Immunological aspects of the pathophysiology of periodontitis

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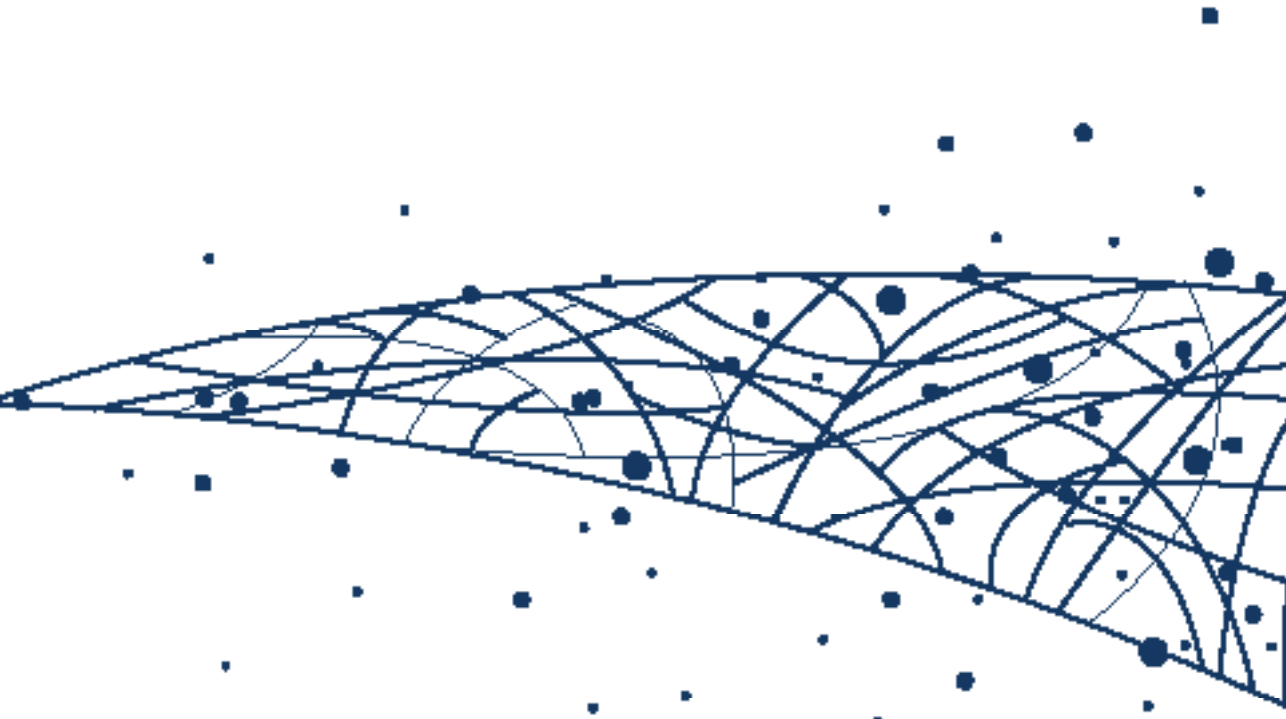
General introduction



In this thesis, several immunological aspects of the pathophysiology of periodontitis are described. In this introductory chapter, various general aspects needed to assist further reading will be introduced. First of all, general aspects of inflammation will be highlighted. Then, the major tissue resident cells of the periodontium (i.e. gingival fibroblasts) are described, after which the infiltrating innate and adaptive immune cells of the periodontium are introduced. Another section is dedicated to the bone-resorbing cells responsible for the pathological alveolar bone resorption occurring in periodontitis. Finally, the aims and outline of this thesis are summarized.

Inflammation

Inflammation is a protective response of the body involving host cells, blood vessels, and mediators which all aim to eliminate the initial cause of tissue injury and to initiate the process of repair. Inflammation can be initiated by physical or chemical trauma or by foreign substances including microbes. Without inflammation, spreading of infections can occur and healing would be a slower process. Although inflammation helps to remove harmful stimuli while facilitating repair, the inflammatory reaction combined with the subsequent repair process has the potential to cause widespread tissue damage (1). The same mechanisms intended to kill microbes and clear damaged tissue also have the potential to damage healthy tissue. Inflammation is induced by chemical mediators which are produced by host cells in response to injurious or foreign stimuli. When a microbe or its components (e.g. lipopolysaccharides; LPS) infiltrates a tissue, the presence of the bacterial challenge or damage is sensed by resident cells such as polymorphonuclear leukocytes (PMNs), macrophages, dendritic cells and mast cells. These cells then secrete molecules, such as cytokines and chemokines, which are important in paracrine and autocrine signaling which induce and regulate the inflammatory immune response. The purpose of an inflammatory reaction is also to facilitate the access of immune cells and molecules circulating in the blood, including circulatory white blood cells (i.e. leukocytes) and plasma proteins, to the damaged or infected tissues. In addition, resident cells of the vascular walls (e.g. endothelial cells) as well as the cells and proteins of the extracellular matrix (ECM) are involved in inflammation and repair (2,3).

Host immune responses

The cells of the immune system are comprised of white blood cells (i.e. leukocytes) and the cells emanating from the hematopoietic lineage which have locally specialized into dendritic cells and macrophages residing in the tissues. Leukocytes are classified in different subgroups; lymphocytes (T, B, and natural killer [NK] cells), granulocytes (neutrophils or polymorphonuclear leukocytes [PMNs], eosinophils, and basophils) and monocytes. Overall, the inflammatory immune response is a multi-facetted process consisting of both innate and adaptive immune responses (4).

The innate immune response is the first line of host defense which includes physical (e.g. mucosal tissues) and chemical (e.g. secreted mucosal fluid containing antibacterial proteins) barriers, as well as innate immune cells which recognize and subsequently kill and eliminate microorganisms. The innate immune response is non-specific, meaning that innate immune cells recognize general features shared by the microbial species and not those specific to a particular microorganism. Phagocytic cells such as PMNs, monocytes, and macrophages, are recruited to the site of inflammation to eliminate foreign agents and slow the bacterial dissemination to avoid any micro-organisms to infiltrate the tissues by executing their antimicrobial functions.

When the innate immune response is insufficient to resolve the inflammation, the more specific and refined defense of the adaptive immune response is initiated. Lymphocytes are adaptive immune responders which are generally present in tissues and release molecules (e.g. cytokines and chemokines) that contribute to the inflammation by inducing the influx of inflammatory cells into the inflamed tissues (5). Specifically, T cell-mediated adaptive immune responses are highly dependent on the associated antigen presenting cells which produce cytokines in response to microbial-derived antigen recognition and capture, which activate the lymphocytes. If the challenging/invasive agent cannot be quickly eliminated, the situation may develop into chronic inflammation, which can have serious pathological consequences and can result in collateral damage.

Periodontal disease

Numerous microorganisms inhabit the oral cavity. In individuals presenting good periodontal health, controlled host-microbe homeostasis is apparent, where a symbiotic biofilm is present in the submarginal and subgingival regions surrounding the teeth (Fig. 1). However, with increasing prolonged accumulations of bacterial biofilm, commonly referred to as dental plaque, a host protective response is initiated – leading to gingival inflammation (i.e. gingivitis). Gingivitis is regarded as a minor, tolerant, and reversible inflammatory host response of the gingival tissues. It is characterized by redness, swelling, and provoked bleeding of the gingival tissues (6). However, in cases of uncontrolled, unresolved, and chronic inflammation, gingivitis can potentially progress into periodontitis, which is characterized by osteoclast-mediated alveolar bone resorption (7) (Fig. 1).

Periodontitis

Periodontitis is a complex chronic inflammatory disease presenting a nonlinear progression and is caused by various factors. These factors may have simultaneous roles and compounding effects while interact with each other (8). The prevalence of severe periodontitis ranges from approximately 7-14% of the Western-European and North-American population (9,10). The risk factors potentially playing simultaneous roles are environmental factors (i.e. the microbiological biofilm), genetic and epigenetic variations,

systemic diseases and lifestyle factors (11–14). It has been established that periodontitis is associated with other diseases such as diabetes (15,16) and atherosclerotic cardiovascular disease (ACVD) (17–20). Thus far, the biological mechanisms and clinical relevance of these associations are still under investigation (21,22).

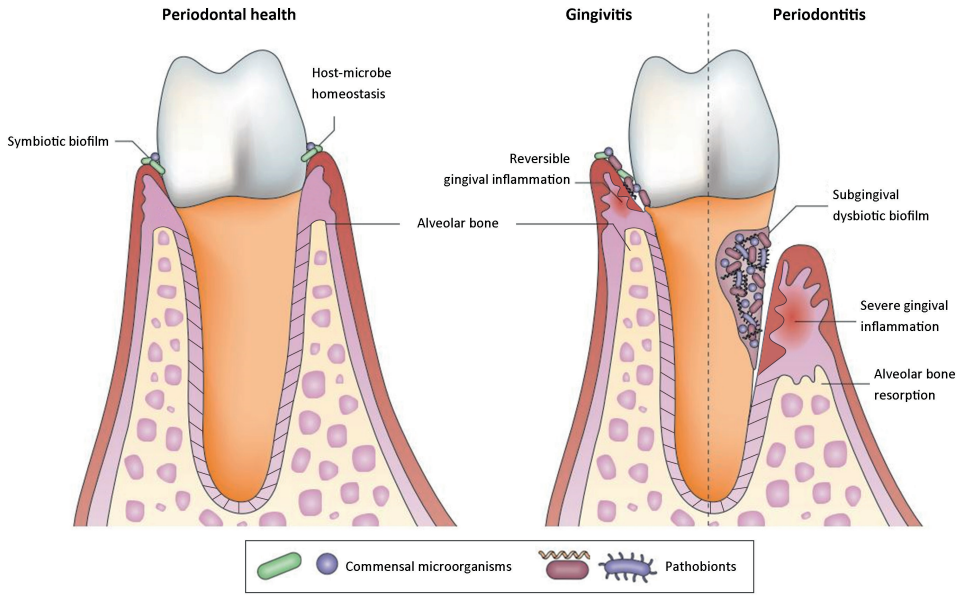


Figure 1: Periodontal health, gingivitis, and periodontitis. Periodontal health (left) is characterized by healthy gingiva without overt signs of inflammation and a symbiotic biofilm present around the teeth containing commensal bacteria. Gingivitis (right figure, left half) is characterized by red and swollen gingiva (modest inflammation) and the presence of inflammophilic pathobionts. The alveolar bone is intact in both aforementioned states. Periodontitis (right figure, right half) is characterized by a periodontal pocket of > 3 mm that harbors a dysbiotic biofilm. Typical for periodontitis is the resorbed alveolar bone that potentially leads to tooth loss. This figure is adapted from the publication 'Periodontitis: from microbial immune subversion to systemic inflammation' by G. Hajishengallis (2015) (7).

The exact causes of periodontitis are still under investigation, however, it is suggested that an aberrant inflammatory response (inherited and acquired during life) results in a hyper- or hyporesponsiveness and/or lack of sufficient resolution of inflammation (23). In health, the dental microbiome is symbiotic, composed of aerobic and anaerobic as well as both Gram-positive and Gram-negative commensal organisms. Importantly, potentially disease-causing microorganisms, primarily anaerobic Gram-negative bacteria (i.e. pathobionts), are also present in the microbiome (6). Pathobionts are present in a symbiotic microflora at low colonization levels and therefore not per se pathogenic bacteria (6). However, an aberrant and chronic inflammatory immune response, typically induced by factors such as smoking, systemic diseases, and genetics, can change in the ecology of the subgingival environment

creating a favorable habitat and thus facilitating the outgrowth and multiplication of these pathobionts (8,23). This, in turn, leads to an imbalance in the microbial ecology after which pathobionts become the dominant periodontal pocket inhabitants, potentially forming a pathogenic entity, and propagate periodontal inflammation. This shift in the ecology of the subgingival environment can further activate (chronic) inflammatory immune responses, including the production of pro-inflammatory cytokines, in a range of host cells such as gingival fibroblasts (GFs), gingival epithelial cells, monocytes, macrophages, and PMNs (24). Finally, this can also result in a pathogenic positive feedback loop where the dysbiotic biofilm and a chronic and exacerbated inflammatory immune response reinforce each other (8).

Microorganisms in the context of periodontal disease

Although the presence of microorganisms is necessary for periodontal disease development, the presence of specific microbes does not guarantee disease initiation or progression. However, in the literature, approximately 10, mainly Gram-negative anaerobic, bacterial species have been frequently identified in the subgingival microbiome of periodontitis patients. The three main Gram-negative bacteria most often observed in periodontitis patients are *Aggregatibacter actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg),* and *Tannerella forsythia (Tf)* (24,25). Of significance, *Pg* is a commensal bacterium, however, at higher colonization levels it can disrupt the homeostatic microbiota and thereby facilitate the shift to a dysbiotic microflora (26). However, microbiome studies have dampened the specific importance of these bacteria and showed a multitude of bacterial species associated with periodontitis (27).

Periodontal therapy aims to reduce the total bacterial load and to suppress pathogenic species in the subgingival microbiome by non-surgical mechanical disruption and removal of the subgingival biofilm and in some cases with the adjunctive use of systemic antibiotics (28). If left untreated, periodontitis potentially leads to the irreversible periodontal ligament and alveolar bone destruction, and ultimately tooth loss (12). The resorption of tooth-adjacent bone is accompanied by a heterogeneous chronic inflammatory effector cell infiltration of leukocytes (5). These leukocytes interact with the resident cells of the periodontium thereby compounding the chronic nature of the inflammatory response (12,29).

Innate immune response: microbial recognition by Toll-like receptors

The innate immune response is the first line of host defense in response to micro-organisms and is comprised of three sequential events: (i) microbial recognition, (ii) activation of signaling pathways, and (iii) activation of effector mechanisms. A determining factor relating to the activation of the host response is how tissue and/or immune cells recognize the presence of potentially harmful agents such as microorganisms. During an inflammatory response,

host cells constantly interact with the dysbiotic microflora. These cells express receptors that are designed to sense the presence of microbes and numerous substances released by dead cells. These receptors are referred to as 'pattern recognition receptors' (PRR) because they recognize structures (i.e. molecular patterns) which are common to many microbes (30). These PRRs bind to so-called 'pathogen-associated molecular patterns (PAMPS)' such as lipoproteins, lipopolysaccharides (LPS), flagellins, and microbial nucleic acids, which are expressed on microorganisms (31). One of the most important PRR families are the Toll-like receptors (TLRs) which sense and recognize PAMPS. A TLR protein is a transmembrane polypeptide with a Toll-interleukin receptor (TIR) signaling domain on the cytoplasmic side of the membrane and a horseshoe-shaped sensor domain on the other side (32). Ten mammalian TLRs have been identified so far. For example, TLR4 can bind LPS, which is a main outer membrane component of Gram-negative bacteria. Microbial recognition of *Pg* occurs mainly through interaction with TLR2 or TLR4 (33–36), both of which are expressed by GFs (35,37) and monocytes (38). In addition to microbial recognition, ligand-bound TLRs activate transcription factors that stimulate the production of pro-inflammatory cytokines (such as tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) (39)) and activate lymphocytes (40) in order to prevent the occurrence of microbial infection. In addition to driving inflammatory responses, TLRs also regulate cell proliferation and survival (41). However, excessive cell proliferation and the production of pro-inflammatory cytokines, due to the chronic stimulation of TLRs, may eventually lead to tissue destruction.

(Immune) cells in the periodontium

The innate immune responses of the periodontium consist of several mechanisms: (i) the flushing action of gingival crevicular fluid and saliva, (ii) the rapid epithelial turnover in conjunction with the multiple layers that are tightly attached, making epithelium virtually impenetrable for bacteria, (iii) the influx of immune cells (e.g. circulatory lymphocytes and leukocytes) to the periodontium, and (iv) the continuous migration of PMNs into the periodontal sulcus and oral mucosal tissues.

Gingival fibroblasts

The gingival fibroblast (GFs) is the predominant cell type of the alveolar bone-lining mucosa (i.e. gingiva). The role of GFs in intact periodontium is to maintain the collagenous fibrous network, necessary for the proper anchorage of the epithelium to the bone, epithelium to the tooth, and anchoring of adjacent teeth. Apart from this main function, GFs interact with innate and adaptive immune responders and thereby are capable of triggering initial inflammatory events. These events, such as sensing and recognition of bacterial antigens and other signals, are followed by the secretion of mediators which trigger the subsequent vascular and cellular events of inflammation (42).

GFs could also play a role in the balance between osteoclastogenesis (resorption of bone) and osteoblastogenesis (bone formation). Accordingly, GFs have been shown to induce differentiation of osteoclast precursors into osteoclasts (37,43). Importantly, cell-cell contact between GF and pre-osteoclasts is required for this differentiation (44). Cellular interactions are thus important for the survival and differentiation of pre-osteoclasts, where GF could play a key role in providing the required survival and differentiation signals. Whether, in this specific context, other immune cells such as T cells play a role in mediating this survival and differentiation of pre-osteoclasts is still unclear.

In healthy gingival tissue, a predominance of T cells, minimal B cells, and a large presence of PMNs, various antigen presenting cells and a small population of innate lymphoid cells are present (5). During chronic inflammation, the products released by leukocytes in order to kill microbes can injure normal host tissues. Therefore, host defense includes checks and balances that ensure that leukocytes are only recruited and activated when and where they are needed. In the next paragraph, the major innate responders of the periodontium; the PMNs, will be further introduced.

Polymorphonuclear leukocytes

The most abundant immune cell type constantly recruited into the periodontium and oral cavity is the PMN. PMNs are myeloid lineage cells originating from the hematopoietic stem cells from the bone marrow and transit through the peripheral blood circulation as circulatory PMNs (cPMNs) (45,46). They are characterized by their cytoplasmic granules which contain proteolytic and antibacterial proteins (lysozymes, cathepsins, lactoferrin, and defensins) (47). cPMNs are the short-lived cells of the innate immunity that rapidly mobilize to enter inflammatory sites, and are specialized in the capture, engulfment, and killing of microorganisms.

In health, approximately 30,000 oral PMNs (oPMNs) transit per minute from the circulation into the oral mucosal tissues and gingival crevices (48,49). The oral cavity harbors over 700 different species of colonizing bacteria, priming and activating the PMNs, which originate from a nearly sterile blood circulation (25,50). In the gingival crevices, oPMNs form a barrier between the epithelium and the dental biofilm to protect the periodontal tissue and to maintain its homeostasis (7,51,52). In periodontal lesions, a substantially higher number of PMNs are recruited (53). Due to the chronic transmigration of oral bacteria from the inflamed periodontal tissues into the blood circulation, oPMNs populating the periodontal lesions, gingival crevices, and oral cavity are in a (hyper)activated state (54,55). Traditionally, cPMNs migrate towards sites of tissue damage, inflamed and infected sites to perform various immune surveillance functions and interact with the micro-organism-biofilm in order to maintain homeostasis and support oral health (56–58).

Antimicrobial functions of PMNs

PMNs sense, migrate towards, immobilize, ingest, and kill microbes intra- and extracellularly (45,59) in order to neutralize and eliminate both microbes and damaged cells (60,61). The recruitment and migration is termed "chemotaxis" and is mediated by endogenous and exogenous chemoattractants such as interleukins such as IL-8 and bacterially derived molecules such as LPS and N-formyl-methionyl-leucyl-phenylalanine (fMLP). These chemoattractants initiate signal transduction events, leading to a multitude of cellular processes including endothelial adhesion, followed by chemotaxis and transmigration of PMNs (62,63).

One of the fundamental processes for the removal of any microorganism and its products is phagocytosis by phagocytes such as PMNs and macrophages. A phagocyte is a cell that eats ('phago' = eat in Greek). Neutrophils were historically called 'microphages' because they are smaller than macrophages, which are the long-lived tissue-resident phagocytic cells. PMNs possess a range of phagocytic receptors that recognize microbial products. After recognition, PMNs adhere to and engulf bacteria, a process known as phagocytosis, which occurs when the plasma membrane engulfs attached organisms (64). When a PMN phagocytoses a bacterium, it will be internalized into the formed phagosome, after which phagosomes are fused with lysosomes and PMN granules into phagolysosomes. The membrane of the intracellular phagolysosomes compartments uses either NADPH oxygenase dependent mechanisms (reactive oxygen species generation) or granule-derived antimicrobial proteins and enzymes to kill the microorganism. Cases where PMNs exhibit impaired phagocytic capacities typically lead to the accumulation of bacteria, a delay in bacterial clearance and a disturbance of oral microbial homeostasis (65). In addition to these mechanisms, it should be noted that microbial destruction is for a large part accomplished through reactive oxygen species (ROS) generation by activated PMNs (46).

PMNs are non-proliferating, short-lived cells which are pre-programmed to die. Before they do so, however, PMNs deploy an ultimate attempt to limit bacterial dissemination by immobilizing and killing microbes in their neutrophil extracellular traps (NETs) (66–69). NETs are web-like structures which are released into the extracellular space by PMNs and are composed of a core DNA element, decondensed nuclear chromatin decorated with histones, combined with various antimicrobial compounds released from the PMN granules (66,70). Among the proteins present in these NETs are bactericidal defensins, several proteases, and calprotectin, which impairs the growth of fungi. The formation of NETs is induced by various stimuli such as phorbol myristate acetate (PMA), microbes and their components like LPS, as well as host factors, immune complexes, and other stimuli binding to the receptors present on PMNs (71).

NETs, therefore, play a key role in protecting the host from bacterial dissemination. In addition to their initially proposed beneficial role, abnormal NET formation and/or accumulation due to insufficient NET degradation may trigger auto-inflammatory responses (72–74). Accordingly, NETs have been suggested as possible players in the development or exacerbation of autoimmune diseases such as rheumatoid arthritis (RA) (71), and systemic lupus erythematosus (SLE) (74–76). This has yet to be described for periodontitis but it is conceivable that auto-inflammatory responses could also play a role in periodontitis.

In healthy individuals, NETs are degraded by multiple enzymes, in particular, plasma deoxyribonucleases (DNases), which are endonucleases secreted by the pancreas. DNaseI degrades the phosphodiester linkages of the DNA backbone thereby degrading both single- and double-stranded DNA (77,78). Accordingly, excessive accumulation of cytotoxic NET-associated compounds such as antimicrobial peptides, auto-immunogenic DNA (citrullinated histones and single-stranded DNA), enzymes (myeloperoxidase and elastase), and entrapped bacteria, can amplify (chronic) immune reactions and potentially trigger the presentation of auto-antigens in the host, eventually leading to tissue damage (71,79). NETs also induce endothelial dysfunction by activation and damage of endothelial cells. These complications are risk factors for atherosclerotic cardiovascular disease events (80,81).

Peripheral blood lymphocytes

During gingival inflammation, resident cells of the periodontium (e.g. GF) interact with heterogeneous effector cell populations of the innate and adaptive immune response that have infiltrated the periodontal soft tissues (82). Next to PMNs, also other leukocytes like monocytes, T and B cells populate inflamed periodontal lesions (5,83). Specifically, T cells are abundantly present in these lesions. The infiltration of T cells is initiated by interactions between microorganisms and PRRs which are expressed on immune cells such as innate immune cells and also gingival fibroblasts. Furthermore, antigen presenting cells (monocytes, macrophages, B cells, and dendritic cells) interact with T cells. Altogether, this may lead to the activation, proliferation, and differentiation of peripheral blood lymphocytes (PBL) (84). Cell proliferation is critical for immune cell expansion, resolution of inflammatory responses, and tissue repair or regeneration processes. Lymphocyte activation is accompanied by the increased production of pro-inflammatory cytokines such as TNF-α and IL-1β, which, in turn, play a role in T cell proliferation (85,86).

Osteoclasts

Irreversible alveolar bone resorption, the hallmark of periodontitis progression, is mediated by bone-resorbing cells: the multinucleated osteoclasts (12,29). Osteoclasts are derived from fused monocyte/macrophage precursors and are specialized in bone resorption. Monocyte differentiation into osteoclasts is regulated primarily by the receptor activator of nuclear factor κ B ligand (RANKL) (87,88), but other cytokines such as in IL-1β, IL-6, and TNF-α can

also activate osteoclastogenesis. Importantly, the differentiation of pre-osteoclasts requires RANKL when added to osteoclast precursor cells. Alternatively, osteoclast differentiation can be achieved in cocultures of tooth associated cells such as periodontal ligament fibroblasts and gingival fibroblasts, through a direct cell-to-cell interaction (44). The decoy receptor osteoprotegerin binds to RANKL, thereby inactivating its activity.

RANKL expression has been observed on a wide variety of cells present in the periodontium, including T cells, B cells (89–92), periodontal ligament and GFs (93). RANKL expression has also recently been discovered on alveolar bone's osteocytes (94). Interestingly, high numbers of PMNs have been found at sites of bone resorption (95). Though never reported for periodontitis patients, RANKL has also been discovered on cPMNs and synovial fluid-derived PMNs from rheumatoid arthritis patients (96).

Aims and outline of this thesis

The main aim of this thesis was to expand our understanding of the immunological aspects of PMNs and fibroblasts in the pathophysiology of periodontitis. This thesis is subdivided into 5 experimental chapters, covering several immunological aspects of the pathophysiology of periodontitis. The first experimental chapter of this thesis (**Chapter 2**) describes the different characteristics of oral and circulatory PMNs. The differences between these two populations of PMNs were characterized in terms of chemotaxis, phagocytosis, and NET formation properties. Next, the effects of periodontal therapy on *in vitro* NET degradation in periodontitis patients was reported in **Chapter 3**. Subsequently, a bridge was made between PMN biology and osteoimmunology in **Chapter 4**. Here, it was assessed whether PMN could express RANKL and whether stimulated PMNs could contribute to osteoclastogenesis. In **Chapter 5**, the interplay between GFs and peripheral blood leukocytes (T, B, NK cells and monocytes), such as prevalent in periodontitis lesions, was described. Accordingly, it was investigated how GFs play a role in osteoclastogenesis and in the survival, retention, and proliferation of lymphocytes. In the last experimental chapter of this thesis (**Chapter 6**), we reported how lymphocytes are affected in terms of proliferation via persistently present TLR agonists in the presence and absence of GF, mimicking chronic inflammatory situations. Finally, the overall findings of this thesis are discussed in **Chapter 7,** which places the main results in a broader perspective and addresses the limitations of this thesis. Summaries of this thesis are presented in **Chapter 8** (English) and **Chapter 9** (Dutch).

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