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STUDIES ON MYELOID CELL FUNCTIONS IN PERIODONTITIS

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Cover image: Immunohistochemical staining for CD68 in periodontitis gingival tissue. CD68 is a marker for monocytes/macrophages. Magnification: 40x. Image acquired by Reuben Clark.

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Studies on myeloid cell functions in periodontitis

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To my beloved family

ABSTRACT

Periodontitis (PD) is a chronic inflammatory disease characterized by destruction of the tooth supporting tissues; gingiva, periodontal ligament, and alveolar bone. Myeloid cells, including monocytes and macrophages, play a crucial part in the inflammatory host response by clearing pathogens and producing a vast array of inflammatory mediators. However, during chronic inflammation, the actions of these cells can contribute to tissue degradation. By utilizing clinical samples and different cell culture systems, this thesis aimed to investigate the role of monocytes and macrophages, along with factors that regulate them in PD.

In **study I**, we found elevated gene and protein expression of MMP-12 in PD gingival tissue and the expression was mainly attributed to $CD68^{+}CD64^{+}CD14^{+}$ monocyte-derived cells. These cells displayed low expression of the co-stimulatory molecule CD200R. Induced inflammation in oral tissue cultures reduced the CD200R and increased the MMP-12 expression in monocyte-derived cells. CSF-2 was found to potently induce MMP-12 and a CD200 ligand reduced MMP-12 production in monocyte-derived cells, suggesting that the CD200/CD200R pathway is a potential target to modulate harmful inflammatory processes in PD.

In **study II**, the expression of S100A12 was found to be high in monocytes, decreased during macrophage differentiation, and unaltered upon macrophage polarization. Analysis of S100A12 in different monocyte subsets revealed that classical monocytes had the highest expression followed by the intermediate and non-classical subsets. Peripheral blood monocytes from PD patients were more frequently positive for S100A12 and showed higher secretion *in vitro* compared with controls. The importance of monocytes for S100A12 production was demonstrated in oral tissue cultures. The protein expression of S100A12 was increased in gingival tissue along with higher frequencies of $S100A12^{+}$ monocyte-derived cells. S100A12 levels in saliva reflected the severity of PD, suggesting A100A12 as a potential biomarker for disease.

The expression of the macrophage growth factors CSF-1 and IL-34 in gingival tissue and gingival fibroblasts (GFs) was investigated in **study III**. Elevated CSF-1 protein expression was found in gingival tissue from PD patients while the levels of IL-34 were similar in PD and control tissue. CSF-1 and IL-34 were expressed and produced by GFs constitutively and pro-inflammatory stimuli induced their secretion. CSF-1 and IL-34 secretion did not differ in GFs isolated from PD patients compared with controls in unstimulated condition or in response to TNF-α or IL-1β stimuli.

In **study IV**, we found elevated expression of CSF-1R in gingiva from PD patients. $HLA\text{-}DR^{+}CD64^{+}$ macrophages displayed similar frequencies in PD and controls with no difference regarding CSF-1R expression. In peripheral blood mononuclear cells, CSF-1R inhibition did not alter the monocyte subsets or the expression of the myeloid markers CD64, CD206 or CD163. However, CSF-1R blockade attenuated MMP production in PD gingival explants. Thus, CSF-1R may contribute to the inflammatory processes leading to tissue degradation in PD.

In summary, these studies investigated the involvement of monocytes and macrophages in PD as well as the expression of factors that regulate their functions. Furthermore, strategies to target tissue-destructive myeloid cell related processes were explored.

LIST OF SCIENTIFIC PAPERS

I. Sofia Björnfot Holmström, **Reuben Clark**, Stephanie Zwicker, Daniela Bureik, Egle Kvedaraite, Eric Bernasconi, Anh Thu Nguyen Hoang, Gunnar Johannsen, Benjamin J. Marsland, Elisabeth A. Boström, Mattias Svensson. Gingival tissue inflammation promotes matrix metalloproteinase-12 production by CD200R^{low} monocyte derived cells in periodontitis.
The Journal of Immunology (2017) 199, doi: 10.4049/jimmunol.1700672.

II. Ronaldo Lira-Junior, Sofia Björnfot Holmström, **Reuben Clark,** Stephanie Zwicker, Mirjam Majster, Gunnar Johannsen, Björn Axtelius, Sigvard Åkerman, Mattias Svensson, Björn Klinge, Elisabeth A. Boström. S100A12 expression is modulated during monocyte differentiation and reflects periodontitis severity.
Frontiers in Immunology (2020) 11: 86. doi: 10.3389/fimmu.2020.00086.

III. **Reuben Clark**, Stephanie Zwicker, Daniela Bureik, Gunnar Johannsen, Elisabeth A. Boström. Expression of colony-stimulating factor 1 and interleukin-34 in gingival tissue and gingival fibroblasts from periodontitis patients and controls.
Journal of Periodontology (2019) doi: 10.1002/JPER.19-0296.

IV. **Reuben Clark**, Ronaldo Lira-Junior, Gunnar Johannsen, Elisabeth A. Boström. Colony-stimulating factor 1 receptor blockade attenuates inflammation in gingival explants from periodontitis patients.
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

BOP	Bleeding on probing
CAL	Clinical attachment loss
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
c-fms	CSF-1 receptor tyrosine kinase
cMoP	Common monocyte progenitor
CMP	Common myeloid progenitor
COX	Cyclooxygenase
CSF	Colony stimulating factor
CXCR	C-X-C chemokine receptor
DAB	Diaminobenzidine
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle medium
ELISA	Enzyme-linked linked immunosorbent assay
ERK	Extracellular signal regulated kinase
FBS	Fetal bovine serum
GCF	Gingival crevicular fluid
GF	Gingival fibroblast
GMP	Granulocyte/monocyte progenitor
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JE	Junctional epithelium
JMD	Juxtamembrane domain

LPS	Lipopolysaccharide
MDP	Macrophage/Dendritic cell progenitor
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor-kappa B
OKF6-TERT-2	TERT-immortalized normal human oral keratinocyte line
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD	Periodontitis
PDLF	Periodontal ligament fibroblast
PGE2	Prostaglandin E2
PI3K	Phosphoinositol 3-kinase
PMN	Polymorphonuclear leukocytes
PRR	Pathogen recognition receptor
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor Kappa B
RANKL	Receptor activator of nuclear factor Kappa B ligand
SE	Sulcular epithelium
SLE	Systemic lupus erythematosus
TBS-T	Tris buffered saline with tween
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF	Tumor necrosis factor
αMEM	Medium essential medium Eagle- alpha modification

1 INTRODUCTION

1.1 INNATE IMMUNITY OF THE ORAL MUCOSA

The oral mucosa is constantly challenged with a particularly diverse microbiota. To prevent colonization of the periodontal tissues, the immune system needs to eliminate infectious pathogens, and at the same time keep the commensal microbiota in balance. The first line of defense is comprised of physical and chemical barriers, complement system, and innate myeloid and lymphoid cells. In the oral cavity, the constant flow of saliva and gingival crevicular fluid (GCF) disturbs the colonization of microbial biofilms. These fluids exhibit antimicrobial properties through enzymes, immunoglobulins, complement system factors, and leukocytes. Tissue resident cells e.g. epithelial cells and fibroblasts provide the structure and integrity of the mucosal barrier. Epithelial cells form the outer layer of the mucosa and are connected to each other through intercellular junctions. Aside from forming a physical barrier, epithelial cells recognize pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs). Fibroblasts are the most numerous cell type in the connective tissue and are important regulators of the extracellular matrix. In response to bacterial insults, epithelial cells and fibroblasts produce antimicrobial peptides and inflammatory mediators that attract and activate immune cells. Innate immune cells, including monocytes, macrophages and dendritic cells (DCs) are situated within the mucosal barrier and play an important role as scavengers. These cells are also equipped with PRRs and play an important part in clearing pathogens that have breached the epithelium. Innate immune cells exert several functions to eliminate the pathogens, including phagocytosis and production of antimicrobial peptides and inflammatory mediators that eliminate bacteria and attract more leukocytes to the site of infection. Furthermore, they also take part in activation of the adaptive immune response by engulfing and processing pathogens for presentation to T-lymphocytes. Proinflammatory mediators produced by innate immune cells in response to infectious stimuli, may in a chronic inflammatory setting lead to detrimental effects on host tissues[1,2].

1.2 PERIODONTITIS (PD)

Inflammatory diseases of the periodontium are common conditions. Plaque-induced gingivitis is initiated by the microbiota and confined to the soft tissues. It is diagnosed by bleeding on probing (BOP) assessed as the proportion of bleeding sites and can be reversed by removing the dental plaque[3]. However, if the microbial biofilm remains, gingivitis may advance into periodontitis (PD) in susceptible individuals[4]. The chronic inflammatory disease PD is characterized by irreversible destruction of the tooth supporting tissues; e.g. gingiva, periodontal ligament, and alveolar bone. If left untreated, the chronic inflammation eventually results in tooth mobility and tooth loss[4]. The prevalence of PD is high with approximately 29% of individuals aged 20-80 years in Jönköping, Sweden, reported to be affected by a moderate form of the disease, and 11% with severe disease[5]. The global prevalence of severe PD has

been reported to be 11.2%[6]. The quality of life is negatively impacted by PD due to compromised tooth function and aesthetics[7], and PD may also be a risk factor for general health by impacting the onset and severity of systemic diseases, e.g. cardiovascular disease and diabetes[8].

The diagnosis of PD is based on several parameters: measurement of BOP, probing depth, clinical attachment loss (CAL), and radiographic bone loss. In the 1999 classification of PD, the extent of disease was characterized as local if ≤ 30% of sites were involved and generalized if ≥ 30% of sites were involved. Severity of disease was based on CAL and classified as slight, moderate and severe (advanced)[9]. A new classification system was presented in 2017, classifying PD in stages (I-IV) defined by severity, complexity, and extent and distribution. The progression and prognosis are determined by grades (A-C) as defined by evidence of, or risk for rapid progression, anticipated treatment response, and effects on systemic health[10]. The treatment of PD includes motivating the patient to remove supragingival plaque by maintaining oral hygiene procedures, as well as non-surgical and surgical debridement followed by supportive periodontal care[11].

1.2.1 The periodontium

The periodontium is comprised of the tissues that surround and support the teeth. These include the root cementum, periodontal ligament, gingiva, and alveolar bone. The root cementum is a hard, avascular structure covering the roots, and its main function is to anchor the periodontal ligament fibers to the teeth. The alveolar process is the bone that forms the sockets housing the teeth, and the specialized connective tissue located between the root cementum and the alveolar bone makes up the periodontal ligament. It consists of a diverse cell population as well as collagen fiber bundles anchored to the teeth and the bone, providing support during forces of mastication[12]. The gingiva covers the alveolar bone and surrounds the teeth and is comprised of epithelium and underlying connective tissue. The epithelium is divided into three different types based on location and function; the gingival epithelium, sulcular epithelium (SE), and the junctional epithelium (JE). The gingival epithelium extends from the muco-gingival junction to the gingival crest. The SE is in contact with the tooth without being attached, forming the gingival sulcus, or gingival crevice. The SE merges with the JE which is attached to the tooth surface (Figure 1). The highly vascularized connective tissue adjacent to the JE provides a constant infiltration of leukocytes, both during homeostasis and disease[12].

The dento-gingival junction may be the most vulnerable part of the periodontium, constituting the border between the periodontal tissues and the oral cavity. The highly specialized JE is of great importance as it is attached to the tooth and forms a protective barrier against bacteria in the sulcus[13]. The high permeability due to its wide, fluid-filled intercellular spaces allows for infiltration of leukocytes and release of antimicrobial molecules into the sulcus and oral cavity with the GCF[13,14]. The composition of immune cells in healthy gingiva is primarily constituted by a rich T cell and neutrophil infiltrate along with monocytes, and macrophages[15-17]. During

homeostasis, this defense system keeps the commensal microbiota in balance with the host and prevents colonization of the periodontal tissues.

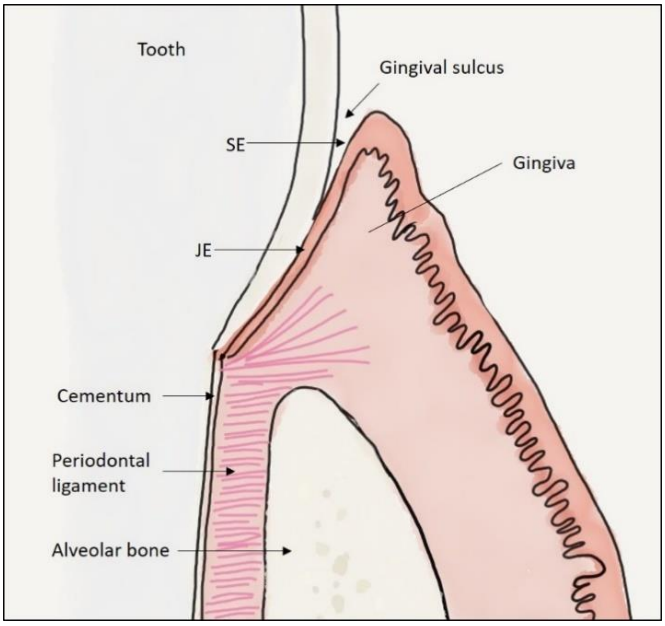

Figure 1. Schematic illustration of the periodontium. The sulcular epithelium (SE) merges with the junctional epithelium (JE) which is attached to the tooth surface. The space between the sulcular epithelium and the tooth is called the gingival sulcus, or gingival crevice and holds the gingival crevicular fluid (GCF). The periodontal ligament is attached to the root cementum and the alveolar bone and provides support during mastication. Illustration by Julia Landmark Clark.

1.2.2 **Pathogenesis of PD**

The formation of a pathological periodontal pocket is characterized by apical migration of the JE, converting to pocket epithelium. Pathological pockets are likely formed due to intra-epithelial cleavage caused by migration of leukocytes and direct interaction with bacterial products, followed by degradation of the connective tissue apically to the JE[13]. Recently acquired knowledge suggests that this is due to a disrupted balance between the host and the commensal microbiota. Keystone pathobionts such as *Porphyromonas gingivalis* subvert the immune response and impairs innate immunity. The host-mediated immunopathology that ensues leads to tissue degradation which provides a recess for colonization. The inflammation causes release of nutritional factors favoring pathobionts resulting in a shift in microbial composition[18,19]. Thus, microbial insult initiates the inflammation, but the development and progression of PD is considered to be mediated by a dysregulated immune response. Several risk factors are known to affect the onset and progression of disease, e.g. immunodeficiency, systemic disease, smoking, stress, diet and genetic risk factors[4] (Figure 2).

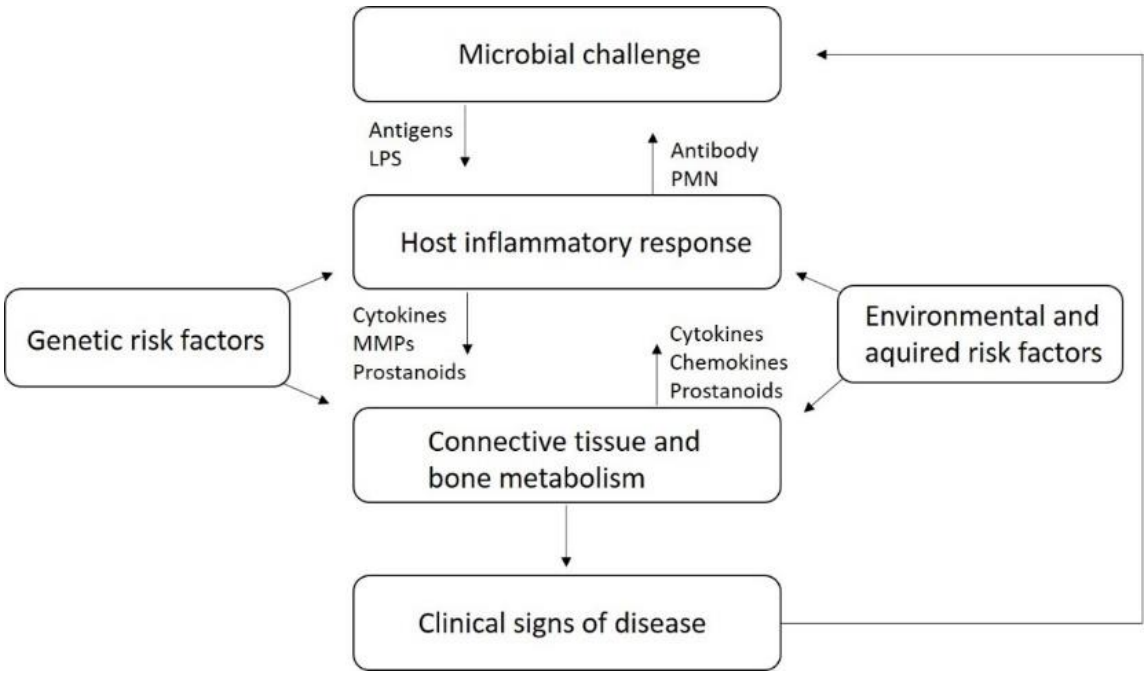

Figure 2. Schematic illustration of the pathogenesis of PD. Adapted from Page and Kornman 1997[20]. The pathogenesis of PD is multifactorial and involves dysbiosis, genetic and environmental risk factors, and a dysregulated immune response. Abbreviations: LPS; lipopolysaccharide, PMN; polymorphonuclear leukocytes, MMPs; matrix metalloproteinases.

The histopathological changes in the gingiva during the progression of PD were originally described in four stages by Page and Schroeder including the *initial lesion* dominated by neutrophils, the *early lesion* displaying large numbers of T-cells and macrophages, and lastly the *established* and *advanced lesions*, dominated by plasma cells[21].
In the initiating step, toxins released by bacteria stimulate epithelial cells, gingival fibroblasts (GFs) and macrophages to produce a vast array of cytokines and chemokines. These events lead to the upregulation of adhesion molecules on endothelial cells, increased vascular permeability, and recruitment of leukocytes to the site of infection[22]. Among the first responders are neutrophils, migrating into the tissue over a chemoattractant gradient. They combat the bacterial insult by utilizing phagocytosis and microbicidal functions, including the release of reactive oxygen species and antimicrobial peptides. Aside from killing bacteria, neutrophil-activity also causes connective tissue degradation through production matrix metalloproteinases (MMPs)[23]. As the inflammation ensues, more leukocytes are attracted. Lymphocytes, monocytes and macrophages also accumulate in the tissue at this stage of the disease[22]. In the enhanced inflammatory environment, the production of cytokines and proteolytic enzymes is maintained, leading to deepened periodontal pockets due to the degradation of the connective tissue and formation of the apically proliferating pocket epithelium. In the established periodontal lesion, B-cells and antibody producing plasma cells can be observed in great numbers in the affected connective tissue. As the lesion progresses, osteoclast precursors are accumulated and differentiate into bone resorbing osteoclasts[22]. Although the histopathological changes during progression of PD have been extensively

studied, it is still unclear what changes in the cellular composition causes the inflammation to "tip over" and become destructive. Previous studies have reported that increasing numbers of B cells characterizes PD lesions compared with gingivitis[24,25]. Further, when comparing the cellular infiltrate of PD and gingivitis, Thorbert Mros et al. found that PD lesions contained larger numbers of macrophages, plasma cells, as well as B cells expressing cluster of differentiation (CD) 5[26]. By utilizing multi-color flow cytometry, more in-depth analysis of different immune cell populations in gingiva can be performed, and possibly link certain phenotypes to disease development and progression. For example, Dutzan et al. analyzed the frequency of different immune cell populations in PD and healthy gingiva and found similar frequencies of T-cells, B-cells and DC-Mac (dendritic cells, monocytes, macrophages), but an increased proportion of granulocytes in PD[15]. With the notion that interleukin (IL)-17 is a key mediator of inflammatory bone loss and associated with accumulation of neutrophils in animal models of PD, the IL-17 expressing cells were characterized. The authors found that IL-17 expression is mainly attributed to $CD4^+$ T cells and that the percentage of this population producing IL-17 is increased in PD[15]. Thus, identifying specific immune cell phenotypes and their functions is important in order to understand the events that lead to initiation and progression of PD.

1.3 INFLAMMATORY MEDIATORS IN PD

During the complex inflammatory processes of PD, leukocytes and tissue resident cells interact with each other to orchestrate the host response against the microbiota. The secretion of a vast number of inflammatory mediators is induced by bacterial products such as lipopolysaccharides (LPS) binding to toll-like receptors (TLRs) on host cells[27,28]. Expression of several cytokines involved in regulating inflammatory processes has been found to be elevated in gingival tissue and GCF from PD-affected sites, including tumor necrosis factor-α (TNF-α), IL-1β, IL-6, IL-10, and receptor activator of nuclear factor kappa-B ligand (RANKL)[29-34]. TNF-α and IL-1β are produced by many different cell types, and especially by monocytes and macrophages. They are key pro-inflammatory cytokines with wide biological effects, including regulation of cytokines, chemokines, growth factors, and proteolytic enzymes[35]. Studies in animal models of PD have demonstrated that targeting TNF-α and IL-1β reduces inflammatory mediator production, leukocyte recruitment, and alveolar bone resorption[36-38]. In humans, targeting TNF-α is an established treatment for several chronic inflammatory diseases such as rheumatoid arthritis (RA), and it has been shown to have a positive effect on periodontal parameters in RA patients with PD[39-42]. Another pleiotropic cytokine is IL-6 which plays an important role in acute-phase responses, hematopoiesis and immune reactions[43]. It is believed to have an important function in the initiation of PD, but also takes part in osteoclast differentiation and stimulates production of inflammatory mediators by gingival cells[44-46]. The anti-inflammatory cytokine IL-10 is crucial for the regulation of the immune response and limiting tissue damage[47]. In experimental PD, IL-10 deficient mice exhibit accelerated alveolar bone resorption, demonstrating its tissue protective role during

periodontal inflammation[48]. Chemokines are a large family of cytokines that regulate the migration of immune cells to the site of infection or injury[49]. The chemokines C-C motif chemokine ligand (CCL) 2 and IL-8 are crucial for the recruitment of monocytes and neutrophils respectively and are increased in gingival tissue during PD[50,51].

MMPs are a family of proteolytic enzymes that play important roles in tissue modulation by orchestrating extracellular matrix turnover. They also take part in modulating the immune response by cleaving cytokines and chemokines[52]. MMP activity is controlled by endogenous tissue inhibitors of metalloproteinases (TIMPs), and an imbalance between MMP and TIMP production may have consequences for periodontal tissue integrity during PD[53]. MMPs are divided into different subclasses based on their structure and target substrate. Among them are MMP-1, -8, and 13 (collagenases), MMP-2 and -9 (gelatinases), MMP-3 and -10 (stromelysins), and MMP-7 and -26 (matrilysins). These MMPs degrade various matrix components such as collagen, gelatin, fibronectin, and elastin[54]. Another MMP not belonging to these groups is MMP-12, or macrophage metalloelastase, which is produced my monocytes and macrophages and targets elastin and its precursor tropoelastin[55,56]. Elevated expression of several MMPs have been reported in gingival tissue, GCF, and saliva during PD[57-59], and their involvement in inflammatory destructive processes has been demonstrated in animal models. Ramamurthy and colleagues utilized a rat model of PD and reported decreased MMP activity and cytokine levels in the gingival tissue, as well as reduced alveolar bone loss following administration of MMP inhibitors[60]. Furthermore, in humans, a low dose of doxycycline has been shown to have tissue protective effects by inhibiting MMP activity[61]. Macrophages produce MMPs abundantly in response to proinflammatory stimuli which may contribute to tissue destruction during chronic inflammation[62].

The S100 protein family consists of 25 proteins involved in a wide range of cellular processes including proliferation, differentiation, migration and inflammation[63]. S100A12 is a protein that belongs to a subgroup of this family termed calgranulins, and induces cellular activation and production of proinflammatory cytokines by binding to receptor for advanced glycation end-products and TLR4[64,65]. It is also a potent chemoattractant for monocytes and neutrophils[66]. S100A12 is elevated in inflammatory diseases such as RA[67], and its levels in saliva has been shown to correlate with gingival inflammation[68].

The colony stimulating factors (CSFs) CSF-1, IL-34, and CSF-2 are crucial for the differentiation, proliferation and survival of monocytes, macrophages, and dendritic cells (DCs)[69]. However, if overexpressed, they can contribute to harmful inflammatory processes[70]. Studies have investigated the expression of these growth factors in oral fluids to assess their potential involvement in PD. Previous studies from our group demonstrated increased CSF-1 levels and decreased IL-34 in saliva from PD patients[71,72]. Elevated CSF-2 levels have been reported in GCF from PD patients, along with increased gene expression levels in gingival tissue[73].

1.4 THE ROLE OF FIBROBLASTS IN PD

GFs are the most abundant cells in the gingiva and play an important role in producing and remodeling the collagenous extracellular matrix. They actively take part in the host response by responding to bacterial products through TLRs and sustain inflammation by exhibiting LPS tolerance[74,75]. Moreover, GFs can also target bacteria directly by producing antimicrobial peptides[76]. In response to inflammatory stimuli *in vitro*, and in co-cultures with peripheral blood mononuclear cells (PBMCs), fibroblasts from the periodontal tissues display elevated expression of the osteoclastogenic factors CSF-1, IL-34, RANKL, TNF-α, and IL-1β, and contain the capacity to induce osteoclast formation[77-80]. Co-culture experiments have also demonstrated that GFs are important for the retention, survival and proliferation of both lymphocyte and monocyte populations[80].

Exposure to inflammatory mediators may result in epigenetic alterations with changes in gene activation and cell phenotype as a result[81]. Fibroblasts display great plasticity and can undergo phenotypic changes in response to different stimulations *in vitro*[82,83]. In line with this, a number of studies have reported altered characteristics of fibroblasts isolated from PD-affected periodontal tissues. Periodontal ligament fibroblasts (PDLFs) from PD patients have been reported to exhibit an enhanced gene expression of several inflammatory mediators including IL-6, and sustain their inflammatory profile when isolated and cultured *in vitro* without bacterial stimuli[84]. In another study, Sokos et al. reported that *P. gingivalis* induces genes related to osteoclastogenesis in PDLFs[85]. Moreover, the authors found that in co-cultures of PDLFs from control subjects and PBMCs, osteoclast differentiation was decreased after *P. gingivalis* challenge. However this was not observed in cultures with PDLFs from PD patients, likely caused by desensitization to *P.gingivalis*. In another study, GFs isolated from inflamed gingiva were shown to quickly upregulate a high expression of the proinflammatory cytokines IL-6, IL-8, IL-1β, and TNF-α in response to *P. gingivalis* LPS compared to GFs from controls[86]. On the other hand, a compromised immune response in GFs isolated from inflamed gingiva has also been reported, with lower IL-6 production[87]. Taken together, the immune regulatory functions of GFs and their possible altered inflammatory characteristics in inflamed gingiva marks them as interesting targets for investigation in PD.

1.5 MONOCYTES

Monocytes belong to the mononuclear phagocyte system together with macrophages and conventional DCs. They derive from hematopoietic stem cells in the bone marrow and go through a series of differentiation stages during their development: a common myeloid progenitor (CMP), a granulocyte-macrophage progenitor (GMP), a common macrophage and DC precursor (MDP), and lastly a committed monocyte progenitor (cMoP)[88-90]. After development, monocytes are released into circulation where they make up approximately 10% of the leukocyte population. They are key effector cells during inflammation by exerting microbicidal functions, but also take part in tissue remodeling[91]. Three monocyte subsets have

been identified based on the differential cell surface expression of the LPS co-receptor CD14 and the Fc gamma receptor IIIa (CD16). Classical monocytes ($CD14^{++}CD16^{-}$) are the most numerous in the peripheral circulation making up 80-90% of the monocyte population and stay in the blood stream for approximately one day. Intermediate monocytes ($CD14^{+}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$) constitute the remaining 10-20% and remain in circulation for approximately 4 and 7 days respectively[92,93]. Recent evidence from gene expression profiling and fate-mapping studies suggests that the intermediate and non-classical subsets are derived from classical monocytes that undergo differentiation and transformation in the blood stream, and thus are not derived from the bone marrow like classical monocytes[93-95].

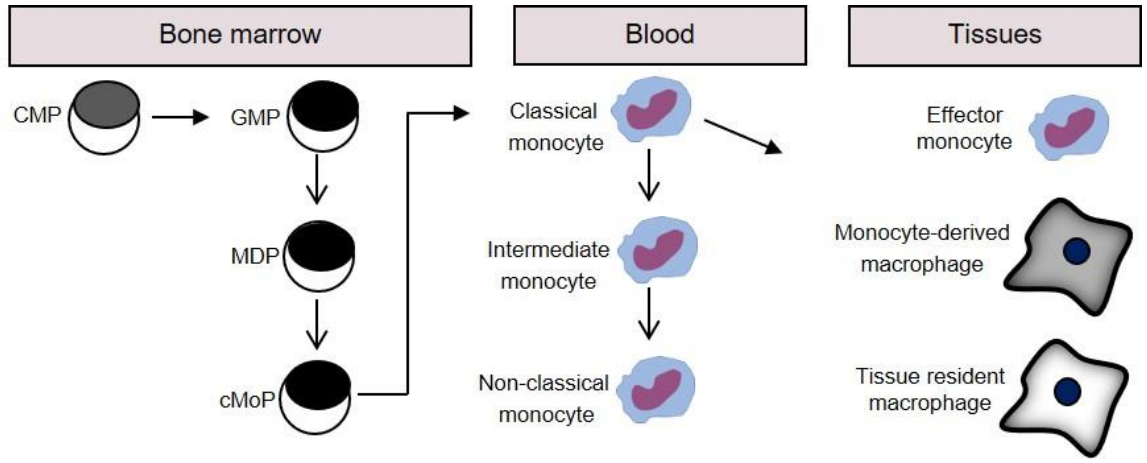

Figure 3. The development of monocytes and macrophages starts in the bone marrow. A common myeloid progenitor gives rise to a granulocyte-macrophage progenitor (GMP). The GMP differentiates into a macrophage and DC precursor (MDP), which in turn differentiates into a committed monocyte progenitor (cMoP). Classical monocytes are differentiated from the cMoP and released into the blood stream and further differentiate into intermediate and non-classical monocytes. Classical monocytes are also recruited to the tissues and take on the role as effector monocytes or differentiate into monocyte-derived macrophages or DCs. Illustration by Reuben Clark.

The monocyte subsets exhibit some distinct features, although varying results have been reported regarding their functions and gene expression. The classical monocyte population has been reported to express high levels of chemokine receptors such as CCR1, CCR2, and C-X-C chemokine receptor (CXCR) 1 which enables them to quickly respond to infection or injury and migrate into the tissue over chemokine gradients[95,96]. This subpopulation also expresses high levels of PRRs and secretes pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 when stimulated with PRR-agonists[97]. Classical monocytes possess high potential for phagocytosis[98,99], as well as antimicrobial activity by producing myeloperoxidase, lysozymes, and S100 proteins[100]. They have been shown to express lower levels of antigen-presenting molecules than the other subsets but strongly upregulate the expression of MHC class II molecules in response to inflammatory stimuli[101]. Their antigen-presentation ability has further

been demonstrated in mice, showing their ability to take up antigens, and recirculate to the lymph nodes[102].

In a study by Wong and colleagues it was reported that intermediate monocytes express a majority of genes and surface proteins at levels between the classical and non-classical subsets[95]. However this subset stands out with a high constitutive expression of MHC II, and thus likely plays an important role in antigen-presentation[101]. Other markers that stand out are CCR5 and TNF receptors, suggesting that they are highly responsive to chemokine and cytokine signaling[103]. Intermediate monocytes also exhibit high expression levels of pro-inflammatory mediators such as IL1β, IL-12, IL-18, IL-23, cathepsins and MMPs[104], and are sometimes referred to as "inflammatory monocytes" due to their expansion in the circulation in several inflammatory diseases[105-107].

The non-classical subset patrols the blood vessels for pathogens or tissue damage and takes part in wound healing processes[108,109]. This patrolling behavior has been shown to be dependent on lymphocyte function-associated antigen 1 and the chemokine receptor CX_3CR1[108]. Non-classical monocytes express lower levels of proinflammatory cytokines in response to TLR-agonists compared with the other subsets and exhibits low expression of genes related to bacterial phagocytosis[97,104]. On the other hand, this subset expresses high levels of the complement component C1QA and CD16A which are involved in antibody mediated phagocytosis[104]. Another feature that stands out with the non-classical monocytes is their role in the inflammatory response against viruses and nucleic acids, which is mediated via TLR7 and TLR8[98].

The intermediate and non-classical monocyte subsets express high levels of CSF-1R compared with classical monocytes and are highly dependent on CSF-1R signaling for their survival and maintenance, which is evident by the reduction of these subsets in RA patients treated with a monoclonal antibody against CSF-1[110].

1.6 MACROPHAGES

Macrophages are distributed throughout the tissues, and for a long time it was believed that they derive solely from circulating blood monocytes[111]. However, studies have shown that tissue-resident macrophages in many tissues-compartments throughout the body are seeded prior to birth and maintain themselves without replenishment from blood monocytes[94,112]. During infection, tissue resident macrophages are supplemented by classical monocytes, which migrate in large numbers into the tissue and take on the role as effector monocytes/inflammatory macrophages. These cells produce high levels of TNF-α and inducible nitric oxide synthase (iNOS) and are crucial players in the clearance of pathogens and orchestrating the immune response. The fate of monocytes is highly dependent on the tissue environment, and they may also differentiate into monocyte-derived DCs or a macrophage phenotype involved in efferocytosis and tissue healing[113].

Regardless of origin, macrophages display great plasticity and undergo polarization in response to different environmental cues. They are generally categorized into two different subtypes: classically activated/M1 and alternatively activated/M2 macrophages. Classically activated macrophages possess crucial antimicrobial properties. They induce and sustain inflammation which in the long run can have harmful effects on host tissues. Alternatively activated macrophages are known as anti-inflammatory and play a key role in phagocytosis, immune regulation, and tissue remodeling[114] (Figure 4).

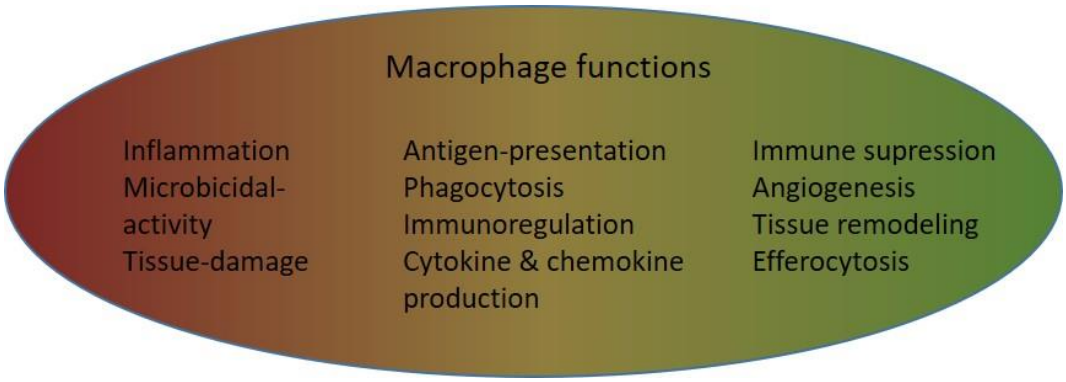

Figure 4. Macrophages are plastic cells that undergo polarization in response to environmental cues. They exert a wide array of functions in inflammation and homeostasis. Illustration by Reuben Clark.

The concept of M1/M2 macrophages stems from the Th1/Th2 polarization of T cells, where IFN-γ producing Th1 cells and IL-4 producing Th2 cells were shown to activate different effector functions in macrophages[115]. Macrophages are commonly polarized into an M1 phenotype with LPS/ IFN-γ and M2 with the Th2 cytokines IL-4/IL13 *in vitro*[116]. Macrophage polarization has also been shown to be impacted by the differentiation stimuli, where CSF-1 and CSF-2 induce anti-inflammatory and proinflammatory features respectively[116]. Among the markers upregulated during M1 polarization are the co-stimulatory molecules CD64, CD80, CD86, and CD40[116,117]. The M1 phenotype produces pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6[116,118]. Apart from IL-4/IL-13, additional stimulus inducing M2 polarization has been found, inducing different gene expression profiles. This has led to further subgrouping of the alternatively activated macrophages into M2a, M2b, and M2c[119]. M2 polarized macrophages express high levels of the scavenger receptors CD206 and CD163[116], as well as the co-inhibitory molecule CD200R, which is an important regulator of the immune response through ligation with CD200[120,121]. More specifically, in terms of the M2a, M2b, M2c concept, *in vitro* studies have demonstrated that CD206 and CD200R expression is up-regulated on M2a macrophages polarized with IL-4/IL-13[120]. Induction of the M2b subset with immune complexes downregulates CD163 and CD206 and induces both pro and anti-inflammatory cytokines such as TNF-α and IL-10[122]. The M2c subtype is polarized with IL-10 and glucocorticoids and expresses high levels of CD163[120]. The three subsets have been associated with different functions including cell growth and tissue regeneration (M2a), immunoregulation (M2b), and clearing of apoptotic cells through efferocytosis (M2c)[122-124]. However, one must keep in mind that the M1/M2 classification is highly simplified, and in the

in vivo situation, macrophages are subjected to a vast array of differentiation and polarization stimuli. Thus, these cells display great plasticity and exist in a continuum of activation states.

1.7 THE CSF-1/IL-34/CSF-1R SYSTEM

The CSF-1R, also known as c-fms or CD115, controls the development and survival of myeloid linage cells, which include a broad range of cell types such as monocytes, tissue macrophages, osteoclasts, dendritic cells, and microglia. It is a class III receptor tyrosine kinase belonging to the platelet-derived growth factor family, and is activated by two ligands; the macrophage growth factors CSF-1 and IL-34[69]. CSF-1R is comprised of an extracellular portion containing five immunoglobulin domains as well as a transmembrane domain, and an intracellular domain. The intracellular domain consists of a juxtamembrane domain (JMD) and an intracellular kinase domain divided by a kinase insert domain. The last component is a C-terminal domain. Upon binding one of its ligands, dimerization and activation of the receptor ensues with autophosphorylation of cytoplasmic tyrosine residues. These are used as docking sites for various signaling proteins such as phosphoinositol 3-kinase (PI3K), extracellular signal regulated kinases (ERK), Src kinases, and nuclear factor-κB (NF-κB) that regulate differentiation, proliferation, survival and activation of macrophages[69,125] (Figure 5).

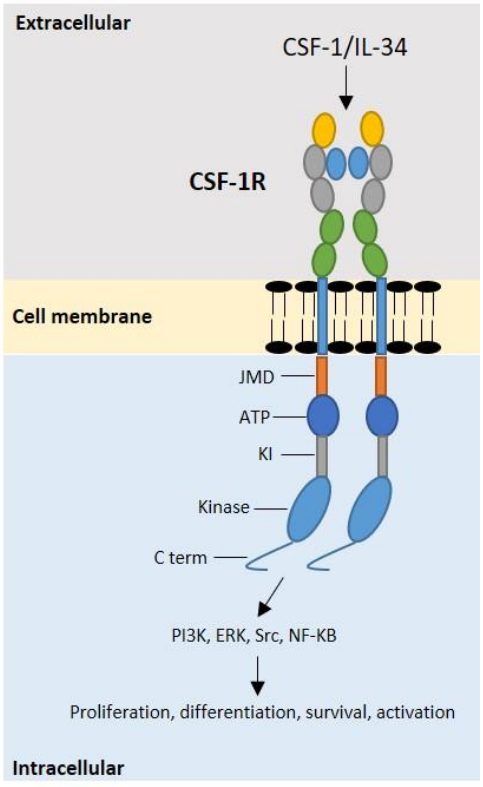

Figure 5. Schematic illustration of the structure of CSF-1R. Illustration adapted from Fleetwood et al. 2016[125]. Abbreviations: CSF-1; colony-stimulating factor 1, IL-34; interleukin-34, JMD; juxtamembrane domain, ATP; adenosine triphosphate binding, KI; kinase insert, C term; C-terminal domain, PI3K; phosphoinositol 3-kinase, ERK; extracellular signal regulated kinases, Src; Src kinases, NF-KB; nuclear factor-κB.

CSF-1 was the first of the CSFs to be discovered, and was shown to stimulate formation of macrophage colonies by murine bone marrow cells[126]. Although CSF-1 and IL-34 share no similarities in DNA sequence, IL-34 was identified in 2008 as an alternative ligand to CSF-1R, capable of differentiating and maintaining mononuclear phagocyte linage cells in a similar

fashion to CSF-1[127]. This important finding explained the more severe phenotype observed in mice lacking CSF-1R (CSF-1R $^{-/-}$) compared with mice lacking CSF-1 (CSF-1$^{op/op}$)[128].

Beyond binding to CSF-1R, IL-34 also interacts with two other receptors; receptor type-protein tyrosine phosphatase zeta and syndecan-1, implying that this cytokine may exert additional functions[129,130]. CSF-1 and IL-34 display overlapping functions however, differences have been found regarding their expression patterns in tissues. Studies in mice have demonstrated that CSF-1 is broadly expressed and crucial for the maintenance of macrophages and regulation of osteoclastogenesis[128]. IL-34 on the other hand, is reportedly high in neuronal tissues and skin, and important for the development of microglia and Langerhans cells[131]. On the other hand, IL-34 induces osteoclast formation and bone resorption together with RANKL and reduces trabecular bone mass in mice when administered systemically, suggesting that it also plays an important role in osteolytic processes[132]. While CSF-1 and IL-34 possess similar ability to differentiate and maintain macrophages, differences have been reported regarding their signal activity[133]. IL-34 induces a stronger tyrosine phosphorylation and downregulation of the CSF-1R compared to CSF-1. Furthermore, macrophages differentiated with either CSF-1 or IL-34 also exhibit differences in their secretome, where CSF-1 induces high secretion of CCL2, whereas IL-34 strongly upregulates CCL11[133]. Boulakirba and colleagues assessed the secretome and potential to phagocytose bacteria by CSF-1 or IL-34 differentiated macrophages in response to polarization[134]. They found that, compared to CSF-1 derived macrophages, IL-34 differentiated M1 and M2 macrophages secrete high levels of CCL17 and IL-10 respectively. Furthermore, CSF-1 differentiated M2 macrophages displayed a higher capability of phagocytosing bacteria in comparison to IL-34 derived M2 macrophages. Moreover, the authors assessed the relevance of the variations in the secretome and showed that CSF-1 and IL-34 derived macrophages differ in their ability to polarize T-cells.

The CSF-1/IL-34/CSF-1R system is implicated in several chronic inflammatory diseases exhibiting tissue destruction. Increased expression of CSF-1 and IL-34 has been reported in synovial fluid from RA patients[135,136], as well as in inflamed RA synovial tissue together with increased CSF-1R expression[137,138]. Other studies have reported elevated levels of CSF-1 and IL-34 in inflammatory bowel disease, and in serum from patients with systemic lupus erythematosus (SLE) [139-142]. Furthermore, in a study on Sjögren's syndrome, IL-34 expression was found to be increased in inflamed salivary glands[143].

CSF-1 concentrations are reportedly higher in the circulation of patients with PD compared with controls[144]. Furthermore, these authors showed that PBMCs isolated from PD patients were able to induce osteoclastogenesis in the presence of RANKL alone, whereas PBMCs isolated from control individuals needed CSF-1 supplementation. These findings suggest a role for CSF-1 in priming PBMCs for osteoclastogenesis. Another group reported reduced osteoclast differentiation and alveolar bone loss in a mouse model treated with a CSF-1R neutralizing antibody[145], further suggesting that the CSF-1/CSF-1R system may play an important role in inflammatory bone resorption during PD. Thus, although CSF-1R signaling

plays a crucial role for the development and maintenance of myeloid cells, overexpression of these factors during chronic inflammation may have harmful effects on host tissues.

1.8 MONOCYTES AND MACROPHAGES IN PD

Researchers have used different experimental approaches in attempt to delineate the role of macrophages in PD. In a mouse model of *P. gingivalis* induced PD, it was demonstrated that $CD86^+$ M1 macrophages are the predominant phenotype in the gingival inflammatory infiltrate as opposed to $CD206^+$ M2 macrophages[146]. Other *in vitro* studies have also reported a shift towards M1 polarization by this periodontal pathogen. Utilizing mouse macrophages, Yu and colleagues demonstrated that *P. gingivalis* induces M1 activation and inhibits the production of α-ketoglutarate which is involved in M2 activation[147].

Several strategies to deplete macrophages or inhibit their activation have also been tested in animal models. For example, liposomes containing the bisphosphonate clodronate induces apoptosis in macrophages when engulfed and phagocytosed[148]. In animal models of PD, treatment with clodronate liposomes depletes gingival macrophages and reduces alveolar bone loss[146,149]. However, since macrophages are also important players in tissue regeneration and healing, depleting them completely may have a negative impact on host tissues. The plasticity and overlapping functions of macrophage phenotypes makes it challenging to target pathological macrophages specifically[150]. Thus, a more beneficial approach may be to induce a phenotype associated with resolution of inflammation and healing. In a mouse model of PD, Viniegra et al. induced a pro-resolving $CD206^+$ macrophage phenotype using the peroxisome proliferator-activated receptor-γ agonist rosiglitazone, which resulted in reduced bone loss during PD and enhanced bone regeneration during the healing phase[149]. This effect was found to be partly mediated by the actions of M2 derived cystatin C on osteoblasts and osteoclasts.

The importance of the chemokine CCL2 in periodontal inflammation is emphasized by the correlation of its levels in GCF with CAL[151]. Furthermore, in an experimental PD model utilizing rats, CCL2 was found to correlate with inflammatory cell infiltration in the gingiva as well as alveolar bone loss[152]. These findings indicate that monocytes are recruited from the circulation and contribute to tissue degradation in PD. Studies investigating monocyte subsets in humans have shown increased proportions of the intermediate and non-classical subsets in the circulation of PD patients[153-155]. However, there are also reports of unaltered proportions in the circulation during PD[156]. These conflicting results emphasize the need for more studies to delineate the characteristics of monocytes in the circulation of PD patients. Characterization of monocyte subsets has also been performed in gingiva showing elevated frequencies of intermediate and non-classical subsets[154,157].

Isolation and culture of monocytes from PD patients and controls have rendered interesting results regarding their activation state during PD. In a study by Cheng et al. *P. gingivalis* induced the activation markers CD40, CD54, and HLA-DR on monocytes. Furthermore, monocytes isolated from PD patients induced significantly higher IL-17 production by T cells

in co-culture compared with monocytes from control individuals[158]. In another study on whole blood samples, mass cytometry analysis revealed heightened TLR2/TLR4 signaling responses to *P. gingivalis* LPS by monocytes from PD patients[159]. Together, these findings indicate that peripheral blood monocytes display an activated state and are primed for inflammation in PD.

Enhanced osteoclast activity and bone resorption are hallmarks of PD. In line with this, Herrera et al reported that PBMCs isolated from PD patients are primed for osteoclast differentiation and display high resorption activity when differentiated into osteoclasts compared with PBMCs from controls[144]. Moreover, PBMCs from PD patients exhibited elevated production of inflammatory mediators, including TNF-α, IL-1β and CCL2, further supporting that these cells display an enhanced activation state in PD.

Increased proportions and numbers of $CD68^+$ macrophages have been reported in PD-affected gingiva with correlations to collagen destruction[26,160,161]. Studies attempting to characterize macrophage phenotypes have presented varying results. Comparing typical markers used for identifying classical and alternative macrophages, including iNOS, CD80, CD206, and CD163, as well as analysis of pro- and anti-inflammatory cytokine expression have resulted in reports of higher proinflammatory macrophage activity in PD[157,162]. On the other hand, in a study by Garaicoa-Pazmino and colleagues, no difference in macrophage polarization was found in gingiva from PD patients and control individuals[163].

The role of monocytes and macrophages, as well as factors that regulate them in PD remain to be fully elucidated. In the studies included in this thesis we utilized human samples (gingival tissue, peripheral blood, saliva and isolated cells) with the aim to investigate monocyte and macrophage functions in PD.

2 AIMS OF THE THESIS

The overall aim of the thesis was to investigate the involvement of monocytes and macrophages and factors that regulate them in periodontitis (PD).

Specific aims:

- To explore monocytes/macrophages as well as myeloid-related inflammatory mediators in clinical samples from individuals with and without PD.

- To assess functions of monocyte-derived cells in inflammation and tissue degradation using oral tissue cultures.

- To investigate the role of gingival fibroblasts regarding production of growth factors that regulate monocyte/macrophage function.

- To explore potential strategies to dampen monocyte/macrophage mediated inflammatory processes.

3 METHODS

This section will briefly describe the methods used for the experiments in this thesis.

3.1 ETHICAL CONSIDERATIONS

The collection of human gingival tissue samples, buffy-coated blood from anonymous donors (**study I-IV**), and peripheral blood from PD patients and controls (**study II**) was approved by the regional ethics committee in Stockholm, Sweden. The collection of saliva samples (**study II**) was approved by the regional ethics committee at Lund University, Sweden. All the studies were carried out in accordance with the declaration of Helsinki. A written, informed consent was given by all participants before inclusion in the studies.

3.2 RESEARCH SUBJECTS

For all studies, gingival tissue biopsies were harvested during planned periodontal surgery (PD-patients) or non-periodontal surgery, e.g. surgical extractions or dental implant surgery (controls). The inclusion criteria for PD patients were ≥ 4 teeth with a probing depth of ≥ 6 mm and a persistent pocket of ≥ 6 mm at the site of biopsy collection. The inclusion criteria for the controls were no pockets with probing depth of ≥ 4 mm and no alveolar bone loss exceeding 1/4 of the root length. Exclusion criteria for both groups included subjects with systemic or genetic diseases or antibiotic treatment in the last three months prior to oral surgery.

Gingival tissue used for immunohistochemistry was collected in histocon and the samples were embedded in optimal cutting temperature compound on the collection day. Gingival tissue samples were collected in RNAlater for mRNA-analysis and stored in 4°C for 1-3 days. The RNAlater was then removed and samples stored in -80°C until RNA extraction. For western blot, gingival tissue samples were snap frozen using dry ice and then stored in -80°C until homogenization and protein extraction. Tissue to be used for flow cytometry was collected in complete RPMI media and stored on ice. Digestion of the tissue was performed 2-4 h after collection.

For **study II**, a cohort of individuals living in Kalmar county, Sweden was recruited, and all participants answered a questionnaire regarding sex, smoking habits, and the presence of diseases. The participants were orally examined and provided a sample of stimulated saliva.

Peripheral blood was obtained from PD patients and controls recruited at the student dental clinic at Karolinska Institutet (**study II**). Buffy-coated blood was obtained from anonymous donors at the Karolinska University hospital, Stockholm (**study I, II, and IV**). The blood was used for PBMC and monocyte isolation.

3.3 CELL AND TISSUE CULTURE

3.3.1 **Gingival fibroblast isolation and culture (study III)**

Gingival tissue explants used for primary GF isolation were placed in αMEM supplemented with fetal bovine serum (FBS), glutamine, penicillin streptomycin (pen-strep), and Antibiotic-antimycotic after excision. Using an explant method, the biopsies were cut into small pieces and placed under cover glasses in 6 well plates and incubated in 37°C with 5% CO_2. When cultures of GFs were established, the gingival tissue was removed. Cells were trypsinized and transferred to cell culture flasks. Dulbeccos's Modified Eagle Medium (DMEM/F12), supplemented with FBS, glutamine and pen-strep was added and changed every other day. GFs were passaged when 80% confluent. For experiments, cells in passages 4-6 were cultured in 6 or 12 well plates. The cells were treated with TNF-α, IL-1β, *P. gingivalis* or *Escherichia coli* LPS and untreated cells were used as controls. Cell lysates were collected for RNA isolation and mRNA analysis and supernatants were collected for analysis of protein expression with ELISA. GFs were also cultured on chamber slides. When 80% confluent, cells were fixed in ice cold acetone and subjected to immunofluorescent staining.

3.3.2 **Isolation and culture of PBMCs and monocytes (study I, II, and IV)**

In **study I**, **II** and **IV**, PBMCs were isolated from buffy-coated blood using Ficoll-Hypaque gradient centrifugation. The cells were used for flow cytometry analysis directly or cultured in complete RPMI 1640 media supplemented with different combinations of CSF-1, IL-34 or *E. coli* LPS in 37°C with 5% CO_2. Cells were also incubated with a CSF-1R neutralizing antibody or isotype control antibody. At the end of experiments, supernatants were collected for analysis of cytokine secretion and cells were subjected to flow cytometry analysis.

Monocytes were isolated from PBMCs from buffy-coated blood (**study I** and **II**) and from peripheral blood from PD and control donors (**study II**), using a monocyte enrichment kit without CD16 depletion. The cells were processed for mRNA expression analysis, used for monolayer experiments, and differentiation of macrophages. Monocytes were also implemented in the oral tissue cultures. Supernatants from monocytes were collected for measurement of various cytokines with enzyme-linked immunosorbent assay (ELISA). Monocytes and macrophages were further subjected to flow cytometry analysis.

3.3.3 **Oral mucosa tissue cultures (study I and II)**

An oral mucosa tissue culture was developed to study the interaction between monocytes, fibroblasts and epithelial cells. The membrane of transwell culture inserts were coated with an acellular collagenous layer (collagen I) and placed in 6-well plates. Next, primary GFs were trypsinized and suspended in complete DMEM and diluted in a collagen/DMEM suspension. The suspension containing GFs was then added to the acellular layer in the inserts, followed

by addition of 2 ml complete DMEM media to the wells after 2 h incubation in 5% CO_2 at 37°C. The models were incubated for 5-7 days letting the fibroblasts remodel the collagen. Media was changed every other day. Thereafter, the media in the wells was removed and replaced with 1.5 ml complete DMEM. Monocytes isolated from PBMCs and suspended in 50 µl complete RPMI were added on top of the fibroblasts and incubated for 1.5 h. Next, 1,5 ml complete DMEM was carefully added to the inserts followed by a 24 h incubation to let the monocytes attach. The media was then removed from the inserts and the media in the wells was replaced with 1.5 ml complete DMEM. TERT-immortalized normal human oral keratinocytes (OKF6/TERT-2 cell line)[164] were suspended in 50 µl complete keratinocyte serum free (K-SFM) media, added on top of the monocyte/fibroblast-layer, and incubated for 1.5 h. Then, 2ml of K-SFM media was added to the inserts followed by a 48 h incubation. The models were then air-exposed by removing all media and adding 1.8 ml complete K-SFM to the wells, letting the epithelium to stratify. The oral tissue cultures were air-exposed for 3-4 days with media change every second day. The tissue cultures were subsequently subjected to stimulations with *E. coli* LPS and IFN-γ in the inserts. For experiments assessing the effect of COX2 inhibition, a COX2 inhibitor was added to the wells 30 min before LPS stimulation. Oral mucosa tissue cultures without monocytes were also stimulated with rhMMP12 in the inserts. Supernatants were collected for analysis of cytokine production and the oral tissue cultures were prepared for flow cytometry, mRNA or histological analysis.

3.3.4 **Gingival explant experiments (study IV)**

Gingival tissue from PD patients was collected in complete RPMI media and cut into small pieces using a biopsy punch and scalpel. The tissue was then incubated in complete RPMI media with a CSF-1R neutralizing antibody or IgG1 isotype control antibody for 72 h in a 96 well plate. Supernatants were collected after 24 h and 72 h for protein analysis. At the end of the 72 h time-point, the gingival tissue was washed in PBS and placed in a protein lysis buffer supplemented with a protease and phosphatase inhibitor cocktail. The tissue was homogenized using a tissue lyser and the total protein of each tissue piece was measured with a colorimetric assay. MMPs in the supernatants were analyzed with a bead-based multiplex array and normalized to the total protein of the tissue. Gingival explants were also subjected to flow cytometry analysis.

3.4 GENE EXPRESSION ANALYSIS

3.4.1 **RNA isolation, cDNA synthesis and quantitative real-time PCR**

RNA extraction from gingival tissue and cells was performed with different methods. The RNA quantity and purity were measured using a spectrophotometer and subsequently reversed transcribed into complementary DNA (cDNA) and used for quantitative real-time PCR (qRT-PCR) analyses.

For qRT-PCR, SYBR Green with specific primers (**study II**, **III**, and **IV**, Table 1) or TaqMan gene expression assays (**study I**) were used. The relative mRNA expression of specific genes was calculated using the comparative threshold (ΔΔCt) method. First the Ct values of each sample was normalized to the housekeeping gene GAPDH (CT target- CT housekeeping gene = ΔCt). The ΔΔCt (ΔCt sample – ΔCt control average) was calculated, and fold change in mRNA expression calculated using the formula: 2^(-ΔΔCt).

Table 1. Primer sequences used for RT-qPCR with SYBR Green.

Gene	Forward	Reverse
CD206	GGGTTGCTATCACTCTCTATGC	TTTCTTGTCTGTTGCCGTAGTT
CD64	GCATGGGAAAGCATCGCTAC	GCAAGAGCAACTTTGTTTCACA
CD68	GTCCACCTCGACCTGCTCT	CACTGGGGCAGGAGAAACT
CSF1	GTGGAACTGCCAGTGTAGAGG	TGGAGGGCAGACCACATT
CSF1R	ATGCTACCACCAAGGACACA	AGCCTCCTGGGTTTCTGG
IL34	GCCACCCATCCTGGAAGTA	GACAACACGGATTCCACCTT
S100A12	CACATTCCTGTGCATTGAGG	GGTGTCAAAATGCCCCTTC
GAPDH	TCCACTGGCGTCTTCACC	GGCAGAGATGATGACCCTTTT

In **study I**, a multiplex real-time PCR was used with custom primers and probes to analyze the mRNA expression of specific genes in gingival tissue from PD and controls. Genes analyzed were: *TNF*, *COX2*, *CXCL11*, *IL10*, *IL1RN*, *IDO*, *MRC1*, *DCSIGN*, *PDGFD*, *MMP12*, and *TIMP1*. *GNB2L1* was used as endogenous control. Absolute quantification was performed using a set of purified amplicons as standards.

3.5 PROTEIN EXPRESSION ANALYSIS

3.5.1 **Western blot**

Total protein was extracted from gingival tissue for protein analysis using a protein extraction buffer, and the tissue was homogenized using a tissue lyser. Fifteen micrograms of total protein per lane were loaded and separated by precast polyacrylamide gels and subsequently transferred to nitrocellulose membranes. The membranes were blocked with TBS-T containing non-fat milk powder and subsequently washed in TBS-T. Primary antibodies for MMP-12, S100A12, CSF-1, IL-34, and CSF-1R were incubated in 4°C overnight followed by incubation with secondary antibodies. The membranes were developed with a chemiluminescent solution and an imaging system and software were used for detection. The membranes were then washed, blocked and stained for β-actin which was used as loading control. For quantification of bands, the volume intensity of the protein of target was normalized to the loading control.

3.5.2 **Immunohistochemistry and immunofluorescence**

In **study II**, **III**, and **IV**, immunohistochemistry was utilized to analyze the expression and localization of inflammatory mediators and cell markers in gingival tissue. Acetone was used as fixative and the washing procedure was performed with PBS containing non-fat milk and bovine serum albumin. To block endogenous peroxidase, the tissue sections were incubated in methanol containing H_2O_2 followed by incubation with blocking buffer. Primary antibodies for S100A12, CSF-1, IL-34, and CSF-1R were incubated in 4°C overnight followed by incubation with secondary antibodies. Avidin-Biotin complex was added to the tissue sections. Development was performed with diaminobenzidine (DAB) solution and counterstaining with Mayer's hemalum. Lastly, the sections were dehydrated with ethanol and xylene, and then mounted with Pertex mounting medium. A light microscope was used for development.

Immunofluorescence was used in **study I** and **II** for double labeling of cells and analysis of structural proteins in gingival tissue and oral tissue cultures. Frozen sections of gingival tissue or oral tissue cultures were fixed using acetone or formaldehyde. Sections were incubated with Image It Fx signal enhancer and blocked with serum and Background Buster. The sections were incubated with primary antibodies in 4°C overnight in a wet chamber. Staining was detected by fluorochrome-labeled secondary antibodies and cell nuclei counter staining was performed with DAPI. The sections were mounted with Pro Gold Antifade mounting medium. A fluorescence microscope or confocal microscope was used for visualization.

3.5.3 **Enzyme-linked immunosorbent assay (ELISA)**

In all studies, commercial ELISA kits were used to measure protein concentrations in supernatants from *in vitro* cell culture experiments or saliva. Concentrations of CSF-1, S100A12, TNF-α, IL-1β, IL-6, IL-8, and MMP-9 were measured with sandwich ELISA: a

capture antibody for the protein of interest was coated in 96-well plates and incubated overnight in room temperature. Next, the plates were washed to remove unbound antibodies, and subsequently incubated with the samples and standards. A biotinylated detection antibody that binds to the target protein was then added to the wells forming and antibody-antigen complex. Unbound antibodies were washed away and streptavidin-HRP added to the wells, binding to the detection antibody. The washing step was repeated followed by development with substrate solution. The development was stopped with 2N H_2SO_4 and the optical density of the wells was determined with a microplate spectrophotometer. Concentrations of IL-34 and MMP-12 were measured with pre-coated sandwich ELISAs.

3.5.4 **Bead-based multiplex array (study IV)**

Supernatants from gingival explants were analyzed with a Bio-Plex Pro Human MMP 9-plex panel kit. The MMPs included in the panel were: MMP-1, -2, -3, -7, -8, -9, -10, -12, and 13. The fluorescently dyed magnetic beads each have a unique color code to discriminate between the different analytes. Capture antibodies are covalently coupled to beads and bind the targets of interest. The beads were added to the wells of a microplate and washed, followed by incubation of standards and samples. Following washing, a detection antibody was added to the wells. The addition of the secondary antibody creates a sandwich complex. Next, the wells were incubated with streptavidin-PE which creates the final detection complex (Figure 6). The beads were then resuspended in assay buffer and data was acquired with a bio-plex system. The bio-plex uses two lasers during data acquisition; one for bead-classification and one to excite the PE to generate a reporter signal.

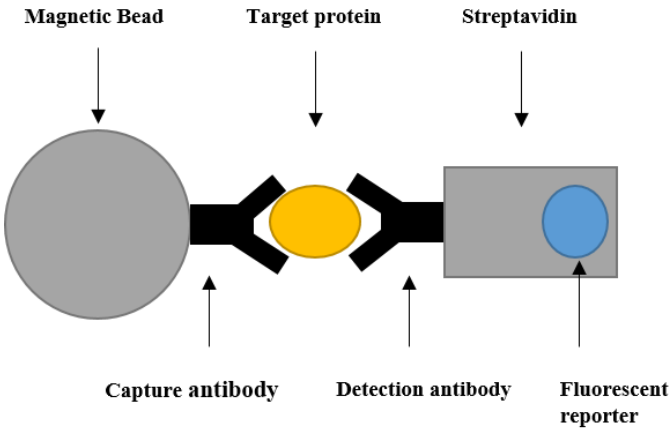

Figure 6. Schematic illustration of a bio-plex sandwich complex used in the bead based multiplex array. Illustration by Reuben Clark

3.5.5 **Flow cytometry (study I, II, and IV)**

Analysis of the cell surface and intracellular protein expression of individual cells in digested gingiva or cells isolated from blood was performed with flow cytometry.

Flow cytometry works by labeling cells in a suspension with fluorochrome-conjugated antibodies targeting specific cell components. When labeled cells are passaged in a single file through the flow cytometer, the fluorochromes are excited by lasers and emit light at different wavelengths. The emitted light is passed through optical filters and measured by detectors. Thus, if light of a certain wavelength is detected, it means that the cell was labeled with a specific fluorochrome-conjugated antibody. Determination of the size and granularity of cells is performed by measuring forward and side scattering of light.

Gingival tissue harvested during surgery was cut into small pieces and enzymatically digested with Dnase I and collagenase II. After digestion, the suspension was filtered through a mesh filter and centrifuged. First, the cells were subjected to viability stain followed by Fc block. The cells were then incubated with different combinations of fluorochrome-conjugated antibodies on ice. Next, the cells were fixed and analyzed, or permeabilized and stained with intracellular antibodies. The samples were analyzed with a flow cytometer and data processed with FlowJo software.

3.6 STATISTICAL ANALYSIS

Data analysis was performed with GraphPad prism or SPSS statistical software. The statistical methods used in the studies are briefly outlined below. Detailed descriptions of the statistical methods used for each experiment are provided in the studies.

In **study I,** Mann-Whitney *U* test or unpaired t-test was applied when appropriate for comparison of two groups. Friedman test with Dunn's multiple comparisons test was used to compare three groups or more.

In **study II**, Mann-Whitney U test or Wilcoxon was used for comparison of two groups. For comparison of three groups or more, Friedman test with Dunn's multiple comparisons test, or Kruskal-Wallis-test with Dunn's multiple comparisons test was applied. Spearman rank correlation coefficient was applied to analyze correlations.

In **study III**, Mann-Whitney *U* test was applied to compare protein expression in gingival tissue and mRNA expression in GFs. Kruskal-Wallis test with Dunn's multiple comparisons test was used for comparison of ELISA data.

In **study IV**, Mann-Whitney *U* test or paired t test was applied to compare two groups. One-way or two-way ANOVA with Sidak's multiple comparisons test was used when appropriate to compare three groups or more.

4 RESULTS AND DISCUSSION

The following section will give an overview of the results in this thesis.

4.1 STUDY I

4.1.1 MMP-12 expression in gingival tissue

To assess genes related to myeloid cell functions in gingival tissue from PD patients and controls, a mRNA multiplex array was utilized. This revealed an up-regulation of *MMP12*, *COX2*, *TNF*, and dendritic cell specific intercellular adhesion molecule-3 grabbing non-integrin in PD. Several MMPs are elevated in PD, and they have been proposed to be potential biomarkers for the disease[53,165]. Therefore, MMP-12 protein expression was further analyzed in gingival tissue using western blot, which revealed elevated MMP-12 in PD-affected gingiva. In a later study by our group, salivary levels of MMP-12 were reported to be associated with periodontal inflammation[68], further suggesting its involvement in PD.

In vitro experiments have shown that MMP-12 contains a potent ability to degrade the elastin precursor tropoelastin[56]. Interestingly, the expression of tropoelastin was significantly lower in gingival tissue from PD patients, suggesting that MMPs targeting elastin, such as MMP-12, are involved in undermining the oral mucosa during inflammation. RA is another chronic inflammatory disease exhibiting tissue degradation, and previous findings have demonstrated that MMP-12 is elevated in the synovium during RA and expressed by macrophages[55]. In consonance with this, we found that the expression of MMP-12 was primarily associated with $CD68^+$ cells at inflamed sites in gingival tissue. Flow cytometry analysis of digested gingival tissue further revealed that the increased expression of MMP-12 in PD was attributed to a $CD68^+CD14^+CD64^+$ cell population, likely to be monocyte-derived cells. These cells displayed low surface molecule expression of the co-inhibitory receptor CD200R. This is interesting considering that low CD200R expression on monocytes and macrophages has been found in other inflammatory diseases, such as RA and sarcoidosis, and is associated with an enhanced inflammatory state[166,167].

4.1.2 Oral tissue cultures to study monocyte-derived cells

Co-culture systems provide the opportunity to study cellular functions in a setting resembling the tissue environment. We developed an organotypic three-dimensional oral tissue culture consisting of primary gingival fibroblasts embedded in collagen I, monocytes isolated from PBMCs, and the oral keratinocyte cell line, OKF6/TERT-2[164]. This system allowed us to study the function of monocyte-derived cells during oral tissue inflammation. Histologically, the oral tissue cultures with implanted monocytes resembled normal gingival tissue morphology with

a stratified epithelium on top of the fibroblasts/collagen matrix (Figure 7). The models also displayed a similar expression pattern of structural proteins as seen in normal gingival tissue.

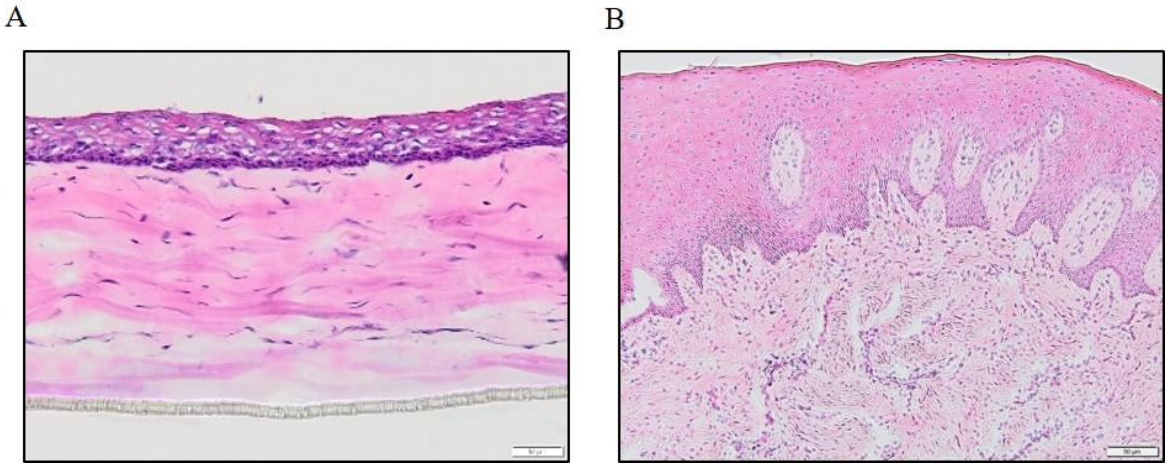

Figure 7. Hematoxylin and eosin staining showing morphology of A) Oral mucosa tissue culture. B) Normal gingival tissue. The morphology of the oral mucosa tissue cultures resembles that of normal gingival tissue, with a stratified squamous epithelium and a basement membrane separating the epithelium from the underlying connective tissue. Scale bars 50µm. 20x magnification. Photos by Reuben Clark.

A constitutive production of CSF-1 along with up-regulation of the markers CD14, CD68 and CD163 over time indicated that the model promoted monocyte survival and macrophage differentiation. Classical macrophage activation with LPS and IFN-γ has been reported to upregulate the expression of CD80 and downregulate CD200R in monocultured macrophages[168]. In line with this, LPS/IFN-γ stimulation induced inflammation in the tissue cultures, resulting in upregulation of CD80 and CD14 expression and downregulation of CD200R on monocyte-derived cells. The downregulation of CD200R is in line with the phenotype of monocyte-derived cells we observed in PD-affected gingiva.

Utilizing multiplex real-time PCR, we analyzed the expression of genes associated with monocyte/macrophage function in oral tissue cultures with and without monocytes. LPS/IFN-γ stimulation in cultures with monocytes induced an inflammatory profile with significantly increased mRNA expression of *MMP12*, *TNF* and *COX2* compared with unstimulated cultures, thus resembling the gene expression profile of inflamed gingival tissue. In contrast, tissue cultures without monocytes exhibited low expression of these markers and the expression was not induced in response to inflammatory stimulation. This demonstrates the importance of monocytes in inducing an inflammatory gene expression profile in the oral tissue cultures in response to proinflammatory stimuli. This was further confirmed with ELISA demonstrating the need for monocytes for the secretion of MMP-12 and TNF-α. Co-culture experiments have shown that GFs maintain both lymphocyte and monocyte populations and that the interaction between fibroblasts and PBMCs induce proinflammatory cytokine production[80,169]. Another study reported enhanced production of MMP-3 and MMP-9 in response to LPS stimulation in co-cultures of GFs and macrophages[170]. Thus, the crosstalk between non-immune cells and monocyte-derived cells is important for a wide array of functions and studying these cells in co-culture systems may reveal functions otherwise missed in a monoculture setting. In line with

the previously found effect of MMP-12 on matrix components[56,171], we found that stimulating the oral mucosa with recombinant MMP-12 resulted in detachment of the epithelium from the fibroblast matrix, and lower tropoelastin and fibronectin expression in the oral mucosa tissue cultures, thereby adding to the notion that MMP-12 plays a role in degrading the oral mucosa.

4.1.3 Modulation of the CD200/CD200R pathway in monocyte-derived cells

Oral tissue cultures and monocytes cultured in monolayer were used to investigate the inflammatory pathways regulating MMP-12 production. The cyclooxygenase (COX)-2/prostaglandin E2 (PGE2) pathway has previously been reported to be an important regulator of MMP expression in macrophages[172]. In the presence of LPS and a COX2 inhibitor, the PGE2 production was attenuated. However, the production of MMP-12 remained unaffected, leading to the conclusion that MMP-12 production is not mediated via the COX2 pathway. CSF-2 is elevated in PD[73], and we found increased CSF2 gene expression in the oral mucosa tissue cultures containing monocyte-derived cells when inducing inflammation using IFN-γ and LPS. Previous studies have demonstrated that CSF-2 stimulation induces MMP-12 production by monocytes[173], and in line with this, we observed induced MMP-12 production by monocyte-derived in response to CSF-2 stimulation.

CD200 is an important regulator of monocytes and macrophages through interaction with CD200R[174]. Moreover, CD200 has been shown to inhibit the RANKL signaling pathway as well as osteoclast maturation[175], which may have implications in osteolytic diseases such as PD. The low expression of CD200R on monocyte-derived cells in PD could possibly lead to an enhanced inflammatory state due to the lack of CD200 mediated inhibition, with elevated MMP-12 production as a result. In support of this, addition of a CD200 Fc chimera significantly reduced the MMP-12 production by monocyte-derived cells. Taken together, these results suggest that MMP-12 produced by monocyte-derived cells is involved in the tissue-degrading events during PD, and that the CD200/CD200R pathway is a potential target to reduce the impact of MMP-12 on host tissues.

4.2 STUDY II

4.2.1 S100A12 expression in gingival tissue and saliva

S100A12 belongs to the S100 family of calcium binding proteins and plays an important part in the initiation of innate immune responses by acting as a pro-inflammatory alarmin[176]. We aimed to assess the expression of this protein in samples from subjects with and without PD. In gingiva, histological assessment revealed abundant S100A12 staining in proximity to the inflammatory infiltrate in the connective tissue, and western blot analysis demonstrated increased S100A12 protein in PD gingiva compared with controls. Elevated levels of S100A12 have been detected in serum and synovial tissue from RA patients as well as in fecal samples

from IBD patients, suggesting that this protein potentially could be utilized as a biomarker of inflammation[67,177]. In a large cohort of orally examined participants, we found that the salivary levels of S100A12 were increased with BOP and presence of gingival pockets. Furthermore, S100A12 was significantly higher in patients with severe stages (III and IV) of PD compared with periodontally healthy participants and individuals with gingivitis. Individuals with PD stages I and II showed a tendency towards increased S100A12 compared with the healthy/gingivitis group. These results are supported by a previous study conducted by our group, showing that salivary levels of S100A12 correlate with the severity of periodontal inflammation[68]. Besides being regulated in saliva, S100A12 is also elevated in serum and GCF from PD patients and correlates with the expression of C-reactive protein[178]. Together, these findings suggest that S100A12 is a potential biomarker for PD. Whether or not it could be targeted as a therapeutic measure remains to be elucidated. However, it has been proposed as a potential target for inflammatory osteolysis due to its capacity to differentiate monocytes into osteoclasts and enhance their bone resorption ability[179].

4.2.2 Expression of S100A12 in monocytes and during macrophage differentiation

We aimed to assess the expression of S100A12 in monocyte subsets as well as during macrophage differentiation and polarization. For this we used PBMCs from buffy-coated blood and isolated monocytes using a monocyte enrichment kit. Flow cytometry was used to identify the subsets with a gating based on CD14 and CD16 expression. According to previous results in peripheral blood, the frequency of classical monocytes was around 85%, intermediate monocytes approximately 7%, and the non-classical subset around 8%[95,180]. Classical monocytes displayed the highest proportion of $S100A12^+$ cells and also had the highest expression as measured by median fluorescence intensity, whereas non-classical monocytes displayed the lowest expression and frequency of $S100A12^+$ cells, which is in line with previous findings[95,181]. Intermediate monocytes expressed S100A12 at levels between the classical and non-classical subset but had a more similar expression to classical monocytes. Classical monocytes are potent responders to bacterial stimuli[95,97], and the high expression of S100A12 by this subset further suggests that S100A12 is involved in supporting inflammation[182].

The gene expression of S100A12 significantly decreased during macrophage differentiation in response to CSF-1 which is also in accordance with previous reports[183]. In our study, we found that the secretion of S100A12 decreases during macrophage differentiation, and thereby follows the gene expression pattern. The decreased expression of S100A12 in non-classical monocytes and during macrophage differentiation is in line with the notion that monocytes are believed to undergo maturation from classical monocytes, to intermediate and lastly the non-classical subset[93]. CSF-1-differentiated macrophages were polarized into classically activated macrophages using LPS and IFN-γ, or alternatively activated macrophages with IL-4 and IL-13. Similar to the results by Shah et al.[183], the S100A12 gene expression was unaltered with

macrophage polarization. Moreover, we found that S100A12 secretion by macrophages remained unaltered with polarization. Oral tissue cultures were prepared either with or without monocytes to assess the contribution of monocyte-derived cells to S100A12 secretion. In oral tissue cultures with monocytes, the S100A12 production was significantly increased over time and in response to LPS/IFN-γ stimulation. In contrast, the tissue cultures without monocytes produced low levels of S100A12 and did not respond to inflammatory stimulation, suggesting that the presence of monocytes is crucial for S100A12 production. We then stimulated monocytes with media from tissue cultures without monocytes to assess whether factors secreted by GFs and epithelial cells influence the S100A12 production. No apparent difference in S100A12 secretion was observed in response to media from unstimulated and IFN-γ/LPS stimulated cultures without monocytes. Thus, direct cell-cell interactions between monocytes and GFs/epithelial cells may play an important role for the production of S100A12 in the oral tissue cultures.

4.2.3 **S100A12 expression in peripheral blood monocytes and gingival macrophages during PD**

Next, we sought to analyze the characteristics of monocytes in the circulation during PD. Peripheral blood was obtained from PD patients and controls to assess possible differences in monocyte subsets and their expression of S100A12. No significant difference in monocyte counts was observed comparing patients and controls. Flow cytometry was used to analyze the monocyte subsets, revealing lower frequencies of non-classical monocytes in PD. This is a conflicting result to what other studies have reported, showing increased proportions of intermediate and non-classical monocytes in the circulation of PD patients[154,157]. On the other hand, one study reported no differences in the frequencies of monocyte subsets in circulation during PD[156]. A possible explanation for these conflicting results could be variations in the patient groups analyzed, such as genetic and lifestyle factors, and severity of PD. Non-classical and intermediate monocytes are increased in peripheral blood in several chronic inflammatory diseases, such as Crohn's disease and RA[184,185], which supports the notion that these subsets are expanded in the circulation during inflammation. One could speculate that the reported increased proportion of non-classical and intermediate monocytes in the circulation during PD could in part be explained by the migration of classical monocytes into the tissues. Another speculation is that leakage of periodontal pathogens into the circulation upregulates inflammatory mediators and enhances the activation and differentiation state of monocytes in the circulation

We proceeded to assess the S100A12 expression in monocytes from PD patients and controls. Interestingly, higher proportions of $S100A12^{+}$ monocytes were observed in PD. In consonance with this, monocytes from PD patients exhibited higher secretion of S100A12 when cultured for 24 h *in vitro*. This is in line with the fact that circulating monocytes have been shown to be primed for inflammation in PD patients, displaying several features indicating that they are

more responsive to bacterial/LPS stimuli[159]. The most prominent difference in S100A12 expression was found in the intermediate followed by non-classical monocytes.

We did not investigate the proportions of monocyte subsets in gingival tissue, however it has been reported that intermediate and non-classical monocytes are increased in PD gingival tissue[154,157]. Since these subsets represent a later maturation state, an explanation for their reported increase in inflamed gingival tissue could be that migrated classical monocytes undergo differentiation in response to CSFs and other factors in the tissue. We assessed the S100A12 expression in macrophage populations in gingival tissue based on the markers CD14 and CD64. Interestingly, the proportion of $S100A12^+$ macrophages was increased in PD-affected gingival tissue. Furthermore, the $S100A12^+$ macrophages in PD were less frequently positive for CD206. Since studies in mice have shown that expression of CD206 is associated with the healing phase of PD[149], this may suggest that $S100A12^+$ macrophages play a role in the tissue destructive processes during periodontal inflammation.

Taken together, the notion that monocytes are important for the production of S100A12 in the oral tissue cultures, and that monocytes and macrophages display enhanced S100A12 expression in PD, suggests that they are in part responsible for the abundant expression of S100A12 in PD-affected gingival tissue. However, in this study we did not investigate other immune cell populations, and S100A12 is abundantly produced by neutrophils as well[182]. Thus, expanding the oral tissue culture experiments to include other immune cell populations would further delineate the expression and function of S100A12 in oral tissue inflammation.

4.3 STUDY III

4.3.1 CSF-1 and IL-34 expression in gingival tissue

The macrophage growth factors CSF-1 and IL-34 are crucial for the proliferation, differentiation, and survival of myeloid cells but may contribute to tissue degradation in chronic inflammation[70]. To assess and quantify the expression of CSF-1 and IL-34 in gingiva, samples were collected from PD patients and controls. The tissue was homogenized and subjected to western blot analysis. The results revealed elevated levels of CSF-1 in PD gingiva whereas IL-34 levels displayed similar levels in the two groups. In a previous study from our group, elevated CSF-1 but decreased IL-34 salivary levels were found in PD[71]. In that study, CSF-1 was also found to correlate with MMP-8, and both CSF-1 and MMP-8 correlated negatively with IL-34. CSF-1 expression may have clinical relevance as its levels in saliva are significantly associated with the percentage of probing depths ≤ 4 mm[72]. Furthermore, CSF-1 has been shown to be increased in the circulation of PD patients and possibly prime PBMCs for osteoclast differentiation[144]. The finding that CSF-1 but not IL-34 is upregulated in PD-affected gingiva adds to the notion that CSF-1 and IL-34 may be differentially regulated during periodontal inflammation. However, of note, increased IL-34 levels have been reported in GCF from PD patients[186]. The authors did not investigate the expression of CSF-1, and thus more studies are needed to compare the expression of these two cytokines in GCF. A possible

differential expression could in part be explained by different tissue expression patterns. In mice, it has been shown that CSF-1 is broadly expressed in many tissues whereas IL-34 seems to play a crucial role in some specific tissue types e.g. neuronal tissue and skin[131,187]. On the other hand, elevated IL-34 expression has been shown in several chronic inflammatory diseases such as RA, IBD and Sjögren's syndrome, indicating a role for this cytokine in inflammatory processes[139,143,188]. To further assess the expression of these growth factors in gingiva, we used immunohistochemistry to stain gingival tissue from PD patients which revealed expression CSF-1 and IL-34 in the connective tissue. The abundance of fibroblasts in the connective tissue led us to hypothesize that these cells are important sources of CSF-1 and IL-34.

4.3.2 **CSF-1 and IL-34 expression by gingival fibroblasts**

GFs are the most abundant cells in the gingiva, and it has been shown that they play an important part in orchestrating the immune response[75]. TNF-α and IL-1β stimulation activates the NF-KB and MAPK c-jun terminal kinase (JNK) signaling pathways in GFs[189,190]. In a previous study from our group, IL-34 gene expression was found to be upregulated in GFs by TNF-α via the NF-kB and JNK pathways, and through the NF-kB pathway by IL-1β stimulation. TNF-α and IL-1β induced CSF-1 expression was regulated through NF-kB[78].

In study III we wanted to follow up on these results and assess the protein secretion of CSF-1 and IL-34 by GFs under inflammatory and non-inflammatory conditions. The expression of CSF-1 and IL-34 in GFs was confirmed with immunofluorescence stainings of cells cultures in chamber slides. Stimulations with TNF-α, IL-1β, *E. coli*, or *P. gingivalis* LPS significantly increased the secretion of CSF-1 by GFs whereas only *E. coli* LPS significantly elevated IL-34 production. Considering that GFs isolated from PD-affected gingiva have been shown to exhibit an altered inflammatory profile *in vitro*[86,191], we hypothesized that CSF-1 and IL-34 expression and secretion are altered in GFs isolated from PD patients. CSF-1 and IL-34 mRNA did not differ between patient and control GFs, however, both cytokines were constitutively secreted over time with higher CSF-1 concentrations measured. Proinflammatory stimuli increased the secretion of CSF-1 and IL-34, however no difference was observed between patients and controls. We did not assess the effect of LPS stimulation on CSF-1 and IL-34 secretion which, in hindsight, would have been interesting considering the reported differential cytokine secretion by control and PD GFs in response to *P. gingivalis* LPS[86,87]. El-Awady and colleagues reported that PDLFs isolated from PD-affected teeth and cultured *in vitro* sustain their inflammatory profile without inflammatory stimuli[84]. In another study, Baek et al. reported an enhanced inflammatory state in GFs isolated from inflamed gingiva[191]. One important difference between these studies and ours is the age of the study participants, which in our study was quite high. Age impacts the function and characteristics of GFs[192], thus a different result regarding CSF-1 and IL-34 expression may be observed with cells isolated from younger individuals.

4.4 STUDY IV

4.4.1 CSF-1R expression in gingival tissue

The finding of increased CSF-1 in PD-affected gingiva prompted us to also investigate expression of the receptor. Binding of CSF-1 to CSF-1R induces receptor dimerization, activation, and autophosphorylation of cytoplasmic tyrosine residues[69]. This is followed by regulated intramembrane proteolysis resulting in release of the cytoplasmic domain into the cytosol. The activated cytoplasmic domain then moves to the nucleus and regulates gene transcription[193]. To analyze the gene expression of CSF-1R we used qPCR which revealed similar levels in gingival tissue from PD patients and controls. We then went on to analyze the protein expression in homogenized gingival tissue with western blot and found significantly higher levels of the cytoplasmic domain of the receptor in PD. An insignificant increase in the expression of a 140 kDa band was observed in PD samples, likely to be the CSF-1R precursor. A band was also seen in close proximity to the 140 kDa band (around 150 kDa). Whether or not this represents the CSF-1R is difficult to establish since the expected band size of the receptor is 175 kDa. To complement the western blot analysis, immunohistochemistry was performed showing abundant CSF-1R staining in the inflammatory infiltrate in the connective tissue. The number of DAB^+ cells/mm^2 was significantly higher in PD gingival tissue compared with controls. To the best of our knowledge, no other studies have investigated the expression of CSF-1R in human gingival tissue. In contrast, more work has been conducted in the RA field, showing increased CSF-1R expression in inflamed synovial tissue in conjunction with $CD68^+$ macrophages[138], suggesting that CSF-1R takes part in regulating macrophage functions during tissue inflammation.

Based on the results of elevated CSF-1R expression in gingiva, we aimed to assess the expression of the receptor in macrophages using flow cytometry and observed similar levels on cells from PD and controls. Furthermore, we assessed whether the CSF-1R expression could be attributed to an alternatively activated phenotype by assessing the receptor expression on $CD206^+$ and $CD206^-$ macrophages, however no differences between the two populations were found, either in PD or controls.

4.4.2 Assessment of macrophage markers in gingival tissue

The number of macrophages in PD-affected gingiva has been investigated in several histological studies based on markers such as CD68, showing that macrophage counts are increased in PD and that their presence correlates with collagen degradation[26,160]. Utilizing qPCR, we found increased gene expression of CD68 and CD64 in PD gingiva (Figure 8). The proportions of macrophages in gingiva from PD patients and controls were analyzed with flow cytometry based on the markers CD45, HLA-DR, and CD64. The proportions of macrophages was unaltered in PD-affected gingiva which is in line with previous findings[15]. This is also in accordance with the results in study I and II.

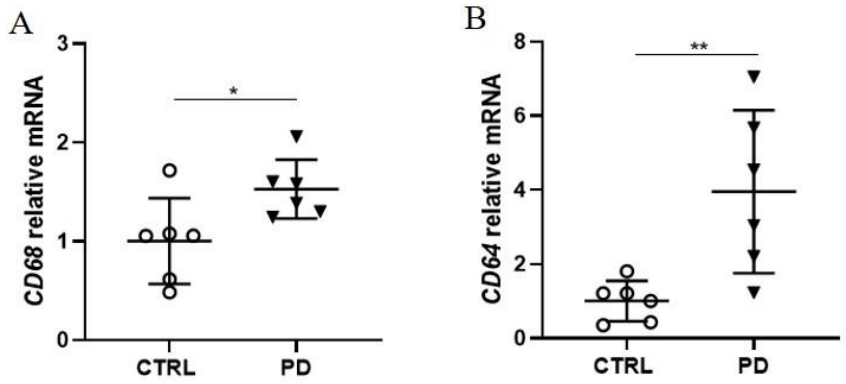

Figure 8. Relative mRNA expression of the macrophage markers A) CD68 and B) CD64 was assessed with qPCR in gingival tissue from PD patients (n=6) and controls (n=6). Mann-Whitney U test was applied to determine statistical significance. *p<0.05, **p<0.01.

We found a slightly increased CD206 mRNA expression in PD-affected gingival tissue and unaltered proportions of $CD206^+$ macrophages comparing PD and controls. In PD, an enhanced M1/M2 ratio has been reported based on the expression of iNOS and CD206 on macrophages[162]. In that study, the absolute number of $CD206^+$ cells was unaltered in PD compared with control gingiva whereas the iNOS expressing cells were elevated. On the other hand, another study using immunofluorescence to quantify polarized macrophages reported unaltered polarization in PD[163]. Moreover, the authors found that macrophage numbers were higher in gingivitis compared with PD lesions, a finding also supported by others[194]. An explanation for this could be a failure of macrophage recruitment and activation in PD[195]. Another reason for the varying results could be that the complex chronic inflammatory milieu during PD may harbor macrophage phenotypes involved in both proinflammatory processes and resolution of inflammation concomitantly. Moreover, variations in experimental approaches to analyze macrophage phenotypes, as well as sample sizes and donor variations in the groups analyzed may also affect the phenotype-profile.

4.4.3 CSF-1R inhibition in PBMCs

To assess the expression of CSF-1R on monocytes, PBMCs from buffy-coated blood were analyzed using flow cytometry. This confirmed previous reports that CSF-1R is primarily expressed by monocytes among immune cell populations in peripheral blood. Non-classical monocytes displayed the highest CSF-1R expression among the subsets followed by the intermediate subset, and lastly the classical monocytes.

After culturing PBMCs *in vitro* for 24 h together with CSF-1, the intermediate monocyte subset predominated. CSF-1R signaling is crucial for the survival of non-classical and intermediate monocytes. This has been shown by the depletion of these subsets in the peripheral blood of

RA patients after treatment with a CSF-1 neutralizing antibody[110]. We aimed to assess whether CSF-1R blockade has an impact on the composition of monocyte subsets in cultures of PBMCs. Addition of a CSF-1R neutralizing antibody reduced the proportions of CSF-1R$^+$ monocytes significantly and resulted in a tendency towards reduced surface expression of CSF-1R but did not affect the proportions of monocyte subsets. A concern regarding CSF-1R inhibition is the possibility of mainly targeting macrophages involved in resolution and tissue remodeling. Indeed, CSF-1 has been shown to polarize macrophages towards and alternatively activated phenotype[196], and likewise, CSF-1R inhibition may in some settings reduce alternatively activated macrophages in tissue[197]. However, in another study on neuropathic pain conducted in mice, CSF-1R inhibition was found to mainly target classically activated CD86$^+$ macrophages[198]. The surface markers CD163 and CD206 are associated with alternative activation and are downregulated in response to LPS/IFN-γ stimulation *in vitro*, whereas CD64 has been shown to be upregulated[116]. To assess whether CSF-1R inhibition has an impact on these markers, we analyzed the expression of CD64, CD163 and CD206 on monocytes after culturing PBMCs for 24 h together with a CSF-1R neutralizing antibody and CSF-1. However, no difference was observed comparing cells subjected to CSF-1R blocking and IgG1/untreated controls.

We hypothesized that the CSF-1/CSF-1R axis takes part in regulating the production of inflammatory mediators in PBMCs. Therefore, we analyzed the concentration of a number of inflammatory mediators known to be regulated in PD in supernatants from PBMCs subjected to CSF-1R inhibition. A significant reduction in IL-8 and MMP-9 levels was observed after receptor inhibition. In line with this, reduced production of IL-8 and MMP-9 has been reported in response to CSF-1R inhibition in RA synovial explants[137]. We then stimulated PBMCs with *E. coli* LPS which led to a predominance of the classical monocyte subset. This effect could be due to a reduction of CD16 in response to LPS stimulation, and the use of more stable markers such as CD38 have been proposed to track activated non-classical monocytes[199]. To further assess phenotypical changes of monocytes after 24 h in culture with LPS, we analyzed the expression of CD64, CD163 and CD206. LPS stimulation resulted in a small increase of CD64 and a slight decrease of CD163 and CD206 expression (Fig 4C-E), however this did not reach statistical significance. The insignificant changes in the expression of these markers could be due to the lack of IFN-γ stimulation (commonly used for classical polarization in combination with LPS). Furthermore, we used a short incubation time of 24 h. A longer incubation of three to eight days to allow for macrophage differentiation may have rendered different results regarding the expression of these markers.

4.4.4 **CSF-1R inhibition in gingival tissue explants**

MMPs are key players in tissue degradation during PD. Therefore, we aimed to investigate their secretion after CSF-1R inhibition in gingival tissue. Two different concentrations of blocking antibody were tested, 0.1 µg/ml and 1 µg/ml. Addition of the lower concentration clearly had an impact on MMP production with significant reduction of MMP-1, MMP-12 and

MMP-13 compared with the isotype controls. Treatment with the higher concentration did not result in the same attenuation of MMPs. It would have been desirable to assess the viability of the cells after CSF-1R inhibition in explants to assess whether this could be attributed to toxic effects, however this was not performed. Moreover, in the study by Garcia and colleagues, blocking CSF-1 and IL-34 individually did not result in changes in the expression of proinflammatory mediators in RA synovial explants, which may be due to the redundant role of these growth factors in maintaining macrophage populations[137]. If the same applies for PD gingival explants remains to be elucidated.

Few studies have investigated the effect of CSF-1R inhibition in PD. In a murine model, Kimura et al. reported that CSF-1R inhibition with a monoclonal antibody reduced osteoclastogenesis and RANK expression with diminished bone resorption as a result[145]. More studies have been conducted in models of other inflammatory diseases. Using a monoclonal antibody against CSF-1R, Toh et al. reported, depletion of osteoclasts, reduced infiltration of macrophages, and bone and cartilage protecting effects in animal models of RA[138]. Furthermore, anti-CSF-1R treatment had protective effects against tissue destructive processes in experimental models of retinal inflammation and SLE[200,201]. Taken together, CSF-1R may be a potential target to reduce destructive inflammatory processes, however more studies are needed to elucidate changes in macrophage functions and phenotypes in response to CSF-1R inhibition during periodontal inflammation.

4.5 MAIN FINDINGS

Periodontitis (PD) is a highly prevalent chronic inflammatory disease characterized by the destruction of tissues supporting the teeth. Monocytes and macrophages are key players in chronic inflammatory tissue destruction. Studying their characteristics and functions in PD is important to delineate possible strategies to modulate their function.

The main findings of this thesis:

- MMP-12 is increased in gingival tissue from PD patients and is primarily expressed by monocyte-derived cells. These cells exhibit low surface expresssion of the co-inhibitory receptor CD200R. CSF-2 induced MMP-12 is regulated by the CD200/CD200R pathway. Oral mucosa tissue cultures can be utilized to study monocyte-derived cells in a miliue that mimics inflamed oral mucosa.

- S100A12 expression is increased in peripheral blood monocytes and macrophages from PD patients. The secretion of S100A12 by monocytes decreases during monocyte maturation. S100A12 is elevated in gingival tissue and reflects PD severity in saliva, and its production is dependent on the presence of monocytes in oral tissue cultures.

- CSF-1 is increased in gingival tissue from PD patients. GFs constitutively produce CSF-1 and IL-34 and enhance the secretion in response to inflammatory stimuli. GFs isolated from PD-affected gingival tissue and controls do not differ in regards to CSF-1 and IL-34 expression and production.

- CSF-1R expression is increased in gingival tissue from PD patients but unaltered in macrophages from PD and control gingiva. CSF-1R inhibition attenuates inflammatory mediator production by PD gingival tissue explants and PBMCs.

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