Inhibition of LPS-induced NLRP3 inflammasome activation by stem cell-conditioned culture media in human gingival epithelial cells

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Abstract. Interleukin (IL)-1β is a pathogenic factor associated with the destruction of periodontal tissue in periodontitis. IL-1β processing is regulated by cytosolic machinery known as the inflammasome. *Porphyromonas gingivalis* infection and lipopolysaccharide (LPS) have an important role in the destruction of periodontal tissue in periodontitis. *P. gingivalis* infection and LPS have been reported to activate the NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome in human oral cells. Stem cell therapy exhibits anti-inflammatory effects and stem cell-conditioned culture media (SCM) shows similar beneficial effects. The present study tested the hypothesis that SCM inhibits activation of the inflammasome and protects human gingival epithelial cells (GECs) against LPS-induced inflammatory damage. Human GECs were treated with or without LPS plus SCM or control cell media. NLPR3 inflammasome components and inflammatory factors were measured by western blotting and immunofluorescence. The present study revealed that LPS induced an increase in the expression of inflammasome components, NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1. Co-immunoprecipitation revealed increased binding of NLRP3 and ASC, and immunofluorescence showed an increased co-localization of ASC and caspase-1, suggesting that LPS stimulated assembly of the NLRP3 inflammasome. SCM inhibited the overexpression and assembly of NLRP3 inflammasome components induced by LPS. Furthermore, SCM blocked the increase in IL-1β production induced by LPS and inhibited the translocation of the inflammatory factor, NF-κB, into the nuclei. Consequently, SCM protected cells against LPS-induced damage, as suggested by the recovery of disturbed E-cadherin staining pattern, which indicates a disruption in epithelial integrity. In conclusion, treatment with SCM may attenuate LPS-induced inflammatory damage in human GECs via inhibition of NLRP3 inflammasome activation, suggesting a potential therapeutic use for SCM.

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Introduction

Periodontitis is an infection-associated chronic inflammatory disease of the periodontium; it is one of the most prevalent chronic diseases and a major cause of tooth loss (1). Interleukin (IL)-1β plays a significant role in pathogenic host defense; however, excessive IL-1β causes pathogenicity, resulting in the destruction of periodontal tissue and thus periodontitis (2,3). The production of IL-1β involves cytosolic machinery named the inflammasome (4,5). The components of the inflammasome consist of NOD-like receptor family pyrin domain-containing protein (NLRP) and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which recruits and activates caspase-1. The activation of caspase-1 causes the cleavage of pro-IL-1β to produce mature IL-1β. Thus, inflammasome activation is required for IL-1β production.

A number of inflammasomes have been identified in humans. They contain different NLRP family proteins, such as NLRP1, NLRP3 and NLRC4, and respond to different stimuli for activation (6). The NLRP3 inflammasome is the inflammasome activated in periodontitis (7). It has been reported that *Porphyromonas gingivalis* infection stimulates activation of the NLRP3 inflammasome and the production of IL-1β (8). Similarly, bacterial endotoxin lipopolysaccharide (LPS) has been shown to activate the NLRP3 inflammasome and stimulate the production of IL-1β in oral epithelial cells (9,10). Gingival epithelial cells (GECs) have been reported to have an important role as a component of innate host response to periodontal bacteria and significantly contribute to gingival health (11). Thus, targeting the inflammasome may be a potential approach for the prevention and treatment of periodontitis.

The use of stem cell-based therapies has been reported in different diseases, such as inflammatory bowel diseases,

multiple sclerosis, myocardial infarction and type 1 diabetes (12). Stem cell therapy exerts beneficial effects predominantly through indirect paracrine actions, rather than direct differentiation and substitution of damaged cells (13-15). It is well documented that stem cells possess immunomodulatory and anti-inflammatory functions (14,16). Studies have demonstrated that stem cell-conditioned culture media (SCM) exhibits protective effects against various pathological conditions (17-19). Notably, administration of SCM shows similar protective effects to stem cell transplantation (15,20). Due to stem cell therapy-associated risks, treatment with SCM has been considered as a promising alternative (21). Considering the anti-inflammatory functions that stem cells possess and that LPS induces inflammasome activation (22-24), it was hypothesized that SCM may inhibit inflammasome activation and protect against LPS-induced inflammatory damage in human GECs. To test the hypothesis, the current study first determined the inhibitory effect of SCM on LPS-induced inflammasome activation, then measured IL-1β production and NF-κB activation, and finally, accessed the damage of the GECs. To the best of our knowledge, the results from the present study were the first to demonstrate that SCM inhibited LPS-induced NLRP3 inflammasome activation and attenuated LPS-induced inflammatory damage in human GECs.

Materials and methods

Cell culture and preparation of SCM. Human GECs were cultured and treated as described previously (25). Briefly, GECs (cat. no. 1626; Beijing Yuhengfeng Technology Co., Ltd.) were cultured in a defined medium (EpiCM; cat. no. 4101; Beijing Yuhengfeng Technology Co., Ltd.), in which 2% FBS, 1% epithelial cell growth supplement, penicillin (100 IU/ml) and streptomycin (100 μg/ml) were supplemented, at 37˚C in a humidified atmosphere containing 5% CO_2. Human mesenchymal stem cells (MSCs) were purchased from Cyagen Biosciences (cat. no. HUXMA-01001; cryopreserved at second passage) and cultured according to the manufacturers' instructions using the defined MSC medium (Human MSC Complete Medium; cat. no. HUXMA-90011; Cyagen Biosciences). After 72 h, the medium was collected and used as SCM. Sixth passage MSCs were used in the present study. Notably, these MSCs have been wildly used in various studies, including in the preparation of SCM (26-34). The control conditioned media (CCM) was obtained from culturing human fibroblast cells (cat. no. 2621; Beijing Yuhengfeng Technology Co., Ltd.) for 72 h using the same medium for the culture of MSCs. The human GECs and fibroblasts were immortalized cells, and experiments were performed using cells at passages 4-6 after initial thawing.

Experimental groups. Upon reaching 80% confluence, the GECs were treated with either vehicle + CCM, LPS (2 μg/ml; cat. no. abx165771; Beijing Yuhengfeng Technology Co., Ltd.) + CCM or LPS + SCM for 16 h. Subsequently, cells were harvested for the isolation of proteins. For immunofluorescence imaging, cells were cultured on glass coverslips. The concentration of LPS was based on previous studies (9,35-37). Using a cell viability assay, these studies concluded that higher concentrations of LPS (>5 μg/ml) were toxic to cells, and that 1, 2 and 2.5 μg/ml LPS did not cause cell death (38,39). LPS was dissolved in H_2O (stock solution 1 mg/ml), 10 μl H_2O (vehicle) or 10 μl LPS stock solution was added into 5 ml culture medium in 60-mm dishes, which gave 2 μg/ml PLS in working solution.

Western blot analysis for levels of NLRP3, ASC, caspase-1 and NF-κB (p65). Cytosolic and nuclear proteins were prepared as described previously (25,40,41). Briefly, cells were washed with PBS and homogenized in ice-cold HEPES buffer (buffer-A) containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10% Nonidet P-40. The homogenate was centrifuged at 1,000 x g for 5 min at 4˚C, and the supernatant and pellet were collected for cytosolic protein preparation and nuclear protein isolation, respectively. The supernatant was centrifuged again at 6,000 x g for 10 min at 4˚C and the resulting cytosolic protein-containing supernatant was used for western blot analysis of NLRP3, ASC and caspase-1.

For nuclear fraction isolation, the pellets from the first centrifugation, which contained cell nuclei, were washed with ice-cold buffer-A, and then incubated on ice with ice-cold HEPES buffer (buffer-B) containing 5 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 300 mM NaCl, 400 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 26% glycerol for 30 min to release nuclear proteins. Next, the reaction mixtures were centrifuged at 14,000 x g for 30 min at 4˚C, and the supernatants were collected and stored at -80˚C until they were used as nuclear extracts for western blot analysis of NF-κB levels.

Western blot analysis was performed as previously described (42). Briefly, protein concentration was measured by a BCA Protein Assay Kit (cat. no. P0010S; Beyotime Institute of Biotechnology), samples (20 μg) were then separated by SDS-PAGE on 10% gels and transferred onto nitrocellulose membranes. The membranes were blocked with the QuickBlock™ blocking buffer (cat. no. P0252; Beyotime Institute of Biotechnology) for 15 min at room temperature and then incubated with primary antibodies at 4˚C overnight, followed by incubation with horseradish peroxidase (HRP)-labeled secondary antibodies at room temperature for 1 h. β-actin and PCNA were used as loading controls for cytosolic and nuclear fractions, respectively. After incubation with ECL detection solution (BeyoECL Plus; cat. No. P0018S, Beyotime Biotechnology), the membranes were visualized using a chemiluminescence imaging system (BeyoImager Luminometers, Beyotime). The intensity of the blots was semi-quantified using ImageJ (1.53e; National Institutes of Health).

Primary antibodies used in the present study included anti-human NLRP3 (1:500; cat. no. sc-134306; mouse monoclonal), ASC (1:1,000; cat. no. sc-514414; mouse monoclonal), caspase-1 (1:3,000; cat. no. sc-1218-R; rabbit polyclonal) and NF-κB (p65) (1:1,000; cat. no. sc-8008, mouse monoclonal), which were purchased from Santa Cruz Biotechnology, Inc. Anti-β-actin (1:3,000, cat. No. sc-47778; mouse; Santa Cruz Biotechnology, Inc.) and anti-PCNA (1:1,000, cat. no. ab92552; rabbit; Abcam) were used as loading controls for cytoplasmic and nuclear protein detection, respectively. Secondary antibodies used in the present study included HRP-conjugated goat-anti-mouse and -rabbit antibodies (1:2,000; cat. nos. A0216 and A0208, respectively; Beyotime Institute of Biotechnology).

Co-immunoprecipitation (Co-IP) of NLRP3 and ASC. Co-IP was performed using the Capturem Co-IP kit (cat. no. 635721; Takara Bio, Beijing) according to the manufacturer's instructions. Briefly, cell lysates (200 μl) were mixed with an antibody against ASC (1 μg; cat. no. sc-514414; mouse; Santa Cruz Biotechnology, Inc.), the mixture was then loaded onto a Protein A column and centrifuged at 1,000 x g for 1 min at room temperature. The flowthrough was reloaded onto the Protein A column and centrifuged again at 1,000 x g for 1 min at room temperature. After washing the Protein A column with 200 μl washing buffer, 50 μl Elution buffer was added, and the eluted sample was collected by 1,000 x g centrifugation for 1 min at room temperature and subjected to western blot analysis with the aforementioned anti-NLRP3 or anti-ASC antibody. 5% input was loaded as the control and a normal mouse IgG_1 was used as an isotype control (cat. no. sc-3877; Santa Cruz Biotechnology, Inc.).

Immunofluorescence microscopy. Immunofluorescence staining was performed in cells cultured on glass coverslips as described previously (25,40). After fixation with 2% paraformaldehyde for 30 min at room temperature, the cells were incubated with anti-ASC (1:100; cat. no. sc-514414; mouse), anti-caspase-1 (1:100; cat. no. sc-1218-R; rabbit), anti-E-cadherin (1:200; cat. no. sc-21791; mouse) or anti-NF-κB (1:200; cat. no. sc-8008; mouse) antibodies (all Santa Cruz Biotechnology, Inc.) at 4˚C overnight. After washing, the slides were incubated with Alexa Fluor 488-labeled goat anti-rabbit (1:500; cat. no. ab150077; Abcam) or Alexa Fluor 555-labeled anti-mouse secondary antibody (1:500; cat. no. ab150113; Abcam) for 1 h at room temperature and then subjected to examination using a Fluoview FV1000 confocal laser scanning microscope (Olympus Corporation). Images were analyzed and the co-localization coefficients were calculated using Image-Pro® Plus (version 7.01; Media Cybernetics, Inc.) (43). Caspase-1 was detected using Alexa Fluor-488 anti-rabbit IgG secondary antibody and ASC, NF-κB or E-cadherin using Alexa Fluor-555 anti-mouse secondary antibody. For the staining of NF-κB, nuclei were counterstained using Nuclear Green DCS1 (cat. no. ab138905; Abcam).

Measurement of IL-1β in cell culture media. IL-1β levels in culture media were measured as described previously (25) using a Valukine ELISA kit (cat. no. VAL101; R&D Systems, Inc.) according to the manufacturer's instruction.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 9.4.1 (Graphpad Software; Dotmatics). Data are presented as the mean ± SEM. The significance of differences in mean values between multiple groups was evaluated using one-way ANOVA followed by a Tukey's multiple range test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of SCM on the expression levels of NLRP3, ASC and caspase-1. LPS significantly increased the protein expression levels of inflammasome components, NLRP3, ASC and caspase-1. SCM inhibited the LPS-induced increases in NLRP3, ASC and caspase-1 expression (Fig. 1). These results demonstrated that LPS stimulated activation of the inflammasome and that SCM blocked activation of the inflammasome induced by LPS.

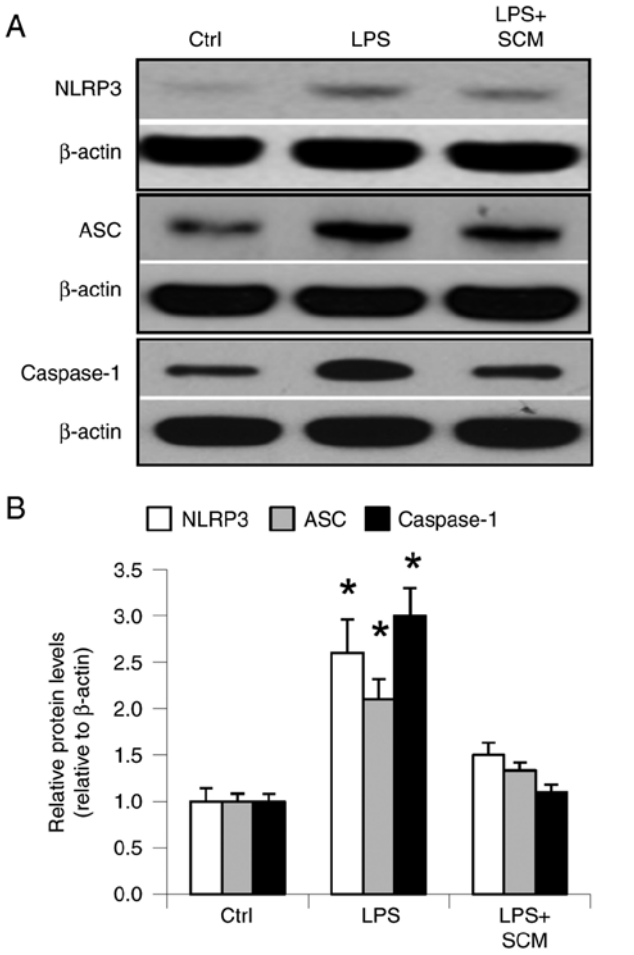

Figure 1. Effect of SCM on the expression levels of inflammasome components NLRP3, ASC and caspase-1, as detected by western blot analysis. (A) Representative western blot of NLRP3, ASC and caspase-1 expression. (B) Blot intensities (normalized to control) showing the expression levels of inflammasome components. n=5, *P<0.05 vs. Ctrl and LPS + SCM groups. No significant difference was observed between the control and LPS + SCM groups. ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; LPS, lipopolysaccharide; NLRP3, NOD-like receptor family pyrin domain-containing protein 3; SCM, stem cell-conditioned culture media.

Effect of SCM on inflammasome assembly. LPS significantly increased the fluorescence intensity and the colocalization of caspase-1 and ASC immunostaining (Fig. 2A and B). In addition, Co-IP analysis suggested that LPS treatment markedly enhanced the binding of NLRP3 and ASC (Fig. 2C and D). SCM inhibited the increase in the co-localization of ASC and caspase-1 as well as the binding of ASC and NLRP3 (Fig. 2). These data indicated that LPS enhanced formation of the inflammasome and the recruitment of caspase-1, further suggesting that LPS induced activation of the inflammasome, and that SCM attenuated LPS-induced inflammasome activation.

Effect of SCM on the production of IL-1β. LPS-treated cells exhibited a 3.6-fold increase in IL-1β levels compared with

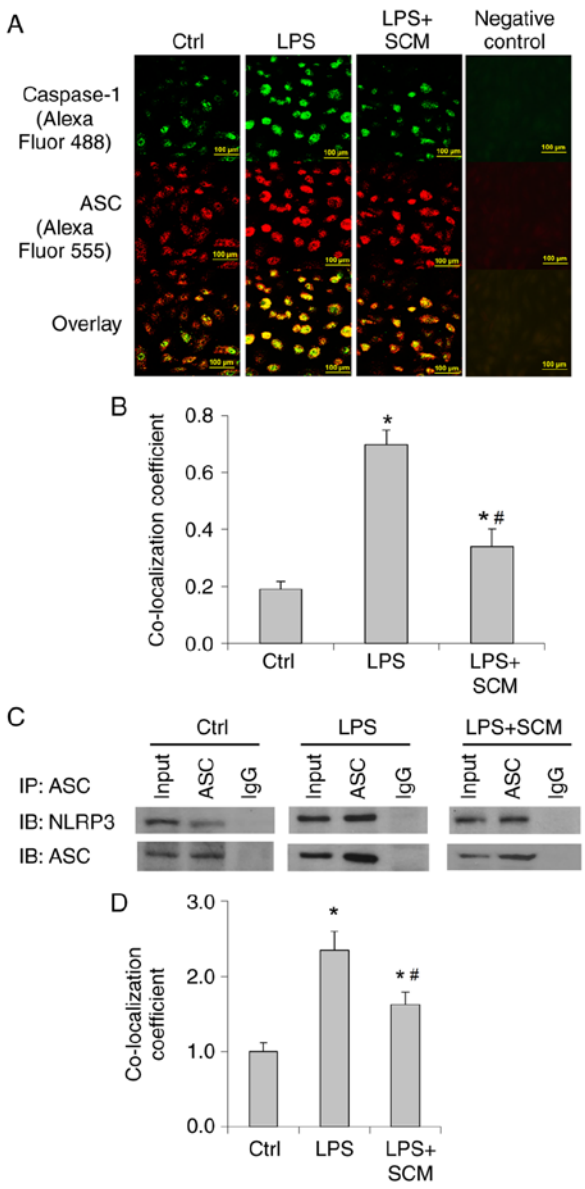

Figure 2. Effect of SCM on inflammasome assembly. (A) Representative photomicrographs of immunofluorescence staining of caspase-1 and ASC. Caspase-1 was detected using a primary antibody followed by an Alexa Fluor-488-labeled secondary antibody (green fluorescence), whereas ASC was detected using a primary antibody followed by an Alexa Fluor-555-labeled secondary antibody (red fluorescence). The negative control was detected without primary antibodies. (B) Co-localization coefficients of the merged images of caspase-1 and ASC calculated using Image-Pro Plus. (C) Representative western blot depicting NLRP3 Co-IP using the ASC antibody. (D) Blot intensity ratio of NLRP3 to ASC in ASC IP samples (normalized to control). n=5, *P<0.05 vs. Ctrl; $^{\#}$P<0.05 vs. LPS. ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; IP, immunoprecipitation; LPS, lipopolysaccharide; NLRP3, NOD-like receptor family pyrin domain-containing protein 3; SCM, stem cell-conditioned culture media.

controls, whereas the LPS-induced increase in IL-1β level was reduced by 74% in LPS + SCM-treated cells (Fig. 3). These data suggested that SCM inhibited activation of the inflammasome and thereby inhibited the excessive production of IL-1β induced by LPS.

Effect of SCM on activation of the inflammatory factor NF-κB. Western blotting results suggested that LPS significantly

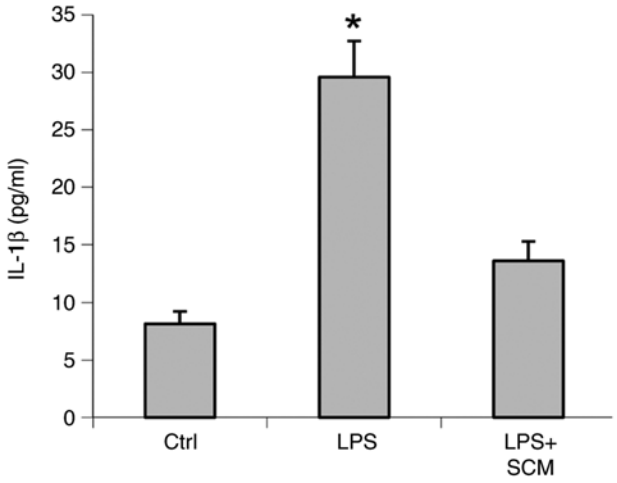

Figure 3. Effect of SCM on IL-1β levels in culture media, as detected by ELISA. n=4-6. *P<0.05 vs. Ctrl and LPS + SCM. No significant difference was observed between the control group and LPS + SCM group. LPS, lipopolysaccharide; SCM, stem cell-conditioned culture media.

upregulated NF-κB levels in the nuclear extracts (Fig. 4A). Fluorescence staining demonstrated that the location of NF-κB was predominantly in the cytoplasm in control cells (Fig. 4B), whereas NF-κB was mainly localized in the nuclear area in LPS-treated cells, suggesting an enhanced activation of NF-κB induced by LPS. SCM appeared to block NF-κB translocation into the nuclei (Fig. 4A and B).

Effect of SCM on cell damage. Immunofluorescence microscopy showed that the immunostaining pattern of E-cadherin, a cell junction protein, was disturbed in LPS-treated cells. However, the LPS-induced disarrangement of E-cadherin was blocked in SCM-treated cells (Fig. 5), suggesting that SCM inhibited NLRP3 inflammasome activation, and therefore reduced the production of its downstream pro-inflammatory effectors, protecting cells against LPS-induced damage.

Discussion

The results of the present study showed that SCM reduced the LPS-induced increase in the expression levels of NLRP3, ASC and caspase-1, inhibited the assembly of inflammasome components, and blocked IL-1β production, as well as activation of the pro-inflammatory factor NF-κB. Furthermore, SCM attenuated the cell damage induced by LPS in cultured human GECs. To the best of our knowledge, these findings suggested, for the first time, that SCM can inhibit inflammasome activation and protect human GECs from LPS-induced inflammatory damage.

Epithelial cells are the first line of defense against pathogens and danger signals, such as bacterial toxins. These cells act as a physical barrier to protect other cells, such as osteoblasts and fibroblasts, from exposure to pathogens. The present study thus chose to assess GECs. Consistent with previous reports (23,25), results from the present study suggested that LPS treatment increased the levels of NLRP3 inflammasome components in GECs. The present results also indicated that LPS not only increased expression levels of the

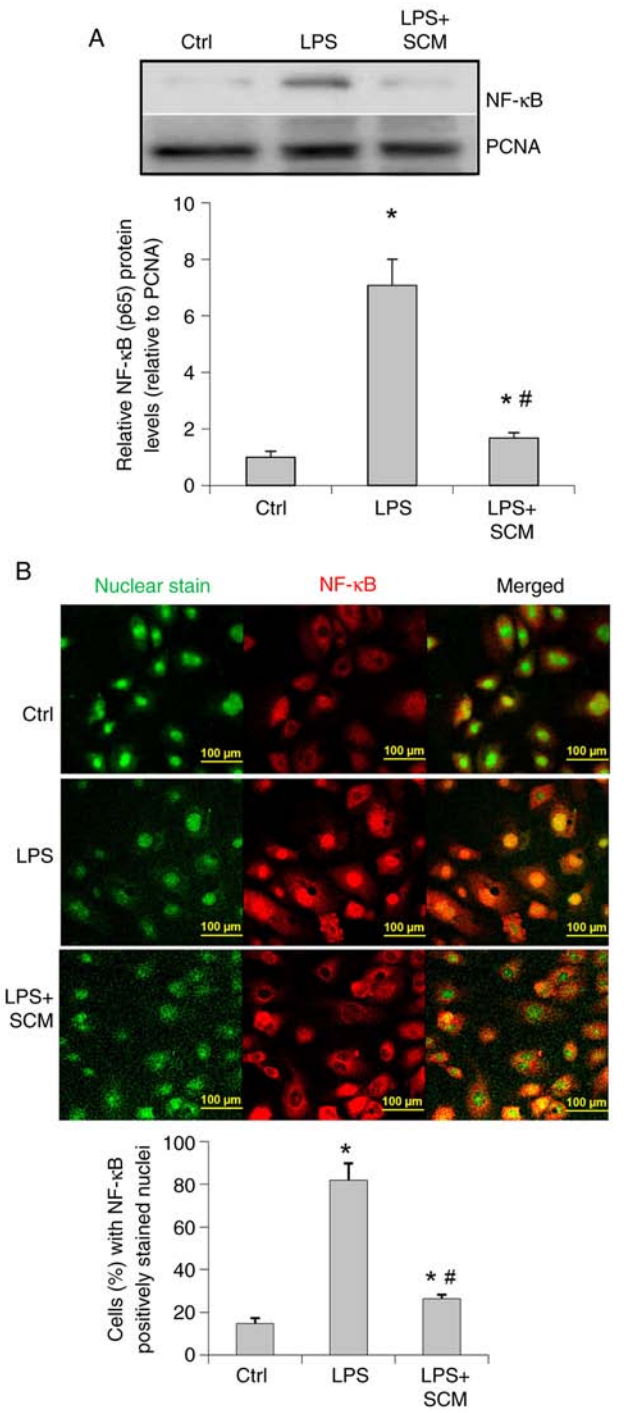

Figure 4. Effect of SCM on NF-κB activation and localization. (A) Protein expression levels of NF-κB (p65) in the nuclear extract. (B) Immunofluorescence showing the localization of NF-κB in the cells. NF-κB was detected using a primary antibody followed by an Alexa Fluor-555-labeled secondary antibody (red fluorescence). Nuclei were stained using Nuclear Green DCS1. n=4-5, *P<0.05 vs. Ctrl; #P<0.05 vs. LPS. LPS, lipopolysaccharide; SCM, stem cell-conditioned culture media.

components of the NLRP3 inflammasome, but also enhanced assembly of the NLRP3 inflammasome, further suggesting that NLRP3 inflammasome activation could be induced by LPS. Consequently, LPS increased the production of IL-1β; however, SCM significantly inhibited LPS-induced increases in the levels of NLRP3 inflammasome components, assembly of the inflammasome and the production of IL-1β, suggesting

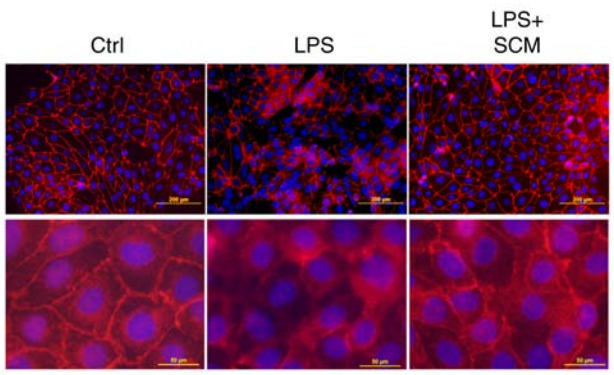

Figure 5. Effect of SCM on the localization of E-cadherin. Immunostaining showing the localization patterns of E-cadherin (red) with double-staining of nuclei using DAPI (blue). Representative images from five repeats. LPS, lipopolysaccharide; SCM, stem cell-conditioned culture media.

that SCM inhibits LPS-induced activation of the NLRP3 inflammasome in GECs.

Previous studies have shown that IL-1β activates NF-κB (44,45) and that IL-1β mediates the activation of NF-κB induced by LPS (24). The present study therefore evaluated the effect of SCM on NF-κB activation. Results from the present study suggested that LPS enhanced the nuclear translocation of NF-κB, as indicated by the elevation of NF-κB levels in the nuclear extract and the increase of NF-κB immunostaining in the nuclei. The present results are consistent with the literature showing that LPS induces the increase in the expression levels of both NF-κB and the NLRP3 inflammasome (46,47). SCM blocked the nuclear translocation of NF-κB induced by LPS. These results indicated that SCM blocked the activation of downstream inflammatory factors associated with the inflammasome. The present data suggested that SCM inhibits NLRP3 inflammasome activation and thereby blocks the downstream inflammatory response induced by LPS.

Inhibition of pro-inflammatory factors IL-1β and NF-κB by SCM is expected to protect cells from LPS-induced damage. The present study examined the staining pattern of E-cadherin, a cell junction protein, to determine epithelial integrity. Consistent with a previous report (48), the present study showed a disarrangement of the staining pattern of E-cadherin, suggesting an LPS-induced disruption of cell junctions. The present results were supported by previous reports showing that NLRP3 inflammasome activation was associated with the reduction of E-cadherin expression (49-51). Notably, SCM blocked the disturbance in the staining pattern of E-cadherin, suggesting that SCM protected the cells from LPS-induced inflammatory damage, probably through inhibition of LPS-induced activation of the NLRP3 inflammasome.

Notably, several studies have shown the inhibition of NLRP3 inflammasome activation and protection against LPS-induced damage in human gingival fibroblasts using agents such as the Vitamin D analog, Eldecalcitol (52), and the antioxidants, Flavocoxid (9) and Fisetin (53). Although those studies were similar in design, the current study provided a number of novel observations compared with the aforementioned studies. First, the current study suggested a new potential approach, SCM, for the management of periodontitis. Second, human GECs were used because GECs are

the first line of defense against pathogens and function as a physical barrier to protect other cells, such as osteoblasts and fibroblasts, from exposure. Therefore, studies using GECs may have better clinical relevance and also are in a different cell type from the previous reports. Third, unlike previous reports, the present study measured not only the expression of NLRP3 inflammasome components, but also the assembly by double staining and Co-IP, which better represented activation of the NLRP3 inflammasome. Moreover, the present study examined the nuclear translocation of NF-κB in addition to the levels of IL-1β, which mapped the changes of the inflammatory cascade with different signals. Examination of the immunostaining pattern produced by E-cadherin showed the morphological integrity of the cells, which is a more reliable indicator of cell damage than quantifying molecular signals alone. Overall, the present study suggested a potential novel approach for the management of periodontitis supported by observations that have, to the best of our knowledge, not been revealed in previous studies.

The current study did not attempt to investigate the mechanisms or molecules produced in SCM that exert the actions revealed in this study. In this regard, SCM has been shown to contain a number of growth factors and cytokines that may contribute to the beneficial effects of SCM (54,55). Recent studies have also shown that SCM contains microvesicles or extracellular vesicles (EVs) and that the EVs obtained from MSCs exert beneficial effects by transferring biological cargo, including cytoskeleton proteins, signaling proteins, lipids, enzymes, transcription factors, mRNA, microRNAs, long non-coding RNAs, DNA and metabolites, to host cells (56,57). However, the exact factors responsible for the effects observed in the present study remain unknown and require further investigation (54). In addition, the findings in the present *in vitro* study require confirmation through *in vivo* studies in the future.

In summary, the present results demonstrated that SCM inhibited activation of the NLRP3 inflammasome induced by LPS, reduced the consequent production of pro-inflammatory factors, and thereby attenuated LPS-induced damage in human GECs. To conclude, SCM protects GECs from LPS-induced inflammatory damage, which provides scientific foundations for testing SCM *in vivo* and may assist development of a potential strategy for the prevention and treatment of chronic periodontitis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL, LS and YW conceived, designed and performed the experiments. HL wrote the paper, LS and YW reviewed and edited the manuscript. All authors read and approved the final manuscript. LS and YW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors confirm that they have no competing interests.

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