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Original Article

T cell and periosteum cooperation in osteoclastogenesis induced by lipopolysaccharide injection in transplanted mouse tibia

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Abstract *Background/purpose:* We previously reported that injedctions of lipopolysaccharide (LPS) into the gingiva of mice induce inflammatory bone resorption that actively involved T cells. Receptor activator of NF-κB ligand (RANKL), which is an essential factor for osteoclastogenesis, was reportedly produced by osteoblasts, fibroblasts, and T cells in vitro; however, it has not been established which cells affect osteoclastogenesis in vivo. Here we determined the roles of T cells and the periosteum on osteoclastogenesis in LPS-induced inflammatory bone resorption.

Materials and methods: Thirty-five BALB/c (wild-type: WT) and 10 BALB/c-nu/nu (nude: Nu) mice congenitally lacking T cells were used. Using inbred WT mice, tibias were transplanted with and without the periostea [(+) and (−), respectively, n = 15 per group] into the dorsal subcutaneous connective tissue of WT or Nu mice. Each group received four injections around the transplanted site: experimental groups were injected with LPS, and control groups were injected with phosphate-buffered saline. Isolated tissues were prepared for histopathological observation of the transplanted bone surface.

Results: Many infiltrating inflammatory cells were present near the surface of the tibias in the LPS-injected groups. Only the WT (+) LPS group showed osteoclasts. The number of mononuclear preosteoclasts and RANKL-positive cells was highest in the WT (+) LPS group, and there were no significant differences among the other three groups.

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Conclusion: T cells and the periosteum are closely involved in osteoclastogenesis in inflammatory bone resorption in vivo.

Introduction

Periodontitis is accompanied by attachment loss and alveolar bone resorption. We previously established an inflammatory bone resorption model using injections of lipopolysaccharide (LPS), which is the structural component of the cell wall of gram negative bacteria, into the gingiva of mice.[1] Using this model, we elucidated that osteoclasts appeared much later in nude (Nu) mice congenitally lacking T cells than in wild-type (WT) mice. Furthermore, osteoclastogenesis occurred earlier when T cells were reconstituted in Nu mice. Thus, T cells actively promote inflammatory bone resorption. Osteoclastogenesis requires receptor activator of NF-κB ligand (RANKL), which is a membrane-bound protein belonging to the tumor necrosis factor superfamily, as an essential factor.[2,3] While there have been authors who have reported that T cells produce soluble RANKL and promote osteoclastogenesis,[4,5] others have reported that RANKL produced by T cells has no effect on osteoclastogenesis.[6] The functional mechanism of T cells accelerating osteoclastogenesis has not been clarified in vivo. Osteoblasts existing on the bone surface in the periosteum have been reported to induce osteoclastogenesis by producing RANKL not only in physiological bone remodeling but also under inflammatory conditions.[7,8] In addition, connective tissue fibroblasts have been reported to induce osteoclastogenesis by expressing RANKL on the cell surface.[9] However, it is not known which cells affect osteoclastogenesis in vivo and whether T cells affect osteoblasts and/or fibroblasts in vivo.

Therefore, the present study examined the effects of T cells and the periosteum on osteoclastogenesis in LPS-induced inflammatory bone resorption. We transplanted inbred tibias with or without periostea into the dorsal subcutaneous connective tissue of WT and Nu mice, induced inflammatory bone resorption on the bone surface using LPS injections around the transplanted bone, and analyzed the histopathology. The results showed that multinuclear osteoclasts can be detected when both T cells and the periosteum were present but not when only the periosteum or T cells were present.

Materials and methods

Mice

Thirty-five 8-week-old male BALB/c (WT) and 10 BALB/c-nu/nu (Nu) mice were purchased from Nippon CLEA (Tokyo, Japan). The mice were maintained under specific pathogen-free conditions in Biomedical Research Center, Center for Frontier Life Sciences, Nagasaki University. The experimental procedures followed the ARRIVE guidelines, the National Institutes of Health guide for the care and use of Laboratory animals, as well as the Guidelines for Animal Experimentation of Nagasaki University. The experimental protocol was approved by the Local Institutional Animal Care and Use Committee of Nagasaki University.

Bone transplantation

The information of animal experimental schedule (date), groups, and sample size(n) was as follows (Fig. 1A). Both tibias of 15 WT mice were used for bone transplantation as follows: mice were sacrificed under ether anesthesia, and tibias were carefully removed. Fifteen tibias were used with the periosteum (+), and the other 15 tibias had the periosteum removed (−). The muscles around the (+) tibia were removed using scissors, and the periosteum was left intact. The muscle and the periosteum around the (−) tibia were completely removed using a scalpel. All processes were performed under aseptic conditions.

For bone transplantation, mice were intraperitoneally injected with combined anesthesia of medetomidine hydrochloride (0.75 mg/kg), midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg).[10] The dorsal skin of the WT mice was shaved prior to transplantation. After administering anesthesia, a 1-cm-long incision was made using scissors on the dorsal skin of all mice. Through the incision, subcutaneous connective tissue was detached from the fascia using a stripper. Thereafter, a (+) tibia was transplanted using tweezers to subcutaneous connective tissue in 10 WT mice and five Nu mice. In the remaining five WT and Nu mice, a (−) tibia was transplanted using the same method. There were four groups of mice: WT (+), WT (−), Nu (+), and Nu (−). Finally, the incisions were closed and sutured using nylon thread.

Five micrograms of *Escherichia coli* LPS (*E. coli* O111: B4; Sigma—Aldrich Corp., St Louis, MO, USA) dissolved in 3 μL of phosphate-buffered saline (PBS) was prepared for injection. LPS injections were administered as previously described.[1] Five mice from the each of the WT (+), WT (−), Nu (+), and Nu (−) groups received four injections of LPS at the area where the tibia was transplanted at 48-h intervals under ether anesthesia, thus creating the WT (+) LPS, WT (−) LPS, Nu(+) LPS, and Nu(−) LPS groups. The remaining 10 WT mice were injected with 3 μL of PBS as controls, forming the WT (+) PBS and WT (−) PBS groups. The PBS injections had not induced inflammatory cell infiltration and changed bone surface condition in the WT (+) PBS and WT (−) PBS groups. For this reason, we did not prepare the Nu (+) PBS and Nu (−) PBS groups. Four injections were used because our previous study indicated the appearance of tartrate-resistant acid phosphatase (TRAP)-positive

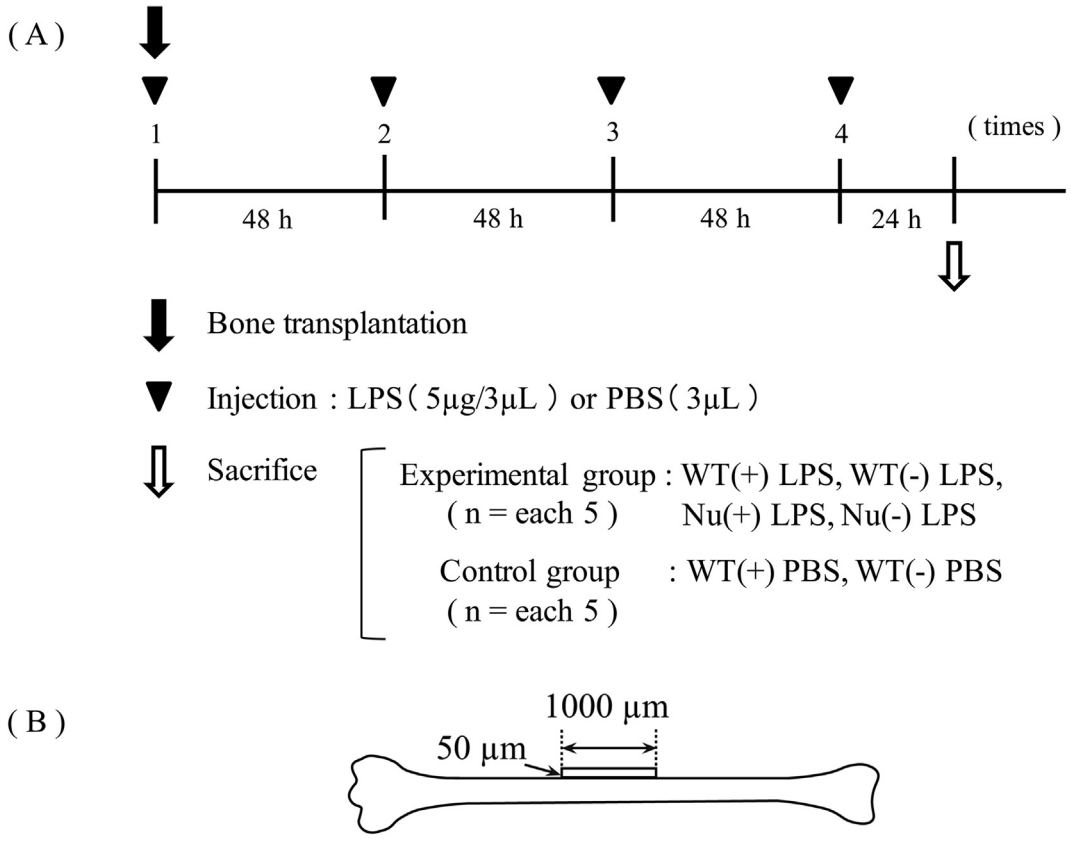

Figure 1 (A) The information of animal experimental schedule (date), groups, and sample size(n). (B) Schema of the tibial surface for histometric analysis. The number of TRAP-positive mononuclear and multinuclear cells, RANKL-positive cells, and CD3-positive cells was counted in a rectangular area (50 × 1000 μm) on the center of the tibia.

multinucleated cells induced by LPS.[1] All mice were sacrificed 24 h after the fourth (final) injection.

Tissue preparation

After sacrificing the mice, the tibias with subcutaneous connective tissue were immediately removed and fixed with 4% paraformaldehyde in PBS for 6 h and decalcified with 10% ethylenediaminetetraacetic acid for 1 week at 4 °C. Tissue samples were then embedded in paraffin using the AMeX method (acetone, methyl benzoate, and xylene).[11] Briefly, samples were fixed in acetone at −20 °C overnight, dehydrated in acetone at 4 °C for 15 min, dehydrated at room temperature for 15 min, cleared using methyl benzoate for 30 min, cleared using xylene for 30 min, penetrated with paraffin for 2 h, and then embedded. Fifty serial 4-μm-thick sections from a block were prepared to examine the longitudinal cross-section of the center of the tibia.

Histopathological and immunohistological staining

Fifty serial sections were divided into five subgroups of 10 subsections. The first subsection of each subgroup was stained with hematoxylin and eosin. The second subsections were stained with TRAP using the procedure described by Katayama et al. to count the number of TRAP-positive mononuclear and multinuclear cells.[12] Briefly, a staining solution was made by mixing 0.5 mL of pararosaniline 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—Aldrich Corp.), 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 each 10-mL aliquot of the staining solution. Sections were incubated in the staining solution for 30 min at 37 °C and then counterstained with hematoxylin. The third subsections from the four LPS-injected groups were used for the immunostaining of RANKL-expressing cells, which enabled the number of these cells near the bone surface to be counted. The fourth subsections from the WT (+) LPS and WT (−) LPS groups were used for immunostaining T cells to count the number of cells around connective tissue. Serial subsections were deparaffinized, and endogenous peroxidase activity was blocked with 0.3% H_2O_2—methanol for 30 min, followed by incubation with normal goat serum (1:20; Dako Cytomation Denmark A/S, Glostrup, Denmark) for 30 min at room temperature. Sections were then immersed in rabbit anti-mouse RANKL polyclonal antibody (1:200; Acris Antibodies, Rockville, MD, USA) and CD3 polyclonal antibody (1:200; Abcam, Cambridge, UK) at 4 °C overnight. Sections were then incubated with biotinylated goat anti-rabbit polyclonal antibody (1:400; Dako Cytomation Denmark A/S) for 30 min at room temperature and then incubated with

peroxidase-labeled streptavidin (KPL, Gaithersburg, MD, USA) for 30 min, followed by incubation with a diaminobenzidine tetroxide solution. Lastly, the sections were counterstained with hematoxylin.

Histological measurement of transplanted bone

A rectangular area of the center of each tibia ($50 \times 1000\,\mu m$) was selected for histometric observations (Fig. 1B). The measurement range was set $50\,\mu m$ from the bone surface because the thickness of the periosteum for each section was less than $50\,\mu m$. The number of TRAP-positive mononuclear and multinuclear cells, RANKL-positive cells, and CD3-positive cells in these areas was counted.

Statistics

StatMate IV (ATMS Co., Ltd., Tokyo, Japan) was used for all statistical analyses. Differences among groups were calculated by one-factor analysis of variance with the Tukey–Kramer test. *P*-values of <0.01 were considered significant.

Results

Histopathological findings

Many infiltrating inflammatory cells, including neutrophils and macrophages, were present in the four LPS-injected groups (Fig. 2A–D). Inflammatory cells infiltrated the periosteum in the WT (+) LPS and Nu(+) LPS groups (Fig. 2A and C), and the tibias of the WT (+) LPS group showed irregular surfaces. In contrast, the PBS-injected groups showed little inflammatory infiltration and smooth tibial surfaces (Fig. 2E and F).

TRAP staining

Some TRAP-positive mononuclear and multinuclear cells were present on the surface of the tibias in the WT (+) LPS group (Fig. 3A). In contrast, only TRAP-positive mononuclear cells were present in the WT (−) LPS and Nu (+) LPS groups (Fig. 3B and C). The Nu (−) group showed few TRAP-positive mononuclear cells (Fig. 3D). There were no TRAP-positive cells in the PBS-injected groups (data not shown).

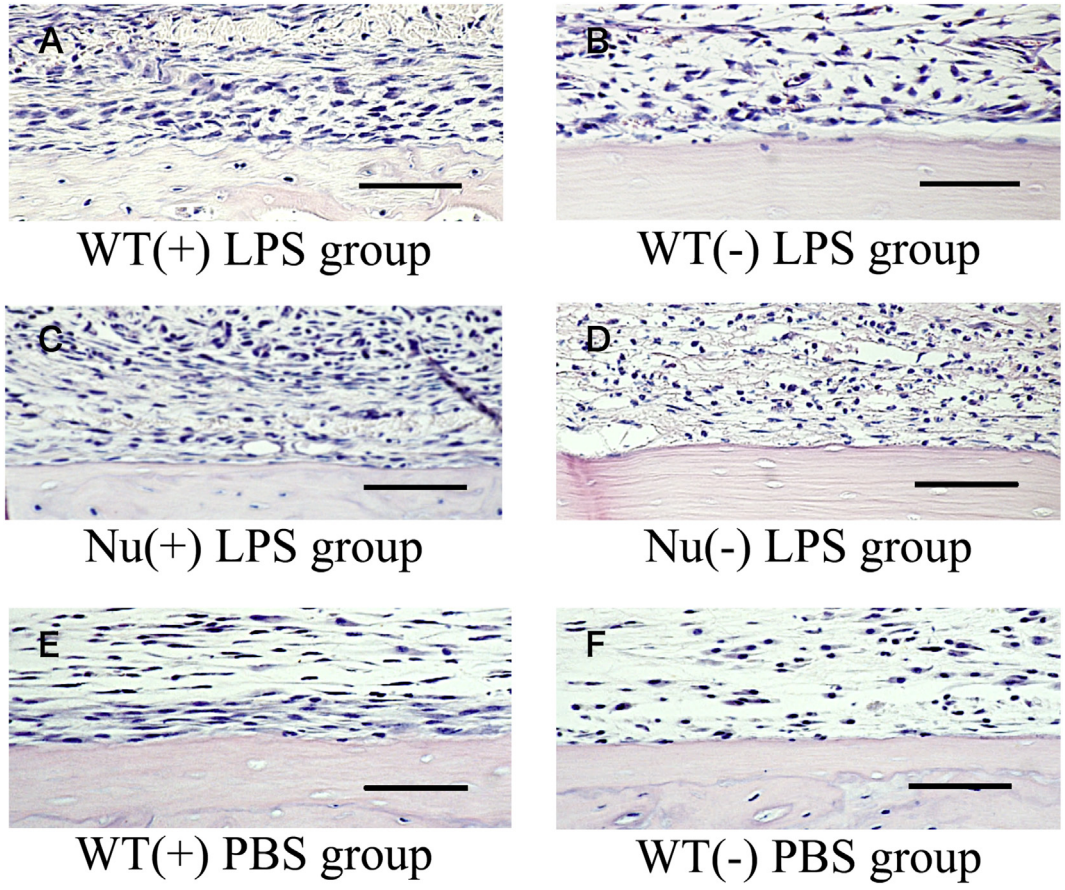

Figure 2 Histopathological findings on the tibial surface. Hematoxylin and eosin staining of specimens from the LPS-injected groups (A–D) and PBS-injected groups (E and F). (A–D) Many inflammatory cells infiltrated the four LPS-injected groups. (E and F) A few inflammatory cells were observed in the PBS-injected groups. Scale bars represent $100\,\mu m$.

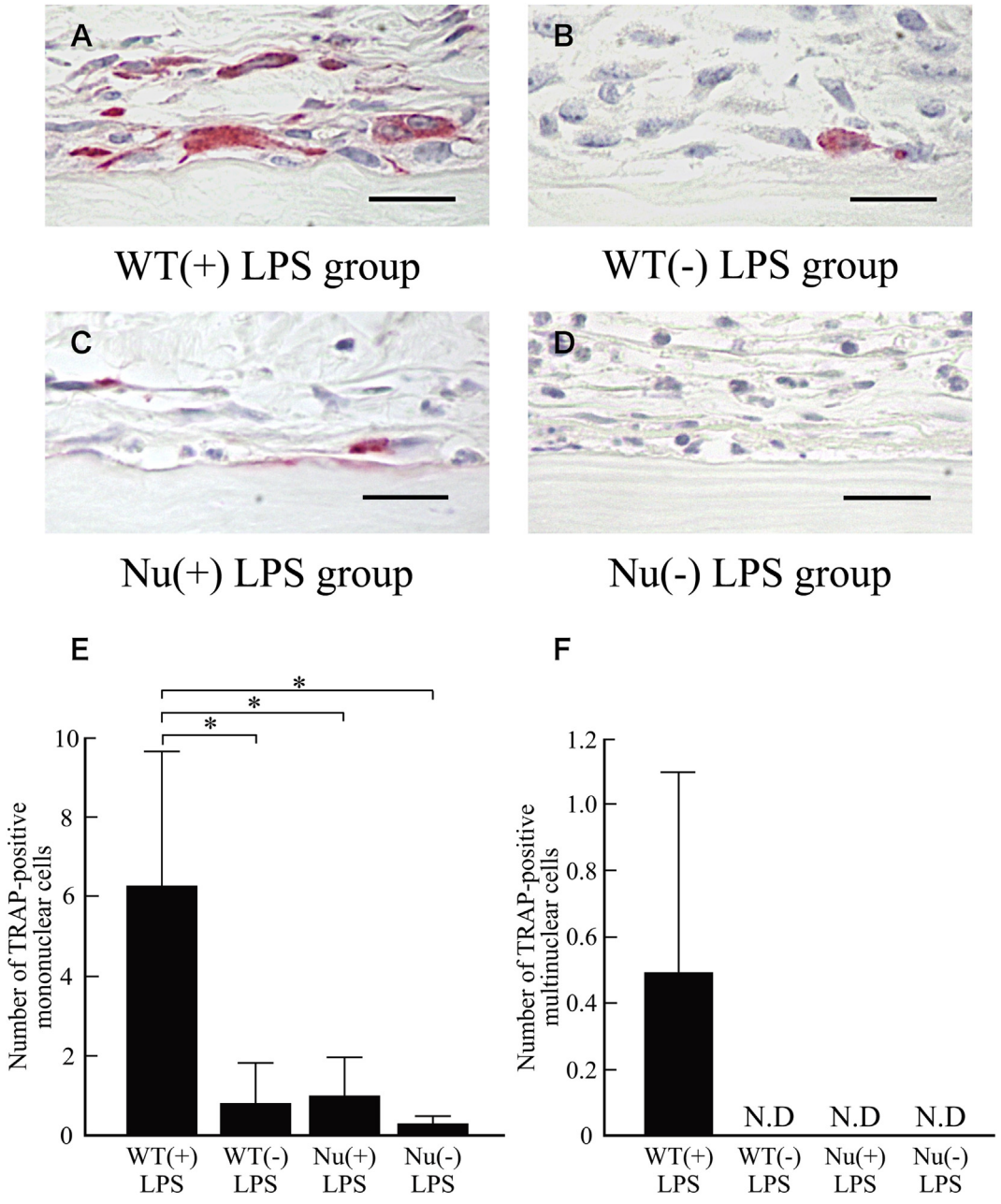

Figure 3 Histopathological findings of TRAP staining (A–D), and the number of TRAP-positive mononuclear and multinuclear cells (E and F). (A) TRAP-positive mononuclear and multinuclear cells were observed in the WT (+) LPS group. (B and C) The WT (−) LPS and Nu(+) LPS groups showed only TRAP-positive mononuclear cells. (D) The Nu (−) LPS group showed few TRAP-positive mononuclear cells. (E) The number of TRAP-positive mononuclear cells was highest in the WT (+) LPS group. (F) TRAP-positive multinuclear cells were only observed in the WT (+) LPS group. Scale bars represent 25 μm. Bars represent mean ± SD. *$P < 0.01$ vs. WT (+) LPS group. ND, not detectable.

The number of TRAP-positive mononuclear cells on the tibia was highest in the WT (+) LPS group (6.08 ± 3.31) (Fig. 3E). The WT (−) LPS and Nu (+) LPS groups showed similar numbers of TRAP-positive mononuclear cells (0.72 ± 1.02 and 0.88 ± 0.99, respectively). There were few TRAP-positive mononuclear cells in the Nu (−) LPS group (0.15 ± 0.33), whereas only TRAP-positive multinuclear cells were observed in the WT (+) LPS group (0.61 ± 0.60) (Fig. 3F).

RANKL immunostaining

RANKL-positive cells appeared in all four LPS-injected four groups (Fig. 4A–D). Cuboid and spindle-shaped RANKL-positive cells were observed near the tibial surface. A few round RANKL-positive cells were observed in the WT (+) LPS and WT (−) LPS groups. The number of RANKL-positive cells was highest in the WT (+) LPS group (24.01 ± 7.41) (Fig. 4E). There was no significant difference in the number

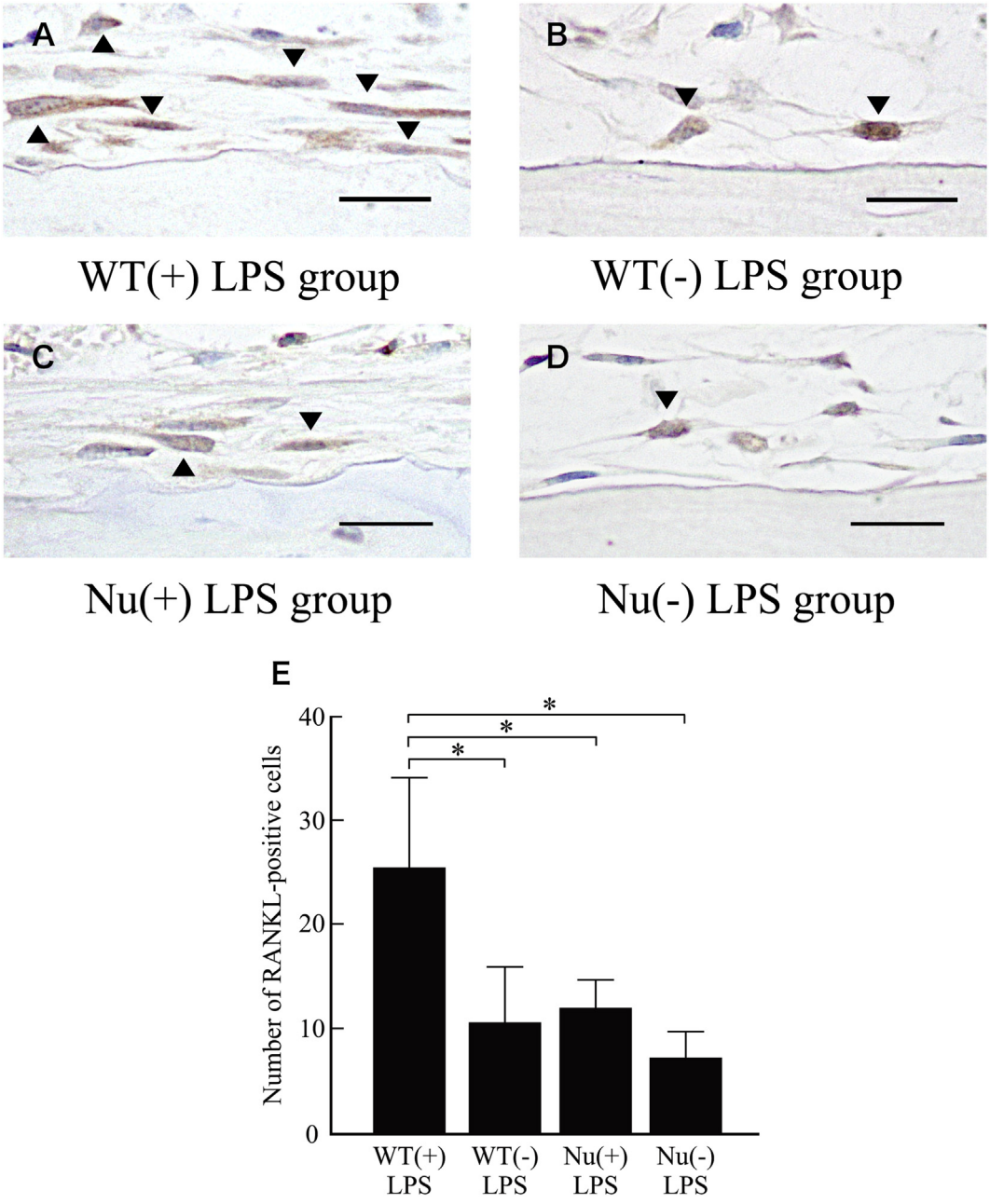

Figure 4 Immunohistological findings (A—D), and the number of RANKL-positive cells (E). (A—D) Arrowheads indicate RANKL-positive cells. (E) The number of RANKL-positive cells was highest in the WT (+) LPS group. Scale bars represent 25 μm. Bars represent mean ± SD. *$P < 0.01$ vs. WT (+) LPS group.

of RANKL-positive cells in the WT (−) LPS (9.92 ± 7.41), Nu (+) LPS (11.12 ± 2.62), and Nu (−) LPS (6.63 ± 2.68) groups.

CD3 immunostaining

CD3-positive cells were observed in the WT (+) LPS and WT (−) LPS groups (Fig. 5A and B). There were some CD3-positive cells near the surface of the tibia in the WT (+) LPS group but few in the WT (−) LPS group. The number of CD3-positive cells in the WT (+) LPS group (6.92 ± 3.35) was significantly higher than that in the WT (−) LPS group (0.86 ± 0.86) (Fig. 5C).

Discussion

In the present study, we transplanted inbred tibias with or without periostea into dorsal subcutaneous connective tissue in WT and Nu mice and injected LPS around the transplanted bone site. We previously reported that TRAP-positive multinuclear cells occurred considerably earlier in WT mice than in Nu mice when LPS was injected into the gingiva.[1] In the present study, TRAP-positive multinuclear cells appeared earlier in the WT (+) LPS group than in the Nu (+) LPS group. Both results showed that TRAP-positive multinuclear cells appeared in a similar

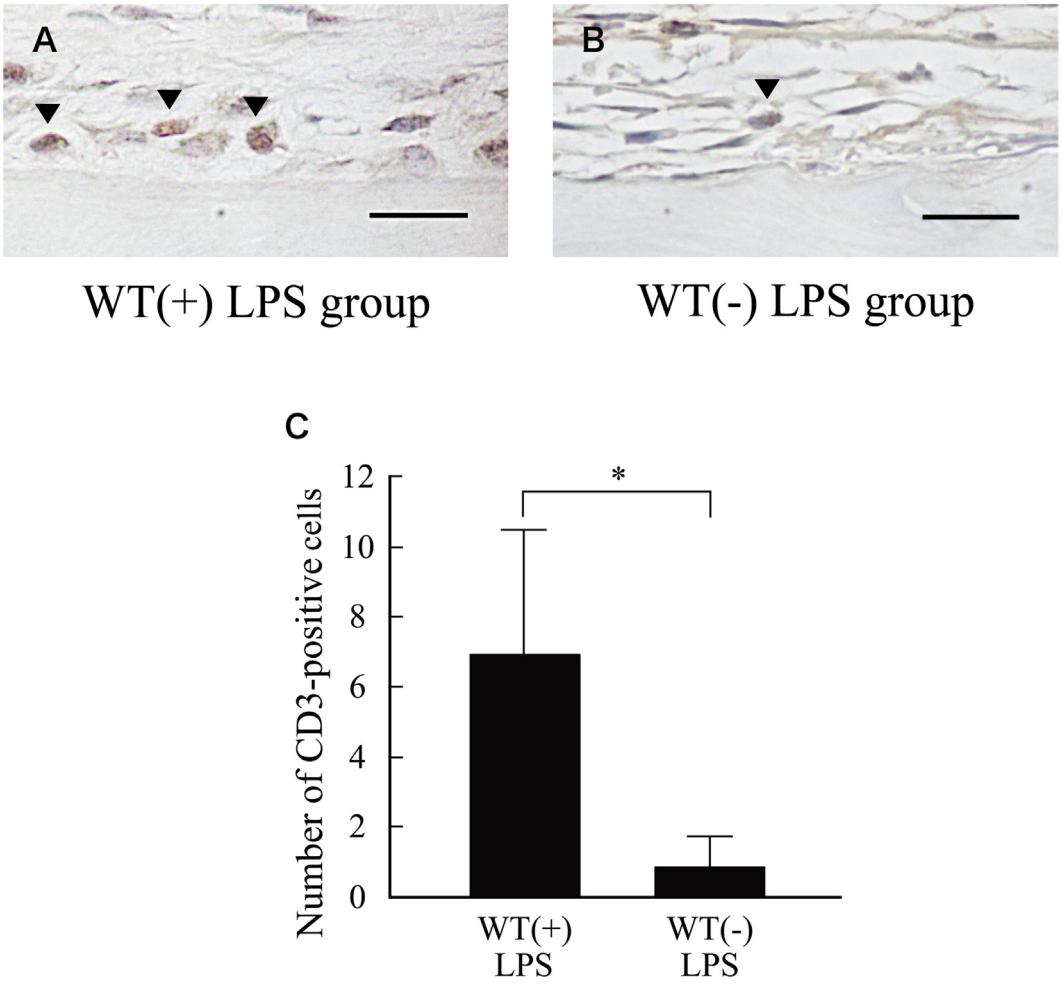

Figure 5 Immunohistological findings (A and B), and the number of CD3-positive cells (C). (A and B) Arrowheads indicate CD3-positive cells. (C) The number of CD3-positive cells in the WT (+) LPS group was significantly higher than that in the WT (−) LPS group. Scale bars represent 25 μm. Bars represent mean ± SD. *$P < 0.01$ vs. WT (+) LPS group.

timing and that osteoclastogenesis can be induced in the dorsal subcutaneous tissue via LPS injection. Furthermore, T cells involved in osteoclastogenesis were promoted in the same area.

We compared the WT (+) LPS and the WT (−) LPS groups to evaluate osteoclastogenesis in periostea with osteoblasts and connective tissue lacking osteoblasts. RANKL is an essential factor for osteoclastogenesis and is expressed by osteoblasts and fibroblasts on the cell surface.[2,3,7–9] LPS binds to Toll-like receptor 4 (TLR4) on the cell surface, and osteoblasts and fibroblasts express TLR4 on their cell surfaces.[13,14] Kikuchi et al. reported that osteoblasts stimulated by *E. coli* LPS induce RANKL expression in vitro.[15] Human fibroblasts stimulated with LPS also produce RANKL in vitro.[16] Although there are no similar reports on studies conducted in vivo, it is likely that osteoblasts and fibroblasts stimulated by LPS express RANKL during inflammatory bone resorption. In the present study, we observed RANKL-positive cells in the periosteum and connective tissue. Furthermore, the number of RANKL-positive cells around the tibia was highest in the WT (+) LPS group. In the same group, only TRAP-positive multinuclear cells were observed; the same group showed the highest number of these cells among all groups. Osteoclastogenesis induced by osteoblasts was significantly higher than that induced by fibroblasts in vitro.[17] We could not confirm the presence of osteoblasts in the periosteum; however, we speculated that RANKL expressed in osteoblasts is an important factor because osteoblasts were present in the periosteum but not connective tissue. Although it is important to measure the cell density of RANKL-positive cells in the periosteum as well as the concentration of RANKL, our results suggested that under our study conditions, the periosteum is more important in osteoclastogenesis than connective tissue.

Finally, we considered the effects of T cells and osteoblasts on osteoclastogenesis. There was no significant difference in the number of TRAP-positive mononuclear and RANKL-positive cells between the Nu (+) LPS, Nu (−) LPS, and WT (−) LPS groups. No significant differences were found between the Nu (+) LPS and Nu (−) LPS groups, indicating that the difference in osteoclastogenesis does not depend only on the presence or absence of the periosteum. Furthermore, no significant differences between the Nu (−) LPS and WT (−) LPS groups indicated that the difference in osteoclastogenesis does not depend only on the presence or absence of T cells. The number of TRAP-positive mononuclear

and RANKL-positive cells in the WT (+) LPS group was significantly higher than that in the WT (−) LPS and Nu (+) LPS groups. A previous study has shown that Th17, which is a subset of T-helper lymphocytes, increased RANKL expression in osteoblasts.[18] In the present study, it was not elucidated how T cells stimulated osteoblasts during osteoclastogenesis; however, we speculated that T-cell infiltration around the tibia stimulated osteoblasts in the periosteum and accelerated osteoclastogenesis.

In conclusion, both T cells and the periosteum are closely involved in osteoclastogenesis during inflammatory bone resorption in vivo.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

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References

1. Ukai T, Hara Y, Kato I. Effects of T cell adoptive transfer into nude mice on alveolar bone resorption induced by endotoxin. *J Periodontal Res* 1996;31:414—22.
2. Lacey DL, Timms E, Tan HL, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998;93:165—76.
3. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. *J Bone Miner Res* 1996;15:2—12.
4. Kong YY, Feige U, Sarosi I, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 1999;402:304—9.
5. Kanamaru F, Iwai H, Ikeda T, Nakajima A, Ishikawa I, Azuma M. Expression of membrane-bound and soluble receptor activator of NF-kappaB ligand (RANKL) in human T cells. *Immunol Lett* 2004;94:239—46.
6. Takayanagi H, Iizuka H, Juji T, et al. Involvement of receptor activator of nuclear factor κB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheumatol* 2000;43:259—69.
7. Capulli M, Paone R, Rucci N. Osteoblast and osteocyte: games without frontiers. *Arch Biochem Biophys* 2014;561:3—12.
8. Udagawa N, Takahashi N, Jimi E, et al. Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor: receptor activator of NF-kappa B ligand. *Bone* 1999;25:517—23.
9. Nakashima T, Hayashi M, Fukunaga T, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med* 2011;17:123—4.
10. Kawai S, Takagi Y, Kaneko S, Kurosawa T. Effect of three types of mixed anesthetic agents alternate to ketamine in mice. *Exp Anim* 2011;60:481—7.
11. Sato Y, Mukai K, Watanabe S, Goto M, Shimosato Y. The AMeX method. A simplified technique of tissue processing and paraffin embedding with improved preservation of antigens for immunostaining. *Am J Pathol* 1986;125:431—5.
12. Katayama I, Li CY, Yam LT. Histochemical study of acid phosphatase isoenzyme in leukemic reticuloendotheliosis. *Cancer* 1972;29:157—64.
13. Takeuchi O, Hoshino K, Kawai T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999;11:443—51.
14. Hatakeyama J, Tamai R, Sugiyama A, Akashi S, Sugawara S, Takada H. Contrasting responses of human gingival and periodontal ligament fibroblasts to bacterial cell-surface components through the CD14/Toll-like receptor system. *Oral Microbiol Immunol* 2003;18:14—23.
15. Kikuchi T, Matsuguchi T, Tsuboi N, et al. Gene expression of osteoclast differentiation factor is induced by lipopolysaccharide in mouse osteoblasts via Toll-like receptors. *J Immunol* 2001;166:3574—9.
16. Wada N, Maeda H, Yoshimine Y, Akamine A. Lipopolysaccharide stimulates expression of osteoprotegerin and receptor activator of NF-kappa B ligand in periodontal ligament fibroblasts through the induction of interleukin-1 beta and tumor necrosis factor-alpha. *Bone* 2004;35:629—35.
17. Costa-Rodrigues J, Teixeira CA, Sampaio P, Fernandes MH. Characterisation of the osteoclastogenic potential of human osteoblastic and fibroblastic conditioned media. *J Cell Biochem* 2010;109:205—16.
18. Sato K, Suematsu A, Okamoto K, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med* 2006;203:2673—82.