Reactivity of neutrophils, monocytes and platelets in periodontitis
Nicu, E.A.

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Chapter 7

Summary and discussion
Periodontitis is a chronic infectious condition in the tooth-supporting tissues, which eventually leads to loss of teeth if left untreated. The host response to bacterial insults leads to chronic inflammation; the features of periodontal inflammation are gingival bleeding, formation of deepened periodontal pockets, and progressive destruction of periodontal ligaments and alveolar bone. While bacteria are essential for the initiation and progression of periodontitis, genetic and lifestyle factors appear to strongly influence the severity of the disease and the response to treatment. New models of how to apply this knowledge for individualized patient care are necessary. An important step forward would be the identification of individuals with heightened susceptibility for periodontitis. They would ultimately require more intensive prevention and treatment strategies.

Since bacteria are central in the initiation and progression of periodontitis, one approach in the direction of identifying modifying factors for susceptibility of periodontitis, is analyzing the immune response to periodontal pathogens. In this thesis we analyzed the importance of phagocytes, i.e. PMNs and monocytes in the defense against periodontal pathogens, and in particular we investigated the function of the receptors in charge with bacterial recognition. In Chapter 2 we analyzed the FcγRIIa on PMNs, with a closer look at one single nucleotide polymorphism in the FcγRIIa-gene (FCG2A). FcγRIIa (one of the types of receptors for the Fc part of immunoglobulins, IgG) mediates phagocytosis and cell activation. Previous studies showed that one of the genetic variants of the FcγRIIa, the FcγRIIa\textsuperscript{131H/H}, is capable of efficiently binding human IgG2, whereas the other two variants, FcγRIIa\textsuperscript{131H/R} or FcγRIIa\textsuperscript{131R/R} do so with reduced affinity. IgG2 is the predominant IgG subclass against periodontal pathogens and opsonization (with IgG, complement, or other plasma proteins) is a pre-requisite for efficient recognition of bacteria by phagocytes. Therefore, we hypothesized that this polymorphism in the FCG2A is modifying the PMN reactivity in response to periodontal pathogens. Our results demonstrate that in response to stimulation with the periodontal pathogen \textit{A. actinomycetemcomitans}, PMNs from FcγRIIa\textsuperscript{131HH} carriers phagocytosed more bacteria. During this phagocytosis step, the FcγRIIa\textsuperscript{131HH}-PMNs released more granular content and more active elastase than the FcγRIIa\textsuperscript{131RR}-PMNs, suggesting a hyper-reactive phenotype of the FcγRIIa\textsuperscript{131HH}-PMNs. In addition to the reactive oxygen-species produced during the respiratory burst, the PMN degranulation products (matrix metallo-proteinases, elastase) may be responsible for a large part of the PMN-induced collateral damage in periodontitis (1,2,3). Therefore, the hyper-reactivity of the FcγRIIa\textsuperscript{131HH}-PMNs resulting in increased...
degranulation might be one of the mechanisms by which periodontitis patients with the FcγRIIa131H/H genotype acquire more severe periodontal destruction compared to patients with the FcγRIIa131H/R or FcγRIIa131R/R genotypes. Indeed, our results give a mechanistic explanation for previous observations of an increased severity of periodontal breakdown in periodontitis patients with the FcγRIIa131H/H genotype (4,5).

The hyper-reactivity of PMNs in periodontitis might result not only from genetically-encoded affinity of their receptors, but also from differential expression of these receptors when compared to periodontally-healthy controls. In Chapter 3 we investigated the cellular expression of IgG-receptors (FcγRI, FcγRIIa, FcγRIIIa and FcγRIIIb), complement-receptor CR3 and the LPS-co-receptor CD14 on PMNs and monocytes, as well as activation of these cells in response to two periodontal pathogens, A. actinomycetemcomitans and P. gingivalis.

Our results demonstrate a similar expression of the analyzed receptors on PMNs in periodontitis patients and healthy controls, suggesting that the chronic inflammatory reaction accompanying periodontitis does not affect the PMNs during their formation in the bone marrow, or in their short-lived blood circulation. The monocytes, on the contrary, demonstrated a decreased membrane-bound CD14 (mCD14) expression with concomitant increase in the FcγRIII. These CD14lowFcγRIII+ monocytes have been described in the literature as precursors of a dendritic cell-type; this cell population is expanding in inflammatory conditions such as rheumatoid arthritis (6,7), sepsis (8) or Kawasaki disease (9). The dendritic cells formed from CD14lowFcγRIII+ monocytes have phagocytic and oxidative capacity, but fail to efficiently stimulate T-cells for a definitive resolution of inflammation (10). Therefore, we propose a role for the CD14lowFcγRIII+ monocytes in the chronicization of periodontal inflammation.

Furthermore, our results showed that the subjects culture-positive for A. actinomyctemcomitans had significantly lower expression of monocytic FcγRI and FcγRIIa than P. gingivalis-infected subjects. This lower FcγRs expression by monocytes might be related to a higher susceptibility of a subject to become infected with A. actinomyctemcomitans or to an adaptation to this particular infection. Indeed, PMNs from A. actinomyctemcomitans -infected subjects responded in a hyper-reactive manner in response to bacterial stimulation, in particular when stimulated with A. actinomyctemcomitans. Taken together, these results suggest a hyper-reactivity of the PMNs in A. actinomyctemcomitans –infected individuals for which the immune system
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tries to account for by lowering the FcγRs expression on monocytes. Nevertheless, the hyper-reactive PMNs might contribute to the severe and relatively rapid periodontal destruction reported in early-onset periodontitis patients sub-gingivally colonized with A. actinomycetemcomitans (11).

Nevertheless, we believe that the origin of the PMN hyper-reactivity in subjects colonized with A. actinomycetemcomitans is not clarified yet. We hypothesize that a certain genetic background could make these individuals more prone for A. actinomycetemcomitans infection. This scenario has been observed for subjects with certain genetic variations in the genes coding mannose-binding lectin, vitamin D receptor, mannose-associated serine-protease-2, and Toll-like receptors; these subjects had a greater risk of infection with Mycobacterium tuberculosis, meningococci or gram-negative bacteria (12,13,14,15,16). On the other hand, it could be the infection with A. actinomycetemcomitans itself responsible for the phenotypic changes of the PMNs. Future intervention studies that would eliminate A. actinomycetemcomitans from the sub-gingival microflora might offer the answer to this question.

In addition to the cellular expression of phagocytic receptors, in Chapter 4 we analyzed the plasmatic levels of the soluble form of CD14 (sCD14) in periodontitis patients and healthy controls. sCD14 can be the result of shedding / cleavage of mCD14 from monocytes / macrophages or be produced in the liver. Acting as a soluble LPS-receptor, sCD14 helps inducing responses in cells that naturally lack CD14, such as endothelial, epithelial and smooth muscle cells (17). Another role is the neutralization of LPS in Gram-negative infectious states. Acting as a decoy receptor for serum LPS, sCD14 prevents extreme pro-inflammatory responses from monocytes/macrophages (18). Also periodontitis is a condition associated with Gram-negative pathogens that gain access to the systemic blood circulation and are responsible for endotoxemia in periodontitis patients (19). Our results demonstrated an increase in sCD14 plasma levels that positively correlated with severity of periodontal destruction. This association points into the direction that the sCD14 increases with the need to remove LPS from circulation. Given the results from Chapter 3, where we demonstrated a decreased membrane-bound CD14 expression on monocytes from periodontitis patients, the increase in sCD14 might be partially attributable to the shedding of the membrane-bound form of CD14 from monocytes. However, we also showed that the increase in sCD14 was positively correlated with systemic inflammatory markers, such as CRP and numbers of leukocytes. Therefore,
we can assume that also an increased sCD14 production by the liver is accompanying periodontitis. Interestingly, cleavage of mCD14 by bacterial proteases like the \textit{P. gingivalis} gingipains has been described \textit{in vitro} (20). However, our results do not support the hypothesis of a direct proteolytic cleavage of mCD14 by gingipains since in our bacterial stimulation assays \textit{A. actinomycetemcomitans} and \textit{P. gingivalis} were similarly able to induce upregulation of mCD14 on monocytes (\textbf{Chapter 3}). In a future study, an assay distinguishing between the three forms of sCD14 (resulting from shedding, cleavage or liver-produced) might bring more insight in the periodontitis-associated increase in sCD14.

It has been shown that several inflammatory markers predict future vascular events (21,22,23). C-reactive protein (CRP) and number of leukocytes have been identified as markers for cardio-vascular disease (CVD) (24,25). Elevated levels of CRP and leukocytes in patients with CVD may be the result of chronic infectious and inflammatory processes (26,27,28). Periodontitis is one of the chronic infectious processes that has been associated with CVD in several epidemiologic studies (29) and elevated blood levels of CRP and leukocytes have been found in patients with periodontitis (30,31). The underlying mechanism of CVD is atherosclerosis. CD14 binds LPS in conjunction with LPS-binding-protein (LBP); the CD14-LPS-LBP complex can directly activate endothelial and smooth muscle cells (32,33), leading to elevated expression of cell adhesion molecules, thereby increasing procoagulant activity, and exacerbating inflammation in atherosclerotic lesions (34). Based on our results in \textbf{Chapter 4} that showed increased sCD14 levels in periodontitis, concomitant with elevated CRP and leukocyte levels, we propose a role for sCD14 in the recurrent episodes of accelerated activity within atherosclerotic plaques in patients at risk for CVD. We hypothesize that sCD14-mediated signaling is one of the pathways leading to increased atherogenic activity, and thus, increased risk for CVD in periodontitis patients.

Not only PMNs and monocytes are capable of interacting with bacteria, also platelets respond to bacteria by releasing anti-microbial peptides (35), and exposing pro-inflammatory receptors (36). In \textbf{Chapters 5} and \textbf{6} we analyzed the activation status of circulating platelets in periodontitis patients, as well as the platelet response to periodontal pathogens and the interplay between platelets, PMNs and monocytes during stimulation with bacteria. Periodontitis patients demonstrated increased platelet activation, as revealed by elevated plasma levels of sP-selectin and increased expression of the glycoprotein IIb-IIIa, both abundantly expressed receptors, and responsible for adhesive properties of
platelets. P-selectin is released from platelets within minutes after activation and is found in plasma as soluble (s)P-selectin (37). Platelet activation was more pronounced in the patients with more severe periodontal disease, showing a severity-dependence. Moreover, in Chapter 6 we showed that platelets from periodontitis patients are more sensitive to oral bacteria than cells from periodontally healthy controls. In response to A. actinomycetemcomitans, P. gingivalis, and S. sanguis, but not T. forsythia, platelets from patients expressed more P-selectin than those from controls. Moreover, more platelet-monocyte complexes were formed in blood from patients in response to oral bacteria than in blood from controls. In a phagocytosis assay, platelet-PMN and platelet-monocyte complexes phagocytosed more bacteria than platelet-free cells, suggesting that the complexes contain primed cells.

Periodontitis is a condition in which due to breaching of the epithelial lining, small-scale, frequent bacteremias occur during regular activities like chewing or tooth brushing (38,39). These bacteremic episodes underlie endotoxemia and a chronic systemic inflammatory reaction in periodontitis patients (40,31,19). In epidemiologic studies, periodontitis has been associated with increased risk for myocardial infarction and stroke (29), but the responsible mechanisms are still obscure. Based on our results, we suggest that the pathologic processes in periodontitis induce platelet and monocyte priming, a repeatable process with virtually every bacteremic episode of periodontal origin. Activated platelets produce vast amounts of proinflammatory mediators stored in their α-granules and dense body systems. Promptly released upon platelet activation, the pro-inflammatory mediators and receptors induce platelet binding to endothelial cells lining vessel walls (36). Once adhered, platelets create a platform onto which monocytes can roll and adhere firmly, leading to increased monocyte recruitment. Monocytes in the subendothelial space will excessively accumulate intracellular low density lipoprotein leading to accumulation of intracellular lipid droplets and formation of the foam cells. These functions make activated platelets and monocytes essential participants in both thrombosis and atherogenesis and we hypothesize that they explain, at least partially, the epidemiologically-increased risk for CVD in periodontitis patients.

It is currently unclear whether the increased platelet reactivity in periodontitis patients is intrinsic, the result of a specific genetic make-up, or extrinsic, the result of priming by bacterial products streaming from periodontal lesions or by the cytokines produced as a part of the systemic inflammatory reaction in periodontitis. Future studies, assessing platelet activation status and reactivity after successful treatment of periodontitis
will shed light on this matter. One published intervention study showed that treatment of patients with periodontitis is followed by an improvement of endothelial function (41); the biological basis of this improvement of the vascular condition is as yet unknown. However, a reduction in the state of platelet activation and in the pro-coagulant state may be part of the explanation.

**Concluding remarks**

In this thesis we have investigated the reactivity of PMNs, monocytes and platelets in periodontitis patients. Individuals at higher risk for periodontal breakdown were represented by the subjects with the FcγRIIa<sup>131H/H</sup> genotype and the subjects sub-gingivally colonized with *A. actinomycetemcomitans*. These groups of subjects demonstrated hyper-reactive PMNs in functional assays using periodontal pathogenic bacteria as stimuli. Whereas for the FcγRIIa genotypic variants, the origin is evidently genetic and the therapeutic approach goes more in the direction of better prevention of periodontitis, for the subjects colonized with *A. actinomycetemcomitans* the answer is not so straightforward. Future intervention studies that would eliminate *A. actinomycetemcomitans* from the sub-gingival microflora might offer the answer to this question.

Furthermore, we have demonstrated that platelets and monocytes from periodontitis patients are more sensitive to activation by oral bacterial species, such as *A. actinomycetemcomitans*, *P. gingivalis*, and *S. sanguis* than cells from healthy controls. Periodontal therapy results in a reduction of the bacterial burden from the periodontal pockets, as well as in a reduction of the systemic inflammatory reaction (40). Given the roles of platelets and monocytes in thrombosis and atherosclerosis, periodontal therapy might have an added value not only in improving the patients’ oral health, but also as prevention measure to reduce their overall risk for myocardial infarction and stroke.
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