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Agomelatine, a MT_1/MT_2 melatonergic receptor agonist with serotonin 5-HT_{2C} receptor antagonistic properties, suppresses *Prevotella intermedia* lipopolysaccharide-induced production of proinflammatory mediators in murine macrophages

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ABSTRACT

Objective: This study was performed in an attempt to examine the influence of agomelatine in mitigating the generation of proinflammatory mediators in RAW264.7 murine macrophages exposed to lipopolysaccharide (LPS) obtained from *Prevotella intermedia*, a gram-negative anaerobic bacterium that is related with various types of periodontal diseases, and the molecular mechanisms behind its effects.

Design: LPS from *P. intermedia* strain ATCC 25611 was prepared employing the conventional phenol-water procedure. Conditioned culture media were analyzed for the levels of nitric oxide (NO), interleukin-1β (IL-1β) and IL-6. Real-time PCR analysis was carried out to determine the mRNA levels of inducible NO synthase (iNOS), IL-1β, IL-6 and SOCS1. Protein expression levels were evaluated by immunoblot test. NF-κB-dependent SEAP reporter assay was performed using a reporter cell line. DNA-binding activities of NF-κB subunits were analyzed utilizing the ELISA-based kits.

Results: Agomelatine was found to down-regulate significantly the generation of iNOS-derived NO, IL-1β and IL-6 as well as the expression of their mRNAs in cells activated with *P. intermedia* LPS. Agomelatine decreased NF-κB-dependent SEAP release caused by *P. intermedia* LPS. Agomelatine did not inhibit NF-κB transcription induced by LPS at the level of IκB-α degradation. Instead, LPS-induced nuclear translocation and DNA binding of NF-κB p50 subunit was blocked by agomelatine. *P. intermedia* LPS-elicited activation of STAT1 and STAT3 was reduced notably by co-treatment with agomelatine. Agomelatine showed a tendency to enhance mRNA level of SOCS1 in LPS-activated cells as well.

Conclusions: Agomelatine merits further evaluation to reveal its usefulness on the host modulation of periodontal disease.

1. Introduction

Periodontal disease is an inflammatory disorder affecting the tooth-supporting structures and several gram-negative anaerobic bacterial species are key etiological agents (Williams, 1990). Periodontal disease has also been demonstrated to be a possible risk factor for a number of systemic diseases, which include cardiovascular diseases (Stewart & West, 2016), type 2 diabetes (Bascones-Martínez, González-Febles, & Sanz-Esporrín, 2014) and stroke (Lafon et al., 2014). It is well recognized that tissue breakdown in periodontal disease is primarily dependent on the immune-inflammatory response of host to several potential periodontopathogens found in the subgingival bacterial biofilm. Thus, various treatment strategies have been introduced lately to modulate the deleterious host responses that are

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responsible for periodontal tissue destruction (Preshaw, 2008; Reddy, Geurs, & Gunsolley, 2003).

Prevotella intermedia, a gram-negative anaerobic bacterium, has long been known to be related with various kinds of periodontal diseases, including chronic periodontitis (Braga et al., 2010; Estrela, Pimenta, Alencar, Ruiz, & Estrela, 2010), aggressive periodontitis (Kamma, Nakou, Gmur, & Baehni, 2004), necrotizing ulcerative gingivitis (Chung, Nisengard, Slots, & Genco, 1983) and hormone-induced pregnancy gingivitis (Kornman & Loesche, 1980). In addition to periodontal disease, this bacterium has also been shown to be associated with endodontic infections (Jacinto, Gomes, Ferraz, Zaia, & Filho, 2003) and noma (Bolivar et al., 2012). Several lines of evidence support the notion that *P. intermedia* induces various inflammatory and immune responses pivotal in periodontal tissue destruction (Guan et al., 2009; Jansen, Grenier, & Van der Hoeven, 1995; Okuda, Ono, & Kato, 1989).

Lipopolysaccharide (LPS) is the major cell wall element of gram-negative bacterial species. It is one of the most effective inducers of proinflammatory mediators, which include nitric oxide (NO), interleukin 1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α), in macrophages and mononuclear cells (Morrison & Ryan, 1987). Our previous studies provide evidence that *P. intermedia* LPS has the capacity to promote the generation of NO and inflammatory cytokines in macrophages (Kim, Ha, Choi, Choi, & Choi, 2004; Kim et al., 2007; Choi et al., 2011; Choi, Jin, Choi, Choi, & Kim, 2014).

Hamada et al. (1990) found that the function and structure of *P. intermedia* LPS is distinct from those of LPS prepared from *Escherichia coli* and *Salmonella* species. Additionally, the findings of Kirikae et al. (1999) show that, quite unlike *Salmonella* LPS, LPS prepared from *P. intermedia* induced the activation of macrophages of LPS-resistant C3H/HeJ mice. Besides, whereas the biological activities of LPS prepared from enteric bacteria were blocked by antimicrobial peptide polymyxin B, *P. intermedia* LPS was not susceptible to this antimicrobial peptide (Kirikae et al., 1999).

Agomelatine, a structural analog of melatonin, is the first melatonergic antidepressant drug (Olie & Kasper, 2007; de Bodinat et al., 2010). It has both melatonin MT_1 and MT_2 receptor agonistic and serotonin 5-HT_{2C} receptor antagonistic activities (Conway et al., 2000; Millan et al., 2003). Our previous study (Choi et al., 2011) showed that melatonin strongly attenuates NO and IL-6 production at both gene transcription and translation levels in RAW264.7 cells activated by *P. intermedia* LPS. LPS-induced nuclear translocation and DNA binding activity of NF-κB p50 subunit was blocked by melatonin. Melatonin attenuated STAT1 signaling as well.

Agomelatine shows a longer half-life and a comparatively higher affinity as an agonist at MT_1 and MT_2 melatonin receptors compared to melatonin (Delagrange & Boutin, 2006). This compound has been reported to possess potentially beneficial properties similar to those of melatonin, such as antioxidant and anti-inflammatory activities (Aguiar et al., 2013; Molteni et al., 2013). Thus, this melatonin analog may useful as putative drug for the treatment of periodontal disease via attenuating inflammatory response. However, the efficacy of agomelatine on inflammatory periodontal disease has not been studied so far. In the present work, we tried to assess the influences and potential mechanisms of agomelatine in mitigating the generation of inflammatory mediators in murine macrophages activated by LPS obtained from *P. intermedia*, a pathogenic bacterial species that is closely associated with various types of periodontal diseases.

2. Material and methods

2.1. Reagents

Agomelatine was acquired from Selleckchem (Houston, TX, USA). Primary antibodies recognizing p38 (#9212), phospho-p38 (#9215), JNK (#9258), phospho-JNK (#4668), IκB-α (#4814), STAT1 (#9172), phospho-STAT1 (#7649), STAT3 (#9139) and phospho-STAT3 (#9145) were bought from Cell Signaling Technology (Beverly, MA, USA), while antibodies against iNOS (sc-650), NF-κB p50 (sc-7178), NF-κB p65 (sc-372), β-actin (sc-1616) and PCNA (sc-7907) were the products of Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the other reagents were purchased from Sigma-Aldrich except stated otherwise.

2.2. Bacterial culture and purification of LPS

P. intermedia strain ATCC 25611 was grown anaerobically at 37 °C in GAM broth (Nissui, Tokyo, Japan). As previously described (Choi et al., 2011), LPS was purified from lyophilized *P. intermedia* cells utilizing the conventional phenol-water extraction procedure described by Westphal and Jann (1965).

2.3. Cell culture and cytotoxicity assay

RAW264.7 murine macrophage cell line, which was obtained from the American Type Culture Collection (Rockville, MD, USA), was cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated FBS and antibiotics (100 μg/ml of streptomycin and 100 U/ml of penicillin) at 37 °C in a 5% CO_2 humidified atmosphere, as reported previously (Choi et al., 2011). The effect of agomelatine on the cell viability was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in accordance with the manufacturer's protocols. In brief, cells were incubated with different dosages of agomelatine with or without *P. intermedia* LPS for 24 h, and MTT was added to the cultures. After incubation for 2 h, the cells were solubilized in dimethyl sulfoxide. Then, the conversion of MTT to formazan within the cells was estimated by measuring the optical density at a wavelength of 570 nm.

2.4. Assay of NO generation

The levels of NO in the culture supernatants were analyzed by determining the quantity of nitrite (NO_2^-), a stable metabolite of NO, following a procedure described (Choi et al., 2011). Briefly, cells were plated at a density of 5×10^5 cells per well in 24-well culture plates, followed by incubation with different dosages of agomelatine (0, 100, 200, 400 and 600 μM) with or without *P. intermedia* LPS (10 μg/ml) for 24 h. An incubation time of 24 h was selected because *P. intermedia* LPS-elicited generation of NO reached a maximum at that time point (data not shown). Following incubation, aliquots of 100 μl of culture supernatant were reacted with the equivalent volume of Griess reagent for 10 min, and the absorbance was determined at a wavelength of 540 nm. Nitrite concentration was estimated by employing a calibration curve prepared with known concentration of $NaNO_2$.

2.5. Assay of IL-1β and IL-6 generation

Cells were plated at a density of 5×10^5 cells per well in 24-well culture plates, followed by incubation with different dosages of agomelatine (0, 100, 200, 400 and 600 μM) with or without *P. intermedia* LPS (10 μg/ml) for 24 h (for IL-6) or 48 h (for IL-1β), after which the levels of cytokines in the culture supernatants were estimated by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (OptEIA; BD Pharmingen, San Diego, CA, USA). The incubation times were selected because *P. intermedia* LPS-elicited generation of IL-6 and IL-1β reached a maximum at those time points (data not shown).

2.6. Real-time polymerase chain reaction analysis

Cells were plated in 100 mm tissue culture dishes at a density of 1×10^7 cells per dish and treated with indicated dosages of agomelatine (0, 100, 200, 400 and 600 μM) in the absence or presence of *P.*

intermedia LPS (10 μg/ml) for 1 h (for SOCS1), 6 h (for IL-1β) or 24 h (for iNOS and IL-6). These incubation times were chosen because maximal effects were found at those time points (data not shown). Following incubation, real-time PCR analysis was performed by a previously described procedure (Choi et al., 2011). Briefly, total RNA was extracted from the treated cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the maker's protocols. Extracted RNA (1 μg) was reverse transcribed into cDNA utilizing an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), and cDNA was amplified by using the CFX96 real-time PCR detection system (Bio-Rad) and SsoFast EvaGreen Supermix (Bio-Rad). The ensuing real-time PCR requirements were applied: After denaturing at 98 °C for 30 s, PCR was carried out for 45 cycles, each of which consisted of denaturing at 95 °C for 1 s, annealing/extending at 60 °C for 5 s. The sequences for the specific oligonucleotide primers utilized were as follows: iNOS (130 bp) forward, 5′-GCACCACCCTCCTCGTTCAG-3′ and reverse, 5′-TCCACAACTCGCTCCAAGATTCC-3′; IL-1β (131 bp) forward, 5′-TTCAGGCAGGCAGTATCA-3′ and reverse, 5′-AGGATGGGCTCTTCTTCAA-3′; IL-6 (162 bp) forward, 5′-GCCAGAGTCCTTCAGAGAGATACAG-3′ and reverse, 5′-GAATTGGATGGTCTTGGTCCTTAGC-3′; SOCS1 (133 bp) forward, 5′-CACTTCTGGCTGGAGACC-3′ and reverse, 5′-TGGAGAGGTAGGAGTGGAA-3′; β-actin (149 bp) forward, 5′-TGAGAGGGAAATCGTGCGTGAC-3′ and reverse, 5′- GCTCGTTGCCAATAGTGATGACC-3′. The levels of the target gene expression were normalized against that of β-actin.

2.7. Immunoblot analysis

Cells were plated in 60-mm tissue culture dishes at a density of 4×10^6 cells per dish and incubated with indicated dosages of agomelatine (0, 100, 200, 400 and 600 μM) with or without *P. intermedia* LPS (10 μg/ml) for 15 min (for p38), 30 min (for JNK, IκB-α and NF-κB p65), 4 h (for STAT1 and STAT3), 8 h (for NF-κB p50) or 24 h (for iNOS). These incubation times were selected because maximum effects were achieved at those time points (data not shown). Then, immunoblot analysis was performed according to conventional methods as described (Choi et al., 2011). Briefly, nuclear proteins were isolated from the incubated cells employing the nuclear extract kit (Active Motif, Carlsbad, CA, USA). To isolate whole-cell lysates, cells were lysed by incubating for 30 min in cold lysis buffer (50 mM Tris·Cl, 150 mM NaCl, 0.002% sodium azide, 0.1% SDS, 1% Nonidet P-40) containing protease inhibitors. Protein extracts (30 μg) were subjected to separation on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. Immunoblots were probed with the appropriate primary antibodies, after which they were treated with secondary antibodies. Development of blots were carried out utilizing the enhanced chemiluminescence reagent (Cell Signaling Technology).

2.8. NF-κB-dependent secretory alkaline phosphatase (SEAP) reporter assay

RAW-Blue cells (Invivogen, San Diego, CA, USA), RAW264.7 cells stably expressing the gene for secreted embryonic alkaline phosphatase (SEAP) inducible by NF-κB transcription factor, were cultivated in DMEM under the selection of Zeocin (Invivogen). Cells were plated in 96-well culture plates at a density of 1×10^5 cells per well and treated with indicated dosages of agomelatine (0, 100, 200, 400 and 600 μM) with or without *P. intermedia* LPS (10 μg/ml) for 24 h. Then, the culture supernatants were subjected to SEAP secretion assay according to the protocols supplied by the manufacturer. Briefly, aliquots of 50 μl of cultured supernatant were reacted with 150 μl of SEAP detection medium (Quanti-Blue; Invivogen) in 96-well culture plate for 30 min at 37 °C to allow color development. Then, the optical density was estimated at a wavelength of 630 nm.

2.9. DNA-binding of NF-κB subunits

Cells were treated with indicated dosages of agomelatine (0, 400, and 600 μM) with or without *P. intermedia* LPS (10 μg/ml) for 30 min (for NF-κB p65) or 8 h (for NF-κB p50). These incubation times were chosen because maximal DNA-binding activities were found at those time points after LPS addition (data not shown). Following incubation, nuclear extracts were isolated and assessed for DNA-binding utilizing the ELISA-based TransAM NF-κB Transcription Factor Assay Kits (Active Motif) (Choi et al., 2011).

2.10. Statistical analysis

Data are depicted as means ± S.E.M. One-way analysis of variance (ANOVA) was utilized to test the significant differences within the groups. The comparison between two groups was ascertained by Tukey's *post-hoc* comparisons. P values of < 0.05 were considered statistically significant.

3. Results

3.1. Effects of agomelatine on the generation of NO, IL-1β and IL-6 by P. intermedia LPS-treated cells

The amounts of NO, IL-1β and IL-6 were remarkably enhanced by stimulation of RAW264.7 cells with *P. intermedia* LPS (10 μg/ml) as compared to untreated cells, and treatment of LPS-stimulated cells with agomelatine effectively decreased the synthesis of these proinflammatory mediators in a dosage-dependent mode (Fig. 1). The suppression of NO and IL-6 generation at 600 μM of agomelatine was about 67 and 90%, respectively, whereas this compound reduced IL-1β release in LPS-stimulated macrophages up to 87% at 400 μM. As agomelatine did not have any appreciable influence on cell viability at the concentrations up to 600 μM as determined by MTT assay, it is unlikely that the suppressive effect of agomelatine results from its direct cytotoxic effect (data not shown). Consistent with the observed inhibitory effects on NO release, agomelatine treatment also leads to a dose-dependent suppression of iNOS protein that was strongly augmented by *P. intermedia* LPS stimulation (Fig. 2). Additionally, similar to its influences on *P. intermedia* LPS-triggered release of NO, IL-1β and IL-6, agomelatine dose-dependently repressed the mRNA levels of these inflammatory mediators too (Fig. 3).

3.2. Effects of agomelatine on P. intermedia LPS-induced p38 and JNK phosphorylation

The p38, JNK, NF-κB and JAK2/STAT1 pathways have been implicated in *P. intermedia* LPS-elicited generation of NO and IL-6 in RAW264.7 cells (Choi et al., 2011). These signaling pathways, except JNK, also mediate IL-1β synthesis triggered by LPS from *P. intermedia* (Choi et al., 2015). In addition, the STAT3 pathway takes part in *P. intermedia* LPS-elicited release of NO, IL-1β and IL-6 (Choi et al., 2015). First, we determined whether agomelatine is capable of disrupting the activation of p38 and JNK elicited by *P. intermedia* LPS. As shown in Fig. 4, immunoblot analysis of cell lysates demonstrated that agomelatine did not interfere with p38 and JNK phosphorylation elicited by LPS, suggesting that the inhibitory effects of this melatonin analog on *P. intermedia* LPS-elicited release of NO, IL-1β and IL-6 are not due to the inactivation of these signaling molecules.

3.3. Effects of agomelatine on P. intermedia LPS-induced activation of NF-κB

We also tested the effect of agomelatine on NF-κB signaling that is known to mediate *P. intermedia* LPS-induced synthesis of NO, IL-1β and IL-6. NF-κB-dependent SEAP reporter assay was carried out using RAW-

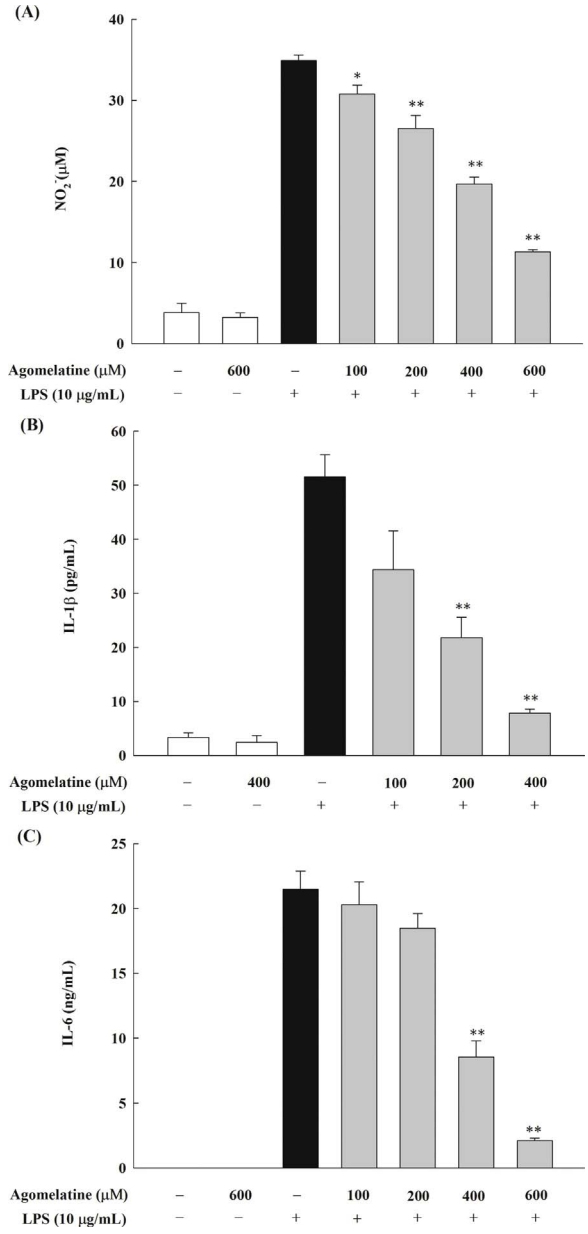

Fig. 1. Effects of agomelatine on the production of NO (A), IL-1β (B) and IL-6 (C) in RAW264.7 cells activated by *Prevotella intermedia* LPS. Cells were incubated with various concentrations of agomelatine in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 24 h (for NO and IL-6) or 48 h (for IL-1β), after which the levels of NO, IL-1β and IL-6 in the culture media were determined. The results are means ± S.E.M. of three independent experiments. $^{*}P < 0.05$ versus *P. intermedia* LPS alone; $^{**}P < 0.01$ versus *P. intermedia* LPS alone.

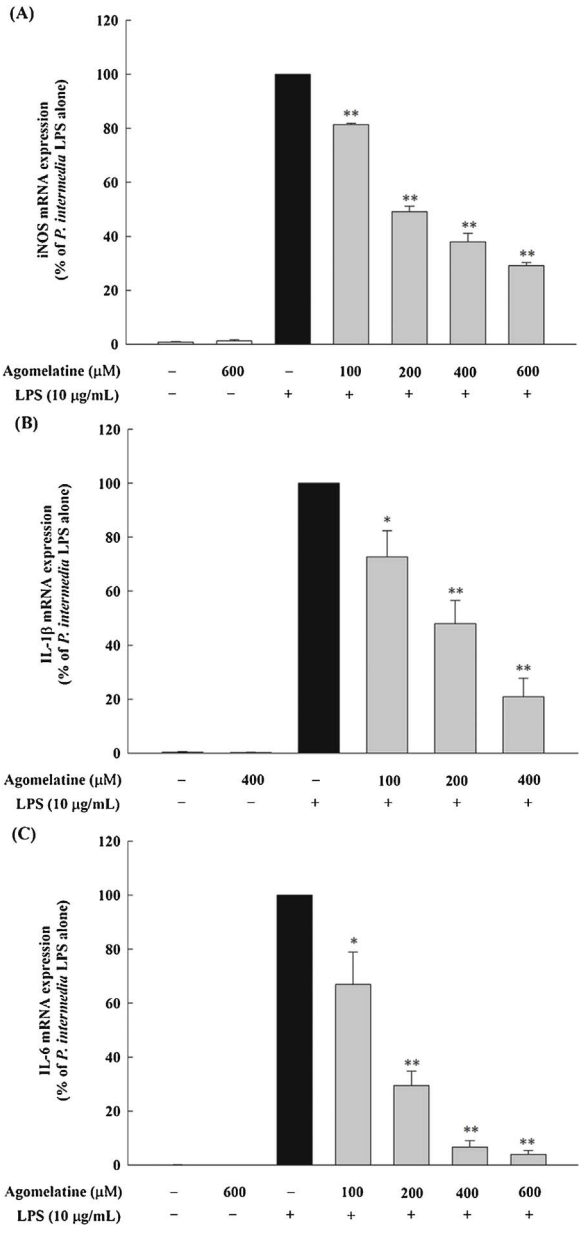

Fig. 3. Effects of agomelatine on iNOS (A), IL-1β (B) and IL-6 (C) mRNA expression induced by *Prevotella intermedia* LPS in RAW264.7 cells. Cells were incubated with various concentrations of agomelatine in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 6 h (for IL-1β) or 24 h (for iNOS and IL-6), after which real-time PCR was performed with EvaGreen Supermix. The results are means ± S.E.M. of three independent experiments. $^{*}P < 0.05$ versus *P. intermedia* LPS alone; $^{**}P < 0.01$ versus *P. intermedia* LPS alone.

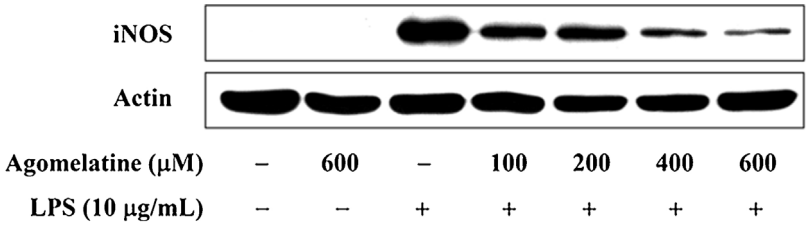

Fig. 2. Effect of agomelatine on iNOS protein expression induced by *Prevotella intermedia* LPS in RAW264.7 cells. Cells were incubated with various concentrations of agomelatine in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 24 h, after which iNOS protein synthesis was determined by immunoblot analysis of cell lysates using iNOS-specific antibody. A representative immunoblot from three separate experiments with similar results is shown.

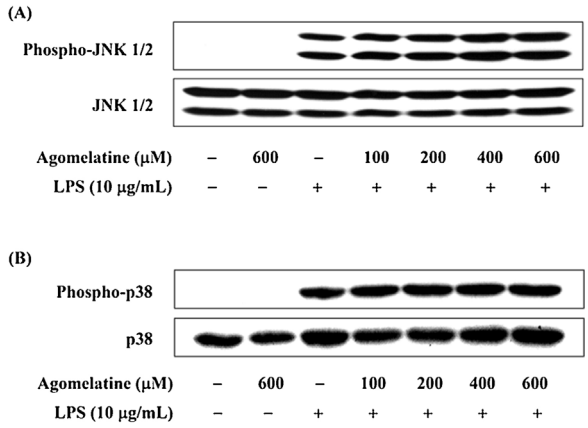

Fig. 4. Effects of agomelatine on the phosphorylation of p38 and JNK induced by *Prevotella intermedia* LPS in RAW264.7 cells. Cells were incubated with various concentrations of agomelatine in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 15 min (for p38) or 30 min (for JNK), after which cells lysates were subjected to immunoblot analysis using specific antibodies. A representative immunoblot from three separate experiments with similar results is shown.

Blue reporter cell line to determine if agomelatine is capable of suppressing NF-κB transcriptional activity. The data presented in Fig. 5A clearly show that incubation of RAW-Blue cells with LPS isolated from *P. intermedia* for 24 h enhanced NF-κB-dependent SEAP release by about 15-fold compared with the resting cells and agomelatine dose-dependently decreased SEAP release induced by LPS. In addition, immunoblot analysis was performed to ascertain whether agomelatine inhibited *P. intermedia* LPS-elicited generation of inflammatory mediators by its effect on IκB-α degradation. Evaluation of the cytoplasmic content of IκB-α protein indicated that IκB-α degradation induced by LPS from *P. intermedia* was not hampered by agomelatine (Fig. 5B). We next determined the influence of agomelatine on the nuclear content of NF-κB subunits in cells activated with *P. intermedia* LPS. Whereas agomelatine dosage-dependently blocked *P. intermedia* LPS-induced translocation of NF-κB p50 subunit into the nucleus, nuclear translocation of p65 elicited by LPS was not impeded in the presence of agomelatine (Fig. 5C). We further investigated whether agomelatine could interfere with the DNA-binding of NF-κB subunits in *P. intermedia* LPS-stimulated cells. p65 and p50 binding to DNA was notably enhanced by *P. intermedia* LPS treatment (Fig. 5D). While treatment with agomelatine induced a notable reduction in DNA-binding of p50 induced by LPS, agomelatine did not influence p65 binding activity (Fig. 5D).

3.4. Effects of agomelatine on P. intermedia LPS-induced phosphorylation of STAT1 and STAT3

We further examined the possibility that agomelatine attenuates elaboration of NO, IL-1β and IL-6 elicited by *P. intermedia* LPS by a mechanism that involves inhibition of STAT1 and STAT3. LPS-tirggered activation of STAT1 and STAT3 was reduced profoundly by co-treatment with agomelatine, indicating the potential role of these members of STAT family in the possible mechanism of agomelatine in suppressing NO, IL-1β and IL-6 release (Fig. 6).

3.5. Effect of agomelatine on P. intermedia LPS-induced expression of SOCS1

Finally, we tested whether agomelatine could regulate the SOCS signaling that is known to provide negative feedback to cytokine signal transduction. Agomelatine showed a tendency to enhance, in a dose-dependent mode, SOCS1 mRNA induction in *P. intermedia* LPS-activated cells (Fig. 7).

4. Discussion

Host's immune response against periodontal pathogenic bacteria and their virulence factors results in excessive secretion of an array of inflammatory mediators, which include NO, IL-1β, IL-6 and TNF-α, by the immune and resident cells of periodontal tissues (Rossomando, Kennedy, & Hadjimichael, 1990; Stashenko et al., 1991; Geivelis, Turner, Pederson, & Lamberts, 1993; Matejka, Partyka, Ulm, Solar, & Sinzinger, 1998; Mogi et al., 1999), and current evidence shows that these mediators have a definitive role in bone destruction (Tatakis, 1993; Kobayashi et al., 2000; Pelt, Zimmermann, Ulbrich, & Bernimoulin, 2002; Liu, Kirschenbaum, Yao, & Levine, 2005). Accordingly, host modulatory strategies targeting these damaging mediators are thought to be crucial in the preventive and therapeutic intervention of periodontal disease.

This study analyzed the impacts of agomelatine on the generation of proinflammatory mediators in *P. intermedia* LPS-activated murine macrophages. *P. intermedia* LPS-induced generation of proinflammatory mediators in RAW264.7 cells attained a maximum at a concentration of 10 μg/ml. Thus, 10 μg/ml of *P. intermedia* LPS was chosen as an inflammatory stimulus in this study. In this work, agomelatine was found to down-regulate significantly the release of iNOS-derived NO, IL-1β and IL-6 as well as the expression of their mRNAs in murine macrophages activated with *P. intermedia* LPS. Our results indicate that agomelatine suppressed these proinflammatory mediators at both gene transcription and translation levels. In this study, we also tested whether agomelatine plays a role in regulating *P. intermedia* LPS-induced generation of TNF-α. However, agomelatine didn't suppress TNF-α production (data not shown).

Our previous study shows that the JNK and p38 pathways are implicated in the generation of NO and IL-6 in murine macrophages activated by *P. intermedia* LPS (Choi et al., 2011). The p38 pathway also mediates *P. intermedia* LPS-elicited elaboration of IL-1β in macrophages (Choi et al., 2015). Accordingly, we analyzed the likelihood that the inhibitory influencies of agomelatine on LPS-elicited elaboration of NO, IL-1β and Il-6 are due to the blockade of p38 and JNK phosphorylation. However, agomelatine did not hamper the activation of p38 and JNK elicited by LPS prepared from *P. intermedia*, indicating that these pathways are not related with the suppressive influences of agomelatine on the generation of proinflammatory mediators.

As a critical transcription factor, the NF-κB family of proteins plays a critical role in the expression of several proinflammatory genes (Baeuerle & Henkel, 1994; Rothwarf & Karin, 1999; Karin & Ben-Neriah, 2000). The modulation of this pathway by bioactive substances could be a potential application in the therapy of inflammatory diseases. In this study, agomelatine decreased NF-κB-dependent SEAP release elicited by *P. intermedia* LPS. Agomelatine notably reduced *P. intermedia* LPS-induced nuclear translocation and DNA binding activity of p50 subunits without affecting IκB-α degradation.

Another important transcription factor that has been implicated to play pivotal roles in the regulation of inflammatory reactions induced by bacterial LPS is signal transducer and activator of transcription (STAT) (Pfitzner, Kliem, Baus, & Litterst, 2004). Among the STAT members, STAT 1 and STAT3 are major regulators which modulate the transcription of target genes encoding diverse proinflammatory mediators in LPS-triggered macrophages (Gao et al., 1998; Samavati et al., 2009). Members of the STAT signaling are typically triggered through Janus kinases (JAKs) through the tyrosine phosphorylation cascade. We found that agomelatine suppressed the generation of NO, IL-1β and IL-6 from *P. intermedia* LPS-activated RAW264.7 cells by via blockade of STAT1 and STAT3 phosphorylation. Inhibition of STAT1 and STAT3 activation by agomelatine may be due to the inactivation of JAK2, the kinase primarily responsible for phosphorylation of these transcription factors.

The suppressors of cytokine signaling (SOCS) family, which suppresses cytokine signaling, has eight members (Alexander, 2002;

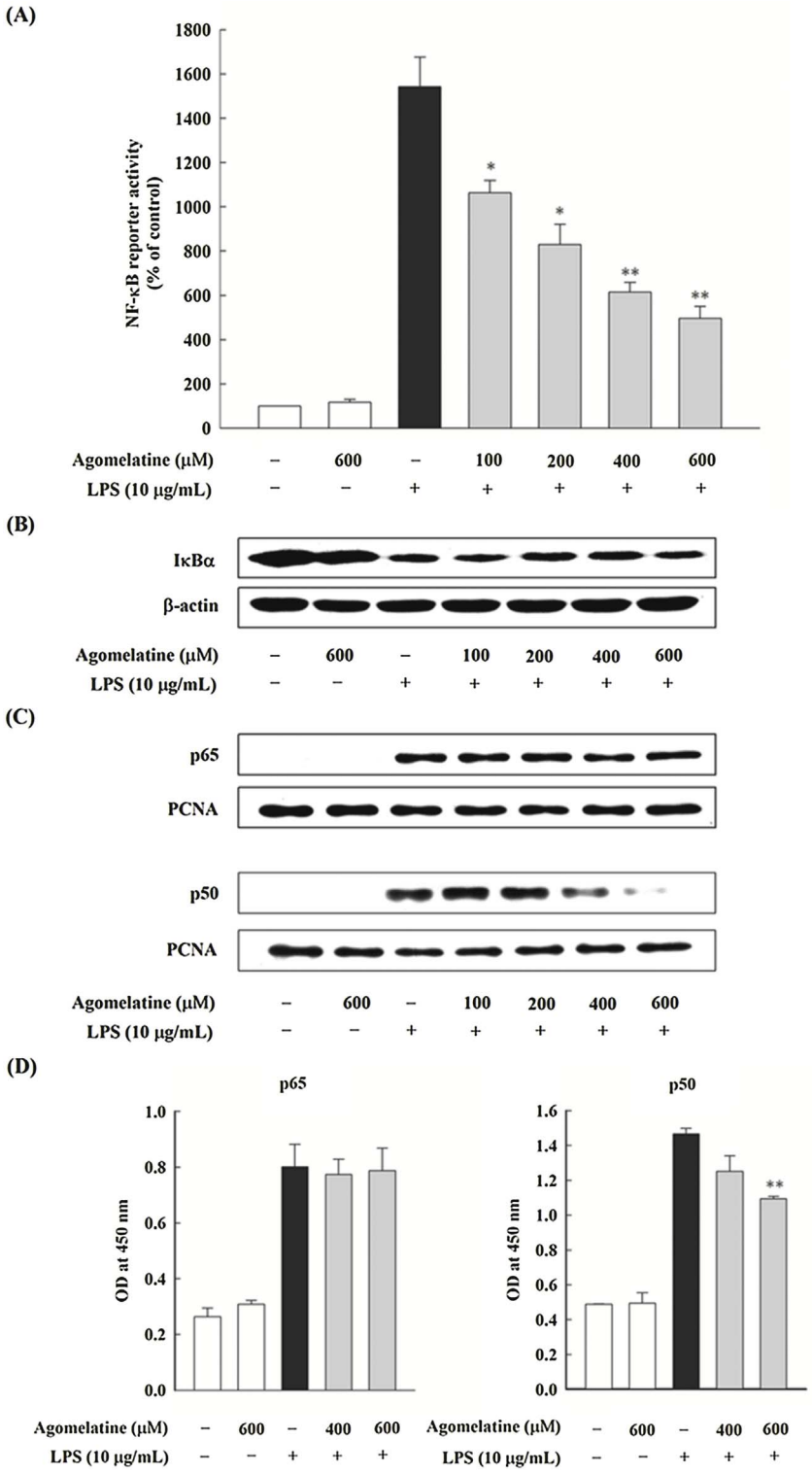

Fig. 5. Effects of agomelatine on activation of NF-κB induced by *Prevotella intermedia* LPS in RAW264.7 cells. (A) RAW-Blue cells, RAW264.7 cells stably expressing the gene for SEAP inducible by NF-κB transcription factors, were cultivated in DMEM under the selection of Zeocin. Cells were treated with different concentrations of agomelatine in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 24 h, after which the supernatants were collected for SEAP secretion assay. The results are means ± S.E.M. of three independent experiments. * $P < 0.05$ versus *P. intermedia* LPS alone; ** $P < 0.01$ versus *P. intermedia* LPS alone. (B, C) RAW264.7 cells were incubated with various concentrations of agomelatine in the absence or presence of *P. intermedia* LPS (10 μg/ml). (B) After 30 min of incubation, IκB-α degradation was determined by immunoblot analysis of cell lysates using antibody. A representative immunoblot from three separate experiments with similar results is shown. (C) After 30 min (for NF-κB p65) or 8 h (for NF-κB p50) of incubation, the nuclear fraction was isolated from cells. Nuclear translocation of NF-κB subunits was analyzed by immunoblot analysis using antibodies against NF-κB p65 and p50. A representative immunoblot from three separate experiments with similar results is shown. (D) Cells were incubated with agomelatine (400 and 600 μM) in the absence or presence of *P. intermedia* LPS (10 μg/ml). After 30 min (for NF-κB p65) or 8 h (for NF-κB p50) of incubation, the nuclear fraction was isolated from cells. DNA-binding activities of NF-κB subunits in nuclear fraction were assessed by using the ELISA-based NF-κB p65/NF-κB p50 transcription factor assay kits. The results are means ± S.E.M. of two independent experiments. ** $P < 0.01$ versus *P. intermedia* LPS alone.

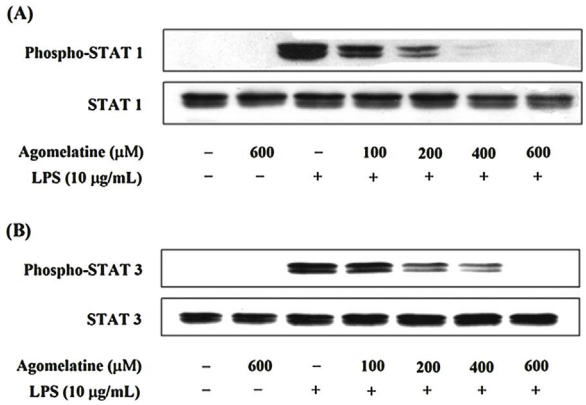

Fig. 6. Effect of agomelatine on the phosphorylation of STAT1 and STAT3 induced by *Prevotella intermedia* LPS in RAW264.7 cells. Cells were incubated with various concentrations of agomelatine in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 4 h, after which phosphorylation of STAT1 and STAT3 was determined by immunoblot analysis of cell lysates. A representative immunoblot from three separate experiments with similar results is shown.

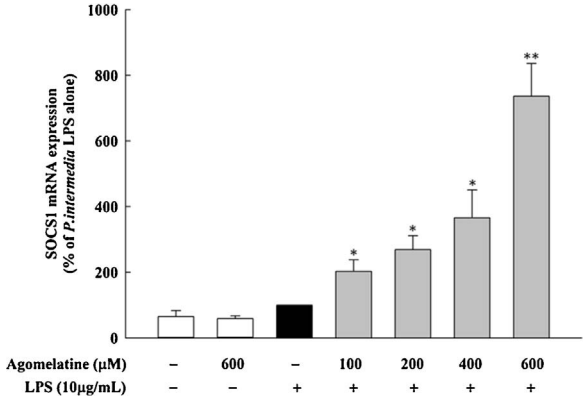

Fig. 7. Effect of agomelatine on SOCS1 expression in RAW264.7 cells activated by *Prevotella intermedia* LPS. Cells were treated with different concentrations of agomelatine in the absence or presence of *P. intermedia* LPS (10 μg/ml) for 1 h, after which real-time PCR was carried out with EvaGreen Supermix, β-actin being used as an endogenous control. The results are means ± S.E.M. of three independent experiments. $^{*}P < 0.05$ versus *P. intermedia* LPS alone; $^{**}P < 0.01$ versus *P. intermedia* LPS alone.

Alexander & Hilton, 2004). Studies have demonstrated that SOCS can be elicited by a variety of cytokines, as well as by toll-like receptor (TLR) ligands, like LPS (Cassatella et al., 1999; Stoiber et al., 1999). SOCS proteins are primarily increased upon the activation of JAK/STAT signaling and give negative feedback to cytokine signal transduction by repressing the interactions of activated JAK or STAT with receptors (Tamiya, Kashiwagi, Takahashi, Yasukawa, & Yoshimura, 2011). In particular, SOCS-1 has been reported to directly bind to JAK2 and exerts anti-inflammatory properties (Waiboci et al., 2007). Additionally, SOCS-1 also suppresses TLR activation and NF-κB-dependent transcription by diminishing the stability of p65 subunit (Strebovsky, Walker, Lang, & Dalpke, 2011). This study tested the likelihood that the inhibitory influences of agomelatine on *P. intermedia* LPS-elicited generation of NO, IL-1β and IL-6 are due to the inducement of SOCS1 protein. We showed that agomelatine notably augmented SOCS1 mRNA expression in cells activated with *P. intermedia* LPS. It was reported by Garlet et al. (2006) that SOCS1 mRNA expression is more elevated in periodontally diseased tissues than in healthy gingival biopsies of control subjects, suggesting a potential role for SOCS1 in the pathology of chronic periodontitis. Further, it has been reported that the kinetics of SOCS1 expression in a rat model of experimental periodontitis was closely associated with severity of inflammation and bone destruction, as well as with the state of STAT1 and STAT3 activation (de Souza et al., 2011). Hence, the induction of SOCS1 in response to agomelatine could be beneficial in the host modulation of periodontal diseases. This is, to our knowledge, the first observation indicating the potential role of this melatonin analog in SOCS modulation.

Macrophage cell lines have been utilized as in vitro models to study compounds for potential anti-inflammatory effects. In the present study, we examined the effects of agomelatine on *P. intermedia* LPS-induced production of proinflammatory mediators in the well-characterized RAW264.7 murine macrophage cell line. However, the responsiveness to LPS might be species-specific. It would be interesting to test the effects of agomelatine on primary human macrophages.

Concerns have been raised regarding the potential risk posed by agomelatine of inducing liver injury. However, this melatonin analog demonstrated a favorable safety profile and no undesirable side effects were reported in a large sample of patients with major depression (Ivanov & Samushiya, 2014; Kennedy, Avedisova, Belaïdi, Picarel-Blanchot, & de Bodinat, 2016). Agomelatine merits further evaluation to reveal its usefulness on the host modulation of periodontal disease.

5. Conclusions

In summary, agomelatine, a structural analog of melatonin, was found to exert inhibitory effects on the generation of NO, IL-1β and IL-6 in *P. intermedia* LPS-activated murine macrophages via the repression of NF-κB, STAT1 and STAT3 activation and SOCS1 induction. Here, we found evidence suggesting that agomelatine may represent an advantage in its therapeutic use for the control of inflammatory periodontal disease. Agomelatine merits further evaluation to reveal its usefulness on the host modulation of periodontal disease.

Conflict of interest

None.

Ethical approval

None.

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References

Aguiar, C. C., Almeida, A. B., Araújo, P. V., Vasconcelos, G. S., Chaves, E. M., do Vale, O. C., et al. (2013). Effects of agomelatine on oxidative stress in the brain of mice after chemically-induced seizures. *Cellular and Molecular Neurobiology*, *33*(6), 825–835.

Alexander, W. S., & Hilton, D. J. (2004). The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Annual Review of Immunology*, *22*, 503–529.

Alexander, W. S. (2002). Suppressors of cytokine signaling (SOCS) in the immune system. *Nature Reviews Immunology*, *2*(6), 410–416.

Baeuerle, P. A., & Henkel, T. (1994). Function and activation of NF-κB in the immune system. *Annual Review of Immunology*, *12*, 141–179.

Bascones-Martínez, A., González-Febles, J., & Sanz-Esporrín, J. (2014). Diabetes and periodontal disease. Review of the literature. *American Journal of Dentistry*, *27*(2), 63–67.

Bolivar, I., Whiteson, K., Stadelmann, B., Baratti-Mayer, M., Gizard, Y., Mombelli, A., et al. (2012). Bacterial diversity in oral samples of children in Niger with acute noma, acute necrotizing gingivitis, and healthy controls. *PLoS Neglected Tropical Diseases*, *6*(3), e1556.

Braga, R. R., Carvalho, M. A., Bruña-Romero, O., Teixeira, R. E., Costa, J. E., Mendes, E. N., et al. (2010). Quantification of five putative periodontal pathogens in female patients with and without chronic periodontitis by real-time polymerase chain reaction. *Anaerobe*, *16*(3), 234–239.

Cassatella, M. A., Gasperini, S., Bovolenta, C., Calzetti, F., Vollebregt, M., Scapini, P., et al. (1999). Interleukin-10 (IL-10) selectively enhances CIS3/SOCS3 mRNA expression in human neutrophils: Evidence for an IL-10-induced pathway that is independent of STAT protein activation. *Blood*, *94*(8), 2880–2889.

Choi, E. Y., Jin, J. Y., Lee, J. Y., Choi, J. I., Choi, I. S., & Kim, S. J. (2011). Melatonin inhibits *Prevotella intermedia* lipopolysaccharide-induced production of nitric oxide and interleukin-6 in murine macrophages by suppressing NF-κB and STAT1 activity.

Journal of Pineal Research, *50*(2), 197–206.
Choi, E. Y., Jin, J. Y., Choi, J. I., Choi, I. S., & Kim, S. J. (2014). DHA suppresses *Prevotella intermedia* lipopolysaccharide-induced production of proinflammatory mediators in murine macrophages. *British Journal of Nutrition*, *111*(7), 1221–1230.
Choi, E. Y., Choe, S. H., Hyeon, J. Y., Choi, J. I., Choi, I. S., & Kim, S. J. (2015). Effect of caffeic acid phenethyl ester on *Prevotella intermedia* lipopolysaccharide-induced production of proinflammatory mediators in murine macrophages. *Journal of Periodontal Research*, *50*(6), 737–747.
Chung, C. P., Nisengard, R. J., Slots, J., & Genco, R. J. (1983). Bacterial IgG and IgM antibody titers in acute necrotizing ulcerative gingivitis. *Journal of Periodontology*, *54*(9), 557–562.
Conway, S., Canning, S. J., Howell, H. E., Mowat, E. S., Barrett, P., Drew, J. E., et al. (2000). Characterisation of human melatonin mt_1 and MT_2 receptors by CRE-luciferase reporter assay. *European Journal of Pharmacology*, *390*(1–2), 15–24.
Delagrange, P., & Boutin, J. A. (2006). Therapeutic potential of melatonin ligands. *Chronobiology International*, *23*(1-2), 413–418.
de Bodinat, C., Guardiola-Lemaitre, B., Mocaër, E., Renard, P., Muñoz, C., & Millan, M. J. (2010). Agomelatine, the first melatonergic antidepressant: Discovery, characterization and development. *Nature Reviews Drug Discovery*, *9*(8), 628–642.
de Souza, J. A., Nogueira, A. V., de Souza, P. P., Cirelli, J. A., Garlet, G. P., & Rossa, C., Jr. (2011). Expression of suppressor of cytokine signaling 1 and 3 in ligature-induced periodontitis in rats. *Archives of Oral Biology*, *56*(10), 1120–1128.
Estrela, C. R., Pimenta, F. C., Alencar, A. H., Ruiz, L. F., & Estrela, C. (2010). Detection of selected bacterial species in intraoral sites of patients with chronic periodontitis using multiplex polymerase chain reaction. *Journal of Applied Oral Science*, *18*(4), 426–431.
Gao, J. J., Filla, M. B., Fultz, M. J., Vogel, S. N., Russell, S. W., & Murphy, W. J. (1998). Autocrine/paracrine IFN-αβ mediates the lipopolysaccharide-induced activation of transcription factor Stat1α in mouse macrophages: Pivotal role of Stat1α in induction of the inducible nitric oxide synthase gene. *Journal of Immunology*, *161*(9), 4803–4810.
Garlet, G. P., Cardoso, C. R., Campanelli, A. P., Martins, W., Jr., & Silva, J. S. (2006). Expression of suppressors of cytokine signaling in diseased periodontal tissues: A stop signal for disease progression? *Journal of Periodontal Research*, *41*(6), 580–584.
Geivelis, M., Turner, D. W., Pederson, E. D., & Lamberts, B. L. (1993). Measurements of interleukin-6 in gingival crevicular fluid from adults with destructive periodontal disease. *Journal of Periodontology*, *64*(10), 980–983.
Guan, S. M., Shu, L., Fu, S. M., Liu, B., Xu, X. L., & Wu, J. Z. (2009). *Prevotella intermedia* upregulates MMP-1 and MMP-8 expression in human periodontal ligament cells. *FEMS Microbiology Letters*, *299*(2), 214–222.
Hamada, S., Takada, H., Ogawa, T., Fujiwara, T., & Mihara, J. (1990). Lipopolysaccharides of oral anaerobes associated with chronic inflammation: Chemical and immunomodulating properties. *International Reviews of Immunology*, *6*(4), 247–261.
Ivanov, S. V., & Samushiya, M. A. (2014). Agomelatine in the treatment of depressive disorders in clinical practice: Multicenter observational CHRONOS study. *Neuropsychiatric Disease and Treatment*, *10*, 631–639.
Jacinto, R. C., Gomes, B. P., Ferraz, C. C., Zaia, A. A., & Filho, F. J. (2003). Microbiological analysis of infected root canals from symptomatic and asymptomatic teeth with periapical periodontitis and the antimicrobial susceptibility of some isolated anaerobic bacteria. *Oral Microbiology and Immunology*, *18*(5), 285–292.
Jansen, H. J., Grenier, D., & Van der Hoeven, J. S. (1995). Characterization of immunoglobulin G-degrading proteases of Prevotella intermedia and *Prevotella nigrescens*. *Oral Microbiology and Immunology*, *10*(3), 138–145.
Kamma, J. J., Nakou, M., Gmur, R., & Baehni, P. C. (2004). Microbiological profile of early onset/aggressive periodontitis patients. *Oral Microbiology and Immunology*, *19*(5), 314–321.
Karin, M., & Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: The control of NF-κB activity. *Annual Review of Immunology*, *18*, 621–663.
Kennedy, S. H., Avedisova, A., Belaïdi, C., Picarel-Blanchot, F., & de Bodinat, C. (2016). Sustained efficacy of agomelatine 10 mg, 25 mg, and 25–50 mg on depressive symptoms and functional outcomes in patients with major depressive disorder. A placebo-controlled study over 6 months. *European Neuropsychopharmacology*, *26*(2), 378–389.
Kim, S. J., Ha, M. S., Choi, E. Y., Choi, J. I., & Choi, I. S. (2004). *Prevotella intermedia* lipopolysaccharide stimulates release of nitric oxide by inducing expression of inducible nitric oxide synthase. *Journal of Periodontal Research*, *39*(6), 424–431.
Kim, S. J., Choi, E. Y., Kim, E. G., Shin, S. H., Lee, J. Y., Choi, J. I., et al. (2007). *Prevotella intermedia* lipopolysaccharide stimulates release of tumor necrosis factor-alpha through mitogen-activated protein kinase signaling pathways in monocyte-derived macrophages. *FEMS Immunology and Medical Microbiology*, *51*(2), 407–413.
Kirikae, T., Nitta, T., Kirikae, F., Suda, Y., Kusumoto, S., Qureshi, N., et al. (1999). Lipopolysaccharides (LPS) of oral black-pigmented bacteria induce tumor necrosis factor production by LPS-refractory C3H/HeJ macrophages in a way different from that of Salmonella LPS. *Infection and Immunity*, *67*(4), 1736–1742.
Kobayashi, K., Takahashi, N., Jimi, E., Udagawa, N., Takami, M., Kotake, S., et al. (2000). Tumor necrosis factor α stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. *Journal of Experimental Medicine*, *191*(2), 275–286.
Kornman, K. S., & Loesche, W. J. (1980). The subgingival microbial flora during pregnancy. *Journal of Periodontal Research*, *15*(2), 111–122.
Lafon, A., Pereira, B., Dufour, T., Rigouby, V., Giroud, M., Béjot, Y., et al. (2014). Periodontal disease and stroke: A meta-analysis of cohort studies. *European Journal of Neurology*, *21*(9), 1155–1161.
Liu, X. H., Kirschenbaum, A., Yao, S., & Levine, A. C. (2005). Cross-talk between the interleukin-6 and prostaglandin E2 signaling systems results in enhancement of osteoclastogenesis through effects on the osteoprotegerin/receptor activator of NF-κB (RANK) ligand/RANK system. *Endocrinology*, *146*(4), 1991–1998.
Matejka, M., Partyka, L., Ulm, C., Solar, P., & Sinzinger, H. (1998). Nitric oxide synthesis is increased in periodontal disease. *Journal of Periodontal Research*, *33*(8), 517–518.
Millan, M. J., Gobert, A., Lejeune, F., Dekeyne, A., Newman-Tancredi, A., Pasteau, V., et al. (2003). The novel melatonin agonist agomelatine (S20098) is an antagonist at 5-hydroxytryptamine$_{2C}$ receptors, blockade of which enhances the activity of frontocortical dopaminergic and adrenergic pathways. *Journal of Pharmacology and Experimental Therapeutics*, *306*(3), 954–964.
Mogi, M., Otogoto, J., Ota, N., Inagaki, H., Minami, M., & Kojima, K. (1999). Interleukin 1β, interleukin 6, β2-microglobulin, and transforming growth factor-α in gingival crevicular fluid from human periodontal disease. *Archives of Oral Biology*, *44*(6), 535–539.
Molteni, R., Macchi, F., Zecchillo, C., Dell'agli, M., Colombo, E., Calabrese, F., et al. (2013). Modulation of the inflammatory response in rats chronically treated with the antidepressant agomelatine. *European Neuropsychopharmacology*, *23*(11), 1645–1655.
Morrison, D. C., & Ryan, J. L. (1987). Endotoxins and disease mechanisms. *Annual Review of Medicine*, *38*, 417–432.
Okuda, K., Ono, M., & Kato, T. (1989). Neuraminidase-enhanced attachment of Bacteroides intermedius to human erythrocytes and buccal epithelial cells. *Infection and Immunity*, *57*(5), 1635–1637.
Olie, J. P., & Kasper, S. (2007). Efficacy of agomelatine, a MT_1/MT_2 receptor agonist with 5-HT_{2C} antagonistic properties, in major depressive disorder. *International Journal of Neuropsychopharmacology*, *10*(5), 661–673.
Pelt, P., Zimmermann, B., Ulbrich, N., & Bernimoulin, J. P. (2002). Effects of lipopolysaccharide extracted from *Prevotella intermedia* on bone formation and on the release of osteolytic mediators by fetal mouse osteoblasts in vitro. *Archives of Oral Biology*, *47*(12), 859–866.
Pfitzner, E., Kliem, S., Baus, D., & Litterst, C. M. (2004). The role of STATs in inflammation and inflammatory diseases. *Current Pharmaceutical Design*, *10*(23), 2839–2850.
Preshaw, P. M. (2008). Host response modulation in periodontics. *Periodontology 2000*, *48*, 92–110.
Reddy, M. S., Geurs, N. C., & Gunsolley, J. C. (2003). Periodontal host modulation with antiproteinase, anti-inflammatory, and bone-sparing agents. A systematic review. *Annals of Periodontology*, *8*(1), 12–37.
Rossomando, E. F., Kennedy, J. E., & Hadjimichael, J. (1990). Tumor necrosis factor alpha in gingival crevicular fluid as a possible indicator of periodontal disease in humans. *Archives of Oral Biology*, *35*(6), 431–434.
Rothwarf, D. M., & Karin, M. (1999). The NF-κB activation pathway: A paradigm in information transfer from membrane to nucleus. *Science's STKE*, *1999*(5).
Samavati, L., Rastogi, R., Du, W., Hüttemann, M., Fite, A., & Franchi, L. (2009). STAT3 tyrosine phosphorylation is critical for interleukin 1 beta and interleukin-6 production in response to lipopolysaccharide and live bacteria. *Molecular Immunology*, *46*(8–9), 1867–1877.
Stashenko, P., Fujiyoshi, P., Obernesser, M. S., Prostak, L., Haffajee, A. D., & Socransky, S. S. (1991). Levels of interleukin 1β in tissues from sites of active periodontal disease. *Journal of Clinical Periodontology*, *18*(7), 548–554.
Stewart, R., & West, M. (2016). Increasing evidence for an association between periodontitis and cardiovascular disease. *Circulation*, *133*(6), 549–551.
Stoiber, D., Kovarik, P., Cohney, S., Johnston, J. A., Steinlein, P., & Decker, T. (1999). Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN-γ. *Journal of Immunology*, *163*(5), 2640–2647.
Strebovsky, J., Walker, P., Lang, R., & Dalpke, A. H. (2011). Suppressor of cytokine signaling 1 (SOCS1) limits NF-κB signaling by decreasing p65 stability within the cell nucleus. *FASEB Journal*, *25*(3), 863–874.
Tamiya, T., Kashiwagi, I., Takahashi, R., Yasukawa, H., & Yoshimura, A. (2011). Suppressors of cytokine signaling (SOCS) proteins and JAK/STAT pathways: Regulation of T-cell inflammation by SOCS1 and SOCS3. *Arteriosclerosis Thrombosis and Vascular Biology*, *31*(5), 980–985.
Tatakis, D. N. (1993). Interleukin-1 and bone metabolism: A review. *Journal of Periodontology*, *64*(5 Suppl), 416–431.
Waiboci, L. W., Ahmed, C. M., Mujtaba, M. G., Flowers, L. O., Martin, J. P., Haider, M. I., & Johnson, H. M. (2007). Both the suppressor of cytokine signaling 1 (SOCS-1) kinase inhibitory region and SOCS-1 mimetic bind to JAK2 autophosphorylation site: Implications for the development of a SOCS-1 antagonist. *Journal of Immunology*, *178*(8), 5058–5068.
Westphal, O., Jann, K. (1965). Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. In: Methods in Carbohydrate Chemistry. Whistler RL ed., Academic Press New York, pp 83–91.
Williams, R. C. (1990). Periodontal disease. *New England Journal of Medicine*, *322*(6), 373–382.