

# Muramyl dipeptide activates human beta defensin 2 and pro-inflammatory mediators through Toll-like receptors and NLRP3 inflammasomes in human dental pulp cells

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## Abstract

**Purpose** The expression levels of intracellular pyrin domain-containing 3 (NLRP3) and microbial pattern-recognition receptors, such as nucleotide-binding oligomerization domain 2 (NOD2), have been reported in human dental pulp cells (HDPCs) and inflamed dental pulp tissue, but the role of NLRP3 and Toll-like receptors (TLRs) in the production of human beta defensin 2 (hBD2) and inflammatory cytokines against invading pathogens remains poorly defined. The aim of this study was to determine whether the NOD2 ligand muramyl dipeptide (MDP) upregulates hBD2 and inflammatory cytokines and whether this response is dependent on TLRs and NLRP3 inflammasomes in HDPCs.

**Methodology** The effects of MDP on the expression of hBD2, TLRs, inflammasomes, and pro-inflammatory mediators in HDPCs were examined using Western blotting and reverse transcription–polymerase chain reaction. Levels of pro-

inflammatory cytokines, such as nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), were determined by enzyme-linked immunosorbent assay.

**Results** MDP upregulated hBD2, TLR2, and TLR4 mRNAs and protein levels in a dose- and time-dependent manner. TLR2 and TLR4 neutralizing blocking antibodies and NOD2- and hBD2-specific small interfering RNAs (siRNAs) attenuated the MDP-induced production of NO, PGE<sub>2</sub>, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and IL-8 and upregulated inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) in HDPCs. Additionally, MDP activated inflammasome-related genes, such as NLRP3, caspase 1, apoptotic speck protein containing a caspase recruitment domain, and IL-1 $\beta$ . Furthermore, silencing of the NLRP3 gene using a siRNA significantly decreased the MDP-induced expression of hBD2 and cytokines, such as iNOS-derived NO, COX2, PGE<sub>2</sub>, TNF- $\alpha$ , IL-6, and IL-8.

**Conclusion** These results suggest that NOD2 activates the TLR2, TLR4, and NLRP3 inflammasome-signaling pathways in HDPCs to induce the production of multiple inflammatory mediators and antimicrobial peptides, which in turn promote pulp immune defense against microbial challenge.

**Clinical relevance** The TLR and NLRP3 inflammasome pathways may represent an important modulatory mechanism of immune defense responses during the progression of pulpitis. Our results suggest that local inhibition of NLRP3 and TLRs may reduce the impact of cytokine-mediated host destructive processes in pulpitis.

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TLR · NLRP3 · Defense

## Introduction

Host defense in dental pulp is achieved by innate and adaptive immunity. The innate immune system provides the first line of

defense as it recognizes pathogen-associated molecular patterns (PAMPs) via pattern-recognition receptors (PRRs) [1, 2]. Microbial PRRs that recognize PAMPs, such as Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD), are essential molecules in the innate immune response [2]. Among cell-surface receptors, TLR2 is crucial for recognition of peptidoglycans, lipoproteins, and lipoteichoic acid, whereas TLR4 plays a major role in detecting lipopolysaccharides (LPSs) [1].

Similar to TLRs, nucleotide-binding oligomerization domain 2 (NOD2), a member of the Apaf1/Nod protein family, is an intracellular sensor of muramyl dipeptide (MDP) and activates nuclear factor- $\kappa$ B, a key transcription factor of inflammatory gene expression [3]. NOD2 consists of two N-terminal caspase-recruitment domains, a centrally located nucleotide-binding domain, and C-terminal leucine-rich repeats that act as a signal-transducing adaptor [4]. The NOD2 protein is expressed in human dental pulp tissue and human dental pulp cells (HDPCs), suggesting an important role in the immune defense of the pulp dentin complex [5]. In addition, a NOD2 agonist acts synergistically with TLR2, not TLR4, to stimulate the production of pro-inflammatory mediators in HDPCs [6]. These findings suggest that TLRs and NOD2 are functionally predominant receptors in HDPCs and play important roles in pulp immune responses, leading to progressive pulpitis [6].

One of the best-characterized and most versatile members of the innate immune Nod-like receptor (NLR) family is intracellular pyrin domain-containing 3 (NLRP3). NLRP3 assembles into a multi-protein structure called the inflammasome following activation, and this complex regulates the bioactivity of several cytokines [7]. NLRP3 detects a wide range of microbial stimuli [8] and is highly expressed in healthy human dental pulp tissues, particularly in odontoblasts and vascular endothelial cells [9]. However, the role and function of NLRP3 inflammasomes in HDPCs are not completely understood.

Activation of an innate immune response after exposure to the external environment leads to the production of inducible antimicrobial peptides [10]. One class of these peptides is  $\beta$ -defensins, which are small cationic peptides containing sulfide bonds produced by various epithelial cells, such as those in the skin, respiratory tract, gastrointestinal tract, and cornea [11]. Human  $\beta$ -defensin 1 (hBD1) is constitutively expressed in epithelial cells, whereas hBD2 is poorly expressed in normal epithelial cells and is induced by bacterial infection, LPS, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin (IL)-1 $\beta$  [12, 13]. hBD2 stimulation leads to the upregulation of IL-6, IL-8, and cytosolic phospholipase-A-2 mRNA levels, suggesting that synthesis of hBD2 in odontoblast cells enhances the immuno-inflammatory reactivity of dental pulp [14]. We reported that TNF- $\alpha$  and IL-1 $\alpha$  synergistically upregulate hBD2 expression and activity in HDPCs [15]. In addition, we demonstrated that LPS and heat stress upregulate the expression of host immune defense molecules—including

antimicrobial peptides (hBD2) and defense molecules (HO1)—in HDPCs through effects on SIRT1 signaling [16].

MDP, the minimal essential structural unit responsible for the immunoadjuvant activity of peptidoglycans, is distributed ubiquitously in the cell walls of both gram-negative and gram-positive bacteria [17]. MDP stimulates murine and human macrophages and monocytes to produce cytokines and chemokines [18] and acts synergistically with LPS to stimulate the release of both pro- and anti-inflammatory cytokines by myeloid cells [19]. Although the combination of signaling through TLRs and NODs leads to synergistic activation of pro-inflammatory cytokines in human monocytic cells [18], the roles of inflammasomes and hBD2 in host defense and the inflammatory response to MDP have not been investigated. The aim of this study was to investigate the role of hBD2 and NLRP3 inflammasomes in the host defense and inflammatory responses to the NOD2 ligand in HDPCs as nonimmune cells.

## Materials and methods

### Reagents

$\alpha$ -Modified Eagle's medium ( $\alpha$ -MEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco-BRL Co. (Grand Island, NY, USA). Ultrapure LPS from *Porphyromonas gingivalis* and *Escherichia coli* were obtained from Invitrogen (Mountain View, CA, USA). MDP and other chemicals were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

### Cell culture

HDPCs were immortalized by transfection with the telomerase catalytic subunit of the human telomerase reverse transcriptase [20]. The cells were kindly provided by Professor Takashi Takata (Hiroshima University, Japan). The cells were cultured in  $\alpha$ -MEM supplemented with 10 % FBS, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin in a humidified atmosphere of 5 % CO<sub>2</sub> at 37 °C.

### Stimulation of HDPCs

For stimulation experiments, the cells were seeded into culture dishes and then cultured in  $\alpha$ -MEM containing 10 % FBS for 2 days until reaching 70 % confluency. The medium was then replaced by serum-free medium to minimize any serum-induced effects. Cells were incubated with MDP (1–20  $\mu$ g/mL) for 72 h and with 10  $\mu$ g/mL MDP for the indicated times. In addition, cells were stimulated with MDP (10  $\mu$ g mg/mL) and/or *P. gingivalis* LPS (1 mg/mL) and *E. coli* LPS (1 mg/mL) for 72 h. For experiments using neutralizing monoclonal antibodies, cells were preincubated with TLR2 (10 mg/mL)

and TLR4 (10 mg/mL) neutralizing antibody for 1 h, followed by stimulation with 10 mg/mL MDP for 72 h. hBD2 and NOD2 (80 nM) short interfering RNAs (siRNAs) were added to cells 24 h before stimulation with MDP (10 mg/mL) for an additional 72 h. All treatments were performed in triplicate.

Determination of NO and PGE2 levels

Enzyme-linked immunosorbent assay kits were used to quantify NO (R&D Systems, Minneapolis, MN, USA) and PGE<sub>2</sub> (Cayman Chemical Co., Ann Arbor, MI, USA) in cell culture supernatants.

NOD2 and HBD2 siRNA transfection

siRNA was used for transient gene knockdown studies. Human NOD2 siRNA (cat. no. SC-35437) and hBD2 siRNA (cat. no. SC-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HDPCs were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Concentration of siRNA was chosen on the basis of dose–response studies. Briefly, cell cultures at 50–60 % confluence were prepared in six-well plates. Then, 4 μL siRNA (5 μM solution) and 3 μL Lipofectamine 2000 were diluted separately with 100 μL Opti-MEM I Reduced Serum Medium and kept at room temperature for 5 min. The

diluted siRNA and Lipofectamine 2000 were mixed gently, followed by incubation for 20 min at room temperature, after which 200 μL of the siRNA–Lipofectamine complex was added to each well containing 800 μL α-MEM without antibiotics. After 5 h of incubation of the siRNA–Lipofectamine 2000 complex, the medium was replaced with normal α-MEM, and cells were maintained for an additional 19 h. Cells were treated with MDP (10 mg/mL) for 72 h. To monitor the transfection efficiency, siRNA targeting green fluorescent protein (GFP; Santa Cruz Biotechnology) was transfected into cells in parallel in all transfections. A transfection efficiency of at least 50 % was achieved. Cells were also transfected with non-targeting, negative control siRNA (Bioneer, Daejeon, South Korea), which allows for the assessment of target specificity and any nonspecific gene silencing effects. Gene silencing effects were verified by reverse transcription–polymerase chain reaction (RT-PCR).

RNA isolation and RT-PCR

Total RNA was prepared using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA), and cDNA was prepared using random hexamer primers (Invitrogen Life Technologies, Grand Island, NY, USA). The primer sequences are detailed in Table 1. After optimization of the PCR cycle number for each experimental condition and primer set, PCR was performed, and semiquantitative analysis was conducted during the linear phase

**Table 1** Reverse transcriptase-polymerase chain reaction (RT-PCR) primers and conditions

Genes	Primer sequence (5'–3')	Annealing temperature (°C)	Cycle number	Product size (bp)
NOD2	F : 5'-CAGAAGGCCGAGCCGCACAA-3' R : 5'-CCAGACACCAGCGGGCAGAG-3'	60	35	145
hBD-2	F : 5'-CATGAGGGTCTGTATCTCCTCT-3' R : 5'-CCTCCTCATGGCTTTTTGCAGC-3'	55	34	201
TLR2	F : 5'-GGAATCGGTGAGGTCTGTCTG-3' R : 5'-GGCGTCACATGCAGAAAGCCC-3'	60	30	296
TLR4	F : 5'-AAGTGTCTGAACTCCCTCCAGG-3' R : 5'-ATGGTCTTATTCATCTGACAGGTGATA-3'	55	35	278
TNF-α	F: 5'- CTC TGG CCC AGG CAG TCA GA -3' R: 5'- GGC GTT TGG GAA GGT TGG AT -3'	60	35	519
IL-1β	F : 5'- GGA TAT GGA GCA ACA AGT GG -3' R : 5'- ATG TAC CAG TTG GGG AAC TG -3'	60	35	288
IL-6	F: 5'-ATGAACTCCTTCTCCACAAGC-3' R: 5'-CTACATTTGCCGAAGAGCCC-3'	55	34	639
IL-8	F: 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' R: 5'-TCTCAGCCCTCTTCAAAAACCTTCTC-3'	62	25	289
NLRP3	F: 5'-CATTCGGAGATTGTGGTT-3' R: 5'-GTTGCCTCGCAGGTTAAAG-3'	52	30	454
ASC	F: 5'-CTCCTCAGTCGGCAGCCAAG-3' R: 5'-CAAGTCCTTGCAGGTCCAGT-3'	62	25	221
Caspase-1	F: 5'-ACAAGACCTCTGACAGCACG-3' R: 5'-CACTTCCTGCCCACAGACAT-3'	59	30	583
β-actin	F : 5'-CATGGATGATGATATCGCCGCG-3' R : 5'-ACATGATCTGGGTCATCTTCTCG-3'	55	34	371

of the reaction. The PCR products were resolved in a 1.5 % agarose gel and stained with ethidium bromide. All experiments were performed in triplicate, with the results of one representative experiment presented here.

### Western blotting

Treated cells were lysed with lysis buffer (1 % Nonidet P-40, 0.5 % sodium deoxycholate, 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 1× protease inhibitor cocktail; Roche Diagnostics GmbH, Mannheim, Germany) containing phosphatase inhibitors, NaF (100 mM), and  $\text{Na}_3\text{VO}_4$  (100 mM). Cell lysates were subjected to electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gels. The proteins were transferred to a polyvinylidene difluoride membrane, probed with specific antibodies, and visualized using an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK).

### Statistical analysis

Differences among groups were analyzed using one-way analysis of variance combined with the Bonferroni test. NO and  $\text{PGE}_2$  values are expressed as means±standard deviations ( $n=4$ ) within a single representative experiment. Statistical differences were considered significant at  $P<0.05$ .

## Results

### Effects of the NOD2 ligand MDP on hBD2 expression in HDPCs

To investigate whether the NOD ligand MDP could induce hBD2 production in HDPCs, the cells were stimulated with various concentrations of MDP for 72 h. As shown in Fig. 1, NOD2 and hBD2 mRNA and protein levels were dose-

dependently increased by MDP, with maximum increases occurring in the 10- and 20- $\mu\text{g}/\text{mL}$ -MDP treatments. Moreover, a time course experiment revealed that 10  $\mu\text{g}/\text{mL}$  MDP increased NOD2 and hBD2 mRNA (Fig. 1c) and protein (Fig. 1d) levels in HDPCs compared with controls, with maximum induction after 72 h of incubation.

### Combined effects of MDP and LPS on hBD2 expression in HDPCs

We examined the ability of the NOD ligand to induce hBD2 production in combination with various bacterial ligands known to interact with the pattern recognition receptors TLR2 (*P. gingivalis* LPS) and TLR4 (*E. coli* LPS). Figure 2 shows that NOD2 and hBD2 mRNA levels were significantly higher in cells treated with MDP plus the TLR ligands than in cells treated with MDP and either the TLR2 or the TLR4 ligand. The increases in NOD2 and hBD2 protein levels appeared to correspond to increased mRNA transcription levels in HDPCs.

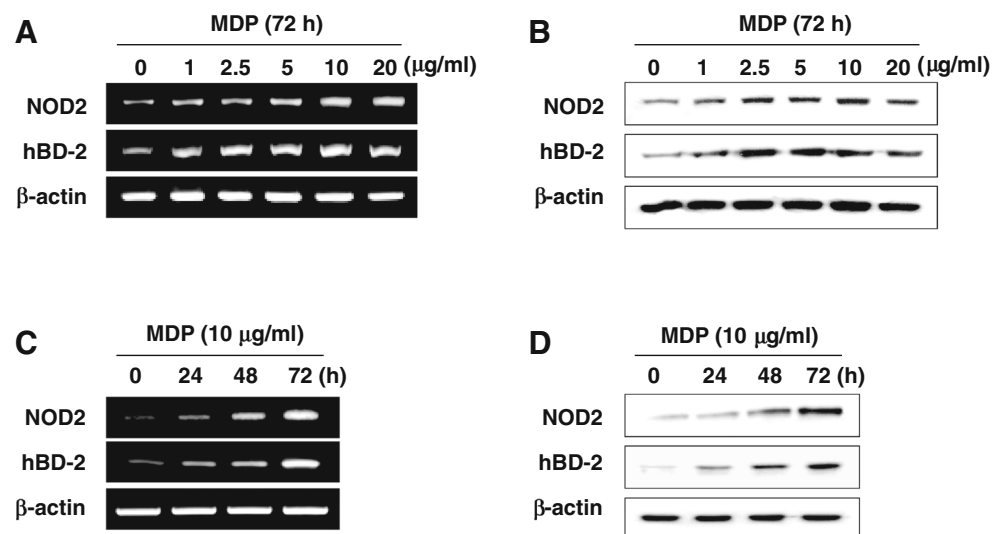
### Effects of MDP on TLR2 and TLR4 expression in HDPCs

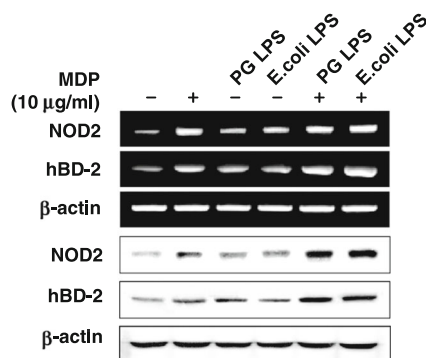
To determine the effects of MDP on TLR2 and TLR4 expression, HDPCs were stimulated with various concentrations of MDP for 72 h. Following exposure to MDP, TLR2 and TLR4 mRNA (Fig. 3a, c) and protein (Fig. 3b, d) levels were upregulated in a dose- and time-dependent manner, with the maximum effect occurring with 10  $\mu\text{g}/\text{mL}$  MDP after 72 h.

### Effects of blocking TLR2 and TLR4 antibodies on MDP-induced hBD2 and inflammatory mediators in HDPCs

To investigate whether MDP-induced hBD2 production is dependent on TLR2 and TLR4, HDPCs were preincubated for 1 h

**Fig. 1** Effects of the nucleotide-binding oligomerization domain (NOD) 2 ligand muramyl dipeptide (MDP) on the expression of NOD2 and human beta defensin (hBD) 2 mRNA (a, b) and protein (b, d) in human dental pulp cells. Cells were incubated with the indicated dose of MDP for 72 h (a, b) and with 10  $\mu\text{g}/\text{mL}$  MDP for the indicated times (c, d). mRNA and protein levels were determined by reverse transcription–polymerase chain reaction and Western blotting, respectively. Data are representative of three independent experiments





**Fig. 2** Combined effects of the nucleotide-binding oligomerization domain (NOD) 2 ligand muramyl dipeptide (MDP) and Toll-like receptor (TLR) agonists (*P. gingivalis* lipopolysaccharide (LPS) and *E. coli* LPS) on human beta defensin (hBD) 2 mRNA and protein levels in human dental pulp cells. Cells were stimulated with or without the TLR2 and TLR4 agonists *P. gingivalis* LPS (1 mg/mL) and *E. coli* LPS (1 mg/mL) and the NOD2 ligand MDP (10 µg/mL) for 72 h. Similar Western blot and reverse transcription–polymerase chain reaction data were obtained from three independent experiments

with neutralizing monoclonal antibodies against TLR2 and TLR4 and then treated with MDP (10 µg/mL) for 72 h. Antibody specificity was assessed by incubation with control IgG from nonimmunized animals (Fig. 4a, b). The anti-TLR4 and anti-TLR2 antibodies (10 µg/mL) decreased MDP-enhanced NOD2 and hBD2 mRNA and protein levels (Fig. 4b). In addition, the anti-TLR4 and anti-TLR2 antibodies attenuated MDP-induced COX2 and iNOS expression, as well as PGE<sub>2</sub> and NO production, and upregulated the levels of inflammatory cytokines, such as TNF-α, IL-6, and IL-8 (Fig. 4c–f).

Effects of NOD2 and hBD2 siRNA on MDP-induced pro-inflammatory mediators and TLR expression in HDPCs

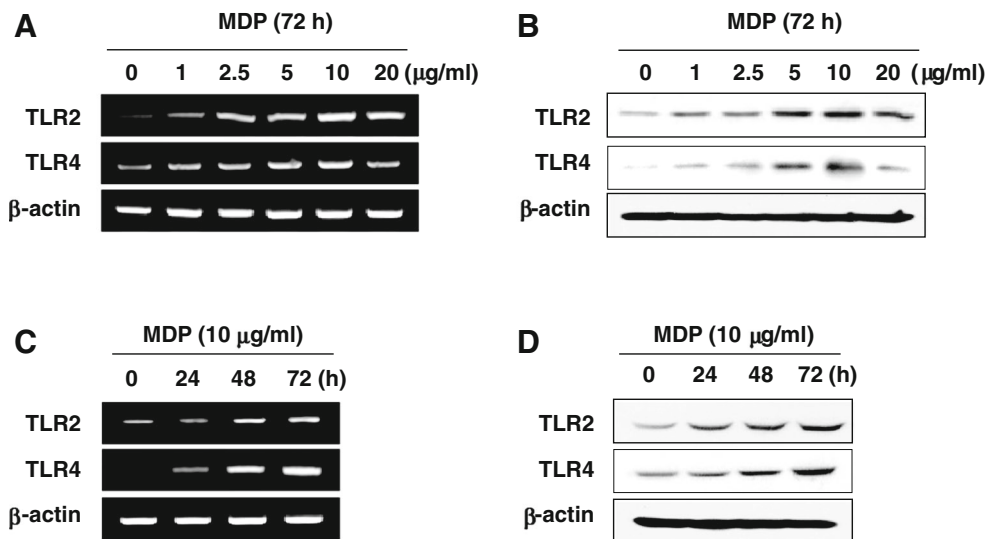
To further explore the role of hBD2 and NOD2 on the MDP-induced inflammatory response, cells were

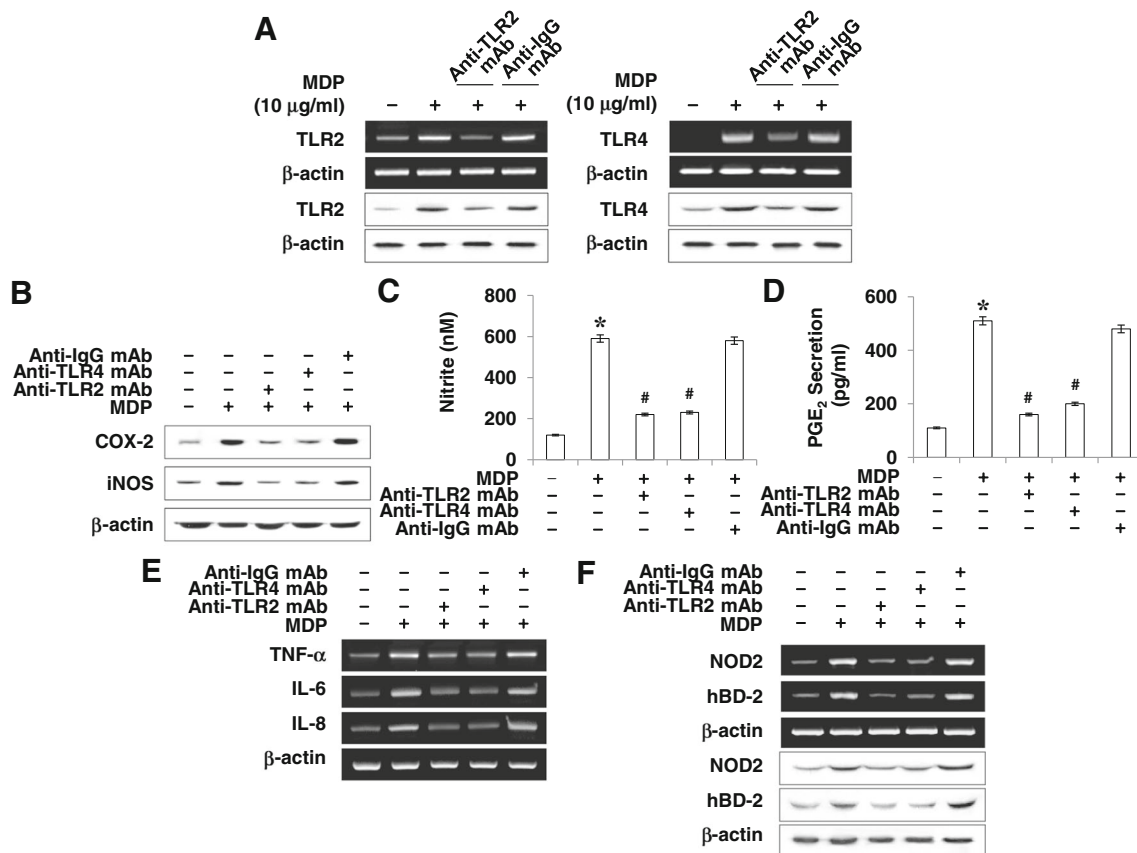
transfected with specific siRNA against the NOD2 and hBD2 genes and stimulated with MDP. As expected, transfection with siRNA specific for NOD2 and hBD2 lowered the MDP-induced expression of NOD2 and hBD-2 (Fig. 5a, b). In addition, NOD2 and hBD2 siRNA decreased MDP-induced COX2 and iNOS expression, as well as PGE<sub>2</sub> and NO production, and upregulated inflammatory cytokines (Fig. 5c–f). Moreover, MDP-induced TLR2 and TLR4 expression levels were attenuated by transfection with NOD2 and hBD2 siRNAs (Fig. 5g).

Involvement of NLRP3 inflammasomes in the MDP-induced inflammatory response

To determine whether MDP could induce NLRP3 inflammasomes and their cytokine targets, the mRNA levels of inflammasome components and IL-1β in MDP-treated HDPCs were examined. MDP treatment dose- and time-dependently increased the expression of IL-β and inflammasome-related genes, such as NLRP3, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), and caspase-1 (Fig. 6a, b). To verify involvement of NLRP3 inflammasomes in the MDP-induced inflammatory response, expression of the NLRP3 receptor, the crucial component of NLRP3 inflammasomes, was silenced in HDPCs using NLRP3-targeted siRNA. Silencing of the NLRP3 gene attenuated MDP-induced COX2 and iNOS expression, as well as PGE<sub>2</sub> and NO production, and decreased inflammatory cytokines, such as TNF-α, IL-6, and IL-8 mRNA (Fig. 6b–g). In addition, MDP-induced hBD2 mRNA and protein levels were attenuated by NLRP3 siRNA (Fig. 6h). However, no significant inhibition of expression was observed in cells transfected with a negative control siRNA.

**Fig. 3** Effects of muramyl dipeptide (MDP) on Toll-like receptor 2 (TLR2) and TLR4 mRNA (a, c) and protein (b, d) levels in human dental pulp cells. Cells were incubated with the indicated dose of MDP for 72 h (a, b) and with 10 µg/mL MDP for the indicated times (c, d). Similar data were obtained from three independent experiments





**Fig. 4 a–f** Effects of Toll-like receptor 2 (*TLR2*) and *TLR4* blocking antibodies on muramyl dipeptide (*MDP*)-induced nucleotide-binding oligomerization domain (NOD) 2 and human beta defensin (hBD) 2 expression (f), production of nitrite and prostaglandin (PG)<sub>E</sub><sub>2</sub> (c, d), expression of cyclooxygenase 2 (COX2), inducible nitric oxide synthase (iNOS) (b), and pro-inflammatory cytokines in human dental pulp cells. The cells were treated with human monoclonal *TLR2* (10 mg/mL) and *TLR4* (10 mg/mL) neutralizing antibodies for 1 h, followed by

stimulation with 10 µg/mL *MDP* for 72 h. mRNA and protein levels were examined by reverse transcription–polymerase chain reaction analysis and Western blotting, respectively. Nitrite and PGE<sub>2</sub> concentrations were determined by enzyme-linked immunosorbent assay ( $n = 4$ ). Data are representative of three independent experiments. Asterisk significantly different compared with the control,  $P < 0.05$ . Number sign significantly different compared with the *MDP*-treated group,  $P < 0.05$

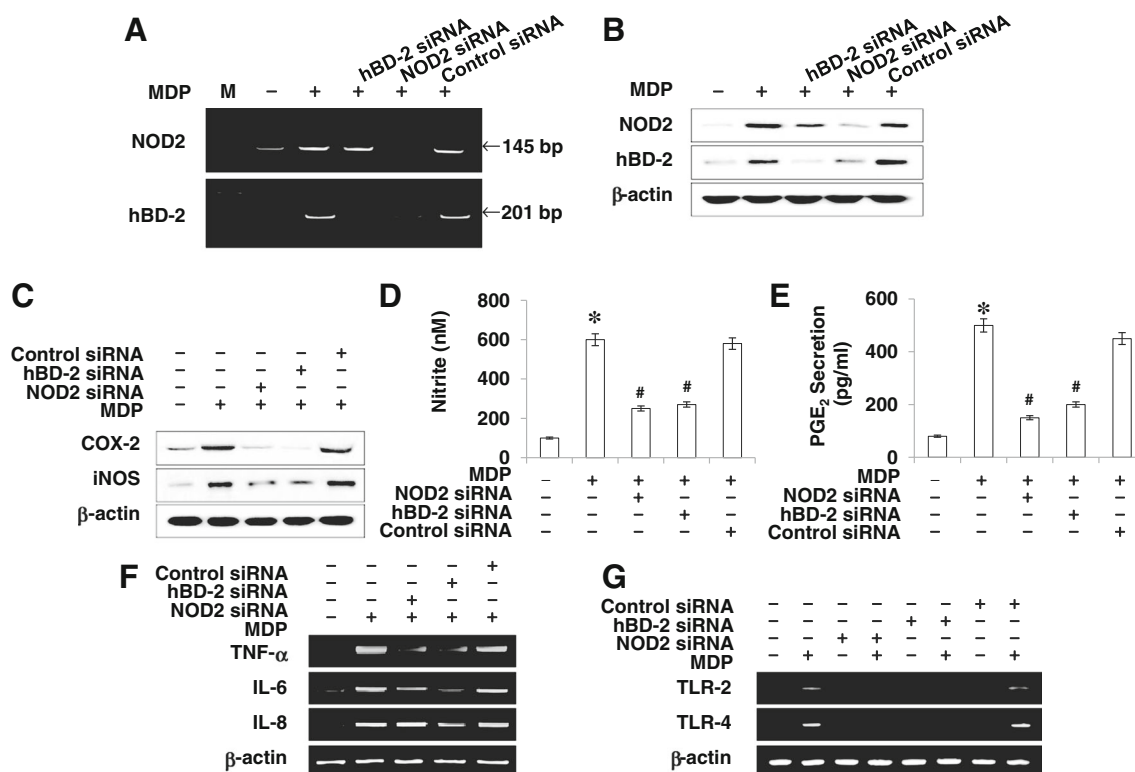
## Discussion

The progression of a carious infection through dental hard tissues and into soft pulpal tissue elicits immune and inflammatory responses. Cytokines that are particularly well characterized as regulators of the immune response and play key roles in the response of the pulp to carious infection include TNF- $\alpha$  and IL-1 $\beta$  [21]. hBD2 plays an important role in the innate host defense against bacterial invasion by promoting the adaptive immune response in human dental pulp [15, 16]. Because TLRs and NLRP3 are expressed in HDPCs [6, 9], we postulated that they might be involved in the inflammatory response against *MDP*.

HDPCs are a major source of many cytokines and chemokines involved in immune responses [5, 6, 9]. However, primary HDPCs undergo senescence when cultured in vitro after several subcultivations. Reproducible results are difficult to obtain and clarify using primary cells. To overcome this problem, we used immortalized HDPCs, which replicate

readily and are easy to maintain in culture, allowing for convenient and cost-effective experimentation. Primary and immortalized cells from single donors have illustrated the qualitative differences in pattern recognition receptor profiles and pathogen responsiveness [22, 23]. Immortalization was previously reported to lead to alterations in both TLR expression and cytokine elaboration [24]. In primary epithelial cells, a general reduction in cytokine production in response to TNF- $\alpha$  occurs compared with immortalized epithelial cell lines [25]. The high concentrations of cytokines produced by the cell lines may be due to the transformed characteristics of the cell lines [25].

NOD2 is involved in the recognition of PAMPs present in the bacterial cell wall, but little is known about its role during the host response to pathogenic bacteria in dental pulp. Basal hBD2 concentration in the oral epithelium is relatively low but increases rapidly in the presence of cytokines, bacteria, or fungi [11]. In contrast, hBD2 is weakly expressed in both healthy and inflamed pulp tissue, which may be due to



**Fig. 5** a–g Effects of nucleotide-binding oligomerization domain (NOD) 2 and human beta defensin (hBD) 2 siRNAs on muramyl dipeptide (MDP)-induced NOD2 and hBD2 (a), pro-inflammatory mediators (c–e), and TLR2 and TLR4 (f) expression in human dental pulp cells. Cells were transiently transfected with control siRNA or NOD2 and hBD2 siRNAs (80 nM) for 24 h and then stimulated with MDP (10 µg/mL) for 72 h (a–f). mRNA (a, e, f) levels were examined using reverse

transcription–polymerase chain reaction. Inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) protein levels (b) and nitrite and prostaglandin (PG)E<sub>2</sub> concentrations (c, d) were determined by Western blotting and enzyme-linked immunosorbent assay (n =4), respectively. Data are representative of three independent experiments. Asterisk significantly different compared with the control, P <0.05. Number sign significantly different compared with the MDP-treated group, P <0.05

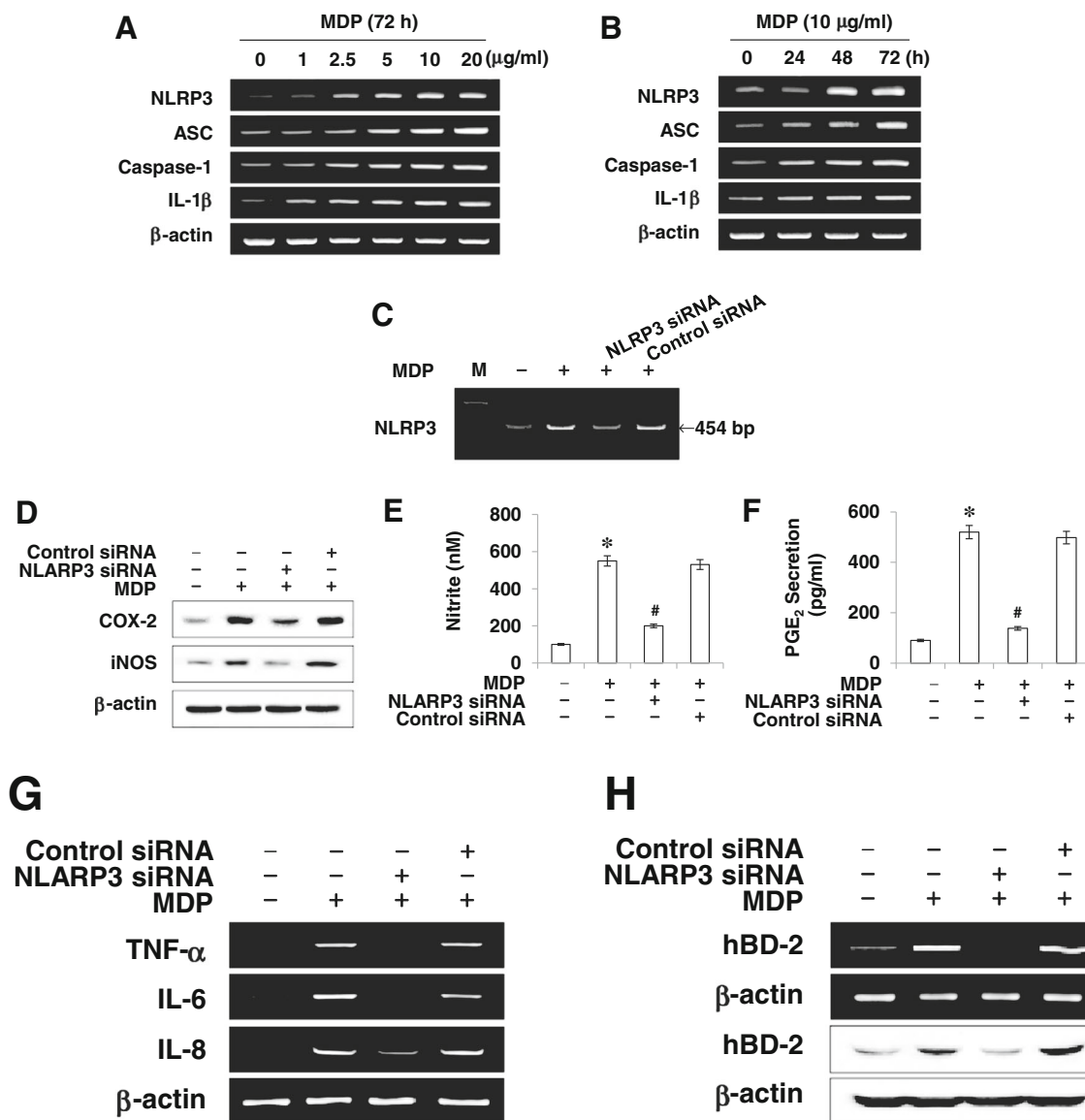
necrotic areas [26]. Our results indicate that treatment of HDPCs with the NOD2 ligand MDP resulted in the upregulation of hBD2 mRNA and protein levels. These results are consistent with previous reports in human oral epithelial cell lines [27] and studies on TNF-α and IL-1α in HDPCs [15].

TLR4 is expressed in the odontoblast layer and pulp tissues, but the TLR2 mRNA level was 30-fold higher than TLR4 9 h after infection in a murine model [28]. TLR2 and NOD2 are functionally predominant receptors stimulating the production of pro-inflammatory mediators, suggesting that these receptors play important roles in pulpal immune responses, leading to progressive pulpitis [6]. Moreover, NOD2 activation potentiated TLR2-mediated production of pro- and anti-inflammatory cytokines by monocytes and macrophages [29]. In this study, MDP upregulated both TLR2 and TLR4 in HDPCs, although bacterial infection mainly stimulates TLR2, not TLR4 [30]. These results suggest that TLR2 and TLR4 participate in the recognition of MDP. Similar TLR2 and TLR4 levels are also observed in human dendritic cells exposed to MDP derivatives [31].

Recent reports indicate that NOD2 is responsible for the cooperative effects with agonists of TLRs, including TLR2 and TLR4 [2]. We demonstrated that the TLR2 and TLR4

ligand treatment attenuated MDP-induced upregulation of hBD2 mRNA and protein levels to similar extents. Consistent with our results, the expression of hBD2 is regulated by TLR4- and TLR2-dependent pathways in intestinal epithelial cells [32]. Our results indicate that upregulation of TLR2 and TLR4 may lead to increased bacterial reactivity, resulting in the secretion of antimicrobial peptides. However, MDP was reported to act synergistically with a TLR2 ligand to induce IL-8 and IL-6 production in HDPCs [6]. In addition, interactions between the TLR4 and NOD2 intracellular pathways in HDPCs have been reported to have an additive effect [6]. These differences might be due to the source of TLR ligands or the experimental conditions.

Various inflammatory mediators may be generated in inflamed pulp tissue. COX2-derived PGE<sub>2</sub> and iNOS-derived NO are two major inflammatory mediators elevated during the progression of pulpitis [33]. Pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-8, have been reported in experimentally induced rat pulpitis [34], human pulpitis [35], and periapical disease [21]. HDPCs are highly responsive to bacterial PAMPs through TLRs and NODs with respect to the production of pro-inflammatory cytokines [6]. In this study, HDPCs showed enhanced production of PGE<sub>2</sub>, NO, TNF-α,



**Fig. 6 a–h** Effects of muramyl dipeptide (MDP) on mRNA levels of inflammasome-related genes (**a**, **b**). Effects of intracellular pyrin domain-containing 3 (NLRP3) siRNA on MDP-induced NLRP3 (**c**), pro-inflammatory mediators (**d–g**), and human beta defensin (hBD) 2 (**H**) expression in human dental pulp cells. Cells were incubated with the indicated dose of MDP for 72 h (**a**) and with 10 μg/mL MDP for the

indicated times (**b**). Cells were transiently transfected with control siRNA or NLRP3 siRNA (150 nM) and then stimulated with MDP (10 μg/mL) for 72 h (**c–g**). Data are representative of three independent experiments. *Asterisk* significantly different compared with the control,  $P < 0.05$ . *Number sign* significantly different compared with the MDP-treated group,  $P < 0.05$

IL-6, and IL-8 as pro-inflammatory mediators against NOD ligands, which is consistent with periodontal ligament cells [36]. Furthermore, MDP-induced production of hBD2 and inflammatory molecules, such as COX2-derived PGE<sub>2</sub>, iNOS-derived NO, TNF-α, IL-6, and IL-8, was downregulated by TLR2- and TLR4-blocking antibodies and NOD2 siRNA. These results were further supported by our recent report in which NOD2 silencing blocked MDP-induced osteoclastogenic cytokines in HDPCs [37]. Similarly, TLR2 neutralizing antibody attenuates *Staphylococcus aureus* bacterial lipoprotein-induced production of IL-6, IL-8, and hBD2 in human corneal epithelial cells [38]. Moreover, NOD2-

specific siRNA blocks MDP-induced hBD2 induction in primary keratinocytes [39] and MDP-induced upregulation of IL-6 and IL-8 in mouse macrophages [40]. Our findings also show that hBD2 siRNA attenuated MDP-induced cytokines and TLR production, suggesting possible signaling cross-talk between hBD2 and TLRs in HDPCs. Furthermore, we recently reported that cytokine-induced upregulation of IL-6, IL-8, IL-17, and the defense gene heme oxygenase-1 is inhibited by hBD2 siRNA in HDPCs [15]. These data suggest that NOD2 and hBD2 contribute to the host immune response by activating pro-inflammatory cytokines in HDPCs.



The regulation of hBD expression by bacteria, which is part of the innate immune response, must be initiated through the recognition of unique microbial molecular patterns by PRRs [1]. The best-characterized PRRs belong to the TLR family [2]. TLR2 and NOD2 signaling are linked, and a function of NOD2 is the regulation of TLR2 signaling [41]. For example, MDP signaling via NOD2 has downregulatory effects on TLR2 signaling, particularly the pathway leading to the induction of IL-12 secretion [42]. To investigate whether TLR expression is affected by knockdown of hBD2 or NOD2 siRNA, we compared the mRNA expression levels of TLR2 and TLR4 in response to MDP stimulation when hBD2 or NOD2 is knocked down. Our results revealed that TLR2 and TLR4 expression decreased in response to MDP when hBD-2 or NOD2 was knocked down. These findings suggest that the expression levels of TLR2 and TLR4 are dependent on the activation of NOD2 and hBD2 in HDPCs. Our results are consistent with those of a previous study reporting that MDP signaling via NOD2 has global inhibitory effects in that it inhibits not only TLR2 and IL-12 but also other TLR responses and other cytokine responses [42]. Further study will be necessary to elucidate the cross-communication among TLRs, NOD, and hBD2 signaling in HDPC responses to bacteria to induce appropriate innate immune responses.

IL-1 $\beta$  is a classic pro-inflammatory cytokine and an important mediator of inflammation in inflamed dental pulp [43]. In addition, HDPCs produce various inflammatory cytokines, including IL-1 $\beta$ , upon stimulation with LPS [44]. Inflammasomes are multi-protein complexes composed of NLRs, including the NLR family, NLRP3, or the HIN200 protein AIM2, and additionally contain the adapter molecule ASC and caspase-1 for cleavage of pro-IL-1 $\beta$  and pro-IL-18 [8]. Upregulation of *NLRP3* receptor mRNA was recently proposed as an important checkpoint preceding activation of NLRP3 inflammasomes [45]. In this study, we found that MDP upregulated the expression of the inflammasome-related genes NLRP3, caspase-1, ASC, and IL-1 $\beta$ . Indeed NLRP3-specific siRNA, the initiator of NLRP3 inflammasome assembly, attenuated MDP-induced production of inflammatory molecules and hBD2 in HDPCs. These observations suggest that NLRP3 is essential for the inflammatory response activated by MDP in HDPCs. Similarly, siRNA-mediated knockdown of NLRP3 blocked *Streptococcus pneumoniae*-stimulated IL-1 $\beta$  release from human bone marrow-derived macrophages [46] and cholesterol crystal-induced IL-1 $\beta$  secretion from THP-1 macrophages [47].

## Conclusion

This is the first study to demonstrate that the NOD2 ligand MDP induces pro-inflammatory mediators and hBD2 by activating TLR2, TLR4, and NLRP3 inflammasomes in

HDPCs. These findings suggest that TLRs and inflammasomes play crucial roles in the NOD signaling pathways that induce inflammatory and immune responses associated with pulpitis. In addition, local inhibition of NLRP3 and TLRs might be useful as a therapeutic target for treating pulp and periapical diseases associated with carious infection.

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**Conflict of interest** The authors declare no conflicts of interest.

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