



Original Article

A histopathologic study of the controlling role of T cells on experimental periodontitis in rats



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Abstract *Background/purpose:* The onset and progression of periodontitis involve bacterial infection and the immune response. T cells function in the immune response and reportedly induce bone resorption in inflammatory bone loss. However, the exact role of T cells in periodontal destruction remains unclear. Using our experimental model of periodontitis, we aimed to investigate the influence of T cells on periodontal destruction.

Materials and methods: Male athymic nude (Nu) and euthymic wild-type (WT) rats were divided into the immunized (I-Nu and I-WT), non-immunized (nI-Nu and nI-WT). The immunized groups were immunized intraperitoneally with lipopolysaccharide (LPS). The non-immunized groups received phosphate-buffered saline (PBS). Nothing was administered to the non-treated groups. LPS was applied to the right palatal gingival sulcus in the immunized and non-immunized groups daily for 20 days. Loss of attachment, numbers of inflammatory cells and osteoclasts, and levels of alveolar bone were investigated histopathologically and histometrically. Osteoclasts were stained with tartrate-resistant acid phosphatase. The numbers of IL-4-positive cells were evaluated immunohistologically.

Results: Loss of attachment, numbers of inflammatory cells, levels of alveolar bone, and the number of osteoclasts were significantly increased in the nI-WT group compared with the nI-Nu group. However, the parameters were significantly increased in the I-Nu group compared with the I-WT group. The number of IL-4-positive cells was greater in the I-WT group than in the I-Nu group.

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Conclusion: T cells promote inflammation in non-immunized animals; however, they regulate these processes in immunized animals.

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Introduction

Periodontitis is a chronic inflammatory disease characterized by periodontal pocket formation and alveolar bone resorption. Bacterial infection and the host immune response are considered responsible for the onset and progression of periodontal destruction.^{1,2} Several studies have elucidated the contribution of bacterial components to the progression of periodontitis;^{3,4} however, the contribution of the immune response remains unclear.

T cells play a central role in the immune response and have been implicated in periodontal destruction by many studies. One investigation suggested that T cells suppress bone loss: periodontal bone loss in nude rats, which are congenitally deficient in T cells, is significantly increased compared with that in normal rats, but decreases when T cells from normal rats are transplanted into nude rats.⁵ In contrast, other studies have suggested that T cells are activated by antigen-presenting cells and express receptor activator of nuclear factor κ -B ligand and tumor necrosis factor- α (TNF- α) induce bone resorption in inflammatory bone loss.^{6,7} Previously, we showed that T cells promote alveolar bone resorption when *Escherichia coli* lipopolysaccharide (LPS) is repeatedly injected into the mouse gingiva.⁸ However, the exact function of T cells in periodontal disease remains unclear.

Recently, we reported that alternate topical application of *E. coli* LPS as an antigen and its specific antibody to rat gingival sulcus induces loss of attachment and alveolar bone resorption.⁹ Additionally, we reported that topical application of *E. coli* LPS into the gingival sulci of LPS-immunized rats induces loss of attachment and alveolar bone resorption when the serum level of anti-LPS immunoglobulin (Ig) G was elevated after immunization of LPS, and represents an experimental model of periodontitis.¹⁰ This model enables elimination of the mechanical stimulation caused by ligature. Using this model, we investigated the influence of T cells on periodontal destruction in periodontitis.

Materials and methods

Animals

Male F344/NJcl (wild-type [WT]) and T cell-deficient F344/NJcl-*rnu/rnu* (Nu) rats were purchased from Charles River Laboratories Japan, Inc. (Tokyo, Japan) and maintained under specific pathogen-free conditions at the Biomedical Research Center, Center for Frontier Life Sciences (Nagasaki University, Nagasaki, Japan). Animal care and experimental procedures were carried out in accordance with the

Guidelines for Animal Experimentation of Nagasaki University and with approval from the Institutional Animal Care and Use Committee.

Experimental design

Male WT and Nu rats were divided into two groups: an immunized (I) group comprising the I-WT and I-Nu groups; a non-immunized (NI) group, comprising the NI-WT and NI-Nu groups. Each group comprised five rats. The immunized groups received intraperitoneal injections of 0.5 mg/kg *E. coli* LPS (O111, B4; Sigma–Aldrich Corp., St. Louis, MO, USA) suspended in phosphate-buffered saline (PBS) emulsified in complete Freund's adjuvant, followed by a booster injection of LPS emulsified in incomplete Freund's adjuvant 28 days later. The non-immunized groups received intraperitoneal injections of Freund's adjuvant with PBS, followed by an injection of incomplete Freund's adjuvant 28 days later. From Day 1 after booster injection, the animals were challenged daily with a topical application of *E. coli* LPS (50 μ g/ μ l) suspended in PBS to the palatal gingival sulcus.¹⁰ Briefly, the animals were anesthetized with isoflurane and then *E. coli* LPS (50 μ g/ μ l) suspended in PBS was applied using a micropipette to the palatal gingival sulcus of the right maxillary first molar (LPS side), and PBS was applied to the palatal gingival sulcus of the left maxillary first molar (PBS side). In total, 21 μ l (3 μ l seven times, with 5-min intervals between each application) of LPS or PBS was administered within a 35 min time period daily for 20 days. Blood samples were collected at the first intraperitoneal injection, booster injection, and immediately after the topical application of LPS or PBS on days 5, 10, and 20. The levels of anti-LPS IgG in individual serum samples were determined by indirect enzyme-linked immunosorbent assay. All animals were sacrificed under isoflurane anesthesia 1 h after the last application of LPS or PBS.

Tissue preparation

The maxilla of each rat was removed immediately after death, fixed in 4% paraformaldehyde in PBS at 4 °C for 10 h, and decalcified with 10% ethylenediaminetetraacetic acid for 3 weeks. The first molars on both sides of the maxilla were separated and embedded in paraffin using the AMeX method.¹¹ Briefly, the specimens were dehydrated in acetone, cleared in methyl benzoate for 30 min and xylene for 30 min, and penetrated with paraffin at 60 °C for 2 h. The penetrated specimens were embedded into paraffin blocks, and bucco-lingually oriented serial sections (4- μ m thick) at the level of the central roots of the upper first molar were obtained.

Histopathologic and immunohistologic staining

Five groups of serial sections, each containing 10 subsections, were obtained from each specimen. The first subsections from each group of serial sections were stained with hematoxylin and eosin for histopathologic observation.

To identify osteoclasts, the second subsections from each group were stained with tartrate-resistant acid phosphatase (TRAP).¹² Briefly, a staining solution was made by mixing 0.5 ml of pararosaniline solution (1 g of pararosaniline in 20 ml of distilled water and 5 ml of concentrated hydrochloric acid), 0.5 ml of 4% sodium nitrite solution, 10 ml of 0.1 M acetate buffer (pH 5.0), and 10 mg of naphthol AS-BI phosphate (Sigma–Aldrich Corp.) dissolved in 8 ml of distilled water. The mixture was adjusted to pH 5.0 using concentrated 1 M NaOH, and filtered through Whatman® Grade-1 Filter Paper (Whatman Fisher Scientific, Houston, TX, USA). After adding 150 mg of L-(+)-tartaric acid to a 10 ml aliquot of the mixture to give a final tartrate concentration of 0.1 M, the solution was adjusted to pH 5.0 with concentrated NaOH. After the second subsections were incubated within the staining solution for 30 min at 37 °C, they were counterstained with hematoxylin.

Interleukin (IL)-4, an anti-inflammatory cytokine produced by Th2 cells, was also stained immunohistologically. Briefly, after the third subsections from each group were deparaffinized, endogenous peroxidase activity was blocked with 0.3% H₂O₂ in methanol for 30 min, followed by incubation in normal goat serum for 30 min at room

temperature. These sections were then immersed in rabbit polyclonal anti-IL-4 antibody (ab9811; Abcam, Cambridge, UK) overnight. The sections were then incubated with biotinylated goat anti-rabbit polyclonal Ig (Dako Denmark A/S) for 30 min. Finally, the sections were incubated with peroxidase-conjugated Streptavidin (Dako Denmark A/S) for 30 min, incubated with diaminobenzidine tetraoxide solution, and counterstained with hematoxylin.

Histomorphometric analysis

We performed histomorphometric analysis of the tissue sections stained with hematoxylin and eosin using image analysis software (ImageJ; National Institutes of Health, Bethesda, MD, USA). The distance between the cemento–enamel junction (CEJ) and coronal position of the junctional epithelium (JE) attached to the root surface was measured to yield the loss of attachment. The distance between the CEJ and the alveolar bone crest was measured to evaluate alveolar bone resorption. The numbers of inflammatory cells in four unit areas (50 × 50 μm) of connective tissue adjacent to the JE were counted at a magnification of × 400 and averaged. The number of TRAP-positive multinucleate cells in an area 500-μm wide on the surface of the alveolar bone crest was counted. The numbers of IL-4-positive cells in eight unit areas (50 × 50 μm) of connective tissue adjacent to the JE and surface of the alveolar bone crest were counted at a magnification of × 400 and averaged (Fig. 1).

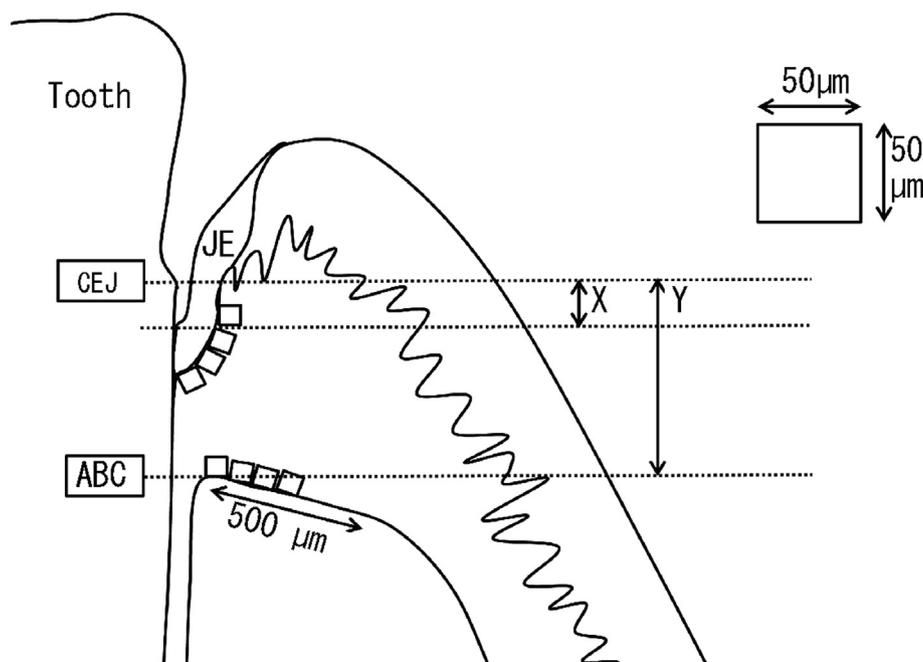


Figure 1 Schema of the histometric analysis of rat periodontal tissue. Loss of attachment (X) was calculated by measuring the distance between the cemento–enamel junction (CEJ) and coronal position of the junctional epithelium (JE). Alveolar bone level (Y) was calculated by measuring the distance between the CEJ and the alveolar bone crest (ABC). The numbers of inflammatory cells in four unit areas (50 × 50 μm) of connective tissue adjacent to the JE were calculated at a magnification of × 400 and averaged. The number of tartrate-resistant acid phosphatase-positive multinucleate cells in an area 500-μm wide on the surface of the alveolar bone was counted. The numbers of interleukin-4-positive cells in eight unit areas (50 × 50 μm) of connective tissue adjacent to the JE and alveolar bone crest were counted at a magnification of × 400 and averaged. CEJ: cemento–enamel junction.

Statistical analysis

Statistical analyses were performed using StatMate IV (ATMS Co., Ltd., Tokyo, Japan). Differences between the non-immunized and immunized groups were evaluated using the Mann–Whitney *U* test. The level of statistical significance was set at $P < 0.001$. We compared histometric results between the WT groups and between the Nu groups. Then, we compared results between the non-immunized and between the immunized groups. Because it was uninformative to compare results between the I-WT and nI-Nu groups and the I-Nu and nI-WT groups, we did not conduct these comparisons. Differences in each parameter between the I-WT and nI-WT, I-Nu and nI-Nu, I-WT and I-Nu, and nI-WT and nI-Nu groups were evaluated using the Mann–Whitney *U* test. The level of statistical significance was set at $P < 0.01$ and $P < 0.001$.

Results

The serum levels of anti-LPS IgG were significantly elevated in the immunized groups at Day 5 and persisted until Day 20. Serum levels of anti-LPS IgG were slightly elevated in the non-immunized groups on Day 20 (data not shown).

Histopathologic findings

On the PBS side in the immunized and non-immunized groups, there were no changes compared with normal rats. The apical portion of the JE was located at the CEJ in all four groups. Few inflammatory cells had infiltrated the JE and surrounding connective tissue and no osteoclasts were evident on the alveolar bone surface. In contrast, in the immunized groups, the LPS side showed a marked loss of attachment and infiltration of inflammatory cells into the JE and surrounding connective tissue, and many osteoclasts were present on the alveolar bone surface. Non-immunized groups exhibited a slight loss of attachment with infiltration of inflammatory cells into the JE and surrounding connective tissue, and some osteoclasts were visible on the alveolar bone surface (Fig. 2).

Histometric analysis

Loss of attachment was only observed on the LPS sides. The loss of attachment in the immunized groups was higher than that in the non-immunized groups for both WT and Nu rats. Of the non-immunized groups, the nI-WT group showed a significantly higher loss of attachment than the nI-Nu group. Conversely, in the immunized groups, the loss of

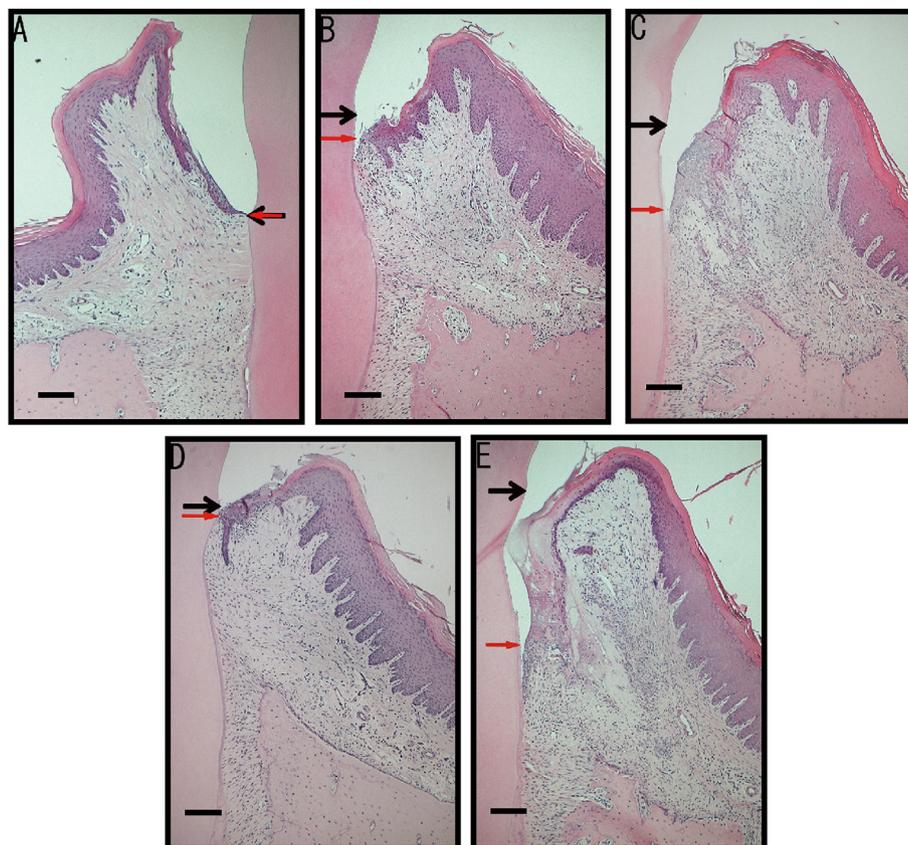


Figure 2 Histopathologic findings of the palatal side of the first molar stained with hematoxylin and eosin. (A) Phosphate-buffered saline side of the non-immunized groups. Greater changes were observed on the lipopolysaccharide side. (B) Non-immunized (nI)-wild-type (WT) group. (C) Immunized (I)-WT group. (D) nI-nude (Nu) group. (E) I-Nu group. Loss of attachment was obvious in the immunized group. Black arrow indicates the cemento–enamel junction. Red arrow indicates the coronal portion of the junctional epithelium attachment. Scale bar = 100 μ m.

attachment was significantly higher in the I-Nu group than in the I-WT group (Fig. 3A).

Alveolar bone resorption and the numbers of inflammatory cells showed similar tendency to loss of attachment. Of the non-immunized groups, they were significantly greater in the nl-WT group than in the nl-Nu one. In contrast, in the immunized groups, they were significantly greater in the I-Nu group than in the I-WT one (Figs. 3B and 4). In addition, the numbers of inflammatory cells on the LPS side were significantly greater than those of the PBS side in all groups (data not shown).

Osteoclasts were only detected on the alveolar bone surface on the LPS sides. In the non-immunized groups, the number of osteoclasts present in the nl-WT group was significantly greater than that present in the nl-Nu group. In contrast, of the immunized groups, the I-Nu group exhibited significantly more osteoclasts than the I-WT group (Fig. 5).

IL-4-positive cells were negligible on the PBS sides, but numerous on the LPS sides. The numbers of IL-4-positive cells evident in the immunized groups exceeded those evident in the non-immunized groups. The I-WT group showed significantly more IL-4-positive cells than the I-Nu group (Fig. 6).

Discussion

In this study, serum levels of anti-LPS IgG were significantly elevated in the immunized groups like our former studies.^{10,13–15} Loss of attachment, numbers of inflammatory

cells, and alveolar bone resorption were significantly greater in the I-WT group than in the nl-WT one. Likewise, loss of attachment, numbers of inflammatory cells, and alveolar bone resorption were elevated in the I-Nu group relative to the nl-Nu group. In addition, the number of osteoclasts evident in the I-Nu group was significantly greater than that in the nl-Nu group. These findings support those of our previous study: namely, that anti-LPS IgG in the serum and LPS applied to the gingival sulcus form immune complexes and induce periodontal destruction.^{9,10} Reportedly, Complement components 3a (C3a) and 5a (C5a) are expressed via the classical complement pathway, which is activated by immune complex formation, and induce neutrophil infiltration.¹⁶ Neutrophils are thought to contribute to periodontal destruction by expressing reactive oxygen and elastase.^{17–19} Furthermore, it has been reported that C3a and C5a induce osteoclastogenesis,²⁰ and that neutrophils produce TNF-alpha and receptor activator of nuclear factor κ -B ligand following inflammatory stimulation to induce bone resorption.²¹ Therefore, in this study, immune complex formation in the immunized groups was considered to induce inflammatory cell infiltration and contribute to periodontal destruction.^{9,10}

Among the non-immunized groups, loss of attachment, numbers of inflammatory cells, alveolar bone resorption, and the number of osteoclasts were significantly greater in the nl-WT group than in the nl-Nu one. It has been suggested that T cells promote bone resorption following LPS injection into the mouse calvaria.²² We also reported that inflammatory cell infiltration and alveolar bone resorption in response to injection of LPS into the gingiva

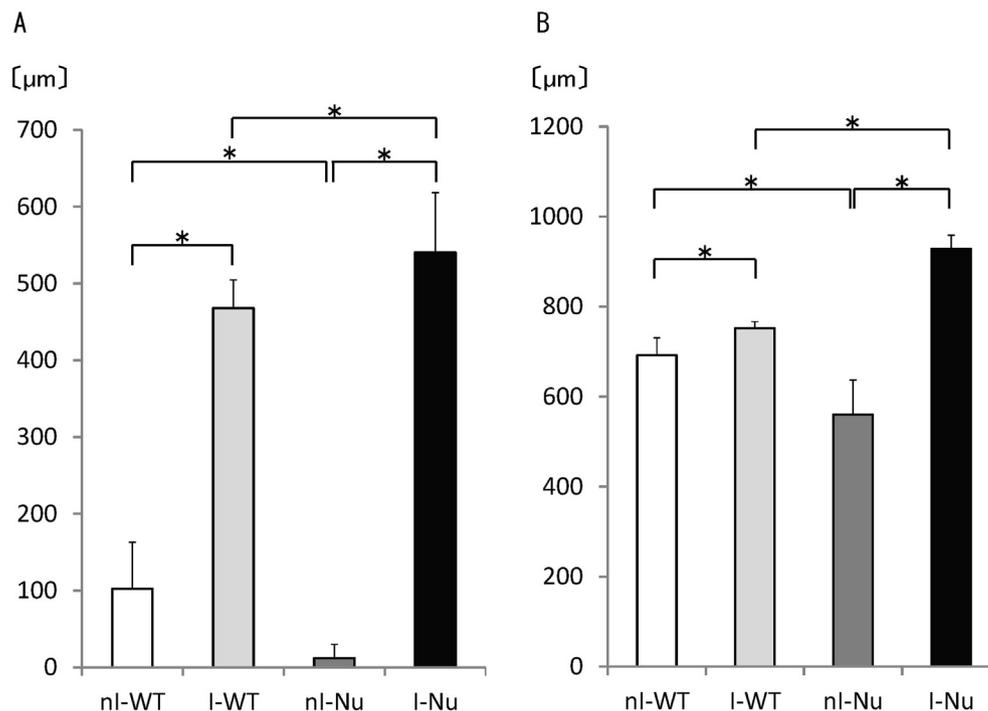


Figure 3 Histometric analysis of loss of attachment and alveolar bone resorption on the lipopolysaccharide side. (A) The distance from the cemento–enamel junction (CEJ) to the coronal portion of the junctional epithelium attachment (loss of attachment) was analyzed histometrically. Loss of attachment was greater in the immunized groups. (B) The distance from the CEJ to the alveolar bone crest was analyzed histometrically. Alveolar bone resorption was significantly greater in the immunized groups. Each bar represents the mean \pm standard deviation. * $P < 0.001$, Mann–Whitney U test on the lipopolysaccharide side.

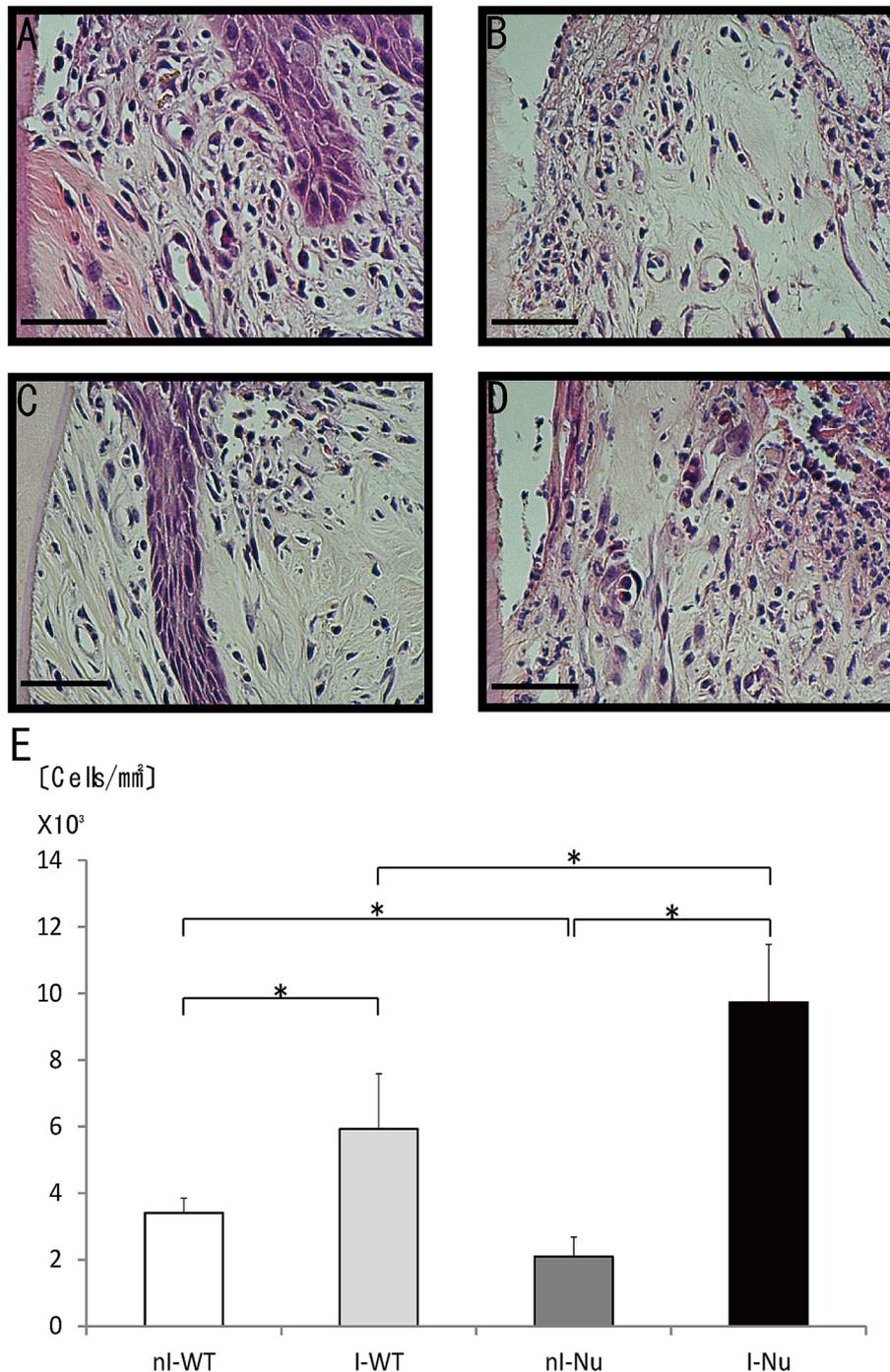


Figure 4 Histopathologic findings of the connective tissue adjacent to the JE stained with hematoxylin and eosin and the number of inflammatory cells on the lipopolysaccharide side in each group. (A) Non-immunized (nl)-wild-type (WT) group. (B) Immunized (I)-WT group. (C) nl-nude (Nu) group. (D) I-Nu group. (E) The numbers of inflammatory cells in the connective tissue adjacent to the junctional epithelium on the lipopolysaccharide side, presented as the cell number/unit squared (mm^2). The numbers of inflammatory cells were significantly greater in the immunized groups. Each bar represents the mean \pm standard deviation. * $P < 0.001$, Mann–Whitney U test. Scale bar = 50 μm .

are inhibited in nude mice, furthermore, when T cells from normal mice are transferred into nude mice, inflammation and bone resorption increases.²³ The same result was obtained following the transfer of T cells from normal mice into T cell- and B cell-deficient severe combined immune deficiency mice.²⁴ Dendritic cells and

macrophages stimulated by LPS are believed to increase production of TNF- α , IL-6, and IL-12, which induces differentiation of naive cluster of differentiation (CD) 4 positive T cells into Th1 cells.^{25–27} Th1 cells induce macrophages to produce pro-inflammatory cytokines such as IL-1 and TNF- α ,^{28,29} moreover, Th1 cells

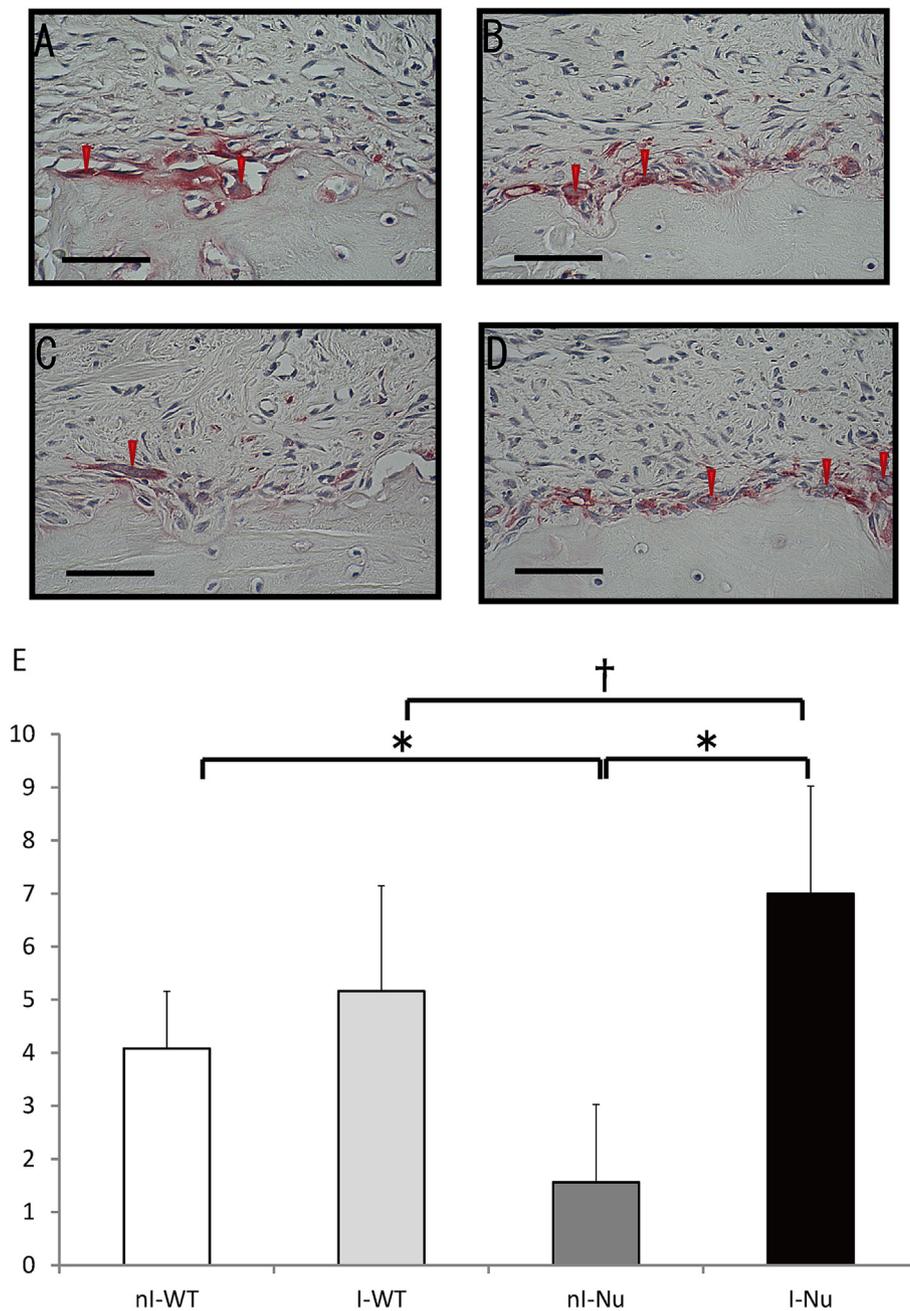


Figure 5 Histopathologic findings of tartrate-resistant acid phosphatase staining and the number of tartrate-resistant acid phosphatase-positive cells (red arrowhead) on the lipopolysaccharide side in each group. (A) Non-immunized (nl)-wild-type (WT) group. (B) Immunized (I)-WT group. (C) nl-nude (Nu) group. (D) I-Nu group. (E) The number of osteoclasts on the alveolar bone surface on the lipopolysaccharide side. The number of tartrate-resistant acid phosphatase-positive cells was significantly greater in the I-Nu group. Each bar represents the mean \pm standard deviation. * $P < 0.001$, † $P < 0.01$, Mann–Whitney U test. Scale bar = 100 μm .

induce osteoclastogenesis.³⁰ Therefore, we supposed that T cells and macrophages cooperated to induce inflammatory cell infiltration and alveolar bone resorption in the nl-WT group. However, the lack of T cells in rats in the nl-Nu group prevented the acceleration of inflammation and bone resorption.

Conversely, among the immunized groups, loss of attachment, numbers of inflammatory cells, alveolar bone resorption, and the number of osteoclasts were significantly greater in the I-Nu group than in the I-WT one.

Immunized status is characterized by the presence of antigen-specific antibodies. Previous studies have shown that immune complex induces naïve T cells to become Th2 cells.^{31,32} Expressed by Th2 cells, IL-4 reportedly reduces production of TNF-alpha and IL-12 by macrophages.³³ In this study, there was no significant difference in the number of IL-4-positive cells between the non-immunized groups; however, among the immunized groups, the number was significantly greater in the I-WT group than in the I-Nu group. Therefore, we suggest that, in the I-WT

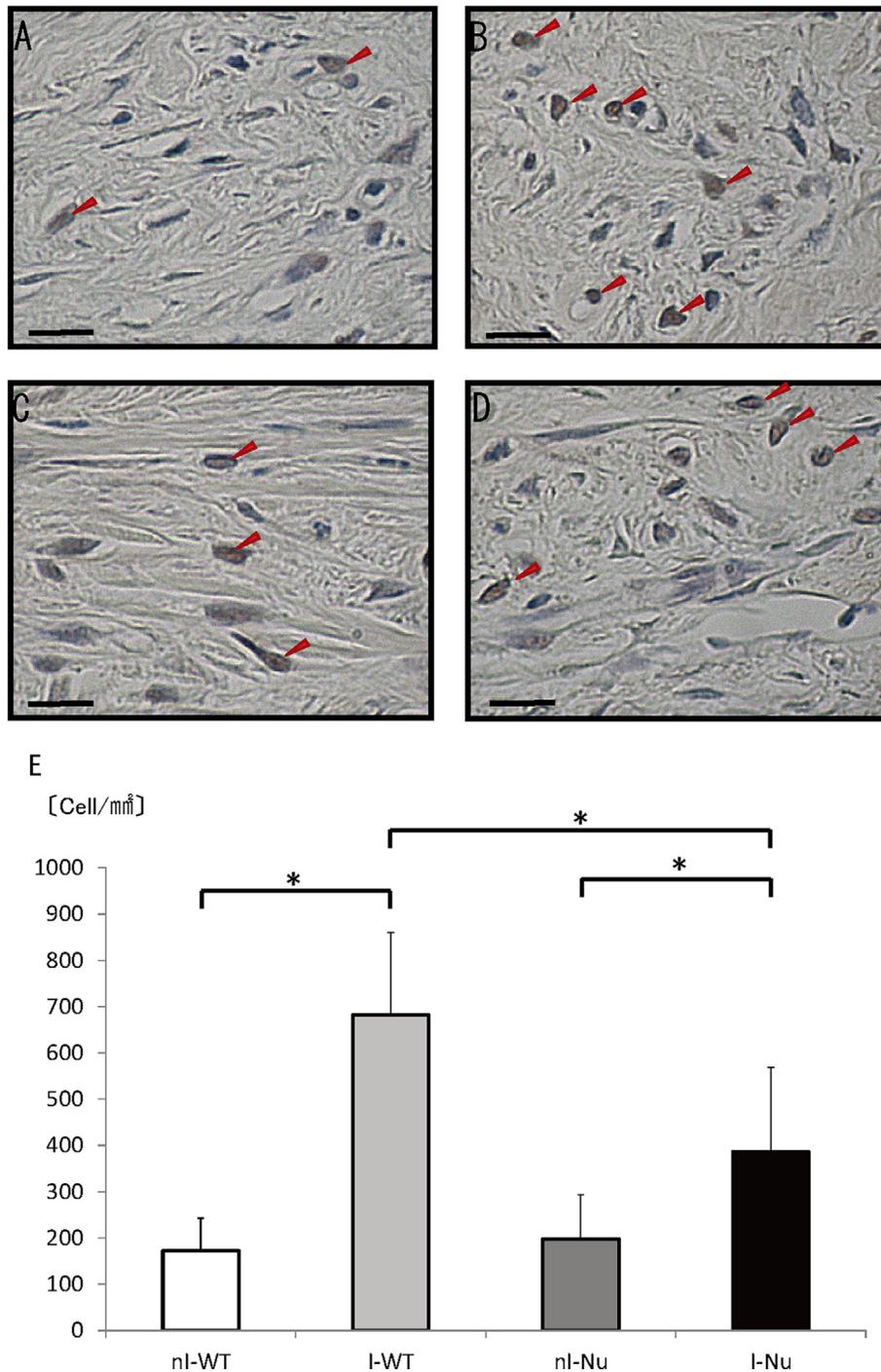


Figure 6 Immunohistologic localization of interleukin-4. Interleukin (IL-4)-positive cells, stained dark red (red arrowhead), were localized in the connective tissue adjacent to the junctional epithelium and surface of the alveolar bone crest. (A) Non-immunized (nl)-wild-type (WT) group. (B) Immunized (I)-WT group. (C) nl-nude (Nu) group. (D) I-Nu group. (E) The number of IL-4-positive cells on the lipopolysaccharide side. The number of IL-4-positive cells was significantly greater in the I-WT group. Each bar represents the mean \pm standard deviation. * $P < 0.001$, Mann-Whitney U test. Scale bar = 25 μ m.

group, T cells differentiated into both Th1 and Th2 cells. Subsequently, inflammation and bone resorption were cooperatively regulated by Th1 and Th2 cells in the I-WT group. However, in the I-Nu group, the lack of T cells equated to a lack of regulation by T cells; hence, infiltration of inflammatory cells and alveolar bone resorption

accelerated after immune complex formation and subsequent complement pathway activation. The influence of immune complex formation is obvious when the results of the nl-Nu and I-Nu groups are compared: the I-Nu group showed greater periodontal destruction than the nl-Nu group.

Treg cells, a subset of T cells, have been suggested to reduce the immune response and osteoclastogenesis through the production of anti-inflammatory cytokines such as IL-10 and TGF- β .^{34,35} It is possible that Treg cells affected the findings of this study. We attempted to stain IL-10 and Foxp3, which is a marker of Tregs, immunohistologically, however, it did not succeed. Regarding the involvement of Treg cells in periodontitis, a further study is required.

In conclusion, we have demonstrated that immune complex formation contributes to aspects of periodontal destruction such as the loss of attachment and alveolar bone resorption. Our results show that, in non-immunized status, T cells promote periodontal destruction, whereas in immunized status, T cells regulate periodontal destruction.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

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