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Bacteria-reactive immune response may induce RANKL-expressing T-cells in the mouse periapical bone loss lesion

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Abstract

Introduction—The present study investigated if T-cells infiltrating the periapical lesion produce RANKL and whether bacteria infecting the root canal can activate T-cells to produce RANKL.

Methods—Using a mouse model of periapical lesion induced by artificial dental pulp exposure, the presence of RANKL-positive T-cells and osteoclasts in the periapical lesion was examined by an immuno-histochemical approach. The bacteria colonizing the exposed root canal were identified by 16S ribosomal RNA (rRNA) sequence analysis. The isolated endodontic bacteria were further immunized to normal mice, and sRANKL production by the T-cells isolated from the immunized mice was evaluated by *ex vivo* culture system.

Results—RANKL-positive T-cells, along with TARP+ osteoclasts, were identified in periapical bone resorption lesions. The Gram-negative bacterium *Pasterurella pnumotropica (P. pnumotropica)*, which was most frequently detected from root canal of exposed pulp, showed remarkably elevated serum IgG antibody response in pulp-exposed mice compared to control non-treated mice. Immunization of mice with *P. pneumotropica* induced not only serum IgG antibody but also primed bacteria reactive T-cells that produced sRANKL in response to *ex vivo* exposure to *P. pneumotropica*.

Conclusion—T-cells infiltrating the periapical region express RANKL, and the endodontic bacteria colonizing the root canal appear to induce RANKL expression from bacteria-reactive T-cells, suggesting the possible pathogenic engagement of immune response to endodontic bacteria in the context of developing boneresorptive periapical lesions.

Introduction

Periapical periodontitis is caused by bacterial infection of the dental pulp that leads to the development of a pathogenic bone resorption lesion (1, 2), which is characterized by the infiltration of inflammatory cells predominantly composed of macrophages and T cells (1, 3, 4), in conjunction with the production of proinflammatory cytokines (2, 5, 6). The current paradigm of bone resorption supports the hypothesis that receptor activator of nuclear factor k B-ligand (RANKL) directly induces osteoclast differentiation and activation (7), while its soluble decoy-receptor osteoprotegerin (OPG) neutralizes such osteoclastogenic effects by RANKL (8). The level of RANKL mRNA expression in human periapical lesion is significantly higher than healthy periapical tissue (9, 10), indicating that locally produced RANKL also elicits periapical bone resorption. However, the biological mechanism underlying such increase of RANKL in the periapical lesion with attendant pathogenic bone resorption remains unclear.

It is reported that RANKL produced by T-cells, but not monocyte linage cells, appears to be engaged in the bone loss exhibited in marginal periodontitis as a result of an adaptive immune response to the bacterium (11, 12). In a mouse model of periodontal disease, bacteria reactive T-cells are demonstrated as the source of RANKL (13). On the other hand, while the kinetics of RANKL-expressing cells were reported to parallel to that of emergent osteoclasts in the rodent model of periapical lesion (14), the cellular source of RANKL is unclear, especially whether T lymphocytes produce RANKL in the periapical lesion.

Therefore, in the present study, we investigated the localization of RANKL-producing T-cells using a mouse model of periapical lesion induced by artificial dental pulp exposure. The bacteria colonizing in the exposed root canal were identified by 16S ribosomal RNA (rRNA) sequencing. The isolated endodontic bacteria were further immunized to normal mice, and sRANKL production by the T-cells isolated from the immunized mice was evaluated by *ex vivo* culture system.

Materials & Methods

Animals

C57BL/6j mice (8-week-old males) were kept in the Forsyth Animal Facility with 12h light-dark cycle at constant temperature. The experimental protocols used in this study were approved by the Forsyth IACUC.

Induction of periapical lesion

Periapical lesions were induced by pulp exposure of the mandibular first molar pulps of mice (8–10-week-old males, n=5/group) following the protocol published previously (15). Both sides of mandibular first molars were exposed using a ¼-size dental round bur. Healthy non-treated mice served as baseline control. Blood serum was collected on day-0 and -14.

Histochemical analyses

The mandibles from mice were fixed in 4% paraformaldehyde and decalcified in 10% EDTA solution. These mandibles were embedded in Tissue-Tek OTC compound (Sakura, Torrance, CA), and sections (8 μm in thickness) of specimens were cut in a buccal-lingual direction by using a cryostat. The sections were stained with 0.1% Mayers Hematoxylin and 0.5% Eosin or with TRAP (13), and the nuclei were counter-stained with methyl green.

Immunofluorescent Laser-Scanning confocal microscopy

Frozen sections were reacted with the anti-mouse CD3 ε chain conjugated with fluorescein isothiocyanate (FITC) (2.5 μg/ml) (BD Biosciences, San Jose, CA) or biotinylated-OPG-Fc (10 μg/ml) followed by Texas Red-Avidin (10 μg/ml) (Invitrogen, Carlsbad, CA) (12). The staining pattern was analyzed by using a Leica TCS/SP-2 confocal microscope (Leica, Wetzlar, Germany).

16S rRNA-based identification of mouse endodontic bacteria

At 14 days after pulp exposure, microbial specimens were collected from the root canal space of anesthetized mice placing paper points for 30 s. The bacteria recovered on paper points were cultured on sheep blood agar plates in an anaerobic chamber at 37°C for 2 days. The total genomic DNA was extracted from all cultivable bacterial isolates, using QIAmp DNA mini kit (Qiagen, Valencia, CA), and subjected to 16S rRNA-based bacterial identification (13, 16).

Immunization of mice with bacterial antigen

C57BL/6j mice (8-week-old males, n=5/group) were immunized with or without heat-killed *Pasteurella pneumotropica (P. pneumotropica)* or *Enterococcus saccharolyticus* (*E. saccharolyticus*) (3×10^8 CFU/mouse, subcutaneous [s.c.] injection) in Freund's adjuvant following the method previously published (17). Four days after booster injection (s.c.) of bacteria in PBS, lymph nodes and blood serum were isolated. Injection of PBS in a mixture of Freund's adjuvant constituted a control.

The measurement of serum IgG antibody responses to bacterial antigens

Sera isolated from mice were reacted to heat-killed bacteria (10^7/ml) coated on ELISA plates, and the reacted serum IgG antibodies were measured by ELISA (17).

The bacterial antigen-specific memory T-cell response

CD11c-positive dendritic cells (DCs) developed by incubation of bone marrow cells of C57BL/6j mice with GM-CSF (20 ng/ml) for 7 days were purified using MACS beads (Miltenyi, Auburn, CA). T-cells (2×10^5 cells/well) isolated from the lymph nodes of mice immunized with or without *P. pneumotropica* or *E. saccharolyticus* were co-cultured *in vitro* with Mitomycin C (20 μg/ml, Sigma)-treated DCs (2×10^4 cells/well) in the presence or absence of fixed *P. pneumotropica* or *E. saccharolyticus* (10^7 CFU/well) in RPMI 1640 medium containing 10% FBS for 3 days (17). The proliferation of T-cells was evaluated by the [^{3}H] thymidine incorporation assay (18). The sRANKL production was monitored using an ELISA kit (PeproTech, Rocky Hill, NJ) (17).

Statistical analysis

Differences between the two groups were analyzed with Student's *t*-test.

Results

Pulp exposure induces inflammatory tissue disruption in periapical lesions

On day-3 after pulp exposure, the periodontal ligament space at the apex of teeth was enlarged (Fig. 1B) compared to control healthy tissue (Fig. 1A). Newly formed blood vessels were also observed in the periapical lesion (Fig. 1B), indicating the induction of primary inflammatory response by the pulp exposure. At day-7, remarkable inflammatory cell infiltration was observed in the periapical lesion (Fig. 1C). Furthermore, massive bone

resorption was found at the apical area at day-14 (Fig. 1D) accompanied by the emergence of TRAP+ osteoclasts on the alveolar bone surface of the periapical area (Fig. 1F).

Temporal changes of RANKL$^+$/CD3$^+$ T-cells in the mouse periapical lesions

According to the double-color confocal microscopy that stained RANKL (red) and T lymphocyte-specific CD3 (green) in the periapical tissue, few or no CD3$^+$ T-cells were detected in control healthy periapical area (Fig. 2D), whereas, in the periapical lesion, remarkable infiltration of CD3$^+$ T-cells was observed at both day-3 (Fig. 2A and 2D) and day-7 (Fig. 2B and 2D), followed by the remission of CD3$^+$ T-cell infiltration at day-14 (Fig. 2C and 2D). Importantly, more than 50% of CD3$^+$ cells, which existed in the periapical lesion, expressed RANKL (Fig. 2A, 2B and 2E). Interestingly, on day-7, the RANKL-expressing CD3$^+$ T-cells were also found in medullary cavity of alveolar bone (Fig. 2B). These findings indicated that RANKL-producing T-cells infiltrate into the periapical lesion in response to pulp exposure.

Orally colonizing bacteria induce RANKL-producing, bacteria-specific activated T lymphocytes

Using 16S rRNA sequencing, only two bacterial strains, *E. saccharolyticus* and *P. pneumotropica*, were found in root canals of all five mice receiving pulp exposure. Other bacteria, such as *Klebsiella pneumoniae* and *Staphylococcus sciuri*, were detected in a few, but not all, exposed roots. *E. saccharolyticus* and *P. pneumotropica* infections in the exposed pulp were further evaluated by the serum IgG response to these two bacteria (Fig. 3A and 3B). Interestingly, serum IgG antibody to *P. pneumotropica* was significantly elevated at 14 days post-pulp exposure compared to that of control baseline level (Fig. 3B), whereas serum IgG antibody to *E. saccharolyticus* showed no significant difference, either before or after pulp exposure (Fig. 3A), suggesting that *P. pneumotropica* invading the root canal was more actively recognized by adaptive immune response than *E. saccharolyticus*. Furthermore, mice immunized with *P. pneumotropica* or *E. saccharolyticus* were examined for their ability to induce T-cell-mediated adaptive immune response. The lymph node T-cells isolated from *P. pneumotropica*-immunized mice showed remarkable increase of T-cell proliferation in response to *in vitro P. pneumotropica*-antigen presentation by DCs (Fig. 3C), while *E. saccharolyticus*-immunized mice showed no induction of such *in vitro* T-cell response to *E. saccharolyticus*-antigen presentation (Fig. 3C). Finally, and most importantly, sRANKL production from these T-cells in response to respective bacterial antigen presentation was remarkably higher in *P. pneumotropica*-reactive T-cells than *saccharolyticus*-reactive T-cells (Fig. 3D), suggesting that the T-cell response is species specific and the T cells activation represented by their proliferation correlates to the amount of sRANKL produced by these bacterial antigen-reactive T cells.

Discussion

In the present study, we revealed that CD3$^+$ T lymphocytes produce RANKL in the periapical lesion with resulting pathogenic bone resorption. The infiltration of RANKL-expressing CD3+ T-cells in the periapical lesion peaked between day-3 and day-7, while such infiltration remitted to baseline level by day-14 after pulp exposure. The inflammatory cell infiltration in the lesion that occurred within the first 3 days after pulp exposure appears to be composed of innate immune cells (Fig. 1B), some of which, probably macrophages, are considered to present bacterial antigen to T cells. The FOXP3$^+$ Treg cells that emerge in the lesion during the later stage (day-7 to day-14)(15) are considered to suppress RANKL expression by CD3$^+$ T cells. Therefore, it is conceivable that CD3$^+$/RANKL$^+$ cells found in this study are adaptive immune T cells, not Treg cells, and that they are engaged in bone

resorption by the production of RANKL in a manner similar to that reported in rheumatoid arthritis and periodontal disease (12, 19).

We previously reported that mucosal immune tolerance appeared to be elicited by *P. pneumotropica* that inhabits the mouse oral cavity (13). More specifically, immune tolerance was represented by low antibody response and minimal T-cell response to *P. pneumotropica*. However, *P. pneumotropica* that invaded exposed root canals induced IgG antibody response, suggesting that immune tolerance to *P. pneumotropica* was breached by the bacterial challenge from the root canal or periradicular tissue. However, the other endodontic bacterium found in this study, *E. saccharolyticus*, which was also isolated from exposed pulp, did not show such remarkable immune responses. Although, the nature and mechanism underlying the adaptive immune recognition and immune tolerance to oral bacteria remains to be elucidated, the different immune responses detected between *E. saccharolyticus* and *P. pneumotropica*, suggested that immune responses induced against endodotic bacteria are regulated in a manner specific to bacterial species..

We did not find human endodontic pathogens, such as *Fusobacterium*, *Porphyromonas*, *Prevotella*, or *Streptococcus* strains in the mouse (20–22). Nonetheless, the adaptive immune response to the endodontic bacterium *P. pneumotropica* was shown to result in RANKL production from bacteria-reactive T cells, which, in turn, contributed to osteoclast-mediated bone resorption. This line of evidence strongly suggests the pathogenic roles of the T-cell mediated immune responses elicited against endodontic bacteria and clearly supports the necessity of removing endodontic bacteria and bacterial components from an affected tooth during endodontic treatment.

Conclusion

Specific bacterial invasion into root canals activates adaptive immune response which results in the induction of RANKL-producing, bacteria-specific memory T-cells, and possibly contributing to osteoclast-mediated bone resorption in the periapical lesion.

Acknowledgments

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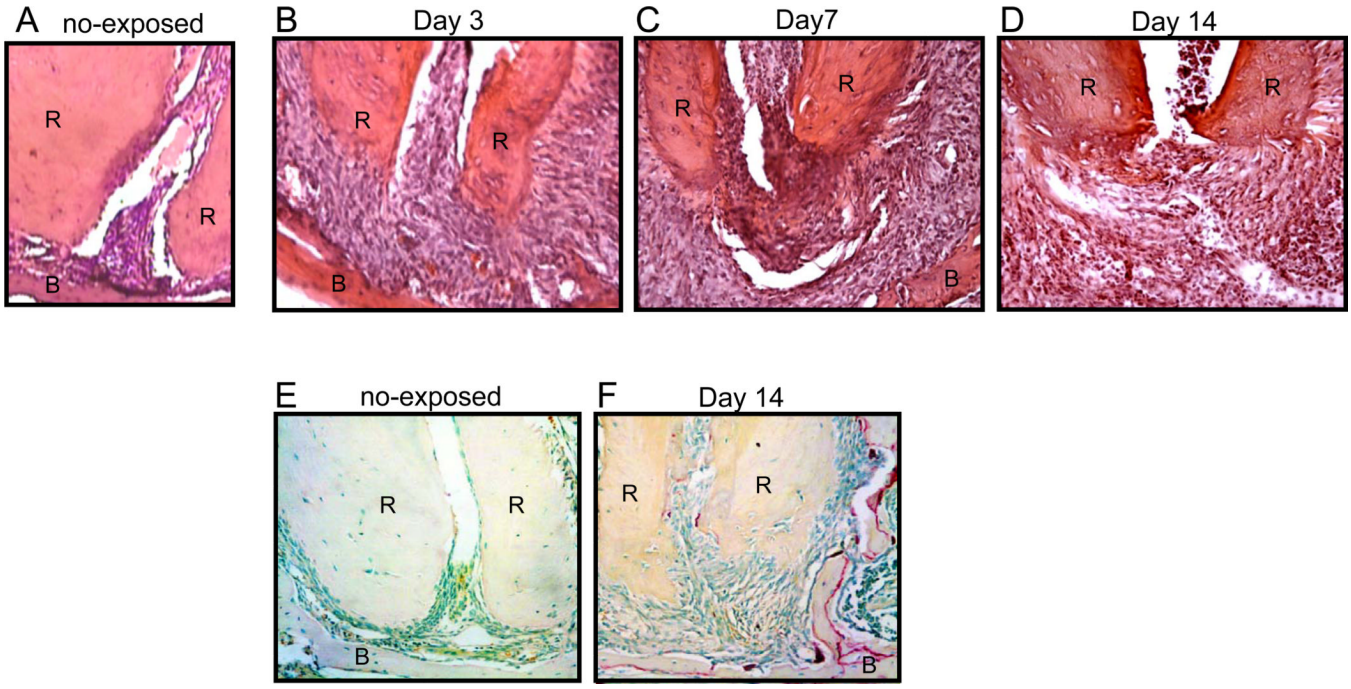

Figure 1.

Pulp exposure induces inflammatory tissue disruption and osteoclastogenesis at the periapical area. Periapical tissues isolated from non-exposed mice (A) or pulp-exposed mice on day-3 (B), day-7 (C), and day-14 (D) were decalcified and stained with H&E. Root (R) and alveolar bone (B) are indicated by the upper case letters. Decalcified periapical tissues of first mandibular molars sampled from (E) control non-treated mice or (F) pulp-exposed mice were stained for TRAP+ osteoclasts. Original magnification: (A) ×400 and (B–F) ×200.

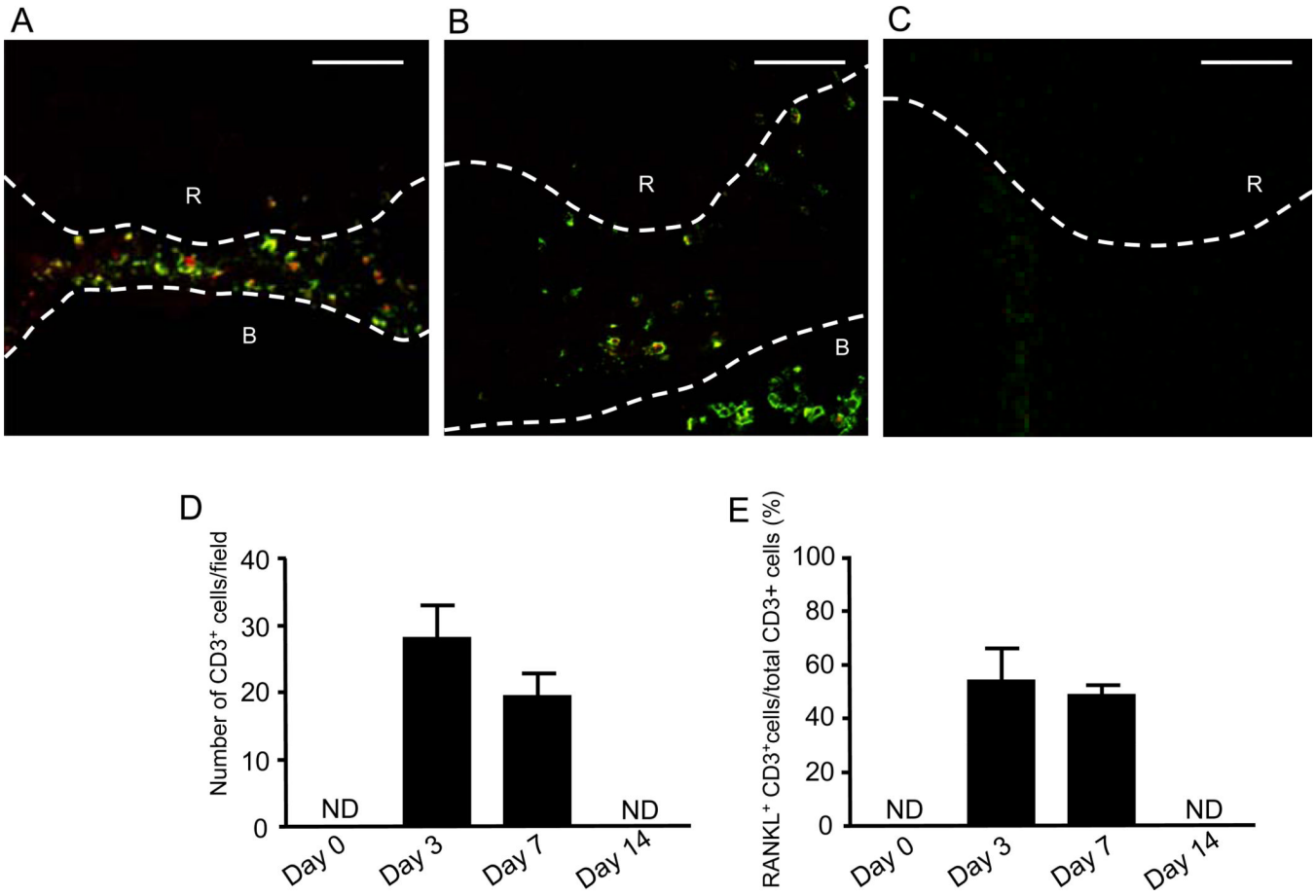

Figure 2.

Chronology of RANKL+/CD3+ T-cells localization pattern in periapical lesions in pulp exposure mouse model. (A–C) The periapical connective tissues at 3 (A), 7 (B) and 14 (C) days after pulp exposure was stained with anti-CD3 mouse antibody-FITC (green) for identification of T-cells and OPG-Fc-biotin/TexasRed-avidin (red) for RANKL. All fluorescent images were captured using a laser scanning confocal microscope. Root (R) and alveolar bone (B) are indicated by the upper case white letters. The white broken line demonstrates the border of root and alveolar bone. Scale bars: 50μm. (D and E) Quantitative assessment of total number of CD3+ T-cells (D) and percentage of RANKL+/CD3+ T-cells in the total CD3+ T-cells (E) are shown in the histograms. The column and bar indicate means ± SD; *, significantly higher than control non-pulp exposure group (day-0) by Student's *t*-test ($p < 0.05$). NA: data not available.

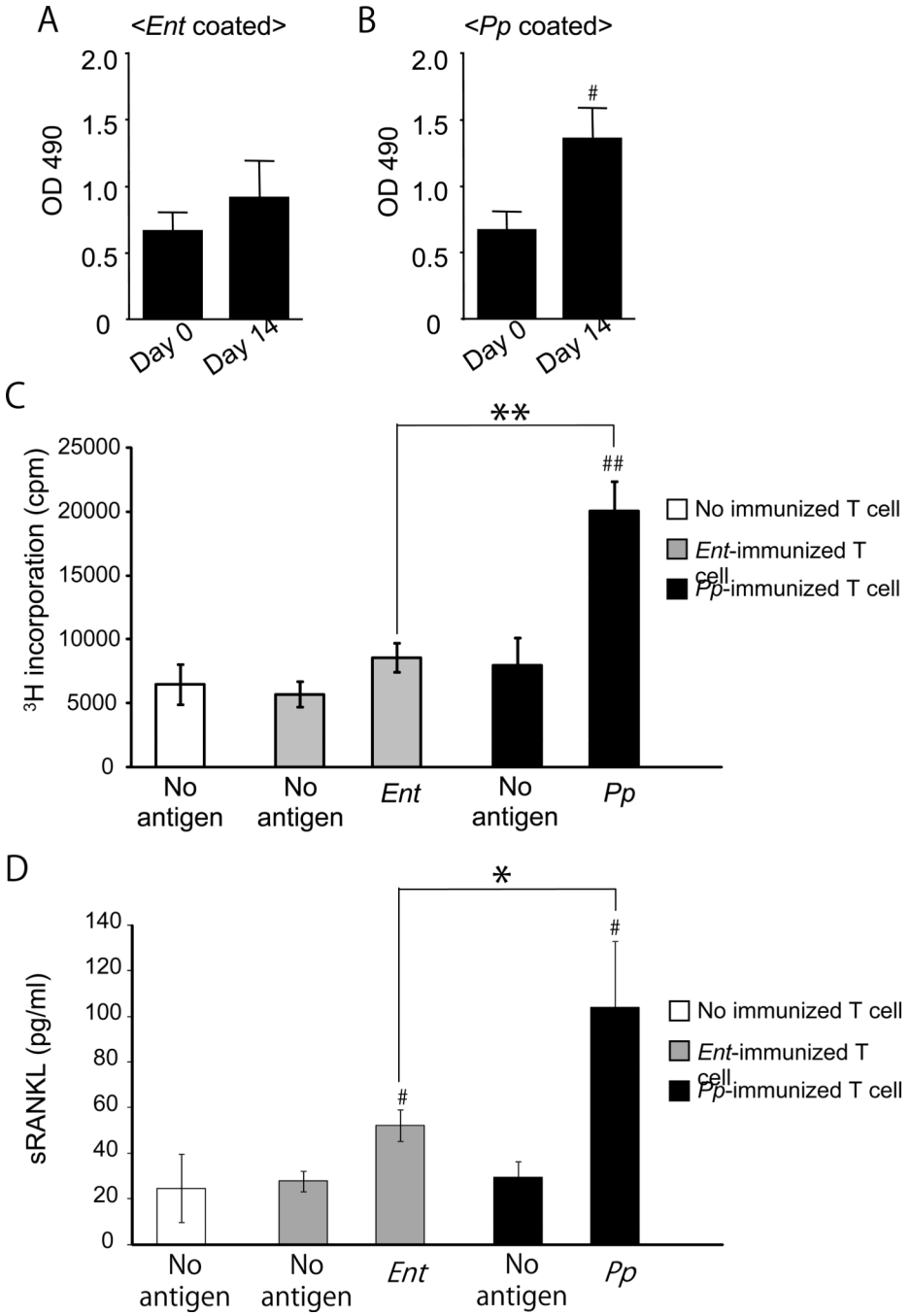

Figure 3.

Orally colonizing bacteria induce RANKL-producing, bacteria-specific activated T lymphocytes. (A and B) Blood serum was collected on day-0 and day-14 after pulp exposure. (n=5/group). The serum IgG antibody response to *E.* saccharolyticus (A) or *P. pneumotropica* (B) was measured using ELISA, as described in Materials and Methods. Raw readout of ELISA measured at OD490 absorption is shown. Data indicate the mean ± SD of five different mice. * Significantly higher than day-0 mice sera IgG by Student's *t*-test ($P < 0.05$). (C and D) The T-cells isolated from lymph nodes of mice immunized with *E. saccharolyticus*, *P. pneumotropica*, or control non-immunized mice, were cultured with or without heat-killed *E. saccharolyticus* or *P. pneumotropica* in the presence of MMC-treated

dendritic cells for 3 days to measure the expressions of sRANKL, using ELISA, or for 4 days to assess the proliferation, using a [^{3}H] thymidine incorporation assay. Proliferation of T lymphocytes (C) and production of sRANKL in culture supernatant are shown in histograms. Data indicate the mean ± SD of three cultures. $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, significantly higher than control non-immunized T-cells by Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, significantly higher than *E. saccharolyticus*-immunized T-cells with each bacterial antigen by Student's *t*-test.