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Expression of NOD2 is increased in inflamed human dental pulps and lipoteichoic acid-stimulated odontoblast-like cells

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Human odontoblasts trigger immune response s to oral bacteria that invade dental tissues during the caries process. To date, their ability to regulate the expression of the nucleotide-binding domain leucine-rich repeat containing receptor NOD2 when challenged by Gram-positive bacteria is unknown. In this study, we investigated NOD2 expression in healthy and inflamed human dental pulps challenged by bacteria, and in cultured odontoblast-like cells stimulated with lipoteichoic acid (LTA), a Toll-like receptor (TLR) 2 agonist which is specific for Gram-positive bacteria. We found that *NOD2* gene expression was significantly up-regulated in pulps with acute inflammation compared to healthy ones. *In vitro*, LTA augmented *NOD2* gene expression and protein level in odontoblast-like cells. The increase was more pronounced in odontoblast-like cells compared to dental pulp fibroblasts. Blocking experiments in odontoblast-like cells with anti-TLR2 antibody strongly reduced the *NOD2* gene expression increase, whereas stimulation with the synthetic TLR2 ligand Pam_2CSK_4 confirmed *NOD2* gene up-regulation following TLR2 engagement. These data suggest that NOD2 up-regulation is part of the odontoblast immune response to Gram-positive bacteria and might be important in protecting human dental pulp from the deleterious effects of cariogenic pathogens.

Keywords: dental pulp, innate immunity, lipoteichoic acid, odontoblast, pattern recognition molecule

Introduction

Successful defense against invading pathogens involves their rapid sensing through specialized pattern recognition molecules (PRMs) including Toll-like receptors (TLRs) which are expressed at the cell surface or within endosomes and nucleotide-binding domain leucine-rich repeat containing receptors (NLRs) located in the cytosol.[1–4] In humans, the NLR family encompasses 23 cytosolic proteins that sense various intracellular pathogens and microbial by-products.[2,5,6] Among them, considerable attention has been focused on nucleotide-binding oligomerisation domain 2 (NOD2) that was identified as the first susceptibility gene for Crohn's disease.[7] NOD2 detects muramyl dipeptide (MDP), a subunit of peptidoglycan from the cell wall of Gram-positive and Gram-negative bacteria. Studies have demonstrated that NOD2 co-operates with TLRs in shaping the host response to bacteria to increase the sensitivity for pathogen detection and potentiate the cellular response against invading bacteria.[8–11] In particular, NOD2 activated by MDP was shown to act

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synergistically with TLR2 engaged by specific agonists. NOD2 activation potentiated TLR2-mediated production of pro- and anti-inflammatory cytokines by monocytes and macrophages.[12–15] NOD2 is up-regulated in human keratinocytes upon TLR2 engagement.[16] Collectively, these findings suggest that, during bacterial insult, various ligands, acting through cell surface TLR2 and cytoplasmic NOD2, act in synergy to potentiate the innate immune response and influence the nature and direction of the adaptive immune response.

Odontoblasts are neural crest-derived, dentin-producing, mesenchymal cells aligned at the periphery of the connective tissue located in the centre of the tooth, the dental pulp. They become exposed to cariogenic oral bacteria as these progressively demineralize enamel then dentin to gain access to the pulp. Due to their situation at the dentin–pulp interface, odontoblasts are the first cells encountered by invading pathogens and/or their released components, and represent, in the tooth, the first line of cellular defense for the host. More centrally located pulp fibroblasts are subsequently challenged by bacteria.[17–20] We have shown that *in vitro* differentiated human odontoblasts express *TLR1–6* and *TLR9* genes.[17] Toll-like receptor 2 can be activated by its own ligand, lipoteichoic acid (LTA), a cell wall component from Gram-positive bacteria. Toll-like receptor 2 engagement leads to an increase in *TLR2* expression, TLR2 protein accumulation in the cell membrane, nuclear factor (NF)-κB nuclear translocation, chemokine production and immature dendritic cell recruitment.[17–19,21] We also found that odontoblasts and dental pulp fibroblasts differ in their response to LTA by differential up-regulation of TLR2, production of chemokines, and ability to attract dendritic cells.[18] In particular, TLR2 was up-regulated by LTA in odontoblasts but not in pulp fibroblasts. To date, the existence of interplay between NOD2 and TLR pathways in both cell types challenged with Gram-positive bacteria is unknown.

The aim of this study was to investigate *NOD2* gene expression in healthy and inflamed human dental pulps and determine whether *NOD2* gene and NOD2 protein levels are modified in odontoblasts upon TLR2 engagement through LTA. Odontoblast response was compared to that of dental pulp fibroblasts. Toll-like receptor 2 engagement was confirmed in odontoblasts by blocking experiments and cell stimulation with the synthetic TLR2 agonist Pam_2CSK_4.

Materials and Methods

Dental pulp samples

Molars were collected with informed consent of the patients, in accordance with the Declaration of Helsinki and following a protocol approved by the local ethics committee. Inflamed pulps were taken from decayed teeth with clinical features of irreversible acute pulpitis (deep dentin caries lesions, severe spontaneous dental pain for at least 12 h, no sensitivity to vertical or horizontal percussion, lack of peri-apical lesions) and in the absence of anti-inflammatory treatment.

Reagents

Purified *Staphylococcus aureus* LTA was purchased from InvivoGen (Toulouse, France). Nuclear factor-κB-secretory embryonic alkaline phosphatase reporter gene assays performed by the TLR screening service of InvivoGen showed that LTA activates human embryonic kidney (HEK) 293 cells stably transfected with a TLR2-expressing vector from a concentration of 0.1 μg/ml. It failed to activate HEK293 cells transfected with TLR4- or NOD2-expressing vectors in the 0.01–10 μg/ml range, indicating that the preparation was devoid of contaminants able to activate TLR4- and NOD2-mediated pathways either directly or significantly. Mouse anti-TLR2 blocking monoclonal antibody (clone T2.5) and the synthetic TLR2 agonist Pam_2CSK_4 were from InvivoGen. Mouse IgG1 isotype (MOPC21) was from Sigma-Aldrich (St Louis, MO, USA).

Cell stimulation

Odontoblast-like cells and pulp fibroblasts were obtained from non-erupted healthy human third molars by culturing pulp explants as described.[22] After 4 weeks, cultures were stimulated for 1, 2, 4 or 8 h with 1 μg/ml or 10 μg/ml LTA. To confirm TLR2 engagement in odontoblast-like cells, inhibition experiments were performed by adding 20 μg/ml anti-TLR2 antibody to the culture medium 1 h before stimulating cells with 10 μg/ml LTA for 4 h. Control was performed using the mouse IgG1 isotype at the same concentration. Odontoblast-like cells were also treated for 4 h with 1 μg/ml or 10 μg/ml Pam_2CSK_4.

Real-time PCR

Ribonucleic acid extraction, reverse transcription and real-time PCR were performed essentially as described.[21] Total RNA was extracted from healthy and inflamed dental pulps and from stimulated and control odontoblast-like cell and pulp fibroblast cultures with a Nucleospin RNA II kit (Macherey-Nagel; Düren, Germany) according to the manufacturer's instructions. Ribonucleic acid samples (1 μg) were then converted to first-strand cDNAs using 500 ng oligo$(dT)_{15}$ primers

Table 1. Primers used for real-time PCR analysis

Gene	Forward primer	Reverse primer	Annealing temperature
TNF-α	GGCGTGGAGCTGAGAGATAAC	GGTGTGGGTGAGGAGCACAT	64°C
NOD2	TCAGTTAAGCCTTTGGAAACAG	CATCAACCAGAAGCCTAGTGAG	70°C
CXCL8	CTGGCCGTGGCTCTCTTG	CCTTGGCAAAACTGCACCTT	68°C
Cyclophilin A	ATGGCACTGGTGGCAAGTCC	TTGCCATTCCTGGACCCAAA	58°C

(Roche Diagnostics; Meylan, France) and SuperScript III Reverse Transcriptase (Invitrogen Life Technologies; Grand Island, NY, USA). Real-time PCR was performed in a Light Cycler instrument (Roche) with the FastStart DNA Master SYBR Green I kit according to the manufacturer's specifications. All runs were performed in duplicate. NOD2 primer sequences were obtained from Dr B. Sperandio (Institut Pasteur, Paris). Cyclophilin A house-keeping gene was used for sample normalization. Analysis of tumor necrosis factor-α (*TNF-α*) gene expression was performed to confirm the pulp inflammatory state. *CXCL8* gene expression was used to monitor odontoblast-like cell immune response to LTA in inhibition as well as Pam_2CSK_4 stimulation experiments. Primer sets and annealing temperatures are listed in Table 1. For each target gene, relative expression was determined after normalization using the RelQuant software (Roche). Results were expressed as fold-change values relative to healthy pulp for *in vivo* analysis and to unstimulated control samples for *in vitro* analysis.

Flow cytometry

Cells were obtained following trypsin/EDTA treatment of cultures then stained in Fix&Perm reagent (Invitrogen) for 30 min with anti-human NOD2 rat monoclonal antibody 6F6.[23] Staining was revealed by goat anti-rat IgG-FITC (Invitrogen). Negative controls were performed with isotype-matched rat IgGs (Vector laboratories; Burlingame, CA, USA). Data were acquired on a Dako cytometer and analyzed with WinMDI v.2.8 software (Scripps Institute; La Jolla, CA, USA).

Statistical analysis

Results were expressed as mean values ± SD obtained from five healthy pulps and five inflamed pulps from different donors for *in vivo* analysis, and from three odontoblast-like cell and three pulp fibroblast cultures for *in vitro* experiments. Statistical analysis was determined with Student's *t*-test.

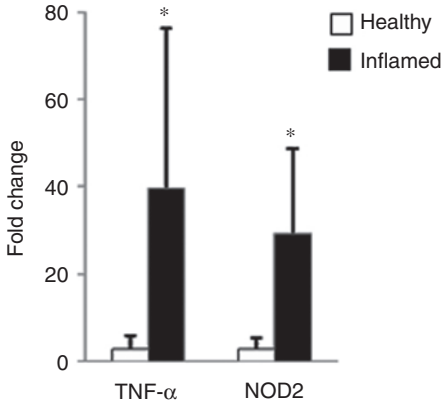

Fig. 1. *TNF-α* and *NOD2* gene expression is up-regulated in inflamed human dental pulps compared to healthy ones. Ribonucleic acid was extracted and retrotranscribed, then gene expression was analyzed by real-time PCR. Results were normalized to the cyclophilin A gene and are expressed as fold-change values relative to healthy pulps. Data represent the mean ± SD obtained from five healthy and five inflamed pulps from 10 different donors. $*P = 0.05$.

RESULTS

A significant up-regulation of *TNF-α* and *NOD2* genes (14.8- and 10-fold, respectively) was observed in pulps affected by acute inflammation compared to healthy ones (Fig. 1). Analysis of *NOD2* gene regulation *in vitro* showed that addition of 1 μg/ml LTA had no effect on odontoblast-like cells and pulp fibroblasts, but that stimulation with 10 μg/ml LTA augmented *NOD2* gene expression in a time-dependent manner (Fig. 2). Expression significantly increased after 2 h of LTA stimulation in odontoblast-like cells and pulp fibroblasts. It was maximal in both cell types after 4 h. The mRNA level of *NOD2* was significantly higher in odontoblast-like cells than in pulp fibroblasts after 4 h of LTA stimulation (15.9-fold versus 6.6-fold; $P = 0.05$). Adding anti-TLR2 antibody to odontoblast-like cell cultures 1 h before stimulating cells with 10 μg/ml LTA for 4 h inhibited LTA-induced increase in *CXCL8* and *NOD2* gene expression by 84.6% and 86%, respectively (Fig. 3). Control antibody did not affect *CXCL8* and *NOD2* gene up-regulation by LTA. Addition of 1 μg/ml or 10 μg/ml Pam_2CSK_4 markedly increased *CXCL8* and *NOD2* mRNA levels after 4 h of stimulation (Fig. 4).

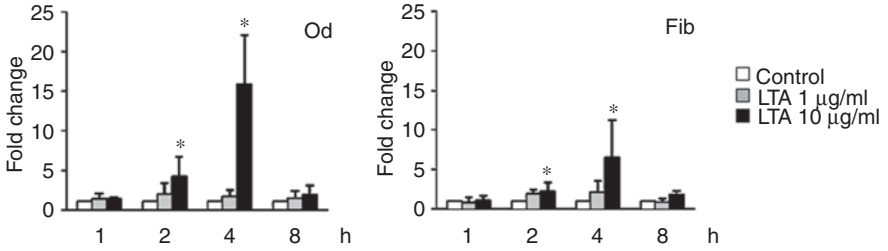

Fig. 2. Expression of *NOD2* gene in LTA-stimulated odontoblast-like cells and dental pulp fibroblasts. Cells were incubated with 1 µg/ml or 10 µg/ml LTA for the indicated times. Ribonucleic acid was extracted and retrotranscribed, then gene expression was analyzed by real-time PCR. Results were normalized to the cyclophilin A gene and are expressed as fold-change values relative to control unstimulated cells. Data represent the mean ± SD obtained from three independent experiments. $*P = 0.05$. Od, odontoblast-like cells; Fib, fibroblasts.

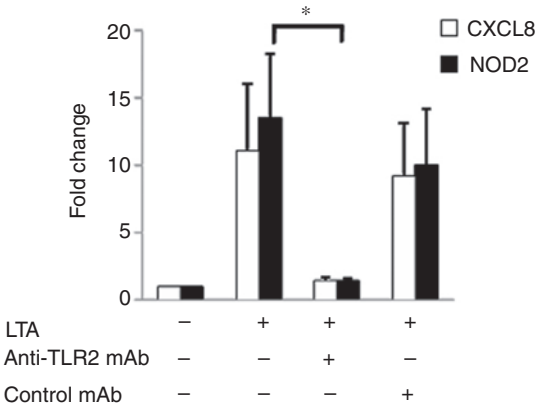

Fig. 3. Effect of TLR2 inhibition on *CXCL8* and *NOD2* gene expression in odontoblast-like cells. Anti-TLR2 blocking monoclonal antibody was added to odontoblast-like cell cultures 1 h before stimulating cells with 10 µg/ml LTA for 4 h. Mouse IgG1 isotype was used in controls. Ribonucleic acid was extracted and retrotranscribed, then gene expression was analyzed by real-time PCR. Results were normalized to the cyclophilin A gene and expressed as fold-change values relative to control unstimulated cells. Data represent the mean ± SD obtained from three independent experiments. $*P = 0.05$.

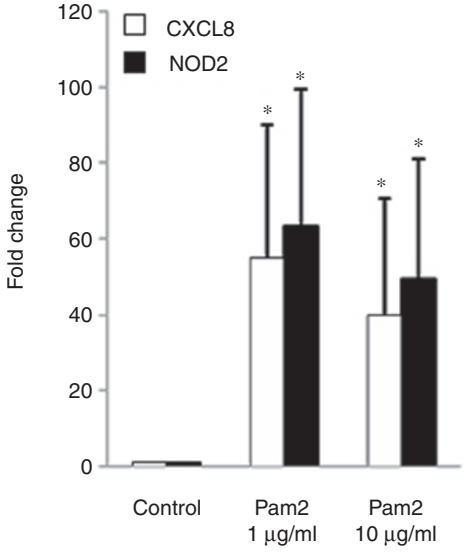

Fig. 4. Pam_2CSK_4 induces increase of *CXCL8* and *NOD2* gene expression in odontoblast-like cells. Cells were incubated with 1 µg/ml or 10 µg/ml Pam_2CSK_4 for 4 h. Ribonucleic acid was extracted and retrotranscribed, then gene expression was analyzed by real-time PCR. Results were normalized to the cyclophilin A gene and are expressed as fold-change values relative to control unstimulated cells. Data represent the mean ± SD obtained from three independent experiments. $*P = 0.05$.

At the protein level, a clear NOD2 up-regulation was observed in odontoblast-like cells stimulated with 10 µg/ml LTA but not in pulp fibroblasts (Fig. 5).

Discussion

In this study, we demonstrated that NOD2 expression is increased upon TLR2 engagement by LTA in human odontoblast-like cells. Lipoteichoic acid stimulated NOD2 expression at high concentration (10 µg/ml), which might raise the concern of possible LTA contamination by NOD2 or TLR4 ligands such as MDP or LPS.[13,24] However, routine NF-κB–alkaline phosphatase reporter gene assays indicated that the purified LTA used in this study only activated HEK293 cells transfected with a TLR2-expressing vector, from a concentration of 0.1 µg/ml. It failed to activate HEK293 cells stably transfected with TLR4- or NOD2-expressing vectors in the 0.01–10 µg/ml range, indicating that the preparation was devoid of contaminants able to activate, directly and significantly, TLR4- and NOD2-mediated pathways. Blocking TLR2 in odontoblast-like cells had a clear inhibitory effect on LTA-induced *NOD2* gene expression, and the synthetic TLR2 agonist Pam_2CSK_4 induced a marked up-regulation of *NOD2* mRNA level compared to LTA. Taken together, these data confirmed that the effect observed was due to TLR2 engagement. We previously showed that LTA concentrations starting from 1 µg/ml can activate TLR2 in odontoblast-like

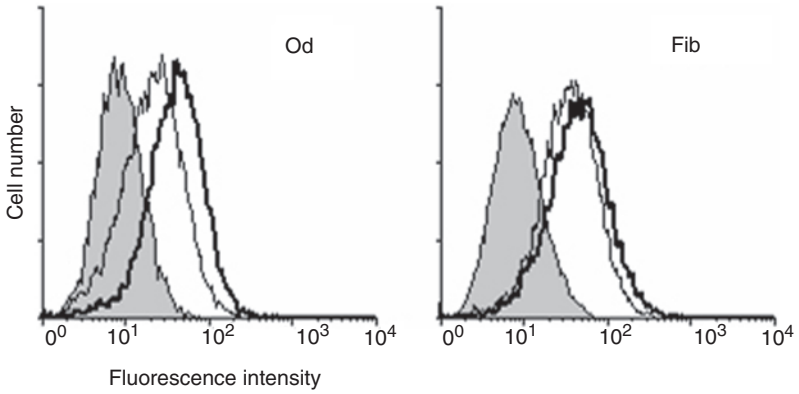

Fig. 5. Detection of NOD2 protein in LTA-stimulated odontoblast-like cells and dental pulp fibroblasts by flow cytometry. Cells were obtained following trypsin/EDTA treatment of cultures and examined for NOD2 protein in the absence (regular line) or the presence of 10 μg/ml LTA (bold line) for 16 h. Filled histograms denote isotype controls. Histograms shown are representative of two independent experiments. Od, odontoblast-like cells; Fib, fibroblasts.

cells, which is sufficient to induce TLR2 up-regulation and accumulation to the cell membrane, chemokine production and attraction of immature dendritic cells.[17–19,21] In the present study, a higher concentration (10 μg/ml) was required to increase NOD2 expression significantly. This suggests that NOD2 is engaged by LTA when the amount of Gram-positive bacteria which come into contact with odontoblasts is high and/or when peptidoglycan-released MDPs are present in high amount in the odontoblast extracellular milieu. NOD2 protein up-regulation in pulp fibroblasts was very weak, indicating a higher responsiveness of odontoblasts to TLR2 ligands, as we previously demonstrated.[18] It is likely that the NOD2 protein level is tightly controlled within odontoblast-like cells. Indeed, a decreased tendency of *NOD2* gene expression was observed in odontoblast-like cells between 4–8 h of stimulation with LTA, or when the Pam_2CSK_4 concentration is increased from 1 μg/ml to 10 μg/ml. This suggests that feedback mechanisms are rapidly triggered after *NOD2* gene up-regulation to maintain a moderate level of intracellular NOD2 protein, possibly as long as its ligand MDP has not been detected in the cytosol. Further experiments are necessary to confirm this hypothesis.

NOD2 and TLR2 engagement induce secretion of pro- and anti-inflammatory cytokines and chemokines.[8–11] We have previously shown that chemokines including CXCL1, CXCL2, CXCL8, CXCL10 and CCL2 are up-regulated in odontoblast-like cells stimulated by LTA. Conversely, TGF-β1 is down-regulated, which has led us to suggest that activation of TGF-β1 and TLR2 signaling pathways in odontoblasts has antagonistic effects, as shown in other cell types.[17] A recent paper has confirmed this hypothesis by showing that TGF-β1 negatively regulates TLR2 expression and resulting CXCL8 secretion in two odontoblast-like cell clones derived from human dental pulp cells.[25] Whether NOD2 engagement contributes to chemokine up-regulation and/or TGF-β1 down-regulation in odontoblasts requires further investigation.

We recently showed that healthy human dental pulp expresses the 10 PRMs of the TLR family and thus is well-equipped to sense pathogens that gain access to the pulp.[19] We report in this study that another PRM, NOD2, is expressed in healthy dental pulp and up-regulated in inflamed pulps challenged by cariogenic bacteria. This suggests that NOD2 is involved in the pulp response to pathogens that invade enamel, then dentin, during the caries process. A recent immunohistochemical analysis of healthy human dental pulp showed that odontoblasts express NOD2.[26] Therefore the authors suggested that these cells might be able to respond, through this PRM, to bacteria that gain access to the pulp from infected dentin. The TLR2 ligand-induced up-regulation of NOD2 we report in this study might be part of this response and represent a modulatory mechanism by which odontoblasts sense pathogens and provide additional protection to the host against bacterial invasion.

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Jean-François Keller and Florence Carrouel contributed equally to this work.

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