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ORIGINAL ARTICLE

Gasdermin-d Played a Critical Role in the Cyclic Stretch–Induced Inflammatory Reaction in Human Periodontal Ligament Cells

Jiabao Zhuang,[1,2,3] Yingying Wang,[1,2,3] Fang Qu,[1,2,3] Yaqin Wu,[1,2,3] Dan Zhao,[1,2,3] and Chun Xu[1,2,3,4]

Abstract—It has been shown that cyclic stretch could induce inflammatory response such as pyroptosis and the release of IL-1β in human periodontal ligament cells, through activating inflammasome and related caspases. Though gasdermin-d (GSDMD) has been reported to be present in some inflammatory diseases and function as a crucial executioner of pyroptosis, the role of GSDMD in the stretch-induced inflammatory response in human periodontal ligament cells (HPDLCs) has not been well clarified. In this study, it was found that GSDMD was activated by cyclic stretch, and its activation affected the pyroptotic rate in HPDLCs, leading to the maturation and secretion of IL-1β and IL-18 ultimately. In addition, GSDMD was found to be regulated by caspase-1 directly. Nevertheless, the exact relationship between inflammasomes and GSDMD in the stretch-induced inflammatory response still needs to be further elucidated.

KEY WORDS: cyclic stretch; human periodontal ligament cells; gasdermin-d; inflammation.

INTRODUCTION

In the periodontal milieu, various and complicated regulatory modalities coordinate in the process to achieve periodontal homeostasis. Among which, physiological mechanical stimulations have been proved to be of great importance in maintaining periodontal health [1]. Periodontal ligament is sensitive to mechanical stimulations, and the periodontal ligament cells distributed among the periodontal ligament fibers are able to perceive the mechanical stress applied to the tooth [2]. Periodontal ligament cells have been identified as cells with multipotency, regulating osseous remolding and ligament formation [3, 4]. Mechanical stimulations are important in the process of periodontal reconstruction and tooth movement mediated by periodontal ligament cells *via* mechanical signal transduction. However, aberrant mechanical stimulations may bring harmful effect to periodontal tissues. For example, when the occlusal force decreases abnormally due to the extraction of teeth in the opposite jaw, the periodontal tissues around the residual teeth may undergo degeneration along with alveolar bone trabecular absorption [5]. On the other hand, excessive mechanical stimulations aroused from occlusal overloading or improper orthodontic treatment may induce inflammatory response and ultimately result in pathological injuries of periodontium [6, 7]. Furthermore, several studies suggested that excessive occlusal

Jiabao Zhuang and Yingying Wang contributed equally to this work.

[1] Department of Prosthodontics, Shanghai Ninth People's Hospital, College of Stomatology, Shanghai Jiao Tong University School of Medicine, 639 Zhizaoju Road, Shanghai, 200011, China
[2] National Clinical Research Center for Oral Diseases, Shanghai, China
[3] Shanghai Key Laboratory of Stomatology & Shanghai Research Institute of Stomatology, Shanghai, China
[4] To whom correspondence should be addressed at Department of Prosthodontics, Shanghai Ninth People's Hospital, College of Stomatology, Shanghai Jiao Tong University School of Medicine, 639 Zhizaoju Road, Shanghai, 200011, China. E-mail: imxuchun@163.com

force and orthodontic malpractice were major etiological factors in the destruction of the periodontal tissues [1, 8, 9]. Therefore, it is vital to figure out the mechanism of periodontal tissue reconstruction and its transformation to injury in the condition of mechanical stimulations.

Previous studies reported that mechanical stimulation induced the release of diverse inflammatory mediators including pro-inflammatory cytokines, adenosine triphosphate (ATP), prostaglandin E_2 (PGE_2), and nitric oxide (NO) in various cell types such as vascular endothelial cell and alveolar macrophage [10–12]. Recently, it has been proved that under the circumstance of mechanical stress, both animal and human periodontal ligament cells released inflammatory cytokines (such as IL-18, IL-6, IL-1β, TNF-α) [13–15]. Similarly, these inflammatory factors were also found highly expressed in the gingival tissues and crevicular fluid of patients receiving orthodontic treatments [16, 17]. In addition, Zhao *et al*. further illustrated that the release of mature IL-1β in human periodontal ligament cells (HPDLCs) in response to mechanical stretch was related to the activation of NLRP1 and NLRP3 inflammasomes [18]. Being a multisubunit complex, inflammasome consists of pro-inflammatory caspase(s) and nucleotide-binding oligomerization domain-like receptor family members containing pyrin domain (NLRP) along with adaptor protein ASC and is closely connected with pyroptosis [19, 20].

The term pyroptosis was initially named in 2001 as a new form of programmed cell death and was distinguished from apoptosis and necrosis by its close relationship with inflammation [21]. Several studies have reported that stimuli as pathogens or mechanical stretch could induce pyroptosis in various cell types (macrophages, epithelial cells, and periodontal ligament cells), with activation of caspase-1 in NLRP1/3 inflammasomes and the maturation of IL-1β and IL-18 afterward [18, 22, 23]. Besides, the discovery of gasdermin-d (GSDMD) and its role in the pyroptotic cascade enriched the definition of pyroptosis, making it the pore-forming–mediated programmed cell death [24].

GSDMD is considered a crucial effector that mediates pyroptosis mainly in immune cells to protect hosts from intracellular bacteria [24]. Activated inflammatory caspases (caspase-1, caspase-4, caspase-5, caspase-11) cleave GSDMD in macrophages, separating its N-terminal domain from the inhibitory C-terminal domain. The N-terminal fragments then form oligomers and insert in the plasma membrane to induce membrane pore formation and release of inflammatory cytokines such as IL-1β [25–28]. Though inflammatory reactions as pyroptosis and release of IL-1β have been reported to be present in periodontal ligament cells under the circumstance of mechanical stretch loading [18], the expression of GSDMD in non-macrophage cells as HPDLCs and its exact regulatory mechanisms in the stretch-induced inflammatory reaction of HPDLCs remain unknown. Therefore, the present study aimed to investigate the role of GSDMD in the inflammatory reaction of HPDLCs stimulated by *in vitro* stretch.

MATERIALS AND METHODS

Cell Cultures

Human periodontal ligament (PDL) tissues were acquired from extracted premolars of teenagers (11–16 years old) undergoing orthodontic treatments, after the informed consents were obtained from their parents. This protocol was approved by the Ethics Committee of Shanghai Ninth People's Hospital, College of Stomatology, Shanghai Jiao Tong University School of Medicine (Reference: [2017]96). Pieces of PDL tissues were scraped from the middle of the root surface with a sterile scalpel and then attached to a cell culture dish and were incubated with high-glucose Dulbecco's modification of Eagle's medium (DMEM, HyClone, Logan, UT, USA) supplemented with 20% (*v*/*v*) fetal bovine serum (FBS, Carlsbad, CA, USA) and fivefold reinforced antibiotics (500 U/ml penicillin and 500 μg/ml streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2 in air. When cells grew out from the tissue pieces and reached 80–90% confluence, they were passaged and cultured in DMEM supplemented with 10% (*v*/*v*) FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells at passage 4 to 6 were used for the following experiments.

Cyclic Stretch Loading

The HPDLCs were passaged onto the six-well BioFlex plates (Flexcell International, Hillsborough, NC, USA) at a concentration of 2×10^5 cells per well. Once reached 70–80% confluence, the cells were subjected to cyclic stretch using the Flexcell Tension Plus system (FX-500T, Flexcell International) with 20% strain for 6 or 24 h. The loading frequency was 6 cycles per minute with 5-s stretch and 5-s relaxation. Cells were cultured on the same kind of plates but without stretch loading served as time-matched non-stretched control. Three independent loading experiments were carried out in every group. It has been reported that cyclic stretch less than 24% is appropriate to mimic the strain confronted by HPDLCs *in vivo* [29]. In addition, our recent studies demonstrated that the early and the late programmed cell deaths were apparently induced

after 6- and 24-h cyclic stretches with 20% strains, respectively [30, 31]. Moreover, Agarwal and Nokhbehsaim defined 20% cyclic stretch as high-magnitude stimulation, which had the tendency to induce pro-inflammatory cytokines [32, 33]. Therefore, the cultured cells were stretched by 20% cyclic stretch strain for 6 or 24 h in the present study, to mimic the *in vivo* state of HPDLCs subjected to excessive mechanical stimulation. The loading frequency of 6 cycle/min (5-s stretch and 5-s relaxation) was the same as that in our previous studies [30, 31, 34].

RNA Isolation and Quantitative Real-time Polymerase Chain Reaction

After the cyclic stretch loading accomplished, total RNA was isolated from HPDLCs using Trizol reagent (Life Technologies, Carlsbad, CA, USA) and cDNA was synthesized with Reverse Transcription Kit (Toyobo, Tokyo, Japan), according to the standard protocol. Amplification and measurement were carried out by using the Light Cycler 480 Real-Time PCR System (Version LCS480 1.5.1.62 software, Roche, Basel, Switzerland). The amount of target mRNA in relevant samples was measured and normalized to GAPDH, and the designs of PCR primers are shown in Table 1.

Western Blot Analysis

Total protein was extracted from the collected cells and processed with RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were assayed by using Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China). Equal amount of protein that mixed with appropriate volume of SDS sampling buffer was separated by 12% SDS-PAGE, and then, the protein was transferred to a 0.22-μm PVDF membrane. After being blocked, the membranes were incubated with primary antibodies at 4 °C overnight. Primary antibodies consisted of GAPDH (1:1000, rabbit, Weiao, Shanghai, China), caspase-1 (1:1000, mouse, Santa Cruz Biotechnology, Dallas, TX, USA), GSDMD (1:500, rabbit, Santa Cruz Biotechnology), IL-1β (1:200, rabbit, Proteintech, Rosemont, IL, USA), and IL-18 (1:200, rabbit, Proteintech). Next, the membranes were washed three times (5 min each) with PBS containing 0.1% Tween 20 (PBST) and then incubated with the corresponding secondary antibodies (anti-mouse, 1:2000; anti-rabbit, 1:2000; Weiao) at room temperature for 1 h. Subsequently, the membranes were washed with PBST three times (15 min each), and then, the protein bands were detected by enhanced chemiluminescence.

Cytokine ELISA

After the application of cyclic stretch, the supernatants from cultured HPDLCs were collected and then estimated using the IL-1β (R&D Systems, Minneapolis, MN, USA) and IL-18 ELISA kit (Anogen, Mississauga, Canada), according to the manufacturers' instructions. The expression level of IL-1β and IL-18 were shown as picograms per milliliter.

Annexin V-FITC and Propidium Iodide Labeling

The supernatant and the cells of the sample were collected and were centrifuged in 1200 r/min for 5 min to get the sediment. The sediment was re-suspended and washed with PBS twice and the cells were stained with FITC-coupled Annexin V and propidium iodide (PI) (BD Biosciences, San Jose, CA, USA) simultaneously for 15 min at room temperature in darkness. Samples were analyzed by using a FACStar Plus flow cytometer (BD Pharmingen, Oakville, ON, USA) within 1 h.

Small Interference RNA Transfection and Inhibitor Treatment

The HPDLCs were passaged onto the six-well BioFlex plates (Flexcell International) at a concentration of 1×10^5 cells per well. To silence the expression of GSDMD in HPDLCs, 2 μg double-stranded siRNA-target human GSDMD (Santa Cruz Biotechnology), 5 μl Transfection Regent (Santa Cruz Biotechnology), and 1 ml Opti-MEM (Gibco, Grand Island, NY, USA) were mixed and added into each well and incubated with cells for 6–8 h when cells reached 60–80% confluence, according to the manufacturers' instructions. One mini liter medium with double fetal bovine serum was added into each well and incubated for another 12 h. Then, the culture medium was changed to normal medium for 24-h incubation until subjected to cyclic stretch. The knockdown efficiency of GSDMD was further analyzed by qRT-PCR. To inhibit the expression of caspase-1, HPDLCs were pre-incubated with 2 mM z-YVAD-FMK (Biovision Research Products, Mountain View, CA, USA) for 1 h before cyclic stretching. HPDLCs under the same conditions but without siRNA transfection or inhibitor treatment served as non-inhibited control.

Data Analysis

The results were expressed as means ± standard deviation ($n = 3$) and were statistically analyzed with one-way analysis of variance (ANOVA) followed with Student-

Table 1. PCR Primers

Gene	Sequence
GAPDH	F: 5′GGG AAG GTG AAG GTC GGA G 3′ R: 5′GGG GTC ATT GAT GGC AAC A 3′
GSDMD	F: 5′AGC CAG AAG AAG ACG GTC A 3′ R: 5′TCC AAG TCA GAG TCA ATA ACC A3′

New-Keuls post hoc test, and P values less than 0.05 were considered statistically significant.

RESULTS

Cyclic Stretch Induced the Activation of GSDMD in HPDLCs

Real-time PCR result revealed that the expression of GSDMD mRNA in HPDLCs increased in response to 6-h and 24-h cyclic stretch ($P < 0.05$ *vs* control for 6 h, $P < 0.01$ *vs* control for 24 h) (Fig. 1a). Additionally, western blot analysis detected the increased expression of active 31kd GSDMD N-fragment in response to 6-h and 24-h cyclic stretch ($P < 0.05$ *vs* control), whereas the expression of 51-kd GSDMD-FL presented no statistical difference among 6-h and 24-h cyclic stretched cells and control ($P > 0.05$) (Fig. 1b).

The Stretch-Induced Pyroptotic Rate in HPDLCs Was Inhibited by Blocking GSDMD

Flow cytometric analysis demonstrated that the pyroptotic rate of HPDLCs in response to 6-h cyclic stretch increased dramatically, compared with non-stretched control ($P < 0.05$). However, the increased pyroptotic rate was significantly inhibited with the addition of GSDMD siRNA, in comparison with the non-inhibited 6-h stretched cells ($P < 0.05$). Annexin V/PI double-staining method was used to differentiate healthy (FITC−/PI−, the lower left quadrant in the scatter plot), apoptotic (FITC+/PI−, the lower right quadrant in the scatter plot), and necrotic/pyroptotic (FITC+/PI+, the upper right quadrant in the scatter plot) cells (Fig. 2).

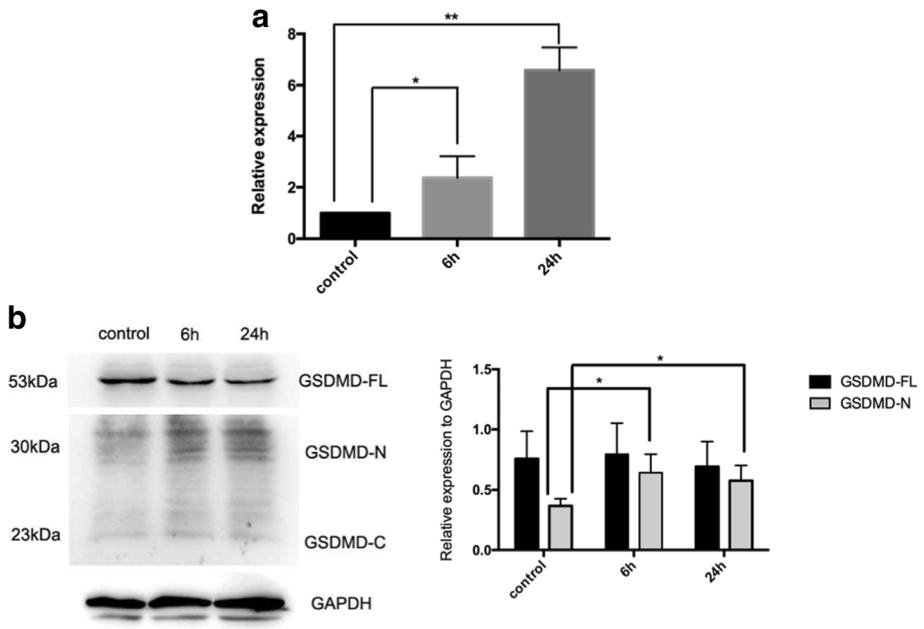


Fig. 1. GSDMD was expressed and was activated in HPDLCs in response to cyclic stretch. **a** Real-time PCR result showed that GSDMD mRNA increased in response to the 6-h and 24-h cyclic stretch, when compared to control group. **b** Western blot analysis revealed that the 6-h and 24-h cyclic stretch could increase the expression of active 31kd GSDMD N-fragment, in comparison with control group. Bars represent standard deviations ($n = 3$). $^{**}P < 0.01$ *vs* non-stretched control; $^{*}P < 0.05$ *vs* non-stretched control.

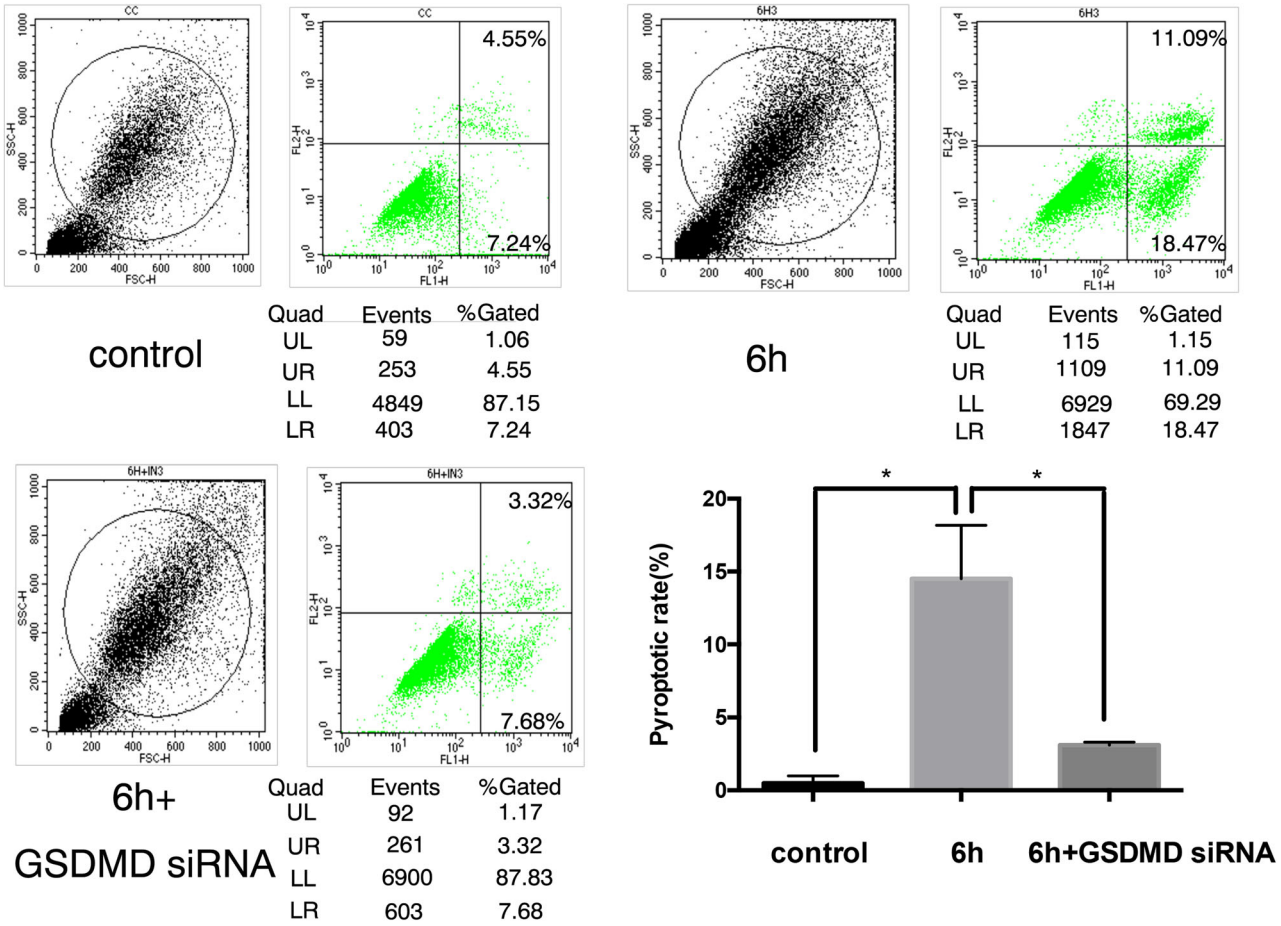


Fig. 2. GSDMD blocking decreased the stretch-induced pyroptotic rate in HPDLCs. The pyroptotic rate of HPDLCs in response to 6-h cyclic stretch increased significantly, compared with non-stretched control. Yet, the pyroptotic rate then dropped dramatically after the addition of the GSDMD siRNA, compared with the 6-h non-inhibited stretched cells. Annexin V/PI double-staining method was used to differentiate healthy (FITC−/PI−), apoptotic (FITC+/PI−), and necrotic/pyroptotic (FITC+/PI+) cells. Bars represent standard deviations ($n = 3$). *$P < 0.05$.

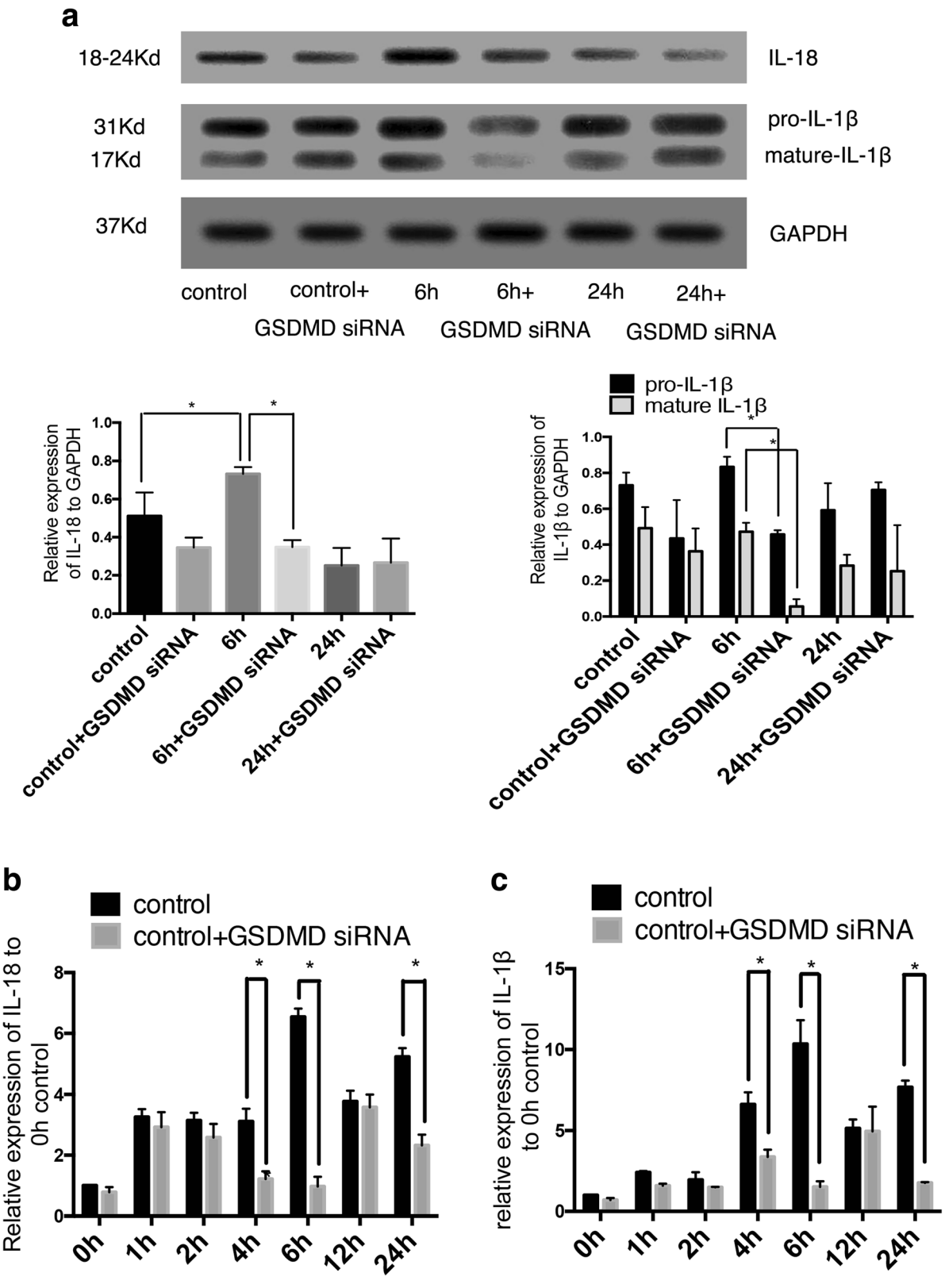
The Stretch-Induced Secretion of IL-1β and IL-18 in HPDLCs Was Inhibited by Blocking GSDMD

Western blot analysis showed that 6-h cyclic stretch increased the expression of the matured IL-1β and IL-18 moderately, yet only IL-18 had statistically significant difference, while the protein level of matured IL-1β and IL-18 then declined sharply after the addition of GSDMD siRNA in response to 6-h cyclic stretch ($P < 0.05$) (Fig. 3a).

The results of ELISA revealed that the appearance of IL-1β and IL-18 in the cell culture medium of HPDLCs varied in response to 1-, 2-, 4-, 6-, 12-, and 24-h cyclic stretches, both of which reached the highest point after 6-h cyclic stretch ($P < 0.05$ *vs* non-stretched control). In addition, the concentration of IL-1β and IL-18 in the cell culture medium declined with the addition of GSDMD siRNA in the 4-h, 6-h, and 24-h stretched cells ($P < 0.05$ *vs* non-inhibited control) (Fig. 3b, c).

The Stretch-Induced Activation of GSDMD in HPDLCs Was Inhibited with the Addition of Caspase-1 Inhibitor

Compared with the non-inhibited 6-h stretched cells, the expression of the activated GSDMD (N-fragment) decreased with the addition of caspase-1 inhibitor, z-YVAD-FMK ($P < 0.05$). Nevertheless, no statistical difference was found between the expressions of the activated GSDMD (N-fragment) in the inhibited and non-inhibited 24-h stretched cells ($P > 0.05$) (Fig. 4).


a
18-24Kd
IL-18
31Kd
17Kd
pro-IL-1β
mature-IL-1β
37Kd
GAPDH
control
control+
6h
6h+
24h
24h+
GSDMD siRNA
GSDMD siRNA
GSDMD siRNA
Relative expression of IL-18 to GAPDH
control
control+GSDMD siRNA
6h
6h+GSDMD siRNA
24h
24h+GSDMD siRNA
pro-IL-1β
mature IL-1β
Relative expression of IL-1β to GAPDH
b
control
control+GSDMD siRNA
Relative expression of IL-18 to 0h control
0h
1h
2h
4h
6h
12h
24h
c
control
control+GSDMD siRNA
relative expression of IL-1β to 0h control

◀ **Fig. 3.** GSDMD blocking inhibited the stretch-induced secretion of IL-18 and IL-1β in HPDLCs. **a** Western blot analysis revealed that the 6-h cyclic stretch increased the expression of the matured IL-18 and IL-1β, yet only IL-18 had statistical difference. The protein level of matured IL-18 and IL-1β then declined sharply after the addition of GSDMD siRNA in response to the 6-h cyclic stretch. **b**, **c** ELISA assay verified the appearance of IL-1β and IL-18 in the cell culture medium of HPDLCs in response to 1-, 2-, 4-, 6-, 12-, 24-h cyclic stretches, both of which reached a peak in response to the 6-h stretch. The concentration of IL-1β and IL-18 in the cell culture medium declined with the addition of GSDMD siRNA in the 4-h, 6-h, and 24-h stretched cells. The results were quantified from at least three independent experiments and expressed as mean ± S.E. Statistical significance was calculated using one-way ANOVA with multiple comparisons. $*P < 0.05$.

DISCUSSION

After Glickman and Smulow firstly proposed the theory in the early 1960s that a traumatogenic occlusion could act as a co-factor in the progression of periodontitis, further studies showed the harmful effect of hyperocclusal forces as well as orthodontic malpractice on the periodontal ligament and their close relationship with the dental plaque–induced periodontitis [33, 35, 36]. Nevertheless, the specific mechanism concerning how mechanical stimulations finally lead to inflammatory process in periodontium remains unclear.

Nowadays, plenty of studies indicate that inflammasome-mediated inflammatory process might be a critical mediator in gingivitis, chronic periodontitis, and aggressive periodontitis [37, 38]. For instance, the expressions of NLRP3, NLRP6, and AIM2 inflammasomes were found elevated in relative tissues of periodontitis [38, 39]. A recent study showed that microbial pathogens (*e.g.*, lipopolysaccharide) activated caspase-1–mediated pyroptosis and promoted the production of pro-inflammatory cytokines such as IL-1β, MCP-1, IL-6, and IL-8 in human periodontal ligament fibroblasts [40]. Also, we previously demonstrated that cyclic stretch activated NLRP1/3 inflammasomes and induced pyroptosis, along with the release of IL-1β in HPDLCs *via* inflammatory caspase-1–related mechanism [18]. The

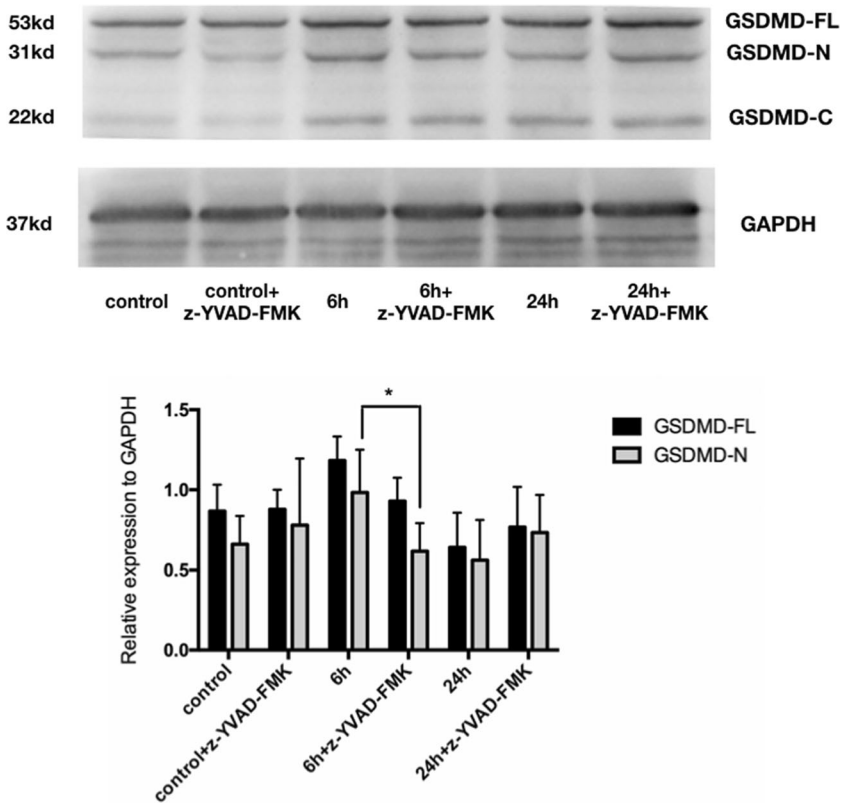


Fig. 4. The stretch-induced activation of GSDMD in HPDLCs was inhibited with the addition of caspase-1 inhibitor. The expression of the activated GSDMD N-fragment upregulated after the 6-h cyclic stretch, yet no statistical difference was found between control and 6-h stretched cells, while, compared with the non-inhibited 6-h stretched cells, the expression of the activated GSDMD N-fragment decreased with the addition of caspase-1 inhibitor, z-YVAD-FMK. Bars represent standard deviations ($n = 3$). $*P < 0.05$.

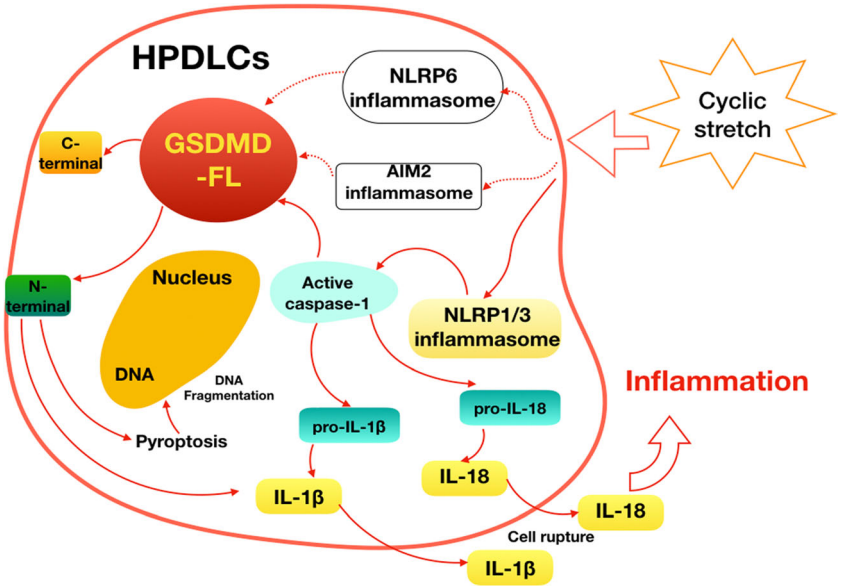


Fig. 5. The schematic drawing of the role that GSDMD plays in cyclic stretch–induced inflammatory reaction in HPDLCs.

present study again proved that 6-h cyclic stretch induced pyroptosis in HPDLCs. The more interesting finding in the present study is that the expressions of GSDMD mRNA and activated protein in HPDLCs increased remarkably in response to 6-h and 24-h cyclic stretch, indicating that cyclic stretch induced the expression and activation of GSDMD.

As a pore-forming protein, GSDMD was recently identified as the significant executioner of pyroptosis, which retained in a state of autoinhibition until the C-terminal of the protein was released upon cleavage by proinflammatory caspases [28, 41, 42]. Once activated, GSDMD translocated to the plasma membrane and oligomerized to form membrane pores, resulting in cellular swelling and membrane rupture, along with extravasation of inflammatory cytokines [43]. Several studies proved that GSDMD was closely related to some inflammatory or autoimmune diseases such as Familial Mediterranean Fever, sepsis, and multiple sclerosis [44–46]. Based on the results of previous and present studies, it is highly likely that GSDMD is involved in the stretch-induced pyroptosis and release of inflammatory cytokines in HPDLCs and might be a critical mediator in the process of mechanical stimulation–related periodontal inflammatory diseases such as gingivitis and periodontitis.

However, the specific relationship between GSDMD and cyclic stretch–induced pyroptosis and release of inflammatory cytokines has not been well inspected previously. The present study showed that the addition of GSDMD siRNA significantly inhibited the 6-h cyclic stretch–induced pyroptosis in HPDLCs. These results highly suggest that GSDMD is critical in the stretch-induced pyroptosis.

Pyroptosis is a lytic form of programmed cell death characterized by cell swelling, pore forming in the cell membrane, and release of the inflammatory cytokines IL-1β and IL-18, both of which are critical inflammatory mediators in periodontitis [47]. IL-1β level in periodontal tissue has been reported to be related to periodontal status, indicating that its levels in periodontal tissue may reflect the severity of periodontal inflammation [38]. Furthermore, high level of IL-1 was found in HPDLCs in response to mechanical stress both *in vivo* an *in vitro* [14, 16, 17]. Taken together, we speculate that mechanical stress–induced inflammation in the periodontal tissues may be one of the causes of periodontitis.

As the most significant effector of pyroptosis, GSDMD is processed by inflammatory caspases and cleaved into an active N-terminal and an autoinhibitory C-terminal fragment. Recently, it has been shown that GSDMD N-terminal can bind to lipids on the inside of the cell membranes and oligomerize to form membrane pores which can disrupt the integrity of cell membrane, thus regulating the release and secretion of inflammatory cytokines [27, 48]. However, whether and how GSDMD affected the release of inflammatory cytokines IL-1β and IL-18 during the stretch-induced pyroptosis in HPDLCs remains unknown.

In the present study, the ELISA result indicated that the 6-h stretch-induced secretion of both IL-1β and IL-18 in HPDLCs was inhibited by blocking GSDMD with siRNA. These results highly suggest that GSDMD may be an important regulating effector in the secretion of inflammatory cytokines during the process of stretch-induced pyroptosis in HPDLCs.

It has been reported that GSDMD can be cleaved and thereafter activated by different inflammatory caspases, including human caspase-1, caspase-4, caspase-5, and murine caspase-11 [41]. As mentioned before, NLRP1/3 inflammasomes were both found activated in the process of stretch-induced pyroptosis in HPDLCs, with caspase-1 involved [18]. Therefore, the present study explored the exact relationship between caspase-1 and GSDMD in mechanically stretched HPDLCs. The results showed that along with the inhibition of caspase-1 by z-YVAD-FMK, the 6-h stretch-induced activation of GSDMD in HPDLCs was inhibited. This illustrates that the mechanical stretch–induced activation of GSDMD is caspase-1 dependent.

In the present study, we firstly illustrated the role of GSDMD in cyclic stretch–induced inflammatory reaction and pyroptosis *via* caspase-1–related mechanism in HPDLCs (Fig. 5), which may provide a new clew for the causes of periodontitis. However, as a limitation, the direct connection between GSDMD and a definitive inflammasome has not been clarified. One probable reason may be that several inflammasomes, such as NLRP1, NLRP3, NLRP6, and AIM2 [38, 39], may be involved in such signaling pathway and no inflammasome works out in this signaling pathway independently (Fig. 5). Therefore, further studies are required to elucidate the predominant inflammasome/s as well as its/their regulating mechanisms in such signaling pathway.

CONCLUSION

The present study demonstrates that GSDMD was activated in HPDLCs in response to cyclic stretch, and it might have played a critical role in cyclic stretch–induced pyroptosis. In addition, GSDMD may be an important regulating effector in the secretion of inflammatory cytokines IL-1β and IL-18 during the process of stretch-induced pyroptosis and may be regulated by caspase-1. These findings indicate a more precise signaling of stretch-induced inflammatory response in HPDLCs.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

Ethics Statement. The experimental protocol was reviewed and approved by the Ethics Committee of Shanghai Ninth People's Hospital, College of Stomatology, Shanghai Jiao Tong University School of Medicine. The relevant judgment's reference number is [2017]96.

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