Original Article
http://dx.doi.org/10.1590/1678-7757-2022-0404

JAOS
JOURNAL OF APPLIED ORAL SCIENCE

C5aR antagonist inhibits LPS-induced inflammation in human gingival fibroblasts via NF-κB and MAPK signaling pathways

Yan CHEN[1,2#]
Yang LIU[3#]
Hao LI[2]
Risu HUNA[4]
Xiaohan TAN[5]
Ning LI[6]
Yiying ZHANG[4]
Xiaohui JIAO[1*]
Mingyue LIU[5*]

Abstract

Objective: Abnormal complement activation is associated with periodontitis. W54011 is a novel non-peptide C5aR antagonist (C5aRA) that exhibits favorable anti-inflammatory effects in various inflammatory models. However, whether W54011 inhibits periodontitis has not yet been fully elucidated. To address this, we have investigated the probable anti-inflammatory mechanism of W54011 in LPS-treated inflammation in human gingival fibroblasts (HGFs). Methodology: HGFs were isolated from healthy gingival tissue samples using the tissue block method and were identified with immunofluorescence staining. The CCK8 assay and reverse transcription-PCR (RT-PCR) were used to select the optimal induction conditions for Lipopolysaccharide (LPS) and C5aRA (according to supplementary data S1, S2 and S3). The levels of inflammatory cytokines, C5aR, and the activation of NF-κB/MAPK signaling pathways were determined by RT-quantitative PCR (RT-qPCR) and Western blotting. Results: Immunofluorescence results showed that vimentin and FSP-1 were positive in HGFs and Keratin was negative in HGFs. Immunofluorescence staining demonstrated that C5aRA inhibited LPS-stimulated nuclear translocation of p-p65. RT-qPCR and Western blotting showed that C5aRA reduced the expression of IL-1β, IL-6, TNF-α, C5aR, p-p65, p-IκBα, p-JNK, p-c-JUN, and TLR4 in LPS-induced HGFs. Conclusion: These findings suggested that C5aRA attenuated the release of inflammatory cytokines in LPS-induced HGFs by blocking the activation of the NF-κB and MAPK signaling pathways.

Keywords: C5aR antagonist. Gingival fibroblasts. Periodontitis. NF-κB. MAPK.

Corresponding address:
Xiaohui Jiao
The First Affiliated Hospital of Harbin Medical University - Department of Oral Maxillofacial Surgery - Harbin - Heilongjiang 150001 - China.
Phone: +86-0451-84816772
e-mail: doctorjiaoxiaohui@163.com
Mingyue Liu
The Second Affiliated Hospital of Harbin Medical University - Department of Prosthodontics - Harbin - Heilongjiang 150086 - China.
Phone: +86-0451-84816772
e-mail: drliumingyue@163.com

Received: October 14, 2021
Revised: December 16, 2022
Accepted: December 21, 2022

Editor: Linda Wang
Associate Editor: Ana Carolina Morandini

[1]The First Affiliated Hospital of Harbin Medical University, Department of Oral Maxillofacial Surgery, Harbin, Heilongjiang, China.
[2]The Fourth Affiliated Hospital of Heilongjiang University of Traditional Chinese Medicine, Department of Stomatology, Harbin, Heilongjiang, China.
[3]Heilongjiang Provincial Hospital, Department of Stomatology, Harbin, Heilongjiang, China.
[4]The Second Affiliated Hospital of Harbin Medical University, Oral Implant Center, Harbin, Heilongjiang, China.
[5]The Second Affiliated Hospital of Harbin Medical University, Department of Prosthodontics, Harbin, Heilongjiang, China.
[6]The Second Affiliated Hospital of Harbin Medical University, Department of Cardiology, Harbin, Heilongjiang, China.

# Yan Chen and Yang Liu Contributed equally.
* Xiaohui Jiao and Mingyue Liu Contributed equally.

Introduction

Periodontal disease begins as a bacterial infection. Since it destroys the tissue surrounding the teeth, it may cause tooth loss if untreated. Periodontitis is regarded as the second most common dental disease worldwide, affecting more than 50% of the world's population.[1] The presence of bacteria is the primary etiological factor for periodontal diseases, but the susceptibility of the host is another important factor to consider, as their immune response is closely related to pathogen clearance. If the immune response in the host is too strong, the pathogen may be cleared, but this will cost excessive inflammatory damage to the host tissue.[2] A successful host immune response, thus, generally depends on the balance of pro-and anti-inflammatory cytokines, which are carefully adjusted to eliminate the pathogen while limiting host damage. A previous study showed that the occurrence of periodontitis is closely related to the activation of the complement system. The treatment chosen for periodontitis needs to determine the precise complement-associated signaling pathways with protective effects. The complement pathway with defensive functions can play a protective role and achieve the purpose of effective immunity.[3] When the complement system is activated, anaphylatoxins are released, including complement components 3a (C3a), 4a (C4a), and 5a (C5a). Among them, C5a reported the most powerful effect, being 2500 and 20 times as potent as C4a and C3a, respectively. C5a is a glycoprotein composed of 74 amino acids, and it functions by binding to C5aR on the cell membranes of target cells.[4] C5a can then chemotactic neutrophils and macrophages, promote inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and promote T-cell proliferation and B-cell production of antibodies to enhance the damaging effects of the inflammation.[5,6]

Lipopolysaccharide (LPS), also known as endotoxin, is an essential component of the outer membrane of gram-negative bacteria. LPS can cause inflammation in various organs and tissues throughout the body, including periodontal tissue. Previous studies showed that LPS can be used as an inflammation model in human gingival fibroblasts.[7-9] In the early identification of a pathogen, innate immune responses are activated by Toll-like receptors (TLRs), which can sense specific molecular patterns.[10] Toll-like receptor 4 (TLR4), as a transmembrane protein and member of the Toll-like receptor family, is activated by LPS. The downstream signaling then cascades through the myd88-dependent pathway to initiate multiple signaling pathways, such as the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways. This process induces the activation of downstream transcription factors such as NF-κB and activator protein-1 (AP-1) and the production of various pro-inflammatory factors, including IL-6, IL-1β, and TNF-α.[11] Despite its protective role, complement stimulated by LPS may cause or exacerbate infection including periodontitis when excessively activated or deregulated.[3] This process may not only directly trigger the cascade reaction of the C5a-c5aR axis, but also crosstalk and regulate other host pathways, disrupting the balance of immune and inflammatory responses in periodontal tissue, consequently aggravating the destruction of connective tissue in it, which may lead to tooth loosening and missing.[12] Current treatment methods for periodontal disease include both surgical and non-surgical approaches, although the long-term use of antibiotics can lead to an increase in resistant strains of resident bacteria. Thus, it is crucial that we develop safe and effective drugs for the treatment of periodontitis.[13]

C5aRA (PMX53) and C5aRA (PMX205) are cyclic hexapeptides, which are non-competitive inhibitors of C5aR.[14] PMX53 and PMX205 are widely used as non-peptide antagonists. Studies showed that they could significantly inhibit C5a-mediated inflammatory diseases, although the oral bioavailability of PMX205 and PMX53 is only 23% and 9%.[15-17] W54011 is an orally active non-peptide C5a receptor antagonist (C5aRA) developed by Mitsubishi Pharmaceutical. It can inhibit C5a activation by inhibiting the binding of C5a to its receptor C5aR. W-54011 is a competitive antagonist with stronger affinity than non-competitive antagonists and high oral bioavailability and is known as the most effective small C5aRA molecule.[18] Previous research showed that C5aRA (W54011) can reduce kidney damage by inhibiting the MAPK signaling pathway,[19] and it can also be used as a novel therapeutic to improve sleep deprivation-mediated spatial memory impairment by inhibiting neuroinflammation and adult neurogenesis decline in the hippocampus.[20] W54011 can also protect lung cells and tissues from LPS-induced damage by alleviating inflammation and pyroptosis.[21] These studies suggest that W54011 may

help to treat inflammatory diseases mediated by C5a. However, whether C5aRA (W54011) can inhibit the inflammatory response of gingival fibroblasts to treat periodontitis has not yet been reported.

Therefore, we used LPS as an inflammatory model to induce gingival fibroblasts and aimed to ensure the inhibition of C5aRA (W54011) on the C5a-C5aR axis, which subsequently inhibits the expression of inflammatory cytokines in LPS-stimulated HGFs. Furthermore, we also aimed to clarify the anti-inflammatory effect and mechanism of C5aRA by verifying the involvement of the classical inflammatory response signaling pathways NF-κB and MAPK.

Methodology

Isolation and culture of human gingival fibroblasts

Human gingival fibroblasts (HGFs) were isolated from gingival tissues taken from the area around impacted third molars or periodontal healthy premolars, which were primarily extracted by orthodontics with the consent of the patient and/or guardian. The selected donors were aged from 18 to 25, in good health, without systemic diseases, without genetic diseases, non-smokers, antibiotic-free in the past six months, and had good oral hygiene. The male-female ratio was 1:1. This study was approved by the Institutional Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. A total of 30 gum samples were collected for three consecutive days. Considering that the cells of different patients may have differences, the samples obtained were mixed and extracted every day. Contamination occurred in six samples on the second day, and cells with similar morphology were obtained within 14 days from 24 tissue samples without contamination. Specific steps are as follows: the freshly excised gingival tissue was immediately placed in pre-cooled Dulbecco's Modified Eagle Medium (DMEM)-F12 (containing 1% penicillin and 1% streptomycin) (HyClone; GE Healthcare Life Sciences) and soaked for later use. The collected gingival tissues were stored in a refrigerator at 4 °C and experimentally extracted within 4 h of sampling. The samples were first washed 3–4 times with 5 × antibiotics (containing 1% penicillin and 1% streptomycin) in phosphate-buffered saline (PBS). They were cut into approximately 1.0 mm^3 pieces (1mL of DMEM-F12 medium on the tissue block when cutting was added), and a dental probe was used to evenly spread the tissue block on the wall of a 25 cm^2 sterile culture flask. The flask was turned over and incubated at 37 °C, 5% CO_2 for 1 h. After that, 2 mL of DMEM-F12 supplemented with 10% fetal bovine serum (Biological Industries), penicillin (100 U/mL), and streptomycin (100 μg/mL) was added in the flip-back flask at 37 °C with 5% CO_2. On the fourth day of culture, 2 mL DMEM-F12 medium was supplemented. The medium was changed for the first time on day seven, using a pipette to gently aspirate the culture medium, to ensure that the pipette did not contact the tissue. During this process, the tissue block was attached to the bottom of the culture bottle. The medium was then replaced every second day. As the adherent cells increased, the tissue pieces floating alongside them also had to be removed when changing the medium. Primary cells were passaged when they were 80–90% confluent. Cell adherence was observed using an inverted microscope.

Purification of human gingival fibroblasts

All primary cells were cultured for no longer than 14 days to avoid cell senescence. Therefore, when the earliest extracted sample was cultured for 14 days, we digested all primary cells (observe that the number of cells in the culture flask with the least amount of cells also reached 75% confluent) according to the differential digestion method, then mixed resuspended cells for passage. Specific steps are as follows: enzymatic digestion was used to purify the HGFs. Under the action of trypsin at 25 °C for 3–4 minutes, fibroblasts peeled off first, while epithelial cells required more time to digest before peeling off. The supernatant was carefully collected and then centrifuged at 1,000 rpm for 5 min (Cell count approximately 3.6×10^7). The resuspended cell pellet was transferred to a flask, which was then filled up to 4 mL with the DMEM-F12 medium. After the second-generation cells are congested, some will be used for subsequent research such as identification and induction, and some will be frozen for future use.

Identification of gingival fibroblasts using immunofluorescence

The second-passage cells were plated in 24-well plates at a density of 8000 cells per well. When the cells reached 50–70% confluence, they were washed twice with PBS and fixed with 4% paraformaldehyde

for 15 min. The 0.5% Triton X-100 (in PBS) was used for 20 minutes at 25 °C to permeabilize. Normal goat serum was used to block non-specific binding at 25 °C for 30 min. Then, the blocking solution was aspirated, and vimentin (rabbit anti-human, 1:250, Abcam, UK), keratin (rabbit anti-human, 1:250, Abcam, UK), and fibroblast-specific protein (FSP-1, also known as S100A-4, rabbit anti-human, 1:250, Abcam, Britain) were added overnight at 4 °C. Goat anti-rabbit Alexa Fluor 488 (1:300, Abcam, UK) or Alexa Fluor 594 (1:300, Abcam, Britain)-coupled secondary antibodies were then added and incubated in the dark for 2 h. The nuclei were stained via incubation with DAPI (1:150) in the dark for 5 min, and the slides were sealed with mounting liquid containing an anti-fluorescence quencher. Images were obtained using a fluorescence microscope (DMI4000B, Leica, Germany).

NF-κB activation-nuclear transport assay

The NF-κB Activation-Nuclear Transport Assay Kit (SN368, Beyotime Biotechnology Limited Company, Shanghai, China) was used to detect p-p65, under the guidance of the manufacturer's instructions. Fourth HGFs were cultured in a 24-well plate containing cell slides (8000 cells/well). After the cells securely adhered, they were cultured in serum-free DMEM-F12 for 24 h and were then divided into three groups, as follows: i) 1 μg/mL LPS (48 h) + C5aRA (48 h); ii) serum-free DMEM-F12 alone for 4 days (negative control group); and iii) 1 μg/mL LPS (48 h). Cells in each group were rinsed with PBS for 3 × 5 min, fixed with a fixative for 10 min, then washed for 3 × 5 min. An immunostaining blocking solution was used to block cells at 25 °C for 1 h. The p-p65 antibody was incubated overnight at 4°C. The secondary antibody (anti-rabbit Cy3) was then added and incubated at 25 °C for 1 h. The cells were stained with a nucleus staining agent (DAPI) at 25 °C for approximately 5 min.

Treatment groups and culture conditions

According to supplementary data S1, S2 and S3 the cultured cells were divided into four groups, as follows: i) 1 μg/mL LPS for 48h, then add 1 μg/mL C5aRA (W54011, HY-16992A; MedChemExpress, USA) for another 48h; ii) serum-free culture medium (96 h) as the control group; iii) serum-free medium for 48 h at first, then add 1 μg/mL LPS for 48 h; and iv) serum-free medium for 48 h at first, then add 1 μg/mL C5aRA for 48h. All groups were starved for 24 hours with the serum-free medium at first, and then added LPS or C5aRA according to the grouping described above.

ELISA assay

Cells were divided into four groups as described before, and the cell supernatant was extracted. Following the manufacturer's protocol, the activation of C5a in cell supernatant was performed using a C5a enzyme immunoassay kit (Abcam, Cambridge, UK), as previously described.[21] Finally, the absorbance at 450 nm was measured with a microplate reader.

Real-Time Quantitative PCR analysis

The fourth generation cells were split into four 100mm^2 plates. When the cell fusion reached 80-90%,

Figure 1- Primer sequences used for RT-PCR and RT-qPCR

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
C5aR	CGCTTTCTGCTGGTGTTTA	GTTTGTCGTGGCTGTAGTCC
IL-1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
IL-6	GAGGAGACTTGCCTGGTG	GCATTTGTGGTTGGGTCA
TNF-α	CCCCAGGGACCTCTCTCTAATC	GGTTTGCTACAACATGGGCTACA
p-p65	AACAGAGAGGATTTCGTTTCCG	TTTGACCTGAGGGTAAGACTTCT
p65	ATGTGGAGATCATTGAGCAGC	CCTGGTCCTGTGTAGCCATT
p-IκBα	AGACCTGTCCCTGAACCCTAT	CGATGGACTTCTAAACCAGCCA
IκBα	CTCCGAGACTTTCGAGGAAATAC	GCCATTGTAGTTGGTAGCCTTCA
JNK	TGTGTGGAATCAAGCACCTTC	AGGCGTCATCATAAAACTCGTTC
p-JNK	TGTGTGGAATCAAGCACCTTC	AGGCGTCATCATAAAACTCGTTC
c-JUN	TCCAAGTGCCGAAAAAGGAAG	CGAGTTCTGAGCTTTCAAGGT
p-c-JUN	TCCAAGTGCCGAAAAAGGAAG	CGAGTTCTGAGCTTTCAAGGT
TLR4	CCTCGGCGGCAACTTCATAA	AGAGCGGATCTGGTTGTACTG
Actin	AAGGAGCCCCACGAGAAAAAT	ACCGAACTTGCATTGATTCCAG

the cells were divided into four groups following the description above. These steps were performed three times. The real-time quantitative PCR (RT-qPCR) was completed using the FastStart Universal SYBR-Green Master Mix (Roche, USA). RT-qPCR reaction conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, then 72 °C for 5 min. Three repetitions were conducted for each sample. Actin was used for the normalization of mRNA. The relative quantity of mRNA expression was shown by the 2^-ΔΔCt method (Livak et al., 2001). The primers for RT-qPCR were presented in Figure 1.

Western blotting analysis

Cells were grown to 80–90% confluence in a 100 mm^2 dish. The cells were added with RIPA lysis buffer supplemented with 1% PMSF (Beyotime, China) to extract total proteins. Cells were ultrasonically shaken for 1 min (2 s shaking, 1 s stop, ampl 40%) and then centrifuged at 13,300 × g for 15 min at 4 °C. The supernatant was subjected to the Pierce™ BCA Protein Assay kit (Beyotime, China) for protein concentration. A sample of 10 μg was added to each lane of the gel separately. SDS-PAGE (10%) was performed for C5aR, p-IκBα , IκBα , p-p65, p65, c-JUN, p-c-JUN, JNK, p-JNK, TLR4, and actin antibodies, and 15% SDS-PAGE was performed for IL-1β, IL-6, and TNF-α antibodies. Separated proteins were transferred onto PVDF membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% skimmed milk at 25 °C for 1 h and incubated with primary antibodies (1:1000 dilutions in TBST) at 4°C overnight: C5aR rabbit polyclonal antibody (ProteinTech Group, Inc.), IL-6 mouse monoclonal antibody (ProteinTech Group, Inc), IL-1β rabbit monoclonal antibody (ProteinTech Group, Inc), TNF-α rabbit monoclonal antibody (ProteinTech Group, Inc), p-IκBα rabbit monoclonal antibody (Abcam, Britain), IκBα rabbit monoclonal antibody (Abcam, Britain), p65 rabbit monoclonal antibody (Abcam, Britain), p-p65 rabbit monoclonal antibody (Abcam, Britain), c-JUN rabbit monoclonal antibody (Abcam, Britain), p-c-JUN rabbit monoclonal antibody (Abcam, Britain), JNK rabbit monoclonal antibody (Abcam, Britain), p-JNK rabbit monoclonal antibody (Abcam, Britain), TLR-4 rabbit monoclonal antibody (ProteinTech Group, Inc.). After washing with TBST, the membranes were exposed to secondary antibodies at 25 °C for 1 h, using goat anti-mouse IgG and goat anti-rabbit IgG (1:10,000; ProteinTech Group, Inc.). After washing the membrane with TBST three times, the bands were visualized using enhanced chemiluminescence (Ultrasensitive ECL Detection Kit, ProteinTech Group, Inc.). β-Actin mouse monoclonal antibody (1:5,000; ProteinTech Group, Inc.) was used as a loading control. The bands were quantified using the ImageJ program (NIH, USA).

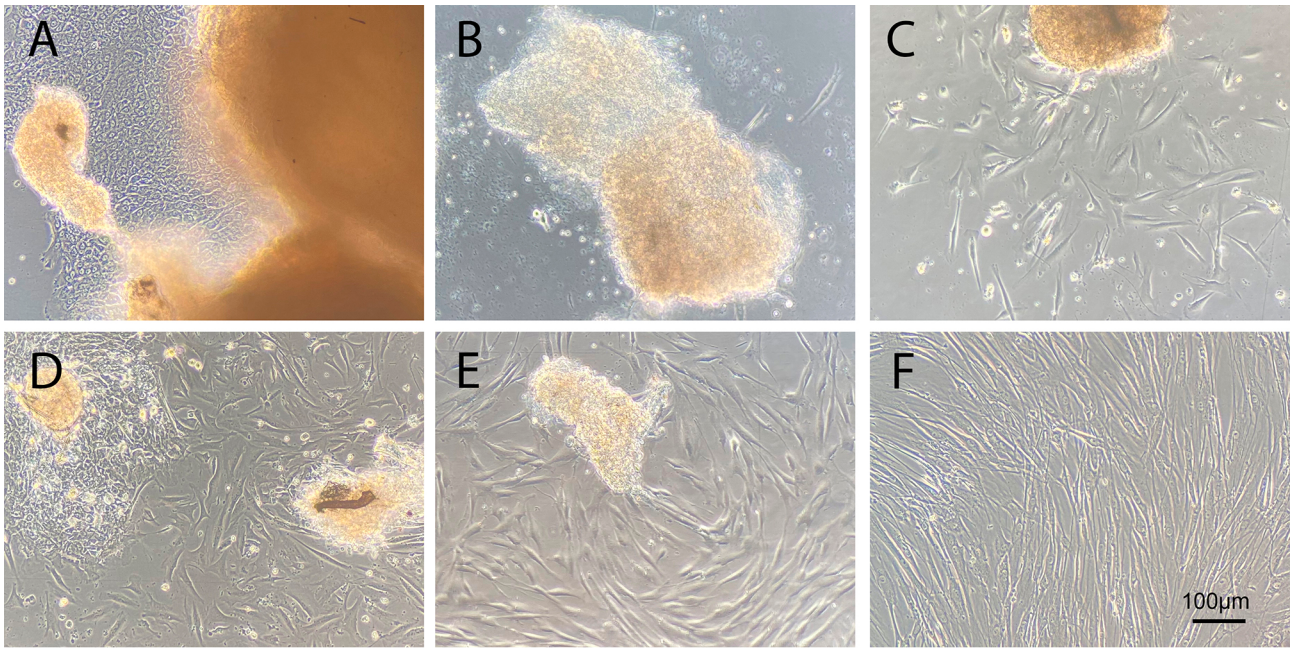

Figure 2- Isolation and culture of human gingival fibroblasts (HGFs).(A) The appearance of primary HGFs cultured for two days.(B) The appearance of primary HGFs cultured for four days.(C) The appearance of primary HGFs cultured for seven days.(D) The appearance of primary HGFs cultured for10 days.(E) After 14 days of culture, the primary HGFs reached 80-90% confluency.(F) The appearance of HGFs after passaging. Scale bar = 100μm.

Statistical analysis

Data were analyzed for normality distribution using the Shapiro-Wilk test. Comparisons between the groups were assessed using one-way ANOVA, followed by Bonferroni multiple comparisons using GraphPad 8. All values of this study are expressed as the mean ± SEM. $P < 0.05$ was considered statistically significant.

Results

Isolation and culture of human gingival fibroblasts

We successfully isolated HGFs from 24 test samples using the tissue block adherence method. On the second day, we observed the flat polygonal cells isolated from gingival tissue blocks and tightly packed them into clone-like clumps (Figure 2A). After four days of culture, a small number of spindle-shaped or short-fusiform adherent cells could be observed from other tissue blocks (Figure 2B). We observed a significant increase in the number of spindle-shaped or short-fusiform adherent cells on day seven of the culture (Figure 2C). On day 10, the number of adherent cells increased significantly, and two obvious cell shapes were observed: flat polygonal and long spindles (Figure 2D). On day 14, almost no clear boundary between the two cell types was observed. At this time, the proliferating cell clusters were close to each other, and cells reaching 80%–90% confluence could be passaged (Figure 2E). Cells were digested with trypsin for 3–4 minutes at 25 °C. At this point, almost all fibroblast-like cells curled up and detached

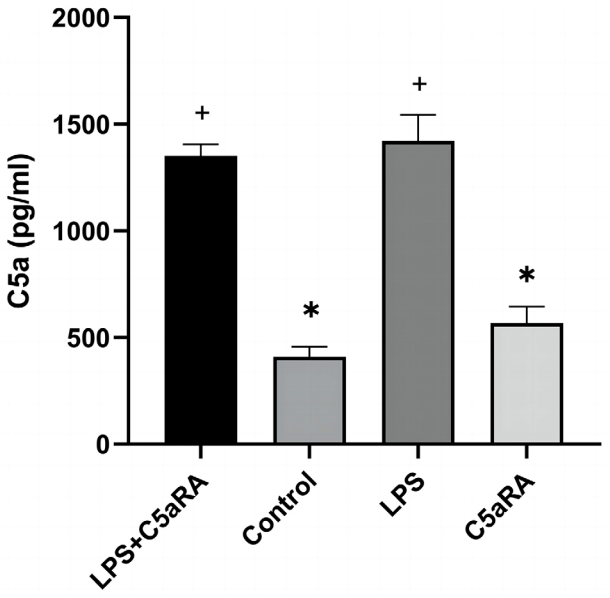

Figure 4- The content of C5a in the cell supernatant of the different group was measured by the ELISA assay. Data represent the mean ± SEM of three independent experiments. +$P < 0.05$ vs. the control group. *$P < 0.05$ vs. the LPS+C5aRA group

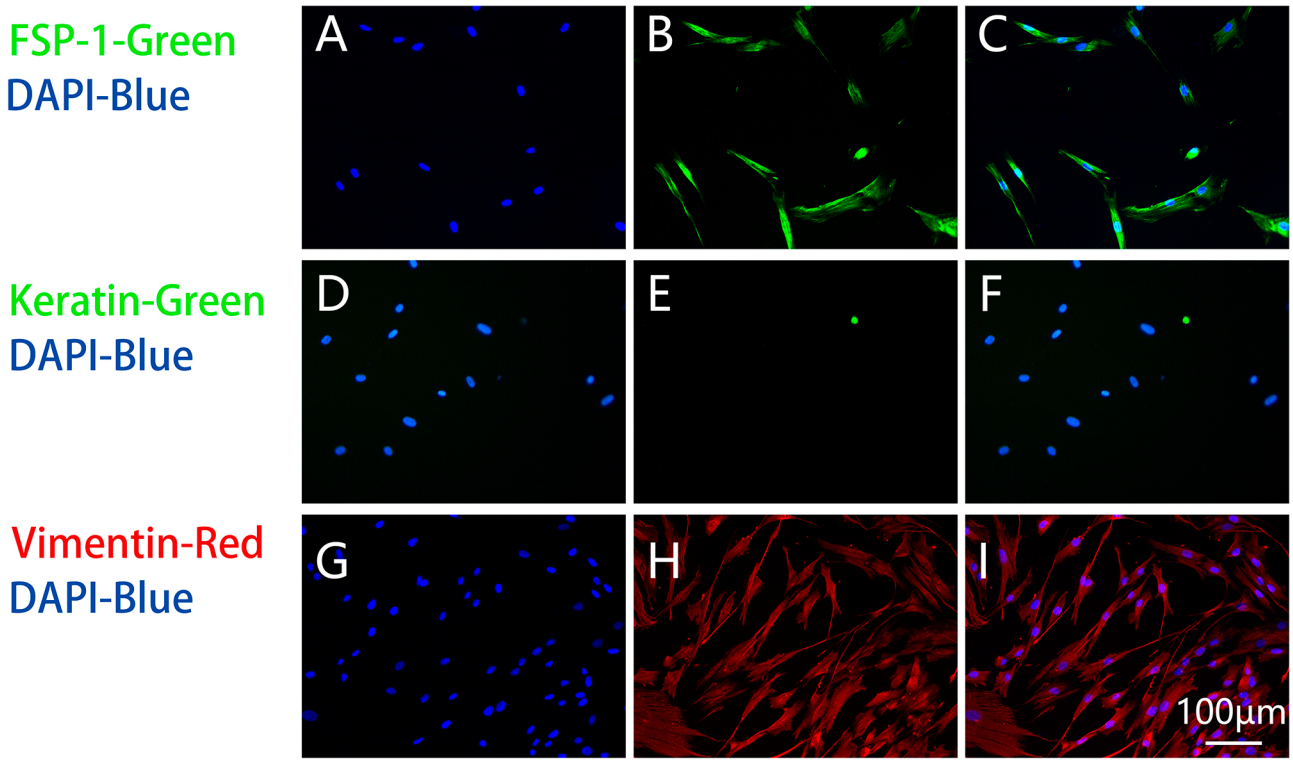

Figure 3- Identification of the second passage cells from HGFs with immunofluorescent staining.(A-C) The green positive expression of FSP-1 in the cytoplasm of HGFs.(D-F) Keratin is unstained in the cytoplasm.(G-I) The positive staining of vimentin in the cytoplasm of HGFs was red fluorescence. The positive staining of nucleus of HGFs was blue fluorescence. Scale bar =100μm. Fluorescence microscope × 200

from the culture flask. However, polygonal cells retained their original morphology. After passage, the morphology and size of the fibroblast-like cells were relatively uniform and typical for their cell type (Figure 2F). The cells proliferated rapidly and were passaged every 3–4 days.

Identification of the source of human gingival fibroblasts

Fluorescence microscopy showed that second-generation cells had a single long spindle shape arranged in parallel, and the volume of the passaged cells was slightly larger than that of the primary cells

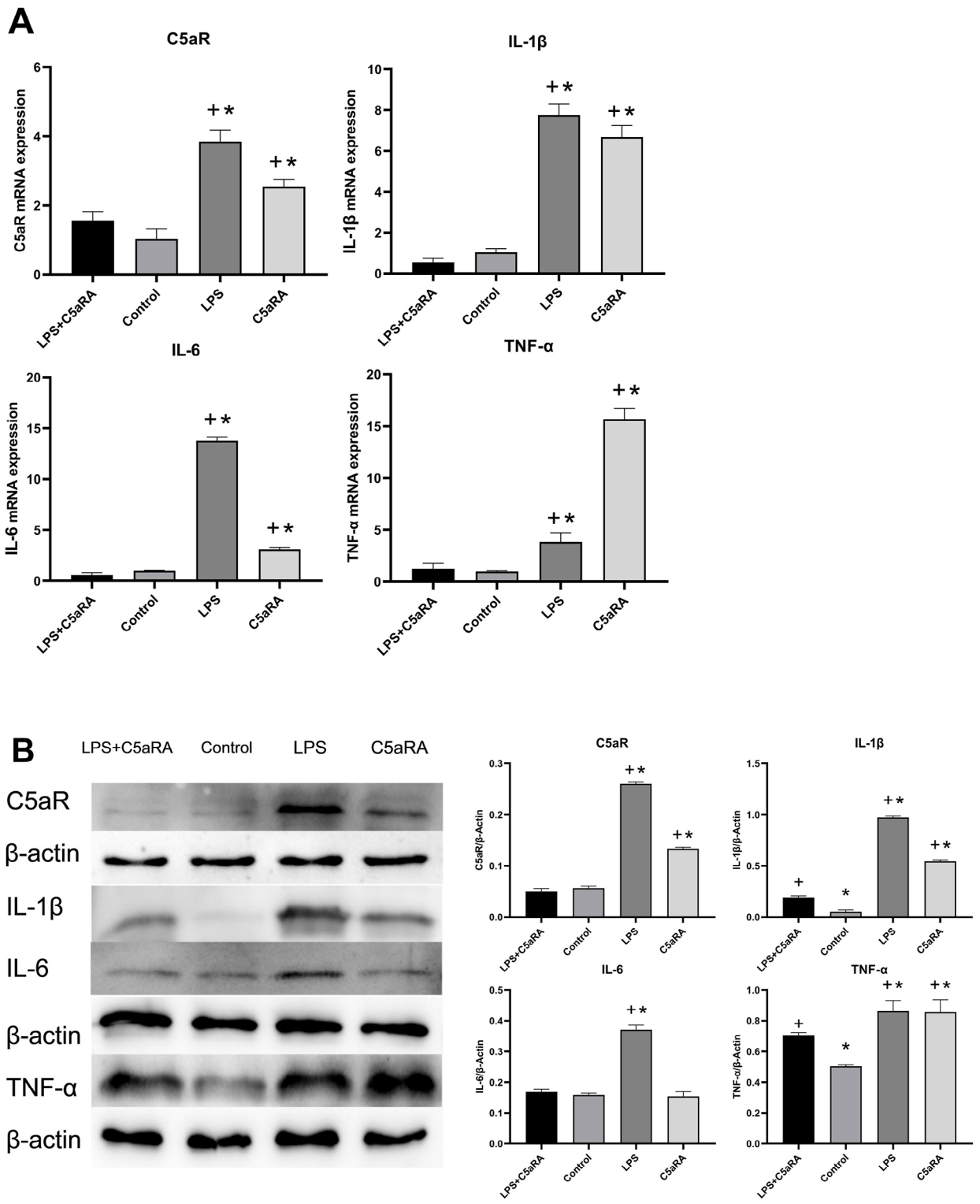

Figure 5- Effects of C5aRA on the expression of C5aR and inflammatory factors of LPS-induced HGFs by RT-qPCR and Western blotting. (A) C5aRA inhibited expression of C5aR, TNF-α, IL-1β, and IL-6 mRNA in LPS-stimulated HGFs. (B) C5aRA inhibited expression of C5aR, TNF-α, IL-1β, and IL-6 protein in LPS-stimulated HGFs. Data represent the mean ± SEM of three independent experiments. +P < 0.05 vs. the control group. *P < 0.05 vs. the LPS+C5aRA group

(Figure 3). Immunofluorescence detection showed that the positive staining of vimentin in the cytoplasm and nucleus of the HGFs was red and blue, respectively. Keratin was unstained in the cytoplasm, and the nuclei were blue. FSP-1 immunofluorescence results showed positive green expression in the cytoplasm of the tested cells.

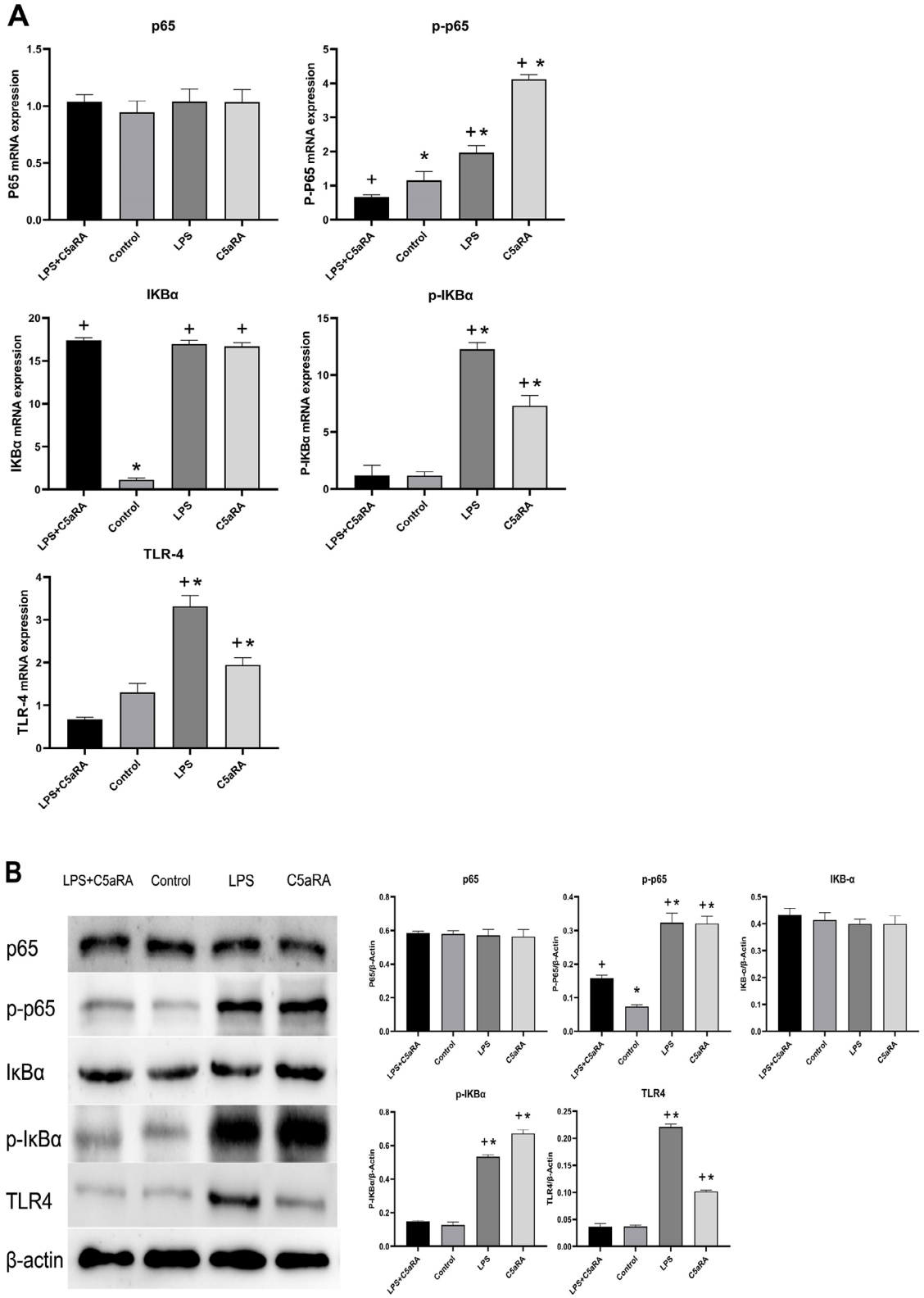

Figure 6- Effects of C5aRA on NF-κB signaling pathway activation and TLR4 expression in LPS-stimulated HGFs.(A) Effects of C5aRA on mRNA levels of p65, p-p65, IκBα, p-IκBα and TLR4 in LPS-stimulated HGFs.(B) Effects of C5aRA on protein levels of p65, p-p65, IκBα, p-IκBα and TLR4 in LPS-stimulated HGFs. Data represent the mean ± SEM of three independent experiments. +P<0.05 vs. the control group. *P<0.05 vs. the LPS+C5aRA group

C5aRA affected the expression of C5a, C5aR, and inflammatory factors in HGFs treated by LPS

ELISA results showed that compared with the control group, the concentration of C5a in the LPS group was significantly increased and that there was no significant difference between the LPS + C5aRA group and the LPS group. The expression of C5a in the C5aRA group alone was not significantly different from that in the control group (Figure 4).

The trends in the results of RT-qPCR and Western blotting analysis were the same (Figure 5). They showed that the mRNA and protein expression levels for inflammatory factors IL-1β, IL-6, TNF-α , and C5aR in the LPS group expressed a significantly higher level of the inflammatory factor than the control group. Then, the LPS + C5aRA group significantly downregulated the mRNA and protein expression levels of IL-1β, IL-6, TNF-α , and C5aR. This indicates that C5aRA significantly inhibited LPS-induced inflammation in human gingival fibroblasts. Furthermore, compared to the LPS + C5aRA group, the C5aRA alone group showed upregulated mRNA and protein expression levels for IL-1β, IL-6, TNF-α , and C5aR.

C5aRA affected TLR4/NF-κB signaling pathway after being treated by LPS

Western blotting and RT-qPCR analyses showed that the expression levels of TLR4, p-p65, and p-IκBα in the LPS group were significantly higher than those in the control group and that the C5aRA treatment could further increase the expression levels of TLR4, p-p65, and p-IκBα (Figure 6). In addition, the expression of TLR4, p-p65, and p-IκBα in the C5aRA treatment group was higher than that in the LPS + C5aRA group. The groups showed no difference in the mRNA and protein expression levels for the p65. Nevertheless, the expression trends for the IκBα mRNA and protein differed. The expression of the IκBα mRNA in the control group was lower than in all other groups. However, the expression of the IκBα protein showed no statistically significant difference among the groups. To further validate these results, we used immunofluorescence to detect the nuclear translocation of p-p65. The results showed that bright red fluorescence in the LPS group was mainly expressed in the nucleus, although a small fraction was observable in the cytoplasm. Compared to the LPS group, the C5aRA treatment group showed significantly reduced expression of red fluorescent cells in the nucleus, as they were mainly in the cytoplasm. In the control group, bright red fluorescence was observed in the cytoplasm but not

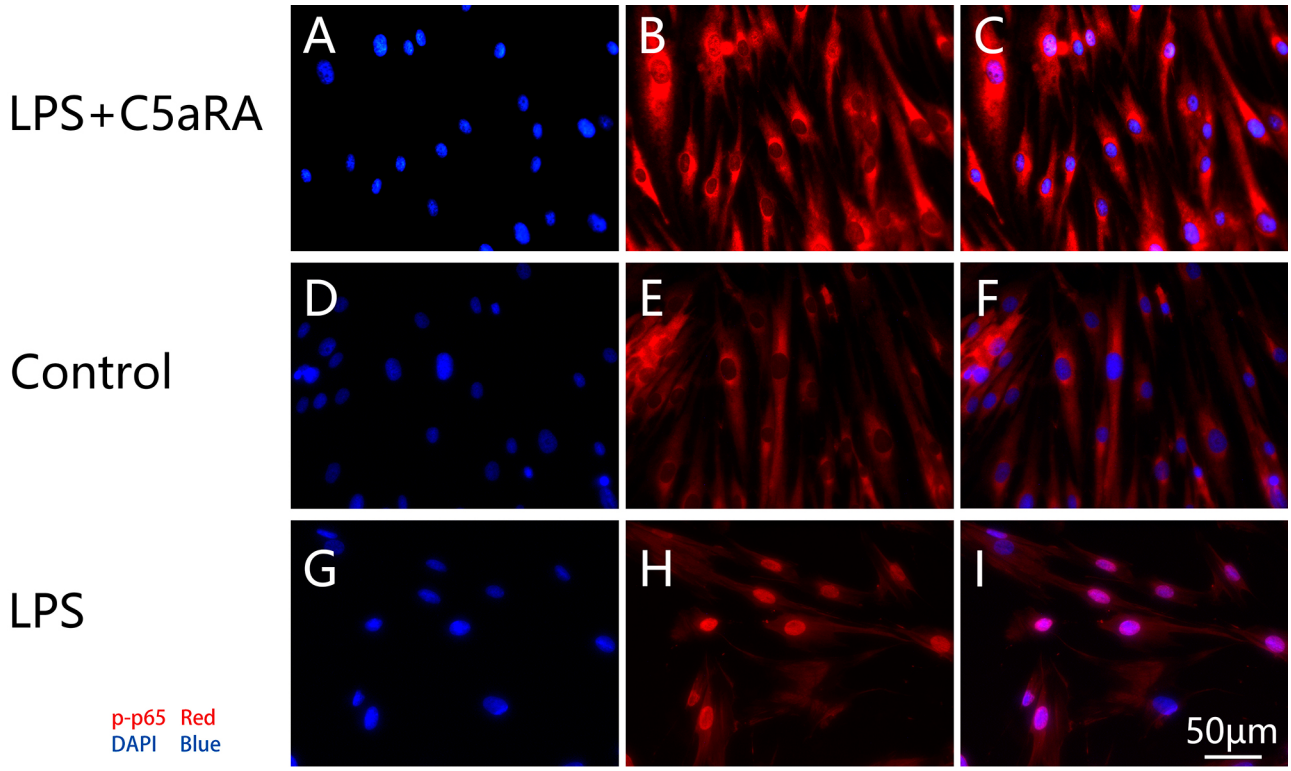

Figure 7- The effects of C5aRA on p-p65 translocation into the nucleus were used to detect by immunofluorescence staining.(A-C) LPS+C5aRA group.(D-F) The control group.(G-I) LPS group. The blue staining represented the nucleus; the green staining was p-p65. Scale bar = 50μm. Fluorescence microscope × 400

in the nucleus (Figure 7).

C5aRA affected MAPK signaling pathway after being treated with LPS

We used Western blotting and RT-qPCR analyses to detect the expression of MAPK pathway-related factors. The LPS stimulation group showed significantly upregulated mRNA and protein expression levels for p-JNK and p-c-JUN, and the C5aRA treatment group showed significantly downregulated mRNA and protein expression levels for p-JNK and p-c-JUN, compared with the control (Figure 8). The mRNA and protein expression levels of the p-JNK and p-c-JUN in the C5aRA treatment group were also higher than those

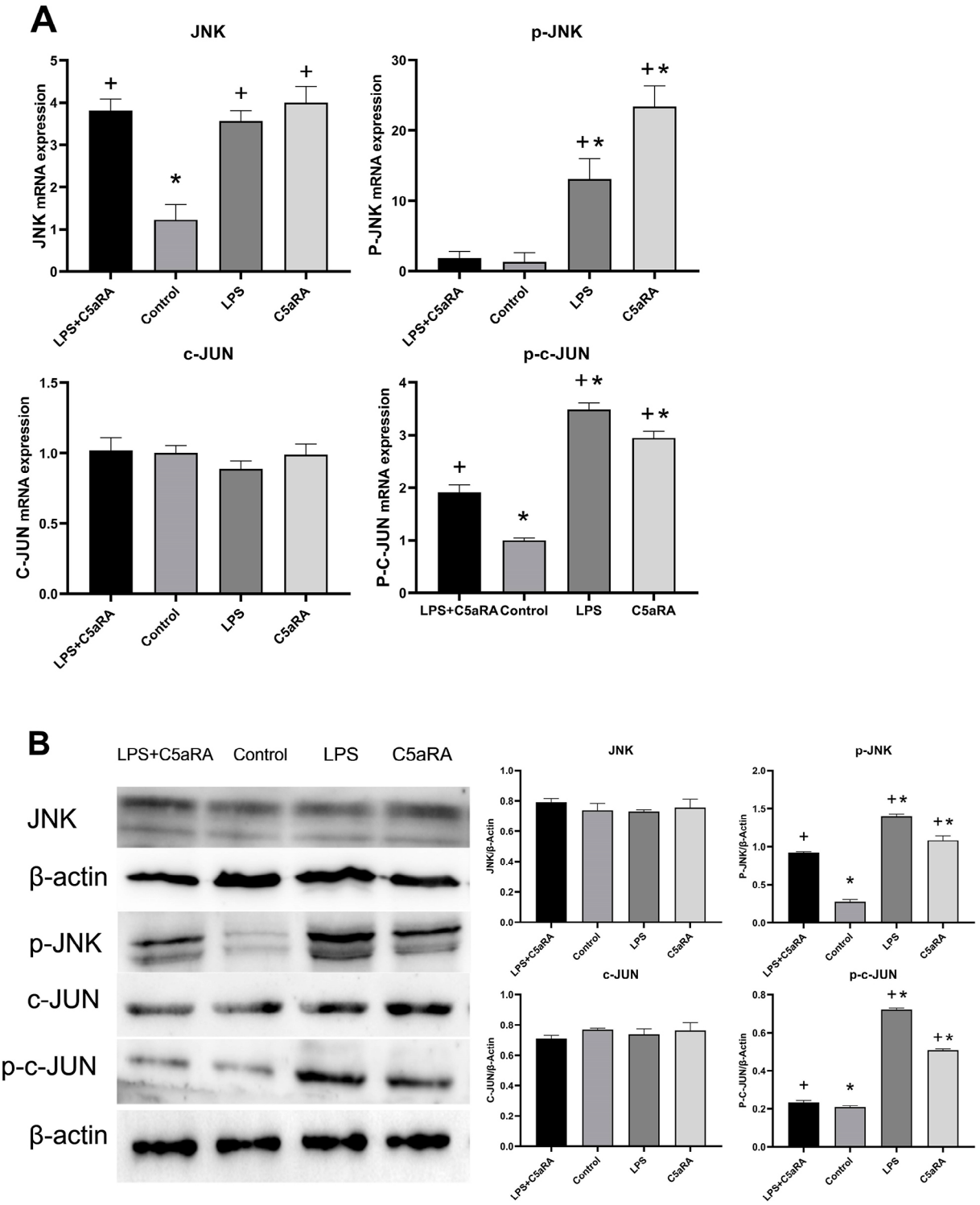

Figure 8- Effects of C5aRA on MAPK signal pathway activation in LPS-stimulated HGFs.(A) Effects of C5aRA on mRNA levels of JNK, p-JNK, c-JUN, and p-c-JUN in LPS-stimulated HGFs. (B) Effects of C5aRA on protein levels of JNK, p-JNK, c-JUN, and p-c-JUN in LPS-stimulated HGFs. Data represent the mean ± SEM. of three independent experiments. +P<0.05 vs. the control group. *P<0.05 vs. the LPS+C5aRA group

in the LPS + C5aRA group. RT-qPCR showed that the expression of JNK mRNA in the control group was the lowest among the groups ($P<0.05$). However, JNK protein expression showed no difference between the groups, and we found no statistically significant difference in the expression of the c-Jun mRNA or protein between the groups.

Discussion

HGFs are the most abundant resident cells of gingival connective tissue, which can directly provide the signal to mediate host immune responses and recruit inflammatory cells to clear infection in periodontal injury. Inflammatory cytokines can also be secreted from chemotactic immune cells during HGFs immune responses. Thus, HGFs play a key role in periodontitis pathogenesis.[22] Therefore, we selected it as the focus of this study. As previously reported, the fibroblast-like cells were successfully obtained from human gingival tissues using the tissue block method, which is simple, fast, reproducible, and maintains cell viability and function.[23] Our results agree with previous studies which reported the typical fibroblast-like morphology of cells isolated from human gingival tissues.[24]

The main skeletal protein of keratinocytes is Keratin, which is mainly present in epithelial cells. Its main function is to maintain epithelial integrity and continuity. Vimentin is an intermediate filament protein mainly present in the cytoskeleton of mesenchymal cells, and FSP-1 (also known as S100A-4) is a member of the calbindin S100 family, which plays an important role in cell fibrosis, cell proliferation, and motility, and is also commonly used as fibroblast markers. We identified HGFs by detecting the positive expression of vimentin and FSP-1 and the negative expression of keratin using immunofluorescence. The results showed that the cultured cells were from mesodermal mesenchymal tissues and that they exhibited fibroblast characteristics. Combined with the sampling site and histological characteristics, we confirmed that the cells obtained and cultured in our study were human gingival fibroblasts. Gingival tissue also has a resident stem cell population derived from mesenchyme. However, we did not specifically study the ratio of fibroblasts to stem cells in our experiments. On the one hand, the number of stem cells is very low under normal circumstances. On the other hand, as Denu et al. said, fibroblasts and mesenchymal stromal/stem cells are phenotypically indistinguishable and have almost the same immune regulatory properties.[25] Therefore, even if fibroblasts and stem cells were not isolated separately, it would not affect our experimental results.

The complement system is an important part of the immune system and the first line of defense against pathogens and stressed host cells. Complement C5a, the most potent anaphylatoxin, exerts its main pro-inflammatory activity by interacting with its specific receptor C5aR and becomes the main factor triggering the inflammatory response in various diseases.[26] A previous report showed that the complement C5a is an important factor in periodontal disease and can cause inflammation and tissue damage. Even if the dentist effectively removes plaque microorganisms, it is difficult to completely alter the periodontal immune microenvironment. High expression levels of C5a activate the immune process, resulting in the destruction of periodontal tissue, which may be one of the reasons for highly recurrent periodontitis.[16] Moreover, the destructive influences of the inflammatory factors IL-6, IL-1β, and TNF-α on periodontal tissue during periodontitis have been well-established in previous studies.[27] Our experiment evaluated the activation of the C5a-C5aR signaling axis by analyzing the variations of C5aR expression levels. In addition, many studies have used LPS to establish an *in vitro* periodontitis cell model. As a potent activator of inflammation, LPS leads to the over-release of inflammatory cytokines, such as IL-6, IL-1β, and TNF-α.[28] CCK8 was used to determine the optimal concentration and time for LPS administration to generate periodontitis cell models *in vitro*. Based on the differences in the mRNA expression levels of C5aR, IL-6, IL-1β, and TNF-α , the 1 μg/mL LPS for 48 h was the optimal stimulation condition (supplementary data S1 and S2). These results also showed that the group of 0.1 μg/mL LPS had better cell viability than the control group. The results indicate that HGFs can promote cell proliferation when stimulated by a very low concentration of LPS, which may be related to the high recovery ability of the gingival tissue itself.

However, not all immune responses are destructive, and complement is also crucial for the defense against infection. Therefore, we chose a C5aR antagonist (W54011) to target periodontitis, which can not only specifically inhibit the biological activity of C5aR but can

also gain therapeutic benefits without compromising the protective immune response. To better study the mechanism by which C5aRA inhibits inflammation, we screened the concentrations and durations of action for C5aRA (supplementary data S1 and S3). The CCK8 assay and RT-PCR determined that the treatment with 1 μg/mL C5aRA for 48 h unaffected cell activity but significantly reduced the mRNA expression of C5aR, IL-6, IL-1β, and TNF-α .

The expression results of C5aR and inflammatory factors detected by RT-qPCR and Western blot analysis indicate that LPS can activate the complement system in HGFs and promote the inflammatory response. The ELISA result of C5a also showed the effectiveness of activation of the complement system stimulated by LPS. Therefore, we suggest that LPS can be considered a typical "*in vitro* model" of gingival fibroblast inflammation. Additionally, after LPS-stimulated HGFs were treated with C5aRA, the expression of C5aR, IL-6, IL-1β, and TNF-α significantly decreased. However, there was no significant change in the expression of C5a. This indicates that C5aRA did not affect C5a expression. More importantly, C5aRA can effectively inhibit C5aR expression, thus reducing the binding efficiency of C5a to C5aR and inhibiting the activation of the complement system to control inflammation.

TLR4 recognizes bacterial lipopolysaccharide (LPS) and participates in and mediates the activation of the NF-κB signaling pathway.[29] The NF-κB signaling pathway exists in almost all cell types and can be rapidly activated when an organism is subjected to harmful internal and external stimuli, and this helps to regulate many life processes and diseases in cells and organisms. The binding of NF-κB to its inhibitor IκB proteins exists in the cytoplasm in the resting state. Once activated, IκB is phosphorylated by proteases, and free p-p65 is transported into the nucleus to mediate the transcription of inflammatory genes. Growing studies suggest that TLR4-regulated NF-κB signaling is critical to inflammatory responses. For example, prediabetes-enhanced periodontal inflammation in rats can inversely activate TLR4 and increase the accumulation of p-p65 in the nucleus, while baicalin inhibits experimental rat teeth by downregulating TLR4 expression and inhibiting the nuclear translocation of p-p65 to inhibit the development of periarthritis. LPS-induced acute lung injury can be prevented by Athyrium multidentate extract, which blocks the TLR4 signaling pathway.[30-32] Agreeing with previous findings, our results showed that LPS stimulation upregulated the expression of TLR4, p-p65, and p-IκBα in HGFs and increased the nuclear translocation of p-p65.[9] These studies showed that LPS can activate TLR4, induce the phosphorylation of p65 and IκBα proteins. In contrast, the C5aRA treatment reversed the increased expression of TLR4, p-p65, and p-IκBα and inhibited the nuclear translocation of p-p65. Taken together, this shows that C5aR antagonists inhibit LPS recognition by TLR4 and inactivated the NF-κB pathway.

After TLR4 recognizes LPS, it triggers an intercellular signaling cascade, which activates the downstream NF-κB pathway, as well as MAPK pathway.[33,34] C-JUN NH2-terminal kinases (JNKs) are a subfamily of MAPKs, and previous studies showed that JNK activation is related to the phosphorylation of the JNK-dependent transcription factor c-JUN (a subunit of AP-1). Phosphorylation of JNK activates the MAPK signaling pathway, which partially activates the AP-1 transcription factor by initiating the phosphorylation of c-JUN, thus promoting the secretion of inflammatory factors.[35] Such process has also been confirmed by many studies; for example, tormentic acid inhibits LPS-stimulated inflammation by preventing the activation of TLR4-mediated NF-κB and p38 MAPK pathways; prolyl hydroxylases positively regulate the inflammatory effects of LPS-induced HGFs via TLR4-mediated NF-κB and MAPK pathways;[36] and sulforaphane may inhibit the inflammatory effects of LPS-activated microglia by inhibiting the JNK/AP-1/NF-κB signaling pathway.[37] To further investigate the regulatory mechanism of C5aRA, we first stimulated HGFs with LPS. RT-qPCR and Western blotting results showed that the expression of p-JNK and p-c-JUN significantly increased. The expression levels of p-JNK and p-c-JUN were significantly downregulated after the C5aRA treatment, indicating that C5aRA significantly prevented the phosphorylation of JNK and c-JUN. Our results show that C5aRA blocks the MAPK signaling pathway and the activation of its downstream transcription factor AP-1.

Unusually, results displayed that the mRNA expression of IκBα and JNK in the control group was significantly lower than those in the other groups, whereas the total expression for the IκBα and JNK proteins did not change significantly in each group. On the one hand, this may be because after the cells were stimulated, the gene expression was instantaneously

high, which may be reduced after the stress. On the other hand, protein synthesis may also be complex, and the processes of mRNA transcription, translation, and modification that enable protein expression, are affected by many factors.[38] However, our follow-up period was relatively short, requiring further research to confirm these findings.

Our study showed some interesting results. The C5aRA group not only upregulated the expression of C5aR and IL-6, IL-1β, and TNF-α but also activated NF-κB and MAPK signaling pathways when compared with the LPS + C5aRA group. This may be due to an imbalance in the original complement system caused by the C5aRA treatment of the HGFs. However, the specific reasons for this require further investigation. Previous research has confirmed that the MAPK signaling pathway is closely associated with regulating the AP-1 pathway.[33,34] Furthermore, studies also showed that although NF-κB and AP-1 transcription factors are mediated by various mechanisms, the fact that activation of the inflammatory cytokines or MAPK signaling pathways is usually accompanied by nuclear translocation of NF-κB, suggests that the transcription factor AP-1 and NF-κB signaling pathway often have a synergistic effect.[39] Furthermore, studies have speculated that a clue to the interaction between AP-1 and NF-κB activation pathways is the discovery that the activation of the MAPK pathway leads to the activation of the JNK and IκB kinase complexes.[40] Our experimental results confirm this hypothesis. After HGFs were stimulated by LPS, both the NF-κB and MAPK signaling pathways were activated, and the AP-1 signaling pathway was activated by the expression of p-c-JUN, which in turn led to the secretion of IL-1β, IL-6, and TNF-α. We speculate that this may be because many pathogenic stimuli that trigger the activation of the NF-κB pathway also induce cascades such as the MAPK signaling pathway, and these signaling pathways cooperate, resulting in the phosphorylation of the transcription factor AP-1, which promotes the release of inflammatory factors.

Conclusions

In short, our findings suggest that while C5aR antagonists inhibit the binding of C5a and C5aR, they also inhibit TLR4-mediated activation of the NF-κB/MAPK signaling pathway, thus inhibiting the expression of IL-6, IL-1β, and TNF-α. C5aR antagonists may also regulate the balance of the immune system by eliminating the synergistic effect of C5a and TLR4 on immune inflammation in periodontal tissues, ultimately inhibiting inflammation. The proposed mechanism illustration was presented in Supplementary Figure. Additionally, whether the C5aR antagonist (W54011) promotes a protective immune response needs further study. Thus, the results not only highlight the vital role of the complement system in the development of periodontitis but also provide new evidence for the potential value of a C5aR antagonist (W54011) as a novel therapeutic approach for complement-dependent inflammatory diseases.

Funding Statement

This study was supported by the Second-Class Postdoctoral Science Foundation of Heilongjiang Province, China, No. LBH-Z20075 and No. LBH-Z20079.

Acknowledgments

The authors thanks the Key Laboratory of Myocardial Ischemia of the Ministry of Education (The Second Affiliated Hospital of Harbin Medical University, Harbin, China) for providing the facilities we conducted this study.

Conflict of interest

The authors declare no conflict of interest

Data availability statement

The datasets generated during and/or analyzed during the current study are available in the SciELO Data repository, [DOI – 10.48331/scielodata.XR5TEV].

Authors' contributions

Jiao, Xiaohui: Conceptualization (Equal); Resources (Equal); Supervision (Equal); Validation (Equal); Writing – review & editing (Equal). **Chen, Yan:** Data curation (Equal); Project administration (Equal); Software (Equal); Validation (Equal); Writing – original draft (Equal). **Liu, Yang:** Data curation (Equal); Formal analysis (Equal); Investigation (Equal); Software (Equal); Writing – original draft (Equal). **Li, Hao:** Data curation (Equal); Investigation (Equal); Software (Equal); Validation (Equal); Visualization (Equal). **Huna, Risu:** Formal analysis (Equal); Investigation (Equal); Software (Equal). **Tan, Xiaohan:** Data curation (Equal); Software (Equal); Supervision (Equal); Visualization (Equal).

Li, Ning: Data curation (Equal); Software (Equal); Validation (Equal); Visualization (Equal). **Zhang, Yiying:** Formal analysis (Equal); Investigation (Equal); Software (Equal); Visualization (Equal). **Liu, Mingyue:** Conceptualization (Equal); Methodology (Equal); Project administration (Equal); Resources (Equal); Supervision (Equal); Writing – review & editing (Equal).

References

1- Eke PI, Dye BA, Wei L, Slade GD, Thornton-Evans GO, Borgnakke WS, et al. Update on prevalence of periodontitis in adults in the United States: NHANES 2009 to 2012. J Periodontol. 2015;86(5):611-22. doi: 10.1902/jop.2015.140520

2- Kwon T, Lamster IB, Levin L. Current concepts in the management of periodontitis. Int Dent J. 2021;71(6):462-76. doi: 10.1111/idj.12630

3- Hajishengallis G. Complement and periodontitis. Biochem Pharmacol. 2010;80(12):1992-2001. doi: 10.1016/j.bcp.2010.06.017

4- Liu M, Mu H, Peng W, Zhao L, Hu W, Jiang Z, et al. Time-dependent C5a and C5aR expression in dental pulp cells following stimulation with LTA and LPS. Int J Mol Med. 2019;44(3):823-34. doi: 10.3892/ijmm.2019.4246

5- Manthey HD, Woodruff TM, Taylor SM, Monk PN. Complement component 5a (C5a). Int J Biochem Cell Biol. 2009;41(11):2114-7. doi: 10.1016/j.biocel.2009.04.005

6- Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, Köhl J. The role of the anaphylatoxins in health and disease. Mol Immunol. 2009;46(14):2753-66. doi: 10.1016/j.molimm.2009.04.027

7- Shang L, Wang T, Tong D, Kang W, Liang Q, Ge S. Prolyl hydroxylases positively regulated LPS-induced inflammation in human gingival fibroblasts via TLR4/MyD88-mediated AKT/NF-κB and MAPK pathways. Cell Prolif. 2018;51(6):e12516. doi: 10.1111/cpr.12516

8- Xiong G, Ji W, Wang F, Zhang F, Xue P, Cheng M, et al. Quercetin inhibits inflammatory response induced by LPS from *Porphyromonas gingivalis* in human gingival fibroblasts via suppressing NF-κB signaling pathway. Biomed Res Int. 2019;2019:6282635. doi: 10.1155/2019/6282635

9- Yu T, Xie W, Sun Y. Oridonin inhibits LPS-induced inflammation in human gingival fibroblasts by activating PPARγ. Int Immunopharmacol. 2019;72:301-7. doi: 10.1016/j.intimp.2019.04.006

10- Wallet SM, Puri V, Gibson FC. Linkage of Infection to adverse systemic complications: periodontal disease, toll-like receptors, and other pattern recognition systems. Vaccines (Basel). 2018;6(2):21. doi: 10.3390/vaccines6020021

11- Assuma R, Oates T, Cochran D, Amar S, Graves DT. IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. J Immunol. 1998;160(1):403-9.

12- Li G, Pan J, Tang Q, Liu X, Wang L, et al. Anti-inflammatory effects of PMX205 in mouse macrophage periodontitis model. Iran J Immunol. 2018;15(2):84-96.

13- Souza JA, Rossa C Jr, Garlet GP, Nogueira AV, Cirelli JA. Modulation of host cell signaling pathways as a therapeutic approach in periodontal disease. J Appl Oral Sci. 2012;20(2):128-38. doi: 10.1590/s1678-77572012000200002

14- Allegretti M, Moriconi A, Beccari AR, Di Bitondo R, Bizzarri C, Bertini R, et al. Targeting C5a: recent advances in drug discovery. Curr Med Chem. 2005;12(2):217-36. doi: 10.2174/0929867053363379

15- Kumar V, Lee JD, Clark RJ, Noakes PG, Taylor SM, et al. Preclinical pharmacokinetics of complement c5a receptor antagonists PMX53 and PMX205 in mice. ACS Omega. 2020;5(5):2345-54. doi: 10.1021/acsomega.9b03735

16- Breivik T, Gundersen Y, Gjermo P, Taylor SM, Woodruff TM, Opstad PK. Oral treatment with complement factor C5a receptor (CD88) antagonists inhibits experimental periodontitis in rats. J Periodontal Res. 2011;46(6):643-7. doi: 10.1111/j.1600-0765.2011.01383.x.

17- Cui CS, Kumar V, Gorman DM, Clark RJ, Lee JD, Woodruff TM. *In vivo* pharmacodynamic method to assess complement C5a receptor antagonist efficacy. ACS Pharmacol Transl Sci. 2021;5(1):41-51. doi: 10.1021/acsptsci.1c00227

18- Sumichika H, Sakata K, Sato N, Takeshita S, Ishibuchi S, Nakamura M, et al. Identification of a potent and orally active non-peptide C5a receptor antagonist. J Biol Chem. 2002;277(51):49403-7. doi: 10.1074/jbc.M209672200

19- Zhang JX, Zha WS, Ye LP, Wang F, Wang H, Shen T, et al. Complement C5a-C5aR interaction enhances MAPK signaling pathway activities to mediate renal injury in trichloroethylene sensitized BALB/c mice. J Appl Toxicol. 2016;36(2):271-84. doi: 10.1002/jat.3179

20- Wadhwa M, Prabhakar A, Anand JP, Ray K, Prasad D, Kumar B, et al. Complement activation sustains neuroinflammation and deteriorates adult neurogenesis and spatial memory impairment in rat hippocampus following sleep deprivation. Brain Behav Immun. 2019;82:129-44. doi: 10.1016/j.bbi.2019.08.004

21- Wang R, Wang Y, Hu L, Lu Z, Wang X. Inhibition of complement C5a receptor protects lung cells and tissues against lipopolysaccharide-induced injury via blocking pyroptosis. Aging (Albany NY). 2021;13(6):8588-98. doi: 10.18632/aging.202671

22- Buckley CD, Pilling D, Lord JM, Akbar AN, Scheel-Toellner D, Salmon M. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. Trends Immunol. 2001;22(4):199-204. doi: 10.1016/s1471-4906(01)01863-4

23- Diar-Bakirly S, El-Bialy T. Human gingival fibroblasts: isolation, characterization, and evaluation of CD146 expression. Saudi J Biol Sci. 2021;28(4):2518-26. doi: 10.1016/j.sjbs.2021.01.053

24- Fournier BP, Larjava H, Häkkinen L. Gingiva as a source of stem cells with therapeutic potential. Stem Cells Dev. 2013;22(24):3157-77. doi: 10.1089/scd.2013.0015

25- Denu RA, Nemcek S, Bloom DD, Goodrich AD, Kim J, Mosher DF, et al. Fibroblasts and mesenchymal stromal/stem cells are phenotypically indistinguishable. Acta Haematol. 2016;136(2):85-97. doi: 10.1159/000445096

26- Roumenina LT, Daugan MV, Petitprez F, Sautès-Fridman C, Fridman WH. Context-dependent roles of complement in cancer. Nat Rev Cancer. 2019;19(12):698-715. doi: 10.1038/s41568-019-0210-0

27- Graves D. Cytokines that promote periodontal tissue destruction. J Periodontol. 2008;79(8 Suppl):1585-91. doi: 10.1902/jop.2008.080183.

28- Liu P, Cui L, Shen L. Knockdown of TRIM52 alleviates LPS-induced inflammatory injury in human periodontal ligament cells through the TLR4/NF-κB pathway. Biosci Rep. 2020;40(8):BSR20201223. doi: 10.1042/BSR20201223

29- Huang C, Yang X, Huang J, Liu X, Yang X, Jin H, et al. porcine beta-defensin 2 provides protection against bacterial infection by a direct bactericidal activity and alleviates inflammation via interference with the TLR4/NF-κB pathway. Front Immunol. 2019;10:1673. doi: 10.3389/fimmu.2019.01673

30- Han XZ, Ma R, Chen Q, Jin X, Jin YZ, An RB, et al. Anti-inflammatory action of *Athyrium multidentatum* extract suppresses the LPS-induced TLR4 signaling pathway. J Ethnopharmacol. 2018;217:220-7. doi: 10.1016/j.jep.2018.02.031

31- Huang Y, Guo W, Zeng J, Chen G, Sun W, Zhang X, et al. Prediabetes enhances periodontal inflammation consistent with activation of toll-like receptor-mediated nuclear factor-κB pathway in rats. J Periodontol. 2016;87(5):e64-74. doi: 10.1902/jop.2015.150522

32- Sun JY, Li DL, Dong Y, Zhu CH, Liu J, Li JD, Zhou T, Gou JZ, Li A, Zang WJ. Baicalin inhibits toll-like receptor 2/4 expression and downstream signaling in rat experimental periodontitis. Int Immunopharmacol. 2016;36:86-93. doi: 10.1016/j.intimp.2016.04.012

33- Ishikawa T, Morris PL. Interleukin-1beta signals through a c-Jun N-terminal kinase-dependent inducible nitric oxide synthase and nitric oxide production pathway in Sertoli epithelial cells. Endocrinology. 2006;147(11):5424-30. doi: 10.1210/en.2006-0643

34- Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy: from molecular mechanisms to therapeutic benefits. Biochim Biophys Acta. 2005;1754(1-2):253-62. doi: 10.1016/j.bbapap.2005.08.017

35- Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opin Cell Biol. 2007;19(2):142-9. doi: 10.1016/j.ceb.2007.02.001

36- Jian CX, Li MZ, Zheng WY, He Y, Ren Y, Wu ZM, et al. Tormentic acid inhibits LPS-induced inflammatory response in human gingival fibroblasts via inhibition of TLR4-mediated NF-κB and MAPK signalling pathway. Arch Oral Biol. 2015;60(9):1327-32. doi: 10.1016/j.archoralbio.2015.05.005

37- Subedi L, Lee JH, Yumnam S, Ji E, Kim SY. Anti-Inflammatory effect of sulforaphane on LPS-activated microglia potentially through JNK/AP-1/NF-κB inhibition and Nrf2/HO-1 activation. Cells. 2019;8(2):194. doi: 10.3390/cells8020194

38- de Klerk E, 't Hoen PA. Alternative mRNA transcription, processing, and translation: insights from RNA sequencing. Trends Genet. 2015;31(3):128-39. doi: 10.1016/j.tig.2015.01.001

39- Fujioka S, Niu J, Schmidt C, Sclabas GM, Peng B, Uwagawa T, et al. NF-kappaB and AP-1 connection: mechanism of NF-kappaB-dependent regulation of AP-1 activity. Mol Cell Biol. 2004;24(17):7806-19. doi: 10.1128/MCB.24.17.7806-7819.2004

40- Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. Genes Dev. 1995;9(22):2723-35. doi: 10.1101/gad.9.22.2723