Original Research

Possible Protective Role of NLRC4 Inflammasome in Periodontal Diseases: A Preliminary Study

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Abstract

Introduction: Inflammasomes are multiprotein complexes, which regulate proinflammatory cytokines, Interleukin-18 (IL-18), and Interleukin-1β (IL-1β) that are associated with periodontal breakdown. This study investigated the expression of NOD-like receptor pyrin domain-containing-3 (NLRP3) and NOD-like receptor family CARD domain-containing protein 4 (NLRC4) inflammasomes in different periodontal diseases in humans and their potential association with IL-18 release in gingival crevicular fluid (GCF). **Materials and Methods:** A total of 45 participants (21 males and 24 females) divided into four groups; periodontally healthy (H), gingivitis (G), chronic periodontitis (CP), and aggressive periodontitis (AgP) based on periodontal examination. NLRC4 and NLRP3 expression were detected by immunohistochemistry in gingival tissue samples for all groups. Expression percentage (%) and staining intensity distribution score (SID) were calculated for both NLRC4 and NLRP3. IL-18 was measured in GCF via enzyme linked immunosorbent assay (ELISA). **Results:** Positive immunoreactivity was seen for NLRC4 and NLRP3 across groups. No differences were found for NLRC4 expression %, but SID scores were slightly higher in G and AgP compared to other groups ($P > 0.05$). Results showed a significant increase of NRLP3 expression % in group CP compared to group H ($P < 0.05$) without affecting SID scores ($P > 0.05$). IL-18 levels were significantly higher in AgP and CP groups compared to H and G groups ($P < 0.05$). IL-18 significantly and positively correlated with clinical attachment levels across groups. **Conclusion:** Within the limitations of this preliminary study, we suggest that the NLRC4 platform may have a protective role contrary to the NLRP3 platform influencing IL-18 release and associated periodontal tissue breakdown.

Keywords: Inflammasome, interleukin 18, NLR proteins, periodontal disease

Introduction

Periodontal disease is a chronic inflammatory condition triggered by an abnormal host response to periodontal pathogens, and subsequent release of proinflammatory cytokines such as Interleukin-1 (IL-1) and Interleukin-18 (IL-18) from host cells.[1] IL-1β and IL-18 are produced in response to pathogens-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) as inactive precursors, pro-IL-1β and pro-IL-18.[2] These function through their pattern recognition receptors (PRRs) such as nucleotide binding oligomerization domain (NOD)-like receptors (NLRs).[2] The conversion of IL-1β and IL-18 from inactive precursors to active forms of IL-1β and IL-18 is facilitated by a complex innate immune inflammasome platform consisting of an NLR, an adaptor protein ASC [apoptosis-related speck-like protein containing a caspase activation and recruitment domain (CARD)] and caspase-1.[3,4]

The inflammasome platform modulates innate immune processes in response to PAMPs and DAMPs.[5] Several inflammasomes including NLR family pyrin domain containing 2 and 3 (NLRP2), (NLRP3, and AIM2 (Absent in Melanoma 2) have been suggested to be involved in periodontal disease.[6] Among them, the most

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extensively studied inflammasome is NLRP3[6]. NLRP3 inflammasome is activated by a variety of stimuli including bacterial lipopolysaccharides (LPS) and host molecules released during tissue injury.[7] The role of NLRP3 inflammasome in periodontal diseases has been evaluated in *in vitro,* animal and *clinical studies*.[8,9] NLRP3 expression has been detected at higher levels in the gingiva and saliva of periodontal disease patients.[10-12] *In vitro* research also reported the ability of periodontal pathogens to promote NLRP3 activation and thus influence pro-IL-1β and IL-18 expression.

NLRC4 (NLR family CARD domain containing 4; IPAF) inflammasome, like NLRP3 and AIM2, shown to be activated by microbial stimuli including gram negative bacteria and DAMPs, resulting in release of biologically active IL-1β and IL-18. [13-15] *In vitro* research reported that *P. gingivalis* alone was unable to activate NLRC4 levels in human gingival fibroblasts[15] and a monocytic cell line (THP-1).[8] Further several inflammatory mediators have been assessed recently regarding their potential biomarker status in periodontal diseases. [16-18]

However, till date, no in-vivo study has evaluated the levels of NLRC4 inflammasome in different types of periodontal diseases.

Hence, this preliminary study is the first study to evaluate the in vivo expression of NLRC4 inflammasome in different types of human periodontal diseases. [5] Further we investigated the production of NLRP3 inflammasomes in different types of periodontal disease and explored its possible effects on IL-18 release in gingival crevicular fluid (GCF) in different types of periodontal disease. The hypothesis of this preliminary study was periodontal pathogens and or tissue injury products influenced NLRP3 and NLRC4 inflammasome production that resulted in IL-18 release.

Materials and Methods

Forty-five participants (21 males and 24 females, between 18 and 65 years) presenting to Department of Periodontics, SRM Dental College, Ramapuram, Chennai, India were enrolled in this study between December 2013 and September 2015. Prior to enrolment, subjects received written information and discussed with investigators the nature of the study. Eligible subjects were asked to provide written informed consent. This study was approved by Institutional scientific and ethical review board of the university (/2013/ Student/506).

Periodontal examination

All participants underwent a standard periodontal examination including gingival index, probing pocket depth, clinical attachment level (CAL) recorded at six sites per tooth by using a University of North Carolina −15 probe (Hu-Friedy, Chicago, IL, USA) and appropriate radiographic investigations.[19]

Study groups

This preliminary study included four groups; periodontally healthy participants (Group H: $n = 5$, 3 males and 2 females) and untreated patients for other groups; gingivitis (Group G: $n = 15$, 10 females and 5 males); chronic periodontitis (Group CP: $n = 15$, 8 females and 7 males); aggressive periodontitis (Group AgP: $n = 10$, 3 females and 7 males). Since this is a preliminary study, the sample size is based on feasible considerations such as budgetary constraints of immunohistochemistry procedure and reagents, patient in flow and the number of participants needed to reasonably evaluate practicability goals. [20]

Inclusion criteria

Selection of participants was according to the clinical and or radiographic criteria of 1999 World Workshop for classification of periodontal diseases or conditions.[21] The inclusion criteria for group H were individuals with clinically healthy periodontium without any signs of gingival inflammation and clinical attachment loss (CAL). For Group G, patients with active gingival inflammation and without CAL. Chronic periodontitis (CP) was classified based on the presence of radiographic evidence of moderate-to-severe bone loss, probing pocket depth (PPD) ≥ 5 mm, CAL ≥ 5 mm at multiple sites in all quadrants. The amount of destruction to be proportional to the quantity of local factors. Aggressive periodontitis (AgP) was classified based on the radiographic evidence of severe bone loss, PPD ≥ 5 mm, CAL ≥ 5 mm, on at least three permanent teeth other than incisors or first molars, periodontal destruction being disproportionate with the quantity of local factors and evidence of familial tendency.[22]

A single calibrated examiner (VKN) recorded all the periodontal parameters. The calibration was performed and calculated with testing of intra-examiner variability using kappa test (correlation coefficient > 0.85).

Exclusion criteria

The exclusion criteria for the participants were the presence of a systemic disease, smoking, pregnancy, lactation, a history of periodontal treatment, the use of antibiotics within 6 months, or the use of systemic medications affecting periodontium.[10]

Gingival tissue sampling

Gingival tissue samples were taken for group H, from areas of crown lengthening procedures or orthodontic extractions. For group G, patients undergoing orthodontic tooth extraction or third molar extraction. For group CP and AgP, from areas of periodontal surgery or during extraction of periodontally compromised grade III mobile teeth by a calibrated clinician (SP).[19] Tissue samples were immediately washed with sterile normal saline solution and transferred to the sterile tubes containing 10% buffered formalin prior to analysis.

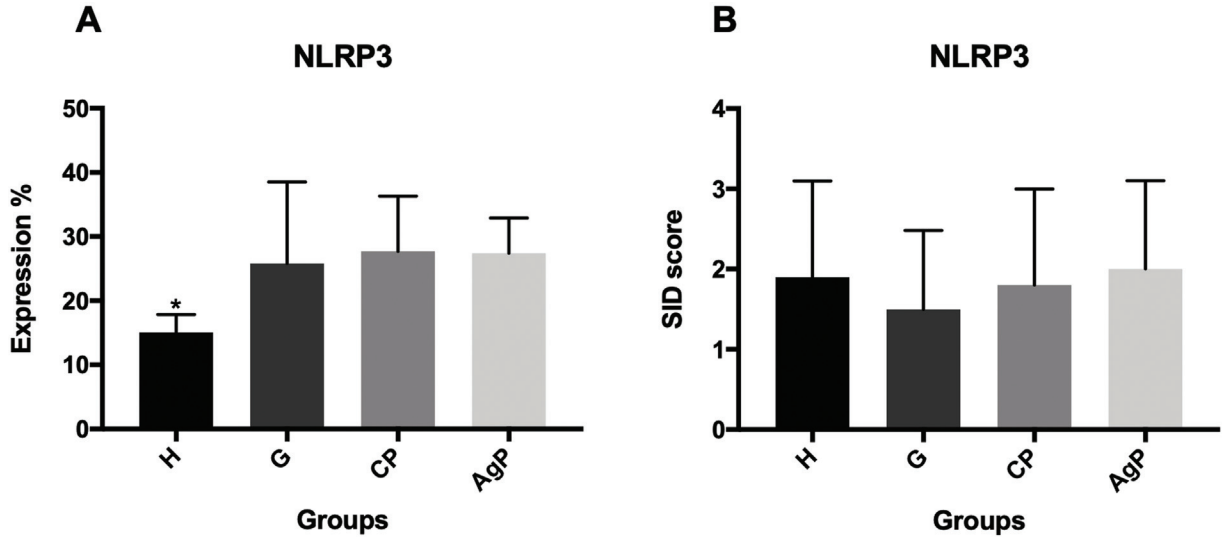

Figure 1: The expression of NLRP3 between groups. (a) The expression percentage of NLRP3 was significantly lower in group H compared to group CP (*$P < 0.05$). (b) Qualitative evaluation of NLRP3 by using Staining-Intensity-Distribution score. No differences were detected between any of groups ($P > 0.05$). CP = chronic periodontitis, SID = staining intensity distribution.

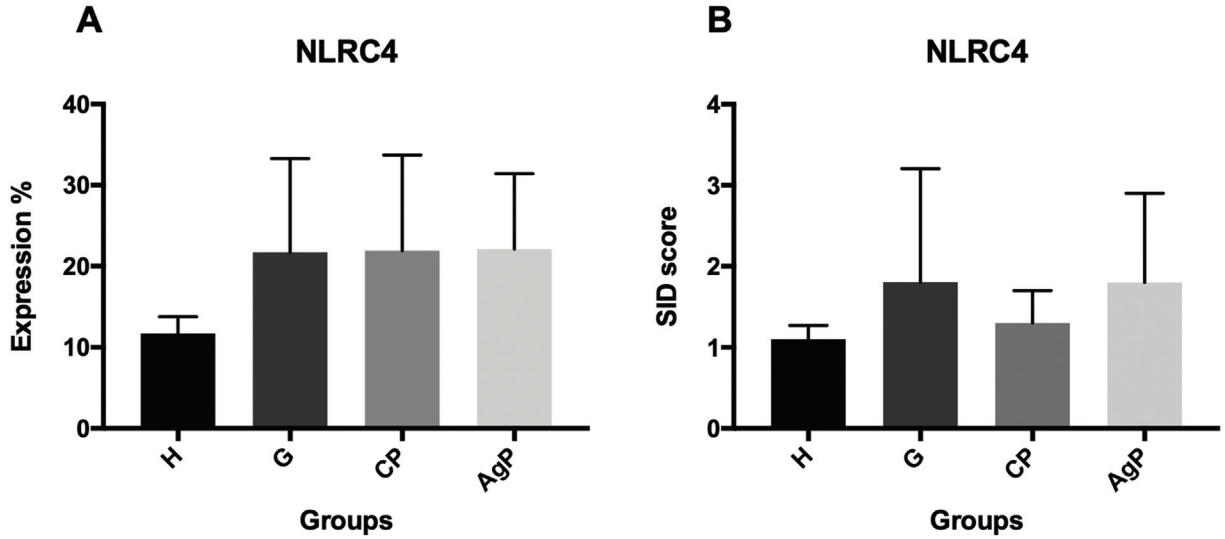

Figure 2: The expression of NLRC4 between groups. No differences were found between groups for NLRP3 expression both (a) quantitatively (expression percentage) (b) or qualitatively (SID) ($P > 0.05$). SID = staining intensity distribution.

Gingival crevicular fluid sampling

GCF samples were collected from all subjects prior to tissue sampling. The areas designated for sampling were cleaned using a curette, dried, and isolated using cotton rolls to prevent contamination. A standardized volume of 1–5 μL of GCF was collected by using a calibrated volumetric microcapillary pipettes (Sigma-Aldrich, St. Louis, MO, USA). The GCF collection was performed for up to 10 minutes, sites were excluded if insufficient GCF was collected over this time or was contaminated by blood or saliva. Samples were transferred into sterile Eppendorf tubes and stored at −80°C until analysis.[23]

Immunohistochemistry

Immunohistochemistry staining was accomplished using a polyclonal antibody against NLRP3 (NBP2-12446SS) and NLRC4 (NBP1-78979SS) proteins in the formalin fixed and paraffin infiltrated, microtomed gingival tissue sections of 3–4 μm thickness. The sections were then deparaffinized and rehydrated to remove crosslinking produced by formalin fixation, before carrying out antigen retrieval. Primary antibody kit contained a vial with 0.025 rabbit polyclonal antibody in 1 mL of PBS with 0.05% sodium azide and 30% glycerol, which was diluted to 1:50 for NLRP3 and 1:400 for NLRC4. Antigen retrieval was performed by consulting the product datasheet recommendation to follow a validated protocol.

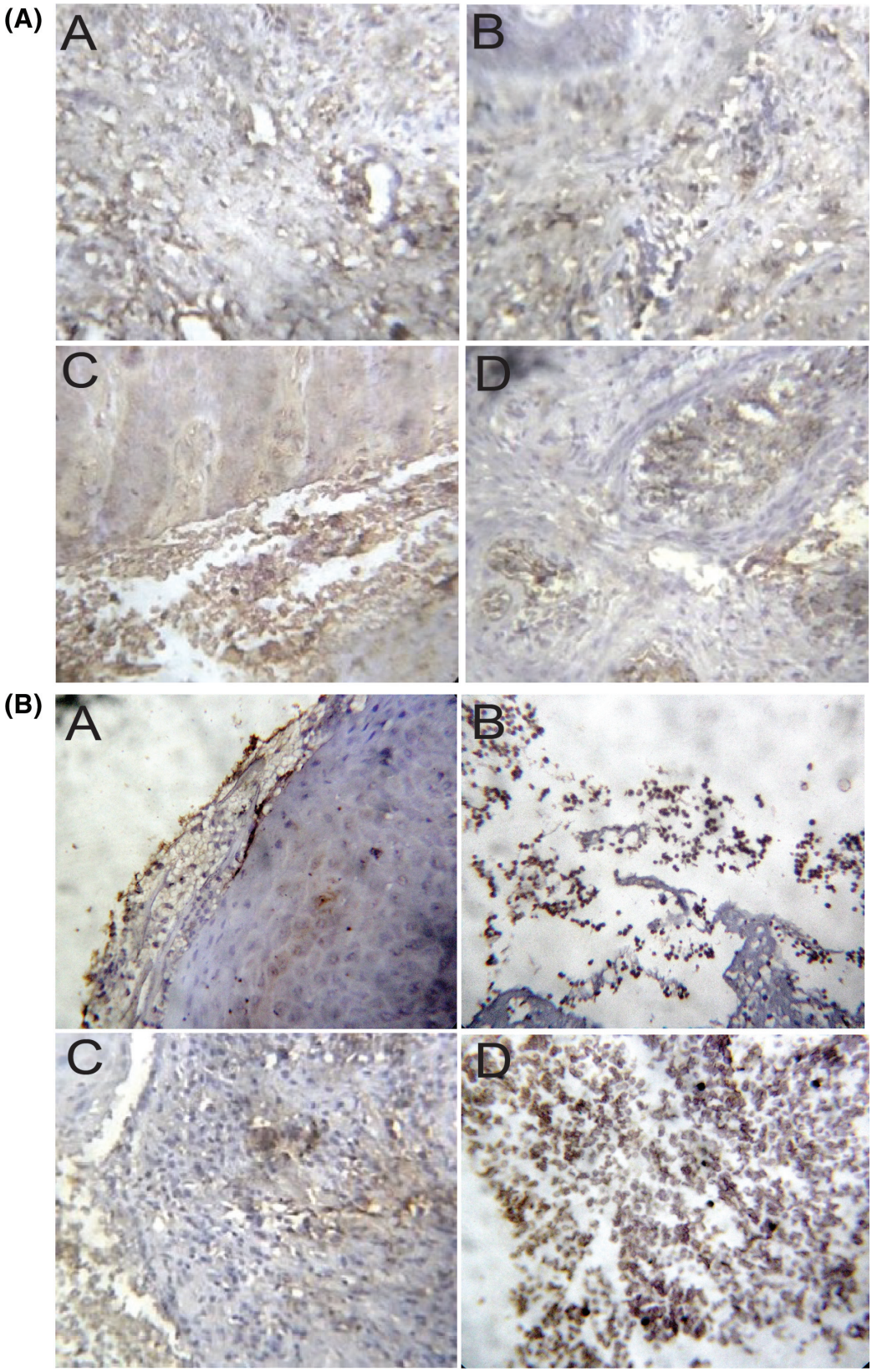

Figure 3: (a) NLRP3 in the gingiva of healthy (A), G (B), CP (C), and AgP (D) groups was assessed using immunohistochemistry (x40). CP = chronic periodontitis, AgP = aggressive periodontitis, G = gingivitis. (b) NLRC4 in the gingiva of healthy (a), G (b), CP (c), and AgP (d) groups was assessed using immunohistochemistry (x40). AgP = aggressive periodontitis, CP = chronic periodontitis, G = gingivitis.

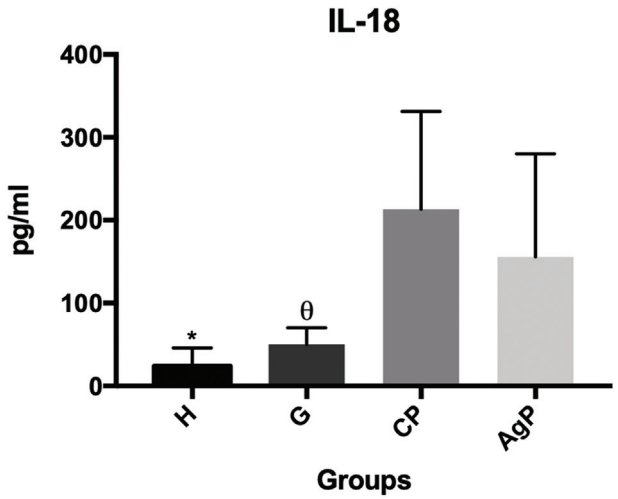

Figure 4: GCF levels of IL-18 between groups. IL-18 levels in GCF were significantly higher in groups CP and AgP compared to group H and group G but found similar between groups G and H. *$P < 0.001$, $P < 0.05$. AgP = aggressive periodontitis, CP = chronic periodontitis, GCF = gingival crevicular fluid.

The components of secondary antibody kit were peroxide block, power block, substrate DAB buffer, chromogen-DAB-3,3' diaminobenzidine, super enhancer, and poly-Horseradish Peroxidase (HRP). The product datasheet was consulted during the procedure to obtain consistent results. Following antigen retrieval, peroxide block was used for 10 to 15 minutes to quench the peroxidase activity. Following this, tissue sections were washed, incubated with primary and secondary antibodies, washed yet again, and exposed to staining protocol for 1 minute. The slides were then examined by two trained, experienced, oral pathologists and were blinded to the slides. Five random fields at a magnification of ×40 were quantified.[20] Each field was for the proportion of stained cells and the intensity of overall staining.

Quantitative scoring was performed according to the proportion of stained cells: 0 = no stained cells, 1 = less than 25% stained cells, 2 = 25 to 50% stained cells, and 3 = greater than 50% stained cells. The semiquantitative analysis was used to determine if staining was localized and occurred in cell nucleus, cytoplasm, or cytoplasmic membrane. The staining intensity in each field was evaluated and compared with that of the control to classify each sample as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = intense staining as previously described.[24] A staining-intensity-distribution (SID) score was calculated by multiplying the stained cell proportion score by the staining intensity score in each field. The mean of five fields was used as the SID score for that case.[24] This approach transformed qualitative to semiquantitative evaluation.

ELISA analysis

IL-18 levels in the GCF were analyzed by using an ELISA kit (Ray Biotech, Georgia, USA) in accordance with the instructions of the manufacturer. A microplate reader was used to monitor color development, and the optical density was read at 450 nm. The total levels of IL-18 were as pg/mL according to the calibration curve determined.

Statistical analysis

The normal distribution of the data was determined via the Shapiro–Wilk test. The non-normality distributed variables were assessed by nonparametric tests. Significant differences between the groups were examined using parametric or nonparametric methods. Inter-group differences were compared using analysis of variance (ANOVA) and post hoc comparison tests for the demographic and clinical variables. A Mann Whitney U test was used for nonparametric variables. Spearman correlation analysis was used to analyze the correlations between NLRP3, NLRC4, and IL-18 levels and clinical periodontal parameters. Statistical analyses were conducted using statistical analysis software (SPSS, Version 21.0, Chicago, IL, USA). $P < 0.05$ were considered statistically significant.

Results

NLRP3 and NLRC4 expression in gingiva

Demographic data and clinical characteristics of participants are shown in Table 1. Positive immunoreactivity for both NLRP3 and NLRC4 was detected in both epithelium and connective tissue cells across all groups. The expression percentage of tissue samples for NLRP3 was significantly higher in all disease groups compared to controls ($P < 0.05$) [Figure 1a]. SID score, the semiquantitative evaluation for NLRP3 expression were almost similar across groups [Figures 1b and 3a] ($P>0.05$). The expression percentage of NLRC4 was similar across all diseased groups and SID score, the semiquantitative evaluation for NLRC4 was slightly higher in G and AgP compared to other groups but reached no statistical significance [Figures 2a and b] [Figure 3b] ($P > 0.05$). Correlation analysis results showed that GI and NLRC4 expression percentage was significantly and negatively correlated in group CP ($r = -0.556$, $P = 0.031$). CAL and NLRP3 expression percentage ($r = -0.581$, $P = 0.023$), and NLRP3 SID score ($r = -0.632$, $P = 0.011$) were significantly and negatively correlated in CP patients; however, the same parameters were significantly and positively correlated in gingiva of AgP patients (expression percentage $r = 0.872$, $P = 0.001$; SID score $r = 0.825$, $P = 0.003$) (Table 2)

IL-18 levels in GCF

IL-18 levels in GCF were significantly higher in groups CP ($P<0.001$) and AgP ($P<0.05$) compared to groups H and G however no significant differences were found between group G and H for IL-18 levels [Figure 4] ($P>0.05$). Correlation analysis results showed that IL-18 was significantly and

positively correlated with CAL levels in all participants (Table 3) ($P<0.05$).

Discussion

Inflammasomes are suggested to be the central signaling platform of the innate immune system and has been reported to play a significant role in the pathogenesis of periodontal disease by promoting IL-18 and IL-1β expression, which has been associated with periodontal attachment loss.[12] The current study investigated NLRC4 and NLRP3 expression using IHC procedure in different types of periodontal disease and its possible effects on GCF IL-18 levels.

The findings of our study indicated that the NLRC4 and NLERP3 expressed more in epithelium of diseased sites when compared to healthy controls and IL-18 levels were elevated in disease groups compared to controls. The NLRC4 expression decreased with increasing gingival inflammation in gingivitis, CP and AgP group and showed a positive relationship with the IL-18 levels. The expression of NLRP3 and NLRC4 varied in different forms of periodontal diseases. Further, we observed an inverse relationship between NLRP3 expression and CAL in CP group, on the contrary there was a positive relationship between the NLRP3 expression and CAL in AgP group. The IL-18 levels increased with attachment loss.

Current results support the earlier reports of elevated NLRP3 production in chronic periodontitis patients and increased IL-18 release in GCF. Further, a significant positive correlation was also detected between IL-18 and CAL, which supports earlier reports in the literature. Another aim of our study was to evaluate the possible role of NLRC4 inflammasome in periodontal diseases that has never been evaluated in periodontal diseases in humans till date. Although current study revealed similar expression of NLRC4 across groups, without reaching a statistical significance, further studies with larger sample size are warranted in different population to confirm or refute this preliminary data.

NLRP3 inflammasome has been evaluated by several authors in the pathogenesis of the periodontal disease.[12] Moore *et al.*[20] evaluated the levels of NLRP3 gene and protein expression in gingiva of periodontitis patients and reported that both were elevated in CP and AgP groups compared to healthy controls. The current study showed similar results in terms of the overexpression of NLRP3 present in the gingiva of the CP group compared to healthy controls [Figures 1a and b]. Cheng *et al.*[7] reported an overexpression for NLRP3 levels determined by immunohistochemistry in gingiva of CP patients compared to healthy controls. Park *et al.*[8] also found NLRP3 protein expression using immunoblotting technique in a CP group compared to healthy subjects. Further, Isaza-guzman *et al.*[11] suggested an overexpression of NLRP3 levels in saliva of CP and AgP patients compared to a group of healthy patients. In addition, Bostanci *et al.*[10] reported an increase for NLRP3 and IL-18 gene expression in gingiva of G, CP, and AgP patients compared to gingiva of healthy subjects. Similar to other studies, the current study also detected a significant overexpression for NLRP3 protein in gingiva with elevated IL-18 levels in the GCF of the chronic periodontitis group compared to periodontally healthy controls. ($P < 0.05$). Garcia-Hernandez *et al.*[25] reported that Type II diabetes mellitus patients with CP expressed more NLRP3 and IL-18 compared to diabetic patients periodontally healthy controls. Moreover, IL-18 levels were elevated in GCF of periodontal disease patients compared to healthy participants.[26] In addition, the gingival levels of IL-18 also reported to be positively correlated with PD.[26] Our current results showed a significant increase of NLRP3 inflammasome production and IL-18 release and positively correlated with CAL in chronic periodontitis. Therefore, it can be suggested that NLRP3 inflammasome increases IL-18 release in GCF and contribute towards periodontal attachment loss in chronic periodontitis patients.

NLRC4 inflammasome activation in response to *Salmonella typhimurium* and *Legionella pneumophila* infections has been suggested to trigger caspase-1 independently from NLRP3.[33] NLRC4-dependent activation of caspase-1 was found to occur in the absence of surface Toll-like receptors such as TLR5, which recognizes extracellular flagellin.[27] Although TLR5 ligation only activates synthesis of pro-IL-1β but not its secretion, NRLC4 ligation initiates both cytokine synthesis and its processing by caspase-1.[27] Previously, *in vitro* research evaluated the possible role of NLRC4 in different cells stimulated by *P. gingivalis*. However, in human gingival epithelial[15] and a THP-1 cell line, *P. gingivalis* was also found unable to trigger NLRC4 inflammasome activation. A recent animal study reported increased inflammatory bone resorption in NLRC4 knockout mice.[27] In the current study, although NLRC4 activation was increased in gingiva of the periodontal disease groups, it did not reach significance [Figures 2a and b] ($P > 0.05$). However, the percentage expression of NLRP3 was significantly higher in the gingiva of the CP group compared to group H ($P < 0.05$) [Figure 1a]. It has been suggested that NLRP3 can be activated by a variety of stimuli including bacterial LPS, extracellular ATP, and hyaluronan, but NLRC4 can only be triggered by bacterial type III flagellin system.[13,28] Therefore, the release of IL-18 in periodontitis may be primarily associated with the overactivity of NLRP3 rather than NLRC4 that may be associated with a protecting role.NLRC4 inflammasome was reported to be activated in response to flagellin proteins of gram-negative bacteria.[28] *Trepenoma denticola,* a key subgingival pathogen in periodontitis is reported to have three bacterial flaggellar protein FLABs and a periplasmic flagellum that is essential for cell motility and strongly involved with the pathogenicity of bacteria.[29] Furthermore, nonflagellated strains of *T. denticola* are reported to their inability to penetrate oral epithelial cells,

a key process in periodontal disease pathogenesis. Therefore, *T. denticola* might activate NLRC4 with its flagellins.

Although current study did not show statistically significant results with relation to NLRC4 expression, it certainly noted immunoreactivity NLRC4 in gingiva of periodontitis patients and this is the first study to report this preliminary finding [Figures 2a and b]. Further studies needs to explore the quantity and quality of bacteria in subgingival biofilm along with NLRC4 expression. This preliminary data supports the previous reports of NLRC4 expression may not be associated with inflammation, rather it has protective role.[28]

Conclusion

Within the limitations of the current study, we suggest that NLRC4 inflammasome may not be involved in periodontal tissue destruction but may display a protective role, which needs further investigation. Further, periodontal pathogens or damage-associated molecular patterns may increase NLRP3 inflammasome, which may trigger IL-18 release in GCF that may be involved in periodontal attachment breakdown.

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Conflicts of interest

There are no conflicts of interest.

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