doi:10.1111/j.1365-2591.2008.01429.x

# Phosphatidylinositol-3-kinase inhibitor LY 294002 blocks *Streptococcus mutans*-induced interleukin (IL)-6 and IL-8 gene expression in odontoblast-like cells

**H. Dommisch[1,2], M. Steglich[1], J. Eberhard[3], J. Winter[1] & S. Jepsen[1]**

[1]Department of Periodontology, Operative and Preventive Dentistry, University Hospital Bonn, Bonn, Germany; [2]Department of Oral Biology, University of Washington, Seattle, WA, USA; and [3]Department of Operative Dentistry and Periodontology, University Hospital Schleswig-Holstein, Campus Kiel, Germany

## Abstract

**Dommisch H, Steglich M, Eberhard J, Winter J, Jepsen S.** Phosphatidylinositol-3-kinase inhibitor LY 294002 blocks *Streptococcus mutans*-induced interleukin (IL)-6 and IL-8 gene expression in odontoblast-like cells. *International Endodontic Journal*, **41**, 763–771, 2008.

**Aim** To investigate the involvement of the phosphatidylinositol-3-kinase (PI3K) in interleukin-6 (IL-6) and interleukin-8 (IL-8) gene expression in odontoblast-like cells when exposed to heat-killed *Streptococcus mutans*.

**Methodology** Cultured human odontoblast-like cells (Dulbecco's modified Eagle's medium) were pre-treated with a specific inhibitor for PI3K (LY 294002) and subsequently stimulated with heat-killed *S. mutans* for 6 and 24 h. After stimulation, RNA was extracted from the cells and cDNA synthesis was performed. Gene expression of IL-6 and IL-8 was analysed by real-time polymerase chain reaction and normalized to the gene expression of beta-actin. Cell survival was determined for stimulation experiments.

**Results** The gene expression of IL-6 and IL-8 was significantly increased in response to heat-killed *S. mutans* ($P = 0.002$ and $P < 0.001$, respectively). After 6 h, the mRNA expression of IL-6 and IL-8 was significantly higher than after 24 h of stimulation ($P = 0.019$ and $P < 0.001$, respectively). Pre-treatment with the inhibitor LY 294002 blocked the induced gene expression of IL-6 and IL-8 ($P = 0.002$ and $P < 0.001$, respectively). No differences in viable cell counts were found after stimulation with heat-killed *S. mutans* and/or pre-treatment with LY 294002 compared with the unstimulated control.

**Conclusion** Gene expression of IL-6 and IL-8 was induced by heat-killed *S. mutans* via signalling pathways mediated by PI3K. These findings indicate that odontoblasts respond to cariogenic bacteria with increased gene expression of pro-inflammatory mediators and hence participate in immune processes.

**Keywords:** interleukin-6, interleukin-8, odontoblast-like cells, phosphatidylinositol-3-kinase, real-time PCR, *Streptococcus mutans.*

*Received 17 October 2007; accepted 25 March 2008*

## Introduction

The dental pulp is a complex tissue represented by various cell types that are entirely surrounded by mineralized tissue. Demineralization and bacterial colonization of the dental hard tissue (caries) are accompanied by inflammatory reactions of the dentine–pulp complex (Izumi *et al.* 1995).

Cytokines are mediators that play an important role during pro-inflammatory reactions and general immune responses against pathogenic microorganisms. Interleukin (IL)-6 belongs to the class of cytokines and is synthesized by monocytes, lymphocytes, fibroblasts, epithelial and endothelial cells (Akira *et al.* 1990), but

Correspondence: Dr Henrik Dommisch, Department of Periodontology, Operative and Preventive Dentistry, University Hospital Bonn, Welschnonnenstraße 17, 53111 Bonn, Germany (Tel.: 0049 228 287 22480; fax: 0049 228 287 22161; e-mail: henrik.dommisch@ukb.uni-bonn.de).

also odontoblasts and dental fibroblasts (Lin *et al.* 2002, Dommisch *et al.* 2007a, Pääkkönen *et al.* 2007, Veerayutthwilai *et al.* 2007). In dental pulp fibroblasts, IL-6 is rapidly inducible by black-pigmented *Bacteroides* (*Porphyromonas endodontalis*, *Porphyromonas gingivalis*, *Prevotella intermedia*) (Yang *et al.* 2003), LPS from *Escherichia coli* (Coil *et al.* 2004), substance P and calcitonin gene-related peptide (Yamaguchi *et al.* 2004, Tokuda *et al.* 2005). In addition, it has been shown that IL-6 was increased in dental pulp fibroblasts when exposed to the pro-inflammatory mediators IL-1 beta and tumour necrosis factor alpha (TNF-alpha) (Lin *et al.* 2002). In cultured odontoblast-like cells, the mRNA expression of IL-6 was increased dose-dependently in response to human beta-defensin-2 (hBD-2) (Dommisch *et al.* 2007a), which exhibits direct antimicrobial activity against pathogenic microorganisms (Lehrer 2004).

The chemokine IL-8 represents an inducible chemotactic factor for a number of immune cells and is specifically involved in the activation of polymorphonuclear leucocytes (neutrophils) (Schröder & Christophers 1992). IL-8 is synthesized in macrophages, monocytes, fibroblasts, epithelial cells (Schröder & Christophers 1992) and dental pulp fibroblasts (Patel *et al.* 2003) as wells as odontoblasts (Levin *et al.* 1999, Dommisch *et al.* 2007a). In experiments using dental pulp fibroblasts, IL-8 protein levels were increased in response to *Prevotella intermedia* (Tokuda *et al.* 2002), *Fusobacterium nucleatum* (Thaweboon *et al.* 2006), LPS from *E. coli* (Chang *et al.* 2005), substance P and calcitonin gene-related peptide (Patel *et al.* 2003). The expression of IL-8 was demonstrated in the odontoblast cell layer after stimulation with LPS from *E. coli* (Levin *et al.* 1999) and in dental pulp-derived cells in response to *Streptococcus mutans* (Engels-Deutsch *et al.* 2003). Similar to IL-6, the mRNA expression of IL-8 was increased in cultured odontoblast-like cells when exposed to the antimicrobial peptide hBD-2 (Dommisch *et al.* 2007a).

*Streptococcus mutans* is recognized as a major aetiologic factor in caries development and progression (Donkersloot & Hoerman 1974). Progressive and invasive carious lesions lead to interaction of *S. mutans* and dental pulp cells, which may be accompanied with inflammatory reactions of the dentine–pulp complex (Jontell *et al.* 1998, Hahn *et al.* 2000). Immunohistological analysis of dental pulp tissue showed increased expression of pro-inflammatory mediators such as IL-1 beta, TNF-alpha, IL-6 and IL-8 in tissue from carious teeth compared with tissue from caries-free teeth (McLachlan *et al.* 2004). Elevated expression of IL-6 and IL-8 in response to *S. mutans* was shown in epithelial cells, endothelial cells (Vernier *et al.* 1996), monocytes (Soell *et al.* 1994), human plasma (Chia *et al.* 2002) and in dental pulp-derived cells (Engels-Deutsch *et al.* 2003).

Phosphatidylinositol-3-kinase (PI3K) represents an important enzyme involved in a number of processes including intracellular trafficking. Previous studies on a number of different cell types showed that the PI3K takes part in intracellular signalling with subsequent IL-6 and IL-8 expression (Xu *et al.* 2004, Yadav *et al.* 2006, Yeh *et al.* 2006, Gobert *et al.* 2007, Turner *et al.* 2007, Wrann *et al.* 2007). Currently, there are no data available showing the effects of inhibiting PI3K-mediated pathways in odontoblasts.

The purpose of this study was to show mRNA expression of IL-6 and IL-8 in response to heat-killed *S. mutans* stimulation of cultured odontoblast-like cells. Furthermore, the hypothesis that PI3K-dependent pathways mediate the gene expression of IL-6 and IL-8 in response to heat-killed *S. mutans* was tested.

## Methods

### Cell culture

Human odontoblast-like cells were cultured from freshly extracted, intact third molars with immature roots. Donors were healthy patients (age 20–25 years) that underwent routine surgical procedures in the Department of Oral Surgery, University Hospital Bonn (Germany). The study was conducted in full accordance with the declared principles (World Medical Association Declaration of Helsiki, version VI, 2002) and was approved by the Ethics Committee of the University of Bonn. All patients had been informed about this study and signed a letter of informed consent. Tooth preparation and subsequent culturing of odontoblast-like cells were performed according to established and described protocols (Tjaderhane *et al.* 1998, Dommisch *et al.* 2007a, Veerayutthwilai *et al.* 2007). Briefly, before disinfection using 70% (v/v) ethanol, each tooth surface was cleaned from blood and periodontal ligament. To dissect the root fragment from the crown, teeth were cut perpendicular to their long axis apically to the cemento-enamel junction using sterile instruments. The dental pulp tissue was then removed from the crown by means of sterile forceps. Crowns with attached odontoblasts were placed in Dulbecco's modified Eagle's medium (DMEM, 10% fetal

 

calf serum, penicillin, streptomycin, amphotericin; PAA, Cölbe, Germany) and cultured at 37 °C in a humidified atmosphere (5% $CO_2$). In this study, two teeth derived from one donor were included for stimulation experiments. For verification of the odontoblast phenotype and cell differentiation, gene expression of the dentine sialophosphoprotein (DSPP) was measured, and an increased gene expression after 24 h (Fig. 1) was shown.

### *S. mutans* culture and cell treatment

Wild-type *S. mutans* (kindly provided by Prof. Dr Sahl) was cultured in LB-medium (10 g trypton, 5 g yeast extract, 10 g NaCl; Roth, Karlsruhe, Germany) overnight at 37 °C (16 h). Bacterial numbers were estimated by absorbance measurement. To avoid bacterial growth during stimulation experiments, *S. mutans* (including culture supernatant) was heat-killed in boiling water for 3 min.

The odontoblast-like cells were grown to 80% confluence and subsequently stimulated with heat-killed *S. mutans* including culture supernatant using an amount equivalent to a multiplicity of infection (MOI) of 100 : 1. For stimulation with heat-killed *S. mutans*, a MOI of 100 : 1 was determined in preliminary experiments. Clear induction of genes of interest combined with consistent cell survival over the examined time period was considered as crucial criteria for the MOI-evaluation. Cells were then incubated for either 6 or 24 h at 37 °C. The pharmacological inhibitor 4-morpholinyl-8-phenyl-4H-1-benzopyran-4-one (LY294002; Calbiochem, Darmstadt, Germany) was used to specifically block phosphatidylinositol-3-kinase activity. For inhibition experiments, cells were pre-incubated with 163 μmol $L^{-1}$ LY 294002 1 h prior to bacterial stimulation (Huang *et al.* 2005b, 2006). Both Blank bacteria medium and LY 294002 (163 μmol $L^{-1}$) were mixed with DMEM and therefore served as negative controls for stimulation experiments. The amount of control bacteria medium was equivalent to a MOI of 100 : 1 but without bacterial cells. Experimental groups were:

- unstimulated odontoblast-like cells (including DMEM; 6 and 24 h),
- odontoblast-like cells treated with heat-killed *S. mutans* (including DMEM, heat-killed *S. mutans* using a MOI of 100 : 1 and bacteria media; 6 and 24 h),
- odontoblast-like cells treated with bacteria media (including DMEM, blank bacteria media using an amount equivalent to a MOI of 100 : 1; 6 and 24 h),
- pre-treated odontoblast-like cells with LY 294002 (163 μmol $L^{-1}$) and stimulated with heat-killed *S. mutans* (including DMEM, heat-killed *S. mutans* using a MOI of 100 : 1 and bacteria media; 6 h),
- odontoblast-like cells treated with blank bacteria medium and LY 294002 (163 μmol $L^{-1}$) (including DMEM, blank bacteria media using an amount equivalent to a MOI of 100 : 1; 6 h) and
- odontoblast-like cells treated with LY 294002 (163 μmol $L^{-1}$) (including DMEM; 6 h). Each experiment was performed in triplicate ($n$ = 3).

To evaluate odontoblast-like cell survival, control cells and stimulated cells (with and without LY 294002 treatment) were stained with Trypan Blue® (0.4%; Invitrogen, Karlsruhe, Germany) after 6 and 24 h (Thaweboon *et al.* 2006). Cell numbers of unstained (viable) and stained (nonviable) were counted and analysed. Experiments for evaluation of cell survival were performed with six replicates in each group.

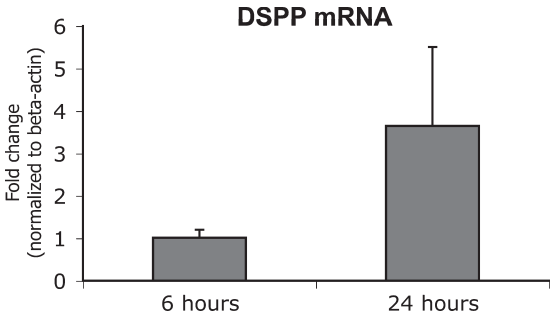


**Figure 1** Gene expression of the dentine sialophosphoprotein (DSPP) in odontoblast-like cells. Cells were cultured in Dulbecco's modified Eagle's medium for 6 and 24 h. The mRNA expression of DSPP was slightly increased (about 3.5-fold) over the time. Bars and error bars represent mean values and standard deviation, respectively. Each bar represents three experimental replications ($n$ = 3).

### Conditions for RT-PCR and real-time PCR

After stimulation, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and the reverse transcription reaction was performed using 500 ng of total RNA. RNA extraction and reverse transcription reaction were carried out following standard protocols as described previously (Dommisch *et al.* 2007b). The reaction mix contained 1× reverse transcriptase (RT) buffer, 250 nmol $L^{-1}$ oligo-(dT)-primer, 10 mmol $L^{-1}$ dNTP mix, 50 U of RT and 13 U of RNase inhibitor (Invitrogen). Experimental controls

without RT enzyme were included with every experiment.

The mRNA expression level of IL-6 and IL-8 was evaluated by means of real-time PCR using the iCycler® (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. In addition, the gene expression of beta-actin (housekeeping gene) was determined and the mRNA expression of the DSPP was analysed for verification of the odontoblast phenotype. PCR reactions were carried out in a total volume of 25 μL, including 1 μL of cDNA and 250 nmol $L^{-1}$ primers. All primer sequences were published elsewhere (Dommisch *et al.* 2007a). Melting curve analysis was performed to confirm that the amplified product was specific. Standard curve analysis was conducted confirming a linear dependency (efficiency) between the cDNA concentration and the threshold cycle (Ct) calculated by the iCycler® software (Bio-Rad). All reactions were carried out in duplicate and average Ct-values were calculated. Sample values were normalized to the expression of the housekeeping gene beta-actin and relative expression was calculated using an established mathematical model (Pfaffl 2001). PCR negative controls were performed using water (1 μL) instead of cDNA.

### Statistical analysis

The data from real-time PCR experiments were statistically analysed using the two-tailed *t*-test for unpaired samples (SPSS, Version 14, Munich, Germany). For analysis of cell survival, nonparametric Kruskal–Wallis *H*-test and Mann–Whitney test were applied (SPSS). With both evaluations, a level of $P \leq 0.05$ was considered as significant.

## Results

### Heat-killed *S. mutans* induced IL-6 and IL-8 in odontoblast-like cells

For stimulation experiments, odontoblast-like cells were exposed to heat-killed *S. mutans* for 6 and 24 h. After 6 h of stimulation, the gene expression of both IL-6 and IL-8 was significantly induced in response to heat-killed *S. mutans* ($P = 0.002$ (mean difference: −1579.9; 95% confidence interval of the difference: −2175.4/−84.5) and $P < 0.001$ (mean difference: −105.5; 95% confidence interval of the difference: −119.8/−92.2), respectively) (Fig. 2a,b). For IL-6, the mRNA expression was significantly higher in odontoblast-like cells when stimulated for 6 h compared with 24 h [$P = 0.019$ (mean difference: 1370.8; 95% confidence interval of the difference: 756.5/1985.1)] (Fig. 2a). The mRNA of IL-8 also showed a significantly higher expression after 6 h compared with 24 h of stimulation with heat-killed *S. mutans* [$P < 0.001$ (mean difference: 94.5; 95% confidence interval of the difference: 76.7/112.2)] (Fig. 2b). Controls using blank bacterial media did not influence the gene expression of IL-6 and IL-8 in odontoblast-like cells (Fig. 2a,b).

### Gene expression of IL-6 and IL-8 was PI3K-dependent

For inhibition experiments, odontoblast-like cells were pre-incubated with the specific inhibitor LY 294002 targeting the PI3K pathway. In response to heat-killed *S. mutans*, the gene expression of IL-6 was significantly reduced in cells pre-incubated with the inhibitor compared with untreated but stimulated odontoblast-like cells [$P = 0.002$ (mean difference: 1533.3; 95% confidence interval of the difference: 937.8/2128.8)]. The mRNA expression of IL-6 in controls using blank bacteria media mixed with LY 294002 (163 μmol $L^{-1}$) and LY 294002 (163 μmol $L^{-1}$) alone was significant lower compared with untreated, but stimulated cells [$P = 0.003$ (mean difference: 1484.7; confidence interval of the difference: 863.7/2105.8) and $P = 0.002$ (mean difference: 1473.9; confidence interval of the difference: 878.3/2069.7), respectively] (Fig. 3a).

The IL-8 mRNA expression was significantly lower in LY 294002 pre-incubated compared with untreated but stimulated odontoblast-like cells when exposed to heat-killed *S. mutans* [$P < 0.001$ (mean difference: 103.9; 95% confidence interval of the difference: 89.6/118.3)] (Fig. 3b). The gene expression of IL-8 in controls using blank bacteria media mixed with LY 294002 (163 μmol $L^{-1}$) and LY 294002 (163 μmol $L^{-1}$) alone was significantly lower compared with untreated, but stimulated cells [$P < 0.001$ (mean difference: 105.4; confidence interval of the difference: 90.9/119.9) and $P < 0.001$ (mean difference: 105.3; confidence interval of the difference: 90.9/119.6), respectively] (Fig. 3b).

### Cell survival

After treatment with LY 294002 and stimulation with heat-killed *S. mutans*, cell survival was tested using the Trypan-Blue® test. Inhibitor treatment and stimulation with heat-killed *S. mutans* did not affect viable cell

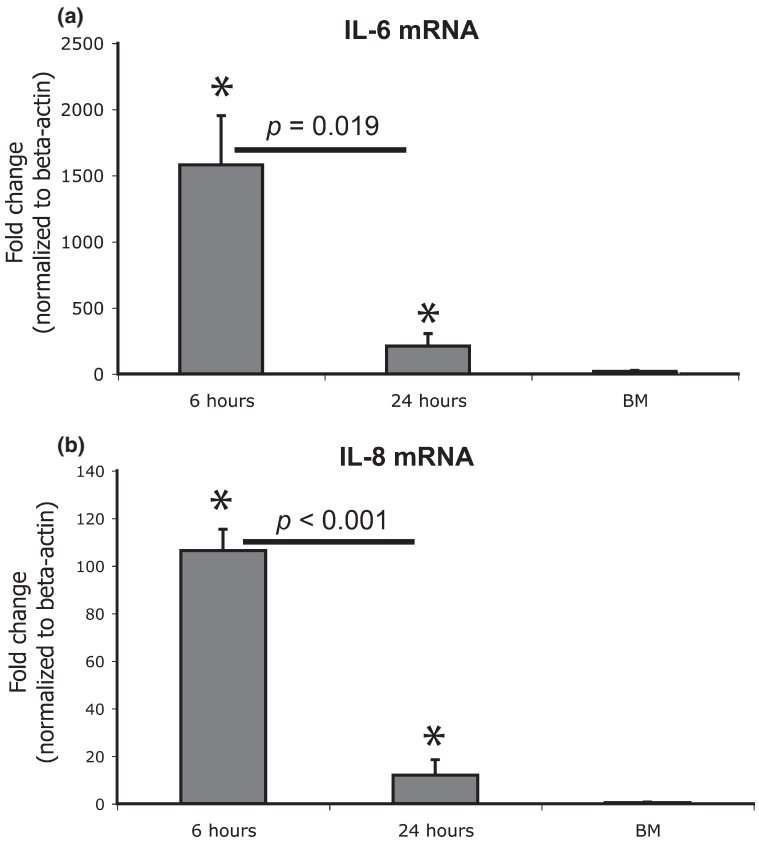


**Figure 2** Gene expression of Interleukin-6 and -8 (IL-6, IL-8) in odontoblast-like cells exposed to heat-killed *Streptococcus mutans*. (a) The IL-6 gene expression was significantly increased after 6 and 24 h ($P = 0.002$ and $P = 0.018$, respectively) in odontoblast-like cells when stimulated with heat-killed *S. mutans*. (b) The IL-8 gene expression was significantly increased after 6 and 24 h ($P < 0.001$ and $P = 0.043$, respectively) in odontoblast-like cells when stimulated with heat-killed *S. mutants*. The mRNA expression of IL-6 and IL-8 was significantly higher after 6 h compared with 24 h of stimulation ($P = 0.019$ and $P < 0.001$, respectively). Controls with blank bacteria medium did not affect the IL-6 or IL-8 gene expression. Bars and error bars represent mean values and standard deviation, respectively. Each bar represents three experimental replications ($n = 3$). BM, blank bacteria medium mixed with DMEM; *significant changes.

counts in comparison with the unstimulated control ($P = 0.944$). No differences in counts of nonviable cell were detected in treatment and control groups ($P = 0.306$). In the control and treatment group, the number of viable cells was significantly higher than the number of nonviable cells after 24 h ($P < 0.002$) (Fig. 4).

## Discussion

Deep carious lesions commonly cause inflammatory reactions of the dentine–pulp complex (Jontell *et al.* 1998, McLachlan *et al.* 2004). Interleukins such as IL-6 and IL-8 are involved in pro-inflammatory reactions and hence are strongly induced as a result of bacterial infections (Akira *et al.* 1990, Schröder & Christophers 1992). Both odontoblasts and dental pulp fibroblasts synthesize IL-6 and IL-8 when stimulated by bacteria or bacterial components (Tokuda *et al.* 2002, Yang *et al.* 2003, Chang *et al.* 2005).

In this study, the mRNA expression of IL-6 and IL-8 was analysed in cultured odontoblast-like cells. The aim was to determine whether the IL-6 and IL-8 mRNA expression is inducible in response to heat-killed *S. mutans*. Furthermore, it was hypothesized that an induction of IL-6 and IL-8 mRNA expression in odontoblast-like cells is mediated via signalling pathways involving PI3K. To address the questions, odontoblast-like cells were cultured from patient samples (third molars) according to established protocols (Tjaderhane *et al.* 1998, Dommisch *et al.* 2007a, Veerayutthwilai *et al.* 2007). The odontoblast phenotype was confirmed by analyzing the gene expression of DSPP (Begue-Kirn *et al.* 1998). Odontoblast-like cells were then stimulated with bacterial preparations of heat-killed *S. mutans*. To exclude false interpretation of stimulation experiments, cell survival was analysed using the Trypan-Blue® test (Thaweboon *et al.* 2006). It could be shown that more than approximately 75% of control and stimulated cells were still viable after 6 and 24 h. It was noticed that there was higher variability in the viable cell counts when cells were exposed to heat-killed *S. mutans*. As only moderate variations were documented in counts of nonviable cells, it may be speculated that cell proliferation was affected by the

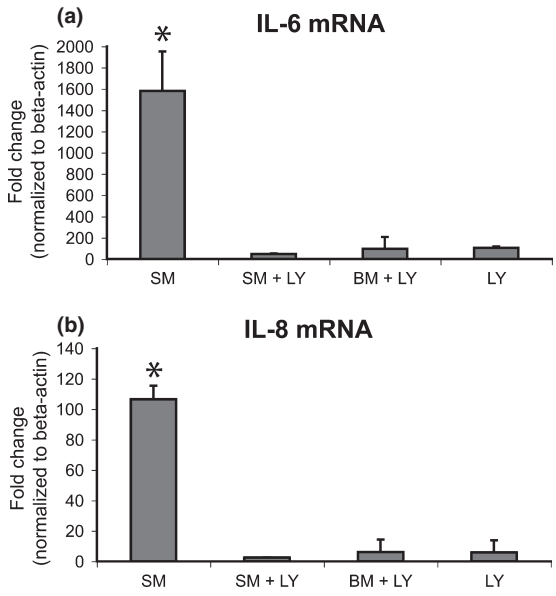


**Figure 3** Gene expression of Interleukin-6 and -8 (IL-6, IL-8) in odontoblast-like cells pre-treated with the PI3K-inhibitor LY 294002 and subsequent exposure to heat-killed *Streptococcus mutans* (6 h). (a) The IL-6 gene expression was significantly higher in cells that were not pre-treated compared with LY 294002-pre-treated odontoblast-like cells when responding to heat-killed *S. mutans* ($P = 0.002$). (b) The IL-8 gene expression was significantly higher in cells that were not pre-treated compared with LY 294002-pre-treated odontoblast-like cells when responding to heat-killed *S. mutans* ($P < 0.001$). The mRNA expression of IL-6 and IL-8 in controls using blank bacteria media mixed with LY 294002 (163 μmol $L^{-1}$) and LY 294002 (163 μmol $L^{-1}$) alone was significantly lower compared with untreated, but stimulated cells ($P < 0.003$ and $P < 0.002$, respectively). Bars and error bars represent mean values and standard deviation, respectively. Each bar represents three experimental replications ($n = 3$). SM, stimulation with *S. mutans*; BM, blank bacteria medium mixed with DMEM; LY, pre-treatment with the PI3K inhibitor LY 294002 mixed with DMEM; *significant changes.

exposure to heat-killed *S. mutans*. Therefore, additional effects on the gene expression of IL-6 and IL-8 because of exceeded cell death as a response to bacterial antigens could be excluded.

Recent studies have demonstrated that odontoblasts and dental pulp fibroblasts take part in regulation of immune processes by expressing pro-inflammatory mediators (Levin *et al.* 1999, Dommisch *et al.* 2007a, Veerayutthwilai *et al.* 2007). Odontoblasts with their processes extending into the dentinal tubuli represent the outer cell layer of the dental pulp adjacent to the dentine. Inflammatory reactions of the dental pulp may occur, even though advanced carious lesions would not have reached the pulp chamber. Bacteria and bacterial components that invade into dentinal tubuli and therefore stimulate odontoblasts and/or dental fibroblasts may cause this reaction.

This study reports increased mRNA expression of IL-6 and IL-8 in odontoblast-like cells in response to heat-killed *S. mutans*. In the time-dependent experiment, the gene expression of IL-6 and IL-8 showed higher levels after 6 h than after 24 h of stimulation suggesting early immune responses of odontoblast-like cells. Increased expression of IL-6 and IL-8 at early time-points could be shown in several studies on dental pulp fibroblasts (Patel *et al.* 2003, Yang *et al.* 2003, Tokuda *et al.* 2005). Stimulation experiments showed that the gene expression of IL-8 was increased in odontoblasts when exposed to LPS from *E. coli* (Levin *et al.* 1999). Recently, it was demonstrated that odontoblasts express pattern-recognition receptors such as toll-like receptor-2 (TLR-2) and TLR-4 (Veerayutthwilai *et al.* 2007). In response to the activation of TLR-2/TLR-4, the gene expression of pro-inflammatory mediators (TNF-alpha, IL-8) and antimicrobial peptides (macrophage-inflammatory-peptide-3-alpha, MIP-3 alpha; hBD-2) were affected in odontoblasts (Veerayutthwilai *et al.* 2007).

It is a recent finding that odontoblasts synthesize antimicrobial peptides (hBD-1, hBD-2) (Dommisch *et al.* 2005), which may also be able to modulate immune reactions of the dental pulp (Dommisch *et al.* 2007a). Stimulation experiments using recombinant hBD-2 led to increased gene expression of IL-6 and IL-8 in cultured odontoblast-like cells (Dommisch *et al.* 2007a). MIP-3 alpha and beta-defensin-2 possess antimicrobial properties against *S. mutans* and *Lactobacilli casei*, which present major aetiologic factors in caries development (Shiba *et al.* 2003).

To test the hypothesis that PI3K is involved in mediating the gene expression of IL-6 and IL-8, cultured odontoblast-like cells were stimulated with heat-killed *S. mutans*. Recent studies have demonstrated elevated levels of IL-6 and IL-8 in several cell types, but not yet in odontoblasts (Soell *et al.* 1994, Vernier *et al.* 1996, Chia *et al.* 2002). In this study, odontoblasts-like cells were treated with a specific inhibitor for PI3K (LY 294002) prior to the stimulation with heat-killed *S. mutans*. It could be demonstrated that LY 294002 blocked the signalling pathway involved in IL-6 and IL-8 gene expression.

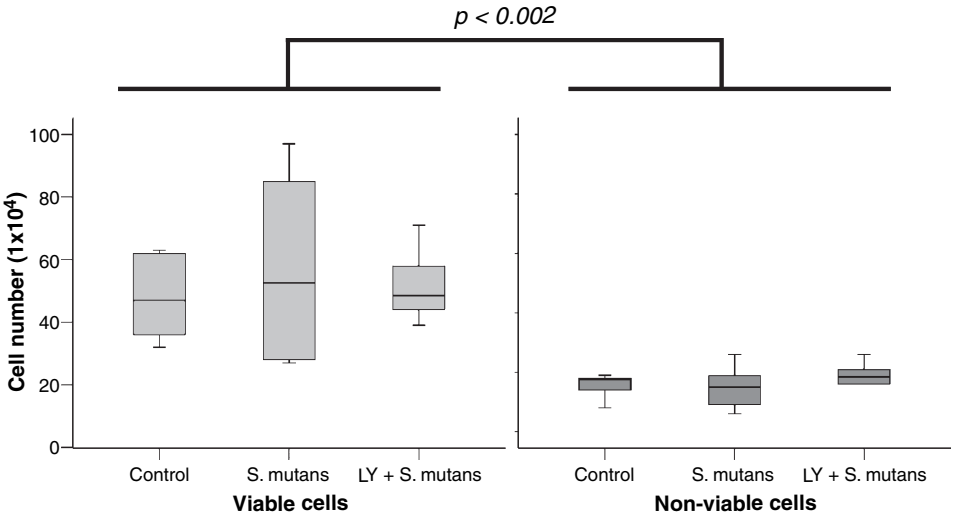


**Figure 4** Comparison of viable and non-viable cell counts. Stimulation with *S. mutans* and pre-treatment with LY 294002 (LY) did not affect viable and non-viable cell counts compared with the unstimulated control ($P = 0.944$ and $P = 0.306$, respectively). Approximately 75% of all cells were still viable in all treatment groups after 24 h compared with untreated and unstimulated cells, respectively ($P < 0.002$). Boxplots show minimum and maximum values (error bars), 25 percentile (lower boxplot line), median (middle line in box) and 75 percentile (upper boxplot line). Each boxplot represents six experimental replications.

These findings are in concert with a recent study on endothelial cells stimulated with viridans group streptococci (Yeh *et al.* 2006). Here, inhibition of PI3K blocked IL-6 and partly IL-8 expression (Yeh *et al.* 2006). So far, involvement of PI3K in IL-6 and IL-8 expression was tested in a number a different cell types (endothelial cells, carcinoma cells, cardial fibroblasts, microglia cells, retinal pigment cells, adipocytes, monocytes, neutrophils) (Bian *et al.* 2004, Xu *et al.* 2004, Ajuwon & Spurlock 2005, Huang *et al.* 2005a, Yadav *et al.* 2006, Tang *et al.* 2007, Turner *et al.* 2007, Wrann *et al.* 2007). Although odontoblasts are covered and therefore protected by dental hard tissues (enamel and dentine), these data indicate that odontoblasts respond to bacterial antigens with increased gene expression of pro-inflammatory mediators, and utilize PI3K as it has been shown for cells, such as monocytes and neutrophils, that actively take part in host immune responses. These studies and this report provide evidence that PI3K may be crucial in pathways signalling the gene expression of IL-6 and IL-8.

In conclusion, this study on cultured odontoblast-like cells showed increased gene expression of the pro-inflammatory mediators IL-6 and IL-8 in response to heat-killed *S. mutans*. The treatment of odontoblast-like cells with the pharmacological inhibitor LY 294002 (specific for PI3K) blocked the *S. mutans*-induced gene expression of both IL-6 and IL-8.

## Conclusion

Odontoblasts represent the first cell type of the dentine–pulp complex that may respond to cariogenic bacteria. The results of this study showed that heat-killed *S. mutans* induces the gene expression of IL-6 and IL-8 in odontoblast-like cells *in vitro*. This induction was blocked when cells were pre-treated with the specific PI3K inhibitor LY 294002. It is suggested that odontoblasts recognize bacteria, respond rapidly with elevated mRNA expression of pro-inflammatory mediators, and therefore actively participate in immune responses.

## Acknowledgement

This study was financially supported by the BONFOR research foundation (University of Bonn).

## References

Ajuwon KM, Spurlock ME (2005) Palmitate activates the NF-kappaB transcription factor and induces IL-6 and TNFalpha expression in 3T3-L1 adipocytes. *Journal of Nutrition* **135**, 1841–6.

Akira S, Hirano T, Taga T, Kishimoto T (1990) Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF). *The Federation of American Societies for Experimental Biology Journal* **4**, 2860–7.

Begue-Kirn C, Krebsbach PH, Bartlett JD, Butler WT (1998) Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. *European Journal of Oral Sciences* **106**, 963–70.

Bian ZM, Elner SG, Yoshida A, Elner VM (2004) Differential involvement of phosphoinositide 3-kinase/Akt in human RPE MCP-1 and IL-8 expression. *Investigative Ophthalmology and Visual Sciences* **45**, 1887–96.

Chang J, Zhang C, Tani-Ishii N, Shi S, Wang CY (2005) NF-kappaB activation in human dental pulp stem cells by TNF and LPS. *Journal of Dental Research* **84**, 994–8.

Chia JS, Lien HT, Hsueh PR, Chen PM, Sun A, Chen JY (2002) Induction of cytokines by glucosyltransferases of streptococcus mutans. *Clinical Diagnostic Laboratory Immunology* **9**, 892–7.

Coil J, Tam E, Waterfield JD (2004) Proinflammatory cytokine profiles in pulp fibroblasts stimulated with lipopolysaccharide and methyl mercaptan. *Journal of Endodontics* **30**, 88–91.

Dommisch H, Winter J, Acil Y, Dunsche A, Tiemann M, Jepsen S (2005) Human beta-defensin (hBD-1, -2) expression in dental pulp. *Oral Microbiology and Immunology* **20**, 163–6.

Dommisch H, Winter J, Willebrand C, Eberhard J, Jepsen S (2007a) Immune regulatory functions of human beta-defensin-2 in odontoblast-like cells. *International Endodontic Journal* **40**, 300–7.

Dommisch H, Chung WO, Rohani MG *et al.* (2007b) Protease-activated receptor 2 mediates human beta-defensin 2 and CC chemokine ligand 20 mRNA expression in response to proteases secreted by Porphyromonas gingivalis. *Infection and Immunity* **75**, 4326–33.

Donkersloot JA, Hoerman KC (1974) Letter: S mutans and caries. *Journal of the American Dental Association* **88**, 466–7.

Engels-Deutsch M, Pini A, Yamashita Y *et al.* (2003) Insertional inactivation of pac and rmlB genes reduces the release of tumor necrosis factor alpha, interleukin-6, and interleukin-8 induced by *Streptococcus mutans* in monocytic, dental pulp, and periodontal ligament cells. *Infection and Immunity* **71**, 5169–77.

Gobert AP, Vareille M, Glasser AL, Hindre T, de Sablet T, Martin C (2007) Shiga toxin produced by enterohemorrhagic *Escherichia coli* inhibits PI3K/NF-kappaB signaling pathway in globotriaosylceramide-3-negative human intestinal epithelial cells. *Journal of Immunology* **178**, 8168–74.

Hahn CL, Best AM, Tew JG (2000) Cytokine induction by *Streptococcus mutans* and pulpal pathogenesis. *Infection and Immunity* **68**, 6785–9.

Huang FC, Li Q, Cherayil BJ (2005a) A phosphatidyl-inositol-3-kinase-dependent anti-inflammatory pathway activated by Salmonella in epithelial cells. *The Federation of European Microbiological Societies Microbiological Letters* **243**, 265–70.

Huang FM, Chen YJ, Chou MY, Chang YC (2005b) Examination of the signal transduction pathways leading to upregulation of tissue type plasminogen activator by *Porphyromonas endodontalis* in human pulp cells. *International Endodontic Journal* **38**, 860–5.

Huang FM, Tsai CH, Chen YJ, Chou MY, Chang YC (2006) Examination of the signal transduction pathways leading to upregulation of tissue type plasminogen activator by interleukin-1alpha in human pulp cells. *Journal of Endodontics* **32**, 30–3.

Izumi T, Kobayashi I, Okamura K, Sakai H (1995) Immunohistochemical study on the immunocompetent cells of the pulp in human non-carious and carious teeth. *Archives of Oral Biology* **40**, 609–14.

Jontell M, Okiji T, Dahlgren U, Bergenholtz G (1998) Immune defense mechanisms of the dental pulp. *Critical Reviews in Oral Biology and Medicine* **9**, 179–200.

Lehrer RI (2004) Primate defensins. *Nature Reviews Microbiology* **2**, 727–38.

Levin LG, Rudd A, Bletsa A, Reisner H (1999) Expression of IL-8 by cells of the odontoblast layer *in vitro*. *European Journal of Oral Sciences* **107**, 131–7.

Lin SK, Kuo MY, Wang JS *et al.* (2002) Differential regulation of interleukin-6 and inducible cyclooxygenase gene expression by cytokines through prostaglandin-dependent and -independent mechanisms in human dental pulp fibroblasts. *Journal of Endodontics* **28**, 197–201.

McLachlan JL, Sloan AJ, Smith AJ, Landini G, Cooper PR (2004) S100 and cytokine expression in caries. *Infection and Immunity* **72**, 4102–8.

Pääkkönen V, Vuoristo JT, Salo T, Tjaderhane L (2007) Effects of TGF-beta 1 on interleukin profile of human dental pulp and odontoblasts. *Cytokine* **40**, 44–51.

Patel T, Park SH, Lin LM, Chiappelli F, Huang GT (2003) Substance P induces interleukin-8 secretion from human dental pulp cells. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **96**, 478–85.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**, e45.

Schröder JM, Christophers E (1992) The biology of NAP-1/IL-8, a neutrophil-activating cytokine. *Immunology Series* **57**, 387–416.

Shiba H, Mouri Y, Komatsuzawa H *et al.* (2003) Macrophage inflammatory protein-3alpha and beta-defensin-2 stimulate dentin sialophosphoprotein gene expression in human pulp cells. *Biochemical Biophysical Research Communication* **306**, 867–71.

Soell M, Holveck F, Scholler M, Wachsmann RD, Klein JP (1994) Binding of *Streptococcus mutans* SR protein to human monocytes: production of tumor necrosis factor, interleukin 1, and interleukin 6. *Infection and Immunity* **62**, 1805–12.

Tang CH, Lu DY, Yang RS *et al.* (2007) Leptin-induced IL-6 production is mediated by leptin receptor, insulin receptor substrate-1, phosphatidylinositol 3-kinase, Akt, NF-kappaB, and p300 pathway in microglia. *Journal of Immunology* **179**, 1292–302.

Thaweboon B, Thaweboon S, Choonharuangdej S, Suppakpatana P (2006) Effects of sonicated *Prevotella intermedia, Fusobacterium nucleatum* and *Lactobacillus casei* extracts on interleukin-8 production by human dental pulp cells. *The Southeast Asian Journal of Tropical Medicine and Public Health* **37**, 523–7.

Tjaderhane L, Salo T, Larjava H, Larmas M, Overall CM (1998) A novel organ culture method to study the function of human odontoblasts *in vitro*: gelatinase expression by odontoblasts is differentially regulated by TGF-beta1. *Journal of Dental Research* **77**, 1486–96.

Tokuda M, Nagaoka S, Torii M (2002) Interleukin-10 inhibits expression of interleukin-6 and -8 mRNA in human dental pulp cell cultures via nuclear factor-kappaB deactivation. *Journal of Endodontics* **28**, 177–80.

Tokuda M, Miyamoto R, Sakuta T, Nagaoka S, Torii M (2005) Substance P activates p38 mitogen-activated protein kinase to promote IL-6 induction in human dental pulp fibroblasts. *Connective Tissue Research* **46**, 153–8.

Turner NA, Mughal RS, Warburton P, O'Regan DJ, Ball SG, Porter KE (2007) Mechanism of TNFalpha-induced IL-1alpha, IL-1beta and IL-6 expression in human cardiac fibroblasts: effects of statins and thiazolidinediones. *Cardiovascular Research* **76**, 81–90.

Veerayutthwilai O, Byers MR, Pham TT, Darveau RP, Dale BA (2007) Differential regulation of immune responses by odontoblasts. *Oral Microbiology and Immunology* **22**, 5–13.

Vernier A, Diab M, Soell M *et al.* (1996) Cytokine production by human epithelial and endothelial cells following exposure to oral viridans streptococci involves lectin interactions between bacteria and cell surface receptors. *Infection and Immunity* **64**, 3016–22.

Wrann CD, Tabriz NA, Barkhausen T *et al.* (2007) The phosphatidylinositol 3-kinase signaling pathway exerts protective effects during sepsis by controlling C5a-mediated activation of innate immune functions. *Journal of Immunology* **178**, 5940–8.

Xu L, Pathak PS, Fukumura D (2004) Hypoxia-induced activation of p38 mitogen-activated protein kinase and phosphatidylinositol 3'-kinase signaling pathways contributes to expression of interleukin 8 in human ovarian carcinoma cells. *Clinical Cancer Research* **10**, 701–7.

Yadav M, Clark L, Schorey JS (2006) Macrophage's proinflammatory response to a mycobacterial infection is dependent on sphingosine kinase-mediated activation of phosphatidylinositol phospholipase C, protein kinase C, ERK1/2, and phosphatidylinositol 3-kinase. *Journal of Immunology* **176**, 5494–503.

Yamaguchi M, Kojima T, Kanekawa M, Aihara N, Nogimura A, Kasai K (2004) Neuropeptides stimulate production of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha in human dental pulp cells. *Inflammation Research* **53**, 199–204.

Yang LC, Tsai CH, Huang FM, Liu CM, Lai CC, Chang YC (2003) Induction of interleukin-6 gene expression by proinflammatory cytokines and black-pigmented Bacteroides in human pulp cell cultures. *International Endodontic Journal* **36**, 352–7.

Yeh CY, Chen JY, Chia JS (2006) Glucosyltransferases of viridans group streptococci modulate interleukin-6 and adhesion molecule expression in endothelial cells and augment monocytic cell adherence. *Infection and Immunity* **74**, 1273–83.