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References

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Lipoteichoic Acid Increases TLR and Functional Chemokine Expression while Reducing Dentin Formation in In Vitro Differentiated Human Odontoblasts

Stéphanie H. Durand,* Vincent Flacher,‡ Annick Roméas,* Florence Carrouel,* Evelyne Colomb,† Claude Vincent,† Henry Magloire,† Marie-Lise Couble,* Françoise Bleicher,* Marie-Jeanne Staquet,* Serge Lebecque,‡ and Jean-Christophe Farges1*

Gram-positive bacteria entering the dentinal tissue during the carious process are suspected to influence the immune response in human dental pulp. Odontoblasts situated at the pulp/dentin interface are the first cells encountered by these bacteria and therefore could play a crucial role in this response. In the present study, we found that in vitro-differentiated odontoblasts constitutively expressed the pattern recognition receptor TLR1–6 and 9 genes but not TLR7, 8, and 10. Furthermore, lipoteichoic acid (LTA), a wall component of Gram-positive bacteria, triggered the activation of the odontoblasts. LTA up-regulated the expression of its own receptor TLR2, as well as the production of several chemokines. In particular, an increased amount of CCL2 and CXCL10 was detected in supernatants from LTA-stimulated odontoblasts, and those supernatants augmented the migration of immature dendritic cells in vitro compared with controls. Clinical relevance of these observations came from immunohistochemical analysis showing that CCL2 was expressed in vivo by odontoblasts and blood vessels present under active carious lesions but not in healthy dental pulps. In contrast with this inflammatory response, gene expression of major dentin matrix components (type I collagen, dentin sialophosphoprotein) and TGF-β1 was sharply down-regulated in odontoblasts by LTA. Taken together, these data suggest that odontoblasts activated through TLR2 by Gram-positive bacteria LTA are able to initiate an innate immune response by secreting chemokines that recruit immature dendritic cells while down-regulating their specialized functions of dentin matrix synthesis and mineralization. The Journal of Immunology, 2006, 176: 2880–2887.
In the present study, we used odontoblasts generated in vitro from cultures of human dental pulp explants (14). In these conditions, dental pulp cells give rise to highly differentiated cells that exhibit many odontoblast features such as cell body polarization, formation of a typical process at the cell pole opposite to the nucleus, and strong expression of specific markers, including type I collagen, dentin sialophosphoprotein (DSP), TGF-β1, phosphate regulating gene with homologies to endopeptidases on the X chromosome, osteoadherin, and reelin (14, 15, 16, 17). Although some differences may exist with in vivo cells, in vitro-differentiated odontoblasts are referred to as odontoblasts from herein.

Our aim here was to determine the TLR gene profile of odontoblasts and their innate immune response to LTA, a PAMP from Gram-positive bacteria and a ligand for TLR2. We stimulated odontoblasts with LTA and analyzed consequences on their phenotype in terms of TLR and chemokine expression, induction of iDCs migration, dentin matrix synthesis, and mineralization.

Materials and Methods

Cell culture

Odontoblasts were generated in vitro from human dental pulp explants as described by Couble et al. (14). Briefly, 20 healthy nonerupted third molars were collected with informed consent of the patients, in accordance with the French Public Health Code and following a protocol approved by the local ethics committee. The pulp tissue was separated from the dentin/enamel mineralized complex, and its apical end was removed to prevent periodontal cell contamination. Pulp cultures (2 mm 3) were grown in H11011 enamel mineralized complex, and its apical end was removed to prevent periodontal cell contamination. Pulp explants (2 mm 3) were grown in

Gene arrays

RNA samples (1 μg each) from LTA-stimulated and control odontoblast cultures were amplified with the MessageAmp aRNA kit (Ambion), according to the manufacturer’s instructions. Amplified RNAs (500 ng) were then used as a template to generate α32P-dCTP-labeled cDNA probes with use of the AmpoLabeling-LPR kit (SuperArray Bioscience). Nylon membrane arrays containing cDNA fragments from human chemokine and receptor genes (GEArray Q Series, catalog no. HS-005.2) were purchased from SuperArray. Detailed information about these arrays is available online (www.superarray.com/). For each target gene, mRNA levels were normalized to the cyclophilin A housekeeping gene.

Real-time PCR

Total RNA was extracted from LTA-stimulated or control odontoblast cultures with the Nucleospin RNA II kit (Macherey-Nagel), according to the manufacturer’s instructions. RNA samples (1 μg) were then converted to first-strand cDNAs using 500 ng oligo (dT)15 primers (Roche Diagnostics) and PowerScript Reverse Transcriptase (BD Clontech). Real-time PCR was performed in a Light Cycler instrument (Roche) with the Fast Start DNA Master SYBR Green I kit, according to the manufacturer’s specifications. Primer sets and annealing temperatures are listed in Table I. The real-time PCR conditions were as follows: 10 min at 95°C, followed by 45 cycles of 95°C for 15 s, annealing temperature for 5 s, 72°C for 20 s. The increase in PCR product was monitored for each amplification cycle by measuring the increase in fluorescence caused by the binding of SYBR Green dye to dsDNA. Amplification specificity was confirmed by analysis of melting curves. Standard curves for target and cyclophilin A housekeeping genes were constructed with serial dilutions of reverse transcription samples. For each target gene, mRNA levels were normalized to the cyclophilin A housekeeping gene with the RelQuant software (Roche). Results were expressed as fold change values relative to control odontoblast samples nonstimulated with LTA. All runs were performed in duplicates and included a negative control without template. PCR amplification was also verified for expected products, mispairing, and primer dimer formation on 2% agarose gels.

Flow cytometry

Odontoblasts were obtained following trypsin/EDTA treatment of cultures and incubated for 30 min with anti-TLR2 mouse mAb (clone TL2.1; Santa Cruz Biotechnology) or isotype-matched mouse IgG2a (clone UPC 10; Sigma-Aldrich) at the same concentration. After washing, cells were incubated with FITC-conjugated F(ab’2) goat anti-mouse Ab (Caltag Laboratories) for 30 min. Cells were washed and resuspended in PBS containing 1% formaldehyde. Data were acquired on a DakoCytomation cytomter (DakoCytomation) and analyzed using the WinMDI 2.8 software (The Scripps Institute).

Table I. Primers used for real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’–3’)</th>
<th>Reverse Primer (5’–3’)</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>CCCCATGCCAGAATCTACCATCATACTTACCTTACCA</td>
<td>TTCTTCTGTTGCTAGGAGATCCATACCA</td>
<td>60</td>
</tr>
<tr>
<td>TLR2</td>
<td>TTCCTCTGGAGAGCTGATGT</td>
<td>TTCTCTGGAGAGCTGATGT</td>
<td>60</td>
</tr>
<tr>
<td>TLR3</td>
<td>TGGTTGGCCACCTTAGAATGAC</td>
<td>TCTCTACTCTGTGCTGATGT</td>
<td>60</td>
</tr>
<tr>
<td>TLR4</td>
<td>CTGGTGATGAATCTAGACCA</td>
<td>TTATCTCTGGAGATGGTGGCAGATCC</td>
<td>60</td>
</tr>
<tr>
<td>TLR5</td>
<td>TCTCTCAGATCTATCTATTAG</td>
<td>CACACATCGAGTGGACGAC</td>
<td>60</td>
</tr>
<tr>
<td>TLR6</td>
<td>CACCTCAACCCACTAGAAGACCC</td>
<td>TGGAGCCTGAGAGCTGATGACTA</td>
<td>60</td>
</tr>
<tr>
<td>TLR8</td>
<td>AACTCCCTGATGCTTACCTCTATCTTATGAC</td>
<td>GGGTGTAGCCACCGACTTAT</td>
<td>60</td>
</tr>
<tr>
<td>TLR9</td>
<td>TGAGACCTGAGGATGACTCATC</td>
<td>TTCACAGGCCACTCATTC</td>
<td>60</td>
</tr>
<tr>
<td>TLR10</td>
<td>TTGATGCTGCTCCTGTGATCTCA</td>
<td>AGTTGCTTCTGTTGTTTCTTCATC</td>
<td>60</td>
</tr>
<tr>
<td>Coll11</td>
<td>TGCCCTACAGATGTTGGCCT</td>
<td>AGGCACATGCTCCTTGCC</td>
<td>62</td>
</tr>
<tr>
<td>DSPP</td>
<td>GCACTCCAGACGCAAGTGGATGA</td>
<td>CTGATCTGCTGCTCTGCTT</td>
<td>58</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>CACCCCTACAGATGCTGATGAT</td>
<td>TGGCCCTTGGGCAAGCCAAA</td>
<td>58</td>
</tr>
<tr>
<td>CCL2</td>
<td>GAGCCCTACAGGCGCTGTG</td>
<td>AACAGGCTTCCAGGAGCTT</td>
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</tr>
<tr>
<td>CCL7</td>
<td>GCACCTGCTGCTGCTGCTGCTG</td>
<td>TACGCTCCAGCCCTGCTT</td>
<td>66</td>
</tr>
<tr>
<td>CXCL2</td>
<td>ACAAGGAATCAAGGAGCCAC</td>
<td>CAGGAACACGACACAATAAG</td>
<td>58</td>
</tr>
<tr>
<td>CXCL10</td>
<td>TGACCTTACAGCCAGGAAA</td>
<td>TACCTCCTTGAAGGGCAGTTT</td>
<td>62</td>
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</table>
expressed as a percentage of cyclophilin A gene expression. Data were reported for genes whose expression level was >5% of the cyclophilin A gene (this threshold corresponding approximately to the visual detection of the spot) and that exhibited at least a 2-fold change of expression between LTA-stimulated and control samples. Only the matched positive and negative results of three experiments are presented.

**Statistical analysis**

Results were expressed as mean values ± SD obtained from three independent experiments. Statistical analysis was determined with Student’s *t* test.

**Protein array**

A Human Chemokine Ab Array I kit was purchased from Raybiotech and used according to the manufacturer’s instructions. Briefly, membranes were blocked for 30 min at room temperature before being incubated for 16 h at 4°C with supernatants recovered from odontoblasts cultured with or without LTA. They were then washed, incubated with biotin-conjugated primary Abs, HRP-conjugated streptavidin, and finally developed by using ECL-type solution. Membranes were scanned with a VersaDoc Imaging System (Bio-Rad), and semiquantitative analysis of the comparative intensity of the spots was performed with the Quantity One 4.4.1 software (Bio-Rad).

**Immunohistochemistry**

Three healthy and seven carious human third molars were fixed in 4% paraformaldehyde-PBS solution for 16 h, rinsed, and cut into 750-μm-thick slices. After demineralization in acetic acid or EDTA for 25 days, slices were routinely treated for paraffin embedding (18). Five-micrometer serial sections were deparaffinized, rehydrated, and treated with an Ag unmasking solution (Vector Laboratories) for 15 min. They were then incubated with 20 μg/ml anti-CCL2 goat polyclonal Ab (R&D Systems). Ab detection was performed using the Vectastain Elite ABC kit (Vector Laboratories), according to the manufacturer’s protocol. Sections were slightly counterstained with toluidine blue.

**Generation of iDCs**

iDCs were generated from human CD34+ hematopoietic progenitors isolated from human umbilical cord blood mononuclear fraction by immunomagnetic selection with miniMACS (Miltenyi Biotec). Purified progenitors (90–98% purity) were cultured in RPMI 1640 supplemented with 5% heat-inactivated FBS (Myolone super plus), 10 mM HEPES, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen Life Technologies), 5 × 10⁻⁶ M 2-ME (Sigma-Aldrich), 200 U/ml recombinant human GM-CSF (kindly provided by Schering-Plough Laboratories), and 50 U/ml recombinant human TNF-α (Genzyme) for 7 days. Under these conditions, cells remain poorly differentiated and display features of iDCs (19).

**Chemotaxis assay**

Cell migration was assessed using Costar Transwell devices with an 8-μm pore size. Supernatants recovered from odontoblasts cultured with or without LTA were diluted (1/4) in RPMI 1640–5% FBS and added to 24-well plates. iDCs (1.5 × 10⁵) suspended in RPMI 1640 supplemented with 5% FBS were applied to Transwell inserts. Plates were incubated for 4 h at 37°C. After removal of the Transwell inserts, migrated cells were recovered and counted. Supernatants of three different control and LTA-stimulated odontoblast cultures were analyzed in duplicate. Buffer alone and buffer + LTA were used as controls. Results were expressed as the number of migrated iDCs in percentage of the input cell number introduced into the upper compartment.

**Results**

**TLR expression in resting and LTA-activated odontoblasts**

We first set out to determine the expression of TLR mRNAs in resting odontoblasts and in response to LTA, a ligand for TLR2. Real-time PCR investigation of the TLR profile of odontoblasts showed that these cells constitutively expressed genes coding for TLR1, 2, 3, 4, 5, 6, and 9 (Fig. 1A). TLR7, 8, and 10 mRNA could not be amplified in odontoblasts, although easily detected in plasmacytoid or myeloid DCS (data not shown). Upon LTA activation, an increase in gene expression level was observed for TLR2 (4.1-fold; *p* = 0.004), TLR3 (2.4-fold; *p* = 0.001), TLR5 (2.1-fold; *p* = 0.02), and TLR9 (2.1-fold; *p* = 0.03). Other TLR genes remained unchanged. TLR2 was then selected for protein analysis given its crucial role in LTA signaling. Flow cytometry revealed that LTA up-regulated TLR2 in odontoblasts (Fig. 1B).

**LTA treatment of odontoblasts modulates chemokine gene and protein expression**

Given that one major consequence of TLR activation is the up-regulation of chemokine synthesis, we then examined the chemokine expression profile of odontoblasts by cDNA array analysis (Table II and Fig. 2A). Data revealed that odontoblasts constitutively expressed 17 genes related to the chemokine pathway, including genes encoding for the chemokines CCL2, CCL26, CXCL4, CXCL12, and CXCL14. The remainder of genes include chemokine receptors (CXCR2, CCR1, and CCR2), leucotriene B4 receptor, brain-derived neurotrophic factor, endothelial mono-}

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**Figure 1.** TLR expression in resting and LTA-activated odontoblasts. A. Real-time PCR analysis of TLR gene expression in human odontoblasts stimulated or not with LTA. Results were normalized to cyclophilin A gene expression and expressed as fold change values relative to control non-stimulated samples. Data represent the mean ± SD obtained from three independent experiments. Odontoblasts in culture constitutively expressed TLR1, 2, 3, 4, 5, 6, and 9 but not TLR7, 8, and 10. A significant increase of gene expression for TLR2 (4.1-fold; *p* = 0.004), TLR3 (2.4-fold; *p* = 0.001), TLR5 (2.1-fold; *p* = 0.02), and TLR9 (2.1-fold; *p* = 0.03) was observed in LTA-stimulated odontoblasts (Od + LTA) compared with controls (Od). B. Flow cytometry analysis of TLR2 protein expression in human odontoblasts stimulated or not with LTA. Isotype controls are shown as open histograms. TLR2 was induced in LTA-stimulated odontoblasts (Od + LTA) compared with controls (Od). Results are representative of two independent experiments.

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significant statistically. The four chemokine genes up-regulated by LTA were selected for verification by real-time PCR with the same cultured samples. Stimulation of genes encoding CCL2 (3-fold; \( p < 0.01 \)), CCL7 (7.6-fold; \( p < 0.02 \)), CXCL2 (10.3-fold; \( p < 0.006 \)), and CXCL10 (6-fold; \( p < 0.04 \)) in LTA-treated cultures was confirmed (Fig. 3). Protein array analysis of culture supernatants showed an increase of 2.3- and 2.7-fold for CCL2 and CXCL10, respectively. The CCL7 level was very low and showed no significant modification (Fig. 4). CXCL2 could not be assessed because of the absence of a specific Ab on the membrane.

**CCL2 is expressed in vivo in carious teeth**

Data from the literature indicating that CCL2 was able to stimulate iDCs migration in vitro (11), and our results showing that CCL2

---

**Table II. Expression profile of chemokine-related genes in odontoblasts as determined by gene array analysis**

<table>
<thead>
<tr>
<th>Position</th>
<th>Gene Name</th>
<th>GenBank Accession No.</th>
<th>Expression Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5C</td>
<td>CXCL4/PF4/SCYB4</td>
<td>NM_002619</td>
<td>+</td>
</tr>
<tr>
<td>6G</td>
<td>CCL2/MCP-1/SCYA2</td>
<td>NM_002982</td>
<td>++</td>
</tr>
<tr>
<td>7F</td>
<td>CCL26/Eotaxin-3/SCYA26</td>
<td>NM_006072</td>
<td>++</td>
</tr>
<tr>
<td>9B</td>
<td>CXCL14/B2AK/SCYB14</td>
<td>NM_004897</td>
<td>+</td>
</tr>
<tr>
<td>10A</td>
<td>CXCL12/SDF1/SCYB12</td>
<td>NM_000609</td>
<td>+</td>
</tr>
<tr>
<td>Other genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1B</td>
<td>C10</td>
<td>NM_138425</td>
<td>+++</td>
</tr>
<tr>
<td>2E</td>
<td>L-CCR/CCRL2</td>
<td>NM_003965</td>
<td>++</td>
</tr>
<tr>
<td>4H</td>
<td>CXCR2/IL8RB</td>
<td>NM_001557</td>
<td>++</td>
</tr>
<tr>
<td>5A</td>
<td>LTBR</td>
<td>NM_181657</td>
<td>+</td>
</tr>
<tr>
<td>6A</td>
<td>BDNF</td>
<td>NM_001709</td>
<td>+</td>
</tr>
<tr>
<td>9H</td>
<td>EMAP2p43/SCYE1</td>
<td>NM_004757</td>
<td>+</td>
</tr>
<tr>
<td>10B</td>
<td>SDF2</td>
<td>NM_006923</td>
<td>+</td>
</tr>
<tr>
<td>10E</td>
<td>CCRL1</td>
<td>NM_016557</td>
<td>+</td>
</tr>
<tr>
<td>10G</td>
<td>CKLFSF3</td>
<td>NM_144601</td>
<td>+</td>
</tr>
<tr>
<td>11B</td>
<td>CKLFSF6</td>
<td>NM_017801</td>
<td>+</td>
</tr>
<tr>
<td>11C</td>
<td>CKLFSF7</td>
<td>NM_138410</td>
<td>+</td>
</tr>
<tr>
<td>11H</td>
<td>HIF1A</td>
<td>NM_020850</td>
<td>++</td>
</tr>
</tbody>
</table>

* Only the matched positive and negative results of three experiments are presented. The gene expression level was normalized to the cyclophilin A gene and presented as the following ratio: (chemokine signal−background)/(cyclophilin A signal−background). +, between 5 and 25% of the expression level of the cyclophilin A gene; ++, between 25 and 100%; ++++, >100%.

---

**FIGURE 2.** Gene expression profile of odontoblasts determined by array analysis and changes induced by LTA treatment. Complete list of genes spotted on the Q Series Human Chemokines and Receptors Gene Array nylon membrane (catalog no. HS-005.2) is available online (www.superarray.com/). A. Odontoblasts (Od) constitutively expressed 17 chemokine-related genes (listed in Table II), including CCL2 (position 6G on the membrane), CCL26 (7F), CXCL4 (5C), CXCL12 (10A), and CXCL14 (9B) chemokine genes. B. After LTA stimulation (Od + LTA), hybridization signals were significantly increased for CCL2 (6G), CCL7 (8E), CXCL2 (4C), CXCL10 (8G), CCRL1 (10E), and CCRL2 (2E) genes. Cyclophilin A tetrads (position 14A-D) were used for normalization. The three pUC18 tetrads used for background subtraction are in the position 13A-C. The autoradiograms shown are representative of three independent experiments. (C) Quantification of hybridization signals on gene arrays. Statistical analysis of three independent experiments showed that six genes were up-regulated >2-fold: three genes whose expression was detected in nonstimulated odontoblasts and three genes whose expression was only detected in LTA-stimulated samples. The former were CCL2 (2.4-fold; \( p < 0.01 \)), CCRL1 (10.7-fold; \( p < 0.002 \)) and CCRL2 (3.9-fold; \( p < 0.02 \)), the latter were CCL7, CXCL10, and CXCL2. Other genes exhibited no change or low changes that were statistically not significant.
was up-regulated at the mRNA and protein levels in LTA-stimulated odontoblasts, prompted us to examine expression of this molecule in vivo in tooth specimens. Analysis of healthy (Fig. 5A) and carious (Fig. 5B) third molars with an anti-CCL2 polyclonal Ab showed an absence of staining in healthy teeth (Fig. 5C), whereas a moderate staining was observed in odontoblasts under active carious lesions (Fig. 5D). Endothelial cells present in the underlying inflammatory tissue were also stained (Fig. 5E), as was previously shown in various inflammatory conditions (22).

LTA-stimulated odontoblasts attract iDCs

To further assess the biological relevance of LTA-induced chemokine response of odontoblasts, LTA-conditioned culture supernatants were tested for their chemotactic effect on iDCs in a Transwell migration assay. In experiments with buffer alone, buffer + LTA (data not shown), and supernatants from nonstimulated odontoblasts, a mean number of 37 ± 9% iDCs migrated (Fig. 6). The migratory response was enhanced significantly (69 ± 11.8%, p = 0.001) when supernatants from LTA-stimulated odontoblasts were added in the lower compartment. Checkboard analysis established that cells attracted by LTA-stimulated odontoblasts migrated in a directional rather than a random fashion (data not shown).

FIGURE 4. Protein array analysis of chemokine release from odontoblasts stimulated or not with LTA. Each anti-chemokine Ab is present on the membrane in duplicate. Chemokines whose gene expression was found to be up-regulated in gene array and real-time PCR experiments are shown, except CXCL2, which could not be assessed because of the absence of a specific Ab on the membrane. After background subtraction (Blank), values were adjusted based on the intensity of control spots on the filters (Pos). CCL2 and CXCL10 showed a protein level increase of 2.3- and 2.7-fold, respectively. The CCL7 level was very low and showed no significant modification (N.S.). Data are representative of two independent experiments.

Down-regulation of dentin matrix protein genes by LTA

Since the main steady functions of odontoblasts are the secretion of the extracellular matrix of dentin and its mineralization, and that this matrix is strongly reduced under active dentin carious lesions, we determined whether LTA stimulation influences these odontoblast specialized functions. Thus, we analyzed gene expression of two dentin matrix components and of TGF-β1, considered to be a crucial regulatory element for dentinogenesis, by real-time PCR. A significant down-regulation of expression was observed for type I collagen α1 chain (3.4-fold; p = 0.001), DSPP (98-fold; p ≤ 0.0001), and TGF-β1 (3.5-fold; p = 0.02) genes in LTA-stimulated cells (Fig. 7).

Discussion

The interface between human teeth and oral cavity presents several unique features that must be balanced to maintain dental pulp sterility. The dentin-pulp complex is protected by an impermeable cap of enamel and confronted to the oral microflora predominantly composed of Gram-positive saprophyte bacteria that are usually well tolerated. However, these bacteria release products that can destroy the enamel and the underlying dentin during the carious process. Dentin-producing odontoblasts then become the first cells run into by pathogens penetrating the living dental tissue. Hence, these cells represent a unique example where neural crest-derived mesenchymal cells must fulfill the role devoted elsewhere to epithelial cells (8). The recent localization of β-defensins 1 and 2 in healthy odontoblasts (23) also argues in favor of this hypothesis.

To study the expression of TLRs by odontoblasts, we used a pure population of cells generated in vitro that are similar in many aspects to in vivo odontoblasts (14, 16). We observed the constitutive expression of TLR1–6 and 9 genes but not TLR7, 8, and 10 genes. This large range of TLRs expressed by odontoblasts appears comparable to what has been reported for cultured epithelial cells,
including keratinocytes (24), intestinal epithelial cells (25), bronchial epithelial cells (26), and gingival epithelial cells (27). Interestingly, the pattern of TLRs expressed by odontoblasts was similar to the one reported for gingival fibroblasts in primary cultures (27). Whether a common TLR expression profile exists for all oral mesenchymal cell types remains to be determined.

Thus, odontoblasts might be involved in the recognition of bacterial products such as triacylated lipoproteins (TLR1+TLR2), LTA (TLR2), diacylated lipoproteins, peptidoglycans (TLR2+TLR6), LPS (TLR4), flagellin (TLR5), and unmethylated CpG motif-containing DNA (TLR9), and also of viral dsRNA through TLR3 (7, 8). Indeed, we found in the present work that odontoblasts responded in vitro to the TLR2 ligand LTA but also to TLR3 and TLR4 ligands (our preliminary data). It remains to be determined whether these cells can actually detect and react to TLR5 and 9 ligands.

Given the predominant role of Gram-positive bacteria in dental caries, we analyzed further the odontoblast response to LTA. LTA was found to strongly up-regulate the expression of its own receptor, TLR2, and to a lesser extent, of TLR3, 5, and 9. The up-regulation of TLR2 by its ligand may increase the sensitivity of odontoblasts, as previously reported in hemopoietic cells (28). The absence of TLR2 detection in resting odontoblasts is probably because the protein is present at a level below the sensitivity threshold of flow cytometry, as reported for iDCs (29).

One well-recognized consequence of TLR2 activation is the up-regulation of chemokine synthesis (30). Using gene arrays, we compared the expression of chemokines by resting vs TLR2-activated odontoblasts. We observed the expression by resting odontoblasts of CCL2, CXCL12, and CXCL14, three chemokines known to drive iDCs recruitment in vitro (11, 31, 32, 33). However, the chemokine the most strongly expressed by resting odontoblasts was CCL26, a natural antagonist for CCR1, CCR2, and CCR5 (34). Therefore, it is tempting to speculate that the balance of odontoblast-derived chemokines may control the homing of iDCs in the human dental pulp in normal conditions. Whether CCL2 could also contribute to iDCs recruitment remains uncertain because we could not detect this protein in the healthy dental pulp by immunohistochemistry.

Following LTA stimulation, four chemokine genes (CCL2, CCL7, CXCL2, and CXCL10) and two corresponding proteins (CCL2 and CXCL10) were clearly up-regulated. Furthermore, odontoblasts present under active dentin carious lesions were found to express the CCL2 protein by immunohistochemistry. CXCL2 is a key inflammatory chemokine produced during microbial infection that attracts iDCs and also monocytes, activated T cells, NK cells, and basophils through CCR1 and CCR2 (11). This chemokine might be responsible for iDCs recruitment into the odontoblast layer, thereby facilitating their interaction with invading Gram-positive bacteria. The detection in vitro of a strong chemotactic activity for iDCs in the supernatant of LTA-stimulated odontoblasts supports this hypothesis. Experiments are going on to identify the chemokines responsible for this migration. Moreover, through the up-regulation of CXCL2 and CXCL10 expression, odontoblasts are likely to contribute to the recruitment of neutrophils and lymphocytes, respectively, during infection (11). CXCR2, a chemokine receptor primarily present on hemopoietic cells (35), was expressed at a high level by odontoblasts. Strikingly, CXCL2, for which the only known receptor is CXCR2, was also expressed by odontoblasts and strongly increased after LTA stimulation. Although this increase remains to be confirmed at the protein level, an autocrine control might occur in odontoblasts via CXCL2 regarding the expression of genes involved in predentin remodeling, as CXCR2 activation was shown to induce matrix metalloprotease-3 release in human chondrocytes (36).

Chemokines not only induce cell locomotion but also influence angiogenesis (37, 38). Among chemokines expressed by odontoblasts, CCL2, CXCL2, and CXCL12 are proangiogenic (11, 39), whereas CXCL4, CXCL10, and CXCL14 are antiangiogenic (40, 41). In vivo, the production of angiostatic chemokines in the healthy dental pulp might be involved in the maintenance of blood vessels out of the odontoblast layer. During inflammation of carious origin, the number of capillaries augments in the pulp under the lesion, and some of them penetrate into the odontoblast layer (42). Regarding the proangiogenic chemokine CXCL2, its expression was strongly up-regulated in LTA-stimulated odontoblasts, and...
INNATE IMMUNE RESPONSE IN HUMAN ODONTOBLASTS

this chemokine might thus contribute to the increased vascularization by binding to CXCR2 that is highly expressed on endothelial cells (41). Altogether, these data suggest that TLR2-activated odontoblasts play a key role in the recruitment of iDCs and inflammatory leukocytes and in the neoangiogenesis featured by the dental pulp during the carious infection.

The main steady functions of odontoblasts are the secretion of the extracellular matrix of dentin, i.e., predentin, and its mineralization (4). Predentin is deposited by odontoblasts at a relatively constant rate throughout the tooth life, but it is strongly reduced in active dentin carious lesions (43). We found that the major predentin structural component, type I collagen, was down-regulated in LTA-stimulated odontoblasts might be necessary for the termination of the dental pulp immune response. In parallel, we observed a clear diminution of TGF-β1 gene expression in odontoblasts. This result might indicate a decrease of TGF-β1 amount in the odontoblast microenvironment in vivo. As TGF-β1 is known to attenuate TLR signaling (48, 49, 50, 51), the TGF-β1 decrease observed in LTA-stimulated odontoblasts might be necessary for an effective innate immune response to take place. Also, as TGF-β1 inhibits Th1 and Th2 cell responses (52) and B cell activation (50), its reduction would allow a protective adaptive immune response to happen within the infected dental pulp.

In summary, the present work provides insights about the role of neural crest-derived odontoblasts in the initiation of immune responses that occur in the human dental pulp in response to carious lesion. Thus, it is possible that coordinated regulatory mechanisms are activated to decrease both predentin matrix synthesis and mineralization during the initial phase of the dental pulp immune response. In parallel, we observed a clear diminution of TGF-β1 gene expression in odontoblasts. This might result in a decrease of TGF-β1 amount in the odontoblast microenvironment in vivo. As TGF-β1 is known to attenuate TLR signaling (48, 49, 50, 51), the TGF-β1 decrease observed in LTA-stimulated odontoblasts might be necessary for an effective innate immune response to take place. Also, as TGF-β1 inhibits Th1 and Th2 cell responses (52) and B cell activation (50), its reduction would allow a protective adaptive immune response to happen within the infected dental pulp.

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