RESEARCH REPORTS
Biological

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J Dent Res 90(5):653-658, 2011

Induction of IL-10-producing CD4+ T-cells in Chronic Periodontitis

ABSTRACT

Precise immunological aspects of inflamed gingival mucosa remain to be elucidated in the murine experimental periodontitis model; therefore, we have characterized the mucosal immune cells in the inflamed gingiva of mice with alveolar bone reduction. Mice were orally infected with *Porphyromonas gingivalis* 15 times over 2 weeks. Gingival mononuclear cells (GMCs) were isolated from *P. gingivalis*- and sham-infected mice 1, 7, 15, and 30 days after the last infection. Although the greatest degree of periodontitis was seen in *P. gingivalis*-infected mice at 30 days after infection, the highest levels of IL-6 and TNF-α production were noted in the GMCs isolated 7 days after infection. Further, the frequency of $RANKL^+CD4^+$ T-cells in GMCs of inflamed gingiva peaked 15 days after infection. Importantly, the number of $Foxp3^+CD4^+$ $CD25^+$ regulatory T (Treg)-cells was increased only in the experimental group 30 days after infection. Thus, intracellular cytokine analysis revealed an increased number of IL-10-producing $CD4^+$ T-cells in inflamed gingiva when compared with the control group. These results suggest that there are potential roles for Treg cells during the chronic stage of periodontitis in the regulation of gingival inflammation and alveolar bone loss.

KEY WORDS: mucosal immunology, periodontal disease, inflammation, bone loss.

DOI: 10.1177/0022034510397838

Received September 20, 2010; Last revision December 6, 2010; Accepted December 7, 2010

INTRODUCTION

The oral cavity is considered to be a type of mucosal tissue that is protected by both the mucosal and systemic arms of the immune system. The induction of mucosal secretory IgA antibodies (Abs) in saliva and plasma-derived IgG in gingival crevicular fluid together defend the oral cavity against invading pathogens (Gilthorpe *et al*., 2003; Graves and Cochran, 2003). Another unique feature of the oral cavity is that it consists of both mucosal and bone tissues when compared with other mucosal compartments such as the gastrointestinal and respiratory tracts (Gilthorpe *et al*., 2003; Graves and Cochran, 2003). Thus, it is clear that the cellular and molecular intranet between mucosal and osteoimmune systems plays a critical role in the regulation of bone homeostasis in the oral cavity. Indeed, it has been shown that RANKL (receptor activator of NF-κB ligand) is a key differentiation factor for osteoclasts (Theill *et al*., 2002; Walsh *et al*., 2006). Furthermore, it was reported that RANKL is expressed on activated T- and B-cells (Teng *et al*., 2000; Theill *et al*., 2002; Walsh *et al*., 2006). These findings clearly show the potential for crosstalk between immune responses and bone turnover. However, cellular and molecular mechanisms of the immune system that influence oral bone metabolism remain to be elucidated, particularly when the immune system has been activated by infection or becomes dysregulated. In conditions such as periodontal disease, infiltrating lymphocytes and other mononuclear cells produce several key factors that alter bone homeostasis regulated by bone formation and resorption (Walsh *et al*., 2006). To this end, we hypothesize that the mucosal immune cells in gingiva of the oral cavity play a critical role in the regulation of osteoimmunopathology during periodontal disease development. Characterization of the mucosal immune response in the inflamed gingival tissues is critical for understanding of the cellular and molecular mechanisms by which periodontal disease causes alveolar bone and tooth loss.

MATERIALS & METHODS

Mice

Six- to 8-week-old female BALB/c mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD, USA). Upon arrival, these mice were housed in micro-isolators, maintained in horizontal laminar flow cabinets, and provided sterile food and water as part of a specific-pathogen-free facility in the University of Alabama at Birmingham (UAB). 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 NIH and UAB institutional guidelines.

Induction of Periodontal Inflammation with Alveolar Bone Loss

Mice were orally infected with *Porphyromonas gingivalis* (ATCC: 33277) as described previously, with minor modifications (Baker *et al*., 1994; Momoi *et al*., 2008). Briefly, mice were given sulfamethoxazole and trimethoprim oral suspension at 10 mL *per* pint of de-ionized water *ad libitum* for 10 days. This was followed by a three-day antibiotic-free period. Mice were then daily administered 10^9 CFU of *P. gingivalis* suspended in 100 μL of PBS with 2% carboxymethylcellulose *via* oral topical application for a total of 15 inoculations. The control group consisted of sham-infected mice that received the antibiotic pre-treatment and carboxymethylcellulose without *P. gingivalis*.

Histological Analysis of Gingival Tissue and Alveolar Bone Loss Measurement

Thirty days after the last infection, *P. gingivalis*-specific 16S rRNA was detected by real-time PCR analysis. Further, the skin of the lower jaws was removed and fixed in 1% paraformaldehyde in PBS for 24 hrs. The lower jaw was decalcified by 150 mM EDTA in PBS for 5-7 days at 4°C and then embedded in paraffin. Four-micrometer-thick serial sections were then prepared and stained with hematoxylin and eosin. Horizontal bone loss around the maxillary molars was assessed by a morphometric method as described previously (Klausen *et al*., 1989). Briefly, after removal of gingival tissue, skulls were immersed overnight in 3% hydrogen peroxide, pulsed for 1 min in bleach, and stained with 1% methylene blue. The distance from the cement-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured at a total of 28 buccal sites *per* mouse (Momoi *et al*., 2008). Measurements were made under a dissecting microscope (x20) fitted with a video image marker measurement system standardized to give measurements in millimeters.

Isolation of Gingival Mononuclear Cells (GMCs)

One, 7, 15, and 30 days after the last *P. gingivalis* infection, mice were sacrificed, and gingival tissues from both upper and lower jaws were carefully removed with the use of microsurgical tweezers under a stereomicroscope. Cells from gingival tissues were prepared by being gently 'teased' through sterile stainless steel screens, followed by an enzymatic dissociation procedure with collagenase type IV (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) (Fujihashi *et al*., 1993, 1996; Yamamoto *et al*., 1997). GMCs were purified on discontinuous Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and re-suspended in RPMI 1640 (Cellgro Mediatech, Washington, DC, USA) supplemented with HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% fetal calf serum (FCS) (complete medium).

Flow Cytometry

GMCs (2×10^5 cells) were stained with combinations of fluorescence-conjugated or biotinylated monoclonal antibodies (mAbs) including anti-CD3, -B220, -CD4, -CD8, -CD11c, -CD11b, -CD25, -CD44, -CD62L, and -RANKL (BD Pharmingen and eBiosciences, San Diego, CA, USA). Samples were subjected to flow cytometry analysis (FACSCalibur™; BD Biosciences). In some experiments, GMCs were incubated with fluorescence-tagged anti-CD4 and -CD25 mAbs and were then intracellularly stained with Foxp3 mAb (eBiosciences). For intracellular IL-10 analysis, cells were incubated with ionomycin (1 μg/mL, Sigma-Aldrich) and phorbol 12-myristate 13-acetate (PMA, 25 ng/mL, Sigma-Aldrich) for 3 hrs in the presence of monensin and then stained for CD4 and CD25 before being stained intracellularly with Alexa Fluor® 488-labeled anti-IL-10 mAb (JES5-16E3).

IL-6, TNF-α, and IL-10-specific ELISAs

GMCs (1×10^6/mL) were cultured in complete medium for 3 days, and the culture supernatants were collected and subjected to IL-6-, TNF-α-, and IL-10-specific ELISAs. Combinations of anti-IL-6 (MP5-20F3 and MP5-32C11) mAbs were used as coating and detection antibodies, respectively. We used a mouse TNF-α and IL-10 immunoassay kit (Quantikine M: R&D Systems, Minneapolis, MN, USA) to detect TNF-α and IL-10 in the culture supernatants.

Statistical Analysis

All results are presented as the mean ± the standard error of the mean (SEM), and experimental groups were compared with controls by an unpaired non-parametric Mann-Whitney *U* test with Statview software (Abacus Concepts, Cary, NY, USA) designed for Macintosh computers. Values of $p < 0.05$ or < 0.01 were considered significant.

RESULTS

P. gingivalis Induced Gingival Inflammation and Alveolar Bone Loss

We initially determined whether our modified oral infection protocol with *P. gingivalis* induced gingival inflammation and alveolar bone loss. Thirty days after the last infection, mononuclear cell infiltration was observed in gingiva of mice infected with *P. gingivalis*, and thus, the marginal gingiva was hypertrophic and contained lymphocytes and fibrotic tissue (Fig. 1A). Further, gingival tissues isolated 30 days after infection contained *P. gingivalis*-specific 16S rRNA, which is equivalent to that isolated from approximately 4×10^3 *P. gingivalis* (Fig. 1B). Cell infiltration was observed even one day after the last infection. Thus, significantly increased numbers of $CD3^+$ T-cells, $B220^+$ B-cells, and $CD11c^+$ dendritic cells were seen in gingiva of *P. gingivalis*-infected mice when compared with that of sham-infected and naïve mice (Fig. 2A). When the distances from the CEJ to the ABC at buccal sites were measured, significant bone resorption was noted in mice orally infected with *P. gingivalis*. In contrast, sham-infected mice showed minimal bone loss when compared with experimental groups (Fig. 2B).

Increased Levels of IL-6 and TNF-α Production by GMCs

To detect inflammatory responses in gingival tissues of mice given oral *P. gingivalis*, we examined IL-6 and TNF-α production by GMCs 1, 7, 15, and 30 days after the last infection. GMCs of

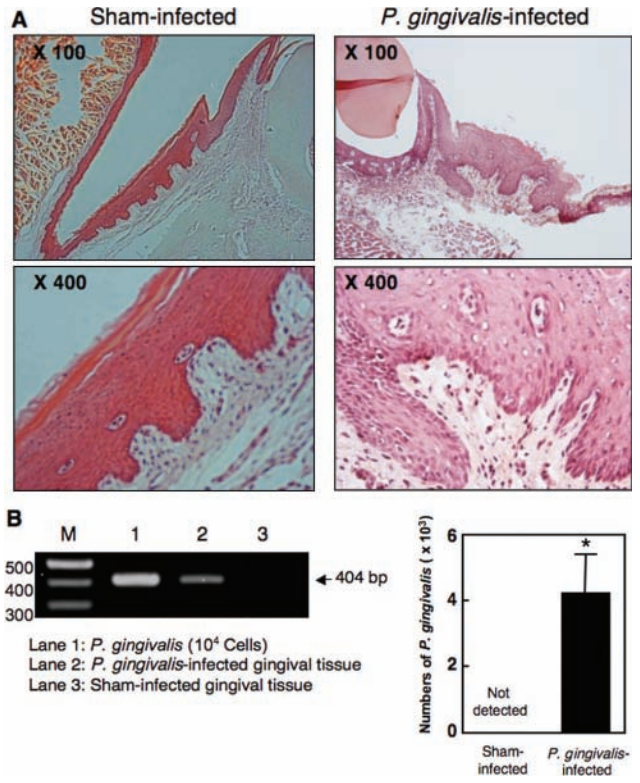

Figure 1. Induction of marginal gingival inflammation in mice infected with *P. gingivalis*. All mice were sacrificed by cervical dislocation after being anesthetized with ketamine and xylazine. **(A)** Histochemical analysis of gingival tissue and alveolar bone loss of mice. Thirty days after the last infection, lower jaws with gingival tissue from mice infected with or without *P. gingivalis* were stained with hematoxylin and eosin. **(B)** Detection of *P. gingivalis*-specific 16S rRNA. DNA was extracted from gingival tissues of *P. gingivalis*- and sham-infected mice 30 days after the last infection. DNA was amplified by real-time quantitative PCR with a pair of primers corresponding to *P. gingivalis*-specific 16S rRNA. Different numbers of DNA from *P. gingivalis* 33277 were used for generating a standard curve. All values are presented as the mean ± SEM of 25 mice in each group; $*p < 0.05$ when compared with sham-infected mice.

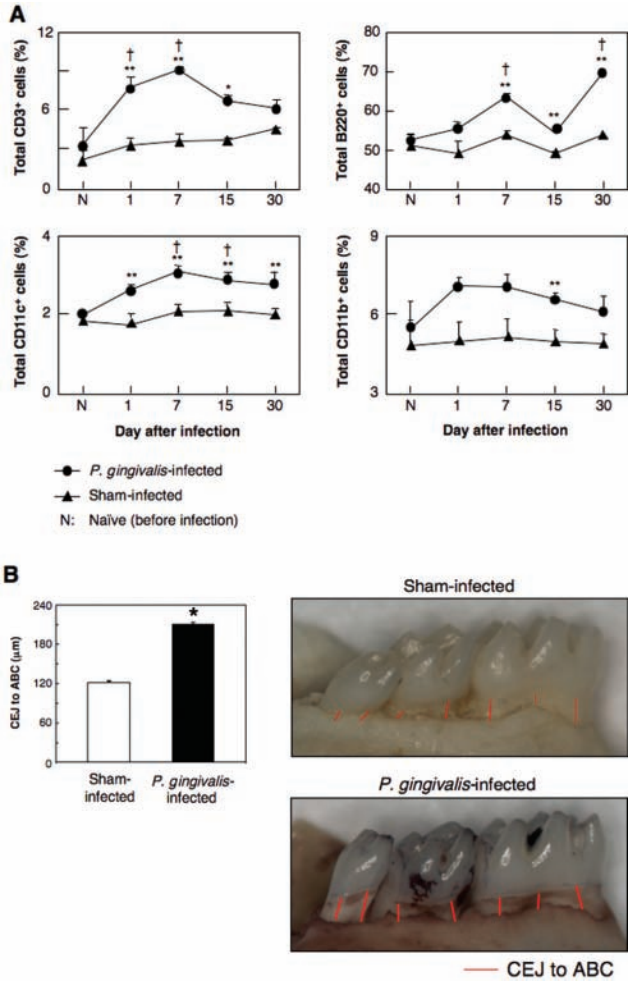

Figure 2. The frequency of lymphocytes and bone loss in mice infected with *P. gingivalis*. All mice were sacrificed at each time-point as described in the legend to Fig. 1. **(A)** Time-points were assessed on days 1, 7, 15, and 30, after the last infection, and gingival mononuclear cells (GMCs) were stained for CD3, B220, CD11c, and CD11b expression. **(B)** Prolonged *P. gingivalis* infection induced alveolar bone loss. Thirty days after the last infection, horizontal bone loss around the maxillary molars was assessed by a morphometric method. Alveolar bone loss was measured at a total of 28 buccal sites *per* mouse (CEJ to ABC). Bone measurements were performed a total of 3 times by two evaluators using a random and blinded protocol. All values are presented as the mean ± SEM of 25 mice in each group at each time-point; $*p < 0.05$; $**p < 0.01$ when compared with sham-infected mice. $^{\dagger}p < 0.05$ when compared with mice before the infection.

P. gingivalis-infected mice produced higher levels of IL-6 and TNF-α than those from sham-infected mice (Fig. 3A). The highest levels of IL-6 and TNF-α were detected 7 days after the last infection (Fig. 3A). IL-6 production decreased over the next 3 wks. Although TNF-α production by *P. gingivalis*-infected GMCs was reduced at day 15, synthesis was essentially maintained at that level for the duration of our analysis (Fig. 3A). These results indicate that acute inflammatory responses were induced in gingival mucosa 7 days after the last infection, and this response shifted into the chronic stage over the next 3 wks.

RANKL Up-regulation in CD4⁺ T-cells in Inflamed Gingiva

As previously stated, alveolar bone loss was induced in *P. gingivalis*-infected mice; therefore, we next examined the frequency of RANKL-expressing $CD4^+$ T-cells in gingival mucosa of *P. gingivalis*-infected mice. Increased numbers of $RANKL^+CD4^+$ T-cells were seen 7, 15, and 30 days after the last infection when compared with those of the sham-infected group. The highest number of $RANKL^+CD4^+$ T-cells was detected 15 days after the last infection and decreased thereafter (Fig. 3B). Importantly, RANKL expression was preferentially up-regulated in $CD44^+$ $CD4^+$ T-cells (Fig. 3B). Thus, CD62L-expressing (naïve) $CD4^+$ T-cells did not show any significant increases of RANKL expression. These results show that $CD4^+$ effector T-cells that can potentially regulate osteoclast development

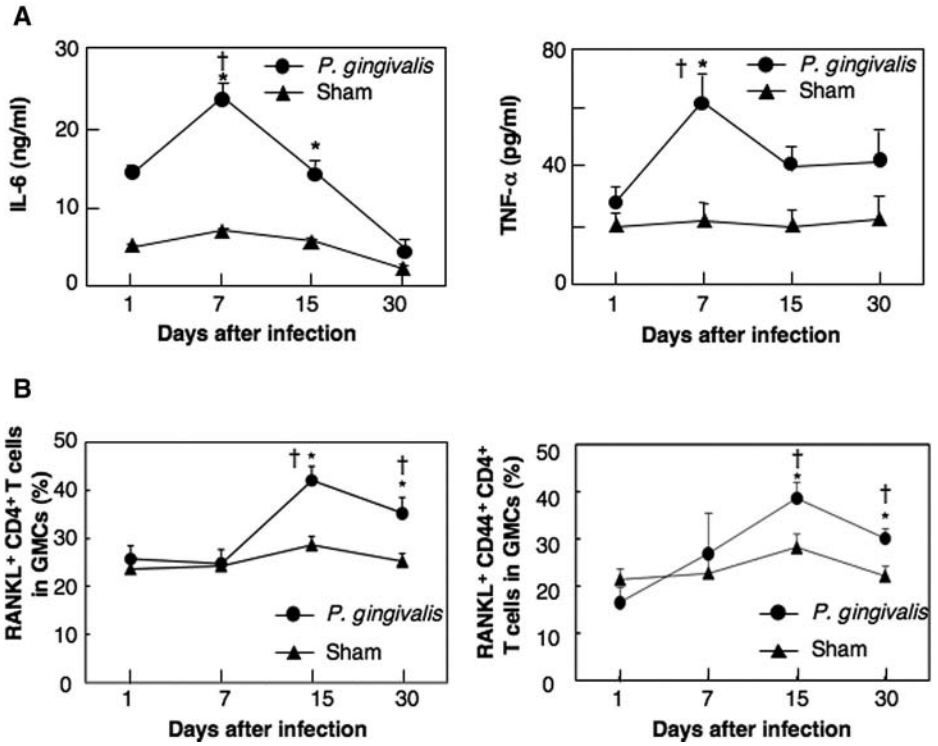

Figure 3. Cytokine profiles and the frequency of RANKL expression by $CD4^+$ T-cells in mice orally infected with *P. gingivalis*. All mice were sacrificed at each time-point as described in the legend to Fig. 1. **(A)** GMCs (1×10^6/mL) were isolated 1, 7, 15, and 30 days after the last infection and were then cultured for 3 days. The culture supernatants were collected and subjected to IL-6 and TNF-α specific ELISA. **(B)** One, 7, 15, and 30 days after the last infection with *P. gingivalis*, the frequency of $RANKL^+CD44^+CD4^+$ T-cells was examined by FACSCalibur™. The values are presented as the mean ± SEM of 20 mice in each group at each time-point; $^*p < 0.05$ when compared with sham-infected mice. $^\dagger p < 0.05$ when compared with mice one day after infection.

were induced in *P. gingivalis*-infected gingival mucosa after the peak of acute inflammation.

Induction of IL-10-producing Tregs in Inflamed Gingiva

We next examined the frequency of Treg cells and IL-10 production in inflamed gingival tissue. IL-10 production by GMCs was essentially the same between *P. gingivalis*- and sham-infected mice until day 15 after infection. However, *P. gingivalis*-infected GMC showed a marked increase in IL-10 synthesis 30 days after the last infection (Fig. 4A). Thus, intracellular IL-10 analysis revealed an increased number of IL-10-producing $CD4^+$ T-cells in inflamed gingiva when compared with control groups (Fig. 4B). Importantly, approximately twice the number of these $CD4^+CD25^+$ T-cells expressed the intracellular Foxp3 molecule 30 days after the last infection, although a change was not found in the initial period (Fig. 4C). These results indicate that regulatory-type $CD4^+$ T-cells begin to appear after the peak of the acute inflammatory response.

DISCUSSION

In this study, we examined the cellular and molecular aspects of GMCs isolated from mice exhibiting significant inflammation in gingival mucosa with bone loss due to oral infection with *P. gingivalis*. The highest levels of IL-6 and TNF-α production were noted during the early stage of disease progression (7 days after the last infection). Over the next 8 days, the frequency of $RANKL^+CD44^+CD4^+$ T-cells reached a maximum and gradually decreased thereafter in the gingival mucosa of *P. gingivalis*-infected mice. In contrast, numbers of Foxp3-expressing $CD4^+CD25^+$ Treg cells and IL-10-producing $CD4^+$ T-cells were increased in the later stage of periodontitis and peaked at 30 days after the last infection with *P. gingivalis*. These results are the first to show changes in mucosal immune cells of the inflamed gingival tissue that may directly influence alveolar bone metabolism during periodontal disease. Thus, our results logically demonstrate the progression of periodontal disease, showing that early inflammatory responses initiate $CD4^+$ effector T-cell activation followed by $CD4^+$ Treg induction that may contribute to the chronic stage of periodontitis.

Although it has been suggested that RANKL-expressing activated $CD4^+$ T-cells may be capable of inducing osteoclast differentiation (Horwood *et al*., 1999; Kong *et al*., 1999), previous studies have shown that activated $CD4^+$ T-cell subsets and their cytokines can have pro- or anti-osteoclastogenic effects (Mosmann and Coffman, 1989; Sakaguchi, 2004; Harrington *et al*., 2005; Takayanagi, 2007; Weaver *et al*., 2007). Thus, both IFN-γ and IL-4 produced by effector $CD4^+$ T-cells would inhibit the pro-osteoclastogenic effects of RANKL-RANK signaling (Walsh *et al*., 2006). Further, IFN-γ-producing, Th1-type $CD4^+$ T-cells would play a crucial role in the prevention of T-cell-mediated osteoclastogenesis (Takayanagi *et al*., 2000). In contrast, it was reported that IL-6- and IL-17-producing $CD4^+$ T-cells indirectly up-regulate osteoclastogenesis by inducing RANKL on mesenchymal cells (Takayanagi, 2007). Since increased levels of IFN-γ, IL-6, and IL-17 production in the gingival mucosa are hallmarks of gingival mucosal inflammation (Fujihashi *et al*., 1996; Johnson *et al*., 2004; Takahashi *et al*., 2005), it is important to determine cytokine profile of RANKL-expressing $CD4^+$ T-cells and roles in the osteoclastogenesis in the gingival mucosa.

Ag-induced Treg cells play an essential role in the regulation of inflammatory responses by producing suppressive cytokines such as IL-10 and TGF-β_1 (Sakaguchi, 2004). In this regard, recent studies showed that inflamed gingival tissue contained a high frequency of $Foxp3^+$ Treg cells (Nakajima *et al*., 2005; Okui

et al., 2008). Indeed, our study showed that the frequency of $Foxp3^+CD4^+CD25^+$ T-cells was significantly increased in the later stage of infection with *P. gingivalis*. Of importance, intracellular IL-10 analysis showed a higher frequency of IL-10-producing $CD4^+$ T-cells in inflamed gingiva than that in normal tissue. These results suggest that gingival Treg cells from mice infected with *P. gingivalis* may be central players in the down-regulation of inflammatory responses through their IL-10 production. To this end, it is essential to determine TGF-β_1 production by these $Foxp3^+CD4^+CD25^+$ T-cells, in addition to IL-10, since TGF-β_1 is another regulatory cytokine produced by Treg cells for the maintenance of immune homeostasis (Sakaguchi, 2004). Further, it has been reported that the TGF-β_1 signal is important for the reduction of osteoclast activity (Koseki *et al*., 2002).

In summary, our findings define the immunological aspects of inflamed gingival mucosa that may up- or down-regulate subsequent induction of alveolar bone loss in periodontal disease. Thus, *P. gingivalis*-induced inflammatory responses can initiate naive $CD4^+$ T-cell differentiation into $RANKL^+CD4^+$ T-helper cells that may contribute to osteoclast differentiation. In contrast, $Foxp3^+$Tregs in the inflamed gingiva may be essential players in the regulation of periodontal inflammation and alveolar bone loss. Additional ongoing studies will provide a better understanding of the cellular and molecular mechanisms of these mucosal immune cells in the inflamed gingiva during the induction and regulation of periodontal disease.

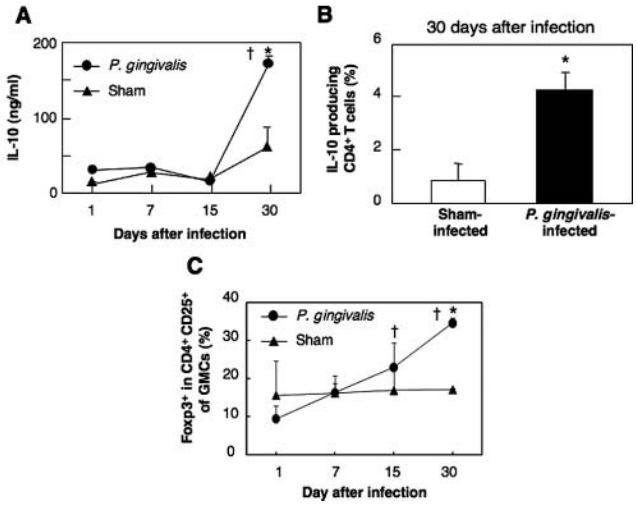

Figure 4. Frequencies of interleukin-10-producing $CD4^+$ T-cells and Foxp3-expressing $CD25^+CD4^+$ T-cells in inflamed gingiva. All mice were sacrificed 1, 7, 15, and 30 days after the last infection, as described in the legend to Fig. 1. **(A)** GMCs (1×10^6/mL) were isolated and cultured for 3 days. The culture supernatants were collected and subjected to an IL-10-specific ELISA. **(B)** Thirty days after the last infection, GMCs were incubated with ionomycin (1 mg/mL) and phorbol 12-myristate 13-acetate (PMA, 25 ng/mL) for 3 hrs and then stained for the surface CD4 and intracellular IL-10 expression. **(C)** GMCs isolated from each time-point were stained with fluorescence-conjugated anti-CD4 and biotinylated anti-CD25 mAbs, followed by PerCP-Cy5.5-streptavidin. Samples were further stained intracellularly with PE-labeled anti-Foxp3 mAb. The values are presented as the mean ± SEM of 20 to 30 mice in each group at each time-point; $^*p < 0.05$ when compared with sham-infected mice. $^\dagger p < 0.05$ when compared with mice one day after infection.

ACKNOWLEDGMENTS

We thank Dr. Jerry R. McGhee for his editorial assistance as well as for his scientific discussion and critiques. This study was supported by NIH grants DE012242 and AG028573.

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