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Endotoxin tolerance induction in human periodontal ligament fibroblasts stimulated with different bacterial lipopolysaccharides

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ABSTRACT

Objective: Human periodontal ligament fibroblasts (hPDLFs) may play an important role in immune responses in the periodontal microenvironment by secreting proinflammatory cytokines. The present study aimed to explore the reaction of hPDLFs to a secondary lipopolysaccharide (LPS) challenge by examining cytokines and innate sensors.

Methods: Primary cultures of hPDLFs were obtained and identified by immunochemistry. Cells were treated with a secondary LPS challenge followed by a primary LPS or no LPS as a control. The levels of cytokines were assayed using cytometric bead array (CBA) kits, and the protein levels of Toll-like receptors (TLRs) were determined using flow cytometry. The messenger RNA (mRNA) levels of TLRs were determined using real-time polymerase chain reaction (PCR).

Results: We show that the initial LPS exposure significantly induced hPDLFs to produce cytokines interleukin-8 (IL-8) and IL-6, whereas the secondary LPS challenge dramatically diminished the levels of cytokines IL-8 and IL-6. The mRNA and protein levels of TLRs were increased by the initial LPS stimulus but decreased by the secondary LPS challenge.

Conclusion: An LPS stimulus induces immune responses in hPDLFs, whereas an LPS challenge exerts endotoxin tolerance by downregulating proinflammatory cytokines and TLR mRNA and protein expression. This process may confer hPDLFs with their essential functions for maintaining oral mucosal immunity homeostasis.

1. Introduction

Periodontal disease is a type of infection-driven chronic inflammatory disease that is ultimately characterized by the destruction of periodontal tissues and adult tooth loss.[1] Among the major periodontopathogens, which include *Porphyromonas gingivalis* (*P. gingivalis*), *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, and *Treponema denticola*, *P. gingivalis* is a gram-negative (G^-) anaerobe that is recognized as a primary etiological agent of chronic periodontal disease.[2] A major constituent of the outer membrane of G^- bacteria, lipopolysaccharide (LPS) is a crucial virulence factor involved in the evocation and evolution of periodontal disease. Host cells can be incited by different types of components and products from microbes to release various cytokines, such as

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tumour necrosis factor (TNF-α), interleukin-6 (IL-6), and IL-8.[2,3] However, if not promptly balanced, uncontrolled inflammation can cause tissue destruction.

The periodontal ligament, a type of tooth-supporting structure and non-mineralized connective tissue between alveolar bone and cementum, plays an important role in the maintenance and renewal of periodontal tissues. Human periodontal ligament fibroblasts (hPDLFs), the primary cells in the periodontal ligament, are involved in innate immune responses to invading bacteria through pattern-recognition receptors.[4] As an important innate immune sensor, Toll-like receptors (TLRs) are type I transmembrane glycoproteins expressed by resident cells and immune cells in the periodontal microenvironment and are involved in the binding of various ligands, such as peptidoglycan, LPS, and flagellin.[5] The recognitions of periodontopathogens by host cells elicit the initial inflammatory events, including the production of proinflammatory cytokines, and subsequently amplify a wide range of immune responses in innate and adaptive immunity.[5,6] Hence, TLRs play pivotal roles in launching host immune responses to maintain periodontal health as well as to evoke disease. Recent studies have considered a principal role for both TLR2 and TLR4 in the signal transduction for different LPS-producing species, such as the periodontopathogen *P. gingivalis* and intestinal pathogen *Escherichia coli* (*E. coli*).[7,8]

Previous stimulation of a host with low levels of pathogenic factors in vivo and in vitro induces a state of attenuated response to subsequent challenge, which is termed as endotoxin tolerance (ET).[9] Many immune cells, including monocytes, macrophages, lymphocytes, and neutrophils, exhibit this phenomenon. Characterized by decreasing inflammatory mediators, ET is known to be a potential mechanism to dampen excessive responses and destruction derived from sustaining a hyperresponsiveness of the host immune system. Different components of bacteria such as LPS and lipopeptide were identified to induce ET.[10] Accumulating evidence has revealed the involvement of TLR pathways, especially TLR2 and TLR4, in ET responding to LPS derived from *P. gingivalis* and *E. coli*.[11–14] Given that the oral cavity is exposed to an extremely extensive abundance and diversity of pathogens, tolerance induced by constant pathogenic stimulations might be an alternative strategy to maintain immune homeostasis in the periodontal microenvironment. Regardless, comparatively very little is known about the exact mechanisms of ET in inflammatory disease, particularly regarding whether the resident cells in periodontal tissues might be able to develop tolerance.

However, except for intestinal epithelial cells, which were identified as acquiring ET to maintain host–microbe symbiosis,[15] thus far, no other resident cells have been found to show ET. Thus, we speculated whether the resident cells in oral mucosa display this characteristic. Although recent study indicates that gingival fibroblasts (GFs) in periodontal tissues do not develop ET, hPDLFs still have not been excluded from this process.[16] Both kinds of cells can be induced by bacterial LPS to secrete IL-6 and prostaglandin E_2.[17] However, compared with GFs, hPDLFs show a higher expression of TLR2 and lower expression of CD14 response to bacterial components.[18] The heterogeneous molecular expression and anatomical location of both cell types may be helpful for hPDLFs to differentially respond to various bacteria and virulence factors. At present, it is not clear whether or to what extent hPDLFs are able to manage specific responses to a secondary challenge with LPS following a previous LPS stimulation. Therefore, in this study, we isolated hPDLFs to compare the responses to different bacterial LPS challenges, mainly focusing on the differential release of proinflammatory cytokines and associated TLRs in vitro. This study will further deepen our understanding of the regulation of ET by periodontopathogens and the homeostasis of oral mucosal immunity.

2. Materials and methods

2.1. Tissue collection

The Institutional Ethic Committee approved our protocol. Periodontal ligament tissues were obtained from the teeth extracted for orthodontic reasons from six periodontally healthy donors under approved guidelines set by the Ethical Committee Board of the Third Military Medical University. Informed parental consent was obtained from all of the patients prior to the experiments. The teeth were examined with a probing depth $\leq$3 mm but without gingival inflammation, bleeding on probing, and loss of attachment.

2.2. Cell isolation and culture of hPDLFs

The primary culture of hPDLFs was performed with reference to the method as previously described.[6,18–20] Briefly, the periodontal ligament tissues attached to the middle third root were exclusively scraped, cut into small pieces, vibrated and disaggregated with 0.1% collagenase I (Sigma, St. Louis, MO, USA) for approximately 45 min at 37 °C, and then inoculated into six-well plates (Corning Incorporated, Corning, NY, USA). The cells were cultured with Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% (v/v) foetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Beyotime, Shanghai, China) in a humidified atmosphere of 5% (v/v) CO_2 at 37 °C. The medium was refreshed every 3 days for the entire duration (10–14 days) of culture. After 80% confluence, the cells were detached with 0.25% (w/v) trypsin (Gibco) and passaged to purify. The cells were used at passages 4–6.

2.3. Immunohistochemistry

First, the cells were attached to coverslips at a density of 2×10^4 cells per well in 24-well plates (Corning) with complete culture medium. After 48 h, the coverslips were gently rinsed with PBS three times and fixed with 4% paraformaldehyde for 20 min. The experiments were then performed according to the manual of the PV-9000 polymer detection system (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China). Finally, chromogenic reaction was performed with a diaminobenzidine (DAB) kit (Beijing Zhongshan Golden Bridge Biotechnology) followed by