

# Inhibition of GSK3 Abolishes Bacterial-Induced Periodontal Bone Loss in Mice

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The tissue destruction that characterizes periodontitis is driven by the host response to bacterial pathogens. Inhibition of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) in innate cells leads to suppression of Toll-like receptor (TLR)-initiated proinflammatory cytokines under nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) p65 transcriptional control and promotion of cyclic adenosine monophosphate response element-binding (CREB)-dependent gene activation. Therefore, we hypothesized that the cell permeable GSK3-specific inhibitor, SB216763, would protect against alveolar bone loss induced by the key periodontal pathogen, *Porphyromonas gingivalis* (*P. gingivalis*), in a murine model. B6129SF2/J mice either were infected orally with *P. gingivalis* ATCC 33277; or treated with SB216763 and infected with *P. gingivalis*; sham infected; or exposed to vehicle only (dimethyl sulfoxide (DMSO)); or to GSK3 inhibitor only (SB216763). Alveolar bone loss and local (neutrophil infiltration and interleukin (IL)-17) and systemic (tumor necrosis factor (TNF), IL-6, IL-1 $\beta$  and IL-12/IL-23 p40) inflammatory indices also were monitored. SB216763 unequivocally abrogated mean *P. gingivalis*-induced bone resorption, measured at 14 predetermined points on the molars of defleshed maxillae as the distance from the cemento-enamel junction to the alveolar bone crest ( $p < 0.05$ ). The systemic cytokine response, the local neutrophil infiltration and the IL-17 expression were suppressed ( $p < 0.001$ ). These data confirm the relevance of prior *in vitro* phenomena and establish GSK3 as a novel, efficacious therapeutic preventing periodontal disease progression in a susceptible host. These findings also may have relevance to other chronic inflammatory diseases and the systemic sequelae associated with periodontal infections.

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## INTRODUCTION

Periodontitis is a highly prevalent chronic inflammatory disease defined by irreversible destruction of the hard and soft tissues surrounding the teeth. *Porphyromonas gingivalis*, a causative factor for periodontitis, is a black-pigmented, asaccharolytic, anaerobic, gram-negative bacterium that produces a wide array of virulence factors and also is associated with several systemic sequelae to periodontitis. Lipopolysaccharide (LPS) and various other microbe-associated molecular patterns (MAMPs) of *P. gingivalis* initiate an innate response primarily through en-

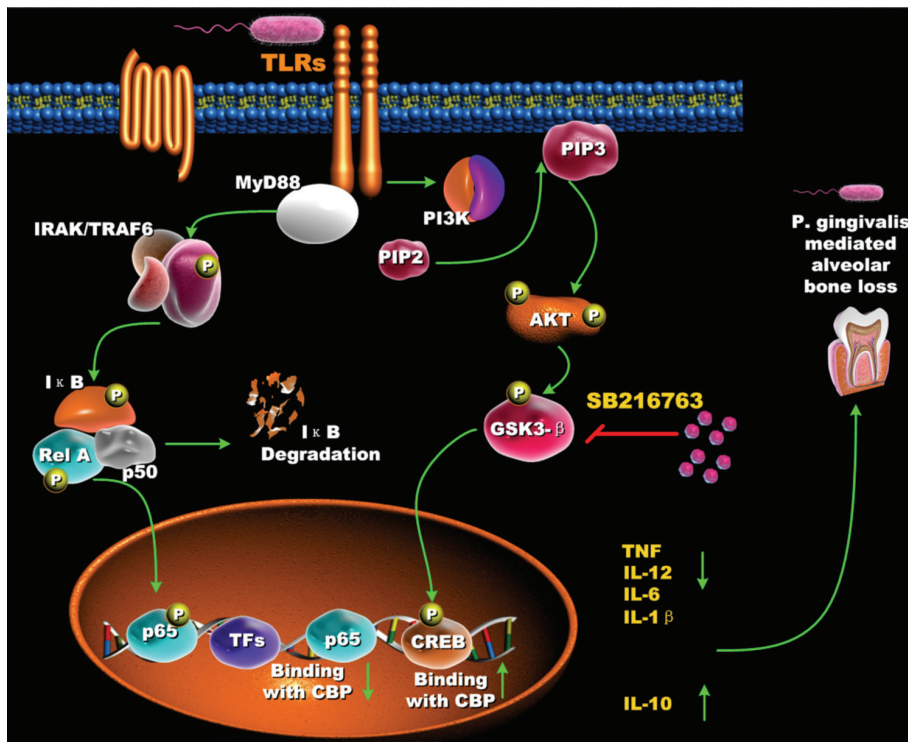
gagement of TLR-2 and -4 (1). Therefore, considerable efforts have been made to delineate intracellular signaling pathways induced upon *P. gingivalis*-TLR interaction to establish novel therapeutic targets for periodontitis (1–4).

GSK3 $\beta$  is a constitutively active serine-threonine kinase that plays a vital role in directing the immune response following TLR stimulation (5). Essentially, GSK3 $\beta$  is known to be a key mediator of proinflammatory cytokine production during bacterial infections and, subsequently, inhibition of GSK3 $\beta$  leads to an innate hyporeactivity to oral, and other, pathogens

(6). The specific mechanisms that drive this suppression of the inflammatory response are not completely understood but are, nevertheless, highly complex. For example, we have shown that GSK3 $\beta$  controls the major immune modulating molecule, IFN- $\beta$  production in LPS (TLR4)-stimulated human macrophages via a c-Jun and activating transcription factor (ATF)-2-dependent mechanism (7). GSK3 $\beta$  also negatively regulates production of the endogenous IL-1 $\beta$  antagonist, IL-1R, via its ability to regulate the mitogen-activated protein kinase (MAPK) extracellular-signal-regulated kinase (ERK)1/2 in LPS-stimulated innate cells by a mechanism that involves modulation of the level of inhibitory residue ser71 on Rac1 which, subsequently, controls the ability of Rac1 to activate p21-activated protein kinase (8). Of particular interest is our recent identification of IFN- $\beta$  as a novel antiinflammatory therapeutic target that stimulates innate production of IL-10 through activation of the

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**Figure 1.** Rationale for targeting GSK3 to prevent TLR-induced alveolar bone loss. There is a large body of existing, predominantly *in vitro* evidence, that, together, provide strong rationale for examining GSK3 as a potential therapeutic target for periodontal diseases. This figure summarizes the key data. GSK3 is a constitutively active serine-threonine kinase that, upon TLR engagement by *P. gingivalis*, acts as a downstream effector molecule in the PI3K pathway that augments the production of proinflammatory cytokines including TNF, IL-6, IL-12 and IL-1 $\beta$ . Such cytokines are known to promote osteoclastogenesis and promote alveolar bone loss. Pharmacological inhibition of GSK3 suppresses the bacterial-induced production of multiple proinflammatory cytokines while concurrently augmenting production of the antiinflammatory cytokine, IL-10. This occurs through a mechanism that leads to a shift in the balance of nuclear NF- $\kappa$ B (p65)- and CREB-driven transcription events. Further details are provided in the recent review by Wang *et al.* (6). Such alterations to the pro- and antiinflammatory cytokine balance would be expected to suppress the progression of periodontitis. Therefore, it is hypothesized that the GSK3 inhibitor, SB216763, will protect susceptible mice from *P. gingivalis*-mediated alveolar bone loss. Those parts of the pathway that are relevant *in vivo* remain to be clarified. This report, which confirms that pharmacological inhibition of GSK3 $\beta$  suppresses pathogen-induced periodontal inflammation and alveolar bone loss, represents a first step in this process.

Janus kinase 1 (JAK1)/phosphatidylinositol 3 kinase (PI3K)/protein kinase B1 (Akt1)/GSK3 $\beta$  signaling cascade (9). Antoniv and Ivashikiv have shown that the PI3K/Akt/GSK3 signaling axis controls the activation of specific IL-10 inducible genes in TLR-stimulated macrophages (10). We also have shown that p85S6K-associated GSK3 $\beta$  is a kinase target for mammalian target of rapamycin complex

1 (mTORC1), allowing control (inactivation) of GSK3 $\beta$  activity and, thus, regulation of the pro- versus antiinflammatory cytokine balance in TLR-4 stimulated innate cells (11). Inhibitors of GSK3 also suppress signal transducer and activator of transcription (STAT)3 and STAT5 activation, providing a further mechanism of differential inflammatory response regulation (12). Stimulation of

innate  $\alpha$ 7 nicotinic acetylcholine ( $\alpha$ 7nAChR) receptors results in the convergence of the cholinergic and GSK3 $\beta$  antiinflammatory pathways (13). Thus,  $\alpha$ 7nAChR has been determined to be a potent, endogenous amplifier of GSK3 $\beta$  antiinflammatory events (enhancement of IL-10 and inhibition of NF- $\kappa$ B controlled proinflammatory cytokines) in TLR4- and whole *P. gingivalis*-stimulated innate cells (13).

As the potential importance of GSK3 $\beta$  in regulating periodontal inflammation has been clearly established *in vitro*, as recently reviewed (6) and as summarized in Figure 1, we hypothesized that the cell permeable GSK3-specific inhibitor, SB216763, would protect against alveolar bone loss induced by the key periodontal pathogen, *P. gingivalis*. We set out to test this hypothesis in an established murine model of periodontitis.

## MATERIALS AND METHODS

### Materials

B6129SF2/J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). *Porphyromonas gingivalis* ATCC 33277 was obtained from the American Type Culture Collection (Manassas, VA, USA). Trypticase soy broth (TSB) came from BD (Franklin Lakes, NJ, USA). TNF enzyme-linked immunosorbent assays (ELISAs) were purchased from eBioscience (San Diego, CA, USA). SB216763 was purchased from Tocris Bioscience/R&D Systems (Minneapolis, MN, USA). Sulfamethoxazole, trimethoprim, carboxymethylcellulose, dimethyl sulfoxide (DMSO) and paraformaldehyde came from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ly6G (RB6-8C5) and anti-human/mouse IL-17A antibodies came from LifeSpan Biosciences (Seattle, WA, USA) and Santa Cruz Biotech (Santa Cruz, CA, USA), respectively. Alexa Fluor 594-conjugated goat anti-rabbit IgG was purchased from Molecular Probes/Life Technologies (Grand Island, NY, USA). Immunocal solution was bought from Decal Chemical Corpora-

tion (Tallman, NY, USA) and OCT compound came from Fisher Scientific (Pittsburgh, PA, USA). Procarta Mouse Cytokine Assay Kits were purchased from Affymetrix (Santa Clara, CA, USA).

### Growth of *Porphyromonas gingivalis*

*P. gingivalis* ATCC 33277 cells were grown in TSB under anaerobic conditions (85% N<sub>2</sub>, 10% H<sub>2</sub>, 5% CO<sub>2</sub>) at 37°C. SB216763 (0 to 125 μm) did not influence the growth of *P. gingivalis* in planktonic cultures compared with untreated and solvent controls (data not shown).

### *P. gingivalis*-Induced Bone Loss Model

An established *P. gingivalis*-induced periodontal bone loss model (14) was utilized. The oral microflora was suppressed in 10- to 12-wk-old B6129SF2/J mice by sulfamethoxazole (800 μg mL<sup>-1</sup>) and trimethoprim (400 μg mL<sup>-1</sup>) provided *ad libitum* in water for 10 d. The mice then received pure drinking water for 3 d. Alveolar bone loss was induced by oral infection with 1 × 10<sup>9</sup> CFU of live *P. gingivalis* suspended in 100 μL of phosphate-buffered saline with 2% carboxymethylcellulose directly by gavage. Infections were performed five times at 2-d intervals. The experimental group was also administered intraperitoneally (IP) SB216763 (10 mg/kg) 1 d prior to infection and every other day thereafter until euthanization. Sham-infected and vehicle controls also were established. The mice were euthanized with CO<sub>2</sub> and cervical dislocation 42 d after the final infection. Alveolar bone loss was measured in millimeters at 14 predetermined points on the maxillary molars of defleshed maxillae as the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC). Bone loss was visualized by methylene blue/eosin staining and quantified using a Nikon SMX 800 dissecting microscope (40×; Nikon Instruments Inc., Melville, NY, USA) fitted with a Boeckeler VIA-170K video image marker measurement system (Boeckeler Instruments Inc, Tucson, AZ, USA). The results were expressed as

the mean and standard deviation. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee, University of Louisville (IACUC # 10045).

### Evaluation of Periodontal Inflammation

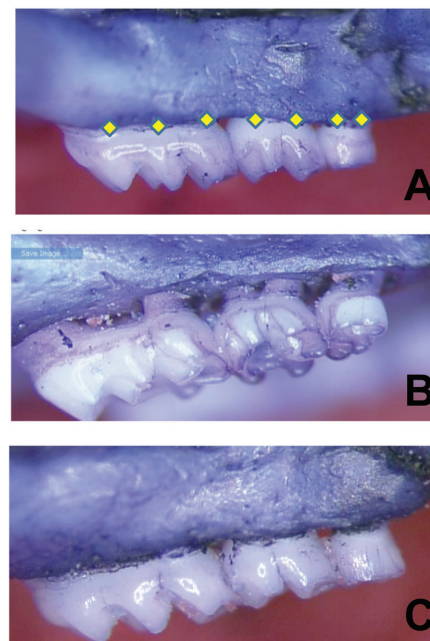
Neutrophil infiltration and IL-17 expression were monitored by immunohistochemistry, as described recently by Eskan *et al.* (15). Jawbones were fixed in 4% paraformaldehyde, decalcified in immunocal solution for 15 d and embedded in OCT compound. 7- to 8-μm mesiodistal sections were stained with FITC-conjugated anti-mouse Ly6G, a neutrophil marker, or with anti-human/mouse IL-17 antibodies visualized using Alexa Fluor 594-conjugated goat anti-rabbit IgG. The specificity of staining was confirmed by using a FITC-conjugated isotype control or normal rabbit IgG followed by Alexa Fluor 594-goat anti-rabbit IgG. Images were captured using a laser-scanning confocal microscope (Olympus FV1000; Olympus America Inc., Center Valley, PA, USA). Quantitative data are presented as mean comparative fluorescence intensity ± standard deviation. Quantification was performed using ImageJ software v1.46 [NIH, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>].

### Evaluation of Systemic Inflammation

Systemic cytokine concentrations (IL-12 p40, IL-1β and IL-6) were determined in mouse serum in duplicate with the Procarta Mouse Cytokine Assay Kit and Luminex 100, according to the manufacturer's instructions (eBioscience). TNF concentrations were determined by ELISA, according to the manufacturer's instructions (eBioscience).

### Statistical Approaches

Statistical significance between groups was evaluated by analysis of variance (ANOVA) and Tukey multiple-comparison test using the InStat program (GraphPad Software, San Diego, CA, USA). Differences between groups were considered significant at the level of  $p < 0.05$ .

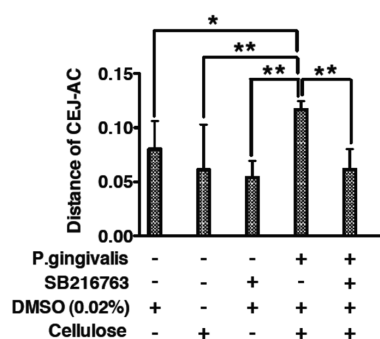


**Figure 2.** Visualization of *P. gingivalis*-induced bone loss. 8- to 12-wk-old B6129SF2/J mice were divided randomly into three control groups and two experimental groups ( $n = 5$  per group). The control groups were treated with cellulose (sham infected), 0.02% DMSO, or SB216763 (10 mg/kg) respectively. The experimental groups were infected orally with *P. gingivalis* 33277 with or without pretreatment with the GSK3 inhibitor, SB216763 (10 mg/kg). Alveolar bone loss was visualized by methylene blue/eosin staining 6 wks later. Typical maxillae from (A) sham-infected, (B) *P. gingivalis*-infected, and (C) SB216763-treated, *P. gingivalis*-infected mice are presented.

## RESULTS

### Inhibition of GSK3β Abrogates *P. gingivalis*-Induced Bone Loss

Typical photographs of pathogen-induced bone loss in methylene blue-stained murine maxillae are presented in Figure 2. As expected, alveolar bone loss was readily induced in B6129SF2/J mice by *P. gingivalis* compared with sham-infected controls. Such hard tissue destruction appeared to be completely abrogated by the systemic administration of the GSK3 inhibitor, SB216763. The seven



**Figure 3.** Quantification of *P. gingivalis*-induced bone loss. The distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured 6 wks after infection at 14 predetermined maxillary buccal sites in 8- to 12-wk-old B6129SF2/J mice divided into three control (cellulose, DMSO, or SB216763 treated) and two experimental (*P. gingivalis*-infected; and SB216763-treated, *P. gingivalis*-infected mice) groups. Data are presented as mean distance CEJ-ABC in mm  $\pm$  s.d. n = 5 mice per group. \* $p < 0.05$  compared with *P. gingivalis*-treated group. \*\* $p < 0.01$  compared with *P. gingivalis*-treated group.

predetermined points on the buccal surface of the maxillary molars used to assess alveolar bone loss are highlighted in Figure 2A. Bone loss also was measured on the equivalent points on the opposing buccal surface. As shown in Figure 3, *P. gingivalis*-induced bone loss was significantly greater than each of the three control groups: sham infected (cellulose) ( $p < 0.01$ ); DMSO ( $p < 0.05$ ); and SB216763 only ( $p < 0.01$ ). Critically, pharmacological inhibition of GSK3 reduced pathogen-induced bone destruction to control levels.

### Inhibition of GSK3 $\beta$ Reduces *P. gingivalis*-Induced Periodontal Inflammation

Neutrophil infiltration into gingival tissues, visualized as Ly6G-positive cells, was reduced in mice inoculated with *P. gingivalis* and treated with SB216763, compared with the *P. gingivalis*-induced bone loss group (Figure 4B). Similarly, the proinflammatory cytokine IL-17 was also reduced upon GSK3 inhibition (Figure

4C). Double immunofluorescence staining suggested that neutrophils were a major, but not sole, source of IL-17 in the mice that developed bone loss (Figure 4D).

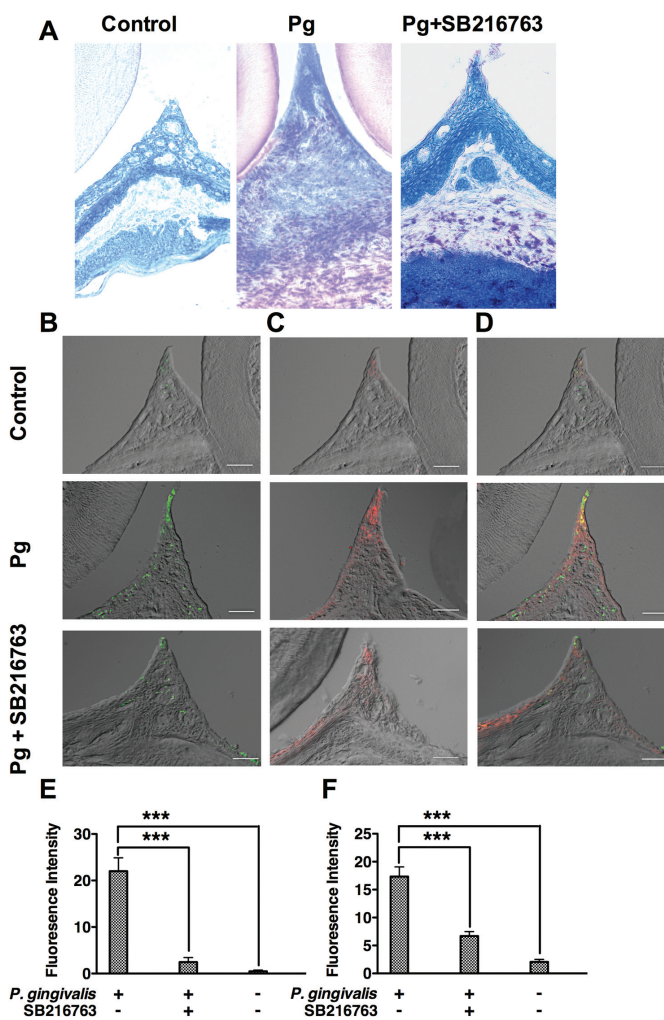
### Inhibition of GSK3 $\beta$ Reduces *P. gingivalis*-Induced Systemic Inflammation

As shown in Figure 5, oral inoculation of mice with *P. gingivalis* led to systemic inflammation, quantified as the serum concentration of proinflammatory cy-

tokines, 42 d after the final bacterial infection. This *P. gingivalis*-induced circulating burden of IL-12 p40, TNF, IL-1 $\beta$  and IL-6 was reduced to levels close to those seen in uninoculated controls on SB216763 treatment.

### DISCUSSION

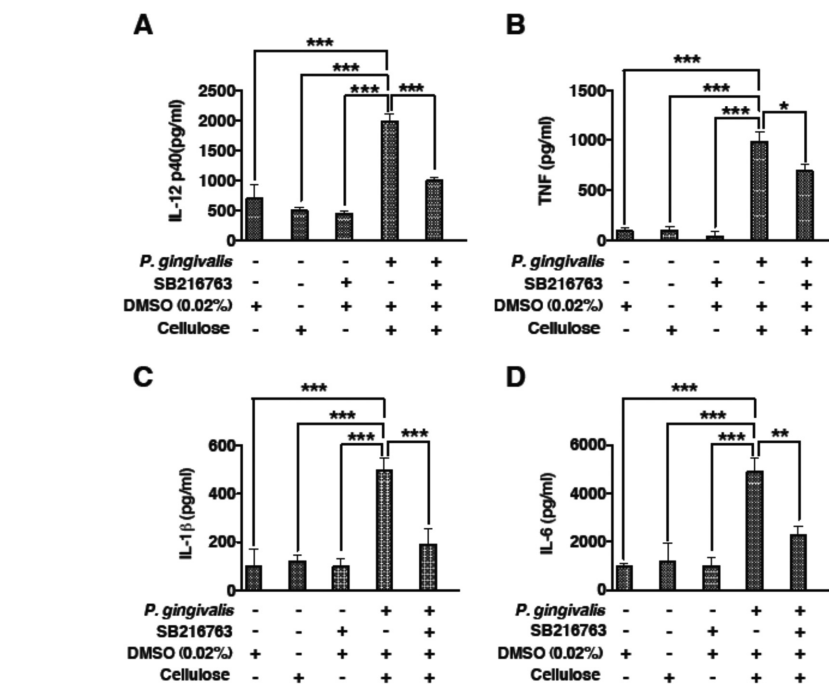
Regulation of TLR signaling is critical in the determination of the qualitative and quantitative ferocity of the inflammatory response to microbial insult (5). We



**Figure 4.** Quantification of maxillary neutrophil infiltration and IL-17 expression. Typical (A) Hematoxylin and eosin (H and E)-stained and (B) confocal microscopy images of Ly6G-positive neutrophil infiltration, (C) IL-17 expression and (D) IL-17 expressing Ly6G-positive cells in periodontal tissues are presented. The mean comparative fluorescence intensity for (E) neutrophils and (F) IL-17 also are presented. Error bars represent the standard deviation. \*\*\* $p < 0.001$  compared with *P. gingivalis* bone loss group. Pg, *P. gingivalis* infected; Pg + SB216763, SB216763 treated and *P. gingivalis* infected. Scale bars, 50  $\mu$ m.

have established previously that the engagement of the  $\alpha 7nAChR$  cholinergic antiinflammatory pathway amplifies the PI3K pathway in a GSK3-dependent manner (13). GSK3 $\beta$  inhibition differentially affects the nuclear amounts of transcription factors NF- $\kappa$ B subunit p65 and CREB interacting with the coactivator calcium-binding protein (CBP) (5). Consequently, TLR-initiated proinflammatory cytokine production (IL-1 $\beta$ , IL-8, TNF, IL-12) in response to *P. gingivalis* is potently suppressed, concurrent with the upregulation of the antiinflammatory cytokine, IL-10 (13). This represents a powerful antiinflammatory mechanism, at least *in vitro*. Endogenously, GSK3 $\beta$  is inactivated by phosphorylation of the Ser<sup>9</sup> residue (16). GSK3 $\beta$  inhibition also can be achieved pharmacologically, for example, by SB216763. Recently, it has been established that Wortmannin, which inhibits PI3K and, thus, reduces GSK3 $\beta$  phosphorylation (preventing the inactivation of this constitutively active kinase), augments liver damage in a murine hepatic ischemia-reperfusion model (17). Similarly, GSK3 $\beta$  inhibition has been shown to reduce chronic intestinal inflammation significantly in a dextran sodium sulfate-induced colitis model (18). Herein, we have established that these phenomena may be active in the oral cavity *in vivo*, as inhibition of GSK with SB216763 reduces periodontal inflammation and abrogates pathogen-induced periodontitis in mice.

Murine alveolar bone loss models have been used to examine the importance of multiple innate cell surface receptors and intracellular signaling molecules, including TLR-2, complement component 3a receptor (C3aR), complement component 5a receptor (C5aR), C-C chemokine receptor type 2 (CCR2), receptor activator of nuclear factor- $\kappa$ B ligand (RANK-L) and MAPK-MAP kinase phosphatase 1 (MPK1) (19–21) in periodontitis. Whereas significant roles in periodontal bone loss were found for several of these inflammatory modulators, the potency of GSK3 inhibition in abrogating *P. gingivalis*-induced periodontal disease progression in this model is striking. Considering the



**Figure 5.** Systemic cytokine response to *P. gingivalis* infection and GSK3 inhibition. Systemic cytokine concentrations were determined in mouse serum collected at euthanization, 42 d from the last infection, using Luminex technology (IL-12 p40, IL-1 $\beta$  and IL-6) or ELISA (TNF). Assays were performed in duplicate with  $n = 5$  mice per group. Data are presented as mean cytokine concentration in pg/mL  $\pm$  s.d. \*\*\* $p < 0.001$  compared with the *P. gingivalis* bone loss group.

complexity of periodontal disease etiology, the efficacy of such a single therapeutic agent is perhaps surprising. On the other hand, validation of the current findings will confirm the critical importance of GSK3 as a central regulator of the inflammatory response to microbial infections.

*P. gingivalis* persistence may not be clinically significant, because the ultimate goal is to prevent and/or treat periodontal disease, thus, many studies testing potential antiinflammatory therapeutics for the inhibition of bacterial-induced alveolar bone loss have not assessed the fate of the inoculum (22–25). Yuan *et al.* showed that immune modulation via RANKL antagonism suppressed *P. gingivalis*-induced inflammation and bone loss *in vivo*, but did not influence pathogen colonization (20). We did not quantify infection with *P. gingivalis*. However, all animals were dosed equally with this pathogen and

inflammatory indices were measured chronically after inoculation. Furthermore, inhibition of GSK3 $\beta$  is known to suppress the immune response, as we have confirmed in our model herein. Thus, we would expect any remaining *P. gingivalis* infection to be higher in the SB216763-treated group. Yet, the inflammatory indices and bone loss are lower in this experimental group. Neutrophils are protective in so much as they control the pathogenic population, attacking bacteria in the gingival crevice, external to the periodontal soft and hard tissues, and they also scavenge cellular debris. As we have recently reviewed (26), however, there is a clear association between neutrophil infiltration into periodontal tissues and the severity and progression of inflammatory periodontal diseases (27–31). The importance of IL-17 in the promotion of osteoclastogenesis, bone loss and periodontitis is now recognized (15,32–36), while *P.*

*gingivalis* has been shown to induce innate cell IL-17 production and promote Th17 polarization (37,38). We establish that SB216763 suppresses the infiltration of neutrophils to the periodontal tissues of *P. gingivalis*-loaded animals. Interestingly, local expression of IL-17 also is suppressed upon GSK3 $\beta$  inhibition. IL-17, recently recognized as a key mediator of inflammatory alveolar bone loss, promotes neutrophil hematopoiesis and attracts and activates neutrophils (15,39,40).

Periodontitis occurs in approximately 50% of the population, resulting in significant debilitation for about half of these persons (41,42), and represents an enormous economic burden that consumes >\$14 billion per annum in the United States alone (43). Furthermore, increasing evidence suggests that periodontitis is associated with increased risk of vascular diseases (including coronary artery disease and stroke), diabetes mellitus, lung diseases (chronic obstructive pulmonary disease [COPD] and pneumonia), and preterm delivery (44,45). Thus, the potential significance and impact of controlling or reducing pathogen-induced periodontal diseases is enormous. In keeping with the concept of increased systemic inflammation during periodontitis, we have shown that *P. gingivalis*-loaded mice exhibit both alveolar bone loss and significantly increased systemic concentrations of TNF and IL-12/IL-23 p40, compared with control mice. These same cytokines are known to be suppressed by GSK3 $\beta$  inhibition *in vitro* and in other animal models, including a murine endotoxic shock model (5). We show, for the first time, that SB216763 reduces not only periodontal inflammation, but also the circulating TNF and IL-12/IL-23 p40 burden. Thus, it is possible that GSK3 $\beta$  inhibition may protect against the chronic, systemic sequelae associated with periodontal diseases. It is interesting that both IL-6 and IL-23 are known to induce Th17 cell differentiation (46). The possibility of systemic sequelae due to persistent *P. gingivalis* will need to be tested in future

studies, although the fact that circulating cytokines are significantly reduced in *P. gingivalis*-inoculated, GSK3 $\beta$ -inhibited animals is a promising sign. Potential side effects of SB216763 will also need to be considered carefully.

## CONCLUSION

To the best of our knowledge, this is the first *in vivo* report of GSK3 as a novel, efficacious therapeutic preventing periodontal disease progression in a susceptible host. It is hoped that these data can serve as a basis for the rational design of intervention therapeutic strategies for manipulating the innate immune response in the periodontium. Future studies will be required to confirm and further elucidate the mechanisms by which SB216763 and other GSK3 inhibitors block periodontal disease progression in response to pathogenic insult.

## ACKNOWLEDGMENTS

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## DISCLOSURE

DA Scott is an inventor of U.S. Patent Application PCT/US2008/054569, "Therapeutic Cotinine Compositions." Cotinine stimulates the cholinergic anti-inflammatory pathway which augments GSK3 $\beta$  anti-inflammatory events (13).

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