RESEARCH ARTICLE

Mycobacterium tuberculosis escapes from the phagosomes of infected human osteoclasts reprograms osteoclast development via dysregulation of cytokines and chemokines

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In the context of spinal tuberculosis a study of the inflammatory responses of human multinucleated osteoclasts infected with virulent Mtb is of interest. Intracellular Mtb infection resulted in the rapid growth of Mtb and production of proinflammatory cytokines. In contrast, highly-fused multinucleated osteoclasts incapacitated the production of these cytokines, Mtb escaped from the endosome/phagosome, and led to a different pattern of osteoclast activation with the production of a set of chemokines. These findings indicate that intracellular Mtb infection in multinuclear osteoclasts reprograms osteoclast development via the dysregulation of cytokines and chemokines.

Keywords
chemokines; chemokine receptors; host defense; inflammation; Mycobacterium tuberculosis; osteoclast.

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Abstract

Spinal tuberculosis is a condition characterized by massive resorption of the spinal vertebrae due to the infection with Mycobacterium tuberculosis (Mtb). However, the pathogenesis of spinal tuberculosis has not been established because it was almost completely eradicated by the establishment of antibiotic treatment in the mid-20th century. In this study, we investigated the inflammatory responses of human multinucleated osteoclasts infected with virulent Mtb strain. We found that the intracellular Mtb infection of multinuclear osteoclasts resulted in the rapid growth of Mtb and production of proinflammatory cytokines. In contrast, highly fused multinucleated osteoclasts incapacitated the production of these cytokines, Mtb escaped from the endosome/phagosome, leading to a different pattern of osteoclast activation, with the production of chemokines such as CCL5, CCL17, CCL20, CCL22, CCL24, and CCL25. Moreover, intracellular infection with an avirulent Mtb strain resulted in diminished production of these chemokines. These findings indicate that intracellular Mtb infection in multinuclear osteoclasts reprograms osteoclast development via the dysregulation of cytokines and chemokines.

Introduction

Inflammatory bone diseases are characterized by the infiltration of immune cells, including lymphocytes, monocytes, polymorphonuclear leukocytes, and even activated osteoclasts. Spinal tuberculosis (also called Pott’s disease) is defined as a chronic inflammatory destruction of spinal bones, mainly induced by the mycobacterial infection of the spinal cavity, and is believed to be initiated by the abnormal activation of osteoclasts in the bone tissue, which leads to inflammatory bone destruction (Haynes, 2004), fracture, and collapse of the vertebrae, resulting in massive kyphotic deformities. Finally, the spinal canal may become narrower because of abscesses, granulation tissue or direct dural invasion, leading to spinal cord compression and neurological deficits.

The mortality from tuberculosis has decreased dramatically in developed countries following the introduction of effective chemotherapeutic agents in the mid-20th century, including isoniazid, rifampicin, pyrazinamide, streptomycin,
and ethambutol, before the molecular biology-based pathological mechanism of the disease had been completely revealed. However, Mycobacterium tuberculosis (Mtb) now affects one-third of the world population, and causes almost 2 million deaths per year; 30% of new cases can be found in India, China, Africa, and South America (WHO, 2012). In addition, current treatments are becoming obsolete because of the emergence of drug-resistant strains of Mtb. Thus, the development of a new, improved vaccine and/or new drugs that tackle the emergence of antibiotic resistance are sorely needed.

There is a question remaining as to whether osteoclastic activation and differentiation are facilitated at the inflammatory sites of spinal tuberculosis. It is well known that activated osteoclasts play an important role in inflammatory bone destruction, such as that occurring due to rheumatoid arthritis and inflammatory osteolysis. Similarly, spinal tuberculosis is believed to develop as a result of the activation of osteoclasts in bone tissue in response to Mtb infection, as an enormous amount of multinuclear osteoclast-like cells surrounding granulomatous caseous necrosis were observed around destroyed bones in a histological analysis. In fact, the development of spinal tuberculosis seems to be related to the infiltration of inflammatory immune cells into the bone tissue. However, there are no data available concerning whether Mtb directly infects the multinuclear osteoclasts intracellularly. Thus, we assumed that the source of Mtb-activated osteoclasts in spinal tuberculosis was as follows; (1) abnormal activation of Mtb-infected macrophages or their precursors in the inflammatory sites following a local infection, and subsequent activation of the RANK-RANKL axis, (2) abnormal induction and extravasation of Mtb-infected mononuclear osteoclast precursors from circulating cells into the inflammatory sites due to systemic infection, resulting in abnormal activation, and (3) unexpected pathogenic activation and reprogramming of multinuclear osteoclasts resident in the tissue in response to Mtb infection, followed by the dysregulation of cytokines and chemokines, causing the pathological destruction of the bone tissue.

Generally, proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, are produced by macrophages in response to bacterial infection; proinflammatory cytokines also promote osteolysis (Boyle et al., 2003). In the case of rheumatoid arthritis, the RANK-RANKL axis plays a principal role in the promotion of osteoclast differentiation. This is because the higher expression of RANKL in synovial fibroblasts, which is strongly induced by the autoreactive T cells that infiltrated into the synovial tissue, directs pathological osteoclast differentiation via the production of proinflammatory cytokines (Takayanagi et al., 2000a, b). Bacterial pathogens surged RANKL via Toll-like receptor (TLR)-mediated signaling (Kikuchi et al., 2001; Jiang et al., 2002; Suda et al., 2002); however, several reports suggested that proinflammatory cytokines (Boyle et al., 2003) and several bacterial molecules act as promoters of osteolysis (Kikuchi et al., 2001; Jiang et al., 2002; Suda et al., 2002), whereas other studies reported that bacterial stimulation suppressed the generation of osteoclast precursors (Takami et al., 2002; Ji et al., 2009). Thus, it is still not clear whether bacterial stimulation promotes osteoclastogenesis.

Recently, chemokines have been recognized to be major factors involved in osteolysis and pathological osteolysis (Oba et al., 2005; Kim et al., 2006). CCL3/MIP-1α induces ectopic osteoclastogenesis in osteolytic lesions of rheumatoid arthritis (Haringman et al., 2006; Menu et al., 2006) and multiple myeloma (Choi et al., 2000; Han et al., 2001; Haringman et al., 2006), implying that CCL3 and its receptor CCR1 act as a crucial chemokine for communication between osteoclasts and osteoblasts (Hoshino et al., 2010). The participation of other axes of chemokine–chemokine receptors such as CCL2-CCR2 axis (Kim et al., 2006; Li et al., 2007; Binder et al., 2009), CCL5-CCR5 axis (Oba et al., 2005; Menu et al., 2006; Hoshino et al., 2009), and CX3CL1-CX3CR1 axis (Saitoh et al., 2007; Koizumi et al., 2009; Hoshino et al., 2013) also play roles in bone remodeling. Nowadays, some types of chemokines are responsible for pathological bone destruction through the regulation of osteoclasts and their precursor cells that are derived from common progenitor cells in bone marrow, thus suggesting that several chemokine antagonists provide a strong rationale for further development of the therapeutic targets of associated osteolytic bone disease such as multiple myeloma and rheumatoid arthritis (Oba et al., 2005; Menu et al., 2006; Dairaghi et al., 2012). These findings are referenced in favor of therapeutic treatments targeting chemokines to prevent pathogenic bone resorption.

To clarify the pathological mechanism underlying the overactivation of osteoclasts observed in tuberculosis-related osteolytic lesions, we stimulated osteoclasts and their precursors with virulent living Mtb and investigated the expression profiles of chemokines specific for tuberculosis infection.

Materials and methods

Ethical guidelines for human studies

All human experiments were performed with the approval of the local ethics committees of the Research Institute of National Center for Global Health and Medicine (No. H21-785).

Cells, materials, and bacteria

*Mycobacterium tuberculosis* H37Rv virulent laboratory strain (ATCC 27294), *Mycobacterium tuberculosis* H37Ra avirulent strain (ATCC 25177), and *Mycobacterium bovis* BCG-Tokyo vaccine strain were cultured at exponential growth phase in Middlebrook 7H9 broth (BD, Sparks, MD) with ADC enrichment (BD) and 0.05% Tween 80 at 37 °C for 7–10 days. Lipopolysaccharide (LPS) from *Escherichia coli* O55B5 and peptidoglycan (PGN) from *Staphylococcus aureus* were purchased from Sigma Aldrich (St. Louis, MO).
and Fluka Chemical (St. Gallen, Switzerland), respectively. Recombinant human M-CSF and RANKL were purchased from R&D Systems Inc. (Minneapolis, MN). Normal human natural mononuclear osteoclast precursors cells (Poietics™ Osteoclast Precursor Cell System) were purchased from Lonza Walkersville, Inc. (Walkersville, MD) and maintained with osteoclast precursor cell basal medium in the presence of 10 ng mL⁻¹ M-CSF (without RANKL) and used as mononuclear osteoclast precursors (precursor mononuclear cells; pMCs). The multinuclear osteoclasts were induced according to the manufacturer’s instructions. Human peripheral blood monocytes were collected from a healthy volunteer, separated using CD11b MicroBeads (Miltenyi Biotec Inc., Bergisch Gladbach, Germany), and stored at −80 °C prior to use.

Osteoclast culture and intracellular Mtb infection

Mononuclear pMCs were cultured with osteoclast precursor cell basal medium (Lonza), and the culture medium was replaced every 3 days. The highly fused multinuclear osteoclasts (multinuclear osteoclasts; mOCs) were induced from mononuclear cells with 10 ng mL⁻¹ M-CSF and 20 ng mL⁻¹ RANKL for 5 days. Intracellular Mtb infection was performed by the co-culture of mononuclear pMCs and multinuclear mOCs (5 × 10⁵ cells per well, 1 mL) with 0.5 μL of Mtb H37Rv broth (equivalent to 1.0 × 10⁶ CFU per well) for 24 h to minimize the influence of bacterial broth, and the excess bacteria were rinsed off with phosphate-buffered saline (PBS). The culture supernatants of stimulated osteoclasts were harvested, irradiated with UV-C (254-nm wavelength) for 30 s, and filtered with Ultrafree-FC™ 0.1-μm membrane filters (Merck Millipore, Darmstadt, Germany) to remove living Mtb. For immunohistochemical staining, osteoclasts were fixed with 4% paraformaldehyde, permeabilized and stained with the indicated specific Abs or Alexa488-labeled phalloidin (Molecular Probes). The images were captured using an IX-81 fluorescent microscopy (Olympus Corp, Tokyo, Japan) equipped with a confocal microscope DSU unit (Olympus) and analyzed by MetaMorph (Universal Imaging corporation, Molecular Devices, Downingtown, PA).

Ziehl–Neelsen staining

For acid-fast bacterial staining, the culture slides (LabTech® chamber slide system, Nunc-Nalgene™, Thermo Fisher Scientific, Waltham, MA) were fixed, stained with steps of 0.3% carbol-fuchsin solution, decolorization with a 3% hydrochloric acid/95% ethanol solution, and counterstaining with Löffler’s methylene blue. The slides were rinsed, allowed to dry, and examined under a binocular microscope with oil immersion.

Electron microscopic examination of infected osteoclasts

Osteoclasts cultured and infected with the Mtb H37Rv strain were fixed with 2.5% glutaraldehyde in 100 mM phosphate buffer (PB, pH 7.4) for 1 h at 4 °C, rinsed three times with PB, and post-fixed with 1% osmium tetroxide. Then, the samples were dehydrated with a graded ethanol series, embedded with Spurr’s resin and polymerized at 70 °C for 16 h. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined with a JEOL JEM-1230 microscope (Yamada et al., 2001).

Real-time PCR analysis

A real-time quantitative PCR analysis was performed using an ABI 7700 sequence detector system with the purchased probe sets (Taqman® gene expression analysis system, Applied Biosystems, Foster City, CA). The sequences were amplified for 40 cycles under the following two-step parameters: denaturation at 95 °C for 15 s, annealing, and extension at 60 °C for 60 s. The relative gene expression levels were normalized by the expression of GAPDH by the 2−ΔΔCt method.

Measurement of cytokines and chemokines, and TRAP activity

The production of chemokines was determined using DuoSet® ELISA kits (R&D systems) for CCL17, CCL20, CCL22, CCL24, and CCL25. The cytokines [TNF-α, IL-1β, IL-6, IL-8, and interferon (IFN)-γ] and chemokines (CCL2, CCL3, and CCL5) were measured using the human 27-plex multiple cytokine detection system (Bio-Rad Corp., Hercules, CA) according to the manufacturer’s instructions. Tartrate-resistant acid phosphatase (TRAP) activity in the culture supernatant was measured by the Osteolinks TRAP assay kit (DS pharma biochemical, Osaka, Japan).

Immunohistochemistry

Paraffin-embedded tissue sections of tuberculosis patients were deparaffinized in xylene, rehydrated in a graded series of ethanol, and immersed in Target Retrieval Solution pH 9.0 (Dako, Glostrup, Denmark) for 20 min boiling for antigen retrieval. Then sections were incubated with 10% normal goat serum to avoid nonspecific reaction, and stained with primary antibodies for 2 h. After inactivating the endogenous peroxidase activity of tissue sections by 3% hydrogen peroxide, the signals were visualized using HRP-conjugated anti-rabbit IgG secondary antibody system (EnVision™ system, Dako) for 15 min incubation, followed by DAB substrate chromogen system (Dako) with nuclear counterstaining using hematoxylin.

Statistical analysis

The data are presented as the means ± SE and statistical significance was determined with a one-factor factorial ANOVA and Tukey-Kramer’s HSD test in the case of multiple comparisons, using the KALEIDAGRAPH® 4.0 program for WINDOWS (Synergy Software, Reading, PA). A significant difference (P < 0.05) is indicated with an asterisk and NS indicates nonsignificant differences.

Osteoclast dysregulation by M. tuberculosis infection

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Results and discussion

Living Mtb were incorporated and expanded inside multinuclear osteoclasts

It is well known that macrophages are designed to kill pathogens inside the phagosome. After the uptake of pathogens into the macrophages by receptor-mediated phagocytosis, the resulting phagosome undergoes a series of fusion and fission events via the endocytosis pathways. It is believed that osteoclast precursors could be infected with Mtb intracellularly, which would be followed by activation in the inflammatory lesions of spinal tissues, as well as activation of a conditional immune response in macrophages. Generally, the pathogenesis of spinal tuberculosis has been reported to rely on an Mtb-derived chaperonin protein that induces osteoclast recruitment and inhibits the proliferation of osteoblast precursor cells (Meghji et al., 1997). However, it remains to be elucidated whether Mtb has the ability to intracellularly infect multinucleated osteoclasts, although osteoclasts may play an important role in the immunopathogenesis of inflammatory bone diseases.

To investigate whether living Mtb were incorporated and expanded inside multinuclear osteoclasts, we cultured living Mtb with mature multinuclear osteoclasts (mOCs) and their precursor mononuclear pre-osteoclasts (pMCs) (Fig. 1a). After co-culture with Mtb, large numbers of bacteria were incorporated into both mononuclear pMCs and multinuclear mOCs at day 3; in addition, Mtb-infected pMCs have ability to multinucleate at day 5 (Fig. 1a). The results indicated that multinuclear osteoclasts also have ability to uptake living Mtb inside, as well as mononuclear cells. Next we...

Fig. 1 Intracellular growth of Mtb in human mononuclear and multinuclear osteoclasts. (a) Human pMCs and mOCs with living Mtb (6.0 × 10⁶ CFU per well) for 3 or 5 days were stained red with Ziehl–Neelsen stain. Nuclei were counterstained with methylene blue. Magnification ×1000 with oil immersion. Scale bar: 40 μm. (b) The CFU numbers of intracellularly infected Mtb by pMCs (open column) and mOCs (filled column) were quantified by bacterial culture using a mycobacteria growth indicator glass tube with Ogawa media. (c,d) Electron microscopic images of multinuclear osteoclasts infected with living Mtb. The low magnification electron micrograph shows Mtb phagocytosed by an mOC (c), and an enlarged image (d) of the area indicated by the rectangle, where five tubercle bacilli reside in the osteoclast cytoplasm without phagosome membrane (black arrows). Free lysosomes are also distributed in the cytoplasm near tubercle bacilli (white arrows). N, nucleus of the osteoclast.
investigated the numbers of intracellular Mtb, since Mtb was reported previously to grow rapidly inside macrophages (van der Wel et al., 2007). The numbers of intracellular Mtb increased after a five day culture of the osteoclasts. Notably, Mtb grew better in multinuclear osteoclasts than in mononuclear cells; there were four times as many intracellular Mtb in the mOCs than in pMCs on day 5 of culture (Fig. 1b). These results suggest that living Mtb has a greater ability to expand intracellularly following the infection of multinuclear osteoclasts than mononuclear osteoclast precursors.

We next evaluated the intracellular localization of the living Mtb in multinuclear osteoclasts using electron microscopic analysis, because the intracellular localization of Mtb inside the macrophage has been a matter of debate in recent years (Welin & Lerm, 2012). Mtb has the ability to grow in macrophages by arresting the normal process of phagosome maturation. As part of their strategy for intracellular survival, mycobacteria prevent the maturation of the phagosomes, in which they reside inside macrophages. Notably, we observed no significant phagosome membrane around the intracellular Mtb in our study (Fig. 1c and d). These data demonstrated that Mtb escapes from osteoclastic phagosomes, whereas the bacteria were arrested in phagosomes of macrophages. Escape from phagosomes could drive the more rapid expansion of Mtb in osteoclasts. During our electron microscopic analysis, we did not observe any significant phagosome membrane formation around intracellular Mtb (Fig. 1d). It is well known that Mtb survives inside macrophage phagosomes, whose normal maturation process is arrested by the bacteria (Fratti et al., 2001; Pethe et al., 2004). Thus, Mtb interrupts the acidification of endosomes, followed by the fusion of acidic endosomes and lysosomes. As a result, the immature endosomes maintain a high pH (> 6.4) (Pethe et al., 2004), which leads to a failure of the cells to supply hydrolytic enzymes and antimicrobial peptides (Rohde et al., 2007a, b). Our previous report also provided support for the hypothesis that Mtb resides inside endosomes to form multilocular necrotic lesions in the lung, liver, and spleen tissues (Yamada et al., 2002), and that phagolysosomal fusion incorporating many tubercle bacilli is prominent (Yamada et al., 2001). In contrast, our current results demonstrated that there are Mtb-containing phagosomes/endosomes inside the multinuclear osteoclasts (see Fig. 1d). The fact that Mtb escapes from the endosome/phagosome might be due to the endosomal acidification; osteoclasts expressed higher amounts of acid-producing vacuolar type H+ATPase (V-ATPase) for osteolysis. Similar bacterial eviction from the endosomes is observed for other mycobacteria species. An acid-fast bacillus, Mycobacterium marinum, which causes a systemic tuberculosis-like disease in its natural hosts, such as fish and frogs, was reported to escape from phagosomes (Stamm et al., 2003). In addition, intracellular pathogens, including Listeria monocytogenes, Shigella flexneri, and Rickettsia rickettsii (Goldberg, 2001) also share the ability to enter the host cell cytoplasm, induce actin polymerization, and use actin-based motility to spread between host cells during intracellular infection.

Living Mtb have the ability to facilitate osteolytic response by multinuclear osteoclasts rather than proinflammatory responses

We investigated whether proinflammatory responses were induced by intracellular Mtb-infected multinuclear osteoclasts. To assess the productivity of proinflammatory cytokines during osteoclastogenesis in response to living Mtb, the production of proinflammatory cytokines (TNF-α and IL-1β) and proinflammatory chemokines (MCP-1/CCL2 and MIP-1α/CCL3) in response to intracellular Mtb infection by mOCs were measured (Fig. 2a and b). We found that Mtb-infected mOCs lost the ability to produce proinflammatory cytokines (TNF-α and IL-1β) and proinflammatory chemokines (MCP-1/CCL2 and MIP-1α/CCL3), whereas Mtb-infected pMCs retained the ability to produce these proinflammatory factors. Surprisingly, the production level of another proinflammatory cytokine IL-6 decreased by half, suggesting that the basal level of IL-6 production of pMCs was dampened by the early phase of Mtb infection. Notably, we detected no IFN-γ production during osteoclastogenesis (data not shown), although IFN-γ is involved in Th1-mediated immune response and is followed by the formation of granulomatous caseous necrosis observed around destroyed bone tissue (Schluger & Rom, 1998). The data demonstrate that the production patterns of proinflammatory cytokines in inflammatory sites with bone destruction in tuberculosis lesions might be different from those of other inflammatory conditions, such as rheumatoid arthritis.

In addition, the enzymatic activity of tartrate-resistant acid phosphatase (TRAP), which is the principal osteolytic enzyme secreted in the culture supernatant, was measured (Fig. 2c). In pMCs, elevated TRAP activity was observed in unstimulated pMCs, although Mtb-infected pMCs showed decreased TRAP production. This was in contrast to several previous reports suggesting that TNF-α is an enhancer of inflammatory osteolysis in bone destructive lesions (Abu-Amer et al., 1997; Lam et al., 2002). As reported previously, bacterial products, LPS (a ligand for TLR4) and Pam3CSK4 (a TLR2 ligand), has the ability to promote TRAP secretion by pMCs (Fig. 2d), indicating that osteoclastogenesis is accelerated not only by TNF-α but also by TLR-ligands. However, intracellular Mtb infection in mOCs sustained, whereas TLR-ligands obstructed, the TRAP secretion (Fig. 2c and d). These findings are in agreement with previous reports that the ligands for TLR4 inhibit osteoclast differentiation (Itoh et al., 2003; Chang et al., 2007).

In contrast to TRAP activity, the transcriptional level of another osteolytic enzyme, cathepsin K, was enhanced by pMCs in response to Mtb (Fig. 2e). Thus, intracellular infection of Mtb resulted in a different osteolytic response than typical microbial responses via TLR-mediated signals. We confirmed that the expression levels of TLR2 and TLR4 were not significantly changed during osteoclastogenesis (data not shown). Inflammatory activation signals in response to intracellular Mtb, which seems to be independent of the TLR-mediated pathways, could interrupt the osteoclast development.
in pMCs and mOCs stimulated with Mtb after Mtb infection. (a,b) The production of (a) the proinflammatory cytokines TNF-α, IL-1β and IL-6, and (b) the proinflammatory chemokines MCP-1/CCL2 and MIP-1α/CCL3 for 24 h after Mtb infection, were measured by an ELISA. The data show the means ± SD (n = 6). (c,d) The levels of Trap5b in pMCs and mOCs stimulated with Mtb (10⁵ CFU mL⁻¹), LPS (10 ng mL⁻¹) and Pam3CSK4 (100 ng mL⁻¹) were measured by an ELISA. The data show the means ± SD (n = 6). (e) The relative expression levels of an osteolytic enzyme, cathepsin K (CTSK), produced by pMCs and mOCs, stimulated with Mtb, were measured by real-time Q-PCR. The data show the means ± SD (n = 4). (f,g) In (f) the area and the numbers of osteoclasts after Mtb (10⁵ CFU mL⁻¹), LPS (10 ng mL⁻¹), and Pam3CSK4 (100 ng mL⁻¹) stimulation were visualized by immunohistochemical staining using an anti-cathepsin K antibody conjugated with Alexa594 (red). F-actin and nuclei were counterstained by phalloidin-AlexaFlour 488 (green) and hoechst33258 (blue), respectively. Magnification ×400. (g) Histograms of the area distribution of multinuclear osteoclasts delimited with phalloidin and of the number of multinuclear osteoclasts in (f). The data are presented as the means ± SD (n = 3). (h) The relative expression levels of the RANKL receptor (RANK) and M-CSF receptor (CSF1R) by pMCs and mOCs stimulated with Mtb were measured by real-time Q-PCR. The data show the means ± SD (n = 4).

We next investigated whether Mtb infection exerts a facilitatory effect on osteoclast formation, because bacterial products are reported to utilize two different pathways via TLR4 in both survival and cytokine production of osteoclasts (Itoh et al., 2003). Compared with mOCs, Mtb infection by pMCs did not facilitate the formation of multinuclear osteoclasts (Fig. 2f and g). Stimulation of pMCs by Pam3CSK4 also resulted in formation of large numbers of large multinuclear osteoclasts. In contrast, in response to LPS, pMCs increased the number of osteoclasts with diminished osteoclast area. The result indicates that LPS facilitates the osteoclast formation but not osteoclastic activation directly via TLR4. Notably, we found that the osteoclast area increased in response to Mtb, although the osteoclast numbers were decreased by Mtb stimulation (Fig. 2g), indicating that intracellular Mtb infection facilitates osteoclast formation but abrogates typical osteoclastic activation. Therefore, intracellular Mtb infection induces irregular osteoclastogenesis by shifting the properties of osteoclasts from osteolytic to inflammatory.

We then investigated whether the changes in the properties of Mtb-infected osteoclasts were due to the dysfunction of the RANK-RANKL axis, which is the principal osteoclastic regulator. Mtb-infected pMCs exhibited decreased expression levels of RANK and c-fms, which encodes CSF-1R, the receptor for M-CSF (Fig. 2h). These data indicated that Mtb-infected pMCs lost their ability to differentiate into osteoclasts in response to osteoclastogenic...
growth factors M-CSF and RANKL as a result of downregulation of their receptors on osteoclasts. Consequently, *Mtb* infection by pMCs leads to dysfunction in the physiological osteoclastogenesis process and facilitates the inflammatory osteoclastic activation. However, it is still unknown why the development of osteoclasts from the precursor cells was inhibited by intracellular *Mtb* infection but not by the TLR-ligands. Various reports have suggested that there are differences in the biological responses induced by TLRs and RANK, although both TLRs and RANK share a common downstream signaling molecule, TRAF-6 (Mansell et al., 2004; Takeda & Akira, 2005). One possible reason for this is that the early secreted antigen, ESAT-6, facilitates the recognition of *Mtb* in cooperation with TLR2 in macrophages (Pathak et al., 2007).

Specific expression profiles of chemokine ligands and their counterpart receptors are induced by *Mtb*-infected osteoclasts

Several reports suggested the participation of chemokines during physiological osteoclastogenesis (Oba et al., 2005; Menu et al., 2006; Hoshino et al., 2009, 2010), which is induced by the combination of M-CSF and RANKL. The fact that intracellular *Mtb* infection disturbs the physiological osteoclastogenesis prompted us to investigate whether intracellular *Mtb* infection could disrupt the chemokine profiles. Therefore, we compared the chemokine expression profiles in response to intracellular *Mtb* infection. We noted that various chemokine ligands are selectively produced by osteoclasts at mRNA transcription level (Supporting Information, Fig. S1). Among these ligands, *CCL5* (also called RANTES) was highly upregulated during osteoclastogenesis (Hoshino et al., 2010). In addition, pMC expressed small amounts of chemokines, namely, *CCL17, CCL20, CCL22, CCL24,* and *CCL25* (Fig. 3a). However, mature mOCs failed to produce these proinflammatory chemokines in response to *Mtb* infection.

To confirm that the immune response to virulent *Mtb* strains is different from the response to non-virulent strains, we measured the transcription of the chemokines by pMCs and mOCs in response to an avirulent *Mtb* strain (H37Ra) and a BCG-Tokyo strain. Both the avirulent H37Ra strain

![Fig. 3](https://academic.oup.com/femspd/article-abstract/70/1/28/450245/0)
and the BCG strain had a decreased ability to induce the production of the chemokine ligands by osteoclasts (Fig. 3a).

We next investigated the expression profile of C-C chemokine receptors during human osteoclastogenesis. In previous murine experiments we reported that the selective upregulation of two chemokine receptors, CCR1 and CCR5, which are required for osteolytic enzyme production (Hoshino et al., 2009), occurred with the downregulation of other chemokine receptors during murine osteoclastogenesis (Hoshino et al., 2009, 2010). Among the chemokine receptors evaluated, CCR1 and CCR5 were both upregulated during human osteoclastogenesis (Fig. 3b). Besides the virulent Mtb strain, H37Rv infection also upregulated the expression of CCR1, CCR7, and CCR9, whereas there was diminished expression of CCR2, CCR3, CCR6, and CX3CR1 (Fig. 3b, Fig. S2). The avirulent Mtb strain (H37Ra) had sustained expression of CCR1 during osteoclastogenesis, whereas the expression of other chemokine receptors decreased. The osteolytic enzyme cathepsin K (CTSK) is also expressed only following infection with the virulent Mtb strain (Fig. 3c). An enzyme matrix metalloproteinase-9 (MMP9), which was secreted mainly by epithelial cells surrounding the growing granuloma and promoted the recruitment of new macrophages to the granuloma (Volkman et al., 2010), was also expressed with the virulent Mtb stimulation. As CCR1 is the most abundant chemokine receptor during osteoclastogenesis, we next investigated the expression of CCR1 and CCR5 in multinuclear cells in a granuloma from a tissue section of the Mtb-infected patient (Fig. 4), and the results suggested that the expression of chemokine receptors plays a role during multinuclear cell formation in the pathological condition. Therefore, intracellular Mtb infection induces the evacuation of Mtb into the cytosol, which leads to irregular osteoclastogenesis as a result of the dysregulation of chemokines, chemokine receptors, and osteoclastic molecules, and finally results in a shift in the properties of osteoclasts from osteolytic to inflammatory.

**Infection of osteoclasts with Mtb produces specific chemokines**

To further investigate whether these chemokine responses are specific for Mtb infection, we measured and compared the production of these chemokines after Mtb infection, and LPS stimulation by pMCs and mOCs (Fig. 5). As previously mentioned, inflammatory chemokines CCL2/MCP-1 and CCL3/MIP-1α are produced in limited numbers by Mtb-infected pMCs (see Fig. 1b). Instead, abundant production of several chemokines by mOCs was observed: (1) a common proinflammatory response to both LPS and intracellular Mtb infection, (2) a *Mycobacteria*-specific chemokine response, and (3) an intracellular virulent Mtb infection-specific chemokine response. The first group of chemokines, CCL5, CCL24, and CCL17, are commonly produced in response to both LPS and intracellular Mtb stimulation (Fig. 5a–c), suggesting that the activation of the CCR5-CCL5, CCR4-CCL17, and CCR3-CCL24 axes is a common infectious response of multinuclear osteoclasts. Notably, the CCL17 production was limited to mOCs (Fig. 5c), implying that the activation of the CCR4-CCL17 axis reflects abnormal osteoclast activation. The second group of chemokines, including CCL22, was enhanced by virulent Mtb H37Rv infection, as well as avirulent H37Ra infection (Fig. 5d). CCL22 was physiologically produced by unstimulated mOCs, and its production was pathologically enhanced by pMCs in response to both LPS and Mtb stimulation, indicating that the aberrant production of CCL22 by pMCs might be a facilitator for the abnormal osteoclastic activation. Interestingly, the production of the third group of chemokines, CCL20 and CCL25, which was enhanced by virulent Mtb infection, was observed only by mOCs (Figs. 5e and f). The data suggested that CCL20 and CCL25 might play pivotal roles in the osteolytic response of virulent Mtb-infected bone tissue. These findings suggest that the pivotal role of chemokines in pathological osteoclastogenesis was via the combined production of osteoclast-specific chemokines, such as CCL17, and Mtb-specific chemokines.

**Fig. 4** Expression of chemokine receptors CCR1 and CCR5 in Mtb-infected multinuclear granuloma in a tuberculosis patient. (Center panel) Immunohistochemical staining of chemokine receptor CCR1 visualized by an anti-human CCR1 antibody and followed by DAB chromogen. Nuclei were counterstained using hematoxylin. Magnification ×400. (Right panel) Immunohistochemical staining of chemokine receptor CCR5 visualized by an anti-human CCR5 antibody conjugated with phycoerythrin (red). Nuclei were counterstained by Cyto13green fluorescent dye in green. Magnification ×400. (Left panel) H&E stain of counterpart section. Scale bar: 100 µm.
including CCL20, CCL22, and CCL25, in response to evacuated Mtb in the cytosol.

The findings of the present study demonstrate that the intracellular infection of multinuclear osteoclasts by Mtb failed to induce the secretion of typical proinflammatory cytokines and proinflammatory chemokines, such as TNF-α, IL-1β, CCL2/MCP-1 or CCL3/MIP-1α (Fig. 2), but resulted in the selective expression of osteoclast-specific chemokines and their receptors (Fig. 3). However, the combination of chemokine ligands and their receptors do not have a one-to-one correspondence; the induction ratio of chemokine receptors after virulent Mtb infection revealed that Mtb infection activates a very limited number of chemokine ligands, namely CCL5, CCL17, CCL19, CCL20, CCL22, CCL24, and CCL25 (Fig. 3a, Fig. S1), which was followed by the induction of their corresponding chemokine receptors, such as CCR1, CCR4, CCR5, CCR7, and CCR9 (see Fig. 3b). Thus, these chemokine axes could be an important chemokine-mediated response induced by intracellular Mtb infection. Among them, the chemokine axes of CCR1/CCR5-CCL5, CCR4-CCL17/CCL22, and CCR9-CCL25 could play roles in the chemokine-mediated response to intracellular Mtb infection. In support of this, both CCR1 and CCR5 were highly expressed in multinuclear granuloma cells by a Mtb-infected patient (Fig. 4). In addition, several clinical papers indicated the involvement of the chemokine...
system in intracellular Mtb infection; CCR7 is related to the progression of several inflammatory bone diseases, such as rheumatoid arthritis (Bugatti et al., 2005; Pickens et al., 2011) and a murine model of tuberculosis (Kahnert et al., 2007; Khader et al., 2009). The involvement of the CCR9–CCL25 axis in rheumatoid arthritis was also reported (Endres et al., 2010; Schmutz et al., 2010). CCL22 and its receptor, CCR4, are highly expressed in the lungs, especially in tuberculosis lungs (Volpe et al., 2006; Okamoto et al., 2007; Wu et al., 2010), and it has also been reported to be produced by human osteoclasts in response to foreign substances, such as titanium particles (Cadosch et al., 2010). These data provide new aspects of how CCR4 switches its ligands, CCL17 and CCL22. The mechanism leading to the switch in the specific chemokine axes could play a pivotal role during pathological osteoclastogenesis via virulent Mtb infection. Further investigations will be needed to clarify the osteolytic mechanism of skeletal tuberculosis.

In conclusion, we have characterized the pathological activation of osteoclasts in response to intracellular Mtb infection. Our data indicate that the inflammatory osteoclastogenesis by Mtb infection is facilitated not only by proinflammatory chemokines such as TNF-α, and by the activation of the RANK-RANKL pathways, but also by the production of specific chemokines in response to intracellular Mtb infection. Notably, we did not detect any TNF-α production by Mtb-infected multinuclear osteoclasts (see Fig. 2a). TNF-α is responsible for granuloma formation and maintenance, and the deficiency of TNF-α resulted in hypersusceptibility to tuberculosis (Ramakrishnan, 2012). Thus, the unresponsiveness of TNF-α might cause the defective immune response during the early stages of Mtb infection. Although early infection of macrophages might be predicted to promote initial host immunity, Mtb impairs antigen presentation and an effective adaptive immune response. Furthermore, we found that the intracellular Mtb inside multinuclear osteoclasts escaped from the endosome/phagosome, and led to dysregulation of osteoclast activation. Aberrant production of chemokines is due to the evacuation of Mtb from the endosome/phagosome, which accomplishes to avoid acidification. Evacuated Mtb in cytosol might lead to dysregulation of cytokines and chemokines, which promotes atypical osteoclast activation, and finally causes pathological bone destruction in the bone tissue. Consequently, our data suggest that the source of Mtb-activated osteoclasts in spinal tuberculosis could be derived from tissue-resident multinuclear osteoclasts that were unexpectedly activated in response to Mtb infection. Our findings provide novel information about an atypical type of inflammation that is independent of proinflammatory cytokine production due, in the case of osteoclast activation to spinal tuberculosis.

The present observations provide further evidence that the chemokine/chemokine receptor axes in bone metabolism play pathological roles, including the functional differentiation of osteoclasts following intracellular infection. Our current findings emphasize the relevance of the chemokine axes as exacerbating factors for bone destruction diseases. The dysregulation of cytokines and chemokines appears to play a pivotal role in the abnormal differentiation of macrophage-lineage cells via reprogramming of the inflammatory responses involved in pathological bone metabolism. Further studies are needed to clarify the mechanism of destruction following intracellular infections with pathogens.

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Authors’ contributions

A.H. and S.H. performed the experiments and analyzed the data for H.Y., Sa.M., and Y.M.; H.Y. and Sa.M. provided the equipment, the Bio Safety Level-3 facility for Mtb experiments, and virulent Mtb strain H37Rv; Sh.M. and M.T. provided the paraffin-embedded tissue section of tuberculosis patients; A.H., Y.M., and K.Y. designed the research; A.H. wrote the paper. Contact authors: Yoshinobu Manome (manome@jikei.ac.jp), Akiyoshi Hoshino (hoshino@nih.go.jp); hoshino.akiyoshi@med.nagoya-u.ac.jp.

References


Supporting Information

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

Fig. S1. The expression levels of chemokine ligands by Mtb-infected monocytes, pre-osteoclasts, and multinuclear osteoclasts.

Fig. S2. The expression levels of chemokine receptors by monocytes, pre-osteoclasts, and multinuclear osteoclasts after Mtb stimulation.

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