Characterization of Gingival Th1, Th2 and Th17 Cells in Murine Periodontitis Model

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Abstract
Cellular and molecular mechanisms of the immune system influencing oral bone metabolism remain to be elucidated. In this study, we characterize the mucosal cytokine production by CD4⁺ T cells in the inflamed gingiva of mice infected with Porphyromonas gingivalis (P. gingivalis). A murine periodontal disease model with alveolar bone loss showed significant levels of FimA-specific salivary secretory IgA and plasma IgG Ab responses. Further, IL-6 and TNF-α production was elevated when compared with sham-infected mice. The frequencies of Th1 (IFN-γ), Th2 (IL-4) and Th17 (IL-17) producing CD4⁺ T cells were also increased in P. gingivalis infected mice. Among these cytokines, a significantly higher frequency of IFN-γ producing CD4⁺ T cells was noted. The kinetics of intracellular cytokine analyses revealed a significantly increased frequency of IFN-γ, IL-4 and IL-17-producing CD4⁺ T cells one day after infection. Further, the frequency of IFN-γ producing CD4⁺ T cells was maintained throughout the experimental period. Although IL-4 and IL-17 production was intact until 15 days after the infection, the numbers of CD4⁺ T cells producing these cytokines were reduced thereafter. These results indicate that Th1-type CD4⁺ T cells play distinct roles in the induction and regulation of periodontal disease when compared with Th2- and Th17-type cytokine-producing CD4⁺ T cells.

Keywords: CD4⁺ T cells, periodontal disease, cytokine, gingival mucosa

Introduction
The pathogenesis of periodontal disease (PD) is an inflammatory process involving both innate and adaptive immune responses (1-4). PD is characterized by the host-mediated destruction of soft tissue caused by the induced production and activation of lytic enzymes and stimulated osteoclastogenesis (5-7). Although it is widely recognized that PD is chronic, the nature of the chronicity has not been established. Thus, it is uncertain whether PD is a continuous process or consists of episodes of exacerbation and remission. However, there is an established relationship between periodontal bone resorption and certain bacteria (3, 4). The inflammatory process occurring in PD is characterized by an infiltration of leukocytes, which limit the level of bacterial invasion. There are a number of factors that promote leukocyte recruitment, including bacterial products, cytokines, chemokines, lipid mediators, complement components and cross-talk between innate and adaptive immune systems (1-4).

The oral cavity is considered to be one type of mucosal tissue that is protected by both the mucosal and systemic arms of the immune system. The induction of mucosal S-IgA antibodies (Abs) in saliva and in the bloodstream, e.g., plasma IgG in gingival crevicular fluid together defend the oral cavity against invading pathogens (8, 9). Another unique feature of the oral cavity is that it consists of both mucosal and bone tissue when compared with other mucosal compartments such as the gastrointestinal and respiratory tracts (8, 9). Thus, it is clear that the cellular and molecular intranet between mucosal and osteoimmune systems play a critical role in the regu-
lation of bone homeostasis. Indeed, it has been shown that a tumor necrosis factor (TNF) family member, RANKL (receptor activator of NF-κB ligand), is a key differentiation factor for osteoclasts (10, 11). Furthermore, it was reported that RANKL is expressed on activated T and B cells (10–12). These findings clearly show the potential for crosstalk between immune responses and bone turnover. However, cellular and molecular mechanisms of the immune system influencing oral bone metabolism remain to be elucidated, particularly when the immune system has been activated by infection or becomes dysregulated. In conditions such as periodontitis, infiltrating CD4+ T cells and their produced cytokines play the key roles and alter bone homeostasis regulated by bone formation (osteoblasts) and resorption (osteoclasts) (11). In this regard, it is important to determine cytokine production by CD4+ T cells in inflamed gingival mucosa, which can largely influence alveolar bone resorption.

**Materials and Methods**

**Mice**

BALB/c mice were purchased from the Frederick Cancer Research facility (National Cancer Institute, National Institutes of Health, Frederick, MD). Mice were housed in microisolators, maintained in horizontal laminar flow cabinets, and provided sterile food and water as part of a specific-pathogen-free facility in the Immunobiology Vaccine Center at the University of Alabama at Birmingham (UAB). The health of the mice was monitored by both serology for bacterial and viral pathogens and immunohistology. All of the mice used in these experiments were free of bacterial and viral pathogens. All of the animal studies were done in accordance with both National Institutes of Health (NIH) in U.S. department and UAB institutional guidelines.

**Induction of periodontal inflammation with alveolar bone loss**

Mice were orally infected with *P. gingivalis* ATCC (American Type Culture Collection) strain 33277 as described previously with minor modifications (13, 14). Briefly, mice were given sulfamethoxazole–trimethoprim (Sulfatrim; Goldline Laboratories, Ft. Lauderdale, Fla.) at 10 ml per pint of deionized water *ad libitum* for 10 days. This was followed by a 3-day antibiotic–free period. Mice were then administered 10⁶ colony–forming units (CFU) of *P. gingivalis* suspended in 100 μl of phosphate buffered saline (PBS) with 2% carboxymethylcellulose via oral topical application over three weeks for a total of 15 inoculations. Control groups include sham–infected mice, which received the antibiotic pretreatment and carboxymethylcellulose without *P. gingivalis*. Thirty days after the last infection, horizontal bone loss around the mandibular molars was confirmed by a morphometric method as described previously (15).

**P. gingivalis Fim–A specific Abs assay**

*P. gingivalis* Fim–A specific Abs in plasma and external secretions were determined by an enzyme–linked immunosorbent assay (ELISA) as previously described (16–18). Briefly, 96–well Falcon microtest assay plates (BD Biosciences, San Jose, CA) were coated with 2 μg/ml Fim–A in PBS. Plates were incubated at 4°C overnight in a humid atmosphere and washed three times with PBS. After blocking with 1% bovine serum albumin (BSA) (Sigma–Aldrich, St. Louis, MO) in PBS for overnight at 4°C. The plates were washed three times with PBS. Serial dilutions of samples were added in duplicate. Starting dilution of each samples were 1: 2° and incubated overnight at 4°C. After plate were washed with PBS-0.05% Tween 20 and horseradish peroxidase (HRP)–labeled goat anti–mouse μ, γ, or α heavy chain–specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added to individual wells. Peroxidase–conjugated goat anti–biotin Abs (Vector Laboratories, Burlingame, CA) were used for detection. The color reaction was developed for 15 min at room temperature (RT) with 100 μl of 1.1 mM [2,2′–azino–bis(3–ethybenzthiazoline–6–sulphonic acid)] (ABTS) (EMD Biosciences, La Jolla, CA) in 0.1 M citrate–phosphate buffer (pH 4.2) containing 0.01% H₂O₂. (Moss, Inc., Pasadena, MD). End point titers were expressed as the reciprocal log₂ of the last
dilution that gave an optical density at 415 nm (OD$_{415}$) of 0.1 greater than background.

**Isolation of gingival mononuclear cells (GMCs)**

Gingival tissues from both upper and lower jaws were carefully removed by using micro-surgical tweezers under a stereoscopic microscope. Cells from gingival tissues were prepared by gentle teasing through sterile stainless steel screens followed by enzymatic dissociation using collagenase type IV (0.5 mg/ml; Sigma–Aldrich, St. Louis, MO) to obtain single-cell preparations. Mononuclear cells were purified on discontinuous Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden). Nine parts Percoll were mixed with one part 10 × Hanks’ balanced salt solution and used as 100% Percoll. Gradients were prepared in 15–ml centrifuge tubes by first placing 2 ml of a 75% Percoll solution diluted in RPMI 1640–2% FCS at the bottom of the tube and then adding the cells suspended in 4 ml of 40% Percoll. Gradients were centrifuged at 20 °C for 20 min at 600 × g. Mononuclear cells in the interface between the 40% and 75% layers were removed, washed and resuspended in RPMI 1640 (Cellgro Mediatech, Washington, DC) supplemented with HEPES buffer (15 mM), L–glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) and 10% fetal calf serum (FCS) (complete RPMI 1640) (3, 19, 20).

**Flow cytometry analysis**

In order to examine the frequency of cytokine-producing CD4$^+$ T cells, harvest GMCs suspended in $1 \times 10^6$ cells by complete RPMI1640 from sham- and *P. gingivalis*-infected mice were incubated with ionomycin (1 μg/ml, Sigma–Aldrich, St. Louis, MO) and phorbol 12-myristate 13-acetate (PMA, 25 ng/ml, Sigma–Aldrich, St. Louis, MO) for 3 hours at 37 °C in a 5% CO$_2$ incubator and wash by 1% BSA–PBS then stained with FITC-labeled anti–CD4 for 15 min at 4 °C. Then wash with PBS buffer and fix the stained cells with 1000 μl of 1% paraformaldehyde for 20 min at RT. After wash with PBS, next these cells were permeabilize fixed with 500 μl of PBS with 0.01% Saponin (Sigma–Aldrich, St. Louis, MO) solution for 10 min at RT. Subsequently, wash with PBS and finally these samples were further stained intracellularly overnight with PE-conjugated anti-interferon (IFN)–γ, -interleukin (IL)-4, -IL-6, -IL-17 and -TNF-α monoclonal Abs for flow cytometric analysis (FACSCalibur; BD Biosciences, San Jose, CA).

**Statistical analysis**

All results are expressed as the mean ± the standard error of the mean (SE), and experimental groups were compared with controls using an unpaired non-parametric Mann-Whitney U test with Statview software (Abacus Concepts, Berkley, CA) designed for Macintosh computers. Values of $p$ of <0.05 or <0.01 were considered significant.

**Results**

**Fim-A Specific Ab Responses in Mice Infected with *P. gingivalis***

Upon confirming alveolar bone loss 30 days after the last infection with *P. gingivalis* (Data not shown), we examined *P. gingivalis*-specific Ab responses in plasma and mucosal secretions. Plasma, saliva, and fecal extract samples were collected 30 days after the last infection and subject to *P. gingivalis*-specific ELISA. Two μg/ml of Fim-A from *P. gingivalis* was used as coating Ag. Fim-A specific plasma IgG responses were seen in *P. gingivalis*-infected mice

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*Fig. 1. Increased level of FimA-specific Ab responses in mice infected with *P. gingivalis*. Plasma and mucosal secretions (saliva, and fecal extract) samples were collected 30 days after the last infection. *P. gingivalis* FimA-specific IgG and IgA Ab responses were examined by ELISA. The values shown are the mean±SE for 20 mice in each experimental group. *p <0.05 when compared with sham-infected mice.*
when compared with those in sham-infected mice (Fig. 1). Further, elevated levels of S-IgA Ab responses were noted in saliva and fecal extracts of mice infected with *P. gingivalis* (Fig. 1). These results show that oral infection with *P. gingivalis* induces Ag-specific immune responses.

**Increased levels of IL-6 and TNF-α production by CD4+ T cells in inflamed gingiva**

In order to detect inflammatory responses in gingival tissues of mice infected with *P. gingivalis* orally, IL-6 and TNF-α production by CD4+ T cells were examined 30 days after the last infection. The numbers of IL-6-producing CD4+ T cells were significantly increased in gingival mononuclear cells (GMCs) of mice infected with *P. gingivalis* when compared with those from sham-infected mice (Fig. 2). Further, higher TNF-α production by CD4+ T cells was also noted in the gingival tissue of *P. gingivalis*-infected mice than those seen in sham-infected mice (Fig. 2). Taken together, these results clearly indicate that *P. gingivalis* infection induces inflammatory responses with IL-6 and TNF-α production by CD4+ T cells in diseased gingival tissue.

**Dominant IFN-γ production by CD4+ T cells of inflamed gingiva**

We next examined the frequencies of Th1-, Th2- and Th17-type CD4+ T cells in the inflamed gingival tissues. Intracellular FACS analyses revealed that increased levels of IFN-γ-, IL-4- and IL-17-producing CD4+ T cells in GMCs of mice infected with *P. gingivalis* when compared with those of sham-infected mice (Fig. 3). IL-4- or IL-17-producing CD4+ T cells were not indicated a significant difference. However, the frequency of IFN-γ-producing CD4+ T cells was significantly higher than that of sham-infected mice. Thus, approximately a 2-fold higher frequency of IFN-γ production was noted in GMCs of inflamed gingival tissues. These results indicate that IFN-γ in gingival tissues are key players in the induction of periodontal disease. (Fig. 3).

**Kinetics of Th1, Th2 and Th17 cytokine production by CD4+ T cells in inflamed gingiva**

In order to define the contribution of Th1-, Th2- and Th17-type CD4+ T cells in the development of inflammatory responses in the gingival mucosa, the kinetics of IFN-γ-, IL-4- and IL-17-producing CD4+ T cells were determined. An increased frequency of CD4+ T cells were noted in the GMCs of *P. gingivalis*–infected mice through the experiment period when compared with sham-infected mice (Table 1). A high
frequency of IFN-γ-producing CD4+ T cells was noted one day after the last infection and was maintained throughout the experimental period. Thus, the numbers of IFN-γ-producing CD4+ T cells are comparable to those seen in GMCs isolated 30 days after the last infection (Fig. 4). Interestingly, significantly increased frequencies of IL-4- and IL-17-producing CD4+ T cells were also noted in inflamed gingival mucosa one day after the last infection (Fig. 4). Thus, approximately 20% of total CD4+ T cells are able to produce IL-4 or IL-17, which is essentially the same frequency seen as IFN-γ-producing CD4+ T cells. The frequencies of IL-4- and IL-17-producing CD4+ T cells were maintained until 15 days after the last infection; however, these CD4+ T cell subsets decreased thereafter. These results show that IFN-γ-producing CD4+ T cells play central roles in the induction and maintenance of inflammatory responses in gingival mucosa.

### Discussion

In this study, we examined cytokine production by CD4+ T cells isolated from inflamed gingiva of mice orally infected with *P. gingivalis* showing significant inflammation in gingival mucosa with bone loss. An increased number of CD4+ T cells was seen in GMCs of inflamed gingiva when compared with normal gingival mucosa. Further, these CD4+ T cells in inflamed gingiva contained higher frequencies of IL-6- and TNF-α-producing cells than gingival CD4+ T cells from sham-infected mice. Th1-, Th2- and Th17-type cytokine analyses revealed a higher number of IFN-γ-producing CD4+ T cells in inflamed gingival tissue. Although numbers of IL-4- and IL-17-producing CD4+ T cells were significantly increased and maintained until 15 days after the infection with *P. gingivalis*, the frequencies of both cell types were significantly reduced at 30 days after the infection. These results are the first to show Th1-, Th2- and Th17-type cytokine profiles in the inflamed gingival tissue which may directly influence periodontal disease–induced alveolar bone loss.

Early studies have shown that the majority of CD4+ T cells isolated from inflamed gingival tissues of adult periodontitis (AP) patients expressed IFN-γ-, and IL-6-specific mRNA (21). Further, it has been shown that increased levels of both IFN-γ and IL-17 production in the gingival mucosa are hallmarks of gingival mucosal inflammation (22-25). These reports clearly showed the importance of these cytokines in the induction of periodontal disease. Indeed, our results show that there are increased frequencies of IL-6-, IL-17- and IFN-γ-producing CD4+ T cells in GMCs of mice infected with *P. gingivalis*. Although IL-17 production in *P. gingivalis*-infected mice was higher than that of

### Table 1. Frequency of CD4+ T cells in gingiva after infection with *P. gingivalis*

<table>
<thead>
<tr>
<th>After final infection</th>
<th>Percents of CD4+ T cells/GMCs</th>
</tr>
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<tbody>
<tr>
<td></td>
<td><em>P. gingivalis</em> infected</td>
</tr>
<tr>
<td>Day 1</td>
<td>6.66±0.57**</td>
</tr>
<tr>
<td>Day 7</td>
<td>6.78±0.63**</td>
</tr>
<tr>
<td>Day 15</td>
<td>4.77±0.93*</td>
</tr>
<tr>
<td>Day 30</td>
<td>5.21±0.45</td>
</tr>
</tbody>
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*p < 0.05, **p < 0.01

Fig. 4. Kinetics of IFN-γ-, IL-4- and IL-17-producing CD4+ T cells. GMCs from *P. gingivalis*– and sham infected mice were isolated 1, 7, 15, and 30 days after the last infection. Cells were treated with ionomycin and PMA and stained as the same manner as described in Fig. 3 legend. The values are shown the mean±SE for 30 mice (sample number indicate that 10 mice for n=1) in each experimental group. *p < 0.05 when compared with sham infected mice.
sham-infected mice, kinetic analysis revealed that significantly high number of IL-17-producing CD4+ T cells were only detected in the early periods of infection (day 1 to 15). In contrast, high numbers of IFN-γ-producing CD4+ T cells were maintained from day 1 to day 30 post infection. These results indicate that there are distinct roles for IFN-γ and IL-17 in the induction of periodontal disease. Thus, IL-17-producing Th17 cells may contribute to the early phase of the inflammatory response, whereas IFN-γ-producing Th1 cells are most likely involved in the initiation and progression of the disease process.

It has been shown that CD4+ T cells and their cytokine production play key roles in the regulation of bone loss. Thus, it has been shown that both IFN-γ and IL-4 produced by effector CD4+ T cells would inhibit the pro-osteoclastogenic effects of RANKL-RANK signaling (11). Most notably, IFN-γ-producing, Th1-type CD4+ T cells would play a crucial role in the prevention of T cell-mediated osteoclastogenesis (26). Further, it was reported that a deficiency in IL-17R resulted in enhanced alveolar bone loss when mice were orally infected with *P. gingivalis* (27). These findings clearly showed that Th1-, Th2-, and Th17-type cytokines possess protective effects in bone resorption. Indeed, our results showed that both numbers of IL-4- and IL-17-producing CD4+ T cells were significantly reduced in the later phases of infection. Thus, significant bone loss at 30 days after the infection may be due to the loss of Th2- and Th17-type cell subsets in the inflamed gingival tissue despite the presence of Th1-type cells.

In summary, our findings provide roles for Th1-, Th2- and Th17-type cytokine-producing CD4+ T cells in the induction and regulation of gingival tissue inflammation and alveolar bone loss. Thus, *P. gingivalis*-activated naïve CD4+ T cells differentiate into IFN-γ-, IL-4- and IL-17-producing effector CD4+ T cells that contribute to the early phase of gingival tissue inflammation. In contrast, reduction of IL-4- and IL-17-producing cells may be essential for the induction of osteoclast differentiation and alveolar bone loss. These findings clearly show that IFN-γ-, IL-4-, and IL-17-producing CD4+ T cells play different roles in terms of gingival tissue inflammation and alveolar bone loss in periodontal disease. Additional ongoing studies will provide better understanding of the cellular and molecular mechanisms of these mucosal immune cells in the inflamed gingiva for the induction and regulation of periodontal disease.

References

chronic inflammatory periodontal tissues produce interleukin (IL)-5 and IL-6 but not IL-2 and IL-4. Am J Pathol, 142: 1239-1250, 1993.