Lipoxin A4 promotes autophagy and inhibits overactivation of macrophage inflammasome activity induced by Pg LPS

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Abstract
Objective: To explore the role of lipoxin A4 (LXA4) on inflammasome and inflammatory activity in macrophages activated by Porphyromonas gingivalis lipopolysaccharide (Pg LPS) one of the major causative agents of chronic periodontitis.

Methods: The mouse macrophage cell line RAW264.7 was used to produce an activated inflammation model. Markers of inflammasome and inflammatory activity and autophagy were assessed by ELISA, reverse transcription polymerase chain reaction (RT-PCR), and Western blot assay.

Results: Markers of inflammasome activity, inflammation and autophagy increased with Pg LPS concentration. They also increased with increasing exposure to Pg LPS up to 12h but decreased at 24h. However, markers of autophagy increased. Phosphorylated NF-κBp65 decreased with LXA4, which was similar to results obtained with the autophagy inducer, rapamycin.

Conclusions: LXA4 promoted autophagy and inhibited activation of inflammasomes and inflammation markers in macrophage inflammation induced by Pg LPS and this action was linked to the phosphorylation of NF-κB.

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Keywords
Inflammasome, autophagy, Pg LPS, LXA4

Date received: 27 February 2020; accepted: 20 November 2020

Introduction
Periodontitis is an inflammatory disease caused by subgingival microbes which can lead to the destruction of the periodontal supporting tissue by the formation of periodontal pockets, absorption of alveolar bone, tooth loosening, tooth displacement, and loss of attachment. It is also an important risk factor for some systemic conditions, such as cardiovascular disease, diabetes, adverse pregnancy outcomes and osteoporosis. Porphyromonas gingivalis is the major causative agent of chronic periodontitis. The lipopolysaccharide (LPS) located on the outer membrane of the pathogen plays an important role in the virulence of P. gingivalis. The P. gingivalis (Pg) LPS reacts with the immune effector cells in the periodontal tissue to activate the innate and acquired immunity of the host.

Macrophages in the periodontal tissue are the main effector cells of Pg LPS. They play a defensive role in the phagocytosis of pathogens and release a large number of inflammatory mediators and cytokines, leading to periodontitis. Research has shown that periodontitis can lead to the activation of the NLRP3 inflammasome. This inflammasome mediates the activation of caspase-1 and boosts the release of interleukin (IL)-1β, which plays an important role in regulating the persistence of oral pathogens and the degree of inflammation. Inflammasome factors such as tumour necrosis factor (TNF)-α and IL-6 can directly cause a sustained immune response, resulting in secondary tissue damage; they can also be used as messenger factors to transmit information to other cells, regulate cell growth and differentiation and regulate the secretion of inflammatory mediators.

Autophagy is a conserved, intracellular degradation pathway and protects cells against various dangerous stimuli such as infection. The autophagy pathway can uphold homeostasis by degrading the damaged macromolecular proteins or organelles. The autophagy process is linked to the formation of proteins beclin-1 and LC3, and the transformation of LC3 I to LC3 II can be used as a marker of autophagy induction. Autophagy has been shown to regulate inflammation and immune response by regulating the activation of inflammasomes. Presently, the relationship between autophagy and inflammasomes in periodontitis is unclear.

Lipoxins (LXs) are a class of arachidonic acid metabolites. LXA4 is one of the most characteristic members of this group and has been found to have a significant regulatory effect on many inflammatory cells and inflammation-related genes. Studies have shown that LXs can inhibit the activation of inflammasomes, and the inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway is involved in many inflammatory models. In most cells NF-κB exists in the cytoplasm in an inactive complex bound to I kappa B kinase (IkB). LXs have also been reported to induce autophagy, thus facilitating the interaction between autophagy and the NF-κB signalling pathway. The role of LXs in regulating the activation of inflammasomes via autophagy in periodontitis has not been determined. Therefore, the aim of our
research was to explore the role of LXA4 on macrophage inflammasome activity stimulated by Pg LPS and investigate the role of the NF-κB signalling pathway.

**Methods**

**Materials**

The mouse macrophage cell line RAW264.7, was purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Foetal bovine serum (FBS) was obtained from Gibco, USA. Pg LPS was purchased from Sigma Chemical Co. (L2630) and dissolved in dimethyl sulphoxide (DMSO) also obtained from Sigma. LXA4 was obtained from Sigma (L0521), as was the autophagy inducer, rapamycin (V900930) which was dissolved in DMSO. The autophagy inhibitor 3-methyladenine (3-MA) was purchased from Sigma (M9281) and dissolved in sterilized water.

**Cell culture**

RAW264.7 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 100 U/ml of penicillin/streptomycin in an incubator at 37°C in 5% CO₂. The well-growing RAW264.7 cells were inoculated into a 6-well cell culture plate (1 × 10⁵ cells/well) and placed in a cell incubator and fostered overnight at 5% CO₂ and 37°C:

Cells were separated into six assessment groups:

1. Pg LPS concentration (control; Pg LPS [1, 5, 10 and 30 μg/ml for 6 h]);
2. Pg LPS incubation time (control; Pg LPS [10 μg/ml for 3, 6, 12, and 24 h]);
3. LXA4 (control; Pg LPS [10 μg/ml for 12 h] after pre-treatment with LXA4 [50, 100, and 150 nM] for 2 h);
4. Rapamycin (control; Pg LPS [10 μg/ml for 12 h]; Pg LPS [10 μg/ml for 12 h] after pre-treatment with rapamycin [50 μg/ml for 30 min]).
5. 3-MA (control; Pg LPS [10 μg/ml for 12 h]; Pg LPS [10 μg/ml for 12 h] after pre-treatment with 3-MA [1 μg/ml for 30 min]).
6. All groups (control; Pg LPS [10 μg/ml for 12 h] after pre-treatment with LXA4 [100, and 150 nM] for 2 h; Pg LPS [10 μg/ml for 12 h] after pre-treatment with rapamycin [50 μg/ml for 30 min]; Pg LPS [10 μg/ml for 12 h] after pre-treatment with 3-MA [1 μg/ml for 30 min])

**Enzyme-linked immunosorbent assay (ELISA)**

Commercially available ELISA kits (Ab208048 and Ab100713, Abcam, San Francisco, CA, USA) were used to detect TNF-α and IL-6 expression. Following drug incubation, the supernatant from the macrophage culture was collected. Absorbance was measured at 492 nm using a multifunctional enzyme standard instrument. The content of TNF-α and IL-6 in the experimental samples was calculated according to the standard curve. Each experiment was repeated eight times.

**Western blot analysis**

Protein was extracted from the RAW264.7 cells using the cell total protein extraction kit (BC3790-50T, Solarbio, Beijing, China). Total protein (100 μg/well) was initially separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then immuno-blotted to polyvinylidene fluoride according to the manufacturer’s instructions. The nitrocellulose membranes were blocked with 5% non-fat dry milk in tris-buffered saline, 0.1% Tween 20 (TBST) buffer for 2 h and then incubated with antibodies against LC3 (2775S, CST, Danvers,
MA, USA), beclin-1 (MA5-15825, ThermoFisher, Shanghai, China), p-NF-κBp65 (Ab183559, Abcam, San Francisco, CA, USA), p-NF-κBp52 (PA5-17385, ThermoFisher, Shanghai, China), IkB (4814T, CST, Danvers, MA, USA), caspase-1 (Ab74279, Abcam, San Francisco, CA, USA) and IL-1β (Ab234437, Abcam, San Francisco, CA, USA) at 4°C overnight. Thereafter, they were washed and incubated with the corresponding goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase in 1:3000-1:5000 in phosphate buffered saline, 0.1% Tween 20 (PBST) for 60 min. The developed protein bands were detected using the enhanced chemiluminescent (ECL) Super Signal reagent (PE0010, Solarbio, Beijing, China). The relative band densities of the marked target proteins were measured from the scanned films using an Omnipotent Gel Imaging and Analysis system. Each experiment was repeated three times. The antibodies were diluted using 5% non-fat dry milk in TBST. Dilution multiples were as follow: LC3 (1:1000), Beclin-1 (1:500), p-NF-κBp65 (1:1000), p-NF-κBp52 (1:1000), IkB (1:1000), caspase-1(1:100) and IL-1β (1:1000).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using an extraction kit (KT204-12, Tiangen Company, Beijing, China) and its concentration and purity were determined by an ultramicro spectrophotometer. Approximately 2μg total RNA was reverse transcribed into cDNAs via the Prime Script TM RT Master Mix kit (Takara Bio, Kyoto, Japan). Primers were designed according to the gene sequences for each gene published in Genbank:

NLRP3 upstream primers: 5’—TGC ATG CCG TAT CTG GTT GT—3’,

and downstream primers: 5’—AGC TGA GCA AGC TAA AGG CT—3’.

IL-1β upstream primers: 5’—GAA ATG CCA CCT TTT GAC AGT G—3’, and downstream primers: 5’—TGG ATG CTC TCA TCA GGA CAG—3’.

GAPDH upstream primers: 5’—TAC CCA CGG CAA GTT CAA CG—3’, and downstream primer: 5’—CAC CAG CAT CAC CCC CCC ATT TG—3’.

Statistical analyses

The analyses were performed using Statistical Package for Social Sciences (SPSS®) for Windows® release 24.0 (IBM Corp. Armonk, NY Released 2016). A t-test was used for paired samples. Differences between control and experimental groups were determined using the one-way analysis of variance (ANOVA) test for post-hoc comparisons. Differences in parameters over time were evaluated using a two-way ANOVA with repeated measures.

Results

Effects of different Pg LPS concentrations

Compared with the control group, TNF-α and IL-6 levels increased as the Pg LPS concentration increased (Table 1). In addition, a markedly increased level of biomarkers (i.e., caspase-1, NLRP3 and IL-1β) was observed at 5, 10 and 30μg/ml Pg LPS compared with the control group (Figures 1 and 2).

Effects of different incubation times for Pg LPS

Compared with the control group, TNF-α and IL-6 levels increased as the Pg LPS
10 μg/ml incubation time increased up to 12 hours. However, at 24h, TNF-α and IL-6 levels decreased (Table 2). Similarly, the inflammasome markers (i.e., caspase-1 and IL-1β) showed increases from control up to 12h but values decreased at 24h (Figure 3A and 3B). However, increases in the autophagy-associated proteins (i.e., LC3 II and beclin-1) were seen at all timepoints (Figure 3A and 3C).

**Effect of pre-treatment with different concentrations of LXA4**

Compared with the control group, the markers of inflammation (i.e., TNF-α and IL-6) were significantly increased in all Pg LPS 10 μg/ml groups (Table 3). However, pre-treatment with increasing concentrations of LXA4 produced a progressive decrease in their levels.

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**Table 1.** Effects of different concentrations of *Porphyromonas gingivalis* lipopolysaccharide (Pg LPS) on tumour necrosis factor (TNF-α) and interleukin (IL)-6 expression in macrophages as determined by ELISA (*n* = 8).

|               | Control    | Pg LPS (1 μg/ml) | Pg LPS (5 μg/ml) | Pg LPS (10 μg/ml) | Pg LPS (30 μg/ml) |
|---------------|------------|------------------|------------------|-------------------|-------------------|
| TNF-α, pg/ml  | 60 ± 21    | 321 ± 170        | 736 ± 228**      | 1408 ± 286**      | 2108 ± 471**      |
| IL-6, pg/ml   | 1.9 ± 0.9  | 6.5 ± 1.8*       | 43 ± 10**        | 84 ± 27**         | 264 ± 77**        |

Values shown as mean ± SD.

ELISA, Enzyme-linked immunosorbent assay.

*P* < 0.01 vs control; **P** < 0.05 vs control.

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**Figure 1.** Effects of different concentrations of *Porphyromonas gingivalis* lipopolysaccharide (Pg LPS) on the expression of caspase-1 and interleukin (IL-1β).

(a) Effects of different concentrations of Pg LPS and control (con) on the expression of caspase-1 and IL-1β in macrophages, observed using Western blot assay by comparison with beta-actin.

(b) Semi-quantitative analysis of caspase-1 and IL-1β levels in the macrophage inflammatory activation index at different concentrations of Pg LPS and control (con) (*n* = 3). Values were normalised to beta-actin.

*P* < 0.01 vs control; **P** < 0.05 vs control.
Compared with the control group, the inflammasome markers (i.e., caspase-1 and IL-1β) significantly increased in the Pg LPS group and were progressively inhibited by increasing concentrations of LXA4 (Figure 4A and 4B). Statistically significant differences from the Pg LPS group were observed in the 100 and 150nM LXA4 groups.

By contrast, compared with controls, the autophagy-associated proteins (i.e., LC3 II and beclin-1) were significantly increased in all Pg LPS groups and pre-treatment with LXA4 induced an increase in their levels (Figure 4A and 4C). Statistically significant differences from the Pg LPS group were observed in the 100 and 150nM LXA4 groups.

Table 2. Effects of different incubation times of Porphyromonas gingivalis lipopolysaccharide (Pg LPS; 10 μg/ml) on tumour necrosis factor (TNF)-α and interleukin (IL)-6 expression in macrophages as determined by ELISA (n = 8).

|          | Control | 3 h    | 6 h    | 12 h   | 24 h   |
|----------|---------|--------|--------|--------|--------|
| TNF-α, pg/ml | 48 ± 10 | 654 ± 176** | 1549 ± 300** | 1971 ± 299** | 1655 ± 398** |
| IL-6, pg/ml  | 1.1 ± 0.4 | 5.5 ± 2.1*  | 83 ± 27**   | 259 ± 80**   | 184 ± 68**   |

Values shown as mean ± SD.
ELISA, Enzyme-linked immunosorbent assay.
*P < 0.01 vs control; **P < 0.05 vs control.
Figure 3. Effects of different incubation times of Porphyromonas gingivalis lipopolysaccharide (Pg LPS). (a) Effects of different incubation times of Pg LPS 10 μg/ml on expression of caspase-1, interleukin (IL)-1β, beclin-1 and LC3 II in macrophages according to Western blot assay by comparison with control (con) and glyceraldehyde-phosphate dehydrogenase (GAPDH). (b) Semi-quantitative analysis of caspase-1 and IL-1β levels in the macrophage inflammatory activation index at different incubation times of Pg LPS 10 μg/ml and control (con) (n = 3). Values were normalised to GAPDH. (c) Semi-quantitative analysis of beclin-1 and LC3 II levels in the macrophage inflammatory activation index at different treatment times of 10 μg/mL of Pg LPS and control (con) (n = 3). Values were normalised to GAPDH.

* P < 0.01 vs control; ** P < 0.05 vs control; # P < 0.05 vs Pg LPS.

Table 3. Effects of different concentrations of LXA4 in addition to Porphyromonas gingivalis lipopolysaccharide (Pg LPS; 10 μg/ml) on tumour necrosis factor (TNF)-α and interleukin (IL)-6 expression in macrophages as determined by ELISA (n=8).

|          | Control | Pg LPS | Pg LPS + 50nM LXA4 | Pg LPS + 100nM LXA4 | Pg LPS + 150nM LXA4 |
|----------|---------|--------|--------------------|---------------------|---------------------|
| TNF-α, pg/ml | 53 ± 16 | 1943 ± 396** | 1698 ± 383** | 978 ± 255***#       | 607 ± 193***#       |
| IL-6, pg/ml   | 1.1 ± 0.6  | 262 ± 67**   | 222 ± 78**       | 170 ± 44**         | 83 ± 23**#         |

Values shown as mean ± SD. ELISA, Enzyme-linked immunosorbent assay. *P < 0.01 vs control; **P < 0.05 vs control; # P < 0.05 vs Pg LPS.
Effect of pre-treatment with the autophagy inhibitor, 3-MA or the autophagy inducer, rapamycin

Compared with the control group, the autophagy-associated proteins (i.e., LC3II and beclin-1) were significantly increased in the Pg LPS 10 μg/ml group (Figure 5A). By comparison with Pg LPS levels, these proteins were significantly inhibited by pre-treatment with 3-MA 1 μg/ml and significantly induced by pre-treatment with rapamycin 50 μg/ml (Figure 5B).

Compared with the control group, the inflammasome markers (i.e., caspase-1 and IL-1β) were significantly increased in the Pg LPS group. By comparison with Pg LPS levels, these markers were significantly inhibited by pre-treatment with rapamycin 50 μg/ml and significantly induced by pre-treatment with 3-MA 1 μg/ml (Figure 5C).

Investigation into the NF-κB signalling pathway

To explore the role of the NF-κB signalling mechanism in autophagy and inflammation, we undertook an extra experiment and compared controls with, Pg LPS, and Pg LPS pre-treated with LAX4 (100, 150 nM), rapamycin (50 μg/ml) or 3-MA (1 μg/ml).
Phosphorylated NF-κB p52 was low in all experimental groups, while phosphorylated NF-κB p65 and IkB expressions varied group to group (Figure 6A).

Phosphorylated NF-κB p65 in the PgLPS group was significantly higher than in the control group (Figure 6B). Compared with the PgLPS group, pre-treatment with LXA4 at both concentrations decreased the phosphorylation of NF-κB p65 and increased IkB levels in a dose-dependent manner.

Compared with the PgLPS group, pre-treatment with rapamycin also lowered phosphorylated NF-κB p65 levels while 3-MA significantly increased levels. By contrast, rapamycin was associated with increased IkB levels whereas 3-MA inhibited IkB levels (Figures 6B and 6C).

**Discussion**

Periodontitis is an oral infectious disease associated with an imbalance of microbial flora and host immune activation. Excessive inflammation is considered to be the main cause of periodontal tissue
destruction and alveolar bone loss.\textsuperscript{23} Compared with healthy gums, the expression of NLRP3, IL-1\textbeta and IL-6 in gingival tissue of patients with gingivitis, chronic periodontitis, or generalized aggressive periodontitis is high.\textsuperscript{24,25} In addition, a positive correlation exists between the expression of NLRP3 and that of IL-1\textbeta in inflammatory periodontal disease.\textsuperscript{25}

We found that the expression of the inflammasome, NLRP3, inflammasome markers (caspase-1, IL-1\textbeta) and inflammation markers (IL-6 and TNF-\textalpha) in macrophages had a positive association with the concentration of Pg LPS. Moreover, levels of inflammasome and inflammation markers increased as exposure to Pg LPS increased, but fell at 24 h. By contrast autophagy-associated proteins (LC3II and beclin-1) increased over the entire 24h study period.

Autophagy is a highly conserved intracellular degradation pathway.\textsuperscript{8} It is a protective mechanism by which cells respond to

![Image](https://example.com/image.png)

**Figure 6.** LXA4 may interfere with autophagy and inhibit inflammatory hyperactivation via activation of the NF-\textkB signalling pathway.

(a) Western blot assay was used to detect the expression of phospho-p52, phospho-p65 and I\textkB in macrophages stimulated by PgLPS (LPS) and pre-treated with different concentrations of LXA4 (100 and 150nM), rapamycin or 3-MA. Data were compared to beta-actin.

(b) By comparison with control (con), semi-quantitative analysis of the phospho-p65 level in macrophages stimulated by LPS and pre-treated with different concentrations of LXA4 (100 and 150nM), rapamycin or 3-MA (n = 3) Values were normalised to beta-actin.

(c) By comparison with control (con), semi-quantitative analysis of I\textkB in in macrophages stimulated by LPS and pre-treated with different concentrations of LXA4 (100 and 150nM), rapamycin or 3-MA (n = 3). Values were normalised to beta-actin.

*P < 0.01 vs control; **P < 0.05 vs control; # P < 0.05 vs Pg LPS.
dangerous stimuli by degrading damaged macromolecular proteins or organelles to maintain intracellular homeostasis. There is increasing evidence of a complex interaction between inflammation and autophagy. Inflammation is at the core of controlling bacterial infection but excessive inflammation can lead to host tissue damage and disease progression. Studies have suggested that autophagy regulates excessive inflammation by degrading intracellular pathogens, capturing inflammatory small molecules and inhibiting the activation of inflammasomes. It has been reported that through the negative regulation of IL-1 and type I IFN, autophagy may be essential in the control of inflammation and fine-tuning of the immune response. Moreover, some pathogens can exploit the inhibition of autophagy upon inflammation to avoid the antimicrobial defence of macrophages.

In our macrophage inflammatory model induced by Pg LPS, we found that autophagy-related factors increased with Pg LPS exposure over a 24 h study period, while inflammasome and inflammation markers increased up to 12 h but decreased at 24 h. When we further explored the effects of autophagy on inflammation using the autophagy inducer, rapamycin and the autophagy inhibitor, 3-MA, we found that inflammation could be inhibited when autophagy was strengthened and increased when autophagy was suppressed. Some studies have found that autophagy plays an important role in the pathogenesis of periodontitis by removing bacteria, promoting the internalization of periodontal-specific pathogens and inhibiting the immune response. Consistent with these findings, our study showed the negative effect of autophagy on inflammation. However, while our study showed that the inflammatory response induced by Pg LPS was associated with an increase in of LC3II expression, a previous study showed that Actinobacillus actinomycetes, another periodontal pathogen, was associated with a decrease in LC3II. These findings suggest that the response of autophagy to infection is related to the type of periodontal bacteria and that not all periodontal pathogens induce autophagy.

Lipoxins are important endogenous lipid mediators, that have been reported to have therapeutic potential in the treatment of acute pancreatitis, atherosclerosis and nervous system diseases. Lipoxins have been shown to inhibit the expression of inflammatory cytokines by regulating the inflammatory signalling pathways such as protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and NF-κB. Their action involves various cell types and the anti-inflammatory effect is extensive and powerful. Lipoxins have been reported to be closely related to autophagy and the induction of autophagy by lipoxins has been found to have beneficial effects on reducing inflammation and inducing cancer cell death. Studies have shown that lipoxins may increase the number of autophagy lysosomes by activating MAPK1, thereby increasing the autophagy process. In this present study, we found that autophagy related markers (beclin-1 and LC3II) increased in a dose-dependent manner whereas the inflammatory factors (TNF-α and IL-6) and inflammasome markers (caspase-1 and IL-1β) decreased after LXA4-pretreated macrophages were exposed to Pg LPS. Therefore, in periodontitis, LXA4 may inhibit the activation of inflammasomes and the release of inflammatory factors which are associated with autophagy activation and therefore, be a negative regulator of inflammation.

The transcriptional regulatory binding site of NF-κB is located in the promoter region of various inflammatory cytokines. NF-κB induces the production of various pro-inflammatory including those encoding cytokines and also plays a role in
inflammasome regulation.\textsuperscript{40} An increased expression of NF-κB in infected periodontal tissues has been shown to lead to periodontal destruction, inflammation and osteoclast differentiation.\textsuperscript{41} Moreover, NF-κB not only plays a key role in inflammation but also plays an important role in cell proliferation, differentiation, apoptosis and autophagy.\textsuperscript{40} Autophagy is closely linked to the NF-κB pathway; an overexpression of the transcription factor NF-κB has been found in autophagy-deficient cells.\textsuperscript{42} Interestingly, autophagy has been shown to inhibit the inflammatory response induced by LPS-TLR4-NF-κB signalling pathway by degrading endogenous signalling molecules such as high-mobility group box 1 (HMGB1) protein.\textsuperscript{43}

We investigated the role of the NF-κB signalling pathway in regulating inflammasome activation when LXA4-pretreated macrophages were exposed to Pg LPS. Our results showed that phosphorylated NF-κBp65 decreased with increasing concentrations of LXA4, which was similar to results obtained with the autophagy inducer, rapamycin and opposite to those obtained with the autophagy inhibitor, 3-MA. By contrast, levels of IκB increased with LXA4 and rapamycin and decreased with 3-MA. Therefore, we suggest that LXA4 has the ability to regulate the NF-κB pathway, and the effect may be associated with the activation of autophagy in macrophages incubated with Pg LPS.

The study had some limitations. For example, our results were obtained from a small number of samples over a short time span. Furthermore, we performed \textit{in vitro} experiments using a cell line mouse macrophage cell line and so further work is needed exploring the effect of LXA4 in tissues from patients with periodontitis.

In conclusion, we showed that LXA4 promoted autophagy and inhibited activation of inflammasomes and inflammation markers in macrophage inflammation induced by PgLPS and this action was linked to the phosphorylation of NF-κB.

\textbf{Acknowledgments}

We would like to express our sincere gratitude to our supervisor, Dr Xiangdong Kuang.

\textbf{Declaration of conflicting interests}

The authors declare that there are no conflicts of interest.

\textbf{Funding}

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Natural Science Foundation of China [grant number 81960012].

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