International Journal of Oral Biology, Vol. 42, No. 1 March 31 2017, p. 17-23
Copyright ⓒ 2017, *The Korean Academy of Oral Biology*
http://dx.doi.org/10.11620/IJOB.2017.42.1.017
pISSN 1226-7155, eISSN 2287-6618

International Journal of Oral Biology

Chracterization of THP-1 Cell Death Induced by *Porphyromonas gingivalis* Infection

YuRi Song, SeYeon Kim, Mee Hee Park, Hee Sam Na and Jin Chung*

Department of Oral Microbiology, School of Dentistry, Pusan National University, Yangsan 50612, Korea

(received February 22, 2017; revised March 09, 2017; accepted March 10, 2017)

Background: Periodontitis is generally a chronic disorder characterized by the breakdown of tooth-supporting tissues. *P. gingivalis*, a Gram-negative anaerobic rod, is one of the major pathogens associated with periodontitis. Frequently, *P. gingivalis* infection leads to cell death. However, the correlation between *P. gingivalis*–induced cell death and periodontal inflammation remains to be elucidated. Among cell deaths, the death of immune cells appears to play a significant role in inflammatory response. Thus, the aim of this study was to examine *P. gingivalis*–induced cell death, focusing on autophagy and apoptosis in THP-1 cells.

Methods: Human acute monocytic leukemia cell line (THP-1) was used for all experiments. Autophagy induced by *P. gingivalis* in THP-1 cells was examined by Cyto ID staining. Intracellular autophagic vacuoles were observed by fluorescence microscopy using staining Acridine orange (AO); and 3-methyladenine (3-MA) was used to inhibit autophagy. Total cell death was measured by LDH assay. Cytokine production was measured by an ELISA method.

Results: *P. gingivalis* induced autophagy in an MOI-dependent manner in THP-1 cells, but 3-MA treatment decreased autophagy and increased the apoptotic blebs. *P. gingivalis* infection did not increase apoptosis compared to the control cells, whereas inhibition of autophagy by 3-MA significantly increased apoptosis in *P. gingivalis*-infected THP-1 cells. Inhibition of autophagy by 3-MA also increased total cell deaths and inflammatory cytokine production, including IL-1β and TNF-α.

Conclusion: *P. gingivalis* induced autophagy in THP-1 cells, but the inhibition of autophagy by 3-MA stimulated apoptosis, leading to increased cell deaths and pro-inflammatory cytokines production. Hence, the modulation of cell deaths may provide a mechanism to fight against invading microorganisms in host cells and could be a promising way to control inflammation.

Key words: *P. gingivalis*, macrophage, cell death, autophagy, apoptosis, periodontitis

Introduction

Periodontitis is the most prevalent chronic inflammatory human disease and is considered as a major problem in the global burden of oral diseases [1]. It is characterized by the breakdown of tooth-supporting tissues producing teeth loss. *Porphyromonas gingivalis* (*P. gingivalis*) is asaccharolytic gram-negative anaerobic rod that is a major etiological agent in the initiation and progression of chronic periodontal disease [2]. *P. gingivalis* is also implicated in certain systemic conditions such as atherosclerosis, aspiration pneumonia, and rheumatoid arthritis [3,4].

*Correspondence to: Jin Chung, Department of Oral Microbiology, School of Dentistry, Pusan National University, Yangsan 50612, Korea
Tel: +82-51-510-8245, Fax: +82-51-510-8246
E-mail: jchung@pusan.ac.kr
ORCID : 0000-0002-6859-615X

This is an Open-Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Before initiating an infection, bacterial pathogens come into contact with the human skin, respiratory or gastro-intestinal system and interact with different host cells such as epithelial cell, PMN cell, and macrophage. As sentinels of infection, macrophages are one of the first cell types to encounter pathogens and the frontline of defense when combating bacterial infection [5]. During the host cell-pathogen interaction, macrophages can die in many ways such as apoptosis, necrosis, pyroptosis and autophagy, and sometimes they are intertwined involving with different and complex underlying mechanisms [6]. Cell death appears to play a significant role in macrophage and triggers an inflammatory response. Programmed cell death (PCD) different from necrosis is an active cell death that is mediated by a series of gene expression events [7,8]. PCD can be further classified into apoptosis, autophagy, programmed necrosis (necroptosis), and pyroptosis [9]. Although PCD is involved in many inflammatory diseases, its correlation with periodontitis is unclear. The purpose of the present study aimed to evaluate macrophage cell death in response to *P. gingivalis* infection.

Materials and Methods

Cell treatment

The human monocyte cell line, THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and were cultured at 37°C in 5% CO_2 incubator. To induce cell death, THP-1 cells were infected with *P. gingivalis* for 24 h. To inhibit autophagic cell death, THP-1 cells were pretreated with 5 mM of 3-methyladenine (3-MA; sigma, MO, USA) for 30 min and were infected with *P. gingivalis* for 24 hr. For confocal microscopy, THP-1 cells were differentiated into macrophage-like cells with 50 ng/ml of Phorbol 12-mystristate 13-acetate (PMA; sigma, MO, USA).

Bacterial culture

P. gingivalis (strain 381) were grown in gifu anaerobic medium (GAM; Nissui, Japan) broth, which contained hemin (5mg/mL) and 3-phytyl-menadione (vitamin K, 0.5mg/ml) at 37°C in an anaerobic chamber under an atmosphere containing 90% N_2, 5% H_2, and 5% CO_2. An optical density (OD) of 1.0 (650 nm) was determined to correlate to $1x10^9$ colony forming units/mL. To prepare the bacteria for infection, an overnight culture was diluted to an OD 650 nm of 1.0 in GAM broth. The bacteria were washed, resuspended in RPMI media, and were used to infect THP-1 cells at a multiplicity of infection (MOI) of 50 and 100.

Autophagy determined by flow cytometry

Cells were harvested and autophagy flux was determined using a Cyto-ID autophagy detection kit (Enzo Life Sciences, NY, USA) according to the manufacturer's instructions. Briefly, the cells were collected by centrifuge and stained in a solution with Cyto-ID Detection Reagent covered from light for 30 min at 37°C. Cells were then analyzed by FACS verse (BD bioscience, NJ, USA). Data were presented as the mean fluorescence intensity of Cyto-ID.

Quantifying autophagy with AO staining

THP-1 cells were seeded in 8-well-chamber slides. Cells were pre-treated with or without 3-MA for 30 min followed by infection with *P. gingivalis* (MOI 50, 100) for 24 hr and stained with 100 μg/ml of AO in serum-free medium at 37 °C for 15 min. Then, cells were washed with PBS and fluorescent micrographs were obtained using confocal laser-scanning microscope (LSM 700, Carl Zeiss, Germany).

Apoptosis analyses with flow cytometry

Apoptosis was analyzed by flow cytometry using annexin-V and propium iodine (PI) double staining according to the manufacturer's protocol (Invitrogen, CA, USA). Briefly, THP-1 cells were infected *with P. gingivalis* for 24 h. THP-1 cells were collected and were washed with cold PBS and annexin-binding buffer. Then, annexin V and PI working solution were added to cells. After incubated at room temperature for 15 min, the stained cells were analyzed with flow cytometry (FACS verse, BD, NJ, USA).

Cell death assay

Cell death was measured with a lactate dehydrogenase (LDH) Cytotoxicity assay kit (CytoTox 96 non radio active cytotoxicity assay; Promega, WI, USA). The percent cytotoxicity was calculated by the following formula: 100 X [(experimental LDH release - spontaneous LDH release)/(maximal LDH release - spontaneous LDH release)]. To determine the maximal LDH release, cells were treated with 1% Triton X-100

Cytokine analysis

The amounts of TNF-α and IL-1β released into the culture media after *P. gingivalis* infection were analyzed by using an

ELISA kit purchased from Biolegend (San Diego, CA, USA). Cytokine level was measured by the manufacturer's instruction. The plates were read in an ELISA reader (Tecan, Männedorf, Switzerland) at 450/570nm.

Statistics

Statistically significant differences between samples were analyzed with the SPSS 13.0 statistical software program (SPSS Inc., IL, USA). The data were shown as the mean ± SD. p value of < 0.05 was considered statically significant. * p < 0.05 vs control, # p<0.05 vs *P. g* MOI 100. *p < 0.05, **p < 0.001, and ***p < 0.005.

Results

1. *P. gingivalis* induced autophagosome formation in THP-1 cells.

To determine if *P. gingivalis* induce autophagy in THP-1 cells, autophagosome formation was examined by cyto-ID staining and was quantitated by FACS. THP-1 cells infected with *P. gingivalis* showed significantly increased mean fluorescence intensity suggesting autophagosome formation was induced by *P. gingivalis* infection. Also, 3-MA, which inhibit autophagy, decreased the autophagosome formation induced by *P. gingivalis* infection (Fig. 1).

To confirm autophagosome formation by *P. gingivalis*, *P. gingivalis*-infected THP-1 cells were stained with acridine orange. As shown in Fig. 2A, control cells primarily displayed green fluorescence with minimal red fluorescence, indicating a lack of acidic vesicular organelles (AVOs). In *P. gingivalis*-infected THP-1 cells, Cells showed an increased red fluorescent AVOs in both MOI 50 and 100 (Fig 2). The numbers of *P. gingivalis*-induced AVOs forming cells were decreased by 3-MA treatment (Fig 2). Moreover, in bright field apoptotic blebs were significantly increased in *P. gingivalis*-infected THP-1 cells treated with 3-MA. Taken together, *P. gingivalis* infection induced autophagy in THP-1 cells and inhibiting autophagy seemed to drive cell death into apoptosis.

2. Inhibition of autophagy by 3-MA increased *P. gingivalis*-induced apoptosis in THP-1 cells.

To test the effect of inhibition of autophagy by 3-MA, cell apoptosis was detected by annexin-V and PI staining. As shown in Fig. 3, the infection of THP-1 cells by *P. gingivalis* showed similar apoptosis rate compared to the control. In THP-1 cells treated with 3-MA and infected with *P. gingivalis*, significantly higher proportion of cells were positive for early and late apoptosis (Fig 3). These results indicate that *P. gingivalis* does not directly induce apoptosis, but apoptosis is significantly induced by inhibiting autophagy in THP-1 cells infected with *P. gingivalis*.

3. Effect of inhibiting autophagy by 3-MA on *P. gingivalis*-induced cell death and cytokine production.

To determine total cell death, we analyzed LDH release in the cell supernatant. Compared with control, *P. gingivalis* infection significantly increased cell cytotoxicity in a MOI-dependent manner. When 3-MA was pretreated, LDH release was significantly increased compared to *P. gingivalis*

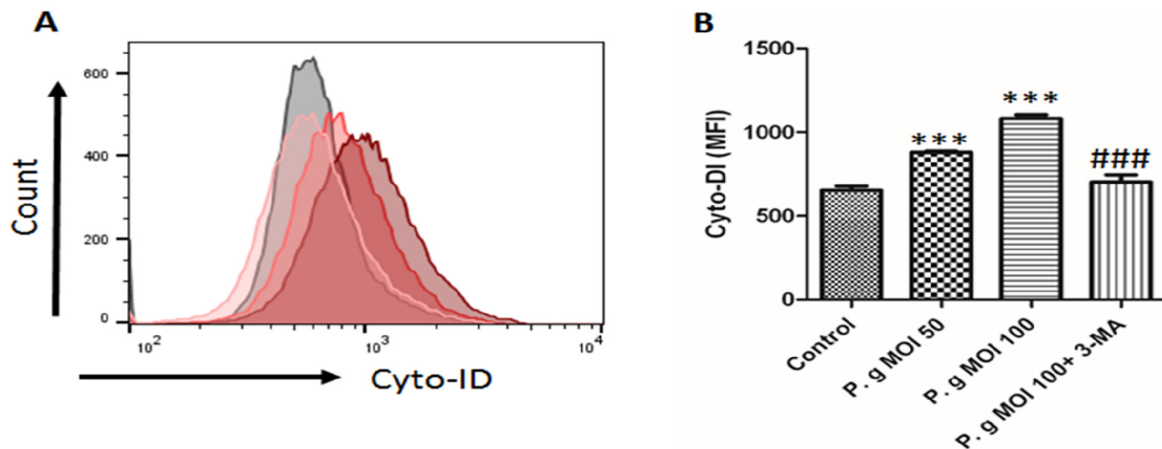

Fig 1. *P. gingivalis* induced autophagosome in THP-1 cells. THP-1 cells were infected with *P. gingivalis* (MOI 50 or 100) or in combination with 3-MA(5mM) for 24 h. (A) Autophagy was determined by cyto-ID and analyzed by FACS. (B) Cyto-ID mean fluorescent intensity of each group determined and has been shown in bar graphs. * p < 0.05 vs control, # p<0.05 vs P. g MOI 100.

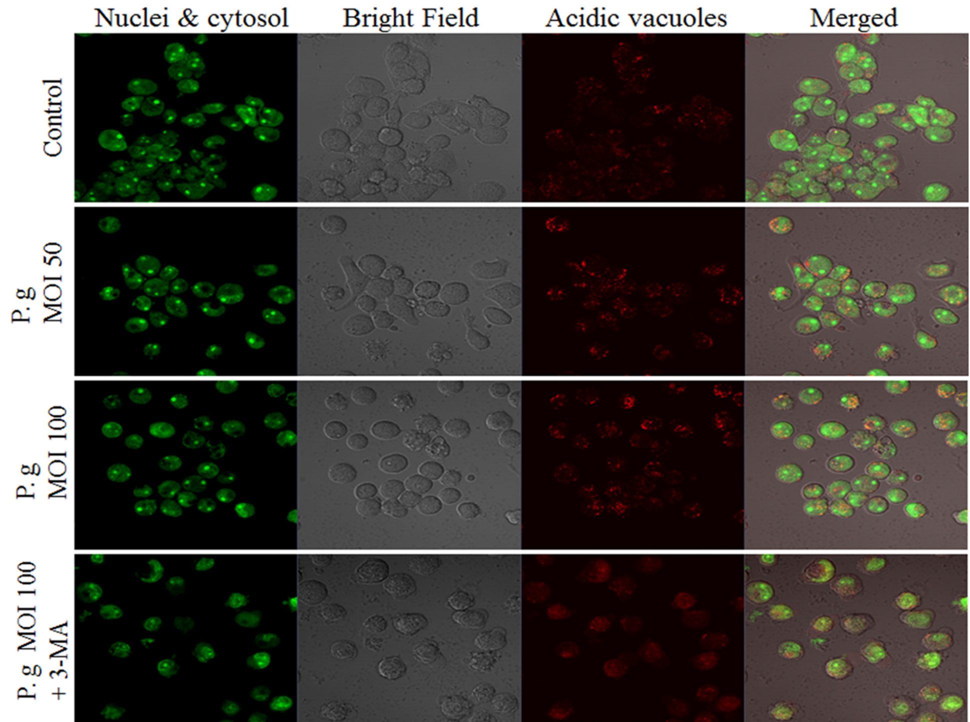

Fig 2. Visulization of intracellular autophagic vacuoles in THP-1-derived macrophages. THP-1 cells were infected with *P. gingivalis* (MOI 50, 100 for 24h) or in combination with 3-MA (5mM). THP-1 cells were stained with acridine orange and were photographed by flurescence confocal microscopy. (× 400 magnification)

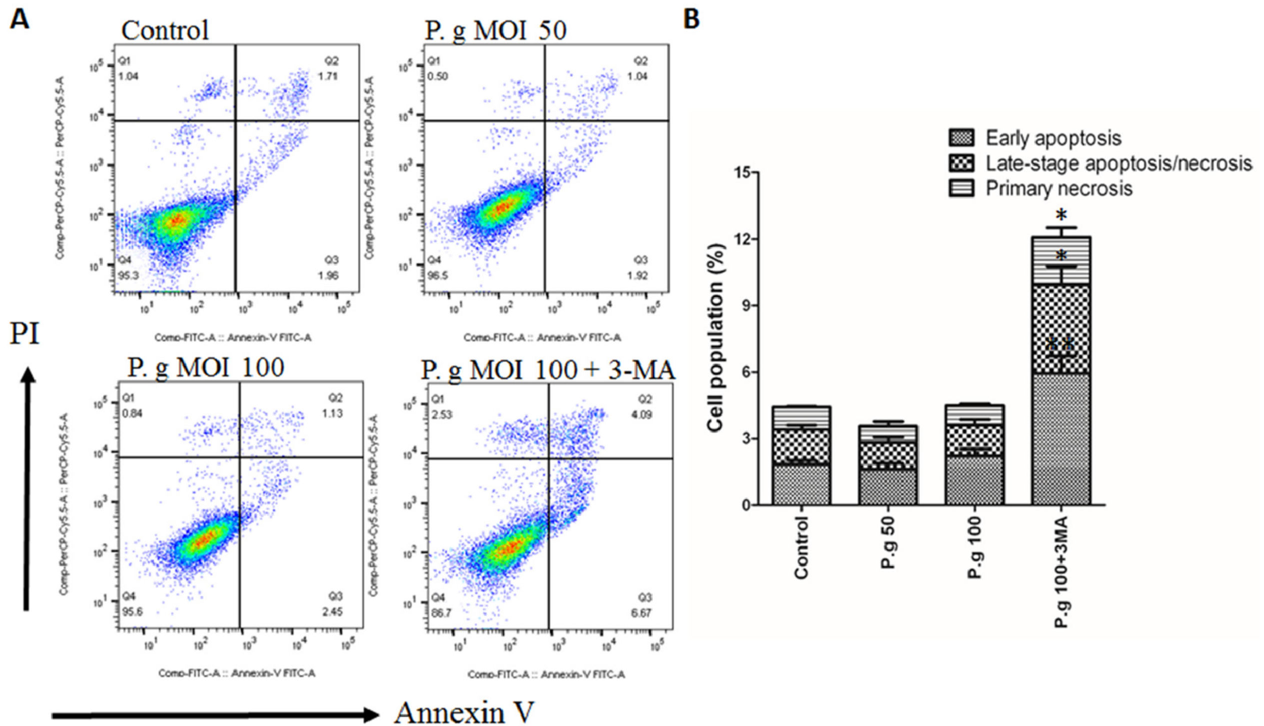

Fig 3. Inhibition of autophagy by 3-MA enchanced *P. gingivalis*-induced apoptosis in THP-1 cells. (A) Apoptosis was assessed by Annexin V/PI and Flow cytometry. THP-1 cells were infection with *P. gingivalis* (MOI 50, 100 for 24h) or in combination with 3-MA (5mM). (B) The cell population of apoptotic proportions (Annexin V positive cells) are shown in bar graphs. The error bars represent mean ± SD. *$p < 0.05$, **$p < 0.001$, and ***$p < 0.005$.

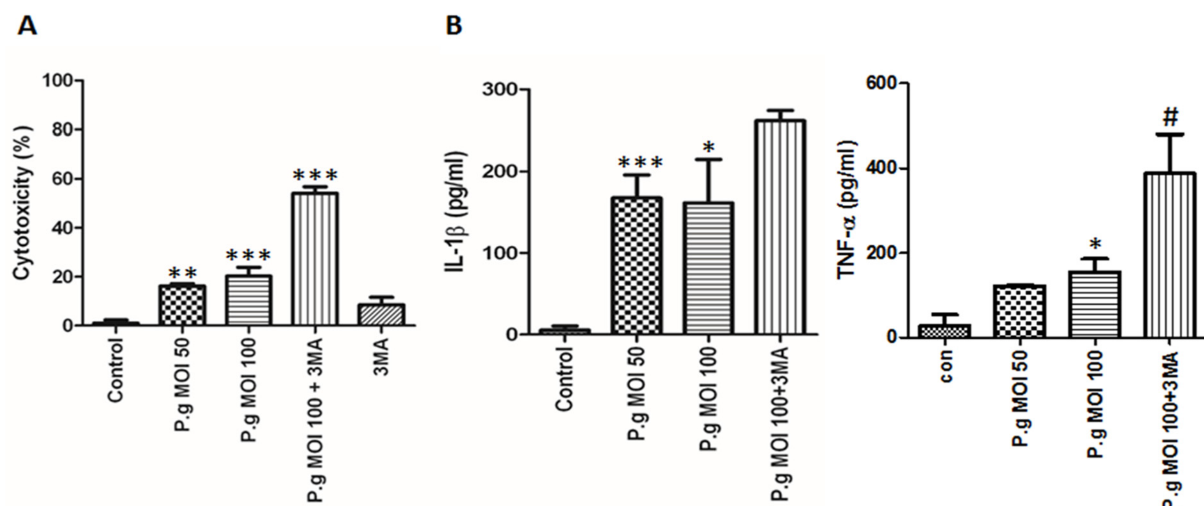

Fig 4. Inhibition of autophagy by 3-MA enhanced *P. gingivalis*-induced IL-1β and TNF-α production and increased cell death. THP-1 cells were infected with *P. gingivalis* (MOI 50 or 100) only or in combination with 3-MA (5mM) for 24 h. Cell culture supernatant was collected to determine the concentration of the cytokines. (A) The cytoplasmic enzyme LDH released was measured with an LDH-cytotoxicity assay kit. (B) The production of cytokines was determined by ELISA. The data represent mean ± SD values (n=3). * $p < 0.05$ vs control, ***$p < 0.005$ # $p<0.05$ vs P. g MOI 100.

infection (Fig. 4A).

Finally, we tested the relation between cell death and inflammatory response in *P. gingivalis*-infected THP-1 cells. *P. gingivalis* infection significantly induced IL-1β and TNF-α production in THP-1 cells and inhibiting autophagy by 3-MA slightly increased IL-1β production while TNF-α production was significantly increased compared to *P. gingivalis* infection (Fig 4B). Taken together, *P. gingivalis* can induce inflammatory cytokine production and cell death in THP-1 cells and autophagy inhibition significantly increased inflammatory cytokine production and cell death.

Discussion

The maintenance of a balance between the host response and microorganisms is important to preserve health. Bacteria-triggered cell death is an intrinsic immune defense mechanism in response to microbial infection. Understanding the cell death by bacterial pathogens will provide insight into new therapeutic approaches for the control of infection [12]. Programmed cell death (PCD) different from necrosis is an active cell death [7,8]. and is classified into apoptosis, autophagy, necroptosis, and pyroptosis [9]. Until now, the relationship between PCD and inflammatory diseases, especially periodontitis is not specified. Thus, in this stidy, we examine macrophage cell death in response to *P. gingivalis* infection, because macrophage plays key roles in inflammation and immune responses.

We previously reported that *P. gingivalis* induces autophagy in PMA-differentiated THP-1-derived macrophages and in turn, macrophages eliminate *P. gingivalis* through an autophagic response, which can lead to the restriction of an excessive inflammatory response by downregulating interleukin-1β production [1]. Autophagy is a physiologically essential cellular process for the degradation of long-lived proteins and damaged organelles in lysosomes. Autophagy also modulates immunity through the direct elimination of microorganisms, the control of inflammation and adaptive immunity, and the secretion of immune mediators [13]. Many studies demonstrated a function of autophagy *in vitro* in defense against invading pathogens including *Streptococcus, Shigella flexneri, Mycobacterium tuberculosis, Salmonella typhimurium*, and *Toxoplasma gondii* [14,15]. Induction of autophagy inhibited *M. tuberculosis* survival in infected macrophages [16]. In the study, we used CytoID to quantify autophagosome formation in THP-1 cells infected with *P. gingivalis*. Cyto-ID reagent which specifically fluorescense in autophagic vesicles, detects autophagic flux in lysosomally inhibited live cells using a novel dye that selectively labels accumulated autophagic vacuoles [17]. *P. gingivalis* induced autophagy in a dose-dependent manner (Fig 1). To visualize autophagosome formation, THP-1 cells were stained with acridine orange. Acridine orange is a lysotropic dye that accumulates in acidic organelles in a pH-dependent manner. At neutral pH, acridine orange is a hydrophobic green

fluorescent molecule. However, within acidic vesicles, acridine orange becomes protonated and trapped within the organelle and forms aggregates that emit bright red fluorescence [18]. Autophagosome formation was significantly increased in *P. gingivalis* infected THP-1 cells. Treatment of 3-MA, an autophagy inhibitor, inhibited autophagy formation when detected both by CytoID and by acridine orange staining (Fig. 1 and Fig. 2). Taken together, THP-1 cells induced autophagy to resist against *P. gingivalis* infection.

When autophagosome formation was observed under confocal microscopy, THP-1 cells treated with 3-MA and *P. gingivalis* showed bleb formation (Fig. 2). To examine the effect of autophagy inhibition on apoptosis, cells were stained with annexin-V and propium iodine. The inhibition of autophagy by 3-MA significantly enhanced *P. gingivalis*-induced cell apoptosis, although *P. gingivalis* infection did not induce any apoptosis, *P. gingivalis* has been reported to be anti-apoptotic for epithelial [19] while heat killed *P. gingivalis* is pro-apoptotic. *P. gingivalis* cell wall components such as gingipain proteases can also directly induce apoptosis [20]. Taken together, although *P. gingivalis* has the ability to induce apoptosis in THP-1 cells, autophagic host response may be the first line defense mechanism to resist apoptotic cell death. When autophagy is inhibited, *P. gingivalis* may induce host cell apoptosis. Apoptosis of fibroblasts has been suggest to be associated with inflammation in human gingiva [21]. *In vitro* studies show that *P. gingivalis* can modulate apoptosis in the following cell types: fibroblasts, endothelial cells and lymphocytes and apoptosis has been proposed as a mechanism to explain the extensive tissue destruction in chronic periodontitis lesions [22].

To determine total cell death, LDH assay was carried out in THP-1 cells infected with *P. gingivalis*. *P. gingivalis*-infected THP-1 cells with 3MA pretreatment showed significantly increased LDH release compared to *P. gingivalis*-infected THP-1 cells. Inhibition of autophagy by 3-MA should have increased total cell death through the increase of cellular apoptosis (Fig 4A). Cell death plays an important role in the regulation of inflammation. The maintenance of tissue homeostasis necessitates both the recognition and removal of invading microbial pathogens as well as the clearance of dying cells [23].

To determine inflammatory response, cytokines were determined in THP-1 cells infected with *P. gingivalis. P. gingivalis* infection significantly induced both IL-1β and TNF-α production. When 3-MA was pretreated to THP-1 cells, *P. gingivalis* infection increased IL-1β and TNF-α production. Excessive IL-1β and TNF-α production can lead to tissue damage and inflammatory disease including periodontitis [24]. Thus, autophagic host response may be the first line of defense mechanism for macrophage cells to resist bacterial infection and minimize host inflammatory response.

In summary, THP-1 cells infected with *P. gingivalis* induced autophagy. Inhibition of autophagy induced apoptotic cell death and increased IL-1β and TNF-α production in THP-1 cells infected with *P. gingivalis*. Cellular analysis for autophagy and apoptosis may provide a new understanding on the interaction between host macrophages and *P. gingivalis* and may provide a new strategy for prophylactic and therapeutic treatment of *P. gingivalis* infection.

Acknowledgements

A National Research Foundation of Korea (NRF) grant funded by the Korea Ministry of Education, Science and Technology (MEST; No. 2015R1A2A2A01007404) supported this research.

Conflict of interest

The author's declare that there is no conflict of interest that would prejudice the impartiality of this work.

References

1. Park MH, Jeong SY, Na HS, Chung J. *Porphyromonas gingivalis* induces autophagy in THP-1-derived macrophages. Mol Oral Microbiol. 2017;32(1):48-59. doi:http://dx.doi.org/10.1111/omi.12153.
2. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev. 1998;62(4):1244-63.
3. Hajishengallis G. Immune evasion strategies of *Porphyromonas gingivalis*. J Oral Biosci. 2011;53(3):233-40. doi:http://dx.doi.org/10.2330/joralbiosci.53.233.
4. Lundberg K, Wegner N, Yucel-Lindberg T, Venables PJ. Periodontitis in RA-the citrullinated enolase connection. Nat Rev Rheumatol. 2010;6(12):727-30. doi:http://dx.doi.org/10.1038/nrrheum.2010.139.
5. Lai XH, Xu Y, Chen XM, Ren Y. Macrophage cell death upon

intracellular bacterial infection. Macrophage (Houst). 2015; 2:e779. doi:http://dx.doi.org/10. 14800/Macrophage.779.

6. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ. 2012; 19(1):107-20. doi:http://dx.doi.org/10.1038/cdd.2011.96.
7. Song B, Zhou T, Yang WL, Liu J, Shao LQ. Programmed cell death in periodontitis: recent advances and future perspectives. Oral Dis. 2016. doi:http://dx.doi.org/10.1111/odi.12574.
8. Foller M, Huber SM, Lang F. Erythrocyte programmed cell death. IUBMB Life. 2008;60(10):661-8.
9. Cabon L, Martinez-Torres AC, Susin SA. [Programmed cell death comes in many flavors]. Med Sci (Paris). 2013;29 (12):1117-24. doi:http://dx.doi.org/10.1051/medsci/20132912015.
10. Trindade SC, Olczak T, Gomes-Filho IS, Moura-Costa LF, Vale VL, Galdino-Neto M, et al. *Porphyromonas gingivalis* antigens differently participate in the proliferation and cell death of human PBMC. Arch Oral Biol. 2012;57(3):314-20. doi:http://dx.doi.org/10.1016/j.archoralbio.2011.09.003.
11. Ivanyi L, Wilton JM, Lehner T. Cell-mediated immunity in periodontal disease; cytotoxicity, migration inhibition and lymphocyte transformation studies. Immunology. 1972; 22(1):141-5.
12. Ashida H, Mimuro H, Ogawa M, Kobayashi T, Sanada T, Kim M, et al. Cell death and infection: a double-edged sword for host and pathogen survival. J Cell Biol. 2011;195(6):931-42.
13. Deretic V, Saitoh T, Akira S. Autophagy in infection, inflammation and immunity. Nat Rev Immunol. 2013; 13(10):722-37.
14. Orvedahl A, Levine B. Eating the enemy within: autophagy in infectious diseases. Cell Death Differ. 2009;16(1):57-69. doi:http://dx.doi.org/10.1038/cdd.2008.130.
15. Virgin HW, Levine B. Autophagy genes in immunity. Nat Immunol. 2009;10(5):461-70.
16. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell. 2004;119(6):753-66. doi:http://dx.doi.org/10.1016/j.cell.2004.11.038.
17. Wei RJ, Lin SS, Wu WR, Chen LR, Li CF, Chen HD, et al. A microtubule inhibitor, ABT-751, induces autophagy and delays apoptosis in Huh-7 cells. Toxicology and applied pharmacology. 2016;311:88-98. doi:http://dx.doi.org/10.1016/j.taap.2016.09.021.
18. Kundu S, Kim TH, Yoon JH, Shin HS, Lee J, Jung JH, et al. Viriditoxin regulates apoptosis and autophagy via mitotic catastrophe and microtubule formation in human prostate cancer cells. Int J Oncol. 2014;45(6):2331-40. doi:http://dx.doi.org/10.3892/ijo.2014.2659.
19. Nakhjiri SF, Park Y, Yilmaz O, Chung WO, Watanabe K, El-Sabaeny A, et al. Inhibition of epithelial cell apoptosis by *Porphyromonas gingivalis*. FEMS Microbiol Lett. 2001; 200(2):145-9.
20. Kurita-Ochiai T, Ochiai K, Fukushima K. Volatile fatty acid, metabolic by-product of periodontopathic bacteria, induces apoptosis in WEHI 231 and RAJI B lymphoma cells and splenic B cells. Infect Immun. 1998;66(6):2587-94.
21. Tonetti MS, Cortellini D, Lang NP. In situ detection of apoptosis at sites of chronic bacterially induced inflammation in human gingiva. Infect Immun. 1998;66(11):5190-5.
22. Stathopoulou PG, Galicia JC, Benakanakere MR, Garcia CA, Potempa J, Kinane DF. *Porphyromonas gingivalis* induce apoptosis in human gingival epithelial cells through a gingipain-dependent mechanism. BMC Microbiol. 2009; 9:107. doi:http://dx.doi.org/10.1186/1471-2180-9-107.
23. Yang Y, Jiang G, Zhang P, Fan J. Programmed cell death and its role in inflammation. Mil Med Res. 2015;2:12. doi: http://dx.doi.org/10.1186/s40779-015-0039-0.
24. Park E, Na HS, Song YR, Shin SY, Kim YM, Chung J. Activation of NLRP3 and AIM2 inflammasomes by *Porphyromonas gingivalis* infection. Infect Immun. 2014; 82(1):112-23. doi:http://dx.doi.org/10.1128/IAI.00862-13.