A p38α Selective Mitogen-Activated Protein Kinase Inhibitor Prevents Periodontal Bone Loss

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ABSTRACT

In the oral microbial environment, Gram-negative bacterial derived lipopolysaccharide (LPS) can initiate inflammatory bone loss as seen in periodontal diseases. p38 Mitogen-activated protein kinase (MAPK) signaling is critical to inflammatory cytokine and LPS-induced cytokine expression, which may contribute toward periodontal bone loss. The purpose of this proof-of-principle study was to evaluate the ability of an orally active p38α MAPK inhibitor (SD-282) to reduce periopathogenic LPS-induced alveolar bone loss. The results from this in vivo study suggest that orally active p38 MAPK inhibitors can reduce LPS-induced inflammatory cytokine production and osteoclast formation and protect against LPS-stimulated alveolar bone loss.

Periodontal disease initiation and progression occurs as a consequence of the host immune inflammatory response to oral pathogens. Periodontal pathogen-derived lipopolysaccharide (LPS) is considered a key factor in the development of chronic inflammation leading to bone loss, the main hallmark feature of periodontitis. LPS-induced alveolar bone loss results from a local host response in gingival tissues through recruitment of inflammatory cells, generation of prostanoids and cytokines, elaboration of proteolytic enzymes, and activation of osteoclasts resulting in alveolar bone destruction and eventual tooth loss (Baker, 2000; Madianos et al., 2005).

Activated monocytes, macrophages, and fibroblasts all produce cytokines, such as TNF-α, IL-1β, prostaglandin E2, and IL-6, within periodontal lesions (Lee et al., 1995; Reddi et al., 1996), and have all been found to be significantly elevated in diseased periodontal sites compared with healthy or inactive sites. Multiple inflammatory signals can modulate receptor activator of NF-κB ligand (RANKL), RANK, or osteoprotegerin (OPG)—three novel members of the TNF ligand and receptor superfamilies, which modulate osteoclastogenesis (Aubin and Bonnelye, 2000; Hofbauer and Heufelder, 2001). For osteoclastogenesis to occur, RANKL must bind to its cognate receptor, RANK, a receptor on the cell surface of osteoclasts and osteoclast precursors, to stimulate proliferation and differentiation of osteoclasts resulting in alveolar bone destruction and eventual tooth loss (Baker, 2000; Madianos et al., 2005). A p38α selective mitogen-activated protein kinase inhibitor (SD-282) showed significant protection from LPS-induced bone loss. Bone area and volumetric analysis of maxillas injected with LPS showed significant loss of bone volume with LPS treatment, which was blocked with the p38 inhibitor. Histological examination indicated significantly fewer tartate-resistant acid phosphatase-positive osteoclasts and a significant decrease in interleukin (IL)-6, IL-1β, and tumor necrosis factor α expression in p38 inhibitor-treated groups compared with LPS groups by immunostaining. Results from this in vivo study suggest that orally active p38 MAPK inhibitors can reduce LPS-induced inflammatory cytokine production and osteoclast formation and protect against LPS-stimulated alveolar bone loss.
of cells from the monocyte/macrophage lineage to form the functional osteoclasts. OPG, a soluble decoy receptor produced by osteoblasts, marrow stromal cells, and other cells, profoundly modifies the effects of RANKL by inhibiting RANKL/RANK interaction (Simonet et al., 1997) and has shown promising results for the treatment of bone-related diseases (Kostenuik et al., 2004). Within the diseased periodontal tissues, activated osteoclasts are an integral component of bone destruction (As-suma et al., 1998; Crottì et al., 2003).

One of the major intracellular pathways activated by environmental stimuli, including periopathogenic LPS, is the mitogen-activated protein kinase (MAPK) pathway. MAPKs are divided into three major subgroups: the extracellular signal-regulated kinases 1/2, c-jun N-terminal kinases, and p38. Mitogens and growth factors primarily activate extracellular signal-regulated kinases 1/2, whereas the proinflammatory cytokines IL-1 and TNF-α and cell stress-inducing factors, such as LPS, heat shock, osmotic shock, ultraviolet radiation, and oxygen radicals, chiefly activate c-jun N-terminal kinases and p38. The three MAPKs control the activation of many transcription factors, including AP-1 (ho-modimer or heterodimer of the proteins c-fos and c-jun), NF-κB, or CAAT-enhancer-binding protein. MAPKs, most notably p38, can activate NF-κB and NF-κB.

Aggregatibacter actinomycetemcomitans (formerly known as Actinobacillus actinomycetemcomitans) is highly associated with localized aggressive periodontitis (Slots and Ting, 1999). Within periodontal resident cell types, including tissue macrophages and other periodontal cells, MAPKs are activated by A. actinomycetemcomitans LPS (Patil et al., 2006). p38 MAPK, most notably its p38α isoform, is activated mainly within cells involved in the inflammatory process. Activation of p38 induces synthesis of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1, IL-6, and IL-8, either via direct activation of gene transcription or via mRNA stabilization (Adams et al., 2001; Hoffmann et al., 2002; Kirkwood et al., 2003; Patil et al., 2004). p38 MAPK stabilizes mRNA via the enzyme substrate MAPK-activated protein kinase 2, which may act on one or more proteins capable of binding to mRNA (Holtmann et al., 1999). In addition, p38 MAPK controls the synthesis of other compounds, including chemokines, metalloproteinases, and prostaglandins (Lee et al., 2000). Recently, our research group has shown that IL-1β and TNF-α-induced RANKL expression in bone marrow stromal cells requires p38 signaling for maximal expression (Rossa et al., 2006). Collectively, these data suggest that p38 inhibitors may be beneficial to target bacterial induced alveolar bone loss—the hallmark of periodontitis. Recently, a p38α-specific inhibitor, SD-282, has been shown to be efficacious in reducing and reversing bone and cartilage destruction in an experimental arthritis model (Medicherla et al., 2006). The purpose of the present in vivo study was to determine whether this orally active p38α inhibitor can prevent alveolar bone loss initiated by A. actinomycetemcomitans LPS in an experimental rat model.

Materials and Methods

LPS Preparation. A. actinomycetemcomitans strain Y4 (serotype B) was grown in brain heart infusion media at 37°C, 5% CO2. LPS was extracted by the hot phenol-water method as described previously (Wilson and Hamilton, 1992). The bacteria were sequentially treated with lysozyme, DNase, RNase, and proteases to extract and isolate the lipopolysaccharide. The LPS used in the present study contained <0.01% nucleic acid by spectrophotometry and ca. 0.7% protein by BCA protein assay. The absence of protein in the LPS preparations was confirmed by polyacrylamide gel electrophoresis of extract samples and subsequent staining with silver nitrate and Coomassie Blue.

Chemical Description of SD-282. SD-282 is an indole-5-carboxamide, an ATP-competitive inhibitor of p38 MAPK kinase (Medicherla et al., 2006). It is a small-molecule orally active inhibitor of p38α MAPK.

Animals. Female adult Sprague-Dawley rats (approximately 250 g) were housed under specific pathogen-free conditions in pairs with food and tap water ad libitum. Once weekly, animals were weighed to ensure proper growth and nutrition. Five groups of Sprague-Dawley rats received one of the following treatments: 2 μl of a 10-μg/ml solution of A. actinomycetemcomitans LPS delivered to the palatal gingiva via a 33-gauge Hamilton syringe between the maxillary 1st and 2nd molars three times per week for 8 weeks (480 μg of LPS over the 8-week period; n = 12), LPS plus two doses of SD-282 (15 or 45 mg/kg) twice daily by oral gavage (n = 8 per group), or control groups given drug vehicle (1% polyethylene glycol; n = 6) or SD-282 (45 mg/kg; n = 6) only. For the injection procedures, anesthesia was induced with 4 to 5% isoflurane and maintained with 1 to 2% isoflurane. Mock injection control was phosphate-buffered saline (PBS) delivered between the 1st and 2nd and 2nd and 3rd molars three times per week for 8 weeks in SD-282 (45 mg/kg) only. At the end of the experimental period, animals were sacrificed by carbon dioxide asphyxiation. The maxillas were hemisected, and posterior block sections were immersed directly in 10% buffered formalin fixative solution for at least 72 h. All protocols were approved by the University Committee on the Use and Care of Animals (UCUCA) at the University of Michigan.

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

Loss of bone volume was assessed using three-dimensional isoform displays. 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 cup tips to root apices. Depth was equal to the buco-lingual size of the teeth plus 100 voxels (1.8 mm³). After establishing the threshold, the bone volume fraction was calculated as the percentage of bone within the ROI.

To compare the inner proximal bone loss within the treatment groups, a bone area fraction comparison was used. To create the ROI, anatomical landmarks were used to discern differences between rats. The spline feature was used within GE MicroView that allowed for several points to dictate the total area. The spline started at the CEJ of the 1st molar and followed in an apical direction to the root apex and then continued to the apex of the 2nd molar and extended in a coronal direction along the root surface to the CEJ of the 2nd molar. The spline was then connected from the bottom of the 2nd molar CEJ to the 1st molars CEJ. After the area was defined, the bone area fraction was taken using an established threshold of 1621. Examiners (D.D.C. and F.L.) were trained at the University of Michigan Core Center for Musculoskeletal Disorders by J.K. who performed all μCT scans in a blinded manner.

Immunohistochemistry and Tartate-Resistant Acid Phosphatase Staining. Formalin-fixed specimens were decalcified in a
10% EDTA solution for 2 weeks at 4°C. EDTA solution was changed three times per week. The maxillas were paraffin-embedded, and sagittal sections of 5 μm were prepared. Some slides were stained with hematoxylin and eosin for descriptive histology.

Immunohistochemical staining for rat phospho-p38α, IL-6, IL-1β, and TNF-α was performed on harvested maxillary tissue sections. Deparaffinized ethanol-dehydrated tissue sections were placed in a pressure chamber (Biocare Medical, Concord, CA) for 15 min in an antigen retrieval buffer (DAKO, Glostrup, Denmark) and allowed to cool to room temperature. Primary antibodies of anti phospho-p38α monoclonal antibody (1:100; Cell Signaling, Beverly, MA), anti-rat IL-6 monoclonal antibody, anti-rat IL-1β, and anti-rat TNF-α (1:200 each antibody; R&D Systems, Minneapolis, MN) were used to evaluate the extent of immunoreactive cytokine expression. Cytokine presence was detected using Vectastain Elite ABC reagent and Nova Red (Vector Laboratories, Burlingame, CA) per manufacturer’s instructions. Coverslips were seated with Permount mounting media. Control sections were incubated with preimmunoserum to assess background staining. Images were captured using an inverted scope (Nikon TS100) and mega pixel camera (Nikon CCD camera with 5.1).

For enumeration of osteoclasts, tartate-resistant acid phosphatase (TRAP) staining was performed from all of the groups using a leucocyte acid phosphatase kit (Sigma, St. Louis, MO). Active osteoclasts were defined as multinucleated TRAP-positive cells in contact with the bone surface. Slides from approximately the same sagittal sections were used to enumerate TRAP-positive cells.

All digitized slide images were displayed on a computer screen for scoring by two independent examiners. Calibration was done by comparing screen images to a standardized score sheet displaying representative slides of 1, 2, 3, or 4 color intensity. The examiners then assigned a score of 0, 1, 2, 3, or 4 to each image, where 0 represented the absence of red stain and 4 represented the greatest intensity red stain.

Statistical Analysis. Data were analyzed by Student’s t test or one-way analysis of variance and post hoc Bonferroni’s tests were indicated.

Results
SD-282 Reduces Inflammatory Infiltrate in LPS-Induced Alveolar Bone Loss. Maxillas were fixed and embedded for histological staining by hematoxylin and eosin. A significant amount of inflammatory cell infiltrate, primarily neutrophils with scattered lymphocytes and macrophages along with proliferating endothelial cells, was noted in the connective tissue proximal to the junctional epithelium and surrounding osseous tissue of rats injected with A. actinomycetemcomitans LPS after 8 weeks compared with mock (saline)-injected control animals. Representative hematoxylin and eosin-stained sections are presented in Fig. 1. In contrast, SD-282 with LPS-treated rats had less inflammatory cells with demonstrable lack of neutrophil infiltrate.

SD-282 Reduces LPS-Induced Phospho-p38 Expression. Immunostaining for the active phosphorylated p38 MAPK (P-p38) was performed on histological sections in the periodontal tissues. Data indicate that there was a modest amounts of P-p38 in mock-injected control animals but significantly higher in LPS-induced periodontal tissues (p < 0.001). SD-282 reduced the extent of P-p38 staining in these tissues. Figure 2 displays representative slides of immunostained tissues along with the graphical display where significantly less immunostained P-p38 was found in SD-282-treated periodontal tissues compared with LPS-injected animals (p < 0.001).
SD-282 Significantly Reduces Alveolar Bone Loss in Experimental Periodontitis. A substantial amount of preclinical data from arthritis models of disease supports the role of p38 in the disease process. To gain insight into the role of p38 signaling and in periodontitis, we investigated the ability of an orally active p38α inhibitor (SD-282) to prevent *A. actinomycetemcomitans* LPS-induced alveolar bone loss in a rat model. To evaluate the extent of alveolar bone loss, μCT was employed. Three dimensional reformatted μCT images from 8-week specimens revealed a dramatic difference in the amount of bone destruction of the maxilla (Fig. 3A). In the rats that received vehicle only, there was no bone loss compared with noninjected rats (data not shown). *A. actinomycetemcomitans* LPS-injection resulted in significant destruction of the palatal and interproximal bone with the greatest vertical destruction corresponding to the site of LPS injection. Figure 3B depicts linear measurements of alveolar bone loss from CEJ to the ABC. These data show a 0.405-mm mean CEJ-ABC distance in the control animals and a 1.008 ± 0.154-mm mean bone loss in the LPS-injected animals (*p* < 0.01). In animals receiving the p38α inhibitor, the CEJ-ABC distance was reduced to 0.518 mm in SD-282- (15 mg/kg) and 0.551 mm in SD-282-treated (45 mg/kg) animals resulting in significant protection from LPS-induced periodontal bone loss (*p* < 0.01 for SD-282 (15 mg/kg) and *p* < 0.05 for SD-282 (45 mg/kg)).

Linear bone loss measurements were verified by two additional independent measures of bone loss via μCT: bone area analysis and bone volume analysis. Using GEHC MicroView software, the spline feature was used, which permitted several points to be connected to determine the total area. Figure 4A depicts the area of measure included in the analysis that was the interproximal (between the molar roots) area of bone. Data were expressed as the bone area fraction (BAF; Fig. 4B). As shown in this figure, the average BAF was 0.337 ± 0.033, which was reduced in LPS-injected animals (0.228 ± 0.017). In SD-282-treated rats with LPS-induced periodontal bone loss, there was a significant protection of interproximal area bone loss (0.387 ± 0.029; *p* < 0.01 for SD-282 (15 mg/kg) and 0.406 ± 0.021; *p* < 0.01 for SD-282 (45 mg/kg)). In addition, the bone volume fraction (BVF) was calculated by a standard volumetric analysis of the ROI, as depicted as the highlighted areas (Fig. 5, A from control, and B, from LPS-injected animal), and was used to define the
mean volumetric measurement. Using this unbiased approach, the BVF was determined to be 0.421 ± 0.009 for the vehicle group and 0.374 ± 0.008 for the LPS group (Fig. 5C). In SD-282-treated rats with LPS-induced periodontal bone loss, there was a significant protection of periodontal bone loss [BVF of 0.403 ± 0.001 for SD-282 (15 mg/kg); n = 8] and *, p < 0.05 for SD-282 (45 mg/kg; n = 8)].

**SD-282 Reduces LPS-Induced Inflammatory Cytokine Expression.** Immunohistochemistry (IHC) was performed on rat periodontal tissue sections to detect key inflammatory cytokines associated with periodontitis. Marked differences among LPS, control, and treatment groups were observed. Figure 6 displays the IHC values obtained using a graded scoring system recently established (Rogers et al., 2006). Proinflammatory IHC scores were all higher in A. actinomycetemcomitans LPS-injected animals with significance reached with IL-1β (p < 0.01) and TNF-α (p < 0.05) but not IL-6. However, both doses of SD-282 significantly reduced inflammatory cytokine expression in periodontal tissues. TNF-α and IL-6 levels were significantly reduced (p < 0.05) in 15 and 45 mg/kg SD-282 groups compared with LPS-injected animals. IL-1β IHC scores were reduced more significantly with the lower dose of SD-282 (*, p < 0.01) than the higher dose (p < 0.05).

**SD-282 Reduces Periopathogenic LPS-Induced Osteoclastogenesis.** Histological examination and TRAP staining was performed to quantify osteoclastogenesis. Without A. actinomycetemcomitans LPS injection, few osteoclasts were detected (data not shown). However, in LPS-injected animals, significantly more active osteoclasts were much more prevalent (p < 0.001) after 8 weeks of A. actinomycetemcomitans LPS (Fig. 7). Osteoclast numbers were significantly reduced with SD-282 at both 15 and 45 mg/kg (p < 0.001) compared with LPS-induced bone loss.

**Discussion**

Periodontal disease and rheumatoid arthritis have remarkably similar inflammatory mediator profiles (Green-
wald and Kirkwood, 1999; Mercado et al., 2003; Kirkwood et al., 2006). A variety of immune-associated cell populations are responsible for the pathogenesis of periodontal diseases. Within periodontal lesions, activated monocytes, macrophages, and fibroblasts all produce cytokines, such as TNF-α, IL-1β, prostaglandin E2, and IL-6, and have all been found to be significantly elevated in diseased periodontal sites compared with healthy or inactive sites (Stashenko et al., 1991; Lee et al., 1995; Ejeil et al., 2003). These cytokines orchestrate the cascade of destructive events that occur in the periodontal tissues and trigger the production of an array of inflammatory enzymes and mediators, including matrix metalloproteinases, prostaglandins, and osteoclasts, thus resulting in irreversible hard and soft tissue damage (Assuma et al., 1998; Graves, 1999). Due to the similarity of pathogenesis between periodontitis and rheumatoid arthritis, p38 inhibitors have the potential to effectively manage periodontal disease progression.

In this study, an experimental rat model of alveolar bone loss was used to assess the effect of inhibiting p38 MAPK on inflammatory alveolar bone loss. Our study demonstrates for the first time that an orally active p38α inhibitor, SD-282, can prevent *A. actinomycetemcomitans* LPS-induced periodontal bone loss over an 8-week experimental period. *A. actinomycetemcomitans* LPS induced a massive infiltration of inflammatory cells consistent with human pathology (Page, 1991). Histological assessments comparing LPS + SD-282-treated animals to LPS-alone animals revealed striking differences between the amounts of inflammation (quantity of neutrophils and macrophages) noted in periodontal tissues (Fig. 1). These data are consistent with the relative lack of inflammatory bone loss observed in these animals.

Previous data from our laboratory have established that the p38α isoform is clearly required for matrix metalloproteinase-13, IL-6, and RANKL expression in periodontally relevant cell types, including osteoblasts and periodontal ligament fibroblasts (Patil et al., 2004, 2006; Rossa et al., 2005, 2006). Data presented here support the role of p38 signaling is required in *A. actinomycetemcomitans* LPS-induced alveolar bone loss progression. Phosphorylated levels of p38 were higher after 8 weeks of LPS-induced pathology (Fig. 2). In this same animal model, we have observed that active (phosphorylated) p38 was observed as early as 24 h postinjection of periopathogenic LPS (data not shown). In the present study, phosphorylated levels of p38 were indeed suppressed in LPS-injected tissues in the presence of SD-282. This orally active therapeutic has previously been shown to demonstrate selectivity for the α-isofrom, showing 14.3 to >1000 times greater

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**Fig. 5.** SD-282 spares maxillary bone volume in experimental periodontitis. Reformatted μCT isoforms display showing volumetric ROI used for analysis in control (A) and LPS-injected maxillas (B). Analysis of μCT volumes were assessed using GE Healthcare Biosciences software (C). Data are presented as bone volume fraction (mean ± S.E.M.). Significant bone loss (***, *p* < 0.001) was observed between control (n = 6) and *A. actinomycetemcomitans* LPS-injected rats (n = 12). In SD-282-treated rats, significant protection of LPS-induced periodontal bone loss was observed in both treatment groups (*, *p* < 0.05 and **, *p* < 0.01; n = 8 per group).
activity against p38α than p38β, p38γ, or p38δ isoforms (Medicherla et al., 2006). Moreover, the phosphospecific antibody for p38 recognizes the all p38 isoforms. Taken together, these data suggest that the α-isoform is the predominant isoform expressed in LPS-induced periodontal disease pathology.

Evidence from μCT data indicates that SD-282 has a potent protective effect in experimental periodontitis. All three parameters of bone loss measured in this study indicated significant differences in reduction of alveolar bone loss in the presence of SD-282. Linear measurement data can vary significantly depending upon the site chosen to measure bone loss from any two points adjacent to molar areas. Furthermore, the character of LPS-induced bone loss did vary slightly in this animal model due to the local nature of LPS injections in the palatal regions. Because these issues would potentially skew the data if only one parameter was used, two other independent measures of bone loss were included to corroborate linear measurement data. Both area and volumetric analysis of inflammatory bone loss in this model indicated that SD-282 prevented periodontal bone loss (Figs. 4 and 5). This type of data analysis represents a relatively novel way to quantitate periodontal bone loss in small animal models of periodontitis, which has only recently been used to measure alveolar bone loss (Wilensky et al., 2005). Interestingly, in all of the μCT data, we observed a small but consistent increase in bone parameters from SD-282 only animals, suggesting that there may be some proanabolic properties associated with this therapeutic agent. Future studies will address this aspect in periodontal bone regeneration models.

Several mediators of periodontal bone resorption and remodeling have been identified, which contribute toward loss of bone and connective tissue loss, including proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6 (Williams, 1990; Graves and Cochran, 2003). All of these key cytokines have been shown to be expressed in inflamed periodontal tissues and gingival cervicular fluid (Tsai et al., 1995; Chen et al., 1998). In this study, SD-282 significantly reduced A. actinomycetemcomitans LPS-induced expression of IL-1β, TNF-α, and IL-6. From the immunostaining performed as part of these studies, the majority of inflammatory cytokine-
stained cell types seem to be primarily monocytes and macrophages (data not shown). These data are consistent with recent data showing proinflammatory cytokine suppression in an arthritis model with SD-282 (Medicherla et al., 2006). RANKL is the main stimulatory factor for the differentiation and activation of osteoclasts, which is counterbalanced by OPG. In periodontal disease, as well as other inflammatory bone disorders, the imbalance of this system dictates bone turnover (Rodan and Martin, 2000). In periodontal tissues, RANKL expression has been found to be increased in severe periodontitis, and the ratio of RANKL/OPG may determine disease severity (Teng et al., 2000; Crotti et al., 2003). The essential role of p38 MAPK in RANKL-induced osteoclastogenesis has been elucidated in macrophage osteoclast precursor cells (Matsumoto et al., 2000); however, the role of p38 regulation in stromal/osteoblastic-derived RANKL expression has only recently been addressed (Ishida et al., 2002; Wei et al., 2005; Dai et al., 2006). Our group recently showed that MAPK kinase kinase-3/p38 signaling is necessary for IL-1β-induced RANKL expression involving a transcriptional mechanism that does not require the proximal RANKL promoter relative to the transcriptional start site (Rossa et al., 2006). In addition, the LPS used in these studies can directly support osteoclastogenesis (M. Liu and K. Kirkwood, unpublished data). Information presented in these studies is consistent with in vitro findings where p38 inhibitors can reduce LPS-induced osteoclastogenesis.

In summary, this proof-of-principle study supports the role of p38 inhibitors to have potential beneficial effects in LPS-induced alveolar bone loss. Although p38 inhibitors should be evaluated in infectious periodontal disease models, these data suggest that use of these agents may be considered as novel host modulatory agents in the treatment and management of human chronic periodontitis.

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