Received: 28 March 2019 | Revised: 26 July 2019 | Accepted: 11 September 2019

DOI: 10.1111/odi.13198

ORIGINAL ARTICLE

ORAL DISEASES WILEY

miR-155 promotes macrophage pyroptosis induced by *Porphyromonas gingivalis* through regulating the NLRP3 inflammasome

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Funding information
National Nature Science Foundation of China, Grant/Award Number: 81470744; Natural Science Foundation of Liaoning Province, Grant/Award Number: 2014021067

Abstract

Objective: The aim of this study is to detect pyroptosis in macrophages stimulated with *Porphyromonas gingivalis* and elucidate the mechanism by which *P. gingivalis* induces pyroptosis in macrophages.

Methods: The immortalized human monocyte cell line U937 was stimulated with *P. gingivalis* W83. Flow cytometry was carried out to detect pyroptosis in macrophages. The expression of miR-155 was detected by real-time PCR and inhibited using RNAi. Suppressor of cytokine signaling (SOCS) 1, cleaved GSDMD, caspase (CAS)-1, caspase-11, apoptosis-associated speck-like protein (ASC), and NOD-like receptor protein 3 (NLRP3) were detected by Western blotting, and IL-1β and IL-18 were detected by ELISA.

Results: The rate of pyroptosis in macrophages and the expression of miR-155 increased upon stimulation with *P. gingivalis* and pyroptosis rate decreased when miR-155 was silenced. GSDMD-NT, CAS-11, CAS-1, ASC, NLRP3, IL-1β, and IL-18 levels increased, but SOCS1 decreased in U937 cells after stimulated with *P. gingivalis*. These changes were weakened in *P. gingivalis*-stimulated U937 macrophages transfected with lentiviruses carrying miR-155 shRNA compared to those transfected with non-targeting control sequence. However, there was no significant difference in ASC expression between *P. gingivalis*-stimulated shCont and shMiR-155 cells.

Conclusions: *Porphyromonas gingivalis* promotes pyroptosis in macrophages during early infection. miR-155 is involved in this process through regulating the NLRP3 inflammasome.

KEYWORDS
macrophages, miR-155, NLRP3, *Porphyromonas gingivalis*, pyroptosis

1 | INTRODUCTION

Periodontitis, which refers to inflammation of the periodontal support tissues, is generally characterized as a chronic, non-specific inflammatory response to infection by a variety of pathogens (Salminen et al., 2014). Periodontitis is known as a multifactorial disease. It is caused directly by the damage inflicted by plaque microbes and is also associated with the innate and adaptive immune responses (Lei, Li, Yan, Li, & Xiao, 2011; Mesa et al., 2014; Okinaga,

Li and Yin equal contribution.

Ariyoshi, & Nishihara, 2015). The inflammatory response plays an important role in the pathogenesis of periodontal disease (Li, Tian, & Wang, 2013; Zhang et al., 2012). The accumulation of pathogenic bacteria promotes the continuous secretion of inflammatory response factors and triggers an inflammatory response cascade. Periodontitis and its relevant systemic diseases, including cardiovascular disease, diabetes mellitus, low premature birth weight, and rheumatoid arthritis, have become a research hot spot because they seriously influence patient quality of life (Ahmed & Johnson, 2013; Chapple & Wilson, 2014; Tanner & Izard, 2006). Thus, understanding the mechanisms underlying the development and progression of periodontitis is of great significance.

Pyroptosis is a form of programmed cell death that depends on caspase-1 in inflammatory cells such as macrophages. Caspase-1 regulates pyroptosis by cleaving gasdermin D (GSDMD) into N- and C-terminal domains. Pyroptosis is accompanied by the release and activation of many pro-inflammatory cytokines. It is induced by external triggers, such as viruses, foreign substances, bacteria, and their metabolic products (Case et al., 2013). Unlike apoptosis, pyroptosis presents a unique morphology characterized by the formation of 1.1–2.4-nm pores on the cell membrane, which disturb the ionic gradient. Permeabilization of the cell membrane results in an influx of water and cell swelling. Meanwhile, intracellular material, including pro-inflammatory cytokines, is released through the pores (Byrne, Dubuisson, Joshi, Persson, & Swanson, 2013). Additionally, DNA is cleaved in the process and dispersed through the nucleus (Labbe & Saleh, 2008). Finally, whole cell lysis occurs, and the cytosolic contents are released into the extracellular space (Brodsky & Medzhitov, 2011). NOD-like receptors (NLRs), which constitute a family of receptors involved in the inflammatory response, are active in the pyroptosis process. NLRs sense pathogen-associated molecular patterns (PAMPs) and activate caspase-1, which acts upon its substrates and ultimately processes pro-IL-1β and pro-IL-18 (Chapple & Wilson, 2014; Huang et al., 2009). As an intracellular sensor of pathogenic microbes, the NOD-like receptor protein 3 (NLRP3) inflammasome is a crucial component of the innate immune inflammatory response (Li, Zhang, et al., 2014; Van Opdenbosch et al., 2014). This inflammasome consists of NLRP3, apoptosis-associated speck-like protein (ASC), and caspase-1 precursor (Inagaki-Ohara, Kondo, Ito, & Yoshimura, 2013). The NLRP3 inflammasome is activated by several factors, including ATP, sodium urate, cholesterol crystals, and bacteria. It is involved in the innate immune response and activates caspase-1, which cleaves and processes pro-IL-1β and pro-IL-18 into mature inflammatory cytokines (Nikaido, 2003; Prasadarao et al., 1996; Sthitmatee et al., 2013). IL-1β and IL-18 are the major factors secreted in an inflammatory response to periodontal infection (Belibasakis & Johansson, 2012). During this process, ASC binds with NLRP3 and pro-caspase to mediate the activation of caspase-1 (Sagulenko et al., 2013; Satoh, Kambe, & Matsue, 2013).

Pyroptosis can be induced when macrophages are stimulated with bacteria. *Porphyromonas gingivalis*, one of the major players in chronic periodontitis, regulates innate immunity by modulating NLRP3 inflammasome activation (Yoshida et al., 2017), which may promote macrophage pyroptosis and release cytokines leading to inflammation. The activation of the NLRP3 inflammasome is regulated by caspases. Caspase-11 upregulates NLRP3 inflammasome activation, while suppressor of cytokine signaling 1 (SOCS1) is a potent negative regulator of pro-inflammatory cytokine signaling, which can inhibit NLRP3 inflammasome formation.

MicroRNA (miRNA) is a type of small RNA that is 18–25 nt in length. miRNAs are highly expressed, non-coding, and highly conserved and are associated with various diseases, such as cancer and inflammation. Xie, Shu, Jiang, Liu, & Zhang, 2011 compared differences in miRNAs between severe periodontitis patients and healthy periodontal subjects and found that miR-155 was significantly upregulated in severe periodontitis patients. O'Connell, Taganov, Boldin, Cheng, & Baltimore, 2007. found that miR-155 plays an important role in pathogen-induced host cell proliferation, differentiation, and apoptosis disorders and is an important inflammation mediator in macrophages. Additionally, miR-155 is one of the important regulatory molecules during macrophage polarization. *P. gingivalis*-stimulated human monocytes upregulate miR-155 (Nayar et al., 2016). However, the relationship between miR-155 in *P. gingivalis* infection and the NLRP3 inflammasome remains unknown.

In this study, U937 macrophages were stimulated with *P. gingivalis* W83. Pyroptosis and miR-155 expression were detected by flow cytometry and real-time PCR, respectively. To clarify the mechanism by which *P. gingivalis* W83 promotes pyroptosis in U937 cells, miR-155 was inhibited using lentivirus-mediated RNA interference (RNAi). Suppressor of cytokine signaling (SOCS) 1, GSDMD N-terminal (GSDMD-NT), caspase-1, caspase-11, ASC, and NLRP3 were detected by Western blotting, while IL-1β and IL-18 were detected by ELISA. The results showed that *P. gingivalis* promoted pyroptosis in macrophages through miR-155-mediated regulation of the NLRP3 inflammasome.

2 | MATERIALS AND METHODS

2.1 | Culture of *P. gingivalis*

Porphyromonas gingivalis W83 (ATCC BAA-308) was cultured in brain heart infusion with 5 μg/ml hemin, 1 μg/ml menadione, and 1 mg/ml yeast extract at 37°C under anaerobic conditions (80% N_2, 10% H_2, and 10% CO_2). For blood agar plates, trypticase soy broth medium was supplemented with 1.5% agar and 5% sheep blood (Beiruite Biotechnology Co.) (Li et al., 2012).

2.2 | Cell culture and U937 stimulation with *P. gingivalis* W83

Immortalized human monocyte U937 (ATCC CRL-1593.2) cells were purchased from American Type Culture Collection. The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2. The concentration was adjusted to 1×10^6 cells/ml and seeded into 6-well culture plates. Phorbol myristic acid (PMA, 10 ng/ml; Sigma) was added to induce differentiation into macrophage-like cells. After 72 hr, the medium

was replaced with fresh medium without PMA or antibiotics, and the differentiated cells were incubated for an additional 24 hr. After 24 hr, the supernatants were discarded, and *P. gingivalis* W83 (1×10^8 cells/ml) was added for 6 and 24 hr. The multiplicity of infection (MOI) was 100 (Gmiterek et al., 2016). *Escherichia coli* (*E. coli*) DH5α was used for DNA cloning. *E. coli* DH5α was cultured in Luria–Bertani (LB) broth and on LB agar plates supplemented with appropriate antibiotics.

2.3 | Flow cytometry

Flow cytometry was carried out to detect pyroptosis in macrophages. After cells were treated as described above, caspase-1 activity was identified using an FAM FLICA Caspase-1 Assay Kit following the manufacturer's protocol (AbD Serotec). Macrophages were incubated with FLICA solution for 1 hr. After washing with wash buffer, the cells were treated with PI for 10 min, fixative was added, and the cells were incubated for 15 min in the dark. The cells were detected using an LSRFortessa Flow Cytometer (Becton Dickson) and analyzed using FlowJo Single Cell Analysis Software version 10 (FlowJo) (Wang, Rahman, & Lehner, 2012).

2.4 | Transmission electron microscope (TEM)

U937 cells were incubated with *P. gingivalis* W83 (MOI = 100) for 6 hr at 37°C. The cells were collected and fixed for 2 hr in 2.5% glutaraldehyde, postfixed with 1% osmic acid for 2 hr, dehydrated through a graded series of ethanol (50, 70, 90, and 100% for 15 min and then three times at 100%), and embedded in Poly/Bed 812 resin. Ultrathin sections were double-stained with 1% lead citrate and 0.5% uranyl acetate and examined with a TEM (H-7650, HITACHI) at 80 kV (Cheong, Kim, Eom, Jeung, & Min, 2016).

2.5 | Real-time PCR

miRNA was extracted from macrophages using TRIzol reagent (Invitrogen) and reverse-transcribed using a TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. The expression of miR-155 was measured using TaqMan Universal Master Mix II and TaqMan MicroRNA Assay kits (Life Technologies). The following primers were used: miR-155 F: CCGCGCTTAATGCTAATCG; miR-155 R: GTGCAGGGTCCGAGGTATTC; U6 F: CTCGCTTCGGCAGCACA; and U6 R: AACGCTTCACGAATTTGCGT. Fluorescence real-time PCR was performed on an ABI 7500 Fast Real-Time PCR System (Life Technologies). The expression level of miR-155 was evaluated using the $2^{-\Delta\Delta Ct}$ method using the U6 gene as an internal control. miR-155 expression in U937 cells without stimulation by *P. gingivalis* was used for calibration.

2.6 | Short hairpin RNA transfection

To determine the ability of miR-155 to modulate the pyroptosis in macrophage, RNAi was utilized to silence the expression of miR-155 in U937 macrophages. Briefly, two single-stranded DNA oligos were synthesized. The 4620hsa-miR-155-5p-inhibitor-Top (BamHI) oligo sequence was gatccGACGGCGCTAGGATCATCAACACCCCTATCACGAATCTTTAGCATTAACAAGTATTCTGGTCACAGAATACAACACCCCTATCACGAATCTTTAGCATTAACAAGATGATCCTAGCGCCGTCTTTTTTG. The 4620hsa-miR-155-5p-inhibitor-Bot (EcoRI) sequence was aattcAAAAAAGACGGCGCTAGGATCATCTTGTTAATGCTAAAGATTCGTGATAGGGGTGTTGTATTCTGTGACCAGAATACTTGTTAATGCTAAAGATTCGTGATAGGGGTGTTGATGATCCTAGCGCCGTCg. These oligos were annealed into a double-stranded oligo and inserted into a recombinant plasmid construct. The plasmid was used to transform competent DH5α cells. The transformed DH5α cells were cultured, and positive clones were selected. The carrier was linearized by digestion with BamH1 and EcoR1 restriction enzymes, and the sequence was inserted into a pGMLV-MI7 lentiviral vector construct. The expression vector and packaging plasmids were cotransfected into 293T cells to package the lentivirus with the miR-155 silencing shRNA. The virus was collected from the supernatant and concentrated by ultracentrifugation. U937-differentiated macrophages cells were infected with the virus (MOI = 100) with 5 μl/ml polybrene, and non-transfected cells (NTC) were used as a control. The silencing efficiency was measured using fluorescent microscope after 72 hr infection and found to be 59%. The result of miR-155 silencing was evaluated by Western blotting for the downstream protein SOCS1. For all experiments, three groups of cells were utilized: (1) normally cultured U937 cells (control), (2) U937 cells subjected to lentiviral transfection with NTC, named shCont, and (3) U937 macrophages transfected with lentiviruses carrying miR-155 shRNA, named shMiR-155.

2.7 | Western blot analysis

Macrophages were stimulated with *P. gingivalis* W83 for 6 hr. The cells were washed twice with cold PBS, lysed with radioimmunoprecipitation assay lysis buffer supplemented with phenylmethylsulfonyl fluoride (PMSF, protease inhibitor), and separated by centrifugation (4°C, 13500 *g*) for 5 min. Then, the supernatants were collected and prepared for analysis. The total protein concentration in each cell lysate was determined with a BCA Protein Assay Kit (Beyotime). After quantification, the proteins were separated by 10% SDS-PAGE and electrotransferred (90 min, 4°C, 80 V) to polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk for 60 min and incubated with primary rabbit antibodies, including anti-SOCS1, anti-GSDMD-NT, anti-caspase-11, anti-caspase-1, anti-ASC, anti-NLRP3, and anti-β-actin (Abcam), at a 1:1,000 dilution at 4°C overnight. Then, the membranes were washed four times with Tris–Tween buffer saline for 5 min per wash cycle. The washed membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (diluted 1:1,000) at room temperature for 1 hr. Proteins were detected using enhanced chemiluminescence (ECL) with a Gel-Pro Analyzer system. The Western blot results were quantified using Quantity One software (Bio-Rad), and band intensities were expressed relative to the control (Li et al., 2017).

2.8 | Measurement of IL-1β and IL-18 by ELISA

Macrophages were stimulated with *P. gingivalis* W83 for 6 hr. The concentrations of IL-1β and IL-18 in the supernatants of each group were detected using IL-1β and IL-18 ELISA Kits (Lianke Biotechnology Co., Ltd) according to the manufacturer's instructions for the respective kits (Xin, Wang, Zhang, & Wang, 2017).

2.9 | Statistical analyses

All data were analyzed using SPSS 17.0 (SPSS). Data were expressed as the means ± *SD* of at least 3 independent experiments. The Student's *t* tests or analysis of variance (ANOVA) was performed to compare differences between the groups. Statistical significance was defined as $p < .05$.

3 | RESULTS

3.1 | *Porphyromonas gingivalis* induces pyroptosis in U937 cells

To evaluate macrophage pyroptosis, flow cytometry was performed by staining for active caspase-1 and with propidium iodide (PI) in cells, and cells undergoing pyroptosis were positive for both caspase-1 and PI. The rate of U937 pyroptosis without *P. gingivalis* stimulation was 9.8%, while the percentage increased to 31.6% when U937 cells were stimulated with *P. gingivalis* for 6 hr ($p < .01$) and decreased to 24.8% upon stimulation for 24 hr ($p < .01$) (Figure 1a). The observation of TEM showed that pyroptotic macrophage showed chromatin margination and condensation as well as mitochondria remained intact but exhibited swelling (Figure 1b).

3.2 | *Porphyromonas gingivalis* promotes miR-155 expression in U937 cells, and miR-155 silencing inhibits pyroptosis in U937 cells stimulated with *P. gingivalis*

As shown in Figure 2a, the expression of miR-155 in U937 cells was significantly higher (4.3-fold) after stimulation with *P. gingivalis* W83 than in the absence of *P. gingivalis* W83 stimulation ($p < .01$). To determine the role of miR-155 in pyroptosis of macrophages stimulated with *P. gingivalis*, miR-155 was silenced by lentivirus-mediated RNAi. The expression of SOCS1 was detected by Western blotting to confirm the silencing of miR-155 and showed that it was significantly increased in shMiR-155 cells compared with shCont cells ($p < .05$) (Figure 2b). After stimulation by *P. gingivalis* W83, the percentage of pyroptosis was significantly lower in shMiR-155 cells (15.3%) compared with shCont cells (28.1%) ($p < .01$) (Figure 3a). The expression of GSDMD-NT was also detected by Western

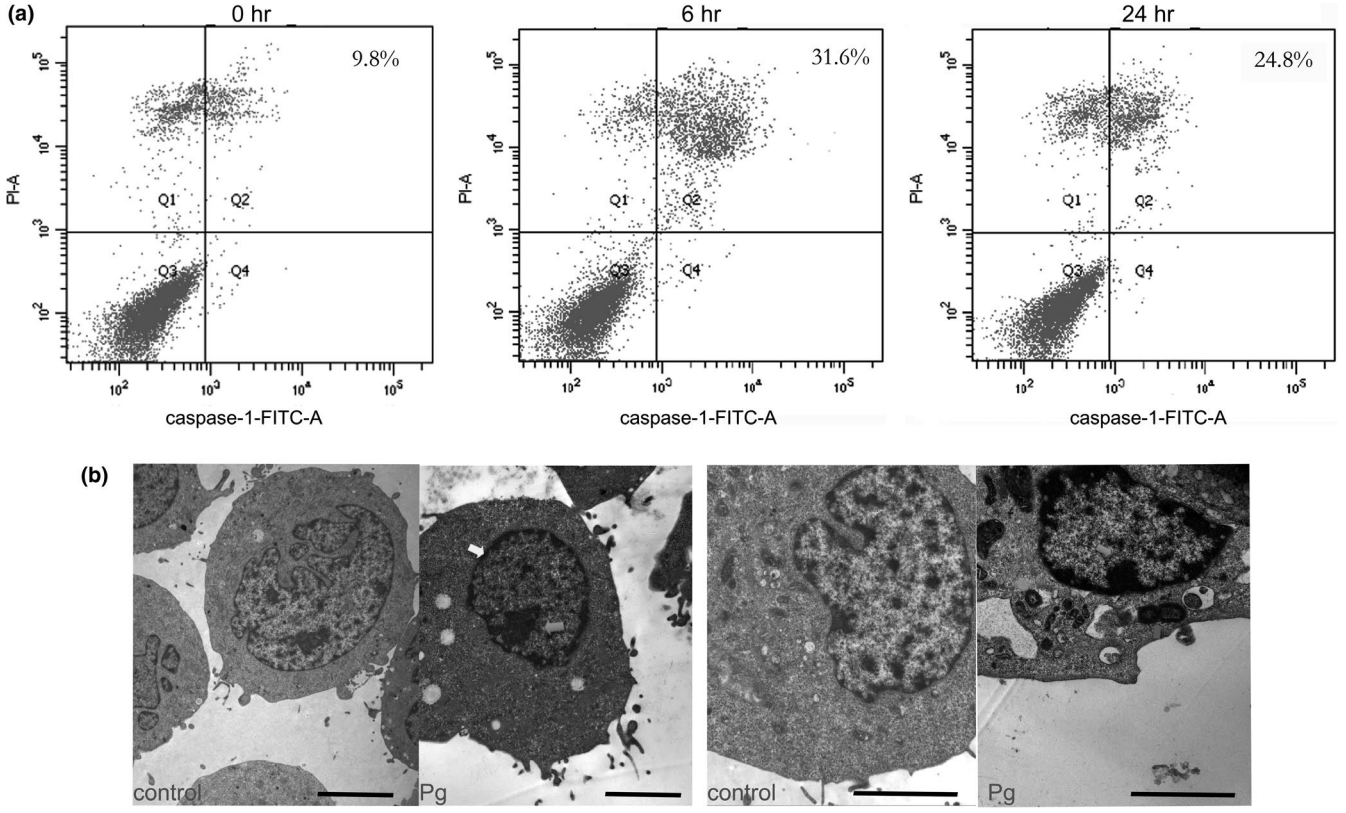

FIGURE 1 Detection of pyroptosis in *Porphyromonas gingivalis* -stimulated U937 cells. (a) Pyroptosis rates in U937 cells stimulated with *P. gingivalis* W83 at MOI 100:1 for 0 hr, 6 hr, and 24 hr detected by flow cytometry. x-axis: FITC-A-labeled caspase 1; y-axis: PI. (b) TEM observation of U937 stimulated with *P. gingivalis* W83. Pyroptotic macrophage showed chromatin margination and condensation (blue arrow) as well as mitochondria remained intact but exhibited swelling (yellow arrow), and *P. gingivalis* can also be found in U937 (red arrow) [Colour figure can be viewed at wileyonlinelibrary.com]

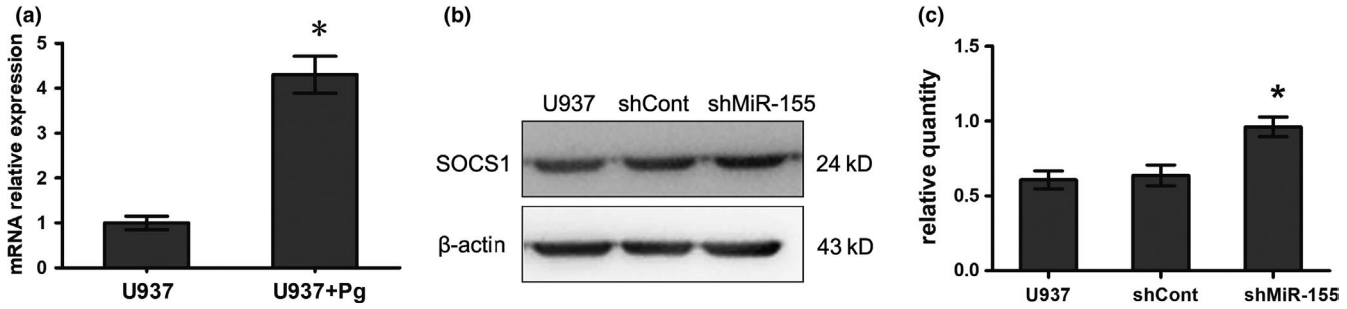

FIGURE 2 miRNA-155 expression and silencing. (a) miRNA-155 expression levels in U937 cells stimulated with *Porphyromonas gingivalis* W83 by real-time PCR. *versus U937 cells without any stimulation, $p < .01$. (b) and (c) Western blotting detection of the expression of SOCS1, the downstream protein of miR-155, before and after miR-155 silencing. *versus shCont stimulated with *P. gingivalis* W83, $p < .05$

blotting, and the results showed that it was significantly decreased in shMiR-155 cells compared with shCont cells after stimulation with *P. gingivalis* W83 ($p < .01$) (Figure 3b,c).

3.3 | *Porphyromonas gingivalis*. increases IL-1β and IL-18 levels in U937 cells, and miR-155 silencing attenuates this increase

Both IL-1β and IL-18 levels increased upon stimulation with *P. gingivalis* W83 in U937, shCont, and shMiR-155 cells. Compared with U937 and shCont cells, IL-1β and IL-18 levels in shMiR-155 cells were significantly lower ($p < .05$) (Figure 4).

3.4 | miR-155 modified the expression of SOCS1, CAS-11, CAS-1, and NLRP3 in U937 cells stimulated with *P. gingivalis*

As shown in Figure 5, CAS-11, CAS-1, ASC, and NLRP3 levels increased and SOCS1 levels decreased in U937 cells stimulated with *P. gingivalis* compared with the levels in U937 cells without any

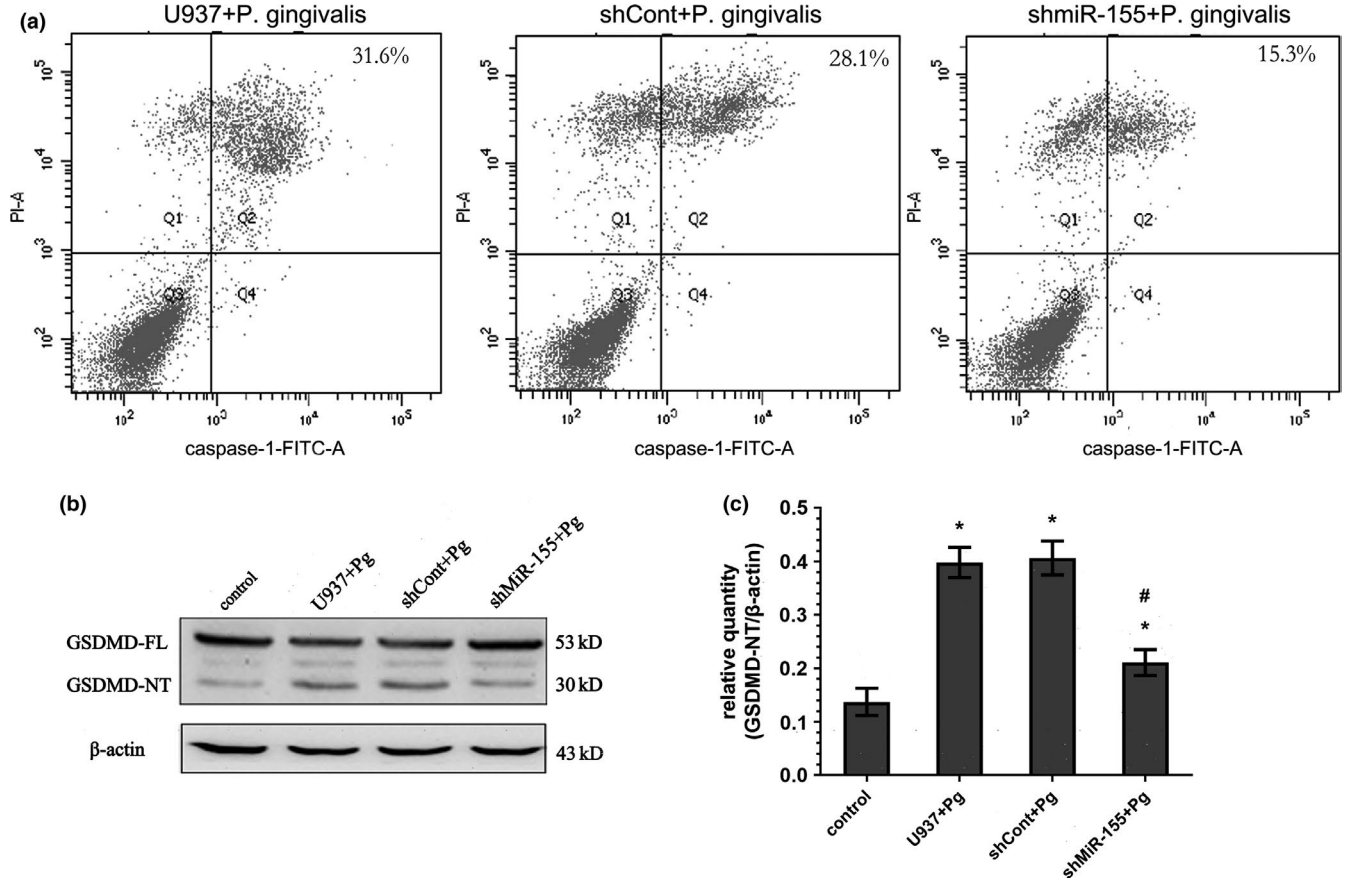

FIGURE 3 Pyroptosis rates and GSDMD-NT expression in different U937 cells stimulated with *Porphyromonas gingivalis* W83. (a) Pyroptosis rates in U937 cells, shCont, and shMiR-155 detected by flow cytometry. *x*-axis: FITC-A-labeled caspase 1; y-axis: PI. (b) and (c) Western blotting detection of the expression of GSDMD-NT before and after miR-155 silencing. *versus U937 cells without any stimulation, $p < .05$; ♯versus shCont stimulated with *P. gingivalis* W83, $p < .05$ [Colour figure can be viewed at wileyonlinelibrary.com]

stimulation. SOCS1 expression increased and CAS-11, CAS-1, and NLRP3 expression decreased in shMiR-155 cells compared with the expressions in shCont cells. There was no significant difference in ASC expression in all cells mentioned.

4 | DISCUSSION

Pyroptosis is one of the programmed cell deaths. It combines the characteristics of both apoptosis and necrosis. Pyroptosis is similar to necrosis in terms of morphology, pore formation on the cell membrane, and the release of cytosolic contents (Shi et al., 2015). At the molecular level, pyroptosis is more similar to apoptosis in that the terminal deoxynucleotidyl transferase positively mediates chromosome disruption and dUTP biotin nick end labeling in both pyroptosis and apoptosis. The difference between pyroptosis and apoptosis is the lack of cytosolic content release in apoptosis; therefore, apoptosis is unable to induce an inflammatory response. Pyroptosis is a unique type of cell death that is dependent on caspase-1 activation and followed by production of the inflammatory cytokines IL-1β and IL-18 (Wang et al., 2018). Based on the previously described characteristics of pyroptosis, we detected pyroptosis in macrophages stimulated with *P. gingivalis* by flow cytometry in this study. PI staining was used to detect cell integrity, and an FAM FLICA Caspase-1 Assay Kit was used to detect caspase-1 activity in macrophages. In this assay, macrophages underwent pyroptosis if they were both PI- and caspase-1-positive. We also detected the observation of cell morphology by TEM and found that pyroptotic macrophage showed chromatin margination and condensation as well as mitochondria remained intact but exhibited swelling.

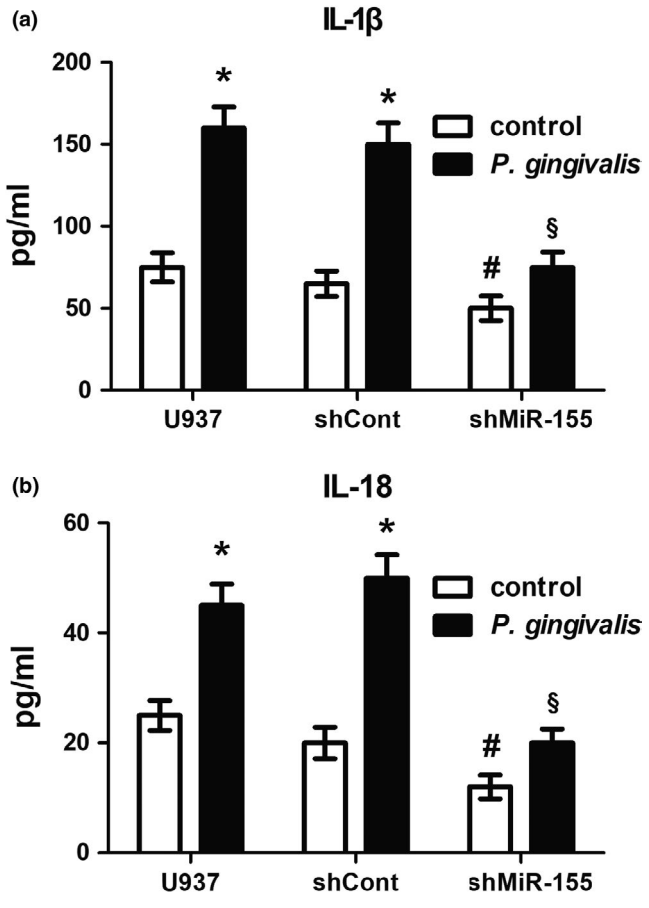

FIGURE 4 Enzyme-linked immunosorbent assays (ELISAs) for IL-1β (a) and IL-18 (b) in different U937 cells stimulated by *Porphyromonas gingivalis* W83. *Versus control group, $p < .01$; ♯versus shCont cells without any stimulation, $p < .05$; §versus shCont cells stimulated with *P. gingivalis* W83, $p < .01$

In vivo and in vitro examinations, pyroptosis has been found to be associated with atherosclerosis, nervous system diseases, and metabolic disease (Duewell et al., 2010; Li, Du, et al., 2014; Luo et al., 2014). Recently, more evidence has indicated that pyroptosis is related to inflammatory diseases (Strowig, Henao-Mejia, Elinav, & Flavell, 2012). Infections by certain bacteria, such as *Salmonella enterica* and *Legionella pneumophila*, induce cell pyroptosis, which is employed by the host to eliminate invading pathogens and maintain homeostasis (Katagiri, Shobuike, Chang, Kukita, & Miyamoto, 2012; Martins et al., 2013). Some bacteria, such as *Salmonella typhimurium*, are unable to induce pyroptosis in specific cases. (Diamond et al., 2017). Additional studies have focused on the relationship between *P. gingivalis* and host cell pyroptosis, while the results were different (Fleetwood et al., 2017; Park et al., 2014). The underlying reason for the diverse results of these different studies involves the use of various *P. gingivalis* strains, different sources of macrophages, diverse MOIs, and differing infection times. Based on the results in our study, we found that the rate of pyroptosis in macrophages increased dramatically upon stimulation with *P. gingivalis* at an MOI of 100 for 6 hr and 24 hr. Notably, the percentage of cells in pyroptosis decreased at 24 hr relative to the higher percentage at 6 hr. One potential explanation for this profile is that there are several distinct cell death processes, including necroptosis, apoptosis, pyroptosis, efferocytosis, NETosis, and autophagic cell death (Gurung, Lukens, & Kanneganti, 2015), and these processes can influence one another. For example, autophagy can decrease pyroptosis by negatively regulating NLRP3 inflammasome activation. In addition, the percentage of cells in pyroptosis may be associated with the cell cycle. The mechanism underlying this observation should be elucidated in a further study.

The inflammasome is a macromolecular protein complex that induces cell death by pyroptosis (Mathur, Hayward, & Man, 2018). The inflammasome consists of three components: a scaffolding protein containing a nucleotide-binding domain (NBD) and leucine-rich repeats (LRRs), called the NBD-LRR (NLR) superfamily; ASC, which is an adaptor protein with a caspase recruitment domain; and the caspase-1 precursor (von Moltke, Ayres, Kofoed, Chavarria-Smith, & Vance, 2013). NLRP3 is a typical representative of the NLRP protein family; it is involved in various host immune and inflammatory responses to pathogen invasion and non-infectious stimuli (Labbe & Saleh, 2008; Lee et al., 2015; Zhang, Ting, Marcu, & Bliska, 2005). Furthermore, it binds to uric acid, ATP, endotoxins, cell lysates, crystalized endogenous molecules (e.g., cholesterol), or other ligands through the LRR to generate an immune response, activate caspase-1, and generate and release mature pro-inflammatory

factors, such as IL-1β and IL-18 (Case et al., 2013). After binding of the ligand to the LRR domain, NLRP3 changes its conformation, exposes the NOD domain, and recruits ASC and caspase to generate an inflammasome complex (Ghorpade, Leyland, Kurowska-Stolarska, Patil, & Balaji, 2012; Wang et al., 2014). As expected, our results showed that NLRP3 was increased in macrophages stimulated by *P. gingivalis*. ASC, caspase-1, and caspase-11, as well as the cytokines IL-1β and IL-18, were all increased. These results suggest that *P. gingivalis* exerted a positive effect on the NLRP3 inflammasome and promoted the expression of IL-1β and IL-18 through NLRP3 activation. In addition, a recent similar study reported that NLRP6 can induce pyroptosis by activating caspase-1 in fibroblasts (Liu, Liu, Wang, Wang, & Ouyang, 2018).

Bacterial infection modulates miRNA expression to subvert any innate immune response. One study reported by Chen, Constantinides, Kebschull, & Papapanou, 2016. showed that five miRNAs, including miR-155, were significantly increased in gingival epithelial cells upon stimulation with *P. gingivalis*. Another study showed increased expression of miR-155 in macrophages stimulated with *P. gingivalis*. The real-time PCR results in our study demonstrated that the expression of miR-155 in macrophages dramatically increased by approximately 4.3-fold over 6 hr of infection, indicating that miR-155 was involved in the interaction between *P. gingivalis* and the macrophages. Notably, the rate of pyroptosis and NLRP3, caspase-1, IL-1β, and IL-18 levels in macrophages stimulated with *P. gingivalis* decreased when the expression of miR-155 was silenced. This result indicates that miR-155 regulates pyroptosis in macrophages stimulated with *P. gingivalis*.

Next, we wanted to uncover how miR-155 regulates pyroptosis in macrophages. First, the TargetScan (http://www.targetscan.org/vert_50/) and miRDB (http://mirdb.org/miRDB/download.html)

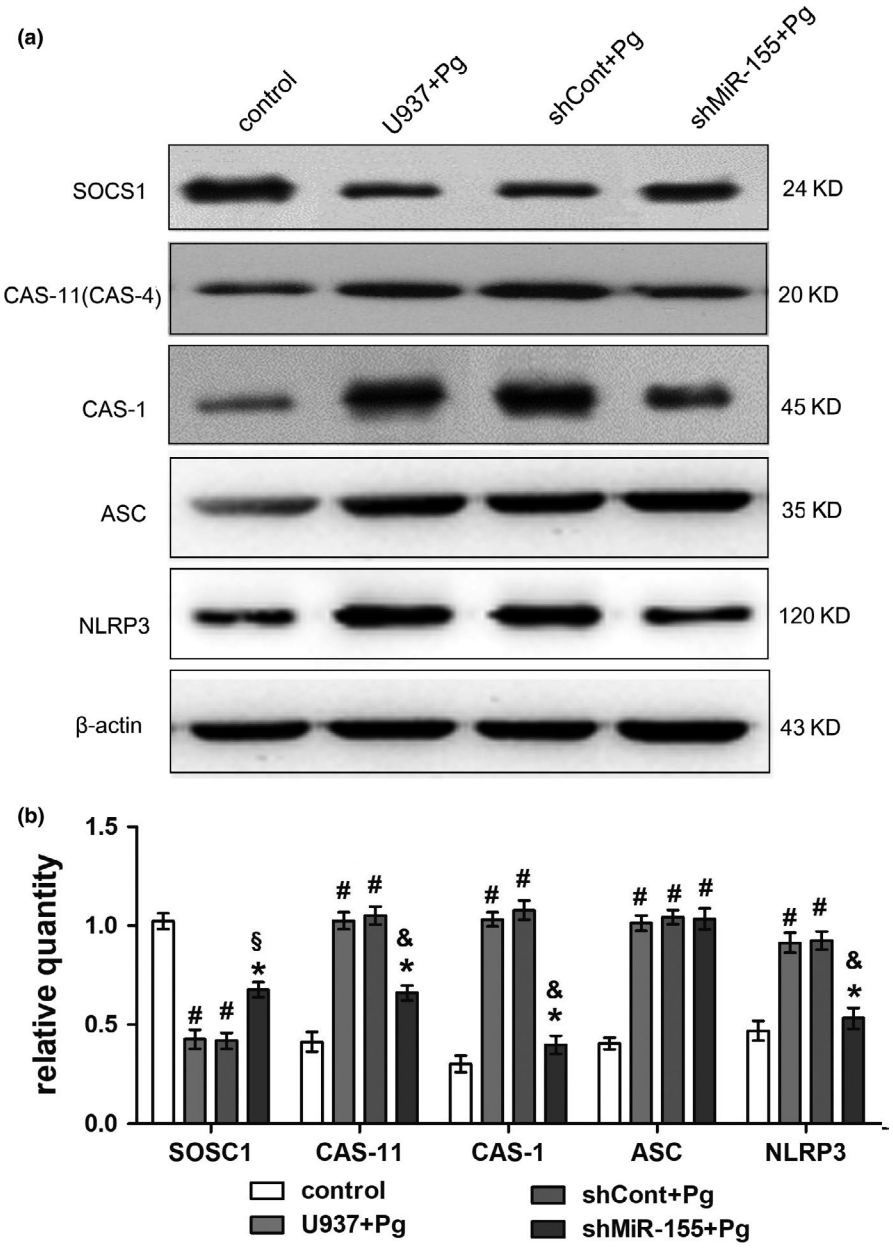

FIGURE 5 SOCS1, caspase-11, caspase-1, ASC, and NLRP3 expression levels in different U937 cells stimulated with *Porphyromonas gingivalis* W83 by Western blotting. (a) Bands from Western blotting; (b) the ratio plot of each protein to β-actin. *versus U937 cells without any stimulation, $p < .05$; #versus U937 cells without any stimulation, $p < .01$; §versus shCont cells stimulated with *P. gingivalis* W83, $p < .05$; &versus shCont cells stimulated with *P. gingivalis* W83, $p < .01$

databases were used to predict the interactions between miR-155 and NLRP3, caspase-1, or GSDMD, respectively. It was not possible to predict any interactions. Second, we constructed luciferase reporter plasmids and confirmed that there were no interactions between miR-155 and NLRP3, caspase-1, or GSDMD (data not shown). Therefore, we deduced that miR-155 indirectly regulates the inflammasome and pyroptosis in macrophages. Several studies found that miR-155 downregulates SOCS1 expression (Cardoso, Guedes, Pereira de Almeida, & Pedroso de Lima, 2012; Wang et al., 2016), and SOCS1 inhibits the macrophage inflammatory response by regulating the JAK/STAT pathway (Xu et al., 2014) and promotes the transformation from pro-caspase-11 to caspase-11 (Schauvliege, Vanrobaeys, Schotte, & Beyaert, 2002), which increases NLRP3 expression (Rathinam et al., 2012) and eventually promotes pyroptosis in macrophages. The results in this study showed that SOCS1 expression increased, while caspase-11, caspase-1, NLRP3, GSDMD-NT, IL-1β, and IL-18 expression decreased in miR-155-silenced macrophages stimulated with *P. gingivalis*. Thus, we concluded that miR-155 promotes pyroptosis induced by *P. gingivalis* through regulating SOCS1, caspase-11, caspase-1, and NLRP3 expression. This potential mechanism may explain how miR-155 regulates pyroptosis in macrophages stimulated with *P. gingivalis*.

Pyroptosis, a type of inflammatory programmed cell death, notably decreases the ability of macrophages to clear pathogens. In this study, we found that *P. gingivalis* promotes pyroptosis in macrophages during early infection. During this period, *P. gingivalis* easily escapes being engulfed by macrophages and enters into deeper tissue. We also found that miR-155 promoted macrophage pyroptosis induced by *P. gingivalis* through regulating the NLRP3 inflammasome. The results in this study elucidated the mechanism by which *P. gingivalis* promotes pyroptosis in macrophages and showed that inhibiting miR-155 will decrease the rate of pyroptosis in macrophages stimulated with *P. gingivalis*, increasing the ability of macrophages to engulf these bacteria. This knowledge will provide new targets and methodologies to prevent and treat periodontal disease.

ACKNOWLEDGEMENTS

This study was supported by the National Nature Science Foundation of China (No. 81470744), Beijing, China, and the Natural Science Foundation of Liaoning Province (No. 2014021067), Shenyang, Liaoning, China.

CONFLICT OF INTEREST

The authors report no conflict of interest related to this study.

AUTHOR CONTRIBUTION

CL, YP, and LL conceived and designed the experiments. CL, WY, DZ, HZ, JL, and JCL performed the experiments. CL, NY, and LL analyzed the data. CL and WY wrote the manuscript.

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How to cite this article: Li C, Yin W, Yu N, et al. miR-155 promotes macrophage pyroptosis induced by *Porphyromonas gingivalis* through regulating the NLRP3 inflammasome. *Oral Dis*. 2019;25:2030–2039. https://doi.org/10.1111/odi.13198