Tumor Necrosis Factor-α from macrophage in response to bacteria via

Toll-like Receptor 2 is responsible for osteoclastogenesis

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Running title

Osteoclastogenesis by Bacterial Infected Macrophage

Abstract

Receptor activator of NF-κB ligand (RANKL) and pro-inflammatory cytokines are believed to play important roles in osetoclastogenesis. We recently reported that the innate immune recognition receptor, toll-like receptor (TLR) 2 is crucial for inflammatory bone loss in response to *Porphyromonas gingivalis,* the primary organism associated with chronic inflammatory periodontal disease. However, the contribution of the TLRs of the macrophage in osteoclastogenesis has not been defined. In this study we defined a requirement for TLR2 in tumor necrosis factor (TNF)-α elicited osteoclastogenesis in response to exposure with *P. gingivalis.* Culture supernatant (CS) fluids from *P. gingivalis*-stimulated macrophages induced bone marrow macrophages-derived osteoclastogenesis. This activity was dependent on TNF-α and occurred independent of RANKL, interleukin (IL) -1β, and IL-6. CS fluids from *P. gingivalis*-stimulated TLR2$^{-/-}$ macrophages failed to express TNF- α and these fluids induced significantly less osteoclast formation as compared with wild type or TLR4$^{-/-}$ macrophages. In addition, *P. gingivalis*-induced the up-regulation of cell surface expression of TLR2 on macrophages, which was demonstrated to functionally react to re-exposure with *P. gingivalis* as measured by a further increase in TNF-α production. These results demonstrate that macrophage-dependent TLR2 signaling is crucial for TNF-α-dependent / RANKL-independent osteoclastogenesis in response to *P. gingivalis*. Furthermore, the ability of *P. gingivalis* to induced cell surface expression of TLR2 may contribute to the chronic inflammatory state induced by this pathogen.

Introduction

Bone metabolism is a complex process, and in healthy individuals, a homeostatic balance is achieved between bone resorption and new bone formation (7). Osteoclasts play an important role in bone resorption and originate from the fusion of precursors belonging to the monocyte/macrophage lineage. Receptor activator of nuclear factor κB ligand (RANKL), a member of the tumor necrosis factor (TNF) ligand super family, is a major factor involved in osteoclast generation. Both soluble and transmembrane forms of RANKL induce osteoclast formation when these cells are cultured with macrophage colony- stimulating factor (M-CSF) (16). RANKL-induced osteoclast formation is inhibited by osteoprotegerin (OPG), a decoy receptor of RANKL (31, 38). Osteoclast differentiation also occurs through a mechanism that is independent of RANKL-RANK interaction and is dependent on TNF-α (3, 20). It has been reported that TNF-α dependent osteoclastogenesis occurs when permissive levels of RANKL exist (22). Recently, however, Kim *et al.* (19) reported that transforming growth factor (TGF)-β treated bone marrow (BM) macrophages could be differentiated into osteoclast-like cells when cultured with TNF-α by a mechanism that was independent of RANKL-RANK suggesting that TNF-α also plays an important role in osteoclastogenesis.

In chronic inflammatory bone diseases including rheumatoid arthritis (RA), RANKL as well as pro-inflammatory cytokines such as TNF-α, interleukin (IL)-1β and IL-6 have been shown to be important for disease progression (14). Histological evaluations have revealed that the cellular composition of

inflammatory bone lesions in RA primarily consists of T and B cells, as well as macrophages (27, 32).   T cells and B cells have been reported to contribute to the acceleration of bone resorption by production of cytokines and RANKL (12, 21, 37).   Macrophages in inflamed tissues can not only differentiate to osteoclasts (1), but also appear to accelerate bone resorption through production of pro-inflammatory cytokines (8).

Toll-like receptors (TLRs) are a group of pathogen-associated pattern recognition receptors, which have been identified as key participants in innate recognition for pathogens (23, 33).   TLR2 is activated by bacterial lipoproteins, peptidoglycans, and *Staphylococcus aureus* lipoteichoic acid (SLTA) (2, 29, 30). TLR4 recognizes enteric lipopolysaccharide (LPS) (34).   Monocytes and macrophages express both TLR2 and TLR4 (15).   Following TLR engagement macrophages produce pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α (17, 18, 29).   A recent study reported that peripheral blood mononuclear cells of RA patients expressed increased TLR2 and TLR4 when compared to levels expressed in cells obtained from healthy subjects (28).   Iwahashi *et al.* (18) have also shown that both blood monocytes and synovial tissue macrophages in RA patients expressed high levels of TLR2.   While these studies have correlated macrophage TLR expression with inflammatory tissue damage associated with bone destruction, a requirement for macrophage-specific TLR signaling influencing osteoclast formation has not been defined.

Periodontal disease is a well characterized chronic inflammatory bone destructive disease induced by bacterial infection.   While periodontal disease

and RA are different diseases initiated by distinct causes, it has been suggested that similar pathological process are involved in the resulting inflammatory bone destruction (14). Histological analysis of local inflammatory lesions obtained from severe periodontal disease patients possess increased expression of TLR2 and TLR4 from macrophages as compared to lesions obtained from patients with mild periodontal disease (25). Others, on the other hand, have reported there is significant down-regulation of TLR2 mRNA by real-time PCR in chronic periodontitis (26). We and others (6, 11) have reported that TLR2 is crucial for inflammatory bone loss in response to the Gram-negative pathogen *Porphyromonas gingivalis* (*P. gingivalis*), the primary organism associated with chronic inflammatory periodontal disease. However, the contribution of the innate immune recognition system of the macrophage in inflammatory bone loss has not been defined. In this study, we examined the requirement for TLR2 and TLR4 in *P. gingivalis* infected macrophage induced osteoclastogenesis. Using murine macrophages, we detail a mechanism by which TLR2-mediated signaling to *P. gingivalis* stimulates a potent soluble pro-inflammatory response which stimulates bone marrow macrophage (BMM) to undergo osteoclastogenesis via TNF-α induction. Furthermore we demonstrate that *P. gingivalis*-induced the up-regulation of cell surface expression of TLR2 on macrophages, which was demonstrated to functionally react to re-infection with *P. gingivalis* as measured by a further increase in TNF-α production. The ability of *P. gingivalis* to induce cell surface expression of TLR2 may further contribute to chronic inflammation, which is characteristic of human periodontal disease.

MATERIALS AND METHODS

Animals

C57BL/6 wild-type (WT) (Jackson Laboratory, Bar Harbor, Me), TLR2-knochout (TLR2$^{-/-}$) and TLR4-knochout (TLR4$^{-/-}$) mice (originally provided by S. Akira, Osaka University) were cared for in accordance with Boston University Institutional Animal Care and Use Committee procedures.

Bacteria and growth conditions

P. gingivalis strain 381 was grown on anaerobic blood agar plates (BBL media; Becton Dickinson Co.) and used to seed Brain Heart Infusion broth (BHI; Difco) (pH 7.4) supplemented with yeast extract (Difco), hemin (1μg /ml; Sigma) and menadione (1 μg/ml; Sigma). Organism numbers were standardized using a spectrophotometer (DU 7500, Beckman) at an optical density of 1 at 660 nm (equivalent to 1x10^9 CFU/ml).

Peritoneal macrophage cultures

Male mice (8-10 wk of age) were injected intraperitoneally with 3.0 ml of a sterile thioglycollate solution (Remel). After 4 days, peritoneal exudates cells were harvested by lavage with RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) and 50 μg/ml of gentamicin (Sigma). After the cells were cultured in plate dish for 24 h, non-adherent cells were removed. The adherent cells were cultured with medium not containing antibiotics for one day and were used as peritoneal macrophages.

Macrophage *P. gingivalis* stimulation assays

Peritoneal macrophages from WT, TLR2$^{-/-}$, or TLR4$^{-/-}$ mice were cultured with *P. gingivalis* at a various multiplicity of infection (MOI) for 6 or 24 hours in 12- (for FACS or osteoclast formation) or 96- (for ELISA) well plate dishes. In some experiments, cells were stimulated with 2 μg/ml of *Staphylococcus aureus* lipoteichoic acid (SLTA) (InvivoGen) or 100ng/ml of *Escherichia. Coli* (*E. coli*) LPS (InvivoGen). Some wells were incubated with either 20 μg/ml of murine TLR2 blocking antibody (T2.5) or isotype-matched rat IgG (eBioscience) before stimulation of cells. Cells were collected for FACS analysis. The culture supernatant (CS) fluids centrifuged to eliminate the bacteria were collected and keep in –70°C until used for ELISA or osteoclast formation. Unstimulated macrophages were used as a control.

TLR functional assay

Peritoneal macrophages were cultured with *P. gingivalis* (MOI = 10) in 96-well culture plates for 1 h. Non-adherent bacteria were then removed by washing, and these cells were cultured with medium for 5 hrs. After medium change, these *P. gingivalis* infected cells were re-exposed with *P. gingivalis* (MOI = 10) for an additional 24 hrs. CS fluids and cells were collected for ELISA or FACS analysis as described below.

Cytokines and RANKL assay

Concentrations of IL-1β, IL-6, TNF-α (BD Biosciences), and RANKL (R&D system) in CS fluids were determined using commercially available murine

enzyme-linked immunosorbent assay (ELISA) kits.

Flow cytometry analysis

Fluorescence-activated cell sorter (FACS) analysis was performed on macrophage cultures by first washing cells in buffer (0.2%BSA/PBS), followed by incubation with Fc receptor blocker (eBioscience). The cells were then labeled with fluoescein isothiocyanate (FITC) conjugated anti-mouse TLR2 monoclonal antibody (mAb) (6C2, eBioscience), phycoerythrin (PE)-conjugated anti-mouse TLR4 mAb (MTS510, eBioscience), PE-conjugated anti-mouse RANKL mAb (IK22/5, eBioscience) or isotype controls.   The cells were washed and 10,000 events were analyzed by flow cytometry using a FACScan flowcytometer (Becton Dickinson). Geometric means of three independent experiments were utilized for statistical analysis.

Osteoclast formation

C57BL/6 mouse BM cells were cultured in α-minimum essential medium (α-MEM) (Gibco) containing 10% FBS, 100 μg/ml of streptomycin, 100 IU of penicillin (Cellgro) and 5ng/ml of mouse recombinant M-CSF (R&D Systems) for 12h in 100-mm diameter dishes.   Non-adherent cells were harvested and cultured with 30ng/ml of M-CSF in 100-mm diameter dishes for an additional 24 hours.   Non-adherent cells were washed out and adherent cells were collected and used as BMMs (3).   BMMs (3×10^4 cells/0.5ml/well) were cultured for 5 days in α-MEM containing 10% FBS, antibiotics, 30ng/ml of M-CSF and 0.2 ml of CS fluids from macrophages of WT, TLR2$^{-/-}$ or TLR4$^{-/-}$ mice challenged with *P.*

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gingivalis (MOI = 10) for 24 hours in 8-well Lab-Tek chamber slide (Nalge Nunc international). To some wells were added 300ng/ml of OPG, or 5 μg/ml of anti-mouse TNF-α IL-1β IL-6 polyclonal antibody, or normal goat IgG (R&D system). On day 3, the culture medium and all reagents were replaced. To identify osteoclasts, cells were fixed in 4% paraformaldehyde and stained with tartrate-resistant acid phosphatase (TRAP) (Sigma). TRAP-positive multi-nucleated cells (more than three unstained nuclei) were defined as osteoclast, and the number was determined by microscopic counts.

Pit formation assay

BMMs (2 x 10^4 cells/0.25ml/ well) were plated on dentine disks (OsteoSite) in 96-well culture dishes and cultured in α-MEM containing 10% FBS, antibiotics and 100 μl of CS fluids from peritoneal macrophages of WT, TLR2$^{-/-}$ or TLR4$^{-/-}$ mice infected with *P. gingivalis* in the presence of M-CSF (30ng/ml) for 7 days. Medium and all reagents were replaced every 3 days. After culturing, the dentine disks were cleaned by ultrasonication in 1M of NH_4OH to remove adherent cells. After rinsing, dentine disks were stained with hematoxylin for visualizing resorption lacunae (20).

Statistical analysis

Statistical analysis was carried out with Statview software (SAS Institute Inc.). Unpaired *t*-test, one- or two-factor analysis of variance (ANOVA) with Tukey-Kramer test was performed to assess differences among groups. *p*<0.05 was considered as statistically significant.

RESULTS

***P. gingivalis* infected macrophages produce the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, but not RANKL**

While a few studies have examined the pro-inflammatory response of mouse macrophages to *P. gingivalis*, little is known about the expression of pro-inflammatory cytokines believed to play a role in osteoclastogenesis following exposure with live *P. gingivalis*. Thus in our initial studies we examined the production of TNF-α, IL-1β, IL-6 and soluble RANKL in CS-fluids from peritoneal macrophages of WT mice in response to live *P. gingivalis* at different MOI. There is the possibility that some enzyme from *P. gingivalis* can affect the activities of cytokines in culture medium. In our present study, we focused the affect live bacteria with some enzyme but not only their components, because a lot of components of bacteria will influence *in vivo*. To examine the affect of live bacteria, we use the medium from un-stimulated macrophage as control. Significantly elevated levels of TNF-α, IL-1β, and IL-6 were detected 24 hours post-exposure with *P. gingivalis* at MOIs of 10 and 100 (Fig. 1A-C); however, RANKL production was not increased when macrophages were cultured with *P. gingivalis* (Fig. 1D). We also examined challenged macrophages for membrane bound RANKL expression by FACS. The expression of membrane RANKL was similar between challenged and unchallenged macrophages (data no shown). Since it has been reported that TGF-β can influence osteoclastogenesis [8], we also measured TGF-β levels in CS-fluids. TGF-β levels in CS-fluids from live *P. gingivalis* stimulated cells were

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not significantly different from unstimulated cells (*p*>0.05; data not shown). These results indicate that macrophages cultured with *P. gingivalis* express pro-inflammatory cytokines but do not produce soluble or membrane-bound RANKL.

TLR2 partially mediates TNF-α, production by murine macrophages in response to *P. gingivalis*.

We and others (6, 11) recently reported that TLR2 is crucial for inflammatory bone loss in response to *P. gingivalis* in a mouse model of oral bone loss. To assess the requirements for TLR signaling pathways in pro-inflammatory cytokine production in response to *P. gingivalis*, we cultured macrophages from WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice with live *P. gingivalis* and assessed the levels of each cytokine in CS-fluids.   Expectedly, macrophages from TLR2$^{-/-}$ and TLR4$^{-/-}$ mice did not produce cytokines in response to their cognate ligands (SLTA and *E. coli* LPS, respectively), while macrophages obtained from WT mice responded well to stimulation with both antigens (Fig. 2A-C). TNF-α production by TLR2$^{-/-}$ mouse macrophages cultured with *P. gingivalis* was clearly reduced when compared to WT or TLR4$^{-/-}$ mice (Fig. 2A). IL-1ß and IL-6 production were similar among all strains of mouse macrophages cultured with *P. gingivalis* (Fig. 2B, 2C). To verify the requirement for TLR2 in eliciting pro-inflammatory cytokines from macrophages in response to *P. gingivalis*, we incubated WT macrophages with anti-mouse TLR2 blocking antibody followed by exposure with *P. gingivalis*. We observed that blocking antibody treatment inhibited SLTA-induced cytokine production, but failed to inhibit stimulation of macrophages cultured with *E. coli*

LPS. Macrophages cultured with *P. gingivalis*, after anti-TLR2 antibody, demonstrated a marked reduction in TNF-α production, but showed no changes in IL-1β and IL-6 levels (Fig. 2D-F).   Collectively, these results indicate that the TLR2 signaling is involved in mediating the TNF-α, response to live *P. gingivalis* and that the observed IL-1β and IL-6 response are mediated via TLR2 independent mechanisms.

Live *P. gingivalis* stimulation sensitizes murine macrophages making them hyper-responsive to subsequent exposure

We have previously reported that *P. gingivalis* infection results in the up regulation of cell surface TLRs on endothelial cells (39).   We postulated that the ability of *P. gingivalis* to stimulate cell surface expression of TLRs on the endothelial cell could serve as a mechanism to sensitize these cells to other TLRs ligands and could play a role in chronic inflammation induced by this pathogen.   To determine if a similar response occurred in macrophages, we cultured peritoneal macrophages with *P. gingivalis* and examined the temporal expression of TLR2 and TLR4.   As shown in Figure 4, macrophages in response with *P. gingivalis* expressed increased cell surface TLR2 as compared to un-stimulated macrophage cultures at both 6 and 24 hours post-stimulation. However, we did not observe changes in TLR4 surface expression following incubation of macrophages with *P. gingivalis* at either time point (Fig. 3A).

We next examined if the increased expression of TLR2 on the surface of macrophages elicited by *P. gingivalis* could enhance TNF-α production to subsequent exposure of macrophages to this organism.   We confirmed that

TLR2 but not TLR4 expression was significantly up regulated before re-exposure (Fig. 3B).   Exposure of macrophages with *P. gingivalis*, prior to re-exposure with the organism, resulted in a significant increase in TNF-α production as compared to macrophages which had not been exposed to *P. gingivalis*.   SLTA, TLR2 agonist, stimulation also increased TNF-α, production when macrophages were exposed to *P. gingivalis* (Fig. 3C). Collectively these results indicate that similar to that observed in endothelial cells, live *P. gingivalis* exposure can induce cell surface expression of TLR2 which results in sensitization of the macrophage as measured by TNF-α production following re-exposure with this organism.

CS fluids collected from macrophages cultured with *P. gingivalis* induced osteoclast formation is mediated by TNF-α but not RANKL.

The differentiation of BMMs into osteoclasts has previously been demonstrated by RANKL or TNF-α, in conjunction with M-CSF (3, 20).   To examine if CS fluids collected from peritoneal macrophages stimulated with *P. gingivalis* could induce osteoclast formation from BMMs, we stimulated BMMs with CS fluids from WT mouse peritoneal macrophages in response with *P. gingivalis* for 24 hours.   As shown in Figure 5, formation of osteoclast-like cells was induced by the CS fluids from *P. gingivalis* challenged macrophages, but not by unchallenged macrophages. TNF-α blocking antibody, but not OPG or control goat IgG, completely blocked osteoclastogenesis induced by the CS fluids from peritoneal macrophages cultured with *P. gingivalis* (Fig. 4A and 5B).   We

confirmed that OPG or anti-TNF-α antibody completely blocked the osteoclast formation in response to their cognate ligands (RANKL or TNF-α, respectively) (p<0.0001, data not shown). In addition, we demonstrated that neither IL-1β nor IL-6, in conjunction with M-CSF, could induce osteoclast formation from BMMs (p=0.9955 or p=0.9991, respectively, data not shown).   We also attempted to block osteoclastogenesis using anti-mouse IL-1β or anti-mouse IL-6 blocking antibodies; however, these did not alter osteoclastogenesis elicited by CS fluids collected from *P. gingivalis* challenged macrophages (p=0.3152 or p=0.1783, respectively, data not shown).   These results indicate that osteoclast-like cell formation is dependent upon TNF-α, but not IL-1β, IL-6 or soluble RANKL that was present in the CS fluids collected from WT macrophages cultured with *P. gingivalis*.

We next assessed the functional activity of osteoclast-like cells to resorb dentine.   CS fluids of macrophages in response to *P. gingivalis*, but not un stimulated cells also induced dentine resorption. Resorption of dentine by osteoclast-like cells elicited using CS fluids collected from macrophages cultured with *P. gingivalis* was completely blocked by anti-TNF-α antibody, but not with OPG or control goat IgG (Fig. 4C).   Taken together these results indicate that the CS fluids from live *P. gingivalis* stimulsted peritoneal macrophages can promote functional osteoclast formation from BMMs.

Role for TLR2 in osteoclast formation induction by CS fluids from macrophages in response to *P. gingivalis*.

To define the role of TLRs in macrophage cultured with *P. gingivalis* induced osteoclast formation, we next compared the ability of *P. gingivalis* stimulated WT, $TLR2^{-/-}$, $TLR4^{-/-}$ peritoneal macrophages to induce osteoclastogenesis *in vitro*. CS fluids from live *P. gingivalis* stimulated macrophages were able to induce osteoclast formation in all mouse strains examined; however, CS fluids obtained from $TLR2^{-/-}$ macrophages induced the differentiation of much fewer osteoclasts when compared to CS fluids obtained from WT or $TLR4^{-/-}$ *P. gingivalis* stimulated macrophages (Fig 5. A and B). As expected, TNF-α levels in CS fluids obtained from *P. gingivalis* stimulated $TLR2^{-/-}$ macrophages were lower (1.9 ± 0.8ng/ml) than that observed in CS fluids obtained from *P. gingivalis* infected WT (17.4 ± 2.5ng/ml) and $TLR4^{-/-}$ macrophages (17.5 ± 8.0ng/ml). These results indicate that the reduced formation of osteoclasts following incubation with CS fluids from $TLR2^{-/-}$ macrophages appears to result from low levels of TNF-α production. We also observed that anti-TNF-α blocking antibody, but not OPG and control goat IgG, added to the infected CS fluids, completely inhibited osteoclast-like cell formation in all mouse strains tested further confirming the importance of TNF-α in mediating osteoclast formation by CS fluids obtained *P. gingivalis* stimulated macrophage. (Fig. 5B).

Resorption lacunae on the dentine were rarely observed with cells cultured with CS fluids from *P. gingivalis* stimulated macrophages obtained from $TLR2^{-/-}$ mice. Conversely, dentine resorption induced by CS fluids from *P. gingivalis* stimulated macrophages from $TLR4^{-/-}$ mice was similar to that observed in the CS-fluids from WT mice macrophages (Fig. 5C). These results indicate that *P. gingivalis* stimulated macrophages obtained from $TLR4^{-/-}$ mice also can induce

functional osteoclast formation. Collectively, these results indicate that TLR2 signaling plays an important role in live *P. gingivalis* induced osteoclastogenesis. Furthermore, TLR4 signaling does not appear to be critical for osteoclastogenesis and pit formation elicited by BMMs stimulated with CS fluids from macrophages in response to *P. gingivalis.*

DISCUSSION

In this study we have defined a requirement for TLR2 in TNF-α elicited osteoclastogenesis in response to with live *P. gingivalis.* Furthermore, we determined that osteoclastogenesis in response to *P. gingivalis* while dependent on TNF-α was independent of RANKL, IL-1β, and IL-6. In addition, we also demonstrate that *P. gingivalis*-induced the up-regulation of cell surface expression of TLR2 on macrophages, which was shown to functionally react to re-infection with *P. gingivalis* as measured by a further increase in TNF-α production. The ability of *P. gingivalis* to induce cell surface expression of TLR2 may thus contribute to the chronic inflammatory state induced by this pathogen.

While several reports have documented that both T and B cells play an important role in inflammatory oral bone loss (4, 12, 21, 35), it is clear that macrophages are also important immune cells in the initial innate immune recognition of bacteria. Our results demonstrate the contribution of the macrophage in response with *P. gingivalis* and the resulting induction of osteoclastogenesis. Histological studies have reported that RANKL-bearing macrophages are present in diseased tissue obtained from patients with periodontal disease (9, 24). However, very little is known about the macrophage production of RANKL *in vitro*. One study demonstrated that low levels of RANKL could be detected in culture supernatants of unstimulated human macrophages differentiated from blood monocytes; however, the functional activity of RANKL was not examined (10). In our study, we found that osteoclastogenesis and dentine pit formation induced by *P. gingivalis* stimulated macrophage CS fluids was not affected by OPG. These results indicate that the

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induction of osteoclastogenesis by CS fluids was independent of soluble RANKL. RANKL exists not only as soluble form but also as a membrane bound form and both membrane and soluble RANKL are capable of stimulating osteoclastogenesis. However, we did not observe a difference in the expression of membrane bound RANKL on macrophages following stimulation with *P. gingivalis*. Gingipain, systein protease, from *P. gingivalis* should be exist in culture medium and this may affect RANKL form and its activity. Even if RANKL from macrophage was degraded by gingipain, it is no doubt *P. gingivalis* stimulated-macrophage induced osteoclast formation in independent of RANKL because the formation could not be blocked with OPG. Collectively these results suggest that RANKL does not play an important role in osteoclast formation induced by live *P. gingivalis*-stimulated macrophages.

TNF-α has been previously reported to stimulate osteoclastogenesis by a RANKL-independent mechanism (3, 19, 20). Kim *et al.* (19) reported that TNF-α was capable of mediated BMM osteoclastogenesis; however, TGF-β was reported to also play a role in this process. While we did detect low levels of TGF-β in the fluids of unstimulated macrophages, we did not observe significant differences in TGF-β production between unstimulated or *P. gingivalis*-stimulated macrophages. Since our BMMs were exposed to FBS prior to incubation with CS fluids and it is known that TGF-β is present in FBS, we cannot definitively rule out a role for TGF-β in *P. gingivalis* induced osteoclastogenesis in our macrophage system.

TNF-α is frequently detected in inflamed tissues from patients with

periodontal disease (36). TNF-α has been demonstrated to contribute to oral bone loss in a *P. gingivalis* induced inflammatory oral bone loss mouse model (13). *P. gingivalis* has also been demonstrated by others to induce TNF-α following stimulation of monocyte/macrophage (5) and recent studies have documented a role for TLR2 in this response (40). Our results are in agreement with these studies and further demonstrated that TNF-α production in response to *P. gingivalis* was independent of TLR4 signaling. Moreover, we also observed that both IL-1β and IL-6 production from *P. gingivalis* stimulated macrophages occurred via a TLR2-independent signaling mechanism. We and others recently reported that TLR2 deficient mice were resistant to inflammatory bone loss following oral infection with *P. gingivalis* (6. 11). We also demonstrated that TLR4 is not absolutely required for *P. gingivalis* induced oral inflammatory bone loss (11). Thus it appears that TLR2 is an essential signaling receptor for *P. gingivalis* as demonstrated in both *in vitro* and *in vivo* models of inflammation.

Another important observation in this study was that live *P. gingivalis* induced the up-regulation of TLR2 surface expression on macrophages. Furthermore, we demonstrated that macrophages, which were exposed to *P. gingivalis*, responded upon subsequent exposure with *P. gingivalis* or SLTA as measured by an increase in TNF-α production. Muthukuru et al have reported that re-stimulation with *P. gingivalis* LPS induce down-regulation of TLR2 and TLR4 on human monocyte and reduction of cytokines secretion (26). In present study, we used live *P. gingivalis* but not their components for stimulation of murine macrophages. The effect of TLRs regulation of live *P. gingivalis* may be different from that of their each component. Collectively these results indicate

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that similar to that observed in endothelial cells, live *P. gingivalis* exposure can induce cell surface expression of TLR2 which results in sensitization of the macrophage as measured by TNF-α production following re-exposure with this organism. The ability of *P. gingivalis* to induce cell surface expression of TLR2 may contribute to the chronic inflammatory state induced by this pathogen. Many chronic infectious diseases such as periodontal disease induced by various bacterial infections, therefore, other bacteria may also act similar to *P. gingivalis.*

In conclusion we have demonstrated the importance of TLR2 signaling in macrophage-induced osteoclastogenesis following live bacterial stimulation *in vitro.* Furthermore we observed that this response is mediated via TNF-α. In addition, we have confirmed that *P. gingivalis* stimulation can induce cell surface expression of TLR2, which results in sensitization of the macrophage as measured by TNF-α production. It is tempting to speculate that chronic inflammation in response to *P. gingivalis* infection of the macrophage results from the increased expression of TLR2 which in turn leads to increased expression of TNF-α.

Acknowledgements

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Footnotes

Figure legends

FIGURE 1. Comparison of pro-inflammatory cytokines and RANKL production from peritoneal macrophages cultured with *P. gingivalis*. Peritoneal macrophages from WT mice were cultured with *P. gingivalis* at the indicated MOIs for 6 or 24hours. CS fluids were then harvested for ELISA of TNF-α (A), IL-1β (B), IL-6 (C) and RANKL (D). Control cultures were incubated with culture medium alone (unstimulated). Data are shown as the mean and SDs of two independent experiments each performed in triplicate. * = $p<0.05$, ** = $p<0.01$ compared with uninfected controls at each time points. ND = not detectable.

FIGURE 2. *P. gingivalis* induces TNF-α production in macrophages via a TLR2 dependent pathway. Peritoneal macrophages from WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were cultured with *P. gingivalis* (MOI = 10) (*P.g.*) for 24hours (A-C). In another experiment, peritoneal macrophages from WT mice were pre-treated with anti-TLR2 blocking antibody, isotype control Ab for 1 hour or untreated followed by culture with *P. gingivalis* (MOI = 10) (*P.g.*) for 24hours (D-F). Cells were also stimulated with SLTA (2 μg/ml) (SLTA) or *E. coli* LPS (100 ng/ml) (*E.c. LPS*) as a control. CS fluids were harvested for ELISA analysis of TNF-α. (A, D), IL-1β (B, E), or IL-6 (C, F). Control cultures were incubated with culture media only (unstimulated). Data are shown as the mean and SDs of two independent experiments each performed in triplicate. * = $p<0.01$ compared

with each of the WT macrophages. † = $p<0.01$ compared with *P.g.* of TLR4$^{-/-}$ macrophages. ** = $p<0.01$ compared with each of the isotype control.

FIGURE 3. *P. gingivalis* up-regulates TLR2 surface expression on murine macrophages.   (A) Peritoneal macrophages from WT mice were cultured with *P. gingivalis* (MOI = 10) or with medium alone (uninfected) and temporal expression of TLR2 and TLR4 were determined by FACS analysis at 6 and 24 hours.   Data is expressed as the geo-mean fluorescence intensity and SDs of 3 independent experiments.   * = $p<0.01$ by unpaired *t*-test.   (B) Peritoneal macrophages from wild-type mice were cultured with *P. gingivalis* (MOI = 10) or with medium alone for 1 hour, and then non-adherent bacteria were removed by washing out cells.   The cells were then incubated with medium alone for 5 hours and the temporal expression of TLR2 and TLR4 were determined by FACS analysis. Data is expressed as the geo-mean fluorescence intensity and SDs of 3 independent experiments.   * = $P<0.01$ by unpaired *t*-test. (C) Non-pre-exposed peritoneal macrophages or macrophages pre-exposed with *P. gingivalis* (MOI = 10) for 1hour, were washed and incubated with medium alone for 5 hours. Following medium change, cells were then incubated with medium, *P. gingivalis* (MOI = 10) or SLTA (2µg/ml) for 24 hours and TNF-α production in the CS fluids was assessed by ELISA.   Data are shown as the mean and SDs of two independent experiments each performed in triplicate.   * = $p<0.01$ by unpaired *t*-test.

FIGURE 4.   CS fluids of *P. gingivalis* infected peritoneal macrophages

induce osteoclastogenesis. (A) Osteoclast formation. BMMs from WT mice were cultured with CS fluids from peritoneal macrophages that were previously infected with *P. gingivalis* (*P.g.*CS fluids) or CS fluids from uninfected macrophages (control CS fluids). Some CS fluids were supplemented with OPG (300ng/ml) or anti-TNF-α blocking antibody (5 μg/ml) or control goat IgG (5 μg/ml) as indicated in each panel. After 5 days of culture, cells were stained with TRAP for identification of osteoclasts. Each representative photograph is shown. Bars = 100 μm. (B) Osteoclast numbers in cell cultures. BMMs were cultured under the same conditions as described above. TRAP positive multinucleated cells were identified and counted. Data are shown as the mean and SDs of two independent experiments each performed in triplicate. * = *p*<0.01 compared with control CS fluids. (C) Pit formation assay. BMMs were cultured on dentine disks under the same conditions as described above. After 7 days, cells were removed and the dentine disks were stained with hematoxylin. Representative photograph of duplicate is shown. Bars = 400 μm. All cultures (A-C) were incubated in the presence of M-CSF (30ng/ml). Every 3 days, the medium and reagents were replaced.

FIGURE 5. Osteoclastogenesis induced by CS fluids of *P. gingivalis* infected peritoneal macrophages from TLR2$^{-/-}$ and TLR4$^{-/-}$ mice. (A) Osteoclast formation. BMMs from WT mice were cultured with CS fluids from peritoneal macrophages obtained from WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice that were previously infected with *P. gingivalis* (*P.g.* CS fluids), or uninfected macrophages (control CS fluids). After 5 days of culture, cells were stained with TRAP. Each

representative photograph is shown.   Bars = 100 μm.   (B) Osteoclast numbers.

BMMs were cultured under the same conditions as described above.   Some

macrophages were also cultured in the presence of OPG (300ng/ml), anti-TNF-α

blocking antibody (5μg/ml) or control goat IgG (5μg/ml).   TRAP positive

multinucleated cells were counted in each well.   Data are shown as the mean

and SDs of two independent experiments each performed in triplicate.   * =

p<0.01 compared with *P.g.* CS fluids each mouse group.   ** = p<0.01 compared

with *P.g.* CS fluids of WT.   † = p<0.01 compared with *P.g.* CS fluids of TLR4$^{-/-}$.

(C) Pit formation assay.   BMMs were cultured on dentine disks under the same

conditions as described above.   After 7 days, cells were removed and the

dentine disks were stained with hematoxylin.   Representative photograph of

duplicate is shown.   Bars = 400 μm.   All cultures (A-C) were incubated in the

presence of M-CSF.   Every 3 days, the medium and all reagents were replaced.
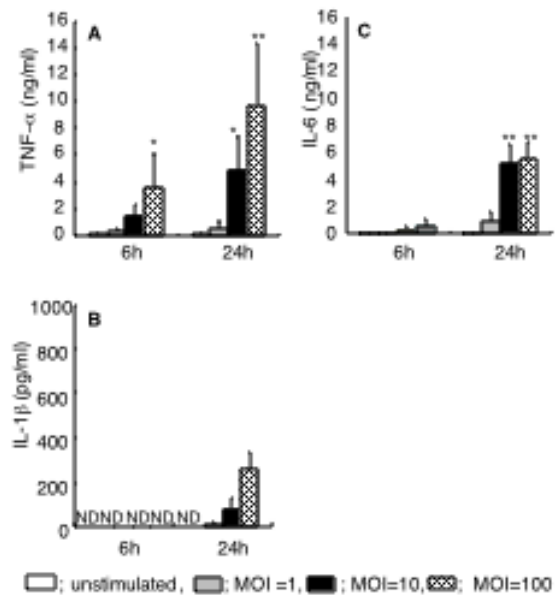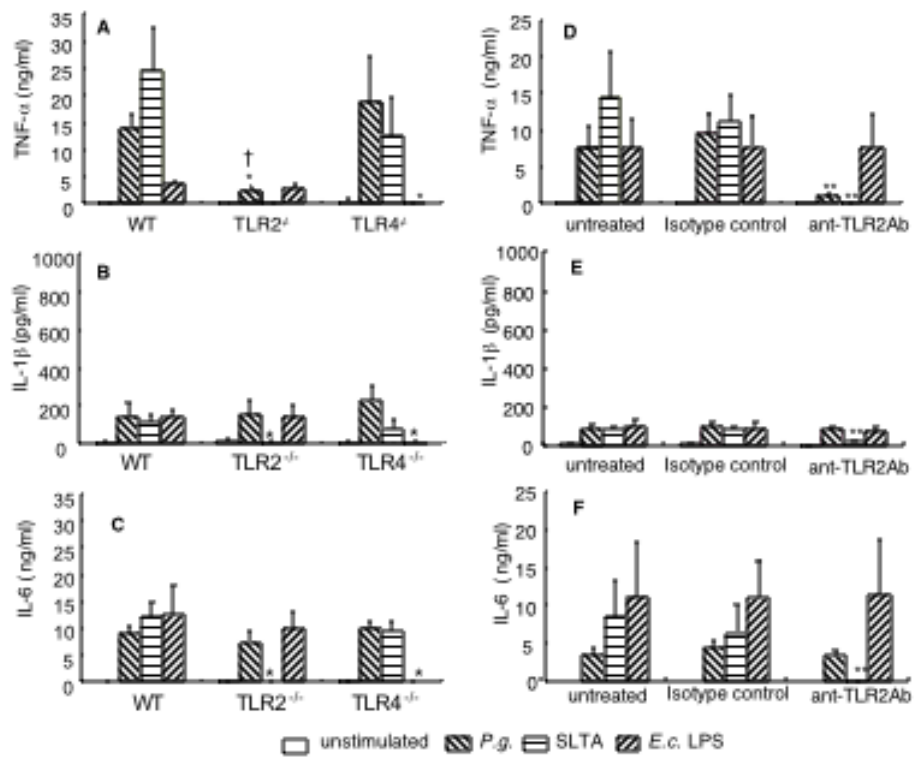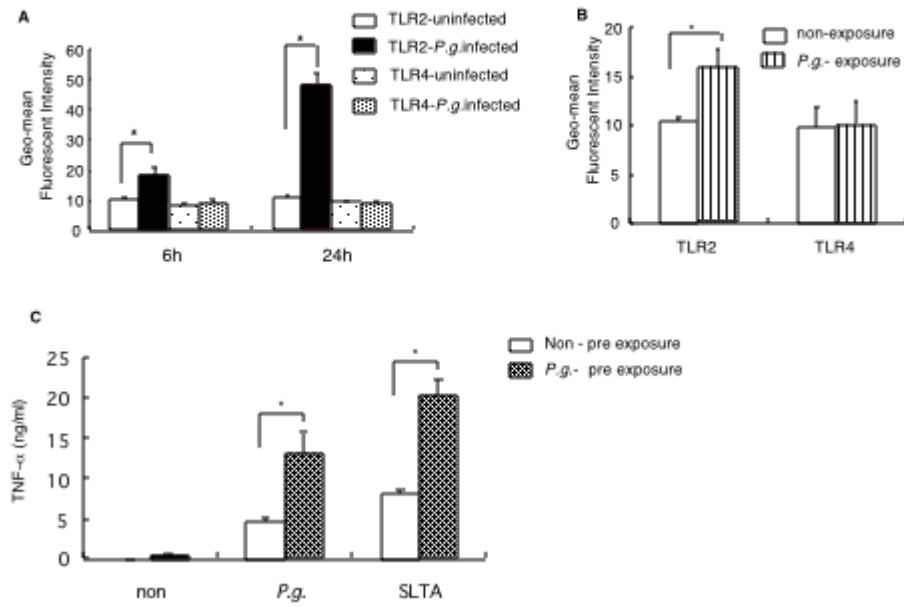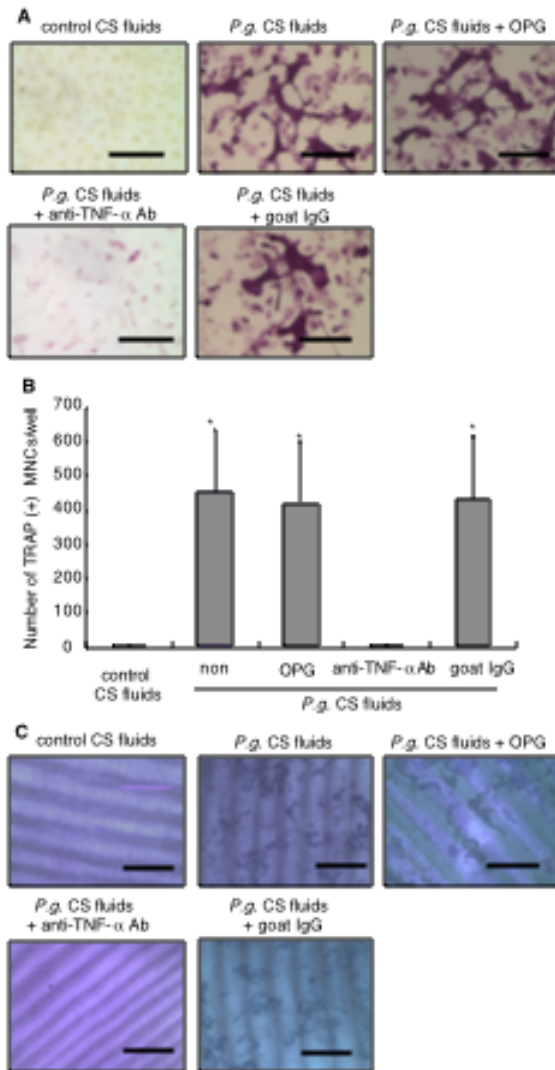
Figure 4, Takashi Ukai *et al*

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