Human neutrophil heterogeneity: subsets, markers and autoantibodies

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To my father

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ABSTRACT

Neutrophils are phagocytic white blood cells that play essential roles in innate immunity and inflammation. Neutrophils have traditionally been viewed as a homogenous cell population, where all cells carry the same molecular cargo and have identical functions. Lately, this view is changing and distinct subsets of neutrophils are being recognized. The aim of this thesis was to characterize neutrophil subsets and investigate the role of their markers as anti-neutrophil cytoplasmic (ANCA) antigens.

The first paper investigated the recently discovered neutrophil subsets defined by the presence or absence of Olfactomedin-4 (OLFM4). We found that OLFM4 is indeed expressed by only a portion of the neutrophils from a given individual and that OLFM4 is a specific granule protein that can be exposed on neutrophil extracellular traps (NETs). We did not find any functional differences between the subsets in vitro, and the subsets migrated equally well into tissue in vivo, both to synovial fluid of arthritis patients and to aseptic experimental skin chamber on healthy skin.

During routine testing for the presence of ANCA in the circulation of patients with suspected autoimmune disorders, we found sera from two patients with diffuse inflammatory symptoms that displayed unusual staining patterns, reacting with only a subset of neutrophils. In the second paper, we identified the target antigen as OLFM4, and this was the first report of autoantibodies towards this subset marker.

The third study began with two additional patient sera that gave rise to subset-restricted ANCA staining patterns. This time, we identified the target antigen as CD177, which is a known neutrophil subset marker and a known target of alloantibodies. CD177 has previously been shown to be of importance for trans-migration of neutrophils in vitro. However, using synovial fluid from arthritis patients and the experimental skin blister model, we found no evidence for a major general role of CD177 in in vivo neutrophil transmigration. In summary, we have characterized two bona fide neutrophil subsets and identified OLFM4 and CD177 as ANCA antigens.

Keywords: ANCA, OLFM4, CD177, neutrophils

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Sammanfattning på svenska

Neutrofiler är vita blodkroppar som spelar en mycket viktig roll vid inflammationsprocessen genom att vandra till infektionsställen och äta upp och förstöra mikroorganismer. Neutrofiler har traditionellt setts som en homogen grupp av celler, där alla celler bär på samma molekyler och har identiska funktioner. På senare tid har denna syn förändrats och distinkta subtyper av neutrofiler har upptäckts. Patienter med autoimmuna sjukdomar såsom exempelvis inflammation i små blodkärl kan bilda antikroppar som binder till molekyler hos neutrofiler, s.k. anti-neutrofila cytoplasmatiska antikroppar (ANCA), vilka tros bidra till sjukdomsutvecklingen. Syftet med denna avhandling var att karakterisera subtyper av neutrofiler och undersöka vilken roll de molekyler som definierar subtyper har som ANCA-antigener.

I den första studien undersöktes de nyupptäckta undergrupperna av neutrofiler som definieras genom närvaro eller frånvaro av proteinet Olfactomedin-4 (OLFM4). Vi fann att OLFM4 faktiskt uttrycks i endast en del av neutrofilerna från en given individ. OLFM4 finns i blåsor inuti cellen som kallas specifika granule och proteinet kan exponeras på de extracellulära DNA-nät som neutrofilerna kan släppa ut utanför cellen (sk. neutrophil extracellular traps; NETs). Vi kunde dock inte hitta några funktionella skillnader mellan neutrofiler som var positiva och negativa för OLFM4 när cellerna undersöktes i laboratoriet och de två grupperna av celler vandrade i samma grad ut i vävnad, både till ledvätska hos patienter med inflammatorisk artrit och till hudblåsor i en aseptisk modell av akut inflammation på frisk hud.

Under rutintestning för närvaron av ANCA i blodomloppet hos patienter med misstänkta autoimmuna sjukdomar fann vi serumprover från två patienter med diffusa symptom på inflammation som visade ovanliga färgningsmönster genom att endast reagera med en del av neutrofilerna i testet. I det andra delarbetet visade vi att antikroppar i serumprover från dessa patienter reagerade med just OLFM4, och detta var den första rapporten om ANCA mot detta protein hos patienter.

I den tredje studien identifierade vi serumprover från två ytterligare patienter som gav upphov till liknande partiella ANCA färgningsmönster. Vi visade att antikroppar i dessa prover band till CD177, som är ett välkänt protein hos en subtyp av neutrofiler och ett känt mål för alloantikroppar. CD177 har tidigare visat sig vara av betydelse för vandring av neutrofiler i experimentella provrörstester. När vi undersökte neutrofilernas förmåga att vandra i vävnader genom att analysera ledvätska från patienter med inflammatorisk artrit och vätska från experimentella blåsor på hud, fann vi dock inga bevis för en större allmän betydelse av CD177 för utvandring av neutrofiler i vävnad.

Sammanfattningsvis har vi karaktäriserat två undergrupper av neutrofiler och identifierat respektive bekräftat OLFM4 och CD177 som ANCA-antigener.

List of papers

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

I. Welin A, Amirbeagi F, Christenson K, Björkman L, Björnsdottir H, Forsman H, Dahlgren C, Karlsson A, Bylund J. The human neutrophil subsets defined by the presence or absence of OLFM4 both transmigrate into tissue in vivo and give rise to distinct NETs in vitro.
PloS One. 2013 Jul 29; 8 (7):e69575.

II. Amirbeagi F, Thulin P, Pullerits R, Pedersen B, Andersson BA, Dahlgren C, Welin A, Bylund J. Olfactomedin-4 autoantibodies give unusual c-ANCA staining patterns with reactivity to a subpopulation of neutrophils.
J. Leukoc. Biol. 2015 Jan 97(1):181-9.

III. Amirbeagi F, Thulin P, Björkman L, Andersson BA, Saalman R, Pullerits R, Welin A, Bylund J. CD177 (HNA-2a, NB1): Bimodal ANCA pattern of patient autoantibodies and investigations of involvement in neutrophil extravsation.
In Manuscript.

Abbreviations

AAV	ANCA associated vasculitis
ALP	alkaline phosphatase
ANCA	anti-neutrophil cytoplasmic antibody
BPI	bactericidal permeability-increasing protein
CF	cystic fibrosis
CGD	chronic granulomatous disease
CR	complement receptor
EGPA	eosinophilic granulomatosis with polyangiitis
G-CSF	granulocyte colony-stimulating factor
G-MDSC	granulocytic myeloid-derived suppressor cells
GPA	granulomatosis with polyangiit
GPI	glycosylphosphatidylinositol
HNA	human neutrophil antigen
HOCl	hypochlorous acid
IBD	inflammatory bowel disease
IIF	indirect immunofluorescence
LDN	low-density neutrophils
MMP	matrix metalloproteinases
MPA	microscopic polyangiitis
MPO	myeloperoxidase
mPR3	membrane-associated PR3
NETs	neutrophil extracellular traps
NGAL	neutrophil gelatinase-associated lipocalin
OLFM4	olfactomedin-4
PECAM-1	platelet and endothelial cell adhesion molecule 1
PR3	proteinase 3
PRR	pattern recognition receptors
ROS	reactive oxygen species
SVV	small vessel vasculitis

Content

Introduction 1
The immune system 3
Inflammation 4
Neutrophils 5
Maturation and release 5
Storage organelles - granules and vesicles 6
Azurophil granules 6
Specific granules 7
Gelatinase granules 7
Secretory vesicles 7
Neutrophil transmigration 8
Granule mobilization and priming during transmigration 9
In vivo models of transmigration and priming 9
Microbial eradication mechanisms 10
Oxygen-independent antimicrobials 11
Reactive oxygen species (ROS) 11
Neutrophil extracellular traps 11
Neutrophil subsets 12
Olfactomedin-4 (OLFM4) 13
CD177 15
Other bona fide subset markers 17
Anti neutrophil cytoplasmic antibodies (ANCA) 19
Vasculitis 19
Classic ANCA antigens and their detection 20
The role of ANCA in vasculitis 21
Atypical ANCAs 23
Subset ANCAs 23
OLFM4-ANCA 25
CD177-ANCA 26
Finding additional neutrophil subsets… 28
Concluding remarks and future perspectives 29
Acknowledgements 31
References 33

Introduction

We are constantly surrounded by microorganisms and countless different species live their lives in or on our bodies. Despite this we are relatively rarely sick from serious infections and the key to our general well-being is our immune system.

A potent immune system is crucial for protection against infectious diseases, but there are also cases where seemingly over-reactive immune responses may in themselves cause inflammatory and autoimmune diseases. Thus as most often in biology, balance is crucial for maintaining health.

During an inflammatory process, neutrophils, the phagocytic cells, are the first line immune cells to reach the site of inflammation. They are armed with a vast number of antimicrobial substances that help them to degrade the invaders.

These cells have traditionally been viewed as a homogenous cell population, which all carry the same molecular cargo and are capable of carrying out the same functions. Lately, this view is changing and distinct subset subpopulations are being recognized.

This thesis deals with characterization of neutrophil subsets and investigates the role of their markers as anti-neutrophil cytoplasmic (ANCA) antigens.

The immune system

The immune system is a collection of cells, tissues and molecules that are engaged in resistance, prevention and eradication of infections. In mammals, it consists of two overlapping parts often referred to as the innate and the adaptive immune system. While adaptive immunity is characterized by a gradual development of high-specific recognition and the formation of immunological memory, the innate system is less specific and relies on fixed recognition by germline-encoded receptor structures that are present from birth. Both systems work hand in hand with the goal of keeping invading microbes in check.

Adaptive immune responses are started by the recognition of antigens presented by antigen presenting cells such as macrophages and dendritic cells. The recognition structures are represented by humoral components (antibodies) and cellular receptors (on the surface of B- and T-cells). Given time, the specificity of recognition gradually increases and there is a continuous development leading to higher and higher affinity for the antigens. Briefly, during cell-mediated immunity, activated T-cells attack (often infected or malignant) host cells presenting the antigen for which the T-cell carries receptors [1, 2]. During humoral, or antibody-mediated responses, antibodies directly bind and neutralize e.g., viral particles or toxins, but they may also promote the actions of innate immunity by e.g., opsonization of microbes so that phagocytosis is facilitated. On the other hand, failure to discriminate between self and non-self by the immune system results in an immune response to the body's own tissues and cells. This autoreactivity against self-antigens may result in autoimmune diseases, which simplistically could be seen as the immune system trying to attack and eradicate our own tissues.

During the time it takes for adaptive immunity to develop high-specific recognition, innate immunity almost instantaneously responds and keeps the threat under control. Besides cytokines, chemokines and complement factors, innate immunity consists of epithelial cells, sentinel cells in tissues (macrophages, dendritic cells), natural killer cells, and polymorphonuclear leukocytes, i.e., basophils, eosinophils, and neutrophils. Many of these cells and molecules come together in a rapid and orchestrated process known as inflammation.

Inflammation

Inflammation may be considered a classic biological stimulation–response system [3]. Inflammation is a process by which our body's immune cells, and substances that they produce, protect us against harmful infections. Importantly, both infectious (microorganisms) and non-infectious irritants of different kinds can initiate inflammation. Upon recognition of the triggering agent, innate immune cells become activated and release pro-inflammatory molecules that increase local blood flow and vessel permeability in order to facilitate exudation of serum proteins and the recruitment of leukocytes. This gives rise to the cardinal symptoms of inflammation; pain, heat, redness, swelling and loss of function [3].

It is of great importance that the inflammatory process is regulated and resolved as a prolonged inflammation may cause harm to surrounding tissues. Optimally, inflammation should be fast and efficient and as soon as the initial threat is neutralized and/or removed, it should transit into a healing phase where homeostasis is gradually restored. However, inflammation is a very powerful reaction that unavoidably causes collateral damage to surrounding cells and tissues. Prolonged and/or misdirected inflammation is the hallmark of many debilitating disease states [4].

One of the most powerful cell types that are part of acute inflammation is neutrophils. As will be described in the coming section, neutrophils are the dominating cell type to arrive to inflamed tissues and these cells are equipped with an impressive arsenal of anti-microbial compounds. These microbicidal agents are not only central for killing invading microbes, but also have the potential to cause severe inflammatory tissue damage.

Neutrophils

Neutrophils are phagocytic white blood cells, belonging to the family of polymorphonuclear granulocytes that play an essential role in innate immunity and inflammation. The nucleus of these cells is divided into 2-5 lobes and small membrane-enclosed organelles (granules) are present in their cytoplasm. As opposed to most other leukocytes, mature neutrophils have a limited ability to synthesize new proteins. They store most of the molecules that they need during their limited life-span in a variety of storage organelles or granules; hence the term granulocytes. Neutrophils are the most abundant white blood cells in human circulation (constituting around 50-75% of all circulating leukocytes) and as will be described below they are key players during acute inflammation [5, 6].

Maturation and release

The bone marrow is the site of formation and maturation of neutrophils. This process is regulated by various signaling molecules, such as the cytokine granulocyte colony-stimulating factor (G-CSF), directing haematopoietic stem cell differentiation [7]. During proliferation and differentiation in the bone marrow, the first cells of granulopoiesis, the myeloblasts, differentiate to promyelocytes that eventually continues to form myelocytes at which stage proliferation stops. The following maturation stages of terminal differentiation consist of metamyelocytes, band cells and segmented neutrophils that are subsequently released to circulation as mature neutrophils [8].

Mature neutrophils are released continuously into circulation and in case they do not transmigrate out into inflamed tissues, aged neutrophils are ultimately cleared in the spleen, liver or the bone marrow itself [9, 10]. Besides circulating neutrophils in vessels and the reserve pool of mature cells in the bone marrow, marginating neutrophils that are loosely attached to the vascular endothelium form a third pool of these cells [11].

Storage organelles - granules and vesicles

Intracellular granules and vesicles are central to neutrophil physiology. These organelles are membrane-enclosed and packed with pre-formed molecules that can be used during different phases [12]. The granules are formed during different stages of granulopoiesis. Their formation is described as a continuum from the earliest-formed azurophil (primary) granules created during the early promyelocyte stage, to specific (secondary) granules during the myelocyte-metamyelocyte stage, gelatinase (tertiary) granules during the band cell stage, to the latest-formed secretory vesicles formed in segmented neutrophils [13]. The granules are filled with different proteins synthesized during the time of their formation, according to the targeting-by-timing hypothesis [14].

One reason for segregating certain proteins into different types of granules is that the content of the different granules may be needed at different times and places. As will be described, the granule types formed during the later phases of differentiation are more prone to extracellular - than those formed in the early stages. The early-formed granules fuse mainly with phagosomes where they empty their contents [15, 16]. Another reason for this granule segregation can be that some proteins cannot coexist in the same compartment, examples of this being proteins that would be digested if stored in the same granules as proteases [17].

In order to study neutrophil granules in this study, we have isolated granules, by so-called subcellular fractionation, after cellular disruption using nitrogen cavitation [18]. This method, and variants thereof, has since it was first described been an excellent tool for the isolation and further study of the different granule types [19]. Isolated granules contain both soluble matrix proteins and membrane-anchored molecules and the different types of granules will be more closely described below.

Azurophil granules

The first type of granules to be formed during neutrophil maturation is peroxidase positive granules referred to as azurophil granules due to their affinity for the basic dye azure A [20]. These granules contain a large amount of myeloperoxidase (MPO), which is an important enzyme that contributes to the generation of oxygen radicals and is the primary marker of these granules (Paper I and II). Azurophil granules are also the main storage compartment for serine proteases such as proteinase 3 (PR3), elastase, and cathepsin G. They also store antimicrobial peptides and proteins (e.g. defensins and bactericidal permeability-increasing protein (BPI)) [21].

The azurophil granules contain many antimicrobial components that are of importance for the killing of ingested microbes. These agents would also be of potential danger for surrounding cells and tissues. In line with this, the azurophil granules rarely fuse with the plasma membrane and secrete their matrix proteins. Instead they fuse with internal membranes, normally that of a phagosome and empty their contents onto ingested microbes. Besides soluble matrix proteins, the membranes of azurophil granules contain different membrane markers such as CD63 [22], and CD68 [23].

Specific granules

The next granules formed during neutrophil maturation are specific granules which are mainly defined by their content of lactoferrin, a multifunctional protein with antimicrobial effects [24]. Besides a high concentration of lactoferrin, specific granules also contain collagenase, vitamin B12-binding protein and neutrophil gelatinase-associated lipocalin (NGAL) [25]. As for membrane proteins, specific granules contain a variety of chemoattractant receptors and the b-cytochrome part of the phagocyte oxidase [26] and (Paper I and II).

Similar to azurophil granules, the specific granules fuse primarily with phagosomal membranes, but may also fuse with azurophil granules [27, 28]. During subcellular fractionation, lactoferrin and b-cytochrome are traditionally used as markers for specific granules (Paper I and II).

Gelatinase granules

Granules with a high content of gelatinase are formed during the metamyelocyte and band cell stage, after which granule formation ceases [29]. The gelatinase granules contain hydrolytic proteases also known as matrix metalloproteinases (MMPs) that help degrading extracellular matrix during extravasation of neutrophils in the tissue [30, 31]. Accordingly, these granules do not normally fuse with phagosomes, but upon cellular stimulation they are rather mobilized to the plasma membrane resulting in secretion of their content. The gelatinase granule membranes contain for example integrins, meaning that secretion of these granules is a way for neutrophils to upregulate the surface expression of adhesion molecules. Additionally, gelatinase granule membranes contain the b-cytochrome that has been used as a marker in Paper I and II.

Secretory vesicles

The most easily mobilized neutrophil subcellular compartment is the secretory vesicles [32]. These vesicles contain plasma proteins like albumin in their ma-

trix, but on the other hand their membranes constitute a rich source of important receptors and/or adhesion molecules. Notable examples are the complement receptors (CRs) CR1 (CD35) and CR3 (CD11b/CD18), that play important adhesive roles [33-35] during the initial stages of neutrophil transmigration over the vessel walls (as will be described later in Section Neutrophil transmigration). Secretory vesicles require rather limited stimulation in order to be exocytosed [36].

During subcellular fractionation, secretory vesicles may be identified by serum albumin content, or by the activity of alkaline phosphatase (ALP) (Paper I and II). ALP is presented on the luminal side of the secretory vesicles, whereas fused to the membrane, the ALP is translocated to the outside of the plasma membrane. The enzymatic activity of ALP present in secretory vesicles can only be assayed in the presence of a detergent [37] (Paper I and II) making it possible to distinguish plasma membrane fractions where ALP activity is measured in the absence of detergent.

Neutrophil transmigration

The main task of neutrophils in our body is to fight invading microbes and to eliminate them. To execute their crucial functions, they are often required to transmigrate into infected tissues from the blood stream. The initial alarm signals from infected tissues arise when sentinel cells including macrophages recognize microbes (and/or damaged tissues) by various pattern recognition receptors (PRR) [38, 39]. As a result of this recognition, cytokines are released leading to activation of nearby vascular endothelium [40]. Upon activation, the vascular endothelium upregulates adhesion molecules (e.g., P- and E-selectin) on the luminal surface of the endothelial layer [41]. The selectins mediate interactions with circulating neutrophils that pass by, primarily by binding to glycosylated ligands, leading to so-called tethering [42].

Gradually, the interactions between neutrophils and the endothelial layer become stronger and the neutrophils slow down and start rolling along the vessel wall [43]. The interactions between the neutrophils and endothelial cells mediate mobilization of secretory vesicles, leading to upregulation of CR1 and CR3 and the loose interaction is gradually replaced by stronger, integrin-mediated firm adhesion. Concomitant with this, adhesion receptors such as L-selectin (CD62L) are cleaved off as a result of cell activation. Additionally, once the firmly adherent neutrophils start crossing the endothelial layer, further mobilization of gelatinase granules take place which supplies the cells with surface receptors needed for

chemotaxis towards the inflammatory focus and also release proteolytic enzymes capable of degrading the extracellular matrix and facilitate chemotaxis through the tissues [44, 45].

Granule mobilization and priming during transmigration

In healthy individuals, neutrophils circulate in a resting state waiting for inflammatory signals to start their journey to the inflamed tissue. During extravasation (the migration from blood to tissues), neutrophils undergo several changes that increase their responsiveness to subsequent stimulation. This process is referred to as priming and results in an altered cell with greater antimicrobial capacity to face the microbial intruders [46, 47].

Priming is in part mediated by mobilization of secretory vesicles and gelatinase granules which supply the plasma membranes with additional surface receptors. In vitro, the priming process can be mimicked by stimulation with e.g., bacterial endotoxin or various pro-inflammatory mediators [47] and it generally results in the shedding of L-selectin [13, 48] and Paper I.

In vivo models of transmigration and priming

The study of neutrophil priming in vivo relies on access to transmigrated tissue neutrophils. This is not a trivial matter in human subjects and the isolation of tissue neutrophils suffers from many limitations. One such limitation is the small number of cells that can be obtained from naturally occurring neutrophil exudates. Another is that many neutrophil-rich exudates are often associated with bacterial infections and the presence of bacteria may in itself affect the activation state and viability of the cells. To overcome these obstacles, and to study tissue neutrophils regarding their phenotype changes after transmigration, there are also aseptic models available [49, 50].

Experimental generation of local inflammatory reactions in the skin provides a relatively easy and controlled system for the isolation of tissue neutrophils [50]. Skin blisters can be generated by the application of negative pressure on the forearms of healthy individuals [49, 50]. The negative pressure causes separation of the epidermis from the underlying tissues, resulting in the formation of exudate filled blisters, and the leukocytes that arrive to the blisters can be isolated and studied directly (Paper III). This is a quite physiological model of acute inflammation and depending on the time point at which the blisters are emptied,

the cellular composition varies with neutrophils dominating at early time points (Paper III). Alternatively, the blister roofs can be removed and collection chambers filled with autologous serum (or another source of chemoattractants) are placed on top of the non-bleeding blister floors (Paper I). This chamber method, although perhaps more artificial, yields high numbers of neutrophils and relatively little contamination by other leukocytes [51]. These skin methods have been used to establish that neutrophils are in fact primed during transmigration in vivo [49, 51, 52].

It is also possible to use patient material for the study of in vivo transmigration and one good source is inflamed synovial fluid from patients with inflammatory arthritis (Paper I and III). Synovial fluid is a viscous fluid found in the cavities of synovial joint. This fluid provides lubrication for joints and prevents bone friction during movement. In a healthy joint, the synovial fluid does not contain any leukocytes, but during inflammatory episodes, it can be filled with substantial amounts of different leukocytes. Although neutrophils are normally not the dominating leukocyte in the joints of patients with inflammatory arthritis, e.g., rheumatoid arthritis, flares of acute inflammation may occur in these patients where the synovial fluid may be filled with substantial numbers of neutrophils [53] and (Paper I and II). To reach the inflamed site, neutrophils transmigrate from blood through the inflamed synovium and end up in synovial fluid [53].

Microbial eradication mechanisms

Once neutrophils have migrated to the inflamed tissues, they are primed and well-equipped to fight microbial invaders using a wide variety of different antimicrobial substances (described below). The general mechanism by which neutrophils kill microbes is through engulfment of the prey, phagocytosis.

Phagocytosis was first described by Metchnikov late in the 19th century [54, 55]. Through this process, foreign particles (e.g. microbes) are engulfed by phagocytes after contact is made either directly through their surface receptors, or indirectly as microbes may be opsonized by complement factors or antibodies. Following recognition and attachment, the microbe is internalized into a vacuole called a phagosome [56]. The neutrophil phagosome then gradually matures as it fuses with azurophil and specific granules and form a phagolysosome. In the phagolysosome the different antimicrobial agents of neutrophils come together and the local concentrations of granule contents will be extremely high. The multiple different antimicrobial agents are usually divided into two groups depending on their requirement for oxygen [56].

Oxygen-independent antimicrobials

Neutrophils are armed with very strong antimicrobial agents including proteolytic enzymes (i.e. serine proteases) and antimicrobial peptides (e.g., defensins) and proteins (e.g., BPI). As described above, most of these agents are stored in azurophil and/or specific granules and their release into phagosomes help killing and degrading of the ingested microbes [56]. The defensins display antimicrobial activity against a broad range of bacteria and fungi, and exert their antimicrobial effect through the disruption of the microbial membranes [57].

Reactive oxygen species (ROS)

During phagocytosis, the consumption of oxygen increases tremendously in neutrophils, a process known as the respiratory burst. Neutrophils are equipped to produce reactive oxygen species (ROS) and this formation explains the respiratory burst. The enzyme underlying ROS production is the phagocyte NADPH-oxidase, a multi-component enzyme consisting of both membrane-bound (the b-cytochrome described above) and several cytoplasmic components [58, 59] that shuttles electrons across membranes to form superoxide anion at the expense of molecular oxygen. The super-oxide spontaneously dismutates into hydrogen peroxide that may be further processed to form a wide variety of different ROS. In the phago-some, MPO (originally from the azurophil granules) catalyzes the form-ation of known antimicrobials such as hypochlorous acid (HOCl) [60].

The importance of ROS as a part of neutrophils antimicrobial defence is seen in patients with the rare condition of chronic granulomatous disease (CGD). These patients are extremely susceptible to (bacterial and fungal) infections as a result of non-functional NADPH-oxidase genes [61].

Neutrophil extracellular traps

Besides the long-known mechanisms of phagocytosis, the formation of so-called neutrophil extracellular traps (NETs) has more recently been described as one additional strategy that neutrophils use to eliminate microorganisms extracellularly [62]. NETs are networks of extracellular fibres, primarily composed of DNA extruded from neutrophils, which have the ability to bind and possibly kill extracellular microbes. Covering the DNA in NETs are proteins from the different types of neutrophil granules [62, 63] and (Paper I and II). Interestingly, ROS formation and MPO activity plays a central role in NET formation [64, 65], but there are also ROS-independent modes of NET formation [66-68].

It has been suggested that NETs may not only contribute to host defense by trapping and killing microorganisms, but that they also may play roles in non-infectious conditions such as autoimmune diseases [69, 70]. Since NET formation is associated with the extracellular exposure of otherwise intracellular granule components, it could be of importance for the development of certain autoantibodies that are central for pathology in some autoimmune disorders [71]. These matters will be discussed below.

Neutrophil subsets

Until recently [72], neutrophils from healthy individuals have been viewed as a homogenous population of cells. They were all considered to carry out the same functions, exhibit the same properties, and to be equipped with the same granule proteins. However, this view is gradually changing and it is becoming clear that not all neutrophils are identical and that more or less defined subsets that can be identified by the presence or absence of certain markers, may in fact exist side by side.

Lately, a lot of focus has been put on somewhat ill-defined cells called low-density neutrophils (LDNs) and/or granulocytic myeloid-derived suppressor cells (G-MDSCs) that can be found during various pathological conditions [73, 74]. Today there is no consensus as to whether these cells truly represent distinct neutrophil subsets or rather reflects maturation- and or activation-induced cellular changes [75].

As described above, neutrophil priming is characterized by an altered composition of cell surface molecules such as those brought about by mobilization of granules. Especially sensitive to priming-induced expression changes include L-selectin and CR3. These markers are present on all neutrophils but differentially displayed depending on the degree of priming (Paper I). Importantly, priming is not restricted to transmigration to the tissues, but a certain degree of granule mobilization may also take place on circulating cells. Theoretically, if partial priming of circulating neutrophils is triggered, for instance by increased levels of pro-inflammatory cytokines, a blood sample could contain a mixture of resting and primed cells which would appear distinct from each other on the basis of surface molecule expression [76, 77].

Many of the described neutrophil subsets may thus reflect cells that differ in terms of maturity and/or priming status and as such are not really true subsets. However, there are also certain subset markers (described below) that are (or are

not) expressed by neutrophils of a given donor independently of maturity and priming. To distinguish these from the maturation- or priming-induced subsets, we call these bona fide neutrophil subsets and define them as neutrophils characterized by the presence or absence of distinct markers, the expression of which is independent of maturation stage and/or priming. Additionally, bona fide neutrophil subsets can be found simultaneously in circulation of healthy donors (Paper I, II and III). We (Paper I, II and III) and others [78-80] have studied primarily two distinct bona fide subset markers, Olfactomedin-4 (OLFM4) and CD177.

Olfactomedin-4 (OLFM4)

Human Olfactomedin-4 (OLFM4), known as GW112 or hGC-1 is a member of the Olfactomedin family with an Olfactomedin domain in the C terminus. The biological function of this domain and its potential binding partners are still largely unknown [81]. The OLFM4 gene is mapped to chromosome 13q14.3 and translates into a protein containing 510 amino acids with no transmembrane domains [82]. The protein has a molecular mass of approximately 64 kDa in glycosylated form [83], or even higher (Paper I and II), and the size is decreased to 54 kDa after deglycosylation treatment [82].

OLFM4 was initially cloned from hematopoetic stem cells subjected to in vitro differentiation with G-CSF [82] and has been found to be expressed in various tissues such as gastro intestinal tissues, prostate, and bone marrow [82]. In contrast, other Olfactomedin-related proteins are mainly expressed in the brain and nervous system [82]. Even though the function of OLFM4 is unknown, it is up-regulated during tumorigenesis [84] and increased serum levels can be used as a diagnostic marker of early stages of gastric and colorectal cancer [84-87].

All myelopoietic cells in the bone marrow of healthy individuals contain and express the OLFM4 gene, mostly at the myelocyte/metamyelocyte stage of differentiation [82]. Based on this it is quite surprising that only a subset of mature circulating neutrophils express the protein [78] and (Paper I). In neutro-phils, OLFM4 shows a very distinct bimodal expression, meaning that a fraction of the cells express the protein, whereas the rest lack it. In blood samples from healthy donors, the proportion of OLFM4 expressing neutrophils is reportedly between 5-40% [78] and 8-57% (Paper I), and it is a granule protein primarily localized to specific granules. In line with this, we found that in skin chamber neutrophils, cells that have undergone in vivo priming and mobilized secretory vesicles as well as gelatinase granules, OLFM4 was still retained (Paper I).

It was however, possible to trigger secretion of OLFM4 in vitro by stimulating cells with a highly potent (and rather non-physiologic) secretagogue. Further,

both subsets showed equal migration tendency into tissue and no privilege in transmigration between these two subsets into pathologically inflamed synovial fluid or acute inflammation induced in healthy skin could be detected. Noteworthy to mention is that the proportion of OLFM4-positive blood neutrophils did not differ markedly between inflammatory arthritis patients and healthy controls (Paper I). This observation argues that whatever the function of OLFM4 in neutrophils, its expression does not influence the ability of these cells to transmigrate to tissues.

In search for functional differences between the two neutrophil subsets differing in OLFM4 expression, we investigated phagocytic ability, and regulation of cell death. We did not detect any differences in phagocytosis of Mycobacterium tuberculosis by the two subsets, using both sera opsonized or non-opsonized bacteria (Paper I). We also found no differ-ences concerning the propensity to undergo apoptosis after 4 respective 24 hours. Given that OLFM4 is a matrix protein of primarily the specific granules, it could be released into phagosomes and possibly assist in microbial killing. We did not observe any direct antibacterial effects of recombinant OLFM4, which is in agreement with findings by others [88].

We also tested whether extracellular recombinant OLFM4 could mediate any effects on neutrophils in vitro; at least at micrograms/ml concentrations, recombinant OLFM4 did not affect priming or cell death regulation (Paper I). Lack of effects observed with recombinant OLFM4 can be explained by the lack of post-translational modification of the recombinant protein. We cannot rule out that endogenous, properly glycosylated, OLFM4 could have effects in our systems.

Additionally, after inducing NET formation in vitro we could detect OLFM4 on a subset of NETs, which is in concordance with OLFM4 granule localization (Paper I). As will be discussed later (Paper II), the presence of OLFM4 on NETs could possibly explain the development of autoantibodies towards this particular protein.

A recently published study by Alder et al. showed that neutrophil OLFM4 expression may be of importance during sepsis [89]. It was reported that OLFM4 is the most highly expressed gene in non-survivors of septic shock, compared with survivors and also that patients with a complicated course had significantly higher proportions of OLFM4-positive neutrophils as compared to patients with less complicated courses of disease. This indicate that the OLFM4-positive neutrophil subset may play important roles during severe sepsis and raise the ques-

tion about the interplay between OLFM4 and other factors that may affect the immune system in response to severe infections.

As mentioned earlier, serine proteases are known to play significant roles in bacterial killing. In mice, neutrophil OLFM4 has been shown to bind and inactivate cathepsin C, the activity of which is required to activate downstream proteases (cathepsin G, PR3, and elastase). Both in vitro and in vivo studies show higher cathepsin C activity, bacterial killing (such as Staphylococcus aureus, Helicobacter pylori, and Escherichia coli) and clearance capabilities in OLFM4-deficient mice than wild type mice [88]. So far there are no published data addressing the existence of heterogenous OLFM4 expression (subsets) in mice.

The questions about the role of OLFM4 in human neutrophils and why we have evolved to carry two subsets of neutrophils that differ in expression of this protein remain unanswered.

CD177

Another established bona fide neutrophil subset marker, CD177 (also known as HNA-2a or NB1) is a 58- to 64-kDa glycosylphosphatidyl-inositol (GPI)-anchored glycoprotein, lacking the capacity of mediating signal transduction [90]. The expression of CD177 is restricted to neutrophils where it is expressed on the plasma- and granule membranes of 0-100% of the neutrophils depending on the donor [90-92] and (Paper III). About 3-5% of healthy individuals lack CD177 expression completely and these may be at risk for developing CD177 alloantibodies (described later). CD177 deficiency was observed for the first time in 1971 [93], but the genetic mechanism behind the presence or absence of this molecule is not yet completely understood and the expression is seemingly regulated by a number of complex genetic mechanisms [94].

We investigated whether leukocytes other than neutrophils express CD177. The results showed a bimodal expression of CD177 on neutrophils, whereas CD177 was completely absent from the surface of monocytes and lymphocytes (Paper III). When analyzing CD177 expression on neutrophils, leukocytes from 13 healthy donors were stained for surface CD177; two donors lacked CD177-expressing neutrophils completely and the remaining individuals had 20-90% CD177-positive neutrophils. These data are in concordance with published reports [92, 93, 95]. Further, the proportion of CD177-positive neutrophils was relatively constant when the same individuals were sampled on two different days. Importantly, the two individuals who lacked CD177 altogether on their neutrophils were negative for the molecule on both sampling occasions.

While proportions of CD177-expressing neutrophils in circulation are relatively constant in the same individual [91, 96] over time (Paper III), the CD177-expressing subset increases during pregnancy [97] and during severe bacterial infections [98].

It has been shown that CD177 has the ability to bind and present the serine protease PR3 on the plasma membrane [79, 99] and these two molecules are expressed on the plasma membrane of the same subset of neutrophils [79]. Studies have also shown that proportions of neutrophils with membrane-associated PR3 (mPR3) and CD177-expressing neutro-phils are increased in certain autoimmune conditions (described later).

In vitro studies implicate CD177 to be an adhesion molecule that is of importance during transendothelial migration, through direct interactions between CD177 and PECAM-1 on endothelial cells [100] or through the presentation of enzymatically active PR3, which could degrade extracellular matrix proteins and/or junctional proteins, facilitating transmigration [101].

Considering these observations, we wanted to determine whether CD177 expression implicated any recruitment advantage of neutrophils into inflamed tissues (Paper III). For this reason, we investigated neutrophils in synovial fluid from patients with inflammatory arthritis (described above). Based on the previous studies indicating a role for CD177 in neutrophil transmigration [100, 101] our hypothesis was that the proportion of CD177-expressing neutrophils in synovial fluid would be higher than in corresponding peripheral blood samples from the same patient. Cells from synovial fluid and isolated blood leukocytes from the same patient showed similar proportions of CD177-positive and -negative neutrophils, indicating that CD177 expression does not provide neutrophils with any recruitment advantage to this pathologically inflamed site.

Our data on synovial fluid neutrophils clearly demonstrate that CD177 is not necessary for neutrophil transmigration to inflamed joints, and also imply that expression of CD177 does not give neutrophils any advantage regarding in vivo transmigration to this site. In an attempt to study in vivo transmigration in a more controlled setting, we also employed the aseptic skin blister technique (described above) where the migration of immune cells can be followed over time in a controlled manner [49]. Also in skin blisters on healthy skin, the proportions of CD177-expressing neutrophils were similar as in circulation of the same subjects (Paper III), indicating that CD177 does not confer any recruitment advantage to this site.

Our results are in line with a report by Wang et al. [80] who showed that during human peritonitis, the proportion of CD177-positive neutrophils was very similar in the blood and peritoneal fluid of 10 patients. On the other hand, in a murine model, CD177-deficient neutrophils were shown to accumulate less in response to local inflammation triggered by bacteria [102].

Although it is not clear what the underlying cause of peritonitis was in the patients investigated by Wang and co-workers [80], the sites tested in Paper III have in common that they are aseptic and not driven by the presence of microbes. It may be that CD177 is of importance specifically during microbe-driven transmigration, although the fact that individuals lacking the protein altogether (3-5% of a healthy population) do not seem overly susceptible to infections of any kind argues against this [103].

Other bona fide subset markers

In addition to the bona fide neutrophil subsets defined by OLFM4 and CD177, there are some reports of other subset markers seemingly independent of neutrophil maturation and/or priming. However, to our knowledge these reports have not been independently confirmed. One suggested subset marker is the transcription factor RORγT, needed for production of pro-inflammatory cytokines of the IL-17 family that was reportedly present in only a subset of neutrophils from human and mice [104]. Other suggested subset markers are the taste receptors TAS1R and TAS2R which were demonstrated to be expressed in 40-60% of circulating neutrophils [105], and the same research group has also demonstrated subset-restricted expression of the trace amine receptors TAAR1 and TAAR2, as well as differences between the subsets regarding chemotactic migration towards amines [106].

Anti neutrophil cytoplasmic antibodies (ANCA)

Anti neutrophil cytoplasmic antibodies (ANCA) are autoantibodies directed against antigens in the cytoplasm of neutrophils. Despite the name, most ANCA antigens are not cytoplasmic, but rather granule-localized proteins. ANCAs are generally of IgG type and two major target antigens for ANCA are PR3 and MPO in the neutrophil granules. Autoantibodies directed against PR3 and MPO are particularly associated with a group of systemic small vessel vasculitis, so called ANCA-associated vasculitis (AAV), and the presence of ANCA in patient serum is used as a diagnostic and prognostic marker of AAV [107].

Vasculitis

Vasculitides is a group of diseases characterized by inflammation of the blood vessels leading to necrosis of the vessel wall as well as narrowing and blockage of the vessel lumen [108]. Diagnosis of vasculitis is rather challenging due to the wide range of specific types of diseases, which require very specific treatment strategies. Vasculitis can affect blood vessels of any organ, and depending on the size of the affected vessels, it can be divided into large-, medium-, and small-vessel vasculitis (SVV). AAV is a disorder that mainly involves small vessels, and is therefore classified as a type of SVV [109]. Distinguishing AAV from immune complex vasculitis, which is another type of SVV, AAV is a necrotizing vasculitis characterized by the absence or scarcity of immunoglobulin deposition in the vessel walls [110]. AAV is further sub-classified into three major categories based on clinical manifestations and ANCA specificity; microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA, formerly called Wegener's granulomatosis) and eosinophilic granulomatosis with polyangiitis (EGPA, formerly called Churg–Strauss Syndrome) [110]. ANCA is present in approximately 90% of patients with systemic GPA and MPA, whereas only 50-60% of patients with EGPA have ANCA. Despite decades of research, it is not fully understood what causes AAV and what the role of ANCA is in its pathogenesis [111].

Classic ANCA antigens and their detection

Among the vast number of proteins stored within neutrophil granules, MPO and PR3 are the most common and clinically important target antigens of the ANCA antibodies in AAV. Some less common ANCA antigens include cathepsin G, BPI, elastase, lactoferrin and lysozyme. MPA is usually associated with antibodies against MPO [112] while GPA is usually characterized by antibodies against PR3 [113]. Common for all classic ANCA antigens is that the targeted antigen is expressed in the granules of all neutrophils uniformly.

Today, reactivity in sera for the classic ANCA targets, MPO and PR3, is tested primarily by ELISA-based methods, but these methods are often used in combination with the first established diagnostic method for ANCA detection in patient serum: indirect immunofluorescence (IIF). Incubation of the serum with ethanol-fixed and -permeabilized human neutrophils on microscope slides, followed by incubation with FITC-labeled secondary anti-human antibodies (Figure 1), gives rise to two distinct binding patterns that can be visualized by fluorescence microscopy.

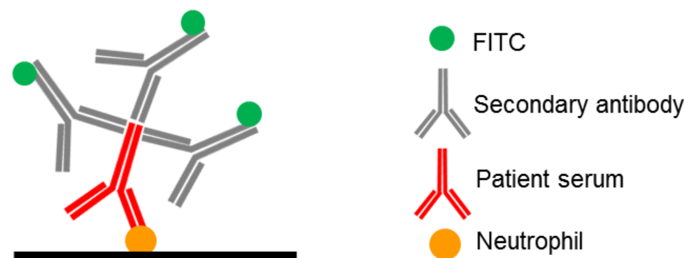

Figure 1. A schematic representation of indirect immunofluorescence (IIF).

A granular cytoplasmic (c-ANCA) pattern is usually associated with anti-PR3 ANCA, whereas anti-MPO ANCA generally results in a perinuclear ANCA (p-ANCA) pattern. These distinguished patterns are the artefact of ethanol fixation of the neutrophils on the slide.

Upon ethanol fixation, cationic granule proteins such as MPO are redistributed from their site in the granules onto the anionic nucleus by ionic attraction, thus displaying a perinuclear pattern. In contrast, the neutral or weakly cationic PR3 is retained inside the granules in the cytoplasm, thereby producing a cytoplasmic

pattern [114] (Figure 2). Since all neutrophils express MPO and PR3, the resulting staining pattern is homogenous, i.e. displayed in 100% of the cells on the slide.

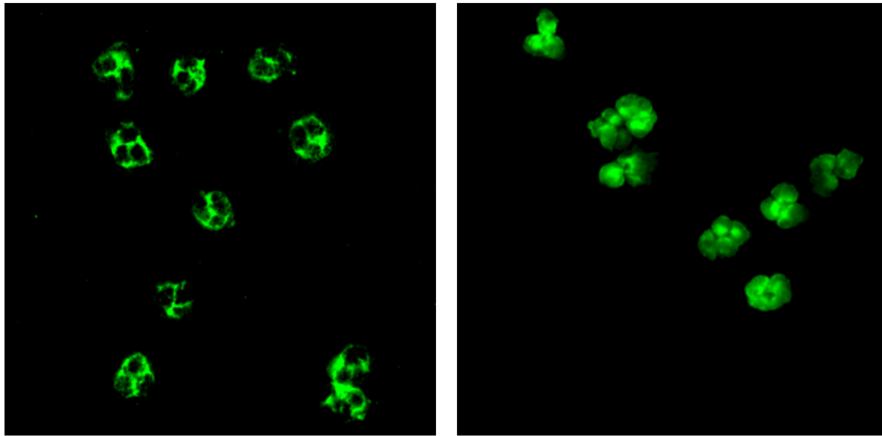

Figure 2. c-ANCA (left) and p-ANCA (right) staining pattern on ethanol fixed-human neutrophils.

The role of ANCA in vasculitis

The pathogenesis of AAV is still not fully understood [115], but as mentioned either MPO- or PR3-specific ANCA is present in a majority of AAV patients [107]. Several hypotheses concerning the origin of ANCAs and the autoantigens that give rise to them have been proposed.

One hypothesis concerning the origin of ANCAs is molecular mimicry between microorganisms and self-antigens, leading to the production of antibodies faultily targeted against the self-antigens. This process has been described for both S. aureus [116] and Ross River virus [117] antigens. Another hypothesis concerning ANCA origin is that ANCA antigens can be displayed during the process of neutrophil cell death or through NET formation, resulting in the production of antibodies against autoantigens that are not usually displayed [70, 118].

A major question in recent years has been whether the anti-MPO and anti-PR3 antibodies themselves have a direct role in the pathogenesis of AAV [115], and evidence supporting a pathogenic role of ANCA has accumulated in recent

years. Several in vitro studies have shown that ANCA IgG directed against PR3 can activate primed neutrophils, which have released PR3 from intracellular stores, making the cells produce ROS and destructive granule components [119-121]. The binding of ANCA to the neutrophil surface probably occurs either through the binding of the ANCA F(ab′)2 to the antigen when present on the surface [120, 122] or by engaging Fcγ receptors (FcγR) on neutrophils [123]. The activated neutrophils can in turn release factors that lead to the activation of the alternative complement pathway [124, 125], generating C5a. C5a is a chemoattractant for neutrophils, and can also prime the newly arrived neutrophils for more activation by ANCAs [125, 126].

As a result of activation, the neutrophils will adhere to and penetrate the blood vessel wall and release their toxic radicals and enzymes, causing apoptosis and necrosis of the neutrophils themselves as well as of the adjacent vessel wall cells and matrix [121, 127-129] leading to inflammation in these vessels. Activation of neutrophils by ANCA in vitro causes injury and death of adjacent cultured monolayers of endothelial cells, a finding which also supports the role of ANCAs in the pathogenesis of vasculitis [115].

Additionally, an association has been found between the presence of membrane-associated PR3 (mPR3) on the neutrophil cell surface, possibly presented by CD177 [79], and PR3-specific ANCA binding to the neutrophils leading to ANCA-mediated vessel damage [119]. Studies have shown that proportions of both mPR3- and CD177-expressing neutrophils are increased in AAV patients as compared to healthy controls and patients with other autoimmune conditions (e.g., rheumatoid arthritis) [130]. Also, high proportions of mPR3high neutrophils are a risk factor for relapse in GPA [92, 95, 131].

Meanwhile, other studies show that primed neutrophils from CD177-negative individuals also express mPR3 and are prone to anti-PR3 mediated oxidative burst, suggesting that surface presentation of CD177-independent mPR3 is also involved in anti-PR3 induced neutrophil activation [132]. It has been found that PR3 and CD177 recruit additional molecules, such as transmembrane receptor CR3, to form a larger signaling complex that allows PR3-ANCA to induce stronger superoxide generation and degranulation in CD177pos/mPR3high human neutrophils compared with the CD177neg/mPR3low counterparts [133]. Thus, if PR3-ANCA-induced activation of mPR3 positive neutrophils is involved in driving the pathology of AAV, it would seem likely that the CD177-expressing neutrophil subset could be of special importance for AAV.

Moreover, therapies that reduce the levels of autoantibodies or deplete B cells, as well as plasma exchange in order to remove autoantibodies, are effective treatments in AAV, providing further support of the theory that ANCA antibodies are involved in AAV pathogenesis [134, 135].

On the other hand, several facts argue against the pathogenicity of ANCA. Firstly, natural autoantibodies against PR3 and MPO, although in lower titers and with significantly lower avidity, may be detected in healthy individuals without any signs of vasculitis [136]. Secondly, a proportion of patients without detectable anti-PR3 or anti-MPO antibodies still has classical symptoms of small vessel vasculitis and fulfils diagnostic criteria for AAV [137]. Finally, the correlation between ANCA titers and the clinical disease activity is not strong, although anti-PR3 and anti-MPO titers are often used in the setting of clinical follow-up of AAV patients. This discrepancy, however, might be explained by the fact that ANCAs directed against different epitopes on the MPO and PR3 molecule have different pathogenic properties [138].

Atypical ANCAs

Atypical ANCAs are defined as ANCAs detected by IIF that are not directed to predominantly PR3 or MPO [139]. Sera containing antibodies against other neutrophil proteins like elastase have been shown in previous studies [140]. Another rare, but important ANCA specificity is against BPI (an antibacterial protein stored in azurophil granules), commonly found in patients with cystic fibrosis (CF) and Pseudomonas infections [141]. These ANCAs have also been found in patients with inflammatory bowel disease (IBD) [142]. Further, ANCA against cathepsin G (a serine protease stored in azurophil granules) have been detected in patients with rheumatoid arthritis [143], CF, and acute lung injury [144]. With regards to staining patterns, BPI ANCA gives rise to c-ANCA patterns, whereas lactoferrin and elastase ANCA display p-ANCA patterns [139, 145, 146].

Similar to classic ANCAs, the antigens of atypical ANCAs are expressed uniformly in all neutrophils of a given individual and the staining (be it c-ANCA or p-ANCA) is the same in all cells.

Subset ANCAs

Thousands of patients’ sera are analyzed routinely for ANCAs in order to help diagnosis of AVV, by a combination of IIF and ELISAs specific for PR3 or

MPO. A common feature for all conventional ANCA antigens is that, regardless of the staining pattern displayed by individual ANCA-positive cells, the targeted antigens are expressed uniformly by all neutrophils and all cells on an IIF slide are stained similarly. During routine analyses of patients' sera by IIF, we occasionally detect samples that give staining of only a subset of neutrophils (Paper II and III). Subset ANCAs are defined by us as serum antibodies that give rise to IIF staining of some, but not all neutrophils in a sample, i.e., that recognize neutrophil subsets (Figure 3). The origin, as well as the potential pathogenic roles of these antibodies in patients is still unknown.

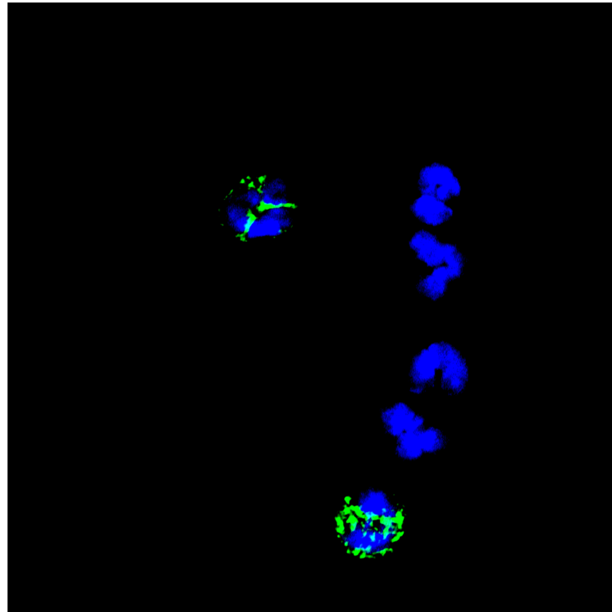

Figure 3. Subset-staining of ethanol-fixed neutrophils produced by patient serum.

OLFM4-ANCA

During our routine analyses of ANCAs, we found sera from two patients with diffuse inflammatory disorders, giving rise to an unusual ANCA pattern on ANCA slides (Paper II). An ELISA-based method confirmed that the antibodies in the sera were not directed against MPO or PR3. The antibodies reacted to only 30% of ethanol-fixed neutrophils on IIF slides with a c-ANCA pattern and were of the IgG1 subclass.

We verified our staining results on commercially available IIF slides using home-made ethanol fixed and permeabilized neutrophils from healthy donors and observed the same staining pattern with both patients' sera and a similar mean percentage of ANCA- positive neutrophils. This indicated that the patient ANCAs were in fact subset ANCAs. We next observed extracellular release (or rather intracellular disappearance) of the subset ANCA antigen upon strong artificial secretion stimuli, indicating that the antigen can be secreted and is lost from neutrophils after the triggering of granule secretion and is probably a granule protein. It is been suggested that interaction between ANCAs and intracellular antigens takes place extracellularly when the antigens are present on NETs [147]. Patient serum reacted to a subset of NETs (induced in vitro) from healthy control neutrophils.

The next step in identifying our intracellular antigen was to localize the ANCA antigen in neutrophil granules and for this purpose subcellular fractionation of neutrophil granules was performed. Subjecting granule fractions to immunoblotting, using patients' sera, indicated the expression of the antigen in specific/gelatinase granules with both sera. Importantly, an ANCA-negative control serum did not react with any proteins in the granule fractions. On basis of Paper I, we of course noted the many similarities between OLFM4 and the antigen recognized by the patients' sera. A proteomics approach, with LC-MS/MS analysis of the protein band from granule fractions, identified OLFM4 as a major protein of the immunoreactive band. Finally, co-staining of neutrophils with antibodies against the two known bona fide neutrophil subset antigens (OLFM4 and CD177) and patients' sera showed a 100% co-localization of the ANCA antigen and OLFM4.

As mentioned earlier, the function of the OLFM4 protein itself in human neutrophils is still unknown and if and how these OLFM4-specific subset ANCAs are involved in patients' disease pathology is not obvious. One could speculate that binding of ANCAs to OLFM4 expressed on NETs could perhaps prevent the degradation of NETs in a manner similar to what has been shown for other NET-

bound autoantibodies [148]. Accumulation and/or persistence of NETs and their toxic components at the site of inflammation would likely harm adjacent tissues [149]. One could also imagine that large OLFM4-clad structures such as NETs could be opsonized by the ANCA which could result in frustrated phagocytosis with extracellular secretion of toxic granule agents and ROS [150]. The presentation of granule proteins on NETs has been suggested to explain the generation of ANCA specific for granule proteins that are not normally secreted [147]. Thus our findings that OLFM4 is a NETs constituent (Paper III) could possibly explain the appearance of OLFM4-specific subset ANCA (Paper II).

CD177-ANCA

As mentioned above, complete lack of CD177 expression is relatively common (around 3-5%) [93] in healthy individuals and these may be at risk for developing CD177 alloantibodies during pregnancy or blood/plasma transfusion. CD177 alloantibodies can lead to neonatal neutropenia (i.e., abnormally low concentration of circulating neutrophils) during pregnancy [93]. A CD177-negative mother bearing a CD177-positive child may develop antibodies against the child's CD177-positive neutrophils. These antibodies can pass the placenta and cause neutropenia of the newborn [93]. Moreover, alloantibodies directed to CD177 can be involved in transfusion-related acute lung injury (TRALI) following transfusion of blood or plasma [151]. Alloantibodies arise after introduction of an antigen into the system of an individual lacking that particular antigen, whereas autoantibodies are specific for antigens present in the individual that produce the antibodies. As will be described, it is not always easy to determine whether reactivity is caused by allo- or autoantibodies.

Our identification of OLFM4 ANCAs (Paper II) drew our attention to additional subset ANCAs. All along the screening for ANCAs, sera from two other patients (described thoroughly in Paper III) were identified that showed distinct c-ANCA staining patterns in a subset of the neutrophils on ANCA-slides. Double staining of ethanol-fixed neutrophils with patient sera and known subset markers (CD177 and OLFM4) showed that the neutrophil subset stained by the patient sera coincided with the subset expressing CD177. On the other hand, OLFM4 antibodies stained a different subset. Immunoblotting using one of the patient's serum revealed that the ANCA reacted with both whole cell lysates from healthy control leukocytes and recombinant CD177, but not with whole cell lysates from the patient. Another set of immunoblots, using a commercial monoclonal anti-CD177 antibody, gave identical results. This indicated that the patient ANCA reacted with CD177 and also indicated that this antigen was not expressed by the patient's leukocytes.

The lack of CD177 on the patient's leukocytes was confirmed by flow cytometric analysis of patient neutrophils. These results indicate that patient antibodies directed to CD177 can give rise to subset-specific staining patterns on ANCA slides.

Detecting antibodies against CD177 in sera of two different patients awakened the question about the origin of these antibodies -are they allo- or autoantibodies? Although none of the patients had any clinical signs of neutropenia or TRALI, both had a clinical history of either bone marrow/liver transplantation and/or multiple blood transfusions suggesting that the anti-CD177 antibodies were in fact alloantibodies developed against CD177 positive neutrophils from donor blood.

On the other hand, antibodies transfused from donor to recipient will eventually degrade, a phenomenon that was not accurate in any of cases since we could detect the antibodies continuously in our patients' sera.

In one case, (patient#1 that was diagnosed with acute myeloid leukemia at early age and went through stem cell transplantation treatment), we had the opportunity to analyze a blood sample from the stem cell donor regarding CD177 expression. Donor neutrophils lacked CD177 expression completely (data not shown), similar to the findings of the recipient/patient. However, this finding is difficult to interpret without genetic analyses, since the origin of the patient's circulating neutrophils is quite likely from the bone marrow of the donor. We still cannot completely rule out that the anti-CD177 antibodies in patient sera are autoantibodies. One could argue that such autoantibodies would lead to the clearance of CD177-expressing cells (such as during neonatal neutropenia) and those would then not be available in a blood sample, i.e., all subjects with anti-CD177 antibodies in circulation would appear CD177-deficient. Given that the original CD177-expressing fraction would be small enough, this might also not be seen as neutropenia.

We are currently trying to understand how the antibodies towards CD177 developed in the patients in Paper III and whether the presence of these antibodies could explain any of the clinical findings.

Finding additional neutrophil subsets through screening for subset ANCAs

Throughout the work with this thesis, it has become clear that screening for ANCA by IIF could be a powerful strategy for the detection of subset-restricted ANCA and possibly lead to the identification of novel, yet undiscovered, bona fide neutrophil subsets.

Aside from the patient sera described in Paper II and Paper III, we have found additional subset ANCA that does not react with either OLFM4 or CD177. Furthermore, another unusual subset has been found in serum from a patient with suspected CLIPPERS syndrome. CLIPPERS is an acronym for Chronic Lymphocytic Inflammation with Pontine Perivascular Enhancement Responsive to Steroids and is an inflammatory disorder of the central nervous system, associated with neurological symptoms and lesions in the pons [152]. Serum from this patient displays reactivity to a subset of ethanol-fixed neutrophils on ANCA slides (Figure 4). However, the subcellular localization of the yet unknown target antigen seems markedly different from those described for OLFM4 (Paper II) or CD177 (Paper III). In addition, co-staining experiments with OLFM4 or CD177 clearly demonstrate that the subset of neutrophils that react with CLIPPERS serum is not any of the subsets defined by OLFM4 or CD177 (Figure 4). Our future aim is to identify the target antigen of the CLIPPERS serum and thus potentially identify a novel neutrophil subset marker.

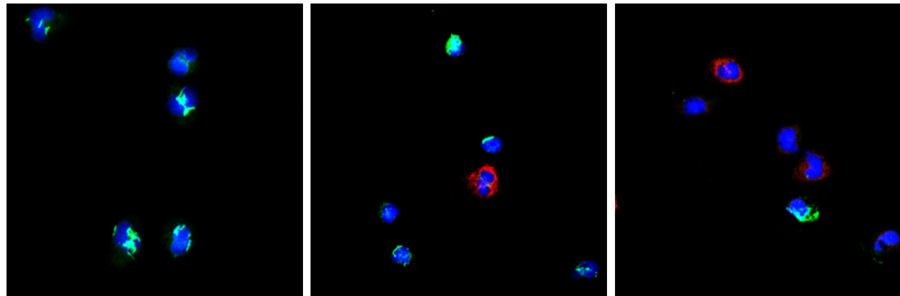

Figure 4. ANCA staining of CLIPPERS serum (green) alone (left) or in combination with staining for known bona fide subset markers (red) OLFM4 (middle) and CD177 (right). All nuclei are DAPI stained (blue).

Concluding remarks and future perspectives

Neutrophils have traditionally been viewed as a homogenous cell population, where all cells contain the same molecular cargo and carry out the same functions. This view is however changing due to the identification of specific protein markers expressed simultaneously on some, but not all neutrophils of a given individual. Two distinct subset markers have been in focus of this thesis; OLFM4 and CD177. Even though the underlying mechanisms to explain the bimodal expression of OLFM4 and CD177 are unknown, the simultaneous existence (in circul-ation of healthy donors) of several bona fide neutrophil subsets is a fact.

Neutrophil heterogeneity is a relatively new idea, and the biologic finesse behind the existence of distinct neutrophil subsets is still not clear. Until now, limited numbers of bona fide subset markers have been characterized and defined, but the analyses of ANCA sera with bimodal reactivity (i.e., subset ANCA) will likely lead to the identification of additional, novel subsets of human neutrophils in the future.

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