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Periodontal inflammation and alveolar bone loss induced by *Aggregatibacter actinomycetemcomitans* is attenuated in sphingosine kinase 1-deficient mice

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Abstract

Background and Objective—Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid, which is generated by activation of sphingosine kinase (SK) 1 and/or 2 in most mammalian cells with various stimuli, including the oral pathogen *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*). S1P signaling has been shown to regulate the migration of monocytes and macrophages (osteoclast precursors) from the circulation to bone tissues and affect bone homeostasis. We aimed to determine the effects of SK1 deficiency on S1P generation, proinflammatory cytokine production, chemotaxis of monocytes and macrophages, and periodontitis induced by *A. actinomycetemcomitans*.

Material and Methods—Murine bone marrow-derived monocytes and macrophages (BMMs) from SK1 knockout (KO) mice or wild type (WT) mice were either untreated or exposed to *A. actinomycetemcomitans*. The mRNA levels of SK1, SK2, and intracellular sphingolipid levels were quantified. Also, murine WT BMMs were treated with vehicle, S1P, with or without *A. actinomycetemcomitans* and the mRNA levels of cyclooxygenase 2 (COX-2), interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF) were quantified. The protein levels of prostaglandin E2 (PGE2), IL-1 β , IL-6, and TNF- α were quantified in the cell media of SK1 KO BMMs or WT BMMs with or without bacterial stimulation. Furthermore, a transwell migration assay was performed and the number of migrated WT BMMs in the presence of vehicle, bacteria-stimulated media, with or without S1P was quantified. Finally, *in vivo* studies were performed on SK1 KO and WT mice by injecting either PBS or *A. actinomycetemcomitans* in the periodontal tissues. The mice maxillae were scanned by micro-computed tomography (μ -CT), and alveolar bone volume was analyzed. The number of periodontal leukocytes and osteoclasts were quantified in maxillary tissue sections.

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Results—SK1 mRNA levels significantly increased after *A. actinomycetemcomitans* stimulation in murine WT BMMs, but were undetectable in SK1 KO BMMs. Deficiency of SK1 in murine BMMs resulted in decreased S1P generation induced by *A. actinomycetemcomitans* as compared with WT BMMs. Additionally, low levels of S1P (1 μ M) did not have a significant impact on the mRNA production of COX-2, IL-1 β , IL-6, and TNF in murine BMMs with or without the presence of *A. actinomycetemcomitans*. There were no significant differences in PGE2, IL-1 β , IL-6, and TNF- α protein levels in the media between SK1 KO BMMs and WT BMMs with or without bacterial stimulation. Importantly, low levels of S1P (1 μ M) dose-dependently promoted the chemotaxis of BMMs. The bacteria-stimulated media derived from SK1 BMMs significantly reduced the chemotaxis response compared with WT control. Finally, SK1 KO mice showed significantly attenuated alveolar bone loss stimulated by *A. actinomycetemcomitans* compared with WT mice treated with *A. actinomycetemcomitans*. Histological analysis of periodontal tissue sections revealed that SK1 KO mice treated with *A. actinomycetemcomitans* significantly reduced the number of infiltrated periodontal leukocytes and mature osteoclasts attached on the alveolar bone compared with WT mice.

Conclusion—Our studies support that SK1 and S1P play an important role in the inflammatory bone loss response induced by the oral pathogen *A. actinomycetemcomitans*. Reducing S1P generation by inhibiting SK1 has potential as a novel therapeutic strategy for periodontitis and other inflammatory bone loss diseases.

Keywords

sphingosine-1-phosphate; sphingosine kinase; periodontal disease; *Aggregatibacter actinomycetemcomitans*; cytokine; osteoclast

Periodontitis is a bacteria-driven inflammatory bone loss disease. Oral pathogens, such as *A. actinomycetemcomitans*, the pathogen associated with localized aggressive periodontitis, initiate a proinflammatory response leading to periodontal soft tissue damage, alveolar bone resorption, and subsequent tooth loss. The mechanisms associated with the inflammatory bone loss response induced by oral pathogens have not been completely elucidated.

Our previous studies demonstrated that *A. actinomycetemcomitans* stimulated the generation of S1P in RAW 264.7 cells (1). S1P is derived from the sphingolipid ceramide (Fig. 1A). Ceramide can be hydrolyzed by ceramidase to release sphingosine, which can be subsequently phosphorylated by sphingosine kinase (SK) 1 and/or 2 to generate S1P. Although both SK1 and SK2 share overall homology and produce the same product, S1P, they display different subcellular locations and possibly have distinct and overlapping functions (2). SK1 normally resides in the cytosolic compartments and can be translocated to the plasma membrane after activation (3, 4). SK1 appears to be a major determinant of S1P production within the cytoplasm and membrane (5). In contrast, SK2 is mainly localized in the nucleus with low levels found in the cytoplasm (3, 4). SK2 mainly accounts for nuclear S1P formation (5). In response to agonist treatment, SK2 can be phosphorylated and exported from the nucleus into the cytoplasm (4, 6). Although SK1 and SK2 double KO mice experience embryonic death owing to severe defects in neurogenesis and angiogenesis (7), SK1 KO mice and SK2 KO mice have no obvious abnormality, which suggested a compensatory role in S1P generation (3, 8).

S1P can be produced in most mammalian cells (5). Constitutive S1P levels in tissues are very low because S1P can be degraded by S1P lyase or can be dephosphorylated by S1P phosphatase (Fig. 1A) (9). In contrast, S1P levels in blood are very high (low μM range) because erythrocytes and platelets produce abundant S1P (9, 10). However, they lack both S1P lyase and S1P phosphatase activities (9, 10). The sharp gradient between the levels of S1P in the circulation and those in the tissues is crucial for the migration of immune cells (9). The intracellular S1P can be exported to extracellular space by specific transporters. S1P binds to five G protein-coupled S1P receptors (S1PR1-5) on the plasma membrane initiating various cellular signaling pathways (2, 9). Additionally, S1P can serve as an intracellular messenger, which triggers calcium release and modulates the immune response (11). S1P plays an essential role in the pathogenesis of many diseases, including cancer, atherosclerosis, rheumatoid arthritis, diabetes, and osteoporosis (12, 13). However, the role of S1P in the pathogenesis of periodontitis has not been determined.

Previous studies demonstrated that S1P signaling was critical in regulating inflammatory diseases and bone homeostasis. The synovial fluid of rheumatoid arthritis patients exhibited significantly higher levels of S1P than their non-inflammatory osteoarthritis counterparts (14). Additionally, S1P signaling controlled the migration of monocytes and macrophages from blood circulation to bone tissues and affected bone homeostasis (15, 16). These monocytes and macrophages are osteoclast precursors, which can fuse to form multinucleated mature osteoclasts leading to bone loss (17). Furthermore, high circulating S1P levels observed in postmenopausal women were positively correlated with their bone resorption markers (18), and transgenic human TNF- α /SK1 KO mice exhibited significant less synovial inflammation and joint erosion compared with WT control (19). Although our previous study showed that the oral pathogen *A. actinomycetemcomitans* stimulated the generation of S1P (1), the mechanisms by which *A. actinomycetemcomitans* stimulated the generation of S1P and S1P modulated the immune response to oral pathogens remained unknown. In this study, we determined the effects of SK1 deficiency on S1P generation, proinflammatory cytokine production, and chemotaxis of monocytes and macrophages. Additionally, we evaluated the effects of SK1 deficiency in the pathogenesis of periodontitis using an *A. actinomycetemcomitans*-induced periodontitis animal model.

Materials and methods

Experimental animals

Eight-week-old male SK1 KO and WT mice (20) were obtained from the COBRE Lipidomics and Pathobiology Animal Core at the Medical University of South Carolina. The mice were housed with a 12-hour light/12-hour dark cycle and had free access to food and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina. The animal study was performed in accordance with ARRIVE guidelines for animal research.

S1P preparation

S1P was purchased from Cayman Chemical (Ann Arbor, MI, USA), dissolved in methanol in a sonicated water bath for 1 h, air dried by nitrogen, and stored at -80°C . 100 μM of stock

S1P was freshly prepared by dissolving S1P in Dulbecco's PBS containing 4 mg/mL fatty acid-free bovine serum albumin (BSA).

Culture of *A. actinomycetemcomitans*

A. actinomycetemcomitans (ATCC 43718, serotype b, strain Y4) was purchased from American Type Culture Collection. Bacterial colonies were grown on Difco™ brain heart infusion agar plates (BD Biosciences, Sparks, MD, USA) and cultured in Bacto™ brain heart infusion broth (BD Biosciences) at 37°C with 10% CO₂ for 24 h. Bacteria were centrifuged, washed with PBS with 5% glycerol, and resuspended in PBS with 5% glycerol. Bacterial concentration was determined by measuring optical density and followed by plating on brain heart infusion agar plates (OD₆₀₀=1, about 3 × 10⁷ colony forming units, CFU/mL).

Bone marrow-derived monocytes and macrophages (BMMs)

Bone marrow cells were harvested from mice as previously described (21). To allow bone marrow progenitor cells to differentiate into BMMs, bone marrow cells were cultured for seven days in minimal essential media (MEM)-alpha media (Life Technologies, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 ng/mL recombinant murine macrophage colony-stimulating factor (M-CSF) (R&D systems, Minneapolis, MN, USA). After seven days, the suspended cells were discarded and the attached BMMs were plated in tissue culture dishes.

BMM treatment

BMMs (1 × 10⁶ or 3 × 10⁶ cells/well) derived from either SK1 KO or WT mice were plated in tissue culture dishes in MEM-alpha media with 5% FBS and antibiotics. BMMs from SK1 KO or WT mice were either untreated or stimulated for 1 to 8 h with *A.*

actinomycetemcomitans (1.5 CFU/cell) for SK1 and SK2 mRNA analyses. The WT BMMs were treated for 4 h with either vehicle (PBS containing 4mg/mL of fatty acid-free BSA), various doses of S1P (0.25 to 5 µM), *A. actinomycetemcomitans* (1.5 CFU/cell) alone, or with both S1P (0.25 to 5 µM) and *A. actinomycetemcomitans* (1.5 CFU/cell) for COX-2, IL-1β, IL-6, and TNF mRNA analyses. The SK1 KO BMMs or WT BMMs were either untreated or stimulated for 8 h with *A. actinomycetemcomitans* (1.5 CFU/cell) for PGE₂, IL-1β, IL-6, and TNF-α protein analyses. BMMs (3 × 10⁶ cells/well) derived from SK1 KO or WT mice were either untreated or stimulated for 4 to 8 h with *A. actinomycetemcomitans* (1.5 CFU/cell) for sphingolipid analyses.

RNA extraction and real time PCR

The RNA extraction and RT-qPCR were performed as previously described (1). The following amplicon primers were obtained from Life Technologies: SK1 (Mm00448841_g1), SK2 (Mm00445021_m1), COX-2 (Mm00478374_m1), IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), TNF (Mm00443258_m1), and GAPDH (Mm99999915_g1). Sample mRNA levels were normalized to an endogenous control GAPDH expression and expressed as relative mRNA levels compared with control groups.

Mass spectrometry analyses for sphingolipids

BMMs (3×10^6 cells) were lysed in 150 μ L RIPA protein lysis buffer (Cell Signaling Technology, Beverly, MA, USA). Periodontal tissues from mice were homogenized in 1 mL RIPA protein lysis buffer. Sphingolipids in cell lysate, tissue lysate, or serum were extracted from the samples by the Lipidomics Core Facility using the Bligh Dyer technique. Sphingolipid analysis was performed using Electrospray Ionization /Tandem Mass Spectrometry (ESI-MS/MS) on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer. This technique was previously described by Bielawski et al. (22). The sphingolipid levels in the cell lysate were normalized to protein concentrations in samples.

Cytokine protein assay

PGE₂, IL-1 β , IL-6, and TNF- α protein levels in the cell culture media of SK1 KO BMMs or WT BMMs with or without bacterial stimulation were determined by ELISA. The PGE₂, IL-1 β , IL-6, and TNF- α ELISA kits were purchased from R & D systems (Minneapolis, MN, USA).

Transwell migration assay

WT BMMs (1×10^5 /well) were put into the upper chambers of transwell plates (8.0 μ M, Corning Incorporated, Corning, NY, USA) in MEM- α media with 5% FBS. The lower chambers were filled with either 1) vehicle, 2) S1P (125 to 1000 nM), 3) vehicle with media derived from bacteria-stimulated WT BMMs (derived from filter sterilized WT BMM culture media stimulated with *A. actinomycetemcomitans* for 24 h), 4) S1P (125-1000 nM) with media derived from bacteria-stimulated WT BMMs (derived from filter sterilized WT BMM culture media stimulated with *A. actinomycetemcomitans* for 24 h), 5) SK1 KO BMM media without bacterial stimulation, 6) WT BMM media without bacterial stimulation, 7) media derived from bacteria-stimulated SK1 KO BMMs (derived from filter sterilized media from SK1 KO BMMs stimulated with *A. actinomycetemcomitans* for 8 h), or 8) media derived from bacteria-stimulated WT BMMs (derived from filter sterilized media from WT BMMs stimulated with *A. actinomycetemcomitans* for 8 h). After incubation for 24 h, the cells in the bottom chamber were quantified by light microscopy. Cell number was quantified in ten 400 $\times$ magnification views for each well. The average number of cells per 400 $\times$ magnification view served as migration index.

In vivo animal study

Eight-week-old SK1 KO mice (n=20) and WT mice (n=20) were injected with either *A. actinomycetemcomitans* (2×10^5 CFU, 2 μ L, n=10) or PBS containing 5% glycerol (2 μ L, n=10) at both sides of the palatal gingival rugae near the first maxillary molar using an Olympus SZ61 dissecting microscope (Olympus, Center Valley, PA, USA) three times per week for four weeks. All the mice were euthanized at the end of four weeks, two days after the last injection of either *A. actinomycetemcomitans* or PBS. The animal periodontal gingival tissues were harvested from the left side for S1P analysis. The left side maxillae, the right side of animal maxillae and adjacent soft tissues were fixed in formalin. Blood was collected from the heart of all animals after animal euthanasia.

Micro-computed tomography (μ -CT) and bone volume fraction (BVF) analysis

Both sides of animal maxillae were scanned by a cone beam μ -CT40 system (Scanco Medical AG, Switzerland). Each scan was reconstructed at a mesh size of $18 \times 18 \times 18 \mu\text{m}$, and three-dimensional digitized images were generated for each specimen. Using GEHC MicroView software, the images were rotated into a standard orientation and threshold to distinguish between mineralized and nonmineralized tissues. Alveolar bone losses expressed as bone volume fraction (BVF) were measured as previously described (23).

Histological tissue processing and pathological evaluation

Formalin-fixed specimens were decalcified in a 10% EDTA solution for 4 weeks and processed for histological analysis. Seven μm sagittal paraffin tissue sections were cut and stained with hematoxylin and eosin (H&E) for descriptive histology, or stained with tartrate-resistant acid phosphatase (TRAP) for osteoclasts using a leukocyte acid phosphatase kit (Sigma Aldrich, St. Louis, MO, USA). The tissue sections were counterstained with hematoxylin after TRAP staining. The number of leukocytes in the periodontal tissues and the number of multinucleated (more than 3 nuclei) TRAP⁺ osteoclasts in contact with alveolar bone was evaluated and quantified by an experienced pathologist.

Statistical analyses

Data were analyzed by Mann-Whitney test for analysis of two groups of samples and Kruskal-Wallis ANOVA test for analysis of more than three groups of samples. All statistical tests were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla CA, USA). Values are expressed as mean $\pm$ SEM. A *P* value of 0.05 or less was considered significant.

Results

SK1 deficiency in BMMs significantly decreased S1P generation induced by *A. actinomycetemcomitans*

Previous studies demonstrated that SK1 could be activated by cytokines and lipopolysaccharide (2, 24). To determine if SK1 can be activated by *A. actinomycetemcomitans*, BMMs from SK1 KO or WT mice were either left unstimulated or stimulated with *A. actinomycetemcomitans* (1.5 CFU/cell) for 1 to 8 h. As shown in Fig. 1B, SK1 mRNA levels significantly increased 4 h after *A. actinomycetemcomitans* stimulation in WT BMMs, but were undetectable in SK1 KO BMMs. In contrast, SK2 mRNA levels remained similar to basal level after *A. actinomycetemcomitans* stimulation in both SK1 KO and WT BMMs (Fig. 1C). *A. actinomycetemcomitans* stimulated the generation of S1P (Fig. 1D). Intracellular S1P levels were increased in WT BMMs 4 h and 8 h after *A. actinomycetemcomitans* stimulation. There were significantly reduced S1P levels in SK1 KO BMMs compared with those in WT BMMs 4 and 8 h post *A. actinomycetemcomitans* stimulation. However, we also observed a moderate increase of S1P in SK1 KO BMMs 4 h after *A. actinomycetemcomitans* stimulation, which suggested a role of SK2 contributing to the S1P production. Additionally, we observed significantly higher levels of sphingosine in SK1 KO BMMs compared with those in WT BMMs after bacterial stimulation (Fig. 1E).

This was likely caused by a decrease in phosphorylation of sphingosine by SK1 in SK1 KO cells. There were no significant differences of total ceramide levels between SK1 KO and WT cells before or after bacterial stimulation (data not shown). These data demonstrated that *A. actinomycetemcomitans* activated SK1 leading to S1P generation. Deficiency of SK1 in BMMs resulted in significantly reduced S1P levels induced by bacterial stimulation compared with WT controls.

SK1 deficiency in BMMs had no significant impact on the proinflammatory cytokine levels induced by *A. actinomycetemcomitans*

Monocytes and macrophages are major sources of proinflammatory cytokines in periodontitis. To determine the role of S1P in modulating the inflammatory response, murine WT BMMs were treated with either vehicle (PBS containing 4mg/mL of fatty acid-free BSA), various doses of S1P (0.25 to 5 μ M), *A. actinomycetemcomitans* (1.5 CFU/cell) alone, or with both S1P (0.25 to 5 μ M) and *A. actinomycetemcomitans* (1.5 CFU/cell) for 4 h. As shown in Fig. 2 A-D, low doses of S1P (1 μ M) did not induce a significant inflammatory response 4 h with or without the presence of *A. actinomycetemcomitans*. In contrast, high doses of S1P (2.5 μ M) dose-dependently increased IL-1 β , IL-6, and TNF mRNA expressions in WT BMMs compared with vehicle treatment. With the presence of *A. actinomycetemcomitans*, high doses of S1P (2.5 μ M) also dose-dependently increased COX-2, IL-1 β , IL-6, and TNF mRNA levels compared with those in cells treated with vehicle and *A. actinomycetemcomitans*. These data supported that low doses of S1P (1 μ M) did not have a significant impact on proinflammatory cytokine production 4 h after stimulation, but high doses of S1P (2.5 μ M) could induce a proinflammatory response 4 h after stimulation. To further determine if SK1 deficiency could influence proinflammatory cytokine production in BMMs after bacterial stimulation, SK1 BMMs or WT BMMs were either untreated or stimulated with *A. actinomycetemcomitans* (1.5 CFU/cell) for 8 h. PGE2, IL-1 β , IL-6, and TNF- α protein levels in the cell culture media were quantified by ELISA. As shown in Fig. 2 E-H, we did not observe significant differences of PGE2, IL-1 β , IL-6, and TNF- α protein expressions between the SK1 BMM media and WT BMM media before or after bacterial stimulation. Our data suggested that the S1P concentration in the media of bacteria-stimulated BMMs was probably below 1 μ M, and did not have a significant impact on the proinflammatory cytokine levels induced by *A. actinomycetemcomitans*.

SK1 deficiency significantly attenuated the chemotaxis of BMMs

Previous studies demonstrated that S1P signaling controlled the migration of monocytes and macrophages from the blood to the bones (15, 16). To determine the role of S1P and its interplay with *A. actinomycetemcomitans* in modulating the migration of monocytes and macrophages, we performed a transwell migration assay. First, we put WT BMMs (1×10^5 / well) with culture media in the upper chamber of transwell plates, and put either culture media with vehicle or the media with various concentrations of S1P (125 to 1000 nM) in the lower chamber. After 24 h, we observed a dose-dependent increase of the number of cells that migrated from the top chamber to the bottom chamber (Fig. 3A-B), which demonstrated that low doses of S1P (1 μ M) promoted the chemotaxis of BMMs. Next, we put WT BMMs (1×10^5 /well) with culture media in the upper chamber of transwell plates, and put either *A. actinomycetemcomitans*-stimulated conditioned media derived from WT BMMs, or the *A.*

actinomycetemcomitans-stimulated media with various concentrations of S1P (125 to 1000 nM) in the lower chamber. The addition of both S1P and *A. actinomycetemcomitans*-stimulated media dose-dependently enhanced the number of migrated BMMs compared with that in cells treated with *A. actinomycetemcomitans*-stimulated media alone (Fig. 3A-B). To further determine if SK1 deficiency could affect the chemotaxis of BMMs, we put WT BMMs (1×10^5 /well) with culture media in the upper chamber of transwell plates, and put either the conditioned media derived from SK1 BMMs or WT BMMs with or without *A. actinomycetemcomitans* stimulation for 8 h in the lower chamber. As shown in Fig. 3 C-D, the conditioned media derived from either SK1 KO BMMs or WT BMMs without bacterial stimulation had only a mild effect on the chemotaxis of BMMs. In contrast, *A. actinomycetemcomitans*-stimulated conditioned media derived from WT BMMs significantly enhanced the number of migrated BMMs. Importantly, *A. actinomycetemcomitans*-stimulated conditioned media derived from SK1 KO BMMs significantly attenuated the chemotaxis of BMMs compared with the WT control. These data supported that SK1 deficiency resulted in decreased S1P levels induced by bacterial stimulation, subsequently reducing the migration of monocytes and macrophages (osteoclast precursors).

SK1 KO mice displayed significantly less alveolar bone loss induced by *A. actinomycetemcomitans* compared with WT control mice

As SK1 deficient cells had decreased S1P levels in response to *A. actinomycetemcomitans* compared with WT cells (Fig. 1), we further determined the effects of SK1 deficiency on the pathogenesis of periodontitis. Eight-week-old SK1 KO mice (n=20) and WT mice (n=20) were injected with either *A. actinomycetemcomitans* (2×10^5 CFU, 2 μ L, n=10) or PBS containing 5% glycerol (2 μ L, n=10) at both sides of the palatal gingival rugae near the first maxillary molar three times per week for four weeks. Following this, both sides of the maxillary alveolar bones were scanned by μ -CT and the volume of alveolar bone was quantified. As shown in Fig. 4A, there was no significant bone resorption in either WT or KO mice injected with PBS. In contrast, there was more alveolar bone resorption in WT mice treated with *A. actinomycetemcomitans* compared with SK1 KO mice treated with *A. actinomycetemcomitans*. Alveolar bone volume analysis revealed that SK1 KO mice significantly attenuated alveolar bone loss compared with WT mice after bacterial stimulation (Fig. 4B). These data show that SK1 KO mice attenuated alveolar bone loss induced by *A. actinomycetemcomitans* compared with WT mice.

SK1 KO mice exhibited a significant decrease in the number of periodontal leukocytes and osteoclasts induced by *A. actinomycetemcomitans* compared with WT control mice

To determine the mechanisms associated with the decreased alveolar bone loss induced by *A. actinomycetemcomitans* in SK1 KO mice, histological analysis was performed in periodontal tissue sections derived from SK1 KO and WT mice. H&E and TRAP staining of periodontal tissue sections showed that *A. actinomycetemcomitans* stimulated periodontal leukocyte infiltration and osteoclastogenesis in both SK1 KO and WT mice, as compared with PBS injection (Fig. 5A&B). Importantly, the number of leukocytes infiltrated into periodontal tissues was significantly reduced in *A. actinomycetemcomitans*-treated SK1 KO mice compared with *A. actinomycetemcomitans*-treated WT mice (Fig. 5A&C). In addition,

the number of TRAP-stained multinucleated osteoclasts attached on the alveolar bone was significantly decreased in *A. actinomycetemcomitans*-treated SK1 KO mice compared with *A. actinomycetemcomitans*-treated WT mice (Fig. 5B&D). These data demonstrate that SK1 KO mice attenuated periodontal leukocyte infiltration and osteoclastogenesis induced by *A. actinomycetemcomitans*, as compared with WT mice.

Discussion

SK1 plays an important role in immune response. In the current study, we observed increased SK1 mRNA levels in WT BMMs 2 to 8 h after *A. actinomycetemcomitans* stimulation. In contrast, SK2 mRNA levels remained similar to the basal level after *A. actinomycetemcomitans* stimulation. This difference might be attributable to the localization of the two SK enzymes. SK1 is mostly a cytosolic enzyme in unstimulated cells, and can be translocated to the plasma membrane when cells are stimulated (25). In contrast, SK2 is mainly localized in the nucleus with low levels found in the intracellular compartments (11). This restricted location might limit its accessibility to the stimulation induced by *A. actinomycetemcomitans*. SK1 mRNA levels did not increase immediately following bacterial stimulation. Instead, we observed increased SK1 mRNA levels 2 h after bacterial stimulation. The significant increase of SK1 mRNA following *A. actinomycetemcomitans* stimulation might be associated with the SK1 translocation from the cytoplasm to the plasma membrane. A previous study showed that phosphorylation of SK1 at serine 225 caused the SK1 translocation to the plasma membrane, which resulted in a 14-fold increase of SK1 catalytic activity and subsequent increases in both intracellular and extracellular S1P levels (26). In this study, WT BMMs exhibited significantly higher intracellular S1P levels 4 and 8 h post *A. actinomycetemcomitans* stimulation compared with the S1P levels in SK1 KO BMMs, which was consistent with the higher SK1 mRNA levels in the WT BMMs.

However, we also observed a moderate increase of intracellular S1P in SK1 KO BMMs 4 h after *A. actinomycetemcomitans* stimulation. This moderate increase of S1P in SK1 KO BMMs might be caused by the SK2 activity after bacterial stimulation. Previous studies demonstrated that SK2 shuttled between the nucleus and the cytoplasm, and phosphorylation of SK2 in a nuclear export region caused the translocation of SK2 from the nucleus to the cytoplasm (4, 6). In this study, we only quantified SK2 mRNA levels, but not SK2 protein levels in BMMs. Although *A. actinomycetemcomitans* did not significantly increase SK2 mRNA production, *A. actinomycetemcomitans* might cause the phosphorylation of SK2, leading to exportation of SK2 from the nucleus to the cytoplasm and subsequently influencing SK2 cytoplasmic catalytic activity. However, S1P levels in SK1 KO BMMs decreased 8 h after *A. actinomycetemcomitans* stimulation. Therefore at 8 h after *A. actinomycetemcomitans* stimulation, the phosphorylation of SK2 might be reduced in SK1 KO BMMs, which might cause SK2 to shuttle back from the cytoplasm to the nucleus and subsequently decrease SK2 cytoplasmic catalytic activity. Although we observed significant differences of S1P and sphingosine levels between SK1 KO BMMs and WT BMMs after bacterial stimulation, there were no significant differences of total ceramide levels (data not shown). This could be explained by the quick metabolic transition of ceramide to other sphingolipids. Besides sphingosine, ceramide can be transformed to sphingomyelin,

ceramide-1-phosphate, and glucosylceramide (27, 28). By 4 and 8 h after bacterial stimulation, ceramide could be transformed to other sphingolipids.

In vitro, we demonstrated that low doses of S1P (1 μ M) did not induce a significant inflammatory response after 4 h treatment. However, Eskan et al. (29) showed that although a low dose of S1P (100 nM) did not significantly increase IL-6 and IL-8 production, S1P (100 nM) cooperatively with lipopolysaccharide (1 μ g) enhanced IL-6 and IL-8 levels after a long-term (24 h) treatment in human gingival epithelial cells. Although SK1 KO BMMs had lower levels of S1P after bacterial stimulation, the SK1 KO BMMs had higher levels of sphingosine than WT control. Sphingosine is also a bioactive sphingolipid, which could induce cell apoptosis and might contribute to the proinflammatory response (27). Therefore, the lack of significant differences of proinflammatory cytokine expressions between SK1 KO BMM media and WT BMM media might be caused to some extent by the high levels of sphingosine in SK1 KO cells. In addition, the S1P concentration in the media might be below 1 μ M. Therefore, it might not have a significant impact on the proinflammatory cytokine production.

In contrast, we demonstrated that low doses of S1P (1 μ M) significantly enhanced the chemotaxis of BMMs. SK1 deficiency significantly reduced the chemotaxis of BMMs compared with WT control. Our data agree with previous studies (15, 16) supporting that S1P signaling is critical for modulating the migration of monocytes and macrophages (osteoclast precursors). *In vivo*, we observed a significant decrease of periodontal leukocytes and osteoclasts in *A. actinomycetemcomitans*-treated SK1 KO animals compared with those in *A. actinomycetemcomitans*-treated WT mice. This might be caused mainly by the chemotaxis effect of S1P. An increase of S1P in the periodontal tissues induced by *A. actinomycetemcomitans* might change the S1P gradient between the blood and the periodontal tissues, resulting in migration of monocytes and macrophages from blood and bone marrow to periodontal tissues. These cells could further differentiate and fuse to form multinucleated osteoclasts.

Previous studies demonstrated that the migration of monocytes and macrophages was mainly regulated by S1PR1 and S1PR2 (16, 30). S1PR1 mediated the positive chemotaxis toward a high concentration of S1P, whereas S1PR2 directed a negative chemotaxis (or chemorepulsion) response, which promoted the migration of osteoclast precursors from the blood to the bone tissues (16, 30). Therefore, the expression of S1PR1 and S1PR2 also impact the migration of monocytes and macrophages between the blood and the bone tissues. Additionally, previous studies showed that lipopolysaccharide or TNF- α increased S1PR2 expression, but not S1PR1 expression in human endothelial cells (31). Furthermore, IL-6 induced the mRNA expression of S1PR2, but not S1PR1 mRNA expression in osteoclast precursors (32). Therefore, the inflammatory bone loss response in this study might also be influenced by the expression of S1PR1 and S1PR2 in the periodontal tissues. Future studies are needed to determine if *A. actinomycetemcomitans* might up-regulate S1PR2 expression and subsequently influence the migration of osteoclast precursors.

Besides controlling the migration of osteoclast precursors, previous studies reported that S1P served as a regulator for osteoclast differentiation and osteoclast-osteoblast coupling

(33). S1P increased the expression of the receptor activator of NF-kappaB ligand (RANKL) in osteoblasts (33), synoviocytes from rheumatoid arthritis patients, and CD4⁺ T cells (34). RANKL is an essential osteoclastogenic factor, which binds with RANK on osteoclast precursors, initiating the differentiation of osteoclasts (35). Additionally, Ryu et al. showed that S1P enhanced osteoclastogenesis in a co-culture of osteoblasts with BMMs (33). However, others have demonstrated that S1P also increased the expression of osteoprotegerin (OPG), a soluble decoy receptor of RANKL, in osteoblasts (36). Future studies are needed to determine how S1P regulates osteoclastogenesis and which of the S1PRs play a major role in modulating bone homeostasis.

Previously, Baker et al. (19) demonstrated that SK1 deficiency significantly decreased synovial inflammation and joint erosions in murine TNF- α -induced arthritis. They (19) showed that human TNF/SK1 KO mice had less synovial and periarticular inflammation, significantly fewer mature osteoclasts in the ankle joints, and markedly decreased bone erosion compared with control WT mice. Our study was in agreement with Baker et al., and we further demonstrated that deficiency of SK1 in mice attenuated periodontal leukocyte infiltration and alveolar bone loss induced by *A. actinomycetemcomitans*. Additionally, Snider et al. demonstrated that SK1 KO mice significantly reduced dextran sulfate sodium-induced colitis compared with WT mice (37). These studies demonstrated the important role of SK1 in modulating the inflammatory response. Although we did not observe significant differences of proinflammatory cytokine levels *in vitro*, we observed fewer infiltrated leukocytes in the periodontal tissues of SK1 KO mice compared with WT control, which could affect the proinflammatory cytokine production *in vivo*. Furthermore, mice treated with a SK1 inhibitor, N, N-dimethylsphingosine, inhibited collagen-induced arthritis (38). A selective SK2 inhibitor, ABC249640, also attenuated both collagen-induced arthritis in mice, as well as adjuvant-induced arthritis in rats (39). These studies support that inhibition of SK activity might be a promising therapeutic approach for inflammatory bone loss diseases.

In this study, the mice were sacrificed two days after the last *A. actinomycetemcomitans* or PBS injection. The S1P levels in periodontal tissues were below detection (data not shown). As shown previously, the S1P levels peaked at 4 h and were reduced at 8 h after bacterial stimulation, which suggested that S1P was mainly produced during an acute phase of inflammation. The inflammatory response might diminish two days after the last bacterial injection, which could cause a reduction in S1P generation. In addition, S1P in tissues could be degraded by S1P lyase or converted to sphingosine by S1P phosphatase two days after the last bacterial injection. In this study, although there was a trend toward higher S1P levels in the serum of WT mice, there were no significant differences of serum S1P levels between SK1 KO and WT animals in different treatment groups (data not shown), which suggested that localized periodontal inflammation did not affect the S1P generation in the blood.

In humans, periodontal diseases initiate with the adherence and colonization of oral pathogens on the gingival epithelium. With disease progression, the inflammatory response induced by oral pathogens leads to soft tissue damage and alveolar bone loss. By injecting the oral pathogen *A. actinomycetemcomitans* in the periodontal tissues of mice, we induced a quick localized inflammatory bone loss response in animals. However, our animal model

has a limited ability to determine the role of S1P on bacterial adherence and colonization on the gingival epithelium. Previous studies demonstrated that S1P cooperated with bacterial lipopolysaccharide to induce the expressions of cell adhesion molecules in human endothelial cells, which enhanced leukocyte attachment to endothelial cells (40). Additionally, the enhanced expressions of cell adhesion molecules in stimulated endothelial cells promoted the adherence of the oral pathogen *Porphyromonas gingivalis* and leukocyte attachment to endothelial cells (41-43). Future studies are needed to determine if S1P is able to affect the expression of cell adhesion molecules in gingival epithelial cells and subsequently influence bacterial adherence and leukocyte attachment to gingival epithelial cells.

In summary, our study is the first to demonstrate that oral pathogen *A. actinomycetemcomitans* activated SK1 leading to S1P generation. *In vitro*, deficiency of SK1 significantly attenuated S1P generation induced by *A. actinomycetemcomitans* and significantly decreased the chemotaxis of monocytes and macrophages. *In vivo*, SK1 KO mice showed significantly attenuated alveolar bone resorption induced by *A. actinomycetemcomitans* stimulation compared with *A. actinomycetemcomitans*-treated WT animals. Histological analysis revealed that SK1 deficiency significantly reduced the number of periodontal leukocytes and mature osteoclasts induced by *A. actinomycetemcomitans* stimulation compared with *A. actinomycetemcomitans*-treated WT animals. Our studies support that SK1 and S1P play an important role in regulating the inflammatory bone loss response induced by oral pathogen *A. actinomycetemcomitans*. Therefore, decreasing S1P generation by inhibiting SK1 might be a novel therapeutic strategy for periodontitis and other inflammatory bone loss diseases.

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Fig. 1. SK1 deficiency significantly decreased S1P production induced by *A. actinomycetemcomitans* (Aa) in BMMs. Murine BMMs derived from SK1 KO or WT mice were either untreated or stimulated with *A. actinomycetemcomitans* (1.5 CFU/cell) for 1 to 8 h. (A) S1P biosynthesis and degradation pathways. (B) SK1 mRNA expression in BMMs. (C) SK2 mRNA expression in BMMs. (D) Intracellular S1P levels in BMMs. (E) Intracellular sphingosine levels in BMMs. The sample mRNA levels were normalized to GAPDH mRNA expression. S1P and sphingosine levels were normalized by protein concentrations in the cell lysate. The data are representatives from three separate experiments (n=3, * $P < 0.05$).

Fig. 2.

SK1 deficiency in BMMs had no significant impact on proinflammatory cytokine levels induced by *A. actinomycetemcomitans* (Aa). Murine WT BMMs were treated for 4 h with either vehicle (PBS containing 4mg/mL of fatty acid-free BSA), various concentrations of S1P (0.25 to 5 μM), with or without *A. actinomycetemcomitans* (1.5 CFU/cell) for COX-2, IL-1β, IL-6, and TNF mRNA assays. SK1 KO BMMs or WT BMM were either untreated or stimulated for 8 h with *A. actinomycetemcomitans* (1.5 CFU/cell) for PGE2, IL-1β, IL-6, and TNF-α protein assays. (A) COX-2 mRNA expression in BMMs. (B) IL-1β mRNA

expression in BMMs. (C) IL-6 mRNA expression in BMMs. (D) TNF mRNA expression in BMMs. (E) PGE2 protein expression in the media of BMMs. (F) IL-1 β protein expression in the media of BMMs. (G) IL-6 protein expression in the media of BMMs. (H) TNF- α protein expression in the media of BMMs. The sample mRNA levels were normalized to GAPDH mRNA expression. The data are representatives from three separate experiments (n=3, * $P < 0.05$).

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Fig. 3.

SK1 deficiency significantly attenuated the chemotaxis of BMMs. The transwell migration assay was performed as described in Materials and Methods. (A) Representative images of migrated BMMs treated with vehicle or various concentrations of S1P, with or without *Aa*-stimulated media from WT BMMs. (B) Migration index of BMMs treated with vehicle, S1P (125 to 1000 nM), with or without the presence of *Aa*-stimulated media from WT BMMs. (C) Representative images of migrated BMMs treated with the conditioned media derived from SK1 KO BMMs or WT BMMs with or without bacterial stimulation. (D) Migration index of BMMs treated with the conditioned media derived from SK1 KO BMMs or WT BMMs with or without bacterial stimulation. Pictures were taken with 400 $\times$ magnification. The data are representatives from three separate experiments ($n=3$, $*P < 0.05$; $**P < 0.01$).

Fig. 4. SK1 KO mice showed significantly attenuated alveolar bone loss induced by *A. actinomycetemcomitans* (Aa) as compared with *A. actinomycetemcomitans*-treated WT mice. SK1 WT and KO mice were injected with PBS or *A. actinomycetemcomitans* in the periodontal tissues for 4 weeks to induce periodontitis as described in Materials and Methods. (A) Representative μ -CT images of alveolar bone. (B) Bone volume fraction of alveolar bone (n=10, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$).

Fig. 5.

SK1 KO mice exhibited a significant decrease in the number of periodontal leukocytes and osteoclasts induced by *A. actinomycetemcomitans* (Aa) as compared with *A. actinomycetemcomitans*-treated WT mice. SK1 WT and KO mice were injected with PBS or *A. actinomycetemcomitans* in the periodontal tissues for 4 weeks to induce periodontitis as described in Materials and Methods. (A) Representative images of H&E staining of periodontal tissue sections. (B) Representative images of TRAP staining of periodontal tissue sections. Pictures were taken under 200× magnification. The scale bars represent 50 μ M. (C) Number of periodontal leukocytes. (D) Number of TRAP⁺ multinucleated osteoclasts on the surface of alveolar bone (n=10, * P < 0.05; ** P < 0.01; *** P < 0.001).