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Thymus-expressed chemokine enhances *Porphyromonas gingivalis* LPS-induced osteoclast formation via NFATc1 activation

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ABSTRACT

Objective: *P. gingivalis* is a gram-negative anaerobic bacterium and a major periodontal pathogen. LPS produced by *P. gingivalis* promotes osteoclast formation. TECK is a CC chemokine whose expression is increased in gingival epithelial cells exposed to *P. gingivalis* LPS. In this study, we investigated the effect of TECK in osteoclastogenesis induced by *P. gingivalis* LPS.

Designs: Real time reverse transcriptase polymerase chain reaction (RTPCR) analysis and western blotting were performed to confirm TECK in MG63, human osteoblast cell line and primary murine osteoblasts and CCR9 in RAW 264.7 cells and murine bone marrow macrophages (BMMs) as osteoclast precursors. *P. gingivalis* LPS-treated BMMs and Raw 264.7 cells were cultured with or without TECK or TECK antibody to examine the effect of TECK on osteoclast formation. Cocultures with murine osteoblasts and bone marrow cells were also treated with or without TECK or TECK antibody. Luciferase assay and western blotting were used to determine whether TECK-CCR9 induced osteoclastogenesis was mediated through NFATc1 or NF-kB signaling.

Results: TECK was shown to be expressed by osteoblasts, and its receptor, CCR9, by osteoclast precursors. TECK increased *P. gingivalis* LPS-induced osteoclast numbers in an in vitro osteoclast formation assay using osteoclast precursors. The enhanced osteoclast formation by TECK was mediated by NFATc1, but not by NF-kB signaling.

Conclusion: TECK may be a novel regulator of osteoclast formation induced by *P. gingivalis* LPS in periodontitis.

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1. Introduction

Inflammatory bone diseases such as rheumatoid arthritis and periodontitis are characterized by imbalances that lead to excessive bone resorption and tissue destruction (Takayanagi, 2005). Periodontitis is a dental plaque-induced inflammatory disease of the periodontal tissues that results in bone loss around the teeth. The primary etiologic organisms in periodontitis are gram-negative anaerobic bacteria residing within subgingival biofilms (Nishihara & Koseki, 2004). Lipopolysaccharide (LPS) is a cell-wall component of gram-negative microorganisms that induces polymorphonuclear leukocyte infiltration, edema, and vascular dilatation in inflamed periodontal tissues (Bainbridge & Darveau, 2001). LPS also plays an important role in the destruction of periodontal tissue, including the gingiva, periodontal ligament, and alveolar bone, by stimulating the production of inflammatory cytokines such as interleukin-1b (IL-1b) and tumor necrosis factor-α (TNF-α), which induce bone resorption (Wada, Maeda, Yoshimine, & Akamine, 2004). Both the late stage of osteoclastogenesis and the survival and activation of osteoclasts are enhanced by LPS (Liu et al., 2009; Takami, Kim, Rho, & Choi, 2002; Zou & Bar-Shavit, 2002). The gram-negative anaerobic bacterium *Porphyromonas gingivalis* (*P. gingivalis*) is a major periodontal pathogen (Ximénez-Fyvie, Haffajee, & Socransky, 2000). Surface components

Abbreviations: BMMs, bone marrow macrophages; CCR9, CCchemokine receptor 9; LPS, lipopolysaccharide; NFATc1, nuclear factor of activated T cells cytoplasmic 1; NF-κB, nuclear factor κB; *P. gingivalis*, *Porphyromonas gingivalis*; RANKL, receptor activator of nuclear factor kappa B ligand; TECK, thymus-expressed chemokine.

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of *P. gingivalis*, including LPS, interact with host-expressed toll-like receptors, which are key control elements involved in the destruction of periodontal tissue (Shaddox et al., 2013). *P. gingivalis* differentially affects osteoclast differentiation by bone marrow macrophages (BMMs) depending on their differentiation state (Zhang et al., 2011).

Chemokines are bioactive peptides that regulate leukocyte activation and migration during infections by microorganisms (Silva, Garlet, Fukada, Silva, & Cunha, 2007), including in gingival tissue and gingival crevicular fluid (GCF) (Tsai, Ho, & Chen, 1995). One of the most abundantly expressed chemokines is macrophage inflammatory protein-1α (MIP-1α/CCL3), which is localized in the connective tissue subjacent to the gingival epithelium (Gemmell, Carter, & Seymour, 2001). Several chemokines, such as monocyte chemotactic protein-1 (MCP-1) (Lu et al., 2007), MIP-1α (Toh et al., 2004), and stromal cell-derived factor-1 (SDF-1), were shown to regulate both the migration of osteoclast precursor cells and osteoclast differentiation (Yu, Collin-Osdoby, & Osdoby, 2003). However, the chemokines and their corresponding receptors that play important roles in osteoclast formation in periodontitis have not been identified.

Thymus-expressed chemokine (TECK), also known as chemokine (C—C motif) ligand (CCL) 25, is a CC chemokine with strong chemotactic activity (Vicari et al., 1997; Youn, Kim, Smith, & Broxmeyer, 1999). TECK is selectively expressed in thymocytes and dendritic cells, and in epithelial cells of the intestine and oral mucosa (Otten, Dragoo, Wang, & Klein, 2003; Wurbel et al., 2000; Zabel et al., 1999). The TECK receptor is CC chemokine receptor (CCR) 9 (Youn et al., 1999; Zaballos, Gutiérrez, Varona, Ardavín, & Márquez, 1999). CCR9 mRNA has been detected in immature and mature thymocytes but not in mature T cells (Wurbel et al., 2000; Yu, Peden, Zaitseva, Golding, & Farber, 2000; Zabel et al., 1999). In gingival epithelial cells, *P. gingivalis* LPS increases TECK expression (Ekhlassi et al., 2008); however, the role of TECK in the LPS-induced osteoclast formation responsible for bone destruction in periodontitis is unknown.

In this study, we investigated the effect of TECK on osteoclastogenesis induced by *P. gingivalis* LPS by examining the expression of TECK and CCR9 in osteoblasts and osteoclast precursors in vitro and the molecular mechanism underlying TECK-induced osteoclastogenesis.

2. Materials and methods

2.1. Reagents and antibodies

Receptor activator of nuclear factor kappa B ligand (RANKL), used in the osteoclast formation assay, was from Oriental Yeast (Tokyo, Japan). Human macrophage colony-stimulating factor (M-CSF), the mouse anti-TECK and CCR9 and anti-goat IgG antibodies used in western blotting and neutralizing studies, and mouse recombinant TECK used in the osteoclast formation and luciferase assays were purchased from R&D Systems (Minneapolis, MN, USA). LPS of *P. gingivalis* was from InvivoGen (San Diego, CA, USA), which is standard grade as Toll-like receptor (TLR) 2 and TLR4 agonist. Anti-nuclear factor of activated T cells cytoplasmic 1 (NFATc1) antibody, normal goat IgG, and anti-β-actin antibody for western blotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The phospho-nuclear factor (NF)-κB p65 antibody used in western blotting was from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture

The human gingival epithelial cell line Ca9-22 and the human osteoblast cell line MG63, derived from an osteosarcoma, were purchased from the RIKEN BioResource Center (Ibaraki, Japan) and cultured according to our previous study (Fujihara et al., 2014; Usui et al., 2015). Briefly, Ca9-22 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sigma, Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Gibco, NY, USA) and supplemented with 1% antibiotics (50 U penicillin/mL and 50 μg streptomycin/mL; Wako, Tokyo, Japan). MG63 cells were cultured in minimal essential medium (MEM; Sigma) containing 10% FBS and supplemented with 1% antibiotics as described above. RAW264.7, a mouse monocyte/macrophage cell line, used in this study as osteoclast precursor cells, were obtained from the American Type Culture Collection (Virginia, USA). RAW264.7 cells were either maintained in DMEM containing 10% FBS or, for osteoclast generation, were cultured in α-MEM containing 10% FBS.

2.3. LPS-induced osteoclast formation assay

BMMs were flushed from the femurs and tibiae of 8-week-old BALB/cA mice (CLEA Japan, Tokyo, Japan), suspended in α-MEM containing 10% FBS, and cultured in the presence of 30 ng/ml macrophage colony-stimulating factor (M-CSF)). After 72 h of culture, non-adherent cells were completely removed. To generate osteoclasts in vitro, the cells were pre-treated with RANKL (50 ng/ml) in the presence of M-CSF (30 ng/ml) for 20 h and then with various combination of *P. gingivalis* LPS (5 μg/ml), TECK (100 ng/ml), and TECK antibody (100 ng/ml) in the presence of M-CSF (30 ng/ml) and the absence of RANKL. Raw 264.7 cells were also pre-treated with RANKL (50 ng/ml) for 20 h and then treated with various combination of *P. gingivalis* LPS (5 μg/ml), TECK (100 ng/ml), and TECK antibody (100 ng/ml). After 72 h, the cultures were stained TRAP (tartrate-resistant acid phosphatase) solution. Osteoclast formation assay in the co-culture was determined in cultures of calvarial osteoblasts and bone marrow cells (BMCs) from BALB/cA mice (CLEA Japan). These cells were isolated according to previous our study (Usui et al., 2002). They were cocultured for 5 days in a 24 well plates with α-MEM containing 10% FBS in the absence or presence of *P. gingivalis* LPS (5 μg/ml). The medium were changed every 2 days. To detect osteoclasts, the cultures were stained with TRAP solution at 5 days. TRAP-positive multinucleated cells with more than three nuclei were counted as osteoclasts, while TRAP positive cells with one or two nuclei were defined as pre-osteoclasts. This study was approved by the Animal Research Committee of Showa University (#18065, 18066).

2.4. Luciferase assay

RAW264.7 cells were co-transfected with 0.45 μg of the reporter plasmids NFAT-RE or NF-kB-RE, carrying the respective response elements (REs) (Promega Madison WI USA), and 0.05 μg of pRL-Renilla vector, as an internal control, using FuGENE6 (Roche Indianapolis, IN, USA). The cells were primed with RANKL (50 ng/ml) for 20 h and then treated with TECK (100 ng/ml), RANKL (100 ng/ml), and/or *P. gingivalis* LPS (5 μg/ml) for 24 h. Luciferase activities were assayed using a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

2.5. Real-time quantitative PCR

Total RNA was extracted using RNeasy mini kits (Qiagen, Valencia, USA) according to the manufacturer's protocol. Polymerase chain reactions (PCRs) were performed using an ABI Step One system (Applied Biosystems), and the analysis was carried out using the sequence detection software supplied by the manufacturer. Each reaction mixture contained 15 μl of TaqMan Universal PCR master mix with uracil-N-glycosylase (UNG) (Applied Biosystems), 1 μl each of the sense and antisense primers

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