Cellular crosstalk mechanism of Toll-like receptors in gingival overgrowth (Review)

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Abstract. Gingival overgrowth is an undesirable outcome of systemic medication and is evidenced by the accretion of collagenous components in gingival connective tissues along with diverse degrees of inflammation. Phenytoin therapy has been found to induce the most fibrotic lesions in gingiva, cyclosporine caused the least fibrotic lesions, and nifedipine induced intermediate fibrosis in drug-induced gingival overgrowth. In drug-induced gingival overgrowth, efficient oral hygiene is compromised and has negative consequences for the systemic health of the patients. Toll-like receptors (TLRs) are involved in the effective recognition of microbial agents and play a vital role in innate immunity and inflammatory signaling responses. TLRs stimulate fibrosis and tissue repairs in several settings, although with evident differences between organs. In particular, TLRs exert a distinct effect on fibrosis in organs with greater exposure to TLR ligands, such as the gingiva. Cumulative evidence from diverse sources suggested that TLRs can affect gingival overgrowth in several ways. Numerous studies have demonstrated the expression of TLRs in gingival tissues and suggested its potential role in gingival inflammation, cell proliferation and synthesis of the extracellular matrix which is crucial to the development of gingival overgrowth. In the present review, we assessed the role of TLRs on individual cell populations in gingival tissues that contribute to the progression of gingival inflammation, and the involvement of TLRs in the development of gingival overgrowth. These observations suggest that TLRs provide new insight into the connection among infection, inflammation, drugs and gingival fibrosis, and are therefore efficient therapeutic target molecules. We hypothesize that TLRs are critical for the development and progression of gingival overgrowth, and thus blocking TLR expression may serve as a novel target for antifibrotic therapy.

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Contents

1. Introduction

Drug-induced gingival overgrowth is an adverse reaction mostly associated with three types of regularly recommended drugs, i.e., immunosuppressants (cyclosporine) (1), antiepileptic drugs (phenytoin) (2) and calcium channel blockers (verapamil, diltiazem and nifedipine) (3-6). As gingival enlargement develops, regular oral hygiene practice is disturbed and progressively severe pain develops, often leading to disfiguration. Several etiological factors triggering gingival overgrowth have been studied and it was shown that the degrees of inflammation, fibrosis, dose, duration, quality of oral hygiene and identity of the drug, local bacterial plaque accumulation, individual susceptibility and environmental influences contribute to the progression of gingival overgrowth (7,8). Bacterial biofilm, one of the primary etiologic factors, harbors several hundred diverse bacterial species that colonize the sulcus and periodontal pockets, and initiate inflammation in the gingival tissues, leading to gingivitis and periodontitis. These bacteria have a number of structural components that directly destroy the periodontal tissues, or stimulate host cells to initiate a wide range of inflammatory responses. These inflammatory

responses are envisioned to overcome the microbial challenge, however, they are frequently responsible for further tissue destruction. The changes in the morphology of gingiva during gingival overgrowth lead to the retention of dental plaque which stimulates the inflammation (8). At the molecular level, an excess of mediators are released following gingival inflammation that contribute to the regulation of fibrogenic and regenerative signals (8). Out of several fibrogenic promoters, platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) play a vital role in the progression of fibrosis. It has been reported that PDGF and TGF-β were overexpressed in the promotion of spontaneous fibrosis of the kidney, liver, lung and gingiva (9-12). In addition to PDGF and TGF-β, a number of other mediators such as angiotensin II, endothelin-1, chemokines, leptin, interleukin-4 (IL-4), IL-6 and IL-13 play vital roles in fibrogenesis (13). Despite their protuberant role in fibrogenesis, recent studies indicate Toll-like receptors (TLRs), a group of receptors that regulate innate and adaptive immune responses, as significant modulators of inflammation during fibrosis (14-16). TLRs recognize pathogen-associated molecular patterns (PAMPs) and activate innate and adaptive immune responses to confiscate pathogens. In this review, we analyzed the role of TLRs in gingival overgrowth and discussed the possibility that TLRs may reveal innovative targets for the prevention or treatment of gingival overgrowth.

2. TLR expression and signaling in gingival tissues

Microbial biofilm induces the inflammatory process of gingival tissues and supporting structures, leading to gingivitis and periodontitis. Oral pathogens present in the biofilm initiate periodontal diseases by stimulating the host immune response. Epithelial cells and connective tissues of gingiva express TLRs, which are crucial in the inflammatory response (17). TLRs stimulate signaling via two main pathways. The first one is myeloid differentiation factor 88 (MyD88)-dependent, while and the second one is MyD88-independent. Following initiation, pro-inflammatory cytokines and type I interferons (IFNs) are produced. The production of pro-inflammatory cytokines is dependent on adaptor molecules MyD88 and TIR-associated protein (TIRAP), whereas IFNs are produced by TIR domain-containing adaptor protein-inducing IFN-β (TRIF) and TRIF-related adaptor molecules (TRAM) (18-21). Generally, bacterial triacylated lipopeptides are recognized by heterodimers of the TLR1/TLR2 complex (22). Bacterial lipoproteins and peptidoglycans derived from Gram-positive bacteria, are recognized by TLR-2. Endogenous ligands such as heat shock proteins are also recognized by TLR-2 (23). Double-strand DNA and its synthetic analogue polyinosine-deoxycytidylic acid are recognized by TLR-3, a potent stimulator of type I IFNs (24). The extensively studied TLR-4 recognizes a wide range of ligands mainly associated with Gram-negative bacteria. Apart from microbial pathogens, TLR-4 also recognizes a series of endogenous ligands in the circulation that are released during necrosis and cell stress. Following stimulation, TLR-4 activates a complex downstream signaling pathway leading to the stimulation of transcription factors, principally nuclear factor-κB (NF-κB) induces the stimulation of inflammatory genes, such as tumor necrosis factor-α (TNF-α), IL-1, IL-6 and IL-8 (25,26). The constant domain D1 present in the monomeric flagellin of bacteria is recognized by TLR-5. The bacterial triacylated lipopeptides are recognized by the TLR1/TLR6 complex (22) and the bacterial DNA is recognized by TLR-9 through unmethylated CpG motifs (27). A summary of TLR ligands, cellular, gene location and the effector molecules induced are listed in Table I.

In gingiva, bacteria that inhabit the sulcus and pockets are attached to the gingival epithelial cells by using their fimbriae. These epithelial cells protect the organism from potentially lethal oral pathogens and offer a surface that tolerates microorganisms and favors the exchange of nutrients. The gingival epithelial cells express almost all TLRs, such as TLR1-9 (27). The oral epithelial cells that harbor TLRs have the potential to stimulate pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α following ligation with their respective ligands (28). Furthermore, IL-8, a well-known chemoattractant enhances the migration of neutrophils from the circulation (29). Simultaneously, epithelial cells produced matrix metalloproteinases (MMPs) in response to PAMP, causing direct damage to periodontal tissues (30). These MMPs are structurally associated with endopeptidases and capable of degrading virtually all extracellular matrix (ECM) and basement membrane components.

Gingival connective tissues predominantly constituted by gingival fibroblasts that produce ECM are involved in tissue regeneration by replacing the injured or disrupted tissue. The expression of TLRs on gingival fibroblasts has generated substantial interest towards this cell population in inflammation in addition to their well-documented fibrogenic effects. It has been demonstrated that the innate immune responses of gingival fibroblasts produced various inflammatory cytokines, such as IL-1, IL-6 and IL-8, following stimulation with lipopolysaccharides from periodontopathic bacteria (31,32). Gingival fibroblast stimulation with lipopolysaccharides induces the production of chemokines such as RANTES, IP-10, MCP-1, MIP-1α and MIP-1β (33). Additionally, the stimulated gingival fibroblasts produce large amounts of collagen and ECM proteins (Fig. 1). Endothelial cells also strongly participate in pathogen recognition, which, due to their blood-exposed location, assigns these cells a key role in the initial responses to pathogens that have reached the blood. Accumulating evidence suggests a significant role for gingival endothelial cell involvement in innate immunity and in controlling the host responses to pathogens. As indicated in previous investigations, endothelial cells express functional TLR-2, TLR-4 and TLR-9 (34,35), while the hypothesized contribution of endothelial cell activities induced via TLRs in pathological processes such as gingivitis and chronic periodontitis has been suggested.

3. TLRs and drug-induced gingival overgrowth

The pathogenesis of drug-induced gingival overgrowth seems complex, especially the involvement of quality of plaque control. Animal and human models have shown that plaque-mediated inflammatory changes have a substantial role in the pathogenesis of drug-induced gingival overgrowth (36-39). The drug may be an important factor in the pathogenesis of gingival fibrosis by reducing cell signaling,

Table I. Summary of predominant cell location, encoding gene location, ligands, and the effector molecules induced by TLRs.

Toll-like receptor	Predominant cellular location (membrane)	Encoding gene location (chromosomes)	Ligands	Effector molecules induced (Inflammatory cytokines)
TLR-1	Plasma	4	Triacyl lipopeptides, modulin	TNF-α and IL-6
TLR-2	Plasma	4	Glycolipids, triacyl lipopeptides, heat shock proteins, high mobility group box 1 protein, rare LPS species (*Porphyromonas gingivalis*), lipopeptides, lipoteichoic acid, measles haemagglutinin, mannuronic acids, neisseria porins, peptidoglycan, zymosan (β-glucan), bacterial fimbriae, *Yersinia* virulence factors, CMV virions, saturated fatty acids	TNF-α, IL-4, -5, -6 and -21
TLR-3	Endosomal	4	dsRNA (self and viral)	TNF-α, IL-4 and -6; type I INFs
TLR-4	Plasma	9	Lipopolysaccharides, saturated free fatty acids, fibrinogen, fibronectin, heat shock proteins, flavolipins, *Streptococcus pneumoniae* pneumolysin, heparan sulfate, hyaluronic acid, high mobility group box 1 protein, MMTV envelope proteins, nickel, paclitaxel, RSV fusion protein, respiratory syncytial virus coat protein, mannuronic acid polymers, teichuronic acids, bacterial fimbriae, surfactant protein A, β-defensin 2	TNF-α, IL-4, -5, -6 and -13); type I INFs
TLR-5	Plasma	1	Flagellin	TNF-α and IL-6
TLR-6	Plasma	4	Diacyl lipopeptides, bacterial cell wall components, modulin	TNF-α and IL-6
TLR-7	Endosomal	X	Self ssRNA, ssRNA, bropirimine, loxoribine, imidazoquinoline	TNF-α and IL-6; type I INFs
TLR-8	Endosomal	X	Self ssRNA, small synthetic compounds, imidazoquinoline	TNF-α and IL-6 type I INFs
TLR-9	Endosomal	3	Self DNA, unmethylated CpG DNA	TNF-α and IL-6; type I INFs

TLRs, Toll-like receptors; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

altering the inflammatory response in gingival tissues and favoring bacterial invasion and proliferation. Elimination of the microbial biofilm resulted in reduction of the inflammatory infiltrate and alteration in connective tissue composition of the gingival tissues of patients with drug-induced gingival overgrowth (40). PAMPs are recognized by pattern recognition receptors (PRRs) presented on several cells, including fibroblasts (41). The interaction between PAMPs and PRRs provides a first line of defense during infection and activates numerous proinflammatory chemokines as well as cytokine responses that modulate fibroblast proliferation and ECM synthesis.

Inflammatory and immune mechanisms triggered by non-infectious as well as, possibly even infectious agents, may be important in the development of fibrosis (42). TLR ligands induce NF-κB activation, resulting in the transcription of various types of genes involved in the inflammatory and proliferative responses of cells crucial to gingival overgrowth and ultimately leading to the synthesis and release of inflammatory cytokines which provide a critical link to adaptive immunity (34,43). These inflammatory mediators can exert various fibrogenic effects involving the expression of adhesion molecules on endothelial cells, proliferation of fibroblasts, activation of immune cells, and stimulation of the acute-phase response. In addition, TLR ligands can directly activate fibroblasts and promote fibrogenesis (44-46). TLR-2 and TLR-4 stimulate a series of events including NF-κB activation following the recognition of the cell-wall component lipoproteins and peptidoglycan, and recognition of the outer membrane component lipopolysaccharides, respectively. NF-κB activation leads to cytokine production and expression of adhesion molecules in gingival fibroblasts (27). Therefore, considering the role of

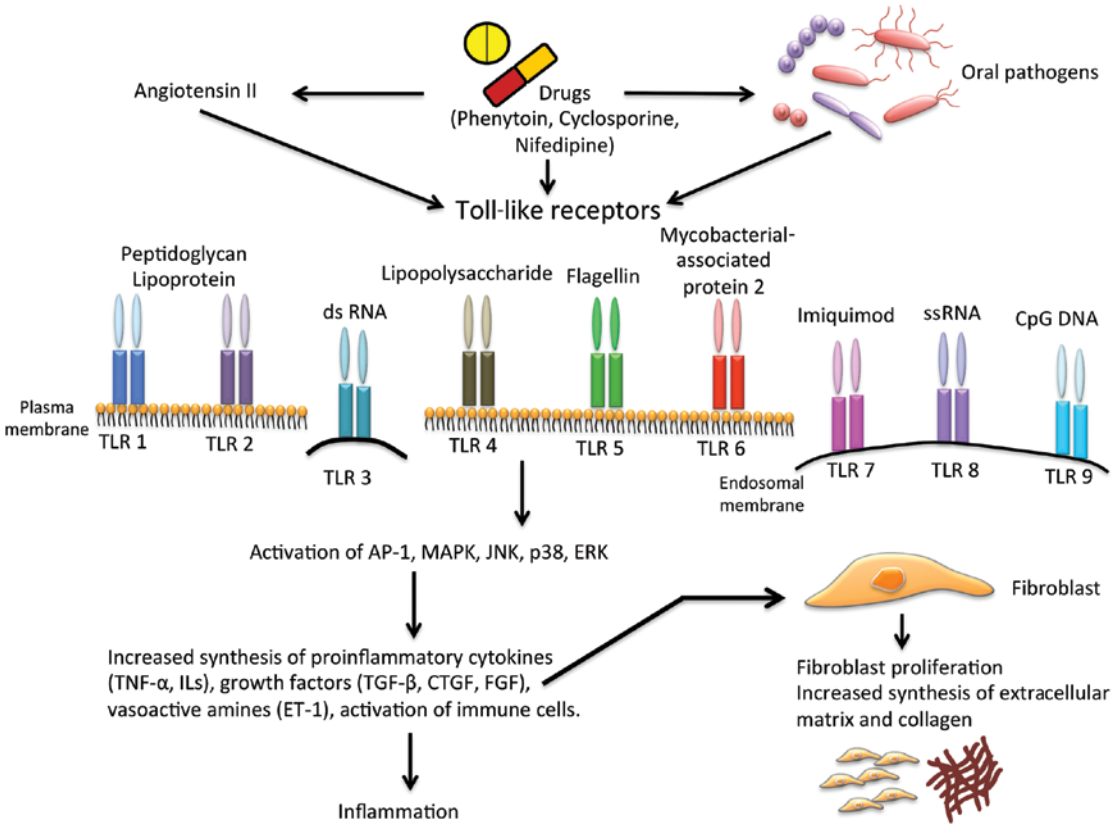

Figure 1. Schematic representation of Toll-like receptor (TLR) signaling in drug-induced gingival overgrowth. TLRs stimulated by endogenous and exogenous ligands and TLRs activate adaptor molecules, which trigger conserved signaling pathways culminating in a profibrotic response consisting of the induction of inflammatory and fibrotic genes that leads to proliferation of gingival fibroblast, accumulation of extracellular matrix (ECM) and collagen in gingival tissues.

TLRs in the recognition of bacterial components and initiation of host response via the release of several inflammatory mediators, TLRs have a strong role in the pathogenesis of gingival fibrosis. An *in vitro* study using hamster cells treated with phenytoin and cyclosporine showed that cyclosporine increased signaling by TLR2 and TLR4, while phenytoin decreased this signaling with a decreased expression of adhesion molecules such as CD54 (47).

The reduction in cell signaling induced by drugs such as phenytoin may alter the inflammatory response in gingival tissues, favoring bacterial invasion and proliferation and, therefore, may be an important factor in the pathogenesis of gingival fibrosis (48). Furthermore, cyclosporine-induced gingival overgrowth patients had a significantly higher number of TLR-4 expressing cells in the basal cell layer of the epithelium, as well as in connective tissue compared to healthy subjects (42). Lim *et al* (49) demonstrated an association between cyclosporine-induced renal injury and activation of innate immunity through TLR-2 and TLR-4 expression in renal tissues of rats and reported an increased TLR-2 and TLR-4 mRNA and protein expression in rat kidney. Another study by Suzuki *et al* (50) showed that TLR-mediated inflammatory responses were positively regulated by cyclosporine in human gingival fibroblasts. Moreover, it has been reported that deficiency of MyD88, the common adaptor for all TLRs except TLR-3, protects mice from inflammation and fibrosis (51). In contrast to TLR-2 and TLR-4, TLR-9 appears to promote lung fibrosis (52). Those results were confirmed by another study, in which TLR-4 deficient mice exhibited a significant reduction in fibroblast accumulation and renal fibrosis (53). Together together, those studies suggest that TLRs, via their common adaptor MyD88, negatively affect tissue remodeling and fibrotic development.

TLRs activate immune cells including mast cells. The activated mast cells degranulate and release various mediators such as ILs, TNF-α and protease enzymes such as chymase and tryptase (54). Findings of our earlier studies suggest that an increased expression of mast cells and its mediators was observed in drug-induced gingival overgrowth compared to healthy gingival tissues (55). Mast-cell chymase actively participate in the production of locally expressed angiotensin II (Ang II) and endothelin-1 in gingiva. Ang II is the effector peptide of the renin angiotensin system, which acts as a major role in mediating contractile activity of vascular smooth muscle, aldosterone release in the adrenal gland, regulation of collagen synthesis and growth-modulating effects on fibroblasts (54,56,57). Furthermore, Ang II activates TLR4 through AT1 receptor and subsequent extracellular signal-regulated kinase (ERK)1/2 and mediates NF-κB to initiate the expression of cytokines involved in the inflammatory and profibrotic response. Our previous study showed elevated TLR4 expression in gingival tissues (17) and the significant increase of Ang II in cyclosporine-induced gingival overgrowth (58). Consequently, TLR4 is closely involved in the Ang II-induced inflammatory response and drug-induced gingival overgrowth. On the other hand, endothelin-1, a potent vasoconstrictor secreted from endothelial cells (59,60), can induce ECM production (61,62). The source of ET-1 is not

restricted to endothelial cells. Human macrophages have been shown to produce ET-1 in response to lipopolysaccharide (63), and human monocyte-derived dendritic cells secrete ET-1 in response to TLR2 and TLR4 agonists (64). Previously we showed a significant increase of ET-1 in cyclosporine-treated human gingival fibroblast cells (65). ET-1 stimulates the synthesis of collagen by gingival fibroblasts from different species, including humans (66-70). Further profibrotic effects of ET-1 occur at the level of MMPs, with evidence that ET-1, acting via the ETA receptor, can reduce collagenase activity (71). These findings showed the potential role of the TLRs-dependent signaling pathway in modulating fibrotic events in drug-induced gingival overgrowth.

TLR activation also upregulates many growth factors such as TGF-β, VEGF, CXCR4, and adhesion molecules such as ICAM-1 (70-73). TGF-β has been the most intensively studied regulator of the ECM and has been associated with the development of fibrosis in a number of diseases (74-77). Once activated, TGF-β signals trigger signaling intermediates known as Smad proteins via transmembrane receptors and modulate the transcription of vital target genes including procollagen I and III (78). Gingival fibrosis is reduced in Smad-deficient mice, confirming the significant role for the TGF-β signaling pathway (79). Furthermore, it has been reported that loss of TGF-β signaling in fibroblasts triggers intraepithelial neoplasia, suggesting that TGF-β1 signaling critically regulates the activity of fibroblasts as well as the oncogenic potential of neighboring epithelial cells (80). In pulmonary fibrosis, alveolar macrophages are thought to produce almost all of the active TGF-β (81). Nevertheless, Smad3/TGF-β1-independent mechanisms of fibrosis have been demonstrated in lung and other tissues (82-84), suggesting that profibrotic mediators such as IL-4, IL-5, IL-13 and IL-21 can act independently from the TGFβ/Smad-signaling pathway to stimulate collagen deposition. The connection between TGF-β and ET-1 has been established, and several lines of evidence indicate that transdifferentiation of fibroblasts occurs in response to the concerted actions of TGF-β, ET-1 and Ang II (85). Activation of AP-1 and the MAPKs c-jun N-terminal kinase (JNK), p38 and ERK1/2 are other classical signals regulated by TLR signaling (86,87). AP-1 transcriptional complexes play a pivotal role in drug-induced gingival overgrowth. AP-1 can be activated through the TLR Myd88-dependent pathway by a variety of growth factors and cytokines (88). AP-1 induced the activation of FOS and Jun, which are observed in many fibrotic conditions (89). Our group previously demonstrated the elevated expression of Jun and Fos in cyclosporine-induced gingival overgrowth (90). The expression of Jun and Fos activates proliferation and ECM synthesis in gingival fibroblasts. Taken together, there is a significant amount of evidence for the involvement of TLRs in drug-induced gingival overgrowth.

4. Current development in TLR-targeted therapeutics

Evidence of the involvement of TLRs in gingival fibrosis largely comes from overexpression in fibrosis and their activation triggering enhancement in the pathogenesis of diseases. Inflammatory mediators such as TNF-α and ILs, which are produced as a consequence of the activation of TLRs have been successfully targeted in an effort to treat inflammatory diseases. Additionally, targeting central upstream mediators in the inflammatory cascades such as the TLRs may modulate pathway activation at an earlier point and are therefore also likely to be effective in the manipulation of the immune system to reduce disease severity. A substantial amount of research has been conducted aiming to develop TLR-targeted drugs for human use; however, only a few have been approved thus far. The ubiquitin-modifying enzyme and zinc-finger protein, A20, has been reported to regulate TLR-4 signaling (91,92). A20 can suppress both TLR-2- and TLR-4-induced IL-8 expression in airway epithelial cells (93). OPN305 is a humanized anti-TLR-2 monoclonal antibody that has potential to block TLR-1/2- and TLR-2/6-mediated signaling and decrease TLR-2-mediated pro-inflammatory cytokine production (94). NI0101 is a TLR-4 epitope-specific antibody targeted towards TLR-4 and inhibits TLR-4 dimerization and decreases pro-inflammatory cytokine production. This antibody remains in the preclinical developmental stage and has some potential indications including rheumatoid arthritis, asthma, acute lung injury and acute respiratory distress syndrome (95). AV411 is another TLR-4-targeted antagonist that has potential utility for the treatment of neurological indications (96). In addition to antibodies, a synthetic analog of lipid A eritoran is targeted TLR-4, which inhibited the production of LPS-induced TNF-α and IL-6 (97,98). IMO3100 is a dual TLR-7/TLR-9 antagonist that inhibits TLR ligand-induced gene expression. Additionally, IMO8400 is a drug capable of antagonising TLR-7, TLR-8 and TLR-9, that has shown efficacy in mouse models of lupus (99). Conserning existing conventional therapies, evidence suggests that many of these approaches directly or indirectly affect TLR-mediated responses (100). Although no specific published data are available on drug-induced gingival overgrowth, there is a substantial evidence suggesting that TLRs are a good target for drug-induced gingival overgrowth. By understanding in more detail the manner in which these modulate the activity of TLRs to good effect, we can design TLR-directed interventions that selectively inhibit the inflammatory component of the cascade while retaining the anti-microbial component.

5. Conclusion and future directions

Evidence of the contribution of TLRs in fibrosis greatly extends this understanding beyond innate immunity, and provides important insights into body responses to diseases. As a major portal of entry for microbes, the gingiva is a key component of the innate immune system. Oral pathogens encounter a number of effective defense mechanisms designed to rapidly counteract potential damage, inhibit colonization and protect against invasion by pathogens. The existence of TLRs equips gingival tissues with a uniquely designed mechanism for controlling microbial infection. However, drug-induced gingival overgrowth is a disease in which the gingival epithelial cells, endothelial cells and fibroblasts stimulate inflammatory mediators and profibrotic mediators through TLRs and facilitate the accumulation of collagen and ECM in gingiva. Conflicting roles of TLRs in various organs and different forms of tissue response make it virtually impossible to outline distinct greater functions for individual TLRs in drug-induced gingival overgrowth. A possible explanation may be the differential

contribution of endogenous and exogenous ligands to TLR activation, which likely depends on the anatomical localization and the related exposure to microbes. Furthermore, drugs such as cyclosporine stimulate TLR expression in gingival tissues. Thus modulation of TLRs was important in drug-induced gingival overgrowth. Suppression of TLRs responses by the use of appropriate inhibitors may reduce the chronic inflammatory characteristic of this disease. Thus, new therapeutics designed to selectively activate or inhibit TLR function specifically and reversibly are powerful tools for the prevention and treatment of the drug-induced gingival overgrowth.

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