

T cells are able to promote LPS-induced bone resorption under the condition of absence of B cells in mice.

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Running title: T cells in LPS-induced bone resorption

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Abstract

Background and Objective: T cells and their cytokines have been believed to be key factors in periodontal disease and bone resorption. We previously showed that T cells transferred to nude mice were related to inflammatory bone resorption *in vivo*. However, it has not been clarified whether T cells can induce bone resorption under the condition of absence of B cells. In this study, we therefore investigated the ability of T cells to induce bone resorption without B cells using both T cell and B cell-deficient SCID mice.

Materials and Methods: *Escherichia coli* lipopolysaccharide (LPS) was injected into the gingivae of SCID mice reconstituted by T cells (SCID+T mice). C.B-17 mice (wild-type mice) and SCID mice were used as controls. Alveolar bone resorption and production of cytokines in the gingivae were then compared histopathologically and immunohistologically.

Results: The degree of bone resorption in SCID+T mice was significantly greater than that in SCID mice but less than wild-type mice. The same tendency was found for expression of receptor activator of nuclear factor kappa B ligand (RANKL). The number of interferon-gamma-positive cells in SCID+T mice was highest among the three groups. On the other hand, interleukin-4-positive cells were detected in wild-type

mice but not in SCID+T and SCID mice.

Conclusion: The results suggest that T cells are able to promote LPS-induced bone resorption under the condition of absence of B cells. The expressions of cytokines in the presence of B cells are quite different.

Introduction

Periodontitis is an infectious disease caused by periodontopathic bacteria. Lipopolysaccharide (LPS), one of their components, and immune cells, especially T cells, have been believed to be key factors in periodontitis and bone resorption [1-11]. T cell subpopulations, Th1 cells producing IFN-gamma and Th2 cells producing IL-4, also regulate immune reactions. We previously reported that IFN-gamma-positive cells were more predominant than IL-4-positive cells in severe periodontitis lesions [12]. Furthermore, Teng et al. reported that CD4 T cells producing IFN-gamma accelerate alveolar bone resorption [13]. These reports suggest that T cells and their cytokines have important roles in periodontitis and bone resorption. However, the role of T cells themselves in bone resorption has still not been clarified, because T cells are activated by other cells, such as macrophages, B cells and fibroblasts, in inflamed lesions. B cells regulate immune responses with T cells and B cells also accelerate bone resorption by themselves.

T cells and B cells have been reported to produce RANKL [6-11, 14]. RANKL is a member of the tumor necrosis factor (TNF) superfamily. It activates osteoclast differentiation, stimulates osteoclast activation and increases osteoclast survival by binding with RANK of osteoclast precursors and mature osteoclasts [15-20]. We

previously showed that B cells promoted bone resorption when B cells were transferred to SCID mice lacking both T cells and B cells [21]. We also showed that T cells promoted LPS-induced bone resorption when T cells were transferred to nude mice congenitally lacking T cells [22]. However, it is still unclear whether T cells by themselves induce bone resorption without B cells, since it is likely that T cells transferred to nude mice cooperated with B cells congenitally existing. It is therefore important to clarify the characteristics of T cells and the role of T cells in bone resorption under the condition of absence of B cells *in vivo*. Clarification of these aspects of T cells will provide information about the influence of T cells on periodontitis.

We have already made a model of LPS-induced bone resorption using *Escherichia coli* (*E. coli*) LPS [21-24]. In the present study, we transferred T cells to SCID mice and observed bone resorption in the mice. Then we investigated the role of T cells in LPS-induced bone resorption under the condition of absence of B cells.

Materials and Methods

Mice

For the experiment, 15 male C.B-17 mice (C.B-17/Icr-+/+ Jcl) and 10 male SCID mice (C.B-17/Icr-SCID Jcl), 6-8 weeks of age, were purchased from Nihon Clear (Tokyo, Japan). They were maintained under specific pathogen-free conditions in the Biomedical Research Center, Center for Frontier Life Sciences, Nagasaki University. Animal care and experimental procedures were in accordance with the Guidelines for Animal Experimentation of Nagasaki University and approved by the Institutional Animal Care and Use Committee.

Isolation of spleen T cells and adoptive transfer

Whole spleen cell suspensions were prepared from CB-17 mice by mincing spleen tissue with scissors and teasing the cells between two frosted microscope slides. T cells were purified by standard negative selection using the StemSepTM (Stem Cell Technologies, Vancouver, Canada) magnetic separation technique in accordance with the manufacturer's instructions. Purity (90-96%) was confirmed by flow-cytometric analysis using antibodies against CD3. T cell-enriched spleen cells were injected intravenously into recipient SCID mice at a concentration of 1×10^7 cells/ml.

Flow cytometric analysis

Flow cytometric analysis was carried out to confirm the purity of separated cells. Purity was confirmed by using fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 monoclonal antibody to examine the presence of T cells. Specific staining was compared to fluorescence using irrelevant isotype controls. All antibodies were purchased from PharMingen (San Diego, CA). Cells were stained according to standard protocols and analyzed by flow cytometry using a FACScanTM instrument with CellQuestTM software (Becton Dickinson, Mountain View, CA).

LPS injection

SCID mice received T cells (SCID+T mice) one week before LPS injection. C.B-17 mice (wild-type mice) and SCID mice were used as controls. Each group consisted of 5 mice, and alveolar bone resorption was induced as in previously described models [21-24]. Every mouse in each group received 13 injections of 5 µg of *E. coli* LPS (*E. coli* O111: B4; Sigma, St. Louis, MO) in 3 µl of phosphate-buffered saline (PBS) under ether anesthesia; each injection was delivered into the mesial gingiva of the first molar of the left mandible with an interval of 48 hours between

injections. In our previous study using the same model [24, 25], bone resorption was shown to reach a peak after the 10th injection of LPS and to plateau thereafter. Mice were killed 24 hours after the 13th injection, and the spleens and left mandibles were removed. Furthermore, 13 times injection of PBS was performed to wild type (PBS group).

Specimen preparation

The removed spleens and left mandibles were fixed in 4% paraformaldehyde in PBS at 4°C for 6 hours. The left mandible was decalcified with 10% ethylenediaminetetraacetic acid (EDTA) for one week. Tissue samples were embedded in paraffin using the AMeX method (acetone, methyl benzoate, and xylene) [26]. Serial sections (4 µm in thickness) of the mesiodistal region of the left first molar were prepared. Three groups of serial sections, each containing 10 subsections, were obtained from each specimen and were subjected to hematoxylin and eosin (HE) staining, tartrate-resistant acid phosphatase (TRAP) staining and immunohistological staining.

The 1st subsections from each group of serial sections were stained with HE for observing the surface of alveolar bone histopathologically.

The 2nd subsections from each group were stained with TRAP using the

procedure of Katayama et al. [27] to examine the osteoclasts. Briefly, a staining solution was made by mixing 0.5 ml of pararosanilin solution, 0.5 ml of 4% sodium nitrite solution, 10 ml of 0.1 M acetate buffer (pH 5.0), 10 mg of naphthol AS-BI phosphate (Sigma), and 8 ml of distilled water. The mixture was adjusted to pH 5.0 using concentrated NaOH and then filtered. Then 150 mg of L (+)-tartaric acid was added to a 10 ml aliquot of the staining solution. After incubating the sections in the staining solution for 30 minutes at 37°C, they were counterstained with hematoxylin. Osteoclasts were identified as TRAP-positive multinucleated cells on the bone surface.

The 3rd to 6th subsections from each group were used for immunohistological staining of CD3, RANKL, IFN-gamma and interleukin-4 (IL-4). The spleen sections were used for immunohistological staining of CD3 and confirmed to be successfully transferred. Serial subsections were deparaffinized, and endogenous peroxidase activity was blocked with 0.3% H₂O₂/methanol for 30 minutes. These subsections were incubated with normal serum for 30 minutes at room temperature. Normal rabbit serum was used for immunostaining of CD3, RANKL and IL-4 and normal goat serum was used for immunostaining of IFN-gamma. The sections were then immersed in primary antibody solution at 4°C overnight. The primary antibodies used goat anti-mouse CD3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-mouse

RANKL antibody (N-19) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-mouse IFN-gamma polyclonal antibody (PBL Biomedical Laboratories Inc., USA), and anti-mouse IL-4 monoclonal antibody (R&D Systems). Then the sections were incubated with a secondary antibody for 30 minutes at room temperature. As secondary antibody, biotinylated rabbit anti-goat polyclonal antibody (Dako, Glostrup, Denmark) was used for staining of CD3 and RANKL. Biotinylated goat anti-rabbit polyclonal antibody (Dako, Glostrup, Denmark) was used for staining of IFN-gamma, and biotinylated rabbit anti-rat polyclonal antibody (VECTOR Laboratories Inc., Burlingame, CA) was used for staining of IL-4. Finally, the sections were incubated with peroxidase-conjugated streptavidin (Dako) for 30 minutes, followed by incubation with diaminobenzidine tetraoxide solution, and then counterstained with hematoxylin.

Bone histomorphometry

Due to the difficulty of quantitatively evaluating total bone resorption, the percentage of bone surface (regardless of the presence or absence of resorption lacunae) in intimate contact with osteoclasts (active resorption surface, ARS) was evaluated to quantitatively evaluate the progression of bone resorption [21-25]. After counting the number of points of intersection of the bone surface with the line of a micrometer

(Olympus, Tokyo, Japan) in 25 μm graduations at x400 magnification, the ratio of ARS to total points of intersection was calculated (Fig. 1).

Immunohistological observation

Numbers of CD3-, RANKL-, IFN-gamma- and IL-4-positive cells in four areas of 250 μm x 500 μm on the surface of alveolar bone was counted.

Statistical analysis

The significance of differences was analyzed using the Mann-Whitney U-test.

Results

Histopathological findings and ARS

There was a few inflammatory infiltration and no bone resorption in the PBS group (Fig. 2A and B). In wild-type mice, the alveolar bone surface was irregular and many TRAP-positive multinucleated cells were observed. Many inflammatory cells, including plasma cells, had infiltrated the connective tissue (Fig. 2C and D). On the other hand, irregularity of the alveolar bone surface and infiltration of inflammatory cells were rare and there were no plasma cells in SCID mice (Fig. 2E and F). SCID+T mice showed an irregular alveolar bone surface and TRAP-positive multinucleated cells as observed in wild-type mice (Fig. 2G and H). Plasma cells were not observed in SCID and SCID+T mice.

Wild-type mice showed the highest ARS (19.0 ± 2.62). ARS in SCID+T mice (10.5 ± 1.77) was significantly higher than that in SCID mice (1.3 ± 1.20) (Fig. 3).

Immunohistological findings and histomorphometry

Diffuse infiltration of CD3-positive cells was observed in wild type (16.12 ± 4.29) and SCID+T mice (8.16 ± 1.84) at injection of *E.coli* LPS. The number of CD3-positive cells in wild-type mice was significantly larger than that in SCID+T mice (Fig. 4). CD3

positive cells were a few observed in PBS injected wild type mice (4.42 ± 1.13).

RANKL-positive cells were observed in all groups. RANKL-positive reactions were mainly observed in fibroblastic and lymphoid cells in connective tissue in all three groups. Although we could not separate fibroblast with osteoblast, almost nothing RANKL positive osteoblastic cells were observed in all groups. The number of RANKL-positive cells in wild-type mice (38.08 ± 4.09) was significantly larger than the numbers in the other three groups. The number in SCID+T mice (24.43 ± 6.81) was significantly larger than that in SCID mice (11.24 ± 3.06). There are very few RANKL positive cells in PBS injection mice (1.19 ± 1.09) (Fig. 5).

IFN-gamma-positive reaction was observed in macrophage-like cells in connective tissue in all groups. Lymphoid cells that had diffusely infiltrated in wild-type (11.78 ± 2.70) and SCID+T mice (22.75 ± 5.75) also showed IFN-gamma-positive reaction. The number of IFN-gamma-positive cells was largest in SCID+T mice. The number in wild-type mice was larger than that in SCID mice (6.56 ± 0.61) and PBS injection mice (5.61 ± 0.80) (Fig. 6).

IL-4-positive reaction was observed mainly in lymphoid cells in wild-type mice (40.17 ± 22.25). Very a few IL-4 positive cells were observed in both SCID+T and SCID mice. No IL-4 positive cells were detected in PBS injection mice (Fig. 7).

Discussion

In this study, bone resorption was induced in wild-type and SCID+T mice. ARS was highest in wild-type mice and that in SCID+T mice was significantly higher than that in SCID mice. These results are related to infiltration of CD3-positive cells in the area of bone resorption. We previously reported that T cells induced bone resorption when they had been transferred to nude mice [22]. However, there is no report of T cells inducing bone resorption under the condition of absence of B cells. The results of the present study strongly suggest that T cells are involved in bone resorption without B cells.

T cells produce RANKL and promote bone resorption. Activated T cells are involved in bone resorption through RANKL [7], and CD4-positive T cells induce osteoclastogenesis by expressing RANKL [15]. Weitzmann et al. [8] and Kotake et al. [9] reported that human T cells cause osteoclastogenesis via RANKL. Moreover, Brunetti et al. [11] and Kawai et al. [14] reported that T cells caused osteoclastogenesis by expressing RANKL in periodontitis patients. In the present study, the number of RANKL-positive cells was largest in wild-type mice and that in SCID+T mice was significantly larger than that in SCID mice. These findings are in agreement with the results for ARS. These results suggest that T cells promote bone resorption by

producing RANKL and/or inducing RANKL production by other cells.

There was a large difference in IFN-gamma production and IL-4 production in the present studies, although ARS and the number of RANKL-positive cells were correlated in wild-type mice and SCID+T mice. SCID+T mice showed more IFN-gamma-positive cells and less IL-4-positive cells than those in wild-type mice. These findings suggest that T helper 1 (Th1) cells are dominant under the condition of absence of B cells *in vivo*. IFN-gamma directly interrupts osteoclastogenesis induced by RANKL *in vitro* [28]. Resting T cells control osteoclastogenesis via IFN-gamma [29], and IFN-gamma is the main cause of the inhibitory effect of activated T cells on osteoclastogenesis [30]. On the other hand, Takayanagi et al. [5] reported that T cells regulate osteoclastogenesis by signaling cross-talk between RANKL and IFN-gamma. Moreover, Gao et al. [31] reported that the balance of direct anti-osteoclastogenetic and indirect pro-osteoclastogenetic properties of IFN-gamma influences bone resorption. In the present study, many IFN-gamma-positive cells and severe bone resorption were observed more in SCID+T mice than in SCID mice. These results suggest that infiltrating IFN-gamma-positive cells are involved in LPS-induced bone resorption *in vivo*. The reason why the number of IFN-gamma positive cell was highest is uncertain. However, it is likely that T cells differentiated into the IFN-gamma positive cell by the

lack of B cells. It is further investigation is necessary.

In the present study, many IL-4-positive cells were observed in wild-type mice, although there were few IL-4-positive cells in SCID+T and SCID mice. It is well known that T cells and B cells mutually regulate their differentiation and proliferation. B cells act as antigen-presenting cells for T cells, and then T cells differentiate into IL-4-producing T cells. These facts explain the reason why few IL-4-positive cells were observed in SCID+T and SCID mice in the present study. There have been many reports on the inhibitory effects of IL-4 on osteoclastogenesis. IL-4 was shown to inhibit osteoclastogenesis by interrupting the path of STAT6 dependence [32, 33], and IL-4 treatment decreased the turnover of bone in ovariectomized mice [34]. In the present study, ARS was highest in wild-type mice, although there were many IL-4-positive cells in wild-type mice. This suggests that IL-4-producing T helper 2 (Th2) cells do not suppress osteoclastogenesis *in vivo*. Further investigation about the relation between Th1 and Th2 cells with bone resorption is necessary.

In this study, we use mouse bone resorption model injected with *E. coli* LPS. Although all cases of bone resorption in clinical periodontitis are not showed in this model, this is thought to be adequate model for examination of bone resorption mechanism because bacterial LPS is one of most important factor for induction of bone

loss in periodontitis. In conclusion, T cells solely promote LPS-induced bone resorption and T cells produce IFN-gamma but not IL-4 under the condition of absence of B cells. Furthermore, ARS in the wild-type mice was higher than that in the SCID+T mice, indicating that T cells and B cells cooperate to exacerbate bone resorption. The effect of interaction between T cells and B cells should be studied in order to clarify the mechanisms of inflammatory bone loss.

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interleukin-4 rapidly inhibits bone formation in normal and ovariectomized mice. *Bone*
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Figure Legends

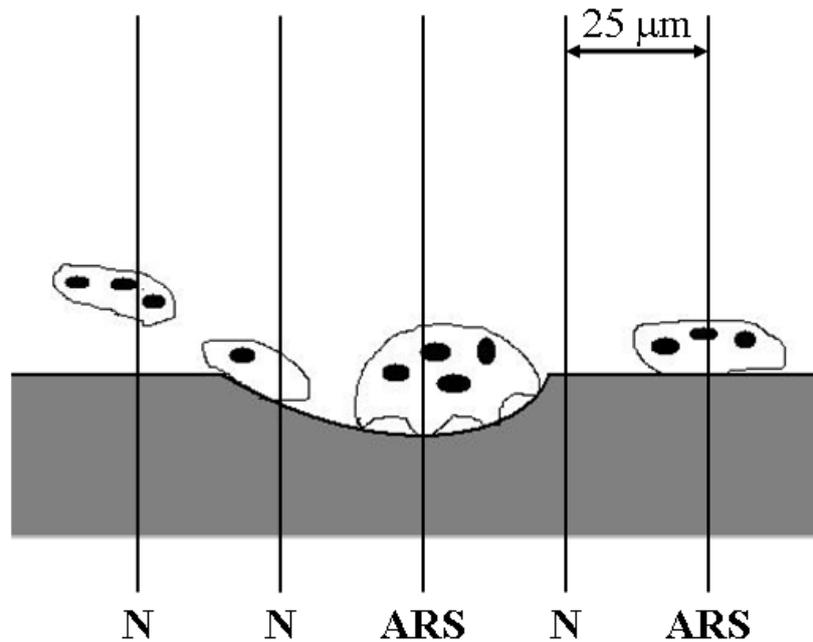


Fig. 1 Definition of active resorption. Bone surfaces in contact with TRAP-positive multinucleated cells were defined as sites of active resorption surface (ARS); other sites were classified as normal (N). The percentage of ARS was derived by dividing the number of sites of ARS by the number of all intersecting points between bone surfaces on a 25 μm scale.

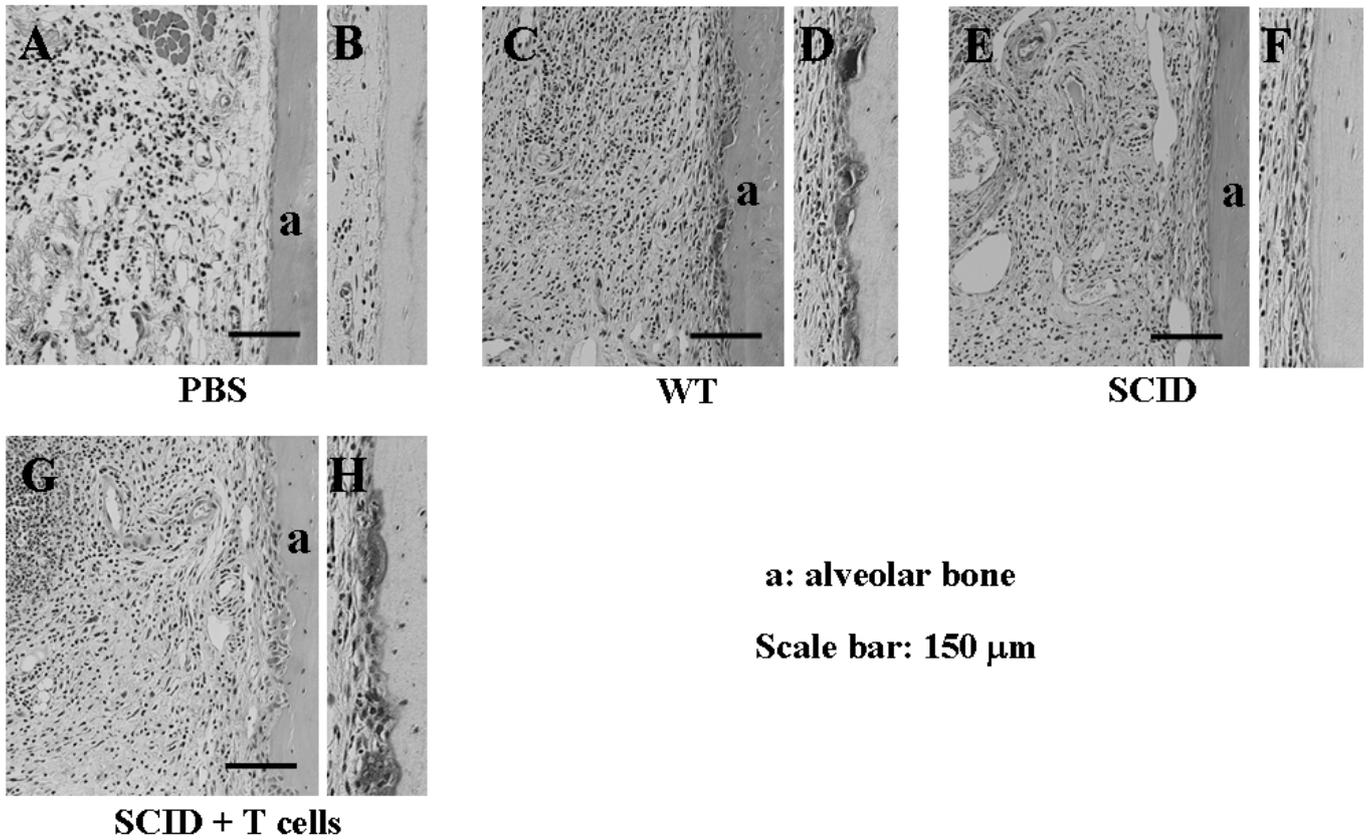


Fig. 2 Histopathological findings of alveolar bone. A few inflammatory cells and bone resorption were observed in PBS group (A, B). In wild-type mice, infiltration of many inflammatory cells and formation of osteoclasts were observed (C, D). In SCID mice, there were few inflammatory cells and almost no osteoclasts (E, F). In SCID+T mice, many inflammatory cells and osteoclasts were observed as in wild-type mice (G, H). A, C, E, and G: HE staining. B, D, F, and H: TRAP staining.

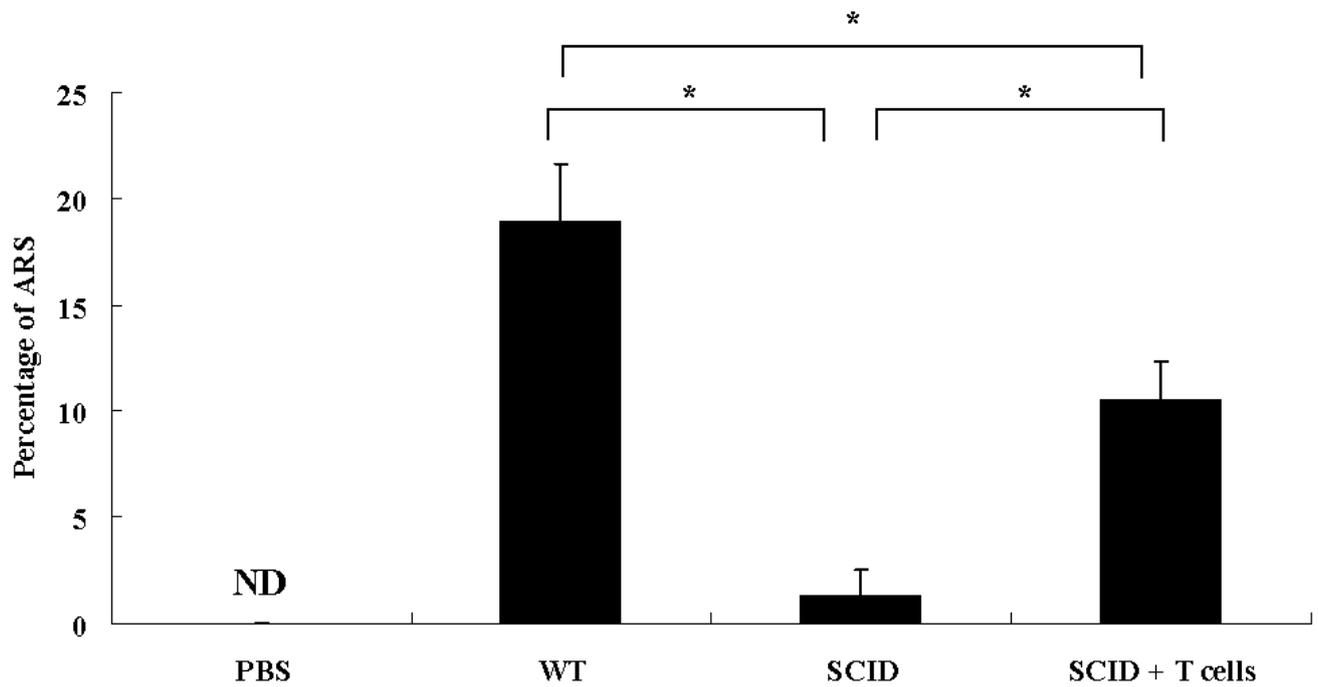


Fig. 3 Percentage of active resorption surfaces (ARS) in each group. ARS was highest in wild-type mice and higher in SCID+T mice than in SCID mice. Significant differences were found between these three groups. Bars represent means \pm SD; ND; not detected, $*P < 0.05$.

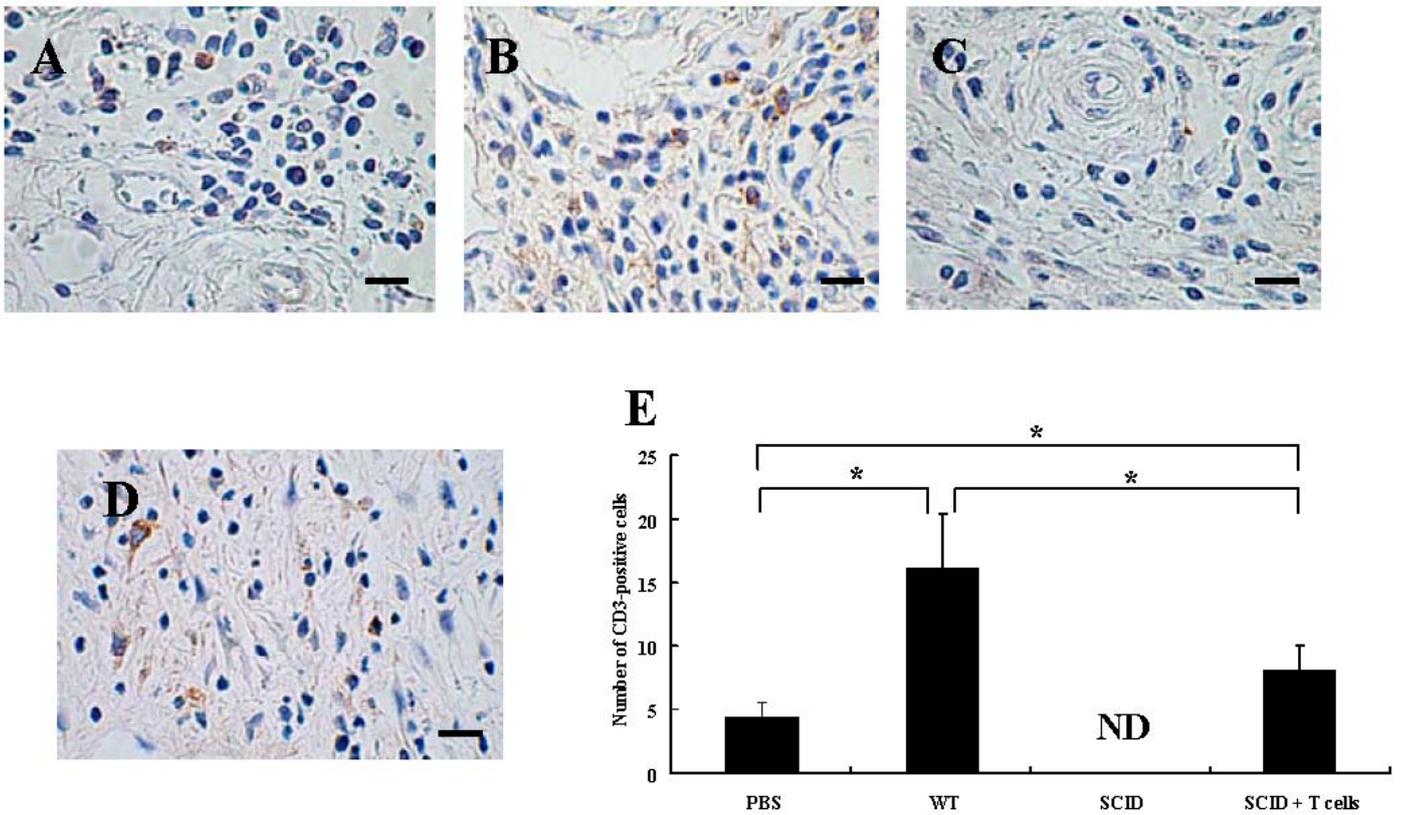


Fig. 4 Immunohistological findings and numbers of CD3. A, B, C, and D are immunostaining view. A; PBS injection mice, B; wild-type mice, C; SCID mice, D; SCID+T mice. E; The number of CD3-positive cells. The number of CD3-positive cells was largerest in wild-type mice. A, B, C, and D; Scale bar means 60 μ m. E; Bars represent means \pm SD; * P <0.05.

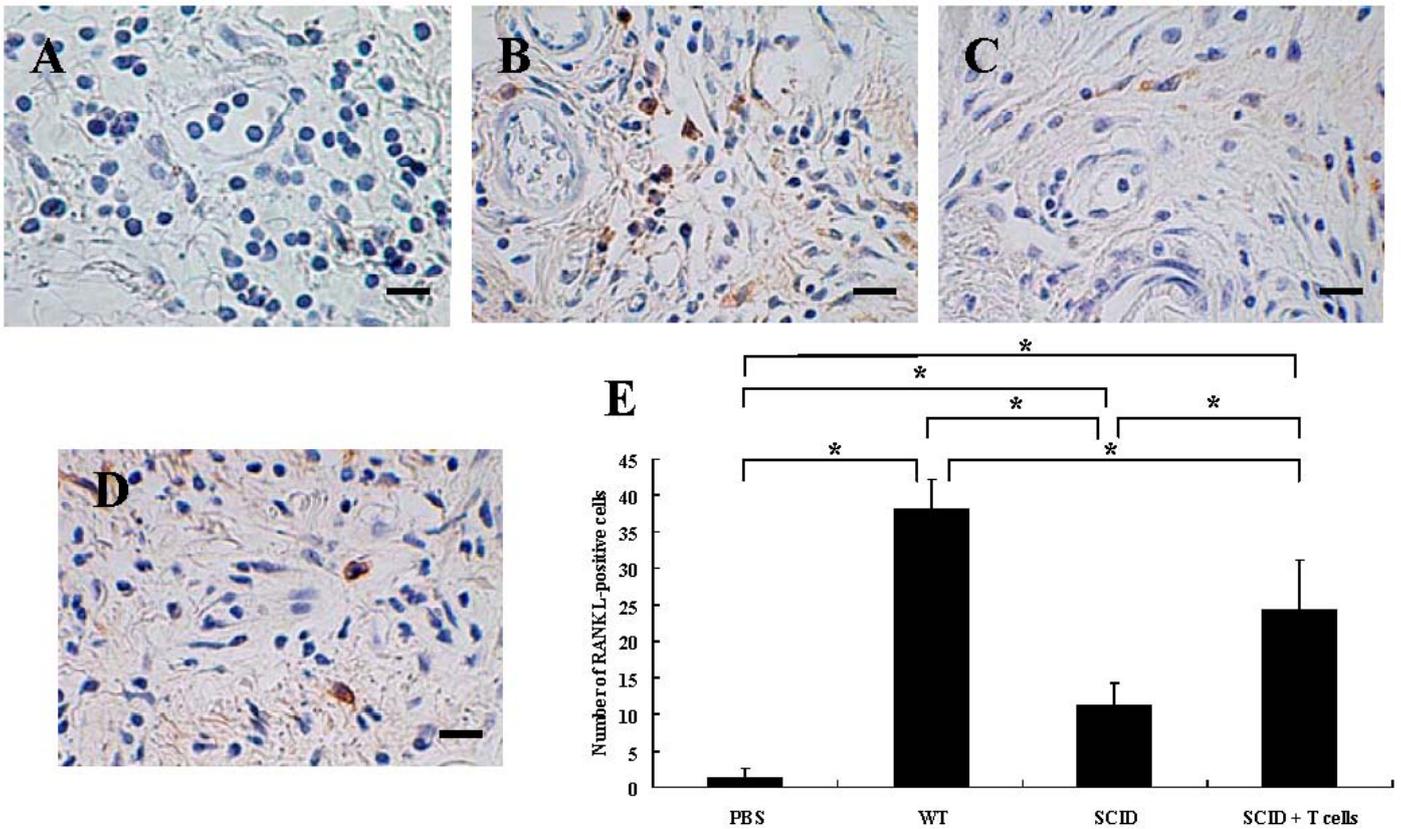


Fig. 5 Immunostainings and numbers of RANKL-positive cells. A, B, C, and D are immunostaining view. A; PBS injection mice, B; wild-type mice, C; SCID mice, D; SCID+T mice. E is the number of RANKL-positive cells. The number of RANKL-positive cells was largest in wild-type mice. Significant differences were found between four groups. A, B, C, and D; Scale bar means 60 μ m. E; Bars represent means \pm SD; * P <0.05.

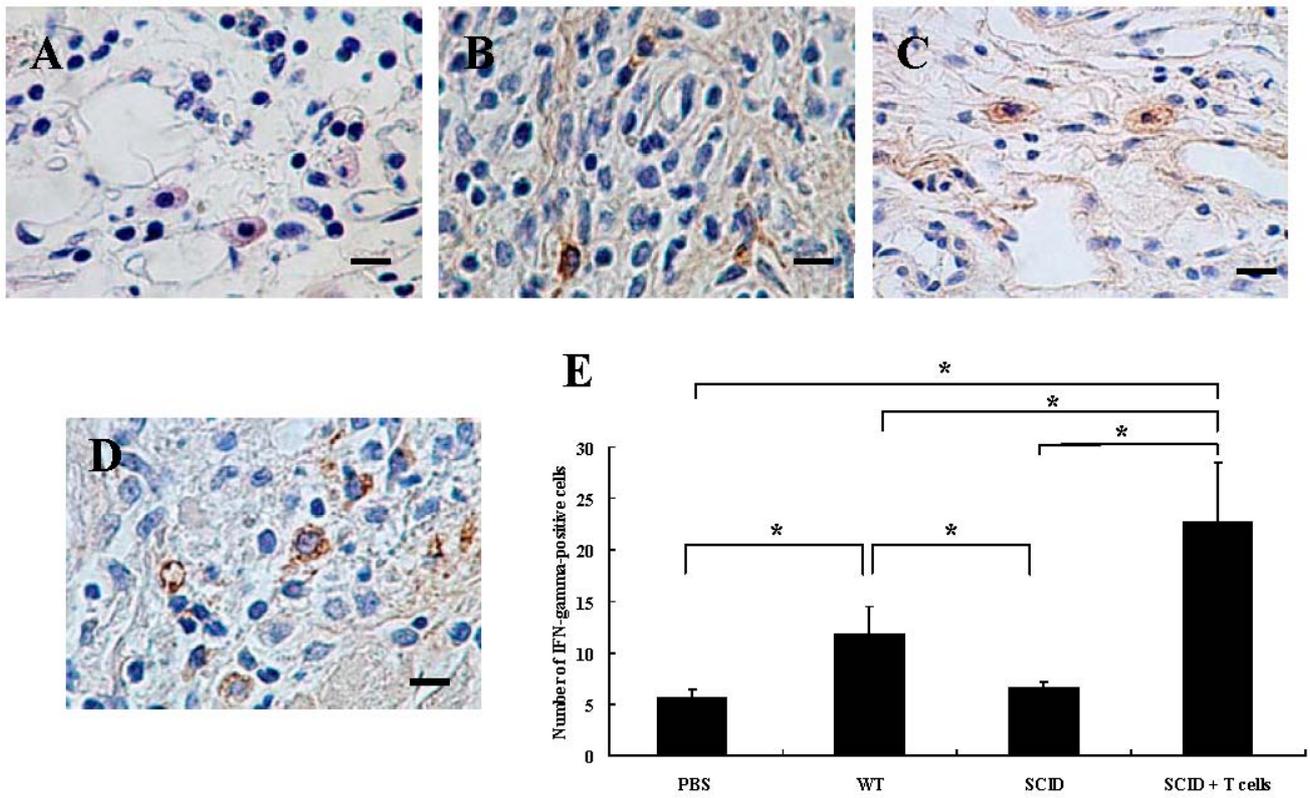


Fig. 6 Immunostainings and numbers of IFN-gamma-positive cells. A, B, C, and D are immunostaining view. A; PBS injection mice, B; wild-type mice, C; SCID mice, D; SCID+T mice. E; The number of IFN-gamma-positive cells. The number of IFN-gamma-positive cells was largest in SCID+T mice. A significant difference was found between four groups. A, B, C, and D; Scale bar means 60 μ m. E; Bars represent means \pm SD; * P <0.05.

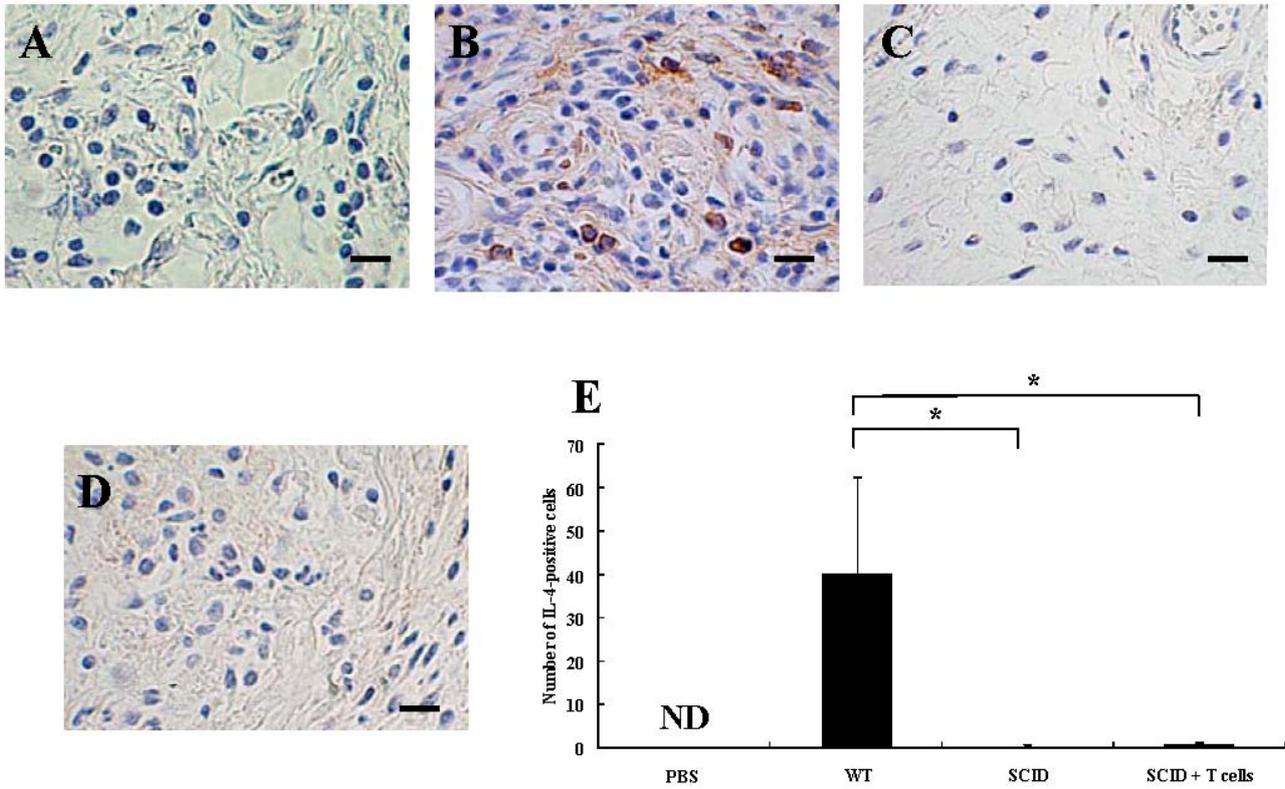


Fig. 7 Immunostainings and numbers of IL-4-positive cells. A, B, C, and D are immunostaining view. A; PBS injection mice, B; wild-type mice, C; SCID mice, D; SCID+T mice. E; The number of IL-4-positive cells. The number of IL-4-positive cells was largest in wild-type mice. In SCID+T and SCID mice, few IL-4-positive cells were observed. No IL-4 positive cells were detected in PBS injection mice. Significant differences were found between PBS vs. WT and WT vs. SCID+T. A, B, C, and D; Scale bar means 60 μ m. E; Bars represent means \pm SD; ND; not detected, * P <0.05.