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Regulation of caries-induced pulp inflammation by NLRP3 inflammasome: A laboratory-based investigation

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Abstract

Aim: To evaluate the expression and function of the nod-like receptor pyrin domain containing 3 (NLRP3) inflammasome in caries induced pulpitis.

Methodology: NLRP3 expression was determined with immunohistochemistry in the dental pulp and qPCR in dental pulp cells (DPCs). THP-1 macrophages expressing the apoptosis-related speck-like protein (ASC) and green fluorescent protein (GFP) fusion protein were used to assess NLRP3 inflammasome activation by live cell imaging, following treatment with lipopolysaccharide (LPS) and lipoteichoic acid (LTA). Caspase I inhibitor was used to confirm inflammasome activation. An *ex-vivo* pulpitis model in which the DPCs were co-cultured with THP-1 macrophages was used to study the effect of the NLRP3 inflammasome inhibitor (MCC950), and cytokines were measured using ELISA and multiplex array. Data were analysed using the *t-test* or ANOVA followed by a Bonferroni *post hoc* test with the level of significance set at $p \leq .05$.

Results: NLRP3 inflammasome was differentially expressed in dental pulp of sound and carious teeth. Treatment of DPCs with LTA significantly upregulates NLRP3 and IL-1 β -expression ($p < .05$) and induces more ASC specks formation compared to LPS. IL- β release in response to LTA treatment is significantly reduced with Caspase I inhibitor suggesting inflammasome dependent mechanism ($p < .01$). NLRP3-specific inhibitor, MCC950, significantly reduced IL-1 β and IL-6 in an *ex-vivo* pulpitis model ($p < .01$) but had no effect on IL-8 or matrix metalloproteinase-9 (MMP-9).

Conclusions: Expression and upregulation of NLRP3 inflammasome with caries and LTA treatment suggest a role in caries-induced pulpitis. NLRP3 inhibitor attenuated the release of selective inflammatory cytokines and could be a potential treatment target that merit further investigation.

KEYWORDS

caries bacteria, cell death, NLRP3 inflammasome, pulp death, pulpitis, vital pulp treatment

INTRODUCTION

The innate immune response plays a fundamental role in the regulation of caries-induced pulp inflammation (Hahn & Liewehr, 2007). In deep carious lesions, bacteria and their by-products entering the dental pulp are recognized initially by extracellular pattern recognition receptor (PRRS), such as Toll-like receptors (TLRs), which initiate a signalling cascade leading to the activation of nuclear factor kappa light chain enhancer of activated B cells (NF κ B) and production of inflammatory cytokines (Kawasaki & Kawai, 2014). Among the TLRs, TLR2, and TLR4 are activated by Gram-positive and Gram-negative bacteria, respectively, and their role in caries-induced pulpitis is well documented (Farges et al., 2015; Hirao et al., 2009; Mutoh et al., 2007).

In addition to TLRs, the dental pulp also expresses intracellular PRRs belonging to the nucleotide-binding domain leucine-rich repeat containing (NLRs) family (Staquet et al., 2011). The first NLRs to be identified were NOD1 and NOD2, which recognize peptidoglycan components common to both Gram-positive and Gram-negative bacteria leading to NF- κ B activation and pro-inflammatory cytokine production (Farges, 2009; Hirao et al., 2009; Jang et al., 2015). Other NLR family members belong to a subfamily of large cytoplasmic multi-protein complexes known as inflammasomes that act as important intracellular sensors. The inflammasome consists of three components, a sensor, an adaptor and an effector. Upon sensing pathogens or other danger signals, the upstream sensor component is activated, oligomerizes and recruits the adaptor protein, apoptosis-related speck-like (ASC) protein, containing a caspase recruitment domain (CARD) and procaspase-1, leading to the activation of caspase-1. Active caspase-1 then cleaves the precursor proinflammatory cytokines pro-interleukin (IL)-1 β and pro-IL-18 into their mature secreted forms (Lamkanfi, 2011; Martinon et al., 2002).

Among inflammasomes, NOD- LRR- and pyrin domain-containing protein 3 (NLRP3) is the best studied and characterized. Its activation is highly regulated and involves an initial priming signal, mediated via TLRs, to induce NF κ B activation and transcription of the genes encoding the precursor forms of cytokines IL-1 β and IL-18. This is followed by a second signal that is required for the assembly of NLRP3 inflammasome and the subsequent release of mature IL-1 β and IL-18 (Broz & Dixit, 2016). The release of these inflammatory cytokines is required for clearance of infection and maintenance of cellular homeostasis (Anders & Schaefer, 2014; Wang et al., 2013). However, unregulated inflammasome activation is also responsible for unbalanced, excessive inflammation (de Torre-Minguela et al., 2017). It is not surprising therefore

that balancing such inflammation with inflammasome inhibitors is a contemporary therapeutic strategy for many chronic inflammatory conditions including oral disease (Coll et al., 2015; Kodji et al., 2021; Lv et al., 2019; Marchesan et al., 2020; Ozaki et al., 2015).

In teeth with deep caries and exposed pulp, bacteria directly invade the dental pulp leading to irreversible pulpitis. The microflora in such lesions is mixed but dominated with Gram-positive bacteria (Hahn et al., 1991; Love & Jenkinson, 2002; Zheng et al., 2019) that lead to the development of inflammatory and immune events in the dental pulp, the molecular and cellular determinants of which remain largely unknown (El Karim et al., 2021; Jontell et al., 1998). The NLRP3 inflammasome is expressed in the dental pulp (Song et al., 2012) and plays an important role in sterile pulp inflammation (Al Natour, Lundy, et al., 2021), but, its role in caries-induced pulp inflammation is not fully understood. The aim of the study was therefore to examine the expression of NLRP3 in healthy and inflamed dental pulp and determine its role in pulpitis using an *ex vivo* pulpitis model and specific NLRP3 inhibition.

MATERIALS AND METHODS

The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Figure S1).

Immunohistochemistry

The histological study was performed on serial sections of 3 healthy and 3 carious teeth which were obtained in compliance with French legislation and covered by the Office for Research Ethics Committees (Northern Ireland) ethical approval number 08/NIR03/15. Prior to staining teeth were fixed in 4% formaldehyde solution and demineralized in a solution of 3.4% sodium formate in 17% formic acid until fully decalcified. Samples were routinely processed through a series of graded alcohols and xylene and finally embedded in paraffin wax, and 7- μ m thick paraffin embedded sections were processed for immunohistochemistry as previously described (McMillan et al., 2021; Téclès et al., 2005). Briefly wax sections were rehydrated with decreasing ethanol concentrations. Antigen retrieval was performed at 98°C for 30 min in 1 mmol/L Tris/0.1 mmol/L EDTA/0.5% Tween. Sections were then incubated with phosphate-buffered saline containing 1% bovine serum albumin for 30 min at room temperature to block non-specific binding prior to overnight incubation at 4°C with

50 µg/ml primary antibody to NLRP3 (LS-B432), (LifeSpan Bioscience) or an isotype control. Following washing, slides were incubated for 45 min at room temperature with 2 mg/ml secondary antibody AlexaFluor 594 (red fluorescence) goat anti-rabbit (A32740), (Life Technology) and 4', 6-diamidino-2-phenylindole (DAPI) (1 mg/ml) counterstain (blue fluorescence). Histology and immunofluorescence were performed on 5 randomly selected sections from the first, second and last thirds of each tooth and representative sections were shown in [Figure 1](#).

Cell culture

Dental pulp cells (DPCs) were derived by explant culture from immature permanent third molar teeth from three donors as previously described (McMillan et al., 2021; Rankin et al., 2020). Cells were grown in minimal essential medium with L-glutamine supplemented with 10% fetal bovine serum (FBS), 100 UI/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies) and maintained in an incubator at 37°C and 5% CO₂ throughout. THP-1 cells (Life Technologies) were grown in Roswell Park Memorial Institute medium (RPMI-1640) media supplemented with 10% FBS and differentiated into macrophages by incubation in 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich) for 72 h. THP1-ASC-green fluorescent protein (GFP) reporter cells (InvivoGen) were cultured in RPMI-1640 media supplemented with 10% FBS, 2 mM glutamine, 25 mM HEPES, 100 µg/ml normacin and 50 UI/ml penicillin (Gibco, Life Technologies).

Quantitative real-time PCR

Dental pulp cells were seeded at a density of 4×10^4 cells/ml and in 6-well plates and allowed to grow to 70% confluence prior to treatment with ultrapure *E. coli* lipopolysaccharide (LPS) (Invivogen) (1 or 10 µg/ml) or purified *S. aureus* lipoteichoic acid (LTA) (Invivogen) (1 or 10 µg/ml) for 24 h. RNA was then extracted using the RNeasy kit (Invitrogen). cDNA was synthesized using the Superscript VILO kit (Invitrogen). Gene expression for the NLRP3, IL-1β, IL-18 and the housekeeping genes Glucuronidase Beta (GusB) and Beta-2-Microglobulin (B2M) was carried out using specific primers obtained from TaqMan Gene Expression Assays (Applied Biosystems). The primer details used are shown in [Table S1](#). The qPCR reaction mixture was prepared as per the TaqMan Universal Mastermix II with UNG (Applied Biosystems) protocol. Data was normalized using the ratiometric mean of two house-keeping genes B2M and GUSB. The relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method. Results

are obtained from at least three independent experiments carried out in duplicates and reported as fold change of gene expression compared with the control.

Live cell imaging

The THP-1-ASC-GFP cell line stably expressing the ASC:GFP fusion protein was obtained from Invivogen. This cell line was employed to study the activation of ASC-dependent inflammasomes in real-time. The cells were grown in RPMI supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 µg/ml Normocin™ and Pen-Strep (50 µg/ml). THP-1-ASC-GFP were differentiated into macrophages as previously described (Al Natour, Lundy, et al., 2021). After differentiation, the cells were stimulated with LPS or LTA for 4 h followed by 5 mM adenosine triphosphate (ATP) disodium hydrate (Sigma-Aldrich) for 2 h. Following the 6-h time point, the cells were visualized using a EVOS FL Auto microscope (ThermoFisher Scientific) at 4× magnification. One distinct field of view (image) per well was defined. Image plugins were used to assist quantifying activated positive ASC cells in all field of views of 6 wells representing 3 independent experiments.

Pulpitis co-culture model

A co-culture *in vitro* model for pulpitis was used as previously described (Al-Natour, Rankin, et al., 2021; Rankin et al., 2020). DPCs and THP-1 cells were counted and seeded in 12 well Greiner CELLSTAR® multi-well culture plates at 6×10^4 cell/ml (Greiner Bio-One Ltd.). THP-1 macrophages were seeded into ThinCert™ Cell Culture Inserts with 0.4 µm pore diameter at 1×10^5 cell/ml and differentiated into macrophages. DPCs were seeded at 6×10^4 cell/ml on the bottom chamber of the plate. On the day of cell treatment, the co-culture system was assembled by adding the ThinCerts containing the THP-1 macrophages to their corresponding wells containing the 70% confluent DPC ([Figure 4](#)). The upper and lower chambers were separated only by a porous (0.4 µm pore diameter) membrane. The co culture cells were then treated with 10 µg/ml purified *S. aureus* LTA (Invivogen) with or without the NLRP3 inhibitor, MCC950, for 24 h. The supernatant was collected from both chambers of the co-culture system for cytokine measurement.

Cytokine measurement

THP-1 macrophages were seeded in 12-well plate at density of 1×10^5 cell/ml and differentiated to macrophages

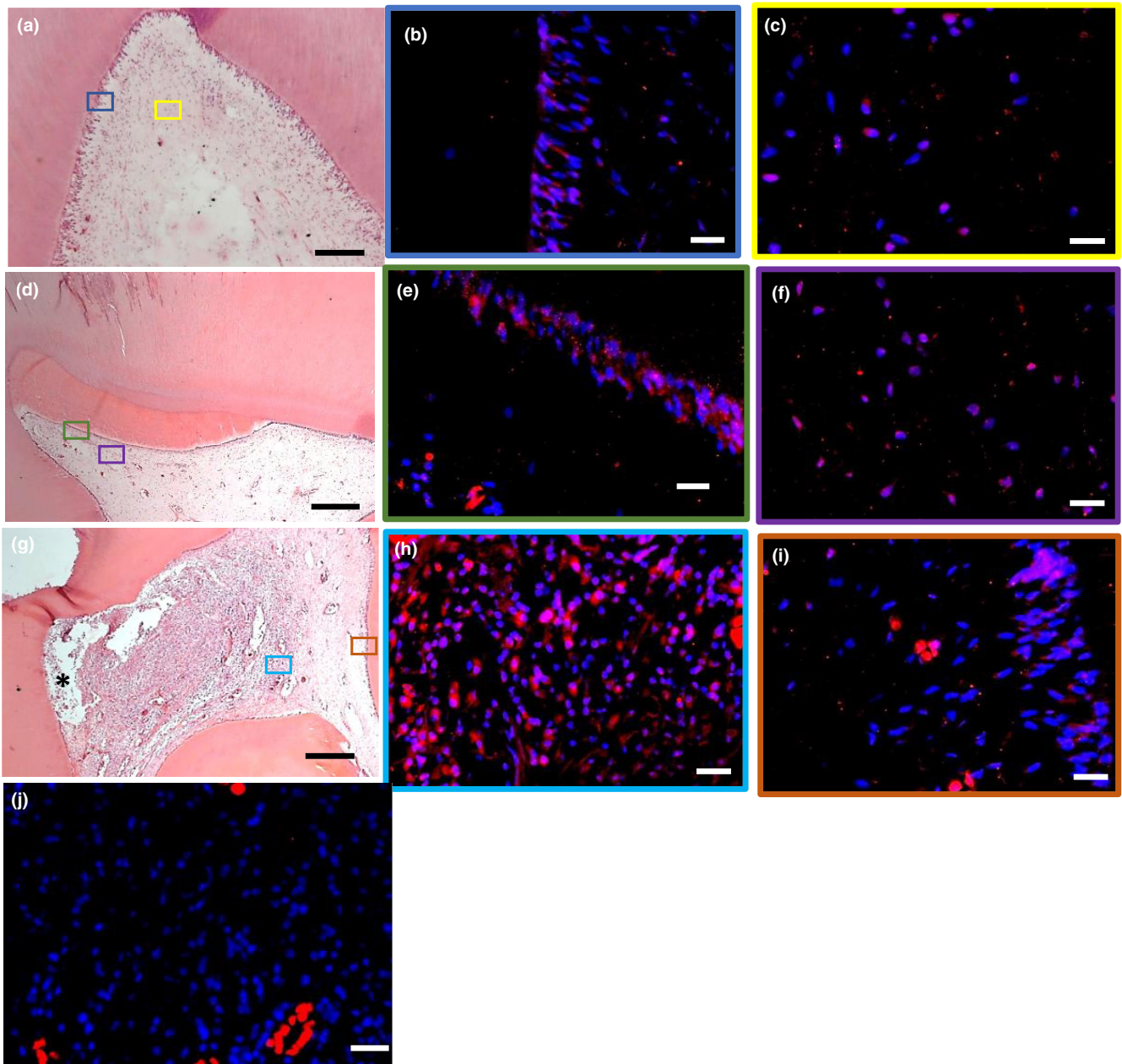


FIGURE 1 Representative images of sections of dental pulp from sound, moderately carious and deeply carious teeth showing expression and localization of NLRP3. (a) Shows an intact odontoblast layer, with NLRP3 minimally expressed in odontoblasts (b) and dental pulp cell (c). (d) Section of tooth with moderate caries shows tertiary dentine formation and slight increase in the NLRP3 expression seen in the odontoblast layer (e) and central pulp cells (f). In the deep caries lesion exposing the pulp, odontoblast layer is disrupted with abscess formation * and infiltrating immune cells (g). NLRP3 expression is intense closer to site of infection (h) and lower in the distal area of the carious teeth (i). (j) Negative controls. Haematoxylin-eosin (H&E) staining (a, d, g). Scale bars: 100 μ m

before treatment with LTA or LPS with or without caspase-1 inhibitor AC-YVAD-CHO for 24h. Supernatant was collected from three independent experiments performed in duplicate for measurement of IL-1 β using the IL-1 β duoSet ELISA kits (R&D Systems). Similarly, for the cytokines released from the pulpitis co-culture model, the supernatant was collected from both co-culture chambers for measurement of the cytokines IL-8, IL-1 β , IL-6 and

MMP-9 simultaneously using Human Magnetic Luminex Assay (R&D Systems).

Statistical analysis

Data were summarized as means \pm standard Error of the mean (SEM) and analysed using GraphPad Prism software,

version 5.0 (GraphPad Software, Inc.). Data normality was tested using Shapiro–Wilk test. Homogeneity of the variance was tested with Bartlett's test and p value $> .05$ indicate variance is equal across the groups. Means were compared using Mann Whitney U test or ANOVA. Results were obtained from at least three independent experiments and *post hoc* power calculation based on one-way ANOVA, two-sided equality showed that the study has 80% power to detect 1.5 difference between groups with type 1 error rate of 5%. The primary outcome measured was change in expression of protein of interest in untreated compared to samples treated with LTA or LPS with or without specific inhibitors. Bonferroni post-test with the level of significance set at $p \leq .05$ was used to correct for multiple testing where applicable.

RESULTS

NLRP3 expression in the dental pulp

Immunohistochemistry showed expression and localization of NLRP3 in the dental pulp of sound teeth and teeth with moderate and deep caries. The expression of NLRP3 appears more intense in teeth with deep caries (Figure 1h,i) compared to sound teeth (Figure 1b,c) and those with moderate caries (Figure 1e,f). The receptor is expressed in dental pulp cells and in inflammatory cells at the site of infection (Figure 1h). To investigate if this change in NLRP3 expression is due to bacterial by-products, the DPCs were treated with LTA and LPS at different concentrations and as shown in Figure 2, LTA treatment results in significant increase in the expression of NLRP3 and IL-1 β . LPS also induced changes in gene expression but this was not significant.

LTA induces ASC speck formation and inflammasome activation

To investigate if LTA activates inflammasome, the inflammasome adaptor ASC formation was visualized in ASC-GFP cells treated for 4 h with LTA at 10 $\mu\text{g/ml}$ or LPS 10 $\mu\text{g/ml}$ as positive control followed by 5 mM ATP for 2 h. As seen in Figure 3, ASC-cell priming and ASC specks can be seen after exposure to LPS or LTA with a significantly higher number of ASC-specks after stimulation with LTA. This is also the case following the stimulation with 5 mM ATP, as LTA + ATP stimulated THP-1 demonstrated a higher number of ASC specks (mean 104, SEM 23.06) compared to untreated (mean 12.3, SEM 1.96), while LPS + ATP stimulated THP-1 showed no significant change compared to untreated (Figure 3b,c).

The formation of ASC specks shown in Figure 3a indicates inflammasome activation with LTA. To further confirm inflammasome activation by LTA, the effect of caspase-1 inhibitor, Ac-YVAD-CHO, on LTA-induced IL-1 β release from differentiated THP-1 macrophages was examined. The results showed a significant increase in IL-1 β level (mean 1523, SEM 186.8), which in turn was significantly reduced following the treatment with caspase-1 inhibitor, Ac-YVAD-CHO (mean 549, SEM 206) (Figure 3e). This confirmed that IL-1 β release from THP-1 macrophages was mediated by caspase-1, and therefore was inflammasome dependent. LPS was used as a positive control (Figure 3d). Our data demonstrated that LTA is a strong first signal for inflammasome activation, compared with the traditional LPS treatment that is generally employed to induce inflammatory responses.

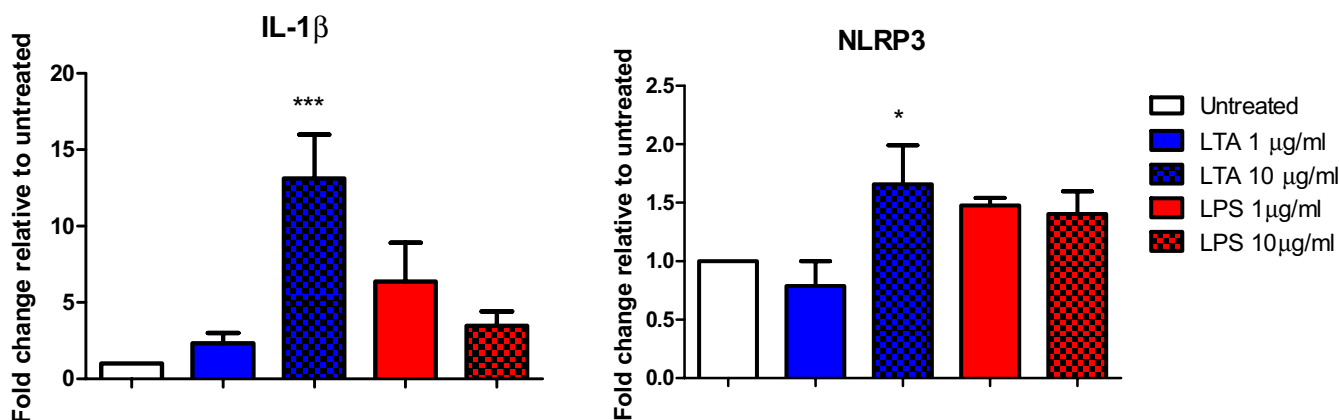


FIGURE 2 Modulation of NLRP3 and IL-1 β gene expression in dental pulp cells (DPCs) following treatment with lipoteichoic acid (LTA) and lipopolysaccharide (LPS). Data represent an average of three independent experiments and presented as mean \pm SEM. * $p < .05$; *** $p < .0001$.

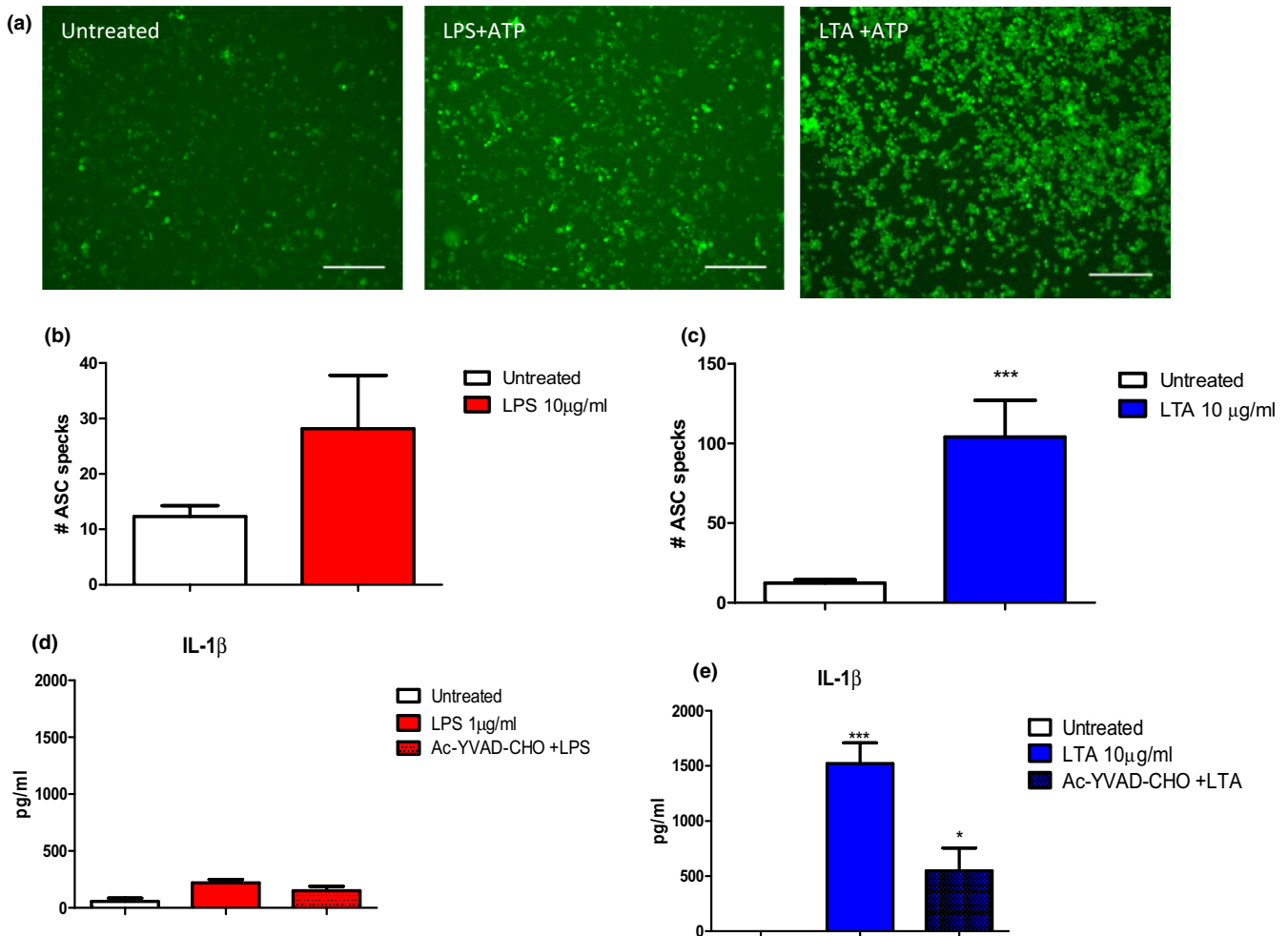


FIGURE 3 Lipoteichoic acid (LTA) induces inflammasome-dependent response in THP-1 macrophages. (a) Fluorescent images showing ASC speck formation in THP-1-GFP-ASC cells following treatment with lipopolysaccharide (LPS) and LTA. (b, c) Quantification of ASC-speck showed significant speck formation following LTA treatment compared to LPS. (d, e) IL-1 β release is significantly increased following LTA treatment and reduced in the presence of caspase-1 inhibitor AC-YVAD-CHO. Data represent an average of three independent experiments and presented as mean \pm SEM and analysed using ANOVA with Bonferroni correction $*p < .05$; $***p < .0001$; scale bar 200 μ m.

NLRP3 inflammasome regulate cytokine release in *ex vivo* pulpitis model

To ascertain whether NLRP3 inflammasome is responsible for cytokine release following LTA treatment, we employed a co-culture model in which immune cells and DPCs were simultaneously treated with LTA in the presence or absence of NLRP3 inflammasome inhibitor MCC950. As shown in Figure 4, LTA treatment significantly increase IL-1 β (Mean 182.4, SEM 29.12) compared to the untreated (Mean 13.77, SEM 0.7) and this is reduced in the presence of MCC950 (Mean 95.83 SEM 3.6). Similarly, IL-6 significantly increased in LTA treated samples (Mean 5204, SEM 1150) compared to untreated (Mean 42.06, SEM 3.3) and this is reduced in the presence of MCC950 (Mean 2060, SEM 194). On the other hand, IL-8 was upregulated with LTA treatment (Mean 21 532, SEM 688) compared to the

untreated (Mean 2068, SEM 723), but this upregulation was not reduced in the presence of MCC950. No change in MMP9 expression was observed with LTA treatment or MCC950 in this model.

DISCUSSION

Advancement in pulp preservation and minimal intervention therapies for managing the cariously exposed pulp requires further understanding of the mechanism of caries-induced inflammation and identification of novel signalling pathways and drug targets. This study investigated the function of the NLRP3 inflammasome, a key component in the innate immune response against bacterial infection. The results showed that the NLRP3 inflammasome is differentially regulated in dental pulp of

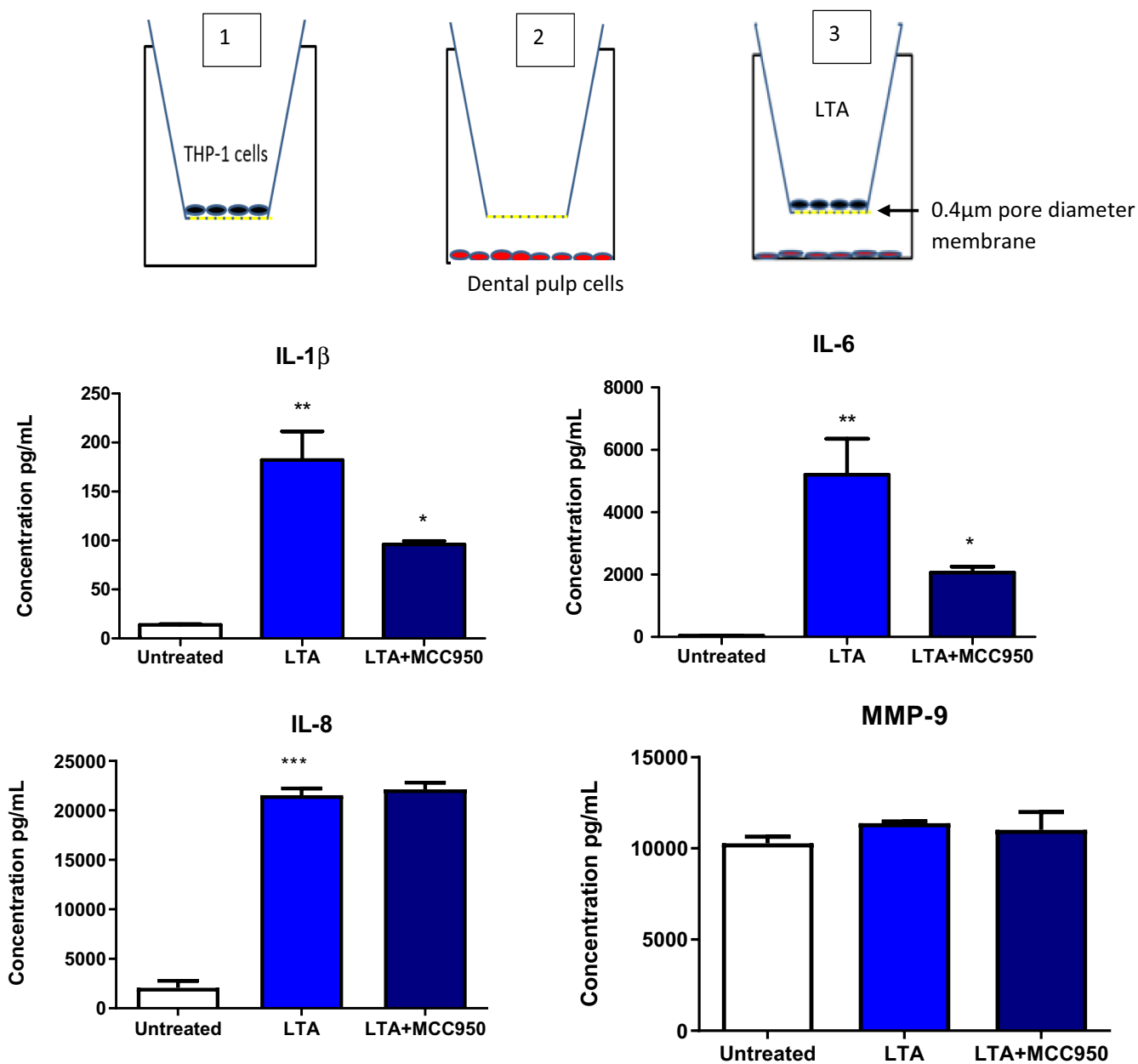


FIGURE 4 Effect of NLRP3 inhibitor on cytokines release in *ex vivo* pulpitis model. Pulpitis co culture model was established in 12 well ThinCert 0.4 μ m pore diameter culture inserts. 1. THP-1 macrophages seeded at 1×10^5 cell/ml on the top chamber 2. DPCs were seeded at 6×10^4 cell/ml on the bottom chamber. 3. The chambers were assembled prior to treatment of the cells with LTA with or without MCC950. Treatment of the co-culture with 10 μ g/ml LTA significantly increases the release of IL-1 β , IL-6, and IL-8. The INLRP3-specific inhibitor MCC950 significantly reduced IL-1 β and IL-6 but had no effect on the IL-8 and MMP-9. Data represent an average of three independent experiments and presented as mean \pm SEM and analysed using ANOVA with Bonferroni correction * $p < .05$; ** $p < .01$, *** $p < .001$.

healthy, moderately carious and deeply carious teeth. In addition, NLRP3 signalling was found to mediate inflammation in response to LTA from Gram-positive bacteria as well as to the more conventional signal, LPS. These results concur with previous findings demonstrating expression of NLRP3 in the dental pulp (Jiang et al., 2015), but showed mechanistically that NLRP3 expression is modulated by LTA, a component of Gram-positive bacteria that was found to dominate the deep carious lesion

and irreversibly inflamed pulp (Hahn et al., 1991; Rôças et al., 2016; Zheng et al., 2019). Previous studies using immune cells and DPCs showed treatment with LPS and ATP significantly increased NLRP3 expression and activation (Jiang et al., 2015; Zhang et al., 2015). In the present study, LPS was used as positive control, and unexpectedly, LTA was found to outperform LPS in term of NLRP3 activation and IL-1 β release. In a recent study, Tian et al. (2021) showed that LTA activates NLRP6, but

not NLRP3; however, in their experiments, LTA was transfected into the cytosol secondary to priming with LPS, which is not the case in this study as LTA was used as a priming agent. Our results clearly showed that LTA has better priming compared to LPS confirming previous report that LTA play an important role in pulp inflammation. Mutoh et al. (2007) investigated the expression of LTA and LPS respective receptors, TLR2 and TLR4, in inflamed dental pulp and found that TLR2 was strongly expressed on macrophages and dendritic cells. TLR4-positive cells were also detected in the pulp, but the number of cells expressing TLR4 was much lower than in the case of TLR2. LTA also was found to upregulate chemokines expression while downregulate dentine matrix synthesis and mineralization (Durand et al., 2006). These findings, together with the results of the current study, further confirm the importance of Gram-positive bacteria in caries-induced pulpitis.

It is known that acute inflammation is an important protective immune response during infections and is essential for healing of cariously inflamed pulp (Cooper et al., 2014; El Karim et al., 2021). However, chronic and excessive inflammation is a driver of host tissue injury seen in many severe infectious diseases, and it must therefore be carefully regulated for an optimal healing response. An important finding from this study is the identification of NLRP3 inflammasome as potential target for control of caries-induced inflammation. The NLRP3 specific inhibitor, MCC950, reduced the level of the inflammatory cytokines IL-1 β and IL-6, known to be upregulated in pulpitis (Al-Natour, Rankin, et al., 2021; Rechenberg et al., 2016). IL-1 β is a master regulator of inflammation via controlling a variety of innate immune responses (Dinarello, 2009), therefore strategies to control excessive IL-1 β release will interrupt the inflammatory vicious circle in favour of healing. Although the data showed reduction of IL-6 with MCC950, it is not clear why no reduction was observed with IL-8 and MMP-9. IL-1 β was shown to induce IL-8 release via P38MAPK pathway (Chang et al., 2019) and not via an interleukin-1 receptor-associated protein kinase (IRAK) suggesting production of IL-8 via mechanism independent of classical IL-1 β signalling pathway in the dental pulp cells.

In addition to their pivotal role in cytokine production, NLRP3 inflammasomes also influence novel mechanisms of programmed cell death known as pyroptosis (Fernandes-Alnemri et al., 2007). Although poorly understood in the dental pulp, pyroptosis is commonly seen in bacterial infections and has dual effects. The process is not only necessary for elimination of bacterial infection but also results in release of alarmins and endogenous danger-associated molecular patterns which augment the inflammatory process particularly if over

activated (Wei et al., 2021). Therefore, controlling excessive inflammasome-induced cell cells is important for healing, particularly in a low compliance environment of the inflamed dental pulp.

In this study, various methods to study expression and activation of NLRP3 in dental pulp tissue and cells have been used. The pulpitis co-culture model represents an improvement over the independent cell culture methods as it simulate more the *in vivo* situation. Co-culturing both DPCs and macrophages results in marked upregulation of inflammatory cytokine production compared with the cells cultured independently (Rankin et al., 2020; Yonehiro et al., 2012), suggesting synergy and interaction between cells as would be expected *in vivo*. Such models provide feasible means for drug screening to help narrow down targets for further investigations (Al Natour, Lundy, et al., 2021; Al-Natour, Rankin, et al., 2021).

CONCLUSION

In conclusion, this study confirmed previous reports on the expression of NLRP3 in dental pulp of carious teeth and showed that LTA is modulator and activator of NLRP3. NLRP3 inhibitors attenuated selective inflammatory cytokines suggesting a role in caries-induced pulp inflammation. Further studies to investigate the utility of MCC950 as a pulp capping agent are warranted.

AUTHOR CONTRIBUTIONS

B Al-N; study design, data acquisition and analysis, FTL & I El-K; study design, data analysis, writing up of manuscript, IA & CJ; data acquisition and revising manuscript, CD, YD; study design and revise manuscript.

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CONFLICT OF INTEREST

All authors explicitly state no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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