Th1 Immune Response Promotes Severe Bone Resorption Caused by *Porphyromonas gingivalis*

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Bacterial infections of the dental pulp result in soft tissue and alveolar bone destruction. It has been suggested that Th1 responses promote disease, whereas Th2 responses are protective. However, other studies have challenged this notion. To address this question, bone destruction was evaluated in mice immunized to develop strong and polarized Th1- or Th2-biased responses to the oral pathogen *Porphyromonas gingivalis*. Th1 bias was confirmed by the presence of high titers of serum IgG2a and the production of high levels of interferon (IFN)- γ and no interleukin (IL)-4 by lymph node cells stimulated with *P. gingivalis* antigens. In contrast, Th2-biased animals had high titer IgG1 and no IgG2a, and their lymph node cells produced high levels of IL-4 but no IFN- γ . Subsequent infection of the dental pulp with *P. gingivalis* caused extensive inflammation and alveolar bone destruction in Th1-biased mice, whereas Th2-biased mice and controls developed minimal lesions. Inflammatory granulomas in Th1-biased mice were heavily infiltrated with osteoclasts and had high local expression of IFN- γ , IL-1 α , and IL-1 β . Little or no IFN- γ /IL-1 α /IL-1 β and no obvious osteoclasts were detected in lesions of Th2-biased and control groups. These results directly demonstrate that specific Th1 responses promote severe infection-stimulated alveolar bone loss. (Am J Pathol 2007, 170:203–213; DOI: 10.2353/ajpath.2007.060597)

Alveolar bone resorption occurs as a consequence of oral infection with bacterial pathogens and is markedly influenced by cytokines produced in the local milieu. Alveolar bone loss is the consequence of two main pathologies. These include periodontal disease, in which

soft tissue and bone loss is caused by bacterial biofilms that colonize the surfaces of teeth and their supporting structures, and periapical periodontitis, caused by bacterial invasion of the dental pulp, resulting in the formation of immune granulomas within alveolar bone proximal to the infected tooth with concomitant bone resorption. The pathogen *Porphyromonas gingivalis* has been associated with active disease in both systems.^{1–6} In periodontal disease, an epithelial barrier separates the microbial biofilm from host tissues, whereas in periapical periodontitis, there is direct tissue invasion and parenteral contact of bacteria with the host immune system. Remarkably, actual microbial invasion of bone rarely occurs in periapical periodontitis, suggesting that the local immune response contains the infection but at the same time mediates bone resorption.⁷

Interleukin (IL)-1 has been strongly associated with alveolar bone resorption via stimulation of receptor activator of nuclear factor κ B ligand (RANKL) expressed by osteoblasts and lymphocytes,^{8–12} although other mediators, particularly those derived from T cells, may also play critical roles by modulating inflammation.^{8,13–17} Both Th1 and Th2 cytokines are expressed after dental pulp infection, with Th1 expression becoming predominant after several weeks.¹⁸ Paradoxically, gene knockouts of the prototype Th1 mediator interferon (IFN)- γ or IFN- γ -inducing cytokines IL-12 and IL-18 have no significant effect on periapical bone destruction,¹⁹ suggesting either a lack of regulatory activity or functional redundancy in pro-inflammatory pathways. In contrast, gene knockouts of Th2 regulatory cytokines IL-10 and IL-6 exhibit increased infection-stimulated bone destruction, indicating nonredundant roles in inhibiting inflammation.^{13,14,20} However, other studies using a subcutaneous chamber model²¹ or oral infection with *P. gingivalis*² suggest the opposite, ie, that IFN- γ and IL-6 mediate bone destruction. Although these conflicting results could be attributed to the use of different animal models, they nevertheless specify that

Supported by National Institutes of Health grant R01-DE-09018 and by a grant from the Krakow Endodontic Research Fund.

Accepted for publication September 15, 2006.

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further studies are needed to define the precise role that Th1 responses play in periapical pathogenesis.

The present work addressed this apparent conflict by evaluating bone resorption caused by pulpal infection with *P. gingivalis* in pre-sensitized mice using protocols that stimulate specific, strong, and polarized Th1 or Th2 responses. Our findings demonstrate that induction of a powerful systemic Th1 response followed by intrapulpal challenge with viable *P. gingivalis* results in massive periapical bone destruction. In contrast, induction of a powerful Th2 response results in minimal disease development.

Materials and Methods

Animals

C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). The mice were maintained under specific pathogen-free conditions and used at 8 to 12 weeks of age. The Animal Care and Use Committee of The Forsyth Institute approved all experiments.

Bacteria and Antigen Preparation

P. gingivalis W83 (BAA-308; American Type Culture Collection, Manassas, VA) was grown in *Mycoplasma* broth (Sigma, St. Louis, MO) medium under anaerobic conditions (80% N₂, 10% H₂, and 10% CO₂), harvested, and suspended in pre-reduced, anaerobically sterilized Ringer's solution under an inert (N₂) atmosphere. The bacteria were washed three times with phosphate-buffered saline (PBS) and suspended to a concentration of ~10⁹ microorganisms/ml, followed by five cycles of freeze/thaw in liquid nitrogen to lyse the bacterial cells. Lysed cells were centrifuged at 10,000 × g to obtain a soluble *P. gingivalis* antigen preparation (Pg lysate). The antigen preparation was concentrated with an Amicon 3 Centriprep concentrator (Amicon, Beverly, MA) to yield a protein concentration of 1 mg/ml as determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Th1/Th2 Immunization Protocols

C57BL/6 mice were bled before and 4 weeks after two subcutaneous (footpad) immunizations (1 month apart) with 10 µg of Pg lysate formulated with either 50 µg of alum (Rehydragel HPA; Reheis, Berkeley Heights, NJ) or with a mixture containing 50 µg of alum plus 1 µg of recombinant mouse IL-12 (Genetic Institute, Cambridge, MA), as previously described to generate a Th2- and a Th1-biased response, respectively.^{22,23} Three weeks after the second immunization, mice were either sacrificed for immunogenicity studies or infected with *P. gingivalis*.

Proliferation and Cytokine Assays

Lymphoid cells were obtained from popliteal lymph nodes harvested from mice inoculated subcutaneously

with 10⁸ live *P. gingivalis*. Cells were cultured at 37°C and 5% CO₂ in the presence of either medium only (RPMI with 10% fetal calf serum and 50 µg/ml gentamicin) or in medium containing specific antigens at the indicated concentrations. For proliferation assay, plates were cultured for 3 days at 37°C in 5% CO₂ and were pulsed with 1 µCi of [³H]thymidine (Amersham, Piscataway, NJ) for an additional 18 hours. Cells were harvested onto filter mats, and incorporated radioactivity was determined by liquid scintillation counting. For cytokine analysis, lymph node cells at 10⁶ cells/well (96-well tissue culture plates) were incubated with or without antigens for 72 hours. Supernatants were harvested and analyzed for IFN-γ, IL-1α, IL-1β, IL-4, and IL-10 by a double sandwich enzyme-linked immunoadsorbent assay (ELISA) using specific monoclonal antibody (PharMingen, San Diego, CA) as described previously.¹⁸ For cytokine analysis in periapical lesions, bone blocks containing periapical tissue were ground using a pre-cooled sterile mortar and pestle, and the tissue fragments were dispersed in 1 ml of lysis buffer consisting of 100 mg/ml bovine albumin (fraction V; Sigma), 0.5% Triton X-100 (Sigma), 50 µg/ml gentamicin (Sigma), 10 mmol/L HEPES buffer (Invitrogen, Carlsbad, CA), 1× protease inhibitor cocktail (Sigma), and 0.1 mmol/L ethylenediamine tetraacetic acid (Invitrogen) in RPMI 1640 (Sigma). The suspensions were incubated for 1 hour on ice and centrifuged to remove debris. Supernatants were collected and stored at -70°C until assay.

Histology and Immunohistochemistry

For histology, bone blocks containing periapical tissue were decalcified and embedded in paraffin. Serial sections, 5 µm thick, were cut on a cryostat (Leica CM 1850), formaldehyde-fixed, and H&E-stained. For immunohistochemistry, tissues sections were treated with citrate buffer, pH 6.0 (Zymed, Fremont, CA), to unmask hidden epitopes followed by overnight incubation at 4°C with cytokine-specific primary antibodies (purified goat anti-mouse IFN-γ or anti-mouse IL-1α/β; R&D Systems, Minneapolis, MN) at an appropriate concentration. Control sections were incubated with isotype control immunoglobulins. After several washings with PBS, endogenous peroxidase was blocked for 20 minutes at room temperature with 0.3% peroxide hydrogen in methanol (Sigma). Sections were washed in PBS and incubated with the detection antibody rabbit anti-goat Ig conjugated to horseradish peroxidase. Reaction product was then developed using diaminobenzidine and hydrogen peroxide (PharMingen) as substrate. Sections were counterstained with Fast Green (Sigma) and mounted with Permount (Fisher Chemicals, Fairlawn, NJ).

Infection with *P. gingivalis*

Adult female mice, 11 weeks of age, were anesthetized via intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) in sterile PBS. Mice were placed on a jaw retraction board, and the pulps of both mandibular first molars were exposed using a no. 1/4 round bur

under a surgical microscope as described previously.²⁴ The exposure size was approximately equivalent to the diameter of the bur. Two microliters of *P. gingivalis* W83 (10^9 cells/ml) in pre-reduced, anaerobically sterilized Ringer's solution was placed into the pulp chamber and introduced into the root canal using a no. 06 endodontic file. The teeth were sealed with composite resin (Zenith, Englewood, NJ) to prevent contamination with microorganisms from the oral cavity.

Microcomputed Tomography

Mice were sacrificed by CO₂ asphyxiation 3 weeks after pulpal infection. Mandibles were isolated, and the left hemi-mandibles were fixed in fresh 4% paraformaldehyde in PBS and analyzed using a compact fan-beam-type tomograph (Micro-CT 20; Scanco Medical AG, Bassersdorf, Switzerland). For each sample, approximately 100 microtomographic slices were acquired at an increment of 17 μ m. The "pivot" slice, representing the central portions of the pulp and root canal showing a patent root canal apex, were selected from the stack of images. The cross-sectional area of periapical bone loss was determined using a pre-drawn template, to limit the coronal extent of the lesion, and a semiautomatic histomorphometric system (Optimas Bioscan; Media Cybernetics, Bethell, WA).²⁵ The images were encoded, randomized, and analyzed independently by two examiners. Each measurement was repeated three times, and the data are expressed in square millimeters of the mean $\pm$ SD.

IgG Isotype ELISA

Mice were bled before and 3 to 4 weeks after infection with *P. gingivalis*, and sera were stored at -20°C until use. The specific serum IgG isotype antibody response was measured by conventional ELISA. Wells of ELISA plates (Costar, Cambridge, MA) were coated with Pg lysate at a concentration of 500 ng/well. Sera were added at twofold serial dilutions followed by washes and addition of biotinylated isotype-specific secondary antibodies (rabbit anti-mouse IgG1 or IgG2a; BD Biosciences PharMingen, San Diego, CA). Wells were then washed and incubated with streptavidin-conjugated horseradish peroxidase (Zymed), after which substrate and chromogen were added, and absorbance was read on an ELISA plate reader (Dynatech, Chantilly, VT).

Statistical Analysis

Statistical analysis was performed using analysis of variance followed by Tukey's multiple comparison test (IN-STAT Software; GraphPad Software Inc., San Diego, CA). All values were considered significantly different at $P < 0.05$.

Results

Severe Local Inflammatory Reaction Induced by Immunization with *P. gingivalis* Antigens Formulated with Th1-Biasing Adjuvant

There is controversy concerning whether the Th1/Th2 paradigm can be applied to periodontal and periapical bone resorption caused by *P. gingivalis*.^{13,14,26,27} To examine this question, mice were initially immunized with a crude extract of *P. gingivalis* antigens formulated either with the adjuvant alum or with alum plus IL-12, which are known to induce strong and polarized Th2 or Th1 re-

Figure 1. Local inflammation in mice immunized with *P. gingivalis* antigens under Th1 or Th2 conditions. C57BL/6 mice (three per group) were immunized in the rear footpads with 10 μ g of *P. gingivalis* a soluble lysate antigens (Pg) preparation mixed with alum (25 μ g) (Th2) or with alum plus the cytokine IL-12 (1 μ g) (Th1). Mice were boosted 4 weeks later using the same antigen/adjuvant formulations used for primary sensitization. Massive swelling was observed in the footpads of mice immunized with the Th1 versus Th2 formulation (**A**). A quantitative measurement of the footpad swelling is depicted in **B**. After immunization, mice were sacrificed, and their feet were amputated and decalcified. **C:** Sections (5 μ m) were obtained from the inflammation site and H&E-stained. Note that the inflammatory cells are predominantly mononuclear and are noticeably more abundant in the Pg plus alum plus IL-12 immunized mice compared with the mice immunized with Pg plus alum.

Figure 2. Isotype-specific antibody response of Th1- and Th2-biased mice. Anti-*P. gingivalis* antibody responses of IgG1 (A) and IgG2a (B) isotypes were measured by ELISA. Sera were obtained from the immunized mice described in Figure 1.

sponses, respectively.^{22,23} Surprisingly, the Th1 formulation caused a severe local inflammation at the inoculation site in the footpad, which was not previously observed for other antigens inoculated either in the footpad with the leishmanial recombinant antigens TSA and LmST11²² or subcutaneously in the nape of the mice neck with HIV gp120 glycoprotein.²³ Macroscopically, intense footpad swelling was observed in the Th1-immunized group compared with mice immunized with *P. gingivalis* antigens formulated with the Th2-biasing adjuvant (Figure 1, A and B). The histology of the inflammation induced by the Th1 adjuvant was characterized by the presence of nonorganized granulomas with predominant mononuclear cell infiltration. In contrast, the local inflammation caused by the Th2-biased adjuvant had a dispersed distribution of mononuclear cells mixed with granulocytes (Figure 1C).

Selective Induction of Th1- or Th2-Biased Immune Response to *P. gingivalis* Antigens

To ascertain whether Th1- or Th2-biased responses were generated, we determined the specific isotype (IgG1 and IgG2a) antibody responses to *P. gingivalis* antigens. IgG1 and IgG2a isotypes of immunoglobulins are surrogates of Th2 and Th1 phenotypes of immune responses, respectively.²⁸ Serum samples were collected before and after immunizations and were used to determine the titer of specific IgG response in an ELISA format. Figure 2 shows that mice immunized with the bacterial antigens formulated with alum alone produced high titers of antigen-specific antibodies predominantly of the IgG1 isotype and little or no IgG2a. In contrast, mice immunized with *P. gingivalis* antigens presented in alum plus IL-12 produced high titers of antigen-specific antibodies of IgG2a and IgG1 isotypes.

To confirm these results at the cellular level, we measured the production of Th1 and Th2 cytokines (IFN- γ and IL-4, respectively) by lymph node cells obtained from

Figure 3. Antigen-induced proliferation of lymph node cells from Th1- and Th2-biased mice. C57BL/6 mice (three per group) were immunized as described in Figure 1. One week after boost, mice were sacrificed, and lymphocytes were obtained from popliteal lymph nodes and cultured for 3 days in the presence of medium containing *P. gingivalis* lysate antigens. Proliferation was measured by incorporation of [³H]thymidine added during the last 12 hours of culture. Results are expressed as the mean $\pm$ SD of triplicate cultures.

Figure 4. Production of cytokines by lymph node cells of Th1- and Th2-biased mice. Culture supernatants harvested at the end of the cultures described in Figure 3 were assayed for the presence of IFN- γ (A) and IL-4 (B) by sandwich ELISA. Bars are the SD of triplicate cultures.

mice immunized with *P. gingivalis* antigens presented with either Th1- or Th2-biased adjuvant formulations. Figure 3 shows that regardless of the adjuvant formulation, lymph node cells proliferated significantly on stimulation with *P. gingivalis* lysate, with Th1-biased cells exhibiting a stronger proliferative response than Th2-biased cells. Lymph node cells obtained from mice immunized with *P. gingivalis* antigens formulated in alum alone produced primarily IL-4 and no detectable IFN- γ . In contrast, mice immunized with *P. gingivalis* antigens in alum plus IL-2 produced predominantly IFN- γ , with little or no IL-4 (Figure 4). These results, taken in conjunction with the high serum IgG2a-specific antibody titers, indicate that immunization of mice with *P. gingivalis* formulated with alum plus IL-12 results in strong and polarized systemic Th1 responses, whereas immunization with the bacteria formulated with alum alone results in strong and specific systemic Th2 responses.

Th1 Response to P. gingivalis Antigens Exacerbates Alveolar Bone Resorption

The consequence of biased Th1 and Th2 responses on the pathological outcome of alveolar bone resorption caused by *P. gingivalis* was then determined. Groups of mice were immunized twice, 1 month apart, with the bacterial antigens formulated with alum alone or with alum plus IL-12. As controls, mice were immunized with alum and saline or with alum plus IL-12 and saline. Mice were then subjected to surgical exposure of the dental pulp in the first mandibular molars, followed by infection with *P. gingivalis*. Alveolar bone resorption was quantified by microcomputed tomography after 21 days. The results are summarized in Figure 5 and show that massive bone loss occurred surrounding the roots of infected teeth in mice previously immunized with *P. gingivalis* antigens formulated with the Th1-biased adjuvant. In contrast, animals that were nonimmunized, inoculated with the adju-

vants alone, or immunized with *P. gingivalis* antigens formulated with Th2-biasing adjuvant had minimal bone destruction. Quantitative measurement of lesion sizes detected by microcomputed tomography revealed that animals immunized with *P. gingivalis* antigens under Th1 conditions developed lesions that were significantly larger than the lesions observed in all other groups (Figure 5).

Severe Alveolar Bone Inflammatory Reaction Caused by P. gingivalis in Th1-Biased Mice

Alveolar bone resorption caused by oral microbial pathogens has traditionally been associated with an infiltrate composed primarily of mononuclear cells and granulocytes. To evaluate the inflammation present in the infrabony lesions in mice previously immunized with *P. gingivalis* antigens formulated with the two adjuvants, histopathological studies were performed. The results are illustrated in Figure 6 and show that a massive inflammatory reaction occurred in Th1-biased mice. Similar to the inflammation seen at subcutaneous sites of immunization, lesions within the alveolar bone in mice immunized with the Th1 adjuvant were characterized by the presence of nonorganized granulomas with a predominant mononuclear cell infiltration. Moreover, many multinucleated osteoclasts were found associated with resorption lacunae in bone adjacent to the granulomas. In contrast, the periapical inflammation observed in Th2-biased mice was moderate and characterized by a disperse distribution of mononuclear cells and granulocytes. Few osteoclasts were seen in these lesions. These results suggest that the presence of *P. gingivalis* antigens emanating from the dental pulp induces a local inflammatory reaction that is dependent on the Th response phenotype induced by the pre-sensitization protocol.

Figure 5. Periapical bone resorption caused by *P. gingivalis* in Th1- and Th2-biased mice. C57BL/6 mice (10 per group) were immunized as described in Figure 1. Four weeks after the second immunization, the mice were subjected to intrapulpal infection with *P. gingivalis*. Alveolar bone resorption was analyzed in the periapical region of the mandibular first molars by microcomputed tomography after 21 days. Representative images are shown. Note the extensive bone resorption (arrow) in Th1-biased mice compared with all other groups. The areas of periapical bone loss per group of mice were calculated from the microcomputed tomography images and are expressed in square millimeters. Bars represent the mean $\pm$ SD ($n = 10$ /group).

Local Production Th1 and Th2 Cytokines

The levels of Th1 and Th2 cytokines expressed within lesions were next determined to evaluate the *in situ* association of the immune response phenotype with the observed bone resorption. In addition, because IL-1 has been implicated in stimulating inflammatory bone resorption, this cytokine was also evaluated. As shown in Figure 7, Th1-biased mice had higher levels of IFN- γ as well as IL-1 α and IL-1 β in infrabony lesions compared with Th2-biased mice. Neither IL-4 nor IL-10 could be detected in the lesion tissues of either Th1- or Th2-biased mice (not shown). The spatial distribution of the cytokines in the lesions was evaluated by immunohistochemistry. Only the cytokines IFN- γ and IL-1 α/β could be evaluated because of the lack of commercially available specific antibodies for mouse IL-4 and IL-10 that are suitable for immunohistochemistry studies in paraffin/formol-fixed tissue samples. In addition, the commercially available anti-IL-1 antiserum (R&D Systems) suitable for these studies reacts with both IL-1 α and IL-1 β . The results, shown in Figure 8, confirm that both IFN- γ and IL-1 α/β were de-

tected within the massive inflammatory periapical lesions present in mice with Th1-biased response to *P. gingivalis* antigens. In contrast, IFN- γ and IL-1 were barely detected in the lesions of mice with a Th2-biased response. Interestingly, both cytokines could be detected in nonimmunized *P. gingivalis*-infected controls, albeit to a much lesser extent than in Th1-biased mice. Together, these results strongly support the notion that the pathogenesis of *P. gingivalis* is mediated to a great extent by the host Th1 immune response to antigens of the bacterium and that a pre-existing Th2 response could interfere with the expression of Th1 cytokines at the site of inflammation and reduce the development of bone resorption.

Discussion

It is generally accepted that infection-induced pathology caused by many microorganisms is associated with the T-helper subset response that is generated during the infectious process. Leishmaniasis is perhaps the foremost example of such a pattern. In this disease, Th1

Figure 6. Histology of periapical tissues of Th1- and Th2-biased mice. Tissues obtained from mice immunized and infected as described in Figure 5 were decalcified and H&E-stained. Note the massive mononuclear cell infiltrate in nonorganized granuloma lesions present in Th1-biased animals. **Arrows** indicate intense infiltration of osteoclasts in lesions of Th1-biased mice. Th2-biased mice had a noticeably less abundant cellular infiltrate and no obvious presence of osteoclasts.

cytokines are associated with resistance, and Th2 cytokines are disease promoters.^{29–37} Conversely, for most helminthic infections, resistance is associated with the emergence of Th2 responses, and susceptibility and pathology are mediated by Th1-specific responses to helminth antigens.^{38–42} Although production of Th1 or Th2 cytokines is a major component of the effector mechanisms of the host immune response to any infectious agent, a polarized association of infection-induced pathology with the Th1/Th2 paradigm, as exemplified above for leishmaniasis and helminthic diseases, has not been clearly described for a variety of other infectious diseases. Bone resorption caused either by an intrapulpal or periodontal infection is among these latter processes.

Here, we use an *in vivo* mouse model of pulpal infection-stimulated alveolar bone resorption to directly evaluate disease development in the context of strong systemic Th1- or Th2-biased responses to the pathogen *P. gingivalis*. Mice immunized with *P. gingivalis* antigens formulated with alum plus IL-12 were Th1-biased, as shown by high serum titers of specific IgG2a antibody response and production of large amounts of IFN- γ and only marginally detectable IL-4, by lymph node cells stimulated *in vitro* with the specific antigen. In contrast, mice immunized with *P. gingivalis* antigens with alum alone produced large amounts of IgG1 but no IgG2a, and their lymph node cells produced large amounts of IL-4 and low IFN- γ . It is important to stress that immunization with both adjuvant formulations resulted in vigorous antigen-induced T-cell proliferative responses *in vitro*. These results demonstrate that these adjuvant regimes induce potent but phenotypically distinct and polarized immune responses to *P. gingivalis*, similar to those seen in other systems.^{22,23}

The biased Th1 response resulted in dramatically increased alveolar bone resorption caused by intrapulpal infection with *P. gingivalis*, whereas resorption in mice with strong Th2 responses was similar to nonimmunized controls. Moreover, *in situ* analysis of cytokines in the periapical tissues showed high levels of IFN- γ , IL-1 α , and IL-1 β in mice biased toward Th1, compared with mice biased toward Th2. This cytokine pattern is probably responsible for the intense infiltration of both mononuclear cells and granulocytes in the periapical lesions of mice with a Th1-biased phenotype. In addition, the number of inflammatory cells, including many osteoclasts, was clearly much larger in the lesions of Th1-biased mice than in the lesions in Th2 or control mice. Unfortunately, direct enumeration of Th1 and Th2 cells was not possible because there are no reliable markers for immunohistochemistry analyses of these cells in tissue sections. Nevertheless, the marked presence of IFN- γ and IL-1 in the lesions of Th1-biased mice (Figure 8) and their absence or low expression in Th2-biased animals strongly indicates that the cells infiltrating the lesions in the infected animals were of the corresponding Th1 or Th2 phenotype. Taken together, these results strongly support the hypothesis that the pathogenesis exerted by *P. gingivalis* is mediated to a great extent by the host Th1 immune response to antigens of this pathogen.

The destructive effects of a Th1-mediated response seen in periapical resorptive lesions are a general mechanism of inflammatory bone loss that can occur in periodontal disease and other osteolytic processes as well. For example, in a rat model of periodontal disease, adoptive transfer of a Th1 clone specific for *Actinobacillus actinomycetemcomitans* outer membrane protein fol-

Figure 7. Local expression of cytokines in the periapical granulomas of Th1- and Th2-biased mice. Proteins were extracted from periapical lesion tissues from the mice described in Figure 5 and were tested for the presence of cytokines by sandwich ELISA. Results are expressed as picograms of cytokine per milligram of periapical tissue and represent the mean $\pm$ SD of the measurements obtained from 10 mice per group. Neither IL-4 nor IL-10 was detected in any sample (not shown).

lowed by intragingival injection of the antigen plus lipopolysaccharide resulted in gingival inflammation and periodontal bone resorption.⁴³ In another study,⁴⁴ pre-immunization of mice with paraformaldehyde-fixed *P. gingivalis* followed by suprapariosteal inoculation of viable organisms over the calvarium resulted in enhanced inflammation and tissue destruction compared with sham-immunized animals. Coincidentally, in recent observations,⁴⁵ we showed that immunization of mice with *P. gingivalis* in the absence of adjuvant induces a predominant Th1 response, providing circumstantial evidence that the calvarial osteolysis caused by *P. gingivalis* is associated with a Th1 response. In addition, protection studies in a murine periodontitis model, using *P. gingivalis* proteinase and adhesin epitopes as antigens, have shown that protected animals developed a predominant systemic *P. gingivalis*-specific IgG1 response, and their lymph nodes produced a higher ratio of IL-4 to IFN- γ in response to antigen stimulation *in vitro*, whereas mice with disease produced an inverse pattern of cytokines.²⁶

Whereas T-cell-mediated responses in periapical versus periodontal bone resorption share similarities, differences seem to exist in cytokine responses that may affect the final pathway(s) that stimulates bone loss. For example, IL-1 α/β was strongly up-regulated in lesions caused by *P. gingivalis* infection in prior studies and in the present study, probably as a result of IFN- γ -induced macrophage activation, but was not increased by infection in a mouse model of periodontal disease.¹⁴ As noted, IL-1 is a powerful inducer of RANKL in osteoblasts/stromal cells.²⁴ Moreover, periodontal bone loss was unaffected by treatment of mice with neutralizing anti-IL-1 α/β or anti-IL-1RI antibodies, suggesting that an IL-1 independent mechanism may operate in periodontal disease, possibly using gingival fibroblasts that stimulate bone resorption via prostaglandin and cyclic AMP-independent mechanisms.⁴⁶ In this regard, activated IFN- γ -producing Th1 cells themselves express RANKL, induce osteoclastogenesis, and mediate infection-stimulated alveolar bone resorption.^{47,48} Additional studies will be needed to precisely determine the pathways leading to bone resorption in Th1-biased animals.

The experiments described here indicate that the pathogenesis of periapical inflammation caused by *P. gingivalis* clearly resembles the lesion and immunological mediators of experimental arthritis.^{49–54} The inflammation present in these diseases, including Lyme-induced arthritis, is characterized by nonorganized granulomas composed predominantly of mononuclear cells. More importantly, the inflammation is mediated by a strong Th1 response to either the antigen used to trigger the disease or antigens of the infectious agent. In contrast, induction of a potent Th2 response not only fails to cause arthritis but regulates lesion development.^{55–58} Therefore, our results add strong experimental evidences in favor of the proposed association between aggressive periodontitis, juvenile idiopathic arthritis, and rheumatoid arthritis.^{59–67}

Finally, these results support the idea that Th1/Th2-inducing antigens of *P. gingivalis* can be identified and assessed for their possible use as immunotherapeutic agents as an adjunct to conventional treatment of peria-

Figure 8. Immunohistochemical staining for IFN- γ and IL-1 α/β in periapical granulomas. Periapical tissues obtained from mice immunized and infected as described in Figure 5 were decalcified, formalin-fixed, and embedded in paraffin. Sections of 5- μ m thickness were obtained and processed as described in Materials and Methods. Note the abundant expression of both IFN- γ and IL-1 α/β (brown and dark brown colors) in Th1-biased mice. R, distal root, first mandibular molar; B, alveolar bone; P, periapical granuloma.

pical and periodontal diseases. We have recently used a murine *P. gingivalis*-specific CD4⁺ T-cell line to screen an expression genomic library of this oral pathogen and identified eight *P. gingivalis* genes coding for T-helper-biased immune responses during infection.⁴⁵ Studies are in progress to evaluate the involvement of each of these proteins in the molecular pathogenesis of bone resorption caused by *P. gingivalis*, in the absence/presence of other *P. gingivalis* components such as lipopolysaccharide that may modulate the response⁶⁸ and to assess their value as prophylactic or therapeutic vaccine candidates. These studies are supported by experiments showing that down-regulation of ongoing Th1-mediated pathology in experimental arthritis by stimulation of the Th2 arm of the immune response with an adjuvant-like alum has the capacity to prevent and/or ameliorate the disease.^{50,52,69}

Acknowledgments

This investigation conducted in a facility renovated with support from Research Facilities Improvement grant C06RR11244 from the National Center for Research Resources, National Institutes of Health.

References

1. Socransky SS, Haffajee AD: The bacterial etiology of destructive periodontal disease: current concepts. *J Periodontol* 1992, 63:322–331
2. Bragd L, Dahlen G, Wikstrom M, Slots J: The capability of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* to indicate progressive periodontitis: a retrospective study. *J Clin Periodontol* 1987, 14:95–99
3. Haffajee AD, Socransky SS, Dzink JL, Taubman MA, Ebersole JL, Smith DJ: Clinical, microbiological and immunological features of subjects with destructive periodontal diseases. *J Clin Periodontol* 1988, 15:240–246
4. van Winkelhoff AJ, van Steenberghe TJ, de Graaff J: The role of black-pigmented *Bacteroides* in human oral infections. *J Clin Periodontol* 1988, 15:145–155
5. Holt SC, Ebersole J, Felton J, Brunsvold M, Kornman KS: Implantation of *Bacteroides gingivalis* in nonhuman primates initiates progression of periodontitis. *Science* 1988, 239:55–57
6. Baker PJ, Evans RT, Roopenian DC: Oral infection with *Porphyromonas gingivalis* and induced alveolar bone loss in immunocompetent and severe combined immunodeficient mice. *Arch Oral Biol* 1994, 39:1035–1040
7. Ricucci D, Bergenholz G: Bacterial status in root-filled teeth exposed to the oral environment by loss of restoration and fracture or caries: a histobacteriological study of treated cases. *Int Endod J* 2003, 36:787–802
8. Tanabe N, Maeno M, Suzuki N, Fujisaki K, Tanaka H, Ogiso B, Ito K: IL-1 alpha stimulates the formation of osteoclast-like cells by increasing M-CSF and PGE2 production and decreasing OPG production by osteoblasts. *Life Sci* 2005, 77:615–626
9. Kwan Tat S, Padriens M, Theoleyre S, Heymann D, Fortin Y: IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. *Cytokine Growth Factor Rev* 2004, 15:49–60
10. Cheung J, Mak YT, Papaioannou S, Evans BA, Fogelman I, Hampson G: Interleukin-6 (IL-6), IL-1, receptor activator of nuclear factor kappaB ligand (RANKL) and osteoprotegerin production by human osteoblastic cells: comparison of the effects of 17-beta oestradiol and raloxifene. *J Endocrinol* 2003, 177:423–433
11. Katagiri T, Takahashi N: Regulatory mechanisms of osteoblast and osteoclast differentiation. *Oral Dis* 2002, 8:147–159
12. Weitzmann MN, Cenci S, Rifas L, Brown C, Pacifici R: Interleukin-7 stimulates osteoclast formation by up-regulating the T-cell production of soluble osteoclastogenic cytokines. *Blood* 2000, 96:1873–1878
13. Sasaki H, Hou L, Belani A, Wang CY, Uchiyama T, Muller R, Stashenko P: IL-10, but not IL-4, suppresses infection-stimulated bone resorption in vivo. *J Immunol* 2000, 165:3626–3630
14. Sasaki H, Okamoto Y, Kawai T, Kent R, Taubman M, Stashenko P: The interleukin-10 knockout mouse is highly susceptible to *Porphyromonas gingivalis*-induced alveolar bone loss. *J Periodontol Res* 2004, 39:432–441
15. Stashenko P, Wang CY, Tani-Ishii N, Yu SM: Pathogenesis of induced

- rat periapical lesions. *Oral Surg Oral Med Oral Pathol* 1994, 78:494–502
16. Wang CY, Stashenko P: The role of interleukin-1 alpha in the pathogenesis of periapical bone destruction in a rat model system. *Oral Microbiol Immunol* 1993, 8:50–56
17. Ishii T, Saito T, Morimoto K, Takeuchi Y, Asano S, Kumegawa M, Ogata E, Matsumoto T: Estrogen stimulates the elaboration of cell/matrix surface-associated inhibitory factor of osteoclastic bone resorption from osteoblastic cells. *Biochem Biophys Res Commun* 1993, 191:495–502
18. Kawashima N, Stashenko P: Expression of bone-resorptive and regulatory cytokines in murine periapical inflammation. *Arch Oral Biol* 1999, 44:55–66
19. Sasaki H, Balto K, Kawashima N, Eastcott J, Hoshino K, Akira S, Stashenko P: Gamma interferon (IFN-gamma) and IFN-gamma-inducing cytokines interleukin-12 (IL-12) and IL-18 do not augment infection-stimulated bone resorption in vivo. *Clin Diagn Lab Immunol* 2004, 11:106–110
20. Balto K, Sasaki H, Stashenko P: Interleukin-6 deficiency increases inflammatory bone destruction. *Infect Immun* 2001, 69:744–750
21. Houri-Haddad Y, Soskolne WA, Shai E, Palmon A, Shapira L: Interferon-gamma deficiency attenuates local P. gingivalis-induced inflammation. *J Dent Res* 2002, 81:395–398
22. Campos-Neto A, Porrozi R, Greeson K, Coler RN, Webb JR, Seiky YA, Reed SG, Grimaldi Jr G: Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. *Infect Immun* 2001, 69:4103–4108
23. Jankovic D, Caspar P, Zweig M, Garcia-Moll M, Showalter SD, Vogel FR, Sher A: Adsorption to aluminum hydroxide promotes the activity of IL-12 as an adjuvant for antibody as well as type 1 cytokine responses to HIV-1 gp120. *J Immunol* 1997, 159:2409–2417
24. Teles R, Wang CY, Stashenko P: Increased susceptibility of RAG-2 SCID mice to dissemination of endodontic infections. *Infect Immun* 1997, 65:3781–3787
25. Balto K, Muller R, Carrington DC, Dobeck J, Stashenko P: Quantification of periapical bone destruction in mice by micro-computed tomography. *J Dent Res* 2000, 79:35–40
26. O'Brien-Simpson NM, Pathirana RD, Paolini RA, Chen YY, Veith PD, Tam V, Ally N, Pike RN, Reynolds EC: An immune response directed to proteinase and adhesin functional epitopes protects against Porphyromonas gingivalis-induced periodontal bone loss. *J Immunol* 2005, 175:3980–3989
27. Gemmell E, Winning TA, Bird PS, Seymour GJ: Cytokine profiles of lesional and splenic T cells in Porphyromonas gingivalis infection in a murine model. *J Periodontol* 1998, 69:1131–1138
28. Stevens TL, Bossie A, Sanders VM, Fernandez-Botran R, Coffman RL, Mosmann TR, Vitetta ES: Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* 1988, 334:255–258
29. Launois P, Tacchini-Cottier F, Parra-Lopez C, Louis JA: Cytokines in parasitic diseases: the example of cutaneous leishmaniasis. *Int Rev Immunol* 1998, 17:157–180
30. Julia V, Rassoulzadegan M, Glaichenhaus N: Resistance to Leishmania major induced by tolerance to a single antigen. *Science* 1996, 274:421–423
31. Güler ML, Gorham JD, Hsieh CS, Mackey AJ, Steen RG, Dietrich WF, Murphy KM: Genetic susceptibility to Leishmania: IL-12 responsiveness in TH1 cell development. *Science* 1996, 271:984–987
32. Pirmez C, Yamamura M, Uyemura K, Paes-Oliveira M, Conceicao-Silva F, Modlin RL: Cytokine patterns in the pathogenesis of human leishmaniasis. *J Clin Invest* 1993, 91:1390–1395
33. Moll H, Rollinghoff M: Resistance to murine cutaneous leishmaniasis is mediated by TH1 cells, but disease-promoting CD4+ cells are different from TH2 cells. *Eur J Immunol* 1990, 20:2067–2074
34. Müller I, Garcia-Sanz JA, Titus R, Behn R, Louis J: Analysis of the cellular parameters of the immune responses contributing to resistance and susceptibility of mice to infection with the intracellular parasite, Leishmania major. *Immunol Rev* 1989, 112:95–113
35. Scott P, Natovitz P, Coffman RL, Pearce E, Sher A: Immunoregulation of cutaneous leishmaniasis: T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J Exp Med* 1988, 168:1675–1684
36. Kopf M, Brombacher F, Kohler G, Kienzle G, Widmann KH, Lefrang K, Humborg C, Ledermann B, Solbach W: IL-4-deficient Balb/c mice resist infection with Leishmania major. *J Exp Med* 1996, 184:1127–1136
37. Swihart K, Fruth U, Messmer N, Hug K, Behn R, Huang S, Del Giudice G, Aguet M, Louis JA: Mice from a genetically resistant background lacking the interferon gamma receptor are susceptible to infection with Leishmania major but mount a polarized T helper cell 1-type CD4+ T cell response. *J Exp Med* 1995, 181:961–971
38. Helmbly H, Takeda K, Akira S, Grencis RK: Interleukin (IL)-18 promotes the development of chronic gastrointestinal helminth infection by downregulating IL-13. *J Exp Med* 2001, 194:355–364
39. Artis D, Humphreys NE, Bancroft AJ, Rothwell NJ, Potten CS, Grencis RK: Tumor necrosis factor alpha is a critical component of interleukin 13-mediated protective T helper cell type 2 responses during helminth infection. *J Exp Med* 1999, 190:953–962
40. Bancroft AJ, Else KJ, Sypek JP, Grencis RK: Interleukin-12 promotes a chronic intestinal nematode infection. *Eur J Immunol* 1997, 27:866–870
41. Curry AJ, Else KJ, Jones F, Bancroft A, Grencis RK, Dunne DW: Evidence that cytokine-mediated immune interactions induced by Schistosoma mansoni alter disease outcome in mice concurrently infected with Trichuris muris. *J Exp Med* 1995, 181:769–774
42. Else KJ, Finkelman FD, Maliszewski CR, Grencis RK: Cytokine-mediated regulation of chronic intestinal helminth infection. *J Exp Med* 1994, 179:347–351
43. Kawai T, Eisen-Lev R, Seki M, Eastcott JW, Wilson ME, Taubman MA: Requirement of B7 costimulation for Th1-mediated inflammatory bone resorption in experimental periodontal disease. *J Immunol* 2000, 164:2102–2109
44. Leone CW, Bokhadhor H, Kuo D, Desta T, Yang J, Siqueira MF, Amar S, Graves DT: Immunization enhances inflammation and tissue destruction in response to Porphyromonas gingivalis. *Infect Immun* 2006, 74:2286–2292
45. Gonçalves RB, Leshem O, Bernards K, Webb JR, Stashenko PP, Campos-Neto A: T-cell expression cloning of Porphyromonas gingivalis genes coding for T helper-biased immune responses during infection. *Infect Immun* 2006, 74:3958–3966
46. Sjöström S, Hanström L, Lerner UH: The bone resorbing activity released by gingival fibroblasts isolated from patients with periodontitis is independent of interleukin-1. *J Periodontol Res* 2000, 35:74–84
47. Kotake S, Nanke Y, Mogi M, Kawamoto M, Furuya T, Yago T, Kobashigawa T, Togari A, Kamatani N: IFN-gamma-producing human T cells directly induce osteoclastogenesis from human monocytes via the expression of RANKL. *Eur J Immunol* 2005, 35:3353–3363
48. Zhang X, Teng YT: Interleukin-10 inhibits gram-negative-microbe-specific human receptor activator of NF- κ B ligand-positive CD4+Th1-cell-associated alveolar bone loss in vivo. *Infect Immun* 2006, 74:4927–4931
49. Hanyecz A, Berlo SE, Szanto S, Broeren CP, Mikecz K, Glant TT: Achievement of a synergistic adjuvant effect on arthritis induction by activation of innate immunity and forcing the immune response toward the Th1 phenotype. *Arthritis Rheum* 2004, 50:1665–1676
50. Finnegan A, Mikecz K, Tao P, Glant TT: Proteoglycan (aggrecan)-induced arthritis in BALB/c mice is a Th1-type disease regulated by Th2 cytokines. *J Immunol* 1999, 163:5383–5390
51. Kageyama Y, Koide Y, Yoshida A, Uchijima M, Arai T, Miyamoto S, Ozeki T, Hiyoshi M, Kushida K, Inoue T: Reduced susceptibility to collagen-induced arthritis in mice deficient in IFN-gamma receptor. *J Immunol* 1998, 161:1542–1548
52. Mauri C, Williams RO, Walmsley M, Feldmann M: Relationship between Th1/Th2 cytokine patterns and the arthritogenic response in collagen-induced arthritis. *Eur J Immunol* 1996, 26:1511–1518
53. Brand DD, Kang AH, Rosloniec EF: Immunopathogenesis of collagen arthritis. *Springer Semin Immunopathol* 2003, 25:3–18
54. Malfait AM, Butler DM, Presky DH, Maini RN, Brennan FM, Feldmann M: Blockade of IL-12 during the induction of collagen-induced arthritis (CIA) markedly attenuates the severity of the arthritis. *Clin Exp Immunol* 1998, 111:377–383
55. Mauri C, Feldmann M, Williams RO: Down-regulation of Th1-mediated pathology in experimental arthritis by stimulation of the Th2 arm of the immune response. *Arthritis Rheum* 2003, 48:839–845
56. Mattsson L, Lorentzen JC, Svelander L, Bucht A, Nyman U, Klareskog L, Larsson P: Immunization with alum-collagen II complex suppresses the development of collagen-induced arthritis in rats by deviating the immune response. *Scand J Immunol* 1997, 46:619–624

57. Doncarli A, Stasiuk LM, Fournier C, Abehsira-Amar O: Conversion in vivo from an early dominant Th0/Th1 response to a Th2 phenotype during the development of collagen-induced arthritis. *Eur J Immunol* 1997, 27:1451–1458
58. Chu CQ, Londei M: Induction of Th2 cytokines and control of collagen-induced arthritis by nondepleting anti-CD4 Abs. *J Immunol* 1996, 157:2685–2689
59. Taubman MA, Kawai T: Involvement of T-lymphocytes in periodontal disease and in direct and indirect induction of bone resorption. *Crit Rev Oral Biol Med* 2001, 12:125–135
60. Havemose-Poulsen A, Westergaard J, Stoltze K, Skjodt H, Danneskiold-Samsøe B, Locht H, Bendtzen K, Holmstrup P: Periodontal and hematological characteristics associated with aggressive periodontitis, juvenile idiopathic arthritis, and rheumatoid arthritis. *J Periodontol* 2006, 77:280–288
61. Havemose-Poulsen A, Sørensen LK, Stoltze K, Bendtzen K, Holmstrup P: Cytokine profiles in peripheral blood and whole blood cell cultures associated with aggressive periodontitis, juvenile idiopathic arthritis, and rheumatoid arthritis. *J Periodontol* 2005, 76:2276–2285
62. Bartold PM, Marshall RI, Haynes DR: Periodontitis and rheumatoid arthritis: a review. *J Periodontol* 2005, 76:2066–2074
63. Ribeiro J, Leao A, Novaes AB: Periodontal infection as a possible severity factor for rheumatoid arthritis. *J Clin Periodontol* 2005, 32:412–416
64. Mercado FB, Marshall RI, Bartold PM: Inter-relationships between rheumatoid arthritis and periodontal disease: a review. *J Clin Periodontol* 2003, 30:761–772
65. Mercado FB, Marshall RI, Klestov AC, Bartold PM: Relationship between rheumatoid arthritis and periodontitis. *J Periodontol* 2001, 72:779–787
66. Mercado F, Marshall RI, Klestov AC, Bartold PM: Is there a relationship between rheumatoid arthritis and periodontal disease? *J Clin Periodontol* 2000, 27:267–272
67. Greenwald RA, Kirkwood K: Adult periodontitis as a model for rheumatoid arthritis (with emphasis on treatment strategies). *J Rheumatol* 1999, 26:1650–1653
68. Darveau RP, Pham TT, Lemley K, Reife RA, Bainbridge BW, Coats SR, Howald WN, Way SS, Hajjar AM: Porphyromonas gingivalis lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect Immun* 2004, 72:5041–5051
69. Salomon I, Netzer N, Wildbaum G, Schiff-Zuck S, Maor G, Karin N: Targeting the function of IFN-gamma-inducible protein 10 suppresses ongoing adjuvant arthritis. *J Immunol* 2002, 169:2685–2693