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Short communication

Gene expression analysis of the CD4$^+$ T-cell clones derived from gingival tissues of periodontitis patients

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The function of T cells infiltrating periodontitis lesions is complex and has not been fully elucidated. Here, we established T-cell clones from the gingival tissues of periodontitis patients and examined their gene expression. A total of 57 and 101 T-cell clones were established by means of immobilized anti-CD3 antibody and IL-2 from gingival tissues and peripheral blood, respectively. The gingival T-cell clones were derived from three patients, and the peripheral blood T-cell clones from two of these patients and a further patient whose gingival T-cell clones were not established. Gingival tissues were also obtained from a further 19 periodontitis patients. The expression of cytokines and molecules related to both regulatory function and tissue destruction were examined by means of reverse-transcription polymerase chain reaction. All the gingival T-cell clones expressed mRNA for TGF-β1, CTLA-4, and CD25, and all the T-cell clones from peripheral blood expressed IFN-γ and TGF-β1 mRNAs. Most but not all the T-cell clones from gingival tissues and peripheral blood expressed mRNA for IFN-γ and, CD25 and CTLA-4, respectively. The frequency of T-cell clones and gingival tissues expressing *FOXP3*, a possible master gene for mouse CD4$^+$CD25$^+$ regulatory T cells, was very high (97%, 93%, and 100% for gingival T-cell clones, peripheral blood T-cell clones, and gingival tissues, respectively). Whereas the frequency of IL-4-expressing T-cell clones was lower for gingival T-cell clones (70% vs. 87%), the frequency of the gingival T-cell clones expressing IL-10 and IL-17 was higher than peripheral blood T-cell clones (75% vs. 62% for IL-10, 51% vs. 11% for IL-17). A similar expression profile was observed for gingival T-cell clones compared with gingival tissue samples with the exception of IL-4 expression, where the frequency of positive samples was lower in the gingival tissues (70% vs. 11%). These results suggest that the individual T cells infiltrating gingival lesions can express mRNA for both Th1 and Th2 cytokines as well as regulatory cytokines simultaneously.

Key words: gene expression; periodontitis; T-cell clones

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T cells are a significant cell component of the inflammatory cell infiltrate in both periodontitis lesions and gingivitis lesions (8). It has been reported that the T cells produce IFN-γ (4, 12, 19), resulting in possible activation of macrophage to release various inflammatory mediators and express receptor activator of nuclear factor (NF)-κB ligand, a key molecule for osteoclast differentiation (11). It has also been reported that auto-reactive T cells with Th1 cytokine profile recognizing self-components such as heat shock protein 60 (20) and collagen type I (15) accumulate in gingival lesions of periodontitis patients. These findings imply that T cells may be actively involved in the immune pathology of periodontal diseases.

In this context, it is reasonable to assume that patient susceptibility to periodontal tissue destruction could be determined at least in part by the balance between autoimmune response mediated by autoreactive T cells and regulatory mechanisms mediated by regulatory T cells.

Although a number of reports have attempted to characterize and clarify the role of T cells in the pathogenesis of periodontal diseases, their functional characteristics have still not been fully elucidated. It is well recognized that a functional analysis of T cells extracted from periodontitis tissues would be important in understanding the precise role of T cells in the periodontal disease process. However, one can predict that it may be difficult to obtain large enough numbers of cells to do such an experiment. Alternatively, the analysis of T-cell clones established directly from the periodontitis lesions would be helpful. In this respect, Gemmell et al. established *Porphyromonas gingivalis*-specific T-cell clones (7) and lines (6, 5), and analyzed their characteristics extensively. Wassenaar et al. also established *Prevotella intermedia*-specific T-cell clones (16). However, these T-cell lines and clones are derived from peripheral blood of periodontitis patients and are, moreover, specific to only two of several hundreds of bacterial species colonizing in the periodontal pocket. Furthermore, the proportion of T-cell clones specific to a particular bacterium in periodontitis lesions is not known. Based on these issues, the aim of the present study was to establish T-cell clones from gingival tissues using anti-CD3 antibody and IL-2 and to analyze the gene expression of various molecules relevant to their regulatory function, comparing them with the expressions of peripheral blood T-cell clones and gingival tissues as controls. The institutional review board of Niigata University Graduate School of Medical and Dental Sciences approved this study and the written informed consent was obtained from all the patients before inclusion in the study.

T-cell clones were established from three patients with moderate to advanced periodontitis aged 45–67 years. Inflamed gingival tissues were obtained at periodontal surgery and cut into small pieces in RPMI1640 containing penicillin, streptomycin, and fungizone, and subjected to Dispase digestion. Tissues were passed through nylon mesh and subsequent single cell suspensions were subjected to Ficoll-Paque PLUS (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density gradient centrifugation. The resulting mononuclear cells were stimulated with immobilized anti-CD3 monoclonal antibody (30 ng/ml, UCHT-1; Immunotech, Marseilles, France) in the presence of irradiated allogenic peripheral blood mononuclear cells (PBMC) (2×10^6/well), EBV-transformed PBMC (2×10^5/well) and 50 U/ml recombinant human interleukin-2 (PEPRO TECH, London, UK) in 2 ml of culture media in a 24-well culture plate (NUNC, Roskilde, Denmark). After expansion of the cells, $CD4^+$ T cells were purified using magnetic beads (Miltenyi Biotec, Auburn, CA). T-cells were then cloned by limiting dilution, and stimulated as described above. Peripheral blood mononuclear cells were also isolated from two subjects whose gingival T-cell clones were established. Peripheral blood T-cell clones were established from one further patient. For peripheral blood T-cell clones, $CD4^+$ T cells were purified from PBMC and subjected for limiting dilution as with gingival T-cell clones. We selected successfully expanded clones for further analysis. A total of 57 gingival and 101 peripheral blood T-cell clones established from four periodontitis patients were analyzed. Nineteen gingival tissues were obtained from other patients undergoing periodontal surgery to compare the gene expression profile with that of the T-cell clones. Total RNA from T-cell clones and gingival tissues (approximately 50 mg) were separated by using Trizol® (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA samples were further purified by successive treatment with DNase I Amplification Grade (Invitrogen). The first strand cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) and 0.3 μg/μl of random hexanucleotides (Takara Shuzo Co., Ltd, Shiga, Japan) from 1–2 μg of total RNA in the reaction buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, supplemented with 0.02 M dithiothreitol and dNTP (each at 1.0 mM). The reaction mixture was incubated at 37°C for 65 min, and then heated at 65°C for 7 min.

cDNA 1 μl from T-cell clones, PBMC samples and gingival tissue-derived

Table 1. Primer sets for reverse transcription-polymerase chain reaction (RT-PCR)

		Sequence	Products (bp)	Ta (°C)	Cycle number	
					T-cell clone	GT
IFN-γ	Forward	5′GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC3′	427	60	30	40
	Reverse	5′CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG3′				
IL-4	Forward	5′ACTGCAAATCGACACCTATT3′	270	57	35	40
	Reverse	5′ATGGTGGCTGTAGAACTGC3′				
IL-17	Forward	5′GGACTGTGATGGTCAACCTGAAC3′	301	60	35	40
	Reverse	5′CCCACGGACACCAGTATCTTCT3′				
RANKL	Forward	5′GCCAGTGGGAGATGTTAG3′	480	55	40	40
	Reverse	5′TTAGCTGCAAGTTTTCCC3′				
CD25	Forward	5′GAATGCAAGAGAGGTTTCCGC3′	301	60	35	40
	Reverse	5′AATAAACCATCTGCCCCACCAC3′				
CTLA-4	Forward	5′AGTATGCATCTCCAGGCAAAGC3′	320	60	35	40
	Reverse	5′CCAGAGGAGGAAGTCAGAATCTG3′				
IL-10	Forward	5′TTGCCAAGCCTTGTCTGAGATG3′	370	58	35	40
	Reverse	5′AGCCCCAGATCCGATTTTGG3′				
TGF-β1	Forward	5′GACATCAACGGGTTCACTACCGG3′	267	60	30	40
	Reverse	5′GAGGCAGAAGTTGGCATGGTAGC3′				
FOXP3	Forward	5′GCAAATGGTGTCTGCAAGTGG3′	215	60	38	40
	Reverse	5′AGTGCCATTTTCCCAGCCA3′				
β-actin	Forward	5′GCGAGAAGATGACCCAGATCATGTT3′	300	55	25	25
	Reverse	5′GCTTCTCCTTAATGTCACGCACGAT3′				

Ta, annealing temperature. GT, gingival tissue.

samples was added to a polymerase chain reaction (PCR) mixture containing 10 × PCR buffer (Promega, Madison, WI), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 420 nM oligonucleotide primers (Table 1) and 0.75 units of *Taq* DNA polymerase (Promega) in a total volume 15 μl. Reaction was performed using a DNA thermal cycler (GeneAmp® PCR System 9700; Applied Biosystems, Foster City, CA). Ten μl of each PCR product was electrophoresed on 2% agarose gel and visualized by ethidium bromide staining. Primers for the T-cell receptor β-chain have been described previously.

To examine the expression of surface molecules on the T-cell clones by flow cytometry, the cells were reacted with ECD-conjugated anti-CD4 (Beckman Coulter, Inc., Miami, FL), FITC-conjugated anti-CD25 (Caltag Laboratories, Burlingame, CA) and PE-conjugated anti-CTLA-4 (eBioscience, San Diego, CA) and respective isotype controls. The expression levels of each surface molecule were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and CellQuest Pro software (BD Bioscience, San Jose, CA).

Representative staining patterns of T-cell clones established from gingival lesion and peripheral blood are shown in Figs 1 and 2, respectively. Phenotypic analysis by flow cytometry revealed that 78.6% of gingival T-cell clones and 80.0% of peripheral blood T-cell clones expressed both CD25 and CTLA-4. However, the level of CD25 expression was not high. As shown in Fig. 3, all T-cell clones established from gingival lesion expressed mRNAs for CD25, TGF-β1 and CTLA-4. IFN-γ was expressed by 100% of the T-cell clones from two patients and 93% of one patient. *FOXP3* was expressed in 95% of the clones from the other two patients and all the clones of one patient. The proportions of positive clones for IL-4, IL-10, IL-17, and RANKL varied from patient to patient. The proportions of mRNA-expressing T-cell clones for these genes were as follows: IL-4: 70%, 55% and 87%,

IL-10: 65%, 73% and 87%,

IL-17: 25%, 82% and 47%,

RANKL: 10%, 14% and 40%.

In peripheral blood T-cell clones, IFN-γ and TGF-β1 were expressed by all of the T-cell clones. For *FOXP3*, CD25, and CTLA-4, the frequency of positive T-cell clones in one patient (patient B) was 79%, 96%, and 96%, respectively. The overall expression of mRNA for IL-17 and RANKL was lower compared with the other cytokines. The frequency and distribution of RANKL mRNA-positive T-cell clones were similar between gingival tissues and peripheral blood. The frequency of IL-17 mRNA-positive T-cell clones from gingival tissues was higher than that

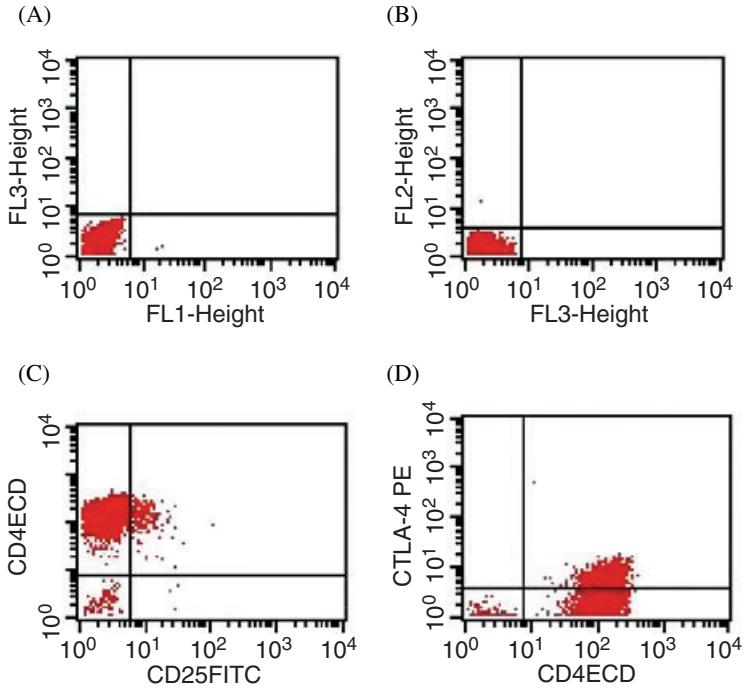

Fig. 1. Surface expression of CD25 and CTLA-4 by a $CD4^+$ T-cell clone established from the gingival lesion of Patient A. Thirteen T-cell clones out of 20 were analyzed and representative flow cytometric profiles are demonstrated. The cells were stained for either CD4/CD25 (C) or CD4/CTLA-4 (D). A and B, isotype controls for C and D, respectively.

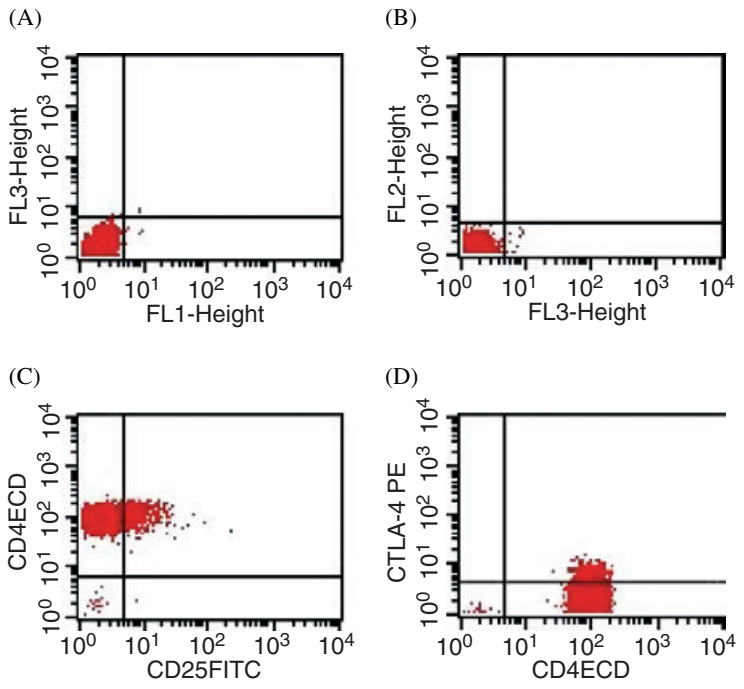

Fig. 2. Surface expression of CD25 and CTLA-4 by a $CD4^+$ T-cell clone established from the peripheral blood of Patient A. Twenty-eight T-cell clones out of 38 were analyzed and representative flow cytometric profiles are demonstrated. The cells were stained for either CD4/CD25 (C) or CD4/CTLA-4 (D). A and B represent isotype controls for C and D, respectively.

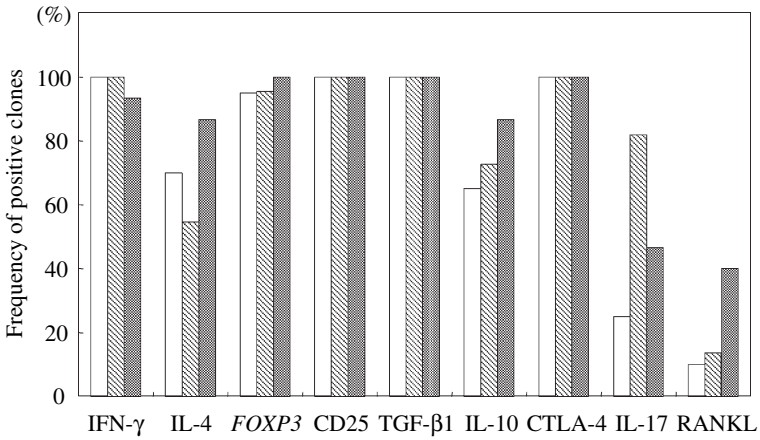

Fig. 3. Prevalence of gingival T-cell clones positive for mRNA expression of each molecule. Each bar represents one patient: □: Patient A (20 clones), ▨: Patient B (22 clones), ■: Patient C (15 clones).

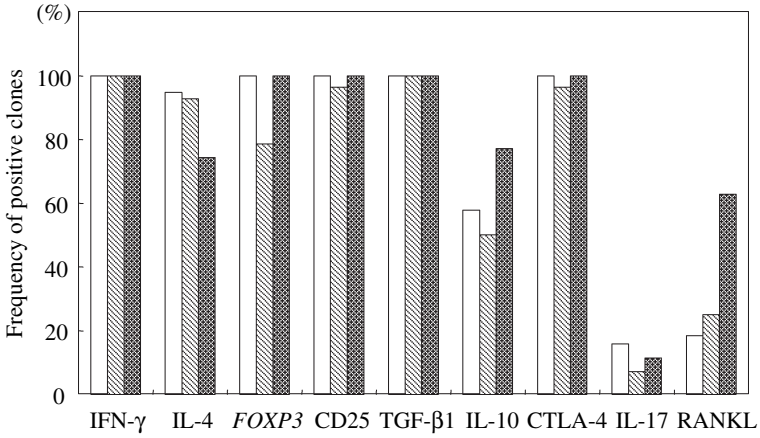

Fig. 4. Prevalence of peripheral blood T-cell clones positive for mRNA expression of each molecule. Each bar represents one patient: □: Patient A (38 clones), ▨: Patient B (28 clones), ■: Patient D (35 clones).

Table 2. T-cell receptor Vβ gene expression of T-cell clones of Patient A

Number of T-cell clones expressing each gene																		
Vβ	**2**	**3**	**4**	**5.1**	**5.2**	**6**	**7**	**8**	**9**	**10**	**12**	**13.1**	**13.2**	**15**	**16**	**17**	**18**	**19**
GT[1](18*)	17	2	0	2	2	2	1	0	1	1	0	4	2	1	0	2	1	1
PB[2](17*)	5	0	4	3	3	4	0	1	0	0	1	3	0	1	1	0	4	0

1: Gingival T-cell clones. 2: Peripheral blood T-cell clones.
*Number of clones analyzed. The number of clones in this table is smaller than that in Fig. 3 and 4 due to the inability to obtain the sufficient amount of cDNA.

from peripheral blood. Although the frequency was highly variable in gingival T-cell clones between patients, ranging from 25% to 82%, that of peripheral blood T-cell clones was consistently low (Fig. 4). TCR β-chain expression was examined as previously described (18) to examine each T-cell clone derived from a multiple population of infiltrating T cells and not from proliferated T-cells. As shown in Table 2, each T-cell clone expressed variable Vβ chains, suggesting that they are in fact derived from different T cells, although some 'T-cell clones' expressed more than 1 Vβ chain (Table 2).

As the expression of the genes examined in the present study is restricted to T-cells, we also analyzed these gene expressions in the RNA samples directly extracted from gingival tissues of periodontitis patients in order to determine whether T-cell clones completely reflect the T-cell population *in situ*. CTLA-4, *FOXP3* TGF-β1, and IL-10 were all expressed in all of the samples, although the expression levels were variable. Transcripts for IFN-γ and CD25 were detected in most samples (18 out of 19) (Fig. 5). In contrast, IL-4 and RANKL were expressed in only 11% and 32% of the samples, respectively. The frequency of IL-17 mRNA-positive samples was 74%.

In the present study, the results of the gene expression profile of gingival T-cell clones indicated that each clone is capable of transcribing both Th1 and Th2 cytokines as well as immunoregulatory cytokines such as IL-10 and TGF-β1. Although the anti-CD3 antibody was used to establish T-cell clones with a wide variety of specificities, this mode of stimulation might modulate their functional characteristics *in vivo*. In spite of this, gingival T-cell clones were different from autologous peripheral blood T-cell clones with respect to the expression of IL-17. Some differences in the gene expression profile between gingival T-cell clones and gingival tissue samples suggest that local environmental factors may modulate T-cell functions in the lesion.

The most interesting finding in the present study is that many of the T-cell clones derived from either gingival tissue or peripheral blood expressed the *FOXP3* gene. Expression of *FOXP3* was also observed in the gingival tissue samples. In addition to CD25 and CTLA-4, *FOXP3* seems to be the most promising marker for $CD4^+CD25^+$ naturally occurring regulatory T cells (Treg) in mice; there is a controversy concerning its use in humans. The expression of CD25 and CTLA-4 is upregulated upon T-cell activation. *FOXP3* is also inducible in $CD4^+CD25^-$ T cells by activation (1, 2, 13). Therefore, the method used to establish the T-cell clones may have affected the gene expression profile. In mice, *Foxp3* mRNA expression is highly enriched within $CD4^+CD25^+$ T cells in the thymus and the periphery, and retroviral transduction of naïve $CD4^+CD25^-$ T cells with *Foxp3* is sufficient to induce $CD4^+CD25^+$ T cells with regulatory activity (3, 9). In humans, it is reported that expression of *FOXP3* is found exclusively in $CD4^+CD25^+$ T cells and is correlated with the suppressive activity of these cells (14). In contrast to the mouse studies, antibody-mediated activation of human $CD4^+CD25^-$ T cells led to the expression of *FOXP3* and resulted in a gain of suppressive function (14). It is not known whether high proportion of $FOXP3^+$ T-cell clones in the present study

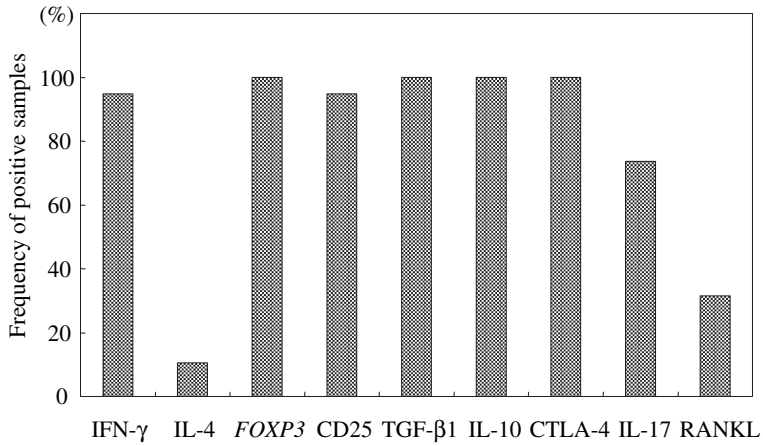

Fig. 5. Percentages of gingival tissue samples positive for mRNA expression of each molecule in 19 tissues.

could be due to nonspecific activation by anti-CD3 and IL-2.

The relevance of *FOXP3* expression to the gain of regulatory function is controversial. Recently, Morgan et al. (10) demonstrated that *FOXP3* expression in humans may not be specific for cells with a regulatory phenotype and may be only a consequence of activation status. On the other hand, Yagi et al. (17) demonstrated that TCR stimulation of $CD4^+CD25^-CD45RO^-$ naïve T cells failed to elicit *FOXP3* expression and that *ex vivo* retroviral gene transfer of *FOXP3* converted the naïve T cells into a regulatory T-cell phenotype similar to $CD4^+CD25^+$ regulatory T cells. Further studies are clearly needed to examine the role of *FOXP3* in the human T-cell response and clarify whether it is a characteristic marker for $CD4^+CD25^+$ regulatory T cells in humans. Nevertheless, there was a high prevalence of T-cell clones positive for regulatory cytokines such as IL-4, IL-10 and TGF-β1, together with CTLA-4.

Although antigen specificities of the T-cell clones remain to be elucidated, the results of the present study suggest that the individual T cells infiltrating in gingival lesion can express mRNA for both Th1 and Th2 cytokines as well as regulatory cytokines simultaneously.

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