T-cell contributions to alveolar bone loss in response to oral infection with *Porphyromonas gingivalis*

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Baker PJ, Garneau J, Howe L, Roopenian DC. T-cell contributions to alveolar bone loss in response to oral infection with *Porphyromonas gingivalis*. Acta Odontol Scand 2001;59:222–225. Oslo. ISSN 0001-6357.

We have previously shown that mice lacking $CD4^+$, but not $CD8^+$, T cells lose less alveolar bone loss in response to oral infection with *Porphyromonas gingivalis* than do immunocompetent mice of the same genetic background, indicating that $CD4^+$ T cells contribute to bone resorption. The $CD4^+$ and $CD8^+$ T-cell knockouts were produced by targeted deletions of, respectively, major histocompatibility complex II (MHCII) or β_2-microglobulin (producing non-expression of MHCI). Because MHC deletions can have other effects in addition to those on T-cell selection, we wanted to confirm that the lessened bone loss was truly an effect of the lack of T cells. Consequently, we repeated our experiments with C57B1/6J-Tcra mice that have a targeted deletion of the alpha chain of the T-cell receptor (Tcra). Six weeks after oral infection with *P. gingivalis* ATCC 53977 the total bone loss at buccal maxillary sites was 0.28 mm in infected immunocompetent mice ($P = 0.002$ compared with sham-infected mice), whereas in Tcra knockouts the bone loss was only 0.08 mm ($P = 0.04$ compared with shams). The T-cell-deficient mice thus lost 70% less bone after infection than did genetically matched immunocompetent mice ($P = 0.003$). These experiments confirm that T cells, and their responses to oral infection with *P. gingivalis*, help to push bone remodeling in the direction of net loss of bone. □ *Alveolar bone resorption; periodontal disease;* Porphyromonas gingivalis*; T lymphocytes*

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Periodontal diseases are chronic inflammatory diseases that lead to destruction of the gingival soft tissue and loss of the periodontal ligament attachment between the tooth root and the alveolar bone of the jaw. In addition, periodontal diseases eventually lead to net resorption of the alveolar bone itself, and it is this outcome of the disease on which our laboratory has focused.

We have developed a mouse model in which we can reliably induce alveolar bone loss by oral infection with *Porphyromonas gingivalis*, a gram-negative black-pigmented bacterial species closely associated with adult periodontitis in humans (1). These are specific-pathogen-free mice. We suppress their normal oral flora by giving the mice an antimicrobial suspension (trimethoprim, 2 mg/ml, and sulfamethoxazole, 0.3 mg/ml) ad libitum in their drinking water for 10 days. After an antibiotic-free period of at least 3 days, and up to 7 days (unpublished data), we infect with *P. gingivalis* A7A1-28 (ATCC 53977). Many, but not all, strains of *P. gingivalis* are effective in inducing bone loss (2), and ATCC strain 53977 is the most virulent in our hands. Oral immunization with killed *P. gingivalis* does not induce bone loss; the bacteria must be alive and must successfully colonize the oral cavity (2).

For the infection, bacteria are grown on blood agar, supplemented with hemin and menadione, in an anaerobic environment (5% H_2, 10% CO_2, and 85% N_2) for 1 week (3). Bacteria are suspended in phosphate-buffered saline to 10^{10} colony-forming units per milliliter, and the suspension is thickened by addition of carboxymethylcellulose w/v to 2% (4). Using a gavage needle, 100 µl of bacterial suspension is put into the esophagus and the oral cavity of each mouse. Controls consist of sham-infected mice that receive the antimicrobial pretreatment and carboxymethylcellulose suspension without *P. gingivalis*. The oral infection is repeated three times at 2-day intervals (3, 4). After a period of 42 days following the last infection the oral microflora is sampled by using paper points, and the mice are euthanized by CO_2 inhalation. Blood is collected and saved for measurement of anti-*P. gingivalis* 53977 titers by enzyme linked immunosorbent assay (ELISA). The heads are defleshed and the distance from the cementoenamel junction to the alveolar bone crest (CEJ to ABC) is measured at 14 sites on the buccal sides of the maxillary molars (5, 6), using a dissecting microscope and a video imaging system (7). The measurements are added together to produce a 14-site total. The CEJ to ABC distance does not change in sham-infected mice in the 47-day period of the experiments, whereas it increases over time in infected mice as alveolar bone is resorbed (8). The total CEJ to ABC from individual mice is subtracted from the mean total CEJ to ABC in sham-infected mice to give the millimeters of change in bone, such that negative values indicate alveolar bone loss (7).

Using this model, we have taken advantage of the rich array of immune knockout mice available, to systematically explore which parts of the immune system may be protective against alveolar bone loss and, alternatively, which may contribute to bone resorption. Indeed, one of the advantages of a mouse model is the large and constantly increasing number of defined mutations avail-

Table 1. Bone loss in T cell or T-cell cytokine knockout mice in response to oral infection with *Porphyromonas gingivalis*, in comparison with bone loss in infected immunocompetent mice of the same genetic background

Immune status of mouse strain	Change (total mm) in bone in infected mice ± standard error of the mean	Different from sham-infected mice of same strain
Immunocompetent	−0.28 ± 0.05	$P = 0.00012$*
T- and B-cell-deficient (SCID)	+0.04 ± 0.05	Not significant
Immunocompetent	−0.33 ± 0.05	$P = 0.0011$
$CD8^+$ T-cell-deficient (β_2-microglobulin knockout)	−0.39 ± 0.12	$P = 0.015$
$CD4^+$ T-cell-deficient (H2-A_β knockout)	−0.018 ± 0.12	Not significant
Immunocompetent	−0.28 ± 0.05	$P = 0.002$
T-cell deficient (Tcra knockout)	−0.08 ± 0.03	$P = 0.04$
Immunocompetent	−0.25 ± 0.08	$P = 0.011$
IL-6 knockout	+0.19 ± 0.04	Not significant
Immunocompetent	−0.18 ± 0.04	$P = 0009$
IFN-γ knockout	−0.06 ± 0.05	Not significant

* P values determined by t tests; with the exception of the Tcra knockouts, data are from Ref. 7.

able. Infected and sham-infected immunocompetent mice are always included simultaneously with any studies on knockout mice. It is important that the immunocompetent mice be of the same genetic background as the knockouts, because different strains of immunocompetent mice differ in their susceptibility to bone loss after *P. gingivalis* infection (6).

The data from our studies on the contributions of T cells and their cytokines are summarized in Table 1. We have shown that severe combined immunodeficient (SCID) mice, with no T or B lymphocytes, lose little or no bone after oral infection, far less bone resorption than occurs in infected immune competent mice (3, 7). These results indicate that T or B lymphocytes, or some combination of the two subsets, have a net destructive effect on bone remodeling in response to oral infection.

We extended those studies by examining mice that lack either $CD8^+$ T cells or $CD4^+$ T cells. These T-cell deficiencies were produced by genetic deletions of either β_2-microglobulin or H2-A_β chain, which block MHCI or MHCII expression, respectively, and thus interfere with either CD8 or CD4 T cell maturation in the thymus (7). When $CD8^+$ T-cell-deficient mice are orally infected with *P. gingivalis*, they lose the same amount of alveolar bone as do infected immunocompetent mice (Table 1), indicating that $CD8^+$ T cells have a neutral effect on post-infection mechanisms leading to bone resorption. In contrast, when mice lacking $CD4^+$ T cells are infected, they lose less bone than do infected immunocompetent mice. In fact, the bone levels in infected $CD4^+$ T-cell-deficient mice are not significantly different from those in sham-infected mice (Table 1). That there is less bone loss when $CD4^+$ T cells are missing than there is when they are present indicates that $CD4^+$ T-cell responses to oral infection with *P. gingivalis* leads in some way to net resorption of the alveolar bone. The diminished bone loss is not due to differences in infection rate; $CD4^+$ or $CD8^+$ T-cell-deficient mice have the same rate of infection as the immunocompetent parent strain (7). There is also no correlation between bone loss and specific antibody titer. Immunocompetent mice develop *P. gingivalis*-specific serum IgG when infected, but $CD4^+$ T-cell-deficient mice do not. $CD8^+$ T-cell-deficient mice have a specific IgG response, but it is lower than that of the immunocompetent parent strain, due to differences in IgG processing related to the β_2-microglobulin deletion (9), and their bone loss is the same as that in immunocompetent mice.

Because non-expression of β_2-microglobulin or of MHC II may have other effects in addition to those on T-cell maturation, we wished to verify our findings in another T-cell knockout mouse strain. We consequently tested mice with a genetic deletion of the alpha chain of their T-cell receptors, Tcra knockouts (C57BL/6J-*Tcra*^{tm1Mom} from The Jackson Laboratory, Bar Harbor, Maine). These mice have the advantage that the gene knockout affects the T cells directly. We have recently shown that infected Tcra knockouts did have bone loss (Table 1), but it was less than in the immunocompetent mice. T tests showed that bone change in infected Tcra knockout was different from bone change in infected immunocompetent at $P = 0.003$. Thus we showed in a knockout of a different pathway that T cells contribute to bone loss after infection with *P. gingivalis*. Because the Tcra knockout affects $CD4^+$ and $CD8^+$ T cells equally, we cannot use this strain to distinguish between the two T-cell subsets.

Normal bone homeostasis is a tightly regulated alternation of bone deposition by osteoblasts, followed by bone resorption by osteoclasts, so that there is no net gain or loss of bone. So how could T cells, particularly T cells responding to an oral infection, affect bone homeostasis? Many immune cytokines affect bone remodeling, and T cells are a known source of some of the cytokines (10–12); therefore, one possibility is via the action of T-cell cytokines.

We examined the effects of deletion of two T-cell cytokines, interleukin-6 (IL-6) and interferon-gamma (IFN-γ). IL-6 is considered a Th2 cytokine, and IFN-γ a Th1 cytokine. We found that mice lacking IL-6 do not lose

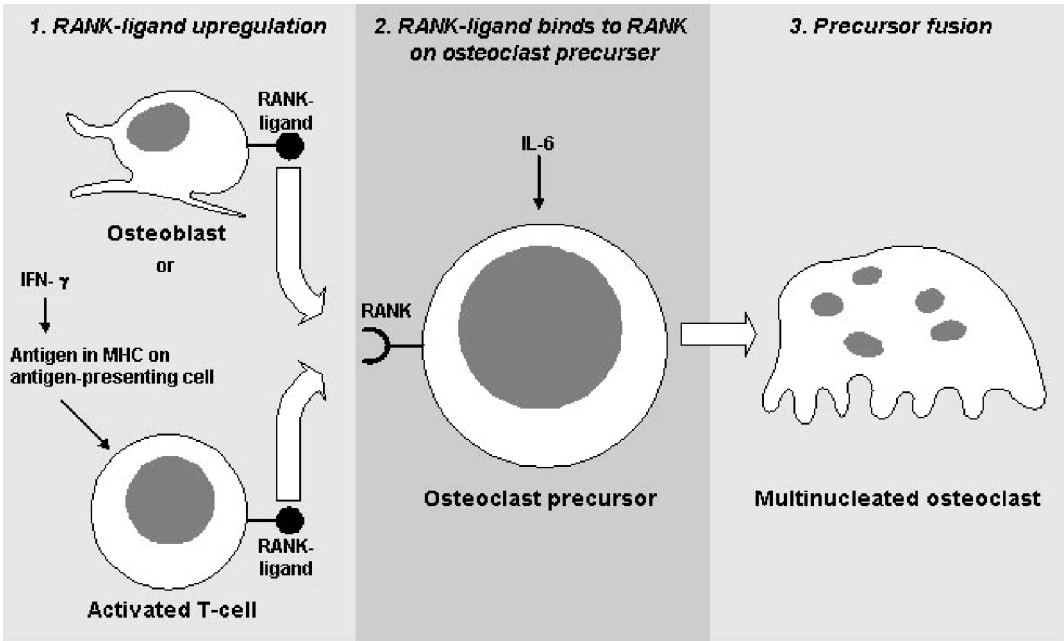

Fig. 1. Possible roles of T cell and two T-cell cytokines, interleukin-6 (IL-6) and interferon gamma (IFN-γ), in tipping bone homeostasis toward net resorption of alveolar bone in response to oral infection with *Porphyromonas gingivalis*. MHC = major histocompatibility complex.

alveolar bone in response to *P. gingivalis* infection; in fact, they actually gain small amounts of alveolar bone, although the change is not statistically significant (Table 1) (7). This finding is in line with in vitro studies that have shown that *P. gingivalis* fimbriae induce IL-6 secretion (13) and that IL-6 induces bone resorption (14). It is also in line with clinical studies that have shown a correlation between the progression of periodontal disease in humans and the presence of cells in inflamed gingiva producing Th2 cytokines, including IL-6 (15–17).

Bone loss does not appear to be exclusively a Th2-mediated response, however. Mice lacking the Th1 cytokine IFN-γ also had decreased bone loss compared with infected immunocompetent mice (Table 1) (7). This finding is in line with reports that suggest that Th1 $CD4^+$ T cells are destructive (18) and that $CD4^+$ T cells from inflamed gingiva of periodontitis patients express mRNA for both IFN-γ and IL-6 (19).

IL-6 increases the number of osteoclasts (Fig. 1), thus tipping bone homeostasis in the direction of net resorption (10). Bone loss induced by interleukin-1 (IL-1) or tumor necrosis factor (TNF) may actually work via IL-6, since anti-IL-6 antibodies block resorption induced by any of these cytokines (12). The mechanisms through which IFN-γ could lead to bone loss are not known and are likely to be less direct. One possible mechanism is by its upregulation of MHC II expression on many types of cells, making them more efficient antigen-presenting cells for the activation of $CD4^+$ T cells (Fig. 1).

Activation of T cells causes them to begin to express RANK ligand, which brings us to another possibility by which T cells could disrupt bone homeostasis. As shown in Fig. 1, osteoblasts normally activate osteoclast precursors by cell-to-cell contact stabilized by the binding of RANK ligand on osteoblasts to RANK on osteoclast precursors. Neither RANK ligand nor RANK is constitutively expressed; each is upregulated by other hormones and cytokines (10, 20, 21). Once both are present, osteoblasts can trigger the fusion of osteoclast precursors into multinucleated osteoclasts, which can actively resorb bone. In addition to osteoblasts, the only other cells known to express RANK ligand are activated and memory T cells (22). As is also shown in Fig. 1, when activated T cells begin to express RANK ligand, they are able to substitute for osteoblasts, triggering fusion of osteoclast precursors into multinucleated osteoclasts (22). It is thought that T cells are not required for normal bone homeostasis because T-cell-deficient mice have normal bone structure and normal tooth eruption but that disease states resulting in T-cell activation can promote net resorption of bone (23). We are currently investigating the effect of oral infection with *P. gingivalis* on MHC II and RANK ligand expression.

In conclusion, our work with T-cell knockout mice shows that because mice respond to an oral infection with *P. gingivalis*, their T cells or T-cell cytokines may be important disrupters of alveolar bone homeostasis.

Acknowledgements.—These studies were supported by United States Public Health Service grants RO1 DE10728 (to P. J. Baker) and RO1 AI24544 (to D. C. Roopenian) and by a grant to Bates College from the Howard Hughes Medical Institute.

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