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Inhibition of *Porphyromonas gingivalis*-induced periodontal bone loss by CXCR4 antagonist treatment

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SUMMARY

Microbial pathogens have evolved mechanisms to proactively manipulate innate immunity, thereby improving their fitness in mammalian hosts. We have previously shown that *Porphyromonas gingivalis* exploits CXC-chemokine receptor-4 (CXCR4) to instigate a subversive crosstalk with Toll-like receptor 2 that inhibits leukocyte killing of this periodontal pathogen. However, whether CXCR4 plays a role in periodontal disease pathogenesis has not been previously addressed. Here, we hypothesized that CXCR4 is required for *P. gingivalis* virulence in the periodontium and that treatment with AMD3100, a potent CXCR4 antagonist, would inhibit *P. gingivalis*-induced periodontitis. Indeed, mice administered AMD3100 via osmotic minipumps became resistant to induction of periodontal bone loss following oral inoculation with *P. gingivalis*. AMD3100 appeared to act in an antimicrobial manner, since mice treated with AMD3100 were protected against *P. gingivalis* colonization and the associated elevation of the total microbiota counts in the periodontal tissue. Moreover, even when administered two weeks post-infection, AMD3100 halted the progression of *P. gingivalis*-induced periodontal bone loss. Therefore, AMD3100 can act in both preventive and therapeutic ways and CXCR4 antagonism could be a promising novel approach to treat human periodontitis.

Keywords

P. gingivalis; CXCR4; AMD3100; periodontitis; bone loss

INTRODUCTION

Toll-like receptors (TLRs) detect and respond to microbial infection via rapid activation of inflammatory and antimicrobial responses in cooperation with other innate immune receptors with which they form multireceptor complexes in membrane lipid rafts of front-line defense cells (*e.g.*, neutrophils and macrophages) (Hajishengallis *et al.*, 2006; Triantafilou *et al.*, 2001). However, the tendency of TLRs to functionally associate with heterotypic receptors poses an opportunity for exploitation by microbial pathogens capable of inducing inappropriate lipid raft recruitment of receptors that could subvert host immunity (Hajishengallis & Lambris, 2011).

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We have previously shown that *Porphyromonas gingivalis,* a keystone pathogen in periodontal disease (Hajishengallis *et al.*, 2011), interacts with several innate immune receptors, including complement receptors and the CXC chemokine receptor 4 (CXCR4), in ways that enhance its own adaptive fitness (Hajishengallis & Harokopakis, 2007; Hajishengallis *et al.*, 2008; Liang *et al.*, 2011; Wang *et al.*, 2010; Wang *et al.*, 2007). With regard to CXCR4, we have shown that *P. gingivalis* uses its surface fimbriae to directly bind and activate CXCR4 to subvert antimicrobial signaling initiated by TLR2 (Hajishengallis *et al.*, 2008; Pierce *et al.*, 2009). Specifically, *P. gingivalis* induces co-association between CXCR4 and TLR2 in lipid rafts, leading to a subversive crosstalk pathway in which cAMP-dependent protein kinase A signaling inhibits intracellular nitric oxide production. This activity, in turn, impairs the killing function of leukocytes (Hajishengallis *et al.*, 2008) suggesting that *P. gingivalis* exploits CXCR4 to evade host immunity and, perhaps, to persist in the periodontal tissue and cause disease.

However, in our previous publications we have not examined whether the exploitation of CXCR4 by *P. gingivalis* enhances its ability to cause periodontitis. To address this hypothesis, we now determined whether a specific and potent antagonist of CXCR4, the bicyclam drug AMD3100 (Donzella *et al.*, 1998), can inhibit *P. gingivalis*-induced periodontitis in the mouse model. Our current results show that AMD3100 impairs the ability of *P. gingivalis* to cause bone loss by interfering with its colonization in the murine periodontal tissue. These findings provide proof of concept that CXCR4 antagonists may be promising therapeutics for the treatment of human periodontitis.

METHODS

Bacteria

P. gingivalis ATCC 33277 was used in this study. The bacterium was grown anaerobically at 37°C in hemin- and menadione-containing Gifu anaerobic medium (Nissui Pharmaceuticals).

Periodontitis model

Periodontal bone loss was induced in 10- to 12-week-old BALB/c mice (The Jackson Laboratory) by oral inoculation with *P. gingivalis* ATCC 33277 as originally described by Baker (Baker *et al.*, 2000) with slight modifications (Wang *et al.*, 2007). Briefly, by means of a ball-ended feeding needle, mice were orally inoculated five times at 2-day intervals with 10^9 CFU *P. gingivalis* suspended in 2% carboxy-methylcellulose vehicle. Sham controls received vehicle alone. The mice were euthanized six weeks after the last oral inoculation. Assessment of periodontal bone loss in defleshed maxillae was performed under a dissecting microscope (x40) fitted with a video image marker measurement system (VIA-170K; Boeckeler Instruments). Specifically, the distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured on 14 predetermined points on the buccal surfaces of the maxillary molars. To calculate bone loss, the 14-site total CEJ-ABC distance for each mouse was subtracted from the mean CEJ-ABC distance of sham-infected mice (Baker *et al.*, 2000). The results were expressed in mm and negative values indicated bone loss relative to sham controls. All animal procedures described in this study were approved by the institutional animal care and use committee, in compliance with established federal and state policies.

Osmotic minipumps

Alzet osmotic minipumps (model #2004; Alza) were subcutaneously implanted through a mid-scapular incision on the back of the mice. The minipumps were placed slightly posterior to the scapulae. The pumps were filled with 20 mg of AMD3100 (Sigma-Aldrich) in 0.2 ml

sterile phosphate-buffered saline (PBS) or PBS alone. The #2004 model pump provides for 4 weeks of continuous infusion and its infusion rate is 0.25 μl/hr. Therefore, when filled with 20 mg of AMD3100 in 0.2 ml PBS, the minipumps would deliver the drug at 600 μg/day, which corresponds to a steady serum level of about 1 μg/ml (Matthys *et al.*, 2001). We found that this concentration effectively blocks CXCR4 in our cell culture experiments (Hajishengallis *et al.*, 2008; Pierce *et al.*, 2009).

Quantitative real-time PCR

Maxillary palatal and buccal gingiva and hard tissue (teeth and immediately surrounding bone) were harvested and placed in ATL lysis buffer from the DNeasy kit (Qiagen). Tissues were lysed overnight at 56°C with occasional agitation. Genomic DNA was isolated using the DNeasy kit and was quantified by NanoDrop spectrometry. The levels of *P. gingivalis* colonization and the number of total bacteria in the periodontal tissue were determined using quantitative real-time PCR of the *ISPg1* gene (*P. gingivalis*) and the 16S rRNA gene (total oral bacteria) (Hajishengallis *et al.*, 2011). *ISPg1* was selected to increase the sensitivity of *P. gingivalis* detection, since this gene is present in 31 copies in the genome *P. gingivalis* ATCC 33277 (the gene copy numbers were thus divided by 31 to obtain genome equivalents) (Naito *et al.*, 2008). Real-time PCR was performed using the ABI 7500 Fast System and TaqMan probes, sense primers, and antisense primers used were purchased from Applied Biosystems. The primer sets used to enumerate *P. gingivalis* copy number and total bacterial load were as follows:

ISPg1 (*P. gingivalis*) (Hajishengallis *et al.*, 2011)

5′-CGCAGACGACAGAGAAGACA-3′

5′-ACGGACAACCTGTTTTTGATAATCCT-3′

5′-FAM-TCCGCCTCGCTCCGAT-TAMRA-3′

16S rRNA (Universal; total bacterial load) (Kuboniwa *et al.*, 2004)

5′TCCTACGGGA GGCAGCAGT-3′

5′-GGACTACCAGGGTATCTAATCCTGTT-3

5′-FAM-CGTATTACCGCGGCTGCTGGCAC-TAMRA-3′

Statistical Analysis

Data were evaluated by analysis of variance and the Dunnett multiple-comparison test using the InStat program (GraphPad Software, San Diego, CA). Where appropriate (comparison of two groups only), two-tailed t tests were performed. $P < 0.05$ was taken as the level of significance.

RESULTS

AMD3100 prevents *P. gingivalis*-induced periodontal bone loss

We hypothesized that AMD3100 can interfere with the virulence of *P. gingivalis* in the periodontal tissue. This hypothesis was based on our previous findings that AMD3100 inhibits the ability of *P. gingivalis* (or purified fimbriae) to bind CXCR4 and evade leukocyte killing (Hajishengallis *et al.*, 2008; Pierce *et al.*, 2009). Therefore, we investigated whether treatment of BALB/c mice with AMD3100 would protect them against *P. gingivalis*-induced periodontal bone loss. The study consisted of four groups of mice, which were treated with AMD3100 or vehicle control (PBS) and were infected with *P. gingivalis* or 2% carboxymethylcellulose vehicle (sham control). AMD3100 was administered systemically by means of osmotic minipumps, which were subcutaneously implanted in the

mice 24 hours prior to *P. gingivalis* infection, involving a total of five oral inoculations at 2-day intervals. Examination of the mice for periodontal bone loss six weeks after the last oral inoculation revealed that only the PBS-treated and *P. gingivalis*-infected mice developed significant bone loss ($P < 0.01$; Fig. 1). Strikingly, the AMD3100-treated and *P. gingivalis*-infected mice were completely protected against bone loss (Fig. 1). Therefore, AMD3100 treatment protects mice from *P. gingivalis*-induced periodontal bone loss when the drug is administered prior to exposure to the pathogen.

AMD3100 eliminates *P. gingivalis* from the murine periodontal tissue

We next hypothesized that the protective effect of AMD3100 against *P. gingivalis*-induced bone loss involved interference with the capacity of *P. gingivalis* to enhance its survival through CXCR4 exploitation (Hajishengallis *et al.*, 2008). If this notion were true in the context of periodontitis, AMD3100 would be expected to inhibit the establishment of *P. gingivalis* in the periodontal tissue. In this regard, we recently showed that *P. gingivalis* stably colonizes the murine periodontal tissue by day 7 post-infection (Hajishengallis *et al.*, 2011). Therefore, mice were treated with AMD3100 (or PBS control) and infected (or not) with *P. gingivalis*, as performed in the Fig. 1 study, and were sacrificed 7 days later. The periodontal tissue was harvested to determine the numbers of *P. gingivalis* and of total periodontal bacteria using quantitative real-time PCR of the *ISPg1* gene or the 16S rRNA gene, respectively.

In the absence of AMD3100 treatment, *P. gingivalis* was readily detected in infected mice at about 4 $\log_{10}$ units lower than total periodontal bacteria (Fig. 2), as seen previously (Hajishengallis *et al.*, 2011). Moreover, in the PBS-treated and *P. gingivalis*-colonized mice, the levels of total periodontal bacteria were significantly ($P < 0.01$) higher as compared to those of PBS-treated and sham-infected mice (Fig. 2), confirming the role of *P. gingivalis* as a keystone pathogen which benefits the entire periodontal biofilm (Hajishengallis *et al.*, 2011). Strikingly, however, treatment with AMD3100 resulted in 97% reduction in the numbers of *P. gingivalis* (Fig. 2). This virtual elimination of *P. gingivalis* from the periodontal tissue due to AMD3100 treatment was accompanied by significant ($P < 0.01$) reduction in the total numbers of periodontal bacteria, which returned to the normal levels seen in mice not colonized by *P. gingivalis* (sham-infected) (Fig. 2). The reduction in the total bacterial numbers was not a direct effect of AMD3100 on the periodontal microbiota at large, since this antagonist failed to affect the total periodontal bacterial numbers in mice not colonized with *P. gingivalis* (*i.e.*, the AMD3100-treated and sham-infected mice) (Fig. 2). Moreover, AMD3100 did not have direct killing activity against *P. gingivalis* (Supporting Fig. 1). Therefore, in the presence of AMD3100, *P. gingivalis* is not capable of colonizing the periodontal tissue and influencing the resident microbiota.

Therapeutic treatment with AMD3100 halts the progression of *P. gingivalis*-induced bone loss

Although treatment with AMD3100 can prevent *P. gingivalis*-induced bone loss when applied prior to *P. gingivalis* infection (Fig. 1), this does not necessarily imply that AMD3100 can also be effective when applied in a therapeutic mode. Therefore, a new experiment was designed to determine if AMD3100 can protect against *P. gingivalis*-induced periodontal bone loss when administered after infection and the onset of bone loss. We first determine the time interval that would be required to observe significant bone loss in *P. gingivalis*-infected mice. To this end, BALB/c mice were orally inoculated with *P. gingivalis* using the standard protocol (*e.g.*, as performed in the Fig. 1 study), and groups of mice were sacrificed at 1, 2, 4, and 6 weeks post-infection. We found that 2 weeks represented the minimum time required to observe significant ($P < 0.05$) *P. gingivalis*-induced bone loss in BALB/c mice (Fig. 3).

Therefore, in a new bone loss study, the mice were first orally infected or not with *P. gingivalis* and, 2 weeks after the last inoculating dose, received AMD3100- or PBS-containing osmotic minipumps through subcutaneous implantation. We found that AMD3100-treated and *P. gingivalis*-infected mice developed significantly ($P < 0.01$) less bone loss than PBS-treated and *P. gingivalis*-infected mice (Fig. 4). These data indicate that AMD3100 inhibits the progression of *P. gingivalis*-induced bone loss and suggest that it could be a promising therapeutic agent against periodontitis.

DISCUSSION

It has recently been proposed that periodontitis fundamentally represents a disruption of host-microbe homeostasis in the periodontal tissue (Darveau, 2010). This notion is supported by mechanistic studies in the mouse model of periodontitis: Alterations either in the composition of the periodontal microbiota or in local regulatory mechanisms that control leukocyte recruitment can cause disruption of periodontal homeostasis which, in turn, may lead to uncontrolled inflammation and periodontal bone loss (Eskan *et al.*, 2012; Hajishengallis *et al.*, 2011). Currently, there is an urgent need to develop innovative adjunctive therapeutic strategies in chronic periodontitis (Hajishengallis, 2009). Indeed, conventional periodontal treatment is often not sufficient by itself to treat destructive inflammation and, moreover, this oral disease appears to increase the patients' risk for atherosclerosis, diabetes, chronic obstructive pulmonary disease, adverse pregnancy outcomes, and possibly rheumatoid arthritis (Genco & Van Dyke, 2010; Lalla & Papapanou, 2011; Lundberg *et al.*, 2010; Pihlstrom *et al.*, 2005; Tonetti *et al.*, 2007).

Several approaches have been successfully tested to inhibit periodontitis in preclinical models including anti-cytokine therapy or the use of agents that promote the resolution of inflammation (Assuma *et al.*, 1998; Hajishengallis, 2009; Hasturk *et al.*, 2007). Another approach to treating periodontitis is to counteract immune evasion or subversion by major periodontal pathogens. Periodontal and other microbial pathogens preferentially target and corrupt innate immunity (Finlay & McFadden, 2006; Hajishengallis & Lambris, 2011). Subversion of innate immunity may additionally undermine the overall host defense, given the instructive role of the innate response in the development of adaptive immunity (Pasare & Medzhitov, 2005). Therefore, understanding the molecular mechanisms whereby microbial pathogens interact with and exploit innate immune receptors may facilitate the development of intervention approaches to inhibit immune evasion and disease pathogenesis.

In this paper, we took advantage of our earlier findings that implicated CXCR4 in *P. gingivalis* immune subversion (Hajishengallis *et al.*, 2008) and showed that a CXCR4 antagonist can protect against *P. gingivalis*-induced periodontal bone loss in both a preventive and therapeutic way. Since *P. gingivalis* uses its fimbriae to exploit CXCR4 (Hajishengallis *et al.*, 2008; Pierce *et al.*, 2009), it is likely that the protective effect of AMD3100 is restricted against fimbriated strains of *P. gingivalis*. The fimbriae of *P. gingivalis* comprise polymerized fimbrillin (FimA) and accessory proteins (FimCDE) encoded by genes of the fimbrial operon (Wang *et al.*, 2007). Since CXCR4 interacts specifically with the accessory protein components (FimCDE) of the fimbriae (Pierce *et al.*, 2009) which, unlike FimA, are well conserved among different fimbriated strains (Kato *et al.*, 2007), the AMD3100 effect may not be restricted to Type I fimbriated *P. gingivalis* strains (as is the strain used in this study).

Interestingly, the expression of CXCR4 was shown by independent groups to be elevated in chronic periodontitis as compared to healthy gingiva (Jotwani *et al.*, 2004; Kebschull *et al.*, 2008). However, it has been uncertain whether CXCR4 plays a role in periodontal

pathogenesis. In this regard, our study is the first to causally link CXCR4 to periodontitis in a preclinical model. The protective effect of AMD3100 against *P. gingivalis*-induced periodontitis may be attributed, at least in great part, to the blockade of a host receptor, CXCR4, which is apparently important for *P. gingivalis* survival in the periodontium. This conclusion is based on the ability of AMD3100 to enhance the killing of *P. gingivalis* by leukocytes (Hajishengallis *et al.*, 2008) and, moreover, to mediate its elimination from the periodontal tissue in vivo (this study).

CXCR4 affects bone metabolism and, in principle, inhibition of this receptor with AMD3100 might have influenced bone resorption in the periodontitis model used in this study. In this regard, CXCR4 activation is known to induce the chemotactic recruitment, development and survival of osteoclasts (Wright *et al.*, 2005). Conversely, another study showed that it is the disruption of CXCR4 that enhances osteoclastogenesis (Hirbe *et al.*, 2007). Yet, another investigation showed that AMD3100 failed to influence osteoclast formation indicating that CXCR4 may not induce osteoclastogenesis (Matthys *et al.*, 2001). Taken together, these findings suggest that the effects of CXCR4 on osteoclastogenesis may be variable, perhaps depending on environmental context. In a similar vein, AMD3100 has complex effects on cell trafficking, since it can block CXCR4-mediated chemotaxis but, on the other hand, can stimulate the mobilization of hematopoietic stem/progenitor cells and granulocytes from the bone marrow (Lee *et al.*, 2009). Since the continuous presence of low colonization levels of *P. gingivalis* in the mouse periodontium is required for induction of bone loss (Hajishengallis *et al.*, 2011), we conclude that the ability of AMD3100 to inhibit the persistence of *P. gingivalis* in the periodontium constitutes the main mechanism responsible for the observed inhibition of periodontal bone loss.

The natural ligand for CXCR4 is the chemokine stromal cell-derived factor-1 (SDF-1), although CXCR4 also functions as a coreceptor with CD4 for the HIV-1 envelope gp120/gp41 complex (Oberlin *et al.*, 1996). In this context, AMD3100, which can also potently antagonize human CXCR4 (Hatse *et al.*, 2002), was shown to block CXCR4-dependent HIV-1 entry and replication (De Clercq, 2005; Donzella *et al.*, 1998). Moreover, AMD3100 can protect against several CXCR4-mediated pathophysiological conditions, such as rheumatoid, infectious, allergic, and malignant diseases, both in humans and in experimental mouse models (De Clercq, 2005; Hogaboam *et al.*, 2005; Lukacs *et al.*, 2002; Matthys *et al.*, 2001). This study adds periodontitis to the list of potential therapeutic applications of AMD3100.

The ability of AMD3100 to inhibit periodontitis by apparently targeting *P. gingivalis* (as this antagonist did not directly influence the periodontal microbiota) has a theoretical basis on the keystone pathogen concept. According to this concept, *P. gingivalis*– at low colonization levels– impairs innate immunity in ways that alter the growth and development of the entire biofilm resulting in dysbiosis that triggers periodontal disease, at least in the mouse model (Hajishengallis *et al.*, 2011). On the other hand, neither the indigenous murine microbiota alone, nor *P. gingivalis* by itself (*i.e.*, in germ-free mice) can initiate pathologic bone loss in young healthy mice (Hajishengallis *et al.*, 2011). In this study, in the presence of AMD3100, *P. gingivalis* failed to support the overgrowth of the total periodontal microbiota which is required for induction of periodontitis. AMD3100 was effective against periodontitis even when the disease was already in progress, suggesting that the continuous presence of *P. gingivalis*, albeit at very low levels compared to the total bacterial counts, is strictly required to sustain dysbiosis and disease progression.

In humans, *P. gingivalis* is also a quantitatively minor component of subgingival pathogenic biofilms, despite its high prevalence, and is associated with progressive bone loss in periodontitis patients (Chaves *et al.*, 2000; Doungudomdacha *et al.*, 2000; Kumar *et al.*,

2006; Moore *et al.*, 1982; Moore *et al.*, 1991). It should be noted that adult chronic periodontitis is associated with multiple etiologies and disease modifiers (Hajishengallis, 2010; Kornman, 2006; Lalla & Papapanou, 2011; Pihlstrom *et al.*, 2005) and, therefore, the presence of *P. gingivalis* may be just one of several etiologic factors. Nevertheless, under favorable environmental conditions, this bacterium has the potential to act as a keystone pathogen to transform an otherwise symbiotic microbiota into a dysbiotic microbial community that can cause periodontitis (Hajishengallis *et al.*, 2011).

In summary, we have established a role for CXCR4 in *P. gingivalis*-induced periodontitis and showed that CXCR4 antagonism using AMD3100 confers protection against the disease through an antimicrobial effect. AMD3100 was shown to be safe in humans with only minimal side effects (typically gastrointestinal in nature) observed at high concentrations of the drug (Hendrix *et al.*, 2000; Schols, 2004). Importantly, AMD3100 was recently approved by the FDA as a drug for stem cell mobilization (Pusic & DiPersio, 2010). Given its safety record, AMD3100, and perhaps other CXCR4 antagonists, could find application as adjunctive therapeutics for the treatment of human periodontitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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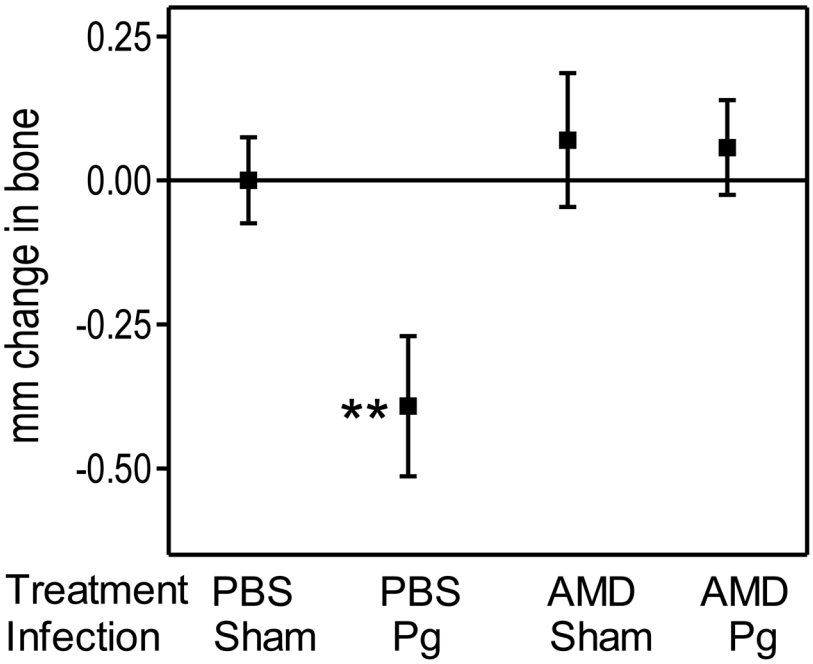

Figure 1. Preventive treatment with AMD3100 abrogates *P. gingivalis*-induced periodontal bone loss

BALB/c mice (10–12 weeks of age) were administered AMD3100 (or PBS control) through osmotic minipumps which were implanted subcutaneously 24 hours prior to oral infection with *P. gingivalis* (or vehicle only; sham) as described in the *Methods*. The mice were euthanized six weeks after the last inoculation with *P. gingivalis*, and bone loss measurements were performed in defleshed maxillae. Data are means ± SD ($n = 5$ mice per group); negative values indicate bone loss in *P. gingivalis*-infected mice relative to sham-infected controls. $^{**}P < 0.01$ compared to control and all other experimental groups. AMD, AMD3100; Pg, *P. gingivalis*.

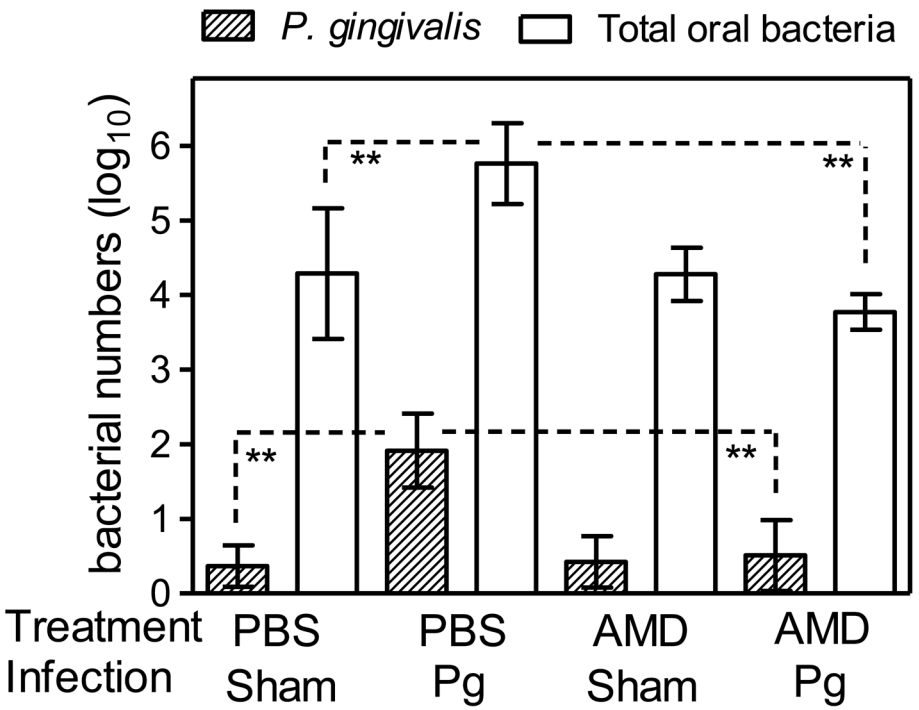

Figure 2. Effect of AMD3100 on the numbers of *P. gingivalis* or total bacteria in the murine periodontal tissue

BALB/c mice (10–12 weeks of age) were treated with AMD3100 (or PBS control) and infected with *P. gingivalis* (or vehicle only; sham) as described in the legend to Figure 1. The mice were sacrificed 7 days after the last inoculation with *P. gingivalis*. The numbers of *P. gingivalis* and of total periodontal bacteria in the periodontal tissue were determined using quantitative real-time PCR of the *ISPg1* gene (*P. gingivalis*) or the 16S rRNA gene (total bacteria). Data are means ± SD ($n = 5$ mice per group). $^{**}P < 0.01$ between the indicated groups. AMD, AMD3100; Pg, *P. gingivalis*.

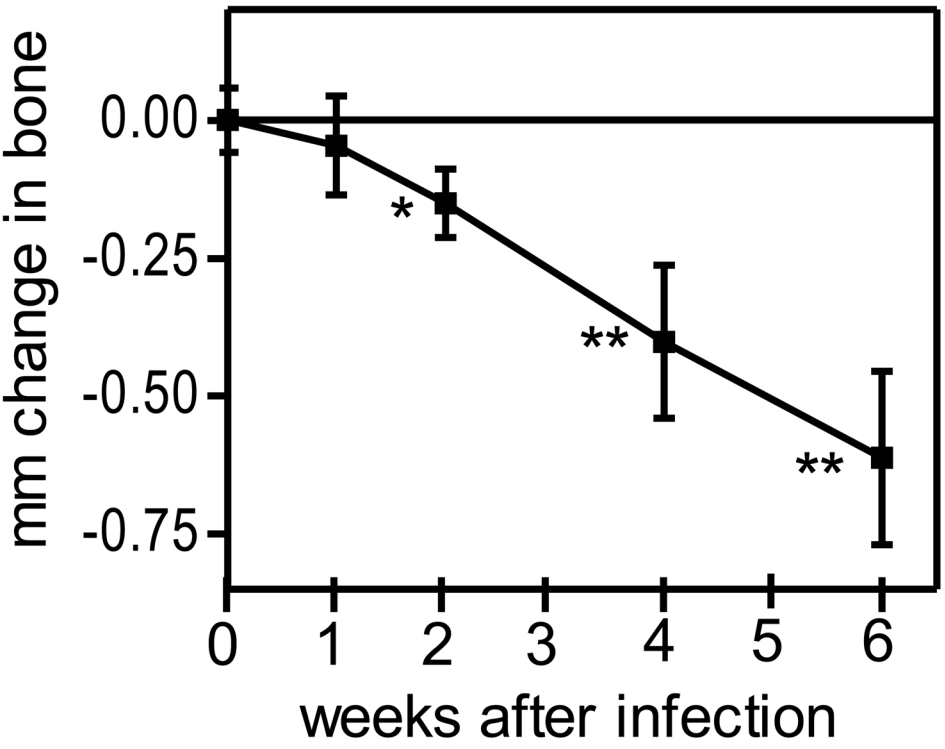

Figure 3. Timecourse of periodontal bone loss induction in BALB/c mice

10- to 12-week-old BALB/c mice were orally infected with *P. gingivalis* as described in *Methods* and euthanized at the indicated times after the last inoculation with *P. gingivalis*. Bone loss measurements were performed in defleshed maxillae. Data are means ± SD ($n = 5$ mice per group); negative values indicate bone loss in *P. gingivalis*-infected mice relative to sham-infected controls. *, $P < 0.05$ and **, $P < 0.01$ vs. time 0.

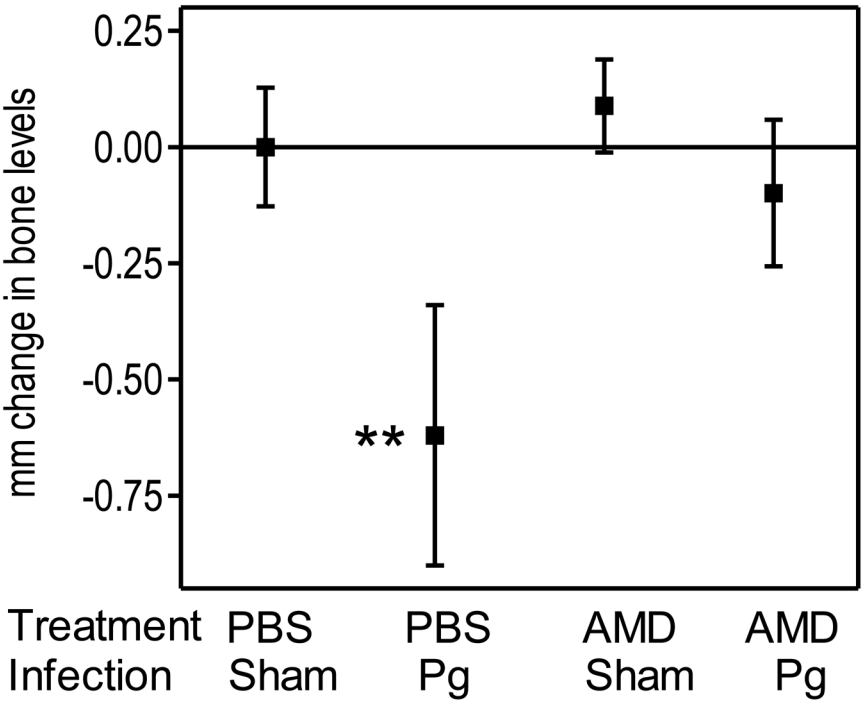

Figure 4. Therapeutic treatment with AMD3100 inhibits *P. gingivalis*-induced periodontal bone loss

BALB/c mice (10–12 weeks of age) were orally infected with *P. gingivalis* (or vehicle only; sham) as described in *Methods*. Two weeks after the last inoculation with *P. gingivalis*, the mice were administered AMD3100 (or PBS control) through subcutaneously implanted osmotic minipumps. The mice were euthanized four weeks later and bone loss measurements were performed in defleshed maxillae. Data are means ± SD ($n = 5$ mice per group); negative values indicate bone loss in *P. gingivalis*-infected mice relative to sham-infected controls. $^{**}P < 0.01$ compared to control and all other experimental or groups. AMD, AMD3100; Pg, *P. gingivalis*.