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TLR2-dependent and independent pyroptosis in dTHP-1 cells induced by *Actinomyces oris* MG-1

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ABSTRACT

In the immune system, the detection of pathogens through various mechanisms triggers immune responses. Several types of specific programmed cell deaths play a role in the inflammatory reaction. This study emphasizes the inflammatory response induced by *Actinomycetes*. *Actinomyces* spp. are resident bacteria in human oral plaque and often serve as a bridge for pathogenic bacteria, which lack affinity to the tooth surface, aiding their colonization of the plaque. We aim to investigate the potential role of *Actinomyces oris* in the early stages of oral diseases from a new perspective. *Actinomyces oris* MG-1 (*A. oris*) was chosen for this research. Differentiated THP-1 (dTHP-1) cells were transiently treated with *A. oris* to model the inflammatory reaction. Cell viability, as well as relative gene and protein expression levels of dTHP-1 cells, were assessed using CCK-8, quantitative real-time polymerase chain reaction (RT-qPCR), enzyme-linked immunosorbent assay (ELISA), and Western blot assay. The treatment decreased cell viability and increased the expression of inflammatory genes such as IL-1R1 and NLRP3. It was also observed to significantly enhance the release of IL-1β/IL-18 into the supernatant. Immunoblot analysis revealed a notable increase in the expression of N-gasdermin D persisting up to 24 h. Conversely, in models pre-treated with TLR2 inhibitors, N-gasdermin D was detectable only 12 h post-treatment and absent at 24 h. These results suggest that *Actinomyces oris* MG-1 induces pyroptosis in dTHP-1 cells via TLR2, but the process is not solely dependent on TLR2.

1. Introduction

In diseases of the oral cavity resulting from bacterial infections, the inflammatory response is consistent throughout the course of the disease. This is a pivotal mechanism of the human immune system, crucial in counteracting external pathogens. A rapid and precise inflammatory reaction is ideal to eliminate these foreign "invaders." Concurrently, this response should be regulated to avert excessive tissue damage. In essence, in oral diseases, while the inflammatory response is a reliable sentinel in the initial stages of infection, if unregulated, it may subsequently contribute to the disease's progressive damage.

The role of macrophages in triggering and modulating inflammatory responses has long been a focal point in the intricate system of the oral cavity. Macrophages function as vital immune cells participating in innate and adaptive immunity, thereby establishing a frontline defense against invading pathogens, including oral bacteria [1]. These cells uphold oral homeostasis by phagocytosing invaders and apoptotic cells and generating signaling molecules, including cytokines and chemokines. Macrophages are pivotal in orchestrating inflammatory responses in conditions, such as gingivitis and periodontitis [2]. Furthermore, macrophages engage in immunosurveillance, identifying pathogens through pattern recognition receptors (PRRs), such as Toll-Like Receptors (TLRs) [3]. Upon activation by PAMPs (Pathogen-Associated Molecular Patterns) or DAMPs (Damage-Associated Molecular Patterns), macrophages undergo transformations leading to either pro-inflammatory or anti-inflammatory outcomes. They can produce nitric oxide, reactive oxygen species, and various cytokines, including TNF-α, IL-1, and IL-6, thereby shaping an inflammatory

Abbreviations: GSDMD, Gasdermin D; HIB, Heart Infusion Broth; THP-1, Human monocytic leukemia cell line; FBS, Fetal bovine serum; PMA, Phorbol 12-myristate 13-acetate; MOI, Multiplicity of infection; CCK-8, Cell Counting Kit-8; TBST, Tris-Buffered Saline and Tween 20; LTA, Lipoteichoic acid; TLR2, Toll-like receptor 2; NLRP3, NOD-like receptor family pyrin domain-containing 3; NF-κB, Nuclear Factor kappa-light-chain-enhancer of activated B cells.

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microenvironment [4]. Within the scope of inflammatory reactions, specific types of programmed cell death are instrumental in directing suitable immune responses and preserving tissue homeostasis. Pyroptosis, a type of programmed cell death, is triggered by specific intracellular pathogens and manifests as cell swelling, plasma membrane rupture, and the release of the pro-inflammatory cytokines IL-1β and IL-18. The gasdermin D (GSDMD) protein is central to pyroptosis; its cleavage creates pores in the cell membrane, causing cell lysis and discharge of inflammatory mediators [5].

Actinomyces spp. are Gram-positive, facultative anaerobic, rod-shaped bacteria that form branching filaments resembling fungal hyphae. Colonies typically exhibit a rough texture and display a molar tooth appearance when cultured [6,7]. Recognized as one of the earliest colonizers of the oral cavity, particularly on tooth surfaces, they play an essential role in the initial establishment and subsequent architecture of the dental biofilm. Their adhesive attributes enable them to function as primary colonizers, establishing a foundation for subsequent secondary and late colonizers, which are often more pathogenic, such as *Porphyromonas gingivalis* and *Treponema denticola*. The interactions between *Actinomyces* spp. and other oral bacteria can be synergistic or antagonistic, influencing the overall health or disease state of the oral cavity [8,9].

Primarily commensals of the human oral cavity, *Actinomyces* spp., can become pathogenic, especially following a mucosal breach, leading to conditions like cervicofacial actinomycosis. Actinomycosis, periodontitis, and peri-implantitis have shown correlation [10,11]. Moreover, distinguishing actinomycosis from localized aggressive periodontitis and peri-implantitis without pathological examination proves challenging.

Actinomyces oris (*A. oris*) MG-1, isolated from a gingivitis patient, was initially classified within the *A. naeslundii/A. viscosus* group [12]. Subsequent research further differentiated it and proposed it as a distinct species. An elevated presence of *A. oris* has been observed in biofilm samples from peri-crownitis sites [13]. It has also been linked to infections beyond the oral cavity, such as in the thoracic and abdominal regions [14,15].

In this study, we examined the inflammatory response triggered by *A. oris* MG-1, specifically to determine its potential to induce cellular pyroptosis.

2. Materials and methods

2.1. Cell and bacterial culture

A. oris MG-1 (ATCC, 43146) [16] was cultured in Heart Infusion Broth (HIB) (Becton, Dickinson and Company, Sparks, MD, USA) or agar plates. The human monocytic leukemia cell line (THP-1) (JCRB0112.1; JCRB Cell Bank, Osaka, Japan) was maintained in RPMI1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) (Cytiva, Logan, UT, USA). These cells were cultured and passaged regularly at 37 °C in a 5% CO_2 incubator. For experimental procedures, THP-1 cells were re-suspended in fresh RPMI1640 medium prior to seeding. Subsequently, THP-1 cells were differentiated into macrophages using 50 ng/mL Phorbol 12-myristate 13-acetate (PMA) for 48 h. The differentiated THP-1 (dTHP-1) cells were left undisturbed without PMA for an additional 48 h. For treatment preparation, single colonies of *A. oris* were selected and cultured in HIB until reaching the logarithmic growth phase. The OD600 value served as a reference to determine the desired concentration (cfu/mL).

2.2. Cell viability assay

THP-1 cells were seeded at 3×10^4 cells/well density in 96-well flat plates. After resting for 48 h post-PMA differentiation, the cells were treated with *A. oris* at varying multiplicities of infection (MOI) for 2 h. The supernatant containing bacteria was aspirated, and the cells were washed three times with PBS. The medium was then replaced with one containing 1% Penicillin-streptomycin to eliminate extracellular *A. oris* [17]. After a 48-h incubation, cell viability was assessed using the Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan), measuring the absorbance at 450 nm. Viability values were calculated as cell viability (%) = [(treated - blank)/(control - blank)] × 100%.

2.3. RT-qPCR analysis

Samples from 0 to 24 h (at intervals of 0, 6, 12, and 24 h) post 2-h treatment with *A. oris* at MOI = 100 were collected. RNA extraction was performed using the RNeasy Mini kit (QIAGEN, Hilden, Germany). RNA quantification was achieved using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The RNA was then reverse-transcribed into cDNA using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Quantitative real-time polymerase chain reaction (RT-qPCR) was performed and evaluated on the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the THUNDERBIRD Next SYBR qPCR Mix (TOYOBO, Osaka, Japan).

Amplification employed gene-specific primers listed in Table 1. *β-actin* served as the housekeeping gene, to which the relative quantification of the target gene expression was normalized. Fold change was determined using the $2^{-\Delta\Delta CT}$ method.

2.4. ELISA

The supernatant from 6-well plates following *A. oris* treatment was collected to assess cytokine secretion.

MMG-11, serving as an antagonist, was used to inhibit the TLR2 to assess whether *A. oris* could still elicit an equivalent level of inflammatory response once this pathway was inhibited. dTHP-1 cells were pretreated with MMG-11(TOCRIS, Bristol, UK) 1 h prior to co-culturing with *A. oris*, and then washed with PBS before initiating co-culture to prevent any impact on the biological activity of *A. oris*. Levels of human IL-1β were determined using the Human IL-1β Uncoated ELISA Kit (Invitrogen, Carlsbad, CA, USA), or Human Total IL-18 ELISA Kit (proteintech, Rosemont, IL, USA) according the manufacturer's instructions.

2.5. Western blot analysis

dTHP-1 cells treated with *A. oris* in 6-well plates were washed with PBS to remove any floating dead cells. Subsequently, we extracted total protein samples at 0, 12, and 24 h post a 2-h treatment with *A. oris* (MOI = 100) and lysed them using SDS lysis buffer. The total proteins were quantified using the BCA Protein Assay Kit (TaKaRa, Kusatsu, Shiga, Japan), according to the manufacturer's instructions. Equal amounts of samples were subjected to SDS-PAGE electrophoresis and electrotransferred to PVDF membranes. The membranes were then blocked using Blocking One (Nacalai Tesque, Kyoto, Japan). After rinsing with TBS containing 0.1% Tween 20 (TBST), membranes were incubated with the Anti-cleaved N-terminal GSDMD (Gasdermin D) antibody (1:1000 dilution) (ab215203, rabbit, abcam, Cambridge, UK) as the primary antibody at 4 °C overnight. After washing, they were exposed to mouse anti-rabbit IgG-HRP (1:5000 dilution) (sc-2357, Santa Cruz Biotechnology, Dallas, TX, USA) for 60 min. Visualization was achieved using the ECL Select Western Blotting Detection Reagent (Cytiva, Logan, UT, USA), with subsequent imaging on the ChemiDoc Imaging System (BioRad, Hercules, CA, USA). β-actin (1:10000 dilution) (GTX110564, rabbit, GenTex, Irvine, CA, USA) was used as an internal control for protein loading. Immunoblots were analyzed with ImageJ (version 1.53t, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

2.6. Statistical analysis

Data analysis was performed using the GraphPad Prism 9.4.0

Table 1
Primers used in the study.

Gene	Forward (5′-3′)	Reverse (5′-3′)
IL-6	CCCACCGGGAACGAAAGAG	CCGAAGGCGCTTGTGGAG
CXCL10	CCACGTGTTGAGATCATTGCTAC	CTGCATCGATTTTGCTCCCC
CD80	GGGAAATGTCGCCTCTCTGA	TGTGGATTTAGTTTCACAGCTTGC
TNF	CCCCAGGGACCTCTCTCTAA	CTCAGCTTGAGGGTTTGCTAC
IL-1R1	ACAAGGCCTTCTCCAAGAAGAATA	GGGTTAAGAGGACAGGGACG
IL-1R2	TGGAGGTGAAAGTCTGGCCT	TTCTGGCAGCCCCTGTGT
IL-1β	TTCGACACTGGGATAACGAGG	TTTTTGCTGTGAGTCCCGGAG
NLRP3	AACATGCCCAAGGAGGAAGA	GGCTGTTCACCAATCCATGA
NLRC4	TTGAAGGCGAGTCTGGCAAAG	TGGAGGTGAAAGTCTGGCCT
β-actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

software. Data are presented as mean ± SD. Statistical significance was assessed using one-way analysis of variance (with Dunnett's or Tukey's multiple-comparisons test) for three or more groups. p-value < 0.05 was considered statistically significant.

3. Results

3.1. A. oris treatment reduced the viability of dTHP-1 cells

Initially, we set up a brief co-culturing with *A. oris* at various MOIs instigated an inflammatory response in dTHP-1 cells, using cell death as an indicator of this response. The data presented at MOIs of 50, 100, and 150 showed a concentration-dependent reduction in dTHP-1 cell viability in response to *A. oris* ($p < 0.05$) (Fig. 1). Considering the practicality of sample extraction from other tests and the model's consistency, we opted for an MOI of 100 for subsequent experiments to procure protein or RNA samples at optimal concentrations.

3.2. A. oris upregulated the expression of inflammation-associated genes in dTHP-1 cells

Samples acquired at 6, 12, and 24 h following a 2-h *A. oris* treatment exhibited a marked upregulation in the mRNA expression of IL-6, CXCL10, CD80, IL-1R1, IL-1R2, IL-1β, TNF, NLRP3, and NLRC4 relative to the control ($p < 0.05$) (Fig. 2). IL-6, CXCL10, and CD80 showed high expression at 24 h compared to the control groups (Fig. 2A).

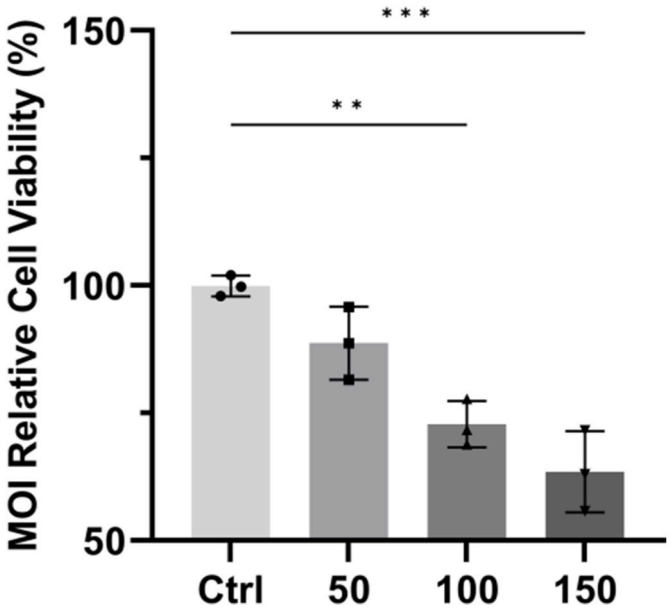

Fig. 1. Viability of dTHP-1 cells. dTHP-1 cells is influenced by *A. oris* treatment and decreases in relation to the MOI of *A. oris*. $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA with Dunnett's or Tukey's multiple-comparisons test; n = 3.

Notably, heightened IL-1R1, IL-1β, and TNF expression is linked with pro-inflammatory reactions. The rise in NLRP3 and NLRC4 expression aligns with the formation of inflammasomes, which facilitate the synthesis and release of IL-1β or activate Caspase-1 within their respective signaling routes. Although IL-1R2 also showed increased expression compared to the control, its function primarily involves curtailing the activity of IL-1β (Fig. 2B).

3.3. MMG-11, a TLR2 inhibitor, suppressed the IL-1β secretion induced by A. oris treatment in dTHP-1 cells

Considering the marked upregulation of the IL-1 gene in our model, we aimed to assess the intensity of the inflammatory response by measuring IL-1β levels in the supernatant using ELISA (Fig. 3A). As a result, we redirected our attention to TLR2, an initial mediator in the inflammatory response cascade and a primary receptor for initiating cellular inflammation in response to Gram-positive bacteria. We selected MMG-11 as the TLR2 inhibitor. The 12- and 24-h periods with the inhibitor showed a significant decline in IL-1β levels in the supernatant ($p < 0.0001$).

IL-18 was also measured in the supernatant under the same conditions, showing a similar change to that of IL-1β (Fig. 3B).

3.4. N-GSDMD expression was triggered in A. oris-treated dTHP-1 cells

The Western blot findings indicate the onset of pyroptosis in the inflammatory response model (Fig. 4). Notably, N-GSDMD was absent in both the control group and the group immediately after *A. oris* treatment (0 h). N-GSDMD presence was discernible at the 12- and 24-h marks during subsequent cultivation in an antibiotic-rich medium post *A. oris* removal. Conversely, when pre-treated with MMG-11 the inhibitor group exhibited negligible downward trends of N-GSDMD at 12 h. N-GSDMD was not detectable by 24 h.

4. Discussion

In this study, we initially established that a brief 2-h treatment of *A. oris* (at MOI = 100) with dTHP-1 cells induced cell death (Fig. 1). Based on the cell viability, the significant upregulation of IL-6, CXCL10, and CD80 indicates that dTHP-1 cells exhibited an M1-like phenotype after being treated with *A. oris* for 2 h (Fig. 2A) [18]. Gram-positive bacteria, such as *Staphylococcus aureus* (*S. aureus*), can instigate host cell death through pyroptosis, apoptosis, necrosis, and autophagy [19]. Subsequently, we quantitatively assessed the gene expression of several common pro-inflammatory genes. Peak expressions for all genes, except NLRP3, occurred at 24 h. NLRP3 and TNF showed peaks at 6 h and 24 h, respectively. As extracellular *A. oris* was removed and cultivation continued in an antibiotic-containing medium, different stimuli sources may have accounted for the two gene upregulations (Fig. 2B). The significant upregulation of IL-1β and IL-1R1 indicates a profoundly active inflammatory response within the dTHP-1 cells that intensifies over time. Although IL-1R2 can bind with IL-1β as an inducible receptor, it

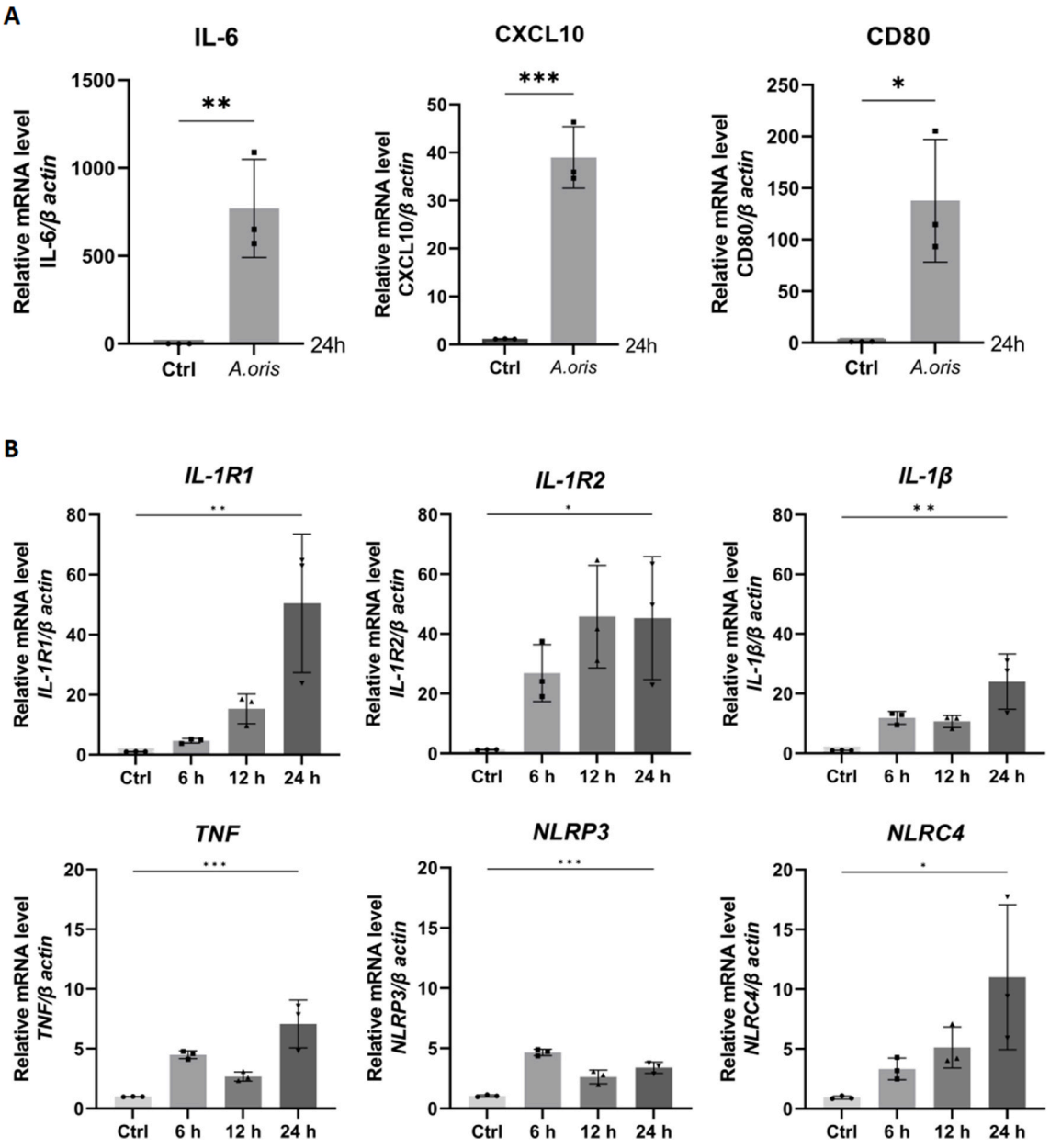

Fig. 2. Expression levels of inflammation-related genes. (**A**) *IL-6, CXCL10* and *CD80* were measured by RT-qPCR at 24 h with or with not post *A. oris* treatment. Values represent means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, T test; n = 3. (**B**) *IL-1R1*, *IL-1R2*, *IL-1β*, *TNF*, *NLRP3*, and *NLRC4* were measured at 6, 12, and 24 h post *A. oris* treatment. Values represent means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one-way ANOVA with Dunnett's or Tukey's multiple-comparisons test; n = 3.

does not elicit any cellular response [20]. This suggests it likely competes with IL-1R1 to mitigate its pro-inflammatory effects. Therefore, the upregulation of IL-1R2 is perceived as a negative feedback mechanism [21]. However, this emphasizes the heightened inflammatory activity within the cells (Fig. 2B) [22]. To determine if the gene upregulations were mirrored at the protein expression level, we quantified the concentration of IL-1β released into the supernatant using ELISA. In data not presented, after ceasing *A. oris* treatment to stop continuous stimulation, the IL-1β concentration in the supernatant persistently rose in a time-dependent fashion.

Based on prior studies, our understanding of PAMPs in Gram-positive and -negative bacteria is as follows: Gram-positive bacteria possess a thick peptidoglycan layer and lack an outer membrane [23]. Consequently, peptidoglycan and lipoteichoic acid (LTA) in the cell wall serve as the primary PAMPs of Gram-positive bacteria. Both peptidoglycan and LTA mainly activate Toll-like receptor 2 (TLR2) on macrophages. PAMPs from both Gram-positive and -negative bacteria invariably activate the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, eliciting an inflammatory response in macrophages. The activation of NF-κB in macrophages initiates a series of intracellular alterations crucially contributing to the pro-inflammatory response. A key consequence is the augmented synthesis of pro-inflammatory cytokines, notably IL-1β and IL-18. IL-1β and IL-18 is primarily produced as an inactive precursor needing further cleavage for activation. This cleavage is expedited by the inflammasome, especially proteins such as NOD-like receptor family pyrin domain-containing 3 (NLRP3) and Caspase-1 [24]. This aligns with our RT-qPCR findings that showed upregulation of NLRP3 mRNA expression with *A. oris* treatment (Fig. 2B). Intriguingly, NLRP3, caspase-1, and IL-1β/IL-18 play central roles in a death route termed inflammatory pyroptosis [5,25,26]. With the identification of GSDMD as a pivotal effector protein in pyroptosis, a novel pathway emerges for exploring how various microbes, including

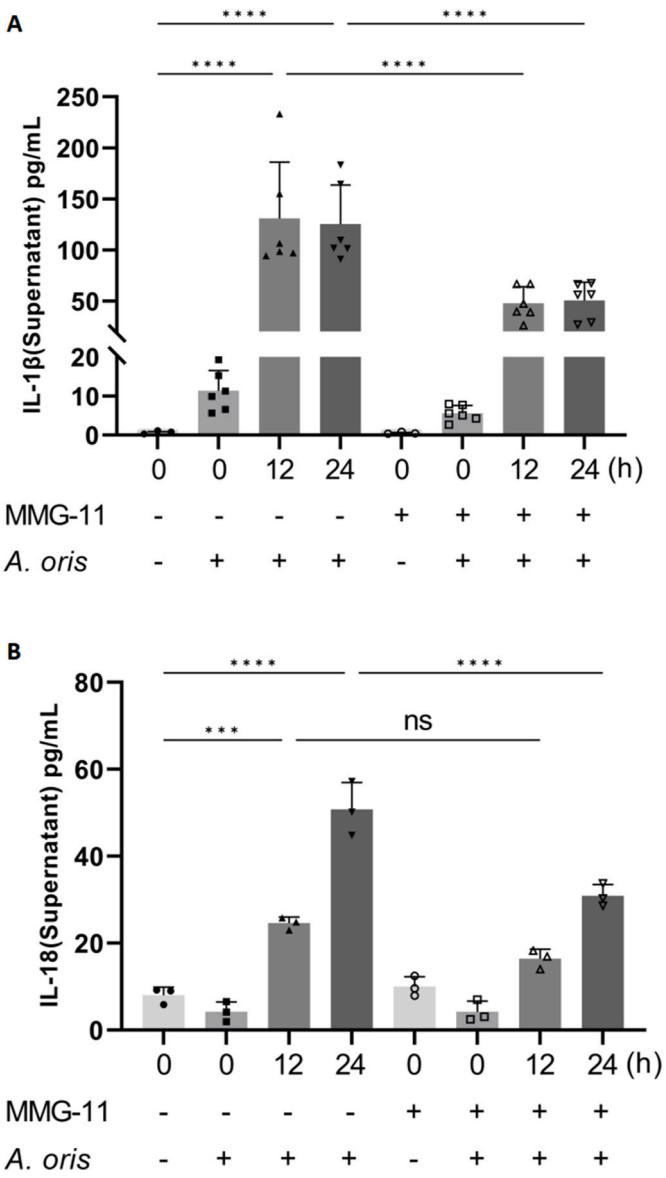

Fig. 3. Secretion concentration of IL-1β/IL-18. A short A. oris treatment raise IL-1β (A)/IL-18 (B) secretion significantly. Otherwise, Pre-treatment of dTHP-1 cells with MMG-11 decreased the secretion concentration of IL-1β/IL-18. Values represent means ± SD. ****p < 0.0001, one-way ANOVA with Dunnett's or Tukey's multiple-comparisons test; (A) n = 6, (B) n = 3.

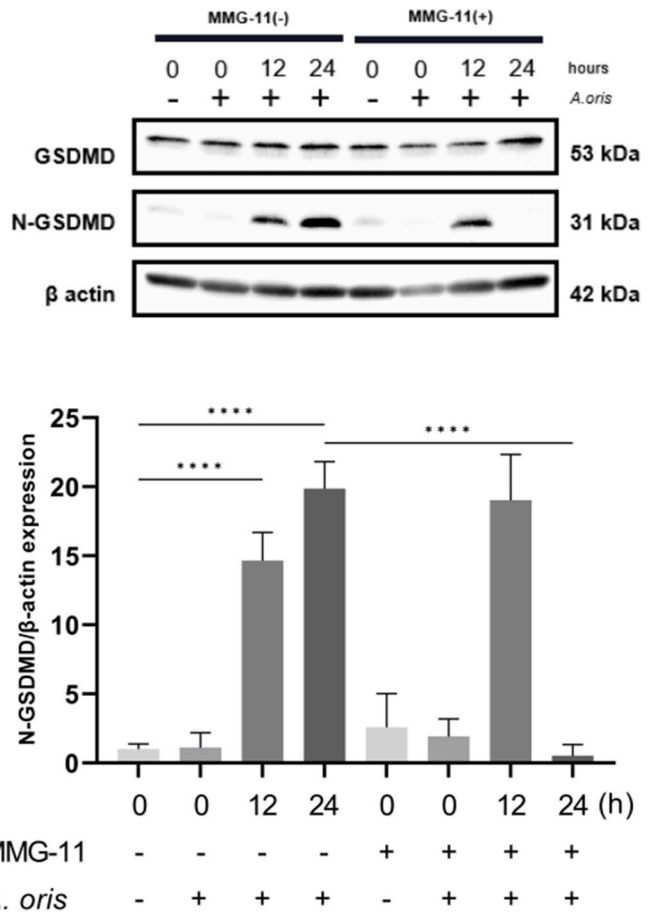

Fig. 4. Analysis of the N-GSDMD. *A. oris* treatment results in the cleavage of GSDMD to N-GSDMD in dTHP-1 cells. Western blot analysis of GSDMD protein expression levels after treating dTHP-1 cells with *A. oris* at 0, 12, and 24 h. The presence of N-GSDMD indicates pyroptosis. At 24 h, a significant difference was observed between groups with and without MMG-11 pre-treatment. ****$p < 0.0001$, one-way ANOVA with Dunnett's or Tukey's multiple-comparisons test; n = 3.

Gram-positive bacteria, instigate pyroptosis in host cells [5,27–29].

Gram-positive bacteria can indeed induce pyroptosis, especially in immune cells like macrophages. A well-studied instance is the induction of pyroptosis by *Staphylococcus aureus*, which releases pore-forming toxins such as α-hemolysin. These toxins can compromise cellular integrity and trigger pyroptosis through NLRP3 inflammasome activation, followed by caspase-1 activation [19,30]. The innate immune response to *S. aureus* is associated with TLR2 and NLRP3 [31]. The precise roles of TLR2 and NLRP3 in shaping host inflammatory response to *S. aureus* infection remain unclear. However, in mice, genetic deficiencies in TLR2 and NLRP3 increase the mortality caused by *S. aureus* [32]. Another example is *Listeria monocytogenes*, a Gram-positive bacterium, which induces pyroptosis through listeriolysin O, a cholesterol-dependent cytolysin. This toxin activates the NLRP3 inflammasome and facilitates pyroptosis [33,34]. In this mechanism, TLR2 plays a pivotal role in recognition, and the NF-κB signaling pathway is disrupted in macrophages lacking TLR2 [35].

While *Actinomyces* spp. serve as pivotal early colonizers in the biofilm, they do not exhibit the pronounced pathogenicity characteristic of other oral pathogens. Nonetheless, their potential as opportunistic pathogens should not be overlooked. Furthermore, the possibility that *Actinomyces* spp. is one of the primary causative bacteria in the early stages of inflammation, subsequently aiding more pathogenic bacteria to colonize the biofilm, cannot be ignored [6]. The role of *Actinomyces viscosus* in triggering TLR2-mediated inflammatory responses has been established [36]. Therefore, in our research on *A. oris*, we initially focused on the TLR2-mediated inflammatory pathway.

We pretreated dTHP-1 cells with a TLR2 inhibitor before *A. oris* treatment to ascertain if IL-1β secretion induced by *A. oris* is associated with the TLR2 signaling pathway. MMG-11 is a recently identified non-toxic competitive antagonist for the TLR2 pathway [37]. By analyzing the supernatant using ELISA, we compared IL-1β concentrations between groups treated with and without the inhibitor(Fig. 3A). At both 12 and 24 h, while the secretion of IL-1β could not be fully suppressed, it was reduced in the supernatant ($p < 0.0001$). Additionally, MMG-11 exhibited a negligible effect on the upregulation of IL-1β secretion in THP-1 cells that were not treated with *A. oris*. Similarly, IL-18 secretion exhibited the same trend in response to *A. oris* treatment (Fig. 3B) ($p < 0.0001$). This demonstrates that in our inflammatory model, *A. oris* induces IL-1β/IL-18 secretion via the TLR2 pathway. When TLR2 was inhibited by MMG-11, IL-1β/IL-18 secretion decreased significantly. However, given that IL-1β/IL-18 secretion did not disappear, its

secretion in this inflammatory model may not be exclusively dependent on TLR2 signaling.

While examining the release of IL-1β/IL-18, we must also consider GSDMD, another protein activated by the inflammasome. The cleaved N-terminal fragment of GSDMD forms pores in the cell membrane. These pores enable the release of mature IL-1β/IL-18 from the cell's interior to the exterior, resulting in an inflammatory cell death termed pyroptosis, which further amplifies the inflammatory response [38]. Western blot analysis showed that cleaved N-GSDMD bands were discernible in the *A. oris*-treated groups at 12 and 24 h. Conversely, almost no cleaved N-GSDMD bands were detected in the control samples or at the 0 h. This emphasizes that *A. oris* treatment can indeed trigger pyroptosis in THP-1 cells (Fig. 4).

Turning to the results from the group where the TLR2 pathway was inhibited by MMG-11, we sought to explore the potential reasons for the suppression of IL-1β/IL-18 secretion at the protein expression level. In the 12-h samples pre-treated with MMG-11, N-GSDMD expression was not notably suppressed, indicating that inhibiting the TLR2 pathway with MMG-11 does not fully obstruct pyroptosis in this inflammatory response model. However, by the 24-h point in the MMG-11 pre-treated samples, N-GSDMD expression became undetectable. This poses a significant difference compared to the expression in samples not pre-treated with MMG-11 (Fig. 4) ($p < 0.0001$). Essentially, while TLR2 inhibition did not halt the onset of pyroptosis, it suppressed its continued manifestation in the inflammation model. This suggests that *A. oris*-induced pyroptosis in THP-1 cells is mediated, at least partially, through the TLR2 pathway.

In this study, we propose that *A. oris* mediates the cleavage of the pivotal pyroptotic protein GSDMD into N-GSDMD through the TLR2 pathway. This implies that *A. oris* induces pyroptosis in dTHP-1 cells, resulting in significant IL-1β/IL-18 secretion and subsequent amplification of the inflammatory response. Caspase-1 plays a role in both GSDMD cleavage and IL-1β/IL-18 secretion [39]. Therefore, we will concentrate on the regulatory relationship between TLR2 and caspase-1 proteins in this inflammatory response model in subsequent studies. Moreover, we will investigate the pathways responsible for sustained IL-1β/IL-18 secretion after TLR2 inhibition and other potential mechanisms mediating GSDMD cleavage beyond TLR2. This will further elucidate the mechanism underlying *A. oris*-induced pyroptosis in dTHP-1 cells.

While *A. oris* is typically considered an opportunistic pathogen, its role in the early stages of oral infectious diseases merits re-evaluation. For example, its participation is frequently observed in the inflammatory responses of gingivitis. The persistent, unresolved gingivitis in these initial stages creates an environment favorable to pathogens typically linked with periodontitis [6]. In experiments not described here, we noted that heat-killed *A. oris* did not trigger IL-1β release in the supernatant of dTHP-1 cells. Consequently, the metabolic activities of *A. oris* and the association of its surface fimbriae with inflammation provide compelling directions for our future research.

5. Conclusion

In this study, *A. oris* was found to induce inflammatory cell death in macrophages, including pyroptosis, with this effect being partially mediated by the TLR2 pathway.

Ethical approval

This study did not involve content requiring ethical approval.

CRediT authorship contribution statement

Zixin Wu: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Hiroki Takigawa:** Formal analysis. **Hugo Maruyama:** Investigation. **Takayuki Nambu:** Data curation. **Chiho Mashimo:** Data curation. **Toshinori Okinaga:** Writing – review & editing, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Toshinori Okinaga reports financial support was provided by Japan Society for the Promotion of Science. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101680.

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