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ORIGINAL ARTICLE

# Presence of *Porphyromonas gingivalis* and plasma cell dominance in gingival tissues with periodontitis

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**OBJECTIVE: *Porphyromonas gingivalis* can invade and survive within its host epithelial cells. The aim of this study was to test our hypothesis that persistent presence of intracellular periodontal pathogens in gingival tissue causes the chronic inflammation and that an inappropriate immune response is a risk factor for periodontitis.**

**METHODS: Together with the presence of *P. gingivalis*, the distribution of B cells, plasma cells, and $CD4^+$, $CD8^+$, and $FOXP3^+$ regulatory T cells was evaluated in gingival tissues from healthy ($n = 7$) and periodontitis ($n = 8$) sites by *in situ* hybridization and immunohistochemistry, respectively.**

**RESULTS: *Porphyromonas gingivalis* was detected in proximity to inflammatory infiltrates in three and seven biopsies from the healthy and periodontitis sites, respectively. Compared with healthy sites, periodontal lesions contained a significantly increased number of each immune cell studied with a relative dominance of plasma cells over T cells.**

**CONCLUSIONS: Persistent bacterial invasion of gingival tissues in combination with a plasma cell-dominant immune response may be involved in the pathogenesis of periodontitis.**

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**Keywords:** periodontium; immunology; inflammatory diseases

## Introduction

Periodontitis is the inflammation of periodontal tissue caused by specific plaque-associated bacteria, which leads to tissue destruction. There is a general consensus at present that the eventual tissue destruction is caused by the host immune response to bacteria rather than the bacteria themselves. However, the nature of the immune response that underlies this pathology remains controversial. There are two conflicting theories on the purported roles of T helper type 1 (Th1) and Th2 responses in periodontal disease (Houri-Haddad *et al*, 2007). One hypothesis is that periodontal lesions can be characterized by delayed-type hypersensitivity and that the Th1 cells are the major mediators of periodontal destruction. The other theory is that a Th2-dominant response leading to the relative dominance of B cells and plasma cells in periodontitis is a risk factor for disease progression (Donati *et al*, 2009). Regulatory T (Treg) cells represent a new subset of effector T cells that has a suppressive function. Two studies have recently reported the presence of Treg cells in periodontitis lesions (Nakajima *et al*, 2005; Cardoso *et al*, 2008) but whether the Treg cells found in periodontitis lesions protect against tissue destruction or contribute to the persistence of periodontal pathogens is not yet clear.

Many periodontopathic bacteria can invade tissues (Lamont and Yilmaz, 2002) and it is noteworthy that among these species *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* also have the capacity to survive and spread to neighbouring cells within the host epithelial cells (Meyer *et al*, 1996; Yilmaz *et al*, 2006). To counter such intracellular pathogens, a cell-mediated immune response is vital. We thus hypothesized that persistent presence of periodontal pathogens in gingival tissue causes chronic inflammation and that a skewed immune response toward humoral immunity is a risk factor for periodontitis. In our present study, we tested this hypothesis by evaluating the distribution of B, plasma, $CD4^+$ T, $CD8^+$ T, and Treg cells, together with the *in situ* presence of *P. gingivalis* in gingival biopsies from periodontitis and clinically healthy sites.

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## Materials and methods

*Patients and biopsies*

Fifteen gingival biopsies were obtained from patients who had undergone periodontal surgery at Kangnam St. Mary's Hospital, Seoul, Korea. Informed consent was obtained from each patient and this study was approved by the institutional review board of the hospital. These biopsied samples were divided into periodontitis and healthy groups. Gingival biopsies ($n = 8$) from seven periodontitis patients (three men and four women; mean age 42.4 years, range 29–59 years) were obtained during resective periodontal surgeries from sites showing signs of tissue destruction (a mean probing pocket depth (PPD) 5.9 mm, range 3.5–7.5 mm) and active inflammation i.e. redness, bleeding on probing (BOP) and swelling. These patients were diagnosed with chronic periodontitis and exhibited generalized loss of attachment (i.e. clinical attachment level; CAL $\geq$ 3 mm), PPD $\geq$ 4 mm, and bone loss of $>$25%, which included the biopsied sites. One patient donated two biopsies from separate surgeries on different quadrants. As controls, clinically healthy biopsies ($n = 7$) were obtained from surgical crown lengthening procedures for aesthetic or prosthetic reasons from seven subjects (two men and five women; mean age 48.6 years, range 22–69 years). Among these seven control subjects, three were in maintenance from treatments for previously diagnosed chronic periodontitis. However, all seven biopsies were from clinically healthy sites defined by the following criteria: no loss of attachment (CAL $<$ 3mm), PPD $\leq$ 3 mm and BOP-negative. Excised tissues were fixed with formalin-free zinc fixative (BD Bioscience, San Diego, CA, USA) and embedded in paraffin.

*In situ hybridization*

To determine the potential presence of bacteria in periodontitis lesions, *P. gingivalis* was selected as a test species from the list of periodontopathic bacteria due to its known ability to invade and then survive inside epithelial cells (Yilmaz *et al*, 2006). A 343-bp DNA fragment of *P. gingivalis* 16S rRNA was amplified by PCR using the following primers: 5′-TGC AAC TTG CCT TAC AGA GG-3′ and 5′-ACT CGT ATC GCC CGT TAT TC-3′. The resulting 343 bp product contains the V1 and V2 hypervariable regions of 16S rRNA (Chakravorty *et al*, 2007). As a negative control, we used a 349-bp DNA fragment of porcine circovirus open reading frame 1 that specifically detects porcine circovirus-1 (Choi and Chae, 1999). Amplifications were performed under the following cycling conditions: 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min 20 s followed by a 5-min extension at 72°C. After purification, the amplified products were labeled by random priming with digoxigenin-dUTP using a commercial kit (Roche Applied Science, Penzberg, Germany) to produce probes.

*In situ* hybridizations were performed as previously described (Choi and Chae, 1999). Briefly, paraffin-embedded sections (4 $\mu$m) were prepared by a sequential deparaffinization in xylene, re-hydration, de-proteinization, digestion with proteinase K, fixation and acetylation. The digoxigenin-labeled probes were diluted in a hybridization buffer [2X SSC containing 50% deionized formamide, 10 mg ml$^{-1}$ salmon sperm DNA (Invitrogen, San Diego, CA, USA), 0.02% sodium dodecyl sulfate, 1% Denhardt's solution, and 10% dextran sulphate solution (Invitrogen)] heated at 100°C for 10 min and quenched on ice. After the probe was applied to the prepared sections, the slides were heated for 10 min at 90°C and hybridized at 42°C overnight. After thorough washing, the tissue sections were blocked with a blocking buffer (Roche Applied Science) for 1 h, and the hybridized probe was detected with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science) and a substrate consisting of nitroblue tetrazolium and 5-bromocresyl-3-indolyphosphate (Roche Applied Science). The sections were then counterstained with 0.5% methyl green and mounted.

To test the specificity of our probes, gDNA was extracted from the following eight oral bacterial species: *Streptococcus sanguinis* NCTC 10904, *S. gordonii* ATCC 10558, *Veillonella atypica* ATCC 17744, *Fusobacterium nucleatum* ATCC 25586, *Prevotella intermedia* ATCC 25611, *P. gingivalis* ATCC 49417, *Tannerella forsythia* ATCC 43407 and *Treponema denticola* ATCC 33521. After denaturation at 100°C for 10 min and quenching on ice, the bacterial gDNA samples (0.5 $\mu$g each) were immobilized to a nylon membrane (Hybond, Amersham Pharmacia Korea, Seoul, Korea). This membrane was then washed with 2 $\times$ SSC, incubated with a blocking buffer at 37°C for 30 min and then subjected to hybridization and detection procedures as described above.

*Antibodies*

The primary mouse monoclonal antibodies and dilutions used for immunohistochemistry are as follows: anti-CD4 clone 4B12 (1:50, Monosan, Uden, the Netherlands), anti-CD8 clone 4B11 (1:50, Serotec, Oxford, UK), anti-CD20 clone 7D1 (1:100, Serotec), anti-CD138 clone B-A38 (1:100, Serotec), and anti-Foxp3 clone 221D/D3 (1:500, Serotec).

*Immunohistochemistry*

Serial paraffin-embedded sections (4 $\mu$m) were deparaffinized in xylene, hydrated and then incubated in a Tris/EDTA pH 9.0 buffer at 125°C for 3 min to expose the antigens. After washing with running water, the sections were immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity and then subjected to immunohistochemistry using an autoimmunostainer (LabVision, Fremont, CA, USA). Briefly, the sections were incubated with primary antibodies at room temperature for 1 h, washed three times with TBS (Tris-buffered saline and Tween 20, pH 7.6) for 5 min, incubated with anti-mouse EnVision+ system-HRP (Dako, Glostrup, Denmark) followed by streptavidin-HRP (Dako) for 30 min, washed with TBS again, and then visualized with 3,3′-diaminobenzidine tetrahydrochloride (Dako). The sections were then

counterstained with Mayer's haematoxylin and mounted. The number of immunolabelled cells per unit area (number of cells $mm^{-2}$) was quantified by computerized morphometry (SPOT version 4.6, Diagnostic Instruments, Inc. Sterling Heights, MI, USA) with an Olympus IX70 microscope (Olympus, Tokyo, Japan). Since CD138 (also known as Syndecan-1) is expressed not only in plasma cells but also in the epithelium (Sanderson *et al*, 1989; Soukka *et al*, 2000), only small round $CD138^+$ cells in the lamina propria were counted as plasma cells. Immunolabelled cells were counted in five randomly chosen microscopic fields per section, and the data for each patient represent the mean of five quantifications.

*Statistics*

The differences between the periodontitis and control groups were analysed with the Mann–Whitney *U*-test using SAS 9.1.3 software. Statistical significance was set at $P < 0.05$.

## Results

*Presence of P. gingivalis within gingival tissues*

A probe against *P. gingivalis* 16S rRNA was found to specifically hybridize to *P. gingivalis* when tested against a gDNA panel of eight oral bacterial species immobilized onto a membrane (Figure 1a). When performing *in situ* hybridization analysis of tissue sections, however, incomplete washing often results in false positive signals. Since a porcine circovirus cannot be detected in human tissues, a specific probe against this virus was used as a negative control. The porcine circovirus-specific probe failed to hybridize any of bacterial gDNAs on the panel (Figure 1a). The integrity of this probe was validated by the positive signals obtained using a porcine superficial inguinal lymph node from an infected animal (Figure 1b). Conversely, the *P. gingivalis*-specific probe did not hybridize to this control porcine tissue (Figure 1b). *In situ* hybridization with the *P. gingivalis*-specific probe revealed the presence of this bacterium within the gingival tissues from seven out of the eight periodontitis biopsies. Positive signals for *P. gingivalis* were associated with cells (Figure 2). Three out of seven healthy biopsies were also found to harbor *P. gingivalis in situ*, but the frequency and intensity of the positive cells was reduced. Interestingly, all three gingival biopsies from the control group showing positive signals for *P. gingivalis* were sampled from the healthy sites of patients who are in maintenance from treatments for previously diagnosed chronic periodontitis. Even the clinically healthy tissues contained a small area of inflammation near to the junctional epithelium in six cases (Figures 2a and 3a). When the *in situ* hybridized sections were compared with the corresponding H&E stained specimens, most *P. gingivalis*-associated cells were found in the connective tissue within or near to the inflammatory infiltrates (Figure 2b and c). The porcine circovirus-specific probe did not produce any positive signals in any of the human gingival tissues examined (Figure 2).

*Immunohistochemical characterization of infiltrating immune cells*

The distribution of immune cells in our gingival tissue samples was analysed by immunohistochemistry using antibodies against CD4, CD8, CD20 and CD138 to detect $CD4^+$ T, $CD8^+$ T, B, and plasma cells, respectively. In addition, Treg cells were detected using an antibody against the transcription factor, FOXP3 (Nik Tavakoli *et al*, 2008). Compared with the clinically healthy tissues where inflammation was found to be restricted to a small area near the junctional epithelium, the periodontal lesions presented with extensive inflammation that reached the oral epithelium (Figure 3a). Whereas the four CD markers stained the plasmic membrane, FOXP3 staining was observed in the nuclei of Treg cells. In contrast to the $CD4^+$ and $CD8^+$ T cells that were observed within the epithelium and the lamina propria of normal tissues without inflammation, B or plasma cells were detected only in the areas of inflammatory infiltration. Similarly, Treg cells were found only within the inflammatory infiltrates (Figure 3b).

In the periodontitis group, in addition to extensive inflammation, we observed an increase in each of the immune cell types studied and a relative dominance of plasma cells over T cells (Table 1). An altered ratio of Treg to $CD4^+$ T cells would likely suggest an imbalance between the suppressor and effector functions of $CD4^+$ T cells but no difference in these ratios between the two groups was evident (Table 1, $P = 0.8323$).

## Discussion

In this study, we demonstrate that the presence of *P. gingivalis* in gingival tissues and a plasma cell-dominant immune response is associated with periodontitis.

*Porphyromonas gingivalis* was detected in inflammatory regions of gingival biopsies from periodontitis lesions as well as from healthy sites of subjects previously treated for periodontitis but not in those from healthy sites of periodontally healthy subjects. The presence of bacteria in gingival tissues with periodontitis was reported in many previous studies. Although *P. gingivalis* in particular has been identified in these lesions using different methods including immunofluorescence, immunohistochemistry, and fluorescence *in situ* hybridization, histological information on the infection sites remains limited (Peković and Fillery, 1984; Saglie *et al*, 1986; Colombo *et al*, 2007). *In situ* hybridization using digoxigenin-labelled DNA probes is a stable method for the detection of infectious micro-organisms in tissues and also provides histological information (Chae, 2004). A drawback of this method is that the series of preparation procedures, including enzyme digestion, can damage the sample tissues. Epithelial tissues in particular were more susceptible to tissue damage than connective tissue and only the cytoplasmic membranes of the epithelium remained. Hence, *P. gingivalis* was mainly detected in the connective tissue. Recently, the intracellular survival of *P. gingivalis* in macrophages has also been reported (Wang and Hajishengallis, 2008),

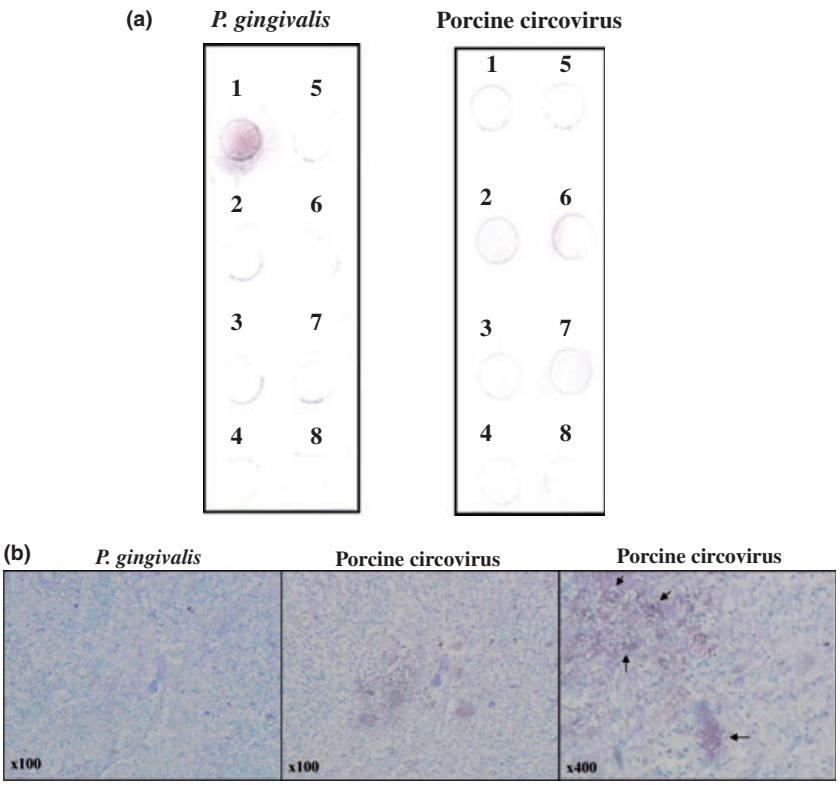


**Figure 1** Specificity of the probes generated against *P. gingivalis* and porcine circovirus. **(a)** Bacterial gDNA from *P. gingivalis* (1), *T. forsythia* (2), *S. sanguinis* (3), *P. intermedia* (4), *T. denticola* (5), *S. gordonii* (6), *F. nucleatum* (7), and *V. atypica* (8) were immobilized onto nylon membranes which were then hybridized with either *P. gingivalis*- or porcine circovirus-specific probes. **(b)** Superficial inguinal lymph node sections from an infected pig were subjected to *in situ* hybridization analysis for the presence of *P. gingivalis* and porcine circovirus. Arrows indicate positive signals

suggesting that the *P. gingivalis*-associated cells we observed may be macrophages. Other invasive oral bacteria in addition to *P. gingivalis* could also be present in gingival tissues, particularly in our samples that contained inflammatory infiltrates but were negative for *P. gingivalis* (one periodontitis and three clinically healthy biopsies). Alternatively, the level of *P. gingivalis* in these biopsies might be below the sensitivity of current methods. In the present study, all three healthy biopsies with positive signals for *P. gingivalis* were from the healthy sites of periodontitis patients. This result coincides with the findings of earlier studies in which spirochetes and *P. gingivalis* were found to be more prevalent in the healthy sites of periodontitis subjects than in the healthy sites of periodontally healthy subjects (Riviere *et al*, 1996; Socransky and Haffajee, 2005).

Although bacterial invasion has been suggested as a potential pathogenic factor of periodontitis for decades (Allenspach-Petrzilka and Guggenheim, 1983), the pathway to eventual tissue destruction remains an open question. In our current study, the association of inflammatory infiltrates with the presence of *P. gingivalis* was evident, indicating that these inflammatory cells may be recruited to the sites in response to bacteria present *in situ*. Furthermore, we found that the periodontitis lesions we analysed contained increased levels of *P. gingivalis* and infiltrates including $CD4^+$ T, $CD8^+$ T, B, plasma, and Treg cells.

Among inflammatory cell types showing increased numbers in advanced periodontitis lesions, the predominance of plasma cells has been well established (Berglundh *et al*, 2007), and was confirmed also in our present experiments. Importantly, neither B nor plasma cells were detectable in the areas without inflammatory infiltrates. Although B cells were recruited to inflammatory regions both in the clinically healthy and periodontitis sites, these cells seem to preferentially differentiate into plasma cells only in periodontitis lesions. It can be speculated that this is due to differences in the cytokine environments.

FOXP3 is a master gene regulator that represses many downstream effector genes of the T cell activation pathways and is sufficient to characterize both naturally occurring and induced Treg cells (Nik Tavakoli *et al*, 2008). Similar to B and plasma cells, $FOXP3^+$ cells were detectable only among inflammatory infiltrates, suggesting that these cells are not required to maintain the tolerogenic properties of the oral mucosa. The number of $FOXP3^+$ cells was also found to be significantly increased in periodontal lesions compared with healthy sites although the frequencies of $FOXP3^+$ cells among the total $CD4^+$ T cells in the two groups were equivalent. Similar to our present results, Nakajima *et al* (2005) previously reported the increased expression of *FOXP3* mRNA in periodontitis compared with gingivitis. Treg cells recruited to infection sites may therefore act to control the immune response and limit the damage to surrounding tissues (Belkaid, 2008). On the other hand, the suppression of effector cells may result in pathogen survival and persistence of infection (Hisaeda *et al*, 2004). Since most $CD4^+$

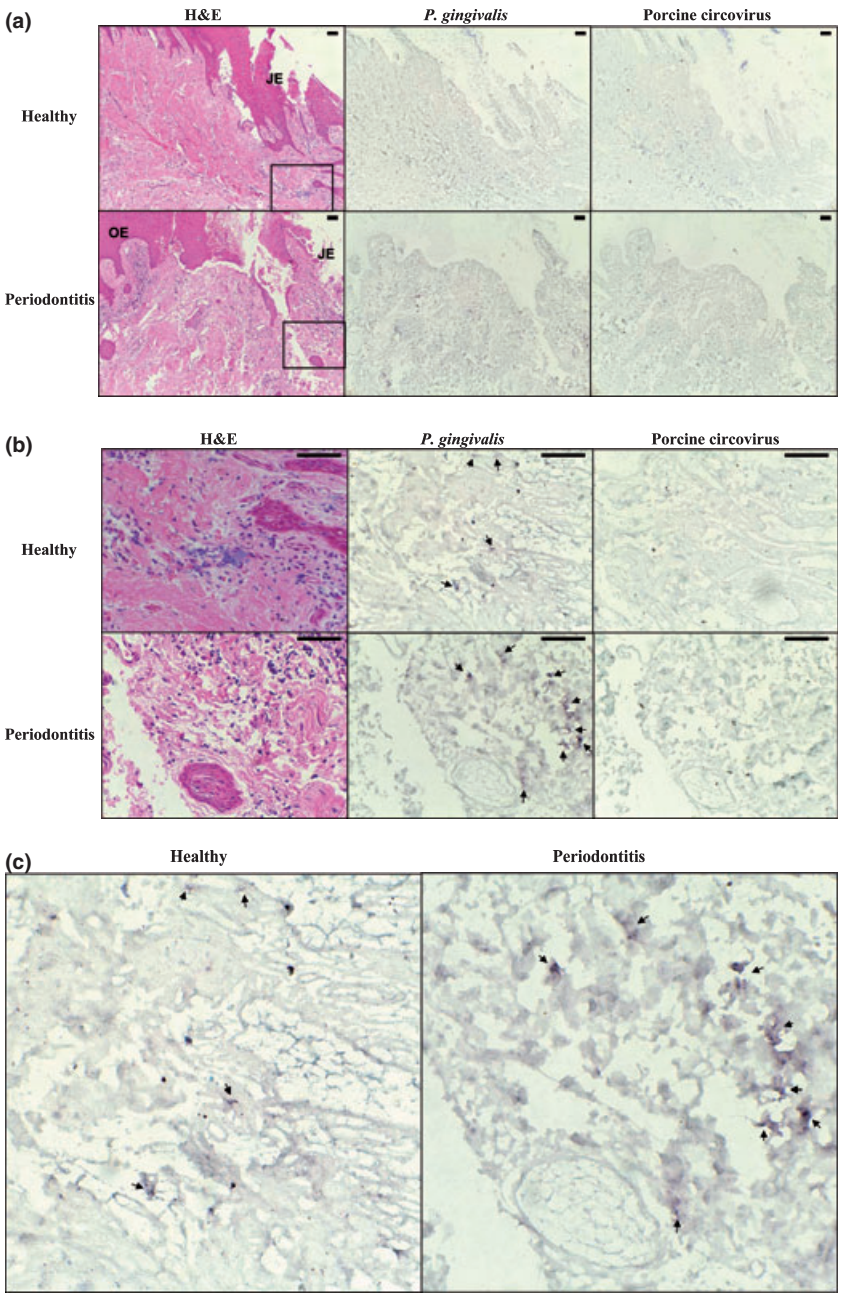


**Figure 2** Detection of *P. gingivalis* in gingival tissues by *in situ* hybridization. Gingival biopsies from clinically healthy and periodontitis sites were subjected to haematoxylin and eosin (H&E) staining and *in situ* hybridization analysis for the presence of *P. gingivalis* and porcine circovirus. The regions examined at a higher magnification in **(b)** are indicated by the boxed areas in **(a)**. The areas with positive signals are shown as enlarged images in **(c)**. Final magnifications, ×100 **(a)** and ×400 **(b, c)**. Bars: 50 $\mu$m; OE: oral epithelium; JE: junctional epithelium. Arrows indicate positive signals stained in violet colour

T cells in periodontitis lesions are activated (Yamazaki *et al*, 1993), the ratios of Treg to effector T cells in periodontitis and healthy sites seem to be similar, thus supporting the former possibility rather than the latter.

Based on our present data, we propose that persistent infection with invasive bacteria causes chronic inflammation and tissue destruction in periodontitis. Subgingival biofilm provides the source of persistent infection, justifying current therapies that are focused on its removal. The ability of periodontal pathogens to survive within host cells must be another important factor that leads to persistent infection. Various immune evasive characteristics of periodontal pathogens have now been documented (Darveau *et al*, 1998; Gemmell *et al*, 2006; Ji *et al*, 2007a,b). We thus speculate that these periodontal pathogens may also modulate the host immune response from a cell-mediated immunity toward a futile humoral immunity which facilitates the survival of intracellular pathogens. The Th1 *vs* Th2 theories, which seem to conflict, may thus both prove to be correct. Th1 cells usually induce more tissue damage than Th2 cells. After infection is cleared, the inflammatory process is programmed to engage in tissue repair. However, persistent infection due to an inappropriate immune response and chronic inflammation may result in unrecoverable periodontal destruction. In this regard, neither Th2 nor plasma cells are suitable for controlling

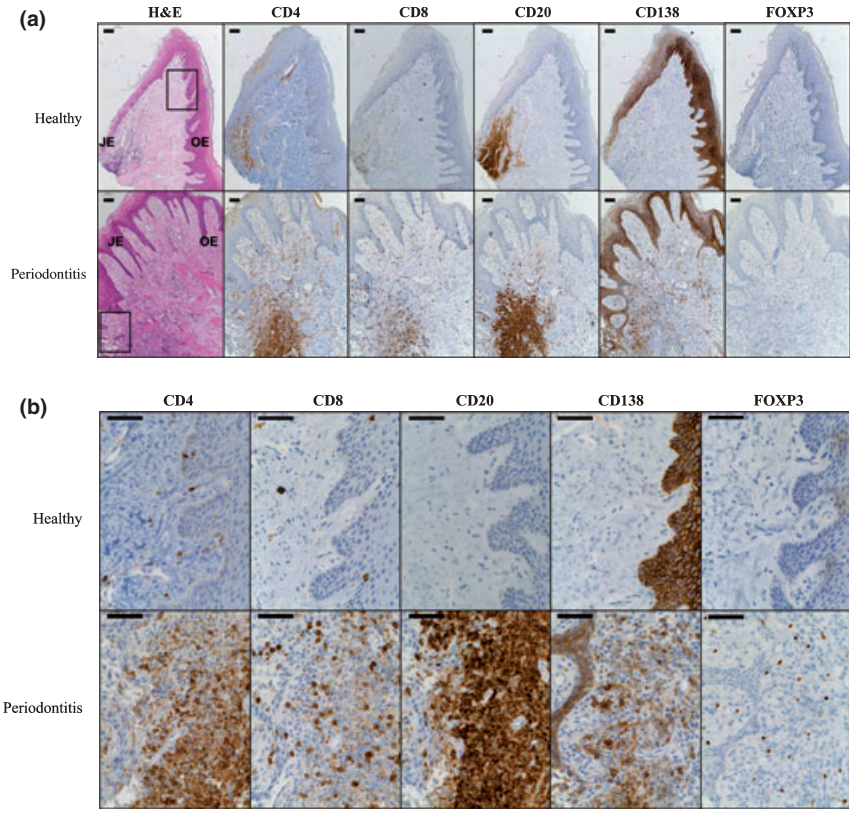


**Figure 3** Immunohistochemical characterization of infiltrating immune cells in gingival tissues. Gingival biopsies from clinically healthy (top panels) and periodontitis (bottom panels) sites were subjected to H&E staining and immunohistochemistry using antibodies against CD4, CD8, CD20, CD138, and FOXP3. The regions examined at a higher magnification in **(b)** are indicated by the boxed areas in **(a)**. Final magnifications, ×100 **(a)** and ×400 **(b)**. Bars: 50 $\mu$m; OE: oral epithelium; JE: junctional epithelium

**Table 1** Quantitative analysis of immunohistochemical results

| *Cell type/ratio* | *Periodontitis*[a] *(*n = *8)* | *Healthy*[a] *(*n = *7)* | P *value* |
|---|---|---|---|
| CD4 | 693.0 (252.1–1193.7) | 305.0 (12. 8–766.7) | 0.0443 |
| CD8 | 418.6 (117.5–757.2) | 111.1 (8.9–304.8) | 0.0043 |
| CD20 | 1026.5 (249.2–1498.5) | 273.0 (0–722.3) | 0.0059 |
| CD138 | 793.0 (196.8–1741.4) | 94.2 (0.7–285.7) | 0.0015 |
| FOXP3 | 43.3 (9.7–135.5) | 14.4 (0–36.5) | 0.0262 |
| B/T[b] | 1.0 (0.2–1.9) | 0.8 (0–2.1) | 0.3408 |
| P/T[c] | 0.9 (0.1–2.2) | 0.2 (0–2.1) | 0.0081 |
| FOXP3/CD4 | 0.057 (0.039–0.13) | 0.062 (0–0.18) | 0.8323 |

[a]Data are expressed as the mean with the range shown in parentheses.
[b]The B to T cell ratio was calculated by CD20/(CD4 + CD8).
[c]The plasma to T cell ratio was calculated by CD138/(CD4 + CD8).

intracellular infections by bacteria such as *P. gingivalis*, and a Th2-dominant immune response may contribute to persistent infection.

Collectively, our current data indicate that the presence of *P. gingivalis* in gingival tissue in combination with plasma cell dominance may play a key role in the development of periodontitis.

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## References

Allenspach-Petrzilka GE, Guggenheim B (1983). Bacterial invasion of the periodontium; an important factor in the pathogenesis of periodontitis? *J Clin Periodontol* **10:** 609–617.

Belkaid Y (2008). Role of Foxp3-positive regulatory T cells during infection. *Eur J Immunol* **38:** 918–921.

Berglundh T, Donati M, Zitzmann N (2007). B cells in periodontitis: friends or enemies? *Periodontol 2000* **45:** 51–66.

Cardoso CR, Garlet GP, Moreira AP, Júnior WM, Rossi MA, Silva JS (2008). Characterization of CD4 + CD25 + natural regulatory T cells in the inflammatory infiltrate of human chronic periodontitis. *J Leukoc Biol* **84:** 311–318.

Chae C (2004). Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. *Vet J* **168:** 41–49.

Chakravorty S, Helb D, Burday M, Connell N, Alland D (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J Microbiol Methods* **69:** 330–339.

Choi C, Chae C (1999). In-situ hybridization for the detection of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. *J Comp Pathol* **121:** 265–270.

Colombo AV, da Silva CM, Haffajee A, Colombo AP (2007). Identification of intracellular oral species within human crevicular epithelial cells from subjects with chronic periodontitis by fluorescence in situ hybridization. *J Periodontal Res* **42:** 236–243.

Darveau RP, Belton CM, Reife RA, Lamont RJ (1998). Local chemokine paralysis, a novel pathogenic mechanism for *Porphyromonas gingivalis*. *Infect Immun* **66:** 1660–1665.

Donati M, Liljenberg B, Zitzmann NU, Berglundh T (2009). B-1a cells and plasma cells in periodontitis lesions. *J Periodontal Res* **44:** 683–688.

Gemmell E, Drysdale KE, Seymour GJ (2006). Gene expression in splenic CD4 and CD8 cells from BALB/c mice immunized with *Porphyromonas gingivalis*. *J Periodontol* **77:** 622–633.

Hisaeda H, Maekawa Y, Iwakawa D *et al* (2004). Escape of malaria parasites from host immunity requires CD4 + CD25 + regulatory T cells. *Nat Med* **10:** 29–30.

Houri-Haddad Y, Wilensky A, Shapira L (2007). T-cell phenotype as a risk factor for periodontal disease. *Periodontol 2000* **45:** 67–75.

Ji S, Hyun J, Park E, Lee BL, Kim KK, Choi Y (2007a). Susceptibility of various oral bacteria to antimicrobial peptides and to phagocytosis by neutrophils. *J Periodontal Res* **42:** 410–419.

Ji S, Kim Y, Min B-Y, Han SH, Choi Y (2007b). Innate immune responses of gingival epithelial cells to nonperiodontopathic and periodontopathic bacteria. *J Periodontal Res* **42:** 503–510.

Lamont RJ, Yilmaz O (2002). In or out: the invasiveness of oral bacteria. *Periodontol 2000* **30:** 61–69.

Meyer DH, Lippmann JE, Fives-Taylor PM (1996). Invasion of epithelial cells by *Actinobacillus actinomycetemcomitans*: a dynamic, multistep process. *Infect Immun* **64:** 2988–2997.

Nakajima T, Ueki-Maruyama K, Oda T *et al* (2005). Regulatory T-cells infiltrate periodontal disease tissues. *J Dent Res* **84:** 639–643.

Nik Tavakoli N, Hambly BD, Sullivan DR, Bao S (2008). Forkhead box protein 3: essential immune regulatory role. *Int J Biochem Cell Biol* **40:** 2369–2373.

Peković DD, Fillery ED (1984). Identification of bacteria in immunopathological mechanisms of human periodontal diseases. *J Periodontal Res* **19:** 329–351.

Riviere GR, Smith KS, Tzagaroulaki E *et al* (1996). Periodontal status and detection frequency of bacteria at sites of periodontal health and gingivitis. *J Periodontol* **67:** 109–115.

Saglie FR, Smith CT, Newman MG *et al* (1986). The presence of bacteria in the oral epithelium in periodontal disease. II. Immunohistochemical identification of bacteria. *J Periodontol* **57:** 492–500.

Sanderson RD, Lalor P, Bernfield M (1989). B lymphocytes express and lose syndecan at specific stages of differentiation. *Cell Regulation* **1:** 27–35.

Socransky SS, Haffajee AD (2005). Periodontal microbial ecology. *Periodontol 2000* **38:** 135–187.

Soukka T, Pohjola J, Inki P, Happonen RP (2000). Reduction of syndecan-1 expression is associated with dysplastic oral epithelium. *J Oral Pathol Med* **29:** 308–313.

Wang M, Hajishengallis G (2008). Lipid raft-dependent uptake, signalling and intracellular fate of *Porphyromonas gingivalis* in mouse macrophages. *Cell Microbiol* **10:** 2029–2042.

Yamazaki K, Nakajima T, Aoyagi T, Hara K (1993). Immunohistological analysis of memory T lymphocytes and activated B lymphocytes in tissues with periodontal disease. *J Periodontal Res* **28:** 324–334.

Yilmaz O, Verbeke P, Lamont RJ, Ojcius DM (2006). Intercellular spreading of *Porphyromonas gingivalis* infection in primary gingival epithelial cells. *Infect Immun* **74:** 703–710.