Neutrophil recruitment in periodontal disease

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ABSTRACT

Neutrophils are the first immune cells to arrive in infected or injured tissues, where they engulf microbes and clean up cell debris. Periodontitis is one of the typical symptoms of both neutropenia and defect neutrophil functionality, suggesting an important role for these cells in maintenance of periodontal health. While representing a minor fraction of the leukocytes in the periodontal lesion, neutrophils are the dominating cell type in the periodontal pocket and gingival crevicular fluid (GCF). The overall aim of this thesis was to characterize factors modulating neutrophil recruitment from blood to GCF in periodontitis.

Neutrophil recruitment to the periodontal pocket is triggered by the bacterial species colonizing this site. Although previous studies have shown that subgingival bacteria trigger neutrophil chemotaxis, the bacterial chemoattractants responsible for this event remained to be identified. The aims of **paper I** and **II** were to identify soluble neutrophil chemoattractants released by the periodontitis associated bacterial species *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, and their corresponding neutrophil receptors. Chemotactic compounds present in culture supernatants of both bacterial species where identified as short chain fatty acids (SCFAs) specifically activating neutrophils via the short chain fatty acid receptor 2 (FFAR2).

CD177 is a neutrophil subtype marker with unknown function, expressed by 1–100% of circulating neutrophils depending on the donor. While CD177 has been proposed to facilitate neutrophil transmigration, this had not yet been demonstrated *in vivo*. The aim of **paper III** was to investigate whether CD177 expression affect neutrophil transmigration to GCF in periodontitis. The $CD177^+$ subtype was enriched in GCF as compared to blood from the same donor, supporting an *in vivo* migration advantage of the $CD177^+$ subtype to this site. Periodontitis patients also exhibited higher levels of $CD177^+$ cells in blood as compared to healthy controls, which resulted in very high proportions of $CD177^+$ cells in GCF. Considering this, functions differing between the subsets could influence destructive inflammation of the periodontal tissues. As CD177 may not be the sole factor contributing to functional differences between the subsets, further proteomic differences between $CD177^+$ and $CD177^-$ neutrophils were investigated in **paper IV**.

In conclusion, this thesis highlights SCFAs signaling via FFAR2 as factors involved in neutrophil chemotaxis triggered by periodontitis associated bacteria. Further, the $CD177^+$ neutrophil subtype is preferentially recruited to GCF and functions specific for this subtype may be of importance for inducing (or suppressing) destructive inflammation in periodontal tissues.

Keywords: Neutrophil, periodontitis, *P. gingivalis*, *F. nucleatum*, SCFA, FFAR2, CD177, A1AT

SAMMANFATTNING PÅ SVENSKA

När neutrofiler nås av signaler om infektion eller vävnadsskada lämnar de blodbanan och tar sig vidare ut i vävnaden för att eliminera mikroorganismer eller städa bort skadade celler. Då parodontit är ett av de typiska symtomen vid både neutropeni och funktionella neutrofildefekter verkar neutrofiler vara viktiga för att bibehålla ett friskt parodontium. Medan neutrofilen endast återfinns i små mängder i parodontitlesionen är de dominerande i gingivalvätskan från djupa tandköttsfickor hos patienter med parodontit. Denna avhandling undersöker faktorer som påverkar rekryteringen av neutrofiler från blodbanan till tandköttsfickan vid parodontit.

Rekrytering av neutrofiler till tandköttsfickan stimuleras av koloniserande, huvudsakligen, anaeroba och gram-negativa bakterier. Tidigare studier har visat att dessa bakterier har förmågan att direkt attrahera neutrofiler, men det var ännu okänt vilka specifika kemotaktiska faktorer som utlöser cellernas rörelse. Syftet med **studie I** och **II** var att identifiera lösliga kemoattraktanter som utsöndras av de parodontitassocierade bakterierna, *Porphyromonas gingivalis* och *Fusobacterium nucleatum*, samt korresponderande neutrofilreceptorer. De kemotaktiska faktorerna visade sig delvis utgöras av korta fettsyror som aktiverar neutrofiler via den neutrofilspecifika receptorn för fria fettsyror (FFAR2).

CD177 är en neutrofil subtypsmarkör med okänd funktion som beroende på donator uttrycks av 1–100% av neutrofilerna i blodbanan. CD177 har föreslagits bidra till rekrytering av neutrofiler från blod till vävnad men detta var ännu inte visat *in vivo*. Syftet med **studie III** var att ta reda på om CD177 uttryck påverkar rekrytering av neutrofiler från blod till tandköttsficka vid parodontit. Studien visade att $CD177^+$ neutrofiler ansamlades i tandköttsfickan vilket stöder teorin om att CD177 underlättar neutrofilrekrytering till denna vävnad. Patienterna hade också generellt högre nivåer av $CD177^+$ celler i blodet jämfört med friska kontroller, vilket resulterade i mycket höga nivåer av $CD177^+$ neutrofiler i tandköttsfickan hos patienterna. Funktioner som skiljer sig mellan subtyperna skulle därför kunna påverka utvecklingen av destruktiv inflammation i parodontiet. Då CD177 uttryck inte nödvändigtvis är den enda faktor som bidrar till funktionella skillnader mellan subtyperna, undersöks ytterligare skillnader på proteinnivå mellan $CD177^+$ and $CD177^-$ neutrofiler i **studie IV**.

Sammanfattningsvis lyfter denna avhandling fram korta fettsyror och FFAR2 som bidragande faktorer till neutrofilrekrytering stimulerad av parodontitassocierade bakterier. Den beskriver även hur den $CD177^+$ neutrofila subtypen ackumuleras i tandköttsfickan vid parodontit och undersöker ytterligare skillnader mellan $CD177^+$ and $CD177^-$ neutrofiler.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. ***Porphyromonas gingivalis*** **produce neutrophil specific chemoattractants including short chain fatty acids**

Dahlstrand Rudin A, Khamzeh A, Venkatakrishnan V, Persson T, Gabl M, Savolainen O, Forsman H, Dahlgren C, Christenson K, Bylund J. *Frontiers in Cellular and Infection Microbiology. 2021 Jan 19. https://doi.org/10.3389/fcimb.2020.620681*

II. **Short chain fatty acids released by** ***Fusobacterium nucleatum*** **are neutrophil chemoattractants acting via free fatty acid receptor 2 (FFAR2)**

Dahlstrand Rudin A, Khamzeh A, Venkatakrishnan V, Basic A, Christenson K, Bylund J. *Cellular Microbiology.* 2021 *Aug;23(8): e13348. https://doi.org/10.1111/cmi.13348*

III. **The neutrophil subset defined by CD177 expression is preferentially recruited to gingival crevicular fluid in periodontitis**

Dahlstrand Rudin A, Amirbeagi F, Davidsson L, Khamzeh A, Thorbert Mros S, Thulin P, Welin A, Björkman L, Christenson K, Bylund J. *Journal of Leukocyte Biology. 2021 Feb;109(2):349-362. https://doi.org/10.1002/JLB.3A0520-081*RR

IV. **Proteomic characterization of neutrophil subsets distinguished by membrane expression of CD177.**

Dahlstrand Rudin A, Sanchez Klose FP, Komic H, Östberg A-K, Venkatakrishnan V, Christenson K, Bylund J. *In manuscript*

CONTENT

ABBREVIATIONS

A1AT	Alpha-1-antitrypsin
AASV	ANCA associated systemic vasculitis
ACPA	Anti-citrullinated protein antibody
ANCA	Anti-neutrophil cytoplasmic autoantibody
BOP	Bleeding on probing
BPI	Bactericidal permeability-increasing protein
C5aR	C5a-receptor
CAL	Clinical attachment loss
CGD	Chronic granulomatous disease
COPD	Chronic obstructive pulmonary disease
DAMP	Danger associated molecular pattern
FFAR	Free fatty acid receptor
FPR	Formyl peptide receptor
GCF	Gingival crevicular fluid
G-CSF	Granulocyte colony stimulating factor
GPCR	G-protein coupled receptor
GPI	Glycosylphosphatidylinositol
HOCl	Hypochlorous acid
IBD	Inflammatory bowel disease
ICAM	Intercellular Adhesion Molecule
IL	Interleukin
LAD	Leukocyte adhesion deficiency
LDN	Low density neutrophil
LFA1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide

LTB_4	Leukotriene B_4
MAC	Membrane attack complex
MAC-1	Macrophage-1 antigen
MPO	Myeloperoxidase
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NGAL	Neutrophil gelatinase-associated lipocalin
OLFM4	Olfactomedin 4
PAD	Peptidyl-arginine deiminase
PAF	Platelet activating factor
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PECAM1	Platelet endothelial cell adhesion molecule 1
PLA_2	Phospholipase A_2
PLS	Papillon-Lefèvre syndrome
PR3	Proteinase 3
PRR	Pattern recognition receptor
PSGL1	P-selectin glycoprotein ligand 1
PV	Polycythemia vera
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SCFA	Short chain fatty acid
SLE	Systemic lupus erythematosus
TLR	Toll like receptor
TNF-α	Tumor necrosis factor-α
VCAM1	Vascular Cell Adhesion Molecule 1
VLA4	Very late antigen 4

INTRODUCTION

Inflammation is our body´s immediate response to microbe invasion or tissue injury. Consisting of an intricate network of inflammatory cells, tissues and biological processes, inflammation is vital for elimination of unwanted intruders and initiation of tissue repair. Neutrophils are the first inflammatory cells to arrive at the infected or injured site, where they engulf microbes and cell debris or capture intruders by release of web-like DNA structures. For these purposes, neutrophils leave the blood stream at the vicinity of the affected tissues and migrate further towards their prey, guided by a series of chemotactic signals. The critical role of neutrophil recruitment from blood to tissue can be illustrated by the fact that genetic defects disrupting this process result in recurrent severe infections. The symptoms of such disorders typically also include destructive inflammation of the tooth-supporting structures, i.e., periodontitis. Periodontitis is a microbe initiated inflammatory disease leading to deepening of gingival pockets and degradation of alveolar bone; which may eventually result in tooth loss. Neutrophils seem to play an important role in the maintenance of periodontal health as insufficient neutrophil numbers or defect neutrophil functionality, as seen in patients with rare genetic defects, often lead to rapidly progressing periodontitis. Moreover, although representing a minor fraction of the leukocytes in the periodontal lesion, neutrophils are the dominating cell type in the inflammatory exudate of the periodontal pocket.

This PhD thesis describes the neutrophil journey from circulation to the periodontal pocket, with the intention of adding new insights regarding the nature of bacteria derived chemotactic signals and neutrophil subsets operating at this site.

THE NEUTROPHIL

Neutrophil granulocytes belong to the family of innate immune cells and the subgroup of polymorphonuclear granulocytes (also including eosinophils and basophils). While the term polymorphonuclear refers to the characteristic multilobulated nuclei exhibited by these cell types (1), the name granulocyte is derived from their cytoplasm being packed with storage granules (2). Constituting 50-70% of all white blood cells in human circulation, neutrophils have a crucial role in our defense against invading microbes and in clearance of injury related cell debris (3). When reached by signals of infection or injury, circulating neutrophils swiftly leave the blood stream, enter the affected tissues and navigate further towards the inflammatory focus where they engulf microbes or disrupted cells (4). The neutrophil granules contain an array of toxic substances aimed for microbe elimination (2). Thus, neutrophil recruitment needs to be tightly regulated as excessive release of granule content may inflict tissue damage (5).

The indispensable role of neutrophils for human immune defense can be exemplified by the fact that a marked decrease in neutrophil numbers, or altered neutrophil functionality, typically results in severe immunodeficiency (6). Insufficient neutrophil numbers in circulation is seen in severe congenital neutropenia; a rare condition that is estimated to occur in 3-8.5 individuals per million (7). This condition is caused by genetic mutations affecting neutrophil differentiation in the bone marrow, with *ELANE* (encoding neutrophil elastase (NE)) mutations being the most prevalent cause. As a result of their unusually low neutrophil blood count, neutropenic patients suffer increased risk of recurrent severe infections and frequently also develop periodontitis (7). Among the most well described neutrophil functional deficiencies are Leukocyte-adhesion deficiency (LAD) and Chronic granulomatous disease (CGD). LAD is characterized by defect neutrophil surface adhesion molecules (β_2-integrins) due to mutations in the gene encoding for the β_2-integrin subunit CD18. These mutations had been reported in just over 300 cases between 1975 and 2017 and is estimated to occur in 1:1 000 000 individuals (8). LAD primarily result in inability of neutrophils to form firm adhesion to the endothelium and thereby

hinder their transmigration to tissues (9). CGD targets neutrophil production of reactive oxygen species (ROS), an important bacterial killing mechanism. These are a group of rare disorders (estimated prevalence ~ 1:250 000) caused by mutations in genes encoding different subunits of the ROS-producing nikotinamid-adenin-dinukleotidfosfat complex (NADPH complex) (10, 11). While patients who suffer from LAD develop similar symptoms as seen in neutropenia (i.e., recurrent infections and periodontitis), CGD patients, although being more sensitive to infections, do not typically present with periodontitis (9, 10). In addition to demonstrating the overall importance of neutrophil functions to human immunity, studies of such specific deficiencies can provide important clues regarding so far unknown pathologic mechanisms underlying development of diseases with neutrophil involvement.

Maturation

Similar to many other blood cells, neutrophil granulocytes are matured in, and released from the bone marrow. Originating from the myeloid progenitor cell, the neutrophil pass through six different developmental stages; myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and mature neutrophil (12, 13). After a maturation period of 14 days the terminally differentiated, non-dividing neutrophil is released into circulation where it will have a relatively short life. Although the longevity of neutrophils in circulation has been debated (14-16), they are generally viewed as having a lifespan of 7-9 hours, which can be greatly extended when exposed to inflammatory stimuli (14). Under healthy conditions, neutrophils remain in circulation for a few hours before they are homed to, and cleared in the bone marrow or die in the vasculature followed by clearance in the liver by resident macrophages (4, 14).

Life cycle

Neutrophils are produced at an impressive rate of about 100 billion cells per day (17). In addition to being continuously released into circulation, a reserve of mature cells is stored in the bone marrow, ready to be quickly released if demanded (13). After release, circulating neutrophils can be divided into two pools; one freely circulating intravascular pool and one marginated pool. The idea of a marginated pool of neutrophils was launched after the discovery that up to

50% of radio-labelled homologous neutrophils injected into the circulation of healthy individuals disappeared from the circulation after a short time period (18), and that the marginated cells re-entered the circulation following injections of adrenalin or physical exercise (19). Later studies have shown a prolonged transit time through the lungs, liver and spleen supporting margination of neutrophils to these organs (17, 20). Whether neutrophil accumulation in these organs have functional implications or if the marginated pool simply consist of cells too rigid to easily pass through narrow blood vessels is not entirely clear (4, 13, 14, 20).

Neutrophil release from the bone marrow into circulation is a regulated process that is in balance with intravascular margination and clearance. This finely tuned system keeps neutrophil blood levels constant at a healthy state, while cell numbers can be rapidly increased in response to inflammatory stimuli (21). The maintenance of homeostasis is of great importance as neutrophils, as well as being indispensable effector cells of innate immunity, also have the capacity to cause tissue damage (17). One factor controlling granulopoiesis is the hematopoietic cytokine granulocyte colony stimulating factor (G-CSF). G-CSF potently stimulates neutrophil proliferation and release (22-24) and show increased serum concentration in response to infection (25). CXCL12 (also known as stromal derived factor 1) is produced by bone marrow stromal cells and is, via its ligation with the neutrophil chemokine receptor 4 (CXCR4), responsible for retaining neutrophils in the bone marrow (17, 26). One of the mechanisms by which G-CSF stimulate neutrophil release from the bone marrow is via inhibition of the CXCR4-CXCL12 interaction (22). It has also been suggested that CXCR4 can be upregulated in mature circulating neutrophils as a signal promoting their return to the bone marrow, where they undergo apoptosis and are subsequently phagocytosed by stromal macrophages (17, 27).

As previously mentioned, neutrophils exert their primary immune functions in tissues after having left the circulation. The general belief is that these tissue neutrophils subsequently die by apoptosis and are cleared by tissue resident macrophages (4). However, studies of animal models indicate that tissue neutrophils may also be able to return to the circulation and thereby prevent tissue damage and facilitate resolution of inflammation (28). In 2006, this phenomenon was visualized *in vivo* in zebrafish following resolution of sterile injury (29). More recently, so-called reverse transmigration of neutrophils was reported to occur

after sterile hepatic injury in a mice model (30). In this study, Wang *et al.* monitored (using intravital imaging) how neutrophils facilitated tissue repair via phagocytosis of cell debris in the injured tissue and subsequently returned to the vasculature. Briefly before returning to the bone marrow to undergo apoptosis, the reverse transmigrated neutrophils were transiently accumulated in the lungs where the homing receptor CXCR4 was upregulated (30). Human neutrophils are reportedly capable of transmigrating in both directions over an endothelial monolayer *in vitro* and these reversely transmigrated cells present with a phenotype distinct from the usual transmigrated cell ($CD54^{high}$ and $CXCR1^{low}$) (31). Increased levels of this phenotype were also detected in blood from patients suffering from inflammatory arthritis and atherosclerosis as compared to healthy controls, indicating the presence of reverse transmigrated cells in these patient groups (31). Reverse transmigration may indeed have positive effects on the resolution of sterile inflammation but it is also speculated that the return of activated neutrophils to circulation could cause dissemination of inflammation leading to multiple organ failure (28, 32). Whether human neutrophils undergo reverse migration *in vivo* and if it occurs following inflammation triggered by infectious stimuli remain to be elucidated (33).

Granules

Circulating neutrophils are terminally differentiated, non-dividing cells with a very limited *de novo* protein synthesis. Although some cytokines (e.g., IL-8) can be synthesized upon neutrophil activation (34), their granules, i.e., small membrane enclosed compartments, are already equipped with most of the proteins needed for microbe elimination (35, 36) (**Fig. 1**). The neutrophil granules are formed by the Golgi complex in a sequential manner during the maturation period, starting with the azurophil granules during the promyelocyte stage. This continues during the myelocyte/metamyelocyte stage with the development of specific- and gelatinase granules. The last membrane enclosed intracellular compartments to be formed are the secretory vesicles, which are formed via endocytosis in mature neutrophils (2, 37). The granules and secretory vesicles are subsequently mobilized in the opposite order to their formation; secretory vesicles being most easily released, followed by gelatinase- and specific granules, while azurophilic granules are rarely released to the extracellular space and instead fuse with the phagosome.

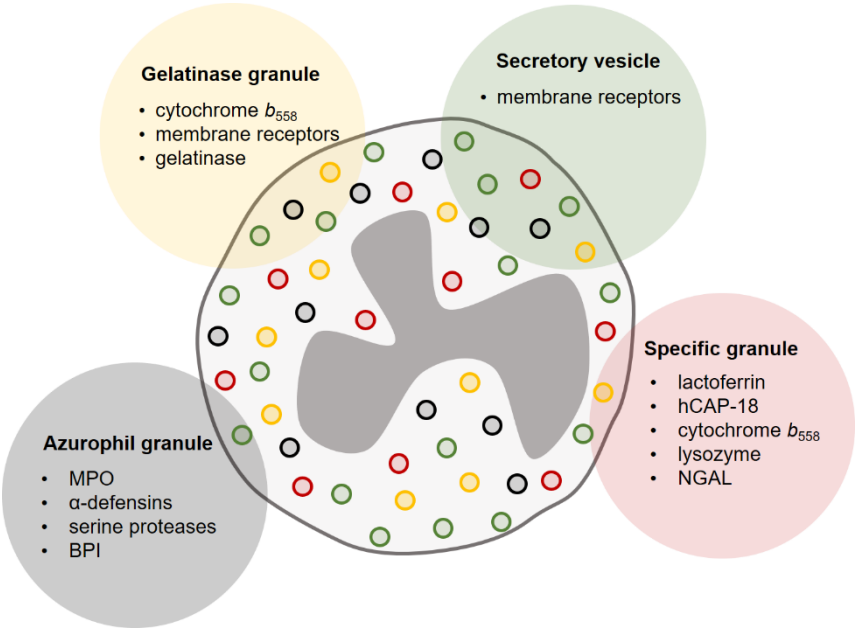

Figure 1. Neutrophil granules and secretory vesicles. *Drawing of the four distinct neutrophil granule and secretory vesicle subsets and examples of their characteristic content.*

Sorting of proteins into the different granule types, each requiring different types of stimuli to be mobilized, allows neutrophils to respond in an optimal way depending on the situation. The distribution of granule proteins in the distinct granule subtypes is a result of both proteins and granules being formed sequentially during neutrophil maturation. Accordingly, granules store the proteins that are synthesized at the time of granule formation, a mechanism that is referred to as 'targeting-by-timing' (38). The 'targeting-by-timing' model explaining the distribution of neutrophil granule proteins has recently also been shown relevant in the context of protein glycosylation patterns, i.e., proteins that are synthesized at a certain time point during granulopoiesis display a characteristic set of oligosaccharides, dubbed 'glycosylation-by-timing' (39).

Azurophil granules

The azurophil granules are formed during the promyelocyte stage and mainly participate in killing of microorganisms in the phagolysosome (36). These

granules are often described as rarely undergoing exocytosis, in order to prevent tissue damage that could otherwise be caused by their toxic content (40). Despite this, proteins derived from azurophil granules can be found extracellularly in inflamed tissues (41). Whether this is a result of exocytosis (42), release of neutrophil extracellular traps (NETs), necrosis or a combination of these events is not entirely clear.

The defining protein of azurophil granules, myeloperoxidase (MPO), contributes to microbial killing in the phagolysosome by increasing the toxic potential of H_2O_2 that is formed by the NADPH oxidase (43). After fusing of azurophil granules with the phagosome, MPO converts H_2O_2 into hypochlorous acid (HOCl). Moreover, and as discussed in more detail below, MPO participate in most cases of neutrophil extracellular trap (NET) formation (44, 45). Other azurophil granule proteins with microbicidal potential are the α-defensins, bactericidal permeability-increasing protein (BPI), and serine proteases (proteinase 3 (PR3), cathepsin G and neutrophil elastase (NE))(36). The serine proteases are particularly interesting in the context of periodontal disease, as a complete lack of active serine proteases result in early onset and rapidly progressing periodontitis. This is seen in the rare autosomal recessive disorder Papillon-Lefèvre syndrome (PLS) (46-48), that will be discussed more in the subsequent chapter on periodontitis.

Specific- and Gelatinase granules

Specific granules and gelatinase granules are both peroxidase- (i.e., MPO) negative but can be subdivided based on their content and formation stage (49). Specific granules, which are formed during the myelocyte stage (37), mainly fuse with the phagosome and contain an array of antibacterial substances including lactoferrin (50), hCAP-18, neutrophil gelatinase-associated lipocalin (NGAL) and lysozyme (36). In contrast to specific granules, gelatinase granules, which are formed during the metamyelocyte stage, rarely fuse with the phagosome and are instead more easily exocytosed (51). This is convenient as they carry matrix degrading enzymes (e.g., the matrix metalloprotease, gelatinase) and adhesion receptors that are required during neutrophil transmigration, in combination with less antimicrobial substances (36). Both specific- and gelatinase granules contain cytochrome b_{558}

(52), which is the membrane bound component of the oxygen radical producing NADPH oxidase (described in more detail below).

Secretory vesicles

Secretory vesicles are the last membrane enclosed intracellular compartments to be formed during granulopoiesis and also the most easily exocytosed upon proinflammatory stimulation. Unlike the neutrophil granules, which are produced by the Golgi complex (53), secretory vesicles are formed through endocytosis, and consequently contain both membrane receptors and plasma proteins (54).

Killing mechanisms

A well-functioning recruitment of neutrophils from circulation, though tissues, and towards the inflammatory focus is inevitably a crucial step in eradication of invading microbes. These processes will be described in more detail in the upcoming chapter on neutrophil transmigration. Once in close contact with invading microbes, the two primary killing mechanisms employed are phagocytosis or NET formation.

Phagocytosis

Phagocytosis is a process during which neutrophils internalize and degrade microbes or cell debris. This is facilitated by bacterial opsonization, i.e., coating of bacterial surfaces with antibodies or complement opsonins, which are recognized by neutrophils receptors (i.e., FC- or CR3 receptors respectively). While antibodies are produced by the B-cells of adaptive immunity, complement opsonins are generated via enzymatic cleavage of complement proteins. The complement system consists of over 30 plasma proteins which when degraded on bacterial surfaces generate powerful inflammatory mediators (i.e., anaphylatoxins) (described in more detail in the chapter on neutrophil chemoattractants), complement opsonins (e.g., C3b) and the membrane lysing 'membrane attack complex' (MAC) (55).

After recognition and attachment of microbes or particles, cytoskeletal rearrangements create a protrusion of the neutrophil plasma membrane, which surrounds and internalizes the prey in an intracellular compartment; i.e., a

phagosome (56, 57). The phagosome is subsequently matured into a phagolysosome, filled with antimicrobial substances, through fusion with azurophil- and specific- granules. Killing of microbes in the phagolysosome is also helped by release of ROS via activation of the membrane bound NADPH oxidase. The NADPH oxidase is an electron transporting enzyme composed of five different sub-units. Two of the subunits ($p22^{phox}$ and $gp91^{phox}$, together making up the so-called cytochrome b_{558}) are bound to the plasma membrane and membranes of specific and gelatinase granules (58, 59), while the complex of the three remaining subunits ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) can be found in the cytosol. Following activation, the membrane bound and cytosolic compartments of NADPH assembles creating an active enzyme complex that transfers electrons over the membrane to O_2 resulting in formation of superoxide (O_2^-). The superoxide anion spontaneously dissociates into H_2O_2, that together with O_2^- are referred to as primary ROS. The toxicity of primary ROS can be further amplified via the previously described reaction catalyzed by MPO (from azurophil granules) resulting in HOCl, and other secondary ROS (60).

The relative importance of the microbicidal factors released and produced in the neutrophil phagolysosome and how these factors influence each other is still not entirely clear (61). As previously mentioned, CGD patients who suffer from genetic mutations in various components of the NADPH complex, display an increased susceptibility to a variety of bacterial and fungal infections and CGD patient neutrophils exhibit decreased bactericidal capacity *in vitro* (62). Thus, the clinical presentation in CGD illustrates that ROS-production is an important mechanism for microbe eradication by neutrophils. Despite this, today many CGD patients survive into adulthood (63), and their neutrophils are able to kill at least some of the pathogens that they encounter (e.g., *Neisseria gonorrhoeae* and *Staphylococcus aureus*) (64, 65), suggesting that microbicidal mechanisms other than ROS production can be functionally overlapping oxygen dependent killing. In contrast to CGD, patients exhibiting complete MPO deficiency rarely show any symptoms, with the exception of diabetic MPO deficient individuals who reportedly suffer from systemic candidiasis (6). This could be interpreted as the MPO dependent conversion of primary ROS into HOCl being a mechanism of minor importance for neutrophil microbial killing. However, *in vitro* studies have demonstrated that MPO deficient neutrophils exhibit decreased capability to kill both fungi (e.g., *Candida albicans*) (66, 67) and several bacterial species including

(e.g., *S aureus* and *Escherichia coli*) (67, 68), as compared to normal neutrophils. It has been argued that although MPO independent antimicrobial mechanisms may be sufficient to kill invading microbes in the majority of cases, HOCl generated via MPO could be critical in cases with extreme bacterial loads or in patients with other immunodeficiencies (69).

In addition to oxygen dependent killing, neutrophils contain a range of antimicrobial proteins stored in azurophil- and specific granules. These bactericidal factors, including α-defensins and serine proteases, are released into the phagosome upon fusion with granules. Although the α-defensins are described as the most potent bactericidal proteins stored in neutrophil granules (70), also neutrophil serine proteases have shown direct bactericidal activities *in vitro* (71, 72). Yet, apart from the characteristic symptoms of rapidly progressing periodontitis and palmoplantar keratosis, patients suffering from PLS (who completely lack active neutrophil serine proteases) are relatively healthy and rarely present with serious infections (46-48, 73). In summary, the described deficiencies display distinct clinical presentations depending on the affected killing mechanism. This indicates that the relative importance of the microbicidal pathways differ depending on situation and nature of the ingested microbe.

Formation of neutrophil extracellular traps

In 2004 Brinkmann *et al.* reported that neutrophils were able to generate large extracellular web-like structures, referred to as neutrophil extracellular traps (NETs), which were able to trap and kill bacteria (74). These structures were composed of decondensed chromatin fibers covered with various granule proteins, including NE, cathepsin G, PR3 and MPO (75). NET formation has been described as a mechanism aimed to neutralize bacteria, fungi, viruses and parasites extracellularly, but it is not entirely clear if microbes are killed or simply trapped and prevented from dissemination by the NET fibers (76, 77).

NET formation was first described as a novel form of programmed cell-death, i.e., NETosis, during which neutrophils release chromatin fibers as a last attempt to eradicate microbes (77, 78). However, it has since then been suggested that neutrophils may also have the capacity to remain viable after NETosis (79). In a majority of cases, ROS, MPO and NE are required for the processing of histones

and disruption of chromatin packaging during NET release (45, 77), but there is also evidence of ROS independent NET formation triggered by *S. aureus* and factors secreted by *S. aureus* (44, 80, 81). In line with NET formation being MPO and NE dependent, neutrophils from patients with complete MPO or NE deficiency (e.g., PLS patients) exhibit defect NETosis *in vitro* (46, 47, 82).

Despite the initial focus on the immune-protective functions of NETs, more recent studies have highlighted NETs as a pathologic factor in a diverse set of medical conditions (77). Isolated NETs have for instance been shown capable of killing both human alveolar epithelial and endothelial cells *in vitro* (83) and tissue damage caused by excess neutrophil infiltration and NET release have been reported to cause acute injury in organs including kidneys, lungs and liver (84). Overt NET release in blood can have lethal effects in sepsis and have also been linked to deep vein thrombosis (85). In the case of thrombosis, NETs are suggested to promote vaso-occlusion by providing a scaffold binding platelets and red blood cells and increasing procoagulant activity (85). Moreover, NETs are implicated in autoimmune diseases involving auto-antibodies against NET derived proteins. One such autoimmune disease is antineutrophil cytoplasmic autoantibody (ANCA) associated vasculitis (AASV), resulting from neutrophil activation by autoantibodies against the NET components MPO or PR3 (86). Another autoimmune disease, rheumatoid arthritis (RA) is associated with autoantibodies directed against citrullinated proteins (ACPAs) and NETosis has been correlated with the presence of ACPAs in these patients. The enzyme catalyzing protein citrullination, peptidyl arginine deiminase (PAD), is expressed at high levels in NETs and this has been suggested to contribute to extracellular protein citrullination, thereby providing access to antigens in tissues (87). Interestingly, the periodontitis associated bacterial species *Porphyromonas gingivalis* has been reported as the only microbial source of PAD (88) (described in more detail in the chapter on *P. gingivalis*). In summary, regulation of release and clearance of NETs appear to be critical as there is a fine line between favorable and harmful effects of this extracellular protection mechanism.

NEUTROPHIL TRANSMIGRATION

The recruitment of blood neutrophils to the site of infection or injury is a pivotal step preceding microbial killing and tissue repair. The importance of a well-functioning recruitment cascade can be exemplified by the fact that rare genetic defects affecting this process results in severe immunodeficiency, as seen in the previously described LAD (8, 9, 89).

Neutrophils circulating the blood stream are constantly surveying tissues for indications of microbe invasion or injury. The first signals of infection are triggered by recognition of conserved microbial molecular patterns by tissue-resident immune cells. Such microbial patterns include lipopolysaccharide (LPS), bacterial flagellin, lipoteichoic acid and peptidoglycan and are referred to as pathogen associated molecular patterns (PAMPS). According to the same principle, tissue injury is recognized via molecular patterns associated with dying or ruptured cells (e.g., the extracellular presence of DNA, mitochondrial proteins and ATP) referred to as damage associated molecular patterns (DAMPS) (90). PAMPS and DAMPS activate pattern recognition receptors (PRRs; e.g., Toll-like receptors and NOD-like receptors) expressed by tissue resident immune cells, such as macrophages and dendritic cells. These cells respond by production and release of proinflammatory factors initiating the recruitment and extravasation of neutrophils. The neutrophil extravasation cascade involves five major steps; tethering, rolling, adhesion, crawling and transmigration, which are illustrated in **Fig. 2** and will be described in detail below

Extravasation

Activation of PRRs on sentinel immune cells induces the release of proinflammatory cytokines (e.g., TNF-α, IL-1) and vasodilators (e.g., nitric oxide (NO)) activating the local blood vessel endothelium. While NO induces vessel dilatation and loosening of endothelial cell junctions, cytokine stimulation of endothelial cells results in upregulation of P- and E-selectins to their luminal surface (90). These selectins capture bypassing neutrophils via ligation to P-

selectin glycoprotein ligand 1 (PSGL-1), which is expressed on the neutrophil cell membrane (35). The transient low-affinity bond between selectins and PSGL-1 allows neutrophils to roll along the vessel wall in a process referred to as tethering. The neutrophil surface glycoprotein L-selectin is also participating in the luminal rolling by its binding to endothelial glycans such as Sialyl LewisX (91, 92). As cellular velocity decreases, integrins bind their endothelial ligands intercellular adhesion molecule 1 and 2 (ICAM1, ICAM2) and vascular cell adhesion molecule 1 (VCAM1); a firm bond resulting in neutrophil adhesion to the endothelium (90, 93). ICAM1 binds to the β_2-integrin lymphocyte function-associated antigen 1 (LFA-1) (consisting of the subunits CD11a and CD18), ICAM2 binds to the β_2-integrin macrophage-1 antigen (MAC-1)/complement receptor 3 (CR3; consisting of the subunits CD11b and CD18) and VCAM1 binds to the β_1-integrin very late antigen 4 (VLA-4). As previously mentioned, LAD associated defects in the β_2-integrin subunit CD18 result in inefficient forming of firm bonds between β_2-integrins and ICAM and thereby hinder neutrophil transmigration (8, 9, 89).

While rolling along the endothelium, neutrophils come in contact with cytokines that induce cell activation (i.e., priming). Neutrophils are circulating the blood stream in a resting state that is characterized by low adherence and minimal responsiveness to stimuli, this in order to prevent excessive responses that might result in tissue damage. During priming, neutrophils undergo phenotypic and functional changes leaving the cells in a hyperresponsive state, i.e., ready to effectively eliminate invading microorganisms. The primed neutrophil is characterized by augmented responses to stimulation, including increased ROS-production, granule mobilization and adhesion, enhanced chemotaxis, and delayed apoptosis (94, 95). Phenotypical changes following priming include rearrangement of surface receptors (e.g., adhesion- and chemotactic receptors) via mobilization of secretory vesicles and shedding of early adhesion molecules. In line with this, shedding of the adhesion molecule L-selectin (CD62L) (92) and upregulation of CR3/MAC-1 (CD11b/CD18) are routinely assessed for determination of neutrophil activation stage (95).

After having established a firm bond to the endothelium, neutrophils start to crawl along the vessel wall in attempt to leave the circulation as close as possible

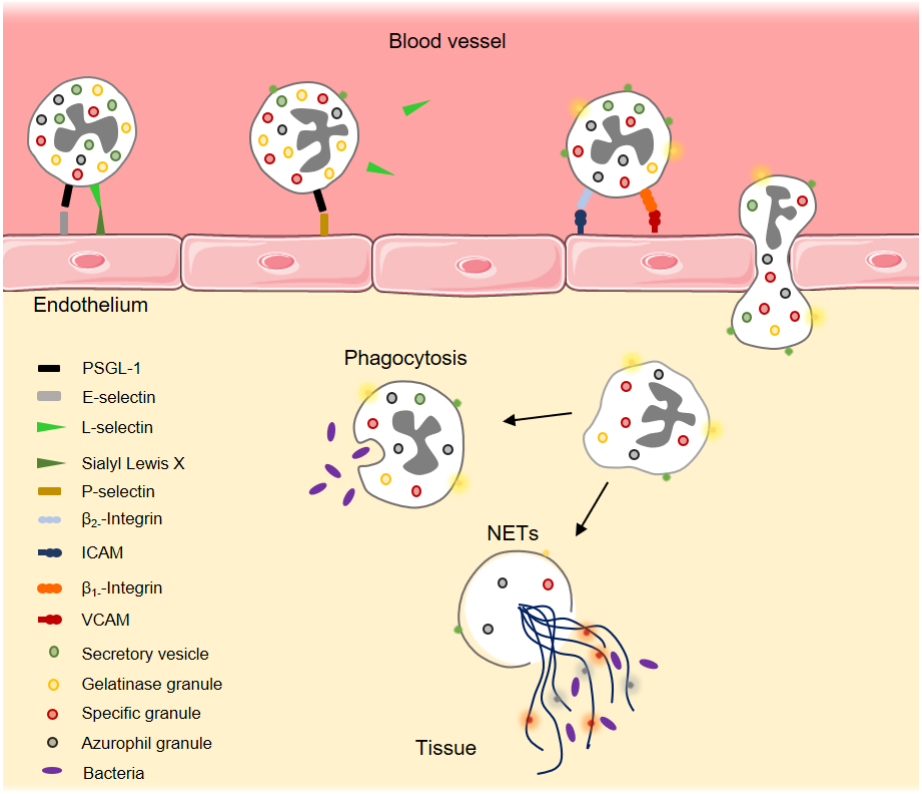

Figure 2. Neutrophil transmigration and microbe elimination. *Microbe stimulation of PRRs on resident immune cells trigger release of cytokines which activate local endothelial cells. P- and E- selectins on the activated endothelium forms low-affinity bonds with neutrophil PSGL-1, resulting in neutrophil rolling along the vessel wall. L-selectin binds to endothelial glycans and is subsequently shredded. As cellular velocity decreases, a firm bond is established between neutrophil integrins and endothelial ICAMs and VCAMs. Neutrophil activation leads to upregulation of surface receptors from secretory vesicles and degranulation of gelatinase granules. Guided by chemotactic gradients, neutrophils transverse the endothelium, migrate further through tissues and eliminate microbes via phagocytosis or NET-release.*

to the site of infection or injury. Crawling is guided by chemokine gradients, i.e., cells move in the direction of increasing chemokine concentration. Although neutrophils preferably transmigrate via endothelial cell-cell junctions (paracellularly), they can also migrate directly through endothelial cells (transcellularly) (96). Paracellular transmigration involves ICAM1, ICAM2, VCAM1 as well as several junctional proteins including platelet endothelial cell adhesion molecule 1 (PECAM1). PECAM1 is expressed on the cell surface of both neutrophils and endothelial cells and its homologous binding facilitates neutrophil transmigration over the endothelium (97). After the endothelial crossing, neutrophils transverse the basal membrane and pericytes surrounding the vessels. This has been attributed to the release of serine proteases (e.g., NE), which degrade the basal membrane laminins and collagen fibers. However, evidence also points at the existence of less dense regions of the basal membrane which are in direct contact with gaps between pericytes, and could represent sites of less resistance for neutrophils to emigrate (98).

Chemotaxis

Once neutrophils have passed through the endothelium and basal membrane, they need to navigate further through tissues towards invading microbes or site of tissue damage. Cell movement towards the inflammatory focus is guided by concentration gradients of soluble chemical signals, i.e., chemoattractants, in a process referred to as chemotaxis (99, 100). Neutrophils express a number of surface receptors that are able to sense chemoattractants released from host cells (i.e., endogenous) or microbes (i.e., bacteria derived), and induce cell movement in the direction of increasing chemoattractant concentration (101). The direction of a chemoattractant gradient is sensed by the detection of differences in the number of occupied chemoattractant receptors (100). When the desired direction of cell movement has been determined, the cell develops a cellular polarity by forming of a surface protrusion in the front (i.e., lamellipodia) while the remaining cell becomes smooth forming a tail (i.e., uropod). The neutrophil movement is subsequently dependent on both cellular adhesion and a combination of actin polymerization in the leading edge and depolymerization in the trailing uropod (100).

Neutrophil chemoattractant receptors

Neutrophil chemotaxis is initiated by agonists binding to G-protein coupled receptors (GPCRs), which are readily expressed on the cell surface or upregulated from granule stores in response to priming. GPCRs, named based on their ability to recruit heterotrimeric G-proteins, are part of a large family of receptors with the common structural feature of seven transmembrane spanning α-helices. The α-helices are linked together with three intra- and three extracellular loops, with an extracellular amino-terminus and a cytoplasmic carboxyl terminus (102). Extracellular agonist binding of these receptors is followed by conformational changes and dissociation of the intracellular G-protein in the Gα and Gβγ subunits (103). Further downstream signaling leads to directional migration, but in many cases also triggers degranulation, shedding of L-selectin, and ROS release (99, 103).

One of the earliest signaling events after GPCR activation is the cytoplasmic release of Ca^{2+}, directly followed by opening of Ca^{2+} channels in the plasma membrane. These events can be monitored as a transient biphasic increase of the cytoplasmic Ca^{2+} using intracellular Ca^{2+} dyes (104). As the transient increase in intracellular Ca^{2+} can be regarded as a valid proxy for GPCR activation, this has become a useful tool in studying neutrophil GPCR signaling (**Paper I, II** and **III**). Moreover, GPCR agonists, antagonists and allosteric modulators are useful pharmacological tools for investigation of specific receptor signaling. Receptor agonists are ligands that bind to GPCRs and induce receptor activation, while receptor antagonists block further activation of the receptor. Allosteric modulators bind the receptor and modify (enhance or decrease) further responses to receptor activation (**Paper I, II**).

To avoid prolonged signaling in the presence of receptor agonists, activated GPCRs need to become non-responsive to their agonists after adequate signals have been transmitted. This process is referred to as homologous receptor desensitization and is subsequently followed by receptor endocytosis, i.e., receptors are removed from the cell surface by internalization. Homologous receptor desensitization can be achieved by binding of β-arrestin proteins which induces a physical separation between the receptor and G-protein, and thereby prevent further signaling. Furthermore, β-arrestins are also suggested to be

involved in receptor endocytosis (105, 106). According to the classic view, recruitment of β-arrestin is a prerequisite for termination of signaling following GPCR activation. However, recent evidence shows that β-arrestin recruitment to a specific receptor can be agonist dependent (107). Hence, β-arrestin recruitment can be utilized as an indication of GPCR activation but the absence of β-arrestin recruitment does not necessarily indicate GPCR inactivity (**Paper I**).

The neutrophil recruitment process is orchestrated by the simultaneous release of several chemoattractants that are both host and microbe derived. Chemoattractants released by host cells (e.g., IL-8, Leukotriene B_4 (LTB_4)) can guide neutrophils from circulation to the general vicinity of infection or tissue damage, and thereby have an intermediary role in the migration process. Neutrophils subsequently need to follow gradients of chemotactic factors originating from the direct inflammatory focus to move further towards the site of infection or injury. These factors can be released by bacteria or damaged host cells (e.g., formylated peptides and short chain fatty acids (SCFAs) that will be described in more detail below), or generated on bacterial surfaces (complement anaphylatoxins, described in more detail below) and are referred to as end-target chemoattractants. When simultaneously encountered with multiple chemotactic gradients, neutrophils need to prioritize end-target attractants over the intermediate counterparts. In support for this, studies have demonstrated that neutrophils favor microbe derived chemoattractants over endogenous ones when they are exposed to competing gradients *in vitro* (108-110).

Intermediate chemoattractants and their receptors

Lipid chemoattractants, including LTB_4 and platelet activating factor (PAF), are produced and secreted rapidly following activation of inflammatory cells. The chemotactic lipid LTB_4 is a product of arachidonic acid, stemming from enzymatic degradation of membrane phospholipids by phospholipase A_2 (PLA_2) in myeloid cells (mainly macrophages) (99). LTB_4 is a strong neutrophil chemoattractant signaling via the neutrophil LTB_4 receptor (111). The lipid chemoattractant PAF, was named after the discovery of its ability to induce aggregation and histamine release in rabbit platelets (112). PAF is a heterogenous class of molecules which are mostly generated from lyso-PAF; an end product of PLA_2 processing of membrane phospholipids (99). The precursor lyso-PAF is

then converted to PAF by further enzymatic processing. Human PAF is produced by neutrophils, monocytes, basophils, endothelial cells and platelets and is a potent neutrophil chemoattractant signaling via the PAF-receptor (PAFR) (113, 114).

Chemokines are a group of low molecular weight (8-12 kDa) cytokines with chemotactic activity on leukocytes. They are produced by a number of different cell types in response to inflammatory stimuli and are classified into four different subgroups; CXC, CC, C and CX (115). The neutrophil attracting chemokines are predominantly of the CXC-type, including CXCL1 to CXCL3 and CXCL5 to CXCL8, which all mediate neutrophil recruitment via the GPCRs CXCR1 and/or CXCR2. CXCL8, also called IL-8, was the first chemokine to be discovered that seemed to specifically attract neutrophils, and all ligands of CXCR1 and CXCR2 are thereby referred to as the CXCL8 family of chemokines (116). IL-8, which is the prototypic CXC-chemokine, is produced by monocytes/macrophages, endothelial- and epithelial cells upon proinflammatory stimulation. This positively charged molecule is immobilized by glycosaminoglycans on the luminal surface of endothelial cells, guiding neutrophils to extravasate at a site close to the inflammatory focus, but also further through the tissue post-extravasation (117). In addition to the members of the CXCL8 family, CXCL12 ligation of CXCR4 is, as mentioned previously, responsible for retention of neutrophils in the bone marrow before release, as well as homing of aged neutrophils to the bone marrow (27).

End-point chemoattractants and their receptors

While protein synthesis in eukaryotic cells is initiated with a methionine, both prokaryotic and mitochondrial protein synthesis starts with a formylated methionine (118, 119). Formylated peptides are released in tissues upon bacterial invasion, or from lysed mitochondria at sites of tissue damage and thereby constitute important patterns for neutrophils to recognize infection (i.e., PAMPs) or injury (i.e., DAMPs). Formylated peptides of both prokaryotic and mitochondrial origin potently stimulate neutrophil chemotaxis (120-124) via their ligation to the formyl peptide receptor 1 (FPR1). Moreover, it is well established that various bacterial species, including *E. coli*, *S. aureus* and *Listeria monocytogenes* release formylated peptides during growth (122-125). The prototypic formylated

peptide fMLF (*N*-formyl-met-leu-phe) that was the first used synthetic FPR1 agonist is still widely used in experimental studies of this receptor (126) (**paper I, II** and **III**). Structurally very similar to FPR1 is the FPR2 receptor, sharing 69% of their sequence identity. Similar to FPR1, FPR2 recognizes formylated peptides of bacterial origin, although generally longer peptides, such as Hp2-20 from *Helicobacter pylori* (127, 128) and phenol soluble modulin (psm-α) peptides from methicillin resistant *S. aureus* (129, 130).

The complement anaphylatoxins (C3a, C4a and C5a) are, as previously mentioned, generated through enzymatic cleavage of complement proteins upon activation of the complement system. The precursors of the complement anaphylatoxins are synthesized in the liver, i.e., have an endogenous origin. However, as the enzymatic activation of these precursors takes place on bacterial surfaces they serve as end-point chemoattractants. Among the complement anaphylatoxins, C5a is the most potent neutrophil chemoattractant signaling by ligation of the G-protein coupled C5a receptor (C5aR) (131).

SCFAs have gained interest as neutrophil activating PAMPs signaling via the recently deorphanized neutrophil GPCR, FFAR2. While SCFAs were initially recognized as end-products of bacterial fermentation of carbohydrates in the gut (132), they are also released during growth of amino acid fermenting oral anaerobic bacteria (133-135) (**paper I** and **II**). As part of a larger group of free fatty acids, SCFAs represent the subgroup exhibiting the shortest carbon core (C2-C6) and include acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and caproate (136). SCFAs trigger intracellular Ca^{2+} signaling and chemotaxis in human neutrophils (137-140), but by themselves do not induce granule mobilization or assembly of NADPH oxidase (otherwise typical neutrophil responses to GPCR activation) (141). While fairly high concentrations of SCFAs are needed to trigger neutrophil signaling, synthetic agonists with enhanced potency have been discovered in recent years (141). Further, the development of FFAR2 specific synthetic antagonists and allosteric modulators have become useful tools for characterization of this receptor (141, 142) (**paper I** and **II**). While the previously described end-point chemoattractant receptors (FPR1, FPR2 and C5aR) are expressed by several cell types (143) and their agonists, in addition to neutrophils, also activate monocytes (121, 144), the FFAR2 receptor is functionally expressed exclusively in neutrophils (141) (**paper**

I). The implications of the neutrophil specific expression of FFAR2 on neutrophil recruitment to the periodontal pocket will be discussed more in the chapter on neutrophil chemoattractants in periodontitis.

Tissue neutrophils

Neutrophils exert many of their vital functions in tissues after having left the vasculature and previous studies show that extravasated neutrophils collected from different human tissues have profoundly different phenotypes as compared to blood- or other tissue cells (51, 145-148). Knowledge about tissue neutrophils is, however, limited due to the difficulties in sampling of such cells. Nevertheless, techniques have been developed enabling collection of tissue neutrophils from sites presenting with inflammation triggered by both aseptic- and microbe derived stimuli; some of which are described below.

Extravasated neutrophils from aseptic inflammation, i.e., triggered by endogenous stimuli, can be acquired in a controlled manner by formation of skin blisters on healthy individuals. According to this technique, application of negative pressure on the forearms of healthy volunteers generates exudate filled skin blisters by separation of epidermis from the underlying tissues. Neutrophils can then be collected directly from the blisters (149), or the blister roofs can be removed followed by application of collection chambers filled with autologous serum (150, 151). When autologous serum is added, neutrophil chemoattractants such as IL-8 and C5a accumulates over time, resulting in excessive neutrophil migration to the collection chambers (151). Although the blister model is less artificial, the skin chamber model can be a useful technique as it yields higher neutrophil numbers (152) (**paper III** and **Fig. 3**).

Extravasated neutrophils can also be collected by sampling of inflammatory exudate from patients suffering from inflammatory disease, such as inflammatory arthritis. The term inflammatory arthritis refers to a group of diseases (including RA, juvenile idiopathic arthritis and spondyloarthritis) with the common feature of longstanding chronic inflammation of the joints (i.e., synovitis), that is interrupted by episodes of acute inflammation. The synovitis is in these conditions triggered by a complement cascade activated by formation of immune complexes in joints, that is, leukocytes are recruited by non-microbial stimuli

(153). The synovium is filled with a viscous fluid that is composed of plasma dialysate and hyaluronic acid, referred to as synovial fluid. The synovial fluid is under healthy conditions acellular but is during acute inflammatory episodes infiltrated by leukocytes (154, 155). While the neutrophils are the most abundant cell type found in this inflammatory exudate, both monocytes and lymphocytes can be present in varying proportions depending on the donor (**Fig 3**.). Aspiration of synovial fluid from inflamed joints is a therapeutic strategy during acute inflammatory flares (156), and neutrophils purified from the fluid collected during these sessions can easily be used for research purpose (145) (**paper III**).

While the two previously described sampling-methods provide useful information regarding neutrophil migration towards aseptic inflammation, other methods are required to study microbe-triggered inflammatory responses. Periodontitis is an inflammatory disease of the tooth-supporting structures, triggered by bacterial colonization of the gingival crevices (described in more detail in the upcoming chapter on periodontitis). This is an attractive model for studying transmigrated neutrophils, as the inflammatory exudate, i.e., gingival crevicular fluid (GCF) contains abundant neutrophils and is readily accessible for collection using minimal-invasive techniques. Sampling of GCF neutrophils can be performed by

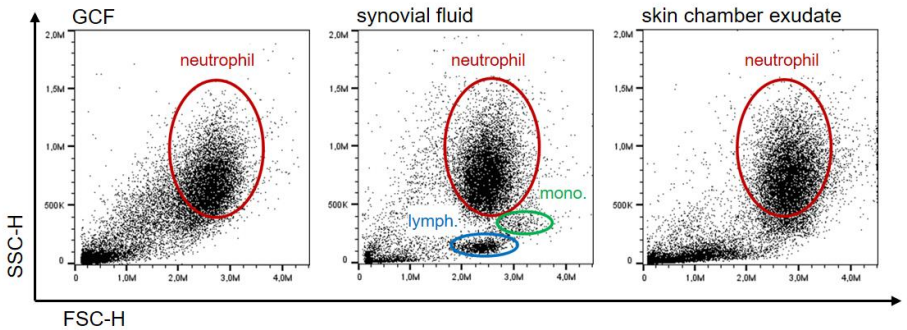

Figure 3. **In vivo** ***transmigrated neutrophils from GCF, synovial fluid and skin chamber exudate.*** *GCF, synovial fluid and skin chamber exudate were sampled as described in the chapter on tissue neutrophils, and analyzed by flow cytometry. Dot plots show distinct neutrophil populations, gated on the basis of forward and side scatter from representative samples of GCF, synovial fluid and skin chamber exudate. While neutrophils (red gate) are the dominating leukocyte in all described exudates, synovial fluid may also contain monocytes (green gate) and lymphocytes (blue gate).*

gently washing gingival pockets with saline solution using a pipette fitted with a gel tip (**Paper III** and **Fig. 3** and **4**).

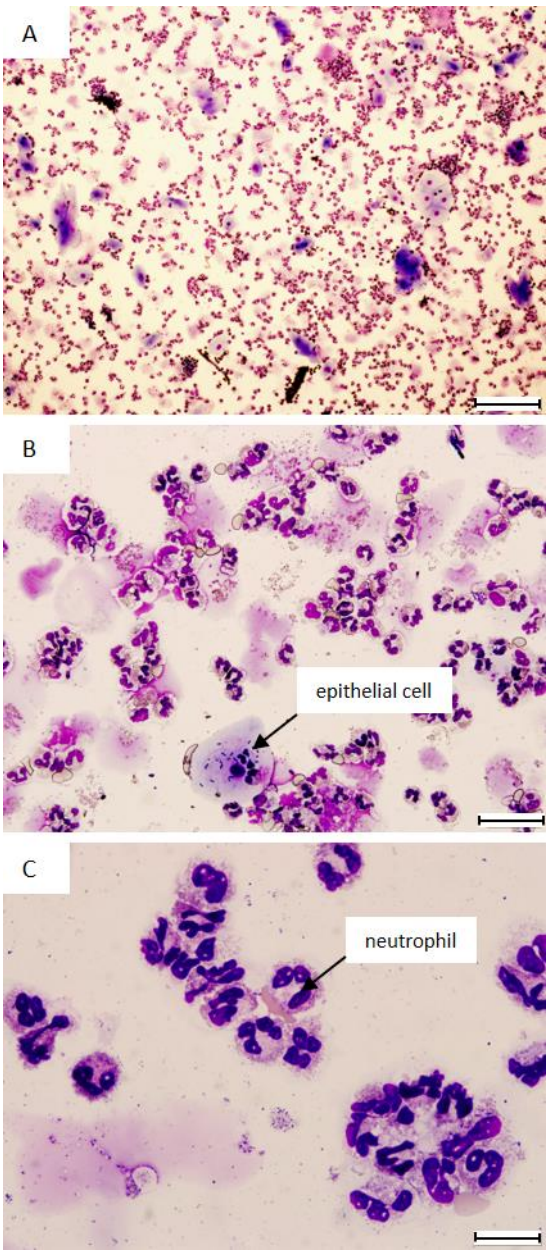

Figure 4. GCF neutrophils. *GCF from a periodontitis patient was cytospun and stained with Giemsa and May-Grünwald. The sample was evaluated microscopically at magnification x10 (A), x40 (B) and x100 (C). Micrographs show abundant neutrophils, epithelial cells and bacteria. The scale bars represent 100 µM (A), 20 µM (B) and 10 µM (C).*

PERIODONTITIS

The oral cavity exhibits a diverse set of habitats suitable for bacterial colonization (e.g., teeth, gingival crevices and tongue) as well as providing a warm and moist environment and accessibility to nutrients such as those ingested, salivary- and GCF proteins. Hence, it is colonized by more than 700 bacterial species (157), which under healthy circumstances coexist in relative harmony with the host (158, 159). However, an undisturbed accumulation of oral bacteria on tooth surfaces along the gingival margin leads to a reversible inflammatory response in the gingival tissues, i.e., gingivitis. Gingivitis presents with clinical signs including erythema, bleeding, tenderness and edema, while not involving loss of tooth supporting tissues (160, 161). If not reversed, gingivitis may progress into periodontitis; a destructive inflammatory condition leading to loss of periodontal ligament, deepening of gingival pockets and degradation of alveolar bone, all of which may ultimately result in tooth loss (162).

Periodontitis is a common disease, affecting 40–50% of the population in all its forms (163-165), while the global prevalence of severe periodontitis was reported to be 11.2 % between 1990–2010 (166), and 9.8% in 2017 (167). Periodontitis has a multifactorial etiology and although a substantial proportion of the interindividual variance in disease phenotype seems to be heritable, i.e., attributable to genetics (168, 169), certain risk factors (including smoking and diabetes) can influence disease development (170). Accumulation of bacterial plaque along the gingival margin inevitably trigger gingival inflammation (171). However, some individuals seem more prone than others to develop severe progressive bone loss (i.e., periodontitis) in response to this inflammation (165). On the other side of the spectrum, certain individuals can present with longstanding gingival inflammation without signs of attachment or bone loss (172). Despite decades of studies in search of single gene variants that predispose for periodontitis, the factors determining the interindividual variation in susceptibility to disease remain unclear (169). Moreover, an accumulating body of evidence supports the epidemiological association of periodontitis with a number of chronic systemic diseases including cardiovascular disease (173), diabetes (174),

chronic obstructive pulmonary disease (COPD) (175), chronic kidney disease (176), RA (88, 177), Alzheimer disease and inflammatory bowel disease (IBD) (178).

Definition, classification and management of periodontitis

The primary characteristic of periodontitis is loss of periodontal tissue as a result of inflammation. Periodontitis is diagnosed based on clinical findings consisting of identification of attachment loss in combination with gingival inflammation. Gingival inflammation can be identified by registration of bleeding on probing (BOP), while severity of attachment loss is assessed either by measurement of clinical attachment loss (CAL) based on probing or by measurement of bone loss based on radiographic images. According to the most recent definition, a periodontitis case can be defined as a patient exhibiting either interdental CAL at ≥ 2 non-adjacent teeth, or buccal or oral CAL ≥3 mm with pocketing > 3 mm at ≥ 2 teeth which cannot be a result of other causes than periodontitis (e.g., traumatic gingival recession, malposition of the third molar, cervical caries, endodontic-periodontal connection or vertical root fracture) (179, 180).

The periodontitis case can be further classified in one of three different forms of periodontitis based on pathophysiology; periodontitis, periodontitis as a manifestation of systemic diseases or necrotizing periodontitis. The two latter forms are differentiated from periodontitis based on the presence of systemic diseases known to cause periodontitis (such as the previously mentioned LAD and PLS) (181), and specific symptoms of necrotizing periodontitis, including papilla necrosis, bleeding and pain (182). The clinical presentation in the individual periodontitis case is characterized using a staging and grading system, in which the stage (I-IV) largely represents severity of disease (including extent and distribution), while the grade provides information regarding progression rate and risk assessment for further progression and treatment outcome (179).

Although gingivitis does not always progress into periodontitis, periodontitis is always preceded by gingivitis and treatment of this condition is therefore an important periodontitis prevention strategy (171). Once periodontitis is established, treatment is primarily based on controlling the accumulation of supra-

and subgingival biofilm, with the intention of resolving the inflammatory response and arrest disease progression. Depending on stage of disease the therapeutic interventions include oral hygiene instructions, professional mechanical removal of supra- and subgingival biofilms, and in the severe cases also surgical intervention (183). Successful periodontal treatment may result in stable conditions with minimal or no disease progression, although stable periodontitis patients remain at increased risk of recurrent disease, and require continuous supportive periodontal care (183).

The oral microbiome and periodontitis

The deep gingival pocket is characterized by its predominantly anaerobic environment, as well as GCF proteins being the main accessible nutritional source. Hence, this site is primarily colonized by gram-negative, anaerobic and proteolytic bacterial species utilizing amino acid fermentation as their main source of energy. During periodontitis development, the gradual transformation of the shallow gingival crevices into deep periodontal pockets, is followed by an outgrowth and enrichment of these anaerobic and proteolytic bacterial species (184).

Theories about the link between the oral microbiome and periodontitis have changed over time, originating at the end of the nineteenth century with the 'non-specific plaque hypothesis', postulating that all bacteria in dental plaque in sufficient amount was the causing agent of disease (185). In the 1970s, this was followed by the 'specific plaque hypothesis' (186), focusing on specific disease-causing bacterial species and proposing that antibiotics could have beneficial effects in periodontitis treatment (187-189). The 'ecological plaque hypothesis', which was proposed in 1990s combined concepts of the previous theories, suggesting that periodontitis is the result of an imbalanced microflora leading to overgrowth of disease associated oral pathogens, referred to as dysbiosis (185, 190). In 1998 Socransky *et al.* identified a complex consisting of the bacterial species *P. gingivalis*, *Treponema denticola* and *Tanerella forsythia* as most strongly associated with periodontitis (191). Although later culture independent studies have expanded the list of periodontitis associated bacteria (also including *Prevotella intermedia*, *Fusobacterium nucleatum*, *Filifactor alocis* and *Eubacterium saphenum*) the

association of the original red complex bacteria to periodontitis has been further corroborated (192).

In 2012 Hajishengallis *et al.* proposed the "keystone pathogen hypothesis" suggesting that specific microbial species can trigger inflammation by increasing the quantity, not only of itself, but also of the total microbial load via manipulation of the innate immunity. This hypothesis was based on the observation that the periodontitis associated species *P. gingivalis,* despite being present in very low amounts (i.e., < 0.01% of the total bacterial count), had the capacity to trigger both compositional alterations and outgrowth of the total commensal oral biofilm in a mouse model, eventually resulting in periodontal breakdown (193). The proposed mechanisms behind the periodontitis inducing properties of *P. gingivalis* include simultaneous anti- and proinflammatory actions, with the intention of avoiding immune clearance by suppressing neutrophil and macrophage killing capacity while maintaining the inflammatory milieu that serves the nutritional needs of amino acid fermenting oral anaerobes (193). These, seemingly contradictory events are achieved by interference with the complement system via gingipain degradation of complement factors, resulting in both increased levels of chemoattractant C5a and modulation of C5a-receptor – Toll like receptor 2 crosstalk, leading to impaired neutrophil and macrophage phagocytosis (161, 194-196). Moreover, transient blocking of gingival epithelial IL-8 production, i.e., "local chemokine paralysis", and expression of atypical LPS that exerts antagonistic effects on TLR4 is suggested to delay neutrophil recruitment to allow for colonization of *P. gingivalis* as well as the outgrowth of other oral commensals (193, 197, 198). Although minimal abundances of *P. gingivalis* have been reported to induce periodontitis in mice models, it is not entirely clear if parallels can be drawn to human periodontal pathology. Moreover, this hypothesis fails to explain why the 23-55% of periodontally healthy subjects who are colonized with *P. gingivalis* do not develop microbial dysbiosis and periodontal breakdown (199-201).

Historically, periodontitis has been viewed as an infectious disease and theories describing the pathogenic mechanisms behind periodontal pathology have primarily focused on the oral microflora as the instigating trigger in disease initiation. In later years, more interest has been directed towards host response as an important factor in periodontitis pathogenesis, and the disease is presently

referred to as a 'chronic inflammatory disease associated with dysbiotic plaque biofilms' (180). In line with this, Van Dyke *et al.* recently proposed the 'Inflammation-mediated polymicrobial-emergence and dysbiotic-exacerbation model', highlighting inflammation as the driver of disease and suggesting that specific periodontitis associated bacteria only play a role in the late stages of disease development (202).

Porphyromonas gingivalis

As a member of Socransky's 'red complex', *P. gingivalis* is one of the bacterial species most highly associated with periodontitis, with up to 87% detection rate at diseased sites (192, 199-201). This assaccharolytic, non-motile, gram-negative, rod-shaped anaerobe acquires energy primarily by amino acid fermentation and requires presence of heme and vitamin K for optimal growth (203, 204). Consequently, *P. gingivalis* thrives in the deep periodontal pocket where oxygen tension is low and inflow of GCF provides continuous accessibility to proteinaceous substrates.

The main end-products stemming from the amino acid fermentation employed by *P. gingivalis* are ammonia and various SCFAs (205-207). In line with this, high concentrations (μM) of SCFAs (primarily acetate, propionate and butyrate) released in culture supernatants of *P. gingivalis* were found to act as chemoattractants specifically activating neutrophils via FFAR2 (discussed more in the chapter on neutrophil chemoattractants in periodontitis) (**paper I**).

P. gingivalis utilizes cysteine proteases (i.e., proteolytic enzymes) for degradation of proteins and peptides, a prerequisite for its ability to take up and utilize these substrates as an energy source. Most (85%) of the proteolytic capacity of *P. gingivalis* can be attributed to a group of cysteine proteases referred to as gingipains (208), which, in addition to providing accessible nutrients, reportedly contribute to invasion of the host immune response via degradation of various immune-regulatory proteins (209). The gingipains in combination with other virulence factors including capsule, fimbriae and atypical LPS, have been proposed to help *P. gingivalis* to effectively avoid and subvert host defense mechanisms rather than acting as a pro-inflammatory bacterium (198, 203).

As previously mentioned, periodontitis is epidemiologically associated with a diverse set of inflammatory disorders including cardiovascular disease (173), diabetes (174), COPD (175), chronic kidney disease (176), RA (88, 177), Alzheimer disease and IBD (178). Low grade systemic inflammation as a result of dissemination of oral derived microbes, such as *P. gingivalis,* has been proposed as a possible pathological mechanism linking periodontitis to some of these comorbidities (178, 210, 211). Moreover, *P. gingivalis* could be a direct mechanistic link between RA and periodontitis based on its previously mentioned expression of PAD, an enzyme known to catalyze protein citrullination. Although, the etiology behind RA is not completely understood, studies support that citrullinated proteins may play a key role in disease initiation (88).

Fusobacterium nucleatum

Fusobacterium nucleatum is a gram-negative, anaerobic, assaccharolytic oral commensal exhibiting a characteristic spindle shaped morphology (212). This anaerobe is the most prevalent gram-negative species found in oral plaque from periodontally healthy sites, with increasing presence following development of gingivitis and periodontitis (184, 212, 213). One of the most important virulence factors of *F. nucleatum* is its expression of various adhesins which bind to both mammalian cells and other bacterial species (213). Based on the unique ability of *F. nucleatum* to form aggregates with both early (e.g., *Streptococcus* spp.) and late colonizers (e.g., *P. gingivalis* and *Aggregatibacter actinomycetemcomitans*), it has been highlighted as an important bridge in the development of oral bacterial plaque (214). Other *F. nucleatum* virulence factors include LPS, serine proteases.

Like most bacterial species colonizing deep periodontal pockets, *F. nucleatum* utilizes amino acid fermentation as main energy source, resulting in end-products including hydrogen sulfide, SCFAs (primarily acetate and butyrate) and ammonia (133, 212, 215, 216). Similar to those derived from *P. gingivalis*, high concentrations of SCFAs (μM-mM) released by *F. nucleatum* acted as FFAR2 dependent neutrophil chemoattractants. Moreover, SCFAs constituted the primary chemoattractants released into culture supernatants of this bacterium (**paper II**), discussed more in the chapter on neutrophil chemoattractants in periodontitis.
Despite primarily being a member of the oral commensal microbiome, *F. nucleatum*, is frequently isolated from extra-oral infections and is thereby regarded

as an opportunistic pathogen. This bacterium has been linked to systemic diseases including atherosclerosis, RA, respiratory tract infections, gastrointestinal disorders (213), colorectal cancer (217) and adverse pregnancy outcomes (218, 219).

Neutrophils in periodontitis

Immune cells are constantly, also under healthy circumstances, recruited to the oral tissues to eliminate microbes and thereby prevent the potentially harmful consequences of their colonization (220). The gingival lesion in periodontitis is dominated by adaptive immune cells (plasma cells, B cells and T helper cells), while neutrophils constitute a relatively small proportion among the leukocytes at this site (172, 221-223). Neutrophils are however found in large quantities on the extraepithelial side of the gingiva (i.e., the gingival pocket and GCF) where they form a 'protective wall' between microbes and host tissues (162, 220, 224). Studies characterizing cell types in GCF have reported on neutrophils constituting 80-98% of the leukocytes present (225-229) (**Paper III**). The neutrophil dominated leukocyte profile of GCF in periodontitis indicate the presence of chemoattractants exclusively attracting this cell type, as will be discussed below.

The critical role of neutrophils for the maintenance of oral health and for avoiding periodontitis can be demonstrated by the fact that the previously described genetic neutrophil deficiencies LAD and PLS as well as congenital neutropenia, predispose to severe and rapidly progressing periodontitis (7-9, 46-48, 224). Similar to what has previously been described for congenital neutropenia and LAD, PLS is an extremely rare disorder with an estimated prevalence of 1-4:1 000 000 individuals (230). The rapidly progressing periodontitis seen in these patients is clearly not directly comparable to the more common form of periodontitis. Despite this, differences in the clinical presentation of inherited deficiencies of distinct neutrophil functions may provide important information regarding neutrophil involvement in periodontal pathology. The rapidly progressing periodontitis seen in patients suffering from congenital neutropenia suggest that a sufficient neutrophil blood count is required to avoid periodontal destruction (7). Resembling what is seen in congenital neutropenia, the deficient neutrophil transmigration in LAD results in insufficient neutrophil numbers at the inflammatory focus. In line with this, LAD patients also suffer from

periodontitis (9). The characteristic periodontal symptoms seen in both congenital neutropenia and LAD have previously been assumed to be a result of defect bacterial killing due to absence of neutrophils in the periodontal pocket. However later studies have revealed that neutrophils, in addition to their bactericidal activities, may also have important immunomodulatory functions, one such being in modulation of T-cell driven periodontal bone loss (231, 232). Further supporting the immunomodulatory role of neutrophils in periodontal pathology is the fact that the previously described CGD patients, who exhibit defect neutrophil microbial killing resulting in an increased susceptibility to severe infections, rarely develop periodontitis (10, 11).

As previously mentioned, PLS is a rare, autosomal recessive disease caused by mutations in the gene encoding cathepsin C (46-48, 73); a cysteine protease that is necessary for end terminal trimming (i.e., activation) of the neutrophil serine proteases NE, PR3 and cathepsin G (233). As a consequence of defect Cathepsin C synthesis, these patients completely lack active neutrophil serine proteases, which results in rapidly progressing periodontitis affecting both the primary and permanent dentition in combination with skin manifestations (46-48). Despite their absence of neutrophil serine proteases, PLS patients do not suffer from severe general immunodeficiency and their neutrophils exhibit intact killing capacity *in vitro* (47), indicating that neutrophil serine proteases may play an important role in protection against periodontal bone destruction that has nothing to do with microbial killing. A common feature for the described periodontitis causing genetic neutrophil defects (congenital neutropenia, LAD and PLS) is the absence of active neutrophil serine proteases at the local inflammatory site, either due to insufficient neutrophil infiltration (neutropenia and LAD) or defects in serine protease activation (PLS). Accordingly, it is tempting to speculate that the absence of these serine proteases may be a factor contributing to periodontitis development in both congenital neutropenia and LAD.

Several studies have shown that blood neutrophils of periodontitis patients are phenotypically different from those of healthy controls. Circulating neutrophils from periodontitis patients reportedly exhibit an increased reactivity in terms of, both stimulated and unstimulated ROS release (234-236) and secretion of pro-inflammatory cytokines (e.g., IL-8, IL-6, TNF-α and IL-1b) (237) as compared to blood neutrophils of healthy controls. This hyperreactivity has been proposed to

contribute to the tissue destruction (ROS) and increased inflammatory responses (cytokine release) that is seen in periodontally diseased patients. Whether the described hyperactivity of neutrophils from periodontitis patients is an innate property or a result of priming by circulating proinflammatory factors (released upon stimulation by oral microbes colonizing the periodontal pockets) in not entirely clear. Similar neutrophil hyperreactivity in terms of enhanced oxidative burst have been reported in blood cells from patients with a diverse set of medical conditions including multiple sclerosis, acute bacteremia and AASV (238-240). The neutrophil hyperreactivity has in these conditions been attributed to increased levels of inflammatory cytokines in circulation (238-240). Moreover, periodontitis patient neutrophils show decreased chemotactic capacity in response to both fMLF and IL-8 *in vitro*, as compared to healthy control neutrophils (241). This may lead to increased neutrophil transit times from blood to the inflammatory site and therefore result in a less effective antimicrobial defense. However, why this periodontitis associated defect in neutrophil chemotaxis does not result in a general overt susceptibility to infection remains to be elucidated.

Neutrophil chemoattractants in periodontitis

To come in close contact with the microbes that colonize the periodontal pocket, neutrophils are guided by coordinated gradients of both endogenous and microbial chemoattractants. The chemokine IL-8 is often proposed to be responsible for chemotaxis to the gingival crevice in periodontitis (242-247), and is indeed capable of mediating neutrophil migration from the vessel wall and through the gingival tissue. However, since IL-8 is an endogenous (intermediate)

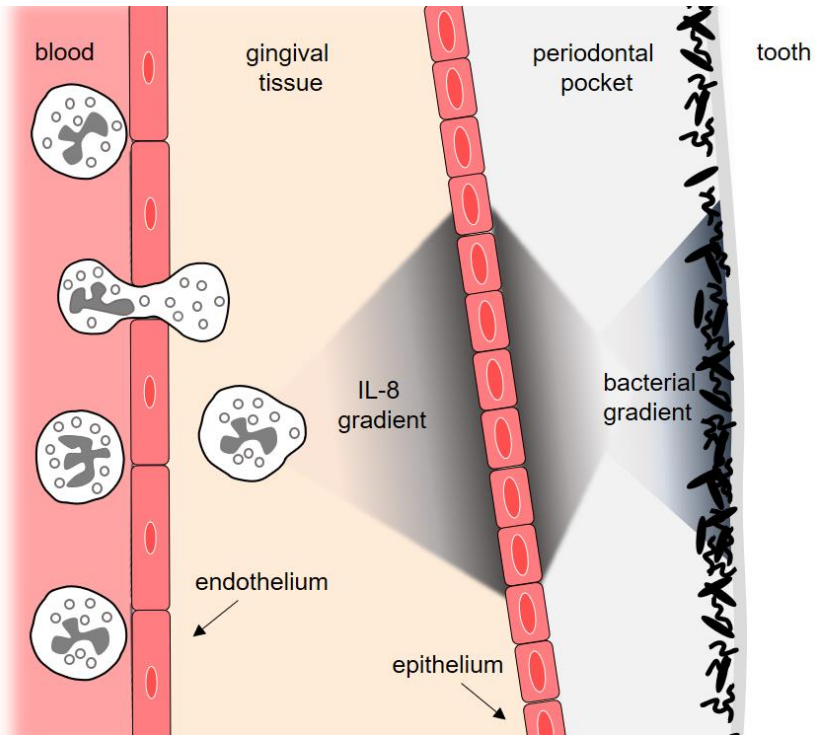

Figure 5. Neutrophil migration from blood to the periodontal pocket. *After endothelial transmigration, neutrophil movement through the gingival tissue is guided by gradients of endogenous chemoattractants, such as IL-8. The concentration of these chemoattractants should be maximal at the epithelium lining the gingival pocket. Thus, further migration over the epithelium may be triggered by chemoattractants released by the anaerobic and amino acid fermenting bacterial species that colonizes the periodontal pocket.*

chemoattractant, produced by tissue residing cells (macrophages, endothelial cells and epithelial cells), the concentration of this chemoattractant should be maximal at the epithelial lining. Thus, bacteria-derived, endpoint chemoattractants should be required to guide neutrophils over the epithelium and further towards the microbes in the gingival pocket (**Fig. 5**).

Although previous studies have shown that both oral subgingival plaque (containing multiple bacterial species) (248) and isolated oral bacterial species (249) (**paper I** and **II**) release factors that attract neutrophils *in vitro,* these chemoattractants have remained uncharacterized. The previously described end-point chemoattractants fMLF and C5a are often mentioned as possible candidates for the chemoattractants released by the anaerobic and proteolytic bacterial species residing in the periodontal pocket. However, the chemoattractant receptors activated by both fMLF and C5a (FPR1 and C5a receptor) are expressed not only by neutrophils, but also monocytes (121, 143, 144). Moreover, their agonists trigger chemotaxis and Ca^{2+} signaling not only in neutrophils, but also in monocytes/macrophages (121, 144) (**paper I**). Therefore, other chemoattractants are needed to explain the neutrophil dominated cellular migration to this inflammatory site. In line with this, purified hydrophilic chemoattractants released by *P. gingivalis* triggered chemotaxis and Ca^{2+} signaling (indicative of GPCR activation) in human neutrophils, while at the same time not activating either monocytes nor lymphocytes (**paper I**). This indicating activation of a GPCR specifically expressed by neutrophils. These chemoattractants were identified (using liquid chromatography and tandem mass spectrometric analysis) as a mixture of SCFAs signaling via the neutrophil GPCR, FFAR2 (**paper I**). As previously mentioned, the FFAR2 receptor is functionally expressed exclusively in neutrophils (141), which explains the neutrophil specific activation capacity of SCFAs. Moreover, SCFAs (primarily acetate and butyrate) acting via FFAR2 were identified as the main chemoattractants released also by *F. nucleatum* (**paper II**).

As previously described, formylated peptides should theoretically be released by all metabolically active prokaryotes. Yet, FPR1 agonists were surprisingly not detected in the bacterial culture supernatant of *P. gingivalis*, at least not agonists capable of inducing β-arrestin recruitment to a detectable extent in a cell line overexpressing FPR1 (**paper I**)**.** A possible explanation to the absence of FPR1 agonists in *P. gingivalis* culture supernatants could be that these bacteria may be

able to degrade and digest formylated peptides secreted as by-products of their own protein synthesis. This as a result of their pronounced proteolytic and amino acid fermenting metabolism.

Although, SCFAs released by *P. gingivalis* did attract neutrophils *in vitro*, the chemotactic activity of these agonists is relatively modest, as compared to other widely studied bacteria derived neutrophil chemoattractants (e.g., fMLF). This in combination with the undetectable levels of FPR1 agonists in culture supernatants of *P. gingivalis* is in line with previous theories describing this bacterium as capable of circumventing host defense mechanisms, rather than being a potent pro-inflammatory bacterium (198, 203). Considering this, one could speculate that a minimal release of FPR1 agonists may constitute a virulence factor that contributes to the overall immune avoiding profile of *P. gingivalis*.

Short chain fatty acids in the periodontal pocket

The release of various SCFAs as end products of the amino acid fermentation employed by oral anaerobes such as *P. gingivalis* (205-207) **(paper I)** and *F. nucleatum* (215, 216, 250, 251) (**paper II**) is well established. SCFAs have also been detected in GCF in varying concentrations depending on severity of periodontal disease, ranging from undetectable in health to millimolar concentrations in severe periodontitis (252, 253). This confirms that SCFAs are indeed produced at high concentrations by oral bacteria also *in vivo*. Moreover, SCFA concentration in GCF is positively correlated to the bacterial load of a number of oral anaerobes including *P. gingivalis* and *F. nucleatum* (253).

Theories linking microbe released SCFAs and gingival inflammation in periodontitis have been around for decades. Singer and Buckner first showed that butyrate and propionate were produced by human dental plaques in concentrations sufficient to effect human cells *in vitro* (inhibition of human fibroblast proliferation) (254). The same authors later showed that pure SCFAs, applied directly on tissues, induced gingival inflammation *in vivo* in beagle dogs (255). Kashket *et al.* subsequently reported that application of SCFAs on human gingival tissues *in vivo* induced an inflammatory response, with one of the outcome measures being an increased neutrophil migration to GCF (256).

In search for the mechanisms underlying SCFA induced gingival inflammation, the effects of SCFAs on various human cell types found in the periodontium have been investigated *in vitro* (255). While, high doses of SCFAs have been described to exert cytotoxic effects on human gingival fibroblasts (254) and epithelial cells (257), no such effects could be seen on endothelial cells exposed to propionate and butyrate (258). SCFAs have also been suggested to inhibit lymphocyte proliferation and cytokine production (259). Regarding neutrophil functions, early studies reported on decreased fMLF or casein induced neutrophil chemotaxis following cell incubation with high concentrations (20-50 mM) of butyrate, succinate and iso-valerate, while incubation with more moderate concentrations of succinate (3 mM), acetate (10 mM) or butyrate (400 μM) had stimulating effects on neutrophil migration towards zymosan activated serum (260-262). Considering the high concentrations of SCFAs detected in GCF from periodontitis patients in combination with the massive neutrophil infiltration to this site, it can be argued that the inhibitory effect of SCFAs on chemotaxis seen in early *in vitro* studies cannot be especially relevant *in vivo*. In support of this notion, later studies have established that pure acetate, propionate and butyrate used as chemotactic stimuli trigger directional migration in neutrophils, and that SCFA stimulated chemotaxis is mediated via the neutrophil FFAR2-receptor (100 uM-1 mM) (137, 138). Moreover, also a mixture of SCFAs in concentrations similar to those detected in culture supernatants of *P. gingivalis* attract neutrophils *in vitro* (**paper I**).

NEUTROPHIL HETEROGENITY

Neutrophils have historically been viewed as a homogenous cell population, with all cells carrying out the same functions and being equipped with similar granule content and surface molecules. However, accumulating evidence supports the simultaneous presence of phenotypically and functionally different sets of neutrophils in the human body and numerous ways of dividing neutrophils into subsets have been proposed in later years (263-265).

In the context of cell heterogeneity, it is important to point out that neutrophils are plastic cells, i.e., they are able to rearrange their expression of surface molecules in response to inflammatory signals (i.e., the priming described in the chapter on neutrophil transmigration) or maturation. Discrepancies in surface molecule expression between cells can thereby represent cells with different activation or maturation status (265). Such surface molecules include the previously mentioned CXCR4 that is expressed in less extent by neutrophils which have recently been released from the bone marrow, but gradually increases in expression during maturation and promotes homing of neutrophils to the bone marrow (26). As a consequence of the circadian oscillations of neutrophil release from the bone marrow, the expression of CXCR4 by circulating neutrophils varies during the day (266). Other surface markers are rearranged based on cellular priming, including shedding of L-selectin (CD62L) and upregulation of the CR3 receptor/MAC-1 (CD11b) (95).

A neutrophil subpopulation that has attracted particular attention in recent years is referred to as 'low density neutrophils' (LDNs) (267, 268). This group of neutrophils are distinguished from 'normal density neutrophils' based on their localization to the peripheral blood mononuclear cell (PBMC) layer (normally consisting of the less dense monocytes and lymphocytes) during neutrophil purification by density gradient centrifugation. Increased numbers of LDNs in blood have been reported from patients with a diverse set of medical conditions including auto-immune diseases such as RA and systemic lupus erythematosus (SLE), cancer, asthma, sepsis and pregnancy. However, it has also been reported

that healthy individuals exhibit a natural spectrum of neutrophil densities (267). The LDNs have been suggested to consist of both immature cells (released from the bone marrow upon emergency granulopoiesis) and activated mature cells, based on increased expression of CD11b (268, 269). Whether LDNs constitute a distinct neutrophil subset or simply represent neutrophils of varying maturation or activation states is still a matter of debate.

Although many of the proposed neutrophil subtypes might simply represent cells in various stages of maturation or activation, evidence also support the existence of true (*bona-fide*) subsets based on the presence or absence of distinct molecular markers (265, 270). These markers are exclusively expressed by a proportion of neutrophils in a given individual, independent of priming or maturation. Two such *bona-fide* neutrophil subset markers are olfactomedin 4 (OLFM4) (271, 272) and CD177 (273-275).

Expression of the glycoprotein OLFM4 has been detected in a range of human tissues including bone marrow, small intestine, stomach, colon and prostate (276). The OLFM4 gene is expressed by all myelopoietic lineage bone marrow cells but, interestingly, only a proportion of mature circulating neutrophils express the protein. The interindividual variation in proportion of OLFM4 expressing blood neutrophils have been reported to be 5-57% (271, 272). Although the precise function of neutrophil OLFM4 is still under investigation, studies have shown that the relative abundance of OLFM4 expressing neutrophils in circulation may affect the immune response to severe infection, such as sepsis (277). Alder *et al.* found high proportions of OLFM4 expressing neutrophils in blood to be a risk factor for massive organ failure and death in pediatric septic shock patients (277). In line with this, complete absence of OLFM4 had a protective function against sepsis in a mouse model (278). Similar to OLFM4 in sepsis, the relative abundance of CD177 expressing neutrophils in circulation may be a modifying factor in several medical conditions that are associated with increased proportions of $CD177^+$ circulating neutrophils (e.g., periodontitis (**paper III**)), which will be further expanded on in the subsequent chapters.

CD177

The granulocyte specific antigen that was later identified as CD177 (273) (also known as NB1 or HNA-2) was first described by Lalezari *et al.* in 1960 while studying new-borns suffering from neutropenia due to maternal isoimmunization (273, 279). CD177 is a ~60 kDa glycoprotein that is anchored to neutrophil plasma- or granule membranes via glycosylphosphatidylinositol (GPI) (280-282). The GPI attachment to the neutrophil membranes suggests that it is inert, i.e., unable to transmit signals intracellularly. The CD177 glycoprotein is exclusively expressed by neutrophils and cannot be detected in other leukocytes, such as monocytes and lymphocytes (273)(**paper III**). Exhibiting a bimodal expression pattern, CD177 can only be found on a proportion of circulating neutrophils in a given individual. The individual expression of CD177 can vary between 0-100% of circulating neutrophils depending on the donor (274, 275, 283)(**paper III** and **Fig. 6.**). The individual proportion of CD177-expressing neutrophils in blood seems to be independent of age, but with a slightly greater expression in women as compared to men (275).

Synthesis of CD177 begins at the metamyelocyte stage (283, 284) in the bone marrow and increases gradually during development into mature neutrophils, with mature bone marrow cells and blood cells exhibiting similar levels of CD177

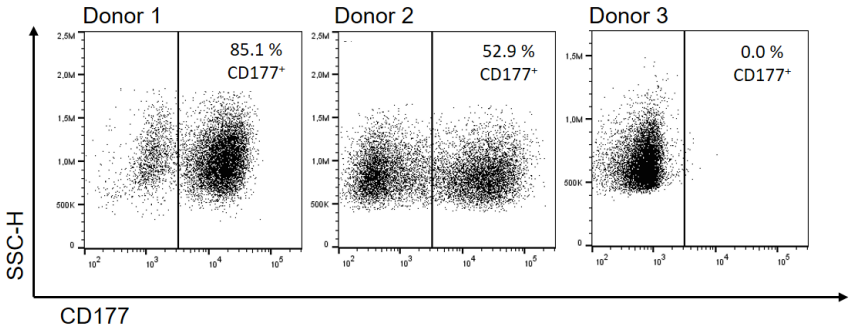

Figure 6. Interindividual variation in proportions of circulating CD177+ neutrophils. *Peripheral blood neutrophils were co-stained with anti-CD45 and anti-CD177 antibodies and analyzed by flow cytometry. Dot plots show the varying proportions of CD177+ neutrophils in circulation of three healthy donors.*

expression (283). Inflammatory stimulation (e.g., with fMLF or TNF-α) can induce further upregulation of CD177 from granules to the cell surface of $CD177^+$ neutrophils, resulting in an increased membrane expression in this subset (274) (**paper III**). However, a similar upregulation do not occur in $CD177^-$ neutrophils (274) (**paper III**). Accordingly, the proportions of $CD177^+$ and $CD177^-$ subsets in an individual remains relatively stable over time (274, 285), with the exception of transiently increased blood levels of $CD177^+$ neutrophils reported in pregnancy (286, 287) and during treatment with G-CSF (288, 289). While a majority of donors exhibit a bimodal expression pattern (with one $CD177^+$ and one $CD177^-$ neutrophil subpopulation), there are individuals also presenting with an intermediate subpopulation, referred to as a trimodal CD177 expression (275) (**paper III**). About 1–10% of individuals completely lack CD177 expressing neutrophils, are referred to as CD177-null and appear to be overall healthy (273-275, 290) (**paper III**). CD177-null individuals are however at risk of complications related to development of anti-CD177 antibodies if exposed to the antigen. Such complications include transfusion related acute lung injury following blood transfusion (291) and neonatal neutropenia affecting CD177 expressing fetuses of CD177-null mothers (279). Induction of a stop codon (originating from a neighboring pseudogene) in the coding region of CD177 has been identified as the genetic factor causing the complete lack of CD177 expression in these CD177-null individuals (292, 293). However, the genetics behind the bimodal expression pattern of CD177 seen in most individuals is still only partially understood, although studies have proposed a combination of complex genetic and epigenetic factors (293-295).

Whereas CD177-null individuals appear to be overall healthy, increased blood levels of $CD177^+$ neutrophils have been associated to various medical conditions, including severe bacterial infections (296), polycythemia vera (PV) (297) and other myeloproliferative disorders (298), AASV (299-301), SLE (299) and periodontitis (**paper III**). Considering these observations, it is tempting to speculate that the relative proportion of $CD177^+$ neutrophils in circulation may be a factor influencing the susceptibility to diseases such as periodontitis, SLE, PV, and AASV. Nonetheless, it should be kept in mind that the increased proportions of $CD177^+$ circulating neutrophils detected in patients with the described conditions may as well be a consequence of unknown factors related to an ongoing inflammation.

Previous studies have established that the percentage of CD177$^+$ cells in blood can be transiently increased by administration of G-CSF to healthy controls (288, 289). As previously mentioned (in the chapter on the neutrophil life cycle), G-CSF regulates baseline levels of neutrophil maturation and release from the bone marrow (22-24) and increased serum levels of this cytokine have been reported in several inflammatory conditions (302). Increased mRNA- and protein expression of G-CSF have also been reported in the gingival tissue of periodontitis patients, as compared to the gingival tissue of healthy controls (303). Considering this, one could speculate that a rise in serum G-CSF levels in response to the ongoing inflammatory process in the periodontitis lesion may result in increased levels of CD177$^+$ neutrophils in circulation of this patient group. Along these lines, Abdgawad *et al.* measured plasma levels of G-CSF in vasculitis patients (who also exhibit increased proportions of CD177$^+$ neutrophils in blood as compared to healthy controls) but did not find increased G-CSF levels in this patient group (300). Moreover, increased levels of G-CSF have been reported in the serum and synovial fluid of patients with RA (304), while this patient group did not exhibit higher levels of CD177$^+$ neutrophils in circulation as compared to healthy controls (300). Thus, it is less likely that increased systemic G-CSF levels are directly responsible for the high proportions of CD177$^+$ neutrophils reported in AASV and other inflammatory conditions such as periodontitis. The pattern of high blood levels of CD177$^+$ neutrophils in these conditions could either be due to yet unknown external factors with the capacity to increase the CD177$^+$ cell proportion, or a result of innate high proportions of CD177$^+$ neutrophils in blood being a factor predisposing to inflammatory disease.

CD177 and neutrophil transmigration

The possible role of CD177 in neutrophil migration have attracted much attention. This based on *in vitro* evidence of an increased capacity of CD177$^+$ neutrophils to migrate over endothelial cell layers, as compared to CD177$^-$ cells (305, 306). The migration advantage of CD177$^+$ neutrophils has in part been attributed to the ability of CD177 to interact with PECAM-1; an important contributor to neutrophil transmigration that is expressed by endothelial cells, platelets, monocytes and granulocytes (307). Furthermore, CD177 has been shown capable of binding and presenting the neutrophil serine protease PR3 on

the cell surface (308, 309). The membrane presentation of PR3 has been proposed to facilitate neutrophil migration via proteolytic degradation of extracellular matrix- or junctional proteins (310). In contrast to the described *in vitro* results, previous studies looking at neutrophil migration to both synovial fluid in RA patients (285) and peritoneal fluid in dialysis patients suffering from peritonitis (311) have not been able to confirm a migration advantage of $CD177^+$ neutrophils *in vivo*.

Interestingly, GCF (sampled as described in the chapter on tissue neutrophils) from patients suffering from periodontitis (stage III-IV) exhibited higher proportions of CD177 expressing cells as compared to blood from the same individual, i.e., the $CD177^+$ neutrophil subtype was enriched in GCF (**paper III**). This phenomenon was not detected either in synovial fluid from inflammatory arthritis patients or inflammatory exudate from experimental skin chambers applied to healthy donors (**paper III**).

The enrichment of $CD177^+$ neutrophils in GCF could not be attributed to general differences in longevity between the subsets, as the $CD177^-$ subset showed similar (in anaerobic environment) or even decreased (in aerobic environment) rate of *in vitro* apoptosis as compared to the $CD177^+$ cells (**paper III**). The $CD177^+$ subtype showed a slight migratory advantage *in vitro* when stimulated with IL-8 or with a culture supernatant prepared from a subgingival bacterial sample from a periodontitis patient. Further, the $CD177^+$ and $CD177^-$ subtypes migrated with equal efficiency towards fMLF (**paper III**). The detected migratory advantage of CD177 expressing neutrophils *in vitro* was not prominent enough to be the only factor explaining the enrichment of $CD177^+$ cells seen in GCF. Consequently, the accumulation of $CD177^+$ cells in this inflammatory exudate may in also be attributed to tissue specific properties. However, the slight migratory advantage of $CD177^+$ neutrophils towards the culture supernatant of the periodontitis bacterial sample indicate that bacterial chemoattractants specific for the periodontal pocket may influence the preferential recruitment of this subtype to GCF in periodontitis. Considering the previously described high concentrations of SCFAs and undetectable levels of FPR1 agonists in culture supernatants of periodontitis associated bacteria (**paper I, II**), the supernatant used to stimulate *in vitro* chemotaxis likely contain similar bacterial end-products. In light of these

observations, it is tempting to speculate that selective CD177$^+$ neutrophil chemotaxis can be promoted by the presence of SCFAs.

Further differences between the neutrophil subtypes defined by CD177 expression?

While studying the neutrophil subtypes defined by the presence or absence of CD177, it is important to keep in mind that CD177 *per se* does not necessarily constitute the only differentiating factor between the CD177$^+$ and CD177$^-$ subsets. There may be additional molecules showing a bimodal expression pattern that completely or partially overlap CD177 expression, and thereby contribute to functional differences between CD177$^+$ and CD177$^-$ neutrophils. As previously mentioned, patients suffering from periodontitis exhibit higher levels of CD177$^+$ neutrophils in circulation, as compared to healthy donors (**paper III**). This, in combination with the further enrichment of the CD177$^+$ subtype in GCF from these patients, resulted in very high proportions of CD177 expressing neutrophils at this pathological site (**paper III**). Considering the high levels of CD177 expressing neutrophils in GCF, functions differing between the subsets could be of importance for induction (or suppression) of destructive inflammation in the periodontal tissues.

A previous comparison of mRNA expression between CD177$^+$ and CD177$^-$ neutrophils showed differential expression of a number of distinct mRNAs including those coding for the granule proteins PR3, MPO, alpha defensin 3, BPI, and Cathepsin G. However, subsequent immunoblotting of these proteins showed no expression differences on protein level between the subsets (284). In view of the minimal *de novo* protein synthesis employed by neutrophils after bone marrow release, mRNA quantification is not likely to appropriately represent the complete proteome profile of these cells and therefore comparisons performed on the protein level are likely more informative.

Relative quantitative proteomics is a method enabling detection of proteins and determination of their relative abundances in several samples simultaneously (312). This method applied to samples of FACS-sorted CD177$^+$ and CD177$^-$ neutrophils from the same individual showed very similar expression levels of the vast majority of detected proteins, but nonetheless, a differential expression

pattern of a limited number of proteins (**paper IV**). CD177 itself was, as expected, the protein that primarily differentiated the subsets. Another top hit, alpha-1-antitrypsin (A1AT), was expressed twice as much in the CD177$^+$ neutrophil subset as compared to the CD177$^-$ subset (**paper IV**). A1AT is an endogenous neutrophil serine protease inhibitor, engaged in defending tissues from proteolytic degradation by excessive release of proteases (313, 314). Constantly present in circulation, A1AT diminish protease activity by complex-formation (1:1) with neutrophil serine proteases. While 80% of the circulating A1AT is produced by hepatocytes in the liver, it is also secreted by immune cells including monocytes, macrophages and circulating neutrophils (315-317). Tissue A1AT levels are significantly increased during an inflammatory response and a majority of this increase is likely a result of local release by resident and infiltrating inflammatory cells (314). The possible effect of an increased A1AT-expression on net protease activity derived from CD177$^+$ cells was further investigated. NE activity was measured in lysates (normalized based on cell number) of FACS-sorted CD177$^+$ and CD177$^-$ cells. In concordance with the increased A1AT expression, CD177$^+$ neutrophils also contained lower NE activity, as compared to the CD177$^-$ cells (**paper IV**). Although the difference in NE activity between the subset may in part be due to a slightly lower (ratio 0.85) expression of the protein in the CD177$^+$ subset, the results indicate an interesting functional difference (net NE activity) between CD177$^+$ and CD177$^-$ cells.

As previously described (in the chapter on periodontitis), a complete lack of active neutrophil serine proteases (as seen in PLS patients) initiates rapidly progressing periodontitis (46-48), indicating that the presence of neutrophil serine proteases constitutes a critical factor for maintenance of periodontal health. Hence, factors disturbing the protease-antiprotease balance at the local inflammatory site may influence destructive inflammatory responses in periodontal tissues. If and how the increased A1AT expression and decreased NE activity seen in CD177$^+$ neutrophils affect local levels of protease activity will be further investigated in **paper IV**.

CONCLUDING REMARKS

Paper I and **II** highlights the SCFA-FFAR2 interaction as a factor mediating neutrophil recruitment from circulation and migration towards the periodontitis associated anaerobes *P. gingivalis* and *F. nucleatum*. As SCFAs are end-products of the amino acid fermenting metabolism that is generally employed by the anaerobic bacterial species colonizing deep periodontal pockets, this finding could be relevant also for other periodontitis associated bacterial species. Given the neutrophil specific expression of FFAR2, SCFA may also contribute to the neutrophil dominated leukocyte profile of GCF in periodontitis. This adds to our understanding of bacteria derived factors triggering neutrophil recruitment to the periodontal pocket.

The results of **paper III** show that the $CD177^+$ neutrophil subtype is enriched in GCF of periodontitis patients, possibly due to a combination of site specific chemoattractants and tissue specific properties. Moreover, and similar to what was previously reported for other inflammatory conditions (299-301), periodontitis patients exhibited increased levels of circulating $CD177^+$ neutrophils as compared to healthy controls. This may be a result of the systemic presence of so far unknown inflammatory factors related to these conditions, but could also indicate that the relative proportion of $CD177^+$ neutrophils in circulation is a factor influencing the susceptibility to inflammatory diseases, such as periodontitis. Whether the increased proportions of $CD177^+$ neutrophils in circulation of these patients are a cause or consequence of disease remains unresolved. To bring clarity to this issue, it would be of interest to monitor inflammatory disease progression in individuals exhibiting varying base line levels of CD177 expression over time.

As a result of both increased levels of $CD177^+$ neutrophils in blood of periodontitis patients and the further enrichment of this subtype after transmigration to the periodontal pocket, $CD177^+$ neutrophils typically constituted a very large proportion of the neutrophils found in GCF of these patients (**paper III**). Accordingly, functions differing between the subtypes may

be influencing destructive inflammation of the periodontal tissues. The preliminary results of **paper IV** show that the neutrophil serine protease inhibitor A1AT is expressed twice as much in the $CD177^+$ neutrophil subset as compared to the $CD177^-$ subset from the same donor. Moreover, results also indicate that $CD177^+$ neutrophils exhibit decreased NE activity as compared to $CD177^-$ neutrophils. Considering that sufficient neutrophil serine protease activity seems to be vital for periodontal health, as demonstrated by the rapid periodontal breakdown seen in PLS, an overt inhibition of this activity may be a modulating factor in periodontal pathology. Along these lines it is tempting to speculate that patients suffering from periodontitis may in fact represent a 'light version' of what is seen in PLS, i.e., present with a deranged protease-antiprotease balance in the local gingival tissue resulting in less-than-optimal serine protease activity. Future studies will have to determine whether local suboptimal protease levels can be a factor influencing periodontal pathology.

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