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ORAL MICROBIOLOGY
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Short communication

Cross-reactive adaptive immune response to oral commensal bacteria results in an induction of receptor activator of nuclear factor-κB ligand (RANKL)-dependent periodontal bone resorption in a mouse model

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Introduction: The present study examined whether induction of an adaptive immune response to orally colonizing non-pathogenic *Pasteurella pneumotropica* by immunization with the phylogenetically closely related bacterium, *Actinobacillus actinomycetemcomitans*, can result in periodontal bone loss in mice.

Methods: BALB/c mice harboring *P. pneumotropica* (*P. pneumotropica*$^+$ mice) in the oral cavity or control *P. pneumotropica*-free mice were immunized with fixed *A. actinomycetemcomitans*. The animals were sacrificed on day 30, and the following measurements were carried out: (i) serum immunoglobulin G and gingival T-cell responses to *A. actinomycetemcomitans* and *P. pneumotropica*; (ii) periodontal bone loss; and (iii) identification of receptor activator of nuclear factor-κB ligand (RANKL) - positive T cells in gingival tissue.

Results: Immunization with *A. actinomycetemcomitans* induced a significantly elevated serum immunoglobulin G response to the 29-kDa *A. actinomycetemcomitans* outer membrane protein (Omp29), which showed strong cross-reactivity with *P. pneumotropica* OmpA compared to results in the control non-immunized mice. The *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mice developed remarkable periodontal bone loss in a RANKL-dependent manner, as determined by the abrogation of bone loss by treatment with osteoprotegerin-Fc. The T cells isolated from the gingival tissue of *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mice showed an *in vitro* proliferative response to both *A. actinomycetemcomitans* and *P. pneumotropica* antigen

Key words: *Actinobacillus actinomycetemcomitans*; immunoglobulin G; 29-kDa outer membrane protein (Omp29); osteoprotegerin-Fc; *Pasteurella pneumotropica*; periodontal bone loss; RANKL; T cells

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presentation, as well as production of soluble(s)RANKL in the culture supernatant. Double-color confocal microscopy demonstrated that the frequency of RANKL$^+$ T cells in the gingival tissue of *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mice was remarkably elevated compared to control mice.

Conclusion: The induction of an adaptive immune response to orally colonizing non-pathogenic *P. pneumotropica* results in RANKL-dependent periodontal bone loss in mice.

The definite pathogenic mechanisms underlying infection by *Actinobacillus actinomycetemcomitans*, recently re-termed *Aggregatibacter actinomycetemcomitans* (19), in the context of the development and progression of localized aggressive periodontitis and adult periodontal diseases are not clear. Nonetheless, these diseases do, in fact, demonstrate that the host adaptive immune reaction to *A. actinomycetemcomitans* is tightly associated with both the development and the progression of these diseases (7, 13, 27). For example, immunoglobulin G (IgG) antibody responses in the serum of patients with localized aggressive periodontitis are prominently elevated to some protein antigens of *A. actinomycetemcomitans* (13), in particular, the 29-kDa outer membrane protein (Omp29) (34). On the other hand, deficiency of neutrophil function and lack of production of innate immune peptide in saliva are both implicated as causal to the onset of periodontal diseases (5, 24, 32). Therefore, it appears that an increase of adaptive immunity, which involves the production of IgG antibody and the induction of T-cell responses, can be associated with the development of periodontal diseases, while diminished innate immune function also appears to be a significant risk factor for the development of periodontal diseases.

Based on the discovery of osteoclast differentiation factor, receptor activator of nuclear factor-*k*B ligand (RANKL), or osteoprotegerin (OPG) ligand (16), it is only recently that the involvement of lymphocytes in bone loss processes has been demonstrated (9, 15). Moreover, we recently discovered that activated T and B cells are the major sources of RANKL in the bone resorptive lesions of human periodontal disease (12). Supporting this finding, we also demonstrated that *A. actinomycetemcomitans* Omp29-specific T helper type 1 cells, or *A. actinomycetemcomitans*-reactive B cells, can trigger periodontal bone resorption in rat models (8, 10, 31). However, it is unclear if induction of an adaptive immune response to non-pathogenic (commensal) bacteria colonizing the oral cavity can elicit RANKL-mediated periodontal bone resorption. In the present study, therefore, we examined whether induction of an adaptive immune response to *Pasteurella pneumotropica*, where colonization in the mouse oral cavity is non-pathogenic, can affect periodontal bone loss in mice.

BALB/c mice maintained in a conventional breeding room at The Forsyth Institute harbor facultative anaerobic gram-negative bacteria in their oral cavity. Similar to *A. actinomycetemcomitans* (26), *P. pneumotropica* was resistant to bacitracin and vancomycin, and only one microorganism was recovered from the BALB/c mouse oral cavity that grew on the agar plate containing this antibiotic combination (Fig. 1A,B). This bacterium was identified as *P. pneumotropica*, using a 16S ribosomal RNA sequence (3, 22, 28). *P. pneumotropica* is closely related to *A. actinomycetemcomitans* because both *P. pneumotropica* and *A. actinomycetemcomitans*, as well as *Haemophilus influenzae*, belong to the phylogeny of the Pasteurellaceae group (Fig. 1C) (19, 20). In general, we found colonization of *P. pneumotropica* in the oral cavity of BALB/c mice, but not in tissues from the skin, vagina, trachea or lung. Although pathogenic opportunistic infection with *P. pneumotropica* was reported in laboratory animals, especially in mice with genetic disorders (1, 18), oral colonization of BALB/c mice with *P. pneumotropica* was latent and did not show any pathogenic features. In support of these non-pathogenic features of *P. pneumotropica* in the BALB/c mice, *Pasteurella* was also reported to be a commensal bacterium in the gingival crevice of the ferret model (4). BALB/c mice supplied by a commercial vendor (Jackson Laboratory, Bar Harbor, ME) were all negative for *P. pneumotropica*. These *P. pneumotropica*-free (*P. pneumotropica*$^-$) mice were also maintained in a specific pathogen-free (SPF) cage system in the Forsyth animal facility. Transmission of *P. pneumotropica* to these SPF BALB/c mice resulted from either co-housing them with *P. pneumotropica*$^+$ mice (for 1 week) or oral inoculation of cultured *P. pneumotropica* (10^8 bacteria/mouse). Importantly, transmission of *P. pneumotropica* to the oral cavity of BALB/c mice derived from the SPF environment did not show any pathogenic outcomes.

To investigate whether systemic immunization of *P. pneumotropica*$^+$ mice with *A. actinomycetemcomitans* can induce any cross-reactive immune response

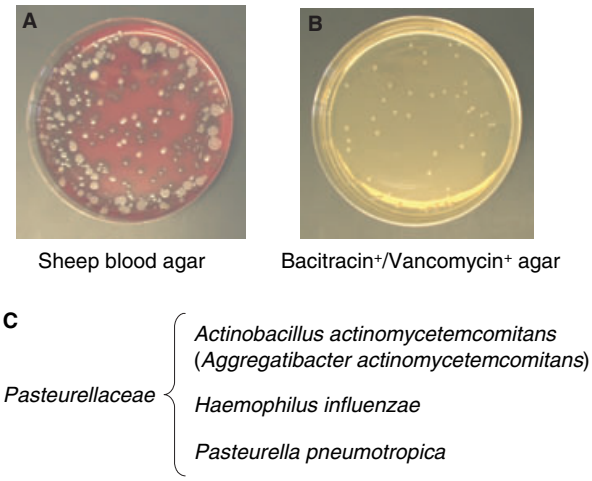


Fig. 1. Pasteurella pneumotropica colonizing the oral cavity of BALB/c mice. An oral swab taken from BALB/c mice maintained in a conventional breeding room was cultured on (A) a sheep blood agar plate or on (B) *Actinobacillus actinomycetemcomitans*-selective agar (bacitracin, 5 μg/ml; vancomycin, 75 μg/ml) (26) in the candle jar at 37 °C for 2 days. Very importantly, only one type of bacterial colony was observed in the *A. actinomycetemcomitans*-selective agar. After 16S ribosomal RNA sequencing (22, 28), the bacterium was identified as *P. pneumotropica*, which is closely related to *A. actinomycetemcomitans* because both *P. pneumotropica* and *A. actinomycetemcomitans*, as well as *Haemophilus influenzae*, belong to the phylogeny of the Pasteurellaceae group (C).

to *P. pneumotropica*, serum IgG responses to *A. actinomycetemcomitans* and *P. pneumotropica* were evaluated using Western blot analyses (Fig. 2), following the previously published protocol (14). Non-immunized *P. pneumotropica*$^{+}$ BALB/c mice (8-week-old females) showed little or no serum IgG response to *A. actinomycetemcomitans* or *P. pneumotropica*. However, 30 days after immunization with formalin-fixed *A. actinomycetemcomitans* Y4 [10^{9} bacteria/100 µl in saline per mouse, subcutaneously (s.c.) on days 0, 2 and 4], remarkable serum IgG responses to both *A. actinomycetemcomitans* and *P. pneumotropica* were observed in *P. pneumotropica*$^{+}$ BALB/c mice (Fig. 2).

Among several antigens of *A. actinomycetemcomitans* determined in the Western blot analysis, Omp29 appeared to be the major antigen recognized by serum IgG isolated from the *A. actinomycetemcomitans*-immunized mice (Fig. 2). Relatively strong IgG responses were also found at 16 kDa and 39 kDa in Western blot membranes of both *A. actinomycetemcomitans* and *P. pneumotropica* (Fig. 2). It is reported that Omp29 possesses epitopes common to the OmpA of the Pasteurellaceae family (14, 17). More specifically, three different mouse monoclonal antibodies (mAbs) specific to *A. actinomycetemcomitans* Omp29 reacted to a 27-kDa antigen present in *P. pneumotropica* (AP7, AP9 and AP14), indicating that the 27-kDa antigen recognized by three mAbs is *P. pneumotropica* OmpA. These three mAbs, developed following the previously published method (11), showed very high specificity for both *A. actinomycetemcomitans* and *P. pneumotropica* and did not react to any other bacteria examined using enzyme-linked immunosorbent assay (ELISA). (The bacterial strains that did not show reactivity with these three mAbs include *Streptococcus sanguis* 10556, *Streptococcus mitis* 49456, *Fusobacterium nucleatum* 25586, *Prevotella intermedia* 25611, *Porphyromonas gingivalis* W83, *Tannerella forsythia*, *Treponema denticola*, *Eikenella corrodens* 23834, *Escherichia coli K12* and *Bacteroides thetaiotaomicron*.)

The sera isolated from *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^{+}$ mice showed significantly higher IgG responses to *A. actinomycetemcomitans* and *P. pneumotropica* than the sera isolated from control non-immunized *P. pneumotropica*$^{+}$ mice (Fig. 2C). Such

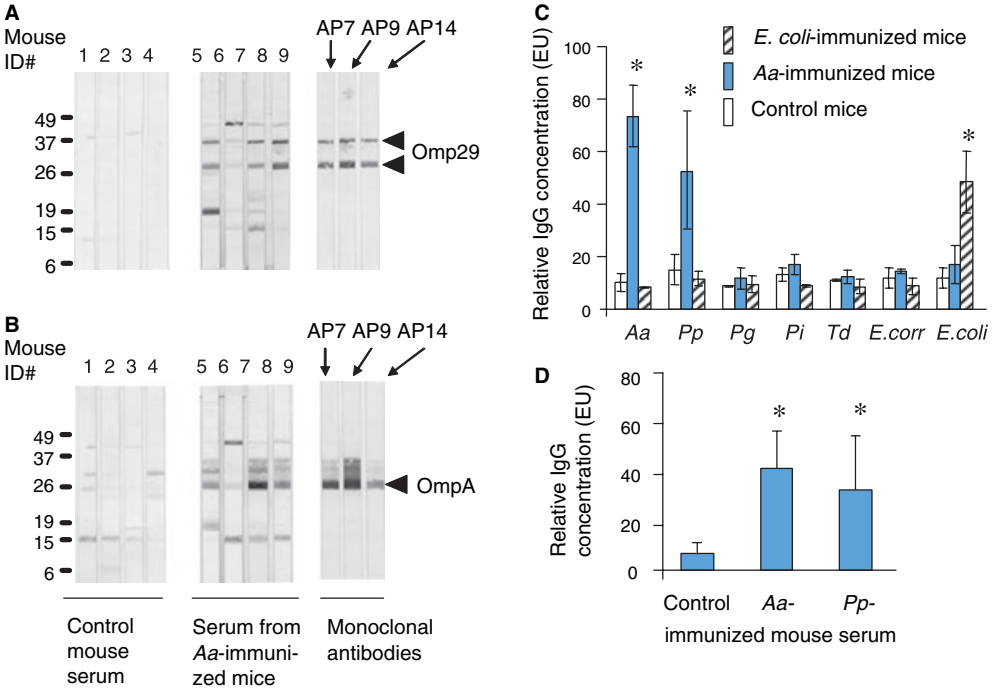


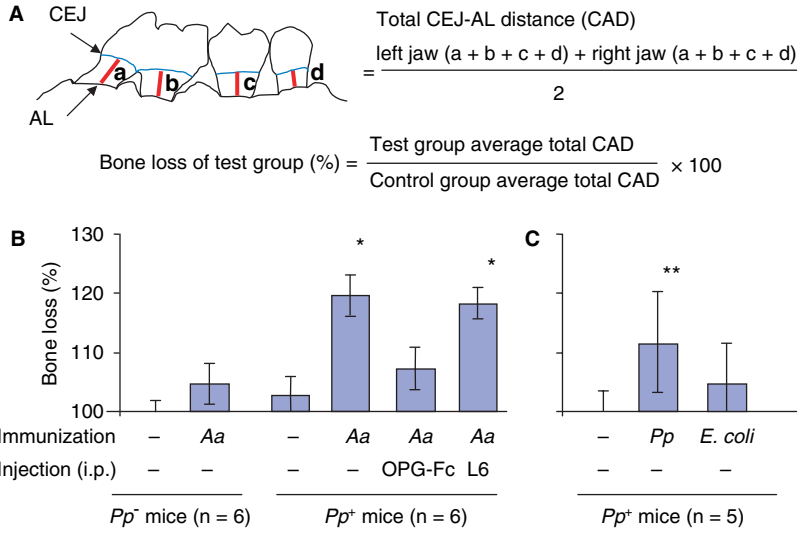
Fig. 2. Systemic immunization of *Pasteurella pneumotropica*$^{+}$ BALB/c mice induced an IgG antibody reacting to *Actinobacillus actinomycetemcomitans* Omp29 as well as *P. pneumotropica* OmpA. BALB/c mice (8-week-old females) harboring *P. pneumotropica* in their oral cavities were immunized with formalin-fixed *A. actinomycetemcomitans* (10^{9} bacteria/100 µl in saline per mouse, s.c. on days 0, 2 and 4, mouse ID# 5–9) or received control saline injection (mouse ID# 1–4). Thirty days after the first injection, animals were sacrificed and blood sera were sampled. *A. actinomycetemcomitans* and *P. pneumotropica* were cultured in *A. actinomycetemcomitans* growth medium (14, 21) and harvested at their mid-log growth phase. The harvested whole *A. actinomycetemcomitans* (A) and *P. pneumotropica* (B) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to nitrocellulose membrane for Western blot (11, 14). The serum isolated from each mouse was diluted (×100) in phosphate-buffered saline with 0.05% Tween-20, then applied to a nitrocellulose membrane that was cut into small strips (11). The specific deposition of IgG on the membrane strips was reacted with horseradish peroxidase-conjugated anti-mouse IgG (Sigma, St Louis, MO), and color was developed using a DAB substrate kit (Vector Laboratories, Burlingame, CA). The three different monoclonal antibodies specific to *A. actinomycetemcomitans* Omp29 (AP7, AP9 and AP14, unpublished) were also reactive with *A. actinomycetemcomitans* and *P. pneumotropica* transferred to nitrocellulose strips. Since the bacteria suspended in the SDS-PAGE loading buffer were treated at 100 °C for 5 min, the two bands of molecular weight 29 kDa and 34 kDa observed were for Omp29 [which is heat-modifiable in SDS-PAGE (14)]. The sera isolated from *P. pneumotropica*$^{+}$ mice immunized with *A. actinomycetemcomitans* or *E. coli* K12 (10^{9} bacteria/100 µl in saline per mouse, s.c. on days 0, 2 and 4, respectively, $n = 3$ per group) were subjected to IgG ELISA for *A. actinomycetemcomitans* (*Aa*), *P. pneumotropica* (*Pp*), *Porphyromonas gingivalis* W83 (*Pg*), *Prevotella intermedia* 25611 (*Pi*), *Treponema denticola* (*Td*), *Eikenella corrodens* 23834 (*E. corr*) or *Escherichia coli* K12 (*E. coli*) (C). Following the same immunization protocol as described above, sera isolated from *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^{+}$ mice, as well as *P. pneumotropica*-immunized *P. pneumotropica*$^{+}$ mice, were subjected to IgG ELISA to purified Omp29 antigen (D). The method for IgG ELISA to bacterial antigens followed those described previously (11). *$P < 0.05$, significantly higher than control non-immunized mice by Student's *t*-test.

sera isolated from the mice immunized with *A. actinomycetemcomitans* did not show any distinguishable IgG reactivity to *P. gingivalis* W83, *P. intermedia* 25611, *T. denticola*, *E. corrodens* 23834 or *E. coli* K12 (Fig. 2C). The *P. pneumotropica*$^+$ mice immunized with *E. coli* K12 showed only a specific IgG response to *E. coli* K12, and not to the other bacteria examined (Fig. 2C), indicating that the mice harboring oral *P. pneumotropica* did not show any IgG cross-reaction between *E. coli* K12 and either *A. actinomycetemcomitans* or *P. pneumotropica.* The serum isolated from *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mice, as well as that from *P. pneumotropica*-immunized *P. pneumotropica*$^+$ mice, showed more significantly elevated IgG responses to purified Omp29 than did control non-immunized *P. pneumotropica*$^+$ mice using an ELISA for IgG reaction to Omp29 (Fig. 2D). Adoptive transfer of *A. actinomycetemcomitans*-reactive T helper type 1 T cells also induced a serum IgG response which reacted to both *A. actinomycetemcomitans* Omp29 and *P. pneumotropica* OmpA (not shown). These results indicated that adaptive immune responses to *A. actinomycetemcomitans* induced in *P. pneumotropica*$^+$ BALB/c mice elicited a serum IgG antibody that was reactive to *A. actinomycetemcomitans* Omp29 and cross-reactive with *P. pneumotropica* OmpA.

Very importantly, the number of *P. pneumotropica* recovered from mouse oral cavities was not significantly affected by *A. actinomycetemcomitans* systemic immunization (78,624 + 11,428 colony-forming units (CFU)/μl for oral swabs from non-immunized control mice versus 65,592 + 12,509 CFU/μl for oral swabs from mice receiving *A. actinomycetemcomitans* systemic immunization on day 30). It is noteworthy that, in the ferret periodontal disease model (4), recovery of *Pasteurella* in the gingival crevice also remained unaffected, irrespective of the development of ligature-induced periodontitis.

P. pneumotropica$^+$ or *P. pneumotropica*$^-$ BALB/c mice were immunized with formalin-fixed *A. actinomycetemcomitans* (10^9 bacteria/100 μl in saline per mouse, s.c. on days 0, 2 and 4; 8-week-old female mice), and the following measurements were carried out: (i) periodontal bone loss ($n = 6$ per group); (ii) determination of tartrate resistant acid phosphatase (TRAP)$^+$ cells on periodontal bone ($n = 3$ per group); (iii) *in vitro* proliferation response of gingival T cells to *A. actinomycetemcomitans* and *P. pneumotropica* antigens ($n = 9$ per group) and their production of soluble(s)RANKL in culture supernatant; and (iv) double-color confocal microscopy for RANKL$^+$ T cells in gingival tissue ($n = 2$ per group).

Bone resorption induced in mice was measured using the previously published method for the rat periodontal disease model with slight modification (8, 10, 31). To distinguish the cement-enamel junction (CEJ), decalcified mouse maxilla



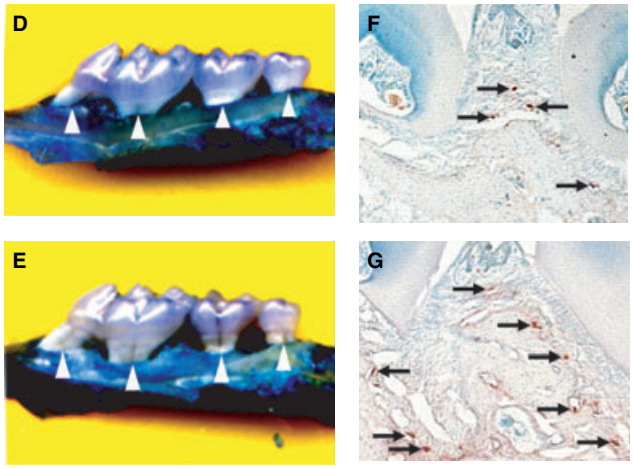


Fig. 3. Systemic immunization of *Pasteurella pneumotropica*$^+$ mice with *Actinobacillus actinomycetemcomitans* induced RANKL-dependent periodontal bone resorption. At 30 days from the initial *A. actinomycetemcomitans* immunization, mice were sacrificed, and the bone resorption rate (%) was measured (A). The rate of bone resorption compared to control non-immunized mice is expressed as a percentage. (B) *P. pneumotropica*$^+$ (*Pp*$^+$) or *P. pneumotropica*$^-$ (*Pp*$^-$) BALB/c mice ($n = 6$) were immunized with formalin-fixed *A. actinomycetemcomitans* (10^9 bacteria/100 μl in saline per mouse, s.c. on days 0, 2 and 4; 8-week-old female mice). Systemic injection of OPG-Fc (100 μg per mouse intraperitoneally every 3 days until day 30) or control L6 fusion protein was administered to the *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ BALB/c mice. $*P < 0.01$, significantly higher than control non-immunized *P. pneumotropica*-free mice by Student's *t*-test. (C) *P. pneumotropica*$^+$ BALB/c mice ($n = 5$) were immunized with formalin-fixed *P. pneumotropica* or *Escherichia coli* K12 (10^9 bacteria/100 μl in saline per mouse, s.c. on days 0, 2 and 4; 8-week-old female mice). At 30 days from initial *P. pneumotropica* immunization, mice were sacrificed, and the bone resorption rate (%) was measured [dimension of *y*-axis is the same as in (A)]. $**P < 0.05$, significantly higher than control non-immunized *P. pneumotropica*$^+$ mice by Student's *t*-test. Defleshed periodontal bone of non-immunized *P. pneumotropica*$^+$ mice and *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mice (D and E, respectively) was stained with methylene blue. The typical alveolar bone loss, which occurred on four roots, is indicated by arrows (E). The decalcified maxillary jaws were stained for TRAP. TRAP$^+$ cells on the alveolar bone of control non-immunized *P. pneumotropica*$^+$ mice and *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mice are indicated by arrows (F and G, respectively).

jaws were stained with methylene blue. The distances between the alveolar ledge (AL) and the CEJ were measured at two roots of the first molar, and at one root of the second and third molars, respectively, at the palatal maxilla (a total of four roots is indicated in Fig. 3A). The total CEJ-AL distance (CAD) of all four roots was calculated for both the left and the right jaws. Thus, the average total CAD of the left and right jaws represents each mouse's bone resorption rate. The average total CAD of the control non-immunized *P. pneumotropica*$^-$ mouse jaw was set as a control baseline level (100%), and the difference of each test group was then compared to this control (Fig. 3A).

The systemic immunization of *P. pneumotropica*$^+$ mice with *A. actinomycetemcomitans* induced significantly increased periodontal bone loss compared to the control *P. pneumotropica*$^+$ mice that did not receive *A. actinomycetemcomitans* immunization, while *A. actinomycetemcomitans* immunization of *P. pneumotropica*$^-$ mice did not cause any change of periodontal bone level, indicating that the bone resorption is dependent on the presence of *P. pneumotropica* in the oral cavity (Fig. 3B). It is also noteworthy that immunization of *P. pneumotropica*$^+$ BALB/c mice with fixed *P. pneumotropica* induced significantly elevated periodontal bone resorption (Fig. 3C). However, immunization of *P. pneumotropica*$^+$ BALB/c mice with the non-cross-reactive bacterium *E. coli* K12 did not induce periodontal bone loss (Fig. 3C). Systemic administration of OPG-Fc (100 μg/mouse, intraperitoneally; a gift from Dr Colin Dunstan, Amgen Inc., Thousand Oaks CA), which was applied every 3 days until day 30, significantly abrogated the bone resorption induced by *A. actinomycetemcomitans* immunization of *P. pneumotropica*$^+$ BALB/c mice (Fig. 3B). Since OPG is the soluble decoy receptor for RANKL (2), the results from OPG-Fc administration indicated that bone resorption induced in *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ BALB/c mice is RANKL-dependent. Systemic immunization of *P. pneumotropica*$^+$ C57BL6 mice with fixed *A. actinomycetemcomitans* also induced significantly increased periodontal bone loss (not shown). The representative pictures of decalcified periodontal bone of the non-immunized *P. pneumotropica*$^+$ mice and the *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mice are shown (Fig. 3D,E).

Maxillary jaws dissected from the sacrificed mice on day 30 were decalcified and embedded in optimum cutting temperature (OCT) compound. The maxilla jaws were then sectioned using a cryostat (8 μm thickness) and stained with TRAP reagent (10, 12). As shown in Fig. 3F,G, an increased number of TRAP$^+$ cells were observed on the alveolar bone of *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mice compared to the control non-immunized *P. pneumotropica*$^+$ mice, indicating that bone resorption induced in *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mice is accompanied by increased numbers of TRAP$^+$ osteoclasts in the periodontal tissues.

T cells isolated from the gingival tissue of *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mice proliferated *in vitro* and produced sRANKL in response to both *A. actinomycetemcomitans*-antigen and *P. pneumotropica*-antigen presentation by mitomycin C-treated spleen antigen-presenting cells (Fig. 4-A,B). However, gingival T cells isolated from non-immunized *P. pneumotropica*$^+$ mice did not show such a proliferative response to either *A. actinomycetemcomitans*-antigen or *P. pneumotropica*-antigen presentation by antigen-presenting cells, nor did they produce sRANKL (Fig. 4-A,B). Therefore, *A. actinomycetemcomitans* immunization appeared to induce a T-cell-mediated adaptive immune response to *A. actinomycetemcomitans*, which also cross-reacted with *P. pneumotropica*.

The decalcified and sectioned maxillary jaws were evaluated for the expression of RANKL on CD3$^+$ T cells. Expression of RANKL was demonstrated by staining with OPG-Fc-biotin followed by Texas Red-conjugated avidin (12). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 mAb (clone 145–2C11, BD Pharmingen, San Diego, CA) was used to

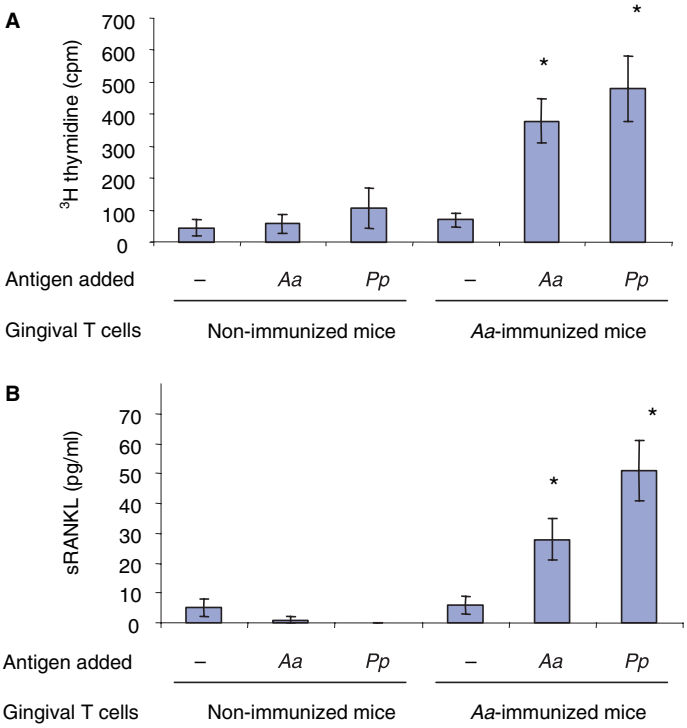


Fig. 4. Antigen-specific proliferation and sRANKL production by T cells isolated from gingival tissue. (A) The gingival tissue of three animals, out of a total of nine mice in each group, was pooled. T cells were isolated from gingival tissues using collagenase type IV for digestion of connective tissues (10). The T cells isolated from gingival tissue (10,000 cells/well in 96-well plate) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum with or without fixed *Actinobacillus actinomycetemcomitans* (*Aa*) or *Pasteurella pneumotropica* (*Pp*) (10^7/well) in the presence of spleen antigen-presenting cells (treated with mitomycin C, 25 μg/ml, for 1 h, 10^5/well) (A, B). [^{3}H]thymidine (0.5 μCi/well) was applied during the last 16 h of a total 4-day incubation. The radioactivity incorporated into the proliferating cells was measured by a scintillation counter (average counts/min (cpm) ± SD of three gingival tissue pools). *$P < 0.01$, significantly higher than control non-immunized *P. pneumotropica*-free mice by Student's *t*-test. (B) Culture supernatants isolated from the T-cell culture described above on day 3 were subjected to sRANKL ELISA (average pg/ml ± SD of three gingival tissue pools). *$P < 0.01$, significantly higher than control non-immunized *P. pneumotropica*-free mice by Student's *t*-test.

identify CD3$^+$ T cells in gingival tissues. The staining patterns of Texas Red and FITC were monitored by laser scan confocal microscopy (DMRXE/TCS/SP-2, Leica), as previously described (10). The number of RANKL$^+$ T cells in the gingival tissues was remarkably increased in the *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mice compared to non-immunized *P. pneumotropica*$^+$ mice (Fig. 4A,B). Using IMAGE J software (publicly accessible in the on-line domain provided by the National Institutes of Health; http://rsb.info.nih.gov/ij/), the intensities of RANKL expression on T cells were converted into the histograms shown in Fig. 5C,D, which indicate that the number of T cells and the intensity of RANKL expression on the T cells were both higher in *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mice (Fig. 5D) than in non-immunized *P. pneumotropica*$^+$ mice (Fig. 5C). Based on the visually enumerated data from the captured images, the actual CD3$^+$ T-cell number of this particular sample was significantly higher in the *A. actinomycetemcomitans*-immunized mouse than in the control non-immunized one (Fig. 5E). The percentage of the total CD3$^+$ T cells that were RANKL-expressing CD3$^+$ T cells was also significantly elevated in the *A. actinomycetemcomitans*-immunized mouse than in the control non-immunized mouse (Fig. 5F).

Taken together, these results demonstrated that an adaptive immune response induced to *A. actinomycetemcomitans* resulted in a cross-reactive IgG response to the *P. pneumotropica* that was colonizing the BALB/c mouse oral cavity. OmpA of *P. pneumotropica*, the homologue of *A. actinomycetemcomitans* Omp29, was a major cross-reactive antigen recognized by *A. actinomycetemcomitans*-immunized mouse serum IgG. Such induction of an IgG response to *P. pneumotropica* was accompanied by RANKL-dependent periodontal bone loss because OPG-Fc could abrogate the induction of bone loss. Remarkably, an increased number of RANKL$^+$ T cells were observed in the periodontal bone resorptive lesion of *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ BALB/c mice. Furthermore, T cells isolated from the gingival tissues of *A. actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ BALB/c mice reacted to both *A. actinomycetemcomitans* and *P. pneumotropica* presentation by spleen antigen-presenting cells and produced sRANKL. Therefore, antigen-specific activation of T cells in local gingival tissue appears to play a key role in the production of RANKL by the activated T cells. This study is the first to demonstrate that cross-reactive immune responses to phylogenetically related bacterial antigens can trigger a pathogenic adaptive immune response that elicits RANKL-dependent periodontal bone resorption.

The significance of the present study is also demonstrated by the finding that mucosal immune tolerance appeared to be elicited to *P. pneumotropica* colonizing the mouse oral cavity. To illustrate this, significantly elevated salivary IgA antibody to *P. pneumotropica* was detected in the non-immunized *P. pneumotropica*$^+$ mice compared to the non-immunized *P. pneumotropica*$^-$ mice (not shown). Irrespective of the presence of salivary IgA antibody to *P. pneumotropica*, little or no serum IgG and T-cell responses were detected, indicating that mucosal tolerance is induced to orally colonizing *P. pneumotropica* (33). In contrast to the well-characterized mucosal tolerance in the gastrointestinal system (30), the tolerance systems in the oral mucosal cavity remain unclear. Mucosal tolerance is defined as the suppression, or down-regulation, of adaptive immune responses mediated by lymphocytes via previous challenge of the antigen through the mucosal surface (33). Since RANKL production by activated lymphocytes appears to be causal for periodontal bone loss (12), mucosal tolerance may play a key role in the down-regulation of such pathogenic immune responses in the oral cavity. Therefore, the mouse model established in this study may be useful in elucidating the regulatory mechanism of oral mucosal tolerance in the context of immune-mediated bone loss processes in periodontal disease.

The rat periodontal disease models that involve oral infection with *A. actinomycetemcomitans* (25) strongly support the pathogenic roles of this bacterium in periodontal disease. Teng et al. (29) reported that adoptive transfer of an *A. actinomycetemcomitans*-specific human T-cell

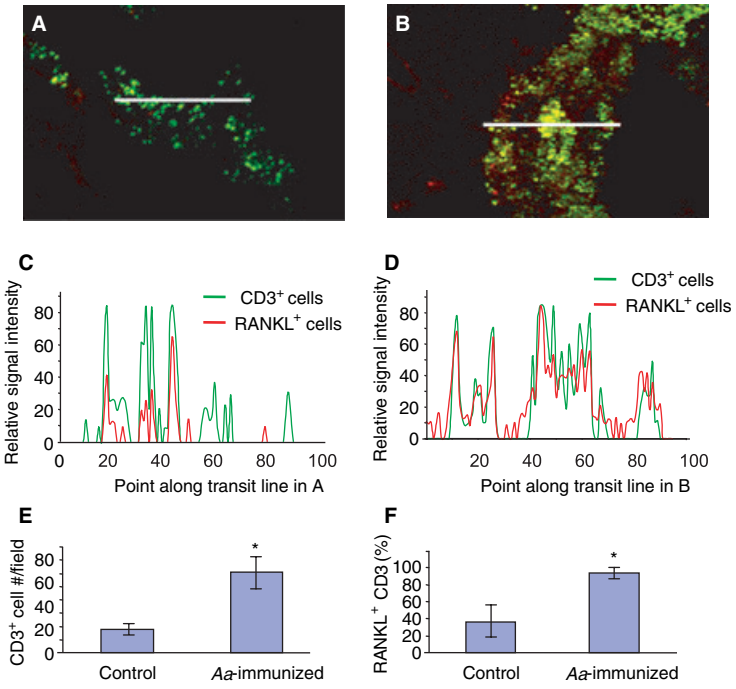


Fig. 5. Confocal microscopy-based RANKL expression pattern by CD3$^+$ T cells in the gingival tissues. Decalcified gingival tissues were sectioned and subjected to double-color confocal microscopic analysis. Non-immunized *Pasteurella pneumotropica*$^+$ mouse gingivae and *Actinobacillus actinomycetemcomitans*-immunized *P. pneumotropica*$^+$ mouse gingivae sampled on day 30 are shown (A and B, respectively). CD3 and RANKL were stained with FITC (green) and Texas Red (Red), respectively. The intensities of the green and red colors were scanned along the transit line drawn in (A) or (B). Then, each color's intensity was converted digitally and expressed in the histograms (C and D). Each peak of CD3 represents individual CD3 T cells. The *x*-axis displays the relative distance that spans the total length of the transit line (100 μm). The actual CD3$^+$ T-cell number in the microscopic field (×1000, $n = 4$) was enumerated and expressed as CD3$^+$ cell number per microscopic field (CD3$^+$ cell #/field) following the method described previously (E) (12). The percentage of RANKL-expressing CD3$^+$ T cells in the total CD3$^+$ T cells in the microscopic field was also measured and expressed as RANKL$^+$ CD3 (%) (F). *$P < 0.01$, significantly higher than control non-immunized mice by Student's *t*-test.

line isolated from patients with aggressive (juvenile) periodontal disease could induce significant periodontal bone loss in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice that were orally inoculated with *A. actinomycetemcomitans* every 3 days (29). This result suggests that the mouse oral cavity is compatible in reproducing human periodontal disease. Garlet et al. demonstrated that *A. actinomycetemcomitans* colonization in C57BL/6 mice induces periodontal bone loss along with an elevated mRNA level of matrix metalloproteinases and RANKL in the gingival tissue (6). We also demonstrated that *A. actinomycetemcomitans* Omp29-specific T helper type 1 cells, or *A. actinomycetemcomitans*-reactive B cells, can trigger periodontal bone resorption in rats in a RANKL-dependent fashion (8, 10, 31). This shows that purified bacterial antigen, or killed bacteria, can elicit pathogenic T-cell-mediated and/or B-cell-mediated periodontal bone loss. The present study additionally demonstrated that induction of an adaptive immune response to orally colonized *P. pneumotropica*, as a consequence of cross-reactive IgG antibody and T-cell responses to *A. actinomycetemcomitans* immunization, results in RANKL-dependent periodontal bone loss in mice. It is noteworthy that, after immunization of *P. pneumotropica*$^{+}$ mice with *A. actinomycetemcomitans*, B-cell infiltration into gingival tissues increased in the bone loss lesions and such infiltrating B cells in the lesions showed remarkably elevated RANKL as determined by confocal microscopy (unpublished data). Since *H. influenzae*, which is another member of the Pasteurellaceae, causes serious life-threatening infections in infants, such as pneumonia and meningitis (23), it is of further interest to know whether the history of *H. influenzae* infection and the resulting adaptive immune responses to this organism are associated with the development of *A. actinomycetemcomitans*-mediated periodontal diseases. In summary, the present findings may offer the insight into the immune-mediated periodontal bone loss mechanisms that will give us the answers to these questions.

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