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Differential regulation of immune responses by odontoblasts

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Odontoblasts (OBs) are cells lining the inner surface of the tooth. Their potential role in host defenses within the tooth is suggested by their production of antimicrobial β-defensins, but their role needs confirmation. The present study sought to define the roles of human OBs in microbial recognition and innate host responses. Toll-like receptor 2 (TLR2) and TLR4, as well as CCR6, were immunolocalized in human OBs and their dentinal processes *in situ*. To examine OB function we used organotypic tooth crown cultures to maintain human OBs within their dentin scaffold. Cells in the OB layer of cultured and non-cultured crown preparations expressed mRNA for several markers of innate immunity including chemokine CCL20, chemokine receptor CCR6, TLR2, TLR4 and the OB marker dentin sialophosphoprotein (DSPP). Expression of human β-defensin 1 (hBD1), hBD2, hBD3, interleukin-8 (IL-8), and CCL20 increased with time in culture. Tooth crown odontoblast (TcOB) cultures were stimulated with agonist that was specific for TLR2 (Pam3CSK4) or TLR4 [*Escherichia coli* lipopolysaccharide (LPS)]. Nuclear factor-κB assays confirmed the TLR2 activity of Pam3CSK4 and the TLR4 activity of LPS. LPS up-regulated IL-1β, tumor necrosis factor-α (TNF-α), CCL20, hBD2, IL-8, TLR2 and TLR4; however, Pam3CSK4 down-regulated these mRNAs. IL-1β, TNF-α, CCL20 were also up-regulated from six-fold to 30-fold in TcOB preparations from decayed teeth. Our results show for the first time that OBs express microbial pattern recognition receptors *in situ*, thus allowing differential responses to gram-positive and gram-negative bacteria, and suggest that pro-inflammatory cytokines and innate immune responses in decayed teeth may result from TLR4 signaling.

Key words: β-defensins; chemokines; dentin; proinflammatory cytokines; toll-like receptors; tooth

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Dental caries and tooth pulp infection are major oral health problems worldwide (35). Innate immunity is the first line of defense against infection, but knowledge about innate immune mechanisms in teeth is still incomplete. The injury to the dental pulp caused by dental caries is unusual in that toxins reach the tissue well ahead of the bacteria that release them. Tooth pulp inflammation can be irreversible once infection reaches the pulp, so that especially effective defense mechanisms are needed to prevent or arrest pulpitis. Several cell types contributing to innate and adaptive immunity are present in the tooth pulp, such as lymphocytes, macrophages and dendritic cells (DCs) (22), and some types of dental innervation and neuropeptides also seem to be involved (4, 51).

Odontoblasts (OBs) make and maintain dentin but they may also be involved in immune defense for the following reasons. First, they are located at the pulp–dentin interface and extend their cell processes far into the dentin where they are the first cell to be encountered by microorganisms or their products, which can easily penetrate through enamel and dentin (5, 50, 52, 53). Second, they have been shown to express antimicrobial peptides (9). Third, they make a partially impermeable barrier as a pseudoepithelial layer (2, 19, 48), and therefore might have innate immune functions similar to those of oral epithelia (7). Fourth, as with epithelia, OBs are closely associated with DCs and T lymphocytes, and all three cell types can respond to caries (56) or dentin injury (36) when transforming growth factor-β1 is released from the damaged dentin (12, 42). Fifth, the OBs may also induce neutrophil chemotaxis *in vivo* via their production of interleukin-8 (IL-8) in response to bacterial lipopolysaccharide (LPS) (27). Thus, a range of OB actions and expressions has been identified, suggesting that they may play a major role in the immune defense of teeth.

Microbial pattern recognition receptors are essential for mammalian innate immunity. These receptors include toll-like receptors (TLRs), LPS-binding protein, peptidoglycan recognition proteins, nucleotide-binding oligomerization domains, CD14, scavenger receptors and C-type lectin which enable mammalian cells to differentially recognize highly conserved microbial structures and consequently mediate innate host responses (6, 13, 39, 41, 46, 49). TLRs constitute a major class of microbial recognition receptors. Their activation regulates the production of antimicrobial peptides, cytokines and chemokines as well as their receptors and consequently controls leukocyte trafficking, T-cell function, and recruitment and maturation of DCs, thereby providing a bridge between innate and adaptive immunity. There are 10 members of the TLR family in humans (1, 46). TLR2 alone or TLR2 heterodimerizing with either TLR1 or TLR6 is crucial for the recognition of gram-positive bacterial cell wall components, including lipoteichoic acid and lipopeptide whereas TLR4 plays a major role in detecting LPS from gram-negative bacteria (1, 46). Dental caries contains both gram-positive and gram-negative bacteria. Gram-positive bacteria (*Streptococcus* and *Lactobacillus* spp.) are common oral microflora, detected in shallow caries or the outer dentinal tubules of deep caries. In contrast, gram-negative bacteria (*Fusobacterium*, *Porphyromonas* and *Prevotella* spp.) are found in deep caries and in the infected root canals. These gram-negative bacteria are associated with pulpal and periradicular inflammation (32, 45).

We have tested the hypotheses that OBs, in a way that is analogous to epithelial cells, form the first line of defense for the tooth pulp by producing antimicrobial peptides, cytokines and chemokines and that these cells have receptors that allow them to recognize tooth-invading microbes, and to initiate antimicrobial capabilities and associated signaling to cells of adaptive immunity. We also propose that OBs can differentially recognize and respond to gram-positive and gram-negative bacteria via expression and utilization of TLR2 and TLR4. Here we employed organotypic tooth crown cultures that predominantly contained human OBs, maintained on their own dentin scaffold. Because this tooth crown odontoblast (TcOB) culture system is relatively new, we have also defined the TcOB cultures to understand the differences between OBs *in vivo* and *in vitro*. We therefore compared cell population, morphology and expression of several innate immune markers in the TcOB cultures before and after culture. Various markers of innate immunity, known to be regulated through TLRs and showing the potential role of OBs in host defenses, were examined here. We also used immunostaining to determine the *in situ* expression of TLR2 and TLR4 as well as CCR6 receptors in human OBs. Finally, we used highly purified ligands specific for TLR2 (Pam3CSK4) or TLR4 (purified *Escherichia coli* LPS) to exclusively activate either TLR2 or TLR4 and tested for differential functions of these receptors in human TcOB cultures. We relate these findings to differences in the expression of innate immune products by TcOB preparation of decayed vs. normal teeth.

Materials and methods

Tooth crown odontoblast (TcOB) preparation and culture

Freshly extracted third molars were collected from patients in the Oral Surgery clinic with consent following an approved protocol of the University of Washington Human Subjects Review Board. Teeth were kept in cold sterile phosphate-buffered saline (PBS) until processed for the TcOB preparation as previously described (27, 34, 47). The crown portion was rinsed with 5.25% NaOCl, then sterile water, and the periodontal tissues were removed under a dissecting microscope. The tooth was rinsed with 70% alcohol, then PBS, and was submerged in PBS while a horizontal groove was made at 1–2 mm above the roots, avoiding pulp exposure (Fig. 1A). The roots were then split off and the loose core of pulp tissue was pulled out (Fig. 1B), leaving the OBs attached to the dentin scaffold (Fig. 1B,C).

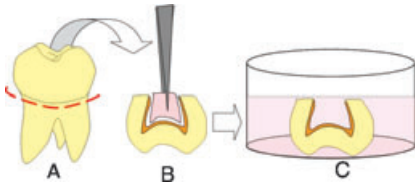

Fig. 1. Tooth crown odontoblast culture. The organotypic tooth crown culture technique was adapted from Tjaderhane et al. (47) to use the stable dentin structure as a scaffold to retain primary human odontoblasts (orange layer in B and C) in culture. Roots were split off at 1–2 mm above the root separation level (red dotted line, A) and the pulp connective tissue core was pulled out using plain forceps with extra fine points (B). The odontoblast layer (orange) remained attached to the dentin scaffold and was maintained in culture (C).

Each TcOB preparation was cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 without HEPES (Gibco, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1 μg/ml vitamin K1 (Sigma-Aldrich, St Louis, MO), 50 μg/ml ascorbic acid (Sigma-Aldrich), 100 IU/ml penicillin G, 100 μg/ml streptomycin sulfate (Gibco), 0.3 μg/ml fungizone (Gibco), and 1% 100× insulin–transferring–selenium-X (Gibco). TcOB cultures were incubated at 37°C in a 5% CO_2 atmosphere for 24 h before stimulation (Fig. 1C). All teeth for culture ($n = 50$) were free of decay. Additional teeth with moderate to deep decay (penetrating one-half to two-thirds of the way through the dentin, $n = 16$) and non-decayed controls ($n = 16$) were similarly prepared for TcOB preparation and immediately processed for RNA extraction.

Activation of TLR2 or TLR4

All reagents were verified to be endotoxin-free (<0.03 endotoxin U/ml) using the *Limulus* amebocyte lysate assay (Pyrotell Associates, Falmouth, MA). Pam3CSK4, a synthetic tripalmitoylated lipopeptide (InvivoGen, San Diego, CA) was used as TLR2 agonist. *E. coli* LPS serotype 0127:B8 (Sigma-Aldrich) was re-purified with phenol extraction as previously described (18) and used as a TLR4 agonist because of its specificity for TLR4. The activity of Pam3CSK4 via TLR2 and *E. coli* LPS through TLR4 was confirmed by nuclear factor-κB assay using HEK 293 cells, transfected with TLR (TLR2 and TLR1 or TLR4) and accessory protein constructs (membrane CD14 and MD2) and the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) as previously described (8, 17). The TLR2+1-expressing HEK293 cells and TLR4-expressing cells were then stimulated with either Pam3CSK4 (0, 10, 100, 1000 ng/ml) or LPS (0, 1, 10, 100, 1000 ng/ml) for 4 h, lysed and measured for luciferase activities. The assays were performed in triplicate.

Stimulation of TcOB cultures

TcOB cultures were unstimulated, LPS-stimulated or Pam3CSK4-stimulated. Each of an unstimulated and a stimulated TcOB pair was from the same donor ($n = 10$ pairs). TcOBs were stimulated with either 100 ng/ml Pam3CSK4 or 100 ng/ml LPS for 24 h. If only one tooth was available

from a donor, cultured TcOB was left unstimulated or RNA was immediately isolated without culture.

RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR) amplification

Total RNA was isolated from the TcOBs with Trizol® Reagent (Invitrogen, Carlsbad, CA) and a Picopure RNA isolation kit (Arcturus, Mountain View, CA). DNase treatment was performed using RNase-free DNase (Qiagen, Valencia, CA). The cDNA was synthesized from 0.5 μg total RNA using a RETROscript® kit (Ambion, Austin, TX) and amplified using 2 μl cDNA in a 25-μl final volume. Specific primers for innate immune products, dentin sialophosphoprotein (DSPP), human leukocyte antigen-DR (HLA-DR), and housekeeping genes [ribosomal phosphoprotein (RPO) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] were as follows: hBD1 sense, 5′-CAT GAG AAC TTC CTA CCT TCT GC-3′; hBD1 antisense, 5′-TCA CTT GCA GCA CTT GGC CTT-3′; hBD2 sense, 5′-CCA GCC ATC AGC CAT GAG GGT-3′; hBD2 antisense, 5′-GGA GCC CTT TCT GAA TCC GCA-3′; hBD3 sense, 5′-CAG TCT CAG CGT GGG GTG AAG-3′; hBD3 antisense, 5′-CAA CAC TCT CGT CAT GTT TCA GGG-3′; another hBD3 sense, 5′-AGC CTA GCA GCT ATG AGG ATC-3′; another hBD3 antisense, 5′-CTT CGG CAG CAT TTT GCG CCA-3′; IL-8 sense, 5′-TTT CTG ATG GAA GAG AGC TCT GTC TGG-3′; IL-8 antisense, 5′-AGT GGA ACA AGG ACT TGT GGA TCC TGG-3′; CCL20 sense, 5′-TGG GCT ATG TCC AAT TCC AT-3′; CCL20 antisense, 5′-GCA AGC AAC TTT GAC TGC TG-3′; IL-1β sense, 5′-AAA AGC TTG GTG ATG TCT GGT CCA-3′; IL-1β antisense, 5′-AGG AGA TCC TCT TAG CAC TAC CCT AAG-3′; tumor necrosis factor-α (TNF-α) sense, 5′-TTC TGC CTG CTG CAC TTT GGA GTG AT-3′; TNF-α antisense, 5′-TTG ATG GCA GAG AGG AGG TTG ACC TT-3′; CCR6 sense, 5′-CCT GGG GAA TAT TCT GGT GGT GA-3′; CCR6 antisense, 5′-CAT CGC TGC CTT GGG TGT TGT AT-3′; TLR2 sense, 5′-GGC CAG CAA ATT ACC TGT GTG-3′; TLR2 antisense, 5′-CCA GGT AGG TCT TGG TGT TCA-3′; TLR4 sense, 5′-CTG CAA TGG ATC AAG GAC CA-3′; TLR4 antisense, 5′-TCC CAC TCC AGG TAA GTG TT-3′; HLA-DR sense, 5′-CCC AAC GTC CTC ATC TGT TT-3′; HLA-DR antisense, 5′-TCA CCT CCA TGT GCC TTA CA-3′; DSPP sense, 5′-GAG GAT AAA GGA CAA CAT GG-3′, DSPP antisense, 5′-AAG AAG CAT CTC CTC GGC-3′; RPO sense, 5′-AGC AGG TGT TCG ACA ATG GCA-3′; RPO antisense, 5′-ACT CTT CCT TGG CTT CAA CCT-3′; GAPDH sense, 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′; GAPDH antisense, 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′; CD14 sense, 5′-TAA AGG ACT GCC AGC CAA GC-3′; CD14 antisense, 5′-AGC CAA GGC AGT TTG AGT CC-3′, MD2 sense, 5′-TTC CAC CCT GTT TTC TTC CA-3′; MD2 antisense 5′-AAT CGT CAT CAG ATC CTC GG-3′. PCR products were visualized by gel electrophoresis using a 1.5% agarose gel with 0.002% ethidium bromide. Gel pictures were taken and the band density of each PCR product was quantified by using the AlphaEase®FC Imaging system and software (Alpha Innotech, San Leandro, CA). For each sample, the relative expression of each marker was determined relative to the housekeeping gene as shown in Fig. 6.

Immunohistochemistry

Six freshly extracted intact human molars were fractured into two halves and immediately fixed with 4% paraformaldehyde in 0.1 M PBS pH 7.4 overnight. Samples were embedded into impression compound (Kerr, Romulus, MI) and cut into 1- to 2-mm-thick slices using a hard-tissue-sectioning saw microtome (Leica SP1600, Bannockburn, IL). Non-cultured tooth slices and 2-day-cultured TcOB were similarly fixed and decalcified with 10% neutral ethylenediaminetetraacetic acid (EDTA) at 4°C for 1–3 months. The decalcified samples were rinsed with PBS and Sorensen's buffer, submerged in 8.5% sucrose in Sorensen's buffer overnight, and embedded in a freezing medium (Tissue-Tek® OCT, Sakura Finetek, Torrance, CA). The samples were cut into 16- to 20-μm-thick sections, and processed for diaminobenzidine immunohistochemistry or double immunofluorescence.

Immunohistochemistry

Floating sections were blocked for endogenous peroxidase and then blocked with 2.5% normal serum (species-matched to secondary antibody) and 2.5% normal human serum in Tris-buffered saline (TBS) for 1 h. Primary antibodies were goat anti-human TLR2 immunoglobulin G (IgG; 4 μg/ml; Capralogics, Hardwick, MA), rabbit anti-human TLR2 IgG (2 μg/ml; ProSci, Poway, CA), rabbit anti-human TLR4 IgG (2 μg/ml; Santa Cruz Biotech, Santa Cruz, CA), goat anti-human CCR6 IgG (4 μg/ml; Capralogics) and mouse anti-HLA-DR (0.86 μg/ml; Zymed, South San Francisco, CA). Specific blocking peptides for TLR2 and CCR6 or non-immunoreactive rabbit IgG were also used to verify the specificity of the staining. The sections were rinsed with TBS and incubated with biotinylated goat anti-rabbit or horse anti-goat IgG (3 μg/ml; Vector Laboratories, Burlingame, CA) plus 2.5% normal serum and 2% normal human serum in TBS at room temperature for 90 min. Antibody binding was detected with avidin–biotin–peroxidase (ABC reagents; Vector, Burlingame, CA) using nickel ammonium sulfate and diaminobenzidine substrate. Reacted sections were then mounted, stained, dehydrated and covered. Omission of primary antibody was used as a negative control.

Double immunofluorescence

Sections were blocked for biotin and avidin (Vector Laboratories) and processed for Texas Red-immunolabeling of HLA-DR (second antibody detected with a streptavidin-Texas Red, 3.33 μg/ml; Vector) and for fluorescein isothiocyanate-immunolabeling of TLR2, TLR4 or CCR6 using the primary antibodies listed above. Secondary antibodies were biotinylated donkey anti-mouse IgG (5 μg/ml; Jackson ImmunoResearch, West Grove, PA), goat anti-mouse IgG (5 μg/ml; Vector Laboratories), fluorescein isothiocyanate-conjugated donkey anti-goat IgG (5 μg/ml; Jackson ImmunoResearch), or goat anti-rabbit IgG (5 μg/ml; Vector Laboratories). Nuclei were labeled with 4′,6-diamidino-2-phenylindole for 10 min (0.4 μg/ml; Sigma-Aldrich). The sections were mounted and coverslipped with Prolong® (Molecular Probe, Eugene, OR). Multi-channel fluorescence images were collected with the SlideBook software (Intelligent Imaging Innovations, Inc., Denver, CO) on a Zeiss Axiovert 200 M microscope and Roper CoolSnap HQ digital camera (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Images from each set of antibody labeling and negative controls were captured using the same exposure time and deconvolved with similar parameters to remove out of focus blurred fluorescence.

Quantitative PCR

RNA samples from TcOB preparations of 16 normal and 16 decayed teeth were

separately pooled and used for quantitative PCR using a BioRad icycler (Hercules, CA). TNF-α and IL-1β primers were from SuperArray Bioscience (Frederick, MD). CCL20 and GAPDH primers were as follows: CCL20 sense, 5′-TTT ATT GTG GGC TTC ACA CG-3′; CCL20 antisense, 5′-GAT TTG CGC ACA CAG ACA AC-3′; GAPDH sense, 5′-CAA AGT TGT CAT GGA TGA CC-3′; GAPDH antisense, 5′-CCA TGG AGA AGG CTG GGG-3′. The cDNA was synthesized using a RETROscript® kit (Ambion) and amplified using SYBR Green PCR Master mix (SuperArray Bioscience), and 2 μM primers. PCR analyses were performed in triplicate. Melt-curve analysis and gel electropheresis confirmed a single specific PCR product from each primer pair. The threshold cycle number was determined during the early log phase of product accumulation at which the fluorescence clearly rises above background in a straight line. Quantification was performed using the comparative threshold cycle method as previously described (38) using the amplification efficiency determined for each primer pair, and compared to GAPDH. The results were presented as fold change of each marker in decayed vs. normal samples.

Results

Markers of innate immunity in the odontoblast layer of normal human teeth

Expression of several types of innate immune markers was assessed in TcOB preparations. These included antimicrobial peptides (hBD2, hBD2, and hBD3); chemokines (IL-8 and CCL20); pro-inflammatory cytokines (IL-1β and TNF-α); and receptors for hBDs and CCL20 (CCR6), lipoteichoic acid/lipopeptide (TLR2), or LPS (TLR4). PCR products for these markers were evaluated relative to the housekeeping gene RPO. TLR2, TLR4 and CCR6 were detected in freshly prepared tooth crowns. Culture for 24–48 h resulted in increased expression of several markers, possibly as a result of the presence of serum and growth factors in the culture media (Fig. 2, Table 1). Serum and growth factors stimulate mitogen-activated protein kinases under these conditions (14, 16) and may also affect the expression of hBD2 and IL-8, regulated through these pathways (23, 24, 40). The mRNAs of hBD1, hBD2 and hBD3 were rarely seen in non-cultured samples but were induced in more than half of the cultured samples (Table 1). IL-8 mRNA was weak or not expressed in normal non-cultured TcOBs but was strongly induced after culture for 24 and 48 h. CCL20 mRNA was detected in almost all of the samples initially but its expression level was highly up-regulated under culture conditions (Fig. 2, Table 1).

OBs and DCs are present in the OB layer of human teeth (Fig. 3A,C). We used

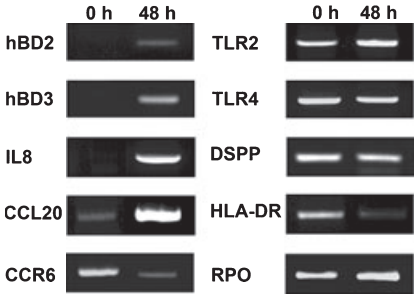

Fig. 2. Markers of innate immunity are present in the odontoblast layer of human tooth crowns. PCR products in the odontoblast layer of non-cultured and cultured tooth crown odontoblast preparations demonstrate up-regulation of hBD2, hBD3, IL-8 and CCL20 but down-regulation of CCR6 and HLA-DR (dendritic cell marker) after culture for 48 h. TLR2, TLR4, DSPP (odontoblast marker) and RPO (housekeeping gene) were highly expressed in both non-cultured and cultured samples.

Table 1. Expression of innate immune markers in the tooth crown odontoblast (TcOB) preparation

Markers of innate immunity	Normal TcOB[1]	
	Not cultured	Cultured
Antimicrobial peptides		
hBD1	4/11 weak	14/26
hBD2	1/11	17/28
hBD3	1/11	17/28
Chemokines		
IL8	3/11 weak	28/28
CCL20	10/11	28/28
Receptors		
CCR6	10/11	27/28
TLR2	11/11	25/28
TLR4	11/11	26/28
OB marker		
DSPP	11/11	28/28
Pulp DC marker		
HLA-DR	10/10	7/10 weak

[1]Number of positive samples containing a detectable PCR product out of total analysed.

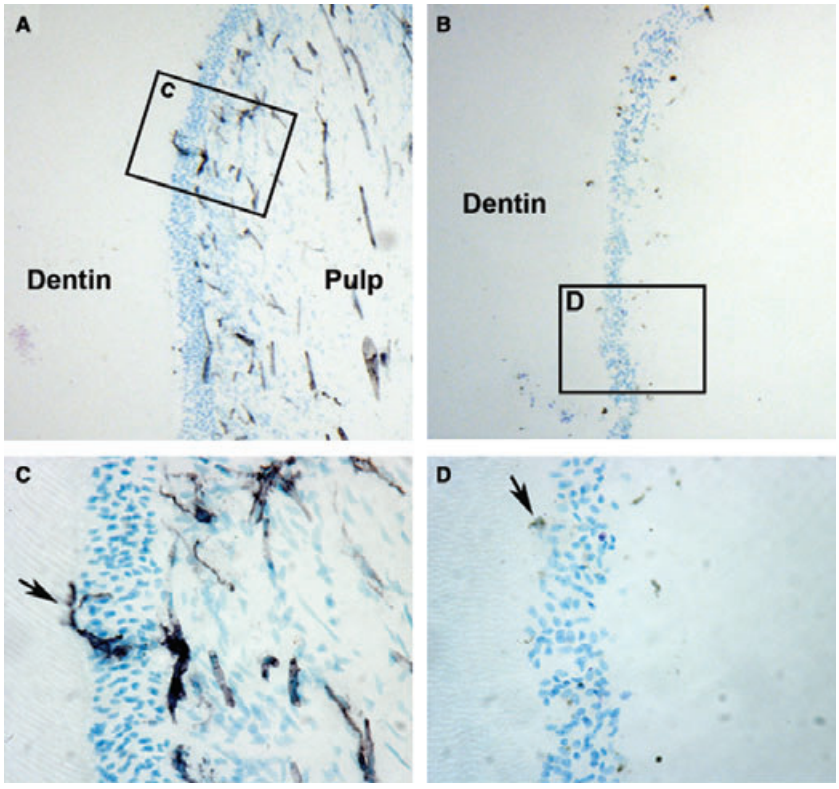

Fig. 3. HLA-DR-positive cells in the odontoblast layer of non-cultured tooth slice and after 2-day culture. Sections of non-cultured tooth slice (A, C) and cultured tooth crown odontoblast (TcOB) preparation (B, D) were stained with antibody to HLA-DR. Many HLA-DR-positive dendritic cells (DCs; black) were present in and below the odontoblast layer of the tooth slice. The cellular processes of some DCs in the odontoblast layer projected into the dentinal tubules (arrow in C). Note that the odontoblast layer was maintained in the TcOB culture but most of the underlying pulp was removed (B). After being cultured for 48 h, only a few HLA-DR-positive structures were retained in the TcOB (B,D) and their morphology (arrow in D) was different from that of DCs in non-cultured tooth slices (arrow in C) and likely to be cell debris.

DSPP as a marker for OBs in this study because DSPP is continuously and exclusively present in OBs but not in other cells of mature human teeth (37). DSPP was highly expressed in all non-cultured and cultured TcOB samples (Fig. 2, Table 1). HLA-DR was used as a marker for the presence of immunocompetent cells, particularly DCs. All non-cultured TcOBs expressed HLA-DR but expression of this marker decreased with culture (Fig. 2, Table 1). However, we found that cells floating in the culture media, which were collected and pooled from 10 TcOB cultures, had HLA-DR mRNA expression and this cell population is likely to include DCs. These results suggest that DCs migrate out of the cultured TcOBs, which is consistent with previous reports (26, 27).

Immunohistochemistry also showed that the OB layer was well preserved on the dentin scaffold but most of the underlying pulp was removed and OBs far outnumbered other cells in the TcOB cultures (Fig. 3B,D). Only a few DCs, underlying pulp cells and remnants of nerves and blood vessels persisted in a small area of the 2-day cultured TcOB (data not shown). HLA-DR-positive structures (arrow in Fig. 3D) in the OB layer of a 2-day TcOB culture were different from HLA-DR-positive DCs in non-cultured tooth slice (arrow in Fig. 3C). Taken together, these findings suggest that the TcOB culture model is largely composed of OBs. DCs and other pulp cells are only a minor population of the total. Thus, OBs are the major source of TLR2, TLR4 and DSPP, which are well expressed in both non-cultured and cultured TcOBs.

In situ expression of CCR6, TLR2 and TLR4 in OBs

CCR6, TLR2 and TLR4 protein expression was verified by immunohistochemistry. CCR6 was localized on the cell surfaces of human OBs (Figs 4A and 5A). Both immunoperoxidase and immunofluorescence demonstrated that TLR2 (Figs 4B and 5B) and TLR4 (Figs 4C and 5C) expression was on cellular processes and cell surfaces, suggesting a capacity of OBs to receive signals from gram-positive and gram-negative bacteria in tooth decay. The TLR2 and TLR4 immunoreactivity in the dentin and OB layer appeared relatively similar and was concentrated in the

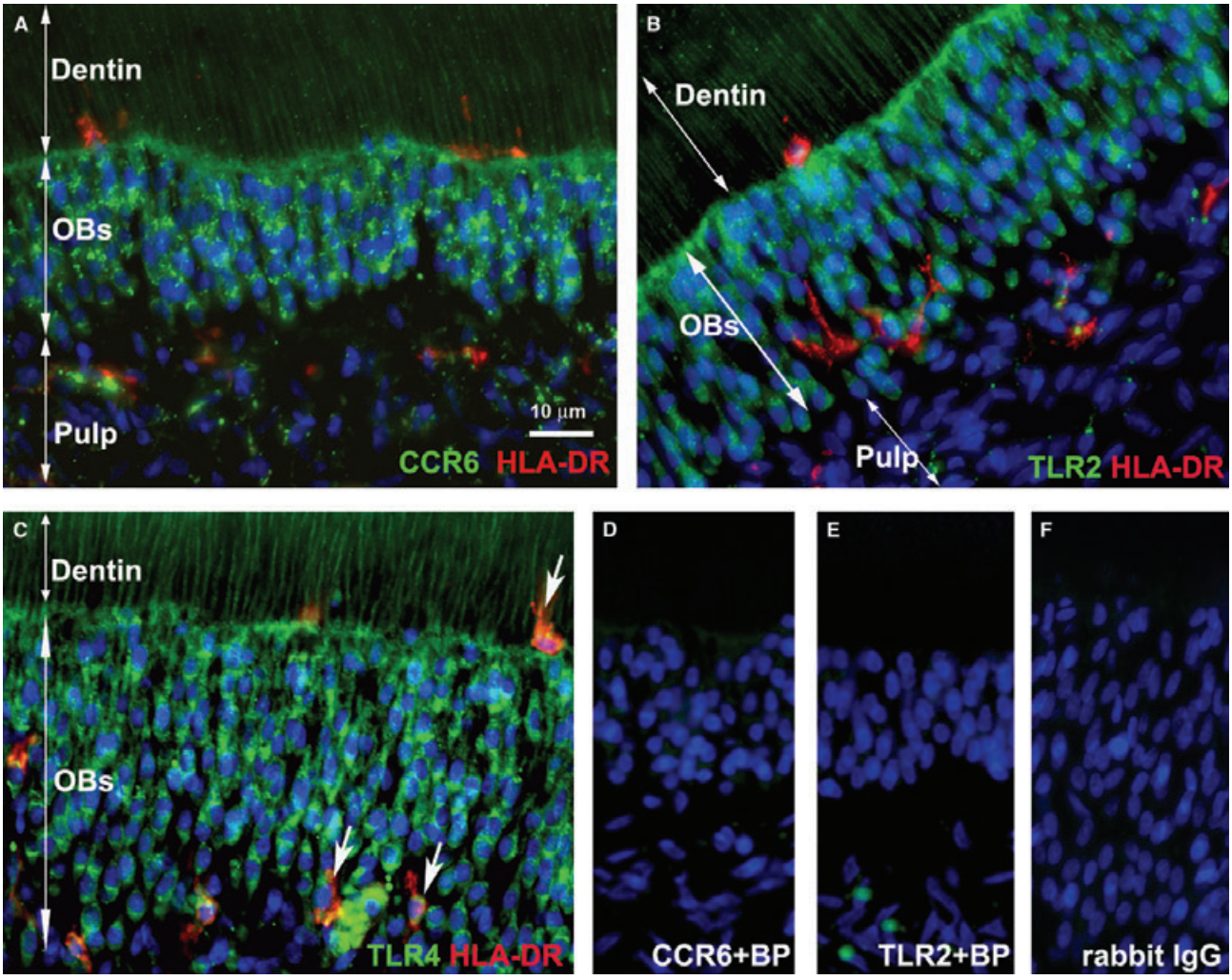

Fig. 4. CCR6, TLR2 and TLR4 were immunolocalized in human odontoblasts (OBs). *In vivo* expression of CCR6 (A; green), TLR2 (B; green), and TLR4 (C; green) was shown on the cell surfaces and cellular processes of human OBs in normal teeth. Staining of CCR6 and TLR2 on some dendritic cells (red; HLA-DR) and other pulp cells was very weak. Pre-incubation of primary antibody with the corresponding blocking peptide (BP) abolished CCR6 and TLR2 staining (D, goat anti-humanCCR6 IgG^+ BP; and E, goat anti-humanTLR2 IgG^+ BP). TLR4 staining was seen in some dendritic cells (arrows in C) as well as in OBs. No staining was seen on human tooth sections incubated with non-immunoreactive rabbit IgG (F). Cellular nuclei are shown in blue.

dentin–pulp interface area. A similar TLR2 staining pattern in OBs was shown by two TLR2 antibodies used in this study. Weaker CCR6, TLR2 and TLR4 staining was also observed in some DCs and in the underlying pulp cells. Specificity of staining for CCR6 and TLR2 was established by using blocking peptides (Fig. 4D,E). No staining was observed on control sections incubated with rabbit IgG (Fig. 4F) or without primary antibody (Fig. 5D).

Differential regulation of innate immune markers by Pam3CSK4 and *E. coli* LPS

To test whether TLR2 and TLR4 expressed in human OBs are functionally active, the TcOB cultures, enriched in OBs, were stimulated for 24 h with ligands for these receptors. We tested several bacterial products including *Enterococcus faecalis* lipoteichoic acid, and a widely used commercial *E. coli* LPS (Sigma-Aldrich). Consistent with previous findings (15, 18), we found that commercial lipoteichoic acid and LPS preparations were contaminated and activated both TLR2 and TLR4 (data not shown). To define exclusive function of each TLR, pure ligands were utilized to activate only TLR2 (Pam3CSK4, a synthetic lipopeptide) or TLR4 (purified *E. coli* LPS). To ensure the purity of these ligands, we determined that the Pam3CSK4 used in our study was endotoxin-free and activated only TLR2, with or without TLR1, whereas purified *E. coli* LPS mediated host responses only through TLR4 and not TLR2 (Fig. 6A).

The TcOB cultures showed differential responses to Pam3CSK4 and LPS (Fig. 6B). Because variation in the level of responses among different donors was evident, we utilized only tooth pairs from the same donor for the unstimulated control and the experimentally stimulated TcOBs. LPS increased mRNA expression for hBD2, IL8, CCL20 and TLR2; and for pro-inflammatory cytokines, IL-1β and TNF-α. In contrast, Pam3CSK4 decreased all of these mRNAs in four out of five samples. Variation in Pam3CSK4 responses was observed in one sample in which hBD2, CCL20 and IL-1β were increased. LPS and Pam3CSK4 both up-regulated CCR6 but down-regulated hBD1 and hBD3 (Fig. 6B). Although consistent results were observed for the change in expression of these markers, the changes between stimulated and unstimulated controls were not statistically significant because of the large standard deviation resulting from individual differences. Nevertheless, statistically significant differences were observed between *E. coli* LPS-stimulated (up-regulated responses) and Pam3CSK4-stimulated (down-regulated responses) expression of CCL20, TNF-α and TLR4. Finally, because CD14 and MD2 are important for LPS recognition through TLR4, we also determined expression of these markers in all the TcOB cultures used for LPS or Pam3CSK4 stimulation. CD14 was uniformly present but MD2 expression level varied among donors (data not shown).

Comparison of immune markers in the OB layer of normal and decayed teeth

Pooled RNA extracted from TcOB preparations of healthy teeth and teeth with moderate to deep decay (one-half to two-thirds of dentin thickness) were also tested for pro-inflammatory cytokines, IL-1β and TNF-α, and for the chemokine CCL20. Decay had extended through approximately one-half of the dentin so that OB processes within the dentin could be expected to have come into contact with bacteria or bacterial components, although decay did not extend into the pulp itself. Quantitative PCR showed a 30.7-fold increase in TNF-α, 6.4-fold increase in IL-1β, and 10.2-fold increase in CCL20 (Fig. 7).

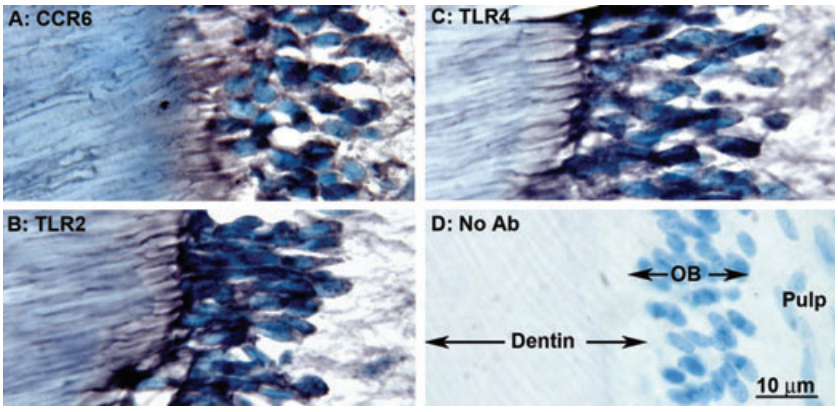

Fig. 5. High magnification view of CCR6, TLR2 and TLR4 staining in the odontoblasts (OBs) of normal human teeth. Immunoperoxidase localization of CCR6 (A), TLR2 (B) and TLR4 (C) on OBs. TLR2 and TLR4 staining was observed on OB cell surfaces and cellular processes and was concentrated in the dentin–pulp interface. No staining was seen in the section processed without primary antibody (D).

Discussion

In this study we show that OBs, the cells lining the hard structure of the tooth at the dentin–pulp border, not only form a physical barrier by producing dentin and by their numerous gap and tight junctions, but also provide an innate immune barrier for the tooth. We demonstrate that OBs *in situ* express TLR2 and TLR4. Immunolocalization shows both TLR2 and TLR4 with especially prominent distribution at the interface of the OB cell body and the dentin layer (Figs 4 and 5). Thus these receptors are in a position to give the earliest response to invading bacteria. We also show, by the use of specific purified reagents, that differential recognition of gram-positive and gram-negative bacterial components by OBs is occurring via TLR2 or TLR4 signaling, respectively.

In our TcOB cultures, we show that hBD1, hBD2, hBD3, IL8, CCL20, IL-1β, TNF-α, CCR6, TLR2 and TLR4 are produced by the OBs. Furthermore, we show that expression of these markers is differentially regulated by cognate ligands for TLR2 and TLR4. These findings suggest the ability of OBs *in situ* to attract neutrophils (via IL-8) and immature DCs and memory T cells (via hBDs and CCL20). We also show that OBs express the CCR6 receptor for hBDs and CCL20 and confirm that OBs express hBD1 and hBD2 in agreement with Dommisch et al. (9). CCL20 is functionally similar to hBD2 (20) and these immunomodulatory peptides are both ligands for CCR6, which is localized on the OB cell surfaces as well as on immature DCs, B cells and memory T cells (28, 29, 55). Thus, CCL20 and hBD secretion from OBs may be involved in recruiting CCR6-expressing immune cells whereas CCR6 expression in OBs also allows other interactions between OBs and these immunocompetent cells. The β-defensins are widely expressed in epithelial cells as part of the antimicrobial barrier (7, 54). The expression of β-defensins, CCL20 and CCR6 in OBs *in situ* is

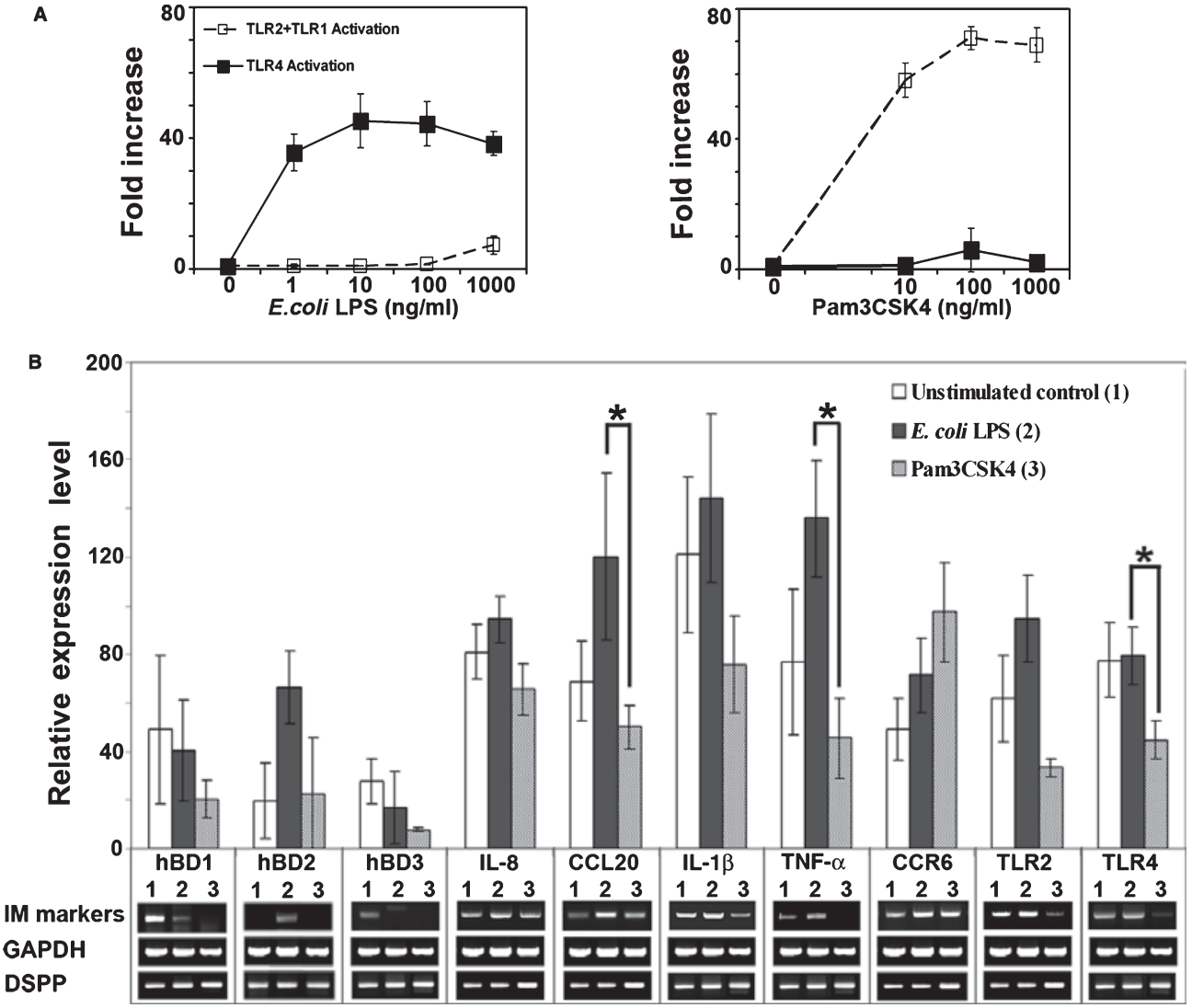

Fig. 6. Innate immune components in the odontoblast layer of human tooth crowns were differentially regulated by TLR2 and TLR4 stimulation. The activities of TLR2 agonist, Pam3CSK4, and TLR4 agonist, *Escherichia coli* LPS, were confirmed by using TLR2 + TLR1-transfected or TLR4-transfected HEK293 cells (A). Semi-quantitative RT-PCR data and gel examples of PCR products from unstimulated, LPS-stimulated, and Pam3CSK4-stimulated TcOB cultures from the same donor demonstrate differential responses to TLR2 or TLR4 activation (B). Band density of each immune (IM) marker in each sample was determined and normalized to the housekeeping gene GAPDH. Data from all samples are presented in the bar graph as mean ± SE. All samples expressed DSPP (a marker for OBs). LPS up-regulated hBD2, IL-8, CCL20, IL1-β, TNF-α and TLR2 whereas Pam3CSK4 down-regulated these markers. Both Pam3CSK4 and LPS decreased hBD1 and hBD3 but increased CCR6 expression. Asterisks indicate statistically significant difference ($P < 0.05$), determined by Mann–Whitney Rank Sum test.

similar to that in intestinal epithelial cells (3) and oral epithelial cells (our unpublished data). The presence of these innate immune molecules suggests that OBs function in orchestrating multiple events to provide a barrier and protect the tooth and underlying pulp tissue from invading microbes, analogous to the barrier in epithelial tissues.

Our data on differential responses in primary OBs in the tooth crown organ cultures are further strengthened by our having purified ligands for TLR4 (LPS) and TLR2 (Pam3CSK4), so that each can only stimulate one set of TLR receptors. Although these two ligands are not from oral bacteria, they have well-defined actions which are essential for the examination of TLR2 and TLR4 functions in OBs. Oral bacteria were not tested in the present study because their actions on human TLRs are not completely understood, and some may activate both TLR2 and TLR4 (8).

Our results suggest that OBs direct differential responses to gram-positive and gram-negative bacteria. LPS-mediated TLR4 activation increased pro-inflammatory cytokines, IL-1β and TNF-α in the TcOB cultures, but Pam3CSK4-mediated TLR2 stimulation decreased these inflammatory markers. The minimal responses to the TLR2 agonist, in contrast to TLR4 agonist, suggests that TLR2 may mediate innate immunotolerance for the tooth while activation of TLR4 by gram-negative bacteria initiates inflammatory responses. The immunotolerance may act to prevent the overwhelming inflammation and pulp tissue damage by gram-positive organisms that is associated with early tooth decay. In contrast, TLR4 signaling may be exaggerated in extensive caries in the presence of gram-negative bacteria, leading to an uncontrolled inflammatory

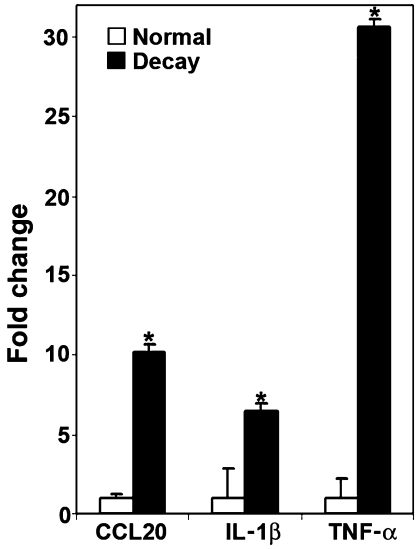

Fig. 7. Expression of CCL20, and pro-inflammatory cytokines, IL-1β and TNF-α increased in the odontoblast layer of teeth with decay. Quantitative (real-time) PCR analyses were performed in triplicate using total RNA from clinically healthy (normal) and decayed TcOB preparations. The results were shown as average fold change in comparison with the normal samples, which were assigned a value of 1. Vertical bars indicate standard error. An asterisk indicates a statistically significant difference of each marker between decayed and normal TcOB preparations ($P < 0.05$), determined by Student's *t*-test.

reaction and consequent irreversible pulp and periradicular tissue damage. Our results showing increases in pro-inflammatory cytokines in TcOB preparations from teeth with decay are consistent with the importance of TLR4 signaling from OBs for pro-inflammatory responses.

Studies in TLR2- and TLR4-deficient mice are also consistent with our study in TcOBs. TLR4-deficient C3H/HeJ mice had less periradicular bone destruction and less expression of pro-inflammatory cytokines (i.e. IL-1α, IL-1β, and IL-12) in periradicular tissues and macrophages than wild-type control mice after their molars were infected with mixed gram-positive and gram-negative anaerobic bacteria (21). In contrast, mice that express TLR4 but are TLR2-null developed higher disease severity and mortality from bacterial meningitis than wild-type mice as a result of higher bacterial load and higher TNF level in the cerebrospinal fluid and stronger meningeal inflammation (11). Although dental infection was not examined in these mice, the tooth and meninges (tissue inside the skull, covering the brain) are both confined anatomical regions in which inflammation can lead to cell death, and it is possible that both tissues need special antimicrobial defenses.

OBs within the tooth represent the first cellular barrier against exogenous pathogens, analogous to the epithelial cells of the mucosa. They both express IL-8 as well as hBDs, CCL20 and their receptor (CCR6). In epithelial cells, expression of these markers is regulated by cell differentiation, bacterial exposure and pro-inflammatory cytokines (3, 25, 30, 31, 33, 43). hBD2, CCL20 and IL-8 share multiple induction mechanisms (3, 25, 30, 44). In contrast, the regulation of expression of hBD1, hBD2 and hBD3 is distinct in epidermis (43) and in our tooth crown culture. We found that hBD2, IL-8 and CCL20 were up-regulated with LPS (TLR4 ligand) but down-regulated with Pam3CSK4 (TLR2 ligand) whereas hBD1 and hBD3 were decreased by both ligands. The findings in this study, however, do not exclude the possibility that pro-inflammatory cytokines, IL-1β and TNF-α released during TLR activation could indirectly affect the regulation of innate immune markers. The detailed mechanisms of differential modulation of innate immune molecules in human OBs and the interaction of OBs with other cells in the tooth pulp for immune defense remains to be explored.

These new observations about the capability for differential regulation of immune responses by human OBs are supported by a recent publication showing specific lipoteichoic acid responses by long-term cultures of *in vitro*-derived OB-like cells that included altered chemokine expression and severely reduced expression of the OB marker gene DSPP (10). *In vitro*-differentiated OB-like cells in long-term cultures can express some OB markers, but they differ from primary OBs which are highly specialized and associated with a dentin scaffold. These cells also have a complex environment *in vivo* with extensive neural, vascular, immune and stem cell support. Although that support would be greatly reduced or absent in the organotypic culture system used here, nevertheless the primary OBs preserved in our TcOB model maintained their expression of DSPP and gave robust differential responses to bacterial challenges.

In conclusion, antimicrobial peptides, hBD1, hBD2 and hBD3; chemokines, CCL20 and IL-8; pro-inflammatory cytokines, IL-1β and TNF-α; chemokine receptor, CCR6; and pathogen-pattern recognition receptors, TLR2 and TLR4, are differentially regulated by OBs upon activation with TLR2 or TLR4 ligands. *In situ* expression of TLR2, TLR4 and CCR6 proteins is localized in OBs and their cellular processes. Taken together, these results show that OBs can recognize and differentially respond to gram-positive and gram-negative bacteria and that they contribute to the innate immune defense of the tooth.

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