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Toll-like receptors 2 and 5 in human gingival epithelial cells co-operate with T-cell cytokine interleukin-17

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Background/aim: Periodontitis begins as the result of perturbation of the gingival epithelial cells caused by subgingival bacteria interacting with the epithelial cells via pattern recognition receptors. Toll-like receptors (TLRs) have been shown to play an important role in the recognition of periodontal pathogens so we have studied the interaction of TLR ligands with TLR2 and TLR5 for cytokine production in the cultures of gingival epithelial cells.
Methods: Immunohistochemistry was used for the localization of TLR2 and TLR5 in tissue specimens. Enzyme-linked immunosorbent assays were performed to detect the levels of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), released from gingival epithelial cell cultures following stimulation with TLR ligand alone or in combination with IL-17.
Results: Both TLR2 and TLR5 were increased in periodontitis (2128 ± 159 vs. 449 ± 59 and 2456 ± 297 vs. 679 ± 103, respectively, $P < 0.001$) including gingival epithelial cells that stained strongly. Cultured gingival epithelial cells stimulated with their respective ligands (HKLM, a TLR2 ligand that is also found in *Porphyromonas gingivalis*, and flagellin, a TLR5 ligand that is also found in *Treponema denticola*) produced both IL-1β and TNF-α. To mimic T-cell help, IL-17 was added. This further greatly enhanced TLR ligand-induced IL-1β ($P < 0.001$) and TNF-α ($P < 0.01$) production.
Conclusions: These findings show how pathogen-associated molecular patterns, shared by many different periodontopathogenic bacteria, stimulate the resident gingival epithelial cells to inflammatory responses in a TLR-dependent manner. This stimulation may be particularly strong in periodontitis and when T helper type 17 cells provide T-cell help in intercellular cooperation.

Key words: immunology; periodontitis; Toll-like receptor 2; Toll-like receptor 5

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The gingival epithelial cells form the first barrier against invasive bacteria and separate the internal host milieu from microbes in the gingival pocket. Epithelial cells also function as sensors for the presence of bacteria. The direct physical contact between the mucosal surface and the bacteria triggers the expression of a variety of immune response mediators from epithelial cells so that protection against bacteria is achieved via sophisticated innate host defense mechanisms (21). This activation of the innate defense system is thought to be controlled by cellular receptors that bind conserved structures of microbial origin. One major class of epithelial cell receptors that recognize pathogen-associated molecular

patterns (PAMPs) is the family of Toll-like receptors (TLRs) (27).

The TLRs may play a role in the host response against those gram-negative and gram-positive plaque bacteria (or actually their PAMPs) that, according to the currently prevailing consensus, cause periodontitis (13). Bacterial cell components can stimulate host cells via TLRs to produce proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), (9). The gingival epithelial cells express several TLRs, including TLR2 and TLR5 (16). Under prolonged exposure to high ligand concentrations, TLR expression is subject to change (20, 28). As the expression of the two aforementioned TLRs was graded higher in periodontal gingival tissues than healthy control tissues in a recent descriptive study (6), these TLRs were subjected to a more detailed study. The pattern-recognizing TLR2 is activated by bacterial lipoproteins, lipoteichoic acid, and, in conjunction with TLR1 or TLR6, by diacylated or triacylated lipopeptides, respectively (2, 23). Ligands for TLR2 are typically found in *Porphyromonas gingivalis* (7). TLR5 can sense flagellin, which is the major subunit of bacterial flagella (3, 11) and is found in *Treponema denticola* (17).

The innate immune response provides the first line of defense against bacteria but it also instructs the T cells of the adaptive immune response. Binding of conserved pathogen-derived molecules to TLRs activates signaling pathways, which in turn results in the transcription of a number of immune response genes that enhance antigen presentation and the development of T-cell responses. Interleukin-17 (IL-17) is a T-cell-derived cytokine that enhances many of the effects of IL-1β and TNF-α and coexists with these cytokines at the sites of the inflammation (15), such as periodontitis (5).

It was hypothesized that ligands typically expressed by periodontopathogenic bacteria and recognized by TLR2 or TLR5 stimulate gingival epithelial cells to produce proinflammatory cytokines and that this process might be further enhanced by epithelial cells cooperating with T cells (or actually a T-cell product).

Material and methods

Patients and gingival tissue samples

Gingival tissue samples were collected from 10 adult patients with periodontitis (age range 31–45 years) during routine periodontal flap operations after the initial phase of periodontal therapy comprising conventional scaling and root planing. Ten healthy control specimens were obtained from the extraction operations performed for retained wisdom teeth (age range 20–37 years). All subjects were in good general health, none of them had used antibiotics within the preceding 6 months and none of them was a smoker. Subjects in each group with a history of drug use associated with gingival overgrowth were excluded. The periodontal status of patients was assessed using plaque and gingival bleeding indices and probing depth (1). The local ethical committee approved the research plan and informed consent was obtained from all patients. After removal, the samples were immediately fixed in phosphate-buffered saline–4% formol solution before processing into paraffin blocks.

Immunohistological stainings

Deparaffinized tissue sections were incubated in 2.6 μg/ml rabbit anti-human TLR2 immunoglobulin G (IgG) or 1.3 μg/ml rabbit anti-human TLR5 IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The avidin–biotin–peroxidase complex method (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) was used for immunohistochemical localization of TLRs in accordance with the manufacturer's instructions. Randomly selected images were captured using × 20 objective magnification. The number of TLR2 and TLR5 immunoreactive cells was counted in three randomly selected fields using semi-automated ANALYSIS image analysis software (Soft Imaging Systems, AnalySIS, Munster, Germany).

Cell culture stimulation

Human gingival epithelial cells isolated using an explant culture technique were maintained in keratinocyte growth medium 2 (PromoCell, C-20211, Heidelberg, Germany) containing the supplement provided by the manufacturer. The cells were grown to confluence in 10-cm tissue culture plates with a medium change every 4–5 days. Thereafter, the confluent cells were reseeded into six-well plates before culture with or without various stimulants. Cells were incubated in the presence of HKLM-TLR2 agonist (10^8 cells/ml, InvivoGen, San Diego, CA), HKLM-TLR2 agonist plus IL-17 as the costimulant (IL-17, 10 ng/ml, R&D Systems Inc., Minneapolis, MN), *Salmonella typhimurium* Flagellin-TLR5 agonist (100 ng/ml, InvivoGen) or *S. typhimurium* Flagellin-TLR5 agonist plus IL-17. Control and conditioned media were collected after 24 h of incubation and stored at −80°C. Cultures were performed in triplicate and the concentrations of cytokines in the supernatants were determined with enzyme-linked immunosorbent assay (ELISA) kits.

ELISA

The levels of IL-1β and TNF-α were analyzed from gingival epithelial cell culture supernatants. The ELISA was performed with commercial colorimetric sandwich ELISA kits in accordance with the manufacturer's instructions (Quantikine, R&D Systems). The kits provided the minimum detection limits as 3.9 pg/ml and 15.6 pg/ml, for IL-1β and TNF-α, respectively.

Statistical analysis

Statistical calculations were made using the PRISM data analysis program (GraphPad Software Inc., San Diego, CA). One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was used to compare stimulated and non-stimulated epithelial cells. *P*-values <0.05 were considered statistically significant. The Mann–Whitney *U*-test was used to analyze the differences between the adult periodontitis patients and the controls.

Results

Expression of cytokines in gingival tissues

In the healthy gingival tissue most of the TLR2 and TLR5 immunoreactive cells were found in the epithelium. The overall intensity of TLR2 and TLR5 immunoreactivity in the epithelium was stronger in periodontitis than in the controls. Evaluation of the total number of TLR2 (2128 ± 159 vs. 449 ± 59) and TLR5 (2456 ± 297 vs. 679 ± 103) immunoreactive cells showed an increase in periodontitis compared with controls, ($P < 0.001$ for both). The TLR immunoreactive cells were, in particular, in periodontitis not only found in epithelial cells but also in subepithelial macrophages, fibroblasts and endothelial cells (Fig. 1A–D). IgG staining controls were negative, confirming the specificity of the staining (data not shown).

Induction of proinflammatory cytokines in human gingival epithelial cells upon stimulation with TLR2 or TLR5 ligands with and without IL-17

The effect of HKLM or Flagellin on the cytokine production of confluent gingival

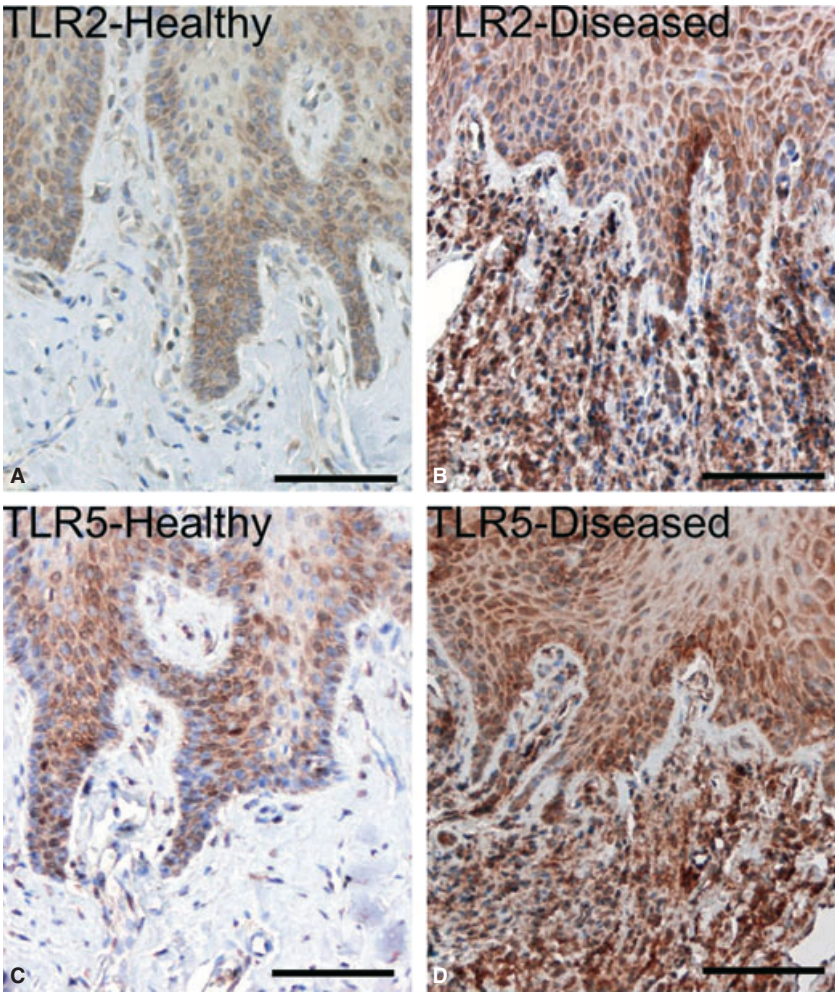

Fig. 1. Immunolocalization of Toll-like receptor 2 (TLR2) and TLR5 in gingival tissue. Total number of TLR2 (2128 ± 159 vs. 449 ± 59) and TLR5 (2456 ± 297 vs. 679 ± 103) immunoreactive cells showed an increase in periodontitis (B, D) compared with controls (A, C), ($P < 0.001$ for both). Scale bar 100 μm, analysis by *t*-tests.

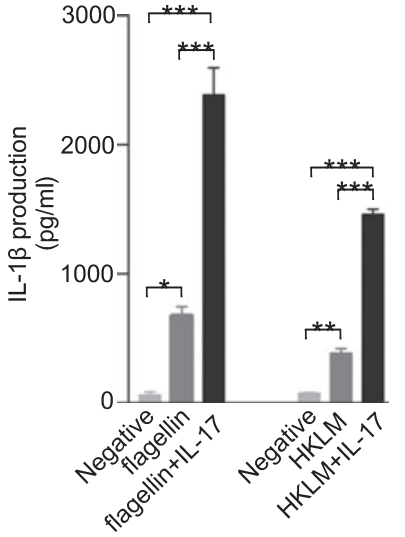

Fig. 2. Effect of flagellin and flagellin plus interleukin-17 (IL-17) or HKLM and HKLM plus IL-17 on induction of IL-1β concentration in gingival epithelial cultures *in vitro*. The results are from triplicate measurements, for both non-stimulated/negative (light gray bars) and stimulated cultures (gray and black bars), mean ± SEM are shown. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, analyses by ANOVA.

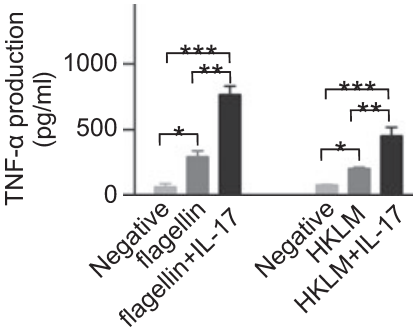

Fig. 3. Effect of flagellin and flagellin plus interleukin-17 (IL-17) or HKLM and HKLM plus IL-17 on induction of tumor necrosis factor-α (TNF-α) concentration in gingival epithelial cultures *in vitro*. The results are from triplicate measurements, for both non-stimulated/negative cultures (light gray bars) and stimulated cultures (gray and black bars), mean ± SEM are shown. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, analyses by ANOVA.

epithelial cells showed that such stimulation significantly increased IL-1β and TNF-α production. The combination of HKLM or Flagellin with IL-17 induced much more IL-1β and TNF-α than the TLR ligands when used alone (Figs 2, 3).

Discussion

The present study demonstrates that TLR2 and TLR5 are expressed on oral epithelial cells and that these receptors have the potential to stimulate production of the proinflammatory cytokines IL-1β and TNF-α upon stimulation with their respective ligands. These results strongly suggest that TLR2 and TLR5 are functional in epithelial cells. This is important because the epithelium forms the first line of defense against bacteria in the gingival pocket.

Gingiva, the outer layer of the oral cavity, is consistently exposed to more than 500 bacterial species of both commensal and pathogenic nature (22). The host immune response employs TLR-dependent and non-TLR-dependent pathways to recognize pathogens and bacteria (17). Continuity of the epithelial cell layer has been thought not only to act as a physical barrier against exogenous noxious stimuli but also to play an active role in the modulation of immune responses (19). We hypothesized that as innate pathogen detectors, TLRs should be expressed by epithelial cells. Our findings that epithelial cells express TLR2 and TLR5 in immunohistochemically stained tissue samples support this hypothesis and extend earlier findings showing TLR2 and TLR5 expression in corneal, gastric and intestinal epithelial cells (8, 24, 31). They also extend a recent study by Kinane et al., who reported TLR messenger RNA expression in gingival epithelial cells (16).

Binding of PAMPs to their pattern-recognizing TLRs activates a proinflammatory cytokine network and up-regulation of costimulatory molecules, which link the rapid innate response to the adaptive immunity (27). In line with such reasoning, stimulation of human gingival epithelial cells with TLR2 or TLR5 ligands greatly increased the production of both IL-1β and TNF-α. Their potent proinflammatory properties mean that the corresponding ligand–receptor interaction *in vivo* might

significantly contribute to gingivitis and periodontitis.

P. gingivalis and *T. denticola* are only two examples out of hundreds of potentially periodontopathogenic bacteria that have been demonstrated in deep gingival pockets and plaques (4, 30). On the other hand, the ligand specificity of the TLRs is very broad as they recognize patterns often shared by tens or hundreds of different microbes. This is exemplified by the fact that in contrast to numerous T-cell receptor specificities, only 10 different pattern-recognizing TLRs have been found in man. Therefore, our findings suggest that participation of relatively few but shared microbial patterns may play a key role in the early epithelial inflammatory response and, via up-regulation of the costimulatory molecules (14, 29), such as IL-1β and TNF-α, also in the late adaptive immune response. Periodontitis might provide a useful model to study inflammatory diseases, which are caused by many microbes, not only by one.

Both *P. gingivalis* and *T. denticola* are anaerobic gram-negative bacteria. Their concentrations are often periodically much increased in periodontitis so they are thought to play an important role in the pathogenesis of periodontal disease (12, 30). This corresponds well with the observations about gingival epithelial cells expressing TLR2 and TLR5 (6, 16), their changed expression pattern in periodontitis as well as the ability of their PAMP-type ligands to stimulate epithelial cells to produce IL-1β and TNF-α (10, 25). These findings support the role of the innate epithelial cell-mediated immune response in the pathogenesis of periodontitis.

Although disease-typical PAMPs greatly enhanced IL-1β and TNF-α production by epithelial cells, the addition of IL-17 markedly further increased their production. These results are in agreement with those of earlier studies demonstrating that IL-17 appears to function in conjunction with IL-1β and TNF-α to exacerbate the inflammatory process in inflammatory diseases (5). IL-17 is a T-cell-derived proinflammatory cytokine that is mainly produced by activated T cells. High levels of IL-17 as well as increased numbers of IL-17-secreting T cells have been detected in periodontitis lesions (26). Our data further demonstrate that stimulation of epithelial cells with TLR ligands combined with IL-17 synergistically enhances IL-1β and TNF-α production. It can be concluded, therefore, that TLR stimulation in the absence of IL-17 stimulation could initiate the host response. Nevertheless, the progression of inflammation leads also to T-cell involvement in the lamina propria, which further aggravates the process and might contribute to severe, destructive periodontitis.

In conclusion epithelial cell stimulation with TLR2 and TLR5 ligands, shared by many potential periodontopathogenic bacteria, leads to an increased production of IL-1β and TNF-α. Furthermore, the synergistic effect of the T cell-derived cytokine IL-17 with such TLR ligands suggests a possible cooperative link between the native and adaptive arms of the host defense, which if excessive and prolonged may contribute to propagation of periodontitis.

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