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Immunohistochemical localization of Toll-like receptors 1–10 in periodontitis

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Background/aim: In periodontitis, bacteria and pathogen-associated molecular patterns are sensed by Toll-like receptors (TLRs), which initiate intracellular signaling cascades that may lead to host inflammation. In this study, the expression and distribution of TLRs (TLR-1 to TLR-10) were immunohistochemically detected in gingival epithelium and connective tissue.

Methods: Immunohistochemistry was used for the localization of TLRs in gingival tissue samples from 10 patients with chronic periodontitis and 10 healthy controls; these samples were obtained during routine periodontal flap operations and during extraction operations performed for retained wisdom teeth, respectively. For the evaluation, epithelial layers were stratified to basal, spinous, and superficial layers, and the percentages of TLR-positive cells were determined.

Results: Both healthy and periodontitis gingival tissues expressed all TLRs except TLR-10. In patients with periodontitis, epithelial cells showed increased TLR expression towards the basal layer. Healthy controls showed more variable cellular TLR expression and distribution between the layers of epithelium. In the connective tissue, consistently higher TLR expression was found within the periodontitis group compared to the healthy group.

Conclusions: For the first time, the cellular expression and distribution of TLR-1 to TLR-10 have been studied in periodontitis, indicating that TLR-1 to TLR-9 are differentially expressed both in connective tissue and epithelial layers. Except for TLR-7 and TLR-8, all the other TLRs showed statistically significant differences between patients with periodontitis and healthy controls, suggesting their involvement in the pathogenesis of periodontitis.

Key words: immunology; inflammatory diseases; periodontium

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Periodontitis is initiated and maintained by a microbial biofilm that forms the dental plaque (14). Dental plaque may contain several hundred different bacterial species (1, 17) so no single periodontopathogenic species has been identified as the sole causative factor for periodontitis (9, 21). It has been recognized that pathogen-associated molecular patterns (PAMPs) from a large array of microbes may stimulate inflammatory and tissue-destructive host responses via a family of membrane-bound Toll-like receptors (TLRs) that belong to the pattern recognizing receptors (24, 25). TLRs form an important and potentially controllable checkpoint for a limited number of PAMPs derived from a large number of different bacterial species. TLRs can be classified according to the types of ligands that they recognize. For example, TLR-1 recognizes triacyl lipopeptides. Lipid-based structures are recognized by TLR-2 and TLR-4. Viral and/or bacterial nucleic acids are recognized by TLR-3, TLR-7, TLR-8, and TLR-9; the flagellin and peptidoglycan ligands are recognized by TLR-5 and TLR-6, respectively (13).

Toll-like receptors lack the sophisticated specificity and diversity of the immune receptors because their specificity is restricted to a limited number of

microbial patterns. TLRs lack memory, which is characteristic to the immune system (6), but they offer an almost instant response and protection against microbial inflammation (26), thereby covering the vulnerable period before full engagement of the immune system. During this time, PAMP-mediated stimulation of TLRs leads to the production of interleukin-1 and tumor necrosis factor-α, i.e. to an acute, protective inflammatory host response (4). This also provides the secondary stimulus for the specific primary immune event based on the processing and presentation of antigen in the context of major histocompatibility complex class II molecule to the T-cell receptor. PAMP–TLR interactions play here an important role by upregulating costimulatory cell surface molecules, e.g. intercellular adhesion molecule-1 on antigen-presenting cells and leukocyte function-associated antigen-1 on T cells (28). The TLR system therefore mediates the first-line defense against infections and provides the secondary stimulus to the adaptive immune response (27). We hypothesized that gingival epithelial cells are equipped with at least some of the recognized TLRs to maintain the normal microbe–host balance. The aim of the study was to clarify this by using a full panel of TLR-specific antibodies (TLR-1 to TLR-10) and immunohistochemistry to localize and evaluate the expression of TLRs in periodontitis.

Material and methods

Patients and samples

Gingival samples were collected from 10 patients with chronic periodontitis during routine periodontal flap operations after the initial phase of periodontal therapy comprising conventional scaling and root planing (age range 31–45 years). The diagnosis of chronic periodontitis was based on pathological values for the gingival index (≥ 2) (12), plaque index (≥ 2) (22), probing pocket depths (>4 mm), which bled upon probing, and clinical attachment loss combined with radiographic evidence of bone loss as detected in orthopantomograms.

The new classification system for periodontal diseases does not consider age discrimination (2); in this study, inclusion criteria for chronic periodontitis comprised patients with at least 20 natural teeth and a minimum of six periodontal pockets >4 mm. In contrast, the inclusion criteria for controls comprised the absence of clinical and radiographic manifestations of periodontal disease, at least 20 teeth present, no history of periodontal treatment, no history of systemic diseases, and no pregnancy.

Periodontal pocket depth (in mm; 4.59 ± 0.5 vs. 1.9 ± 0.3) was measured at six sites per tooth, for periodontitis vs. healthy controls, respectively (mean ± SD). In addition, no clinical attachment loss was detected for the healthy group, whereas the loss for the periodontitis group was 3.01 ± 0.6 mm (mean ± SD).

Ten healthy control specimens were obtained from tooth extraction operations performed for retained wisdom teeth (age range 20–37 years). Patients who had used antibiotics within the preceding 6 months or who had a systemic disease that affects the periodontium were excluded from the study. The study was conducted in accordance with the World Medical Association's Declaration of Helsinki and the research plan was approved by the local ethical committee; an informed consent was obtained from all study subjects. Samples were fixed in phosphate-buffered saline–4% formol and processed to paraffin blocks. Three serial sections were cut and fixed per slide.

Immunohistochemistry

The optimal immunoglobulin G (IgG) concentration for immunohistochemistry was confirmed for each TLR antibody in pilot experiments in which five consecutive deparaffinized tissue sections were stained using a dilution series of the antibody. After this, all periodontitis and gingival tissue control samples were stained using this antibody concentration to allow comparisons between staining intensity. The IgG concentrations used were as follows: 0.8 μg/ml rabbit anti-human TLR-1 IgG, 2.6 μg/ml rabbit anti-human TLR-2 IgG, 2 μg/ml rabbit anti-human TLR-3 IgG, 1.3 μg/ml rabbit anti-human TLR-4 IgG, 1.3 μg/ml rabbit anti-human TLR-5 IgG, 1 μg/ml goat anti-human TLR-6 IgG, 0.8 μg/ml goat anti-human TLR-7 IgG, 1 μg/ml rabbit anti-human TLR-8 IgG, 0.5 μg/ml rabbit anti-human TLR-9 IgG, or 1.6 μg/ml rabbit anti-human TLR-10 IgG (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA). The TLR immunoreactivity was detected using the avidin–biotin–peroxidase complex method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Negatively staining controls were run in parallel with the immunostaining using non-immune rabbit and goat IgG (Jackson ImmunoResearch, West Grove, PA) at the same concentration as and instead of the primary IgG from immunized animals. These staining controls confirmed the specificity of the immunohistochemical staining. Tissue from rheumatoid arthritis synovitis has been described to contain all 10 known human TLRs and such samples were used as positive sample controls, which excluded false-negative staining results. All slides were coded numerically with no other identifying features placed on the slides.

Evaluation of immunostaining

To explain our results clearly they are presented for the epithelial cell layer and connective tissue separately.

For evaluation, the epithelium was also stratified into three different layers (18): the basal, spinous, and superficial cell layers. In the basal layer, cuboidal/columnar cells with nuclei were seen. Above this, the spinous layer was characterized by spherical cells. Cells of the superficial layer were identified in the outermost layers of basal and spinous epithelia. Epithelium covers the entire oral cavity and is supported by connective tissue, which consists of blood and lymphatic vessels, nerves, and a dense network of collagen fiber bundles. In the healthy tissue, the main resident cell type is the gingival fibroblast, which is responsible for the production of extracellular collagen fibers. Mast cells are also resident, whereas the polymorphonuclear cells, monocytes/macrophages, and lymphocytes vary in number and extent, reflecting the need for protection against developing periodontal infection.

Three serial sections were cut and fixed separately for each of the 10 different TLRs, for both healthy and periodontitis group. The slides were coded as 1I, 1II, 1III or 2I, 2II, 2III, and so on, starting with 1 and ending at 20. The slides from 1 to 10 were the sections from healthy samples, whereas the slides from 11 to 20 were the sections from patients with periodontitis.

The number of positive cells and the total number of cells were counted from four random fields. The cell percentages were calculated from the epithelial cell layers and underlying connective tissue separately.

Statistical significances were calculated using analysis of one-way variance (ANOVA) with Bonferroni's post-tests for multiple comparisons. The Mann–Whitney *U*-test was used to analyse the differences in TLR expression in connective tissue between healthy controls and patients with

periodontitis. Statistical calculations were made using the GRAPHPAD PRISM program (GraphPad Software Inc., San Diego, CA) and $P < 0.05$ was considered statistically significant.

Results

Histological analysis of TLRs in the epithelial layers

All TLRs except TLR-10 were found in the gingival epithelia. The mean percentages of TLR-positive cells are shown in Fig. 1. In periodontitis, the number of TLR-1-positive and TLR-5-positive epithelial cells was lower in the superficial layer compared with the healthy group.

The number of TLR-2-expressing cells was similar in patients with periodontitis and healthy controls. In contrast, cells expressing TLR-3 and TLR-9 showed significantly higher frequency in the superficial cell layer in periodontitis compared with healthy samples. TLR-4, TLR-6, and TLR-7 showed similar frequency between periodontitis samples and healthy controls.

The mean percentages of TLR-positive cells in the spinous cell layer varied widely between the healthy and diseased epithelia. The mean percentage of TLR-1-, TLR-4-, TLR-5-, and TLR-8-positive epithelial cells was higher in the healthy tissues, whereas TLR-2, TLR-3, TLR-6, TLR-7, and TLR-9 had higher frequencies in patients with periodontitis. However, only the differences for TLR-1, TLR-2, TLR-4, TLR-6, and TLR-9 reached statistical significance.

In the basal layer, the number of cells expressing all other TLRs except TLR-1 was higher in periodontitis samples compared with healthy controls. The percentages, including TLR-2, TLR-6, and TLR-9 were significantly higher in the basal layer of periodontitis samples. The mean percentages were also higher for TLR-3, TLR-4, TLR-5, TLR-7, and TLR-8 but the differences did not reach to statistical significance.

Taken together, the common finding in all periodontitis samples was that TLR-expressing cells were most frequently found in the basal cell layer, and the frequency tapered off towards the more superficial epithelial layers. Except for TLR-7 and TLR-8, the epithelial cells expressing all other TLRs showed a significant decrease from basal cells towards the upper layer (Fig. 2). In the healthy controls, more variable results were seen in different epithelial layers for each TLR (Fig. 3).

TLR1	*Superficial Cell L.*	*Spinous Cell L.*	*Basal Cell L.*
Healthy (*n* = 10)	93.9 ± 3.5	92.0 ± 2.4	87.1 ± 2.1
Periodontitis (*n* = 10)	76.8 ± 6.4	83.8 ± 5.8	85.1 ± 4.5

TLR2	*Superficial Cell L.*	*Spinous Cell L.*	*Basal Cell L.*
Healthy (*n* = 10)	82.7 ± 6.6	74.1 ± 7.4	77.6 ± 7.6
Periodontitis (*n* = 10)	77.1 ± 6.2	89.8 ± 3.9	88.8 ± 9.1

TLR3	*Superficial Cell L.*	*Spinous Cell L.*	*Basal Cell L.*
Healthy (*n* = 10)	71.5 ± 4.0	75.7 ± 3.1	81.4 ± 6.5
Periodontitis (*n* = 10)	78.7 ± 3.4	79.9 ± 3.2	84.5 ± 4.3

TLR4	*Superficial Cell L.*	*Spinous Cell L.*	*Basal Cell L.*
Healthy (*n* = 10)	56.1 ± 4.9	78.1 ± 2.1	77.8 ± 3.7
Periodontitis (*n* = 10)	61.1 ± 9.6	70.0 ± 4.8	82.0 ± 2.7

TLR5	*Superficial Cell L.*	*Spinous Cell L.*	*Basal Cell L.*
Healthy (*n* = 10)	82.9 ± 6.3	74.9 ± 7.5	75.8 ± 10.1
Periodontitis (*n* = 10)	54.6 ± 10.1	72.2 ± 7.1	83.8 ± 9.2

TLR6	*Superficial Cell L.*	*Spinous Cell L.*	*Basal Cell L.*
Healthy (*n* = 10)	74.9 ± 3.8	75.7 ± 3.0	78.1 ± 2.1
Periodontitis (*n* = 10)	83.5 ± 3.7	93.3 ± 3.9	92.6 ± 4.5

TLR7	*Superficial Cell L.*	*Spinous Cell L.*	*Basal Cell L.*
Healthy (*n* = 10)	84.0 ± 3.0	82.2 ± 8.8	83.3 ± 6.6
Periodontitis (*n* = 10)	87.0 ± 3.2	92.3 ± 3.4	95.6 ± 3.1

TLR8	*Superficial Cell L.*	*Spinous Cell L.*	*Basal Cell L.*
Healthy (*n* = 10)	80.3 ± 3.8	85.7 ± 9.6	87.9 ± 6.7
Periodontitis (*n* = 10)	78.3 ± 5.7	81.8 ± 4.8	84.3 ± 4.3

TLR9	*Superficial Cell L.*	*Spinous Cell L.*	*Basal Cell L.*
Healthy (*n* = 10)	33.8 ± 6.1	51.6 ± 3.2	55.3 ± 3.7
Periodontitis (*n* = 10)	46.1 ± 4.6	54.2 ± 4.8	66.5 ± 6.5

Fig. 1. Percentages of Toll-like receptor-positive cells to total number of cells in superficial, spinous, and basal cell layers of epithelium. The results are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, analyses by analysis of variance; L, layer.

Histological analysis of TLRs in connective tissue

All TLRs except TLR-10 were expressed in many macrophage- and fibroblast-like cells and endothelial cells in connective tissue in both patients with periodontitis and healthy controls. Although TLR-9 expression was weak and restricted to a limited number of cells in healthy controls, the numbers of all TLR-positive cells were much increased in periodontitis ($P < 0.001$). In the connective tissue most TLRs were detected in cells that morphologically resembled macrophages. The mean percentages of TLR-positive cells are shown in Table 1. In both the epithelium and connective tissue, no cells were positive when irrelevant negative control IgGs were used instead of specific TLR IgGs or antibodies (Fig. 4).

Discussion

This is the first study to show the immunocytochemical mapping of TLRs

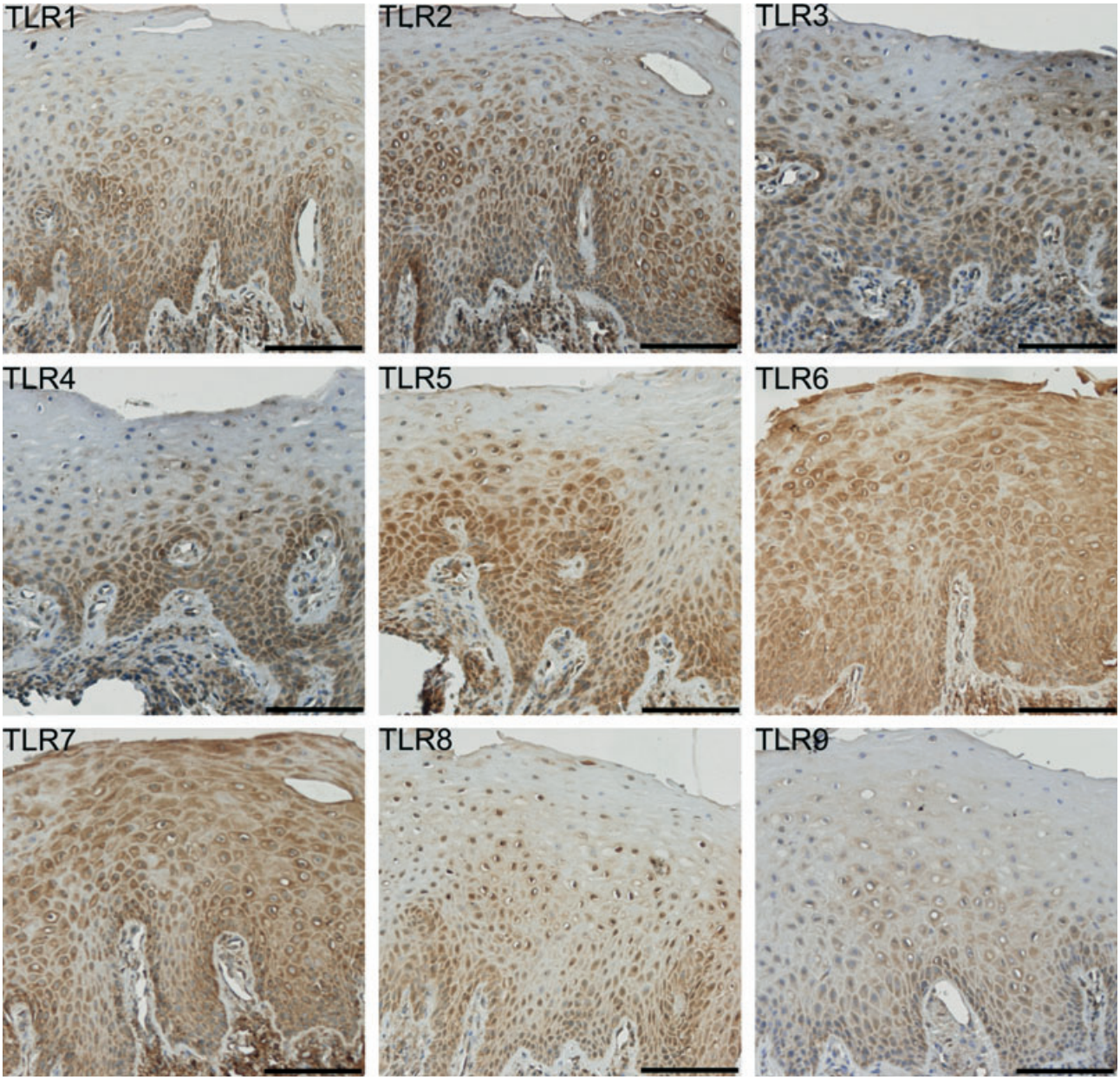

Fig. 2. Immunolocalization of Toll-like receptors (TLRs; 1–9) in the epithelial cell layers of gingival tissues from patients with periodontitis. Distribution of TLRs varies in the basal, spinous, and superficial cell layers of epithelium. Scale bar = 100 μm.

from TLR-1 to TLR-10 in samples from patients with periodontitis and from healthy control subjects, indicating that nine (TLR-1 to TLR-9) out of 10 have been expressed both in the connective tissue and the epithelial cell layers of all samples. Although the expression of TLRs was always much higher in connective tissue, PAMPs on the surface of periodontopathogenic bacteria seemed to differently affect various TLRs on the epithelial cells.

Microbe-typical components, common to a range of bacteria, can stimulate immune inflammatory cascades in healthy epithelium to maintain the normal oral microbial flora–host homeostasis. The functional basis for such a potential is the presence of pattern recognizing TLRs (24). The present work clearly demonstrates the presence of an almost full palette of TLRs in healthy gingival epithelial cells. In this respect our findings confirm and extend those reported by Kinane et al., who used human gingival epithelial cell cultures and reverse transcription–polymerase chain reactions to assess the presence of the components of the TLR system (8). The discrepancies between these two reports are the lack of TLR-8 messenger RNA (mRNA) in epithelial cell cultures but its presence in immunohistochemically stained tissue samples, and the presence of TLR-10 mRNA in cultured cells but the lack of immunoreactivity in tissue sections. The latter may indicate that TLR-10 mRNA is not translated to the corresponding TLR-10 cell surface protein or that the antibody did not work in our samples.

The intensity of immunoreactivities for individual TLRs and the frequency of positive cells varied in a topologically

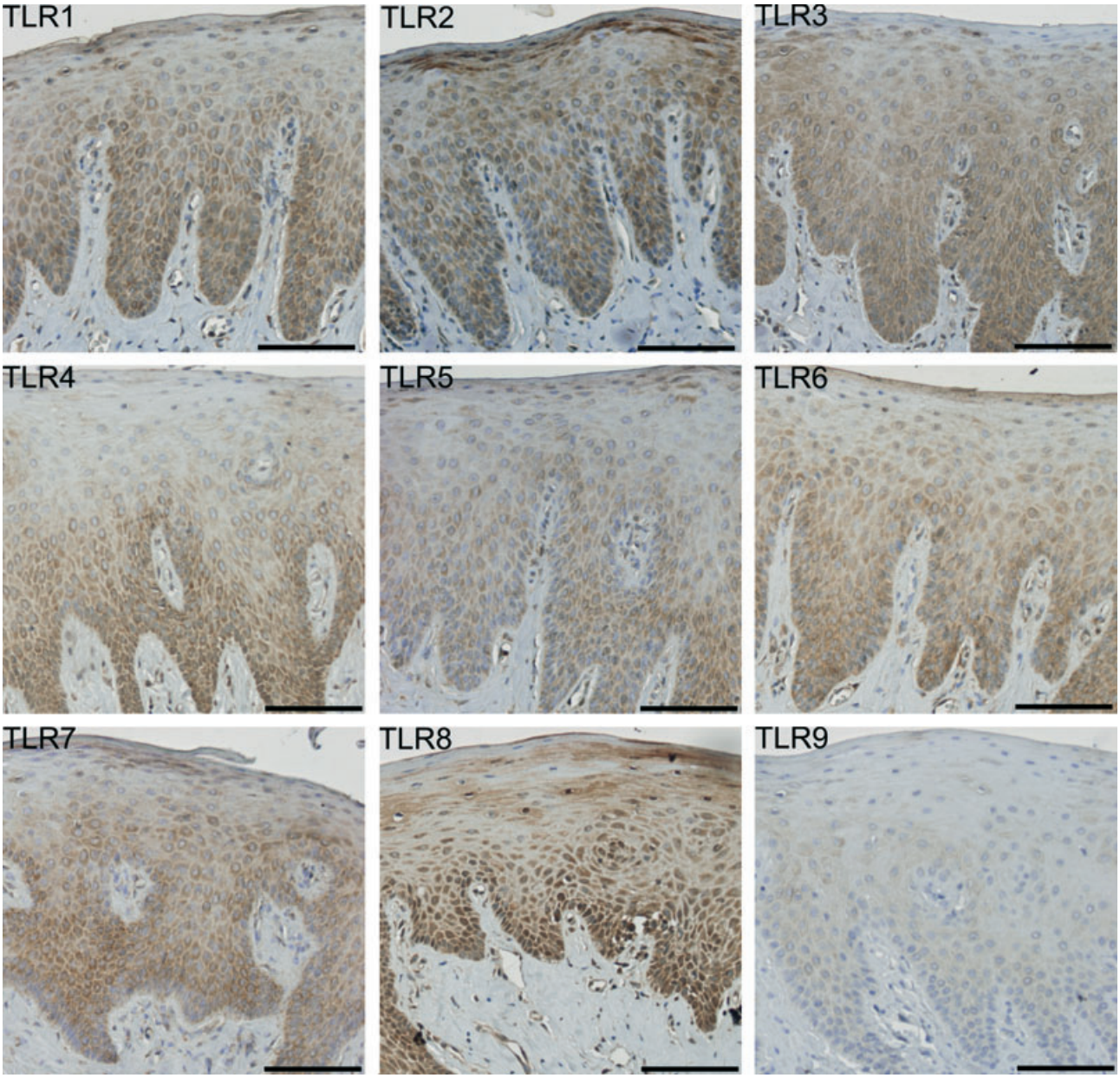

Fig. 3. Immunolocalization of Toll-like receptors (TLRs; 1–9) in the epithelial cell layers of healthy gingival tissues. TLRs are localized in basal, spinous, and superficial cell layers. Scale bar = 100 μm.

Table 1. Toll-like receptor (TLR)-positive cell percentages in the connective tissues of healthy and periodontitis gingival samples

	Healthy samples (n = 10)	Periodontitis samples (n = 10)	P-value[1]
TLR-1	34.7 ± 5.1	82.9 ± 12.3	< 0.001
TLR-2	50.6 ± 6.9	89.7 ± 17.5	< 0.001
TLR-3	40.3 ± 8.4	79.2 ± 13.7	< 0.001
TLR-4	45.7 ± 5.6	86.1 ± 12.6	< 0.001
TLR-5	46.1 ± 10.5	79.2 ± 11.5	< 0.001
TLR-6	42.1 ± 6.5	73.2 ± 13.5	< 0.001
TLR-7	39.5 ± 7.9	74.7 ± 10.5	< 0.001
TLR-8	35.7 ± 7.9	78.2 ± 6.5	< 0.001
TLR-9	12.1 ± 6.5	65.8 ± 9.5	< 0.001

The results are presented as mean ± SEM.
[1]Analyses by Mann–Whitney U-test.

characteristic way. Cells in the epithelial basal layer were most strongly labeled and TLR expression became progressively weaker towards the spinous and superficial layers. This may indicate that TLRs are mostly synthesized in the deeply located basal cells and that their synthesis ceases in the more superficial cell layers. Alternatively, downregulation of TLRs, amply documented in the literature (11), can occur via many different mechanisms affecting signaling pathways, receptor solubilization, intracellular negative regulators, transmembrane protein regulators,

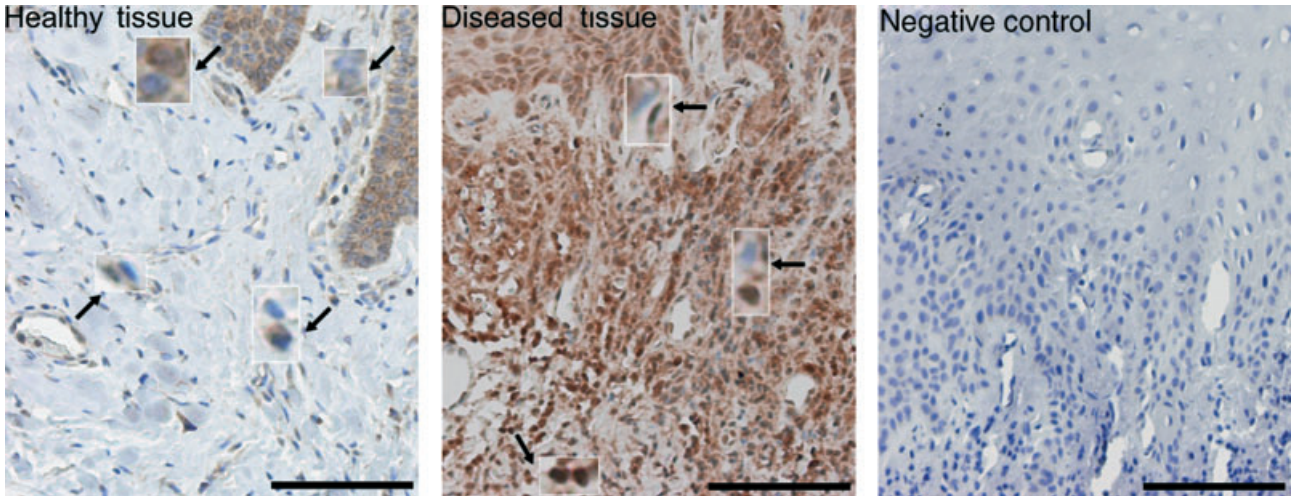

Fig. 4. Representative picture of Toll-like receptors (TLRs) staining in the connective tissue of the gingiva. Similar staining patterns were obtained for immunolocalization of all TLRs (1–9) in the connective tissue. In healthy gingiva, TLRs were expressed in macrophage-like and fibroblast-like cells and endothelial cells, although TLR-9 staining was weak. In periodontitis the numbers of all TLR-positive cells were much increased. Inserts: Sample images of TLR-positive/negative cells both in epithelial layer or connective tissue magnified three times. Scale bar = 100 μm.

reduction of TLR expression, and regulation of TLR effect by apoptosis. The main advantage of downregulation is inhibition of non-specific and potentially harmful inflammatory host responses. This is achieved with root planing, conventional scaling, and good oral hygiene, i.e. by reducing the concentration of dental plaque-derived PAMPs (which affects the ligands). In the future this could also be achieved using medicinal approaches to control the few critical checkpoints via which hundreds of different bacteria via their PAMPs drive only nine different human TLRs (which would affect the receptors). In regular infections the specific immune responses take over host defense after the TLR system has provided early protection and the secondary stimulus (danger signal) to the immune system. *In vivo* complete TLR downregulation is unlikely because new cells are recruited to the response via local cellular proliferation and/or recruitment from the circulation. The responder cells therefore form a heterogeneous population comprising cells during the late and early phases of response. To analyse this further, it would be interesting to study the TLR profile in gingival tissue in animals grown under germ-free conditions.

Analysis of the percentage of TLR-positive cells in different epithelial cell layers of the healthy and periodontitis tissues disclosed some interesting findings. Periodontitis was characterized by diminishing percentages of cells positive for TLR-1, TLR-2, and TLR-5 in the superficial epithelial cell layers. Interestingly, all these TLRs are located on the surface of the epithelial cells (24). This location is favorable for binding of such PAMPs, which are similarly located on the surface of gingival pocket and plaque bacteria (25). The most straightforward way to interpret these findings is to assume that these epithelial cell surface TLRs have been downregulated in periodontitis, and/or masked, by binding to their specific microbial cell surface-associated PAMPs. For example, TLR-1 and TLR-2 form a TLR-1/2 heterodimer that binds triacyl lipopeptides of periodontopathogenic *Mycobacteria* (16) and TLR-5 binds flagellin of *Treponema maltophilum* (10, 13).

TLR expression profiles have been exhaustively examined in many studies. For example, Sarah et al., showed significantly elevated TLR-2 and TLR-4 in tissues of patients with gingivitis and chronic periodontitis compared to controls (20). In another study, compared to healthy controls, increased expression of TLR-2 and TLR-4 by inflamed oral epithelium located at cell borders was found by Sugawara et al. (23). Although a similar technique was used and similar results were found in periodontitis samples, Ren et al. detected only weak expression of TLR-2 and no TLR-4 in healthy gingival tissue (19). In the present study, TLR-2 and TLR-4 were detectable in inflamed and healthy tissues; however, the diseased tissue presented less expression in the most superficial layer compared to the study of Sugawara et al. (23). A possible explanation for the discrepancies between the studies might be the innate immune response to bacterial products (15, 26). Depending upon the external milieu, activation of the epithelial cells may also be different. Epithelium, as a physically protective organ and as part of the innate branch of immune defense, seems to allow the host immunity to respond to the invading pathogens by recognition via TLRs. As a result of the variation in the samples with respect to the health condition of the patient participation of epithelial cells in homeostatic balance and/or bacterial clearance might change from patient to patient. The TLRs that are expressed may later be desensitized or downregulated to avoid tissue destruction by excessive innate immune responses and will no longer be detected on the sample.

Apart from the transmembrane receptors there are also TLRs located within the endosomal compartment of the epithelial cells. TLR-3, TLR-7, TLR-8, and TLR-9 are usually engaged by PAMPs, which in intact microbes are confined to internal microbial compartments. These ligands are double-stranded RNA (binding to TLR-3), single-stranded RNA (binding to TLR-7 and TLR-8), and unmethylated CpG DNA (binding to TLR-9) (24). TLR-7 and TLR-8, which typically bind viral single-stranded RNA, were found to be expressed by epithelial cells at similar frequency in healthy gingiva and in periodontitis. In contrast to TLR-7 and TLR-8, expression patterns of TLR-3 and TLR-9 were lower in the spinous and superficial layers compared with the basal epithelial layer. As epithelial cells are not typically phagocytosing cells a priori phagocytosis and

lysosomal/endosomal degradation and release of viral double-stranded RNA or bacterial unmethylated CpG DNA seems unlikely. Therefore, binding to exogenous PAMPs with subsequent downregulation seems doubtful. It has been shown that these TLRs can bind some endogenous ligands. This is a result of the relatively limited diversity of the TLR system compared to the T-cell receptor system. Pattern recognizing receptors recognize only similarities within patterns, not specific ligands. Indeed, mRNA has been described to activate TLR-3 (7) and mitochondrial DNA binds TLR-9 (5). These ligand–receptor interactions might, to some extent, be facilitated by the gingival epithelial cell apoptosis and/or periodontitis-associated inflammatory cell necrosis. Finally, endogenous ligands are indicated in the diminished expression of TLR-3 and TLR-9 in the superficial cell layers also by the fact that similarly diminished expression was found in healthy gingival tissue samples. As such samples were not affected by periodontitis but were healthy, engagement of TLR-3 and TLR-9 by viral double-stranded RNA and bacterial umethylated CpG DNA, respectively, seems particularly unlikely. The more extensive involvement in periodontitis might indicate the additional burden posed by periodontal inflammation.

As periodontitis is characterized by chronic mononuclear cell infiltrates in connective tissue and immigrant inflammatory cells form the mainstay of the TLR-mediated anti-microbial host defense, the greatly increased numbers of TLR immunolabeled cells in connective tissue was expected. Apart from the immigrant inflammatory cells, many local resident fibroblast-like cells and endothelial cells were also TLR-positive, possibly as a result of locally produced proinflammatory autocrine and paracrine cytokines (3, 13). At first sight, this increased TLR staining of the cells in connective tissue seems paradoxical with respect to the diminished expression just described for the epithelial cells. There is a basic difference between the relatively sessile epithelial cells on their way from the basal to the superficial cell layers and the very mobile monocytes/macrophages, which can be rapidly recruited from the intravascular compartment to the tissue compartment and which can migrate in tissues guided by locally produced chemokines. However, as a result of their exposed location, epithelial TLRs in healthy gingiva seem to be of primary importance for the normal steady-state microbial flora–human host symbiosis. Furthermore, it seems that dental plaque biofilm and calculus actively engage the local epithelial TLR system as the percentage of certain individual TLRs changes in a very characteristic pattern in patients with periodontitis compared with healthy gingival tissues.

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