Published in final edited form as:

Eur J Oral Sci. 2010 August ; 118(4): 333–341. doi:10.1111/j.1600-0722.2010.00751.x.

MyD88 expression in the rat dental follicle: Implications for osteoclastogenesis and tooth eruption

Dawen Liu, Shaomian Yao, and Gary E. Wise¹

Department of Comparative Biomedical Sciences School of Veterinary Medicine Louisiana State University Baton Rouge, Louisiana 70803, USA

Abstract

Myeloid differentiation factor 88 (MyD88) is a key adaptor molecule in the interleukin-1 (IL-1) and IL-18 Toll-like receptor signaling pathway. Because it is present in dental follicle (DF) cells *in vitro*, the purpose of this study was to determine its chronological expression *in vivo*, as well as its possible role in osteoclastogenesis and tooth eruption. An oligo DNA microarray was used to determine gene expression of MyD88 *in vivo* in the DFs from the first mandibular molars of postnatal rats from days 1–11. The results showed that MyD88 was expressed maximally at day 3. Using siRNA to knock down MyD88 expression in the DF cells also reduced the gene expression of nuclear factor-kappa B-1 (NFkB1) and monocyte chemoattractant protein 1 (MCP-1). IL-1 α up-regulated the expression of NFkB1, MCP-1 and receptor activator of nuclear factor kappa B ligand (RANKL), but knockdown of MyD88 nullified this IL-1 α effect. Conditioned medium from DF cells with MyD88 knocked down reduced chemotactic activity for mononuclear cells and reduced osteoclastogenesis as opposed to controls. In conclusion, the maximal expression of MyD88 at day 3 in the DF may contribute to the major burst of osteoclastogenesis needed for eruption by up-regulating MCP-1 and RANKL expression.

Keywords

dental follicle; siRNA; osteoclastogenesis; MyD88; tooth eruption

Osteoclastogenesis and subsequent alveolar bone resorption are essential for creating a pathway for the tooth to erupt (1). For the eruption of the first mandibular molars of rats, mononuclear cells (osteoclast precursors) are recruited into the dental follicle (DF), a loose connective tissue sac that surrounds the unerupted tooth, and differentiate into osteoclasts (2). It has been shown that the recruitment of mononuclear cells into the DF is mediated by monocyte chemoattractant protein-1 (MCP-1) and colony-stimulating factor-1 (CSF-1), which are maximally expressed by the DF at day 3 postnatally and secreted by the DF cells (3–5). Our recent study demonstrated that endothelial monocyte-activating polypeptide II (EMAP-II) also contributes to this recruitment (6). Moreover, a crucial molecule for osteoclastogenesis signaling, receptor activator of nuclear factor-kappa B ligand (RANKL), is expressed in the DF (7). At this time (day 3), osteoprotegerin (OPG), an osteoclastogenesis inhibitor in the DF, is down-regulated by CSF-1 (8). Another osteoclastogenesis inhibitor, secreted frizzled-related protein-1 (SFRP-1), also is down-regulated in the DF at this time (9). With RANKL presence in the DF, a decrease in osteoclastogenesis inhibitors at day 3 provides a favorable microenvironment to promote osteoclastogenesis.

¹Corresponding author: Gary E. Wise, Ph.D., Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803, USA; Tel: +1-225-578-9889, Fax: +1-225-578-9895; gwise@vetmed.lsu.edu.

Myeloid differentiation primary response protein 88 (MyD88) is an adaptor molecule involved in IL-1/IL-18/Toll-like signaling (10). It was originally isolated from M1 differentiation inducible (M1D+) myeloblastic leukemia cell lines after being induced upon IL-6 stimulated terminal differentiation (11). Its expression has been found in primary cultures of myeloid precursor enriched bone marrow cells and other tissues such as skin, muscle, heart, thymus, uterus and ovary (11,12). Structurally, MyD88 is a dual domain molecule, with an N-terminal death domain and a C-terminal Tolls/IL-1Rs (TIR) domain, separated by a shorter linker sequence (13). MyD88 exists as a homodimer of 60 kDa when recruited to the receptor complex (12).

MyD88 acts as an adaptor molecule to link the upstream pathways of IL-1 receptor I (IL-1RI), including IL-1 and IL-18, and toll-like receptors (TIR1 to 9) signaling through its TIR domain to the downstream pathways through its death domain. By interacting with IL-1RI and toll-like receptors, MyD88 recruits IL-1R-associated kinases (IRAKs) and tumor necrosis factor receptor-associated factor 6 (TRAF6), triggering activation of NF- κ B signaling which results in the production of pro-inflammatory cytokines (14).

Using knockout mice, more functions of MyD88 have been revealed. MyD88-deficient mice had no response to IL-1 β stimulation for elevating TNF- α and IL-6 expression (15). Interferon γ (INF γ) production in response to IL-18 stimulation also was abrogated (15). MyD88 also contributes to an innate immune response to invasion of bacteria, yeasts and viruses (10). Studies from the MyD88-deficient mice have shown that MyD88 is essential for induction of interferon α (INF α) and interferon β (INF β) by forming a complex with interferon regulatory factor 7 (IRF-7) and interferon regulatory factor 1 (IRF-1) respectively (16,17). Thus, loss of MyD88 impacts type I interferon production, and results in a weak immune response against pathogens, along with the abolished or greatly suppressed expression of pro-inflammatory cytokines (18–20). Consequently, infections by bacteria, viruses and parasites were lethal to MyD88-deficient mice (20–22). Finally, MyD88 is involved in injury healing, likely through recruitment of leukocytes into the injured area; e.g., in MyD88-deficient mice recruitment of leukocytes into the entorhinal cortex (EC) after brain injury (23) was reduced.

In our previous study, MyD88 was identified in the DF cells by DNA microarray (9). Because of the finding that MyD88-deficient mice exhibited osteopenia with reduced bone resorption and formation (24), MyD88 may be another molecule that could play a role in regulation of tooth eruption via its effect on osteoclastogenesis. To this end, this study was performed to determine in vivo MyD88 expression, and its potential role in regulating gene expression in the DF cells related to tooth eruption. In addition, because MyD88 is required for IL-1 α signaling, and IL-1 α is involved both in osteoclastogenesis and tooth eruption (25,26), the dependence of IL-1 α on MyD88 in the DF cells also was determined in this study.

Materials and Methods

DF isolation and DF cell culture

For in vivo studies of MyD88 gene expression in the DF, rat DFs were isolated surgically from the first mandibular molars of rats (Harlan Sprague-Dawley) at postnatal days 1, 3, 5, 7, 9 and 11 for RNA isolation. The rats were housed in the School of Veterinary Medicine at Louisiana State University in compliance with a protocol approved by the Institute of Animal Care and Use Committee (IACUC). For cell culture, DFs of the first mandibular molars of 5- to 6-d-old rats were cultured after trypsinization in minimum essential medium (MEM) (Sigma-Aldrich, St. Louis, MO, USA) plus 10% (V/V) newborn calf serum, 1 mM

sodium pyruvate, 1% penicillin/streptomycin and 0.2% fungizone as previously reported (27). DF cells of passages 6 to 9 were used in this study.

RNA isolation

Total RNA was isolated from the DF or DF cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) per manufacturer's instructions. The RNA was then treated with DNase I (Ambion, Austin, TX, USA) at 37 °C for 1 h to remove possible DNA contamination. Total RNA was quantified by a spectrophotometer at an absorbance (A) of 260 nm, and RNA purity was confirmed by an A260/A280 ratio of 2.0 or higher.

DNA microarray analysis

DNA microarrays were performed to determine MyD88 expression in vivo, as in our previous study (9). Briefly, total RNA (1–1.5 µg) from the DFs of postnatal day 1 to day 11 rats was labeled with biotin-16-UTP (Roche Applied Science, Indianapolis, IN, USA) to generate complementary RNA (cRNA) using TrueLabeling-AMP 2.0 kit (SA Bioscience, Frederick, MD, USA) per manufacturer's instructions. The labeled cRNA (4 µg) was hybridized to a rat chemokines & receptors oligo DNA microarray containing 113 genes (SA Bioscience) in a hybridization oven at 60 °C for 18 h, followed by two washes at the same temperature. After hybridization, the array was blocked in blocking buffer at room temperature for 40 min, incubated with alkaline phosphatase-conjugated streptavidin for 10 min, and washed 4 times, 5 min each. The array was visualized by incubating with a CDP-Star chemiluminescent substrate (1, 2-dioxetane) (SA Bioscience) at room temperature for 5 min.

The image was acquired after 20 minutes of exposure using a FluoChem 8800 image system (Alpha Innotech, San Leandro, CA, USA). The gene expression data were analyzed using software GEArray Expression Analysis Suite (SA Bioscience) and the gene expression level was expressed as the ratio of MyD88 gene to the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Real-time RT-PCR

To detect gene expression, 2 µg total RNA was reverse transcribed by M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) to synthesize the first strand cDNA per the manufacturer's instructions. The reverse transcription was performed at 37 °C for 1 h, followed by 10 min incubation at 70 °C to inactivate the reverse transcriptase.

Real-time RT-PCR was used to analyze the expression of MyD88, NFκB1, MCP-1 and RANKL in the DF cells. The following primers were designed based on their mRNA sequences in GenBank: 5'-ACCGCATCGAGGAGGACTG-3' and 5'-CTGTGGGACACTGCTCTCCA-3' for MyD88 (GenBank accession No. NM_198130); 5'-GCTTACGGTGGGATTGCATT-3' and 5'-TTATGGTGCCATGGGTGATG-3' for NFκB1 (accession no. XM_342346); 5'-CACGCTTCTGGGCCTGTT-3' and 5'-TGAGACAGCACGTGGATGCT-3' for MCP-1 (accession no. NM_031530); and, 5'-TCCCATCGGGTTCCCATAA-3' and 5'-GGCCCAGCCTCGATCAT-3' for RANKL (accession no. NM_057149). The real-time RT-PCR was performed as our previous study (7) with SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The relative gene expression (RGE) was calculated with β-actin as an internal control using the formula $2^{-\Delta\Delta C_T}$.

In vitro transfection of DF cells with siRNA

Dicer substrate siRNA was designed according to the software provided by Integrated DNA Technologies (Coralville, IA, USA). The siRNA duplex, annealed from sense strand 5'-

rUrGrCrUrGrGrUrUrGrCrGrUrArUrGrUrArUrUrCrUrGrGrCrArG-3' and anti-sense strand 5'-rGrCrCrArGrArArUrArCrArUrUrCrGrCrArArCrCrArGCA-3'), was synthesized by Integrated DNA Technologies, targeting the rat MyD88 mRNA (GenBank accession No. NM_198130) at nucleotides 420–444. The scrambled siRNA sequence, which does not target any mRNA, was used as controls. For transfection, the DF cells were cultured one day before transfection in T-25 flasks in MEM plus 1 mM sodium pyruvate and 10% heat-inactivated fetal calf serum such that the cells were 30–40% confluent at time of transfection. Transfection was carried out using Lipofectamine RNAiMAX per manufacturer's instructions (Invitrogen). Briefly, siRNA was diluted to 50 nM and the transfection reagent was diluted 50-fold using Opti MEM I Reduced Serum Medium (Invitrogen). After 5 min of incubation at room temperature, an equal volume of diluted siRNA and transfection reagent were combined, and incubated at room temperature for another 25 min to form a siRNA-Lipofectamine RNAiMAX complex. One ml of the complex was added into 4 ml cell culture to bring the final siRNA concentration to 5 nM. For controls, the cells were transfected with scrambled siRNA at the same conditions. The culture was incubated at 37 °C with 5% CO₂ for 24, 48 and 72 h. After designated time periods following transfection, the cells were collected for determination of gene expression using real-time RT-PCR.

Immunostaining

For detection of MyD88 expression in vivo, mandibles of postnatal day 5 rats were fixed in 10% neutral-buffered formalin, decalcified, dehydrated, embedded in paraffin, and sectioned at 5 µm thickness. Endogenous peroxidase activity was quenched by incubating sections in 3% hydrogen peroxide for 10 min. After three washes with Tris-buffered saline (TBS) plus 0.025% Triton X-100, the sections were blocked at room temperature for 1 h with 2% normal rabbit serum (Vector Laboratories, Burlingame, CA, USA) in TBS buffer, and then incubated with goat polyclonal anti-human MyD88 primary antibody (Catalog # ab28763) (Abcam, Cambridge, MA, USA), which has cross activity with mouse and rat, at a concentration of 4 µg ml⁻¹ overnight at 4 °C in TBS buffer plus 2% normal rabbit serum. For controls, the primary antibody was replaced by goat IgG. After overnight incubation, the sections were washed three times and incubated with biotinylated rabbit anti-goat IgG secondary antibody (diluted 1: 100 in TBS buffer plus 1% BSA) for 1 h. Following three washes, the sections were incubated with avidin-biotinylated horseradish peroxidase (HRP) (Vector Laboratories) for 30 min and then incubated in 3, 3'-diaminobenzidine (DAB) for 5 min, followed by counterstaining with hematoxylin.

To detect the expression of MCP-1 in the DF cells, the DF cells were transfected either with control siRNA (scrambled siRNA) or MyD88 siRNA as described above. After 48 h the transfected cells were fixed in ice-cold methanol for 5 min and air-dried. Following blocking, the cells were incubated with rabbit anti-rat MCP-1 primary antibody (Catalog # ab7202) (Abcam) at a concentration of 5 µg ml⁻¹. For controls, primary antibody was replaced by rabbit IgG.

Interleukin-1α treatments

The DF cells were treated with interleukin-1 α (IL-1α) at a concentration of 5 ng ml⁻¹ for 1, 3, 6 and 12 h. The cells were then harvested for RNA isolation, followed by real-time RT-PCR analysis with primers described as above to determine MyD88 expression. In addition, IL-1α treatments were also applied to the DF cells with MyD88 expression knocked down and the cells transfected with scrambled siRNA. After 48 h of transfection, the cells were treated with IL-1α (5 ng ml⁻¹) for 1 to 2 h, and the expression of NFκB1, MCP-1 and RANKL was determined using real-time RT-PCR as described above.

Chemotaxis assay and in vitro osteoclastogenesis

The DF cells were transfected with control siRNA (scrambled siRNA) or MyD88 siRNA at a concentration of 5nM for 48 or 72 h as described above. The medium was changed, and the conditioned medium (CM) was collected after 4 h of culture in the absence or presence of IL-1 α (5ng ml⁻¹). Once collected, the CM was stored at -20°C for chemotaxis assays.

The chemotaxis assays were performed in Costar Transwell 24-well plates (Costar Corning, Cambridge, MA, USA), each well of which has a lower chamber and an upper chamber separated by a polycarbonate membrane with 5 μ m pores. The upper chambers were loaded with 100 μ l bone marrow mononuclear cells (1×10^5 cells) prepared from rats as in our previous study (9), while lower chambers were filled with 600 μ l MEM medium (as a blank) or CM. The plates were incubated for 1 h at 37°C with 5% CO₂. After incubation, the medium in the lower chambers containing the migrated cells was collected, centrifuged at 6000 g for 5 min, and resuspended in 100 μ l medium. The cells were counted using a Cellometer Auto T4 (Nexcelom Bioscience LLC, Lawrence, MA, USA) by loading 20 μ l cell suspension in the cell counting chambers. Two counts were performed for each sample. The chemotactic activity was expressed as a migration index; i.e., the ratio of the number of cells migrating into the CM to the number of cells migrating into MEM medium.

For determination of the osteoclastogenic capacity of the mononuclear cells attracted by the conditioned medium of the DF cells, chemotaxis assay were performed as above and the attracted cells in the lower chamber were maintained in the same conditioned medium supplemented with human CSF-1 (75 ng ml⁻¹) (Pepro Tech, Rock Hill, NJ, USA) and mouse RANKL (10 ng ml⁻¹) (R&D Systems, Minneapolis, MN, USA). After 1 d of culture, the CM was replaced by osteoclastogenesis differentiation medium; i.e., α -MEM (Invitrogen) plus the same concentrations of CSF-1 (75 ng ml⁻¹) and RANKL (10 ng ml⁻¹). The medium was changed every other day for seven days. The culture was stained for TRAP (tartrate-resistant acid phosphatase) activity, an osteoclast marker, as described in our previous study (9). The TRAP-positive multinucleated osteoclasts per well were counted under a microscope. The experiments were repeated four times.

Statistical analysis

Data from DNA microarrays, chemotactic assays and in vitro osteoclastogenesis were analyzed using the SAS program (version 9). Analysis of variance (ANOVA) was carried out to evaluate the chronological gene expression, and then the means were separated with the least significant difference (LSD) test at a significance level of $P \leq 0.05$. Data from other experiments were analyzed using paired t-tests at $P \leq 0.05$.

Results

Expression of MyD88 in the DF

To determine MyD88 expression level and chronological changes in the DF, DNA microarrays were performed. As seen in Fig. 1A, MyD88 was expressed in the DF from day 1 to day 11. Chronologically, MyD88 expression was strongest at day 3, which was significantly higher than other days. The expression level at day 3 was 1.7, 1.4 and 1.9 times higher than that at day 1, day 5 and day 7 respectively.

To determine if MyD88 protein was expressed, sections of the first mandibular molars of rats were immunostained for protein expression. As seen in Fig. 1B, the DF stained for MyD88, but there was no staining of the stellate reticulum. Staining was also observed in the alveolar bone. Staining was observed in the apex of ameloblasts. No staining was observed in controls (Fig. 1C).

Up-regulation of MyD88 expression in the DF cells by IL-1 α

Because MyD88 expression was highest at day 3, experiments were conducted to determine what molecule (s) may contribute to its up-regulation. IL-1 α , which is maximally expressed at early days in the stellate reticulum (28), was used to treat the DF cells. As shown in Fig. 2, MyD88 expression was increased in the DF cells after 3 h of treatment, reaching maximal expression after 6 h of treatment. Knocking down EMAP-II, another gene maximally expressed in the DF at day 3 (6), did not affect MyD88 expression, however (data not shown).

Reduction of NFKB1 and MCP-1 expression in the DF cells with MyD88 knocked down

To determine the role of MyD88 in the regulation of gene expression related to tooth eruption, MyD88 was knocked down by Dicer siRNA, and the gene expression of NFKB1, MCP-1 and EMAP-II was examined. The Dicer siRNA knocked down MyD88 expression in the DF cells by 95 to 98% from 24 to 72 h after transfection, as determined by real-time RT-PCR (data not shown). As shown in Fig. 3A, NFKB1 expression was significantly reduced after siRNA transfection from 24 to 72 h. For MCP-1, its expression remained unchanged at 24 h after transfection, but was significantly reduced at 48 and 72 h after transfection (Fig. 3B), with the lowest level at about 40% of the control levels. Regarding EMAP-II, its expression remained unchanged at 24 to 72 h after transfection (data not shown). This suggested that MyD88 contributes to the up-regulation of NFKB1 and MCP-1, but not to EMAP-II.

Because knockdown of MyD88 reduced MCP-1 gene expression, immunostaining was performed to determine if this knockdown also resulted in the reduction of MCP-1 protein. As shown in Fig. 4 A, knockdown of MyD88 resulted in a significant reduction of MCP-1 protein as compared to controls without MyD88 knockdown (Fig. 4 B). Thus, knockdown of MyD88 reduced both mRNA expression and protein expression.

Requirement of MyD88 for IL-1 α enhanced expression of NFKB1, MCP-1 and RANKL

Since MyD88 was reported to be involved in IL-1 α signaling pathway (12), and IL-1 α enhances the expression of NFKB1, MCP-1 and RANKL (5,7,29), experiments were carried out to determine if this enhanced expression is MyD88-dependent. Thus, following MyD88 knockdown by siRNA, the DF cells were then treated with IL-1 α , and the expression of NFKB1, MCP-1 and RANKL was examined. As shown in Fig. 5A–C, IL-1 α enhanced the expression of NFKB1, MCP-1 and RANKL. After MyD88 was knocked down, however, IL-1 α had no effect on the expression of NFKB1 and MCP-1, leaving their expression levels 39% and 60% lower than their base levels and 60% and 75% lower than their enhanced levels, respectively (Fig. 5A, B). Similarly, after MyD88 knockdown, IL-1 α enhanced RANKL expression (5.8 times) was nullified (Fig. 5C). This suggests that MyD88 is required for the IL-1 α enhanced expression of NFKB1, MCP-1 and RANKL.

Reduction of chemotactic activity and in vitro osteoclastogenesis by CM from the DF cells with MyD88 knocked down

Chemotactic assays were performed to determine if MyD88 was involved in mononuclear cell (osteoclast precursor) recruitment by using conditioned medium (CM) from the DF cells with MyD88 knocked down (siMyD88 CM). As seen in Fig. 6A, CM from the DF cells transfected with control siRNA (siControl CM) had a strong chemotactic activity with a migration index of 2.53, whereas siMyD88 CM had a migration index of 1.66, representing a reduction of about 34% in chemotactic activity. For the control CM generated in the presence of IL-1 α (siControl + IL-1 α CM), it had a stronger chemotactic activity with a migration index of 3.39, whereas the siMyD88 CM generated in the presence of IL-1 α

(siMyD88 + IL-1 α CM) had a migration index of 1.77, which was comparable to the siMyD88 CM. Similar results were obtained from the CM after 72 h of transfection (data not shown). These results suggest that MyD88 knockdown reduced chemotactic activity of the CM of DF cells, and that MyD88 is required for the enhanced chemotactic activity by IL-1 α .

To determine if the reduction of chemotactic activity affects osteoclastogenesis, the mononuclear cells attracted by CM were cultured in osteoclastogenesis differentiation medium. As shown in Fig. 6B, siMyD88 CM gave a 46% lower osteoclast number per well as compared to siControl CM. For the siControl + IL-1 α CM, it showed a 41% increase in osteoclast number per well over siControl CM. However, this increase was abolished in siMyD88 C + IL-1 α CM, leaving its osteoclast number per well comparable to that of siMyD88 CM. These results suggest that MyD88 contributes to osteoclastogenesis and its enhancement by IL-1 α .

Discussion

MyD88 is an adaptor molecule involved in IL-1/IL-18/Toll-like signaling (10) and is present in many types of cells and tissues (11,12). Our previous study found that MyD88 was expressed in the DF cells in vitro (9), and this study demonstrates its expression in the DF in vivo. The maximal expression of MyD88 was seen at day 3 (Fig. 1A), which correlates with influx of mononuclear cells in the DF and the major burst of osteoclastogenesis at this time (30,31). Because both bone resorption and formation were impacted in MyD88-deficient mice (24), it is likely that MyD88 also plays a role in regulating osteoclastogenesis in the DF for subsequent tooth eruption. Therefore, further experiments were performed to determine how MyD88 regulates gene expression related to osteoclastogenesis and tooth eruption.

Osteoclastogenesis requires the recruitment of osteoclast precursors, and in the DF this recruitment is regulated by chemoattractant molecules such as CSF-1, MCP-1 and EMAP-II (4,6). In this study, we showed that MCP-1 expression was dependent on MyD88, as knockdown of MyD88 resulted in about 60% reduction of the MCP-1 gene expression (Fig. 3B). In the future, the direct effect of MyD88 on MCP-1 possibly could be determined by transfecting the DF cells with a vector to express the MyD88 gene.

In addition to the possible direct effect of MyD88 on MCP-1 gene expression, it also has an indirect effect. Knocking down MyD88 expression reduced the up-regulation of MCP-1 expression by IL-1 α such that the MCP-1 expression in vitro with MyD88 knocked down was only 25% of its IL-1 α up-regulated level in the absence of a knockdown (Fig. 5B). In vivo, IL-1 α is maximally expressed early in the stellate reticulum, a tissue adjacent to the DF (28). In this study, we found that MyD88 expression was maximal early (day 3) in vivo (Fig. 1A), and IL-1 α up-regulated its expression in vitro (Fig. 2). Therefore, it is possible that IL-1 α from the stellate reticulum contributes to the up-regulation of MyD88 at day 3. The up-regulation of MyD88 also may be the mechanism by which IL-1 α enhances NF κ B1 expression in the DF cells because over-expression of MyD88 in 293T cells results in activation of NF- κ B signaling in the absence of exogenous stimuli such as IL-1 α and TNF- α (12).

The chemotactic assays presented in this study confirm that MyD88 promotes the recruitment of osteoclast precursors (mononuclear cells) by the DF cells. Knocking down the gene expression of MyD88 in the DF cells reduced the capacity of the conditioned medium to recruit mononuclear cells by 34% as compared to conditioned medium of DF cells in which the MyD88 was not knocked down. Moreover, the increase in chemotaxis of the medium of DF cells treated with IL-1 α was negated by MyD88 knockdown. Thus, the reduction in MCP-1 gene expression in the DF cells that is brought about by MyD88

knockdown is reflected in reduced chemotaxis for osteoclast precursors. That the reduction is not 100% is because other chemotactic molecules, such as CSF-1 (4) or EMAP-II (6) also are produced by the DF cells. Thus, as in many other instances of development, there is a redundancy of function among different molecules to bring about a critical developmental event.

The effect of MyD88 on recruitment of osteoclast precursors translates into an effect on osteoclastogenesis. In vitro, reducing the expression of MyD88 in the DF cells results in a conditioned medium with reduced chemotactic activity and hence a reduction in osteoclast formation (Fig. 6B); i.e., there are fewer precursors to form osteoclasts.

Other studies have shown that MyD88 is essential for the elevated MCP-1 expression induced by bacterial infection (32) or by stab injury (23). In the absence of MyD88, MCP-1 was significantly lower in mouse pulmonary transcriptome following bacteria *Chlamydia pneumoniae* infection and in the stab-injured entorhinal cortex. This reduction appeared to affect cell recruitment in that polymorphonuclear neutrophils (PMN) were unable to be recruited into the lungs of MyD88-deficient mice (18), and macrophage recruitment into the stab-injured cortex was 45 to 80% lower in the MyD88-deficient mice (23). Although there was no direct report that osteoclast precursor recruitment was blocked in MyD88-deficient animals, given that MyD88 underlies both the base level and IL-1 α up-regulated MCP-1 level, it is very likely that MyD88 is required for the recruitment of osteoclast precursors through its effect on MCP-1.

Following osteoclast precursor recruitment, cell fusion signaling by RANKL is essential to osteoclastogenesis (33). RANKL is expressed in the DF, and is up-regulated in vitro by IL-1 α , TNF- α and TGF- β (7). By activating NF- κ B pathways, RANKL sequentially activates c-Fos and nuclear factor of activated T cells c1 (NFATc1), leading to osteoclastogenesis (34). In our study, NF κ B1 was shown to be partially dependent on MyD88 (Fig. 3A) in the DF cells. However, osteoclast precursors from MyD88-deficient mice similarly differentiated into osteoclasts as compared to those from wild type mice (24), suggesting that MyD88 may not be directly involved in osteoclastogenesis or that a MyD88-independent NF- κ B signaling may exist in osteoclast precursors. Moreover, our study also showed that after MyD88 was knocked down, IL-1 α enhanced RANKL expression (5.8 times) was nullified (Fig. 5C), indicating that MyD88 is essential to up-regulation of RANKL in response to IL-1 α stimulation. Similar results have been found in osteoblasts from MyD88-deficient mice showing that RANKL expression had no response to stimulation by LPS, diacyl lipopeptide or IL-1 α (24). Thus, it appears that MyD88 is involved in osteoclastogenesis by up-regulating RANKL in osteoblasts or DF cells in response to IL-1 α stimulation, but MyD88 does not directly affect osteoclast precursors. Moreover, as seen in Fig. 5, MyD88 knockdown does not result in a reduced RANKL expression; i.e., it does not affect the base level of RANKL, but it does inhibit the upregulation by IL-1 α .

This study demonstrates that MyD88 knockdown in the DF cells results in the reduced expression of NF κ B1 and MCP-1 (Fig. 3). As NF- κ B protein has been shown to bind to the promoter region of the MCP-1 gene (35), it is likely that the down-regulation of MCP-1 expression in the DF cells was due to NF κ B1 reduction following MyD88 knockdown. This is supported by the observation that NF κ B1 reduction occurs first (as early as 24 h after transfection), whereas MCP-1 reduction is not seen until 48 h after transfection (Fig. 4).

MyD88 may also play a role in oral health. As a crucial adaptor for innate immune responses, MyD88 is involved in the toll-like pathway leading to innate immunity against bacteria, as shown by the finding that MyD88-deficient mice were highly and more

susceptible to bacterial invasion than TLR2-deficient mice (36). Because MyD88, as well as toll-like receptors TL2 and TL4 for LPS and bacterial lipoprotein respectively, is present in the gingival and periodontal ligament fibroblasts (37), it may be possible that MyD88 in periodontal tissues suppresses oral bacteria invasion. Given that the DF is the precursor of the periodontal ligament, it is not surprising that MyD88 is expressed in both tissues.

In conclusion, we have shown that MyD88 is expressed maximally in the DF of the first mandibular molar at day 3, the time of the major burst of osteoclastogenesis, as well as a time of high expression of IL-1 α in the adjacent stellate reticulum (28). Our in vitro studies with DF cells show that knockdown of MyD88 gene expression nullifies the up-regulatory effect of IL-1 α on NF κ B1, MCP-1 and RANKL gene expression. Moreover, knockdown of MyD88 in the DF cells reduces the chemotactic activity of the conditioned medium for osteoclast precursors from these cells in comparison to the controls in which MyD88 is expressed. Both MCP-1 and RANKL promote osteoclastogenesis and the DF regulates the osteoclastogenesis needed for eruption pathway formation. Thus, MyD88 expression in the DF may promote the stimulatory effect of IL-1 α upon genes needed for osteoclastogenesis (MCP-1 and RANKL), as well as possibly directly up-regulate MCP-1 expression.

Acknowledgments

This research was supported by NIH grant 5 RO1 DE008911–18 to G.E.W and S.Y.

References

1. Wise GE. Cellular and molecular basis of tooth eruption. *Orthod Craniofac Res.* 2009; 12:67–73. [PubMed: 19419449]
2. Wise GE, King GJ. Mechanisms of tooth eruption and orthodontic tooth movement. *J Dent Res.* 2008; 87:414–434. [PubMed: 18434571]
3. Wise GE, Lin F. Regulation and localization of colony-stimulating factor-1 mRNA in cultured rat dental follicle cells. *Arch Oral Biol.* 1994; 39:621–627. [PubMed: 7945021]
4. Que BG, Wise GE. Colony-stimulating factor-1 and monocyte chemotactic protein-1 chemotaxis for monocytes in the rat dental follicle. *Arch Oral Biol.* 1997; 42:855–860. [PubMed: 9460539]
5. Wise GE, Que BG, HUANG H. Synthesis and secretion of MCP-1 by dental follicle cells — implications for tooth eruption. *J Dent Res.* 1999; 78:1677–1681. [PubMed: 10576162]
6. Liu D, Wise GE. Expression of endothelial monocyte-activating polypeptide II in the rat dental follicle and its potential role in tooth eruption. *Eur J Oral Sci.* 2008; 116:334–340. [PubMed: 18705801]
7. Liu D, Yao S, Wise GE. Chronology and regulation of gene expression of RANKL in the rat dental follicle. *Eur J Oral Sci.* 2005; 113:404–409. [PubMed: 16202028]
8. Wise GE, Yao S, Odgren PR, Pan F. CSF-1 regulation of osteoclastogenesis for tooth eruption. *J Dent Res.* 2005; 84:837–841. [PubMed: 16109994]
9. Liu DW, Wise GE. A DNA microarray analysis of chemokine and receptor genes in the rat dental follicle — Role of secreted frizzled-related protein-1 in osteoclastogenesis. *Bone.* 2007; 41:266–272. [PubMed: 17540629]
10. O'Neill LAJ, Bowie AG. The family of five: TIR-domain-containing adaptors in toll-like receptors signaling. *Nat Rev Immunol.* 2007; 7:353–364. [PubMed: 17457343]
11. Lord KA, Hoffman-Liebermann B, Lieberman DA. Nucleotide sequence and expression of a cDNA encoding MyD88, a novel myeloid differentiation primary response gene induced by IL6. *Oncogene.* 1990; 5:1095–1097. [PubMed: 2374694]
12. Burns K, Martinon F, Esslinger C, Pahl H, Schneider P, Bodmer JL, Di Marco F, French L, Tschoep J. MyD88, an adaptor protein involved in interleukin-1 signaling. *J Biol Chem.* 1998; 273:12203–12209. [PubMed: 9575168]
13. Slack JL, Schooley K, Bonnert TP, Mitcham JL, Qwarnstrom EE, Sims JE, Dower SK. Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible

- for coupling to proinflammatory signaling pathways. *J Biol Chem.* 2000; 275:4670–4678. [PubMed: 10671496]
14. Akira S, Takeda K. Toll-like receptor signaling. *Nat Rev Immunol.* 2004; 4:499–511. [PubMed: 15229469]
 15. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S. Targeted disruption of the MyD88 gene results in loss of IL-1 and IL-18-mediated function. *Immunity.* 1998; 9:143–150. [PubMed: 9697844]
 16. Kawai T, Sato S, Ishii KJ, Coban C, Hemmi H, Yamamoto M, Terai K, Matsuda M, Inoue J, Uematsu S, Takeuchi O, Akira S. Interferon- α induction through toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nat Immunol.* 2004; 5:1061–1068. [PubMed: 15361868]
 17. Negishi H, Fujita Y, Yanai H, Sakaguchi S, Ouyang X, Shinohara M, Takayanagi H, Ohba Y, Taniguchi T, Honda K. Evidence for licensing of IFN- γ -induced IFN regulatory factor 1 transcription factor by MyD88 in toll-like receptor-dependent gene induction program. *PNAS.* 2006; 103:15136–15141. [PubMed: 17018642]
 18. Rodriguez N, Fend F, Jennen L, Schiemann M, Wantia N, Prazeres Da Costa CU, Durr Sür S, Heinzmann U, Wagner H, Miethke T. Polymorphonuclear neutrophils improve replication of *Chlamydia pneumoniae* in vivo upon MyD88-dependent attraction. *J Immunol.* 2005; 174:4836–4844. [PubMed: 15814710]
 19. Biondo C, Midiri A, Messina L, Tomasello F, Garufi G, Catania MR, Bombaciet M, Beninati C, Teti G, Mancuso G. MyD88 and TLR2, but not TLR4, are required for host defense against *Cryptococcus neoformans*. *Eur J Immunol.* 2005; 35:870–878. [PubMed: 15714580]
 20. Wiersinga WJ, Wieland CW, Roelofs JJ, Van Der Poll T. MyD88 dependent signaling contributes to protective host defense against *Burkholderia pseudomallei*. *PLoS One.* 2008; 3:e3494. [PubMed: 18946505]
 21. Sheahan T, Morrison TE, Funkhouser W, Uematsu S, Akira S, Baric RS, Heise MT. MyD88 is required for protection from lethal infection with a mouse-adapted SARS- CoV. *PLoS Pathogens.* 2008; 4:e1000240. [PubMed: 19079579]
 22. Sukhumavasi W, Egan CE, Warren AL, Taylor GA, Fox BA, Bzik DJ, Denkers EY. TLR adaptor MyD88 is essential for pathogen control during oral *toxoplasma gondii* infection but not adaptive immunity induced by a vaccine strain of the parasite. *J Immunol.* 2008; 181:3464–3473. [PubMed: 18714019]
 23. Babcock AA, Toft-Hansen H, Owens T. Signaling through MyD88 regulates leukocyte recruitment after brain injury. *J Immunol.* 2008; 181:6481–6490. [PubMed: 18941239]
 24. Sato N, Takahashi N, Suda K, Nakamura M, Yamaki M, Ninomiya T, Kobayashi Y, Takada H, Shibata K, Yamamoto M, Takeda K, Akira S, Noguchi T, Udagawa N. MyD88 but not TRIF is essential for osteoclastogenesis induced by lipopolysaccharide, diacyl lipopeptide, and IL-1 α . *J Exp Med.* 2004; 200:601–611. [PubMed: 15353553]
 25. Fukushima H, Jimi E, Okamoto F, Motokawa W, Okabe K. IL-1-induced receptor activator in human periodontal ligament cells involves ERK-dependent PGE2 production. *Bone.* 2005; 36:267–275. [PubMed: 15780952]
 26. Huang H, Wise GE. Delay of tooth eruption in null mice devoid of the type I IL-1R gene. *Eur J Oral Sci.* 2000; 108:297–302. [PubMed: 10946764]
 27. Wise GE, Lin F, Fan W. Culture and characterization of dental follicle cells from rat molars. *Cell Tissue Res.* 1992; 267:483–492. [PubMed: 1571962]
 28. Wise GE, Lin F, Zhao L. Immunolocalization of interleukin-1 α in rat mandibular molars and its enhancement after in vivo injection of epidermal growth factor. *Cell Tissue Res.* 1995; 280:21–26. [PubMed: 7750135]
 29. Que BG, Lumpkin SJ, Wise GE. Implications of tooth eruption of the effect of interleukin-1 α on nuclear factor- κ B gene expression in the rat dental follicle. *Arch Oral Biol.* 1999; 44:961–967. [PubMed: 10580544]
 30. Wise GE, Fan W. Changes in the tartrate-resistant acid phosphatase cell population in dental follicles and bony crypts of rat molars during tooth eruption. *J Dent Res.* 1989; 68:150–156. [PubMed: 2465331]

31. Cielinski, MJ.; Jolie, M.; Wise, GE.; Ando, DG.; Marks, SC, Jr. Colony-stimulating factor-1 (CSF-1) is a potent stimulator of tooth eruption in the rat. In: Davidovitch, Z., editor. The biological mechanisms of tooth eruption, resorption and replacement by implants. Birmingham, AL: EBSCO Media; 1994. p. 429-436.
32. Rodriguez N, Mages J, Dietrich H, Wantia N, Wagner H, Lang R, Miethke T. MyD88-dependent changes in pulmonary transcriptome after infection with *Chlamydia pneumoniae*. *Physiol Genomics*. 2007; 30:134–145. [PubMed: 17374847]
33. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *PNAS*. 1998; 95:3597–3602. [PubMed: 9520411]
34. Yamashita T, Yao Z, Li F, Zhang Q, Badell IR, Schwarz EM, Takeshita S, Wagner EF, Noda M, Matsuo K, Xing L, Boyce BF. NF- κ B p50 and p52 regulate receptor activator of NF- κ B ligand (RANKL) and tumor necrosis factor-induced osteoclast precursor differentiation by activating c-Fos and NFATc1. *J Biol Chem*. 2007; 282:18245–18253. [PubMed: 17485464]
35. Gao X, Iwai M, Inaba S, Tomono Y, Kanno H, Mogi M, Horiuchi M. Attenuation of monocyte chemoattractant protein-1 expression via inhibition of nuclear factor- κ B activity in inflammatory vascular injury. *Am J Hypertens*. 2007; 20:1170–1175. [PubMed: 17954363]
36. Takeuchi O, Hoshino K, Akira S. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J Immunol*. 2000; 165:5392–5396. [PubMed: 11067888]
37. Hatakeyama J, Tamai R, Sugiyama A, Akashi S, Sugawara S, Takada H. Contrasting response of human gingival and periodontal ligament fibroblasts to bacterial cell-surface components through the CD14/toll-like receptor system. *Oral Microbiol Immunol*. 2003; 18:14–23. [PubMed: 12588454]

Fig. 1A

A

Figure 1.

Expression of myeloid differentiation factor 88 (MyD88) mRNA and protein in the dental follicle. (A) mRNA expression of MyD88 in the dental follicle of postnatal day 1 to 11 by DNA microarray. The results are presented as the mean values $\pm$ standard deviations of three independent litters of rats, with the bars without same letter indicating statistical difference from each other. MyD88 expression at day 3 was significantly higher than at other days. (B) Immunostaining of MyD88 in rat first mandibular molar at day 5. MyD88 protein was expressed in the dental follicle (DF), as well in alveolar bone (AB), but not in the stellate reticulum (SR). (C) Immunostaining controls, in which the primary antibody was replaced by immunoglobulin G (IgG). No immunostaining was observed. Scale bar: 50 μ m.

Figure 2.

Up-regulation of MyD88 by interleukin-1 α (IL-1 α). The dental follicle cells were treated with IL-1 α (5 ng ml⁻¹) for 1 to 12 h (repeated three times), and the MyD88 gene expression was determined using real-time RT-PCR. An asterisk (*) indicates a significant difference between IL-1 α treatment and the untreated control for a given time. MyD88 expression was significantly up-regulated by IL-1 α at 3 and 6 h.

Figure 3.

Reduction in expression of NFKB1 (A) and MCP-1(B) in the dental follicle cells with MyD88 knocked down. The dental follicle cells were transfected with siRNA targeting MyD88 mRNA (siRNA) or scrambled siRNA without targeting any RNA (control) at a concentration of 5 nM, and the expression of NFKB1 and MCP-1 was determined at 24, 48 and 72 h using real-time RT-PCR. The experiments were repeated three times, and the data were analyzed using paired t-tests. An asterisk (*) indicates a significant difference between siRNA and control for a given time point. The gene expression was significantly reduced at 24, 48 and 72 h for NFKB1, and at 48 and 72 h for MCP-1 after transfection.

Figure 4. Reduction of MCP-1 protein expression in the DF cells with MyD88 knocked down by siRNA. After 48 h of transfection with either control siRNA or MyD88 siRNA, the cells were immunostained for MCP-1 protein expression. The cells transfected with MyD88 siRNA showed a reduction of MCP-1 protein (A) as compared to the cells transfected with control siRNA (B). For controls, in the presence of IgG no staining was observed in the cells either transfected with MyD88 siRNA (C) or control siRNA (D). Scale bar: 50 μ m.

A

B

C

Figure 5.

Dependence of IL-1 α up-regulated expression of NFKB1, MCP-1 and RANKL on MyD88. The dental follicle cells were transfected with either MyD88 siRNA to knock down MyD88 or scrambled siRNA as a control. After 48 h of transfection, the cells were treated with or without IL-1 α (5 ng ml⁻¹) for 1 h for NFKB1 and RANKL expression, and 2 h for MCP-1 expression. Gene expression was determined using real-time RT-PCR. The experiments were repeated three times, and the data were analyzed using paired t-tests. An asterisk (*) indicates a significant difference between IL-1 α treatment and untreated control. Note that IL-1 α up-regulated the expression of NFKB1, MCP-1 and RANKL expression but that this was nullified in the dental follicle cells after MyD88 was knocked down by siRNA.

A

B

Figure 6.

Chemotaxis assay of the conditioned medium (A) and in vitro osteoclastogenesis from the bone marrow mononuclear cells attracted by the conditioned medium (B). The chemotaxis assay was performed by loading bone marrow mononuclear cells in the upper chamber of the transwell. The cells that migrated into the lower chamber of the transwell in the conditioned medium were used for cell counting or in vitro osteoclastogenesis. The experiments were repeated four times and the results were presented as the mean values $\pm$ standard deviations. The bars in figures labeled without the same letter are significantly different from each other. (A) Knockdown of MyD88 in DF cells reduced the chemotactic activity of the conditioned medium (siMyD88 CM) as compared to the CM from the cells

transfected with control siRNA (siControl CM) ($P \leq 0.05$). The increase in chemotactic activity by siControl + IL-1 α CM was abolished when MyD88 was knocked down (siMyD88 + IL-1 α CM). (B) In vitro osteoclastogenesis as shown by osteoclast number per well was greatly reduced by siMyD88 CM as compared to that by siControl CM ($P \leq 0.05$). Knockdown of MyD88 in the presence of IL-1 α (siMyD88 + IL-1 α CM) abolished the enhanced osteoclastogenesis by siControl + IL-1 α CM.