol was reviewed and approved by the local Committee on Ethics of experiments involving humans. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood using lymphocyte separation medium (H-SMF; Jimro, Gunma, Japan) gradient centrifugation. PBMC were washed with RPMI-1640 (Invitrogen, Carlsbad, CA, USA) and used in several experiments. The plasma samples were kept frozen until assay. In an additional investigation, colonic biopsies from five patients were processed. In each case, two or three small macroscopically inflamed mucosal biopsy specimens were obtained at colonoscopy. The biopsy samples were soaked in RNA*later*™ (Qiagen, Hilden, Germany) and kept frozen until use. Peripheral blood specimens were also obtained from these five patients.

Stimulation of PBMC with recombinant streptococcal mitogenic exotoxin Z-2 (rSMEZ-2) and recombinant toxic shock syndrome toxin 1 (rTSST-1)

PBMC were incubated with 1 ng/ml of rTSST-1 (Toxin Technology, Sarasota, FL, USA) or with 20 pg/ml of rSEMZ-2 (a kind gift from Dr John D. Fraser, University of Auckland, New Zealand) at 1×10^6 cells/ml for 3 days. After 3 days, 20 ng/ml of interleukin (IL)-2 (Shionogi, Osaka, Japan) was added to each well and the cells were incubated for a further 24 h (for rSMEZ-2 stimulation only). The cells were harvested and used for analysis of the TCR repertoire.

Analysis of TCR repertoire

Crude cellular RNA from PBMC, stimulated PBMC or biopsy samples was extracted by using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Adaptor-ligation polymerase chain reaction (PCR) and microplate hybridization assay were performed as previously described [30]. Briefly, 1 μg of total RNA was converted to a double-stranded cDNA with the SuperScript II cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions, except for priming with BSL-18B primer adaptor containing the *Not I* site. The P20EA/P10EA universal adaptors were ligated at the 5′ end of BSL-18B primer cDNA. Three rounds of Cα- and Cβ-specific PCR were performed by using Cα and Cβ sequence-specific oligonucleotide probes (SSOP) to prepare amplified and biotinylated TCR cDNA pools. Hybridization was between biotinylated PCR products and Vα or Vβ SSOP, which were immobilized on a carboxylate-modified enzyme-linked immunosorbent assay (ELISA) plate (Sumitomo Bakelite, Tokyo, Japan). The hybridization was visualized by *p*-nitrophenylphosphate (Nacalai Tesque, Osaka, Japan). The visualized signals were estimated at 405 nm using Multiskan JX (Thermo Labsystems, Helsinki, Finland). The relative expansion of the TCRAV or TCRBV region repertoire was calculated by the following formula: frequency (%) = 100 (the corresponding SSOP signal)/(ΣTCRV SSOP signals).

CDR3 size analysis of TCRBV

By knowing the size of the CDR3 region in TCRBV, it is possible to estimate the polyclonal expansion of T cells [31]. We used this technique to determine the polyclonality of T cells from patients with active UC who had skewed TCRBV4 and PBMC from healthy controls before and after *in vitro* stimulation with SA. The second PCR products described above were labelled by the 20-cycle PCR amplification with fluorescent dye-labelled Cβ–SSOP [31]. After the labelled PCR products were mixed with size marker (CEQ™ DNA Size Standard Kit-600; Beckman Coulter, Fullerton, CA, USA), they were loaded onto a polyacrylamide sequencing

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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).

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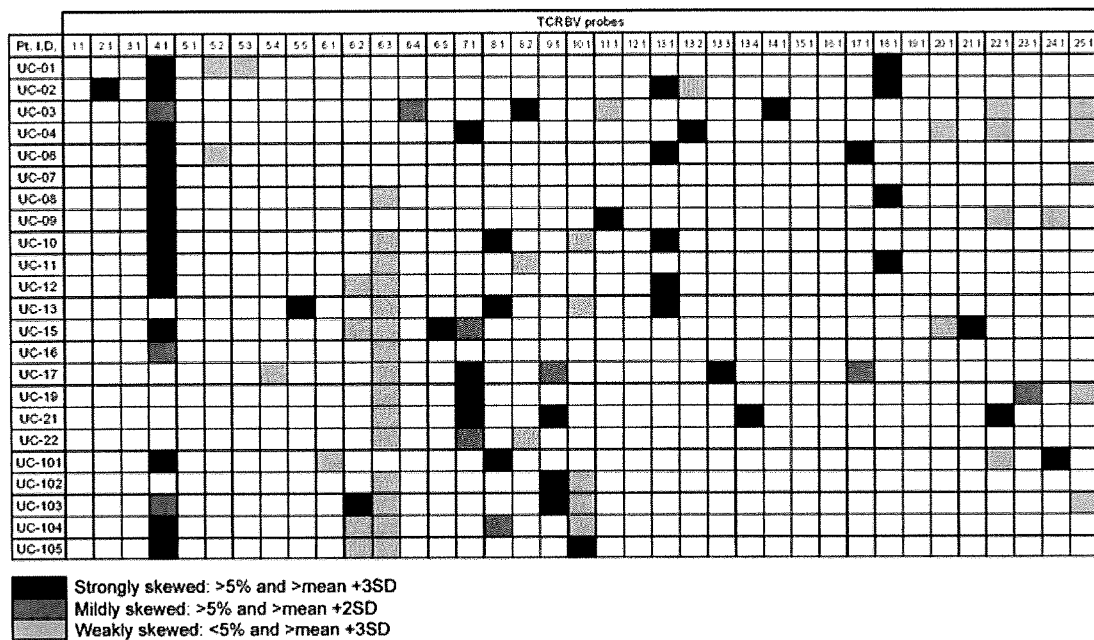

Fig. 1. T cell receptor β-chain (TCRBV) gene expression profile in peripheral blood mononuclear cells (PBMC) of patients with active ulcerative colitis (UC). Total RNA was extracted from PBMC and reverse-transcribed into cDNA and the adaptor was ligated. This adaptor-ligated cDNA was then used as a template 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

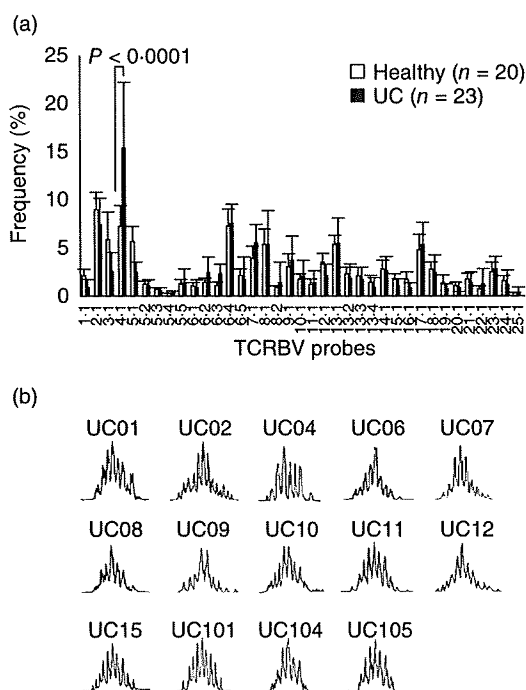

Fig. 2. The polyclonal expansion of T cell receptor β-chain 4 (TCRBV4)-bearing T cells in peripheral blood mononuclear cells (PBMC) of patients with active ulcerative colitis (UC). (a) The percentage of TCRBV4 in PBMC specimens of patients with UC was strikingly high compared with healthy individuals ($P < 0{\cdot}0001$). 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

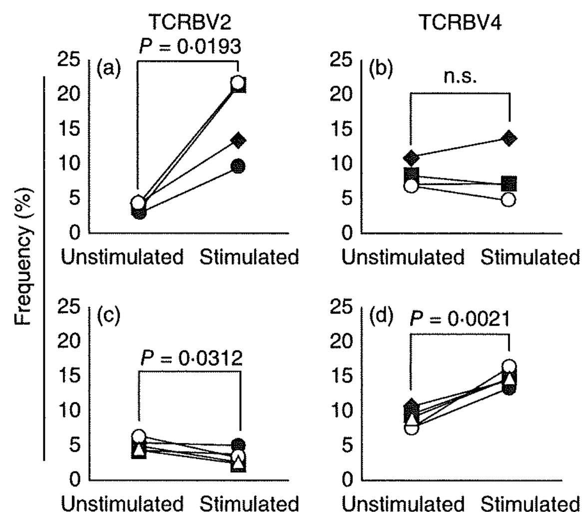

Fig. 3. Changes in the percentage frequency of T cell receptor β-chain (TCRBV) families following stimulation by two different bacterial superantigens (SA), toxic shock syndrome toxin 1 (TSST-1) and streptococcal mitogenic exotoxin Z-2 (SMEZ-2). TSST-1 stimulated TCRBV2-bearing T cells (a) and did not 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.

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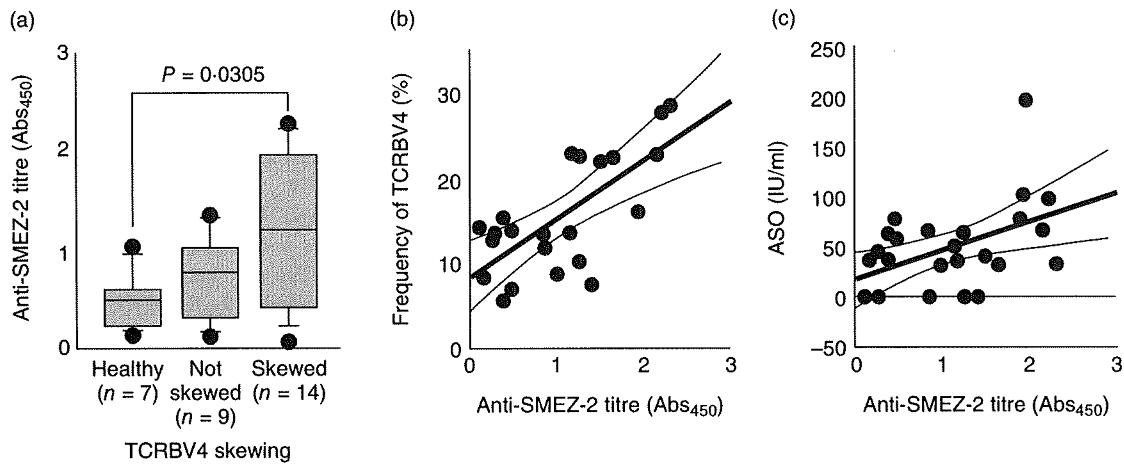

Fig. 4. Elevated anti-streptococcal mitogenic exotoxin Z-2 (SMEZ-2) antibody in patients with active ulcerative colitis (UC) who had skewed T cell receptor β-chain 4 (TCRBV4). (a) The anti-SMEZ-2 titre was significantly higher in patients with skewed TCRBV4 compared with healthy volunteers ($P = 0{\cdot}0305$, 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,

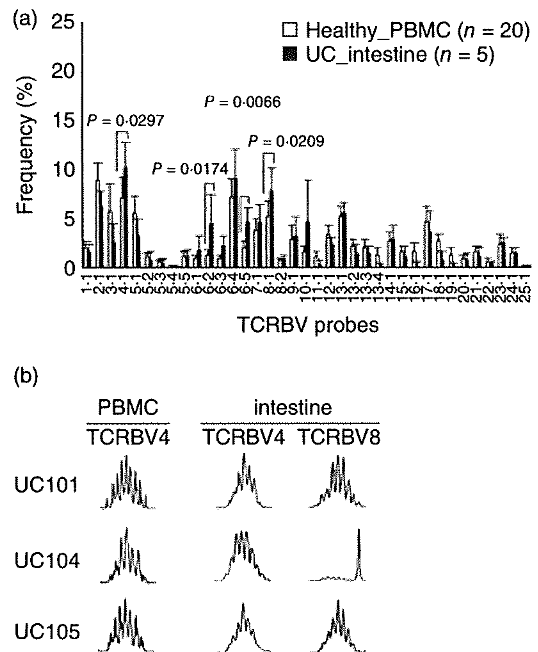

Fig. 5. Polyclonal expansion of T cell receptor β-chain 4 (TCRBV4) and TCRBV8-bearing T cells within the intestinal mucosa of patients with active ulcerative colitis (UC). (a) The percentage of TCRBV4, BV6·2, BV6·5 and BV8 in intestinal samples from patients with UC were significantly higher than the 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.

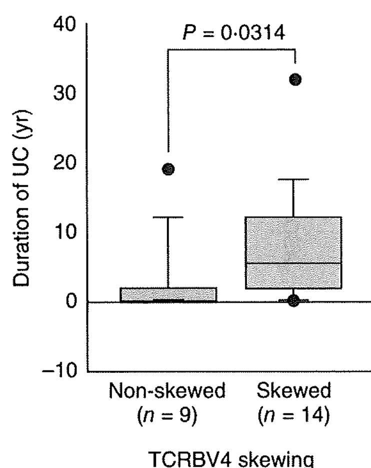

Fig. 6. Significant ($P = 0{\cdot}0314$, by Mann–Whitney U-test) association between duration of ulcerative colitis and T cell receptor β-chain 4 (TCRBV4) skewing. Twenty-three patients were classified into two subgroups based on the skewing of TCRBV4. The skewing was defined to be significant if the 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.

Acknowledgements

We thank our colleague Dr A. R. Saniabadi for editing the manuscript and Mr J. Sato for assisting with the sample processing work.

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International Immunology, Vol. 20, No. 10, pp. 1331–1342
doi:10.1093/intimm/dxn091
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Arthritis and pneumonitis produced by the same T cell clones from mice with spontaneous autoimmune arthritis

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Keywords: animal model, interstitial lung disease, rheumatoid arthritis, T cell clone

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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Transmitting editor: K. Yamamoto

Received 31 March 2008, *accepted* 17 July 2008
Advance Access publication 18 August 2008

FEATURED

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