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Interferon-gamma Production Changes in Parallel with Bacterial Lipopolysaccharide Induced Bone Resorption in Mice: An Immunohistometrical Study

H. Moriyama, T. Ukai, Y. Hara

Department of Periodontology, Nagasaki University School of Dentistry, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan

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Abstract. It has been reported that bacterial lipopolysaccharide (LPS) induces alveolar bone resorption and that the host immune system, especially activated T cells, plays a crucial role in osteoclastogenesis. On the other hand, interferon-gamma (IFN-γ), which is produced by activated T cells, suppresses bone resorption both *in vitro* and *in vivo*. Thus, the question arises as to whether or not IFN-γ production increases with increasing bone resorption. We previously demonstrated that repeated injections of *Escherichia coli* LPS into mouse gingiva causes osteoclast formation in alveolar bone. In the present study we observed changes in the IFN-γ production of infiltrating cells in concurrence with bone resorption. Mice were repeatedly injected with 5 μg LPS 26 times every 48 hours. After the 16th injection, when the alveolar bone resorption reached a plateau, the concentration of LPS was altered (25 μg LPS or PBS alone). The level of bone resorption became significantly elevated, and the number of IFN-γ- and interleukin-1 beta (IL-1β)-bearing cells also increased significantly in relation to bone resorption within the 25 μg LPS-injected group. On the other hand, few tartrate-resistant acid phosphatase positive cells, or IFN-γ- and IL-1β-bearing cells, were seen in the PBS-injected group. These results suggest that alteration in IFN-γ-bearing cells might play a role in counterbalancing LPS-induced bone resorption resulting from osteoclast activating cytokines such as IL-1β.

Key words: Interferon-gamma — Interleukin-1 beta — Bone resorption — Lipopolysaccharide — Histometrical study.

T cells play a key role in bacterial lipopolysaccharide (LPS)-induced bone resorption [1–3]. Activated T cells produce cytokines such as interferon-gamma (IFN-γ) which amplify the stimulation of prostaglandin E2 synthesis by interleukin-1 beta (IL-1β) [4–6]. Moreover, IL-1β is well known to be a strong osteoclast activator [7–13]. On the other hand, IFN-γ suppresses bone resorption both *in vitro* [13, 14] and *in vivo* [15–17]. Some *in vitro* studies have revealed that IFN-γ inhibits osteoclastogenesis [18–20]. In a recent report [21], LPS-induced bone resorption was stronger in mice lacking one of the IFN-γ receptors than in control mice *in vivo*. Furthermore, the culture supernatant of activated T cells induced a decline in the number of tartrate-resistant acid phosphatase (TRAP)-positive cells on a dentine slice. This suppression was deleted by treating the cells with anti-IFN-γ antibody *in vitro*. However, it remains unclear whether IFN-γ production changes in conjunction with increases or decreases in bone resorption. Therefore, in this study we induced bone resorption in mice alveolar bone by the repeated injection of LPS every 48 hours. The number of IFN-γ- and IL-1β-bearing cells was investigated when the number of TRAP-positive cells increased. Furthermore, the dose of repeatedly injected LPS was increased when the number of TRAP-positive cells leveled off after the 16th repeated injection of LPS. Changes in the number of TRAP-positive cells, IFN-γ-, and IL-1β-bearing cells were then investigated. The results showed that changes in the number of IFN-γ-bearing cells paralleled bone resorption and an alteration in the number of IL-1β-bearing cells.

Materials and Methods

Mice

One hundred and eight male BALB/c mice, 7 weeks of age, were used in the present study. The mice were purchased from Charles River Japan and maintained under SPF conditions in the Laboratory Animal Center for Biomedical Research (Nagasaki University School of Medicine). The experimental protocol followed was approved by the Local Institutional Animal Care and Use Committee of Nagasaki University.

Correspondence to: Y. Hara, e-mail: harasen@net.nagasaki-u.ac.jp

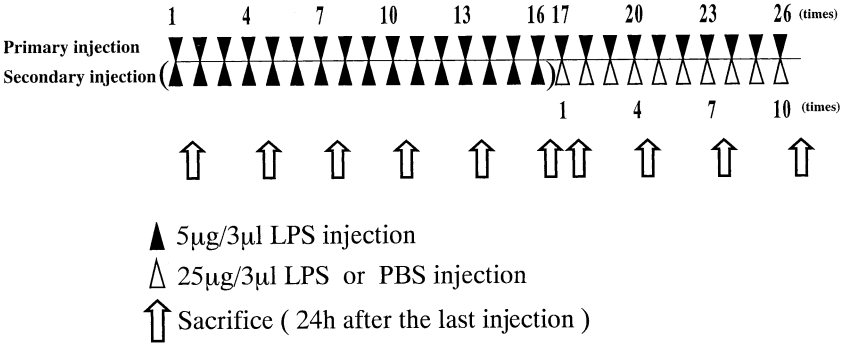


Fig. 1. Experimental schedule. Mice were injected with 5 μg LPS every 48 hours and sacrificed 24 hours after the 1st, 4th, 7th, 10th, 13th, 16th, 17th, 20th, 23rd, and 26th injections of LPS (the primary injection). Forty-eight hours after the 16th injection in the primary injection, the concentration of injected LPS was altered (25 μg of LPS or 3 μl PBS) and the animals were sacrificed 24 hours after the 1st, 4th, 7th, and 10 th injections (the secondary injection).

Preparation of Tissues

Primary Injections. Alveolar bone resorption was induced as in a previously described model [1, 2, 22]. 5 μg of *Escherichia coli* LPS (*E. coli* 0111: B4; Difco, MI) in 3 μl PBS was injected every 48 hours into the mesial gingiva of the 1st molar of the left mandible under ether anesthesia. Groups of six mice were sacrificed 24 hours after the 1st, 4th, 7th, 10th, 13th, 16th, 17th, 20th, 23rd, and 26th injections of LPS, comprising a primary injection group of 60 mice altogether.

Secondary Injections. Forty-eight hours after the 16th injection in the primary injection, at which point the bone resorption had reached a plateau, the concentration of injected LPS was altered (mice within this group comprised a secondary injection group). The secondary injection group was divided into two subgroups. Within these two subgroups, 25 μg of LPS in 3 μl PBS (the 25 μg group) and 3 μl PBS alone (the PBS group) was injected as described above. Groups of six mice from each of the two subgroups were sacrificed 24 hours after the 1st, 4th, 7th, and 10th injections (Fig. 1).

After being sacrificed, the left mandible of each mouse was removed and fixed in 4% paraformaldehyde in PBS at 4°C for 6 hours, decalcified with 10% EDTA for 1 week, and then embedded in paraffin using the AMeX method [23]. They were fixed in acetone at −20°C overnight, dehydrated in acetone at 4°C for 15 minutes, followed by room temperature for 15 minutes, cleared in methyl benzoate for 30 minutes, followed by xylene for 30 minutes, penetrated with paraffin for 2 hours, and then embedded. Serial sections of 4 μm thickness were prepared in order to examine the mesio-distal section of the left first molar.

Histochemical and Immunohistological Staining

Ten groups of serial sections, each group containing five subsections, were obtained from each specimen after receiving a specified number of injections of a given composition. The first subsections from each group of serial sections were stained with hematoxylin and eosin (HE) to observe the surface of alveolar bone histopathologically. In order to examine the osteoclasts, the 2nd subsection from each group was stained with tartrate-resistant acid phosphatase (TRAP) according to the procedure described by Katayama et al. [24]. Briefly, a staining solution was made by mixing 0.5 ml pararosanilin solution (1 g pararosanilin in 20 ml distilled water and 5 ml concentrated hydrochloric acid), 0.5 ml of 4% sodium nitrite solution, 10 ml of 0.1 M acetate buffer of pH 5.0, and 10 mg of naphthol AS-BI phosphate (Sigma, Japan), dissolved in 8 ml DW. The mixture was adjusted to pH 5.0 using concentrated NaOH and filtered through No.1 Whatman filter paper. Furthermore, after adding 150 mg L (+) tartaric acid to a 10 ml aliquot of the mixture solution to a final tartrate concentration of 0.1 M, the solution was adjusted to a pH of 5.0 with concentrated NaOH. After incubating the 2nd subsections within the staining solution for 30 minutes at 37°C, they were counterstained with hematoxylin. The osteoclasts were identified as multinucleated TRAP-positive cells on the bone surface.

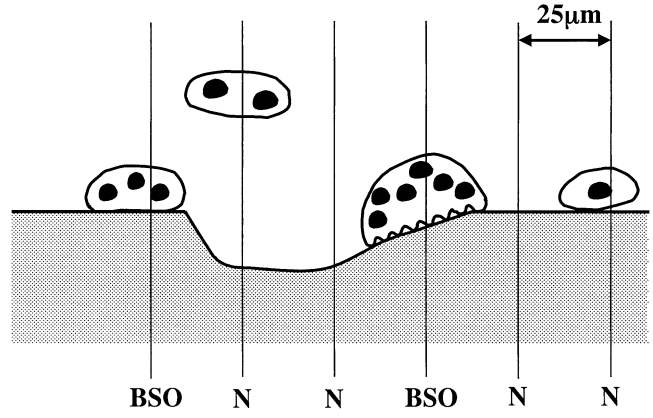


Fig. 2. Bone surfaces, regardless of the presence or absence of resorption lacunae, in contact with TRAP-positive multinucleated cells were defined as sites of Bone surface in contact with osteoclasts (BSO); site of the other (N). Percentage of BSO was derived by dividing the number of sites of BSO by all intersecting points between bone surfaces within a 25 μm scale.

The 3rd and 4th subsections were used for the immunohistological staining of IFN-γ- and IL-1β-bearing cells. Serial subsections were deparaffinized and incubated with normal rabbit or goat serum for 30 minutes at room temperature. They were then immersed in primary rat anti-mouse IFN-γ (PBL Biomedical Laboratories Inc., USA) monoclonal antibody (mAb), or goat anti-mouse IL-1β (Genzyme Corporation, USA) polyclonal antibody at 4°C overnight. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide, followed by incubation with secondary antibody (biotinylated rabbit anti-rat immunoglobulins, Vector Laboratories Inc., USA, or biotinylated rabbit anti-goat immunoglobulins, ICN Pharmaceuticals Inc, Ohio, USA). Finally, these subsections were incubated with peroxidase-conjugated streptavidin (Dako Corporation, Japan), followed by diaminobenzidine tetraoxide solution, and then counterstained with methyl green.

To identify the origin of IFN-γ- and IL-1β-bearing cells, randomly selected sections from the 5th subsections were double-stained with anti-CD3 and anti-IFN-γ antibodies or with anti-IL-1β and anti-macrophage antibodies. After being deparafinized, sections were immersed in goat anti-mouse CD3 (Santa Cruz Biotechnology, Inc., USA) or goat anti-mouse IL-1β polyclonal antibody. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide, followed by incubation with biotinylated rabbit anti goat immunoglobulins (DAKO, Japan). The sections were incubated with peroxidase-conjugated streptavidin, followed by diamino benzidine tetraoxide solution. Following this, rat anti-mouse IFN-γ mAb or rat anti-mouse macrophage mAb (BM8:

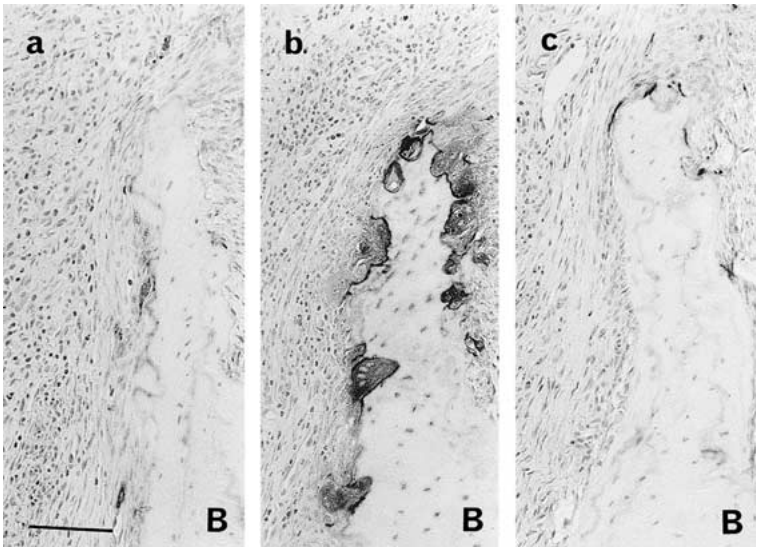
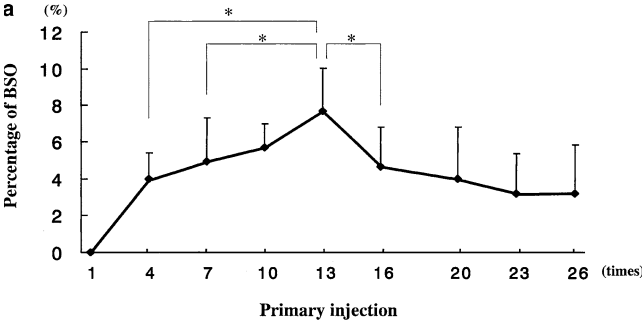
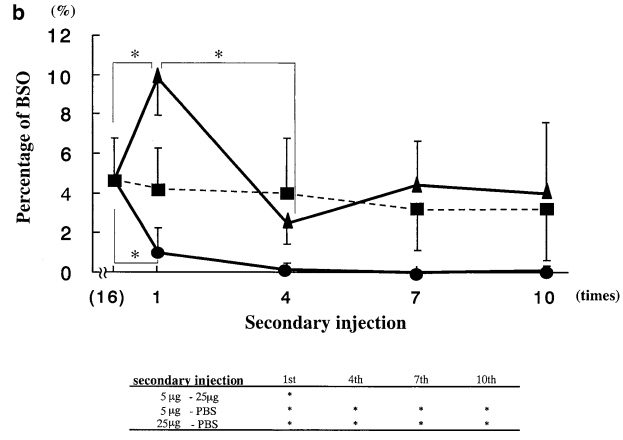


Fig. 3. TRAP-positive multinucleated cells after the 16th injection of the primary injection and the 1st injection of the PBS and 25 μg group (TRAP staining, bar = 100 μm, B = alveolar bone). A number of osteoclasts are present on irregular bone surface after the 16th injection of 5 μg LPS (**a**). Numerous multinucleated cells are found adjacent to the irregular bone surface after 25 μg LPS injection (**b**). Few osteoclasts are observed on smooth bone surface after PBS injection (**c**).





secondary injection	1st	4th	7th	10th
5 μg - 25μg	*			
5 μg - PBS	*	*	*	*
25μg - PBS	*	*	*	*

Fig. 4. (**a**) The percentage of BSO in the primary injection group. The percentage of BSO peaked after the 13th injection. Significant differences were observed between at the 13th and 4th, 7th or 16th injections. Bars represent means ± SD. Significant differences are indicated by an asterisk ($P < 0.05$). (**b**) The percentage of BSO in the secondary injection groups. In the 5 μg group, the percentage of BSO did not change remarkably after the 16th injection (■). Bone resorption significantly increased after the 1st injection in the 25 μg group (▲) and then decreased significantly after the 4th injection. After the 1st injection in the PBS group, few TRAP-positive cells were observed (●). Bars represent means ± SD. Significant differences are indicated by an asterisk ($P < 0.05$).

BMA Biomedicals AG, Switzerland) was applied, respectively. Then sections were incubated with goat anti-rat immunoglobulins (ICN Pharmaceuticals Inc.-Cappel Products, USA) and alkaline phosphatase rat anti-alkaline phosphatase (APAAP) (DAKO, Japan). The APAAP enzyme reaction was developed using a naphthol AS-BI phosphate and fast blue RR salt (Sigma, Japan). Endogenous alkaline phosphatase activity was inhibited by the addition of 1 mM levamisole to this substrate solution. The sections were counterstained by methylgreen.

Bone Histomorphometry

Due to the difficulty of quantitatively evaluating total bone resorption, the percentage of bone surface in intimate contact with osteoclasts (BSO) was evaluated (Fig. 2). After counting the number of points of intersection of the bone surface by a line of a micrometer (Olympus micrometer, Japan) in 25 μm graduations at ×400 magnification, the percentage of BSO to total points of intersection was calculated.

Immunohistological Quantitation. The evaluated field fell within 250 μm from the mesial side of the alveolar bone surface of the first molar of the left mandible. Unit area was defined as a square measuring 250 × 250 μm. The number of IFN-γ-and IL-1β-bearing cells in five unit areas was counted and the average number per unit area was calculated for each section.

Statistics

Averaged data of the percentage of BSO, as well as the immunohistological findings, were obtained from 10 sections of six specimens taken after each set of injections from both the primary and secondary injection groups. Significant differences were analyzed using one-factor ANOVA and a Fisher's PLSD t test between the 1st injection of the secondary injection group and 16th injection of the primary injection group or 4th injection of the secondary injection group, and among each group at the 1st and 4th injections of the secondary injection group. Significance was established at $P < 0.05$.

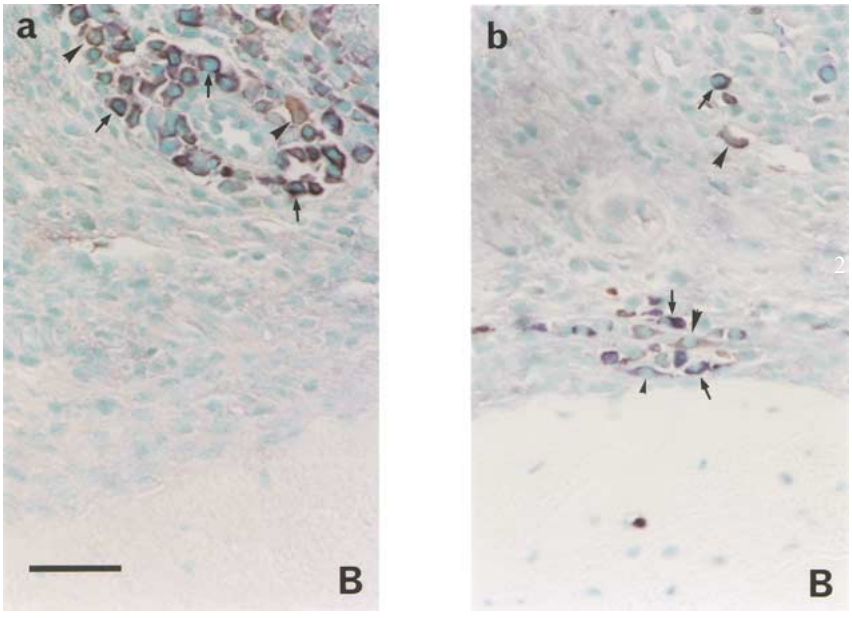


Fig. 5. Immunohistological staining after 1st injection of the 25 μg group (Bar = 50 μm; B = alveolar bone) (**a**) IFN-γ- and CD3-bearing cells. CD3-positive cells were stained with brown (arrowheads) and CD3- and IFN-γ-double-positive cells with dark brown (arrow). There were few IFN-γ-positive and CD3-negative cells that were stained with blue. Most of IFN-γ-bearing cells were included in CD3-bearing cells. (**b**) IL-1β- and anti-macrophage-bearing cells. IL-1β-positive cells were stained with brown (large arrowhead) and anti-macrophage-bearing cells with blue (small arrowhead). IL-1β- and anti-macrophage-double positive cells were stained with dark brown (arrow). Most of IL-1β-bearing cells were macrophages. Double-positive cells were located near the bone surface.

Results

Histopathological and Histometrical Findings

Primary Injection Group. TRAP-positive multinucleated cells appeared after the 4th injection and increased as more injections were given to the primary injection group. The percentage of BSO peaked after the 13th injection and then decreased after the 16th injection. There were significant differences between 13th and 4th, 7th, or 16th injections. Thereafter the percentage of BSO did not change remarkably throughout the length of the experiment (Figs. 3a and 4a).

Secondary Injection Group. In the 25 μg group, the percentage of BSO among mice sacrificed after the 1st injection was significantly higher than in those sacrificed after the 16th injection of primary injection group. It was also higher than that found within the primary injection group. Moreover, it decreased significantly after the 4th injection. Conversely, following the 1st injection of PBS alone, a significantly lower percentage of BSO was observed than in the primary injection group after the 16th injection, or in the other two groups after the 1st injection. Thereafter, few TRAP-positive cells were observed (Figs. 3b, c, and 4b).

Immunohistological Findings

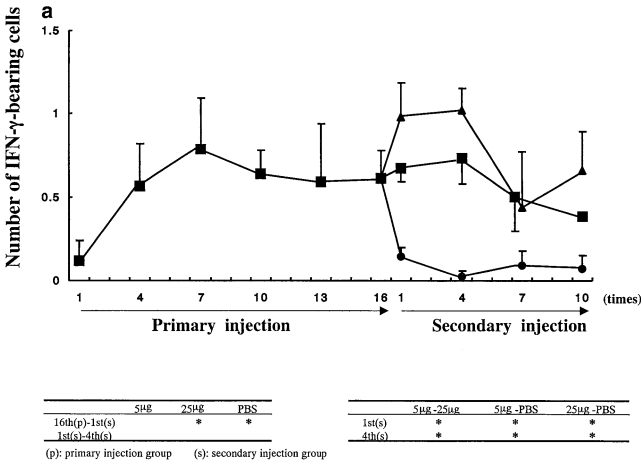
Both IFN-γ-and IL-1β-bearing cells were increased with bone resorption and located near the alveolar bone surface. Most of the IFN-γ-bearing cells were CD3 positive and most of the IL-1β-bearing cells were positively stained with anti-mouse, macrophage antibody (Fig. 5a,b).

The number of IFN-γ-bearing cells peaked after the 7th injection in the primary injection and decreased thereafter. Within the 25 μg group of secondary injection, the number of IFN-γ-bearing cells climbed significantly following the 1st injection and decreased after the 7th injection, whereas only a few IFN-γ-positive cells were observed within the PBS group (Fig. 6a).

The number of IL-1β-bearing cells increased from the 4th to the 10th injection and decreased after the 13th injection in the primary injection group. However, they increased significantly in the 25 μg group of the secondary group after the 1st injection, decreasing again after the 7th and 10th injections. They were located near the alveolar bone after the 1st injection. In the PBS group, there was a significant decrease in IL-1β-bearing cells after the 1st injection and their numbers remained low throughout (Fig. 6b).

Discussion

Bacterial LPS injected into gingiva [1, 2, 22] and calvaria [3] induces bone resorption. Repeated injections of LPS have shown to cause bone resorption to level off after several series of injections. These findings suggest that a balance exists between the challenge presented by LPS and the defense responses of the host system, in chronic inflammatory conditions, and similar findings were observed in bone resorption induced by periodontopathic bacterial LPS [25]. Furthermore, high concentrations of LPS induced a rapid increase in bone resorption, followed by a rapid decrease and leveling off. In addition, the numbers of both IFN-γ-and IL-1β-bearing cells rapidly responded to this challenge. These findings suggest that a secondary immune response comes into play in this circumstance. In order to maintain the established balance between host defense and continuous bacterial LPS challenge, the immune system acted rap-



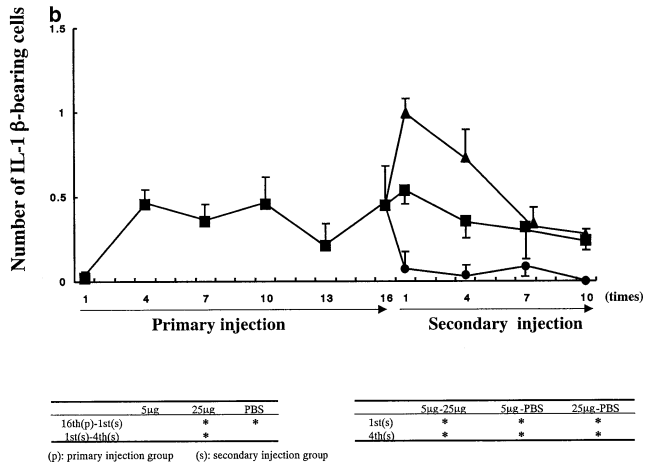


Fig. 6. (**a**) The number of IFN-γ-bearing cells peaked after the 7th injection in the primary injection group (■). In the 25 μg group (▲) the number of IFN-γ-bearing cells increased significantly after the 1st injection and decreased after the 7th injection, whereas only a few positive cells were observed within the PBS group (●). Bars represent means ± SD. Significant differences are indicated by an asterisk ($P < 0.05$). (**b**) The number of IL-1β-bearing cells peaked between the 4th and 10th injection in the primary group (■). In the 25 μg group (▲), the number of IL-1β-bearing cells increased significantly after the 1st injection, whereas they decreased significantly after the 1st injection of PBS alone (●). Bars represent means ± SD. Significant differences are indicated by an asterisk ($P < 0.05$).

idly to reestablish equilibrium when the challenge was strengthened.

IL-1β is a well-known osteoclast activator [7–12, 26]. It has been shown that increases in IL-1β concentration parallel bone resorption after repeated injections of LPS in gingiva [25]. In the present study, the number of IL-1β-bearing cells also changed in concurrence with bone resorption, especially with increases in bone resorption in both the primary and secondary injection groups. These changes within IL-1β levels suggest that IL-1β is involved in bone resorption.

Activated T cells are also essential factors in bone resorption induced by bacterial LPS [1, 2, 3]. T cells express receptor activators for the nuclear factor-kappa B ligand (RANKL), which modulates osteoclastogenesis within RANK osteoclast precursor cells [27–32]. On the other hand, activated T cells produce IFN-γ which suppresses bone resorption *in vitro* [12–14] and *in vivo* [15–17]. A recent report has shown that IFN-γ promotes the degradation of tumor necrosis factor receptor-associated factor 6 (TRAF6) which is key in the RANKL/RANK signaling pathway [21]. Specifically, osteoclastogenesis is suspected to depend on the balance between RANKL and IFN-γ. In the present study, changes in the number of IFN-γ-bearing cells paralleled bone resorption, in addition to changes within the number of IL-1β-bearing cells. This finding suggests that IFN-γ, which has a suppressive effect on bone resorption, is also produced simultaneously with bone resorptive cytokines such as IL-1β. The immune system sometimes simultaneously produces an activator with its suppressor, such as when it produces IL-1 along with an IL-1 receptor antagonist. IFN-γ may be produced to counterbalance bone resorption stimulated by osteoclast-activating cytokines as a negative feedback, and may further inhibit bone resorption from being excessive.

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