Regulatory T-cells Infiltrate Periodontal Disease Tissues
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What is This?
ABSTRACT
CD4+CD25+ regulatory T (Tr) cells are critical in regulating the immune response and thereby play an important role in the defense against infection and control of autoimmune diseases. Our previous studies demonstrated the involvement of autoimmune responses in periodontitis. The aim of this study was to identify CD4+CD25+ Tr cells in periodontitis tissues and compare them with those in gingivitis tissues. Immunohistological analysis of CD4, CD25, and CTLA-4 and the gene expression analysis of FOXP3, TGF-β1, and IL-10 on gingival biopsies revealed the presence of CD4+CD25+ Tr cells in all tissues. In periodontitis, the percentage of CD4+CD25+ Tr cells increased with increasing proportions of B-cells relative to T-cells. FOXP3, a characteristic marker for CD4+CD25+ Tr cells, TGF-β1 and IL-10 were expressed more highly in periodontitis compared with gingivitis. These findings suggest that CD4+CD25+ Tr cells and possibly other regulatory T-cell populations do exist and may play regulatory roles in periodontal diseases.

KEY WORDS: regulatory T-cell, CD25, FOXP3, periodontal disease, immunohistochemistry, real-time PCR.

INTRODUCTION
Although periodontopathic bacteria are the primary etiological agents in periodontal disease, the ultimate determinant of disease progression and clinical outcome is the host’s immune response, which involves the generation of cytokines, the recruitment of inflammatory cells, and the activation of osteoclasts (Seymour et al., 1993). Because of elevated cellular and humoral immune responses to collagen type I (Hirsch et al., 1988; Wassenaar et al., 1995) and self-heat-shock protein 60 (HSP60) (Tabeta et al., 2000; Yamazaki et al., 2002), the immune responses to self-antigens have also been considered as one of the pathogenic pathways in periodontal disease. Therefore, it is reasonable to assume that patients’ susceptibility to periodontal tissue destruction could be determined, at least in part, by the balance between putative autoimmune responses and regulatory mechanisms.

It is becoming evident that T-cells with a suppressive function, the so-called ‘regulatory T (Tr) cells’, represent a crucial element in the regulatory networks that control immune responses and maintain peripheral tolerance (Thompson and Powrie, 2004). CD4+CD25+ Tr cells play a major role in this regulatory process, and, although originally isolated from mice, they have now been reported in humans (Baecher-Allan et al., 2001; Dieckmann et al., 2001). This population has been implicated not only in the inhibition of the spontaneous development of autoimmune diseases, but also in the control of the immune response to infection (McGuirk and Mills, 2002; Lundgren et al., 2003).

CD4+CD25+ Tr cells constitutively express high levels of CD25 and cytotoxic T-lymphocyte-associated antigen (CTLA)-4, and constitute 5-15% of the circulating peripheral CD4+ T-cell population. However, because these phenotypic markers are up-regulated upon activation, they are considered to be insufficient to characterize the CD4+CD25+ Tr cells precisely. Recently, FOXP3, the forkhead/winged helix transcription factor, has been identified as a putative master gene characterizing CD4+CD25+ Tr cells (Hori et al., 2003). We have previously shown that T-cells with suppressive activity, possibly CD4+CD25+ Tr cells expressing elevated levels of CD25 and CTLA-4, can be induced by stimulation with Porphyromonas gingivalis antigens in periodontitis patients (Aoyagi et al., 2000). However, whether or not these unique CD4+CD25+ Tr cells migrate into the periodontal tissues is as yet not known.

Therefore, in the present study, by using both phenotypic marker and gene expression analysis, we investigated the infiltration of CD4+CD25+ Tr cells into periodontal lesions, and whether this T-cell population is elevated in periodontitis lesions compared with gingivitis lesions to maintain immune homeostasis.

MATERIALS & METHODS
Patients and Biopsies
Seventeen patients with moderate to advanced chronic periodontitis, referred to
the Periodontal Clinic of Niigata University Medical and Dental Hospital, took part in this study. Gingival biopsies were obtained at the time of periodontal surgery or the extraction of severely involved teeth. As controls, 9 gingivitis tissues showing no supporting tissue destruction were also obtained from sites requiring extraction for reasons other than periodontitis, such as orthodontic treatment or pericoronitis. A further 20 periodontitis and 21 gingivitis tissues were obtained for gene expression analysis. The clinical status of the biopsy sites is shown in Table 1. The experimental protocol was approved by the Institutional Review Board of Niigata University, and informed consent was obtained from all patients prior to inclusion in this study.

As previously described, serial cryostat sections (5 μm thick) were cut and stored at -20°C until used (Yamazaki et al., 1995).

### Antibodies

Purified monoclonal antibodies (MAb) to human CD4 (Beckman Coulter, Fullerton, CA, USA), CD25 (DAKO, Glostrup, Denmark), CTLA-4 (Pharmingen, San Diego, CA, USA), CD3, CD19 (DAKO), and FITC-conjugated MAb to human CD4 (DAKO) on serial gingival sections with MAbs to CD4, CD25, and CTLA-4 (DAKO), and FITC-conjugated MAb to human CD4 (Pharmingen) were used as described below.

### Immunohistochemistry

Single immunohistochemical staining was performed via an alkaline phosphatase-anti-alkaline phosphatase (APAAP) system (DAKO) on serial gingival sections with MAbs to CD4, CD25, and CTLA-4 as described previously (Yamazaki et al., 1995). Further, double-staining of CD3/CD19, CD4/CD25, and CD4/CTLA-4 was carried out on all specimens by combining an avidin-biotin-alkaline phosphatase-anti-alkaline phosphatase (APAAP) system. In 4 representative samples, triple-staining was performed by the combination of double-immunohistochemistry and immunofluorescent staining for the identification of triple-positive cells for CD4, CD25, and CTLA-4. Briefly, after double-staining of CTLA-4 and CD25 with the APAAP and ABC-PO systems, FITC-conjugated anti-CD4 MAb was added for a further 24 hrs at 4°C in the dark.

### Cell Analysis

The degree of inflammation was confirmed by hematoxylin-eosin (H-E) staining. Cell analysis of selected areas was performed as previously described (Yamazaki et al., 1995). Depending upon the size of the specimen and the infiltrate, 2 or 3 areas of 0.04 mm² with significant round cell infiltration were chosen on the periodontal pocket side, in the center of connective tissue, and on the oral epithelial side of each section. Positively stained cells were counted in each area of serial sections at 400x magnification. We added numbers of CD3⁺ T-cells and CD19⁺ B-cells in serial sections, to estimate the total number of lymphocytes in an area. The B-cell to T-cell (B/T) ratio was defined as CD19⁺ cell number/CD3⁺ cell number.

### RNA Isolation and Real-time Quantitative PCR

Total RNA was isolated from gingival tissues with the use of TRIZOL (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions, and treated with RNase-free DNase I (Invitrogen). The RNA was then reverse-transcribed to cDNA with random primer (TAKARA SHUZO CO., LTD., Shiga, Japan) and M-MLV reverse-transcriptase (Invitrogen). For real-time PCR, primers and probes for FOXP3, IL-10, TGF-β, and GAPDH were all purchased (Applied Biosystems, Foster City, CA, USA). Reactions were conducted in a 25-μL reaction mixture on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), following the pre-developed TaqMan assay reagents protocol (Applied Biosystems). The ABI PRISM SDS 2.0 software (Applied Biosystems) was used to analyze the standards and to carry out the quantifications. The relative quantity of each mRNA was normalized to the relative quantity of GAPDH.

### Statistical Analysis

The difference between the expressions of each molecule was analyzed by the Mann-Whitney U-test. Within each disease group, the numbers of CD3⁺ and CD19⁺ cells were compared by Wilcoxon’s signed-rank test. Correlation coefficients were analyzed between the B/T ratio and the proportion of CD4⁺CD25⁺ or CD4⁺CTLA-4⁺ within the CD4 subset. The statistical significance risk rate was set at p < 0.05.

### RESULTS

**CD4⁺CD25⁺ T-cells and CD4⁺CTLA-4⁺ T-cells in Periodontitis Lesions**

Fifty areas were analyzed. Most specimens showed a dominance of B-cells (median, 122; range, 3-375) over T-cells (median, 65.5; range, 10-333) and CD4⁺ cells (median, 52.5; range, 10-280). There was a statistically significant difference between the numbers of B-cells and T-cells (p = 0.0213). The total number of B-cells tended to be the highest on the periodontal pocket side and the lowest on the oral epithelial side of each section. In contrast, the numbers of T-cells and CD4⁺ cells did not show an apparent distribution pattern. Double-immunohistochemical staining on serial sections demonstrated the presence of individual cells double-positive for either CD4/CD25 (Fig. 1A) or CD4/CTLA-4 (Fig. 1B). Further, the existence of CD4⁺CD25⁺CTLA-4⁺ cells in inflammatory periodontal lesions was confirmed by triple-staining (Figs. 1C, 1D). Although the proportions of both CD4⁺CD25⁺ and CD4⁺CTLA-4⁺ T-cells within the CD4 subset

### Table. Clinical Profile of Gingival Biopsy Sites

<table>
<thead>
<tr>
<th></th>
<th>Periodontitis (n = 17)</th>
<th>Gingivitis (n = 9)</th>
<th>Periodontitis (n = 20)</th>
<th>Gingivitis (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>57.2 ± 11.6*</td>
<td>47.63 ± 19.7</td>
<td>53.1 ± 10.3</td>
<td>30.3 ± 11.7</td>
</tr>
<tr>
<td>Gingival Index</td>
<td>1.12 ± 0.78</td>
<td>0.63 ± 0.52</td>
<td>1.16 ± 0.59</td>
<td>0.29 ± 0.45</td>
</tr>
<tr>
<td>Probing depth (mm)</td>
<td>6.94 ± 2.19</td>
<td>2.50 ± 0.76</td>
<td>7.84 ± 2.52</td>
<td>2.29 ± 0.55</td>
</tr>
<tr>
<td>Loss of attachment (mm)</td>
<td>8.35 ± 2.37</td>
<td>2.88 ± 0.64</td>
<td>8.68 ± 2.60</td>
<td>2.48 ± 0.59</td>
</tr>
<tr>
<td>Tooth mobility</td>
<td>1.76 ± 1.15</td>
<td>0.00 ± 0.00</td>
<td>1.32 ± 0.92</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Bleeding on probing (% site)</td>
<td>47.1</td>
<td>50.0</td>
<td>65.0</td>
<td>14.3</td>
</tr>
<tr>
<td>Bone loss</td>
<td>82.35 ± 24.69</td>
<td>22.86 ± 29.84</td>
<td>72.37 ± 21.24</td>
<td>1.90 ± 3.61</td>
</tr>
</tbody>
</table>

* Data are expressed as mean ± SD.
varied from lesion to lesion, a significantly positive correlation was observed between the proportions of double-positive T-cells and the B/T-cell ratio (r = 0.370, p = 0.0078 for CD4*CD25*; r = 0.359, p = 0.0099 for CD4*CTLA-4*; Figs. 2A, 2C).

Moreover, the proportions of CD4*CD25* cells significantly correlated with CD4*CTLA-4*, suggesting that CD25-expressing CD4 T-cells also express CTLA-4 (r = 0.618, p < 0.0001; Fig. 2E).

**CD4*CD25* T-cells and CD4*CTLA-4* T-cells in Gingivitis Lesions**

Fifteen areas were analyzed. Gingivitis lesions were dominated by T-cells (median, 81; range, 16-157) and CD4* cells (median, 48; range, 18-161) over B-cells (median, 23; range, 2-41). There was a statistically significant difference between the numbers of T-cells and B-cells (p = 0.0007). In contrast to the periodontitis lesions, the number of T-cells was higher on the crevicular side than the oral epithelial side, while the number of B-cells showed no difference between the two areas. Most of the T-cells were CD4*. As with periodontitis lesions, CD4*CD25* T-cells and CD4*CTLA-4* T-cells were also observed in gingivitis lesions. However, it was noted that the proportions of these double-positive cells within the CD4 subset were much lower than those in periodontitis lesions (Figs. 2B, 2D). There was a significantly positive correlation between the proportions of CD4*CD25* and CD4*CTLA-4* T-cells, suggesting again the presence of CD4*CD25*CTLA-4* T-cells in gingivitis lesions (r = 0.514, p = 0.0491; Fig. 2F).

**Comparison of CD4*CD25* and CD4*CTLA-4* T-cells between Periodontitis and Gingivitis**

While CD4*CD25* and CD4*CTLA-4* T-cells were found in both periodontitis and gingivitis lesions, the mean percentage of the double-positive cells within the CD4* cells was higher in periodontitis compared with gingivitis (37.7% vs. 23.7% for CD4*CD25*, 22.8% vs. 12.9% for CD4*CTLA-4*). However, a statistically significant difference was observed only for CD4*CD25* (p = 0.0391; Appendix Fig. 1).

**Analysis of Gene Expression**

The expression of mRNA for FOXP3, TGF-β1, and IL-10 was observed in all tissues of both periodontitis and gingivitis. FOXP3 mRNA expression was significantly higher in periodontitis compared with gingivitis (p = 0.0325; Fig. 3A). Although TGF-β1 mRNA expression tended to be higher in periodontitis than gingivitis (p = 0.0475; Fig. 3B), there was no difference in IL-10 mRNA expression (p = 0.3613; Fig. 3C). When correlations between gene expression were analyzed, there was a positive correlation between FOXP3 and TGF-β1 (r = 0.990, p < 0.0001), FOXP3 and IL-10 (r = 0.942, p < 0.0001), and TGF-β1 and IL-10 in periodontitis (r = 0.963, p < 0.0001; Appendix Fig. 2). In gingivitis, although a significant positive correlation was observed between FOXP3 and TGF-β1 (r = 0.936, p < 0.0001) and between FOXP3 and IL-10 (r = 0.452, p = 0.0388), it was notable that the correlation coefficient of the latter was much lower. In contrast, there was no positive correlation between TGF-β1 and IL-10 (r = 0.329, p = 0.1474; Appendix Fig. 3).

**DISCUSSION**

The results of the present study have demonstrated that putative Tregs, characterized not only phenotypically but also by their gene expression profile, seemed to exist in chronic inflammatory periodontal lesions. Phenotypic markers such as CD25 and CTLA-4, together with other cell-surface molecules such as CD103 and glucocorticoid-induced tumor necrosis factor receptor, are not reliable markers for CD4*CD25* Tregs, because they are inducible upon activation or are also expressed on other cell types. Definitive identification of Treg cells requires the demonstration of FOXP3. Although small numbers of CD25* cells have previously been demonstrated in experimental gingivitis lesions (Seymour et al., 1988), this T-cell population has not been fully characterized. As with other inflammatory lesions of human diseases, such as rheumatoid arthritis (Cao et al., 2003) and inflammatory bowel disease (Makita et al., 2004), CD4*CD25* Treg cells existed in chronic inflammatory periodontal diseases.
The proportion of CD4+CD25+ cells or CD4+CTLA-4+ cells increased with increasing numbers of B-cells in the inflammatory cell infiltrates. Furthermore, the proportions of these double-positive cells correlated well, suggesting that CD4+CD25+ cells also express CTLA-4 and that CD4+CTLA-4+ cells express CD25. This was further confirmed by triple-staining. However, these double-positive or even triple-positive cells cannot necessarily be referred to as 'regulatory T-cells' until functional analyses are carried out. In addition to these phenotypic observations, we clearly demonstrated elevated FOXP3 expression in all periodontitis lesions examined, compared with gingivitis lesions. Considering that FOXP3 is the most characteristic marker for CD4+CD25+ Tr cells at present, it is reasonable to assume that at least some of the CD4+CD25+ or CD4+CTLA-4+ cells are in fact regulatory T-cells.

The precise role of CD4+CD25+ Tr cells in periodontal disease is not known. Since autoantibodies against collagen type I and autologous HSP60 are reported to be produced in periodontitis lesions (Sugawara et al., 1992; Tabeta et al., 2000), at least one of the roles of these CD4+CD25+ Tr cells in periodontitis could be to suppress auto-reactive T-cells, which might be induced or activated during periodontal infection.

In addition to CD4+CD25+ Tr cells, there are other CD4+ regulatory T-cell subsets with distinct phenotypic markers and functions, e.g., Tr1 and Th3 (McGuirk and Mills, 2002). While CD4+CD25+ Tr cells constitutively express CTLA-4 and suppress CD4+CD25+ T-cells in a cell-contact-dependent manner (Read et al., 2000; Takahashi et al., 2000), Tr1 cells are defined as a CD4+CD25+ population which does not express CTLA-4, but which produces IL-10 predominantly. In contrast, Th3 cells are characterized by large amounts of TGF-
β production. The phenotype of this Th3 population is also CD4+CD25+CTLA-4+. In the present study, expression of TGF-β1 mRNA was much higher than that of IL-10. This suggests that, in addition to CD4+CD25+ Tr cells, Th3 cells may also be present in periodontitis lesions. Recently, TGF-β has been shown to induce FOXP3 in activated CD4+CD25+ T-cells, leading to the accumulation of CD4+CD25+ Tr cells (Fantini et al., 2004). This pathway might also be relevant in periodontitis, since CD4+ T-cells in periodontitis lesions are mostly activated memory cells (Yamazaki et al., 1993). In fact, there was a highly positive correlation between TGF-β1 expression and FOXP3 expression in gingivitis and periodontitis.

Further, in the present study, putative CD4+CD25+ Tr cells were also demonstrated in all healthy/gingivitis lesions, even though at lower frequencies than in periodontitis lesions. Considering the vast numbers of bacteria colonizing the gingival sulcus and the lack of tissue destruction in gingivitis, constitutive existence of Tr cells would seem to be a mechanism by which immune responses to these microbes are controlled and periodontal tissue destruction prevented.

It is clear, however, that the differential roles of Tr cells in gingivitis and periodontitis need to be addressed. It is possible to speculate that, in gingivitis lesions, Tr cells control the immune pathology so as to avoid periodontal tissue destruction, whereas, in established periodontitis lesions, Tr cells may be recruited into the lesions in an attempt to suppress tissue destruction through putative autoimmune mechanisms via a negative feedback system. Alternatively, these cells may impede protective immunity to pathogens (Belkaid et al., 2002). Further studies are required, however, to elucidate the respective roles of Tr cells in gingivitis and periodontitis.

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