# TLR4 Mediates LPS-Induced VEGF Expression in Odontoblasts

*Tatiana M. Botero, DDS,* Charles E. Shelburne, PhD,† G. Rex Holland, DDS, MS, PhD,* Carl T. Hanks, DDS, MS, PhD,‡ and Jacques E. Nör, DDS, MS, PhD**

## Abstract

Lipopolysaccharide (LPS) from gram-negative bacteria cell walls such as *Prevotella intermedia* and *Escherichia coli* induce vascular endothelial growth factor (VEGF) expression in odontoblasts, but not in undifferentiated dental pulp cells. CD14 and TLR4 are responsible for LPS signaling in macrophages, but their expression levels and function in dental pulp cells are unknown. We showed here that murine odontoblast-like cells (MDPC-23) express CD14 and TLR4 by immunohistochemistry and flow cytometry. In contrast, undifferentiated dental pulp cells (OD-21) presented low or no expression of these two receptors. MDPC-23 cells showed CD14 and TLR4 up-regulation upon exposure to LPS, as determined by real time PCR. Dominant negative murine TLR4 (DN-mTLR4) transfected MDPC-23 cells did not show upregulated VEGF expression in response to LPS stimulation. These results demonstrate that odontoblast-like cells express CD14 and TLR4, and that LPS-induced VEGF expression is mediated, at least in part, by TLR4 signaling. *(J Endod 2006; 32:951–955)*

## Key Words

Angiogenesis, dental pulp cells, LPS signaling, pulpitis, vascular permeability

From the *Department of Cariology, Restorative Sciences and Endodontics, †Department of Biology and Materials Sciences, and ‡Department of Oral Medicine, Oral Pathology and Oral Oncology, University of Michigan, School of Dentistry, Ann Arbor, Michigan.

Address requests for reprints to Dr. Tatiana M. Botero, Department of Cariology, Restorative Sciences and Endodontics, University of Michigan, School of Dentistry, 1011 N. University, Rm. 2310A, Ann Arbor, MI 48109-1078. E-mail address: tbotero@umich.edu.

0099-2399/$0 - see front matter



doi:10.1016/j.joen.2006.03.018

Lipopolysaccharide (LPS), the major component of the outer membrane in gram-negative bacteria, is largely responsible for the pathogenicity of these organisms (1). Gram-negative bacteria such as *Prevotella intermedia* and *Porphyromonas endodontalis* are prevalent in deep caries and pulpitis (2–4). The odontoblasts, specialized dental pulp mesenchymal cells, have an earlier contact with caries products because of their position as first layer in the dental pulp (5). Odontoblast-like cells (MDPC-23) and macrophages have showed up-regulation of vascular endothelial growth factor (VEGF) after exposure to *Escherichia coli* and *P. intermedia* LPS (6), and streptococcal lipoteichoic acids (7).

VEGF is a key regulatory factor of vascular permeability and angiogenesis (8). It has been found in dentin matrix and may be released during caries and contribute to the reparative response of the pulp-dentinal complex (9). Increased capillary permeability in this low compliance dental pulp environment can lead to vessel collapse leading in turn to necrosis (10). An increase of VEGF expression by the odontoblasts during the bacterial challenge in deep carious progression might contribute to these vascular changes.

LPS recognition and cell activation have been thoroughly studied in immune cells and now there is a significant interest in LPS effects on cells acting as a barrier in the body such as: corneal (11), intestinal (12), and urinary mucosa cells (13). LPS binds to a cell membrane receptor, CD14, a glycosylphosphatidyinositol anchored glycoprotein expressed in monocytes, myeloid cells, macrophages, and neutrophils (14, 15); however, CD14 lacks a transmembrane domain that allows for intracellular signaling (16).

The Toll-like receptor (TLR4) has been reported to mediate LPS-induced cellular signaling (17, 18). Its function was confirmed by positional cloning studies of the LPS gene in hyporesponsiveness of C3H/HeJ and C57BL/10ScCr mice (17). TLR4 is predominantly expressed in macrophages, dendritic, and endothelial cells (19, 20). Until now there are no reports about LPS recognition by TLR4 and CD14 and the signaling processes initiated in dental pulp cells.

We hypothesized that LPS-induced VEGF expression in dental pulp cells is mediated by CD14 and TLR4. The purpose of this study is to evaluate the expression of CD14 and TLR4 and the role of TLR4 signaling in LPS-induced VEGF expression by mouse dental pulp cells.

## Materials and Methods

### Cell Culture

Four mouse cell lines used were: odontoblast-like cells (MDPC-23), undifferentiated dental pulp cells (OD-21) (21), macrophages (RAW 2674; ATCC, Manassas, VA), and gingival fibroblasts (gift from C.T. Hanks) and human embryonic kidney cells (293T, ATCC) were used as controls. The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY), supplemented with 10% fetal bovine serum (Gibco), 200 mM L-glutamine (Gibco), 50-units/ml penicillin (Gibco), and 50 $\mu$g/ml streptomycin (Gibco) in a humidified 5% $CO_2$ incubator at 37°C.

### Flow Cytometry

Untreated samples from each cell line were resuspended in 50 $\mu$l staining buffer (BD Biosciences, San Diego, CA) and incubated with 0.02 $\mu$g/$\mu$l Phycoerythin (PE)-conjugated monoclonal rat anti-mouse TLR4 antibody (BD Biosciences), PE-conju-

gated rat anti-mouse CD14 or IgG antibody as control (BD Biosciences) for 30 min. After two washes, $1 \times 10^4$ cells were analyzed by flow cytometry (FACScan; Becton Dickinson, Raleigh, NC).

### Cytospins and Immunohistochemistry

Untreated cells were resuspended in Hank's balanced salt solution (HBSS) (Gibco BRL) and cytospin preparations of $5 \times 10^4$ cells/slide (100 μl/funnel) were centrifuged in a cytospin machine (Shadon Scientific, Pittsburgh, PA) for 5 min at 500 rpm. Slides were fixed in acetone-methanol, air-dried and stored at −80°C. The fixed cells were incubated for 2 h at 37°C in a humidified chamber with primary rabbit anti-mouse CD14, TLR4, or IgG antibody as control (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). The cells were exposed to two consecutive 30-min incubations with biotinylated secondary antibody universal link (Biocare Medical, Walnut Creek, CA) and streptavidin enzyme conjugates HRP (Biocare Medical). Immunohistochemical reactions were developed with 3-amino-9-ethycarbazole (AEC; Sigma, St. Louis, MO) substrate (5.6 μg/ml) for 5 min, and counterstained with hematoxylin for 10 sec (Sigma).

### Real-Time PCR

Cells were exposed for 30 min to 0 to 20 μg/ml *E. coli* LPS and total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), as recommended by the manufacturer. RNA was cleaned up using RNAeasy Mini Kit and DNAse set (Qiagen, Valencia, CA). The cDNA synthesis reactions (100 μl) were performed with 2 μg total RNA and random hexamers using TaqMan MultiScribe Reverse Transcriptase kit (Applied Biosystems, Foster City, CA), for 35 cycles in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). The 18S rRNA gene was used as a normalizing gene for these experiments and each untreated group as control. A 30 μl PCR reaction was prepared with 1 μl cDNA and 1.5 μl mixture of FAM labeled gene specific probe and gene specific primers in a 20× TaqMan Universal PCR master mix (Applied Biosystems). Thermal cycling conditions were used as recommended by the manufacturer. There were 40 cycles performed using an ABI Prism Sequence Detection System 7700 (Applied Biosystems). Data were analyzed by standard curve method. The sequences of probes used were for CD14: TGGATTTATCCAGGTGTGAAATTGA, for TLR4: TCTACCGACCATGGAGCGTGTGCTT and for 18S: TGGAGGGCAAGTCTGGTGCCAGCAG (Applied Biosystems, Assays on Demand).

### Transfections With DN-TLR4

There were $3 \times 10^4$ cells seeded in 24 well plates and transfected with dominant negative murine TLR4 (DN-TLR4) (gift from Richard Darveau) or with the display plasmid (empty vector) as control. Briefly, 1 μg/ml plasmid and 4 μg/ml lipofectamine 2000 (Invitrogen) were preincubated in Opti-MeM (Gibco BRL) and used to incubate cells for 4 h. After transfection, cells were washed and exposed to 0 to 20 μg/ml *E. coli* LPS for 24 h. The conditioned medium was collected for VEGF analysis by enzyme-linked immunosorbent assay (ELISA) using a Quantikine Murine kit (R & D Systems, Minneapolis, MN), as previously described (6).

### Western Blots

To evaluate the transfection efficiency of DN-TLR4, $6 \times 10^4$ MDPC-23, OD-21, or 293T cells as controls, were cultured and protein extracted 24 h after transfection. Protein was separated on 8% SDS-PAGE gels, blotted into polyvinylidene difluoride membrane (BioScience Inc., Keene, NH), and incubated with 5% milk in Tris buffered saline containing 0.1 % Tween 20 (TBST, pH 7.4). Membranes were incubated overnight at 4°C with primary antibody mouse anti-HA (1:1000 Covance, San Diego, CA), or anti-mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody as control (1:10,000 Chemicon International, Temecula, CA). Membranes were washed in TBST and incubated with goat anti-mouse antibody (1:10,000, Jackson ImmunoResearch) for 1 h at room temperature. After final washes, enhanced chemiluminescence was performed for 5 min with Supersignal substrate (Pierce, Rackford, IL) and detected on X-ray films (Fujifilm, Tokio, Japan).

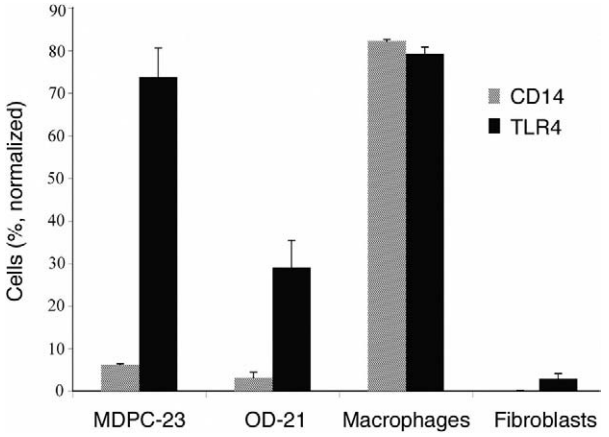


**Figure 1.** Untreated odontoblast-like cells and macrophages express high levels of TLR4. Data from one representative experiment showing the percentage of cells MDPC-23, OD-21, macrophages, and gingival fibroblasts expressing CD14 (light color bars) and TLR4 (black color bars). Data were normalized using cells exposed to IgG antibody as controls.

### Statistical Analyses

Data were analyzed by one-way ANOVA followed by Tukey's test, with Sigmastat 2.0 statistical software (SPSS, Chicago, IL). Three independent experiments for each assay described above were performed to demonstrate reproducibility of the data. Each individual experiment was performed in triplicate to allow for determination of averages and standard deviation. The significance level of the data was determined at $p \leq 0.05$.

## Results

### CD14 and TLR4 Expression by Dental Pulp Cells and Macrophages

The expression of CD14 and TLR4 analyzed by flow cytometry in untreated cells showed in MDPC-23 a low percentage of CD14-positive cells (7.5%), in comparison with CD14-positive macrophages (83.0%) (Fig. 1). A high percentage of MDPC-23 TLR4-positive cells (75.1%) were found as well as macrophages (80.1%) (Fig. 1). Untreated OD-21 had low percentages of CD14-positive (4.0%) and TLR4-positive cells (30.0%) (Fig. 1). Fibroblasts presented low expression levels for both receptors (Fig. 1). These data were confirmed in our immunohistochemical analyses. MDPC-23 and macrophages were found immune-positive for CD14 and TLR4 in comparison to IgG controls (Fig. 2*A–C*, *G–I*). In contrast, OD-21 cells (Fig. 2*D–F*), and fibroblasts (Fig. 2*J–L*) had low or no reaction for CD14 or TLR4.

### CD14 and TLR4 Expressions Were Upregulated in Odontoblast-Like Cells Exposed to *E. coli* LPS in a Dose-Dependent Manner

MDPC-23 presented upregulated CD14 and TLR4 expression levels after exposure to 0 to 2 μg/ml *E. coli* LPS. However, the expression levels went down when MDPC-23 cells were exposed to 20 μg/ml *E. coli* LPS (Fig. 3*A*, *E*), as compared to untreated cells. Interestingly, OD-21 exposed to LPS showed a trend for downregulation of CD14 and TLR4