

Inflammasome dysregulation in human gingival fibroblasts in response to periodontal pathogens

Aral, Kubra; Milward, Michael; Cooper, Paul

DOI:
[10.1111/odi.13760](https://doi.org/10.1111/odi.13760)

Document Version
Peer reviewed version

Citation for published version (Harvard):
Aral, K, Milward, M & Cooper, P 2020, 'Inflammasome dysregulation in human gingival fibroblasts in response to periodontal pathogens', *Oral Diseases*. <https://doi.org/10.1111/odi.13760>

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

This is the peer reviewed version of the following article: Aral, K, Milward, MR, Cooper, PR. Inflammasome dysregulation in human gingival fibroblasts in response to periodontal pathogens. *Oral Dis.* 2021, which has been published in final form at <https://doi.org/10.1111/odi.13760>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

INFLAMMASOME DYSREGULATION IN HUMAN GINGIVAL FIBROBLASTS IN RESPONSE TO PERIODONTAL PATHOGENS

Kübra ARAL^{1,4*}, Michael R MILWARD², Paul R COOPER³

¹ Research Fellow, School of Dentistry, University of Birmingham, Birmingham, UK

² Professor, School of Dentistry, University of Birmingham, Birmingham, UK

³ Professor, Department of Oral Sciences, Faculty of Dentistry, University of Otago,
Dunedin, New Zealand

⁴ Republic of Turkey Ministry of Health, Uskudar Ahmet Yuksel Ozemre Oral and Dental
Health Hospital, Istanbul, Turkey

* **Corresponding Author:** Dr. Kübra Aral, DDS, PhD, Department of Oral Biology, School of Dentistry, University of Birmingham, 5 Mill Pool Way, B5 7EG, Birmingham, United Kingdom, Tel: +44 0121 466 5517, Fax number: +44 0121 625 8815, e-mail: drkubraaral@gmail.com

Running Title: Inflammasome regulation in human gingival fibroblasts

Summary Sentence: Inflammasomes and their regulators may be dysregulated by bacteria in gingival fibroblasts.

ABSTRACT

Objective: Uncontrolled production of Interleukin-1 β (IL-1 β), a major proinflammatory cytokine, is associated with tissue destruction in periodontal disease. IL-1 β production is controlled by inflammasomes which are multiprotein regulatory complexes. The current study aimed to elucidate potential regulatory pathways by monitoring the effects of periodontal pathogens *Fusobacterium nucleatum* (*Fn*) and *Porphyromonas gingivalis* (*Pg*) on inflammasomes and their regulators in human gingival fibroblasts (HGFs) *in vitro*.

Methods: HGFs were exposed to *Fn* and *Pg* alone or in combination for 24h at a multiplicity of infection of 100, +/- 30 min exposure with 5 mM adenosine triphosphate (ATP) incubation. Gene expression of inflammasome components, including Nod-like receptor pyrin domain-containing protein 3 (NLRP3) and Absent in melanoma 2 (AIM2), inflammasome regulatory proteins Pyrin-only protein 1 (POP1), Caspase Recruitment Domain Family Member 16 (CARD16) and Tripartite Motif Containing 16 (TRIM16), and inflammasome components apoptosis-related speck like protein containing a CARD (ASC) and CASPASE 1, and IL-1 β , were evaluated by RT-PCR. Pro- and mature-IL-1 β levels were monitored intracellularly by immunocytochemistry and extracellularly by ELISA.

Results: *Fn*+ATP significantly upregulated NLRP3, AIM2, IL-1 β , ASC and CASPASE 1, however, it downregulated POP1 and TRIM16. *Pg*+ATP downregulated NLRP3, ASC, POP1, but upregulated IL-1 β and CARD16. *Pg*+*Fn*+ATP significantly upregulated AIM2, IL-1 β and CARD16, and downregulated POP1, TRIM16 and CASPASE 1. *Pg*+ATP exposure significantly increased pro- and mature IL-1 β production.

Conclusion: Bacterial exposure with ATP may deregulate IL-1 β by dysregulating inflammasomes and their regulators in HGFs.

Keywords: Inflammasome, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, periodontal disease, interleukin-1 beta, gingival fibroblasts

INTRODUCTION

Periodontal disease is an inflammatory and infectious condition resulting from an excessive host response to dental plaque biofilm which includes the periodontal pathogens *Porphyromonas gingivalis* (*Pg*) and *Fusobacterium nucleatum* (*Fn*) (Kinane & Lappin, 2002; Song, Zhou, Yang, Liu, & Shao, 2017). The host response is characterized by the production of proinflammatory cytokines which elevates the local tissue inflammatory response and also promotes the destruction of the alveolar bone by activation of osteoclast formation and activity (Graves & Cochran, 2003). The release of IL-1 β is a major regulatory step in the immune response due to its ability to induce the expression of a variety of other inflammatory cytokines (Kinane & Lappin, 2002; Song et al., 2017). The monitoring of IL1 β levels in periodontal disease has been proposed as a useful approach for evaluating the host response during disease initiation, progression, and for determining therapeutic outcomes (Yoshinari et al., 2004). Therefore, a better understanding of the regulatory mechanisms responsible for IL-1 β expression may be of benefit for understanding periodontal disease pathogenesis.

IL-1 β expression is controlled by bioactive complexes termed inflammasomes which are central signalling regulators of innate immunity and which trigger the host's response (Lamkanfi & Dixit, 2017) following detection of bacteria and tissue damage. Inflammasomes are multiprotein complexes consisting of a pattern recognition receptor (PRR), an adaptor protein or ASC [apoptosis-related speck like protein containing a CARD (caspase activation and recruitment domain)] and an active form of CASPASE 1 (Schroder & Tschopp, 2010). Inflammasome activation is a two-signal process which requires pathogen associated molecular patterns (PAMPs), such as bacterial LPS and flagellin, as well as danger associated molecular patterns (DAMPs), such as ATP. Cells and tissues are known to transcribe pro-IL-1 β and also NOD-like receptors (NLRs) in response to PAMPs (Schroder & Tschopp, 2010). Consequently, the presence of DAMPs triggers inflammasome assembly that converts pro-

caspase-1 to its active form which then enables the production and release of the mature IL-1 β protein (Schroder & Tschopp, 2010).

Several inflammasomes have so far been defined and found to be dysregulated in periodontal disease, including Nod-like receptor pyrin domain-containing protein 3 (NLRP3) and Absent in melanoma 2 (AIM2) (Aral et al., 2019). The structures controlling inflammasome regulation, including PYD-only proteins (POPs), CARD-only proteins (COPs) and TRIM family proteins (TRIMs), have recently been found to be dysregulated in patients with periodontal disease (Aral et al., 2019). However, to date no study has monitored inflammasome regulators in direct response to *Pg* and *Fn* *in vitro* to better characterise their activation pathway. Therefore, the aim of the current study was to assay the expression of the inflammasomes AIM2 and NLRP3, and their regulators POP1, CARD16 and TRIM16, during IL-1 β transcription, translation and release in human primary gingival fibroblast (HGFs) challenged with *Fn* and or *Pg* in the presence of ATP.

MATERIALS AND METHODS

Bacterial Culture

Fn (*Fn* subsp. *Polymorphum*) (American Type Culture Collection (ATCC) number 10953) and *Pg* (W83) (ATCC BAA-308) were grown anaerobically at 37 °C (80% nitrogen, 10% carbon dioxide and 10% hydrogen) on horse blood agar plates (Oxoid, Basingstoke, UK) at 37 °C, as previously described (Sanchez et al., 2019). Overnight bacterial cell cultures for both bacteria were obtained in Sheadler broth and cells were pelleted by using centrifugation, washed 3 times with sterile PBS and diluted with sterile cell culture media (DMEM/Hams12, Sigma Aldrich, UK). *Escherichia coli* lipopolysaccharide (*E.coli* LPS) (Sigma Aldrich, UK) was also diluted with sterile cell culture media (DMEM/Hams12, Sigma Aldrich, UK).

Cell culture

HGFs were a kind gift from Dr Richard Shelton, School of Dentistry, University of Birmingham (IRAS project ID 171283 and REC reference 15/NW/0079) and were cultured in DMEM/F12 (Sigma-Aldrich, UK) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin and Streptomycin and 1% Amphotericin B (Sigma-Aldrich, UK). Cells were seeded into a 6-well plate at concentrations of 3×10^5 cells/ml and allowed to attach to cultureware overnight. Cell cultures in a serum and antibiotic-free media were infected with either *Fn* and *Pg* alone or in combination at a 100 MOI or *E. coli* LPS at 10 $\mu\text{g/ml}$ (positive control) or media alone (negative control) for 24h (Abdulkareem et al. 2018; Milward et al., 2007) with or without 5mM ATP incubation for 30 min (Sanz & Di Virgilio, 2000). Both assays were repeated 3 times and performed in triplicate.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

For gene expression analysis, total RNA was extracted by using a kit (RNeasy Plus Mini Kit, Qiagen, UK) according to the manufacturer's instructions. The total RNA concentration ($\text{ng}/\mu\text{l}$) was determined using a spectrophotometer (Genova Nano, Jenway, UK) and RNA purity (260/280) was also determined. Total RNA was then reverse transcribed into cDNA by using a kit (Tetro, Bioline, UK) according to the manufacturer's protocol. Relative quantification of genes was performed using reverse transcription polymerase chain reaction (RT-PCR) by using a LightCycler® 480 system (Roche, Switzerland) with a SYBR Green I Master mix (Roche, Switzerland). Cycling conditions were as follows: 95°C for 10 minutes, 45 cycles of 95°C 20 seconds, 60°C 20 seconds and 72°C 30 seconds followed by melt curve analysis. Primers and assay details for the target genes are provided in **Table 1**. PCR efficiency for each primer pair was determined using dilutions of sample cDNA (1:1-1:1000) and CT values were obtained for all genes. To assess differences, the fold-change in expression of the target mRNA was normalized to that of the GAPDH housekeeping control mRNA using the $2^{-\Delta\Delta\text{CT}}$ method.

Immunocytochemistry analysis

HGFs were cultured on μ -Slide 8 Well slides (IbiTreat, IbiDi GmbH, Grafelfing, Germany) and allowed to attach for overnight. Then, cells were incubated either with *Fn* and *Pg* alone or in combination at a 100 MOI or *E. coli* LPS (10 μ g/ml) or only with media (negative control group). ATP groups were also treated with 5mM ATP (Sanz & Di Virgilio, 2000) for the final 30 min of the incubation period. Cultures were then washed with PBS twice and fixed with 4% paraformaldehyde (Thermo-Fisher Scientific, USA), permeabilized with 0.1% Triton X-100 (Thermo-Fisher Scientific, USA) in PBS, incubated with BSA (Sigma Aldrich, UK), washed with PBS twice and incubated with blocking solution (3% donkey serum in 1 % BSA/PBS) for 1 h. After removal of blocking solution, cultures were incubated overnight at 4 °C with 15 μ g/ml anti-human IL-1 β primary antibody (Catno: MAB201-SP, Bio-Techne, Minneapolis, MN) in blocking solution. Samples were washed with PBS and incubated with donkey anti-mouse IgG secondary antibody (Catno: NL007, Bio-Techne, Minneapolis, MN) in blocking solution (1:200 dilution) for 2h at room temperature. Cell nuclei were stained with DAPI (Sigma, UK) and the actin cytoskeleton were stained with fluorescent phalloidin (Cytoskeleton, Denver, USA). Images were captured under an immunofluorescent microscope (Nikon, Japan) at a 20X objective. Cultures with no primary or secondary antibody staining were used to exclude auto-fluorescence and controls with primary Ab only and secondary Ab only were also used to exclude non-specific Ab staining. Relative quantification analysis was performed on fluorescent images to determine the production and localisation of pro- and mature IL-1 β levels. Images from 5 random fields for each group were filtered to remove noise and background, to obtain the true signal for IL-1 β (Kodiha, Banski, & Stochaj, 2011). Fluorescent intensity of nuclei of cells and IL-1 β in each image were calculated. Quantification was determined based on the ratio of total fluorescent intensity of IL-1 β relative to the nuclei (DAPI

stain). Analyses were performed using ImageJ software (NIH, USA) (Selinger et al., 2019) to determine the pro- and mature IL-1 β levels.

ELISA

Cytokine expression of IL-1 β in culture supernatants were analysed by using an ELISA kit (R&D Systems, MN, USA) following the manufacturer's instructions. The optical density was read at 450 and 570 nm by using a microplate reader (Biotek ELX800, USA). The total levels of IL-1 β were determined in picograms (pg) by using a calibration curve. Results were presented as pg/ml.

Statistical analysis

Statistical analyses were conducted using a statistical package program (SPSS 20.0, IBM, Chicago, USA). Mean \pm standard deviations were calculated for all groups. ANOVA, Kruskal-Wallis and student t-test were used for statistical analysis. A p-value < 0.05 was considered statistically significant.

RESULTS

Gene Expression Levels in Response to Bacteria and ATP Exposure

The expression levels of NLRP3, AIM2, CARD16, TRIM16, POP1, CASPASE 1, ASC and IL-1 β were evaluated by using RT-PCR. (Figure 1) All the tested groups except for *Pg*, *Pg*+ATP and *E.coli LPS*+ATP significantly upregulated NLRP3 gene expression levels (Figure 1A). Similarly, *Fn*+ATP in the presence and the absence of *Pg* significantly enhanced AIM2 expression levels (Figure 1B). The upregulation trend was also evident in study groups for IL-1 β expression apart from *Fn*, Control+ATP, and *E.coli LPS* (Figure 1C).

The inflammasome component, CASPASE 1, was downregulated by majority of exposure groups including Control+ATP, *E.coli LPS*, *Pg*, *Fn*+*Pg* and *Fn*+*Pg*+ATP (Figure 1D). ASC, other inflammasome part, was dysregulated differentially by the tested groups. *Fn*

+/- ATP upregulated ASC however *Pg* alone, with *Fn* or ATP significantly downregulated its expression (Figure 1E).

The inflammasome regulator, CARD16, was upregulated in response to tested exposure groups in the presence or absence of ATP except for *Pg* alone (Figure 1F). However other evaluated inflammasome regulator TRIM16 was majorly downregulated by tested groups including *Fn*+/- ATP, *Fn*+*Pg*+ATP or *Pg* alone (Figure 1G). Similar downregulation trend was also detected for POP1 in response to most of the tested groups including by *Fn*+/- ATP, *Pg*+/- ATP or *Fn*+*Pg*+ATP (Figure 1H).

Intracellular IL-1 β levels

Intracellular pro- and mature IL-1 β levels were determined by the quantification and calculation of IL-1 β to nuclei (DAPI) ratio in the fluorescent images captured by fluorescent microscopy (Figure 2). Although the elevation was visible in the images of bacterial exposure groups in the presence and absence of ATP, the only statistically significant difference was found in *Pg*+ATP group (Figure 3)

Extracellular IL-1 β levels

Cytokine levels of IL-1 β was also measured in cell culture supernatants. ELISA analysis did not detect any difference in tested groups compared to control group at 24 h ($p>0.05$).

DISCUSSION

IL-1 β is a major proinflammatory cytokine which plays a significant role in the host response during periodontal disease. Indeed, uncontrolled production of IL-1 β caused by periodontal pathogens, including *Fn* and *Pg*, has been reported to result in tissue destruction in the host during disease pathogenesis. IL-1 β expression is regulated by inflammasome complexes and subsequent dysregulation of these structures may cause uncontrolled IL-1 β release (Aral K, 2019). In our previous work studying inflammasomes, including NLPR3 and AIM2, as well as several inflammasome regulatory proteins, including POPs, COPs and TRIMs, we identified

dysregulation in their levels in gingival tissue samples derived from periodontitis patients (Aral et al., 2019). Therefore, the current study was designed to dissect the potential cell source, stimulants and pathways involved in this dysregulation by monitoring IL-1 β and inflammasome levels of NLRP3 and AIM2 and their regulatory proteins POP1, CARD16 and TRIM16 in HGFs exposed to *Fn* and *Pg*. *Fn* + ATP exposure resulted in an upregulation in NLRP3, AIM2, IL-1 β , ASC, CASPASE 1 expression however it downregulated POP1 and TRIM16 in HGFs. *Pg* + ATP exposure downregulated NLRP3, ASC, POP1, upregulated IL-1 β and CARD16, without significantly influencing CASPASE 1, TRIM16 and AIM2 expression. *Pg*+ATP exposure also increased IL-1 β protein levels. Notably, *Pg* and *Fn* combined exposure significantly upregulated AIM2, IL-1 β and CARD16, but downregulated POP1, TRIM16 and CASPASE 1 without affecting NLRP3 levels. Consequently, it may be concluded that *Pg* and *Fn* in the presence of ATP differentially dysregulate inflammasomes and their regulator molecules in HGFs.

The NLRP3 inflammasome can be activated by PAMPs (Kanneganti et al., 2006; Shibata, 2018) and in addition, DAMPs, including ATP, have also been shown necessary to activate the NLRP3 inflammasome. (Rathinam, Vanaja, & Fitzgerald, 2012) The association between the NLRP3 inflammasome and periodontal disease has been previously studied by *in vitro* (Belibasakis, Guggenheim, & Bostanci, 2013; Bostanci, Meier, Guggenheim, & Belibasakis, 2011; Cheng et al., 2017; Kuo et al., 2016; Xu et al., 2019) and clinical studies (Aral et al., 2019; Bostanci et al., 2009; Cheng et al., 2017; Isaza-Guzman, Medina-Piedrahita, Gutierrez-Henao, & Tobon-Arroyave, 2017; Kim, Park, Song, Na, & Chung, 2016; Park et al., 2014; Xue, Shu, & Xie, 2015). Indeed, NLRP3 expression has been found to be higher in gingiva and saliva from periodontitis patients (Aral et al., 2019; Bostanci et al., 2009; Cheng et al., 2017; Isaza-Guzman et al., 2017; Kim et al., 2016; Park et al., 2014; Xue et al., 2015) compared with periodontally healthy participants. *In vitro* studies have also been performed to

evaluate the NLRP3 inflammasome in response to periodontal pathogens. Cheng et al. (2017) reported a downregulation for NLRP3 expression in HGFs exposed to *Pg* LPS for up to 4h and Bostanci et al. (2011) also reported a downregulation of NLRP3 expression in response to a subgingival biofilm including *Pg* for 6h in HGFs. In addition, Belibasakis et al. (Belibasakis et al., 2013) challenged HGFs with a subgingival biofilm with or without *Pg* for 6h and reported that the presence of *Pg* was required to downregulate NLRP3 levels. In contrast with these findings, Kuo et al. (2016) reported no difference in NLRP3 expression in HGFs challenged with *Pg* at up to 50 MOI for 6h compared with control however the presence of high glucose with *Pg* was found to sufficiently trigger NLRP3 expression. Xu et al. (2019) evaluated the effect of *Pg* LPS for 11h with or without additional ATP incubation in HGFs and reported that *Pg* LPS alone did not trigger NLRP3 expression without ATP. Similar to other reported studies (Belibasakis et al., 2013; Bostanci et al., 2011; Cheng et al., 2017), our findings indicate that *Pg* with or without ATP at a MOI of 100 for 24h was found to downregulate NLRP3 expression in HGFs. The effects of *Fn* with or without *Pg* and/or ATP on NLRP3 expression levels were also monitored in the current study. *Fn* with or without ATP significantly increased NLRP3 expression in HGFs. In addition, *Pg* and *Fn* combined also triggered a significant elevation in NLRP3 expression. Indeed, it may be concluded that *Fn* was a significant promoter of NLRP3 inflammasome in HGFs compared with *Pg*.

AIM2 has been reported to recognise aberrant cytoplasmic dsDNA of viral or bacterial origin (Wang & Yin, 2017), and in gingiva of periodontitis patients an overexpression of AIM2 has been reported (Aral et al., 2019; Park et al., 2014; Xue et al., 2015). *In vitro* studies have also evaluated the effects of pathogens on AIM2 expression levels in HGFs. Belibasakis et al. (Belibasakis et al., 2013) demonstrated that a subgingival biofilm with or without *Pg* did not influence AIM2 expression in HGFs. However, Bostanci et al. (2011) reported a significant upregulation of AIM2 expression in HGFs challenged with both supragingival and subgingival

biofilms (Bostanci et al., 2011). In the current study *Pg* alone significantly downregulated AIM2 expression in HGFs. However, *Fn* with ATP with and without *Pg* significantly upregulated AIM2 expression. It may therefore be concluded that AIM2 may be dysregulated by *Pg* and *Fn* in the presence of ATP differently *in vitro*.

POPs have been proposed to be key regulators of inflammasomes (Aral et al., 2019). POP1, a member of POPs, has been reported to sequester the ASC and NLR connection by interacting with ASC and this process subsequently inhibits inflammasome activation. (Le & Harton, 2013; Stehlik et al., 2003) Previously, we (Aral et al., 2019) demonstrated that POP1 was significantly downregulated in gingival specimens obtained from periodontitis patients. Consequently, we concluded that the downregulation of POP1 may be responsible for increasing NLRP3 and IL-1 β expression levels which associate with periodontal disease. In the current study, *Fn* and *Pg* alone with or without ATP significantly dysregulated POP1 expression in HGFs. In addition, *Fn* and *Pg* together with ATP also caused the same effect. The upregulation of IL-1 β expression by the afore mentioned exposure groups (except for *Fn* alone) also indicate that *Fn* and *Pg* may downregulate POP1 and this may lead to an increase of IL-1 β levels in HGFs.

COPs have also been reported to play a role in inflammasome regulation (Aral et al. 2019). A member of this family, CARD16 (COP/Pseudo-ICE), is an intracellular regulator of CASPASE 1 activation and this molecule can reduce IL-1 β levels by binding CASPASE 1 (Druiilheet et al., 2001). In our previous work we found a downregulation trend in gingiva from periodontitis patients for CARD16 expression levels (Aral et al., 2019). While a downregulation was expected based on the data from our previous clinical study, in our current work we found that CARD16 was significantly upregulated by *Fn* with or without ATP with or without *Pg* and also by *Pg* with ATP. According to our knowledge no study has evaluated

the role of CARD16 in response to periodontal pathogens *in vitro* therefore further studies are now warranted to expand on this work.

TRIM family proteins have also been reported to be involved in inflammasome mediated IL-1 β response (Aral et al., 2019). TRIM16 from TRIMs has been reported to increase IL-1 β production by interacting with pro-caspase-1 (Rathinam et al., 2012). In our previous clinical study, TRIM16 was significantly downregulated in gingiva from periodontitis patients compared with periodontally healthy controls (Aral et al., 2019). In the current study, *Fn* with or without ATP with or without *Pg* significantly downregulated TRIM16 expression however *Fn* with *Pg* caused an opposing response. However, thus far no studies have evaluated the role of TRIM16 in response to *Fn* and or *Pg* and therefore, future studies are needed in this area.

ASC is an inflammasome component that has previously been evaluated in periodontal disease. Clinical data has shown an upregulation in saliva (Isaza-Guzman et al., 2017) and gingival tissues (Aral et al., 2019; Garcia-Hernandez et al., 2019) of periodontitis patients compared with periodontally healthy controls. However other studies have not detected these differences (Aral et al., 2019). *In vitro* data regarding ASC expression in response to periodontal pathogens in HGFs has also been previously reported. Bostanci et al. (2011) described an upregulation of ASC expression in HGFs challenged with a supragingival biofilm for 6h while there was a downregulation in response to higher concentrations of the subgingival biofilm used. Notably, Belibasakis et al. (2013) reported no difference for ASC expression in HGFs in a subgingival biofilm model with or without *Pg*. In the current study, *Fn* with or without ATP upregulated ASC expression although the presence of *Pg* with *Fn* downregulated ASC expression. Interestingly, exposure with *Pg* with or without ATP also downregulated ASC expression in HGFs. Therefore, *Fn* and *Pg* may differentially influence ASC expression in

HGFs and this may support the notion that levels of each perio-pathogen may be important in disease progression.

The other inflammasome component, CASPASE 1, has also been previously investigated in periodontal disease. Wide ranging results have been reported for CASPASE 1 expression in clinical samples from periodontitis patients. Some studies reported an upregulation (Cheng et al., 2017; Garcia-Hernandez et al., 2019; Park et al., 2014) in gingiva from periodontitis patients however others found no differences in levels within the gingiva (Aral et al., 2019) or in the saliva (Isaza-Guzman et al., 2017). *In vitro* studies are also inconsistent. Bostanci et al. (2011) reported an upregulation for CASPASE 1 in HGFs challenged with a subgingival biofilm at relatively low levels, however a downregulation was reported at higher doses. Belibasakis et al. (2013) reported no difference for CASPASE 1 expression in HGFs infected with a subgingival biofilm with or without *Pg*. In the current study, *Fn* with ATP significantly upregulated CASPASE 1 expression however the presence of *Pg* with *Fn* with or without ATP caused a significant downregulation. *Pg* alone also caused a downregulation of CASPASE 1 levels. Therefore, it may be concluded that *Fn* and *Pg* may have different dynamic effects on CASPASE 1 expression in HGFs.

Fn and *Pg* are present in subgingival biofilms during periodontal disease et al., 2005). In addition, *Fn* co-aggregates with *Pg*, and *Fn* plays a significant role in the establishment of *Pg* in the periodontal pocket (Bolstad, Jensen, & Bakken, 1996) by facilitating the growth of *Pg* (Diaz, Zilm, & Rogers, 2002) and increasing the invasion of *Pg* into gingival epithelial cells (Li et al., 2015). Coinfection with *Pg* and *Fn* synergistically increased more bone loss and IL-1 β levels when compared with a mono-infection in rat periodontal tissues (Polak et al., 2009). Therefore, in the current study, a mixed infection of *Fn* and *Pg* in the presence or absence of ATP was applied. IL-1 β levels were significantly higher in the *Fn+Pg+ATP* exposure group compared with the *Fn+ATP* and *Pg+ATP* groups. However, NLPR3 and AIM2 highest

expression levels were detected in the *Fn*+ATP exposure group and this may be because *Pg* has a dominant effect compared with *Fn* in this model system.

In the literature the response of some inflammasomes including NLRP3 and AIM2, and inflammasome parts, CASPASE 1 and ASC were evaluated in supra and subgingival biofilm models (Belibasakis et al., 2013; Bostanci et al., 2011) *in vitro*. The downregulation of NLRP3 expression in response to a 6h of subgingival biofilm challenge including *Pg* and *Fn* was reported (Belibasakis et al., 2013; Bostanci et al., 2011). In addition, Belibasakis et al. (2013) reported the presence of *Pg* is the main factor that cause NLRP3 downregulation. In the current study *Pg* alone significantly reduced NLRP3 expression however *Fn*+/-*Pg* cause a significant upregulation. Therefore within the current findings it may be concluded that *Pg* downregulated NLRP3 however *Fn* caused an upregulation. Belibasakis et al. (2013) reported that AIM2 upregulated in response to a biofilm model including *Pg* and *Fn*, and *Pg* did not directly affect AIM2 expression. However, Bostanci et al. (2011) reported a significant upregulation of AIM2 as a response to a subgingival biofilm model with *Fn* and *Pg*. In our study which evaluates the direct effects of each bacteria on found that *Pg* caused a downregulation of AIM2 however *Fn*+/- *Pg* did not cause any difference. Likewise AIM2 inflammasome Belibasakis et al. (Belibasakis et al., 2013) did not found any difference in ASC in the subgingival biofilm model with or without *Pg*. However Bostanci et al. (Bostanci et al., 2011) reported a downregulation of ASC in the high doses of subgingival biofilm with *Fn* and *Pg*. In our findings, ASC was upregulated by *Fn* alone but downregulated by *Pg*+/-*Fn*. It might be suggested that the presence of *Pg* may be responsible of the ASC downregulation. The other inflammasome part CASPASE 1, was found to be downregulated in response to a higher doses of a subgingival biofilm model with *Fn* and *Pg* by Bostanci et al. (2011) However no difference for CASPASE 1 reported by Belibasakis et al. (2013) as a response to a biofilm model including these pathogens. In the current study we found that *Pg* with or without *Fn* caused a downregulation of CASPASE 1

however *Fn* alone did not affect the expression levels. Nevertheless a full comparison with the current study and Belibasakis and Bostanci et al.s' cannot be performed because of the differences of the bacterial exposure times and amounts.

In the current study both gene and also mature IL-1 β levels were also analyzed. *Pg* and also *Fn* alone or together in the presence or absence of ATP significantly increased IL-1 β gene expression levels. ICC data showed a similar trend of increase were also evident for intracellular pro- and mature IL-1 β levels but only reached significance in *Pg*+ATP group. However the absence of immunoblotting prevented the detection of mature IL-1 β protein levels which was accepted as a limitation of the current work. Therefore, future data is needed to clarify the effect of these pathogens on IL-1 β protein.

CONCLUSION

In clinical periodontal disease, the host responds to a range of pathogenic bacteria present in a complex biofilm. *Fn* and *Pg* are major determinants of this biofilm and play significant roles. Therefore, in the current study the effects of *Fn* and *Pg* were evaluated in HGFs. *Fn* in the presence of ATP increased IL-1 β expression by upregulating NLRP3, AIM2 and by downregulating TRIM16 and POP1 levels. However, *Pg* plus ATP also upregulated IL-1 β expression and production but downregulated NLRP3 and POP1 without influencing the AIM2 inflammasome. *Fn* and *Pg* in the presence of the DAMP, ATP, may differently influence inflammasomes and their regulators in HGFs. These findings may have significant implications for the pathogenesis and diagnostics of periodontal disease.

ACKNOWLEDGEMENTS AND CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article. This study was supported by the School of Dentistry, University of Birmingham. The

corresponding author of this study (K. Aral) was supported with a scholarship by the Scientific and Technological Research Council of Turkey (TÜBİTAK), Ankara, Turkey. The corresponding author thank Dr Dhanak Gupta, University of Birmingham for her assistance during ICC.

REFERENCES

- Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol*, *43*(11), 5721-5732. doi:10.1128/JCM.43.11.5721-5732.2005
- Abdulkareem, A. A., Shelton, R. M., Landini, G., Cooper, P. R., & Milward, M. R. (2018). Periodontal pathogens promote epithelial-mesenchymal transition in oral squamous carcinoma cells in vitro. *Cell Adh Migr*, *12*(2), 127-137. doi:10.1080/19336918.2017.1322253
- Aral, K., Berdeli, E., Cooper, P. R., Milward, M. R., Kapila, Y., Karadede Unal, B., . . . Berdeli, A. (2019). Differential expression of inflammasome regulatory transcripts in periodontal disease. *J Periodontol*. doi:10.1002/JPER.19-0222
- Aral K, M. M., Kapila Y, Berdeli A, Cooper PR. (2019). Inflammasomes and Their Regulation in Periodontal Disease: A Review. *J Periodontal Res*.
- Belibasakis, G. N., Guggenheim, B., & Bostanci, N. (2013). Down-regulation of NLRP3 inflammasome in gingival fibroblasts by subgingival biofilms: involvement of Porphyromonas gingivalis. *Innate Immun*, *19*(1), 3-9. doi:10.1177/1753425912444767
- Bolstad, A. I., Jensen, H. B., & Bakken, V. (1996). Taxonomy, biology, and periodontal aspects of Fusobacterium nucleatum. *Clin Microbiol Rev*, *9*(1), 55-71.
- Bostanci, N., Emingil, G., Saygan, B., Turkoglu, O., Atilla, G., Curtis, M. A., & Belibasakis, G. N. (2009). Expression and regulation of the NALP3 inflammasome complex in periodontal diseases. *Clin Exp Immunol*, *157*(3), 415-422. doi:10.1111/j.1365-2249.2009.03972.x
- Bostanci, N., Meier, A., Guggenheim, B., & Belibasakis, G. N. (2011). Regulation of NLRP3 and AIM2 inflammasome gene expression levels in gingival fibroblasts by oral biofilms. *Cell Immunol*, *270*(1), 88-93. doi:10.1016/j.cellimm.2011.04.002
- Cheng, R., Liu, W., Zhang, R., Feng, Y., Bhowmick, N. A., & Hu, T. (2017). Porphyromonas gingivalis-Derived Lipopolysaccharide Combines Hypoxia to Induce Caspase-1 Activation in Periodontitis. *Front Cell Infect Microbiol*, *7*, 474. doi:10.3389/fcimb.2017.00474
- Diaz, P. I., Zilm, P. S., & Rogers, A. H. (2002). Fusobacterium nucleatum supports the growth of Porphyromonas gingivalis in oxygenated and carbon-dioxide-depleted environments. *Microbiology*, *148*(Pt 2), 467-472. doi:10.1099/00221287-148-2-467
- Druilhe, A., Srinivasula, S. M., Razmara, M., Ahmad, M., & Alnemri, E. S. (2001). Regulation of IL-1beta generation by Pseudo-ICE and ICEBERG, two dominant negative caspase recruitment domain proteins. *Cell Death Differ*, *8*(6), 649-657. doi:10.1038/sj.cdd.4400881
- Garcia-Hernandez, A. L., Munoz-Saavedra, A. E., Gonzalez-Alva, P., Moreno-Fierros, L., Llamosas-Hernandez, F. E., Cifuentes-Mendiola, S. E., & Rubio-Infante, N. (2019). Upregulation of proteins of the NLRP3 inflammasome in patients with periodontitis and uncontrolled type 2 diabetes. *Oral Dis*, *25*(2), 596-608. doi:10.1111/odi.13003
- Graves, D. T., & Cochran, D. (2003). The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *J Periodontol*, *74*(3), 391-401. doi:10.1902/jop.2003.74.3.391
- Isaza-Guzman, D. M., Medina-Piedrahita, V. M., Gutierrez-Henao, C., & Tobon-Arroyave, S. I. (2017). Salivary Levels of NLRP3 Inflammasome-Related Proteins as Potential Biomarkers of Periodontal Clinical Status. *J Periodontol*, *88*(12), 1329-1338. doi:10.1902/jop.2017.170244

- Kanneganti, T. D., Ozoren, N., Body-Malapel, M., Amer, A., Park, J. H., Franchi, L., . . . Nunez, G. (2006). Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature*, *440*(7081), 233-236. doi:10.1038/nature04517
- Kim, S., Park, M. H., Song, Y. R., Na, H. S., & Chung, J. (2016). Aggregatibacter actinomycetemcomitans-Induced AIM2 Inflammasome Activation Is Suppressed by Xylitol in Differentiated THP-1 Macrophages. *J Periodontol*, *87*(6), e116-126. doi:10.1902/jop.2016.150477
- Kinane, D. F., & Lappin, D. F. (2002). Immune processes in periodontal disease: a review. *Ann Periodontol*, *7*(1), 62-71. doi:10.1902/annals.2002.7.1.62
- Kodiha, M., Banski, P., & Stochaj, U. (2011). Computer-based fluorescence quantification: a novel approach to study nucleolar biology. *BMC Cell Biol*, *12*, 25. doi:10.1186/1471-2121-12-25
- Kuo, H. C., Chang, L. C., Chen, T. C., Lee, K. C., Lee, K. F., Chen, C. N., & Yu, H. R. (2016). Sterol Regulatory Element-Binding Protein-1c Regulates Inflammasome Activation in Gingival Fibroblasts Infected with High-Glucose-Treated Porphyromonas gingivalis. *Front Cell Infect Microbiol*, *6*, 195. doi:10.3389/fcimb.2016.00195
- Lamkanfi, M., & Dixit, V. M. (2017). In Retrospect: The inflammasome turns 15. *Nature*, *548*(7669), 534-535. doi:10.1038/548534a
- Le, H. T., & Harton, J. A. (2013). PIRIN- and CARD-only Proteins as Regulators of NLR Functions. *Front Immunol*, *4*, 275. doi:10.3389/fimmu.2013.00275
- Li, Y., Guo, H., Wang, X., Lu, Y., Yang, C., & Yang, P. (2015). Coinfection with Fusobacterium nucleatum can enhance the attachment and invasion of Porphyromonas gingivalis or Aggregatibacter actinomycetemcomitans to human gingival epithelial cells. *Arch Oral Biol*, *60*(9), 1387-1393. doi:10.1016/j.archoralbio.2015.06.017
- Milward, M. R., Chapple, I. L., Wright, H. J., Millard, J. L., Matthews, J. B., & Cooper, P. R. (2007). Differential activation of NF-kappaB and gene expression in oral epithelial cells by periodontal pathogens. *Clin Exp Immunol*, *148*(2), 307-324. doi:10.1111/j.1365-2249.2007.03342.x
- Park, E., Na, H. S., Song, Y. R., Shin, S. Y., Kim, Y. M., & Chung, J. (2014). Activation of NLRP3 and AIM2 inflammasomes by Porphyromonas gingivalis infection. *Infect Immun*, *82*(1), 112-123. doi:10.1128/IAI.00862-13
- Polak, D., Wilensky, A., Shapira, L., Halabi, A., Goldstein, D., Weiss, E. I., & Hourii-Haddad, Y. (2009). Mouse model of experimental periodontitis induced by Porphyromonas gingivalis/Fusobacterium nucleatum infection: bone loss and host response. *J Clin Periodontol*, *36*(5), 406-410. doi:10.1111/j.1600-051X.2009.01393.x
- Rathinam, V. A., Vanaja, S. K., & Fitzgerald, K. A. (2012). Regulation of inflammasome signaling. *Nat Immunol*, *13*(4), 333-342. doi:10.1038/ni.2237
- Sanchez, M. C., Romero-Lastra, P., Ribeiro-Vidal, H., Llama-Palacios, A., Figuero, E., Herrera, D., & Sanz, M. (2019). Comparative gene expression analysis of planktonic Porphyromonas gingivalis ATCC 33277 in the presence of a growing biofilm versus planktonic cells. *BMC Microbiol*, *19*(1), 58. doi:10.1186/s12866-019-1423-9
- Sanz, J. M., & Di Virgilio, F. (2000). Kinetics and mechanism of ATP-dependent IL-1 beta release from microglial cells. *J Immunol*, *164*(9), 4893-4898. doi:10.4049/jimmunol.164.9.4893
- Schroder, K., & Tschopp, J. (2010). The inflammasomes. *Cell*, *140*(6), 821-832. doi:10.1016/j.cell.2010.01.040
- Selinger, M., Tykalova, H., Sterba, J., Vechtova, P., Vavruskova, Z., Lieskovska, J., . . . Grubhoffer, L. (2019). Tick-borne encephalitis virus inhibits rRNA synthesis and host protein production in human cells of neural origin. *PLoS Negl Trop Dis*, *13*(9), e0007745. doi:10.1371/journal.pntd.0007745

- Shibata, K. (2018). Historical aspects of studies on roles of the inflammasome in the pathogenesis of periodontal diseases. *Mol Oral Microbiol*, 33(3), 203-211. doi:10.1111/omi.12217
- Song, B., Zhou, T., Yang, W. L., Liu, J., & Shao, L. Q. (2017). Programmed cell death in periodontitis: recent advances and future perspectives. *Oral Dis*, 23(5), 609-619. doi:10.1111/odi.12574
- Stehlik, C., Krajewska, M., Welsh, K., Krajewski, S., Godzik, A., & Reed, J. C. (2003). The PAAD/PYRIN-only protein POP1/ASC2 is a modulator of ASC-mediated nuclear-factor-kappa B and pro-caspase-1 regulation. *Biochem J*, 373(Pt 1), 101-113. doi:10.1042/BJ20030304
- Wang, B., & Yin, Q. (2017). AIM2 inflammasome activation and regulation: A structural perspective. *J Struct Biol*, 200(3), 279-282. doi:10.1016/j.jsb.2017.08.001
- Xu, S., Zhou, Q., Fan, C., Zhao, H., Wang, Y., Qiu, X., . . . Ji, Q. (2019). Doxycycline inhibits NAct Leucine-rich repeat Protein 3 inflammasome activation and interleukin-1beta production induced by Porphyromonas gingivalis-lipopolysaccharide and adenosine triphosphate in human gingival fibroblasts. *Arch Oral Biol*, 107, 104514. doi:10.1016/j.archoralbio.2019.104514
- Xue, F., Shu, R., & Xie, Y. (2015). The expression of NLRP3, NLRP1 and AIM2 in the gingival tissue of periodontitis patients: RT-PCR study and immunohistochemistry. *Arch Oral Biol*, 60(6), 948-958. doi:10.1016/j.archoralbio.2015.03.005
- Yoshinari, N., Kawase, H., Mitani, A., Ito, M., Sugiishi, S., Matsuoka, M., . . . Noguchi, T. (2004). Effects of scaling and root planing on the amounts of interleukin-1 and interleukin-1 receptor antagonist and the mRNA expression of interleukin-1beta in gingival crevicular fluid and gingival tissues. *J Periodontal Res*, 39(3), 158-167. doi:10.1111/j.1600-0765.2004.00722.x

Table 1: Primer assays used in RT-PCR analysis

Gene	Primer sequence (5'-3')	Genbank Accession Number	Amplicon size (bp)
NLRP3	(F) CACCTGTTGTGCAATCTGAAG (R) GCAAGATCCTGACAACATGC	NM_004895	74
AIM2	(F) GCTGCACCAAAAGTCTCTCCTC (R) CTGCTTGCCTTCTTGGGTCTCA	NM_004833	156
ASC	(F) AGCTCACCGCTAACGTGCTGC (R) GCTTGGCTGCCGACTGAGGAG	NM_013258	129
CASPASE 1	(F) GCTGAGGTTGACATCACAGGCA (R) TGCTGTCAGAGGTCTTGTGCTC	NM_033292	145
IL-1 β	(F) CCACAGACCTTCCAGGAGAATG (R) GTGCAGTTCAGTGATCGTACAGG	NM_000576	131
POP1	(F) GCCTGTGGAATGTACGCATAGC (R) GCACCAGCAATTCCTCCTCATC	NM_001145861	113
TRIM16	(F) TATGGCTGCTCTTTGCCGGA (R) GCACCTCTCCCTCCTGCATT	NM_006470	121
CARD16	(F) ATTCCGAAAGGGGCACAGGCAT (R) TCTGCCTTCTGGGCTTGAGCAT	NM_001017534	150
GAPDH	(F) AGCTGGCCCGATTTCTCCTC (R) ACGGTGCCATGGAATTTGCC	NM_001256799	200

FIGURE LEGENDS

Figure 1. Gene expression analysis in HGFs in the exposure groups studied.

Gene expression differences for the transcripts indicated (A-H) were analysed by RT-PCR for each exposure group. Gene expression analysis of A) NLRP3, B) AIM2, C) IL-1 β , D) CASPASE 1, E) ASC, F) CARD16, G) TRIM16, H) POP1, in the exposure groups. *Fn* and *Pg* in the presence and absence of ATP differentially effected inflammasomes, their regulators and components at 24h at a MOI:100 . * = Statistically significant differences compared with the control group, p<0.05.

Figure 2. Immunocytochemistry analysis for intracellular pro- and mature IL-1 β .

Pro- and mature IL-1 β were determined from immunocytochemistry images captured under fluorescent microscopy with a 20X objective. Nuclei were stained with DAPI (blue) and IL-1 β was stained using primary and secondary antibodies (red) as described. IL-1 β can be observed intracellularly.

Figure 3. Quantification of IL-1 β levels

Relative quantification of pro- and mature IL-1 β was performed by measuring the intensity ratio of the IL-1 β stain normalized with cell nuclei (DAPI) intensity. Compared with the control group, the only significant elevation was detected in the *Pg*+ATP group for IL-1 β protein levels. * = Statistically significance compared to Control group, p<0.05.