Role of periodontal pathogenic bacteria in RANKL-mediated bone destruction in periodontal disease

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Accumulated lines of evidence suggest that hyperimmune responses to periodontal bacteria result in the destruction of periodontal connective tissue and alveolar bone. The etiological roles of periodontal bacteria in the onset and progression of periodontal disease (PD) are well documented. However, the mechanism underlying the engagement of periodontal bacteria in RANKL-mediated alveolar bone resorption remains unclear. Therefore, this review article addresses three critical subjects. First, we discuss earlier studies of immune intervention, ultimately leading to the identification of bacteria-reactive lymphocytes as the cellular source of osteoclast-induction factor lymphokine (now called RANKL) in the context of periodontal bone resorption. Next, we consider (1) the effects of periodontal bacteria on RANKL production from a variety of adaptive immune effector cells, as well as fibroblasts, in inflamed periodontal tissue and (2) the bifunctional roles (upregulation vs. downregulation) of LPS produced from periodontal bacteria in a RANKL-induced osteoclast-signal pathway. Future studies in these two areas could lead to new therapeutic approaches for the management of PD by down-modulating RANKL production and/or RANKL-mediated osteoclastogenesis in the context of host immune responses against periodontal pathogenic bacteria.

Keywords: periodontal pathogenic bacteria; RANKL; bone resorption; osteoimmunology

Periodontal disease (PD) is characterized by chronic inflammation in tissues supporting the tooth. Such inflammation is elicited by host innate and adaptive immune response to a constellation of periodontal biofilm-associated multiple microorganisms (46, 98, 114). Studies have demonstrated that the etiology of PD derives from periodontal pathogenic bacteria, such as Porphyromonas gingivalis or Aggregatibacter actinomyctecomitans (88, 89). While such periodontal pathogens are highly prevalent in periodontally compromised individuals, they are also found in healthy periodontal tissue (19, 30), albeit to a lesser degree, suggesting that host responses to periodontal pathogens play key roles in the onset and progression of PD. To explain this phenomenon, host immune response in healthy individuals seems to facilitate a sufficient protective mechanism against colonization and infection (58). On the other hand, in individuals with PD, host immune response to periodontal pathogens seems to have lost effective control against the bacterial challenge (58, 62, 67). More specifically, both hypo- and hyperimmune responses can result in the pathogenesis of PD. For example, smoking-associated PD appears to be caused by the suppression of the immune system by the effect of nicotine (4, 7), suggesting the engagement of hypoimmune responses in the pathogenesis of PD. On the other hand, hyperimmune responses to bacteria also result in the destruction of periodontal tissues, such as gingiva and alveolar bone, as determined by many studies (see later sections).

In earlier studies of the 1970s and 1980s, elevated IgG antibody titers to multiple bacteria in patients’ sera were declared to be the hallmark immune responses of PD. Induction of IgG antibodies requires the engagement of antigen-specific B cell and T cell responses to periodontal
bacteria (90, 101, 113); therefore, the elevated bacteria-specific IgG antibodies found in patients with PD, compared to healthy subjects, give clear evidence that immune responses are induced to the bacteria. However, it is also true that antibody responses to periodontal bacteria can be detected in the sera of periodontally healthy individuals (21, 22, 97). While the development of B cell-rich lesion containing plasma cells is characteristic of periodontally diseased gingival tissue (64, 77), it still remains unclear if IgG produced from these B cells and plasma cells infiltrating the diseased tissue is protective for periodontal pathogens. In terms of the efficiency of serum IgG antibody reactive to periodontal bacteria, several studies conducted in 1990 showed that the avidity of serum IgG antibody found in the periodontally diseased patient is relatively weak (115). However, it was also found that avidity of serum IgG antibody increases in the patient in response to periodontal treatment (15, 67), suggesting that an antibody produced in the patients with PD may not function efficiently. Although, these results implicated that IgG antibody response may be associated with PD, the molecular mechanism underlying the immune-associated periodontal bone resorption had been unclear until the finding of the receptor activator of nuclear factor-κB ligand (RANKL) in T- and B-lymphocytes infiltrating periodontally diseased tissue (48).

In order to investigate immune responses other than IgG antibody response, a number of studies evaluated the expression patterns of inflammatory cytokines produced from lymphocytes, leukocytes, fibroblasts, and gingival epithelial cells in the context of periodontal host innate and adaptive immune responses (28, 29, 102). As a consequence, several proinflammatory cytokines were identified as key molecules contributing to the destruction of periodontal tissue, including interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-6 (IL-6) and, very importantly, RANKL (60). In contrast to proinflammatory cytokines, including IL-1, IFN-γ, TNF-α, and IL-6, which play roles in the induction and upregulation of inflammatory responses in PD lesion, RANKL was first discovered as a cytokine that directly induces osteoclastogenesis (54, 112). As such, the discovery of increased RANKL production in PD lesion led (48), for the first time, to a plausible explanation for the mechanism underlying alveolar bone resorption in periodontitis. The etiological roles of periodontal bacteria in the onset and progression of PD are well documented (7, 10); however, the mechanism underlying the engagement of periodontal bacteria in RANKL-mediated alveolar bone resorption remains unclear. Therefore, this review article addresses three critical subjects. First, we discuss earlier studies of immune intervention, ultimately leading to the identification of bacteria-reactive lymphocytes as the cellular source of osteoclast-induction factor lymphokine (now called RANKL) in the context of periodontal bone resorption. Next, we consider (1) the effects of periodontal bacteria on RANKL production from a variety of adaptive immune effector cells, as well as fibroblasts, in inflamed periodontal tissue and (2) the bifunctional role of LPS produced from periodontal bacteria in a RANKL-induced osteoclast-signal pathway. Finally, possible new therapeutic approaches for the management of PD by targeting RANKL expression from activated T and B cells are discussed.

**Earlier studies that identified possible immune intervention in periodontal bone resorption**

Before RANKL-mediated osteoclastogenesis was found to be engaged in periodontal bone loss, a number of earlier studies conducted experiments demonstrating that immune responses elicited to oral antigens, including bacteria, were involved in periodontal bone loss (Table 1).

**Animal model of periodontal bone loss caused by oral bacteria**

In 1960, Bear and Newton (8) reported that naturally developed alveolar bone loss in germ-free mice (6–8 months old) was lower than age-matched mice with conventional oral flora, suggesting, for the first time, the possible pathogenic role of oral bacteria in periodontal bone resorption. The first animal model of periodontal bone loss induced by bacterial infection was developed by researchers at The Forsyth Dental Center (currently The Forsyth Institute) (83, 85). Colonization of human gingival plaque bacteria in gnotobiotic mice induced elevated periodontal bone loss in about 5 months compared to the control non-infected gnotobiotic mice (83). In another experiment, the human oral bacterium Streptococcus GS-5 was inoculated to both mice and rats, but only rats retained the Streptococcus GS-5 in the oral cavity. Again, in a follow-up study, an in vivo experiment using rats demonstrated periodontal bone resorption 81 days after bacterial inoculation (85). Although the aspect of immune engagement in the pathogenic process of periodontal bone loss was not addressed, these two studies clearly proved that human oral bacteria can cause periodontal bone loss in animals.

**Induction of immune response to orally colonized bacteria**

It has long been accepted that immune tolerance, especially to food and commensal intestinal bacteria, is induced by gastrointestinal mucosa. As such, investigators asked whether host immune response in oral mucosa could be induced against oral bacteria. In 1963, Hyman and Zeldow (40) used guinea pigs and hamsters to show that an Arthus reaction could be induced in oral cheek mucosa in a manner similar to the induction of an Arthus reaction to the antigen challenged in cutaneous tissue. More specifi-
cally, after systemic immunization with ovalbumin (OVA), the local challenge with OVA-induced Arthus reaction characterized by inflammation accompanied by IgG antibody to OVA as well as C4 complement deposition in the local tissue (40). The investigators did not further examine if such immune induction could lead to periodontal bone resorption. Nonetheless, this study did reveal that protein antigens challenged to oral mucosa could elicit immune response at the challenged site (40). In order to examine if immune response to oral bacteria could be induced in human subjects, a hypersensitivity skin test for oral Actinomyces was carried out in patients with PD compared to healthy subjects (73). It was found that a positive reaction induced by cutaneous injection of Actinomyces antigen was significantly higher in periodontally diseased subjects than subjects with either gingivitis or healthy mucosa (73), suggesting that humans with PD can indeed be immunologically predisposed to oral bacteria.

Periodontal bone loss caused in animals by induction of immune response to orally colonized bacteria: delayed-type hypersensitivity (DTH) versus Arthus-type hypersensitivity

The first experiment that demonstrated immune responses to oral bacteria with subsequent periodontal bone loss was carried out by Guggenheim et al. (27). Germ-free rats systemically immunized (i.v.) with killed Actinomyces viscosus, when compared to control non-immunized germ-free rats, developed a significantly higher level of periodontal bone resorption in response to the oral inoculation of live A. viscosus. Based on the increased serum antibody response to A. viscosus and inflammatory infiltration into the periodontal tissue, it was determined that delayed-type hypersensitivity (DTH or Type-IV hypersensitivity: cell-mediated immune memory response) elicited to A. viscosus resulted in periodontal bone loss (27). Using a primate model of PD, Nisengard et al. (74) also demonstrated that Arthus-type immune responses (or Type-III hypersensitivity: IgG-antigen immune complex-mediated response) induced to topically applied bovine serum albumin (BSA) or ovalbumin (OVA) could cause periodontal bone loss. Based on the elevated level of serum antibody response to BSA or OVA, inflammatory infiltration into the periodontal tissue, and the increased deposition of IgG and C4 complement around the perivascular site of periodontal tissue, it was determined that Arthus-type immune responses caused the periodontal bone loss (74).

During the 1970s, both delayed-type and Arthus-type hypersensitivity were presumed to be the putative pathogenic causes of periodontal bone resorption (61, 75). However, current consensus appears to support the idea that adaptive immune responses mediated by T and B cells account for RANKL-dependent periodontal bone loss.
because RANKL, as a cytokine produced from activated memory T and B cells without engagement of antibody, is now thought to be responsible for periodontal bone loss (18, 32, 98, 103). Therefore, this T and B cell-mediated memory response may be considered an extension of the classic DTH theory, which also results from the recall of cell-mediated memory response (12, 52). However, it is still plausible that Arthus-type hypersensitivity (or IgG-antigen immune complex-mediated response) is also involved in periodontal bone resorption based on a premise that IgG immune complex-mediated Fc receptor (FcR) activation may be able to elicit co-stimulation for RANKL-mediated osteoclastogenesis, which is explained in detail below. In sum, the studies carried out in the 1970s ultimately led to the prediction of an osteoclastogenesis factor, termed lymphokine, which was thought to be produced by activated T cells (61, 75). Some 20 years later, lymphokine was shown to be RANKL, a cytokine that is produced not only by activated T and B cells, but also by osteoblasts and bone marrow stromal cells. As described throughout this review, RANKL plays a pivotal role in both pathogenic and homeostatic bone resorption processes.

An animal model demonstrates that activated T and B cell response to oral bacterial antigens can cause periodontal bone resorption

While the studies described above indicated the association of immune responses to periodontal bone resorption, the direct cellular mechanism underlying immune-mediated periodontal bone loss remained unclear until 1984. At that time, Taubman et al. (101) (Forsyth Dental Center) found that, in a rat model of PD, preimmunization with Actinobacillus actinomycetemcomitans [Actinobacillus actinomycetemcomitans was reclassified in 2006 as Aggregatibacter actinomycetemcomitans (76)] could induce periodontal bone loss in rats orally infected with A. actinomycetemcomitans concomitant with DTH response induced to A. actinomycetemcomitans. Normal rats receiving adoptive transfer of A. actinomycetemcomitans-sensitized T lymphocytes prior to infection with A. actinomycetemcomitans also demonstrated increased DTH and periodontal bone loss (101). Interestingly, congenitally athymic rats (nude) showed more periodontal bone loss than normal littermates in the same A. actinomycetemcomitans-immunization plus an oral A. actinomycetemcomitans-infection model, indicating that the thymus might contain T cells with regulatory function (101). For the first time, this study showed that antigen-primed T cells induced in the context of DTH response to oral bacteria could exert bone destructive effects, whereas a T-cell subset present in the thymus could potentially facilitate regulatory effects.

Yoshie et al. (113), using the same rat model of PD, later demonstrated that periodontal bone loss was increased in nude rats compared to normal rats. Since nude rat periodontal tissue with bone resorption was characterized by B cell-rich lesion, it was suggested that the potentially excessive B cell response to abundant bacterial antigens might also contribute to the development of immune-mediated periodontal bone resorption (113). In support of this theory, thymus cell reconstitution of the nude rats partially abrogated the bone destruction (113), indicating that the thymus may indeed contain the T cells with regulatory function. It should be noted that a recent study revealed that FOXP3+ T regulatory (Treg) cells derived from thymus play a pivotal role in the downregulation of adaptive immune responses (80). While the pathophysiological relevance of Treg cells in PD is still controversial (24, 25, 71), our group found that the prevalence of functionally active Treg cells diminish in the periodontally diseased tissue compared to healthy gingival tissue (24). It was also shown that IL-10, a key regulatory cytokine produced from Treg cells, suppressed both RANKL expression by peripheral blood mononuclear cells (PBMC) activated in vitro in a bacterial antigen-specific manner (24). Thus, these two studies carried out in the 1980s implicated that thymus-derived Treg cells may be involved in the suppression of immune-mediated periodontal bone resorption.

Effects of periodontal bacteria on RANKL production from a variety of host immune cells in inflamed periodontal tissue

Biological basis of RANKL/RANK/OPG interaction

Osteoimmunology is a research field that investigates the interaction between the skeletal and immune systems at the molecular to cellular levels (63, 94). Among a number of inflammatory bone-resorption diseases, PD is a disease whose route to alveolar bone loss by pathophysiological processes can be most appropriately explained in osteoimmunological terms. As noted above, although a constellation of oral microorganisms that infect the gingival crevice is a permissive etiologic factor (87), it is the host immune responses to these microorganisms that react to such periodontal pathogens (31, 100).

The identification and characterization of RANKL, its receptor RANK, and soluble decoy receptor osteoprotegerin (OPG) have significantly contributed to the understanding of the skeletal remodeling mechanism involving differentiation of osteoclasts (osteoclastogenesis) and their activation (Fig. 1) (37, 57, 86). Gene knockout mice deficient in RANKL or RANK, respectively, not only present osteopetrotic phenotypes, but also lack peripheral lymph nodes (20, 55), supporting the idea that both RANKL and RANK play pivotal roles in osteoclastogenesis, as well as in the immune development,
as shown by RANKL expressed on Th1-type cells, which then act on dendritic cells to induce their production of proinflammatory cytokines, such as IL-1β and IL-6 (44). Moreover, when the amount of RANKL produced in the microenvironment surrounding RANK+ preosteoclasts is overwhelming relative to the amount of OPG (a natural antagonist of RANKL), RANKL then becomes available to bind RANK expressed on preosteoclasts, tipping the balance of skeletal remodeling to favor the activation of osteoclast formation and bone resorption. On the other hand, in the condition where relative ratio of RANKL over OPG becomes low, OPG-mediated inhibition of ligation between RANKL and RANK decreases the osteoclastogenesis. The inhibition of RANKL-binding to RANK by OPG also promotes apoptosis of activated mature osteoclasts. Therefore, the relative ratio of RANKL over OPG determines the velocity and intensity of bone resorption mediated by osteoclastogenesis.

**Source of RANKL in periodontitis: RANKL production from fibroblasts stimulated by periodontal pathogens**

The recent discovery of the RANKL/OPG ratio in relation to its effect on osteoclastogenesis, as described above, led investigators to examine the expression pattern of RANKL and OPG in periodontitis (48, 66, 81). Based on a study using human clinical samples, tissue homogenate isolated from diseased periodontal lesion with bone resorption showed significantly higher levels of RANKL protein than gingival tissue taken from healthy subjects, whereas OPG protein detected in diseased tissue was lower than that in healthy gingival tissue (59, 110). Furthermore, the gingival crevicular fluid (GCF) sampled from patients with gingivitis demonstrated an elevated RANKL/OPG ratio compared to gingival tissue from healthy subjects (66). Based on another study using GCF, it was found that the RANKL/OPG ratio...
was significantly elevated in periodontitis compared with healthy subjects or patients with gingivitis (13). Furthermore, the latter study demonstrated a positive correlation between the level of RANKL/OPG ratio and clinical parameters of periodontal pocket depth and clinical attachment loss (13), suggesting that elevated levels of local RANKL/OPG are responsible for periodontal bone resorption. Therefore, any clinical intervention that tips the balance between RANKL and OPG such that periodontal bone resorption is inhibited would lead to a most novel therapeutic regimen (99).

Based on these findings showing elevated expression of RANKL in periodontally diseased tissue, many studies were conducted to identify the cellular source of RANKL or OPG in periodontal tissue. Osteoblasts, which originate from mesenchymal stem cells, had previously been discovered as vital cells that produce RANKL and OPG in bone tissue (14). However, since fibroblasts are also mesenchymal lineage cells, gingival fibroblasts and periodontal ligament fibroblasts are also considered to be a source of RANKL and OPG in periodontal tissue (Fig. 2). Interestingly, periodontal ligament fibroblasts, which play crucial roles in regulating the homeostasis of periodontal ligaments that connect bone and cementum, are reported to express mRNA for both RANKL and OPG (45, 50, 81). The conditioned medium isolated from the culture of periodontal ligament fibroblasts abrogated osteoclast formation from mouse bone marrow macrophages (50), suggesting that periodontal ligament fibroblasts secrete OPG to inhibit RANKL-mediated osteoclast formation (Fig. 2). On the other hand, when human periodontal ligament fibroblasts were co-cultured with peripheral blood mononuclear cells, osteoclastogenesis was induced in the co-culture (45), indicating that human periodontal ligament fibroblasts express functionally active membrane-bound RANKL (Fig. 2). Since periodontal ligament fibroblasts are a source of membrane-bound, but not soluble, RANKL, but concurrently produce OPG, they appear to have properties in common with osteoblasts. For example, in response to IL-1 stimulation, both periodontal ligament fibroblasts and osteoblasts express RANKL and OPG mRNA via PKA and PKC signaling, respectively (38).

Gingival fibroblasts, which are the most abundant cells in gingival connective tissues, showed expression of OPG mRNA, while their expression of RANKL mRNA was weak (38) or undetectable (81). The stimulation of gingival fibroblasts with IL-1 induced expression of OPG mRNA, but not RANKL mRNA, via a signaling cascade involving PKA (38). However, it is also reported that cytolethal

![Fig. 2. Effects of bacteria on the production of RANKL and OPG. Periodontal ligament fibroblasts express membrane bound RANKL (mRANKL), but little OPG, in response to bacterial stimulation, increasing the RANKL/OPG ratio. In contrast to periodontal ligament fibroblasts, it appears that gingival fibroblasts downregulate osteoclastogenesis by their production of OPG, but not RANKL, in response to inflammatory mediators, whereas they may produce RANKL in the periodontal tissue upon stimulation by bacterial virulent factors. Bacterial antigen presentation by professional antigen presenting cells (APC), such as dendritic cells, induces effector T lymphocytes to express both mRANKL and soluble RANKL (sRANKL). Bacterial antigen-engagement of the B cell receptor (BCR) induces B cells to produce not only the IgG antibody but also mRANKL and sRANKL. Furthermore, the activated antigen-specific T cells provide B cells co-stimulatory signals through CD40/CD40L ligation, which also is considered to upregulate the RANKL expression from B cells.](image-url)
distending toxin (CDT) produced from *A. actinomycetemcomitans* can induce *in vitro* RANKL expression from human gingival fibroblasts (11). Arg-gingipains produced from *P. gingivalis* are also indicated to upregulate the RANKL/OPG expression ratio in gingival fibroblasts (10). It was also reported that in a co-culture with human peripheral blood mononuclear cells, gingival fibroblasts, but not ligament fibroblasts, inhibit the induction of osteoclasts (116). Therefore, it appears that gingival fibroblasts downregulate osteoclastogenesis by their production of OPG, but not RANKL, in response to inflammatory mediators, whereas they may also produce RANKL in the periodontal tissue upon stimulation by bacterial virulence factors (Fig. 2).

A large number of *in vitro* studies have examined whether periodontal pathogenic bacteria affect the expressions of RANKL/OPG in fibroblasts. In terms of periodontal ligament fibroblasts, fixed-bacteria, or their constituents, including enzyme or toxin, induced RANKL expression. The *A. actinomycetemcomitans* LPS (79, 105), CTD (11), and *P. gingivalis* Arg-gingipain (13) all stimulated periodontal ligament fibroblasts to produce RANKL mRNA expression. Furthermore, although there are several controversial results, bacterial stimulations reduced OPG expression (17, 105) or increased the ratio of RANKL over OPG (RANKL/OPG ratio) (13) in periodontal ligament fibroblasts (Fig. 2). In relation to the engagement of IL-1 in RANKL/OPG expression as noted above (38), it is reported that LPS stimulates expression of both OPG and RANKL in periodontal ligament fibroblasts via induction of IL-1β and TNF-α (109) (Fig. 2), suggesting that LPS can induce both inflammation and bone resorption concomitantly.

On the other hand, in gingival fibroblasts, LPS enhanced OPG mRNA expression, whereas RANKL mRNA expression was not induced by LPS-stimulation (69). In addition, the conditioned medium isolated from gingival fibroblasts stimulated with LPS inhibited RANKL-induced osteoclastogenesis (69), suggesting that functionally active OPG that can neutralize RANKL is induced in gingival fibroblasts by stimulation with LPS. These findings suggested that periodontal pathogenic bacteria do affect the RANKL/OPG ratio in periodontal ligament fibroblasts in a manner different from gingival fibroblasts (Fig. 2); namely (1) that both RANKL and OPG can be produced by periodontal ligament fibroblasts, with stimulation of periodontal pathogens increasing the RANKL/OPG ratio; and (2) that gingival fibroblasts produce OPG, but not RANKL, with the stimulation of periodontal pathogens decreasing the RANKL/OPG ratio.

However, in contrast to the *in vitro* results demonstrating RANKL expression by bacterially stimulated fibroblasts, it should be noted that RANKL expression by fibroblasts is considerably lower than that of immune cells in the physiological context of human PD or the animal model of PD, as measured using immunohistochemical staining (110). As described below, a high expression of RANKL was detected from T and B lymphocytes, but not from fibroblasts, in human periodontally diseased tissue (48). Based on these lines of evidence, it is speculated that RANKL or OPG derived from fibroblasts may play roles in regulating the homeostasis of healthy bone remodeling, whereas, in the inflamed PD tissue, T and B lymphocytes, rather than fibroblasts, are engaged in bone resorption processes.

### Source of RANKL in PD: bacterial antigen-specific T and B lymphocytes are key players in RANKL-mediated bone resorption

Initial reports demonstrated that RANKL expressed by T lymphocytes can contribute to the bone resorption induced in mouse adjuvant arthritis (54). Based on these findings, the possible engagement of T lymphocytes was explored in the mechanism of periodontal bone resorption as a source of RANKL production in periodontitis. Accordingly, in periodontally diseased gingival tissues, several studies indicated that both T lymphocytes and B lymphocytes express RANKL (16, 48, 59). By using immunohistochemistry analysis, it was revealed that RANKL protein associated with lymphocytes and macrophages was expressed at significantly higher levels in periodontitis tissue (110). Furthermore, our group previously described that abundant RANKL was expressed on CD3+ T cells and CD20+ B cells, but only minimally, or not at all, on CD14+ monocytes in the tissue of patients with chronic periodontitis (48, 65). Otherwise, in healthy gingival tissue, very few RANKL-expressing cells were present (48, 65). Importantly, more than 50% of T cells and 90% of B cells expressed RANKL in periodontitis tissue, whereas less than 20% of either B cells or T cells showed RANKL expression in healthy gingival tissue. The concentrations of RANKL, but not OPG, were significantly higher in periodontitis tissue as opposed to healthy tissue (48). Further, lymphocytes derived from the gingival tissues of patients induced osteoclast differentiation *in vitro* (48). In another study, CD4+ T lymphocytes were the predominant cell type present in the gingival tissues of patients with periodontitis, and they were the main cells responsible for the higher levels of RANKL observed in these patients (108). These findings obtained from gingival tissues isolated from individuals with periodontitis suggested that activated lymphocytes seem to be a particularly critical source of RANKL in PD (Fig. 2).

Since these studies using clinical tissue samples indicated that RANKL derived from lymphocytes seems to play a pivotal role in periodontal bone resorption, many investigators have tried to elucidate the mechanism by which lymphocytes are activated and ultimately cause...
RANKL-mediated bone destruction (Fig. 2). Using a mouse model, Teng et al. demonstrated that RANKL produced by bacteria-reactive activated CD4⁺ T lymphocytes can initiate local alveolar bone resorption (102). In their model, mouse PD was induced by the transplantation of human peripheral blood lymphocytes isolated from periodontitis patients into non-obese diabetic (NOD) severe combined immunodeficiency disease (SCID) mice (102). Reconstitution of human CD4⁺ T cells, but not CD8⁺ T cells, isolated from localized aggressive periodontitis (LAG) in the NOD-SCID mice, which were orally colonized with *A. actinomycetemcomitans*, resulted in local alveolar bone destruction in an RANKL-dependent manner (102). Our group has also developed a periodontal bone resorption model by adaptive transfer of antigen-specific T cells (47, 49). In these studies, to prime T cells, the outer membrane protein (OMP) along with *A. actinomycetemcomitans* LPS were used as T cell antigen and adjuvant, respectively (47, 49). Gingival injection of this antigen/adjuvant mixture could induce local bone resorption after the transfer of antigen-specific Th1-type clone cells, but not Th2-type clone cells. Interestingly, to generate the antigen-specific T cells, both TCR signaling stimulated by antigen presenting cells and CD28 co-stimulation induced by B7, which is expressed in antigen presenting cells, were required (35). For the first time, the idea that T cells could be activated by B7⁺ antigen presenting cells in local gingival tissue was supported. In addition, another study indicated that *P. gingivalis* can cause periodontal bone loss by induction of RANKL from the activation of lymphocytes (43). Most importantly, in these rodent bone loss models, the inhibition of RANKL by OPG reduced periodontal bone destruction. These findings suggested that antigen-specific T lymphocytes, which are activated by antigen presenting cells, trigger the RANKL-mediated bone destruction in local periodontal tissue (Fig. 2).

It is thought that activated B cells in periodontally diseased tissues protect the host from bacterial infection by their production of IgG antibodies. However, it was hypothesized that B cells might also contribute to bone tissue destruction by their production of RANKL because (1) B lymphocytes are abundant in inflamed periodontal lesions (64, 84); (2) excessive B-cell response represented by IgG antibody to abundant bacterial antigens (23, 68, 104) may contribute to the development of immune-mediated periodontal bone resorption, as demonstrated by Taubman et al.’s PD model using nude rats (101, 113); and (3) B cells highly expressed RANKL in human gingival tissue with periodontal bone loss lesion (48). To investigate whether and how RANKL derived from activated B lymphocytes plays a role in bone resorption, adoptive transfer of antigen-specific B cells into a T cell-deficient rat model was developed (31, 33). These studies demonstrated that activated B cells stimulated with *A. actinomycetemcomitans* highly express RANKL and that adoptive transfer of the antigen-specific B lymphocytes can contribute to increased periodontal bone resorption in mice infected with *A. actinomycetemcomitans*. Importantly, administration of OPG-Fc to this B-cell transfer model abrogated the periodontal bone loss (31) in a manner similar to the antigen-specific T-cell transfer model (107), suggesting that the periodontal bone resorption induced by B-cell transfer was RANKL-dependent (31, 33).

It is well known that the CD40 ligand (CD40L) is found almost exclusively on activated, but not naïve CD4⁺, T cells, and that its counter-ligand, CD40, is constitutively expressed on B cells. The ligation of CD40/CD40L initiates both humoral and cellular immune responses, including the expression of numerous cytokines and chemokines, as well as a class switch of immunoglobulin (3, 7). Therefore, we asked whether CD40/CD40L-mediated co-stimulation could induce RANKL production from activated B cells. Very recently, we found that the systemic administration of an anti-CD40L monoclonal antibody reduced the periodontal bone resorption of rats that had received antigen-specific CD4⁺ Th1 cell transfer and gingival injections of antigens (32). These findings suggested that antigen-specific T cells not only produce RANKL, but also induce activation of B cells through CD40/CD40L interaction, which leads to robust RANKL production (Fig. 2). Based on these results, we can conclude that antigen-specific T and B cells stimulated by periodontal pathogenic bacteria, which infiltrate the periodontal lesion close to the bone resorption site, are prominent sources of RANKL in the context of periodontal bone loss.

**Co-stimulatory molecules for RANKL-mediated osteoclastogenesis: possible co-stimulatory contribution by IgG-Ag immune complex**

Osteoclast-associated immunoglobulin-like receptor (OSCAR), along with a triggering receptor expressed on myeloid cells 2 (TREM2), are the recently discovered co-stimulatory molecules in RANKL-mediated osteoclast activation (Fig. 3) (53). As demonstrated in Fig. 3, the main biological event that induces osteoclastogenesis is RANKL binding to its receptor, RANK, in coordination with co-stimulatory signaling provided by OSCAR and/or TREM2 (53). Importantly, OSCAR transmits its signal through an adaptor molecule, FcR common gamma-chain (FcRγ; Fig. 3) (53, 92). In addition to the engagement of FcRγ in osteoclastogenesis, FcRγ is used as an adaptor molecule in order to transmit the signal upon occupation of IgG-FcRs (FcγRIIA and FcγRIIIA) by the Fc-region of IgG in the form of an immune complex (IC; Fig. 3). Upon ligation of OSCAR or FcRs with their respective ligand (the specific ligand for
OSCAR is currently unclear, phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) present in FcRγ up-regulates phospholipase Cγ (PLCγ) and calcium signaling in osteoclast precursors as well as other immune cells (53, 92). For osteoclast precursors, DNAX-activating protein 12 (DAP12; ITAM-harboring adaptor for TREM2) also functions similar to OSCAR-FcRγ in providing co-stimulatory signals during RANKL-induced osteoclastogenesis. In other words, both DAP12 and FcRγ elicit essential co-stimulatory signals to the RANK-induced signaling cascades for terminal differentiation of osteoclasts (53, 92). Therefore, ITAM-dependent co-stimulatory signals induced by multiple different immunoreceptors are considered to play a key role in promoting RANKL-mediated osteoclastogenesis.

Of the three main types of FcγR in mice and humans, the high-affinity receptor FcγRI can bind monomeric IgG, whereas the two low-affinity receptors, FcγRII and FcγRIII, bind polymeric IgG in the form of immune complexes (IC) (92). More specifically, while monomeric IgG cannot induce activation signals, an IC formed of multiple IgG antibodies can induce activation signals in the cells expressing FcγRs by the mechanical force provided by multiple tightly connected IgG antibodies (the so-called capping effects) (6). As shown in Fig. 4, deposition of IgG-IC was found in periodontally diseased gingival tissue, but very little in healthy gingival tissue, using C1q complement as an IgG/C1Ag/C1IC detection module (Fig. 4). Therefore, it is plausible that IgG-IC in the diseased gingival tissues can bind all three FcγRs (FcγRI, FcγRII, and FcγRIII) and may then trigger co-stimulatory signals in the RANKL-stimulated osteoclast precursor cells. Based on our in vitro studies (Fig. 4), in the absence of RANKL, stimulation of FcRs alone with IgG-IC did not induce osteoclast differentiation; whereas, in the presence of RANKL, activation of FcRs mediated by IgG-IC upregulates RANKL-induced osteoclastogenesis. Therefore, we speculate that FcRs provide co-stimulatory signals in RANKL-mediated osteoclastogenesis. The presence of IC in periodontitis patients (26, 106), combined with the expression of FcRγ on osteoclast precursor cells (53), is expected to upregulate osteoclastogenesis triggered by RANKL that, in turn, augments the development of periodontal bone loss lesions.
Immune complex (IC) was developed in immune complex on the RANKL-mediated osteoclastogenesis of IC was found in the gingival epithelium of gingival epithelium appeared to be dendritic cells. Only slight periodontal disease. The IC bearing cells in the patient’s epithelium as well as lamina propria of a patient with C1q reactive IC was monitored in the gingival tissues isolated from healthy subjects or periodontally diseased patients were subjected for immunohistochemical staining for IC. As an IgG-IC detection module, C1q complement (Sigma, St Louis, MO) conjugated with biotin, was reacted with the tissue section, followed by a reaction with streptavidin-peroxidase, color development with DAB, and methyl green nuclear staining. The deposition of C1q reactive IC was stained as dendritic cells. Only slight staining of IC was found in the gingival epithelium of periodontally healthy subjects. (B) Possible effects of IgG-immune complex on the RANKL-mediated osteoclastogenesis. IgG immune complex was developed in vitro by reacting recombinant A. actinomycetemcomitans Omp29 and mouse immune serum IgG antibody reactive to A. actinomycetemcomitans Omp29 (anti-Omp29 IgG-IC). Bone marrow cells isolated from C57BL6/J mice were incubated in the presence of M-CSF (10 ng/ml) with or without (1) RANKL (50 ng/ml), (2) purified anti-Omp29 IgG (1 μg/ml), (3) recombinant Omp29 (1 μg/ml), or (4) anti-Omp29 IgG-IC (1 μg/ml) for 9 days, by partially exchanging medium every 3 days. TRAP+ cells with more than three nuclei were counted as mature osteoclasts. The results showed that anti-Omp29 IgG-IC, but not Omp29 or anti-Omp29 IgG, upregulated the RANKL-mediated osteoclastogenesis, while anti-Omp29 IgG-IC did not induce osteoclastogenesis in the absence of RANKL, suggesting that IgG-IC can provide a co-stimulatory signal to RANKL-mediated osteoclastogenesis. *+, significantly higher than M-CSF/RANKL stimulation alone control (#) by t test (P < .05).

**Fig. 4.** Immune complex (IC) in periodontal disease. (A) Immune complex (IC) detected in human gingival tissue. The gingival tissues isolated from healthy subjects or periodontally diseased patients were subjected for immunohistochemical staining for IC. As an IgG-IC detection module, C1q complement (Sigma, St Louis, MO) conjugated with biotin, was reacted with the tissue section, followed by a reaction with streptavidin-peroxidase, color development with DAB, and methyl green nuclear staining. The deposition of C1q reactive IC was monitored in the gingival tissues isolated from healthy subjects or periodontally diseased patients were subjected for immunohistochemical staining for IC. As an IgG-IC detection module, C1q complement (Sigma, St Louis, MO) conjugated with biotin, was reacted with the tissue section, followed by a reaction with streptavidin-peroxidase, color development with DAB, and methyl green nuclear staining. The deposition of C1q reactive IC was stained as dendritic cells. Only slight staining of IC was found in the gingival epithelium of periodontally healthy subjects. (B) Possible effects of IgG-immune complex on the RANKL-mediated osteoclastogenesis. IgG immune complex was developed in vitro by reacting recombinant A. actinomycetemcomitans Omp29 and mouse immune serum IgG antibody reactive to A. actinomycetemcomitans Omp29 (anti-Omp29 IgG-IC). Bone marrow cells isolated from C57BL6/J mice were incubated in the presence of M-CSF (10 ng/ml) with or without (1) RANKL (50 ng/ml), (2) purified anti-Omp29 IgG (1 μg/ml), (3) recombinant Omp29 (1 μg/ml), or (4) anti-Omp29 IgG-IC (1 μg/ml) for 9 days, by partially exchanging medium every 3 days. TRAP+ cells with more than three nuclei were counted as mature osteoclasts. The results showed that anti-Omp29 IgG-IC, but not Omp29 or anti-Omp29 IgG, upregulated the RANKL-mediated osteoclastogenesis, while anti-Omp29 IgG-IC did not induce osteoclastogenesis in the absence of RANKL, suggesting that IgG-IC can provide a co-stimulatory signal to RANKL-mediated osteoclastogenesis. *+, significantly higher than M-CSF/RANKL stimulation alone control (#) by t test (P < .05).

**LPS-induced innate immune TLR signaling may promote or inhibit osteoclastogenesis**

The LPS, a major constituent of the cell wall of gram-negative bacteria, has long been recognized as a key factor implicated in the development of chronic periodontitis (36, 70). Previously, many studies revealed the effect of LPS on RANKL source cells, as described above, and its role in RANKL-mediated osteoclastogenesis. It is well documented that monocyte lineage cells, such as dendritic cells and macrophages, utilize Toll-like receptor 4 (TLR4) as an innate immune sensor to recognize LPS. Interestingly, osteoclasts, like macrophages, express TLR4, since osteoclasts are derived from same monocyte lineage cells originated from hematopoietic stem cells. Furthermore, the ligation of LPS to TLR4 expressed on preosteoclasts seems to provide co-stimulatory signaling to RANKL-mediated osteoclastogenesis (35). Since signaling caused by TLR4 shares some of the downstream signal transducers, such as TNF receptor-associated factor 6 (TRAF6), NF-kB, and MAPKs, with RANK signaling (2, 42), it was hypothesized that LPS would accelerate RANKL-mediated osteoclastogenesis, enhancing RANK signaling. Indeed, the vast majority of previous studies described LPS as promoting osteoclastogenesis (34, 41, 43, 91, 96), a finding that suggests that LPS may play a role in alveolar bone loss in periodontitis. Interestingly, however, some previous studies also demonstrated a bifunctional role of LPS in osteoclastogenesis. In other words, it was found that LPS can either potentiate or inhibit RANK signaling, depending on the stage of osteoclast precursor differentiation (78, 93). When preosteoclasts were pretreated with RANKL, it was shown that LPS upregulated osteoclastogenesis. On the contrary, when LPS and RANKL were simultaneously added to the cultures of preosteoclasts, LPS stimulation inhibited RANKL-mediated osteoclast differentiation. While this is an interesting finding that may gain insight into the regulatory role of LPS in the pathogenesis of PD, the effects of LPS in the suppression of periodontal bone resorption have, thus far, not received enough attention.

Only very recently has the signal transduction mechanism underlying this dual effect of LPS in osteoclastogenesis been explained in detail by the study of Liu et al. (39). In this study, they demonstrated that LPS abolishes JNK and NF-kB activity in virgin preosteoclasts that were previously exposed to only M-CSF, but not RANKL (Fig. 5) (39). As a consequence, the expression of NFATc1, which is a master transcriptional factor initiating osteoclastogenesis (95), was suppressed by LPS in the virgin preosteoclasts (Fig. 5) (39). On the other hand, when preosteoclasts were pretreated with RANKL, the inhibitory effect of LPS disappeared, and LPS stimulated JNK and NF-kB activity that activated the expression of NFATc1 (Fig. 5) (39). Such results delineate the distinct bifunctional effects of LPS on the fundamental signal
transduction pathways requisite for osteoclastogenesis in different differentiation stages of preosteoclasts; that is, (1) virgin preosteoclasts that are only exposed to M-CSF but not RANKL, and (2) intermediately differentiated pre-osteoclasts that are stimulated with both M-CSF and RANKL (Fig. 5). LPS down-modulates osteoclastogenesis signaling in the virgin preosteoclasts, while it upregulates osteoclastogenesis signaling in intermediately differentiated preosteoclasts (Fig. 5). In addition, several reports revealed that LPS promotes cell survival of RANKL-induced mature multinucleated osteoclasts by activating Akt, NF-κB, and ERK via MyD88 (Fig. 5) (39, 82). These findings suggested that (1) virgin preosteoclasts may turn into phagocytic cells, such as macrophages, in response to LPS, and (2) preosteoclasts may differentiate into osteoclastic lineage cells by RANKL stimulation in the absence of LPS.

In contrast to LPS produced from a majority of Gram (−) bacteria that binds to TLR4 (1), P. gingivalis LPS is known to activate TLR2 (9), which can also induce RANKL expression from cementoblasts (72) and periodontal ligament cells (56). A recent study revealed that P. gingivalis can produce unique sphingolipids that promote RANKL expression from osteoblast cells via ligation to TLR2 (111). Therefore, TLR2 ligands produced from P. gingivalis appear to promote osteoclastogenesis by activating TLR2. However, the effects of P. gingivalis LPS on RANKL-mediated osteoclastogenesis in the light of the above-mentioned bifunctional effects remain unclear. In addition to TLR2 and TLR4, TLR9 was reported to promote osteoclastogenesis (5), while, at the same time, there seems to be a cell signal system where the CpG (TLR9 ligand)-activated genes are subsequently down-regulated (51). In the context of polymicrobial infection in periodontally diseased tissue, it is therefore plausible that multiple different TLR ligands affect RANKL-mediated osteoclastogenesis by activating diverse signaling pathways. Hence, we expect that future studies would

Fig. 5. Possible bifunctional effects of LPS on RANKL-mediated osteoclastogenesis. Bifunctional effects of LPS on the different differentiation stages of preosteoclasts were discovered recently: (1) LPS down-modulates osteoclastogenesis signaling in the virgin preosteoclasts that are only exposed to M-CSF, but not RANKL; and (2) LPS upregulates osteoclastogenesis signaling in intermediately differentiated preosteoclasts that are stimulated with both M-CSF and RANKL. Based on these findings, it is further proposed that (1) virgin preosteoclasts may turn into phagocytic cells, such as macrophages, in response to LPS, and (2) preosteoclasts may differentiate into osteoclastic lineage cells by RANKL stimulation in the absence of LPS.
explore the effects of multiple different TLR ligands released from the polymicrobial biofilm on the RANKL-mediated osteoclastogenesis in the PD.

Possible therapeutic approaches for inhibition of periodontal bone loss by targeting lymphocytes derived RANKL

We know that the production of RANKL from bacterial antigen-specific T and B cells seems to trigger periodontal bone loss and that the suppression of antigen-specific T and B cell responses may sufficiently facilitate down-modulation of periodontal bone destruction. Thus, we might envision an ideal therapy that suppresses the bone destructive consequences of antigen-specific T and B cells while, at the same time, permitting the homeostatic bone remodeling mediated by RANKL-produced from osteoblasts and bone marrow stromal cells. Indeed, we have previously described that interference with co-stimulatory molecules, which are required for induction of antigen-specific T and B cells, abrogated periodontal bone resorption. In other words, systemic administration of CTLA4Ig, a functional antagonist of CD28 binding to B7 (47), or anti-CD40 ligand antibody to inhibit CD40/CD40L (32), could theoretically abrogate bone resorption.

Fig. 6. Immune intervention in the bacteria-associated periodontal of bone resorption. Schematic illustration presents hypothetical mechanisms of immune intervention in the periodontal bone resorption induced by RANKL-mediated osteoclastogenesis. RANKL produced from activated adaptive immune effector cells (i.e. T and B cells) in response to periodontal bacteria play a significant role in pathogenic bone resorption. While the IgG antibody response to periodontal bacteria is thought to protect the host from bacterial challenge, the IgG-immune complex (IgG–IC) developed in diseased tissue with chronic periodontitis may promote inflammatory bone resorption by providing a co-stimulation to RANKL-mediated osteoclastogenesis through its binding to Fc receptors expressed on osteoclasts.
in an animal periodontal model. In addition to these approaches, we are now challenged to establish new therapeutic regimens to mediate the antigen-specific T and B cells. For example, one approach that is attracting wide attention involves the utilization of regulatory CD4+ T cells (Treg cells), which play a suppressive role in inflammation caused by the activation of adaptive T cell responses (80). Accordingly, our group and others have focused on the application of Treg cells to modulate periodontal bone loss. This line of investigation is expected to help in the development of new therapeutic approaches to stop progressive bone resorption induced by oral bacterial challenge.

Summary
In the 1960s through the 1980s, prior to the discovery of RANKL, researchers in the field of periodontology had already predicted the presence of a putative osteoclastogenesis factor produced by bacterially activated lymphocytes. The consensus now holds that RANKL, a cytokine that induces osteoclastogenesis is produced from activated adaptive immune effector cells (i.e. T and B cells) in response to periodontal bacteria and appears to play a significant role in pathogenic bone resorption (Fig. 6). While the IgG antibody response to periodontal bacteria is thought to protect the host from bacterial challenge, the IgG-immune complex (IgG–IC) developed in diseased tissue with chronic periodontitis may, in fact, promote inflammatory bone resorption by providing a co-stimulation to RANKL-mediated osteoclastogenesis through its binding to FcRs expressed on osteoclasts (Fig. 6).

Recent studies indicated the bifunctional effects of LPS on the fundamental signal transduction pathways requisite for osteoclastogenesis in different differentiation stages of preosteoclasts. The LPS downregulates the expression of NFATc1, a master regulator of osteoclastogenesis and, consequently, it inhibits RANKL-induced NFATc1 expression in virgin preosteoclasts that are previously only exposed to M-CSF, but not RANKL. However, LPS upregulates NFATc1 expression in preosteoclasts that are previously exposed to RANKL. These studies implicated the role of innate immune system in the regulation of periodontal bone resorption.

Insights gained from these recent advancements in the study fields of both adaptive and innate immune responses, supported by the discovery of RANKL, have led to the paradigm of immune-mediated periodontal bone loss, a sound scientific platform from which to explore novel therapeutics for the amelioration of PD by regulating immune responses.

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