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Locally administered T cells from mice immunized with lipopolysaccharide (LPS) accelerate LPS-induced bone resorption

Yukio Ozaki [1], Takashi Ukai [1], Masayuki Yamaguchi, Miho Yokoyama, Esperanza R. Ayón Haro, Mayumi Yoshimoto, Takashi Kaneko, Miho Yoshinaga, Hirotaka Nakamura, Chiaki Shiraishi, 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

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ABSTRACT

T cells play important roles in bone destruction and osteoclastogenesis and are found in chronic destructive bone lesions. Lipopolysaccharide (LPS) is one of several pathological factors involved in inflammatory bone destruction. We previously described the importance of T cells in the inflammatory bone resorption that occurs after repeated LPS administration. However, whether local or systemic T cells are important for inflammatory bone resorption and whether immunization of host animals influences bone resorption remain unclear. The present study examines the effects of local extant T cells from LPS-immunized mice on LPS-induced bone resorption. T cells from LPS-immunized or non-immunized mice were injected together with LPS into the gingival tissues of mice with severe combined immunodeficiency disease that lack both T and B cells. We histomorphometrically evaluated bone resorption at sites of T cell injections and examined the influence of T cells from LPS-immunized mice on osteoclastogenesis *in vitro*. We found that locally administered T cells from LPS-immunized but not non-immunized mice accelerated LPS-induced bone resorption *in vivo*. Moreover, T cells from LPS-immunized mice increased osteoclastogenesis *in vitro* induced by receptor activator of NF-κ B ligand and LPS and anti-tumor necrosis factor (TNF)-α antibody inhibited this increase. These results demonstrated that local extant T cells accelerate inflammatory bone resorption. Furthermore, T cells from LPS-immunized mice appear to elevate LPS-induced bone resorption using TNF-α.

Introduction

Control of inflammation and bone resorption is important to prevent and treat inflammatory destructive bone diseases such as periodontitis or rheumatoid arthritis. Osteoclasts comprise the principle cell type involved in bone resorption. Receptor activator of NF-κB ligand (RANKL), a member of the TNF ligand superfamily, is a major factor involved in osteoclast differentiation. Membrane-bound RANKL is mainly expressed on osteoblasts, and T cells produce soluble-type RANKL [1,2], which induces osteoclast formation by binding to RANK on bone marrow macrophages (BMMs) cultured with macrophage colony stimulating factor (M-CSF) [3]. Osteoprotegerin (OPG) binds to RANKL and thus prevents RANKL-RANK binding on osteoclast precursors and thus acts as a soluble decoy receptor for RANKL [4,5]. Tumor necrosis factor-α (TNF-α) also promotes osteoclastogenesis independently of RANKL-RANK interaction [6,7].

Several studies have shown that T cells and T cell infiltration are involved in inflammatory destructive bone diseases *in vivo* such as periodontitis [8–11], suggesting that T cells play an important role in inflammatory bone loss. We also reported that T cells accelerate LPS-induced bone resorption in nude mice with reconstituted T cells or in mice with severe combined immunodeficiency disease (SCID) [12,13]. However, these studies did not determine whether locally infiltrating or systemic extant T cells are more important for bone resorption, which seems important for understanding the role of T cells in osteoclastogenesis.

T cells produce soluble RANKL and promote osteoclastogenesis when activated with phytohaemagglutinin (PHA) and interleukin (IL)-2, or with anti-CD3 and CD28 antibodies [9,14–16]. T cells can also augment RANKL-induced osteoclastogenesis through TNF-α production [17]. On the other hand, resting T cells negatively regulate osteoclast formation *in vitro* [18], and IFN-γ produced by T cells inhibits osteoclast formation *in vitro* but accelerates bone resorption *in vivo* [19]. The mechanisms of T cell involvement in bone resorption are very complex. The effect of activated T cells on osteoclastogenesis probably depends on the method of activation [20].

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria, and is considered to be a cause of periodontitis [21]. Although LPS inhibits RANKL-induced osteoclast formation from BMMs [22], it increases both bone resorption activity and the lifespan of mature osteoclasts [23]. Lipopolysaccharide activates innate immunity through the recognition of antigen-presenting cells such as macrophages, dendritic cells, and B cells [24,25]. We

* Corresponding author. Fax: +81 95 819 7684.
E-mail address: harasen@nagasaki-u.ac.jp (Y. Hara).
[1] These authors have contributed equally to this study.

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studied the involvement of B cells in alveolar bone resorption induced by repeated LPS injections [26] and found that they accelerate LPS-induced bone resorption *in vivo* and that LPS-stimulated B cells accelerate osteoclastogenesis via TNF-α *in vitro*. However, although LPS might not directly induce specific cellular immunity via T cells, dendritic cells that recognize LPS nevertheless activate these cells [27,28]. We have shown that T cells accelerate LPS-induced bone resorption in SCID mice without B cells but with reconstituted T cells [12]. Bone resorption is more severe in nude mice with reconstituted T cells after 13 than after 4 injections of LPS [13]. Thus, T cells in mice immunized with LPS probably influence bone resorption. However, whether T cell in mice immunized or not with LPS is critical or can induce LPS-induced bone resorption remains unclear.

The present study aimed to clarify the effects of local extant T cells in LPS-induced bone resorption and to determine whether T cells from LPS-immunized mice accelerate LPS-induced bone resorption *in vivo* and osteoclastogenesis *in vitro*.

Materials and methods

Mice

Male SCID (CB-17/Icr-scid Jcl) and CB-17 (CB-17/Icr-+/+Jcl) mice purchased from Nihon Clea (Tokyo, Japan) at 7 weeks of age were maintained under specific pathogen-free conditions at the Biomedical Research Center for Frontier Life Sciences, Nagasaki University. Animal care and experiments proceeded according to the Guidelines for Animal Experimentation of Nagasaki University and under the approval of the Institutional Animal Care and Use Committee.

Isolation and activation of spleen T cells

We isolated T cells from the spleens of CB17 mice that had received 13 injections of 5 μg/3 μl of *Escherichia coli* (*E. coli*) LPS (O111:B4; Sigma, St. Louis, MO) into the left mandible every 48 h, which significantly elevated antibody titers against LPS (data not shown). T cells were also obtained from the spleens of non-immunized, age-matched mice that were not administered with LPS. Whole spleen cell suspensions were prepared after red cells were lysed with NH_4Cl. T cells isolated by standard negative selection using StemSep™ magnetic separation (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's instructions. All isolated T cells were activated as described by Ogawa et al. [29]. In brief, T cells from non- or LPS-immunized mice were incubated with PHA (5 μg/ml, SIGMA) and IL-2 (10 ng/ml, SIGMA) for 24 h in RPMI containing 10% fetal bovine serum (FBS).

Flow-cytometric analysis

The purity of the separated cells was confirmed by flow cytometry using a FACScan™ with CellQuest™ software (Becton Dickinson, Mountain View, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 monoclonal antibody (mAb). Specific staining was compared with fluorescence emitted by labeled irrelevant isotype controls. Antibody was purchased from PharMingen (San Diego, CA) and the cells were stained according to standard protocols. The purity of isolated T cells from both LPS-immunized and non-immunized mice was 90–96%.

Preparation of tissues

Eighteen SCID mice were assigned to the following groups: SCID mice without T cell injections (SCID mice), SCID mice injected with T cells from mice that had received 13 LPS injections (L-SCID) and SCID mice injected with T cells from mice that had not been injected with LPS (N-SCID). All mice received 3 injections of *E. coli* LPS (5 μg) in 3 μl of phosphate-buffed saline (PBS) under ether anesthesia into the mesial gingiva of the first molar of the left mandible at 48-h intervals. The 4th injection comprised a mixture of 5×10^5 T cells from non- or LPS-immunized mice and LPS. The SCID mice received only LPS. Control SCID mice received 3 injections of PBS and the 4th comprised T cells from LPS-immunized mice mixed with PBS. Mice were killed 24 h after the 4th injection. In addition, LPS or PBS was injected into 3 SCID mice that were sacrificed before the 4th injection to measure RANKL expression before T cell injection. The left mandibles were removed, fixed in 4% paraformaldehyde in PBS at 4 °C for 6 h, decalcified in 10% ethylenediaminetetraacetic acid for 1 week and then embedded in paraffin using the AMeX method (acetone, methyl benzoate and xylene) [30]. The mesiodistal region of the left first molar was sliced into serial sections (4 μm thick).

Histochemical and immunohistological staining

Four groups of serial sections each comprising 10 subsections were obtained from all specimens. The first set of subsections from each group was stained with hematoxylin and eosin (HE) to histopathologically observe the alveolar bone surface. The second sets of subsections were stained with tartrate-resistant acid phosphatase (TRAP) to identify osteoclasts as TRAP-positive multinucleated cells on the bone surface [26] and then counterstained with hematoxylin. The third and fourth sets of subsections were immunohistologically stained to identify cells expressing CD3 and RANKL. Cells expressing RANKL were also examined in some sections from mice just before the 4th injection. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol for 30 min, followed by incubation with normal rabbit serum for 30 min at room temperature. These sections were then immersed in goat anti-mouse CD3 or RANKL polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight, followed by biotinylated rabbit anti-goat polyclonal antibody (Dako, Glostrup, Denmark) for 30 min, peroxidase-conjugated streptavidin (Dako) for 30 min, diaminobenzidine tetra-oxide for 3–10 min, and final counterstaining with hematoxylin.

Bone histomorphometry

Because quantifying total bone resorption is difficult, we measured the rate (%) of bone surface (regardless of the presence or absence of resorption lacunae) in intimate contact with osteoclasts (active resorption surface, ARS) to evaluate the progression of bone resorption [31]. We calculated the ratio of the ARS to the total points of intersection after counting the number of intersection points of the bone surface with the line of a micrometer (Olympus, Tokyo, Japan) in 25-μm graduations at ×400 magnification.

Immunohistomorphometry of RANKL-positive cells

The numbers of RANKL-positive and total cells in four areas measuring 250 × 500 μm on the surface of alveolar bone were counted using the micrometer and then the ratio of positive cells was calculated.

Osteoclast formation assay

CB17 mouse bone marrow cells were cultured in α-MEM (Gibco, Grand Island NY) containing 10% FBS, 100 μg/ml of streptomycin, 100 IU of penicillin (Gibco) and 5 ng/ml of mouse recombinant M-CSF (R & D Systems, Minneapolis MN) for 12 h in 100-mm dishes. Non-adherent cells were cultured with 30 ng/ml of M-CSF in 100-mm dishes for an additional 24 h. Non-adherent cells were washed out and adherent cells (BMMs) [32] (2×10^4/200 μl/well) were cultured for 3 days in α-MEM containing 10% FBS, antibiotics, 30 ng/ml of M-CSF and various concentrations of RANKL (R & D Systems) in 96-well dishes (Iwaki, Tokyo Japan). In another experiment, BMMs were cultured for 48 h with 30 ng/ml of M-CSF and 1 ng/ml of RANKL, and half of the medium was removed. The BMMs were then co-cultured