# Original Article Activation of autophagy in pulpitis is associated with TLR4

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Abstract: Autophagy, an evolutionarily-conserved cellular process, recycles and degrades proteins and organelles to maintain intracellular homeostasis. Our previous study identified that autophagy is induced by inflammation and infection as a defense mechanism in human dental pulp. Pattern-recognition receptors (PRRs) play an important role in the recognition of invading pathogens and the initiation of inflammation within the human dental pulp. *Toll-like receptor 4* (TLR4), the transmembrane bacterial sensor, has been identified to be associated with pulpitis. Meanwhile, Autophagy is primarily activated by sensors of the innate immunity, as TLR4 signaling. This study hypothesized that TLR4 recognizing cariogenic bacteria may active the autophagy in inflamed human dental pulp. Immunohistochemical analysis revealed that Toll-like receptor 4 (TLR4), nucleotide-binding oligomerization domain-containing protein 2 (NOD2), light chain 3 (LC3) and interleukin-1 $\beta$  were more strongly expressed in the caries and pulpitis groups than in the normal pulp group. Double immunofluorescence analysis indicated distinct colocalization between TLR4, NOD2 and LC3 in the caries and pulpitis groups within the odontoblast layers. TLR4 inhibition decreases autophagy in lipopolysaccharide (LPS)-mouse preodontoblast cell line mDPC6T by Western blot analysis. These results suggest that LPS-induce autophagy is modulated by TLR4 in inflamed odontoblasts. Taken together, TLR4 in human pulp sensing pathogenic bacteria induced inflammatory cytokine production and activated autophagy to protect the host against bacterial invasion.

Keywords: Pulpitis, odontoblast, TLR4, NOD2, autophagy

# Introduction

Pulpitis is the inflammation of the dental pulp that is usually a sequel to caries [1]. Cariesrelated bacteria can cause caries with the demineralization of enamel and dentin and the subsequent deterioration of pulp tissue [2]. However, pulp inflammation is difficult to control and dissipate because of its unique features as the confinement in a hard chamber and its particular blood irrigation and lymphatic circulation [3]. In addition, even in Western societies, the clinical treatment strategies for pulpitis demonstrate relatively high failure rates of 60% after 5 years [4-6]. Thus, finding effective methods to inhibit pulpitis development is extremely urgent and of significant clinical importance.

Host defense in human dental pulp is achieved by innate and adaptive immunity. The innate immune system provides the first line of defense via pattern-recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs). Toll-like receptor 4 (TLR4), the transmembrane bacterial sensor, with a role in recognizing conserved PAMPs and regulating pro-inflammatory cytokine production, has been identified to be associated with pulpitis [7]. TLR4 is the key receptor that manages lipopolysaccharide (LPS) transmembrane signal transduction [8]. Another PRRs, nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is the intracellular sensor of muramyl dipeptide [9]. TLR4 and NOD2 can be produced and released by different cell types and are active in the dental pulp tissues [1012]. In addition, TLR4 and NOD2 have an additive effect in pro-inflammatory mediator production in human dental pulp cells (HDPCs), which implies the synergism between them in pulpitis [13].

Autophagy, an evolutionarily-conserved cellular process, recycles and degrades proteins and organelles to maintain intracellular homeostasis [14]. Autophagy controls inflammation through administrative interactions with innate immune signaling pathways by affecting immune mediator secretion and removing endogenous inflammasome agonists [15]. In oral inflammatory and infection diseases, autophagy can work as a cell-autonomous manner to balance the beneficial and detrimental effects of inflammation and immunity, depending on the disease stage and stress condition [16]. In our previous study, we reported that autophagy is induced by inflammation and infection as a defense mechanism in human dental pulp [17]. Meanwhile, Autophagy is primarily activated by sensors of the innate immunity, as PRR signaling [18, 19]. In particular, TLR4 works as a sensor to induce autophagy associated with innate immunity in macrophage [20]. Hence, we speculated that the activation of autophagy in inflamed human dental pulp is induced by TLR4 by recognizing cariogenic bacteria.

On this basis, the present study aimed to analyze the presence, distribution and colocalization of TLR4, NOD2 and autophagy protein in inflamed human dental pulp by immunohistochemical staining. In order to further elucidate the cross-communication between PRRs and autophagy, we explore the influence of TLR4 on autophagy in LPS-mouse preodontoblast cell line mDPC6T.

# Materials and methods

# Patients' dental pulp samples

Forty seven human third molars were collected from patients at the School and Hospital of Stomatology, Wuhan University. These molars included 16 cases of normal teeth, 16 cases of carious teeth without spontaneous pain, and 15 cases of pulpitis teeth. The diagnosis of carious teeth without spontaneous pain was in accordance with an established protocol [21]. Patients in the caries groups with pulpitis symptoms were excluded. The pulpitis group had a history of spontaneous and intense pain and without periodontal disease. The procedures were performed with permission from the Institutional Ethical Board of Wuhan University (2013-32) and based on the guidelines of the National Institutes of Health regarding the use of human tissues.

# Cell culture and LPS treatment

Self-established mDPC6T is a preodontoblastic cell line, which has most of the phenotypic characteristics and function of odontoblasts such as the expression identification markers of odontoblastic-related genes and mineralization ability [22]. MDPC6T was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL Life Technologies, Paisley, UK) at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Then Cells were incubated with LPS alone (1  $\mu g/\mu L$ ; Sigma, St. Louis, MO, USA) or LPS in combination with TAK-242 (Merck Millipore, Billerica, MA), a TLR4 receptor inhibitor for 12 h. Cells were incubated without LPS as control group.

# Immunohistochemical staining

The immunohistochemical staining was performed with a slight modification as previously described [23]. Three groups of human tissue samples (n=5) respectively were fixed in 4% buffered paraformaldehyde, subsequently dehydrated and embedded in paraffin. Then, the paraffin block-embedded tissue were cut into 5µm sections, deparaffinized, rehydrated and antigen retrieved with gastric enzyme. 3% H<sub>2</sub>O<sub>2</sub> and 2.5% bovine serum albumin (BSA) were used to block the activity of endogenous peroxidase and non-specific binding. After the blocking step, sections were incubated with primary antibodies overnight at 4°C. The antibodies included anti-LC3 (1:50), anti-TLR4 (1:250), anti-NOD2 (1:200), anti-IL-1ß (1:200). Horseradish peroxidase-conjugated secondary antibodies were used to detect antibodies binding using a diaminobenzidine substrate kit (DAKO, Carpinteria, CA, USA) under the guidance of manufacturer's protocol.

# Double immunofluorescence staining

The operation of tissue slices was as in the immunohistochemical staining before using immunohistochemical staining kit. After being blocked with 10% donkey serum for 1 h at



**Figure 1.** Expression of PRRs, inflammation and autophagy in human odontoblastlayer and adjacent tissues. Human teeth samples were sorted into normal, caries, and pulpitis groups (n=5). A-C. HE staining reflects the severity of the inflammation in each group. D-I. TLR4 and NOD2 were increasing in caries and pulpitis groups, and mainly located with odontoblasts and adjacent tissues. J-L. Immunostaining of LC3 could detect in caries and pulpitis groups. M-O. Expression of IL-1β elevated with the progression of tooth decay. Scale bars=50 μm.

37°C, slices were incubated with anti-LC3 (1:200) and anti-TLR4 (1:150) or anti-LC3 (1:200) and anti-NOD2 (1:100) simultaneously overnight at 4°C. After washing with PBS three times for 10 minutes each, the slices were incubated with Cy3-conjugated and Alexa Fluor

488- secondary antibodies (Jackson Immunoresearch West Grove, PA, USA) for 1 h at 37°C. Following washed with PBS three times for 10 minutes each, the slices were mounted in DAPIcontaining quenching sealing agent (ZhongshanGoldenBridge). Finally, a fluorescence

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**Figure 2.** Co-localization of TLR4 and autophagy in human odontoblasts layer. A-F. The expression of TLR4 and LC3 were dramatically increased in odontoblasts layer both caries and pulpitis groups. G-I. Co-localization of TLR4 and LC3 could be observed within odontoblasts layer. Scale bars=50 µm.

microscope (Leica, Germany) was used to observe and photograph the tissue slices.

# Western blot analysis

The total protein content were extracted from cells at indicated time points by using T-PER lysis buffer containing protease and phosphatase inhibitors (Pierce). The protein concentration was measured by using the BCA Protein Assay Kit (Pierce, Biotechnology). Equal amounts of protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked in Trisbuffered saline and Tween 20 (TBST) containing 5% nonfat dry milk for 1 h and then incubated at 4°C overnight with primary antibodies: anti-LC3 (Sigma; L7543), antibeclin1 (Novus; NB500-249), anti-TLR4 (Sigma; SAB1412080), anti-NOD2 (Abcam; ab31488), anti-IL-1ß (SANTA; sc-7884). Following rinsed with TBST 3 times for 10 minutes each, the membrane was incubated for 1 h with peroxidase-conjugated secondary antibodies at room temperature. The protein levels were quantified by densitometry using Image J (National Institutes of Health, Bethesda, MD, USA) and relative expression was calculated by normalized to  $\beta$ -actin.

# Statistical analysis

All experiments were independently repeated at least three times. Data reported as the Mean  $\pm$  SEM. Data were visualized and analyzed using GraphPad Prism 5.0. The significance of the differences between normal, caries and pulpitis groups were determined by using one-way analysis of variance; P<0.05 was regarded as statistically significant.

# Results

Expression of TLR4, NOD2, autophagy-related proteins and IL-1: in inflamed human dental pulp

Human pulpal tissues from normal, caries, and pulpitis groups were stained for TLR4, NOD2,



**Figure 3.** Co-localization of NOD2 and autophagy in human odontoblasts layer. A-F. The expression of NOD2 and LC3 were dramatically increased in odontoblasts layer both caries and pulpitis groups. G-I. Co-localization of NOD2 and LC3 could be observed within odontoblasts layer. Scale bars=50 µm.



**Figure 4.** Inhibition of TLR4 decreases autophagy in LPS- mDPC6T (A, B). Western blot detected the upregulated expression of TLR4, NOD2 and IL-1 $\beta$  in LPS- mDPC6T cells group compared to control group. Activated autophagy was marked by enhanced expression of LC3II/I and beline1. In TLR4 inhibited group, the autophagy related proteins and IL-1 $\beta$  obviously decreased.  $\beta$ -actin was used as a loading control. Experiments were repeated at least three times. Mean ± SEM; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 versus control group; ##P<0.01, ###P<0.001 versus LPS+TAK242 group.

IL-1 $\beta$  and autophagy marker LC3 (Figure 1). Hematoxylin and eosin staining reflected the severity of the inflammation in each group (Figure 1A-C). Inflammatory cells infiltrated, dental pulp cells increased, and dentinal tubules were disrupted as tooth decay progressed. TLR4, NOD2 and LC3 were highly expressed in the caries and pulpitis groups compared with the normal group. Moreover, membranousstain of TLR4 (Figure 1D-F) and cytoplasmic immunostain of NOD2 (Figure 1G-I) and LC3 (Figure 1J-L) were mainly detected in the odontoblastic layer rather than in the dental pulp cell layer. The expression levels of inflammatory indicators IL-1ß elevated with the progression of tooth decay (Figure 1M-O). These results primarily suggest the activation of PRRs (TLR4, NOD2) and autophagy exist simultaneously in inflamed human dental pulp.

# Colocalization of PPRs: TLR4, NOD2 and autophagy in human odontoblast layer

In order to explore the relationship between PRRs and autophagy, we generated double immunofluorescence experiments of TLR4 or NOD2 and autophagy in inflamed dental pulp compared to normal samples. TLR4 (Figure 2A-C) and NOD2 (Figure 3A-C) immunofluorescence in the tissue sections gradually strengthened similar to autophagy marker LC3 (Figures 2D-F and 3D-F). Moreover, distinct colocalization between PPRs (TLR4 and NOD2) and LC3 was obviously observed in the odontoblast layers of the caries and pulpitis groups (Figures 2G-I and 3G-I). These results indicate that the activation of autophagy is associated with PRRs in inflamed dental pulp.

# TLR4 inhibition decreases autophagy in LPSmDPC6T cells

To further illustrate the role of TLR4 in the autophagy of inflamed odontoblasts, we performed a functional assay using TAK 242, a TLR4 special inhibitor [24]. As shown in **Figure 4**, TLR4, NOD2 autophagy related proteins (LC3, belin1) and IL-1 $\beta$  were increased in LPS-mDPC6T group compared to control group by Western blot. When TLR4 was inhibited by TAK 242, the expression of autophagy related proteins obviously decreased. These results suggest that LPS-induce autophagy is modulated by TLR4 in inflamed odontoblasts.

# Discussion

TLR4 and NOD2 are found in various cell types, including neutrophils, macrophages, dendritic cells, epithelial and endothelial cells [25-27] and in malignant tumors [28]. As the key receptors, TLR4 and NOD2 trigger a proinflammatory response under the infectious and noninfectious stimulation. TLR4 or NOD2-mediated inflammation induced by exogenous or endogenous ligandsis involved in several acute and chronic diseases [29, 30]. In this manuscript, status of TLR4, NOD2 and autophagy in human dental pulp samples were upregulated, accompanied by elevated inflammation. These results were detected by immunohistochemicalstaining in human normal, caries and pulpitis samples. It suggests that TLR4 or NOD2-mediated inflammation and autophagy exist simultaneously in pulpitis. In accordance with our founding, TLR4 plays a crucial role in the early pulpitis stage at the same time of the bacterial invasion to dentinal tube [7]. NOD2 expression was significantly upregulated in dental pulps with acute inflammation compared with healthy ones[31]. Moreover, we have reported that autophagy is induced by infection as a defense mechanism in human dental pulp [17].

The results of double immunofluorescence staining reveal that the distinct colocalization between PRRs: TLR4, NOD2 and autophagy was more obviously in the caries and pulpitis groups than normal groups. It prompts that autophagy is associated with PRRs in human dental pulp inflammation. In fact, the interplay of TLR4, NOD2 with autophagy has received adequate attention. TLR4 serves as an environmental sensor for the autophagy-enhancing colocalization of autophagosomes and mycobacteria in macrophage [20]. During bacterial invasion, NOD2 directly interacts with ATG16L1 to induce the formation of autophagosomes [32]. The present study is the first to report the interplay of TLR4, NOD2 with autophagy in inflamed human pulp.

According to immunohistochemical results, TRL4 and NOD2 highly expressed in odontoblasts layer rather than human pulp stroma cells in all groups. In addition, we founded that once the odontoblast layer was disrupted, human pulp cells began to necrosis which contributing to the defense ability of human pulp overall decline. Odontoblasts and fibroblasts are two main types of mesenchymal cells in human dental pulp tissue which differ in location and function. Odontoblasts which situated at the pulp-dentin interface are the first cells to encounter a bacterial infection [33]. Odontoblasts play an important role in pulpal antiinfection and anti-inflammation, and their survivability critically impacts the prognosis of inflamed pulp [34]. Our results illustrate that odontoblast represent not only as the first line of defense but also the most important defense to play a critical role in the anti-infection responses of the dental pulp.

In order to confirm the relationship between TLR4 and autophagy in inflamed dental pulp, the LPS-treated mDPC6T cell line was used for further study. Western blot analysis showed that TLR4 in mDPC6T increased due to LPS treatment, accompanied by the upregulated expression of inflammation factor (IL-1B) and autophagy proteins (LC3II, beclin1). These results suggest the activation of TLR4 and autophagy exist simultaneously in inflamed odontoblasts. This is consistent with the results of series detection in human dental pulp samples by immunofluorescence staining. Western blot analysis also showed the expression of autophagy decreases in LPS-treated mDPC6T when TLR4 was inhibited by TAK 242. This demonstrates that LPS-induce autophagy is modulated by TLR4. Further experiments are necessary to confirm the specific regulatory mechanism. Actually, suppression of TLR4 by TAK 242 can attenuate neuro-inflammation responses and neuronal autophagy in the hippocampus in a rat model of traumatic brain injury [35].

Interestingly, the expression of NOD2 increased in MDPC6T when treated with LPS. This phenomenon prompts NOD2 takes subsidiary function when TLR4 activates autophagy in inflamed odontoblasts. A synergism occurs between TLR4- and NOD2-mediated signaling in cytokine production in other cell experiments. In specific, NOD2 acted in synergy with TLR4 to amplify the production of IL-1 $\beta$ , IL-6, IL-8, IL-10, and tumor necrosis factor (TNF)- $\alpha$  in primary monocytes [36]. However, in vivo, the increased expression of NOD2 in pulpitis is not just because of its assistance of TLR4. Dental pulp inflammation is caused by different types of pathogenic bacteria, such as streptococci, lactobacilli, actinomyces, Porphyromonasgingivalis, and Prevotellaintermedia [37]. TLR4 and NOD2 are involved in responses to the toxin produced by streptococci [13, 38], whereas lactobacilli can be detected by TLR4 and NOD2 in respiratory virus infection and intestinal inflammation [39, 40]. Oral microorganisms are complex pathogens with PAMPs requiring recognition by diverse PRRs. In addition, the same bacteria even cause the activation of multifarious PRRs. These factors are the reason of the simultaneous upregulated expression of TLR4 and NOD2, in inflamed human pulp.

In conclusion, TLR4 in human pulp sensing pathogenic bacteria induce inflammatory cytokine production and activate autophagy to protect the host against bacterial invasion. This finding can provide an immunological rationale for future novel therapies for human dental pulpitis.

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# Disclosure of conflict of interest

# None.

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