Immunological aspects of the pathophysiology of periodontitis

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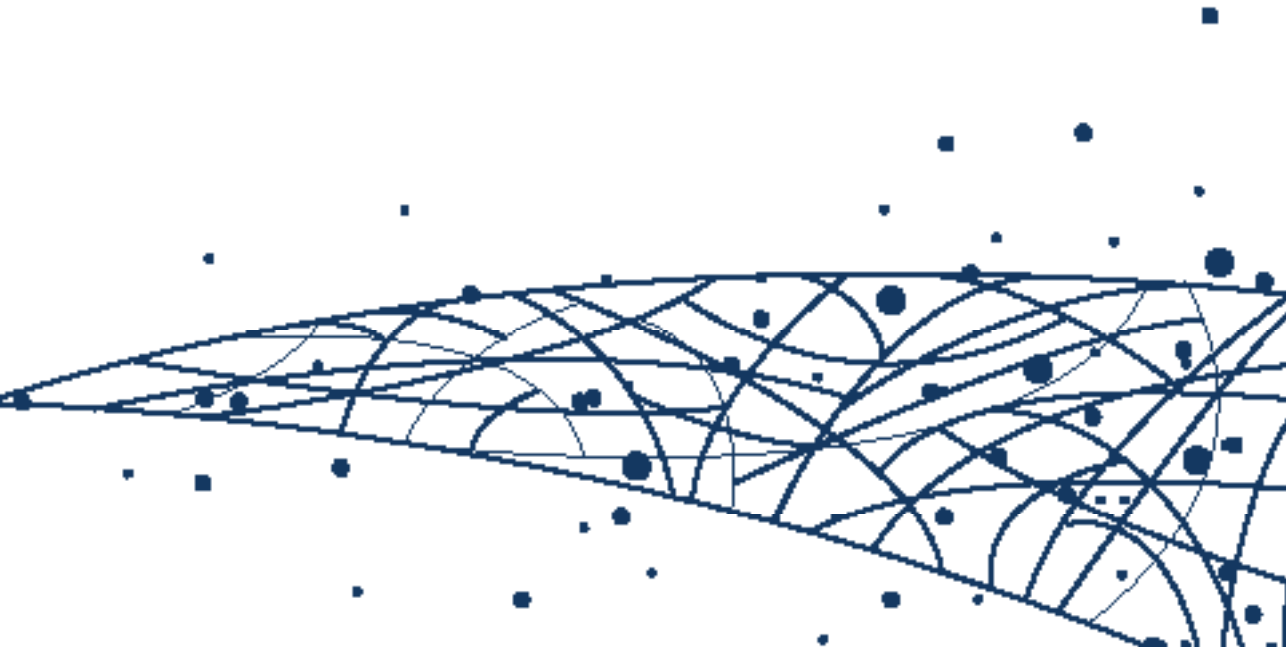
General discussion



GENERAL DISCUSSION

The objective of this thesis was to investigate several immunological aspects of the pathophysiology of periodontitis. In this chapter, the overall research findings are discussed in conjunction with critical reflections. This discussion also offers some suggestions for future directions, in which several challenges need to be overcome.

The characteristics of unstimulated oPMNs

In healthy conditions, approximately 30,000 polymorphonuclear leukocytes (PMNs) transit each minute from the blood circulation through the oral mucosal tissues and gingival crevices and end up in the oral cavity. While circulating PMNs (cPMNs) have been widely studied, little is known about their counterpart in the oral cavity (oPMNs), where these cells may act as important guardians against the numerous commensal and pathogenic microbes that are encountered at this site (1,2).

In **Chapter 2**, we aimed to study the potential of oPMNs in maintaining oral health and compared their chemotactic and antimicrobial functions with those of cPMNs. To establish chemotactic, phagocytic, and NET forming capacities, oPMNs and cPMNs were isolated from healthy subjects without obvious oral inflammation. We described that oPMNs are hyperactive in comparison to cPMNs as indicated by their increased phagocytic and neutrophil extracellular trap (NET) formation capacities but that they have decreased motility towards N-Formylmethionyl-leucyl-phenylalanine (fMLP) (3). These differences could be due to the continuous exposure of oPMNs to microbes, while cPMNs originate from fresh venous blood which represents a largely sterile environment. These differences are one of the main challenges while studying differences between oPMNs and cPMNs.

Clearly, cPMNs have not encountered nearly as many bacterial components as oPMNs which have transited through the periodontium, ending at the oral cavity where eukaryotic cells are vastly outnumbered by bacteria. In our study, the NET formation capacity was studied by exposing PMNs to the well-established NET-inducer phorbol12-myristate 13-acetate (PMA) (4). Our experimental setup included a control condition where PMNs were exposed to culture medium, representing the unstimulated condition. In general, NETs are formed upon activation of PMNs by PMA, pro-inflammatory cytokines (such as interleukin [IL]-8), bacterial products (lipopolysaccharides [LPS]), or bacteria (such as *Phorphorymonas gingivalis [Pg], Candida albicans*, or *Staphylococcus aureus*) (5,6). Furthermore, Mohanty *et al.* demonstrated that saliva induces NET formation in cPMNs (7). As such, exposure to saliva, as an inevitable hallmark for oPMNs, could therefore mean that oPMNs already were in an activated state in terms of NET formation compared to the cPMNs. It is therefore likely that the oPMN control condition was indeed in an activated state since these cells were surrounded by and thus exposed to numerous bacteria from the oral cavity. It can be suggested that pre-incubation of both oPMNs and cPMNs with a cocktail of antibiotics could eliminate the contamination

of oral bacteria prior to killing or NET formation and as such could standardize the starting conditions for both oPMNs and cPMNs. While antibiotics would kill bacteria, they would not stop the stimulation of oPMNs via pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), which are known NET activators (8). Therefore, it appears unlikely that rates of NET induction could be reduced in the presence of these killed microorganisms. While this may not mimic the *in vivo* conditions entirely, it will provide a good starting point for comparing cPMNs and oPMNs. Additionally, this could potentially shed light on the mechanism of NET formation by unstimulated oPMNs which, in all likelihood, and as reported by various groups, can be induced by bacteria (5,9).

Another shortcoming of our NET analysis is that we did not differentiate between vital *versus* suicidal NET formation (10,11). We would then hypothesize that terminally migrated oPMNs are more likely to form suicidal NETs as their 'final attempt' to prevent bacterial dissemination. As previously reported, cPMNs are capable of producing vital NETs (11,12). However, this behavior has not yet been reported for oPMNs.

oPMNs are hyperactive cells

Priming or activation of PMNs changes their characteristics (e.g. expression of markers) and behavior (e.g. effector functions and cytokine production) (13). Activation of PMNs is induced by their environment, lifespan, or interactions via their cellular receptors. Upon activation, cytoplasmic granules of PMNs fuse with their cellular membrane, rendering markers (CD11b, CD63, and CD66b) measurable after activation (14). In **Chapter 2**, we reported that oPMNs are hyperactive in terms of NET formation; unstimulated oPMNs produced 32 times more NETs than unstimulated cPMNs (3). Since 30,000 PMNs enter the oral cavity every minute, and in even greater numbers in periodontitis patients (15), it is conceivable that NETs accumulate in the periodontal tissues and oral cavity, as reported by Hirschfeld *et al.* (16). However, NETs present in the oral cavity are likely rapidly cleared through the natural flow of gingival crevicular fluid and saliva, and swallowed shortly after detachment into the saliva.

The hyperactive state of oPMNs can be partly explained by the findings of Rijkschroeff *et al.* (15). They reported significantly higher expression (3 to 36-fold higher levels) of the activation markers CD11b, CD66b, and CD63 on oPMNs in comparison to cPMNs. Furthermore, unstimulated oPMNs produced 3-5 times more reactive oxygen species (ROS) than unstimulated cPMNs (15). Similarly to our findings, they observed that oPMNs were less viable than cPMNs (60 - 70% *vs*. 95% viability), indicating that oPMNs are more mature cells and probably apoptotic. Altogether, this is in line with our findings of the hyper(re)active NET production characteristic of unstimulated oPMNs. It is highly possible that oPMNs are activated by their migration through oral tissues and transit along the dental biofilms.

Hyperactive phenotype or distinct subsets?

In contrast to other leukocytes, there is limited evidence on the distinct and functional heterogeneity of PMN subsets. Recent research hypothesizes that there may be subsets of PMNs with potentially important functional differences (17–19). It is conceivable that migration through tissues such as the periodontium is selective, meaning that some subsets are more represented in oPMNs than others. If indeed transit through the periodontium after exiting the circulation is shaping their phenotype, these PMNs in periodontal tissues likely represent a mixture of both phenotypes. It is possible that oPMNs represent a distinct subset from cPMNs since oPMNs acquire and lose phenotypic traits during their transmigration process, as described above and in **Chapter 2.** Therefore, it is conceivable that certain combinations of expression markers could distinguish the different subsets of oPMNs. This question is particularly relevant in diseased conditions, e.g. periodontitis, and could be established in future phenotyping studies. For example, if distinct subsets of PMNs are present in the oral cavity and differentially express molecules important in their effector functions (e.g. phagocytosis, ROS, and NET production), and thus in maintaining oral homeostasis, therapeutic targeting of a given subset would be possible without affecting the normal functions of the other subset(s). Nevertheless, it is a challenging and speculative thought and further research into different subsets, distinguished by cell surface markers, functional responses, and/or transcriptional profiles, would certainly solidify the notion whether PMN subsets exist.

The impaired chemotactic capacity of oPMNs towards fMLP

Chemotaxis is the process of directional movement by a cell towards an extracellular chemoattractant gradient and holds an essential role in the recruitment of PMNs towards inflammatory sites. Several studies investigated the antimicrobial functions of cPMNs using transwell systems (20–22). However, the oral cavity is a much more complex cellular environment suggesting that transwell systems do not completely represent the 'journey' made by oPMNs and the environmental factors present in the oral cavity. We, therefore, made use of video microscopy which reflects the moving capacity of cells in a free environment (**Chapter 2**).

In **Chapter 2**, we demonstrated that oPMNs are less motile as shown by their capacity for chemotaxis towards the bacterial product fMLP. The impaired motility of oPMNs could be the result of migration through oral tissues into the oral cavity. We also showed that oPMNs have less fMLP receptor expression, explaining their insensitivity towards fMLP in our experimental setup. However, oPMNs may still be capable of migrating towards other chemokines such as CXCL8 (IL-8), or bacterial products such as LPS. We chose fMLP as a chemoattractant in our studies as it had previously been shown to have a significantly higher chemotactic index, velocity, and accuracy in comparison to the chemotactic response towards CXCL8 by cPMNs from healthy individuals (23). A conceivable physiological

explanation of the impaired chemotactic response by oPMNs would be that these cells have reached their 'final destination' and therefore lose their ability (e.g. chemotactic receptors) to migrate. After arrival in the oral cavity, oPMNs are likely cleared through the natural flow of gingival crevicular fluid and saliva, before being ultimately swallowed.

Interestingly, impaired migration towards a chemoattractant observed in oPMNs from healthy subjects showed similarities with cPMNs from periodontitis patients (23). Impaired chemotactic responses by cPMNs and oPMNs in periodontitis patients may prolong tissue transit times and thus the time required to reach the infected area. This would lead to inadequate microbial clearance in gingival crevices and periodontal pockets, thereby exacerbating the disease. Whether oPMNs from periodontitis patients have different chemotactic capacities than oPMNs from healthy controls represents an interesting subject for future investigations.

Reverse migration of PMNs

Previous work suggests that PMNs migrate to the target tissues in order to perform their antimicrobial functions after which they die and are phagocytosed by macrophages and monocytes (24). Recent studies using intravital microscopy in lungs provided evidence that PMNs not always die at the sites of inflammation, but can migrate back into the circulation as a physiological process potentially to be deactivated or reprogrammed, before selectively migrate back to the bone marrow (25). One could speculate about reverse migration by oPMNs. Despite the attractive biological phenomenon observed in the lung, there are several arguments that reverse migration of oPMNs through the oral tissues back into the circulation would be highly unlikely. Firstly, we show in **Chapter 2** that oPMNs are terminally migrated cells, as shown in our chemotaxis assays. Secondly, oPMNs migrate from the blood circulation into the oral cavity, which is an energy-demanding process. This process likely exhausts the energy required for reverse migration back through the epithelium. Thirdly, due to migration from the circulatory system into the periodontal tissues, oPMNs are in a more mature state than cPMNs and are therefore more likely to undergo apoptosis, and will be cleared through the natural flow of gingival crevicular fluid and saliva, which would render them unable to migrate back into the tissues. Lastly, since the oral cavity is heavily colonized by microorganisms and thus contains (bacterial-derived) chemotactic agents, PMNs migrate from the junctional or pocket epithelium through the crevice into the oral cavity where they contribute to maintaining oral homeostasis by performing their antimicrobial functions. PMNs naturally migrate towards (high concentrations of) chemotactic gradients. In a healthy state, this chemotactic gradient is not present in the oral cavity in reverse direction, making it unlikely that oPMNs migrate back into periodontal tissues. However, in periodontitis patients, microorganisms and their bacterial products can invade the inflamed and highly vascularized gingival lesions. PMNs are recruited to the inflammatory sites, resulting in 4 times higher numbers of oPMNs in periodontitis patients

as in controls (15). Conceivably, cPMNs could be retained and accumulate in the inflamed gingival tissues of periodontitis patients, as found by Dutzan *et al.* (26) and Thorbert-Mros *et al.* (27). Reverse migration of PMNs could be a possible explanation for this finding, however, this has not yet been proven and is therefore a subject for future investigations. Ultimate proof of reverse migration should come from intravital microscopical approaches, or, alternatively, *bona fide* markers present on reverse migrated PMNs.

Periodontal therapy is beneficial for NET degradation

In **Chapter 3**, we aimed to study the NET degradation capacity of plasma from periodontitis patients compared to that of controls (part 1). Our results showed that plasma-induced NET degradation did not differ significantly between periodontitis patients and healthy controls. This was in contrast to a previous study by White *et al.* (28). They compared NET degradation in periodontitis patients and matched controls, and showed that NET degradation and DNaseI levels were lower in periodontitis patients than in healthy individuals (28). In the current unmatched study population about twice the size, we also found a slightly lower mean NET degradation in periodontitis patients compared to controls, but that did not differ significantly between patients and controls, also when we adjusted for variability in patient background characteristics. The differences in NET degradation levels between periodontitis patients and controls found by White *et al.* were explained by different DNAseI levels, the NET-degrading enzyme present in plasma. Since the NET degradation levels were not different between periodontitis patients and controls, we hypothesize that DNAseI levels are similar in these groups. However, this would be a subject for future investigations.

In part 2 of the study of **Chapter 3**, we investigated the effect of non-surgical therapy on NET degradation levels. We demonstrated that non-surgical periodontal therapy improved the NET degradation capacity after 3 months, which was maintained for 6 and 12 months. This beneficial effect occurred irrespective of antibiotic usage, gender, age, or ethnicity. In the present study, we confirmed the beneficial effects of non-surgical periodontal therapy after 3 months, as previously reported by White *et al.* (28). In our study, we investigated the effect of non-surgical therapy with a 3, 6, and 12-month follow-up. We are the first to report that the effect of non-surgical therapy on NET degradation levels remained after 6 and 12 months post non-surgical periodontal therapy.

Non-surgical periodontal therapy (e.g. scaling and root planning) involves the disturbance of the sessile state (adhered to a surface) dental biofilm and thereby creating a brief planktonic state (free floating) in the subgingival space, where after the bacteria will likely disappear within minutes with the gingival crevicular fluid flow which is mixed with the saliva and eventually swallowed. Bacteria display distinct characteristics in a planktonic state than in a biofilm. Importantly, sessile bacteria are 500-5000 times more tolerant towards

antibiotics in comparison to their planktonic state (reviewed by Gupta *et al.* (29)), therefore clinicians prescribe antibiotics in adjunctive to non-surgical therapy in order to maximize the effectiveness of antimicrobials.

In **Chapter 3**, we further demonstrated that adjunctive antimicrobial usage (i.e. killing of bacteria) did not affect increased NET degradation levels after non-surgical therapy Several additional studies explaining the molecular mechanisms of the observed increased NET degradation levels following non-surgical periodontal therapy could be performed to explain our findings. First of all, as performed by White *et al*., DNAseI levels could be measured to confirm that NET degradation levels were improved post-therapy because of an increased presence of NET degrading proteins (28). The beneficial effects of non-surgical therapy on increased NET degradation levels likely results from the overall immune fitness of the subjects. Immune fitness levels have a significant impact on an individual's ability to handle multiple routine immune challenges and perturbations and it also alters the effectiveness of normal inflammation resolving mechanisms (30,31). We have several indications about the immune fitness of the study participants. Although the patient's BMI was a confounding factor for NET degradation levels, the BMI did not change after 3 months ($p = 0.1715$, paired t-test). Furthermore, other factors correlating with the obese profiles of the patients were stable over the 12 months of the study. Accordingly, the waist circumference and weight did not change significantly in these patients over time. In order to address this, periodontists should attempt to motivate their patients to make changes to their lifestyle to support their other conventional methods of treatment. This should not only include their dental cleaning and smoking habits, but also their dietary choices and BMI. Ultimately, making such changes should improve overall immune fitness of the patients.

Interaction between the innate immune responders and cells of the periodontium

In **Chapter 5**, the cellular interactions between gingival fibroblasts (GFs) and peripheral blood mononuclear cells (PBMCs) were investigated in coculture systems. The study aimed to investigate GF's role in the osteoclastogenesis of PBMCs, monocytes, and peripheral blood lymphocytes (PBLs; monocyte-depleted PBMCs) cocultures. After several days of coculturing PBMCs or PBLs with GFs, we observed a firm attachment of PBLS onto the GFs, even after rigorously moving the cell culture plates in a horizontal position. Retention and survival rates for these mononuclear cells were lower in monocultures (without GFs) than in cocultures (with GFs). After this observation, we investigated whether GFs play a role in the survival and retention of lymphocytes and aimed to identify the different cell types present in these cocultures. We further discovered that in addition to their role in osteoclastogenesis, GFs also play a role in the survival and retention of lymphocytes (32).

Since PMNs transit through periodontal tissues, we questioned whether GFs could also play a role in the retention and survival of PMNs. Characterization of the human immune cell network of gingival biopsies originating from periodontitis patients demonstrated a significantly higher number of accumulated inflammatory cells such as PMNs (26). Despite the cPMNs' *lymphocyte function-associated antigen-1* (*LFA-1*) expression (33), which is important for diapedesis and subsequently for firm adhesion to cells expressing intercellular adhesion molecules (ICAMs; expressed by cells such as GFs), cPMNs did not adhere to GFs and died after 3 days (data not shown). Nevertheless, GF-PMN interactions could theoretically lead to the alteration of their phenotype and functionality, which represents an interesting subject for further investigation.

As we show in **Chapter 5**, coculturing of GFs with PBMC/ monocytes led to the differentiation of monocytes into osteoclasts. Indeed, differentiation into macrophages could also represent a differential pathway for monocytes. However, the percentage of CD14+, expressed by monocytes and macrophages, decreased significantly after 7 days (<9%) in cocultures of monocytes/PBMCs with GFs. This more or less coincided with the formation of osteoclast precursors, and subsequently osteoclasts, indicating that only minimal numbers of macrophages were formed in these cocultures. Furthermore, our coculture studies in **Chapters 5** and **6** were performed with GFs obtained from third molars of healthy individuals who did not have any overt signs of gingival inflammation and periodontitis. As GFs from periodontitis patients exhibit inflammatory characteristics *in vitro* (34), it would be interesting to study these GFs to investigate the cellular interactions between GFs and immune cells. This would possibly explain the persistence of inflammatory mediators, accumulation of inflammatory cells, and activation of pathological osteoclastogenesis. However, studies from our own laboratory showed that periodontal ligament fibroblasts originating from periodontitis patients did not induce greater numbers of osteoclasts (35). Nevertheless, GFs could be differentially activated, and of additional importance, a different repertoire of leukocytes could be attained by fibroblasts of periodontitis patients.

T cell alloreactivity

Alloreactivity is defined as: "a strong primary T cell response against allelic variants of major histocompatibility complex (MHC) molecules in the species" (36,37). In **Chapters 5** and **6**, GFs from healthy donors (n = 18, n = 6, respectively) were cocultured with PBMCs from unrelated healthy blood donors (n= 3 in both chapters). The proliferation of T cells in GF-cocultures was observed with and without TLR2 stimulation (**Chapters 6** and **5**, respectively). In general, the percentages of T cell proliferation were relatively low (20-40%) and the overall number of T cells decreased over time in all conditions. Furthermore, only minimal proliferation was observed in the absence of GFs (**Chapter 5**), however, proliferation was significantly increased after stimulation with TLR2 agonists (**Chapter 6**). Therefore, we concluded that, in our experimental setups, T cell alloreactivity reactions were minimal.

Possibly, T cell proliferation could be a result of their activation by alloreactivity rather than through their ability to survive due to GF or TLR stimulation. As such, T cells might recognize GFs as foreign cells (expressing foreign MHC molecules), after which the monocytes and TLR ligands could facilitate T cell activation and proliferation. Despite the proliferation demonstrated in **Chapter 5**, the numbers of CD3+ T cells remained constant over time, suggesting that proliferation and apoptosis were in balance. Since our experimental setup included a high number of replicates, we hypothesize that GFs express growth cytokines (such as tumor necrosis factor alpha (TNF-α) and interleukin 1-beta (IL-1β)) beneficial for the survival, retention an proliferation of peripheral blood lymphocytes (PBLs) and that this is unlikely to be an alloreactivity effect. However, GF and PBMC donor-matched experiments should be performed to exclude the possibility of T cell alloreactivity and to confirm our findings stating that GFs induce T cell proliferation.

Gingival fibroblasts *vs* periodontal ligament fibroblasts

Based on the anatomy of the tooth-surrounding tissues, two functionally different types of fibroblasts can be distinguished: GFs and periodontal ligament fibroblasts (PDL). PDLs are essential to maintain homeostasis of the periodontal ligament, play an important role in the anchoring of teeth to the alveolar bone, and are also involved in processes related to bone remodeling. In **Chapters 5** and **6**, the most abundant structural cells of the periodontium were studied: the GFs. Through this, we demonstrated that GFs play a role in the retention of lymphocytes. However, our observations of retaining T and B cells in cocultures of PBMCs and GFs (**Chapter 5**) were also seen in cocultures with PDLs (results not shown). This means that the retention and survival of lymphocytes is not an effect uniquely attributable to GFs, but rather that other cells from the periodontium may also play a major role in the temporization and clearance of inflammation.

Studying microbe-host interactions

In **Chapters 2**, **4** and **6**, we investigated possible interactions between immune cells and micro-organisms. Host-pathogen interactions can be studied *in vitro* using various experimental setups which employ either whole bacteria (heat-killed and live microbes were used for phagocytosis assays in **Chapter 2**), bacterial products (fMLP was used for chemotaxis assays in **Chapter 2,** LPS for activation of PMNs in **Chapter 4**), or bacterial agonists (TLR agonists were used in **Chapter 6**). Despite the advantage of studying specific responses based on simplified experimental setups using either one type of bacteria or its products, studies investigating cellular interactions using an oral multispecies biofilm would better represent the *in vivo* setting. Using an *in vitro* oral biofilm model, as published by Klug *et al.* (38) and Mira *et al.* (39), would be ideal for studying host-biofilm interactions as studied in **Chapter 6**. However, within each individual, each body compartment, and even inside the oral cavity, there is no uniform microbiome composition, and one can find large differences between the hard and soft tissue microbiomes (1,40). Additionally, other factors

such as salivary composition, pH value variations, host immunity factors, and dietary choices could influence the biofilm composition and are difficult to mimic in an *in* vitro setting using artificial culture medium to feed the biofilm. As a result, studying microbiome/host interactions using these complex experimental setups represents a significant challenge, and therefore, simplified models similar to our experimental setups are still widely applied. Scaling down to species (e.g. *Pg*), or even further down to molecules (specific TLR2 and TLR4 agonists) has the obvious advantage of reproducibility and is mechanistically insightful, pinpointing biological processes down to very precise molecular structures and the cellular reactions thereupon.

Osteoclastogenesis

Alveolar bone loss is typical in periodontitis and is caused by the activation of bone-resorbing osteoclasts which are recruited by chronic inflammation (2,30). Bone-resorbing osteoclasts derive from monocytes via receptor activator of NF-κB ligand (RANKL) stimulation (41–43).

In **Chapter 4**, we aimed to study the possible role of PMNs in osteoclastogenesis based on the findings of Chakravarti *et al*. (44) who reported the role of cPMNs in osteoclastogenesis. They reported that LPS-stimulation of cPMNs led to their expression of RANKL. Furthermore, they cocultured LPS-stimulated cPMNs with monocytes which differentiated into bone-resorbing osteoclasts (44). In contrast to their findings, minimal levels of RANKL were detected on stimulated cPMNs in our experimental setup. Furthermore, we failed to demonstrate that (stimulated) cPMNs were capable of inducing osteoclastogenesis when fixed and cocultured with pre-osteoclasts for 10 days. Despite performing exactly the same protocols in the same laboratory and using the corresponding chemicals and antibodies with the same equipment as described by Chakravarti *et al*., we were not able to reproduce these findings.

A challenge in osteoclastogenesis assays is the long duration (minimal 10 days) of these cultures while PMNs are short-lived cells. To overcome this issue, we fixed PMNs to ensure enduring surface expression of RANKL. However, in *in vivo* situations, a constant influx of PMNs is recruited to the site of inflammation. Therefore, coculturing pre-osteoclasts with daily fresh additions of (stimulated) PMNs would be a suggestion for future research to investigate the possible role of PMNs in osteoclastogenesis.

Despite minimal levels of RANKL expression, no osteoclastogenesis was observed after co-culturing with monocytes as pre-osteoclasts in our study. A number of other studies have further demonstrated the roles of RANKL expressing T and B cells in osteoclastogenesis (45–48). RANKL is expressed by T cells which interact with PMNs by direct cell-cell contact, especially under chronic inflammatory conditions (48,49). As such, cross-talk between PMNs and cells of the adaptive immune system, like T cells, could potentially lead to increased

osteoclastogenesis. We demonstrated that PMNs do not directly induce osteoclastogenesis, but that they could possibly play an indirect role by interacting or activating other immune cells, like T cells.

In **Chapter 5**, we investigated osteoclastogenesis by coculturing GF with PBMCs or monocytes (PBL-depleted PBMCs), while in **Chapter 4**, we investigated osteoclastogenesis by coculturing cPMNs with monocytes. In our experimental setups, we did not include cocultures of PMNs with PBMCs since this would add another possible interfering cell type, like T cells, which are suggested to play a role in osteoclastogenesis (45,48). In **Chapter 5**, no significant differences in the numbers of osteoclasts present in PBMC-GF or monocyte-GF cocultures were found ($p = 0.869$), indicating that PBLs did not significantly contribute to increased osteoclast formation in this experimental setup. It could be, however, that PBLs contain both osteoclastogenesis activating and inhibiting subsets of, for instance, T cells. Further studies could elucidate whether or not and which subsets of T cells modulate osteoclast formation. Our studies have nevertheless demonstrated that T cells interact with osteoclasts under the *in vitro* system conditions that we used.

We initially demonstrated that osteoclast-like cells were formed in GF-PBMC co-cultures, and especially the addition of dexamethasone and vitamin D increased their numbers (50). However, *in vitro* activation of these cells requires the addition of cytokines RANKL and macrophage colony-stimulating factor (M-CSF) to transition these formed osteoclasts to the state of resorbing osteoclasts. Since osteoclast-like cells also form without these additional stimuli, we avoided using these additions, as this introduces additional variables to the experiments. In our study, we merely concentrated on the formation of osteoclasts rather than incorporating the activity aspects. Therefore, as concerns the pathophysiology of periodontitis, it is justified to extrapolate our findings as to the role of GFs and leukocytes in osteoclast formation. In order to accomplish this, it would be worth investigating whether these osteoclast-like cells are indeed active and thus resorb bone.

The role of TLRs in osteoclastogenesis

In **Chapter 6**, we investigated the effects of chronic inflammation, by exposing GF-PBMC cocultures to TLR2 and TLR4 activators for 21 days and assessed whether this influenced PBL retention, survival, and proliferation. Here, we demonstrated that TLR2 stimulation doubled rates of T cell proliferation. As shown in **Chapter 5**, osteoclasts were formed in GF-PBMC cocultures while we did not investigate osteoclastogenesis in **Chapter 6**. However, we could speculate what the effects of TLR activation could ultimately lead to the damaging characteristic of periodontitis: alveolar bone degradation by osteoclasts. Since a significant source of LPS is present in the periodontal pockets of periodontitis patients, and both osteoclastogenesis and TLRs are closely associated with RANKL (51), we hypothesized that monocytes could be stimulated via TLR activation in order to form osteoclasts (Karlis *et*

al. 2019, *in preparation*). LPS binds to TLRs, especially to TLR2 and TLR4, and subsequently induces the production of pro-inflammatory cytokines which sustain chronic inflammation and possibly induce osteoclastogenesis.

Interestingly, as shown in mouse models, especially TLR2, and to a lesser extent TLR4, play a role in periodontal bone loss (52,53). Accordingly, TLR2 knockout mice developed more severe periodontitis, as characterized by greater levels of bone loss, after intraperitoneal infection with *Aggregatibacter actinomycetemcomitans* (*Aa*). This model demonstrated that the innate immune response, including the recruitment of macrophages and PMNs, was impaired when compared to wild-type controls. This highlights the involvement of TLR2 in *Aa* recognition and control (54). Thus, we speculate that TLR activation could lead to two possible outcomes. Firstly, monocytes could differentiate into macrophages, which would then play a role in the clearance of bacterial products, leading to the secretion of IL-1β and TNF-α what would attract T cells which would in turn proliferate. Alternatively, activated monocytes could produce additional pro-inflammatory cytokines, such as TNF-α and IL-1β, which could auto-stimulate monocytes thereby facilitating osteoclast formation and activation. Notwithstanding, these speculations are posited despite the existence of contradictory literature (55). Accordingly, Takami and colleagues state that TLR stimulation of osteoclast precursors inhibited their differentiation into osteoclasts.

Collectively, investigating the cellular players and immune mediators that stimulate alveolar bone resorption in periodontitis will help to unravel its pathogenesis. This thesis has contributed to the more specified understanding of the role played by PMNs in maintaining oral health and the novel role of GFs in retaining leukocytes and by specifically activating T cell proliferation. A better understanding of osteoimmunological processes in which tooth-associated fibroblasts interact with immune cells has led to further insight into the pathogenesis of periodontitis and can be used to achieve the desired homeostasis for periodontal health.

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