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CHARACTERIZATION OF CYTOKINES, MATRIX METALLOPROTEINASES AND TOLL-LIKE RECEPTORS IN HUMAN PERIODONTAL TISSUE DESTRUCTION

Arzu Beklen-Tanzer

ACADEMIC DISSERTATION

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IV. **Beklen A,** Hukkanen M, Richardson R, Konttinen YT: "Immunohistochemical localization of TLRs in Periodontitis". Oral Microbiology And Immunology 2008; 23:425–431.

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The publications are referred to in the text by their roman numerals.

ABBREVIATIONS

ABC	avidin-biotin-peroxidase complex
APMA	aminophenylmercuric acetate
BSA	bovine serum albumin
dsRNA	double-stranded RNA
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GCF	gingival crevicular fluid
HKLM	heat-killed *Listeria monocytogenes*
ICE	interleukin-1 converting enzyme
Ig	immunoglobulin
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IL-17	interleukin-17
kDa	kilodalton
LPS	lipopolysaccharide
MMP	matrix metalloproteinase
NF-kB	nuclear factor kappa beta
NK	natural killer
OCT	optimal compound for tissue embedding
PBS	phosphate buffered saline
PISF	peri-implant sulcular fluid
P. gingivalis	*Porphyromonas gingivalis*
PMN	polymorphonuclear
PRRs	pattern recognition receptors
RANKL	receptor activator of nuclear factor kappa B ligand
RPMI	roswell park memorial institute - basic cell culture medium used
PAMPs	pathogen-associated molecular patterns
ROI	region of interest
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAT-2	tumour-associated trypsinogen-2
TBS	tris buffered saline
TIMP	tissue inhibitor of matrix metalloproteinases
TLR	toll-like receptor
TNF	tumour necrosis factor
TNF-R	tumour necrosis factor-receptor
tPA	tissue plasminogen activator
VCAM-1	vascular cell adhesion molecule-1

ABSTRACT

Periodontal Disease affects the supporting structures of the teeth and is initiated by a microbial biofilm called dental plaque. Severity ranges from superficial inflammation of the gingiva (gingivitis) to extensive destruction of connective tissue and bone, leading to tooth loss (periodontitis). Periodontitis affects 10 - 15% of any population to the extent that they will lose half of their teeth by age 50 (Mariotti 1999). The annual cost of periodontal therapy in the US is more than $14 billion (Brown et *al.* 2002). The link between periodontal disease and a number of systemic diseases including diabetes, atheroma and preterm low birth weight (Teng 2002) underlines the gravity of the condition and the need to elucidate pathogenic mechanisms, which may provide diagnostic tests and novel therapeutic tools.

In periodontitis the destruction of tissue is caused by a cascade of microbial and host factors together with proteolytic enzymes. Matrix metalloproteinases (MMPs) are known to be central mediators of the pathologic destruction in periodontitis. Initially, plaque bacteria provide pathogen-associated molecular patterns (PAMPs), which are sensed by Toll-like receptors (TLRs), and initiate intracellular signaling cascades leading to host inflammation.

The aim of the present study was to characterize tumour necrosis factor-alpha (TNF-α) and its type I and II receptors in periodontal tissues, as well as, the effects of TNF-α, interleukin-1beta (IL-1β) and IL-17 on the production and/or activation of MMP-3, MMP-8 and MMP-9. Furthermore, we mapped TLRs in periodontal tissues and assessed how some of the PAMPs, binding to the key TLRs found in periodontal tissues, affect production of TNF-α and IL-1β by gingival epithelial cells with or without combination of IL-17.

In study (I) TNF-α and its receptors were detected by immunohistochemical staining in third molar pericoronitis. The results showed that increased expression of interleukin-1β and vascular cell adhesion molecule-1 was found as a biological indicator of TNF-α ligand-receptor interaction. Then, in study (II) MMP-3, MMP-8, and MMP-9 were investigated in periodontitis affected human gingival crevicular fluid (GCF), tissues and gingival fibroblasts. Briefly, resident gingival fibroblasts produced pro-MMP-3 in GCF. Partially activated MMP-3, MMP-8, and MMP-9 were found in the GCF samples of periodontitis affected samples. However, cultured gingival fibroblasts released only pro-MMP-3 when stimulated with TNF-α. Since IL-17 has been reported to up-regulate IL-1β and TNF-α, in study (III) the effect of IL-17 was studied on MMP

and pro-inflammatory cytokine production. It was found that IL-17 was increased in periodontitis and up-regulated IL-1β and TNF-α and MMP-1 and MMP-3. These first three studies showed the amplifying inflammatory/tissue destructive process in periodontitis is a cascade involving MMPs. In study (IV) we continued by demonstrating TLRs in gingival tissues with immunohistochemistry. Results showed statistically significant differences between patients with periodontitis and healthy controls, suggesting their involvement in the pathogenesis of periodontitis. In the final study (V), enzyme-linked immunosorbent assays (ELISA) were performed to detect the levels of IL-1β and TNF-α, released from gingival epithelial cell cultures following stimulation with TLR ligand alone or in combination with IL-17. Stimulated cells with the respective ligands produced IL-1β and TNF-α and showed how TLR2 agonist (HKLM) and TLR5 agonist (Flagellin) shared by many different periodontopathogenic bacteria, stimulate the resident gingival cells to inflammatory responses in a TLR-dependent manner (V).

In summary, this thesis demonstrates that TLRs are present in periodontal tissues and present differences in periodontitis compared to healthy controls. The cells of gingival tissues respond to inflammatory process in a TLR-dependent manner by producing pro-inflammatory cytokines (TNF-α and IL-1β). During the destruction of periodontal tissues, the release (IL-1β and TNF-α) and co-operation with other pro-inflammatory cytokines (IL-17), which in turn increase the inflammation and thus be more harmful to the host with the increased presence of MMPs (MMP-1, MMP-3, MMP-8, MMP-9) in diseased over healthy sites.

1. INTRODUCTION

"Periodontology" is a dental research field addressing the tooth-supporting tissues, the "periodontium". The periodontium is consisted of 1) "gingiva" provides a tissue sealing around the cervical portion of the teeth and covers the alveolar process, 2) "periodontal ligament" supports the tooth in the socket and provides nutrients and sensory mechanisms to the tooth, 3) "root cementum" anchors the periodontal ligament to the tooth, 4) "alveolar bone" forms the bony sockets, which provide support and protection to the roots of the tooth (Figure 1). The periodontium is made up of these tissues that surround each tooth and which anchor each tooth into the alveolar process (Latin: para = adjacent to; Greek: odus = tooth). Normal healthy gingival tissues form a protective barrier against infection. In the healthy/balanced situation, the net response of the host is protective, whereas it is destructive if the bacteria –host relationship is unstable. Balanced degradation and repair processes maintain the structural and functional integrity in healthy periodontal tissue compartments.

Dental biofilms provide a shelter for microorganisms to grow and proliferate and, in turn, release bacterial products while provoking an inflammatory host response. If the dental plaque biofilm continue to grow and expand to populate the subgingival space, the compounds will stimulate the gingiva, firstly epithelium to produce bioactive mediators, resulting in further recruitment of a variety of cell types, including neutrophils, T-cells, monocytes, fibroblasts, epithelial cells, etc. (Kinane & Lindhe 1997). The histological appearance of chronic dental inflammation is characterized with a mixed inflammatory cells and enlargement of connective tissue. During chronic inflammation, activated cells of gingiva produces a variety of biologically active mediators, most prominently cytokines such as IL-1β and TNF-α and matrix metalloproteinases. In addition, the epithelium and the cells of connective tissue respond by induction of innate defense system, which include TLRs as well (Gemmell et *al.* 1997, Kinane & Lindhe 1997, Mahanonda & Pichyangkul 2007).

Correlations between tissue degradation and MMPs/cytokines have been investigated intensively in periodontal diseases. However, because of the complexity in the periodontal disease, future investigations are needed for better understanding. In this thesis, we focused to establish the specific relationship between proinflammatory cytokines (IL-1β, TNF-α, IL-17), MMPs (MMP-1, MMP-3, MMP-8, MMP-9) and TLRs in periodontal tissue destruction.

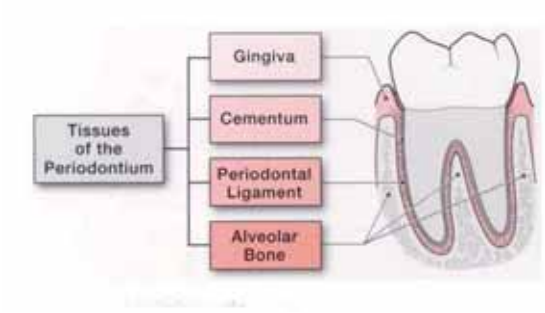

Figure 1. A schematic structure of periodontium (Adapted by the permission from: Nield-Gehrig & Willmann 2007).

2. REVIEW OF THE LITERATURE

2.1 Microscopic features of gingiva

The gingiva consists of a central core of connective tissue covered by epithelium. In gingival epithelium the keratinocytes form the principal cell type. Other cells comprise Langerhans cells, Merkel cells, and melanocytes. The gingival epithelium protects the deep structures while allowing selective interchange with the oral environment. This function is accomplished by proliferation, differentiation and apoptosis of keratinocytes. Cells of gingiva show various layers called stratum basale, stratum spinosum, stratum granulosum and stratum corneum, and a keratin layer where keratinized (Newman et *al.* 1996). The main morphologic change is the progressive flattening of the cells, while at the same time intracellular junctions upon epithelial flow increase and the nucleuses disappear (Schroeder 1981). The oral epithelium undergoes continues renewal in numbers and size. The balance between new cell formation and shedding of old cells maintains the thickness of gingiva (Stern 1967).

In contrast to epithelial layer, the gingival connective tissue below the epithelium (known as lamina propria) has an abundance of extracellular matrix and relatively few cells. The cells, including fibroblasts, immune-inflammatory cells such as neutrophils, macrophages, mast cells and lymphocytes comprise about 5% of the gingival connective tissue (Nield-Gehrig & Willmann 2007). Among these cells, fibroblasts are the preponderant cellular element in the gingiva. They synthesize collagen and elastic fibers and regulate collagen degradation (Newman et *al.* 1996). Mast cells are distributed throughout the body and are found in the connective tissue of gingiva (Carranza & Cabrini 1955, Gemmell et *al.* 2004). Macrophages are present in the gingival tissue as components of mononuclear phagocyte system and they are derived from blood monocytes. In healthy gingiva, lymphocytes are also found in the connective tissue near the base of sulcus. Furthermore, neutrophils can be seen in both the gingival connective tissue and the sulcus (Newman et *al.* 1996). For the extracellular matrix, protein fibers account for about 55 to 65% of the gingival connective tissue, which mostly composed of collagen fibers forming a dense network of strong, rope-like cables to hold and secure the gingival connective tissues together. About 30 to 35% of the gingival connective tissue consists of gel-like material between the cells such as proteoglycans, adhesive glycoproteins and other non-collagenous proteins (Nield-Gehrig & Willmann 2007). Morphologically, connective tissue fibers can be divided to three different types namely collagen, reticular and elastic. Type collagen I is the major type of the collagens in lamina propria and provides the tensile strength to the tissues of gingiva

(Löe & Karring 1969, Itoiz & Carranza 2002). Type IV collagen and laminin networks coupled to each other are found in the basement membrane of the epithelial cell layer and blood vessel walls (Löe & Karring 1969, Chavrier et *al.* 1984, Itoiz & Carranza 2002). In addition to that in periodontal connective tissue, type III collagen is also found (Chavrier et *al.* 1984). The connective tissue of marginal gingiva is formed from dense collagen, containing a prominent system of collagen fiber bundles, which are called gingival fibers. They consist of type I collagen and brace the marginal gingiva firmly against the tooth (Newman et *al.* 1996).

2.2 Periodontal disease

The general term "periodontal disease" refers to inflammatory and recessive changes of the gingiva and periodontium (Page & Schroeder 1976, Armitage 1999). Tooth supporting mechanism is commonly faced with plaque-induced, usually chronic, inflammatory alterations in the gingiva and surrounding periodontal structures. During life, hundreds of different bacterial species are present in and on the human body. These bacteria may be beneficial to the host (or commensal) or can cause injury. So far, more than 500 different bacteria have been identified in the oral cavity, although most of the bacteria stay in ecological balance and do not cause disease. On the other hand, high numbers of certain facultative pathogenic bacteria are occasionally identified in cases of diseases, such as gingivitis and periodontitis (Socransky & Haffajee 1997, Kroes et *al.* 1999).

Gingivitis, which is not as severe as periodontitis, may persist for many years and with good oral hygiene and with an effective plaque removal is completely reversible. On the contrary, periodontitis is partially reversible and develops out of a more or less pronounced gingivitis. Today it is well known that bacteria alone, even the periodontopathic bacteria, can cause gingivitis but not periodontitis in all cases (Page & Schroeder 1976, Armitage 1999). Besides the bacteria, the reaction of the tissues, which involve negative host factors and additional risk factors play an important role for the development of periodontitis (Clarke & Hirsch 1995).

The reasons for why some gingivitis cases progress or not to periodontitis are not completely clarified. Similar to all infections, proliferation of pathogenic microorganisms, toxic potency, and the capacities to penetrate into the tissue (Salvi et *al.* 1997) and furthermore, defects of the acute host response (resulting from functional disturbances of polymorphonuclear granulocytes - PMN), insufficient immunologic reactions and the predominance of pro-inflammatory mediators are among the determining factors. Nowadays, it is established that unhealthy lifestyle factors,

such as smoking, alcohol consumption and unhealthy diets are all risk factors for periodontitis (Wolf et *al.* 2004).

Nevertheless, of all these risk factors mentioned, none is able to directly damage the periodontium. Their main effect derives from the patients' own immune inflammatory system. The delicate balance between attack (bacteria) and defense (host response) is broken in periodontal diseases (Figure 2).

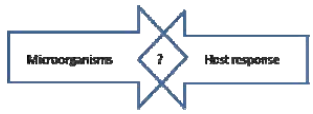

Figure 2. Balance between bacteria and host response is broken in periodontal diseases.

2.2.1 Innate and adaptive immunity

Periodontal disease is initiated by oral bacteria perturbing epithelial cells, which trigger innate, inflammatory, and adaptive immune responses. These processes result in the destruction of the tissues surrounding and supporting the teeth and eventually result in tissue, bone, and, finally, tooth loss. Bacterial plaque has been shown to initiate periodontal diseases (Figure 3). Most studies indicate that the host response, rather than the direct effect of bacteria, is responsible for much of the destruction associated with periodontitis. Previously, the host response to a bacterial challenge was characterized as either acute or chronic inflammation. However, chronic inflammatory diseases, such as periodontitis, have simultaneous acute and chronic components.

The innate immune response developes before the acquired immune response. The innate immune response depends on pattern recognition and is carried out by leucocytes. These cells have receptors such as toll-like receptors. Thus, any molecule that binds to these receptors is recognized as "foreign" and elicits a host response. This response is characterized by the production of inflammatory mediators, including cytokines. Cytokines (such as; TNF-α, IL-1β) stimulate a number of cellular events, including recruitment of phagocytic cells to the site of infection which, taken together, represent the innate immune response. The innate leukocytes include natural killer cells, mast cells, eosinophils, basophils and the phagocytic cells including macrophages, neutrophils and dendritic cells and function within the immune system by identifying and eliminating pathogens that might cause infection (Wang & Ohura 2002, Teng 2003).

Following innate immune, an acquired (adaptive) immune response develops. The adaptive immune response is highly specific for a particular pathogen. The adaptive immune system remembers the infectious agent and can prevent from causing disease later. Briefly, two key features of the adaptive immune response are thus specificity and memory. The cells of the adaptive immune system are a type of leukocyte, called a lymphocyte. B cells and T cells are the major types of lymphocytes and specifically recognize individual pathogens. These cells bind to microorganisms and kill them. B cells and T cells (T-helper, T-cytotoxic, T-regulatory) are derived from the same pluripotential hematopoietic stem cells and are indistinguishable from one another until they are activated. B cells play a large role in the humoral immune response, whereas T-cells are intimately involved in cell-mediated immune responses. B cells combat extracellular pathogens and their products by releasing antibody. T cells have a wider range of activation such as controlling of B lymphocyte development and antibody production, or interact with phagocytic cells to help them destroy pathogens they have taken up or recognize cells infected by virus and destroy them. (Male 2001).

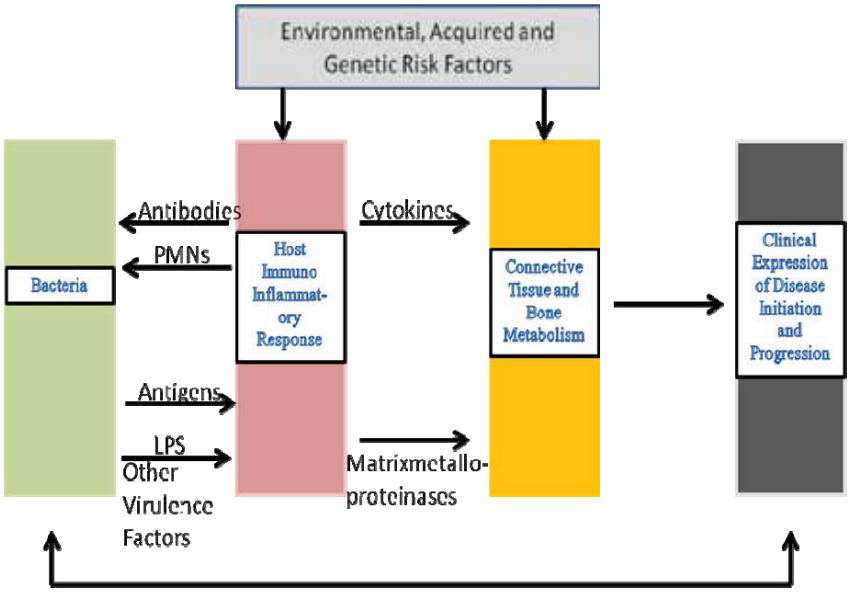

Figure 3. Model of periodontal disease (Adapted by the permission from: Page & Kornman 1997a).

2.2.2 Classification of periodontal diseases

Periodontitis is an infectious disease that leads to the destruction of hard and soft tissues surrounding the teeth. Contrary, in gingivitis the inflammation is confined to the gingiva and is reversible after treatment.

The classification of periodontal diseases was updated by European Federation of Periodontology in collaboration with American Academy of Periodontology in 1999/2000 (Armitage 1999). The old classifications were too much based on the age of the patient at the time for disease initiation. For example, the condition in earlier classification considered "Adult periodontitis" is in fact chronic disease, which can be seen in young patients as well. At the present, there are eight classifications. The table below is brief summary of the classification to assist the readers of this thesis.

Table 1. Brief classification of periodontal diseases (Armitage 1999)

Type I Gingival diseases	Plaque-induced gingival diseases Plaque-induced gingival lesions
Type II Chronic periodontitis	Localized Generalized
Type III Aggressive periodontitis	Localized Generalized
Type IV Periodontitis as a manifestation of systemic disease	Associated with hematological disorders Associated with genetic disorders Not otherwise specified

There are also other types of periodontal diseases (Type V –VIII) described in the literature (Armitage 1999). Because Type I-IV are the most common ones, they are presented in table 1.

The most common form of periodontal disease is chronic periodontitis. Clinically, deepened (> 4 mm) periodontal pockets, reduced attachment level, alveolar bone loss, plaque accumulation and bleeding upon probing are the key characteristics for clinicians. Very often swelling and redness, thickening, fibrosis of gingival margin, and pus formation are seen in the acute stage.

2.3 Pericoronitis

Pericoronitis is an inflammation of the soft and hard tissues surrounding the crown of an erupting or impacted tooth. It is characterized by Gram negative anaerobic bacterial growth (Orbak & Dayi 2003). Bacterial products stimulate host cells to secrete pro-inflammatory

cytokines, which are necessary for host defense, but which may also lead to pain and periodontal tissue destruction (Gemmell et *al.* 1997, Palladino et *al.* 2003). Pericoronitis may occur at any age and in any tooth, but third molars most commonly present with this problem (Orbak & Dayi 2003). Symptoms of pericoronitis vary from localized to general. It occurs when the tissue around the tooth has become infected because bacteria have invaded the area. Poor oral hygiene and mechanical trauma on nearby tissue can cause this inflammation. However, it can be impossible to effectively brush the necessary area and prevent this from occurring due to a partially erupted tooth (Laskaris 2003).

2.4 Dental plaque

Although it is well established that more than 500 bacterial strains can be found in dental plaque, the understanding of the dental plaque has undergone major advances during the years. In the middle of 1900s, all bacteria in the dental plaque were believed to contribute equally to this disease. However, later with the microscopic examination in 1960s different bacterial morphotypes were found in healthy gingiva and specific groups of microorganisms were isolated in periodontal disease and then considered of relevance in this context. Finally, in 1990's with the help of new molecular approaches it was found that a substantially greater diversity of species than earlier expected is found in the periodontal environment (Kroes et *al.* 1999).

Dental plaque can be described as the soft deposits that form the biofilm (well-organized community of bacteria) adhering either to the tooth surface or other hard surfaces in the oral cavity (Bowen 1976). Periodontal pathogens within a biofilm environment behave very differently from free floating planktonic bacteria. This might be one of the explanations why periodontal diseases are so difficult to prevent and to treat. There is a protective extracellular slime matrix making bacteria extremely resistant to antibiotics, antimicrobial agents and host defense mechanisms (Marsh 2004).

Disruption of a balanced state of bacterial population that exists in host tissues causes alterations both in the host and bacterial biofilm. Finally, this results ultimately in the destruction of the tooth supporting connective tissues of periodontium (Newman et *al.* 1996) (Figure 4).

Figure 4. Picture to explain the biological plausibility for the association between bacterial plaque and periodontal disease (reprinted by the permission from the American Academy of Periodontology).

2.5 Gingival crevicular fluid (GCF)

GCF is an exudate found in the periodontal pocket between the tooth and marginal gingiva. GCF is a complex mixture of substances derived from serum, leukocytes, structural cells of periodontium and oral bacteria. Its amount is very small in the healthy gingiva and its constituents participate in the normal maintenance of function of healthy tissues. Quantification of the GCF volume has been used as a measure of the inflammatory status of the periodontal tissues. Because during inflammation the flow rate of GCF increases and its composition starts to resemble an inflammatory exudate (Cimasoni 1983, Nakamura et *al.* 2000, Uitto 2003). The increased GCF flow contributes to host defense by flushing bacterial colonies and metabolites away from the sulcus, which diminishes their penetration into tissues. During inflammation, compositional changes are caused either by bacteria, bacterial metabolites/enzymes, other factors or the inflammatory reactions (Pöllänen et *al.* 2003). More than 65 infection induced enzymes, their inhibitors and regulators have been found in GCF (Armitage 2004) which might be used to diagnose the severity of inflammation in periodontitis (Kinane et *al.* 2003, Sorsa et *al.* 2006). A diagnostic tool/ a chair-side collagenase-2 test stick, has been investigated to see progress, risk and treatment of periodontitis (Mäntylä et *al.* 2003, 2006, Sorsa et *al.* 2009). Increased bacteria- and host-derived products in the GCF have been associated with the initiation and progression

of periodontal disease. Polymorphonuclear neutrophilic leucocytes (PMN) are the major cells that form the most important line of defense (Page et *al.* 1997b).

Cytokines, particularly IL-1, IL-6 and TNF-α in GCF have been associated with periodontal disease. These are secreted into GCF by leucocytes or the cells of the epithelium and their amounts have been shown to be increased in periodontal tissue destruction (Gemmell et *al.* 1997). The presence of MMPs in GCF has also been studied in many different studies. Although most of the proteinases in GCF are of neutrophil origin, other cell groups such as epithelial cells and fibroblasts also release MMPs into the GCF (Uitto et *al.* 2003). So far, it has been shown that MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13, MMP-14 (Kornman et *al.* 1997) MMP-25 and MMP-26 are present in GCF (Emingil et *al.* 2006).

2.6 Structural and functional properties of MMPs

Matrix metalloproteinases are a family of structurally related zinc-dependent enzymes that degrade extracellular matrix (ECM) of interstitial stroma and of basement membrane components. Furthermore, MMPs have various non-matrix substrates, including growth factors, their receptors, cytokines and chemokines (Sternlicht & Werb 2001).

In 1962, the first MMP was discovered in tadpole (frog) tail undergoing resorption (Gross & Lapiere 1962). Since then some other MMPs have been identified. To date, in humans, the MMP family comprises 23 members, which were numbered in order of their discovery, beginning with MMP-1. These groups of 23 human MMPs are structurally related membrane or soluble endopeptidases. Although MMPs have overlapping substrate specificities, MMPs can be divided into five major groups, according to their substrate specificity; 1) collagenases (MMP-1, MMP-8, MMP-13), 2) gelatinases (type IV collagenases; MMP-2 and MMP-9), 3) stromelysins (MMP-3 MMP-10, MMP-11), 4) membrane-type MMPs and 5) other MMPs (MMP-7, MMP-26, MMP-12, MMP-20, MMP-28, MMP-23, MMP-19, MMP-21, MMP-27) (Ahokas et *al.* 2003, 2005, Kelly & Jarjour 2003, Bar-Or et *al.* 2003, Momohara et *al.* 2004, Pardo & Selman 2005) (Table 2).

The collagenases are mainly responsible for degradation of collagen fibers. The gelatinases are able to cleave gelatin (denatured collagen), but also some collagen molecules, particularly reticular type IV collagen of the basement membranes. The stromelysins have an extensive list of substrates, which include elastin, collagen, laminin and proteoglycans. The membrane type MMPs particularly at cell membranes cleave fibronectin, laminin, collagen and proteoglycans

(Brinckerhoff et *al.* 2000, McCawley & Matrisian 2001, Chakraborti et *al.* 2003, Kelly & Jarjour 2003).

MMPs are consequently functionally active and catalytically competent at physiological pH and temperature (Nagase 1997). During healthy conditions, in combination with other extracellular proteinases, MMPs are involved in maintenance of physiological events. These include coagulation, remodelling, apoptosis, wound repair, host defense, regulation of inflammatory responses, reproduction, development and tissue/bone remodelling. In contrast, in disease conditions MMPs are involved in several inflammatory conditions, which can lead to tissue injury in e.g. skin, lungs, eyes, skeletal system and cancer (Owen & Campbell 1999, Sternlicht & Werb 2001, Nyberg et *al.* 2006).

MMPs are secreted by many different cell types. One cell can produce several MMPs. All MMPs are synthesized as inactive proenzymes or zymogens and mostly activated extracellularly. MMP-11, MMP-23, MMP-28 and the transmembrane-type MMPs can be activated intracellulary (Egeblad & Werb 2002, Lijnen 2002). Because MMPs are secreted as proenzymes they have to be cleaved for the activation. Activation occurs by serine proteinases such as trypsin or plasmin (Sorsa et *al.* 1997, Moilanen et *al.* 2003). Other MMPs, microbial proteinases (Sorsa et *al.* 1992) and also other factors such as oxygen-derived free radicals (Saari et *al.* 1990) can lead to activation.

The inactive zymogen/proMMPs have a characteristic multidomain structure common for many MMPs. In general MMPs consist of 1) the signal peptide (prepeptide), 2) the propeptide, 3) the catalytic domain, 4) the hinge region, 5) the hemopexin-like domain, with an order from N to C terminus (Nagase et *al.* 2006). MMP activation requires at least partial removal of the prodomain to change into a lower molecular weight active form through various pathways (Nagase 1997). The signal peptide guides the MMP in the cell, which then during transit form the cell is cut off. The propeptide domain maintains the latency of the proMMP through a cysteine residue that ligates the zinc atom in the active site of the catalytic domain. During activation of proMMP, the linkage between the cysteine residue in the prodomain and the catalytic zinc in the catalytic domain is disrupted. The zinc-containing active site becomes then able to catalyse hydrolysis of peptide bonds. In addition to zinc-containing catalytic site, additional zinc and calcium ions contribute to the maintenance of the three dimensional structure of MMP (Egeblad & Werb 2002, Lijnen 2002).

Table 2. The subgroups of the MMP family members and their sources

	MMP	Source
Collagenases	MMP-1	fibroblasts, endothelial cells, epithelial cells, hepatocytes, keratinocytes, monocytes, osteoblasts
	MMP-8	fibroblasts, endothelial cells, monocytes, neutrophils, B-cells, T-cells
	MMP-13	fibroblasts, bone cells
Gelatinases	MMP-2	fibroblasts, endothelial cells, macrophages, neutrophils, T-cells
	MMP-9	fibroblasts, dentritic cells, endothelial cells, eosinophils, epithelial cells, keratinocytes, macrophages, neutrophils, osteoblasts, T-cells
Stromelysins	MMP-3	fibroblasts, endothelial cells, epithelial cells, monocytes, vascular smooth muscle cells, keratinocytes, chondrocytes
	MMP-10	fibroblasts, epithelial cells, keratinocytes, T-cells, monocytes
	MMP-11	fibroblasts, epithelial cells, B-cells
Membrane-type MMPs	MMP-14	fibroblasts, epithelial cells, macrophages, vascular smooth muscle cells, osteoblasts
	MMP-15	fibroblasts, macrophages, T-cells
	MMP-16	vascular smooth muscle cells, brain, placenta, T-cells
	MMP-17	monocytes, B-cells, brain, reproductive tissues
	MMP-24	T-cells, brain
	MMP-25	neutrophils, monocytes
Other MMP	MMP-7	epithelial cells, monocytes, T-cells, B-cells, mesangial cells
	MMP-12	Macrophages
	MMP-19	monocytes, T-cells
	MMP-20	dental enamel
	MMP-21	epithelial cells, keratinocytes, monocytes, B-cells, T-cells
	MMP-23	monocytes, B-cells, T-cells, ovarium
	MMP-26	B-cells, keratinocytes
	MMP-27	B-cells
	MMP-28	T-cells, cartilage

Ahokas et *al.* 2003, Kelly & Jarjour 2003, Bar-Or et *al.* 2003, Momohara et *al.* 2004, Ahokas et *al.* 2005, Pardo & Selman 2005

2.6.1 MMP-1

MMP-1 (collagenase 1) was the first of the collagenases to be purified. It is also known as interstitial collagenase or fibroblast collagenase (Brinckerhoff & Matrisian 2002, Pardo & Selman 2005). MMP-1 is secreted in a latent 52 kDa form and after activation it is converted to 42 kDa active form (Woessner 1991). It is expressed by a variety of cell types such as fibroblasts, kerotinocytes, endothelial cells and monocytes (Pardo & Selman 2005). Active MMP-1 degrades fibrillar collagens and as well as other matrix molecules including aggrecan, versican, perlecan and gelatin (Brinckerhoff & Matrisian 2002). MMP-1 is effective in hydrolysing type III collagen and plays an important role during the initiation of collagen degradation in periodontal disease in addition to MMP-8, which is another collagenase (Birkedal-Hansen 1993, Ingman et *al.* 1994a, Golub et *al.* 1997). MMP-1 can also degrade cell surface molecules and non-matrix molecules such as IL-1β and proTNF-α (McCawley & Matrisian 2001, Chakraborti et *al.* 2003). MMP-1 is elevated in chronic periodontitis patients in GCF compared to healthy subjects, furthermore, the elevated levels decreased with periodontal treatment (Tüter et *al.* 2002).

2.6.2 MMP-3

MMP-3 (Stromelysin 1) was earlier also known as collagenase-activating protein. It is expressed by gingival connective tissue cells and activates latent proMMP-1, proMMP-8, proMMP-9 and proMMP-13 (Kähäri & Saarialho-Kere 1999, Moilanen et *al.* 2003). ProMMP-3 is released as 53 kDa and after activation extracellulary by plasmin, tryptase, kallikrein, chymase and MMP-3 itself (autocatalytic activation) is converted to 43 kDa active form (Chakraborti et *al.* 2003). The activated MMP-3 has many substrates, including ECM molecules such as collagen III-V and IX, elastin, gelatin, proteoglycans and osteonectin. In addition to that, MMP-3 was shown to cleave also non-matrix molecules proIL-1β, proTNF-α, MMP-1 and MMP-13 (Kähäri & Saarialho-Kere 1999, McCawley & Matrisian 2001, Chakraborti et *al.* 2003, Kelly & Jarjour 2003). The severity of clinical findings of periodontitis correlates with the increased levels of MMP-3 in GCF (Haerian et *al.* 1995).

2.6.3 MMP-8

MMP-8 (collagenase-2, neutrophil collagenase) is synthesized by PMNs during their myelocyte maturation state in bone marrow and stored in intracellular specific granules ready for secretion (Weiss 1989). Later it has been shown that also other cells produce MMP-8. Epithelial cells,

gingival fibroblasts, monocytes/macrophages and plasma cells have been reported as non-PMN sources (Hanemaaijer et al 1997, Tervahartiala et *al.* 2000, Prikk et *al.* 2001, Wahlgren et *al.* 2001, Sorsa et *al.* 2004). MMP-8 is released in a latent form in periodontal inflammation as a result of stimulation by host derived inflammatory mediators such as IL-1β, TNF-α, various periopathogenic bacteria and their virulence factors (Weiss 1989, Sorsa et *al.* 1992, Ryan et *al.* 1996).

The molecular weight of MMP-8 differs a lot according to cell source varying from 85 kDa (sometimes even >100 kDa), to smaller than 20 kDa sizes. The proform of PMN typed MMP-8 can be detected in 75-80 kDa and converted to 65 kDa active form (Ding et *al.* 1996, 1997), whereas non-PMN type MMP-8 is detected in 55 kDa and 45 kDa for latent and active forms, respectively (Moilanen et *al.* 2002, Moilanen et *al.* 2003). Activation can be proteolytic (e.g. by MMP-3) or non-proteolytic (initial activation by oxygen radicals) (Nagase 1997).

Collagens I-III, VII, X, gelatin, proteoglycans, bradykinin, substance P and pro- and anti-inflammatory cytokines/mediators are known substrates of MMP-8 (Sternlicht & Werb 2001). MMP-8 is one of the major collagenases in inflamed human periodontium as well (Sorsa et *al.* 1988, Romanelli 1999). Both GCF (Lee et *al.* 1995, Ingman et *al.* 1996, Golub et *al.* 1997) and extracts of gingival tissues from periodontitis patients presented higher levels of catalytically active MMP-8, compared to samples of healthy subjects (Sorsa et *al.* 1988). Increased GCF collagenase activity has been associated with loss of connective tissue attachment in periodontitis patients (Lee et *al.* 1995). Although MMP-8 is one of the most efficient enzymes in degrading type I collagen (Sorsa et *al.* 2004) in periodontal tissues, MMP-8 has a protective anti-inflammatory role in experimental skin and oral cancer as well as in lung inflammation (Balbin et *al.* 2003, Owen et *al.* 2004, Gueders et *al.* 2005, Korpi et *al.* 2008). Recently its protective role, through the processing anti-inflammatory cytokines and chemokines has been analyzed in periodontal tissue destruction as well (Kuula et *al.* 2009).

2.6.4 MMP-9

MMP-9 (92 kDa type IV collagenase, gelatinase B) is expressed as 92 kDa latent form to be converted into 68-82 kDa active forms during activation (Birkedal-Hansen et *al.* 1993). MMP-9 degrades gelatine, collagens IV, V, VI and X, fibronectin, fibrillin and aggrecan (Senior et *al.* 1991). In periodontitis MMP-9 is the major gelatinase in gingival tissue, dental plaque, saliva and

GCF and its levels in periodontitis affected GCF samples were found to be elevated compared to healthy control samples (Ingman et *al.* 1994b, c, 1996, Sorsa et *al.* 1994, 1995, Golub et *al.* 1995). In GCF samples, active MMP-9 was found in 97.8% percent of the periodontitis samples, whereas only 11.4% of gingivitis samples presented active MMP-9 (Teng et *al.* 1992). In periodontitis, the major source of MMP-9 is PMNs, whereas monocytes and macrophages also form sources to lesser amount as well (Westerlund et *al.* 1996, Pirilä et *al.* 2001). Despite to its destructive effect, it is also shown that MMP-9 is involved in the recruitment of leukocytes to an inflammatory site, by affecting development of allergen-specific T cell responses in asthma (McMillan et *al.* 2004).

2.7 Serine proteinases

Serine proteinases form one of the four different types of proteinases. Similarly to MMPs, they are active at neutral or slight alkaline pH and are neutral endoproteinase. *In vivo* matrix degradation is mainly achieved in extracellular space, which is maintained at neutral pH. Because of this, together with MMPs, serine proteinases also play an important role in matrix degradation (Werb 1989). Serine proteinases such as, neutrophil elastase, cathepsin G, plasmin, tryptase, chymase, and tumour-associated trypsinogen-2 (TAT-2) take role in the activation of proMMPs (Gruber et *al.* 1988, Capodici et *al.* 1989, Weiss 1989, Koivunen et *al.* 1999, Saarinen et *al.* 1994, Sorsa et *al.* 1997, Väänänen et *al.* 2001, Nyberg 2002, Moilanen et *al.* 2003). Among these serine proteinases; PMN elastase and cathepsin G have been widely studied in the tissue destruction in periodontal diseases (Ingman et *al.* 1994a). Elastase and cathepsin G are produced and/or stored by polymorphonuclear leucocytes as an active species and are released by PMN degranulation at sites of inflammation (Weiss 1989, Ding 1998). Both cathepsin G and elastase promote tissue destruction by activating proMMPs, such as proMMP-3 (Okada & Nakanishi 1989, Jenne 1994). Furthermore, both cathepsin G and neutrophil elastase enhance MMP activity by degrading and and inactivating TIMPs (Rice & Banda 1995). Other than these, tPA (tissue type plasminogen activator) is another type of serine proteinase and high levels of tPA has been described as effective proMMP activator in TNF-stimulated gingival fibroblasts (Ueda & Matsushima 2001).

2.8 Regulation of MMPs

In healthy tissues, synthesis and degradation are in balance. This steady state balance is achieved by low level expression of certain MMPs, which are tightly controlled. In general, MMPs are regulated by 1) gene transcription 2) activation of the latent proform and 3) inhibition of their

activity e.g. by TIMPs (Kelly & Jarjour, 2003). Inflammatory cytokines (e.g. TNF-α, IL-1β, IL-17), growth factors, hormones, cell-cell and cell-matrix interactions stimulate the expression of enzymes through changes in transcription (Ravanti et *al.* 1999, Westermarck & Kähäri, 1999, Wassenaar et *al.* 1999, Martelli-junior et *al.* 2003, Jenkins et *al.* 2004, Maldonado et *al.* 2004, Ruwanpura et *al.* 2004, Sakaki et *al.* 2004). Generally, MMPs are not stored by cells (Birkedal Hansen 1993). They are expressed in proform whenever they are needed. In many cases the activation of proform is achieved by the removal of the prodomain (Nagase 1997).

2.8.1 Cell-to-cell interactions

One level of regulation, which leads to the formation of the MMPs is achieved by cell-to-cell interactions between various cells, including epithelial cells, fibroblasts, endothelial cells, monocytes/macrophages and lymphocytes (Huybrechts-Godin et *al.* 1979, Sheppard et *al.* 1992, Burger et *al.* 1998, Hojo et *al.* 2000, Zhu et *al.* 2001). In periodontal disease epithelial cells, fibroblasts and monocytes/macrophages are in close interaction (Tervahartiala et *al.* 2001). Interactions between cells may involve several vascular adhesion molecules (VCAM) such as VCAM-1 (Haskard 1995). The expression of VCAM-1 on monocytes, endothelial cells and fibroblasts are upregulated during inflammation (Elices et *al.* 1990, Haskard 1995, Conran et *al.* 2003, Hosokawa et *al.* 2006). Adhesion molecules play an important role in the pathogenesis of inflammatory diseases due to their increase in response to certain pro-inflammatory cytokines and their ability to act as costimulatory molecules in the activation of immune responses (Haskard 1995). VCAM-1 belongs to the Ig superfamily and is a surface glycoprotein that promotes adhesion and subsequent recruitment of leukocytes (Mojcik & Shevach 1997). VCAM-1 is induced by IL-1β (Joe et *al.* 2001).

2.8.2 Activation of proMMPs / APMA-chemical activator

MMPs are secreted in latent proforms and activated when a small peptide is cleaved from their N-termini. Because of this, MMPs can be recognized both in inactive and active forms based on their molecular weights. As earlier mentioned there are different mechanisms, which have been reported to disrupt the bond between cysteine residue of the propeptide and the Zn-ion in the catalytic domain (Ra & Parks 2007). These mechanisms can involve, autoactivation (Suzuki et *al.* 1990), other soluble MMPs (Ogata et *al.* 1992), some membrane bound MMPs (Strongin et *al.* 1995), furin (Pei & Weiss 1995), plasmin (Devy et *al.* 1997), tumour associated trypsin-2 (Sorsa et *al.* 1997) or several serine proteinases such as kallikrein, elastase and cathepsin G (Saunders et *al.* 2005). In addition, some chemical activators have been demonstrated to activate proMMPs as well (Sorsa et *al.* 1997, Kähäri & Saarialho-Kere 1999). Of the synthetic activators, 4-

aminophenylmercuric acetate (APMA) is the most commonly used organomercurial activator of proMMPs in vitro. Most probably, the activation is based on the interaction of APMA with the cystein residue, which converts it to a nonbinding form (cysteine switch), thereby releasing the active site. MMPs can also be activated by some other factors such as reactive oxygen species (Weiss et *al.* 1985, Saari et *al.* 1990) or periobacterial proteases (Sorsa et *al.* 1992).

2.8.3 TIMPs (Natural inhibitors of MMPs)

MMP activity can be controlled by changes in the balance between the MMPs and their endogenous inhibitors, tissue inhibitors of matrix metalloproteinases. TIMPs are widely distributed in tissues and body fluids and comprise a family of four members: TIMP-1, TIMP-2, TIMP-3, TIMP-4 all sharing common structural features and are secreted by a variety of cell types (Sternlicht & Werb 2001). TIMP-1 and TIMP-2 are expressed in periodontal tissue and they are able to inhibit the activities of most MMPs (Gomez et *al.* 1997, Brew et *al.* 2000). The activation of proenzymes and the inhibition of the activation by TIMPs control the catalytic competence of MMPs. The action of TIMPs on MMPs includes prevention/delay of conversion of proMMPs to active forms and inhibition of catalytic active forms (Uitto et *al.* 2003). TIMPs inhibit the activity of various MMPs with different affinities. At the site of inflammation excessive tissue destruction occurs due to the imbalance between TIMPs and active MMPs (Ingman et *al.* 1996, Sorsa et *al.* 2004). In normal situations TIMPs are found only in tissues which are undergoing remodeling and breakdown (Woessner 1991). In periodontitis affected gingival tissues, TIMPs are expressed by fibroblasts, endothelial cells, mast cells, keratinocytes and macrophage-like cells (Naesse et *al.* 2003).

2.9 Cytokines

Although, bacteria in plaque are required to initiate the periodontal disease process, they are not necessarily responsible for the resultant actual loss of tissue. The indirect role of bacterial plaque products results in an excessive production of inflammatory mediators, such as, cytokines. The name "cytokine" is derived from the Greek ("cyto" - cell, and "kinos" – movement). Cytokines are soluble proteins, which act as messenger molecules transmitting signals to other cells. They have numerous actions, which include initiation and maintenance of immune and inflammatory responses and regulation of growth and differentiation of cells. They mediate cellular effects through interaction with their receptors on the cell membrane. They exert their effects in an intracrine, autocrine, juxtacrine, paracrine or endocrine manner by interacting with specific receptors. They are important for the development and functioning of the innate and adaptive

immune response. During inflammation, they are often secreted by immune cells as a response to a pathogen. So that the activation and recruitment of further immune cells are increased to respond to this pathogen (Gallin & Snyderman 1999, Balkwill 2000).

Cytokines can be classified as pro- or anti-inflammatory according to their role in the inflammatory process. Upregulation or/and downregulation of transcription factors and cytokine genes can result in the production of other cytokines, an increase of surface receptors for other molecules, proteinase production or inhibition of the response by feedback mechanism. During the inflammatory process most of the cytokines are produced by inflammatory cells such as, monocytes/macrophages, lymphocytes, neutrophils etc. However they are also produced by many resident cells such as fibroblasts, epithelial cells and endothelial cells (Scott et *al.* 1994, Julkunen et *al.* 2003).

Cytokines often divided into ten subgroups, lymphokines, interleukins, tumour necrosis factors, interferons, colony stimulating factors, polypeptide growth factors, transforming growth factors, α-chemokines, β-chemokines and stress proteins (Fresno et *al.* 1997). In this PhD thesis TNF-α, IL-1β, IL-17, IL-8 and IL-6 were used to stimulate inflammatory responses or studied for their eventual presence and localization in our samples.

2.9.1 IL-1β

Interleukin-1 (IL-1) is one of the first cytokines ever described. The original members of the IL-1 superfamily are IL-1α, IL-1β, and the IL-1 Receptor antagonist (IL-1RA). IL-1α and -β are pro-inflammatory cytokines involved in immune defense against infection. The IL-1RA is a molecule that competes for receptor binding with IL-1α and IL-1β, blocking their role in immune activation. IL-1α and IL-1β are produced as precursor peptides. In other words, they are made as a long protein that is then processed to release a shorter, active molecule, which is called the mature protein. Mature IL-1β, for instance, is released from Pro-IL-1β following cleavage by a certain member of the caspase family of proteins, called caspase-1 or the interleukin-1 converting enzyme (ICE) (Dinarello et *al.* 1994). Interleukin-1 receptor (IL-1R) is a cytokine receptor, which binds interleukin 1. Two forms of the receptor exist. The type I receptor is primarily responsible for transmitting the inflammatory effects of interleukin-1 (IL-1) while type II receptors may act as a suppressor of IL-1 activity by competing for IL-1 binding (Colotta et *al.* 1993).

IL-1β is one of the two forms of IL-1. It is a potent proinflammatory molecule and can contribute to osteoclast activation. It is released primarily by activated macrophages. In addition to that, other cell types such as lymphocytes, fibroblasts, kerotinocytes, endothelial cells and epithelial cells also release IL-1β (Newman et *al.* 1996). It is synthesized as inactive proIL-1β, which is then converted to active IL-1β in the presence of interleukin1-converting enzyme. IL-1β is a potent mediator to induce synthesis of cytokines, prostaglandins and MMPs (Dinarello 1997, Wassenaar et *al.* 1999). For example, some MMPs, such as MMP-8 are secreted by gingival fibroblasts after stimulation with IL-1β (Abe at *al.* 2001, Cox et *al.* 2006). Through its membrane-bound IL-RI and IL-RII, IL-1-induced responses are mediated (Boraschi et *al.* 1996).

2.9.2 TNF-α and its receptors p55 and p75

TNF- α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. TNF-α has strong pro-inflammatory and immunomodulatory effects. TNF-α induces production of cytokines, prostoglandins and MMPs (Papadakis & Targan 2000). Although monocytes/macrophages are the major sources of TNF-α, some other cells such as lymphocytes, keratinocytes, fibroblasts, epithelial cells and endothelial cells also produce TNF-α (Baud & Karin 2001). TNF-α depended responses are mediated by its two receptors, TNF-R1 (p55) and TNF-R2 (p75) (Vilcek & Lee 1991, Baud & Karin 2001). TNF-α has high affinity to TNF-R1 or TNF-R2, which leads to even that low concentrations of TNF-α are biologically effective (Vilcek & Lee 1991).

TNF-R1 and TNF-R2 are expressed in all nucleated cells. Upon TNF-α binding, a number of adaptor proteins are recruited to the cytoplasmic domains of p55 or p75, which starts complex intracellular events that might cause cell death or cell survival in a context-dependent fashion (Vandenabeele et *al.* 1995, Wallach et *al.* 1999).

Although receptor activator of nuclear factor kappa B ligand (RANKL) is the main stimulator for osteoclast formation, it is well established that these pro-inflammatory cytokines are related to tissue destruction, which involves stimulation of bone resorption and induction of tissue-degrading proteinases (Page 1991, Graves 1999). In addition, TNF-α and IL-1, together with RANKL and M-CSF are key regulators and stimulators of local bone-destruction in a paracrine manner (Konttinen et *al.* 1997). In vivo studies clearly support that IL-1 and TNF-α are key cytokines in the pathogenesis of periodontitis (Masada et *al.* 1990, Stashenko et *al.* 1991). In GCF samples of periodontally diseased patients, IL-1 and TNF-alpha are found in significant

concentrations. In the model of experimental periodontitis, application of IL-1 and TNF antagonists caused 60% reduction in bone loss (Assuma et *al.* 1998).

2.9.3 IL-17

Numerous immune regulatory functions have been reported for the IL-17 family of cytokines, presumably due to their induction of many immune signaling molecules. Lately, more attention has been shown to IL-17. T cells that preferentially produce interleukin-17, are named Th17 cells. Th17 cells produce a group of distinctive cytokines. Interleukin-17 (also called interleukin 17A), interleukin-17F, interleukin-22, and interleukin-21 -all of which participate in orchestrating a specific kind of inflammatory response (Harrington et *al.* 2005, Park et *al.* 2005). IL-17 forms a proinflammatory cytokine family, which has important roles in the inflammatory processes that lead to both autoimmunity and host defense (Yu & Gaffen 2008). IL-17 is a recently discovered cytokine, which is secreted by a limited set of cells (Lubberts & van den Berg 2002). Mostly it is a T-cell cytokine that produce many effects on different cell types such as fibroblasts, endothelial cells and epithelial cells to produce other inflammatory cytokines and chemokines (Rouvier et *al.* 1993, Yao et *al.* 1995). IL-17 can be considered as a potent inducer of TNF-α and IL-1β (Kotake et *al.* 1999), but also enhances inflammation and destruction independent of TNF-α and IL-1β (Koenders et *al.* 2005). IL-17 stimulates production of many cytokines such as TNF-α and IL-1β from macrophages (Jovanovic et *al.* 1998) and IL-6 and IL-8 from human fibroblasts (Yao et *al.* 1995, Fossiez et *al.* 1996). Studies with skin fibroblasts showed that IL-17 enhances the effect of IL-1β and TNF-α on the production of IL-6 and IL-8 (Katz et *al.* 2001). Although it was found in the supernatants of cellular cultures of gingival tissues both in healthy and periodontitis affected situations, cultures of periodontitis patients showed significant results (Vernal et *al.* 2005). Furthermore Takahashi and coworkers claimed that in periodontal lesions, release of IL-17 from T cells exacerbate inflammatory periodontal disease by activating fibroblasts to produce inflammatory mediators (Takahashi et *al.* 2005). Some studies in rheumatoid arthritis suggested IL-1β and TNF-α as major inducers for the cytokines IL-6 and IL-8, and that IL-17 exerts an additive and synergic effect to those two cytokines (Katz et *al.* 2001). However, studies with joint explants suggest that IL-17 is also able to provoke inflammatory responses by itself (Chabaud et *al.* 2001, Lubberts et *al.* 2001).

2.9.4 IL-6

IL-6 is one of the most important mediators of fever and of the acute phase response. For example in muscles, it stimulates energy mobilization that leads to increased body temperature. It acts as both a pro-inflammatory and anti-inflammatory cytokine, where the anti-inflammatory functions are achieved by suppression of IL-1 and TNF-α (Tilg et *al.* 1997). It is produced by different cells, including fibroblasts, activated T cells, activated monocytes or macrophages and endothelial cells, in response to specific microbial molecules. Because it is directly involved in the responses that occur after infection and injury, this may prove to be as important as IL-1 and TNF-α in the regulation of inflammation (Van Snick 1990). IL-6 induces B- and T- cell growth and differentiation, it can also mediate the effects of some other cytokines (Tamm 1989). In gingiva it has been shown that human gingival fibroblasts from a periodontitis affected sites produced higher amounts of IL-6 in vitro than the cells of healthy sites (Dongari-Bagtzoglou & Ebersole 1998). Furthermore, gingival crevicular fluid levels of IL-6 in periodontitis patients were higher than in the healthy controls (Bozkurt et *al.* 2000).

2.9.5 IL-8

Against the invading bacteria, neutrophils are the cells of the first line defense. It functions as a chemoattractant and is also a potent angiogenic factor. IL-8 is a strong chemotactic factor for neutrophils. Macrophages and endothelial cells secrete IL-8, which attracts neutrophils, so that neutrophils marginate and enter the tissue where they are needed especially during inflammation and infection (Baggiolini et *al.* 1994). It was also been shown that IL-17 induces production of IL-6 and IL-8 in rheumatoid synovial fibroblasts (Hwang et *al.* 2004).

2.10 Adhesion Molecules

One of the hallmarks of the early periodontal disease is the recruitment and adhesion of neutrophils, then monocytes and lymphocytes, to the site of endothelial damage (Schwartz et *al.* 1991). A number of surface molecules temporally mediate this process, but, especially in the early stage, the interaction of circulating leukocytes with the activated endothelial cells seems to be mediated by E- and P-selectins (Johnson et *al.* 1997) and, later, in a more stable fashion, by the intercellular adhesion molecule-1 (ICAM-1) and the vascular cell adhesion molecule-1 (VCAM-1) (Cybulsky et *al.* 2001).

Human VCAM-1 is a cell surface protein expressed by activated endothelial cells and certain leukocytes such as macrophages in response to inflammation (Elices et *al.* 1990). It mediates the

adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium. VCAM-1 expression is induced by various cytokines such as IL-1β and TNF-α (Iademarco et *al.* 1992). In immunological and inflammatory reactions, VCAM-1 is an important surface glycoprotein that promotes adhesion and subsequent recruitment of leukocytes (Mojcik & Shevach 1997). It has been reported that VCAM-1 is expressed in periodontally diseased tissue (Del Castillo et *al.* 1996).

2.11 Toll-like receptors (TLRs)

2.11.1 Definition and discovery

From an evolutionary standpoint, the innate immune response developed before the acquired immune response. The innate immune response depends on pattern recognition and is carried out by cells such as polymorphonuclear leukocytes and monocytes or macrophages. These cells have receptors called toll-like receptors, which can discriminate between classes of foreign molecules. Innate immune system possesses a complex system that senses invasion of microbial pathogens by toll-like receptors (TLRs) (Takeda & Akira 2005). TLRs recognize and distinguish highly conserved structures present in and shared by large number of different microorganisms.

In 1985, toll gene product was first discovered and described as being critical for the embryonic development of the fruit fly, Drosophila melanogaster (Anderson et *al.* 1985a,b). In addition, the Toll protein mediates host response to fungal Aspergillus niger infection in adult Drosophila, and binding of Asperillus-derived ligands to this receptor protein induces the release of antimicrobial proteins (Lemaitre et *al.* 1996). In 1991, the cytoplasmic domain of the Toll protein and interleukin 1-receptor were reported to be similar, which is consistent with their involvement in inflammatory responses (Gay & Keith 1991). This cytoplasmic domain is called the Toll-IL-1 receptor (TIR) domain (Medzhitov et *al.* 1997). Then the animal (human) equivalents to Toll were discovered and their cytoplasmic portions were shown to be similar to that of the IL-1 receptor family. Despite to their cytoplasmic similarity, the extracellular ligand binding portions of these molecules are structurally unrelated. The IL-1 receptors possess an immunoglobulin-like domain, whereas TLRs possess leucine-rich repeats (LRRs) in their extracellular domains (Poltorak et *al.* 1998).

Apart from inflammatory responses, activation of innate immunity is a crucial step also in antigen specific acquired (adaptive) immunity. The primary response to pathogens is mediated by

the innate immune system triggered by pattern recognition receptors (PRRs), which bind pathogen-associated molecular patterns (PAMPs) that are found in a broad range of organisms. To date, several classes of PRRs such as Toll-like receptors (TLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) have been identified. These PRRs recognize various PAMPs in various cell compartments and trigger the release of inflammatory cytokines and type I interferons for host defense (Kumar et *al.* 2009). TLRs form an important family of PRRs, which recognize, with a relatively brood range of selectively, a large number of varied and complex PAMPs (Arancibia et *al.* 2007) such as bacterial lipopolysaccharide, peptidoglycan, lipoproteins, bacterial DNA and double-stranded RNA. Therefore, as part of the innate immune system, TLRs sense invasion by microorganisms, such as bacteria, viruses, fungi and protozoa and trigger immune responses, which often lead to the removal of the triggering pathogens (Beutler 2004).

Several different human homologues for Drosophila Toll proteins have been identified in mammals; to date 11 different TLRs have been identified in humans (Zhang et *al.* 2004). The 11 known human TLRs respond to distinctive PAMPs that characterize microbial infections (Ozinsky et *al.* 2000, Hajjar et *al.* 2001). Recognition of microbial components by TLRs initiates signaling transduction pathways that induce expression of pro-inflammatory and antimicrobial genes. In addition TLRs stimulate expression of several costimulatory molecules, which provide the secondary activation stimulus to the immune responses leading to adaptive immune responses. Signaling pathways, activated by TLR ligands lead to activation of NF-kB and MAPK, cytokine gene transcription (eg. IL-6, IL-10 and IL-12), and co-stimulatory molecule expression (Akira et *al.* 2006, Steinman & Hemmi 2006).

2.11.2 Identification of TLR family

The first discovered mammalian TLR was TLR4. After that several proteins which were structurally related to Toll were identified and they are all named toll-like receptors, thirteen TLRs have been identified in mammals so far (Rock et *al.* 1998) but some of them are not functional in humans. For example, TLR10 is presumably functional in humans, whereas non-functional in animals. In contrast, mouse TLR11 is functional, but there is an early stop codon in the human TLR11 gene, which causes lack of production of functionally component human TLR11 (Zhang et *al.* 2004).

2.11.3 TLRs and signaling

PAMP components include combinations of nucleic acids, lipids, carbohydrates and proteins that represent molecular patterns, which are unique to pathogens. Those determinant molecules are essential to integrity, function or replication of microbes or viruses and therefore cannot be changed beyond recognition through mutation. More specifically, these well conserved features in pathogens include bacterial cell-surface lipopolysaccharides (LPS), lipoproteins, lipopeptides and lipoarabinomannan; proteins such as flagellin from bacterial flagella; double-or single stranded RNA of viruses or the unmethylated CpG islands of bacterial and viral DNA etc. (Hemmi et *al.* 2000, Hoebe et *al.* 2003) (see table 3 for a summary of TLR ligands.) TLRs are expressed on various immune cells, including macrophages, dendritic cells, B cells, specific type of T cells and even on nonimmune cells such as fibroblasts and epithelial cells. Expression of TLRs is rapidly modulated in response to pathogens, a variety of cytokines and environmental stresses. After TLR has been in contact to its ligand, the information is transmitted through the intracellular signalling pathways, which then activates innate immune. TLR mediated innate immune response is also crucial for the development responses of adaptive immune responses (Akira et *al.* 2006). TLRs can be organized into several subfamilies according to the PAMPs, which they are recognizing e.g. lipids (TLR1, TLR2, TLR6), nucleic acids (TLR7, TLR8 TLR9) etc. Due to the relatively broad ligand specificity, different types of PAMPs (lipopolysaccharide, heat-shock proteins etc.) can all be recognized by TLR4 (Table 3). It is also known that one set of TLRs is expressed intracellularly and another set extracellularly. It could be noted that TLRs on the cell surface seem to recognize surface components of microbes, whereas the intracellular TLRs recognize nucleic acids (Akira et *al.* 2006).

2.11.4 TLR subfamilies

TLR1, TLR2 and TLR6 recognize lipid and carbohydrates from gram-positive bacteria. TLR2 can combine with TLR1 or TLR6 and recognize triacyl lipopeptides and diacyl lipopeptides, respectively (Kumagai et *al.* 2008). This cooperation is one explanation of the wide spectrum of microbial components recognized by TLR2 (Takeda & Akira 2005). TLR4 is an essential receptor for the recognition of lipopolysaccharide (Hoshino et *al.* 1999). TLR5 is a receptor for bacterial flagellin (Hayashi et *al.* 2001). TLR3, TLR7, TLR8 and TLR9 recognize nucleic acids (Alexopoulou et *al.* 2001, Hemmi et *al.* 2002). TLR3, TLR7, TLR8 and TLR9 are localized in

Table 3. Summary of TLR Ligands (Kumar et *al.* 2009)**.**

Receptor	Location of TLR	Ligand	Effector cytokines induced
TLR1	Cell surface	Triacyl lipopeptides	Inflammatory cytokines (TNF-α, IL-6 etc.)
TLR2	Cell surface	Lipoprotein/lipopeptides Peptidoglycan/lipoteichoic acid Mycobacterial lipoarabinomannan Porphyromonas gingivalis LPS Zymosan Bacteroides fragilis lipopolysaccharide Capnocytophaga ochracea LPS Porphyromonas gingivalis fimbriae	Inflammatory cytokines (TNF-α, IL-6 etc.)
TLR3	Endosome	Double-stranded RNA Polyinosine-polycytidylic acid	Inflammatory cytokines (TNF-α, IL-6 etc.), type I INFs
TLR4	Cell surface	Escherichia coli lipopolysaccharide Porphyromonas gingivalis LPS Actinobacillus actinomycetemcomitans LPS Fusobacterium nucleatum LPS	Inflammatory cytokines (TNF-α, IL-6 etc.), type I INFs
TLR5	Cell surface	Flagellin	Inflammatory cytokines (TNF-α, IL-6 etc.)
TLR6	Cell surface	Peptidoglycan/lipoteichoic acid Diacyl lipopeptides Zymosan	Inflammatory cytokines (TNF-α, IL-6 etc.)
TLR7	Endosome	Imidazoquinoline	I Inflammatory cytokines (TNF-α, IL-6 etc.), type I INFs
TLR8	Endosome	Single-stranded RNA Imidazoquinoline	Inflammatory cytokines (TNF-α, IL-6 etc.), type I INFs
TLR9	Endosome	Bacterial DNA CpG oligodeoxynucleotide	Inflammatory cytokines (TNF-α, IL-6 etc.), type I INFs
TLR10		Not determined	
TLR11	Cell surface	Toxoplasma gondii	Inflammatory cytokines (TNF-α, IL-6 etc.)

cytoplasmic structures like lysosomes and endosomes (Barton et *al.* 2006). TLR3 recognizes double-stranded (ds)RNA, a form of genetic information carried by some viruses such as, rotavirus which is the commonest cause of gastroenteritis in young children (Alexopoulou et *al.* 2001). TLR7 and TLR8 are structurally highly conserved proteins and recognize single-stranded viral RNA (ssRNA). Takeda and Akira reported that although plenty of ssRNA is found in the host, it is usually not recognized by TLR7 and TLR8, because TLR7 and TLR8 are endosomal receptors and host-derived single-stranded RNA is not conveyed to endosomes (Heil et *al.* 2004). TLR9 recognizes DNA with an unmethylated CpG-motif (CpG-DNA). Unmethylated CpG is found in bacterial DNA abundantly, whereas mammalian DNA contains low frequency of unmethylated CpG motifs (Ishii & Akira 2006). Unmethylated CpG is a potent inducer of different pro-inflammatory cytokines and induce type-1 helper T responses in a TLR-9 dependent manner (Krug et *al.* 2001, Verthelyi et *al.* 2001). TLR10 ligand has not been identified yet (Mahanonda & Pichyangkul 2007) and TLR11 has been recognized as nonfunctional because of the presence of a premature stop codon in the human gene (Akira et *al.* 2006).

2.11.4.1 Gingival epithelial cell layer and TLRs

Gingival epithelial cell layer forms a barrier against periodontal pathogens separating them from the underlying periodontal tissues. Periodontal tissues are always exposed to a lot of large number of pathogenic and commensal bacteria. Although TLRs are mediators in inflammation initiated by periodontal pathogens, there is not always any clinically evident inflammation (Mahanonda & Pichyangkul 2007), which keeps the TLR signaling and control mechanism still remain the subject of intense investigation in periodontitis.

Gingival epithelial cells express TLR1 (Onishi et *al.* 2008), TLR2, TLR3, TLR4, TLR5, TLR6 (Kusumoto et *al.* 2004), TLR7 (Uehara et *al.* 2007), TLR9 (Kusumoto et *al.* 2004) and TLR10 (Kinane et *al.* 2006) and are therefore able to respond to hundreds of different microorganisms (Kinane et *al.* 2008).

Encounter of microbial structures detected by TLRs can transfer information into the cell and activate signal cascading leading to the production of proinflammatory cytokines such as TNF-α and IL-1β (Beutler et *al.* 2003). Such productive binding of TLR to its ligand starts the interaction between Toll-like receptor and cytoplasmic adaptor molecules. In most of the TLR cases, the signaling pathways are in particular mediated by the activation of NF-kB. However, cell curface TLR4 and intracellular TLRs (TLR3, TLR7, TLR8 and TLR9) can also activate the cells via IRF-3 or/and IRF-7 (Akira et *al.* 2006).

2.11.4.2 TLR2 in periodontal tissues and in epithelial cells

Gingival epithelium continuously exposed to large numbers of commensal and pathogenic bacteria. TLR2 is capable to recognize a wide range of such microbial components (Takeda et *al.* 2003), namely lipids and carbonhydrate compounds from Gram-positive bacterial cell walls (Takeuchi et *al.* 1999). Apart from the broad ligand reactivity of individual TLR2, this could also in part be explained by the physical and functional interaction of TLR2 with TLR1 and TLR6, which enables recognition of subtle differences in the lipid portion of lipoproteins (Alexopoulou et *al.* 2002). Studies have clarified that TLR1-TLR2 heterodimer recognizes triacylated lipoproteins (Takeuchi et *al.* 2002), whereas diacylated lipoproteins and lipoteichoic acid are recognized by TLR2-TLR6 heterodimer (Takeuchi et *al.* 2001). In addition to that, LPS preparation from Porphyromonas gingivalis was reported to be recognized by TLR2 (Werts et *al.* 2001), but an another study indicates that contaminated LPS preparation activates TLR2, not LPS by itself (Hashimoto et *al.* 2004). However, according to the study by Darveau and coworkers it is presented that LPS of *P. gingivalis* activates cells through either a TLR2 or TLR4 dependent way (Darveau et *al.* 2004).

Studies have demonstrated an association of TLR2 with periodontal diseases by showing their presence in gingival tissues. Significantly higher expression of TLR2 was observed in inflamed gingival tissue compared to healthy tissues (Sugawara et *al.* 2006). Immunostainings revealed prominent expression of TLR2 both in the gingival epithelium of tissue samples and gingival epithelial cells (Kusumoto et *al.* 2004). Upon stimulation with TLR2 ligands, TNF-α (Eskan et *al.* 2007) and IL-1β (Kinane et *al.* 2006) production by epithelial cell lines was stimulated.

Although expression of TLR2 is not uniform throughout the epithelium, clearly more prominent expression of TLR2 is found in the spinous layer than in the basal layer (Kusumoto et *al.* 2004). Moreover high number of TLR2 positive cells was observed in the connective tissue below the pocket epithelium (Mori et *al.* 2003).

In this thesis freeze dried heat-killed preparation of *Listeria monocytogenes* (HKLM) was used as an activator of TLR2. Studies showed that TLR2 can be activated by HKLM and induces rapid activation of NF-kB and strong response characterized by the secretion of proinflammatory cytokines (Flo et *al.* 2000).

2.11.4.3 Flagellin and TLR5 in periodontal tissues

Flagellum (plural=flagella) is a tail-like structure, which projects from the cell body of some prokaryotic and eukaryotic cells. It functions in the locomotion of the cell (Lefebre 2001, Bardy & Ng 2003). The flagellum is a Latin word and refers to a whip. Bacterial flagella is one out of three distinct types of flagella and is composed of the protein flagellin (Silverman & Simon 1974; Meister & Berg 1987). Flagellin is not only a constituent of flagella, but also a potent activator of the innate immune response (Yonekura et *al.* 2003).

TLR5 is responsible for binding PAMPs in flagellin (Hayashi et *al.* 2001). TLR5 is expressed by epithelial cells, monocytes and dendritic cells. Although many gram negative and gram positive bacteria express flagella, there are only few reports of such bacteria in the oral cavity where they are particularly found on spirochetes (Akira et *al.* 2006). The levels of oral spirochetes in the mouth are elevated in patients with periodontal diseases (Moore & Moore 1994). Among these spirochetes *Treponema denticola* a gram-negative oral bacterium, is associated with the incidence and severity of human periodontal disease. It is the most studied periodontopathic bacteria and is considered as one of the main etiological bacteria of periodontitis. The environment in which this bacterium thrives has shaped it into the motile and highly proteolytic bacterium that we know today (Jobin et *al.* 2007). Although the role of TLR5 in the pathogenesis of periodontitis remains to be determined, the increased expression of TLR5 reflects a potentially high reactivity against spirochetes in periodontitis (Kajita et *al.* 2007). One study reported the down-regulation of TLR5 expression (Muthukuru et *al.* 2005) in periodontitis but another one reported high expression for TLR5 compared to TLR2 and TLR7 with no difference between periodontitis and gingivitis (Kajita et *al.* 2007).

2.11.5 TLR regulation mechanisms / negative regulators

TLR activation is a double-edged sword. It is important to raise an innate immune response and to provide the necessary assistance to the adaptive immunity against pathogens. However, due to the generation of their role in inflammatory responses, they are also involved in the pathogenesis of infectious and chronic inflammatory diseases (Cook et *al.* 2004). Therefore, TLR signaling and functions should after the rapid initial inflammatory response and upon development of the more specific and therefore less destructive adaptive immune response be under tight negative regulation. Most probably disease could result either from overaction of TLRs or dysregulation of endogenous TLR-signalling inhibitory processes (Liew et *al.* 2005).

This negative regulation occurs at multiple levels. Some regulatory mechanisms inhibit certain TLRs, whereas others inhibit common components of the TLR signaling, which shut down the effect of all TLR family (Liew et *al.* 2005). So far, five different negative regulation mechanisms have been identified as; soluble decoy TLRs, intracellular negative regulators, transmembrane protein regulators, reduction of TLR expression and regulation of TLR effect by apoptosis. *Soluble decoy TLRs;* Soluble forms of TLRs act as the first defense line to block the TLR signaling. Because both bound and soluble TLRs bind microbial ligands, soluble TLR can compete for microbial ligands, and prevent its interaction with the cell membrane bound signalling TLR and block TLR signaling (Liew et *al.* 2005) (Figure 5). *Intracellular negative regulators;* After TLR-ligand interaction has occurred, intracellular regulators can inhibit TLR signaling pathways and controls TLR signaling. There are many TLR signaling pathways and these seem to be relatively tightly regulated by endogenous regulators at multiple levels (Liew et *al.* 2005). *Reduction of TLR expression;* TLR signaling can also be controlled by reducing the expression of TLRs. This mechanism can work via degradation of already synthesized TLRs by ubiquitylation or by inhibition of TLR gene transcription by anti-inflammatory cytokines (Liew et *al.* 2005). Ubiquitylation is a general mechanism for the regulation of cell-membrane receptor functions by promoting proteolytic degradation of already synthesized proteins (Pickart 2001). Anti-inflammatory cytokines, especially transforming growth factor-β and IL-10, downregulate expression and function of TLRs. For example, TGF-β1 inhibits TLR4 expression via transcription only –or also via other mechanisms (McCartney-Francis et *al.* 2004). In addition to that, TGF-β1 induces MyD88 degradation and down regulates TLR induced signaling (Naiki et *al.* 2005). Similarly, IL-10 blocks the induction of pro-inflammatory cytokine production induced by LPS or other pro-inflammatory cytokines (Muzio et *al.* 2000). *Regulation of TLR effect by apoptosis;* TLRs can function as death receptors, which might be important in the control of dysregulated TLR responses (Aliprantis et *al.* 1999). Although it still remains to be determined how a balanced regulation or even non-responsiveness against commensal microbes of TLR signaling is achieved, it has been shown that for example; TLR2 not only induces NF-kB activation, but also trigger apoptosis using the same pathway. Such downregulation of responsiveness might prevent anti-microbial inflammatory responses against bacteria in the normal flora in the first place and tissue destruction caused by excessive inflammatory reactions against pathogenic microbes in the second place (Hsu et *al.* 2004).

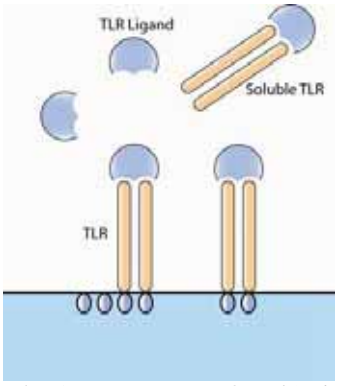

Figure 5. A schematic picture of soluble decoy TLRs (Adapted by the permission from: Liew et *al.* 2005)

3. AIMS OF THE STUDY

1. To investigate the expression and localization of TNF-α and its receptors TNF-R1 and TNF-R2 in pericoronitis affected human gingival tissues.

2. To investigate the levels, molecular forms, expression and activation degree of MMP-3, MMP-8 and MMP-9 in periodontitis affected human GCF, tissues and gingival fibroblasts.

3. To investigate the expression of the cytokines TNF-α, IL-1β and IL-17 in periodontitis affected human tissues and their effect on the expression of proMMP-1 and MMP-3 from gingival fibroblasts; to study IL-17-induced synthesis of IL-6 and IL-8 in gingival fibroblasts and of TNF-α, IL-1β in monocyte/macrophages.

4. To investigate the expression and distribution of TLRs (TLR-1 to TLR-10) in periodontitis affected gingival epithelium and connective tissue.

5. To investigate the induction of proinflammatory cytokines TNF-α, IL-1β in human gingival epithelial cells upon stimulation with TLR2 or TLR5 ligands alone or in combination with IL-17.

4. MATERIAL AND METHODS

4.1 IgGs and reagents

The antibodies, which have been used in this study, are presented in table 4. The reagents used are mentioned in the original articles.

4.2 Controls and patients

From all subjects medical and dental histories were taken. All subjects were 1) healthy, 2) non-smokers, 3) had at least 20 teeth in their mouth, 4) no history of systemic disease, 5) had received no antibiotics or other medication or periodontal treatment within the previous six months. Individuals with a medical condition requiring pre-medication prior to dental procedures or currently using nonsteroidal anti-inflammatory drugs daily or pregnant women were excluded. Ethics Committee of the Institute of Dentistry, University of Helsinki, Helsinki, Finland and the Ethics Committee of the Faculty of Dentistry, Gazi University, Ankara, Turkey approved the study. Participation was voluntary. The purpose of the study was explained to patients and informed consent was obtained from all subjects before collecting data for study.

4.2.1 Control subjects (I-V)

The control samples were collected from periodontally healthy subjects with no systemic diseases and no clinical or radiological signs of periodontal disease. The control groups consisted of n=10 (study I; age, 19-26), n=5 (study II; age, 19-34) and n=10 (study III, IV, V; age, 20-37). Subjects had gingival index of 0 or 1 and probing depth $\leq$ 2 mm. The control group mainly consisted from the dental students who registered into the patient pool of the dental clinics at the Institute of Dentistry, University of Helsinki.

4.2.2 Pericoronitis patients (I)

University students (age, 20-30), n=10 participated to the study. Orthopanthomography showed partially erupted third molars. Students did not suffer from subjective symptoms, but the dentists recognized the pericoronitis. Patients had only redness and swelling as signs of clinical inflammation. Patients of this study were selected from the patients of dental clinics at the Institute of Dentistry, University of Helsinki, Finland.

4.2.3 Chronic (adult) periodontitis patients (II-V)

The clinical diagnosis of periodontitis patients was done based on Gingival Index (≥2) (Löe 1967), Plaque Index (≥2) (Silness & Löe 1964) and Probing Pocket Depths (>4mm) and clinical attachment loss combined with radiologic bone loss. Patients of this study were selected from a group of registered patients for dental treatment at the Institute of Dentistry, University of Helsinki, Finland and University of Gazi, Turkey. In study II seven patients from the dental Faculties of University of Gazi (n=3) or University of Helsinki (n=4) were selected for the study (45-49 years old). Patients suffered from periodontitis and contacted to faculty complaining about their dental problems. Otherwise, the patients were healthy. In studies III, IV, V the number of subjects was increased for these studies. Ten chronic periodontitis patients (31-45 years old) were included into these studies.

4.3 Sample collection

4.3.1 GCF samples

In order to remove supragingival plaque, the crown of tooth was gently cleaned, dried and isolated with cotton rolls not to contaminate the GCF sample with saliva. Filter paper strips were placed to the gingival margin, 1 mm into the sulcus and kept there for 3 minutes avoiding any bleeding and moved into test tubes containing buffer solution. Then the samples were frozen and kept at -20 °C until they were analyzed.

4.3.2 Gingival tissue samples

Gingival tissue samples were collected from patients during routine flap surgery, performed after the initial phase of periodontal surgery comprising conventional scaling and root planning. Tissue samples were collected from the sulcular epithelium and the bottom of the pocket by a surgical incision longitudinally. The collected samples were either snap frozen and/or processed for paraffin blocks using routine methods. Frozen samples were stored at -70˚C. The samples, which were planned to be used for explant cultures, were immediately placed in Eppendorf tubes and transported to the laboratory at +4°C for further processing.

4.4 Immunohistochemical methods (I, II, III, IV, V)

Frozen gingival sections (I) were cut into 6 μm thick cryostat sections and were mounted on glass slides and fixed in cold acetone at +4°C for 5 minutes, whereas the paraffin sections (II, III, IV,V) were deparaffinized and rehydrated. The sections were washed with TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4). Then they were incubated in 0.3% H_2O_2 in methanol at room temperature (30 min) to inhibit endogenous peroxidase. Vectastain® Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used for staining.

The steps can be summarized as follows; Sections were incubated in normal horse serum (diluted 1:50 in PBS containing 0.1% bovine serum albumin) at room temperature for 20 minutes. Excess serum was removed and the sections were treated at room temperature with: a) primary antibodies (diluted in PBS with 0.1% BSA overnight at +4°C) b) biotinylated anti-IgG against the species of the primary IgG antibody (dilution 1:100, Vector) at +22°C for 30 minutes c) avidin-biotin-peroxidase complex (1:100 in PBS; Vector Labs) d) 3,3'- 46 diaminobenzidine tetrahydrochloride (Sigma) in 0.006% H_2O_2 at +22°C for few minutes in darkness. Between each steps, the sections were washed for 3x5 minutes in PBS and counterstained with hematoxylin. Sections were dehydrated in a graded ethanol series, cleared in xylene and mounted in synthetic mounting medium (Diatex, Becker Industrifärg AB, Märsta, Sweden). As a negative staining control, normal IgG of the same subtype but with irrelevant specificity was used instead of and at the same concentration as the primary antibodies to confirm the specificity of the immunohistochemical staining results. The primary antibodies used are presented in table 4.

In study III, after the slides had been fixed and washed, they were installed in DAKO TechMate™ Horizon immunostainer and stained automatically at +22°C as follows: 1) primary antibody which was diluted in DAKO ChemMate™ antibody diluent, for 25 minutes, 2) biotinylated secondary anti-IgG antibody for 25 minutes, 3) peroxidase block for 25 minutes, 4) peroxidase-conjugated streptavidine, 3 times for 3 minutes, 5) horseradish peroxidase (HRP) substrate buffer, and finally 6) substrate working solution containing 3,3'-diaminobenzidine tetrahydrochloride (ChemMate™ detection kit), for 5 minutes. Between steps, the sections were washed with DAKO ChemMate™ washing buffer three times and dried in absorbent pads. Following the staining, the sections were removed from the machine, counterstained with hematoxylin, washed, dehydrated in ethanol series, cleared in xylene and mounted in mounting media.

Table 4. The table is listing all the antibodies used in this thesis.

IgG	Study	Application	Source
Rabbit polyclonal anti-human TNF-α	I, III	IH	Monosan
Mouse monoclonal anti-human TNF-R1	I	IH	Santa Cruz
Mouse monoclonal anti-human TNF-R2	I	IH	Santa Cruz
Rabbit polyclonal anti human IL-1β	I,III	IH	Santa Cruz
Mouse monoclonal anti-human VCAM-1	I	IH	DAKO
Goat polyclonal anti human IL-17	III	IH	R&D
Mouse polyclonal anti-human MMP-1	III	IH	Chemicon
Sheep polyclonal anti-human MMP-3	II	IH, WB	Oncogene
Goat polyclonal anti human MMP3	III	IH	Santa Cruz
Rabbit polyclonal anti human MMP-8	II	WB	Gift by Dr.Michaelis
Rabbit polyclonal anti human MMP-9	II	WB	Gift by Dr.Michaelis
Rabbit polyclonal anti human TLR-1	IV	IH	Santa Cruz
Rabbit polyclonal anti human TLR-2	IV,V	IH	Santa Cruz
Rabbit polyclonal anti human TLR-3	IV	IH	Santa Cruz
Rabbit polyclonal anti human TLR-4	IV	IH	Santa Cruz
Rabbit polyclonal anti human TLR-5	IV,V	IH	Santa Cruz
Goat polyclonal anti human TLR-6	IV	IH	Santa Cruz
Goat polyclonal anti human TLR-7	IV	IH	Santa Cruz
Rabbit polyclonal anti human TLR-8	IV	IH	Santa Cruz
Rabbit polyclonal anti human TLR-9	IV	IH	Santa Cruz
Rabbit polyclonal anti human TLR-10	IV	IH	Santa Cruz

IH: Immunohistochemistry, WB: Western blotting

4.4.1 Microscopic evolution of the samples

Sections were analyzed under an Olympus BX 61 microscope (Olympus, Hachioji-shi, Tokyo, Japan) coupled to an Olympus DP 50 Microscope digital camera (Olympus, Hachioji-shi, Tokyo). Image analysis software (microscopy.info, Harvard, Boston, MA, USA) was used for morphometric evaluation. In each study randomly selected areas at a region of interest (ROI) were analyzed at 200X (I, IV, V) or 400x (I, II) magnification. In study number I, TNF-α/IL-1β/VCAM-1 positive cells in the connective tissue are expressed as percentage of all cells. In study number II, the number of MMP-3 positive cells per fields in the connective tissue was calculated. In study number III, the numbers of MMP-1 and MMP-3 immunoreactive cells were expressed in mm^2 in the connective tissue. In study number IV, percentages of positively stained cells were analyzed separately in the connective tissue and epithelium layers for TLR1-9. In study number V, total number of TLR2 and TLR5 immunoreactive cells was analyzed in epithelium.

The way of data presenting for region of interest has been changed between the studies as percentage or as number of cells at the region. Mainly the idea for presenting the data has been designed to make it as simple as for readers. In the publications of this PhD thesis, the method for presenting the data has been modified as recommended by the reviewers' of the original articles.

4.5 Cell cultures (I,II,III,V)

4.5.1 Cell and explants cultures

In study I fresh healthy tissue samples were weighed and cut into small pieces (approximately 1 mm^3 each) and 6 to 7 explants per well were placed into 96-well plates. Tissues were incubated in RPMI-1640 medium containing 10% fetal calf serum and antibiotics at +37°C in 5% CO_2, either without or with TNF-α (10 ng/mL, R&D Systems Inc., Minneapolis, MN, USA) for 48 hours. Inflamed tissues were similarly prepared and treated without or with TNF-α blocker (Infliximab 10 μg/mL, Centocor, Horsham, PA, USA).

In study II confluent gingival fibroblasts from the 4-7^{th} passage were transferred to serum-free RPMI-1640 for 24 hours before culturing without or with TNF-α (10 ng/mL, R&D Systems Inc., Minneapolis, MN, USA) in serum-free media for 48 hours. Control and conditioned media were collected.

In study III confluent fibroblasts were stimulated for 48 hours with 10 ng/mL IL-1β, TNF-α, or IL-17 (R&D Systems Inc., Minneapolis, MN, USA). Human monocytes were stimulated for 48 hours with 10 ng/mL IL-17 (R&D Systems). Culture supernatants were collected and stored at -70°C.

In study V the confluent cells were reseeded into six well plates before culturing with or without various stimulants. Cells were incubated in the presence of HKLM-TLR2 agonist (10^8cells/ml, InvivoGen, SanDiego, CA), HKLM-TLR2 agonist plus IL-17 as the costimulant (IL-17, 10 ng/ml, R&D SystemsInc., Minneapolis, MN), *Salmonella typhimurium* Flagellin-TLR5 agonist (100ng/ml, InvivoGen) or *Salmonella typhimurium* Flagellin-TLR5 agonist plus IL-17.

4.5.2 Gingival fibroblasts (I, II, III)

Human gingival fibroblasts were isolated by an explant culture technique. The cells were maintained in RPMI-1640 containing 10% fetal calf serum containing 2.3mM glutamine, 117.2

g/mL streptomycin and 117.2 IU/mL penicillin in a humidified 5% CO_2 in air at +37°C. The cells were passaged by a brief incubation with cell-culture-grade trypsin-EDTA (0.5% trypsin and 0.2% EDTA; Gibco-BRL Life Technologies, Gaithersburg, MD). Confluent cells from the 4-7th passage were transferred to serum-free RPMI-1640 before being treated with proper cytokines. Control and conditioned media were collected and kept at -70˚C until used. The samples were used for activity assay, Western blot, zymography and ELISA.

4.5.3 Gingival epithelial cells (V)

An epithelial cell line was a generous gift from Professor Ismo Virtanen, University of Helsinki, Department of Anatomy, Finland. The cells were maintained in keratinocyte growth medium 2 (PromoCell, C20211, Heidelberg, Germany) containing the supplement provided by the manufacturer in a humidified 5% CO_2 in air at 37°C. The cells were passaged by a brief incubation with cell-culture-grade trypsin-EDTA (0.5% trypsin and 0.2% EDTA; Gibco-BRL Life Technologies, Gaithersburg, MD). The cells were grown to confluence in 10 cm diameter tissue culture plates with a change of medium every 4–5 days. Thereafter, the confluent cells were reseeded into six-well plates before culturing with or without various stimulants. Control and conditioned media were collected and kept at -70˚C until use in ELISA.

4.5.4 Monocytes (III)

Human monocytes from healthy persons were isolated from buffy coats by means of Ficoll-Paque density gradient (Pharmacia Biotech, Uppsala, Sweden). Non-adherent cells were removed by repeat PBS (10 mM sodium phosphate buffered, 0.15M NaCl, pH 7.5) washes and adherent cells were used for the experiments.

4.6 Western immunoblotting (II)

Molecular weight of MMP-3, -8, and -9 from GCF samples were analyzed by Western blotting. Before electrophoresis, GCF samples were boiled for 5 minutes in sodium dodecyl sulphate (SDS) gel loading buffer. Electrophoresis was performed in 10 % polyacrylamide gel. Following electrophoresis, the gels were blotted onto nitrocellulose membrane (Bio-Rad Laboratories). The nitrocellulose membrane was blocked overnight using 3 % BSA in TBS. The next day the membrane was washed in a washing buffer containing 0.1 % Tween 20, 50 mM Tris-HCl, 0.5 M NaCl, pH 7.5. Following the washes, the membranes were incubated for one hour in sheep anti-human MMP-3 IgG (2 µg/mL Calbiochem, Oncogene Research Products, San Diego, CA,

USA), rabbit anti-human MMP-8 or MMP-9 IgG (at 1:500 and 1:1000 dilutions, provided by Dr. Jurgen Michaelis, Department of Pathology, Christchurch Medical School, New Zealand, Michaelis et *al.* 1990, and Dr. Lars Kjeldsen, Granulocyte Research Laboratory, University Hospital Copenhagen, Kjeldsen et *al.* 1993, respectively) in washing buffer containing 2 % BSA. This was followed with washes and incubation for one hour with the appropriate alkaline phosphatase-conjugated anti-IgG secondary antibodies at +22°C. The membranes were washed with washing buffer for 30 minutes before alkaline phosphatase-binding sites were revealed in color development solution (Alkaline Phosphatase Conjugate Substrate Kit, Bio-Rad Laboratories) containing a mixture of 5-bromo-4- chloro-3-indolyl phosphate and nitroblue tetrazolium chromogen for 30 minutes. After the reaction was stopped evaluation was performed using a Bio-Rad Model GS-700 Imaging Densitometerand Molecular Analyst/PC program (Bio-Rad, Hercules, CA, USA).

4.7 Enzyme-linked immunosorbent assay (ELISA) (III,V)

Levels of proMMP-1 and total (pro- and active) MMP-3, MMP-8, MMP-9, IL-6 and IL-8 in gingival fibroblast cultures, IL-1β and TNF-α in monocyte/macrophage cultures, IL-1β and TNF-α in gingival epithelial cell culture supernatants were measured using ELISA kits as recommended by the manufacturer. The ELISA kits were Quatikine® (R & D Systems Inc, Minneapolis, MN, USA). Principle of the assay can be summarized as follows: Quantitative sandwich enzyme immunoassay technique, in which a specific capture antibody has been pre-coated onto a microplate. During the experiment, the standards provided and samples were pipetted into the wells so that any MMP or cytokine present was bound by the immobilized antibody. After washing away any unbound sample molecules, an enzyme-linked antibody specific for the MMP or cytokine in question was added to the wells. Following a wash to remove any unbound detection antibody, a colour developing solution was added to the wells so that colour developed in proportion to the amount of total MMP or cytokine bound in the initial step. The colour development was stopped and the intensity of the color was measured.

4.8 Functional assays

4.8.1 Assay of gelatinases by zymography (I,II)

MMP-9 in gingival tissue supernatant and GCF samples were analyzed for gelatinolytic activities by the use of 7.5 % and 11 % SDS-polyacrylamide gels containing 1 mg/mL gelatin substrate. As molecular weight markers, the standard lane was loaded with low range prestained SDS-PAGE

standards (Bio-Rad, Richmond, CA, USA). Following electrophoresis, the gels were washed for 30 minutes in 50 mmol Tris-HCl, 2.5% Tween 80 and 0.02% (v/v) NaN_3, pH 7.5, then for 30 minutes with the same buffer supplemented with 1 mmol/L $ZnCl_2$ and 5 mmol/L $CaCl_2$, 0.02% NaN_3, pH 7.5, at 37°C. Then the gels were incubated in 50 mM Tris-HCl, 5 mM $CaCl_2$, 1μM $ZnCl_2$, 0.02% NaN_3, pH 7.5, at 37°C for 24 hours. Reactions were stopped by staining with Coomassie Brilliant Blue R250. The gelatinolytic activities were seen as white bands against the blue gelatin background.

4.8.2 Activity assay (II)

MMP-3, MMP-8 and MMP-9 activities were measured by a modified pro-urokinase substrate method (Biotrak activity assay systems, Amersham Biosciences, Buckinghamshire, England) (Hanemaaijer et *al.*1998). Briefly, the 96-well plates were coated with antibodies developed by Hanemaaijer and coworkers (Hanemaaijer et *al.* 1998) for 16 hours at +22°C. The wells were washed three times with PBS with 0.05% Tween-20 and incubated with assay buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl2, 1 μM ZnCl2, 0.01% Brij-35, pH 7.6). 15 μL 50 μg/mL modified pro-urokinase containing a MMP-specific activation site and 10 μL 6 mM chromogenic urokinase substrate pyro-Glu-Gly-Arg-*p*-nitroanilide (Chromogenix, Mölndal, Sweden) were added. The color was measured in a photometer (Flow Laboratories, Irvine, Scotland). The results are expressed as U/mL (Verheijen et *al.* 1997; Hanemaaijer et *al.* 1998). In order to activate proforms, samples were incubated with 0.5 mM 4-aminophenylmercuric acetate (APMA) (Sigma) for 2 hours at +37°C and total MMP activity was measured (Verheijen et *al.* 1997).

4.9 Statistical analysis

Statistical calculations were performed using the Prism data analysis program (GraphPad Software Inc., San Diego, CA, USA). In study I, IV, V differences between healthy and diseased groups were tested using Mann-Whitney test. In study II one-way analysis of variance (ANOVA), followed by Bonferroni's multiple-comparison test was used to compare stimulated and non-stimulated fibroblasts. The Mann-Whitney U Test was used to analyze the differences between the adult periodontitis patients and controls. Pearson's correlation test was used to assess the correlations among MMP-3, -8, -9, and their different molecular forms. Correlation between plaque index and pro/active forms of MMP-3 was calculated by the Pearson correlation test. For other studies Student's t-test (III) and ANOVA with Bonferroni's post tests for multiple comparisons (III, IV, V) were used to compare the groups. Significance was set at the $p<0.05$ level.

5. RESULTS

5.1 Immunolocalization of cytokines, MMPs and TLRs in tissues

5.1.1 Immunolocalization of TNF-α and its receptors TNF-R1 and TNF-R2 in pericoronal tissue (I)

In pericoronitis, positively stained cells were more frequent than in controls. In all samples TNF-α staining was found. In general the staining of all cells was obvious, the cellular staining pattern was cytoplasmic and diffuse. In pericoronitis, TNF-α was found in fibroblast- and macrophage-like cells, vascular endothelial cells, and basal and suprabasal epithelial cells. In healthy controls, TNF-α staining was weak, and most of the TNF-α-positive cells were stromal fibroblast-like cells or vascular endothelial cells. The percentage of TNF-α positive cells was clearly increased in pericoronitis samples 62 % (range, 45-72) *vs.* 34 % (range, 10-51), p = 0.0317. TNF-R1 and TNF-R2 positive cells were found in all pericoronitis samples. Additionally, TNF-R1 was also positive in healthy samples, whereas TNF-R2 staining was very weak or absent in healthy controls.

In pericoronitis, TNF-R1 staining was observed in monocyte/macrophage- and fibroblast-like cells, vascular endothelial and basal epithelial cells. TNF-R2 was found in fibroblast- and macrophage-like cells, vascular endothelial cells, and basal epithelial cells. In contrast, in healthy controls, TNF-R1 staining was weak and found mainly in some vascular endothelial cells, stromal fibroblast-like and macrophage-like cells, and basal epithelial cells. TNF-R2 stained only very weakly in a few stromal fibroblast- and macrophage-like cells, vascular endothelial cells, and basal epithelial cells (Figure 6).

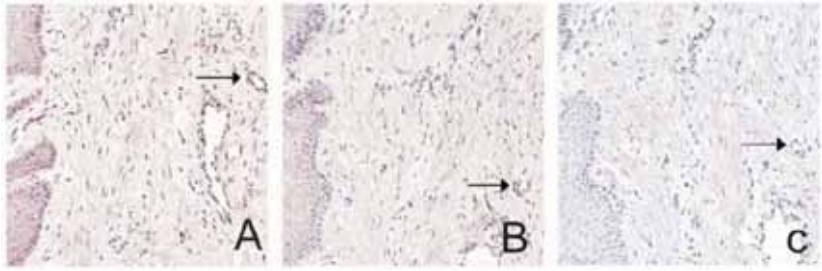

Figure 6. Immunohistochemical staining of TNF- α (A) and its receptors TNF-R1(B) and TNF-R2 (C) in pericoronitis.

5.1.2 Immunolocalization of IL-β and VCAM-1 in pericoronal tissue (I) and IL-1β, TNF-α and IL-17 in gingival tissue (III)

IL-1β and VCAM-1 positive cells were increased in pericoronitis compared to control samples (60 % *vs.* 25 % and 22 % *vs.* 2.4 %, respectively). IL-1β was found in macrophage- and fibroblast-like cells, vascular endothelial cells, and epithelial cells, whereas VCAM-1-positive cells were mainly endothelial cells. In healthy samples, IL-1β staining was weaker and located mostly in vascular endothelium and the number of VCAM-1 stained cells was nearly absent.

In gingival tissues the overall intensity of IL-1β, TNF-α, and IL-17 immunoreactivities were stronger in periodontitis than in the controls. Total number of IL-17 was much lower compared to those of IL-1β and TNF-α positive cells, but a clear increases were revealed for periodontitis versus healthy samples as follows; IL-1β (1726 ± 81 *vs.* 344 ± 20), TNF-α (1594 ± 147 *vs.* 411 ± 17), IL-17 (162 ± 21 *vs.* 26 ± 2) ($p < 0.001$). IL-1β and TNF-α were localized predominantly in macrophage-like cells, fibroblasts-like cells, and epithelial and endothelial cells (Figure 7).

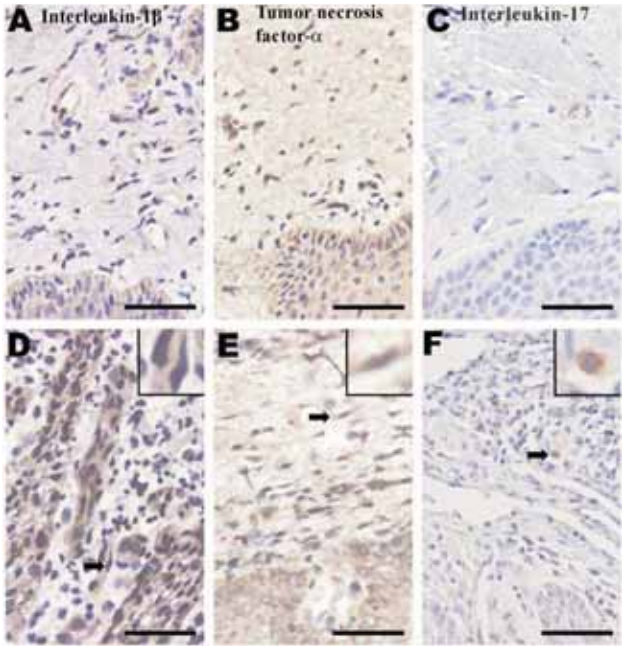

Figure 7. Immunolocalization of cytokines in gingival tissue. In healthy control samples, IL-1β(A), TNF-α(B), and IL-17 (C) immunoreactive cells were less frequent compared with cells in persons with periodontitis (D,E,F).

5.1.3 Immunolocalization of MMP-3 (II, III) and MMP-1 (III)

The number of MMP-1(III) and MMP-3 (II, III) positive cells and their intensity of staining were high in adult periodontitis compared with healthy control gingiva. The immunoreactivity of MMP-1 and MMP-3 was predominantly observed in fibroblast- and macrophage-like cells in periodontitis. Approximately 3-fold MMP-1 and 3-4-fold MMP-3 increases in immunoreactive cells were detected in periodontitis compared to controls.

5.1.4 Immunolocalization of Toll-like receptors in gingival tissues (IV, V TLR2/TLR5)

5.1.4.1 Histological analysis of TLRs in epithelial tissue

All TLRs except TLR-10 were found in the gingival epithelia (The mean percentages of TLR-positive cells are shown in the first figure of article IV). The expression varies in different layers of epithelium for each TLR, but 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. On the contrary, the healthy controls showed more variable results in different epithelial layers for all TLRs.

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 of TLR-positive cells in the spinous cell layer varied widely between the healthy and diseased epithelia. The mean percentages of TLR-1, TLR-4, TLR-5 and TLR-8 positive epithelial cells were higher in the healthy tissues, whereas TLR-2, TLR-3, TLR-6, TLR-7 and TLR-9 had higher frequencies in periodontitis.

In the superficial layer, the number of TLR-1-positive and TLR-5-positive epithelial cells was lower in periodontitis than in healthy controls. In contrast, cells expressing TLR-3 and TLR-9 showed significantly higher frequency in the superficial cell layer in periodontitis. In addition to these, TLR-2, TLR-4, TLR-6, and TLR-7 showed similar frequencies between periodontitis samples and healthy controls.

5.1.4.2 Histological analysis of TLRs in connective tissue

The tissue samples of both patients with periodontitis and healthy controls expressed all TLRs with the exception of TLR-10. Positively stained cells were clearly much more frequent in periodontitis samples than in healthy control samples ($P < 0.001$). Although macrophage- and fibroblast-like cells and endothelial cells were detected in both study group samples, in the healthy group macrophages were the most frequent positively stained cells (IV) (Figure 8).

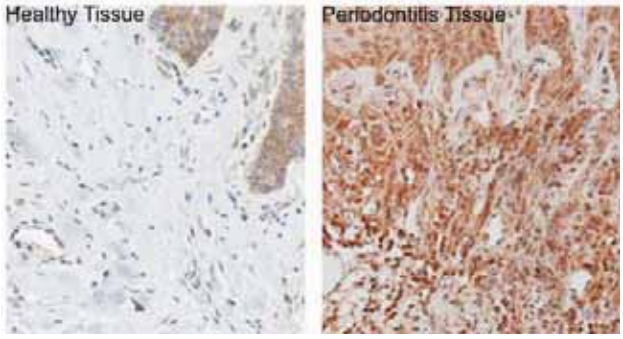

Figure 8. Representative picture of Toll-like receptors (TLR-2) staining in the connective tissue of healthy and periodontitis samples.

5.2 Activities, levels and presence of cytokines, MMPs and TLRs

5.2.1 Gelatin zymography in tissue supernatants for MMP-9 (I), zymography in GCF samples of periodontitis patients for MMP-9 (II) and zymography of fibroblast supernatants (II)

Gelatin zymography of TNF-α (10 ng/mL) stimulated healthy tissue supernatants disclosed two- to five-fold higher proMMP-9 and active MMP-9 expression than the non-stimulated samples. On the contrary, TNF-α blocker down-regulated proMMP-9 and active MMP-9 expression in the supernatants collected from inflamed tissue explant cultures.

Similarly, zymographic analysis of GCF samples of periodontitis patients revealed an elevated tendency for total gelatinolytic activity compared to the GCF samples of healthy samples. Furthermore, correlation between pro- and active molecular forms of MMP-9 was confirmed by this method ($r = 0.950$, $p = 0.013$). In addition to that, zymography of fibroblast supernatants confirmed that all MMP-9 was in the 82-kDa active form.

5.2.2 Western blot for MMP-3, MMP-8, MMP-9 in GCF samples (II)

Western blots revealed that particularly MMP-3 was elevated in periodontitis samples compared to MMP-8 and MMP-9. The ratio of the proMMP to active MMP was 0.5 ± 0.1 for MMP-3, 1.2 ± 0.1 for MMP-8, and 3.3 ± 0.7 for MMP-9 in adult periodontitis (mean ± SEM). However, healthy control GCF samples produced only 55 kDa proMMP- 3 (not any 45 kDa active MMP-3). Latent proMMP-8 and 60 kDa (active) MMP-8 species were observed in periodontitis. Similar to MMP-3, in controls not any active MMP8 was detected. In GCF samples, MMP-9 was also detected in proteolytically degraded low kDa fragments in periodontitis, but not in controls (Figure 9).

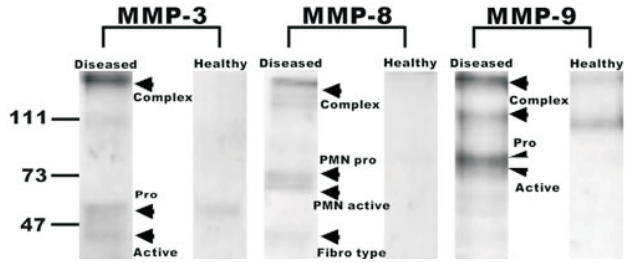

Figure 9. Characterization of various molecular forms of MMP-3, -8, -9 in the gingival crevicular fluid samples of adult periodontitis patients and healthy control samples.

5.2.2.1 Correlation between biochemical parameters (II)

Immunoreactivities showed significant association between pro- and active forms of MMP-3 ($r = 0.957$, $p = 0.0007$), MMP-8 ($r = 0.975$, $p = 0.0002$), and MMP-9 ($r = 0.895$, $p = 0.04$) in adult periodontitis. Furthermore, immunoreactive bands corresponding to the active MMP-3 had a strong positive correlation with active MMP-8 and active MMP-9 in periodontitis. In controls, all MMPs were in their latent pro-enzyme forms, and there were no significant correlations between MMP-3 and MMP-8 or MMP-9.

5.2.3 Activity assay after cell culture for MMP-3, MMP-8 and MMP-9 (II)

After TNF-α stimulation, proMMP-3 synthesis was significantly increased in fibroblasts ($p < 0.001$). Because the experiments were done with and without aminophenylmercuric acetate, both the total and activated MMPs were measured. The results showed that TNF-α increased MMP-3,

-8, and -9 synthesis, but all MMP-3 in the stimulated fibroblast culture supernatant was in the latent proform, whereas MMP-8 and -9 were in their active forms.

5.2.4 Induction of proMMP-1 and MMP-3 in gingival fibroblasts with IL-1β, TNF-α and IL-17 (III)

Both IL-1β and TNF-α increased proMMP-1 ($p < 0.01$) and MMP-3 ($p < 0.001$) synthesis from gingival fibroblasts, whereas IL-17 had less effect ($p < 0.05$) (Figure 10). On the other hand IL-1β and TNF-α did not stimulate MMP-8 or MMP-9 to a detectable degree.

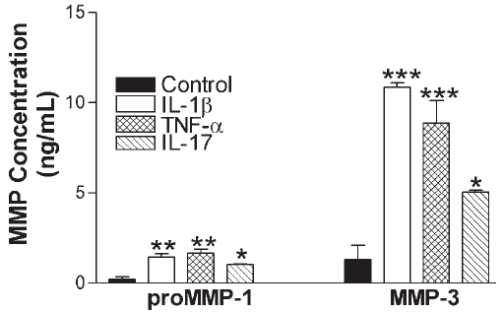

Figure 10. Effects of IL-1β, TNF-α, and IL-17 on induction of proMMP-1 and MMP-3 concentration in gingival fibroblast cultures.

5.2.5 Induction of IL-6 and IL-8 in gingival fibroblasts with IL-17 (III)

IL-1β and TNF-α are the major inducers of IL-6 and IL-8, and in this respect, IL-17 shows a synergistic effect with IL-1β and TNF-α (Katz et al. 2001). Our results showed that both IL-6 and IL-8 release were increased ($p < 0.05$) from gingival fibroblasts with IL-17 stimulation.

5.2.6 Induction of IL-1β and TNF-α in monocytes/macrophages with IL-17 (III)

In gingival connective tissue, monocytes/macrophages are widely seen during inflammation. In our study IL-1β or TNF-α were not released from non-stimulated monocytes/macrophages. However, following IL-17 stimulation, IL-1β (0.59 ± 0.03 ng/mL) and TNF-α (1.19 ± 0.24 ng/mL) were released from monocytes/macrophages ($p < 0.05$ for both).

5.2.7 Induction of proinflammatory cytokines IL-1β and TNF-α in gingival epithelial cells with TLR2 and TLR5 ligands in combination with IL-17 (V)

Stimulation of gingival epithelial cells with HKLM or flagellin increased significantly IL-1β and TNF-α production. However, combination of HKLM or flagellin with IL-17 clearly increased proinflammatory cytokine production more when compared with single TLR ligand stimulation.

6. DISCUSSION

Inflammatory disease of the gingiva and periodontium results in destruction of gingival connective tissue, periodontal ligament and finally alveolar bone. Clinically, inflammation is seen as redness, swelling and bleeding upon probing. However, at molecular and cellular levels, the inflammatory process is defined by cellular infiltrates and the release of a variety of cytokines (Page & Kornman 1997a). Cytokines, in other words "cell proteins" carry the information within one cell but in particular cell to other cells via different mechanisms. After binding to their specific receptors, proinflammatory cytokines like IL-1β, TNF-α trigger intracellular signalling events (Vilcek & Lee 1991).

Upon activation by bacterial products, cells produce TNF-α as a sign of molecular activation (Vilcek & Lee 1991). With this study, we demonstrated the expression and localization of TNF-α and its receptors TNF-R1 and TNF-R2 in pericoronitis. Before our study (I) the phenotype of the local migrant and resident cells had been described in pericoronitis. Based on the high number and more intense positive staining of TNF-α containing cells in periocornitis samples, our study confirmed that the resident cells, fibroblasts, vascular endothelial cells and epithelial cells in pericoronitis tissues are involved in the local inflammatory process and high number of TNF-alpha-containing cells is increased in pericoronitis, as a result of recruitment and/or local activation (I). TNF-α can probably also exert its effect on the cells, which express TNF-α receptors (Springer, 1995). Relatively extensive and intense TNF-R1 and TNF-R2 staining in our pericoronitis samples caused us to conclude, binding of TNF-α to its receptors plays a role in the pericronitis inflammation. Furthermore, in our samples, not only basal cells, but also suprabasal cells presented TNF-α. Maybe bacterial products penetrate the tissue from the intraoral contact surface of the epithelial cells via TLR responses stimulate TNF-α.

We analyzed IL-1β and VCAM-1 in pericoronitis as potential biological indicators of TNF-α TNF-receptor mediated interaction. TNF-α stimulates IL-1β expression (Vilcek & Lee 1991) and, together, these molecules both induce and up-regulate VCAM-1 (Haraldsen et *al.* 1996, Lawson et *al.* 1999). We found more frequent and more intensely IL-1β and VCAM-1 stained cells in pericoronitis than in controls. This shows an independent potential role for IL-1β and VCAM-1 in pericoronitis (Stemerman, 2000), but may also indirectly suggests that TNF-α-TNFR interactions occur in pericoronitis. However, many other stimuli than TNF-α can also induce and upregulate IL-1β and VCAM-1 so their presence in pericoronitis tissue does by no means

prove that TNF-α-TNFR interactions occurred in these tissues although the observation is compatible with such a scenario.

Many previous studies have addressed the MMP-mediated mechanisms of periodontal tissue destruction (Kinane, 1992). There is also intense interest in the ability to block or modulate MMPs to control the progression of tissue destruction and inflammation in periodontal diseases. Human clinical trials with different medications e.g. tetracyclines or low dose doxycycline suggest that when the tissue destructive host MMPs are blocked, also the tissue destruction of periodontitis is blocked (Golub et *al.* 1984). GCF analysis established that during the active stage of periodontal diseases, neutrophil -derived MMP-8 and MMP-9 are actively present (Lee et *al.* 1995). Previous studies showed that MMP-8 and MMP-9 are released as proenzymes (Ding et *al.* 1996, 1997). In our study (II), different than the earlier studies, our current western blot results show that in GCF of infected tissues, these enzymes are in their active forms. In the same study, although only latent form of these enzymes were detected in healthy GCF samples, detection of the active forms of these MMPs in disease samples suggested us these enzymes are somehow processed during the inflammation (II).

In addition to that, based on our *in vitro* fibroblast culture experiments, we revealed that upon stimulation with TNF-α, gingival fibroblasts produced only proform of MMP-3 (II). Since the main cells of GCF are neutrophils and MMP-3 is not derived from PMNs, (Golub et. *al.* 1995), it might be so that fibroblast-derived proMMP3 passes to GCF where our results showed increased levels of active MMP-3 (II). In this situation, it therefore seems that, proMMP-3 must, after secretion, undergo proteolytic conversion from its pro- to the corresponding active form.

We hypothesized that this situation could be explained by co-operation of gingival tissue and GCF (II). Adult periodontitis crevicular fluid contains elevated levels of neutrophil-derived cathepsin G (Tervahartiala et *al.* 1996) and neutrophil elastase (Ingman et *al.* 1994a). Cathepsin G and neutrophil elastase are able to activate proMMP-3 (Okada & Nakanishi 1989, Jenne 1994), which was released from fibroblasts totally in proform. After that, active MMP-3 cleaves off the propeptide or activation peptide and activates proMMP-8 and proMMP-9.

Moreover some of the MMP-8 and MMP-9 was active in GCF. This suggests auto-activation or perhaps plasmin-driven activation (Birkedal-Hansen et *al.* 1993) because, high levels of t-PA, as an effective pro-MMP activator (Ueda & Matsushima 2001), have been described in TNF-stimulated gingival fibroblasts (Xiao et *al.* 1998) (Figure 11).

Figure 11. Mutual MMP activation cascade in periodontitis.

The study indicated that in gingival soft tissue inflammation, TNF-α stimulated, fibroblast mediated synthesis of MMP-3 may play an important role in periodontitis and that MMP-8 and MMP-9 contribute to the same end because they have a large difference between the healthy and diseased samples. To assess the relation of TNF-α to IL-1β and IL-17, and to clarify how they possibly regulate the production and/or activation MMP-3, MMP-8 and MMP-9 a third study was designed.

It was found that the expression of IL-1β, TNF-α and IL-17 was elevated in periodontitis and that human fibroblasts responded to these proinflammatory cytokines by producing proMMP-1, MMP-3, but not MMP-8 and MMP-9 (III). In our third study TNF-α did not increase the release of MMP-8 and MMP-9, whereas in our second study TNF-α increased the release of MMP-8 and MMP-9. Results in our second work were obtained by MMP-activity assay measurement, whereas we used ELISA in the third study. This discrepancy between two results might come from the usage of different technique and different antibodies in two studies.

IL-1β and TNF-α stimulate synthesis and release of MMP-1, MMP-3 (Domeij et *al.* 2002), MMP-8 (Hanemaaijer et *al.* 1997), and MMP-9 (Nee et *al.* 2004). On the other hand, IL-17 functions in conjunction with IL-1β and TNF-α (Ruddy et *al.* 2004) and can regulate some MMPs (Stamp et *al.* 2004). In our study, we confirmed that fibroblasts are capable of producing proMMP-1 and MMP-3 after IL-1β and TNF-α stimulation (Domeij et *al.* 2002). In addition to that, different than the earlier published data (Domeij et *al.* 2002, Stamp et *al.* 2004), we presented that IL-17 was also a potent stimulator of MMP-1 and MMP-3. But compared to IL-1β and TNF-α, IL-17 has less effect. Similar results were also obtained from the staining of periodontitis tissues, in which lower numbers of IL-17 positive cells were detected compared to IL-1β and TNF-α (III). All these results indicate that IL-17 has less effect on MMP production than IL-1β or TNF-α. But considering its IL-6 and IL-8 stimulating effect on fibroblasts (Katz et *al.* 2001, Hwang et *al.* 2004, Takahashi et *al.* 2005), and IL-1β and TNF-α on monocytes/macrophages (Jovanovic et *al.*

1998), it can be thought that apart from its direct effect, IL-17 has an indirect secondary effect on MMP-1 and MMP-3 production (III). This second effect can be explained as a two-stage scenario. In the first stage IL-6 stimulates local production of IL-1β and TNF-α (Ishimi et *al.* 1990), as well as IL-8, which functions as a local chemoattractant for polymorphonuclear leukocytes (Baggiolini et *al.* 1994). Secondly, macrophage released IL-1β and TNF-α can induce production and release of proMMP-1 and MMP-3 from gingival fibroblasts (Birkedal-Hansen et *al.* 1993). Briefly, it can be summarized that IL-1β and TNF-α have a potent effect on the production of MMP-1 and MMP-3, whereas IL-17 seems to exert less potent direct regulatory effect on MMP production, but may function also via induction of IL-1β and TNF-α (III).

Although there are many reasons, which contribute to periodontal diseases, many studies suggest that host responses to the causative bacteria or bacterial biofilms (dental plaque) are the major contributor to disease pathogenesis, which in turn leads to downstream production of proinflammatory cytokines, MMP etc. With a new understanding of host response and periodontal disease pathogenesis, it has become apparent that inhibition of certain host response pathways might be an additional strategy, in addition to suppression of the causative bacteria, to treat periodontal diseases (Mahanonda & Pichyangkul 2007).

TLRs form an important class of proteins, which play a key role in the innate immune system to recognize microbial molecules. 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 (Takeda & Akira 2004a). We thought that they must be present in periodontal tissues to play their role in the inflammatory-tissue destructive effector events. There are no other publications, studied the all TLRs in the gingival tissues. For the fourth study, we decided to map all known TLRs in gingival epithelium and connective tissue. Except TLR10, all nine TLRs (TLR1-TLR9) were expressed both in the connective tissue and in the epithelial cell layers of all samples. The expression in the connective tissue was always higher in periodontitis samples and also on epithelial cells. TLRs were differently expressed in healthy control tissue samples and periodontitis samples (IV).

Periodontitis is characterized by infiltration of chronic mononuclear cell into connective tissue. The immigrant inflammatory cells contribute to TLR-mediated anti-microbial host defense so 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 and more so in periodontitis than in healthy control tissues, most probably because of the locally produced proinflammatory autocrine and paracrine cytokines (Hajishengallis et *al.* 2004, Mahanonda & Pichyangkul 2007).

Microbial components are common for many different bacterial strains. These components stimulate inflammatory cascades to maintain the normal oral microbial flora-host homeostasis in the healthy epithelium (Takeda & Akira 2004a). In the periodontitis affected epithelial layers, the basal layer was most strongly labeled and the expression of TLR became weaker towards the upper layers (IV). There may be different explanations for this. For example TLRs could be mostly synthesized in the basal cells and then degraded or masked during epithelial cell flow or down-regulation of them might increase towards the superficial layers to inhibit potentially harmful inflammatory host responses (Liew et *al.* 2005).

Distribution of TLR positive cells in the different epithelial cell layers presented some interesting findings. In periodontitis, staining of TLR-1, TLR-2, TLR-4 and TLR-5 diminished towards the superficial cell layers but all these TLRs were still located on the surface of the epithelial cells (Takeda & Akira 2004a). This location is favorable for binding of such PAMPs, which are similarly located on the surface of gingival pocket and plaque bacteria (Takeda & Akira 2004b). It might be that, these cell surface TLRs have been downregulated or masked by binding to their specific microbial cell surface-associated PAMPs, which can protect the tissue against damages from excessive innate immune responses (IV). Apart from the transmembrane receptors there are also TLRs located within the endosomal compartment of the epithelial cells (Takeda & Akira 2004a), which showed different distribution characteristics between layers. Whatever the underlying mechanisms are, it seems that epithelium, as a physically protective tissue and as part of the innate branch of the immune defense, seems to allow the host to respond to the invading pathogens by recognition via TLRs (IV).

Gingival epithelium is the outer layer of the oral cavity and is continuously exposed to more than 500 bacterial species (Paster et *al.* 2001). The host immune response uses TLR-dependent or non-dependent pathways in order to recognize and either tolerate (commensals, healthy tissues) or respond against (pathogenic bacteria, periodontitis) pathogens and bacteria (Mahanonda & Pichyangkul 2007). As we already decribed (IV), as innate pathogen detectors, TLRs are expressed by epithelial cells. Due to the presence of bacterial plaque (biofilm), they might respond to relatively few but shared microbial patterns and thus play a key role on the epithelial inflammatory responses and promote the late adaptive immune response, for example by upregulating the costimulatory molecules, including the soluble co-stimulating molecules IL-1β and TNF-α (Vasselon & Detmers 2002, Kaisho & Akira 2006). In the final study, we found that stimulation of epithelial cells with two TLR ligands for TLR2 and TLR5, which are shared by many potential periodontopathogenic bacteria, caused an increased production of proinflammatory cytokines, IL-1β and TNF-α (V). This was important to show that these TLRs

are not only present as epithelial receptor proteins but that they also are functional in epithelial cells and by binding to their PAMPs, activate inflammatory network a rapid innate response which at the same time helps and is necessary for the adaptive immune response to take place (Takeda 2005). In the same study the combination of IL-17 enhanced IL-1β and TNF-α production. This suggests that the progression of inflammation leading to T-cell involvement in lamina propria seem to contribute to a more destructive situation (V). Thus, not only the PAMP-TLR system is helping to mount an adaptive immune response, but vice versa the adaptive response can via T-cell generated IL-17 strengthen the local IL-1β and TNF-α production. In other words, functional characterization of TLRs establishes that the innate immunity of gingival tissue detects invasion of microbial pathogens. Recognition of microbial components by TLRs initiates signals transduction pathways, which triggers expression of further progression. These processes might control innate immune responses and further support development of antigen-specific acquired immunity.

In conclusion, in this research, we focused on the cellular interactions of inflammatory process in periodontitis. Although it is the most extensively studied oral health problem, some questions still need to be addressed, including how our cells respond to various stimulants. Furthermore, it has not been possible to associate any single and specific microbe found in the periodontal pockets with the clinical disease. This study increases our knowledge on a key pathogenic step of periodontitis and microbe-host interactions. In addition to that, this study could also explain, why it has been impossible to ascribe periodontitis to a single bacterial species, providing at the same time information on a limited number of check-points (TLRs), which could be used to stop the pathogenic process on cell cycle check points with therapeutic targets.

Attempts to control the expression and activity of degrading enzymes have been under much attention of periodontal disease treatment. Furthermore, it has been also under much attention that negative regulatory mechanism of TLRs is important for prevention of uncontrolled immune activation in the host response. The results from this thesis may be of importance for future treatment strategies in periodontitis. But, caution should be taken into consideration that the results of this study come from the *in vitro* experiments. The *in vivo* conditions are more complex than the laboratory environment. The future studies are always needed for the expression of MMPs, cytokines and TLRs in individuals with periodontal diseases. Briefly, this PhD study not only increases our knowledge on a key pathogenetic step of periodontitis but also gives deeper information on microbe-host interactions. The ultimate goal of this PhD study is to provide information for a possible novel therapeutics, which are highly safe, more effective, with less adverse effects and less costly, for the people suffering from oral inflammation.

7. CONCLUSIONS

1) In pericoronitis, TNF-α, TNF-R1, TNF-R2 positive cells were found in all samples studied. Immunohistochemical staining of TNF-α, TNF-R1 and TNF-R2 were detected in fibroblast-like stromal, vascular endothelial, and basal and suprabasal epithelial cells.

2) In periodontitis tissue samples, gingival fibroblasts released MMP-3. Although GCF samples of periodontitis patients expressed partially activated MMP-3, MMP-8, and MMP-9, cultured gingival fibroblasts released only proMMP-3 when stimulated with TNF-α.

3) TNF-α, IL-1β and IL-17 levels are elevated in periodontitis. These cytokines induced especially MMP-3 in gingival fibroblasts. IL-17 was less potent as a direct MMP inducer than TNF-α and IL-1β, but it induced TNF-α and IL-1β production from macrophages, and IL-6 and IL-8 from gingival fibroblasts.

4) Toll-like receptors are present in periodontal tissues. TLR-1 to TLR-9 are differentially expressed in connective tissue and epithelial layers. Except for TLR-7 and TLR-8, all the other TLRs express statistically significant differences between patients with periodontitis and healthy controls.

5) Upon stimulation with TLR2 or TLR5 ligands alone or in combination with IL-17, the epithelial cells response to inflammatory process in a TLR-dependent manner by producing TNF-α and IL-1β.

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