Chapter

Innate Immune Response as a New Challenge in Periodontal Inflammation

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Abstract

Gingivitis and periodontitis are induced by numerous pathogenic microbiota hosted in the subgingival biofilm that first trigger the innate immune response. Innate immune response is part of a homeostatic system which is the first line defence and defines the host inherited resistance to infection. Both genetic and environmental factors are involved in variable individual susceptibility to inflammation of periodontal tissues. That is why, although more than 600 bacterial species have been detected in the periodontal plaque, the type of bacteria incriminated in the development of the inflammation is still unclear. Moreover, in the last decade gene polymorphisms have been largely recognised as important conditions associated with increased susceptibility to periodontal diseases. Manipulating the immune response by the development of drugs that inhibit adverse host reactions and promote beneficial effects might be of therapeutic or prophylactic importance. This work intends to assess the importance of Toll-like receptors as main effectors of the innate immune response in the triggering, maintenance and progression of periodontal inflammation, as well as of the involvement of synthetic molecules targeting TLR signalling pathways in treating periodontal diseases.

Keywords: innate immunity, toll-like receptors, periodontal inflammation, therapy

1. Introduction

The surface of the mouth is lined by the oral mucosa which was often considered to be more of a mechanic barrier than an active player involved in the modulation of the host response to various external stimuli in order to maintain mouth homeostasis.

The oral mucosa is permanently insulted by mechanic and chemical factors (like smoke, xenobiotics) as well as a plethora of microorganisms. Both the commensal and pathogen microorganisms, depending on their structure, represent triggers for the immune surveillance players hosted by the oral mucosa which have been intensively studied in the last years. Local defence in the oral cavity is provided by a complex network of cells with their receptors and molecules through which they respond to various ligands (microbial antigens, tissue degradation products,

nucleic acids). The interactions between gingival resident cells and oral microbiome are characteristic of clinical gingival health and homeostasis as well as for gingival inflammation. Host defence takes place in successive, sometimes overlapping stages. The innate immune response represents a homeostatic system which is the first line defence and defines the host inherited resistance to infection.

The immune system has a hierarchical organisation, all the immune and non-immune cells involved being able to sense the pathogens through pattern recognition receptors (PRR) and then develop the immune response triggered by numerous proinflammatory mediators. PRRs detect and respond to conserved patterns originated in microorganisms and stress signals called pathogen- or damage-associated molecular patterns (PAMPS and DAMPS) [1].

Among PRRs, Toll-like receptors (TLRs) are the most intensively studied. In the oral mucosa TLRs are expressed in cells of the immune system including neutrophils, monocytes, macrophages, and not less important, dendritic cells, keratinocytes and endothelial cells. [2] TLR activation induces the local synthesis of cytokines and chemokines through different pathways. Part of these molecules are involved in the development of highly specific adaptive immunity ensured by T and B lymphocytes. Due to this ability, TLRs are considered to be the missing link between innate and acquired immunity.

Manipulating the oral immune response by development of drugs that inhibit adverse host reactions and promote beneficial effects might be of therapeutic or prophylactic importance in periodontal diseases.

This review intends to assess the importance of Toll-like receptors as main effectors of the innate immune response in the triggering, maintenance and progression of periodontal inflammation as well as of the involvement of synthetic molecules targeting TLR signalling networks in treating periodontal diseases.

2. Methodology

This chapter has been designed in order to assess the role of innate immune receptors, especially Toll-like receptors, as main effectors of the innate immune response triggered by various pathogens hosted in the subgingival film which are able to onset periodontal inflammation, as well as the involvement of synthetic molecules targeting TLR signalling pathways in treating periodontal diseases. Information processed has been sourced from the existing scientific literature and supplemented with our personal findings from experimental studies regarding TLR involvement in mediating innate immune response at oral mucosa level. We searched MEDLINE, Web of Science, SCOPUS, without language restriction, following terms „innate immunity" AND „periodontal inflammation" OR „periodontal disease" AND „TLR" OR „TLR therapeutic targets". Identification of specific literature also included manual search and screening of the citations of the relevant studies. The included studies were selected after reviewing the abstract and full-text for eligibility.

3. Oral mucosa cells and the innate immune response

The oral mucosa consists of a stratified oral epithelium formed by keratinocytes lining the inner connective tissue or *lamina propria* which has as main cells the fibroblasts and also proinflammatory cells originated in the bone marrow.

Three distinct phenotypes of oral mucosa are noted: lining, masticatory and specialised mucosa. The lining mucosa cover almost the entire mouth, excepting the rigid structures, such as the alveolar processes and the hard palate, which are covered by the masticatory mucosa, and the dorsal part of the tongue coated by the

specialised mucosa. [3] The crevicular (CE) and junctional epithelium (JE) are the keystones for the antimicrobial barrier function of the oral mucosa. Compared to other types of epithelial cells, JE possesses fewer intercellular junctions, just desmosomes, and more intercellular spaces filled with gingival crevicular fluid (CF). Due to this histophysiology, JE allows the passage of CF, the carrier of inflammatory cells and soluble mediators, from the gingival connective tissue to the crevicular space. The changes of the dental biofilm and the metabolic products of bacteria trigger the synthesis of cytokines and increase the number of polymorphonuclear cells (PMNs) migrating to the initial site of inflammation. This early event, gingivitis, is followed by the appearance of other inflammatory cells (macrophages, lymphocytes, mononuclear and dendritic cells) and the persistence of inflammation of the teeth supporting tissues with progressive attachment loss and bone destruction, namely periodontitis. [4] The proinflammatory cells of the oral mucosa connective tissue are myeloid-originated immune cells such as monocytes, macrophages and dendritic cells which act as the first line of defence during infection, recognising, engulfing and killing bacteria, fungi or viruses. Recent data emphasises that it is not only the immune cells but also the fibroblasts and the keratinocytes that contribute to local immunity through the activation and modulation of specific immune receptors. [5] Fibroblasts activation triggered by the immune cells is involved in the remodelling of the extracellular matrix (ECM); moreover, under the stimulation of the endothelial cells derived signals, fibroblasts are able to differentiate into myofibroblasts important for wound healing of the masticatory mucosa. [6] Knowledge of how immune mechanisms and responses are controlled is essential for understanding the pathogenesis of periodontal inflammation. Bacteria are the most common pathogens in the case of gingivitis, followed by periodontitis, but both pathologies can also be caused by other pathogens like fungi, viruses or by metabolic imbalances. Until recently, innate and specific/adaptive immune responses had been arbitrarily proposed by immunologists as two distinct stages of the immunologic events. Later it was clarified that adaptive or acquired immune response evolved around innate immunity instead of replacing it. The development of acquired immunity is regulated through the activation of some myeloid and antigen presenting cells (APCs). Innate immunity should be considered as an immune system skill developed in order "to buy time" [7] until the acquired, more efficient host immune response, is primed. The immune system has a hierarchical organisation containing immune cells (monocytes, macrophages, neutrophils, dendritic cells (DCs), natural killer cells (NK), mast cells, eosinophils, basophils and newly identified innate lymphoid cells (ILCs), mucosal associated invariant T cells, γδT cells) and humoral circulating components (complement proteins, cytokines and chemokines secreted by those cells, along with various antimicrobial peptides, AMPs). [2, 8–11] All the cells already mentioned are able to sense the pathogens through PRRs and then develop the innate immune response. [12] A limited number of PRRs are able to recognise a large number of pathogens. PRRs detect and respond to conserved motifs originated in microorganisms and stress signals called PAMPS and DAMPS. [1] PAMPs are evolutionarily conserved structures shared among pathogens, but not present in eukaryotes, which confer specificity to the innate immune response. These include lipopolysaccharides, peptidoglycans, bacterial lipoproteins, DNA and double stranded RNA. [7] DAMPs are endogenous ligands derived from host cells (tumour cells, dying cells) or products released from cells in response to various signals which produce inflammation in the absence of infection. DAMPs are often created in environments of trauma, ischemia, or tissue damage involved in various diseases (cancer, autoimmune disease, and atherosclerosis) and do not require pathogens infection. [1, 13] Inside the more generic term microbial-associated molecular patterns (MAMPs), one could consider commensal-associated

molecular patterns (CAMPs), as many microorganisms colonise the human body without causing disease. [14] PAMPs, MAMPs and DAMPs bind to PPRs which include mainly Toll-like receptors (TLRs), transmembrane C-type lectin-like receptors (CLRs), cytoplasmic NOD-like receptors (NLRs) and intracellular retinoic acid-inducible gene-I-like receptors (RLR). [13, 15] PRR-ligand binding and their concomitant conformational changes prompt a cascade of downstream signalling that results in transcriptional and post-translational changes. Among these PRRs, TLRs have been studied most extensively.

4. Toll-like receptors: structure, ligands and signalling

TLRs, so called because they are similar to the product of the Toll gene identified in *Drosophila*, are the best characterised from all the classes of PRRs. Until now, ten human TLRs have been described, the first being reported 26 years ago by Nomura in 2004 [16] and called TLR1. TLRs are evolutionary conserved transmembrane glycoproteins that contain an extracellular (or intra-cytosolic for those intracellular TLRs expressed in endosomes) N-terminal leucin-rich-repeat (LRR) domain, a transmembrane domain and an intra-cytoplasmic Toll/IL-1R (TIR) domain. LRR is responsible for ligand recognition and binding while TIR domain for intracellular signal transfer. [14] It is generally accepted that most TLRs are localised to the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) and recognised extracellular microorganisms and ligands, while TLR3, TLR7, TLR8 and TLR9 are intracellular, hosted into the cytosolic endosomal compartment and recognised molecular patterns that have already passed the cell membrane. [14] Recently has been demonstrated that TLR2 and TLR4are also present inside some cells: DCs, monocytes, epithelial and endothelial cells. [17, 18] The cells expressing TLRs in humans and their ligands are listed in **Table 1**.

Toll-like receptor	Cells	Ligands
TLR 1	Neutrophils, monocytes/macrophages Myeloid and plasmacytoid dendritic cells* B lymphocytes Fibroblasts, Keratinocytes Endothelial cells	Triacyllipopeptides
TLR 2	Neutrophils, monocytes/macrophages Myeloid dendritic cells T lymphocytes Fibroblasts, Keratinocytes Endothelial cells	Lipoproteins Peptidoglycan Lipoteichoic acid Zymosan *P. gingivalis* LPS *C. ochracea* LPS
TLR 3	Myeloid dendritic cells B lymphocytes, Th1/Th2 lymphocytes Fibroblasts, Keratinocytes Endothelial cells	dsRNA, Polyinosine Polycytidilic acid
TLR 4	Neutrophils, monocytes/macrophages Myeloid dendritic cells Fibroblasts, Keratinocytes Endothelial cells	Most bacterial LPS
TLR 5	Neutrophils, monocytes/macrophages Myeloid dendritic cells Th1/Th2 lymphocytes Fibroblasts, Keratinocytes Endothelial cells	Flagellin

Toll-like receptor	Cells	Ligands
TLR 6	Neutrophils, monocytes/macrophages Myeloid and plasmacytoid dendritic cells B lymphocytes Fibroblasts, Keratinocytes Endothelial cells	Peptidoglycan Lipoteichoic acid Diacyllipopeptides Zymosan
TLR 7	Neutrophils, monocytes/macrophages Plasmacytoid dendritic cells B lymphocytes,Fibroblasts	Immunomodulatory drugs (imiquimod, resiquimod)
TLR 8	Neutrophils, monocytes/macrophages Myeloid dendritic cells Treg lymphocytes Fibroblasts	ssRNA Immunomodulatory drugs (imiquimod, resiquimod)
TLR 9	Neutrophils Plasmacytoid dendritic cells B lymphocytes, Th1/Th2 lymphocytes Fibroblasts, Keratinocytes Endothelial cells	Bacterial DNA Viral DNA CpG Oligodeoxynucleotide
TLR 10	Neutrophils Myeloid dendritic cells B lymphocytes Fibroblasts, Keratinocytes	Orphan receptor

**In human blood, two subpopulations of DCs precursors are present – myeloid or conventional and plasmacytoid - different in terms of cell phenotype and morphology, which express various TLRs. Plasmacytoid DCs recognise predominantly viral antigens through the activation of TLR7 and TLR9 [19, 20]. Myeloid or classical DCs recognise both bacterial and viral antigens [21, 22].*

Table 1.
Toll-like receptors expressing cells in humans and their ligands.

In physiologic conditions, the intracellular TLRs have no contact with host cells derived nucleic acids because they are sequestered in endosomes and no innate response is signalled against own nucleic acids. In some pathologic conditions, TLRs signal the synthesis of autoantibodies against host own nucleic acids, for example in rheumatoid polyarthritis, much evidence showing the link between rheumatoid arthritis and periodontal disease [23] which may suggest a similar expression of these receptors in the diseases having in common the destruction of hard tissues.

TLR 1, TLR2, TLR4, TLR5 and TLR6 recognise mainly unique bacterial products and not those produced by the host, which gives them the ability to differentiate microorganisms from the host and conveys some degree of specificity, making them the missing link between the innate and acquired immunity. [24].

TLR2 and TLR4 are the most defined members of this family. TLR2 forms heterodimers with TLR1 and TLR6 and recognises peptidoglycans, lipopeptides and lipoproteins, while TLR4 is the acknowledged PRR for lipopolysaccharide (LPS) and Gram-negative bacteria. Published data is conflicting regarding the ability of TLR4 to recognise *Porphyromonas gingivalis* and *Escherichia coli* LPS, some authors considering that TLR4 is able to detect only *E. coli* product. [25, 26] TLR3 recognises double-stranded RNA (dsRNA), TLR7 and TLR8 are known to recognise single-stranded RNA (ssRNA). TLR5 detects bacterial flagellin and TLR9 recognises cytosine and guanine base-pairing of bacterial and viral DNA [3, 27, 28].

LRR domain of TLRs is involved in the recognition of various ligands. LRR ligand binding induces the formation of TLRs homodimers or heterodimers followed by a conformational change of the TIR domain which allows the interaction between TIRs of adjacent TLRs in order to bind an additional adapter protein essential to trigger the intracellular signal cascade generating chemokines, cytokines and AMP.

In the same cell, the activation of different TLRs induces various proinflammatory responses. For example, the interaction of TLR3 and TLR4 with LPS in DCs triggers the synthesis of IL-12, and respectively type 1 interferon [29].

The same TLR can trigger a different response depending on the upper intracellular adapter protein [30].

To date, two different pathways are recognised for the intracytoplasmic signalling cascade of TLRs: (i) the myeloid differentiation primary response protein 88 (MyD 88) dependent pathway – the most important – essential for the majority of TLR-mediated cell activation, and (ii) MyD88-independent pathway, after TLR3 and TLR4 stimulation takes place. Even if the cells are forced to react to an extensive number of PAMPs, only four adapter molecules have been identified for both pathways: MyD88, toll/interleukin-1 receptor domain-containing adapter protein (TIRAP), toll/interleukin-1 receptor domain-containing adapter-inducing interferon beta (TRIF) and toll/interleukin-1 receptor domain-containing adapter-inducing interferon beta-related adapter molecule (TRAM) [3, 7, 14, 29, 31].

MyD88-dependent signalling predominantly leads to nuclear factor-kB (NF-kB) activation while the TRIF-dependent pathway, MyD88-independent, leads to interferon-regulatory factor 3 (IRF3) and to a lesser extent to NF-kB activation. [31] NF-kB is a crucial transcription factor that promotes the expression of genes encoding proinflammatory and chemotactic cytokines, such as IL-1, IL-12, IFNɣ, CXCL9, CXCL10, costimulatory molecules and other effectors. [32–34] Apart from NF-kB activation, TRIF-dependent pathway induces the expression of type I interferons, mainly IFNβ [31, 35].

5. Toll-like receptors in periodontal diseases

Both in gingivitis and periodontitis a plethora of bacterial germs are accumulated in the gingival mucosa around the tooth, particularly in the gingival crevice. [29] More than 600 species populate the oral cavity [36, 37] with a clearest distinction between those characteristic of shedding epithelia (oral mucosa) and non-shedding surfaces - the tooth biofilm. The complex studies of oral microbiota have demonstrated a conspicuous change of the oral bacterial populations before and during the periodontal disease, making possible the definition of the 'red complex bacteria' including *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola.* [38–40] Recent investigations partially disagree with this paradigm and collectively suggest that the pathogenesis of periodontitis involves not only a polymicrobial synergy and a dysbiosis - the PSD model, but also a host susceptibility to chronic inflammatory disorders leading to an unbalanced immune response, which may trigger the limited success of the antimicrobial treatment [40].

Oral tissues homeostasis involves the balance between the oral mucosa with its immunocompetent cells and the extreme vast oral microbiota. In this paper we do not intend to characterise the oral microbiota but the innate immune response of the oral structures to various germ triggers of inflammatory periodontal inflammation.

Regarding the development of dental plaque two main events imply the importance of the innate immune response. On one hand, gingivitis does not necessarily lead to periodontitis, which suggests that, despite massive dental plaque accumulation, stable gingival inflammation may represent a protective host response. [41] On the other hand, in cases in which gingivitis evolves to periodontitis, plaque accumulation induces the development of a dysbiotic, inflammophilic microbiota. Each of these events depends, in great part, on host ability to control the resolution of the inflammatory processes [40].

Due to the presence of a constantly large number of microorganisms in the oral cavity, TLR expression is essential for the maintenance of oral tissue immune homeostasis. *Porphyromonas gingivalis* (*P. gingivalis*) has recently been described as a" corrupter" of the innate immunity manipulating the host response rather than inducing by itself a chronic inflammation, being a low abundance constituent in rodent and also in human periodontitis associated biofilm. [42, 43] Such a germ is called a"keystone germ" able to impair host defence in order to compromise the homeostasis between the commensal microbiota and the host immune response. Under the influence of a keystone germs, the commensal bacteria become pathobionts, normally harmless symbionts that can become pathogenic under certain environmental conditions. Actually, no bone loss is effective in periodontitis induced *by P. gingivalis* in the absence of commensal microbiota. Even incomplete, an explanation of the corruption effect on TLR innate immune system by the commensal germs could be the observation that commensals and symbionts share similar MAMPs (LPS, peptidoglycans, lipoproteins) with pathogens, so they could induce PRRs activation [44].

In the oral mucosa, TLRs are predominantly expressed in neutrophils, monocytes, macrophages, and equally important DCs. Albeit the macrophages and DCs represent"professional APCs", the first cells responding to PAMPs are the epithelial cells from the oral mucosa, precisely the crevicular lining cells. As APCs, DCs and macrophages are responsible for the presentation of the antigen fragments together with the MHC class II to T cells in order to induce a costimulatory response.

Because TLRs are present in cells from all the structures of the oral mucosa either directly related to the immune local defence or not and their activation leads to redundant local inflammatory reactions, we intend to use a mechanistic approach to their effects in periodontal disease.

5.1 TLR signalling in the oral epithelium

Although the expression of mRNAs for TLR1 to TLR9 has been detected in the oral epithelial cells, the immunolocalisation of the corresponding proteins was variable. Immunohistochemical studies revealed that TLR1 to TLR9 are differentially expressed in the epithelial layers and connective tissue of the gingival samples collected from patients with periodontitis and healthy subjects. [45, 46] Increased TLRs expression towards the basal layer of the epithelium and the connective tissue in samples with periodontitis sustains their involvement in the pathogenesis of this disease. The percentage of TLR4 positive cells was higher in the spinous layer keratinocytes of the healthy tissues in contrast to the periodontitis samples which revealed higher expression in the basal keratinocytes.

TLR2 and TLR4 are the most studied receptors in relation to periodontal tissues. Mori et al. studied the immunohistochemical expression of TLR2, TLR4 and CD14 in samples of oral mucosa classified according to the degree of inflammation. They found that the ratio of TLR4 positive cells was higher in samples from the more severe inflamed tissue and that of TLR2 positive cells was the highest in the connective tissue subjacent to pocket epithelium of the severe inflamed group. [47] Becerik and co-workers reported the same expression of TLR4 in tissue excised from periodontitis sites. [48] Depending on the state of inflammation, TLR2 is highly expressed in the cells of the gingival basal layer and lower reaction was observed in cells of the superficial layers more exposed to pathogens. [33, 45] They presume this expression as a strategy of TLR2 to recognise the pathogens only when they invade the superficial layers of the oral epithelium in order to limit the overexpression of the proinflammatory cytokines and to maintain the oral tissue homeostasis. In bacterial periodontitis, TLR4 expression in gingival cells decreased in order to minimise the damage of oral tissues and bone. [49] This observation is

in accordance with that reported by [50] regarding the decreased number of DCs in the oral epithelium of periodontitis samples compared to non-inflamed mucosa or gingivitis.

We performed an immunohistochemical study for TLR2 and TLR4 expression on samples collected from patients with various degree of gingival inflammation. In samples with gingivitis, we observed that TLR2 is expressed evenly in almost all layers of the gingival epithelium except in the superficial layer (**Figure 1a,b**). In the basal and parabasal layers, we noticed TLR2 positivity in dendritic extensions possibly belonging to Langerhans cells, in addition to an inconstantly positive reaction in keratinocytes (**Figure 1c**). Regarding TLR4 expression, we observed a decrease of positivity in the epithelium from the superficial layers to the basal one (**Figure 1d**). In the connective tissue, we noticed more TLR2 positive proinflammatory cells than TLR4 positive (unpublished data). In periodontitis samples, the immunohistochemical reaction for TLR2 in the epithelium was more intense than in gingivitis, TLR2

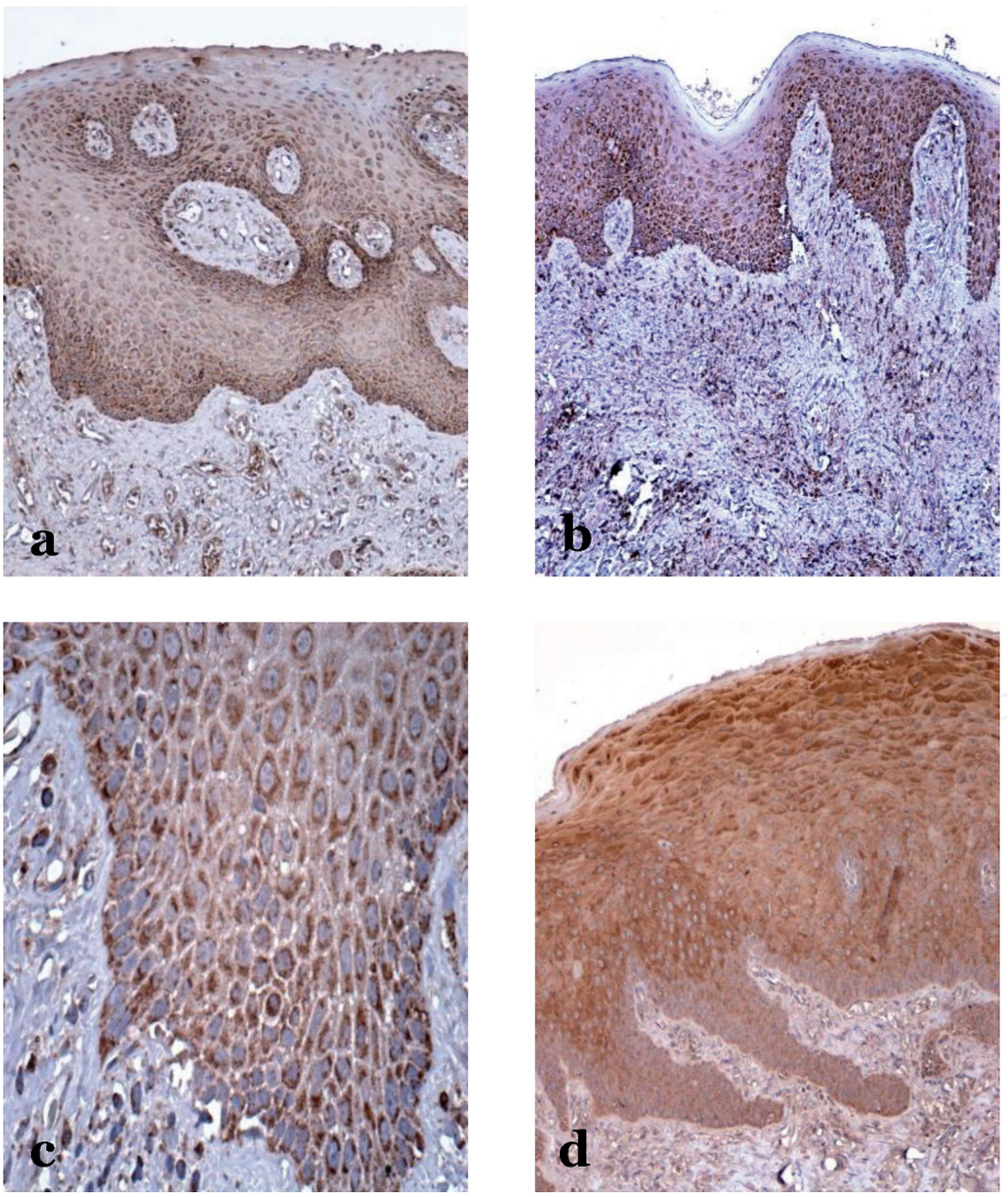

Figure 1.
TLR2 and TLR4 expression in gingivitis. (a) TLR2 positive keratinocytes into the basal and parabasal layers of the gingival epithelium; (b) Same positive reaction in the epithelium and also numerous TLR2 positive cells in the connective tissue, mainly in the vessel's walls; (c) TLR2 positivity in non-keratinocyte cells; (d) Inconstant intensity for TLR4 immune reaction in the epithelium.

being highly expressed in the basal layer (**Figure 2a**). In the connective tissue we noticed faint positivity in fibroblasts and some endothelial cells (**Figure 2b**). Cells from the perivascular concentrated lymphoplasmacytic infiltrate were intensely positive for TLR2. Regarding TLR4 expression, we noticed more intense positive cells in the epithelial spinous layer than in the superficial and basal ones. In the connective tissue, TLR4-positive fibroblasts were increased in number and proinflammatory cells showed discrete positivity (**Figure 2c**).

For TLR7 and TLR8 expression a comparable distribution between healthy and diseased oral tissues was noticed [14].

The antimicrobial effects induced by the activation of TLR2-TLR5 and TLR9 in the oral mucosa is also provided by the secretion of antimicrobial substances, more important being the α-, β-, and θ-defensins. [14] Defensins represent a class of cationic antimicrobial peptides secreted by the epithelial cells and neutrophils to play pivotal roles in innate and adaptive immunity, as well as roles in non-immunological processes. They have evolved to be highly efficient in their antimicrobial responses

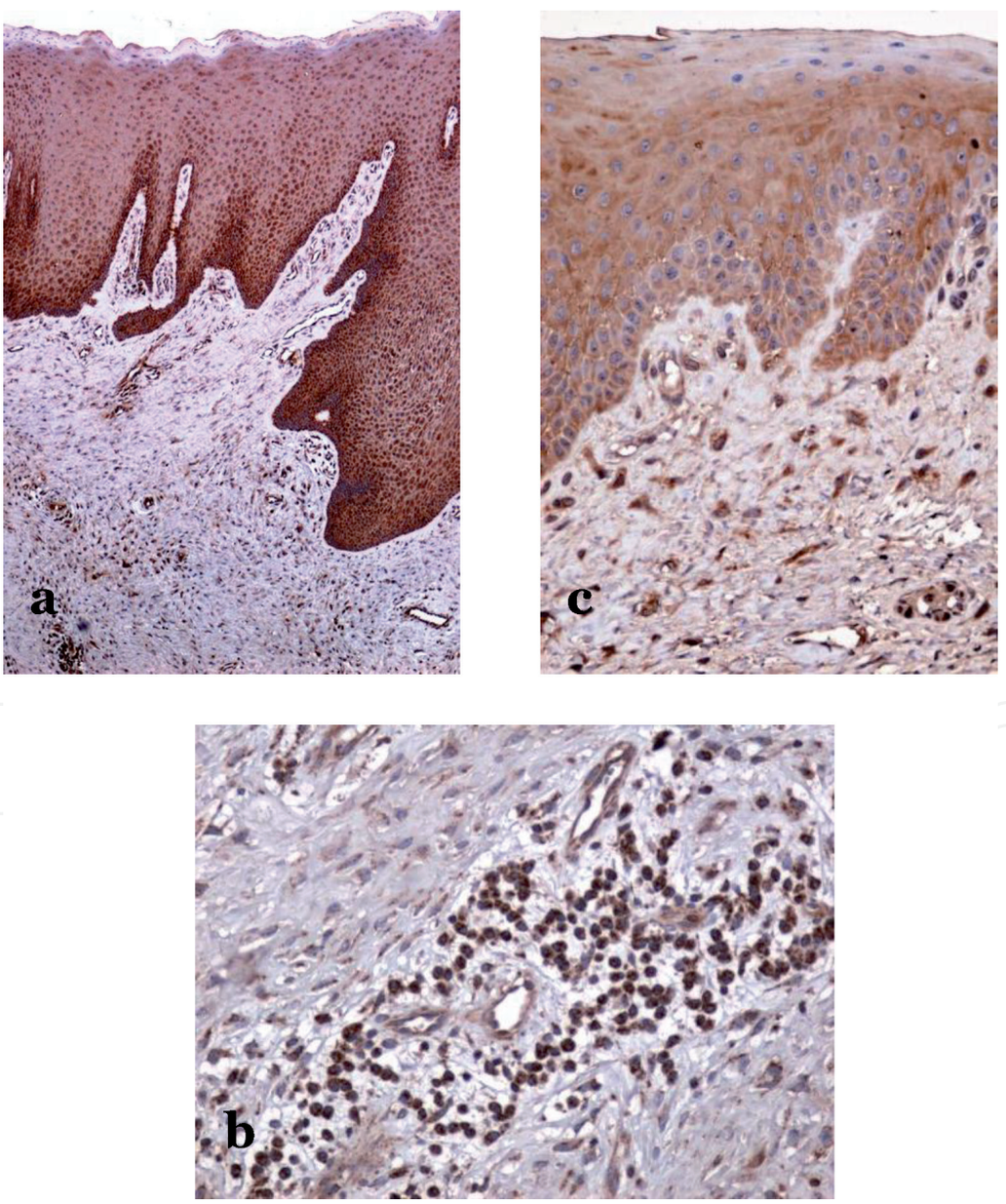

Figure 2.
TLR2 and TLR4 expression in periodontitis (a) Intense TLR2 positive reaction in the epithelial basal layer; (b) TLR2 positive cells spread in the lamina propria and proinflammatory cells concentrated in the proximity of vessels; (c) Different intensity of TLR 4 immune reaction through the keratinocyte layer. Fibroblasts show intense positivity in the superficial lamina propria. Vessels were was also TLR4 positive.

to a vast array of pathogens. [51] mRNAs for β-defensins and their protein products were identified in the human oral epithelium [52].

Reported data sustains that TLR-induced expression of β-defensins is followed by two cellular events: (i) in a positive feedback mechanism, β-defensins themselves stimulate TLR signalling, acting as an epithelial immune inducer and (ii) they contribute to the recruitment/activation of several professional APCs, i.e. DCs and monocytes [52, 53].

5.2 TLR signalling beyond the basal membrane of the oral epithelium

Because the innate immune response is limited, slow and nonspecific for the antimicrobial defence, it is necessary to activate the specific immune response represented by B and T lymphocytes that invade the oral tissues a few days after their activation under DCs stimulation. In the oral epithelium, once activated by various stimuli, including TLR ligands, DCs (also called Langerhans cells by the analogy with those in the epidermis) mature, acquire high phagocytic capacity, express the chemokine receptor 7 (CCR7) and become mobiles. Through the *lamina propria*, they migrate to the neighbouring lymph node where they activate CD4 + T cells (T helper, Th) [54] being the most potent APCs activating naïve T cells and consequently the main messengers between innate and adaptive immune system. Under DCs instructions, CD4 + Th differentiate into various subtypes: Th1, Th2, Th17, T regulatory cells (Treg). [54] The action of DCs on T lymphocytes is a complex process triggered by a various constellation of cytokines dependent on tissue of origin and DCs maturation state. [55] There is little information regarding the subtypes of DCs identified in the *lamina propria* of oral mucosa in humans. DCs have the ability to decide whether or not to respond and what kind of immune response to develop against a particular pathogen or a group of microorganisms. They are activated not only by germs but also by non-immune cells - fibroblasts and keratinocytes, that produce proinflammatory cytokines as a result of exposure to various pathogens. These substances are able to induce different responses in DCs which in turn are provided with the ability to modulate downstream the adaptive immune response.

The number of DCs in gingival mucosa is related to the topographic area and to the degree of inflammation. In normal human mucosa their number in the sulcular epithelium seems to increase with the accumulation of dental plaque. [50, 56, 57] In a model of human gingivitis, some authors showed that the number of DCs decreased by day 21st of inflammation. [58] This variation of incidence is in accordance with the observation that DCs could have a stimulating inflammatory effect in gingival inflammation by the induction of Th1 or Th17 response through IL-12 and IFN ɣ [59] and also a protective effect, cushioning the immune response by stimulating Treg through IL-10 and TGF- βsecretion [20, 60].

A lower number of LCs or a reduced level of CD1a produced by LCs were found in the epithelium during chronic periodontitis compared to gingivitis [59, 61, 62] suggesting that LCs leave the oral epithelium as the inflammation progresses. Other authors did not notice any difference between healthy and inflamed gingiva in periodontitis, or even reported an increased number of DCs. [63, 64] It is difficult to monitor the chronological overlap between the disappearance of LCs and the degree of inflammation and one can suspect that the development of inflammation is due to the low number of DCs. In fact, a link between the high incidence of periodontitis in elderly and the decreased number of DCs in these subjects was highlighted [65].

Chemokines and cytokines produced by DCs in response to their activation through the interaction with pathogens, such as *P. gingivalis*, can attract neutrophils and monocytes to the site of inflammation [20].

Neutrophils and monocytes are the main blood innate immune cells and they pass through the capillary wall into the *lamina propria* of the oral mucosa. IL-8 secreted by the epithelial cells stimulates through TLR4 the adhesion of endothelial cells lining the vessels to monocytes by an increased expression of adhesion molecules E-selectin, Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1). [66] Once in the tissue, the monocytes mature as macrophages or activated macrophages. Depending on the activating substances, the macrophages are phenotypically polarised: M1 macrophages are the classic macrophagic cell, activated by IFN-ɣ and LPS, while the M2 macrophage phenotype is involved in the resolution of inflammation and fibrosis [29].

Both neutrophils and macrophages – phagocytic cells from a myeloid lineage - are considered to represent the first line of defence against microbial pathogens. Neutrophils express TLR 1, 2, 4-10 and macrophages TLR 1, 2, 4-8 (see **Table 1**). When stimulated via TLR, neutrophils display an increased chemotaxis and synthesis of defensins, proinflammatory cytokines (IL-1, IL-6, TNF-α). [67] All these substances are involved in tissue destruction and stimulation of bone resorption. Noteworthy, neutrophils migration into JE was dependent of commensal colonisation and MYD 88 activation pathway. [68, 69] Chang et al. demonstrated that TLR2 and TLR4 are not required for promoting neutrophil trafficking into JE but may substantially contribute to shape the composition of microbiota which may modulate neutrophil homeostasis. Direct recognition of bacteria by TLR2 and TLR4 is not necessary for neutrophil migration into the gingival sulcus [70].

When exposed to PAMPS, monocytes can differentiate into osteoclasts upon direct stimulation of LPS by the help of RANKL [71, 72] and they also produce proinflammatory cytokines. In periodontitis, mature osteoclasts derived from DCs and monocytes are molecularly identical despite the fact that fewer genes must be activated when osteoclasts are formed from DCs than from monocyte precursors [73].

Two possibilities of evolution can be described for gingival inflammation: (i) the return to tissue homeostasis after an acute inflammation, as a protective response to bacterial biofilm, which is an active sequence of events and (ii) the persistence of inflammation leading to activation of gingival fibroblasts and myofibroblasts, in case of failure of resolution pathways, with destruction of ECM and bone, followed by scar formation and fibrosis, preventing the return of tissue homeostasis. [74] Various antibodies could be used to identify the fibroblasts phenotype: vimentin, α-SMA and S100-A4 (FSP1). There is a marked phenotypic heterogeneity of fibroblasts in gingival mucosa, as myofibroblasts are least represented and the cells positive for FSP1 are most commonly present. [75] Fibroblasts are intimately linked with the immune innate system. Uehara and Takada indicated that almost all TLRs from gingival fibroblasts are functionally involved in periodontal inflammatory reactions acting mainly by the MYD88 dependent pathway. [5] Once stimulated by PAMPs, fibroblasts produce proinflammatory cytokines leading directly to periodontal tissue destruction and bone resorption. Fibroblasts involvement in gingival inflammation has been intensively studied in connection to *P. gingivalis* infection. LPS from *P. gingivalis* activates TLRs from gingival fibroblasts and the synthesis of MMP1 and MMP3 to degrade ECM. MMPs in turn induce the expression of IL-1β, IL-6, IL-8 and MCP1 [6] which upregulate the inflammatory response in tissue-dependent manner. When comparing human periodontal ligament fibroblasts with human gingival fibroblasts isolated from the same donor to examine IL-8 responses of the cells to some germs and the possible involvement of the CD14/TLR system, the authors found that gingival fibroblasts strongly expressed CD14 mRNA and CD14 protein while periodontal ligament fibroblasts showed lower levels of expression in both respects.[76] Both types of fibroblasts expressed mRNA of

TLR2, TLR4, MD2 and MyD88, with TLR2 expression more intense in cells from the periodontal ligament. [76] Moreover, gingival fibroblasts exhibited a stronger IL-8 response than those from the periodontal ligament to LPS. The ability of periodontal fibroblasts to develop an adequate secondary immune response to *P. gingivalis* LPS could be compromised with persistence of inflammation.

6. Nucleotide-binding oligomerisation domain (NOD)-like receptors signalling

The NOD-like (NLR) family of innate intracellular receptors detects several PAMPs and endogenous molecules. In humans, this family contains ~20 members classified into five different subfamilies according to their structure: (i) NLRA which has an acidic transactivation domain, (ii) NLRB - with a baculovirus inhibitor of apoptosis protein repeat, (iii) NLRC – contains a CARD domain and includes NOD1 and NOD2, (iv) NLRP – which as a pyrin domain and responds to multiple stimuli forming a multiprotein complex termed NALP – inflammasome, and the last (v) NLRX, containing an uncharacterized domain. [31, 77, 78] A number of putative ligands of NLRs have been reported, but the field of NLRs ligand identification is still open. It is unclear whether NLRs, as well as TLRs, are able to interact directly through the LRR domain with their ligands. [79] NODs are cytosolic PRRs that bind to peptidoglycan from bacterial cell wall. NOD1 and NOD2 are present more or less in the same immune cells as TLR: NOD1 on human mononuclear cells, macrophages, epithelial cells, including those from oral epithelium, and dendritic cells, while NOD2 is present mostly on phagocytic cells: macrophages, DCs, neutrophils [80, 81] and Paneth cells of the small intestine. [82–84] NOD1 is involved in recognising cell wall compounds from Gram-negative bacteria, while NOD2 can sense both Gram-positive and Gram-negative bacterial cell wall components. [82, 85] NOD1 plays an essential role in innate immune response, its downstream signalling inducing the production of proinflammatory cytokines (IL-6, IL-8, TNF-α, hBD-2) and chemokines, as well as compounds with immunoregulatory and antimicrobial properties (IFN-γ, hBD-1).

The inflammasome comprises proteins that are assembled by intracytoplasmic PRRs. A multitude of inflammasomes exist and these can be activated through various mechanisms in order to secrete proinflammatory cytokines. [86] Once activated by PAMPs or DAMPs, the NLRPs undergo conformational changes that trigger the activation of caspase-1. Afterwards, the maturation of proinflammatory cytokines, such as IL-1β and IL-18, to their active forms follows and finally results in inflammation and pyroptosis, a cellular event confirmed in periodontal inflammation. [87, 88] Pyroptosis is a proinflammatory programmed cell death pathway uniquely dependent on caspase-1. [89] The mechanism and outcome of pyroptosis are different from those of apoptosis which actively inhibits inflammation.

Fourteen members of the NLRP subfamily are described. [90] Among these, expression NLRP2 inflammasome was reported to be decreased in gingival epithelia infected by *P. gingivalis.* [91] NLRP1 and NLRP3 are proposed to be involved in inflammasome function in addition to the cytoplasmic receptor absent in melanoma 2 (AIM2). [92] AIM2 is the first non-NLR family member that was identified to mediate inflammasome assembly and activate the caspase-1 pathway [93] having mainly cytosolic dsDNA from viruses, bacteria or the host as ligands. NLRP1, NLRP3 and AIM2 may exhibit inflammasome activity in diseases such as type 2 diabetes, essential hypertension or rheumatoid arthritis [88].

It is confirmed that the expression level of the inflammasome changes as the inflammation destroys the gingival tissues. [91, 94] In an immunohistochemical study, Xue et al. described a dissimilar expression pattern of NLRP1, NLRP3 and AIM2 in chronic and aggressive periodontitis, demonstrating their involvement in the pathogenesis of periodontal diseases to different degrees. [88] NLRP3 was significantly higher in chronic periodontitis and more expressed in the gingival epithelium than in *lamina propria* both in periodontitis and gingivitis. The intensity was gradually weaker from the top to the basal membrane in chronic periodontitis and opposite in aggressive periodontitis, which strengthens the idea that the bacteria outside the gingiva are more important in the pathogenesis of gingivitis when in aggressive periodontitis the host factor may be more importantly involved. [88] NLRP1 seems not to be an important biomarker for distinguishing gingivitis, aggressive and chronic periodontitis, because they observed that NLRP1 was barely expressed in gingival tissues in both conditions.

7. Therapeutic perspectives of TLR targeting in periodontal inflammation

Usual practical approaches to reduce bacterial levels to proportions manageable by the host innate immune system and limit the aggression of oral pathogens on periodontal tissues (scaling, root planning and meticulous oral hygiene techniques) [95] are often inefficient and for this reason adjunctive therapeutic strategies have been proposed in order to modulate host response.

Since their discovery, TLRs have emerged as pivotal mediators of innate host immune and inflammatory response. Due to their role in the recognition of PAMPS and DAMPS of various origins and the triggering of a proinflammatory response, TLR-signalling is regarded as a novel therapeutic target. That is why research was conducted to develop drugs that exploit TLRs signalling in immune therapy, drugs which are already tried to treat diseases, such as asthma and chronic obstructive pulmonary disease [96], AIDS [97], hepatitis B [98, 99], cancer [100, 101].

To date, several directions for targeting TLR-signalling pathways are discussed:

- Negative regulation for prevention of aberrant activation of TLRs;
- Use of synthetic substitutes;
- TLR as vaccine adjuvants;
- TLR agonists;
- TLR antagonists. [102]

Despite the potential of TLRs to activate the synthesis of protective molecules against infection, they can also induce serious immunopathological reactions in case of overstimulation or insufficient control due to the limited action of some negative regulators. An increased number of negative regulators able to dampen the degree and duration of TLR-mediated inflammatory host response were proposed.

Negative regulation of TLR signalling can be exerted extracellularly (inhibition of receptor function) or intracellularly (inhibition of downstream signalling).

Negative regulation occurs through different mechanisms:

- Soluble decoy TLRs, for example isoforms of TLR4, induced by various stimuli are able to block TLRs signalling and production of corresponding cytokines and chemokines; [103]

- Degradation of signal proteins; [104]

- Transcriptional regulation. Several miRNAs are reported to be essential modulators of immune pathways because they target adaptor molecules and their expression varies following TLR activation [104].

Negative regulators fail to control TLRs signalling because requiring a combination of effects, their loss leads to hyperactivation and pathogens develop strategies to evade TLR signalling. [104] TLRs may be novel therapeutic targets in periodontitis since manipulation of their signalling pathways contribute to the control of infection and inflammation and TLR agonists could be tested as vaccine adjuvants in treating periodontal inflammation [105].

Vaccine adjuvants exert their action by increasing antigen delivery to APCs, activating them to produce cytokines and by triggering T lymphocytes response. [102, 106] TLRs can function as adjuvant receptors for the recognition of certain antigens produced by microorganisms, the stimulation and maturation of APCs and the alerting of the immune system [24].

TLR agonists, similar but less toxic than PAMPS, are able to cause DCs maturation. [102] They are small molecules that mimic natural TLR ligands and could have improved pharmacological effects.

TLRs agonists are already clinically tested as vaccine adjuvants for cancer, allergic and infectious diseases [107].

8. Conclusion

Innate immune receptors are critical in maintaining periodontal health as well as in the progression of gingival inflammation by acting on the commensal microbiome and as a link to the activation of adaptive immunity. Due to the fact that TLRs are important in preserving the periodontium state in healthy conditions and TLR-inflammation is responsible for the destructive host reactions in periodontal diseases, the development of drugs that target TLR signalling and promote beneficial local effects would be of great success in such diseases.

Conflict of interest

None.

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