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ABSTRACT

The mitogen-activated protein (MAP) kinase phosphatase (MKP) family plays an important function in regulating the pro-inflammatory cytokines by deactivating MAP kinases. MKP-1 is essential for the dephosphorylation of p38 MAP kinase that regulates expression of IL-6, TNF- α , and IL-1 β . We hypothesized that MKP-1 regulates inflammatory bone loss in experimental periodontitis. Wild-type and *Mkp-1*^{-/-} mice received *A. actinomycetemcomitans* LPS injection in the palatal region or PBS control 3 times/wk for 30 days. Mice were killed, and maxillae were assessed by microcomputed tomography, histological analysis, and TRAP staining for measurement of bone loss, extent of inflammation, and degree of osteoclastogenesis. Results indicated that, in LPS-injected *Mkp-1*^{-/-} mice, significantly greater bone loss occurred with more inflammatory infiltrate and a significant increase in osteoclastogenesis compared with *Mkp-1*^{-/-} control sites or either wild-type group. Analysis of these data indicates that MKP-1 plays a key role in the regulation of inflammatory bone loss.

KEY WORDS: MKP-1, innate immunity, LPS, periodontal disease.

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MAP Kinase Phosphatase-1 Protects against Inflammatory Bone Loss

INTRODUCTION

Bacterial constituents, including Gram-negative-derived lipopolysaccharides (LPS), initiate inflammatory bone loss, as exhibited in periodontal diseases. LPS can stimulate the expression of IL-1 β , TNF- α , IL-6, and RANKL by activating the innate immune response (Nakashima *et al.*, 2000; Jiang *et al.*, 2002; Kikuchi *et al.*, 2003). Within periodontal tissues, TLR-2 and -4 expression is increased in severe disease states, suggesting that these receptors have an increased capacity to signal and influence downstream cytokine expression (Mori *et al.*, 2003). TLR-4 signaling activates MyD88-dependent pathways to subsequent activation of IRAK, TRAF6, and, ultimately, nuclear factor kappa B (NF- κ B), which is required for cytokine induction. Also, TRAF6-dependent pathways are required for recruitment of different adaptor proteins and/or activation of various MAPK cascades such as ERK-1 and -2 (An *et al.*, 2002), JNK and p38 (Schumann *et al.*, 1996), needed for cytokine mRNA transcription and mRNA stabilization. In addition to the important role of p38 MAPK in innate immune responses, p38 MAPK has been shown to be an important signaling link between innate and adaptive immune activation. Our group has recently shown the significance of p38 MAPK signaling in periodontal disease progression, where orally active p38 inhibitors reduced periopathogenic LPS-induced bone destruction in a rat model (Kirkwood *et al.*, 2007; Rogers *et al.*, 2007b).

Negative regulation of MAPK activity is mediated by the MAPK phosphatases (MKPs) that dephosphorylate MAPK proteins. Though there are several classes of phosphatases, a family of dual-specificity phosphatases has been shown to be important for MAPK regulation, due to their ability to dephosphorylate threonine and tyrosine residues, which are necessary for their activation (Keyse, 2000). The founding member of this class of phosphatases is MKP-1. Cloned initially as an early response gene in response to growth factors (Lau and Nathans, 1985), MKP-1 is localized into the nucleus, where it preferentially dephosphorylates activated p38 and JNK compared with ERK MAPK (Wu and Bennett, 2005). Consistent with this activity, *Mkp-1* null mice have sustained p38 and JNK activity in response to stress (Nimah *et al.*, 2005; Zhao *et al.*, 2005, 2006; Chi *et al.*, 2006). Importantly, MKP-1 has been shown to attenuate TNF- α and IL-6 after LPS stimulation (Chen *et al.*, 2002; Shepherd *et al.*, 2004; Nimah *et al.*, 2005). Indeed, MKP-1 plays a crucial role in the regulation of pro-inflammatory cytokine production through the inactivation of p38 signaling (Salojin *et al.*, 2006; Zhao *et al.*, 2006). Thus, MKP-1 acts as a major negative regulator of p38 MAPK phosphorylation.

In vivo, *Mkp-1* null mice have increased sensitivity to endotoxemia, septic shock, and increased alveolar infection in acute models of inflammation and infection (Hammer *et al.*, 2006; Wang *et al.*, 2008). To date, no studies have

examined the role of MKP-1 in bone resorption. In this study, we hypothesized that MKP-1 negatively regulates inflammatory bone loss in experimental periodontitis.

MATERIALS & METHODS

Cells and Materials

The murine macrophage cell line RAW264.7 (ATCC #TIB-71) was cultured in DMEM supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum and maintained in a humidified atmosphere at 37°C and 5% CO₂. LPS from *Aggregatibacter actinomycetemcomitans* (formerly known as *Actinobacillus actinomycetemcomitans*) was extracted from *A. actinomycetemcomitans* strain Y4 (serotype B) by the hot phenol-water method as described (Rogers, 2007b). *A. actinomycetemcomitans* LPS used in the present study was recently characterized as part of other studies from our lab group (Rogers *et al.*, 2007b). The ELISA kit for IL-6 expression in cell culture supernatant was obtained from R&D Systems (Minneapolis, MN, USA). The expression vector containing MKP-1 cDNA in pSRII-Flag, along with the empty vector as a control, was obtained from Dr. Y. Liu (The Ohio State University).

Immunoblot and ELISA Analysis

RAW cells (5×10^4) were grown for 24 hrs in each well of 6-well plates and stimulated with *A. actinomycetemcomitans* LPS at indicated time-points. Whole-cell lysates were used for immunoblot analysis. We detected primary antibodies (total and phospho-p38α and MKP-1) by using HRP-conjugated secondary antibody and a chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA). Digital images and quantitation of the membranes were analyzed via a chemiluminescent documentation system (ChemiDoc XRS, Bio-Rad, Hercules, CA, USA). For IL-6 production experiments, RAW cells were transfected with pSRII control or pSRII-MKP-1 by means of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and then stimulated for 24 hrs with *A. actinomycetemcomitans* LPS. IL-6 ELISA was performed *per* the manufacturer's instructions with cell culture supernatant.

Animals

Mkp-1^{+/+} and *Mkp-1^{-/-}* mice of a mixed genetic background were obtained through a material transfer agreement with Bristol-Myers-Squibb (New York, NY, USA) and housed under specific pathogen-free conditions with food and tap water *ad libitum*. Ten animals were used *per* group. Once weekly, we weighed the animals to ensure proper growth and nutrition. Genotype protocols were used as previously established (Zhao *et al.*, 2006). The University Committee on the Use and Care of Animals at the University of Michigan approved all animal protocols.

Inflammatory Bone Loss Model

To initiate alveolar bone loss, we micro-injected *A. actinomycetemcomitans* LPS (20 µg *per* injection in 2 µL PBS total volume)

directly into the palatal interproximal gingiva between the first and second molars on the left side. The same volume of PBS was injected into the same region on the right side. LPS and PBS injections were repeated 3 times each wk for 4 wks, when all animals were killed by carbon dioxide asphyxiation. Maxillae were hemisected, and posterior block sections were immersed in 10% buffered formalin fixative solution for up to 48 hrs.

Micro-computed Tomography

Non-demineralized rat maxillae were scanned in 70% ethanol by a cone-beam µCT system (GE Healthcare BioSciences; Chalfont St. Giles, UK). Each scan was reconstructed at a mesh size of 18 microns x 18 microns x 18 microns, and three-dimensional digitized images were generated for each specimen. Using GEHC MicroView software (version Viz+ 2.0 build 0029), we rotated the images into a standard orientation and threshold, to distinguish between mineralized and non-mineralized tissue. For each specimen, we generated a grayscale voxel value histogram to determine an optimal threshold value. Linear measurements on bone loss were taken from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC).

Loss of bone volume was assessed by means of 3-D isoform displays as previously described (Kirkwood *et al.*, 2007; Rogers *et al.*, 2007a,b). Briefly, after proper image orientation, the region of interest (ROI) was determined. The width of the ROI was dictated by the height of contour of the molars at the CEJ. Height of the ROI was measured from molar cusp tips to root apices. Depth was equal to the bucco-lingual size of the teeth plus 100 voxels (1.8 mm³). After establishing the threshold, we calculated the bone volume fraction as the percent of bone within the ROI.

TRAP Staining

Formalin-fixed specimens were decalcified in a 10% EDTA solution for 2 wks at 4°C. The EDTA solution was changed 3 times *per* wk. The maxillae were paraffin-embedded, and sagittal sections of 5 µm were prepared. Some slides were stained with hematoxylin and eosin (H&E) for descriptive histology. For enumeration of osteoclasts, tartrate-resistant acid phosphatase (TRAP) staining was performed from all the groups by means of a Leukocyte Acid Phosphatase kit (Sigma, St. Louis, MO, USA). Active osteoclasts were defined as multinucleated (≥ 3) TRAP-positive cells in contact with the bone surface. Slides from approximately the same sagittal sections were used for the enumeration of osteoclasts (as defined previously). Images were captured by means of a Nikon microscope (Nikon Eclipse 80i) and digital camera (Nikon CCD camera).

Statistical Analysis

Ten animals were used *per* experiment ($n = 10$). We used the D'Agostino-Pearson test of normality to determine normality assumption data, either parametric or non-parametric. With normality assumptions satisfied, we could use parametric tests. Data were analyzed by Student's *t* test or one-way analysis of variance and *post hoc* Bonferroni tests where indicated. *P* values < 0.05 were considered significant.

Figure 1. MKP-1 activation coincides with phospho-p38 inactivation and decreases IL-6 production. **(A)** *A. actinomycetemcomitans* LPS stimulated a time-dependent increase in MKP-1 expression in RAW macrophage cells. RAW 264.7 cells were stimulated with *A. actinomycetemcomitans* LPS for the indicated times. Cell lysates were then collected for Western analysis of P-p38, p38, and MKP-1 protein. **(B)** IL-6 attenuation by overexpression of MKP-1 in macrophages. RAW264.7 macrophages were transfected with FLAG-pSR11 (empty vector control) or pSR11-MKP-1 and stimulated 24 hrs later with *A. actinomycetemcomitans* LPS. After 24 hrs, cell culture supernatants were assayed for IL-6 via ELISA. Results from 3 independent experiments are expressed as mean $\pm$ SE (* $p < 0.05$).

RESULTS

MKP-1 Decreases LPS-induced p38 MAPK Phosphorylation and IL-6 Expression

RAW macrophages stimulated with *A. actinomycetemcomitans* LPS RAW (Fig. 1A) showed a rapid induction of phosphorylated p38 in 15 min, but quickly attenuated as MKP-1 levels became elevated by 30 min. These data are consistent with previous studies indicating that MKP-1 expression increases when phospho-p38 MAPK is reciprocally repressed during the same time course. Overexpression of MKP-1 in an expression construct decreased *A. actinomycetemcomitans* LPS-induced IL-6 production in transfected RAW macrophages compared with empty vector transfected control LPS-stimulated cells (Fig. 1B).

LPS-induced Bone Loss is Significantly Increased in *Mkp-1*^{-/-} Mice

To determine the overall contribution of MKP-1 in an inflammatory bone loss model, we subjected age-matched *Mkp-1*^{+/+} and *Mkp-1*^{-/-} mice to LPS-induced alveolar bone loss. Representative μ CT images (Fig. 2A) revealed that *Mkp-1*^{-/-} mice exhibited visibly more bone loss at LPS injection sites compared with the PBS control sites or LPS- or PBS-injected sites in *Mkp-1*^{+/+} mice. Linear and volumetric quantitative analyses of alveolar

Figure 2. MKP-1 protects from LPS-induced bone loss in experimental periodontitis. **(A)** Mouse maxillary images from μ CT. *A. actinomycetemcomitans* LPS-induced bone loss. The Bone Volume Fraction **(B)** and the Linear Bone Loss **(C)** were analyzed by MicroView software (GE Healthcare). Statistical analysis was performed by Student's *t* test. Results are expressed as mean $\pm$ SE ($n = 10$ per group).

bone loss showed significantly less bone volume (Fig. 2B; $p < 0.01$) and significantly more destruction of the interproximal bone on *Mkp-1*^{-/-} mice at the site of LPS injection in comparison with PBS control sites, and also in comparison with either LPS or PBS sites of *Mkp-1*^{+/+} mice (Fig. 2C; $p < 0.001$).

Increased Inflammatory Infiltrate in *Mkp-1*^{-/-} Mice

Histological analysis was performed on the interproximal tissue at the sites of LPS and control injection. Representative H&E-stained sections (Fig. 3) showed that LPS-injected *Mkp-1*^{-/-} mice had a considerable inflammatory cell infiltrate, predominantly leukocytes, compared with either LPS-injected *Mkp-1*^{+/+} mice and PBS-injected sites of either genotype.

Enhanced Osteoclastogenesis Occurs in *Mkp-1*^{-/-} Mice

The number of osteoclasts present on tissue sections was determined after TRAP staining. TRAP-positive multinucleated cells were visually enumerated between maxillary first and second

Figure 3. Histological analysis of experimental periodontitis in wild-type and *Mkp-1*-deficient mice. Representative H&E images from LPS (A,C) or PBS (B,D) contralateral control sites. Boxed area from interproximal areas enlarged to show inflammatory infiltrate. Bars indicate scale; lower-magnification image represents 1 mm, and higher-magnification insert is 10 μ m ($n = 10$ per group).

Figure 4. Increased number of osteoclasts observed in LPS-challenged *Mkp-1*^{-/-} mice. TRAP⁺ cells were enumerated, and values represent the mean $\pm$ SE for all specimens from each group ($n = 10$ per group). Statistical analysis was performed by Student's *t* test.

molars. LPS injections in *Mkp-1*^{-/-} mice resulted in significantly greater numbers of TRAP⁺ cells in comparison with the PBS-injected site in *Mkp-1*^{-/-} mice, as well as in comparison with wild-type controls (Fig. 4).

DISCUSSION

Activation of the innate immune system is critical for lymphocyte activation and other immune cells to help clear infectious micro-organisms. However, exuberant production of pro-inflammatory cytokines leads to severe pathology, including periodontal bone loss, rheumatoid arthritis, and autoimmune diseases (Assuma *et al.*, 1998; Graves, 1999; Delima *et al.*, 2001, 2002; Graves and Cochran, 2003). To help prevent the deleterious effects of TLR activation, several signal-

ing mechanisms have evolved. These mechanisms include the down-regulation of surface TLR receptor expression, transcriptional induction of negative regulators such as IL-1 receptor-associated kinase (IRAK-M), suppressor of cytokine signaling-1 (SOCS1), and SH2-containing inositol phosphatase (SHIP), and production of anti-inflammatory cytokines including IL-10 and TGF- β (Liew *et al.*, 2005). In this report, we addressed the role of MKP-1, an important negative regulator of MAPK activation, in LPS-induced bone loss. We observed that genetic deletion of MKP-1 resulted in increased LPS-induced bone resorption.

Previous studies have shown that MKP-1 expression was necessary to attenuate IL-6 expression (Zhao *et al.*, 2006). In the present study, analysis of the data provided in macrophages supports the role of MKP-1 in negative regulation of *A. actinomycetemcomitans* LPS-induced p38 activation and IL-6 production. These data mimic the effects of anti-inflammatory agents, such as corticosteroids, that selectively induce MKP-1 expression (Zhao *et al.*, 2005). Analysis of these data, collectively, suggests that TLR engagement leads to MAPK pathway activation, producing pro-inflammatory cytokines, and that MKP-1 plays a crucial role in decreasing inflammatory cytokine biosynthesis.

In the LPS-induced inflammatory intra-oral bone loss model, *A. actinomycetemcomitans* LPS is used to induce bone loss in long-term experimental conditions (Rogers

et al., 2007a). This model is distinct from previously reported acute inflammatory models that showed the crucial role of MKP-1, in view of the fact that the dose of LPS was five-fold less and given over a four-week period *vs. via* a single administration (Zhao *et al.*, 2006). In these short-term models, there is unequivocal support for a central role of MKP-1 in the restraint of innate immune responses and the prevention of septic shock syndrome during pathogenic microbial infection. Similarly, analysis of the data presented here suggests a similar protective response in a more chronic model of inflammation and bone loss. After 4 wks of LPS challenge, *Mkp-1*^{-/-} mice exhibited significantly more bone loss than did their wild-type littermates. These same mice also exhibited an increased amount of inflammatory infiltrate in the periodontal areas injected with LPS, consistent with the role of MKP-1 in regulating innate immune cytokine expression. Previous short-term studies reported higher TNF- α , IL-6, and IL-10 in *Mkp-1*^{-/-} macrophages, but attenuated levels of IL-12, a classic Th1 cytokine. It is possible to conclude that MKP-1 is an essential phosphatase to prevent or decrease LPS-induced inflammatory response following short- and long-term LPS exposure. Although the histological appearance is very similar to human periodontitis, this model is not initiated by host response to a subgingival biofilm. Results obtained here may be relevant to human periodontitis, where increased levels of p38 and JNK MAPKs have been observed with an increase in clinical inflammation and attachment loss (our unpublished observations). Further studies are needed to address this issue.

Compared with LPS-challenged wild-type mice, *Mkp-1*^{-/-} mice had a much higher level of inflammatory infiltrate. Similar data have been reported following the systemic administration of LPS in lungs and liver tissue, and are consistent with the increased expression of monocyte chemoattractant protein (MCP)-1/CCL2 following LPS challenge in *Mkp-1*^{-/-} mice (Zhao *et al.*, 2006; Liu *et al.*, 2007). The increased infiltrate into the periodontal micro-environment can probably be attributed to the loss of MKP-1 regulation in the inflammatory cells in terms of cytokine expression and the overall severity of the inflammatory process leading to greater osteoclast recruitment, differentiation, and, ultimately, bone loss in this model.

In summary, these studies indicate the importance of MKP-1 in the development of immune responses that contribute to LPS-induced alveolar bone loss. Through regulation of MAPK pathways, where p38 MAPK and MKP-1 are critical positive and negative regulators of TLR-induced cytokine expression, the loss of MKP-1 disrupts this balance, permitting sustained MAPK activation and immune cytokine production in periodontal disease progression.

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