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Relationship between the presence of periodontopathic bacteria and the expression of chemokine receptor mRNA in inflamed gingival tissues

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Background and Objective: Periodontal disease is a chronic disease characterized by the interaction between periodontopathic bacteria and the host immune response. The aim of this study was to investigate the correlation between periodontopathic bacteria and host immune cell infiltrates.

Material and Methods: Twenty-two patients with chronic periodontitis were included in this study. Gingival tissues were taken at the periodontal surgery after completion of initial therapy. Three types of periodontopathic bacteria were detected by polymerase chain reaction, and the prevalence of mRNA expression of chemokine receptors was examined by reverse-transcription–polymerase chain reaction in the gingival tissues. The infiltration of T and B cells was determined by an immunohistochemical method.

Results: In the patients, both *Porphyromonas gingivalis* and *Tanerella forsythia* were detected, and the mRNA expression of chemokine receptors CXCR1&2, CXCR4, CCR1, CCR2, CCR3 and CCR4 were more prevalent. The mean number of infiltrated B cells was significantly larger than that of T cells in the sites harboring both *P. gingivalis* and *T. forsythia*. Similarly, in the sites where *P. gingivalis* was detected but *T. forsythia* was not, the mean number of B cells was significantly larger than that of T cells. In the sites with mRNA expression of CCR2 and CCR3, the mean number of B cells was significantly larger.

Conclusion: These results suggest that a high proportion of T helper 2-associated chemokine receptor-positive T cells may be associated with the predominance of B cells and may play an important role in the formation of chronic periodontitis in sites where both *P. gingivalis* and *T. forsythia* are detected.

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Periodontal disease is a chronic disease characterized by the interaction between periodontopathic bacteria and the host immune response. Several bacteria have been demonstrated as the key etiological agents of periodontal disease. Although *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Tannerella forsythia* are

thought to be major pathogens of periodontitis, it is considered that periodontal disease is a mixed infection (1). As both *P. gingivalis* and *T. forsythia* were detected more frequently in deeper periodontal pockets, a strong relationship between *P. gingivalis* and *T. forsythia* in subgingival plaque has been suggested (2–4).

It has been reported that periodontitis lesions are characterized by a dense infiltration of B cells and plasma cells, whereas gingivitis lesions display T-cell predominance (5). Polyclonal B-cell activation is believed to be of major significance in the development of B-cell lesions (6). Many polyclonal B-cell activators require T-cell help for activation. T cells expressing the CD4 surface antigen facilitate B-cell maturation and production of antibodies by plasma cells. On the basis of the pattern of cytokine production by CD4 T cells, CD4 T cells can be subdivided into T helper 1 or T helper 2 cells (7). T helper 1 cytokines are important for the generation of cell-mediated immune responses, whereas T helper 2 cytokines are important for the generation of humoral immune responses (8,9). A number of studies have focused on the relative contribution of T helper 1 and T helper 2 cells in periodontal disease by examining the cytokine production profile. There are several studies that examined a T helper cytokine dichotomy in periodontal tissues, peripheral blood and gingival crevicular fluid (10–13). However, patients with chronic periodontitis have elevated levels of both T helper 1 and T helper 2 cytokines. Therefore, the role of T helper 1 and T helper 2 cells in periodontal disease are still controversial. The lack of clear polarization in the cytokine responses in chronic periodontitis might be reflective of the complexity of the polymicrobial infection.

Recent studies have focused on the role of chemokines in the pathogenesis of periodontal disease. Although some chemokines have been found in diseased periodontal tissues, their roles in the pathogenesis of periodontal disease remains unclear (14–17). Chemokines are small secreted proteins involved in the recruitment of well-defined leukocyte subsets and mediate their biological effects after binding to specific receptors. Polarized T helper 1 and T helper 2 populations were shown to have a different chemokine receptor repertoire. For example, CXCR3 and CCR5 are found to be associated with T helper 1 cells, whereas CCR3 and CCR4 are found to be associated with T helper 2 cells. Therefore, the expression of chemokine receptors can account for the formation of specific inflammatory infiltrates. There are some reports about the infiltration of chemokine- and chemokine receptor-expressing cells in periodontal diseases (18,19). However, there are few studies that examine the relationship between the periodontopathic bacteria and the expression of chemokine receptors.

In the present study, to investigate the correlation between periodontopathic bacteria and host immune cell infiltrates, we detected *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythia* in gingival tissue from patients with chronic periodontitis and examined the prevalence of chemokine receptor mRNA expression and the infiltration of T cells and B cells.

Material and methods

Gingival tissue specimens

The institutional review board of Kyushu University Faculty of Dental Science approved this study and informed consent was obtained from all the patients before inclusion in the study.

In total, 24 gingival tissues were obtained from 22 patients referred to the Department of Periodontology, Dental Hospital Kyushu University. All patients (38–66 years old; eight men, 14 women) were categorized into chronic periodontitis groups according to the classification of the American Academy of Periodontology. Gingival tissue was obtained from sites with probing depth > 4 mm at periodontal surgery, after completion of initial therapy including motivation, oral hygiene instruction, scaling and root planing. The gingival tissues were immediately fixed in 10% neutral-buffered formalin and then embedded in paraffin wax. Paraffin-embedded tissues were cut into 4 μm sections.

Detection of periodontopathic bacteria

Total DNA was extracted from paraffin-embedded gingival tissue sections. After deparaffinization, tissue sections were digested with Proteinase K. Then, phenol–chloroform was added and the aqueous phase was transferred to a fresh tube, to which the same volume of isopropanol was added. The solution was incubated for 30 min at −20°C and then centrifuged (20,400 ***g***, 15 min, 4°C). The pellet was washed in ethanol and air-dried at room temperature. DNA samples were resuspended in diethylprocarbonate-treated double distilled-H_2O (Maxim Biotech, Inc., San Francisco, CA, USA). The three species of bacteria (*P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythia*) were detected by polymerase chain reaction (PCR), as previously described (20). The PCR was processed using 5.0 μL of sample added to 45 μL of reaction mixture containing 10× reaction buffer comprising 2.2 mM $MgCl_2$, 0.2 mM dNTP, 2.5 U of *Taq* DNA polymerase and 0.2 μM of each primer. PCR amplification was performed under the following conditions: an initial denaturation step at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 90 s. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel. The agarose gel was stained with ethidium bromide and visualized under ultraviolet illumination.

Detection of chemokine receptor mRNA

The expression of chemokine receptor mRNA was examined by reverse transcription–polymerase chain reaction (RT–PCR) techniques. Total RNA from paraffin-embedded gingival tissue was extracted using the Optimum™ FEPE RNA Isolation Kit (Ambion® Diagnostics, Austin, TX, USA), according to the manufacturer's instructions, and contaminating DNA

was eliminated by incubation with DNase I. The RNA samples were stored at −80°C until use. cDNA was synthesized using 2 μg of RNA through a reverse transcription reaction (RETROscript®; Ambion® Diagnostics). RT–PCR was then performed using the MPCR Kit for Human Chemokine Receptor CXCR and the MPCR Kit for Human Chemokine CCR Set-1 (Maxim Biotech Inc.), according to the manufacturer's instructions. The RT–PCR was processed using 5.0 μL of cDNA added to 45 μL of reaction mixture containing 2× buffer mixture, 10× primers and *Taq* DNA polymerase. PCR amplification was performed under the following conditions: an initial denaturation step at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, and a final elongation step at 72°C for 7 min. The PCR products were analyzed by electrophoresis in 2.5% agarose gel. The agarose gel was stained with ethidium bromide and visualized using ultraviolet illumination.

Immunohistochemical analysis of T and B cells

Serial sections of 4 μm were collected on silane-coated glass slides and used for immunohistochemical staining. Tissue sections were deparaffinized by immersion in xylene and immersed in ethanol for rehydration. Phosphate-buffered saline was used to wash the deparaffinized sections for 5 min. Entire sections were placed in 3% H_2O_2 to block the remaining activity of endoperoxidase. After washing with phosphate-buffered saline, sections were incubated with L.A.B. solution (Polysciences Inc., Warrington, PA, USA) at room temperature for 30 min for the antigen retrieval procedure. Tissue sections were washed with phosphate-buffered saline again and incubated in 10% normal goat serum (Nichirei, Tokyo, Japan) for 30 min to block nonspecific immunoglobulin G binding. CD3 and L26 primary antibodies were used to detect T and B cells, respectively. Slides were then incubated with mouse antihuman primary monoclonal antibodies (CD3 and L26; Nichirei) overnight at 4°C. After rinsing three times in phosphate-buffered saline (5 min per time), sections were incubated with antimouse immunoglobulin G (Nichirei) for 30 min. Following washing three times with phosphate-buffered saline, for 5 min each wash, substrate (DAB Substrate Kit; Nichirei) was added and incubated for ≈15 min. All specimens were washed with distilled water and counterstaining was performed with toluidine blue. Slides were finally dehydrated with ethanol and subsequently mounted with xylene for further microscopic observation and cell counting. The immunostained cells in gingival connective tissue were counted in five squares (0.04 mm^2 each) per section using SCION IMAGE SOFT (Scion Corporation, Frederick, MD, USA).

Statistical analysis

Fisher's exact test or the Student's *t*-test was used to examine the difference between study groups. Values of $p < 0.05$ were considered statistically significant.

Results

Detection of periodontopathic bacteria

The frequencies of periodontopathic bacteria in gingival tissues are shown in Fig. 1A. *P. gingivalis* was detected in 87.5% of the subjects, *T. forsythia* in 58.3% and *A. actinomycetemcomitans* in 4.2%. *P. gingivalis* and *T. forsythia* were both detected in 50% of the subjects; *P. gingivalis*, but not *T. forsythia*, was detected in 37.5% of

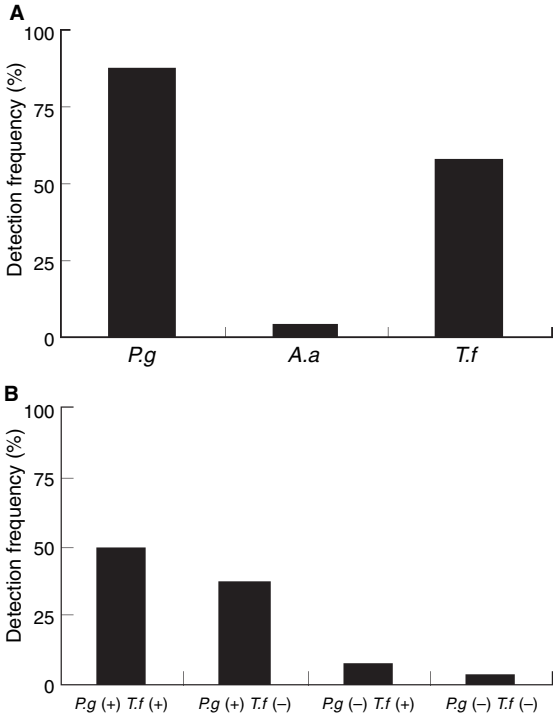

Fig. 1. Detection frequency of periodontopathic bacteria by the polymerase chain reaction. (A) Detection frequency of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Tannerella forsythia*. *P. gingivalis* was detected more frequently (in 87.5% of the subjects), than *T. forsythia* (58.3%) or *A. actinomycetemcomitans* (4.2%). (B) Detection frequency of both *P. gingivalis* and *T. forsythia*. Both *P. gingivalis* and *T. forsythia* [*P.g* (+) *T.f* (+)] were detected in 50% of subjects; *P. gingivalis* alone [*P.g* (+) *T.f* (–)] was detected in 37.5% of subjects; and *P. gingivalis* was undetected in 8.3% [*P.g* (–) *T.f* (+)] and 4.2% *P.g* [(–) *T.f* (–)] of subjects. *A.a*, *Actinobacillus actinomycetemcomitans*; *P.g*, *Porphyromonas gingivalis*; *T.f*, *Tannerella forsythia*.

the subjects; and in a few subjects, *P. gingivalis* was not detected at all (Fig. 1B).

Chemokine receptor mRNA expression

The prevalence of chemokine receptor mRNA was examined in gingival tissues. The mRNAs of CXCR1&2, CXCR4 and CCR3 were more prevalent compared with the mRNAs of the other chemokine receptors studied. CXCR1&2 mRNA was detected in 50% of the subjects, CXCR4 mRNA in 45.8% and CCR3 mRNA in 50% (Fig. 2).

Correlation between periodontopathic bacteria and chemokine receptor mRNA expression

The correlation between the presence of *P. gingivalis* and *T. forsythia* and the prevalence of chemokine receptor mRNA was examined. In the subjects, both *P. gingivalis* and *T. forsythia* were detected; the mRNAs of some chemokine receptors (CXCR1&2, CXCR4, CCR1, CCR2, CCR3, and CCR4) were more prevalent in the presence of both *P. gingivalis* and *T. forsythia* than in the presence of *P. gingivalis* alone (Fig. 3A). There was statistical significance in the prevalences of CXCR1&2, CXCR4 and CCR2 (Fisher exact test; *p*-values of < 0.05 were considered statistically significant).

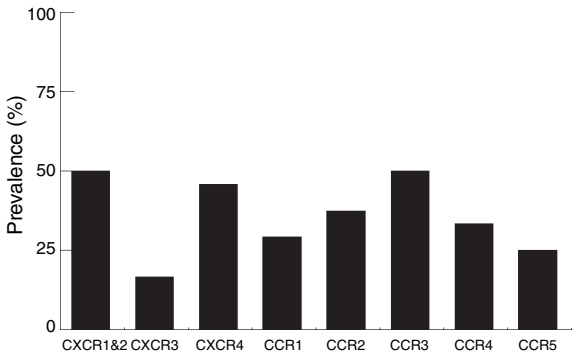

Fig. 2. Prevalence of subjects positive for the expression of chemokine receptor mRNA, as determined by revrese transcription–polymerase chain reaction. CXCR1&2, CXCR4 and CCR3 mRNAs were more prevalent (CXCR1&2 was present in 50%, CXCR4 in 45.8% and CCR3 in 50% of subjects).

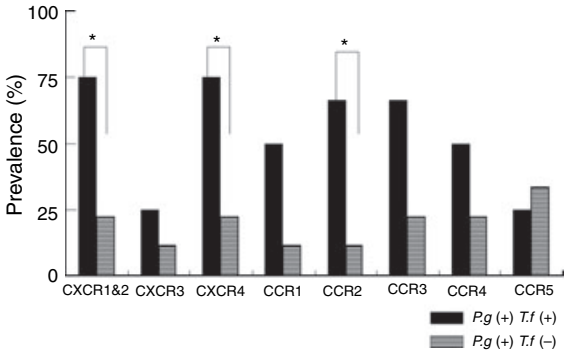

Fig. 3. Relative expression of chemokine receptor mRNA in *P.g* (+) *T.f* (+) sites and in *P.g* (+) *T.f* (–) sites. In *P.g* (+) *T.f* (+) sites, the chemokine receptors CXCR1&2, CXCR4, CCR1, CCR2, CCR3 and CCR4 were more prevalent. There was a significant difference in CXCR1&2, CXCR4 and CCR2 ($p < 0.05$). *P.g*, *Porphyromonas gingivalis*; *T.f*, *Tannerella forsythia*. *, $p < 0.05$.

Immunohistochemical detection of T and B cells

$CD3^+$ and $L26^+$ cells were observed in connective tissues of gingival biopsy. $CD3^+$ cells seemed to infiltrate a large area of connective tissue; by contrast, $L26^+$ cells seemed to infiltrate a small area (data not shown).

Correlation between numbers of T cells and B cells, and periodontopathic and mRNA expression of CC chemokine receptors

$CD3^+$ cells and $L26^+$ cells were counted as stated above. The correlation between the numbers of $CD3^+$ and $L26^+$ cells, and the detection of *P. gingivalis* and *T. forsythia*, were examined. In the subjects, both *P. gingivalis* and *T. forsythia* were detected, and the mean number of $L26^+$ cells was significantly larger than that of $CD3^+$ cells (*t*-test; *p*-values of < 0.01 were considered statistically significant). Similarly, in the subjects, *P. gingivalis* was detected, but *T. forsythia* was not; the mean number of $L26^+$ cells was significantly larger than that of $CD3^+$ cells (*t*-test; *p*-values of < 0.05 were considered statistically significant) (Fig. 4A). In the subjects, CC chemokine receptors were detected, and the mean number of $L26^+$ cells was larger than that of $CD3^+$ cells. There was statistical difference in CCR2 and $CCR3^+$ subjects (*t*-test; *p*-values of < 0.05 were considered statistically significant) (Fig. 4B).

Discussion

P. gingivalis, *A. actinomycetemcomitans* and *T. forsythia* are closely associated with the pathogenesis of periodontitis. These bacteria are detected in the subgingival plaque of periodontitis patients. In the present study, we first tried to detect these bacteria in gingival tissues obtained from chronic periodontitis at periodontal surgery. *P. gingivalis* was detected in 87.5%, *T. forsythia* in 58.3% and *A. actinomycetemcomitans* in 4.2% of tissues, respectively. The results suggested that even after periodontal initial therapy, these bacteria were present in the gingival tissues. The importance of *P. gingivalis* and *T. for-*

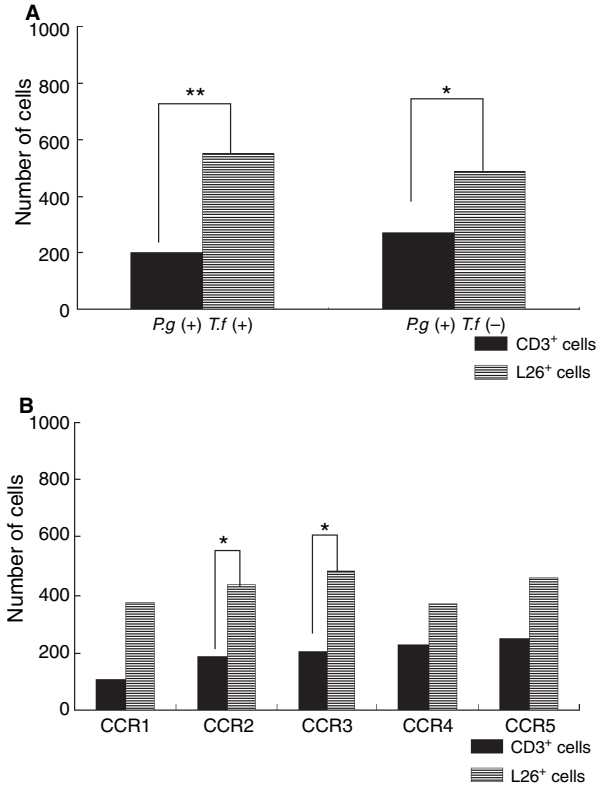

Fig. 4. (A) Mean number of $CD3^+$ and $L26^+$ cells in *P.g* (+) *T.f* (+) sites and *P.g* (+) *T.f* (–) sites. In both *P.g* (+) *T.f* (+) and *P.g* (+) *T.f* (–) sites, there were significantly more $L26^+$ cells than $CD3^+$ cells [$p < 0.01$ in *P.g* (+) *T.f* (+), $p < 0.05$ in *P.g* (+) *T.f* (–)]. (B) The mean number of $CD3^+$ and $L26^+$ cells in CC chemokine mRNA expression sites. In the sites in which CC chemokine receptor was detected, there were more $L26^+$ cells than $CD3^+$ cells. There was statistical difference in subject in whom CCR2 or CCR3 was detected ($p < 0.05$). *P.g*, *Porphyromonas gingivalis*; *T.f*, *Tannerella forsythia*.

sythia in the initiation of chronic periodontitis, as well as the progression to advanced periodontitis, has been reported (2–4). In our study, both *P. gingivalis* and *T. forsythia* were detected in 50% of gingival tissues. Moreover, we detected *A. actinomycetemcomitans* or *T. forsythia* alone in only a few gingival tissues. These results suggest that the co-existence of *P. gingivalis* and *T. forsythia*, or the existence of *P. gingivalis* alone, might be important in the pathogenesis of chronic periodontitis.

Next we examined the prevalence of chemokine receptor mRNA expression in gingival tissue to investigate a T helper 1/T helper 2 balance in chronic periodontitis, because the expression of chemokine receptor mRNA suggests that a T-cell subset is involved. Among CXCR chemokine receptors, CXCR1&2 are expressed in memory T cells, CXCR3 is expressed in T helper 1 cells and CXCR4 is expressed in naïve and memory T cells. Among CCR chemokine receptors, CCR1 and CCR2 are expressed in memory T cells, CCR3 and CCR4 are expressed in T helper 2 cells and CCR5 is expressed in T helper 1 cells. In the present study, we found that CXCR1&2, CXCR4 and CCR3 mRNA were more prevalent. This suggests that there is a tendency towards the development of a predominantly T helper 2 response in chronic periodontitis. On the other hand, Gamonal *et al.* described that CCR5 chemokine receptor-expressing cells were exclusively found in inflamed gingival tissue (18). Taubman *et al.* reported that the number of CCR5- and CXCR3-expressing cells were significantly elevated in inflamed gingival tissues (21). Gartlet *et al.* showed that CCR5 and CXCR3 were more prevalent and expressed more strongly in aggressive periodontitis than in chronic periodontitis. They also reported that the expression of CCR4 was more frequent in chronic periodontitis (19). At the mRNA level, our results suggest the predominance of a T helper 2 response in chronic periodontitis. We tried to examine the distribution of these receptor proteins by immunohistochemistry using specific antibodies. However, we were not able to detect proteins in the paraffin sections. Further studies are required to examine the localization of chemokine receptors.

We examined the correlation between the presence of *P. gingivalis* and *T. forsythia* and the prevalence of chemokine receptor mRNA. CXCR1 &2, CXCR4, CCR1, CCR2, CCR3 and CCR4 mRNAs were more prevalent in gingival tissues in which both *P. gingivalis* and *T. forsythia* were present. This suggests that a tendency towards the development of a predominantly T helper 2 response is intensified in the sites where both *P. gingivalis* and *T. forsythia* exist. It is reported that lipopolysaccharide from *P. gingivalis* induces a T helper 2 effector response in humans, and that fimbriae from *P. gingivalis* induce an inflammatory T helper 1 response (22,23). To date there is no information about lipopolysaccharide from *T. forsythia*. Because it has been reported that the mixed infection of *P. gingivalis* with *T. forsythia* enhances the virulence *in vitro* (24,25), it is possible that some components derived from *T. forsythia* enhance the T helper 2 response induced by *P. gingivalis*.

We examined the relationship between the infiltration of T cells and B cells in inflamed gingival tissues, the presence of periodontopathic bacteria and the prevalence of CC chemokine receptor mRNA. In the gingival tissues co-infected with both *P. gingivalis* and *T. forsythia*, the number of infiltrated B cells was higher than that of T cells. Similarly, in the tissues with *P. gingivalis* alone, the number of infiltrated B cells was larger. However, there was no significant difference between the gingival tissues with both *P. gingivalis* and *T. forsythia* and the tissues with *P. gingivalis* alone. The number of B

cells was larger than that of T cells in the tissues in which CC chemokine receptor mRNA was detected. Furthermore, the expression of chemokine receptor mRNA, associated with the T helper 2 response, was more prevalent. These results suggest that in the gingival tissues where *P. gingivalis* and *T. forsythia* were detected, the expression of CC chemokine receptors might be associated with the predominance of B cells.

In this study, there were no differences in probing pocket depth between sites in which *P. gingivalis* and *T. forsythia* were detected and other sites. This might result from the fact that gingival tissues were obtained from periodontitis patients treated initially with periodontal therapy. Moreover, bleeding on probing was found in all the sites analyzed. Therefore, there were no striking differences in clinical parameters between sites in which *P. gingivalis* and *T. forsythia* were detected and other sites. Further analysis is required to investigate the relationship with local inflammation of gingival tissues obtained from chronic periodontitis patients, such as profiling of inflammatory cytokine expression or qualitative and quantitative analysis of chemokine receptor expression.

In conclusion, a high proportion of T helper 2-associated chemokine receptor-positive T cells may play an important role in the formation of chronic periodontitis after initial periodontal therapy in the sites where *P. gingivalis* and *T. forsythia* are detected.

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