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MyD88 is essential for alveolar bone loss induced by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide in mice

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SUMMARY

Aggregatibacter actinomycetemcomitans **is a Gram-negative bacteria highly associated with localized aggressive periodontitis. The recognition of microbial factors, such as lipopolysaccharide from *A. actinomycetemcomitans* ($_{Aa}$LPS), in the oral environment is made mainly by surface receptors known as Toll-like receptors (TLR). TLR4 is the major LPS receptor. This interaction leads to the production of inflammatory cytokines by myeloid differentiation primary-response protein 88 (MyD88) -dependent and -independent pathways, which may involve the adaptor Toll/interleukin-1 receptor-domain-containing adaptor inducing interferon-β (TRIF). The aim of this study was to assess the involvement of MyD88 in alveolar bone loss induced by $_{Aa}$LPS in mice. C57BL6/J wild-type (WT) mice, MyD88, TRIF or TRIF/MyD88 knockout mice received 10 injections of $_{Aa}$LPS strain FDC Y4 (5 μg in 3 μl), in the palatal gingival tissue of the right first molar, every 48 h. Phosphate-buffered saline was injected in the opposite side and used as control. Animals were sacrificed 24 h after the 10th injection and the maxillae were removed for macroscopic and biochemical analyses. The injections of $_{Aa}$LPS induced significant alveolar bone loss in WT mice. In the absence of MyD88 or TRIF/MyD88 no bone loss induced by $_{Aa}$LPS was observed. In contrast, responses in TRIF$^{-/-}$ mice were similar to those in WT mice. Diminished bone loss in the absence of MyD88 was associated with fewer TRAP-positive cells and increased expression of osteoblast markers, RUNX2 and osteopontin. There was also reduced tumor necrosis factor-α production in MyD88$^{-/-}$ mice. There was less osteoclast differentiation of hematopoietic bone marrow cells from MyD88$^{-/-}$ mice after $_{Aa}$LPS stimulation. Hence, the signaling through MyD88 is pivotal for $_{Aa}$LPS-induced osteoclast formation and alveolar bone loss.**

INTRODUCTION

Periodontal disease is an inflammatory infectious disease of the supporting structures of the teeth and a

significant cause of tooth loss in adults (Brown & Löe, 1993; Preshaw & Taylor, 2011). Some bacteria from the subgingival oral biofilm, known as periodontopathogens, are the etiological agents of periodontal disease. Among them are Gram-negative bacteria such as *Aggregatibacter actinomycetemcomitans*, the main etiological agent of aggressive periodontitis (Fives-Taylor *et al.*, 1999). Lipopolysaccharide from *A. actinomycetemcomitans* ($_{Aa}$LPS) is one of the major virulence factors of *A. actinomycetemcomitans* (Fives-Taylor *et al.*, 1999), given that it is a potent stimulator of innate immune responses, i.e. it activates leukocytes to produce cytokines involved in the destruction of soft and hard tissues (Yoshimura *et al.*, 1997). LPS from *A. actinomycetemcomitans* and *Porphyromonas gingivalis* stimulates alveolar bone resorption *in vivo* (Nishida *et al.*, 2001), but the exact mechanism through which it does so remains unclear. It has been shown that LPS supports the survival of pre-osteoclasts, and induces their fusion to form active multinucleated osteoclasts (Suda *et al.*, 2002).

The recognition of microbial factors, such as LPS, by the host is made by a group of surface receptors known as Toll-like receptors (TLR), among others (Janeway & Medzhitov, 2002; Kawai & Akira, 2010, 2011). TLR4 is the major LPS receptor and its expression has been demonstrated in cells of periodontal tissues, including fibroblasts (Wang *et al.*, 2001), epithelial cells (Mori *et al.*, 2003; Kinane *et al.*, 2006) and osteoclasts (Itoh *et al.*, 2003).

Signaling via TLRs activates the cytoplasmic Toll/Interleukin-1 receptor (TIR) domain, which can be coupled to at least four TIR domain-containing adaptors: myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM; Akira & Takeda, 2004). TLR4 is the only TLR that recruits the four adaptor proteins, but MyD88 seems to be of special interest because it is used by all TLRs (with the exception of TLR3) and members of the interleukin-1 receptor family, culminating in nuclear factor-κB and mitogen-activated protein kinase activation and the induction of inflammatory cytokines (Takeda & Akira, 2004; Kawai & Akira, 2011). The activation of TLR4 can induce the production of inflammatory cytokines via MyD88-dependent and TRIF-dependent pathways, both of which mediate signaling in response to LPS (Kawai *et al.*, 1999, 2001; Kawai & Akira, 2010). Despite such evidence, the involvement of MyD88 and TRIF in alveolar bone loss induced by LPS is not well established. Hence, this work aimed to investigate the involvement of the adaptors MyD88 and TRIF in alveolar bone loss induced by LPS from *A. actinomycetemcomitans* in mice.

METHODS

Experimental animals

Eight-week-old wild-type (C57BL6/J), TRIF knockout (TRIF$^{-/-}$), MyD88$^{-/-}$ and TRIF/MyD88$^{-/-}$ mice were used in this study. These animals were provided by the Transgenose Institute, CNRS, Orléans, France (Doz *et al.*, 2009). We also used MyD88$^{-/-}$ mice from the School of Medicine of Ribeirão Preto (University of São Paulo – USP). The experimental protocol used in this study was approved by the local Institutional Animal Ethics Committee under the protocol number 256/2008.

Purification of *A. actinomycetemcomitans* LPS ($_{Aa}$LPS)

The LPS samples were prepared from *A. actinomycetemcomitans* strain Y4, using the LPS Extraction Kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's procedure. LPS extract was dissolved (10 mM Tris–HCl buffer (pH 7.5), 1 mg ml^{-1} DNase I, and 1 mg ml^{-1} RNase) and incubated at 37°C overnight. The LPS solution was further treated with 2 mg ml^{-1} proteinase K (final concentration) at 37°C overnight. The LPS was collected by ethanol precipitation (20,000 ***g***, 15 min, 4°C) using 0.2 vol of 5 M NaCl and 2 vol of 95% ethanol. After washing with 70% ethanol, LPS was dried, re-treated with the LPS Extraction Kit (iNtRON Biotechnology), and applied to the polymixin B immobilized column (Detoxi-Gel AffinityPak Pre-packed Columns; Pierce, Rockford, IL) for further purification. LPS eluted from the column was collected by ethanol precipitation (20,000 ***g***, 15 min, 4°C) using 0.4 vol of 5 M NaCl and 4 vol. of 95% ethanol. After washing with 70% ethanol, pellets were dried and dissolved in 1 ml of distilled water. LPS concentrations were determined by the malondialdehyde thiobarbituric acid reaction (Waravdekar & Saslaw, 1959). After lyophilization, the sediment obtained was stored at −20°C.

Induction of alveolar bone loss

Alveolar bone resorption was induced as previously described (Iwasaki *et al.*, 1998; Nishida *et al.*, 2001; Nakamura *et al.*, 2008). Briefly, 5 μg of $_{Aa}$LPS in 3 μl of phosphate-buffered saline (PBS) was injected using a microsyringe (Hamilton, Reno, NV) every 48 h into the palatal gingival papilla between the first and the second molar of the right maxilla of wild-type (WT) or knockout ($^{-/-}$) mice under ketamine and xylazine intraperitoneal anesthesia. In addition, PBS was injected in the same way in the left side of the maxilla and this side was used as control. Groups of WT and knockout mice that only received injections of PBS were also investigated. Each group of five WT or knockout mice was housed in separate and appropriate cages under standard conditions and had free access to commercial chow and water. Mice were sacrificed 24 h after the 10th injection of $_{Aa}$LPS.

Quantification of alveolar bone loss

To evaluate the extent of alveolar bone loss we used the modified protocol from Madeira *et al.* (2012). The maxillae were hemisected, exposed overnight to 3% hydrogen peroxide and mechanically defleshed. To distinguish the cement–enamel junction (CEJ), mouse maxilla jaws were stained with 0.3% methylene blue (Kawai *et al.*, 2007). The palatal faces of the molars were photographed with 20 × magnification using a stereomicroscope (Metrimpex Hungary/PZO, Labimex, Budapest, Hungary) and a digital camera (Kodak EasyShare C743, Rochester, NY). The images were analyzed using IMAGE J software (National Institutes of Health, Bethesda, MD). Quantitative analysis was used for the measurement of the disto-mesial area (in mm^2) between the CEJ and the alveolar bone crest (ABC) on the palatal side of the first molar. At each time-point five animals were analysed, and for each animal the alveolar bone loss was defined as the difference of CEJ–ABC between the right and the left arch.

Histological analysis

Five animals selected at random from each group were sacrificed and maxillar tissues obtained were then fixed in 10% formalin at pH 7.4 for 24 h at room temperature. The specimens were demineralized in 14% ethylenediamine tetracetic acid for 2 weeks, dehydrated in graded ethanol and embedded in paraffin. Serial sections (5 μm) were stained for tartrate-resistant acid phosphatase (TRAP; Sigma-Aldrich, St Louis, MO). The alveolar bone crest between the first and the second molars was used for the osteoclast counts, on five fields (magnification: 400 ×) per section. For each animal, three maxillar sections were analysed. All the slides were counted in a blinded manner by a single examiner.

Myeloperoxidase concentration

The activity of myeloperoxidase (MPO) in maxillae was measured as previously described (Madeira *et al.*, 2012). The posterior portions of the maxilla including teeth, periodontal soft tissues and alveolar bone were removed, weighed, homogenized using a Power Gen 1000 homogenizer (Fisher Scientific, Pittsburgh, PA), and snap-frozen in liquid nitrogen. Upon thawing and processing, tissue was assayed for MPO activity by measuring the change in optical density at 450 nm using tetramethylbenzidine. Results were expressed as neutrophil index, which denotes MPO activity related to casein-elicited murine peritoneal neutrophils processed in the same way. One experiment representative of three is presented in the results.

Enzyme-linked immunosorbent assay

The levels of tumor necrosis factor-α (TNF-α) in the maxillae, including the periodontal tissues, teeth and alveolar bone, were measured as previously described (Madeira *et al.*, 2012). For protein extraction, the samples were weighed, homogenized in phosphate-buffered saline (PBS) containing protease inhibitors and 0.05% Tween 20, pH 7.4, using a Power Gen 1000 homogenizer (Fisher Scientific), centrifuged (9000 ***g***, 10 min) and the supernatants were stored at −20°C. The cytokine concentrations in the periodontal extracts were determined by enzyme-linked immunosorbent assay using commercially available kits (R&D Systems, Minneapolis, MN). All assays were carried out according to the manufacturer's instructions. The results are expressed as picograms of cytokine per 100 mg of maxillar tissues for one experiment representative of three.

Osteoclast generation and pit resorption assay

Bone marrow cells from tibiae and femurs of WT or MyD88$^{-/-}$ mice were incubated in Dulbecco's modified Eagle's medium (GIBCO, Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, with soluble macrophage colony-stimulating factor (M-CSF; 50 ng ml^{-1}; Peprotech, London, UK) for 6 days to generate pre-osteoclasts (or macrophage). Soluble receptor activator of nuclear factor-κB ligand (RANKL; 50 ng ml^{-1}; Peprotech) was then added until day 8, when *Aa*LPS (2.5 μg ml^{-1}) was added until day 10. At this time cells were fixed and stained with TRAP. The percentage of TRAP-positive cells was determined by the proportion of TRAP-negative and TRAP-positive cells in 10 fields (magnification: 400 ×) per slide. For pit resorption assay, pre-osteoclasts were added to Corning® Osteo Assay Surface (Corning Life Sciences, Corning Inc., Corning, NY). The resorption pits were assessed according to the manufacturer's instructions. The data are expressed as the number of pits/field.

Quantitative polymerase chain reaction

The mRNA levels in the maxillae were estimated by quantitative polymerase chain reaction (PCR). First, total RNA was extracted from tissues using TRIzol® (Life Technologies, Grand Island, NY) reagent according to the manufacturer's protocol. Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega, Madison, WI) following the manufacturer's instructions. Two micrograms of total RNA were used to make the cDNA. Resultant cDNA was used for real-time PCR as below. Specific primers were designed using Primer Express software and synthesized by IDT (Integrated DNA Technologies, Coralville, IA). Real-time PCR was carried out on a StepOne sequence detection system (Life Technologies) using SYBR Green PCR Master Mix (Life Technologies). The relative levels of gene expression were determined by the $2^{-\Delta\Delta \text{Cycle Threshold}}$ ($2^{-\Delta\Delta CT}$) method as described by the manufacturer, in which data for each sample are normalized to the expression of the 18S rRNA gene and data are shown as fold increase over the negative control (non-infected) group (Livak & Schmittgen, 2001). The specific primer sequences are: 18S rRNA gene forward: CGT TCC ACC AAC TAA GAA CG, reverse: CTC AAC ACG GGA AAC CTC AC; osteopontin forward: GAT TGG CAG TGA TTT GCT TTT G, reverse: GAG CTG CCA GAA TCA GTC ACT TT; RUNX-2 forward: GGC CGG GAA TGA TGA GAA CTA, reverse: CAG ATC GTT GAA CCT GGC TAC TT.

Statistical analysis

Quantitative data were analysed using the statistical features of GraphPad Prism Version 4.0 (GraphPad Inc., San Diego, CA). One-way analysis of variance statistical test, following by Kruskal–Wallis test and Dunn's post-test was used with $P < 0.05$ considered statistically significant.

RESULTS

MyD88 is involved in alveolar bone loss induced by *Aa*LPS

Evident bone resorption was observed in the alveolar bone crest of WT mice 24 h after the 10th *Aa*LPS injection (Fig. 1A). In contrast, there was no significant alveolar bone loss in MyD88$^{-/-}$ (Fig. 1B,E) and TRIF/MyD88$^{-/-}$ mice (Fig. 1D,E). Injection of *Aa*LPS in TRIF$^{-/-}$ mice (Fig. 1C,E) resulted in a phenotype similar to that observed in WT mice. There was no significant alveolar bone loss in PBS groups of WT and knockout mice.

Histological sections of periodontal tissues showed increased numbers of TRAP-positive cells in the alveolar bone crest of WT mice challenged with *Aa*LPS (Fig. 2A,C). In contrast, there were no TRAP-positive cells and there was integrity of alveolar bone crest in MyD88$^{-/-}$ mice after *Aa*LPS challenge (Fig. 2B,C). No significant neutrophil influx was observed in periodontal tissues after *Aa*LPS injection, as assessed by measuring myeloperoxidase activity (WT control: 0.245 ± 0.07; WT LPS: 0.265 ± 0.04; MyD88$^{-/-}$ control: 0.262 ± 0.04; MyD88$^{-/-}$ LPS: 0.382 ± 0.102). Concentrations of TNF-α were increased in periodontal tissues of WT mice, but no significant production of TNF-α was observed in the absence of MyD88 after injection of *Aa*LPS (Fig. 2D).

Expression of markers of osteoblast activity is preserved in MyD88$^{-/-}$ mice

Considering that osteoblasts may interact with osteoclasts to regulate bone turnover, we evaluated

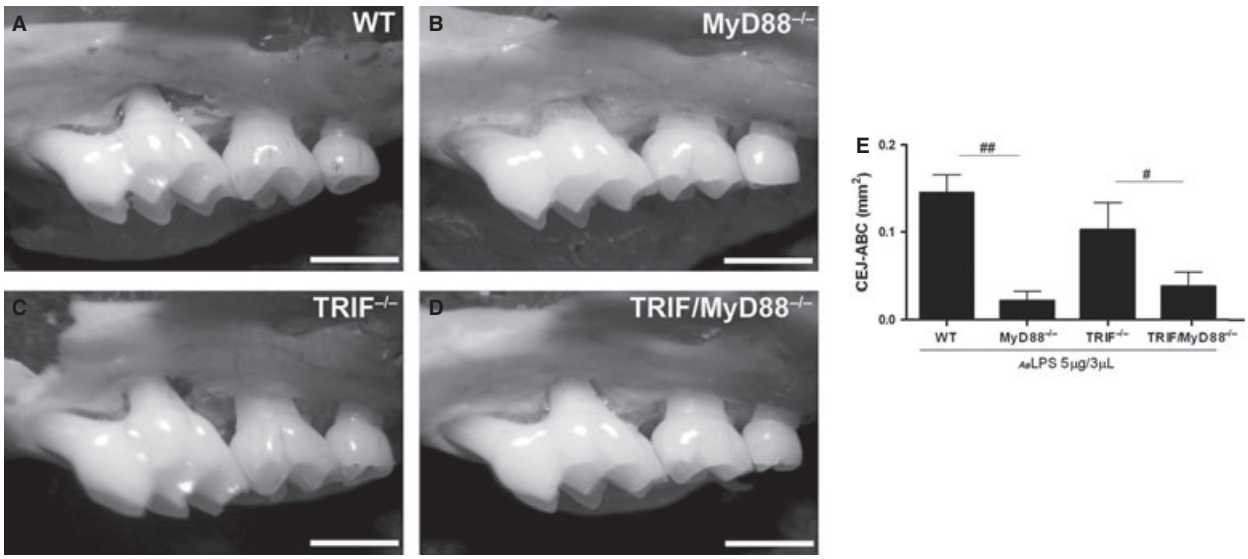

Figure 1 Experimental alveolar bone loss induced by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide (LPS) injection. Bone loss evaluated in wild-type (WT) mice (A) and in mice deficient for myeloid differentiation primary-response protein 88 (MyD88$^{-/-}$; B), Toll/interleukin-1 receptor-domain-containing adaptor inducing interferon-β (TRIF$^{-/-}$; C) or TRIF/MyD88 (TRIF/MyD88$^{-/-}$; D). (A–D) Maxilla palatal aspect of alveolar bone loss in the local of *Aggregatibacter actinomycetemcomitans* lipopolysaccharide ($_{Aa}$LPS) injection, after 10 injections. Bars = 1 mm. (E) Alveolar bone loss quantification was performed through the measurements of cement–enamel junction/alveolar bone crest area in the palatal face of the first molar, with IMAGE J software. Values (mean ± SEM) were obtained from five animals at each point in two independent experiments. Bone resorption was not evident in the control group throughout the study. #$P < 0.05$, ##$P < 0.01$, one-way analysis of variance, Dunn's post test.

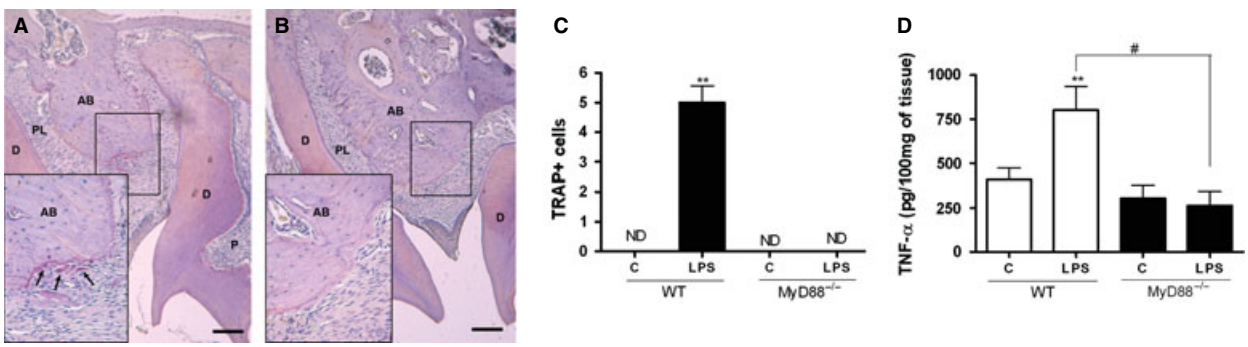

Figure 2 Involvement of myeloid differentiation primary-response protein 88 (MyD88) in osteoclast activity. (A, B) Histological sections stained with tartrate-resistant acid phosphatase (TRAP) showing TRAP-positive cells in alveolar bone crest in wild-type (WT; A) and MyD88$^{-/-}$ mice (B) after 10 injections. Arrows show TRAP-positive cells, Bars = 100 μm. (C) TRAP-positive cells in the alveolar bone crest of WT and MyD88$^{-/-}$ mice ($n = 5$). (D) Levels of tumor necrosis factor-α (TNF-α) in maxilla samples of WT and MyD88$^{-/-}$ mice ($n = 5$). #$P < 0.05$, **$P < 0.01$, one-way analysis of variance, Dunn's post test.

whether the absence of MyD88 could interfere with the expression of markers of osteoblast activity. To this end, we assessed Runt-related transcription factor 2 (RUNX2) and osteopontin expression in periodontal tissues. Our results showed decreased production of RUNX2 (Fig. 3A) and osteopontin (Fig. 3B) in WT mice after $_{Aa}$LPS injections. In contrast, MyD88$^{-/-}$ mice had increased expression of RUNX2 and osteopontin after $_{Aa}$LPS injections in comparison to WT challenged mice (Fig. 3A,B).

MyD88 is important to osteoclast differentiation induced by $_{Aa}$LPS

Given the decreased alveolar bone loss observed in the absence of MyD88, we further evaluated the

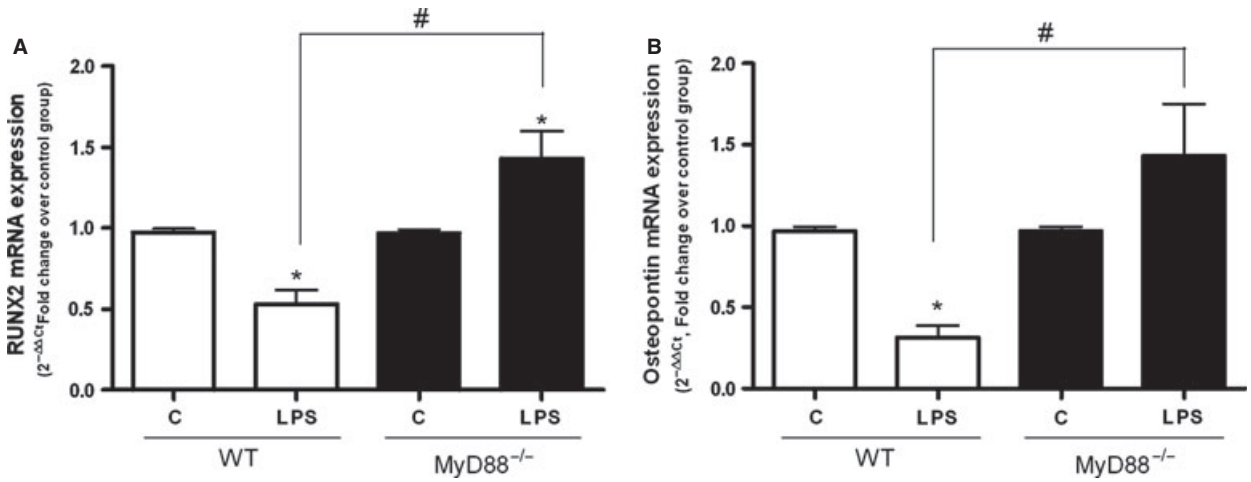

Figure 3 Involvement of myeloid differentiation primary-response protein 88 (MyD88) in osteoblast activity. Levels of RUNX2 (A) and osteopontin (B) in maxillae samples of wild-type (WT) and MyD88$^{-/-}$ mice ($n = 5$). *#$P < 0.05$, one-way analysis of variance, Dunn's post test.

involvement of MyD88 in osteoclast differentiation and activity using *in vitro* assays. Experiments were carried out using bone marrow cells from WT or MyD88$^{-/-}$ mice. As seen in Fig. 4, there were fewer TRAP-positive cells induced by $_{Aa}$LPS in the absence of MyD88 when compared with bone marrow cells from WT mice. In addition, reduced resorption activity was also observed in MyD88$^{-/-}$ cells (Fig. 4F).

DISCUSSION

Gram-negative bacteria such as *P. gingivalis* and *A. actinomycetemcomitans* are major pathogens in adult periodontitis, and their LPS are important inducers of bone resorption (Ishihara *et al.*, 1991; Nair *et al.*, 1996; Sakuma *et al.*, 2000). However, the immune response triggered by LPS from distinct pathogens may drive different inflammatory responses (Pulendran *et al.*, 2001). It was recently demonstrated that the initial inflammatory response to *P. gingivalis* in naive macrophages is MyD88-dependent and requires co-operative signaling of TLR2 and TLR4 (Papadopoulos *et al.*, 2013). Although several reports show the requirement of host TLR2 and TLR4 for *A. actinomycetemcomitans*-induced inflammatory bone loss *in vivo* (Kikkert *et al.*, 2007), there is no evidence regarding the role of specific TLR adaptor proteins during $_{Aa}$LPS-induced alveolar bone loss. The major findings of the current study can be summarized as follows: (i) $_{Aa}$LPS induced significant alveolar bone loss in WT mice, (ii) the effect was dependent on MyD88 but not TRIF, (iii) signaling through MyD88 was fundamental for RANKL-induced osteoclast differentiation and activity after $_{Aa}$LPS stimulation.

Some studies have shown decreased $_{Aa}$LPS-induced bone resorption in C3H/HeJ mice, which are hyporesponsive to LPS in view of their ablated TLR4 receptor function (Hoshino *et al.*, 1999; Nakamura *et al.*, 2008). Despite this evidence, there are no studies that determine which TLR adaptor is indispensable for $_{Aa}$LPS-induced alveolar bone resorption. Takeda & Akira (2004) suggested that the recognition of LPS by TLR4 induced an inflammatory cytokine production mediated by MyD88, although there was also a MyD88-independent component of TLR4-mediated signaling. In the current study, alveolar bone loss induced by $_{Aa}$LPS was dependent on MyD88, but not TRIF. Using other Gram-negative bacteria, Cai *et al.* (2009) suggested that signaling through TRIF and MyD88 contributed to *Klebsiella pneumoniae*-induced cytokine/chemokine production and up-regulation of cell adhesion molecules in the lungs. In fact, recognition of distinct stimuli and microorganisms may involve different host mechanisms (Pulendran *et al.*, 2001) and it seems that MyD88 adaptor is the key component for alveolar bone resorption induced by $_{Aa}$LPS.

After detecting decreased alveolar bone loss in MyD88$^{-/-}$ and TRIF/MyD88$^{-/-}$ mice after $_{Aa}$LPS challenge, we also investigated the involvement of MyD88 in osteoclast recruitment and activity. Bone-related multinucleated cells express major characteristics of osteoclasts in response to LPS, such as TRAP (an

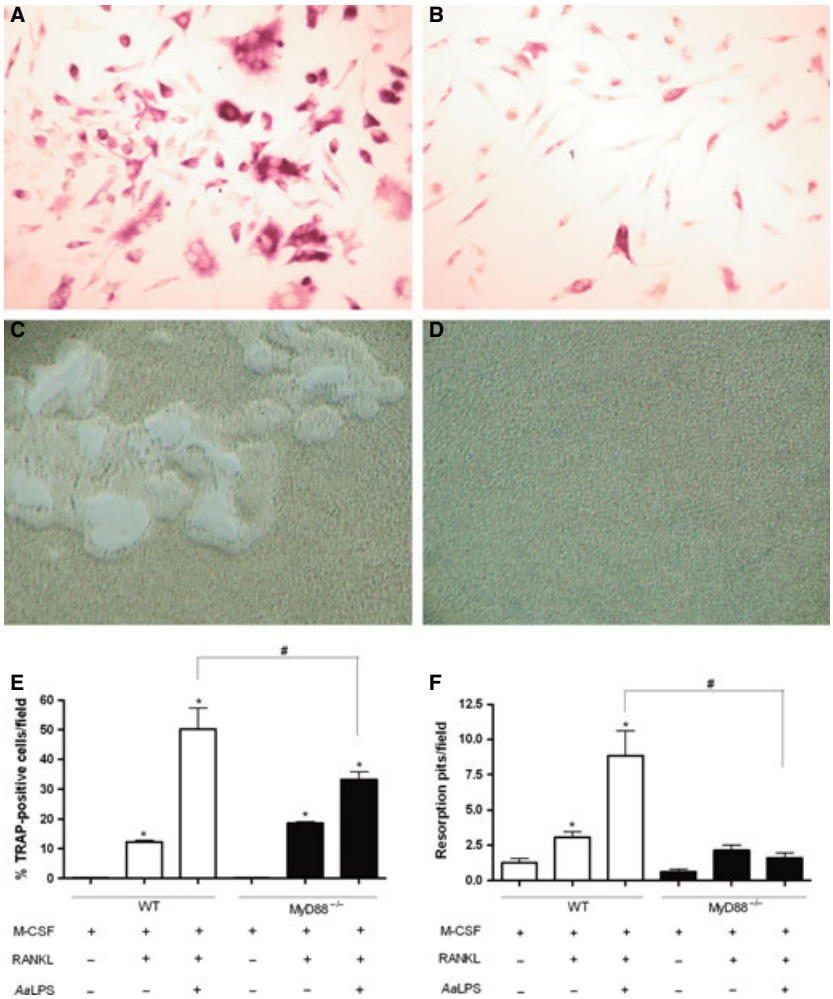

Figure 4 Involvement of myeloid differentiation primary-response protein 88 (MyD88) in osteoclastogenesis. Bone marrow cells from wild-type (WT) and MyD88$^{-/-}$ mice were cultivated in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). (A, E) Number of tartrate-resistant acid phosphatase (TRAP) -positive cells 2 days after *Aggregatibacter actinomycetemcomitans* lipopolysaccharide ($_{Aa}$LPS) treatment in WT cells ($n = 3$). (B, E) Number of TRAP-positive cells 2 days after $_{Aa}$LPS treatment in MyD88$^{-/-}$ cells ($n = 3$). (C, F) Number of resorption pits/field 6 days after $_{Aa}$LPS stimulation in WT cells ($n = 3$). (D, F) Number of resorption pits/field 6 days after $_{Aa}$LPS stimulation in MyD88$^{-/-}$ cells ($n = 3$). The values (means ± SEM) of all *in vitro* experiments were obtained from two independent experiments. *#$P < 0.05$ one-way analysis of variance, Dunn's post test.

enzymatic marker of osteoclasts; Boyle *et al.*, 2003; Itoh *et al.*, 2003). Our results showed TRAP-positive cells in the alveolar bone crest of WT mice after $_{Aa}$LPS injection. In contrast, there was no increase of TRAP-positive cells in MyD88$^{-/-}$ mice under the same conditions. We also observed, *in vitro*, an impairment of osteoclast formation and activity in the absence of MyD88. Accordingly, previous findings have suggested that MyD88-mediated signaling may be essential for osteoclastogenesis (Sato *et al.*, 2004; Zhang *et al.*, 2011).

Besides the influence on bone resorbing osteoclasts, our results also showed that the lack of MyD88 affected osteoblast markers, including RUNX2 and osteopontin. Osteopontin is a protein with cytokine properties produced by distinct cells types. It modulates bone remodeling processes and higher levels of osteopontin are mainly associated with bone formation (Denhardt *et al.*, 2001; Grimm *et al.*, 2010). RUNX2 is a transcription factor required for osteoblast differentiation, and its increased production points to enhanced osteoblast differentiation/activity (Katagiri & Takahashi,

2002). In accordance with the current data, it has been shown that LPS suppresses mRNA expression of RUNX2 in WT but not in the MyD88$^{-/-}$ osteoblasts, suggesting that the inhibitory effect of LPS on osteoblast differentiation may be dependent on MyD88 (Bandow *et al.*, 2010). Nevertheless, further studies are warranted to better understand the mechanisms associated with the increased expression of RUNX2 and osteopontin by $_{Aa}$LPS in the absence of MyD88.

The decreased numbers of osteoclasts, increased expression of osteoblast markers and, consequently, the reduced alveolar bone loss observed in MyD88$^{-/-}$ mice could be related to a smaller production of inflammatory mediators, because MyD88 signaling induced by LPS activates this pathway (Medzhitov *et al.*, 1998). Herein, we evaluated the expression of the pro-resorptive cytokine TNF-α in periodontal tissues and observed that MyD88$^{-/-}$ mice presented lower levels of TNF-α than WT mice, after injection of $_{Aa}$LPS, confirming the involvement of the MyD88-dependent pathway. TNF-α is produced by a variety of cells in inflamed tissues and increases the expression of RANKL and macrophage colony-stimulating factor, factors that regulate the differentiation of osteoclasts (Teitelbaum, 2007). TNF-α also induces the production of enzymes that degrade connective tissues, being involved in the progression of periodontal disease (Azuma *et al.*, 2000; Graves & Cochran, 2003; Garlet *et al.*, 2007; Queiroz-Junior *et al.*, 2011). Therefore, the decreased $_{Aa}$LPS-induced alveolar bone loss observed in the absence of MyD88 may be partially accounted for by the reduction in expression of TNF-α in periodontal tissues.

Overall, this study demonstrates for the first time that MyD88 is involved in the signaling pathway triggered by $_{Aa}$LPS and is crucial for osteoclast differentiation and activity *in vitro*.

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