

# T cells are able to promote lipopolysaccharide-induced bone resorption in mice in the absence of B cells

**M. Yamaguchi, T. Ukai, T. Kaneko, M. Yoshinaga, M. Yokoyama, Y. Ozaki, Y. Hara**

Department of Periodontology, Unit of Translational Medicine, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

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**Background and Objective:** T cells and their cytokines are 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 in the 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 mice with severe combined immune deficiency (SCID).

**Material and Methods:** *Escherichia coli* lipopolysaccharide (LPS) was injected into the gingivae of SCID mice reconstituted by T cells (SCID + T mice). Wild-type C.B-17 mice and SCID mice were used as control animals. 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 that in wild-type mice. The same tendency was found for expression of receptor activator of nuclear factor  $\kappa$ B ligand. The number of interferon- $\gamma$ -positive cells in SCID + T mice was the highest of the three groups. In contrast, 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 in the absence of B cells. The expressions of cytokines in the presence of B cells are quite different.

Yoshitaka Hara, Department of Periodontology, Unit of Translational Medicine, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan  
Tel: +81 95 819 7683  
Fax: +81 95 819 7684  
e-mail: harasen@net.nagasaki-u.ac.jp

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Periodontitis is an infectious disease caused by periodontopathic bacteria. Lipopolysaccharide (LPS), one of their components, and immune cells, especially T cells, are believed to be key factors in periodontitis and bone resorption (1–11). T cell subpopulations, T helper 1 (Th1) cells producing interferon- $\gamma$  (IFN- $\gamma$ ) and T helper 2 (Th2) cells producing interleukin-4 (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 resorp-

tion 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 receptor activator of nuclear factor  $\kappa$ B ligand (RANKL; 6–11,14), which is a member of the

tumor necrosis factor (TNF) superfamily. It activates osteoclast differentiation, stimulates osteoclast activation and increases osteoclast survival by binding with receptor activator of nuclear factor  $\kappa$ B (RANK) of osteoclast precursors and mature osteoclasts (15–20). We previously showed that B cells promoted bone resorption when B cells were transferred to mice with severe combined immune deficiency (SCID), which lack 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 co-operated with B cells that existed congenitally. It is therefore important to clarify the characteristics of T cells and the role of T cells in bone resorption in the 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 in the absence of B cells.

## Material and methods

### Mice

For the experiment, 15 male C.B-17 mice (C.B-17/Icr-<sup>+/+</sup> 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 C.B-17 mice by mincing spleen tissue with scissors and teasing the cells between two frosted microscope slides. The T cells were purified by standard negative selection using the StemSep™ (Stem Cell Technologies, Vancouver, British Columbia, Canada) magnetic separation technique in accordance with the manufacturer's instructions. Purity (90–96%) was confirmed by flow cytometric analysis using antibodies against CD3. The T cell-enriched spleen cells were injected intravenously into recipient SCID mice at a concentration of  $1 \times 10^7$  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-conjugated anti-mouse CD3 monoclonal antibody to determine the presence of T cells. Specific staining was compared to fluorescence using irrelevant isotype controls. All antibodies were purchased from PharMingen (San Diego, CA, USA). Cells were stained according to standard protocols and analyzed by flow cytometry using a FACScan™ instrument with CellQuest™ software (Becton Dickinson, Mountain View, CA, USA).

### Injection of LPS

The SCID mice received T cells (SCID + T mice) 1 week before LPS injection. Wild-type C.B-17 mice and SCID mice were used as control animals. Each group consisted of five mice, and alveolar bone resorption was induced as in previously described models (21–24). Every mouse in each group received 13 injections of 5  $\mu$ g of *E. coli* LPS (*E. coli* O111: B4; Sigma, St Louis, MO, USA) in 3  $\mu$ l 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 h between injections. In our previous

study using the same model (24,25), bone resorption was shown to reach a peak after the tenth injection of LPS and to plateau thereafter. In addition, 13 injections of PBS were given to wild-type mice (PBS group). Mice were killed by cervical dislocation 24 h after the thirteenth injection, and the spleens and left mandibles were removed.

### Specimen preparation

The removed spleens and left mandibles were fixed in 4% paraformaldehyde in PBS at 4°C for 6 h. The left mandible was decalcified with 10% ethylenediaminetetraacetic acid (EDTA) for 1 week. Tissue samples were embedded in paraffin using the AMeX method (acetone, methyl benzoate and xylene; 26). Serial sections (4  $\mu$ m 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 first subsections from each group of serial sections were stained with HE for histopathological observation of the surface of the alveolar bone.

The second 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 incubation of the sections in the staining solution for 30 min at 37°C, they were counterstained with hematoxylin. Osteoclasts were identified as TRAP-positive multinucleated cells on the bone surface.

The third to sixth 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. As a result, it was confirmed that T cell was successfully transferred. Serial subsections were deparaffinized, and endogenous peroxidase activity was blocked with 0.3% H<sub>2</sub>O<sub>2</sub>-methanol for 30 min. These subsections were incubated with normal serum for 30 min 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 were goat anti-mouse CD3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-mouse RANKL antibody (N-19; Santa Cruz Biotechnology), rabbit anti-mouse IFN- $\gamma$  polyclonal antibody (PBL Biomedical Laboratories Inc., Piscataway, NJ, USA), and anti-mouse IL-4 monoclonal antibody (R&D Systems, Minneapolis, MN, USA). Then the sections were incubated with a secondary antibody for 30 min 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) was used for staining of IFN- $\gamma$ , and biotinylated rabbit anti-rat polyclonal antibody (VECTOR Laboratories Inc., Burlingame, CA, USA) was used for staining of IL-4. Finally, the sections were incubated with peroxidase-conjugated streptavidin (Dako) for 30 min, followed by incubation with diaminobenzidine tetraoxide solution, and then counterstained with hematoxylin.

### Bone histomorphometry

Owing 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 assessed to quantitatively evaluate the progression of bone resorption (21–25). After counting the number of points of

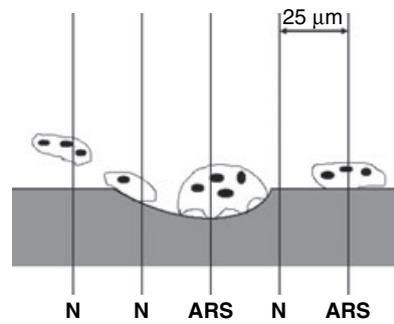


Fig. 1. Definition of active resorption. Bone surfaces in contact with TRAP-positive multinucleated cells were defined as sites of active resorption surface (ARS); other sites were classified as normal (N). The percentage of ARS was derived by dividing the number of sites of ARS by the number of all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

intersection of the bone surface with the line of a micrometer (Olympus, Tokyo, Japan) in 25  $\mu$ m graduations at  $\times 400$  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  $\times$  500  $\mu$ m on the surface of the alveolar bone were counted.

### Statistical analysis

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

## Results

### Histopathological findings and ARS

There was a little inflammatory infiltration and no bone resorption in the PBS group (Fig. 2A,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,D). In SCID mice, in contrast, irregularity of the alveolar bone surface and infiltration of inflammatory cells were rare and there were no plasma cells (Fig. 2E,F). The SCID + T mice showed an irregular alveolar bone surface and TRAP-positive multinucleated cells as observed in wild-type mice (Fig. 2G,H). Plasma cells were not observed in SCID and SCID + T mice.

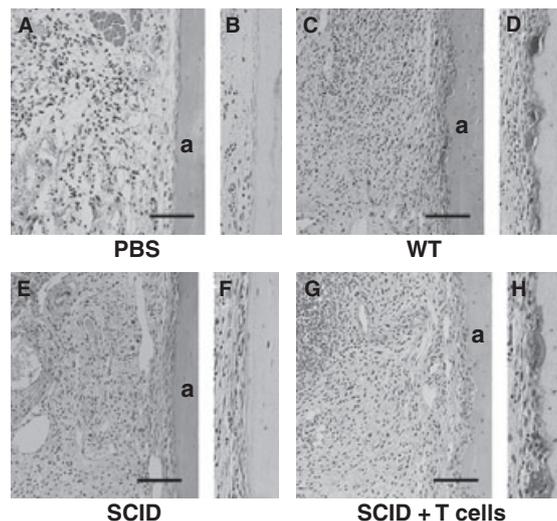


Fig. 2. Histopathological findings in alveolar bone (a). A few inflammatory cells and no bone resorption were observed in the PBS group (A and B). In wild-type mice, infiltration of many inflammatory cells and formation of osteoclasts were observed (C and D). In SCID mice, there were few inflammatory cells and almost no osteoclasts (E and F). In SCID + T mice, many inflammatory cells and osteoclasts were observed, as in wild-type mice (G and H). A, C, E, and G: HE staining. B, D, F, and H: TRAP staining. Scale bars represent 250  $\mu$ m.

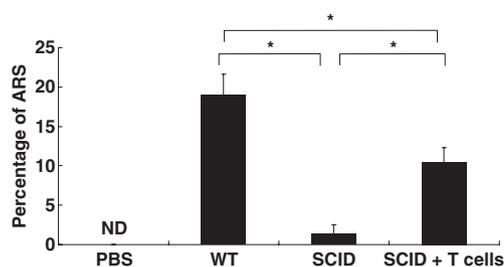


Fig. 3. Percentage of ARS in each group. The percentage of ARS was highest in wild-type mice, and higher in SCID + T mice than in SCID mice. Significant differences were found between these three groups. Bars represent means + SD. WT, wild-type; ND, not detected; \* $p < 0.05$ .

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

#### Immunohistological findings and histomorphometry

Diffuse infiltration of CD3-positive cells was observed in wild-type ( $16.12 \pm 4.29$  cells) and SCID + T mice ( $8.16 \pm 1.84$  cells) following 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). A few CD3-positive cells were observed in PBS-injected wild-type mice ( $4.42 \pm 1.13$  cells).

Some 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 distinguish fibroblasts from osteoblasts, almost no RANKL-positive osteoblastic cells were observed in all groups. The number of RANKL-positive cells in wild-type mice ( $38.08 \pm 4.09$  cells) was significantly larger than the numbers in the other three groups. The number in SCID + T mice ( $24.43 \pm 6.81$  cells) was significantly larger than that in SCID mice ( $11.24 \pm 3.06$ ). There were very few RANKL-positive cells in the mice injected with PBS ( $1.19 \pm 1.09$  cells; Fig. 5).

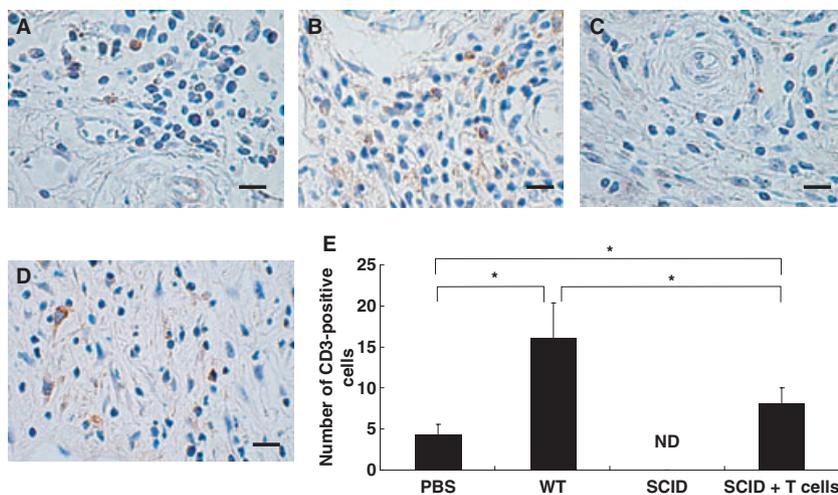


Fig. 4. Immunohistological findings and numbers of CD3-positive cells. A–D are representative immunostained sections. A, section from mouse injected with PBS; B, section from wild-type mouse; C, section from SCID mouse; and D, section from SCID + T mouse. E shows the number of CD3-positive cells. The number of CD3-positive cells was largest in wild-type mice. In A–D, scale bars represent 60  $\mu$ m. In E, bars represent means + SD. WT, wild-type; ND, not detected; \* $p < 0.05$ .

An 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 ( $11.78 \pm 2.70$  cells) and SCID + T mice ( $22.75 \pm 5.75$  cells) also showed an 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 ( $6.56 \pm 0.61$  cells) and in mice injected with PBS ( $5.61 \pm 0.80$  cells; Fig. 6).

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

#### Discussion

In this study, bone resorption was induced in wild-type and SCID + T mice. The 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 in the 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

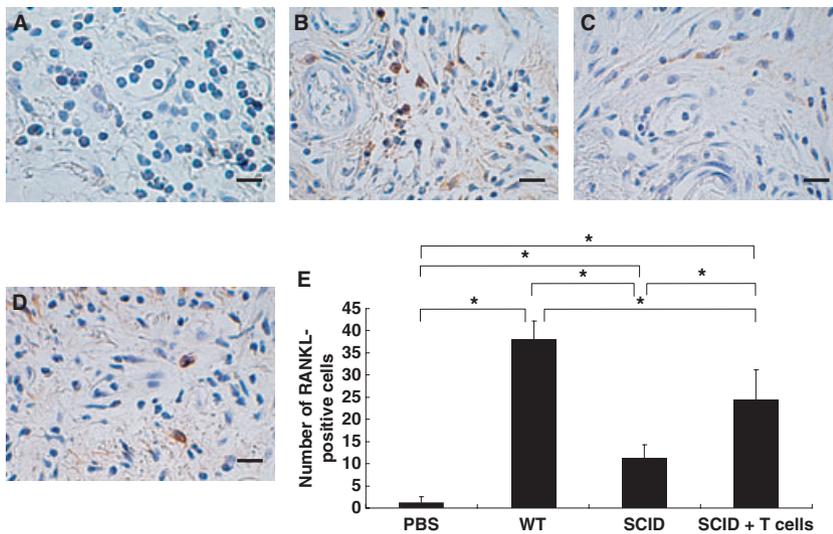


Fig. 5. Immunohistological findings and numbers of RANKL-positive cells. A–D are representative immunostained sections. A, section from mouse injected with PBS; B, section from wild-type mouse; C, section from SCID mouse; and D, section from SCID + T mouse. E shows the number of RANKL-positive cells. The number of RANKL-positive cells was largest in wild-type mice. Significant differences were found between four groups. In A–D, scale bars represent 60  $\mu$ m. In E, bars represent means + SD; WT, wild-type; \* $p$  < 0.05.

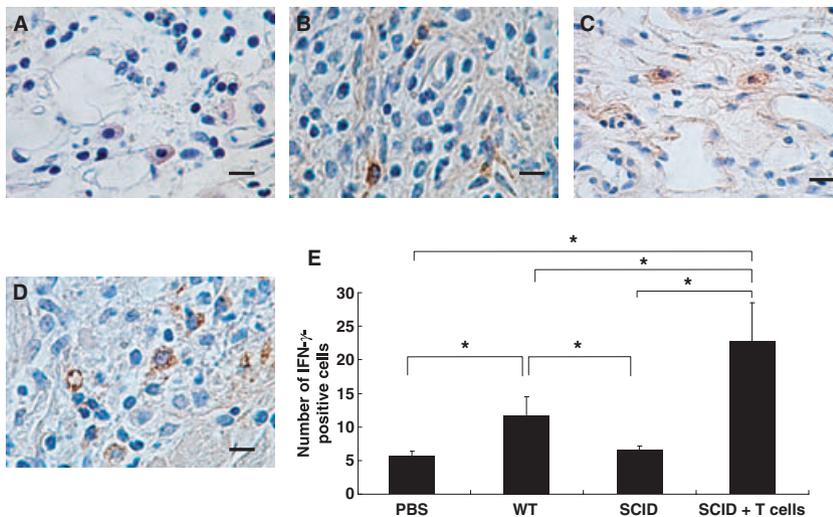


Fig. 6. Immunohistological findings and numbers of IFN- $\gamma$ -positive cells. A–D are representative immunostained sections. A, section from mouse injected with PBS; B, section from wild-type mouse; C, section from SCID mouse; and D, section from SCID + T mouse. E shows the number of IFN- $\gamma$ -positive cells. The number of IFN- $\gamma$ -positive cells was largest in SCID + T mice. A significant difference was found between four groups. In A–D, scale bars represent 60  $\mu$ m. In E, bars represent means + SD. WT, wild-type; \* $p$  < 0.05.

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 study, although ARS and the

number of RANKL-positive cells were correlated in wild-type mice and SCID + T mice. The SCID + T mice showed more IFN- $\gamma$ -positive cells and less IL-4-positive cells than the wild-type mice. These findings suggest that Th1 cells are dominant in the absence of B cells *in vivo*. Interferon- $\gamma$  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). In contrast, 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-osteoclastogenic and indirect pro-osteoclastogenic properties of IFN- $\gamma$  influences bone resorption. In the present study, many more IFN- $\gamma$ -positive cells and more severe bone resorption were observed in SCID + T mice compared with 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 cells was highest in SCID + T mice is uncertain. It is likely that T cells differentiated into IFN- $\gamma$ -positive cells because of the lack of B cells; however, 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. The 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. Interleukin-4 was shown to inhibit osteoclastogenesis by interrupting the path of signal transducer and activator of transcription 6 (STAT6) dependence (32,33), and IL-4 treatment decreased the turnover of bone in ovariectomized mice (34). In the present study, the 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 Th2 cells do not suppress osteoclastogenesis *in vivo*. Further investigation into the relationship between Th1 and Th2 cells with bone resorption is necessary.

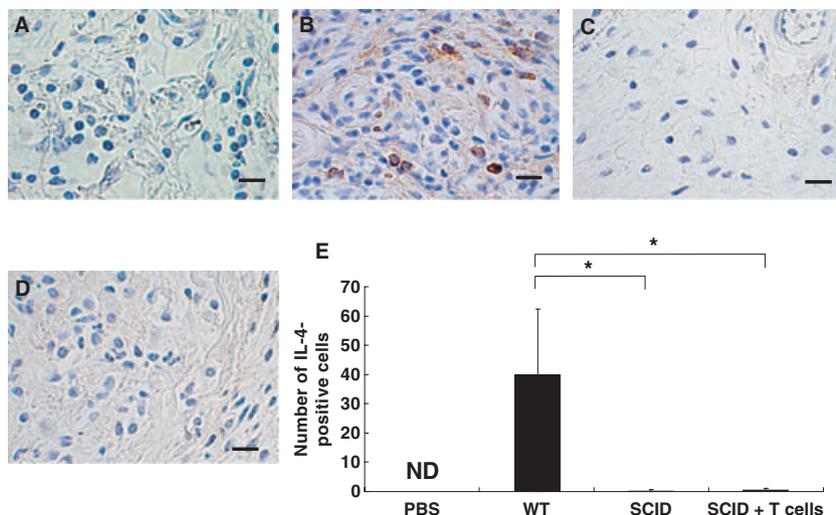


Fig. 7. Immunohistological findings and numbers of IL-4-positive cells. A–D are representative immunostained sections. A, section from mouse injected with PBS; B, section from wild-type mouse; C, section from SCID mouse; and D, section from SCID + T mouse. E shows the number of IL-4-positive cells. The 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 the mice injected with PBS. Significant differences were found between PBS vs. WT and WT vs. SCID + T. In A–D, scale bars represent 60  $\mu$ m. In E, bars represent means + SD. WT, wild-type; ND, not detected; \* $p$  < 0.05.

In this study, we used a mouse bone resorption model injected with *E. coli* LPS. Although all types of bone resorption in clinical periodontitis are not shown in this model, this is thought to be an adequate model for examination of the mechanism of bone resorption because bacterial LPS is one of the most important factors 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 in the 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 co-operate 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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