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Membrane-bound CD40 ligand on T cells from mice injected with lipopolysaccharide accelerates lipopolysaccharide-induced osteoclastogenesis

M. Yokoyama, T. Ukai, E. R. Ayon Haro, T. Kishimoto, Y. Yoshinaga, Y. Hara

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

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Background and Objective: T cells infiltrate the inflammatory site of periodontitis and consequently stimulate the loss of periodontal bone. We previously reported that T cells from lipopolysaccharide (LPS)-injected mice (LPS-T cells) accelerated osteoclastogenesis in the presence of LPS. However, the detailed mechanism of this acceleration is still unclear. In this study, we analyzed the mechanism of osteoclastogenesis accelerated by LPS-T cells.

Material and Methods: We examined the mechanism of osteoclastogenesis acceleration. First, to determine the effect of cell-to-cell contact, we co-cultured T cells and bone marrow macrophages, prestimulated with RANKL for 48 h (R-BMMs), in the presence of LPS for 24 h, in a Transwell. Second, to determine the effect of CD40 ligand (CD40L), we co-cultured T cells and R-BMMs in the presence of LPS and anti-CD40L immunoglobulin. Third, we examined the effect of recombinant mouse CD40L (rCD40L) in the presence of LPS *in vitro* and *in vivo*. Lastly, we examined the expression of membrane-bound CD40L (mCD40L) by fluorescence-activated cell sorting (FACS).

Results: Blocking cell-to-cell contact between LPS-T cells and R-BMMs completely inhibited the acceleration of osteoclastogenesis. Anti-CD40L immunoglobulin also completely inhibited the acceleration of osteoclastogenesis. Moreover, rCD40L accelerated osteoclastogenesis in the presence of LPS *in vitro* and *in vivo*. Finally, the expression of mCD40L on LPS-T cells was higher than that on T cells isolated from mice not injected with LPS.

Conclusion: The results demonstrate that CD40L accelerates osteoclastogenesis in the presence of RANKL and LPS. The results also suggest that mCD40L on LPS-T cells accelerates osteoclastogenesis.

Takashi Ukai, DDS, PhD, Unit of Translational Medicine, Department of Periodontology, 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: ukai@nagasaki-u.ac.jp

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Periodontitis is chronic inflammation induced by bacterial infection. Alveolar bone resorption is one of the characteristics of periodontitis (1), and osteoclasts are the principal type of cell involved in alveolar bone resorption. Macrophage colony-stimulating factor (M-CSF) and RANKL are essential for the differentiation of osteoclasts (2,3). Lipopolysaccharide (LPS) is the major component of the outer membrane of gram-negative bacteria and shows many biological activities (4,5). A previous study suggested that LPS accelerates osteoclastogenesis in the presence of RANKL (6), and it has also been shown that LPS can induce osteoclast formation in RAW cells in the absence of RANKL (7). T cells probably participate in osteoclastogenesis (8). Previous studies have suggested that T cells stimulated with anti-CD3 immunoglobulin and anti-CD28 immunoglobulin produce soluble RANKL and accelerate osteoclastogenesis (9,10). We previously investigated the effect of activated T cells, isolated from LPS-injected mice (LPS-T cells), on the acceleration of osteoclastogenesis, and we found that LPS-T cells showed significantly increased osteoclastogenesis *in vitro* and *in vivo*. Tumor necrosis factor-α (TNF-α) immunoglobulin, but not osteoprotegerin (a decoy receptor of RANKL), completely inhibited this increase *in vitro* (11). The results suggested that LPS-T cells require TNF-α for accelerating osteoclastogenesis. However, we found that the concentration of TNF-α in the media of osteoclast precursors co-cultured with LPS-T cells was not different from the concentration of TNF-α in the culture media without T cells. This suggests that activated LPS-T cells do not produce TNF-α. Although TNF-α is essential for accelerating osteoclastogenesis by LPS-T cells in our culture system, other factors might also be required for the acceleration; however, the detailed mechanism of this is still unclear. The aim of this study was to determine the acceleratory mechanism of osteoclastogenesis in LPS-T cells.

Material and methods

Mice

Male CB-17 (CB-17/Icr-$^{+/+}$ Jcl) and severe combined immunodeficient (SCID) (CB-17/Icr-scid Jcl) mice, purchased from Nihon Clea (Tokyo, Japan), were maintained under specific pathogen-free conditions at the Biomedical Research Center for Frontier Life Sciences, Nagasaki University. Animal care and experiments were carried out according to the Guidelines for Animal Experimentation of Nagasaki University and with the approval of the Institutional Animal Care and Use Committee.

Isolation and activation of spleen T cells

T cells were isolated from the spleens of CB-17 mice that had received 13 injections of 5 μg of *Escherichia coli* LPS (O111:B4; Sigma, St Louis, MO, USA), in a 3-μL volume, into the left mandible every 48 h (11). T cells were also obtained from the spleens of age-matched non-LPS-injected mice. Whole spleen-cell suspensions were prepared after lysing red cells with NH_4Cl. T cells were isolated by standard negative selection using StemSep™ magnetic separation (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. T cells isolated from the LPS-injected mice and from the non-LPS-injected mice were activated with phytohemagglutinin (PHA) (5 μg/mL; Sigma) and interleukin-2 (IL-2) (10 ng/mL; Sigma) for 24 h in RPMI containing 10% fetal bovine serum. The T cells isolated from these two groups of mice were denoted as LPS-T cells and control-T cells respectively.

Osteoclast formation assay

Bone marrow cells of 5-wk-old CB-17 mice were cultured in alpha minimal essential medium (α-MEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum, 100 μg/mL of streptomycin, 100 IU of penicillin (Gibco) and 5 ng/mL of mouse recombinant M-CSF (R&D Systems, Minneapolis, MN, USA) for 12 h in 100-mm dishes. Nonadherent cells were cultured with 30 ng/mL of M-CSF in 100-mm dishes for an additional 36 h. Nonadherent cells were washed out, and adherent cells [bone marrow macrophages (BMMs)] (12) (2×10^4 cells/200 μL/well) were cultured for 48 h with 30 ng/mL of M-CSF and 1 ng/mL of RANKL in a 96-well plate. The RANKL pre-stimulated BMMs (R-BMMs) were cultured with LPS-T cells (2×10^5 cells/well), with control-T cells or without T cells in the presence of 5 ng/mL of LPS for 24 h. For blocking the contact between R-BMMs and T cells, the cells were co-cultured in a Transwell® apparatus (Corning, Lowell, MA, USA) that has a 0.4-μm porosity membrane. R-BMMs (1.2×10^6 cells/well) in the bottom well and T cells (1.2×10^7 cells/well) in the upper well were cultured with 5 ng/mL of LPS for 24 h.

To determine the effect of CD40 ligand (CD40L) on the acceleration of osteoclastogenesis by LPS-T cells, R-BMMs and T cells were co-cultured in the presence of 10 μg/mL of anti-CD40L immunoglobulin (eBioscience, San Diego, CA, USA) or 10 μg/mL of the isotype-control antibody (eBioscience) with 5 ng/mL of LPS for 24 h. Moreover, R-BMMs were cultured with both recombinant mouse CD40L (rCD40L; R&D Systems) and LPS for 24 h in order to determine the effect of CD40L on osteoclastogenesis. To determine the effect of TNF-α on the acceleration of osteoclastogenesis by rCD40L, R-BMMs were incubated with or without anti-mouse TNF-α immunoglobulin (R&D) in the presence of LPS and rCD40L. In all experiments, the cells were fixed in 4% paraformaldehyde and stained with tartate-resistant acid phosphatase (TRAP; Sigma) to identify osteoclasts, defined as TRAP-positive multinucleated (more than three unstained nuclei) cells. Osteoclasts were counted under a microscope.

Resorption assay

R-BMMs were cultured in BD BioCoat™ Osteologic™ Bone Cell Culture

System (BD Biosciences, Bedford, MA, USA). R-BMMs were cultured in the presence or absence of 5 ng/mL of LPS, with or without 100 ng/mL of rCD40L. After 48 h, we removed half of the medium and replaced it with fresh medium. All reagents and cytokines were renewed. To measure the resorption activity of the osteoclasts, after 72 h of culture, we stained the cells with the von Kossa stain and counted the resorption pits, according to the instructions of the system. In the positive control, R-BMMs were cultured with 10 ng/mL of RANKL. Many clear resorption pits were observed in the test culture (Figure S1).

ELISA of cytokines

For detection of cytokines, we collected the supernatant after 3, 6 and 24 h of co-culture of R-BMMs and T cells. The concentration of TNF-α in the supernatant was determined using a DuoSet® ELISA Development System (R&D Systems). The concentration of soluble CD40L (sCD40L) in the supernatant was determined using a PromoKine® ELISA kit (Promocell, Heidelberg, Germany). The examinations were performed according to the instructions of the manufacturers.

PCR amplification of reverse-transcribed mRNA

For RT-PCR analysis, we isolated the total RNA from adherent cells or from T cells, after co-culture for 3 or 6 h, using an RNeasy® Mini kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized from the total RNA using random hexamers and subjected to PCR amplification with EX *Taq* polymerase (Takara Biochemicals, Shiga, Japan) using the following specific PCR primers (Sigma): mouse TNF-α, forward 5′-ATG AGCACAGAAAGCATGATCCGC GAC-3′ and reverse 5′-TCACAGAGC AATGACTCCAAAGTAGACCTG-3′; mouse TNF Receptor1 (TNFR1), forward 5′-CCGGG CCACCTGGTCC G-3′ and reverse 5′-CAAGTAGGTTC CTTTGTG-3′; mouse TNFR2, forward 5′-GTCGCGCTGGTCTTCGA ACTG-3′ and reverse 5′-GGTATACA TGCTTGCCTCA CAGTC-3′; mouse β-actin, forward 5′-GATGACGATA TCGCTGCGCTG-3′ and reverse 5′-G TACGACCAGAGGCATACAGG-3′; and mouse CD40L, forward 5′-ATA CCCACAGTTCCTCCCAGCTTT-3′ and reverse 5′-CACTTGGCTTGCTT CAGTCACGTT-3′. The PCR products were separated by electrophoresis on a 2% agarose gel and were visualized by ethidium bromide staining and illumination under ultraviolet light. We determined the expression of mRNA using ImageJ (NIH, Bethesda, MD, USA).

Flow-cytometric analysis

The expression of membrane-bound CD40L (mCD40L) on T cells before co-culture, and 6 and 24 h after co-culture, was confirmed by flow cytometry. T cells were incubated with either fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-mouse CD40L (Southern Biotech, Birmingham, UK) or FITC-conjugated isotype - control antibody (PharMingen, San Diego, CA, USA). To check the expression of CD40 on R-BMMs using flow cytometry, R-BMMs were incubated with phycoerythrin-conjugated monoclonal anti-mouse CD40 (eBioscience) or with phycoerythrin-conjugated isotype control antibody (eBioscience) after pre-incubation with the anti-mouse CD16/32 immunoglobulin, which blocks the Fc receptors (eBioscience). These cells were analyzed using a FACScan™ with CellQuest™ software (Becton Dickinson, Mountain View, CA, USA).

Preparation of tissues

Twenty SCID mice, at 7 wk of age, were assigned to the following two groups: rCD40L-stimulated mice and phosphate-buffered saline (PBS)-stimulated mice. All mice, under ether anesthesia, received four injections of 5 μg of *E. coli* LPS (in 3 μL of PBS), at 48-h intervals, into the mesial gingiva of the first molar of the left mandible. rCD40L mice were injected with 5 μg of rCD40L (in 5 μL of PBS) together with the fourth injection of LPS. PBS mice were injected with PBS together with the fourth injection of LPS. The mice were killed 24 h after the fourth injection of LPS. The left mandibles were removed, fixed in 4% paraformaldehyde at 4°C for 6 h, decalcified in 10% EDTA for 1 wk and then embedded in paraffin using the AMeX method (acetone, methyl benzoate and xylene) (13). The mesiodistal region of the left first molar was sliced into serial, 4-μm-thick sections.

Histochemical staining

Two groups of serial sections, each comprising 10 subsections, were obtained from all specimens. The first set of subsections from each group was stained with haematoxylin and eosin to histopathologically observe the alveolar bone surface. The second set of subsections was stained with TRAP to identify osteoclasts as TRAP-positive multinucleated cells on the alveolar bone surface (14) and then counterstained with haematoxylin.

Bone histomorphometry

As quantifying total alveolar bone resorption is difficult, the alveolar bone surface (regardless of the presence or absence of resorption lacunae) in intimate contact with osteoclasts [i.e. the active resorption surface (ARS)] was examined to evaluate the progression of alveolar bone resorption (15).We calculated the ratio of ARS to the total points of intersection after counting the number of intersection points of the alveolar bone surface with the line of a micrometer (Olympus, Tokyo, Japan) in 25-μm graduations at ×400 magnification.

Statistics

Data were statistically analyzed using Statmate software (Abacus Concepts Inc., Berkeley, CA, USA). Differences among groups were assessed using one-factor analysis of variance with the Tukey–Kramer test. $p < 0.05$ was considered statistically significant.

Results

Concentration of TNF-α and expression of *TNF*-α and *TNFR* mRNAs were not significantly different among culture groups

We previously demonstrated that LPS-T cells significantly accelerated osteoclastogenesis in the presence of LPS, and we reported that acceleration of osteoclastogenesis by LPS-T cells needed TNF-α because the acceleration was completely inhibited by anti-TNF-α immunoglobulin (11). However, the detailed mechanism of this was not clear. In the present study, we examined, using ELISA, the concentration of TNF-α in the supernatant after co-culture of R-BMMs with T cells for 6 h, and we isolated total RNA from adherent cells or from T cells after co-culture for 6 h and examined, by RT-PCR, the expression of mRNA for *TNF*-α, *TNFR1* and *TNFR2*. No significant difference in the concentration of TNF-α or in the expression of mRNA for *TNF*-α was found among the culture groups in the presence of LPS (Fig. 1A). We confirmed no difference in the concentration among the three groups at 3 and 24 h of culture (Figure S2). TNF-α was not detected in any of the culture groups in the absence of LPS at any time-point (data not shown). There was also no difference in the expression of *TNFR1* or *TNFR2* mRNAs among the culture groups (Fig. 1B). Moreover, the concentration of RANKL was not significantly different among the culture groups (data not shown).

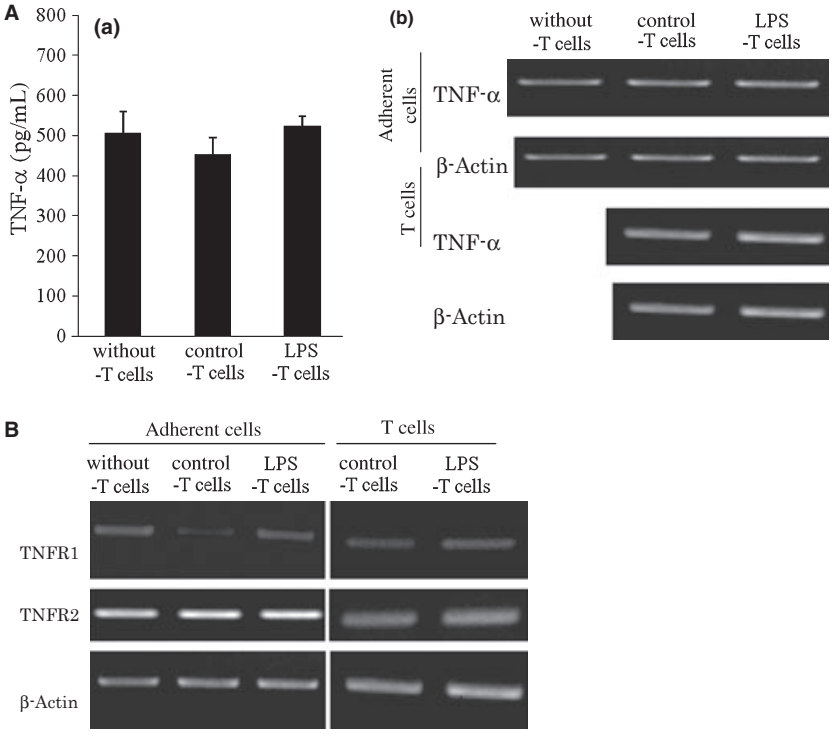

Fig. 1. Concentration of tumor necrosis factor-α (TNF-α) and expression of *TNF*-α mRNA and tumor necrosis factor receptor (*TNFR*) mRNA. (A-a) Bone marrow macrophages (BMMs) were cultured with 30 ng/mL of macrophage colony-stimulating factor (M-CSF) and 1 ng/mL of RANKL. Half of the medium was removed after 48 h and the RANKL pre-stimulated BMMs (R-BMMs) were co-cultured with 100 μL of control-T cells or LPS-T cells (2×10^5 cells/well), or without T cells, together with 5 ng/mL of *Escherichia coli* lipopolysaccharide (LPS). The supernatant was collected and the concentration of TNF-α at 6 h was measured by ELISA. Bars represent means ± standard deviation. Data are representative of three independent experiments. (A-b) Total RNAs of adherent cells and T cells were isolated at 6 h and the expression of *TNF*-α mRNA was examined. Data are representative of three independent experiments. (B) Total RNAs of adherent cells and T cells were collected at 6 h and the expression of *TNFR1* and *TNFR2* mRNAs was examined. Data are representative of three independent experiments.

Blocking the cell-to-cell contact between R-BMMs and LPS-T cells completely inhibited osteoclastogenesis acceleration

We also investigated the effect of the cell-to-cell contact between R-BMMs and LPS-T cells. The number of osteoclasts significantly decreased when R-BMMs were co-cultured with LPS-T cells in a Transwell apparatus compared with co-culture in a normal well. Furthermore, the number of osteoclasts present when R-BMMs were co-cultured with LPS-T cells in a Transwell was similar to the number of osteoclasts found when R-BMMs were co-cultured with control-T cells or without T cells in a normal well or a Transwell (Fig. 2A). These results are confirmed in Fig. 2B: many osteoclasts were observed when R-BMMs were co-cultured with LPS-T cells in a normal well (Fig. 2B-e). However, the number of osteoclasts decreased when R-BMMs were co-cultured with LPS-T cells in a Transwell (Fig. 2B-f).

Anti-CD40L immunoglobulin completely inhibited osteoclastogenesis acceleration by LPS-T cells

We assumed that the cell-to-cell contact is related to the acceleration of osteoclastogenesis by LPS-T cells because the acceleration was inhibited by blocking cell-to-cell contact of R-BMMs and T cells. Activated T cells express mCD40L and make contact with cells through CD40. CD40 is expressed on macrophages and on pre-osteoclasts. Activation of CD40 induces binding to TNF receptor-associated factor 6 (TRAF6), which is an essential adaptor protein of osteoclastogenesis. Therefore, we speculated that CD40 activation can induce the acceleration of osteoclastogenesis. We co-cultured R-BMMs and T cells in the presence of an anti-CD40L immunoglobulin for 24 h to block contact of CD40 with CD40L. The anti-CD40L immunoglobulin completely inhibited

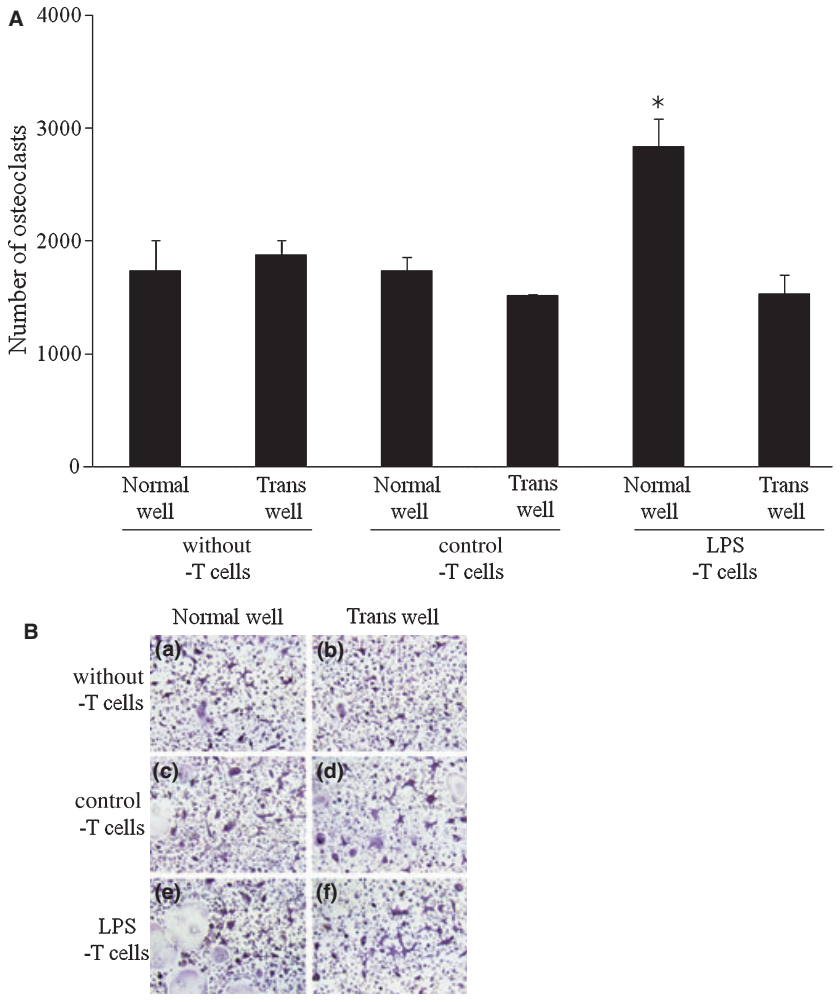

Fig. 2. Number of osteoclasts when blocking cell-to-cell contact using a Transwell. (A) Bone marrow macrophages (BMMs) were cultured with 30 ng/mL of macrophage colony-stimulating factor (M-CSF) and 1 ng/mL of RANKL. Three-hundred microlitres of the medium was removed after 48 h and the RANKL pre-stimulated BMMs (R-BMMs) were co-cultured with 300 μL of T cells (1.2×10^7 cells/well) in a normal well or a Transwell, together with 5 ng/mL of lipopolysaccharide (LPS) for 24 h. R-BMMs were stained with tartrate-resistant acid phosphatase (TRAP) and then the number of multinucleated TRAP-positive cells was counted. Data are representative of three independent experiments. Bars represent means ± standard deviation. The number of osteoclasts in the normal well in co-culture with T cells from LPS-injected mice (LPS-T cells) was significantly higher than in all other groups (*$p < 0.05$). (B) Representative photographs of TRAP-positive cells. Arrows indicate osteoclasts (e). Scale bar = 50 μm.

the acceleration of osteoclastogenesis by LPS-T cells, although the antibody had no effect in the other two culture groups (Fig. 3A). Osteoclasts were observed when R-BMMs were co-cultured with LPS-T cells in the presence of isotype-control antibody (Fig. 3B-e,g). However, the number of osteoclasts decreased when R-BMMs were co-cultured with LPS-T cells in the presence of anti-CD40L immunoglobulin (Fig. 3B-f).

mCD40L expression on LPS-T cells vs. that on control-T cells

To determine mCD40L expression on LPS-T cells, we analyzed mCD40L expression before co-culture using FACS. The mCD40L expression on LPS-T cells (Fig. 4A,B, red line) was slightly higher than that on control-T cells (Fig. 4A,B, blue line) before co-culture (Fig. 4A) and after co-culture for 6 h (Fig. 4B). The expression of mCD40L on LPS-T cells was similar to that on control-T cells after co-culture for 24 h (Fig. 4C). The expression of mCD40L on control-T cells (Fig. 4D, blue line) was similar to that of the isotype control (Fig. 4D, black line). The expression of mCD40L on LPS-T cells (Fig. 4E, red line) was slightly higher than that of the isotype control (Fig. 4E, black line). The mean fluorescence intensity of mCD40L on LPS-T cells before co-culture was higher than that of control-T cells (Fig. 4F; Figure S3). We also examined, by ELISA, the concentration of sCD40L in the supernatant after 24 h of co-culture. sCD40L was not detected in any of the culture groups (data not shown).

We analyzed the expression of *CD40L* mRNA by T cells using RT-PCR. Control-T cells and LPS-T cells expressed *CD40L* mRNA after 3 and 6 h of co-culture in the presence of LPS. The expression of *CD40L* mRNA by LPS-T cells was higher than that of control-T cells (Fig. 4G). We checked the expression of CD40 on R-BMMs using FACS and confirmed that R-BMMs express CD40 (Fig. 4H).

rCD40L significantly accelerated osteoclastogenesis in the presence of LPS

To examine the effect of rCD40L on osteoclastogenesis in the presence of both RANKL and LPS *in vitro*, we cultured R-BMMs with rCD40L in the presence of LPS for 24 h. We found that rCD40L did not accelerate osteoclastogenesis in the absence of LPS. On the other hand, rCD40L, at a concentration of more than 50 ng/mL, significantly accelerated osteoclastogenesis in the presence of LPS (Fig. 5A). The number of osteoclasts increased in the presence of LPS and rCD40L (Fig. 5B-d,e). To examine the effect of TNF-α on the acceleration of osteoclastogenesis by rCD40L in the presence of LPS, we added anti-mouse TNF-α immunoglobulin or isotype-control antibody in the presence of LPS and rCD40L. rCD40L accelerated the osteoclastogenesis and anti-TNF-α immunoglobulin completely inhibited this acceleration (Fig. 5C). The

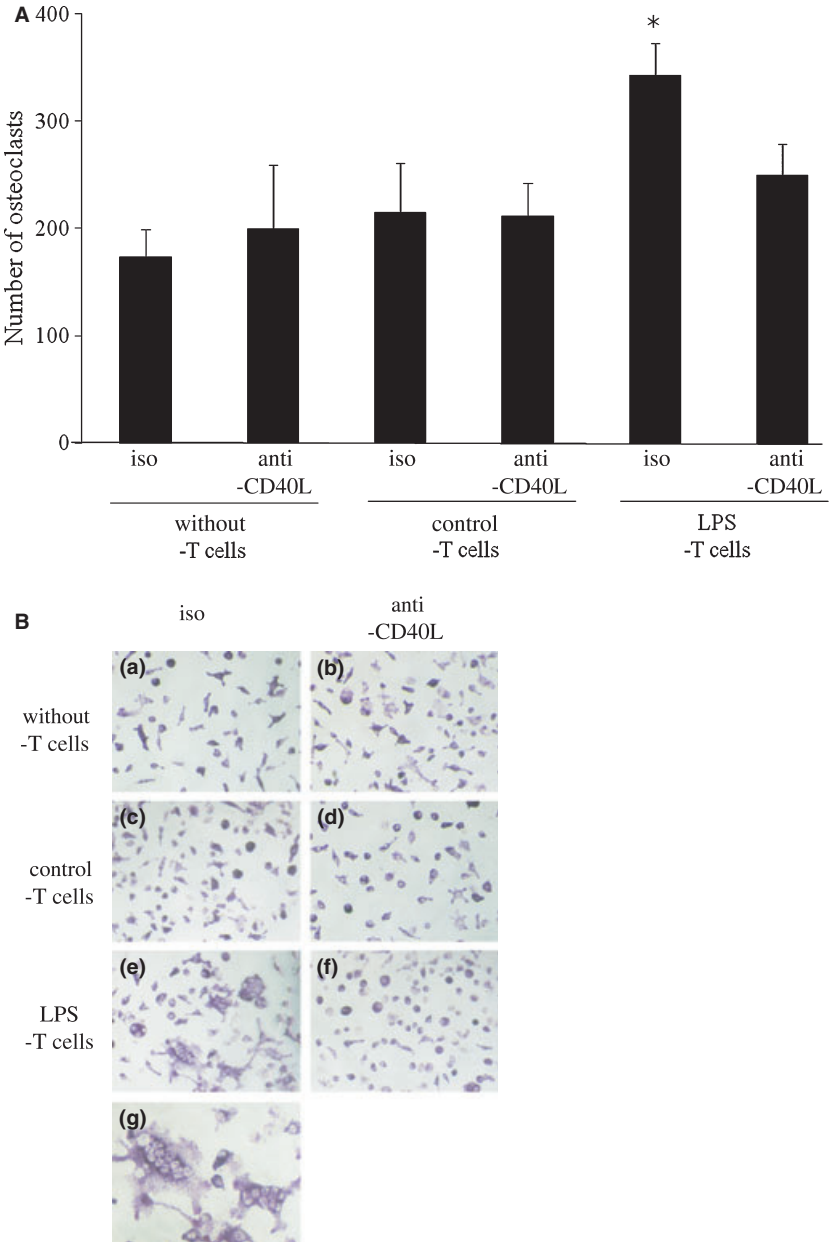

Fig. 3. Number of osteoclasts in the presence of anti-CD40 ligand (CD40L) immunoglobulin. (A) Bone marrow macrophages (BMMs) were cultured with 30 ng/mL of macrophage colony-stimulating factor (M-CSF) and 1 ng/mL of RANKL. Half of the medium was removed after 48 h, and the RANKL pre-stimulated BMMs (R-BMMs) were co-cultured with 100 μL of T cells (2×10^5 cells/well), or without T cells, together with 5 ng/mL of *Escherichia coli* lipopolysaccharide (LPS) and 10 μg/mL of anti-CD40L immunoglobulin (anti-CD40L) or 10 μg/mL of isotype-control antibody (iso) for 24 h. R-BMMs were stained with tartrate-resistant acid phosphatase (TRAP) and then the number of multinucleated TRAP-positive cells was counted. Data are representative of three independent experiments. Bars represent means ± standard deviation. The number of osteoclasts was significantly higher in the co-culture with T cells from LPS-injected mice (LPS-T cells) in the presence of isotype control antibody than in all other groups (*$p < 0.05$). (B) Representative photographs of TRAP-positive cells. Scale bar = 50 μm. Arrows indicate osteoclasts (e). High-magnification image of the osteoclasts in shown in panel B-e. Unstained multinuclei were observed. Scale bar = 25 μm (g).

isotype-control antibody did not influence the acceleration (Fig. 5C). This indicates that acceleration of osteoclastogenesis by rCD40L is required for TNF-α. To examine the resorption activity of osteoclasts, we cultured R-BMMs with rCD40L in the presence of LPS using the BD BioCoat™ Osteologic™ Bone Cell Culture System (16–18). We found that the number of resorption pits in cells cultured in the presence of LPS was significantly higher than in cells cultured without LPS. The number of resorption pits in cells cultured in the presence of rCD40L tended to be higher than in cells cultured in the absence of rCD40L; however, the difference was not significantly different (Fig. 5D). Resorption pits were clearly visible when R-BMMs were cultured with or without rCD40L in the presence of LPS (Fig. 5E-b,c). These results suggest that the osteoclasts formed in the present study have resorption activity in the presence of LPS.

Next, we examined the effect of rCD40L on the acceleration of osteoclastogenesis in the presence of LPS *in vivo*. Five micrograms of LPS was injected (in a 3 μL volume), four times every 48 h, into the gingival tissue of mice. rCD40L or PBS was injected together with the fourth injection of LPS. A few osteoclasts were observed in PBS-treated mice, but many osteoclasts were observed in CD40L-stimulated mice. The bone surface of CD40L-stimulated mice was irregular (Fig. 6A). The ratio of ARS in CD40L-stimulated mice was significantly higher than that in PBS-stimulated mice (Fig. 6B).

Discussion

This study showed that CD40L accelerated osteoclastogenesis in the presence of RANKL and LPS and also suggested that mCD40L on LPS-T cells accelerated osteoclastogenesis.

In a previous study, we found that acceleration of osteoclastogenesis by LPS-T cells required TNF-α. In the present study, the concentration of TNF-α and the expression of *TNF-α*, *TNFR1* and *TNFR2* mRNAs was not significantly different among the culture groups, although TNF-α was

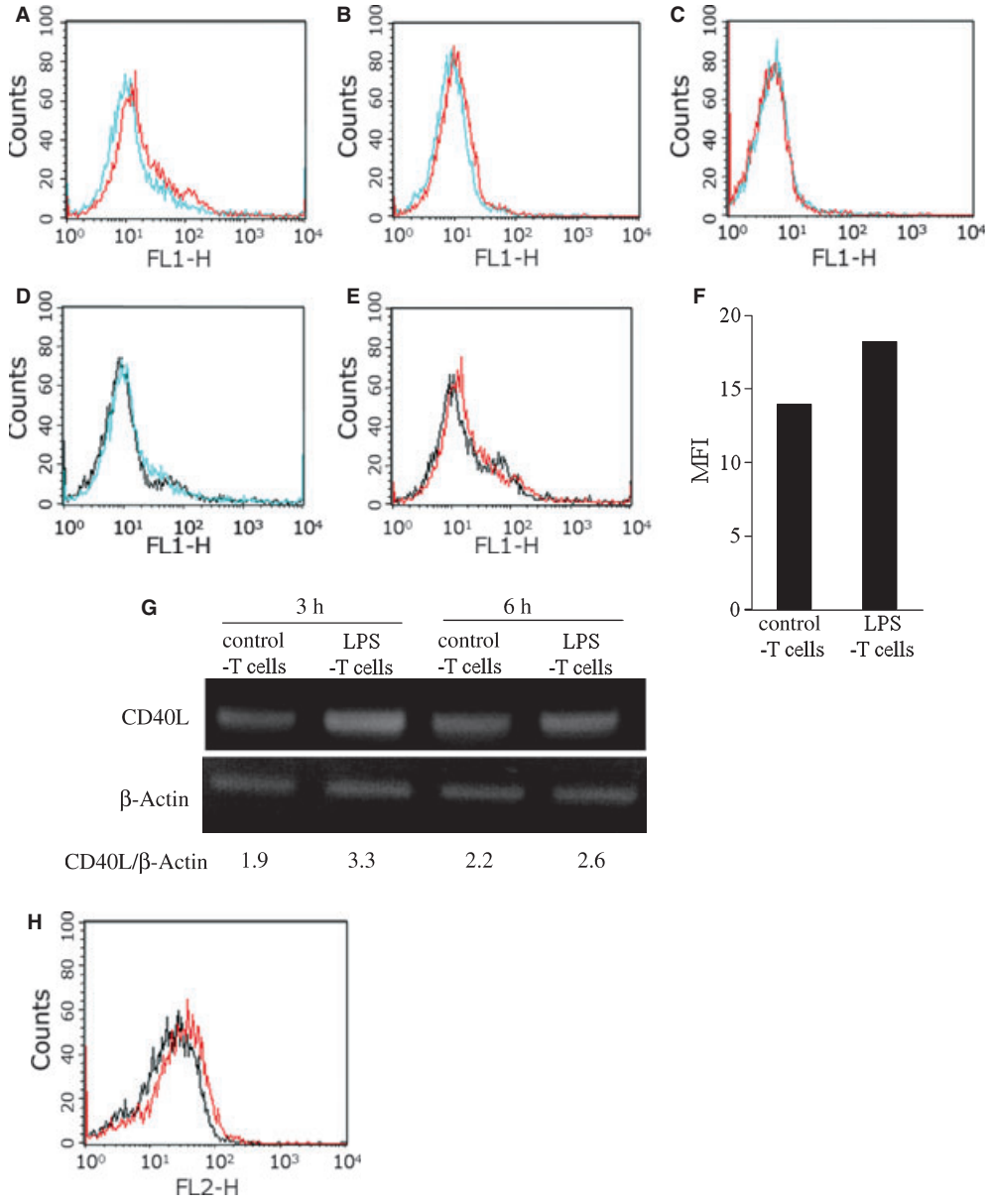

Fig. 4. Expression of membrane-bound CD40 ligand (mCD40L) on T cells from lipopolysaccharide (LPS)-injected mice (LPS-T cells) and on control-T cells. T cells isolated from LPS-injected mice or non-LPS-injected mice were activated with phytohemagglutinin (PHA) and interleukin-2 (IL-2) for 24 h. The cells were incubated with either fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-mouse CD40L or FITC-conjugated isotype-control antibody and were analyzed using a FACScan™. (A) The red line indicates expression of mCD40L on LPS-T cells, and the blue line indicates expression of mCD40L on control-T cells before co-culture. (B) The red line indicates expression of mCD40L on LPS-T cells, and the blue line indicates expression of mCD40L on control-T cells after co-culture for 6 h. (C) The red line indicates expression of mCD40L on LPS-T cells, and the blue line indicates expression of mCD40L on control-T cells after co-culture for 24 h. (D) The blue line indicates expression of mCD40L on control-T cells, and the black line indicates the isotype control. (E) The red line indicates expression of mCD40L on LPS-T cells, and the black line indicates the isotype control. (F) We measured the mean fluorescence intensity (MFI) of mCD40L on T cells. The MFI of LPS-T cells before co-culture was higher than that of control-T cells. Data are representative of three independent experiments. (G) Total RNAs of T cells were isolated after co-culture for 3 or 6 h in the presence of LPS, and the expression of *CD40L* mRNA was examined. We indicated the ratio of *CD40L* mRNA to *β-actin* mRNA. The data are representative of two independent experiments. (H) The red line indicates the expression of CD40 on the RANKL pre-stimulated BMMs (R-BMMs), and the black line indicates isotype-control.

detected. On the other hand, TNF-α was not detected in the culture medium of R-BMMs (i.e. BMMs cultured with 1 ng/mL of RANKL for 48 h) (data not shown). This suggests that mainly R-BMMs stimulated with LPS, but not with T cells, produce TNF-α. The TNF-α is required for acceleration of osteoclastogenesis by LPS-T cells, but

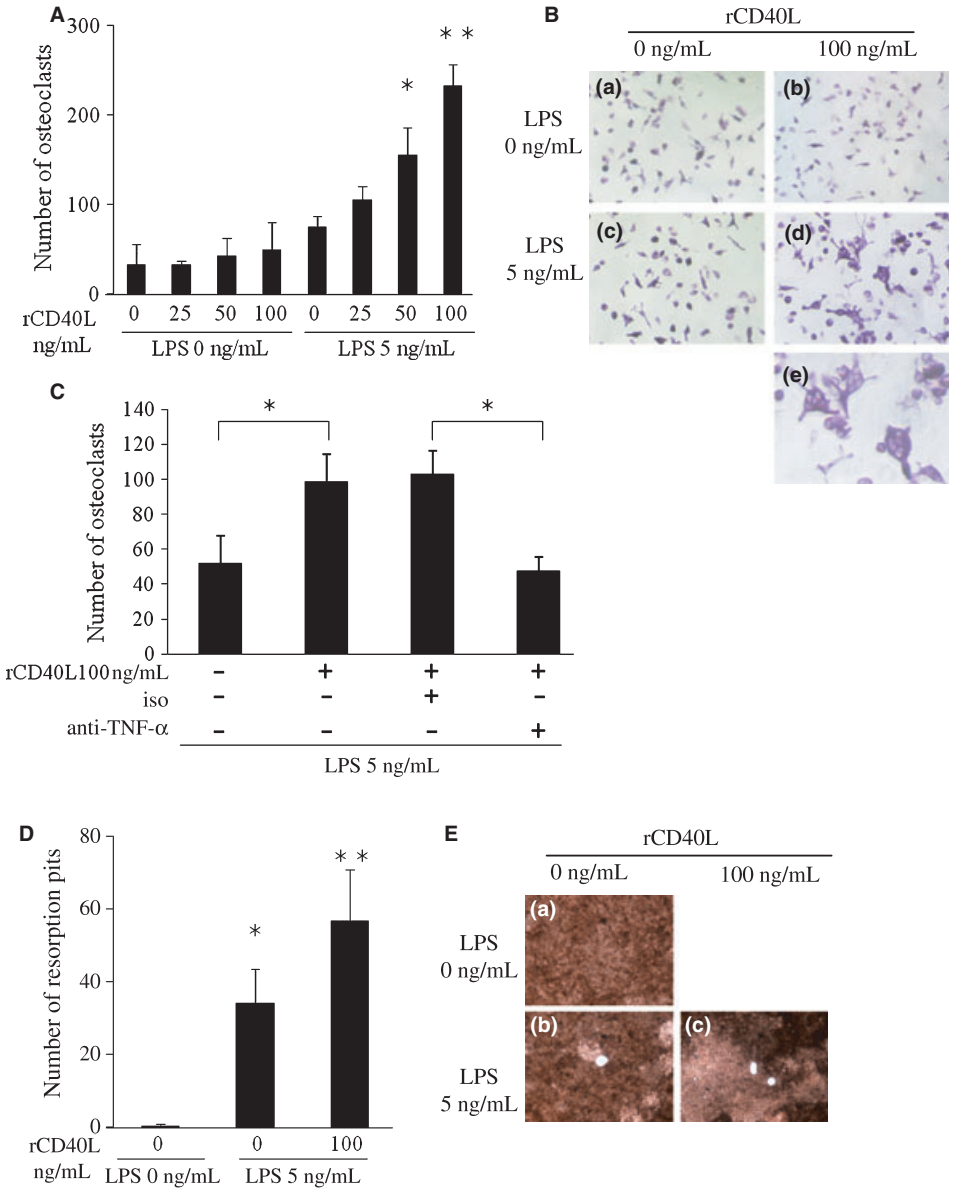

Fig. 5. Osteoclastogenesis in the presence of recombinant mouse CD40 ligand (rCD40L) and lipopolysaccharide (LPS) *in vitro*. Bone marrow macrophages (BMMs) were cultured with 30 ng/mL of macrophage colony-stimulating factor (M-CSF) and 1 ng/mL of RANKL for 48 h. Five nanograms per millilitre of *Escherichia coli* LPS, with or without rCD40L, was added. After 24 h, the culture cells were stained with tartrate-resistant acid phosphatase (TRAP) for 20 min and then multinucleated TRAP-positive cells were counted as osteoclasts. (A) A significantly higher number of osteoclasts was found in the presence of > 50 ng/mL of rCD40L, than without rCD40L, in the presence of LPS. Data are representative of three independent experiments. Bars represent means ± standard deviation (*$p < 0.05$; **$p < 0.001$). (B) Representative photographs of TRAP-positive cells. Scale bar = 50 μm. Arrows indicate osteoclasts (d). Panel B-e shows a high-magnification image of the osteoclasts in the same location as those in panel B-d. Unstained multinuclei were observed. Scale bar = 25 μm. (C) The RANKL pre-stimulated BMMs (R-BMMs) were cultured with rCD40L, with or without anti-tumor necrosis factor-α (TNF-α) immunogloblin, in the presence of LPS. rCD40L significantly accelerated osteoclastogenesis in the presence of LPS. Anti-TNF-α immunogloblin completely inhibited this acceleration of osteoclastogenesis. Bars represent means ± standard deviation (*$p < 0.01$). Data are representative of two independent experiments. (D) BMMs were cultured with 30 ng/mL of M-CSF and 1 ng/mL of RANKL in BD BioCoat™ Osteologic™ Bone Cell Culture System for 48 h. Five nanograms per millilitre of *E. coli* LPS and 100 ng/mL of rCD40L were added at 48 h. After 48 h, we replaced half of the medium with fresh medium and renewed all reagents and cytokines. After 72 h, we stained the cells with von Kossa stain. Bars represent means ± standard deviation. The number of resorption pits with LPS was higher than of without LPS (*$p < 0.05$; **$p < 0.01$). Data are representative of two independent experiments. (E) Representative photographs of resorption pits. Arrows indicate resorption pits. Scale bar = 25 μm.

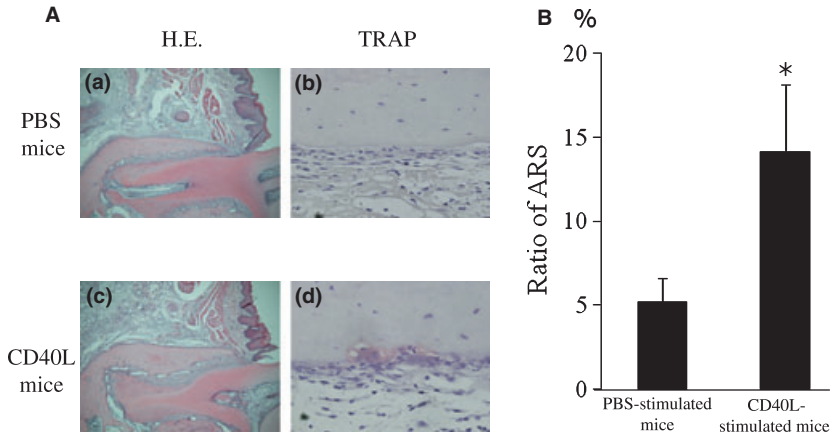

Fig. 6. Osteoclastogenesis in the presence of recombinant mouse CD40 ligand (rCD40L) and lipopolysaccharide (LPS) *in vivo*. (A) Histopathological findings in alveolar bone. Staining with hematoxylin and eosin (H&E) (a, c) or tartrate-resistant acid phosphatase (TRAP) (b, d). In CD40L-stimulated mice, many osteoclasts were observed together with irregularity of the bone surface (c, d). Scale bar = 100 μm (a, c) or 25 μm (b, d). (B) Ratio (%) of the active resorption surface (ARS) in each group. The ARS of CD40L-stimulated mice was significantly higher than that of phosphate-buffered saline (PBS)-stimulated mice. Bars represent mean ± standard deviation (*$p < 0.001$).

a low concentration of TNF-α alone is not sufficient to accelerate osteoclastogenesis. Therefore, we speculated that another factor of LPS-T cells may be related to osteoclastogenesis acceleration.

Some previous studies have shown that cell-to-cell contact plays an important role in osteoclastogenesis. Interaction between lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) is important for osteoclastogenesis between MC3T3/G2-PA6 cells (which are stromal cells) and pre-osteoclasts (19). The 4-1BB/4-1BBL interaction among pre-osteoclasts is also important for osteoclastogenesis (20). In our study, acceleration of osteoclastogenesis by LPS-T cells was completely inhibited by culture in a Transwell. We considered contact between T cells and R-BMMs to be important and therefore we focused on the effect of CD40L on T cells because CD40L stimulates TRAF6, which is an essential adaptor protein of osteoclastogenesis, through CD40 on monocytes/macrophages (21,22). Anti-CD40L immunoglobulin inhibited the acceleration of osteoclastogenesis induced by LPS-T cells. In our cultures, sCD40L was not detected in the supernatant after co-culture, suggesting that sCD40L is not related to the acceleration of osteoclastogenesis induced by LPS-T cells. mCD40L is expressed on the surface of activated T cells (21,22). CD40L plays an important role in the regulation of the immune response (21,22). When antigens are presented by antigen-presenting cells, including macrophages, B cells and dendritic cells, mCD40L on activated T cells makes contact with CD40 on antigen-presenting cells and these cells are stimulated by each other (21,22). Therefore, we speculated that the contact between CD40 on R-BMMs and mCD40L on LPS-T cells is important for the acceleration of osteoclastogenesis by LPS-T cells.

We speculated that a signal via CD40 can induce osteoclastogenesis because TRAF6, which is an essential adaptor protein of osteoclastogenesis, binds to CD40 (21,22). Previous studies have shown that the over-expression of CD40 can induce osteoclastogenesis in the presence of transforming growth factor-β (23,24). However, it has been not reported CD40L accelerated osteoclastogenesis. In our study, we showed that rCD40L accelerated osteoclastogenesis in the presence of LPS *in vitro* and *in vivo*. This is the first study to show that CD40L accelerates osteoclastogenesis. Low concentrations of RANKL, LPS and TNF-α may slightly activate intercellular signals, such as MAPK and nuclear factor-kappaB (NF-κB), to induce osteoclastogenesis, but this is not sufficient to accelerate osteoclast formation. We speculated that osteoclastogenesis was accelerated as a result of activation of these signals by CD40 activation, because CD40 activates MAPK and NF-κB (3,23,24). However, we were unable to clarify the details of the mechanism.

In our study, a tendency to promote the formation of resorption pits was recognized when we added rCD40L in the presence of LPS *in vitro*. The ARS was significantly increased following the administration of rCD40L *in vivo* (by injection), although rCD40L did not significantly accelerate pit formation *in vitro*. We showed that the acceleration of osteoclastogenesis by rCD40L *in vitro* occurred through TNF-α. Kobayashi *et al.* (25) reported that TNF-α induces TRAP-positive multinucleated cells, although these cells do not have resorptive activity. However, we reported that RANKL-positive cells appear around the bone surface after the third LPS injection using the same method of this study (11). RANKL activates osteoclast functions. We considered that rCD40L injection induced an increase in the ARS because of the many RANKL-producing cells present near the bone surface *in vivo*.

mCD40L was expressed more strongly on LPS-T cells than on control-T cells before co-culture. We observed no difference in the expression of mCD40L between control-T cells and LPS-T cells before stimulation with PHA/IL-2 (data not shown). These results suggest that stimulation with PHA/IL-2 up-regulates the expression of mCD40L on LPS-T cells. However, the difference in expression between control-T cells and LPS-T cells was clearly decreased, in a time-dependent manner, after co-culture. It has been reported that mCD40L expression is transiently up-regulated after stimulation of cells (26,27). PHA activates T cells and induces the production of IL-2. The IL-2 also induce the activation, proliferation and dif-

ferentiation of T cells. Expression of mCD40L on activated T cells is induced by αCD3/αCD28 or IL-2 (28,29). The expression of mCD40L induced by αCD3/αCD28 is enhanced by IL-2 restimulation because the responsiveness of activated T cells to IL-2 is enhanced by αCD3/αCD28 (28,29). αCD3/αCD28 stimulates CD3 and CD28 signalling in T cells. When antigen-presenting cells present antigen for T cells, CD3 and CD28 signalling in T cells are stimulated. In our study, repeated injection of LPS may have activated the immune system in which LPS acts as an antigen. Therefore, we consider that the presentation of LPS as antigen activates CD3 and CD28 on the T cells of LPS-injected mice. Because the responsiveness to IL-2 of T cells from LPS-injected mice is enhanced, PHA and IL-2 may induce the expression of mCD40L on LPS-T cells.

It has previously been reported that non-PHA/IL-2-stimulated LPS-T cells did not accelerate osteoclastogenesis (11) and these LPS-T cells did not express mCD40L (data not shown). On the other hand, after stimulation with PHA/IL-2, LPS-T cells expressed mCD40L and were able to accelerate osteoclastogenesis. Furthermore, acceleration of osteoclastogenesis by LPS-T cells was blocked using anti-CD40L immunoglobulin. Therefore, we think that mCD40L is important in the acceleration of osteoclastogenesis by LPS-T cells stimulated with PHA/IL-2. In our experimental system, we cultured RANKL-prestimulated BMMs with LPS-T cells and LPS. The low concentration of RANKL and LPS is not sufficient for acceleration of osteoclastogenesis, which requires the presence of LPS-T cells. We believe that CD40L stimulation activates MAPK or NF-κB, which are important factors for osteoclastogenesis. In our experimental system, multinucleated osteoclasts are observed after co-culture with LPS-T cells for 24 h. This suggests that MAPK and NF-κB induced by mCD40L, LPS and TNF-α are important after co-culture for several hours. Therefore, we believe that it is the mCD40L expressed at the start of co-culture, and not at the end of the culture, that is important for osteoclast formation. We have reported that the acceleration was completely inhibited by anti-TNF-α immunoglobulin. In the present study, acceleration of osteoclastogenesis by rCD40L was also completely inhibited by anti-TNF-α immunoglobulin. This showed that TNF-α is required for the acceleration by rCD40L, similarly to acceleration by LPS-T cells. This supports our idea whereby mCD40L expressed on stimulated LPS-T cells accelerates osteoclastogenesis in the presence of TNF-α.

In this study, our focus was not bacterial LPS, but the immune cell response. LPS is one of the most important bacterial components responsible for periodontal destruction. However, the structures and the receptors of LPS from different species of periodontophathogenic bacteria vary. *Porphyromonas gingivalis (P.gingivalis)* is a well-known periodontopathic bacterium, and its LPS is known to be a toll-like receptor (TLR) 2 agonist. However, Darveau (30) reported that part of the *P. gingivalis* LPS is recognized by TLR4. The LPS of other periodontopathic bacteria, such as *Aggregatibacter actinomycetemcomitans*, is mainly recognized by TLR4. There is no single receptor for periodontopathic bacterial LPS and those that have been identified are not clearly elucidated. In checking a host or cell response against some stimuli, it is important for their receptor to be known. Therefore, in this study, we used *E. coli* LPS, which is a representative TLR4 ligand and is involved in biological activities.

The inflammatory site in periodontitis is exposed by LPS as a result of infection with gram-negative bacteria (1). The levels of expression of RANKL and TNF-α at the site of periodontitis are higher than those at a healthy site (31). Many infiltrating T cells are observed at the inflammatory site in periodontitis (32). We focused on CD40L-positive T cells in the present study. These cells are generally considered as T helper 1 (Th1) cells (33–35). Some studies have reported that T helper 2 (Th2) cells are dominant in periodontitis; however, other studies have detected Th1 cells in periodontitis tissues (36,37). We have also reported that interferon-γ-bearing T cells (Th1 cells) are present at higher levels than IL-4-bearing T cells (Th2 cells) in tissues with severe inflammation or in deep periodontal pockets of periodontitis patients (38). In our mouse model, in which injection of LPS induced bone loss, we found an increase in the number of interferon-γ-positive cells (Th1 cells) in parallel with bone resorption (15). Therefore, CD40L-expressing T cells may be able to stimulate periodontal destruction. In fact, CD40L-positive cells have been detected in the periodontal tissue of periodontitis patients (26). These findings suggest that when CD40L-expressing T cells infiltrate into an inflammatory site, such as that of periodontitis, in which LPS, RANKL and TNF-α are present, osteoclastogenesis will probably be accelerated and bone resorption will be enhanced. Therefore, it is expected that the effect of T cells on the progression of periodontitis will be elucidated by clarifying the effect of CD40L on osteoclastogenesis acceleration.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The resorption pits formation of positive control.

Figure S2. The concentration of TNF-α after co-culture for 3 and 24 h.

Figure S3. The MFI of mCD40L on T cells.

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