Immune Cell Subsets in ANCA-Associated Vasculitis

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Immune Cell Subsets in ANCA-Associated Vasculitis

Sofia Smargianaki

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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on date March 13, 2026, at 09:00 in Lecture Hall 2, Blocket, Skåne University Hospital, Lund

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Abstract:

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) are rare autoimmune diseases, including granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophil granulomatosis with polyangiitis (EGPA). They are three distinct diseases with overlapping symptoms and signs. The ANCAs involved in AAV are mainly directed against myeloperoxidase (MPO) and proteinase 3 (PR3), found in neutrophils and monocytes. The pathogenesis is multifactorial and include genetic and environmental factors e.g. infections. Adult patients with AAV diagnosis were recruited at the time of diagnosis or later when referred to the outpatient clinics of Nephrology or Rheumatology, Skåne University Hospital, Lund Sweden. Patients were classified into GPA, MPA or EGPA. The patient blood samples were collected between 2011 and 2020. Healthy blood donors (HC) were used as controls. Immunophenotyping and function of immune cells were performed by flow cytometry analyses. The aim was to investigate correlations between various cell subsets and cellular functions with disease activity, propensity for relapse and response to rituximab treatment.

In paper I, we identified significant alterations in the B-cell compartment, characterized by reduced frequencies of total B cells and transitional B cells, alongside increased switched memory B cells, plasmablasts and activated B cells in AAV patients compared to healthy controls. The immunoglobulin levels were within normal range.

In paper II, we report increased neutrophil and monocyte subpopulations in AAV patients compared to HC but decreased classical monocytes in MPA patients compared to GPA. During active disease we observed higher concentration of mature neutrophils and decreased total and intermediate monocytes. Moreover, rituximab treatment may influence intermediate and classical monocytes frequencies.

In paper III, we observed decreased phagocytic capacity and alterations to generate reactive oxygen species, related to ANCA subtype, rituximab treatment and infections. Findings from the studies included in this thesis, highlight the important role of immune cells in the pathogenesis and clinical outcome of ANCA-associated vasculitis. However, a clear and comprehensive understanding of the immune cell alterations involved in AAV is still lacking, warranting further investigation.

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II. **Smargianaki S**, Elmér E, Lilliebladh S, Ohlsson S, Pettersson Å, Hellmark T, Johansson ÅC. Disease Activity and Tendency to Relapse in ANCA-Associated Vasculitis Are Reflected in Neutrophil and Intermediate Monocyte Frequencies. J Immunol Res. 2024 Jan 3; 2024:6648265. doi: 10.1155/2024/6648265. PMID: 38213873; PMCID: PMC10781522.

III. **Smargianaki S**, Lilliebladh S, Ohlsson S, Pettersson Å, Hellmark T, Johansson ÅC. Monocyte and granulocyte function and susceptibility to infections in AAV patients under rituximab treatment. *Manuscript*

Abbreviations

AAV	ANCA-associated vasculitis
ACR	American college of Rheumatology
ANCA	Antineutrophil cytoplasmic antibody
APC	Antigen presenting cell
BVAS3	Birmingham Vasculitis Activity Score 3
BCR	B cell receptor
BM	Bone marrow
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DAMPs	Damage-associated molecular patterns
DC	Dendritic cells
EGPA	Eosinophil granulomatosis with polyangiitis
FACS	Fluorescence-activated cell sorting
FSC	Forward scatter
GPA	Granulomatosis with polyangiitis
GSF-1	Colony-stimulating factor 1
HC	Healthy controls
HSC	Hematopoietic stem cell
ILC	Innate Lymphoid cell
MHC	Major histocompatibility complex
MPA	Microscopic polyangiitis
NETs	Neutrophil Extracellular Traps
NK	Natural killer
NO	Nitric Oxide
NLRs	NOD Like receptors
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate Buffered Saline
PDs	Photodiodes
PMA	phorbol 12-myristate 13-acetate
PMTs	Photomultiplier tubes
PRRs	Pattern recognition receptors
ROS	Reactive Oxygen Species
RTX	Rituximab
SSC	Side scatter
TCR	T cell receptor
TGFβ	Transforming Growth Factor-beta
TLRs	Toll Like Receptors

Abstract

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) are rare autoimmune diseases, including granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophil granulomatosis with polyangiitis (EGPA). They are three distinct diseases with overlapping symptoms and signs. The ANCAs involved in AAV are mainly directed against myeloperoxidase (MPO) and proteinase 3 (PR3), found in neutrophils and monocytes. The pathogenesis is multifactorial and include genetic and environmental factors e.g. infections.

Adult patients with AAV diagnosis were recruited at the time of diagnosis or later when referred to the outpatient clinics of Nephrology or Rheumatology, Skåne University Hospital, Lund Sweden. Patients were classified into GPA, MPA or EGPA according to the consensus methodology by Watts et al in 2007 and the American College of Rheumatology (ACR) criteria. The patient blood samples were collected between 2011 and 2020. Healthy blood donors (HC) were used as controls. Immunophenotyping and function of immune cells were performed by flow cytometry analyses. The aim was to investigate correlations between various cell subsets and cellular functions with disease activity, propensity for relapse, and response to rituximab treatment.

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Findings from the studies included in this thesis highlight the important role of immune cells in the pathogenesis and clinical outcome of ANCA-associated vasculitis. However, a clear and comprehensive understanding of the immune cell alterations involved in AAV is still lacking, warranting further investigation.

Populärvetenskaplig sammanfattning

Immunsystemet är kroppens försvarssystem med uppgift att skydda oss mot infektioner och sjukdomar såsom bakterie, virus, svampar, parasiter, cancerceller eller annat som uppfattas som främmande. Immunsystemet består av lymfatiska organ och av högspecialiserade celler, som kommunicerar med varandra. Cellerna är de så kallade vita blodkropparna som kan cirkulera i blodet och i vävnaderna. Lymfatiska organ är benmärg, thymus, lymfkörtlar, mjälte och slemhinnor med lymfatisk funktion (som till exempel tonsiller). Dessutom finns proteiner som samordnar kommunikationen mellan cellerna och organ och de kallas för cytokiner.

När något främmande kommer in i kroppen, kallat antigen, känner immunsystemet det som hot och aktiveras genom att mobilisera sina vita blodkroppar. De vita blodkroppar rör sig till området där problemet finns, angriper den främmande molekyl och eliminerar det. När hotet är eliminerat, lugnar immunsystemet ner sig för att undvika skador på vävnaden.

Det finns olika typer av vita blodkroppar som har olika uppgifter. Det är granulocyter (som i sin tur delas i neutrofiler, eosinofiler och basofiler), monocyter och lymfocyter (av typ B och T). Granulocyter och monocyter kan ”äta upp” de främmande ämnena, och det kallas för fagocytos, och kan även visa de främmande ämnena för lymfocyterna. Lymfocyter kan komma ihåg de ämnen lång tid framöver och kallas för minnesceller. Detta gör att lymfocyter svarar snabbt och effektivt nästa gång de träffar samma antigen igen. Vissa vita blodkroppar producerar antikroppar som känner igen det specifika främmande ämnet och binder upp eller blockerar det, som till exempel efter en infektion eller en vaccination.

Ibland, fungerar immunförsvaret inte som det ska. Det kan börja angripa kroppens egna vävnader genom att bilda antikroppar mot sig självt, så kallade auto-antikroppar, vilket leder till autoimmuna sjukdomar. Immunförsvaret kan också överreagera och orsaka allergier, eller vara för svagt och därmed inte skydda oss mot infektioner.

ANCA-associerade vaskuliter är en grupp av sällsynta kroniska autoimmuna sjukdomar, som inkluderar granulomatos med polyangit (GPA), mikroskopisk polyangit (MPA) och eosinofil granulomatos med polyangit (EGPA). Vaskulit betyder inflammation i kärl och ANCA står för Anti-neutrofil Cytoplasmatiska Antikroppar, en sorts av autoantikroppar. ANCA associerade vaskuliter leder till inflammation inne i kärlen, vilket gör att det blir svårt för blodet att cirkulera. Det kan leda till allvarlig organskada, organsvikt och död, om sjukdomen inte behandlas. Oftast är det njurarna och lungorna som påverkas, men alla organ i kroppen kan skadas, som till exempel huden, öron, näsa, eller nerverna. Sjukdomen är inte smittsam och nuförtiden finns effektiva behandlingar som håller sjukdomen i schack och kan ge en bra livskvalitet för patienterna. Behandlingen syftar till att kontrollera sjukdomen genom att dämpa immunsystemets aktivitet och minska

risken for återfall (skov). Behandlingen kan dock medföra biverkningar som till exempel en ökad risk för infektioner. Ett mycket effektivt läkemedel är Rituximab, en anti-CD20 monoklonal antikropp, som dödar B lymfocyterna och därför blockerar produktion av antikroppar.

Denna avhandling består av tre arbeten som handlar om immunsystemets celler och deras funktion hos patienter med ANCA-associerade vaskuliter.

I första och andra arbete har vi undersökt B lymfocyter (även kallade B celler), granulocyter och monocyter. B celler är celler som producerar antikroppar. Monocyter och granulocyter är immunsystemets första försvar och de kallas omgående till området där mikroorganismer eller skadade celler finns. Deras funktion är att äta upp och rensa bort kroppsfrämmande ämnen. Vi jämförde patienternas celler med celler från friska personer som är blodgivare. Vi samlade blodprover under sjukdomens olika faser, dvs vid diagnos, under aktiv sjukdom, remission, eller vid skov. Vi har även undersökt om medicinering med Rituximab kan påverka immunceller i förhållande till friska blodgivare. Vi noterade att det totala antalet av B-celler samt antalet av vissa undertyper av B-celler avviker hos patienter med ANCA-associerad vaskulit. Även granulocyter och monocyter visade avvikande antal jämfört med friska personer. Vi tror att avvikelserna är kopplade till aktiv sjukdom, skovbenägenhet, sjukdomstyp och till medicinering.

I det tredje arbetet fokuserade vi på funktionen av granulocyter och monocyter och deras förmåga att äta upp och destruera främmande ämne. Även här har vi använt samma patientprover som vid de två föregående arbetena. Vi upptäckte att granulocyter och monocyter har nedsatt funktion, vilket bidrar till infektionsbenägenhet och därför hög infektionsrisk hos patienter med ANCA-associerad vaskulit. Även här verkar Rituximab behandlingen påverka granulocyternas och monocyternas funktion.

Introduction

The Immune System

The human immune system evolved to protect the integrity of the body from external harmful infectious agents such as bacteria, viruses, parasites, and fungi, but also from internal menaces such as cancer. The immune system is also implicated in disease and in therapies like autoimmunity, tissue injury, pathologic inflammation, allergy, transplantation and gene or cell therapy. It consists of the innate or nonspecific immune system and the adaptive or specific immune system and is composed of organs -primary and secondary-, and immune cells. The primary lymphoid organs are bone marrow and thymus, where immune cells are created and where T and B cells first express antigen receptors. The secondary or peripheral lymphoid organs include spleen, the cutaneous and mucosal immune system, lymph nodes and tonsils, where the T and B cells are activated and develop appropriate immune responses. Based on this classification, the immune cells categorized as cells of the innate immune system includes dendritic cells, mast cells, Natural Killer (NK) cells, Innate Lymphoid cells (ILC) and monocytes/macrophages, whereas the T and B cells belongs to the adaptive immune system. The two systems interact with each other constantly to provide an immediate nonspecific defence and later specific reactions by the adaptive immunity.

In the bone marrow, as primary lymphoid organ, the cells are made and multiplied before they migrate to other organs or tissues. All immune cells have a common progenitor, the multipotent hematopoietic stem cell (HSC) from which the common myeloid progenitor and the common lymphoid progenitor derivate. Megakaryocytes, erythrocytes, mast cells, and myeloblasts are all derived from the myeloid progenitor. Myeloblasts differentiates further into monocytes, neutrophils, basophils and eosinophils. Tissue bound macrophages and dendritic cells can be derived from monocytes. The lymphoid progenitor generates large and small lymphocytes. The large lymphocytes are the NKs and ILCs[1] and from the small lymphocytes are divided in T and B cells. Plasma cells are derived from B cells. (fig. 1).

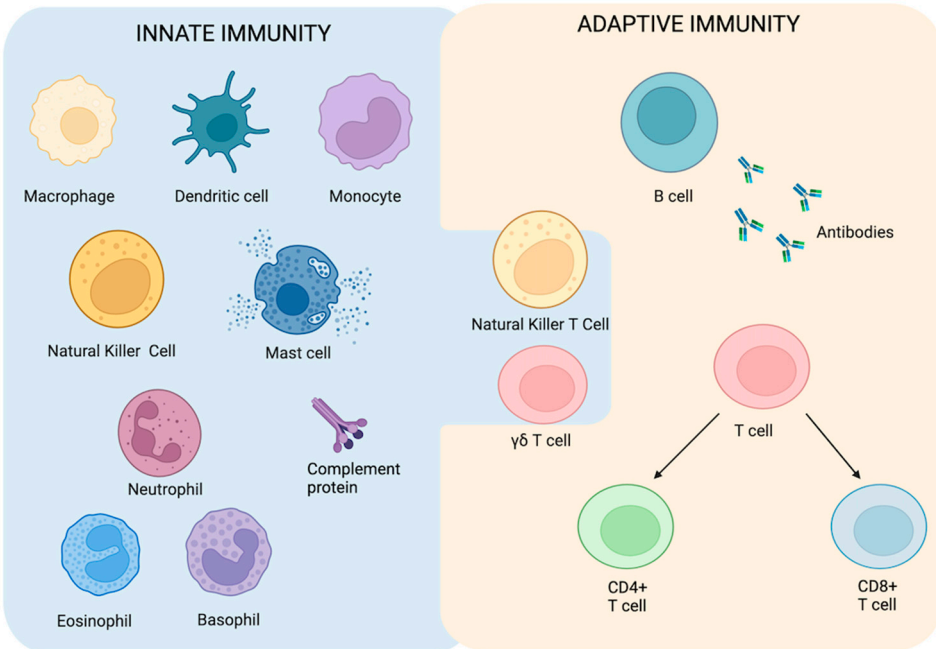

Figure 1. Cells and elements of the innate and adaptive immune systems. Monocytes, macrophages, dendritic cells, neutrophils, eosinophils basophils, mast cells, NK cells and the complement system are the key components of the rapid response of the innate immune system. CD4+ and CD8+ T cells as well as antibody producing B cells participate in the adaptive immune system, which is characterized by a delayed response. Natural killer T cells and γδ T cells serve as a bridge between innate and adaptive immune systems. Created in https://BioRender.com

After proliferation, the cells leave the bone marrow (BM) through the bloodstream, migrate to tissues and organs, for maturation, differentiation, storage, or for direct act. The neutrophils mature in the BM and probably differentiate in the tissues into various functional neutrophil phenotypes[2]. The eosinophils from the BM migrate to primary and secondary lymphoid organs and can infiltrate several organs and tissues during inflammation[3]. The basophils, functionally similar to mast cells, circulate in the blood and can be recruited to inflammatory sites. Monocytes circulate in the blood and differentiate to macrophages locally in the infected or damaged tissue. The mature B cells exit the BM and migrate to secondary lymphoid organs, where they express immunoglobulins on their surface and can recirculate between blood and lymphoid organs. The T cells travel to thymus, a primary lymphoid organ, for maturation and selection and subsequently re-circulate to the body in the secondary lymphoid organs and intestinal tissues searching for Antigen Presenting Cells (APCs)[4].

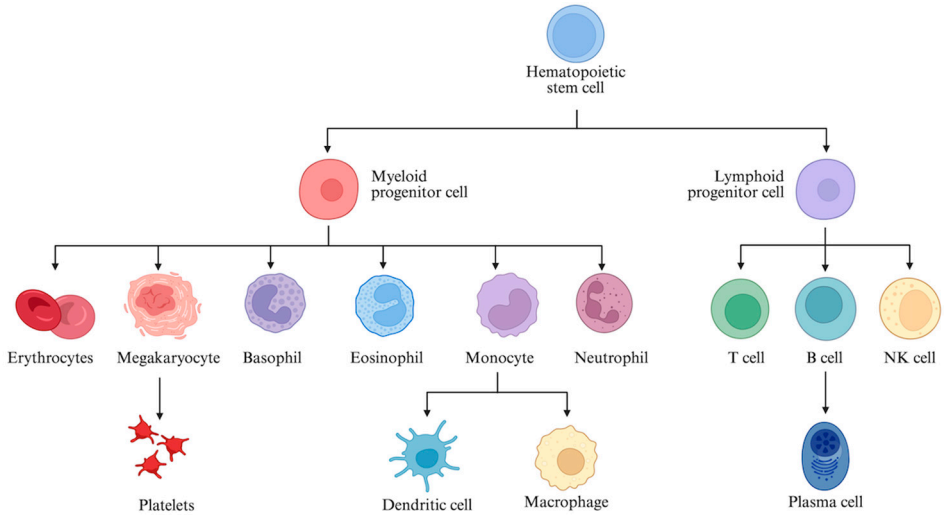

Figure 2. Hematopoiesis in bone marrow. The pluripotent hematopoietic stem cell differentiates into two progenitors: the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). The common myeloid progenitor further differentiates into erythrocytes, neutrophils, basophils, eosinophils, megakaryocytes, and monocytes. Megakaryocytes produce platelets, while monocytes differentiate into macrophages and dendritic cells. The common lymphoid progenitor differentiates into B-cells, T-cells and Natural killer (NK) cells. Plasma cells originate from B-cells. Created in https://BioRender.com

Inflammation

Inflammation is a physiologic reaction to harmful stimulus, as for example physical and chemical injuries, infection or damaged cells. It is characterized by five cardinal signs: pain, heat, redness, swelling, and loss of function, with the main goal to eliminating the cause of tissue or cellular injury. The innate immune system is involved in the acute inflammation, as the first actor in the host defence. The immune cells are activated and produce proinflammatory cytokines, calling the leukocytes to the site. Normally, the resolution of inflammation comes when the stimulus is eliminated. There are cases where inflammation turns to chronic and may become pathologic, such as cardiovascular and rheumatic diseases[5].

Innate immunity

The innate immunity response can react immediately to the infectious agents, providing rapid protection. It does not provide complete protection to the host, partly because board-spectrum first-line defence mechanisms have limited specificity, and partly because many infectious agents have developed resistance to innate immunity responses. The organization of the innate immunity is complex and includes

proteins, cells, antimicrobial peptides, soluble factors, and mechanical barriers that act within minutes or hours of the attack. The aim is to protect by physical blockade, to recognize pathogens or damaged tissue and induce inflammation, to inhibit viral replication, and to stimulate the adaptive immunity.

The mechanisms of defence, directed to reduce the number of pathogens that can enter in the body, include the skin and the gastrointestinal, genitourinary and respiratory tracts mucosa. The antimicrobial peptides in mucus, in secretions and in the skin, the commensal bacteria, the low pH in the gastrointestinal system, the soluble factors such as cytokines, chemokines and the complement factors are the humoral innate immunity. The cellular innate immunity is given by monocytes/macrophages, mast cells, DCs, NK, and ILCs cells[6].

The innate immune system responds to limited antigens; the estimation is about 1000 microbial and damaged cells structures. The pathogen components are called *pathogen-associated molecular patterns* (PAMPs) and include cell wall lipids, nucleic acids, proteins, and carbohydrates. Each type of microbe produces different PAMPs. The recognized microbial structures are often essential for the viability of the microbes. The innate immune system recognizes the PAMPs by the pattern recognition receptors (PRRs) and activates the defence. The endogenous damaged cell structures are also recognized by the innate immunity and are called *damage-associated molecular patterns* (DAMPs). Generally, they are produced by cell damage after infection or by cell injury after mechanical insults. The DAMPs are recognized by the PRRs as well. The PRRs are expressed on the surface of immune cells such as macrophages, neutrophils, DCs, mast cells, and epithelial cells. The activation of the inflammatory processes begins when the PAMPs or DAMPs bind to the PRRs. The PRRs can be cell-associated, as for example Toll Like Receptors (TLRs) and NOD Like receptors (NLRs) or can be soluble as complement factors[6].

The complement system plays an important role in the host defence, in the disposal of waste and is the bridge between the innate and adaptive immunity. The proteins of the complement system are responsible for the opsonization of bacteria, induce chemotaxis, activation of leukocytes and lead to the formation of Membrane Attack Complex (MAC), the final product of the complement cascade that can form pores in pathogen membranes promoting cell lysis.

Chemokines have chemotactic function and are responsible for the leukocyte migration, when they bind to the receptors on the leukocytes. They are produced by most cells after stimulation of proinflammatory cytokines or bacterial structures[7].

Monocytes

Monocytes have a pivotal role in the innate immunity and contribute to the adaptive immunity. Morphologically, their diameter is 10-15 μm with a bean-shaped nuclei and granular cytoplasm. They express major histocompatibility complex (MHC)

class II in the membrane and can act as APC. They represent a small part of leukocytes (2%-8%) and are responsible for phagocytosis, antigen presentation, cytokine production, production of Reactive Oxygen Species (ROS), and Nitric Oxide (NO). In tissues monocytes differentiate in macrophages[8] and contribute to the homeostasis and tissue repair[9].

Monocytes arise in the bone marrow from the common myeloid progenitor (CMP), after stimulation of colony-stimulating factor 1 (CSF-1). Once matured, monocytes circulate in the blood and migrate to the tissues where they display their functional plasticity and dynamic balance[10]. Human monocytes are distinguished in three subsets, defined by the expression of the surface markers CD14 (LPS co-receptor) and CD16 (FcγRIII) in classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and nonclassical $CD14^{+}CD16^{++}$[9]. In addition, other receptors, such as PRRs, complement receptors, chemokine, and scavengers receptors are also expressed on their surface.

Classical monocytes, even called inflammatory monocytes, are most circulating monocytes (80%-90%) and express high levels of CD14, but lack of CD16. The circulating lifespan is one day, and they rapidly migrate to the tissues with signs of infection or injury. Moreover, they produce high levels of cytokines and chemokines[11], and express PAMPs. They also have increased phagocytic capacity and are known to be the most efficient phagocytes. Additionally, it has been shown that they can recruit neutrophils in the early stage of inflammation in mice[12]. In the blood, classical monocytes can differentiate into intermediate monocytes, that express high levels of CD14 and low levels of CD16, undergo apoptosis or be removed from the bloodstream. The life of intermediate monocytes is about four days, and they are implicated in antigen presentation, cytokine production, and T lymphocyte stimulation[13]. In addition, they express higher levels of MHC class II than nonclassical monocytes and are suggested to be a transitional subset between classical and nonclassical monocytes[11]. The intermediate monocytes consist of 5-10% of circulating monocytes. About 1% of classical circulating monocytes become intermediate monocytes that eventually undergo further differentiation to nonclassical monocytes with the role of patrolling[10, 13]. Classical monocytes can convert to nonclassical monocytes with lower expression of CD14 and accentuation of CD16. The proportion of nonclassical monocytes in circulation is 2-5 % of total monocytes; the lifespan is about seven days and they have an pivotal role on the vascular homeostasis as they appear to be caretakers of the vascular tissue[14]. Patrolling monocytes scan the vascular endothelium from the luminal side, respond to viruses, nucleic acids, cell damage, infection, and remove cellular debris from the vasculature[15].

Granulocytes

Granulocytes, including neutrophils, eosinophils, and basophils originate from the myeloid line in the BM.

Eosinophils are derived from the myeloid progenitor and constitute approximately 2-5% of the circulating leukocytes in healthy individuals. Circulating eosinophils have a short lifespan, 8-18 hours, but in the tissues, they could survive for up to 14 days. After maturation in the bone marrow, they migrate to multiple tissues and organs, especially in mucosal linings, where they exert their functions. They are involved in tissue homeostasis and in the host defence of parasitic infections by the release of enzymes from their granules after activation. The granules contain major basic protein (MBP) that neutralizes heparin and is toxic. Additionally, they could interact with the adaptive immunity as APCs, since they are able to phagocyte invading pathogens[3]. Moreover, they are involved in allergic inflammations, in the pathogenesis of asthma and also in autoimmune diseases like Eosinophil Granulomatosis with Polyarthritis (EGPA), where they cause tissue and cell damage[16].

Basophils and mast cells present many phenotypic and functional similarities but are different morphologically. *Basophils* arise from the CMP in the BM and circulate as mature effector cells and do not proliferate after maturation. They represent less than 1% of the circulating leukocytes and live from 1 to 3 days. Their granules contain histamine, tryptase, leukotrienes, and platelet-activating factor (PAF) which contribute to vasodilatation, bronchoconstriction, and acute allergic reactions. Basophils could interact with T-cells by the production of Il-4 and sustain IgE-dependent immune responses[17].

Mast cells also arise in the BM, from the CD34+ pluripotent progenitor cells, and mature, proliferate and reside near to blood vessels in lungs, skin, gastrointestinal tract, and are almost absent in mature form in the blood stream[18]. They have a long lifespan, from weeks to months[19]. Their granules contain histamine, proteoglycans and tryptase and other proteases[20]. Mast cells increase in hypersensitivity reactions, asthma, parasitic infection, tumors[21], are immunomodulatory cells, and implicated in autoimmunity[18]. Both basophils and mast cells express high affinity IgE receptor on their surface and basophilic granules with histamine. In addition, they can release chemical mediators, like cytokines and chemokines, upon activation. However, mast cells are distinct cells with important morphological differences compared to basophils[21].

Neutrophils are the most frequent granulocytes in the blood and comprise 50-70% of circulating leukocytes. They originate from the CMP in the BM, with a circadian release of 1-2 x 10^{11} cells /day. They undergo maturation before they migrate to the bloodstream and the estimated lifespan is still controversial, from hours to some days[22, 23]. It is suggested that the terminal differentiation may occur even outside the BM, as neutrophils and their progenitors have been found in the spleen[24]. They play

an important role in the host defence against bacterial and fungal infections, as they are the first cells to arrive at the infected site, with the ability to kill the pathogens. Moreover, they are also implicated in autoimmune diseases.

Mature neutrophils have a segmented nucleus and a granular cytoplasm with various forms of granule. The granules contain different proteins, like enzymes and antimicrobial peptides. The granules are categorized in primary or azurophilic, secondary or specific, and in tertiary granules. The azurophilic granules contain myeloperoxidase (MPO), proteinase 3 (PR3), defensins, bactericidal/permeability-increasing protein (BPI), cathepsin G, neutrophil elastase, neutrophil serine protease 4 and azurocidin 1. NADPH oxidase, β-glucuronidase, lysozyme, alkaline phosphatase, lactoferrin cathelicidin and collagenase are found in the specific granules. The tertiary granules carry gelatinases, cathepsin, and neutrophil gelatinase-associated lipocalin[25]. Neutrophils express different receptors on their surface, important for the immediate innate immune response such as Fcγ receptors, Toll-like receptors, G protein-coupled receptors which include chemokine receptors, adhesion receptors, and various cytokine receptors including IL-1R and IL-6R[26].

The importance of neutrophils in the first line of host defence during bacterial infections is indisputable. They possess several crucial functions e.g. phagocytosis, formation of Neutrophil Extracellular Traps (called NETosis), Reactive Oxygen Species (ROS) production and degranulation. The neutrophil recruitment to the site of infection is a cascade, starting with the release of inflammatory mediators such as cytokines, chemokines and PRRs-PAMPs complex from cells in contact with the pathogens. These mediators as well as produce changes in the endothelium surface. The endothelium starts to express selectins that are rapidly attracting neutrophils to the affected site, leading to the margination, and rolling of the neutrophils along in the vessel wall. Subsequently, the neutrophils start to crawl and then extravasate between the endothelial cells into the affected tissue by diapedesis [27].

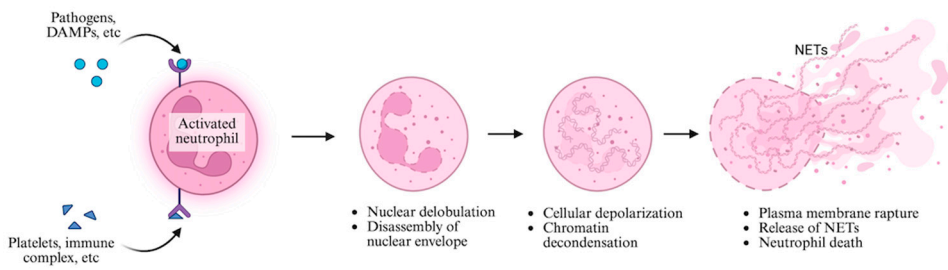

Figure 3. Neutrophil extracellular trap (NET) formation. Adapted in https://BioRender.com

Phagocytosis is one of the killing mechanisms, where neutrophils engulf and degrading pathogens. The neutrophils could recognize both opsonised and non-

opsonised extracellular pathogens by various receptors, as for example the Fcγ receptors. Activated neutrophils form pseudopods that first surround and trap the pathogens and then form the phagosome. Then granules and secondary vesicles of the neutrophils fuse with the phagosome and release their content[28].

In addition, the NADPH oxidase complex assembles in the phagosomal membrane and generates superoxide, which, together with myeloperoxidase (MPO), causes chemical damage to invading microbes[29]. Reactive oxygen species (ROS) are reactive derivates of molecular oxygen that are produced in small amounts in mitochondria during normal cellular metabolism. Upon stimulation, neutrophils activate NADPH oxidase, leading to oxidative stress and increased ROS production[27], which serve as highly effective antimicrobial agents. In neutrophils, the most relevant ROS include superoxide, hydrogen peroxide, and MPO-derived oxydabts[30].

NET formation depends on and is initiated by reactive oxygen species (ROS) production by neutrophils. During this process the nuclear membrane degrades, allowing DNA to be released into the cytoplasm[31]. NETosis results in a form of cell death, that is distinct from apoptosis and necrosis, as it does not involve DNA fragmentation[32]. NETosis is a programmed cell death characterized by distinct morphological changes in neutrophils, including as loss of lobulated nuclear structure; increased permeability of the nuclear and plasma membranes; loss of internal membranes, histone inactivation accompanied by chromatin decondensation and expansion into the cytoplasm; and mixing of chromatin with the granule contents. The DNA is subsequently expelled from the neutrophil forming net-like structures that trap pathogens, with the primary function of immobilizing and killing them. NETs are coated with histones as well as antimicrobial and cytotoxic molecules[33]. In addition, neutrophils interact with other immune cells, such as dendritic cells (DCs) promoting DC recruitment and antigen presentation; macrophages enhancing cytokine production; and B cells, supporting activation and antibody production. Neutrophils can also function as antigen-presenting cells (APCs) for T cells[34], thereby facilitating crosstalk between the innate and the adaptive immunity.

Adaptive Immunity

Adaptive immunity, also called specific immunity, is characterized by the capacity to recognize unique antigens, has immunological memory, and ability to undergo clonal expression. It consists of cell mediated immunity, involving B and T cells that recognize specific antigens and humoral immunity which is mediated by immunoglobulins and cytokines.

B cells

B cells originate in the bone marrow from the common lymphoid progenitor (CLPs) and undergo a highly regulated, multistage development process. These stages include pro-B, pre-B, immature B cells, and ultimately mature or naïve B cells. The mature B cell migrates to secondary lymphoid organs or tissues, such as lymph nodes, spleen and the Peyer´s patches in the small intestine, where the activation occur. All mature B cells express the B cell receptor (BCR), which is a membrane-bound immunoglobulin with the function to recognize different antigens and is unique for every B cell. During the B cell maturation, the B-cell receptor (BCR) is generated through V(D)J recombination -namely the rearrangement of variable (V) diversity (D) and joining (J) gene segments- which ensures the extraordinary diversity of the BCR repertoire[35].

As an immunoglobulin, the BCR is composed of two identical light chains (IgL) and two identical heavy chains (IgH), linked by disulfide bonds. The structure is the classical Y form, with the fragment antigen binding region (Fab) and the fragment crystallizable region (Fc). The Fab is located on the two arms and has high specificity for antigens. The Fc region presents effector functions[36].

The heavy and light chains are made of the variable region and the constant region (C). The association of a single heavy chain variable region with a single light chain variable region generates an individual antigen binding site and underlies the molecular specificity for the antigen recognition. As mentioned, the V(D)J recombination is responsible for the diversity of the BCR, where the VDJ genes code for the variable region in the heavy chains and the VJ genes code for the variable region in the light chains. This means that the light chain recombination is analogous to the heavy chains but lack the D segment.

The V(D)J rearrangement starts in the early stage of the B cells maturation, in the pro-B cell and is the stage where all B cells are screened for autoreactive B cell clones[37]. If recombination of the first heavy chain allele is nonproductive, the developing B cell subsequently attempts rearrangement of the second allele. Failure to generate a productive rearrangement from both alleles results in the induction of apoptotic cell death. The BCR receptor acts as a complex, composted of the homodimer membrane-bound immunoglobulin (mig), responsible for the antigen specificity, and the two signalling subunits, the Igα and the Igβ.[38] The Igα and the Igβ contain immunoreceptor tyrosine-based motifs (ITAMs), that trigger the B-cell activation.

During B cell development, the immature B cells first express surface IgM-class BCR. High levels of IgM on the surface are required for the migration to the secondary lymphoid organs, such as spleen. During the transitional phase from immature to mature B cells, IgD-class BCR appears on the surface, with the co-expression of IgM and IgD[39]. The two BCR classes have different structures and function[40].

In the spleen, the B cells complete the maturation by developing into follicular B cells or marginal zone (MZ) B cells. The follicular B cells recognize antigens, get activated, and present the MHC class II to the T helper cells. Following the activation of the follicular B cells is the proliferation and the formation of germinal centres. In the germinal centres, the B cells undergo somatic hypermutation and immunoglobulin class switch to produce IgG, IgA, or IgE antibodies. Finally, B cells differentiate into either memory B cells with surface IgG and IgE or long-lived plasma cells.

The MZ B cells recognize microbial antigens, and in combination with TLR, differentiate to plasma cells secreting IgM. Their function is to respond quickly to blood-borne pathogens and produce antibodies. However, they may be involved in the loss of immunological tolerance[41].

The antigens that activate B cells can be soluble, like the antigens in the bacterial membrane, or be presented by the T cells, as for example proteins. The activation can be T cell dependent or T cell independent and requires two signals. In the T cell dependent mechanism, a specific antigen binds to the BCR, which promote the MHC class II expression in the B cell surface. MHC class II interact with the TCR of the T cell, generating the B cell activation. During the T cell independent mechanism, the bacteria produce different molecules, such as flagellin or LPS, with cross-linking of critical number of BCR, which leads to accumulation of BCR and cross activation. As mentioned, the B cells are involved in the inflammatory response. However, there are B cells with immunosuppressive action, the regulatory B cells (B regs)[42].

Plasma cells are producing antibodies, e.g. immunoglobulins, do not undergo mitosis, and their lifespan is from some days to years. There are five classes of immunoglobulins: IgG, IgA, IgM, IgE, and IgD, with different structures, dimensions, and lifespan.

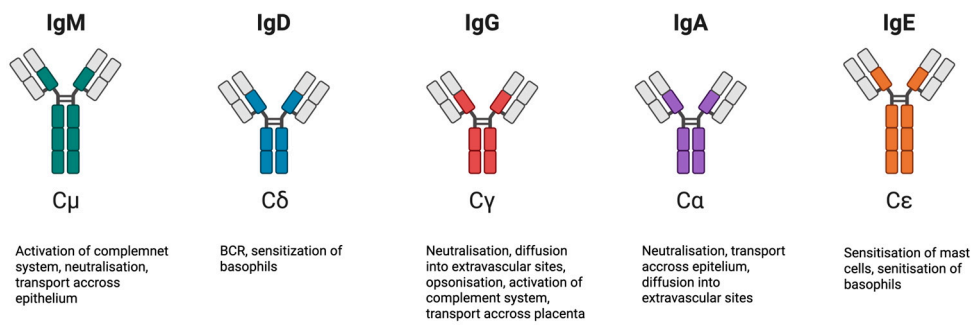

Figure 4. **Immunoglobulins**. Brief presentation of the immunoglobulins and their functions[43]. Created in https://BioRender.com

IgM is released early during an immune response, where it mediates pathogen neutralization and activates the complement system via the classical pathway. It forms a pentamer and despite the low affinity has high avidity. The lifespan is around five days. IgM can be produced in response to antigenic stimulation or can exist as natural IgM[44].

IgD primarily functions as a B-cell receptor and rarely secreted into the serum.

IgG is the most common isotype found in the bloodstream; it is a monomer and consists of four subclasses (IgG1, IgG2, IgG3, IgG4). It is produced later during the immune response, and the circulating half-life is 10 to21 days, depending on the subclass[45]. The IgG are crucial for the neutralization of viruses and toxins, activate complement, opsonize pathogens and is part of the passive immunity of the fetus[46].

IgA is the predominant antibody at mucosal surfaces and breast milk and is the second most abundant antibody in plasma. It exists as a monomer in plasma, and as a dimer in the mucosa[47]. The half-life is four to six days, and the function is to protect the mucosal barriers by neutralizing pathogens without triggering inflammation. It is also the passive immune protection of the new born[48].

IgE is present at very low levels in the serum, and the half-life is two to three days[49]. IgE is involved in defence against parasitic infections and in allergic reactions. When bound to mast cells and basophils, it triggers histamine release upon antigen engagement.

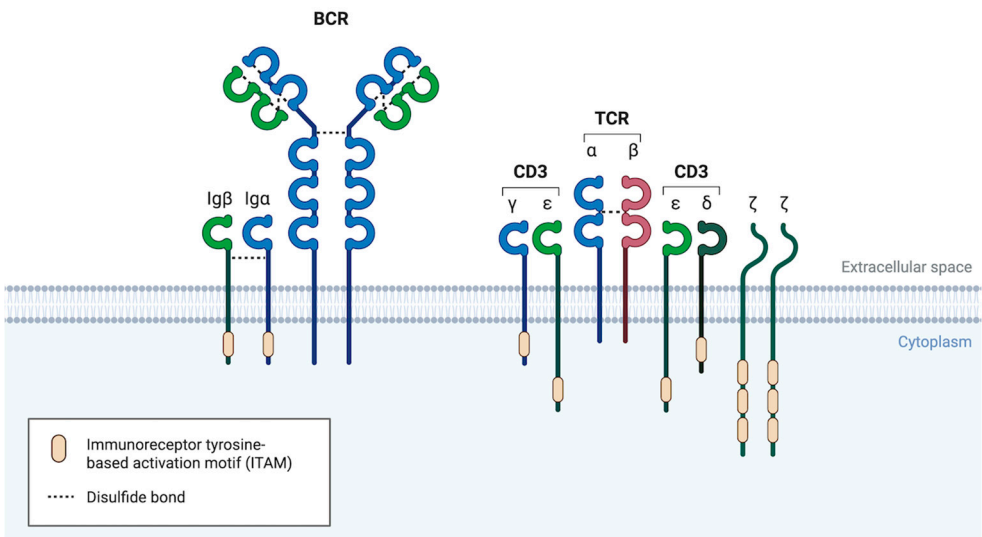

Figure 5. B-cell receptor (BCR) and T-cell receptor (TCR). Created in https://BioRender.com

T cells

T cells, as the B cells, are components of the adaptive immune system and rise from the common lymphoid progenitor in the bone marrow. The bone marrow derived thymocyte progenitors migrate into the thymus for development and differentiation. A pivotal role in the maturation of T cells is the expression of the T cell receptor (TCR) and the CD4 and CD8 co-receptors.

The T Cell receptor (TCR) is an immunoglobulin receptor located in the cell membrane and is composed of two antigen binding chains. These chains are designated α, β, γ and δ and their pairing gives rise to two distinct TCRs: the αβ, or γδ. The two chains are linked by disulfide bonds. Each chain contains the variable (V) region and the constant (C) region. As in B cells, the variable region exhibits substantial diversity, whereas the C region is constant. This diversity is generated through V(D)J rearrangement. The α chain is generated by VJ recombination, while the β chain is generated by VDJ recombination. Most T cells express the αβTCR, while γδ T cells are pronominally found in the gut mucosa[50]. To carry out its function, the αβTCR is in noncovalent association with the CD3 invariant dimers (CD3γε, CD3δε and CD3 ζζ)[51].

The CD4 and CD8 co-receptors are also located on the cell membrane and bind to the MHC complex. Naïve T cells, whether $CD4^+$ or $CD8^+$, are activated by antigen-presenting cells (APCs). This activation requires two signals: the MHC-antigen complex, which engages the TCR, and the co-stimulatory interaction between B7 family proteins (CD80 and CD86) on the APC and the C28 receptor on the T cell surface[52].

Both signals are essential for the naive T-cell activation and ensure the T cell response targets microbial antigens rather than harmless self-antigens. Each antigen stimulates the formation of a distinct population of activated T effector cells- due to the engagement of specific co-receptors.

The CD4+ T cells recognize antigens presented on MHC class II and differentiate into T helper cells, whereas the $CD8^+$ T cells recognize antigens on MHC class I molecules and become T cytotoxic cells. Activated T cells produce cytokines such as IL-2 that act in an autocrine manner to promote T-cell proliferation and function. This step drives T cell proliferation and differentiation into two distinct populations: effector T cells and memory T cells, both of which reside in peripheral tissues. T cells that fail to differentiate undergo apoptosis[53].

The CD4+ T cells produce cytokines that e.g. stimulate phagocytosis by macrophages or induce class switch in B-cells, while and the CD8+ T cells mainly act against virus infected cells or tumour cells.

CD4+ T helper undergo further differentiation into distinct subtypes, the Th1, Th2 Th17, T follicular helper (Tfh) and T regulatory cells (Tregs), in response to specific cytokine milieus. IL-12 and INF-γ drive the differentiation of Th1, which play a

central role in the autoimmunity and the host defense against intracellular pathogens. The Th2 cell differentiation is promoted by IL-4 and IL-2; the Th2 are involved in allergic reactions, asthma and immunity against extracellular parasites. The differentiation of Th17 cells is induced by IL-6, IL-2, IL-23, IL-1β and TGF-β, and the Th17 cells are implicated in autoimmunity and the immunity against the extracellular bacteria and fungi. The Tfh cells arise in response to the Il-6 and IL-21 and promote the B cell maturation in germinal centers and the antibody production including classswitch[54].

T regs are generated by the stimulation of TGF-β and IL-2 and are involved in the immune regulation and tolerance[55]. Tregs are a heterogeneous cell population and are present in different tissues with multiple functions and are essential for life. They suppress inflammation and inflammatory diseases as allergies, regulate the peripheral tolerance and prevent autoimmune diseases. In addition, they aid in tissue repairment after injuries, prevent rejection after transplantation, tolerate the microbiota, and protect stem cells in the follicles. The importance of T regs has gradually evolved after the discovery of forkhead box protein P3 (FOXP3), a transcription factor necessary for their development and function of Foxp3+ T regs[56]. Mutations in FOXP3 lead to the IPEX syndrome (e.g. Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome). The IPEX syndrome impact severely the number of Tregs and their function, with high mortality if not treated.

Different types of T regs are known, but the most studied are the Type 1 regulatory (Tr1) and the Foxp3+ Tregs. Tr1 and Foxp3+ Tregs are different cell subsets but share some common functions[57]. Tregs are activated via TCR and by antigen-specific and non-antigen-specific pathways. Upon activation they release cytokines such as IL-10, TGFβ, Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), and PD-1. IL-10 downregulates the immune response by limiting the MHC class II expression on the cell surface and suppresses the production of pro-inflammatory cytokines with consequent suppression of effector T cells. TGFβ suppresses the T cells proliferation and the differentiation to Th1 and Th2. CTLA-4 and PD-1 also suppress the effector T cells. Tregs express numerous IL-2 receptors in their surface and may reduce the availability for other cells. Moreover, Tregs can regulate the function of APCs[58].

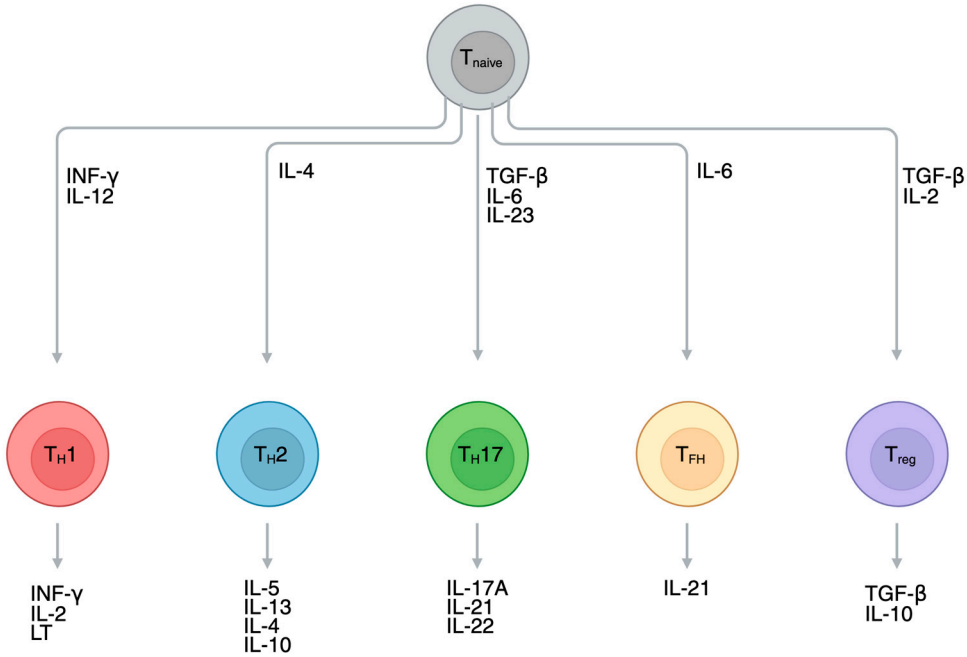

Figure 6. T cell differentiation. Adapted in https://BioRender.com

The CD8+ T cells or cytotoxic T cells play are effector T cells and play an important role in the host defence against infections and tumours, by the expression of cytotoxic molecules as perforin and granzymes, which kill and eliminate pathogens. Upon activation, naïve CD8+ T cells undergo differentiation into functionally specialized effector cells- including Tc1, Tc2, Tc9, Tc2, Tfc and suppressive CD8 T regs- in response to distinct cytokine milieus. Each subset displays distinct phenotypic and functional characteristics[59].

Antigen presenting cells

The antigen presenting cells (APCs) capture, process and display antigens on their surface via MHC class I or class II molecules. These MHC-antigen complexes are recognized by T cells, leading to T-cell activation. APCs can be classified as professional-such as dendritic cells (DCs), macrophages and B cells- or nonprofessional including fibroblasts and hepatocytes.

The professional APCs present exogenous antigens on MHC class II and present them to the CD4+ T cells. They are unique in requiring three signals for full T-cell activation: the MHC-antigen complex, co-stimulatory molecules such as B7, and cytokine secretion[60].

Cytokines

Cytokines are small proteins responsible for the communication between cells during an immune response. Many cells produce cytokines when stimulated by antigens. These cytokines bind to receptors on target cells, triggering activation. Cytokines can signal in three ways: **autocrine,** affecting the cells that produced them; **paracrine**, affecting nearby cells and **endocrine** (travelling through the bloodstream to affect distant cells this allows cytokines to coordinate communication between cells over both short and in long distances[61].

The cytokine family includes interleukins (ILs), Interferons (IFNs), Tumor Necrosis Factor (TNFs), chemokines, colony-stimulating Factors (CSFs) and growth factors. Each cytokine has specific target cells and functions. For example, interferons (INF-α, INF-β, INF-γ) are produced by lymphocytes, dendritic cells, macrophages, fibroblasts, NK cells and they play antiviral, immunomodulatory and antiproliferative roles[62].

Interleukins are released by monocytes, macrophages, lymphocytes, NK cells and each interleukin have its own effect, as for example the IL-2 which is produced by T-cells, has autocrine action and promotes proliferation and differentiation of T cells. Other interleukins are the IL-4, IL-6, IL-12 and more, that promotes the Th cells differentiation[63].

Chemokines, like CXCL1, CXCL2, and CXCL5, induce cell migration, adhesion, and activation.

TNF is produced by macrophages and can, for example, induce fever, stimulate the liver to produce acute phase proteins, and induce apoptosis.

In addition, cytokines can be divided into pro-and anti-inflammatory. The pro-inflammatory cytokines, like IL-1β, IL-6, IL-12, IL-17, can promote cell activation, tissue damage, and necrosis. In contrast, anti-inflammatory cytokines act to counteract inflammation[64] and help resolve the inflammatory response.

Immunological tolerance

Immunological tolerance can be defined as the ability of the immune system to distinguish between the self and non-self and can be divided into two parts, the central and the peripheral. The self-tolerance is induced in the central lymphoid organs (thymus and bone marrow) so called central tolerance, but also in the peripheral tissues, named peripheral tolerance. In the central tolerance the immature lymphocytes meet the self-antigens and typically undergo cell death. As errors may occur, there are peripheral mechanisms that block auto-reactive lymphocytes, inducing peripheral tolerance.

Immature T cells in the thymus are in contact with APCs that carry circulating self-antigens and peripheral tissue cell proteins. Auto-reactive T cells undergo negative selection and apoptosis or undergo development into Tregs. Tregs migrate to peripheral tissues, as part of the peripheral tolerance. The negative selection is regulated by the expression of MHC class I and II, so that both CD8+ and CD4+ T cells are implicated in the tolerance process. In the thymus, the peripheral cell antigens are expressed in medullary thymic epithelial cells (mTECs), and the expression is regulated by the autoimmune regulator protein (AIRE). Mutation in AIRE may cause autoimmune polyglandular syndrome 1 (APS1), a genetic autoimmune condition where several organs are involved including endocrine organs[65]. It is not fully understood why some self-reactive T cells undergo apoptosis and others become T regs, but this mechanism seems to be related to AIRE. It is observed that in the absence of AIRE, the negative selection and the development of Tregs fail with consequent induction of autoimmunity. As mentioned above, Tregs act in the periphery, performing their actions through CTLA-4, IL-10, TGFβ and IL-2, and inducing peripheral tolerance[66].

B cells are also part of the central and peripheral tolerance by inhibiting antibody answers to self-antigens. The immature B cells in the bone marrow express IgM on their surface, e.g. the BCR, recognizing self-antigens with high avidity. The self-reacting B cells undergo apoptosis or anergy, or receptor editing. The receptor editing is regulated by VJ recombination, resulting in a new Ig light chain and formation of a BCR with different specificity. This mechanism is significant for the elimination of auto-reactive B cells. Low avidity of self-antigens may suspend the function of B cells inducing anergy. The destination of B cells in peripheral tissues is once more apoptosis, anergy or regulation. Apoptosis is induced if B cells bind with high avidity the self-antigens. Anergy is the consequence of several stimulations by self-antigens, in which the self-reactive B cells are not capable to responding and becoming inactive. However, B cells able to recognize a self-antigen, experience regulation by inhibition from T regs or by inhibitory receptors[66, 67].

ANCA-associated vasculitis (AAV)

Antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis is a group of rare inflammatory autoimmune diseases with predominant involvement of the small blood vessels. AAV includes three distinct diagnoses, the Granulomatosis with polyangiitis (GPA), Microscopic polyangiitis (MPA), and Eosinophil granulomatosis with polyangiitis (EGPA). The main histologic characteristic is the necrotic inflammation of the small vessels and the formation of granulomas, which leads to tissue damage. Although the most affected organs are the kidneys, the upper and lower respiratory tract, and peripheral nervous system, these conditions are

mainly systemic and may involve any organ. The two ANCAs, Myeloperoxidase (MPO) and proteinase 3 (PR3), play a central role in the clinical development of the AAV, but the aetiology is multifactorial, complex and still not fully understood. The ANCAs association with AAV was first established in the 1980s[68], and since then the diagnostic and therapeutical approach have enhanced the prognosis.

Due to the rapid necrotizing inflammation, the granulomatous and pauci-immune lesions, the ANCA-vasculitis have a poor prognosis and high mortality if not treated on time. The treatment has led that small vessels vasculitis is now considered chronic diseases with potential relapsing attacks and comorbidities. The therapy is aimed at achieving and sustaining remission and is divided into induction and maintenance phases. Induction treatment is necessary and consists of immunosuppression by corticosteroids and other immunosuppressive drugs and may be combined with organ supportive care. Maintenance therapy involves continued corticosteroid and immunosuppressive treatment, with the goal to of sustaining remission while gradually reducing drugs doses. In the long term, very few patients may be able to discontinue immunosuppressive therapy.

Although these diseases present some common overlapping clinical features, they have distinct characteristics of organ involvement. Principally, the inflammatory lesions affect capillary vessels or lead to infarction of the small arterioles followed by ischemia, consequent damage, and finally organ failure.

Granulomatosis with polyangiitis, presented by necrotizing inflammation and granulomas, affects mainly the upper respiratory tracks, the lungs and the kidneys, but may also involve the skin, the heart and the nervous system[67]. In most cases, the ANCA antibody found in patients with GPA is against proteinase-3.

In microscopic polyangiitis, in which the predominant autoantibody is detected against myeloperoxidase, the primary affected organs are the kidneys with pauci-immune glomerulonephritis, and the lungs. Moreover, in MPA can involve the skin, the gastrointestinal track, and the peripheral nervous system[67].

Eosinophil granulomatosis with polyangiitis may present with nonspecific symptoms in the upper respiratory track, such as rhinitis and asthma, but can involve the lungs, the peripheral nervous system and other organs. Here, just 30-40% of patients have ANCA auto-antibodies[69].

ANCA-associated vasculitis are rare diseases and registered on the Orphanet database under the category of rare systemic and rheumatological diseases with the code ORPHA:156152[70]. In a recent meta-analysis[71], the authors reported that the incidence and prevalence of small-vessels vasculitis show an overall increasing trend, particularly pronounced for GPA. This may be explained by better diagnostic tools and revised criteria. The prevalence of AAV is 1-5/10 000 people (based on Orphanet) with variable geographic distribution. GPA and MPA are more prevalent in the north hemisphere, while MPA is more frequent in America and GPA in

Europe. Worldwide the GPA diagnosis exceeds that of MPA and EGPA, and individuals of Caucasian origine represent the most affected population. In the same study, it is reported that the incidence of ANCA-associated vasculitis is 17,2 per million person-year, in a range between 8,1 and 33,0 per million person-year[71].

The aetiology is multifactorial, including genetic associations, environmental factors, immune dysregulation and drugs.

ANCAs

Antineutrophil cytoplasmic autoantibodies play a pathogenetic role in the AAV that is demonstrated by clinical and experimental findings[72]. The two main autoantibodies are against proteins, mainly found in the neutrophils and monocytes: proteinase 3 and myeloperoxidase[73]. Although the presence of ANCAs is associated with the AAV, their presence by itself does not confirm diagnosis. It is necessary with contemporaneous symptomatology and disease characteristics. Additionally, it is shown that other diseases may have ANCA[74, 75]. Conversely, MPO-ANCA and PR3-ANCA seronegative patients exist, that develop typical symptoms and clinical findings that confirm the diagnosis[76, 77, 78].

Myeloperoxidase is located in the azurophil granules of neutrophils and the most abundantly soluble protein with several effects. It is also found in other cells, such as monocytes. MPO is the catalysator enzyme for the production of hypochlorous acid (HOCl), a potent antimicrobial agent, which causes oxidative damage[79] and is released upon neutrophil activation. The intracellular activity of MPO involves the phagocytosis process and the pathogen elimination by reactive oxygen production. The extracellular MPO activity is mediated by degranulation in the extracellular environment or in the NETs, where occurs inactivation of protease inhibitors and release of the lytic enzymes able to degrade structures near neutrophils[80]. A recent study showed that MPO also improves NET formation and stabilization[81]. Myeloperoxidase is involved in the signalling for neutrophil trafficking and in the modulation of the innate immune response by activation of polymorphonuclear cells via autocrine and paracrine action[82].

Proteinase 3 is a serine protease located in the azurophil granules of the neutrophils, but also in lower quantities in monocytes and macrophages. Functionally, PR3 has proteolytic extracellular activity and promotes the diapedesis[83]. It is involved in the degradation of extracellular components at the site of inflammation and modulate inflammatory cytokines and cytokines inhibitors[84]. PR3 is also expressed on the neutrophil surface in the absence of degranulation and is co-expressed with CD177[85]. Cell surface expression of PR3 is giving rise to two distinct neutrophil populations, the mPR3 positive ($mPR3^+$) and the mPR3 negative ($mPR3^-$)[86]. The number of $mPR3^+$ neutrophils is individual and is genetically regulated, which means that individuals with high neutrophil expression of PR3 have higher risk for

GPA[87]. The surface expression is accentuated during apoptosis and may contribute to inflammation and autoimmunity by modulating inflammatory cytokines.

The ANCAs can be detected in the laboratory analysis by indirect immunofluorescence (IIF) and by a solid phase enzyme immunoassay, like Enzyme-Linked Immunosorbent Assay (ELISA). Two patterns can be detected with IIF: the perinuclear ANCA (p-ANCA) associated with antibodies against MPO and the cytoplasmic ANCA (c-ANCA) correlated with antibodies against PR3. The ELISA method permits the quantitative estimation of ANCA[72]. The relation between the presence of antibodies and the disease activity is still not totally understood. The ANCA titers are not directly correlated to the disease activity, and ANCAs can be also found in low titers in healthy individuals. The European Alliance of Associations for Rheumatology (EULAR) recommends that therapeutic decisions should not solely be based on ANCA titers[67]. However, the antibody specificity may play role in the disease prognosis. MPO is mainly found in MPA with mainly renal involvement. PR3 is primarily found in GPA with typical respiratory tract involvement and patients with GPA have higher relapse risk compared to MPA patients[88].

Genetics and epigenetics

Genetic studies have shown the association of HLA class II loci and the risk of developing AAV. In these studies, *HLA-DPB1*0401* is found to be positively associated with PR3-ANCAs and GPA[89]. *HLA-DRB1*0901*, *HLA-DRB1*1302*, *HLA-DQA1*0302* and *HLA-DQB1*0303* are associated to MPO-ANCAs and MPA[90]. A GWAS study on EGPA patients[91] showed that different HLA loci are linked, with the main connection between HLA-*DQ* and MPO positive EGPA. Genetics variations alone do not fully account for the pathogenesis. Epigenetic mechanisms are implicated in phenotypic aberrances without altering the DNA sequence, but with effect on the gene expression. In AAV there is a dysregulated immune response with abnormal ANCA production and atypical expression of myeloperoxidase and proteinase 3[90, 92].

Environmental triggers

As already known, the cause of AAV is multifactorial and not fully understood. Genetics, epigenetics, and immune dysregulation play an key role in the pathogenesis, but triggers are necessary to develop the disease. Viral and bacterial infections, toxins, drugs, solvents, air pollution and more, may act as triggers to the disease development.

Silica, or quartz, is one of the most common minerals in nature, found in rocks, sand, and some plants. It is also used in the constructions, to produce building materials

and in manufacturing of glass, ceramics, silicon and more. The silica dust is associated with AAV and other autoimmune diseases. A meta-analysis of various studies highlighted the elevated risk of AAV after silica exposure[93]. Gregorini et al[94] proposed a possible mechanism including alveolar macrophage activation and silica-induced lesions in the macrophages. In vitro silica dust activates monocytes and macrophages to release cytokines (as IL-1 and TNF), lysosomal enzymes (as PR3 and MPO), and to produce ROS[92].

Viral or bacterial infections may be another trigger to the clinical outcome of small vessel autoimmune vasculitis. GPA patients with elevated risk for relapse, are often found to be nasal carriers of *Staphylococcus aureus*. Antibiotic use showed that the relapse risk was lower[95]. The pathogenetic mechanism is still not completely clear but it seems that the molecular mimicry is involved[96].

Different studies demonstrate the association between infections and autoimmune diseases[97-99]. is often found in GPA patients with high risk for relapse, as nasal carriers of these bacteria. Use of antibiotics as prophylaxis showed that the relapse risk was lower in PR3 positive ANCA vasculitis patients[95]. The *COVID19* infection expanded the research in this subject, with reports for the association between COVID*19* infection and vaccination, and the development of AAV[100-103]. Studies on *Parvovirus B19*[104] and *Hepatitis B*[105] could not confirm any association.

It is shown that some medications and drugs are triggers for the vasculitis onset. As example; cocaine, TNF-inhibitors, sulfasalazine, D-penicillamine, propylthiouracil (PTU), antibiotics, and more[106, 107]. PTU, used against hyperparathyroidism, may e.g. induce acute kidney injury with pauci-immune necrotizing glomerulonephritis.

Loss of tolerance

One of the main functions of the immune system is to distinguish between self and non-self, called tolerance. In autoimmune diseases tolerance against certain self-antigen are lost and the adaptive immune system, e.g. B-cells and T cells can attack specific tissues.

AAV patients has been shown to have decreased number of Tregs with impaired function, and the contemporaneous increase of CD4+ T effector memory cells and activated T cells[108, 109]. Additionally, it is noticed an aberrant expansion of Th17 cells, known to be implicated in autoimmunity[109].

B cells also have a significant role in AAV as APCs, ancestor to plasma cells, and proinflammatory molecules production. B cells can be activated by neutrophils, which release B-cell activating factor (BAFF), thereby promoting B cell activation. BAFF receptors are expressed on the surface of naïve B-cells, memory B cells, and plasmablasts. Dysregulated BAFF signaling is implicated in autoimmune diseases, due to the failure to eliminate autoreactive B cells[110].

Role of neutrophils and monocytes

In addition to neutrophil and monocyte activation by "priming" with pro-inflammatory agents, such as bacterial polysaccharides, TNF-a, C5a, IL-1β and other cytokines, the PR3 and MPO get expressed in higher concentration on the cell surface. The ANCAs, in the form of IgG antibodies, recognize and bind MPO and PR3, leading to ulterior neutrophil and monocyte activation and inflammation with consequent vessel injury.

In addition to increased numbers of PR3 and MPO molecules on primed monocytes, they also express higher levels of CD14, MCH class II and TLR. The activated monocytes release proinflammatory cytokines as TNF-a, IL-1β, IL-6, IL-8 and monocyte chemoattraction protein (MCP-1)[111]. This activation leads to leukocyte recruitment and local inflammation. High monocyte recruitment in glomeruli seems to be crucial for the kidney damage as they are responsible for fibrotic necrosis, T cells and macrophage recruitment, with formation of infiltrates and granulomas. [111, 112].

Moreover, the activated neutrophils can phagocytize, release the enzymatic lytic content of the granules, generate ROS, and activate the complement[72, 113]. Neutrophils undergo necrosis and NETosis during the acute inflammation phase. If the acute inflammation is not solved, segues the chronic phase with monocyte/macrophage involvement, collagen deposition and fibrosis[114].

Role of complement

The complement system, as a part of the innate immune system, is implicated in the acute inflammatory response, by neutrophils and monocytes. In ANCA-associated vasculitis, the complement system is stimulated by the alternative pathway. Activated neutrophils by ANCA, produce and release C5a, causing recruitment and neutrophil priming with C5a. C5a is necessary for complement activation, the increment of vascular permeability, the NETosis, the neutrophil degranulation, and the respiratory burst[113]. The complement is also involved in the disease activity; in active disease the complement factors C3a, C5a, C5b-9 are found in higher serum concentrations, and factor H in lower[115, 116]. Factor H acts as a regulator for C3b-amplification and prevents neutrophil activation and endothelial damage.

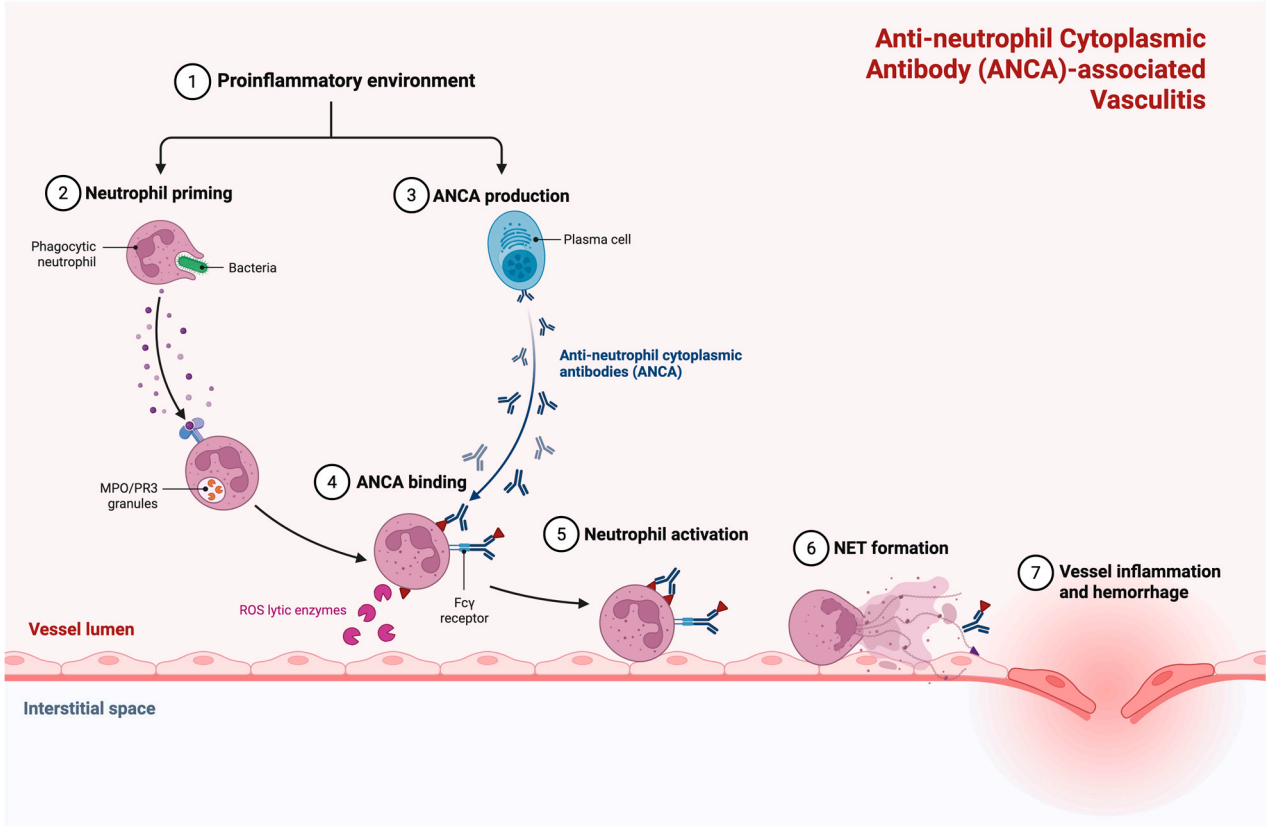

Figure 7. Mechanism of tissue damage in ANCA-associated vasculitis. (1) In a proinflammatory milieu, (2) neutrophils are primed by cytokines and express PR3 and MPO on their surface. At the same time, the proinflammatory milieu activates plasma cells to produce ANCA autoantibodies (3) with consequent neutrophil activation and ROS release (4). Activated neutrophils adhere to the vascular endothelium by rolling (5) and release NETs (6) leading to vessel injury (7). Created in https://BioRender.com

Classification

The ANCA-associated vasculitis is a heterogeneous group of diseases. To identify the largest possible number of patients, different diagnostic and classification criteria have been proposed with some of them currently in use. Diagnostic criteria include signs and symptoms, laboratory and histological findings[117]. The classification criteria are proposed as standardized definitions[117]. Historically, vasculitis symptoms have been reported already in ancient Greece from Hippocrates around the fourth century BC. These symptoms are probably correlated to the now known Behçet's disease[118]. Only during the 20th century, the first classification criteria about necrotizing vasculitis was offered by Zeek[119]. The ACR1990 criteria by the American College of Rheumatology[120], the CHCC1994 classification system by the Chapel Hill consensus conference, revised 2012[121] and the 2022ACR/EULAR classification criteria[122] are mainly in use worldwide. Currently the diseases included in the ANCA-associated vasculitis are granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophil granulomatosis with polyangiitis (EGPA), each one with specific symptoms and signs, but in common some overlapping characteristics.

Symptoms and organ involvement

Granulomatosis with polyangiitis, or previously called Wegener's granulomatosis, is a small and medium vessel vasculitis, with presence of inflammation, granulomatosis, necrotizing lesions which primary affect the upper and lower respiratory tract[123]. Other organs and systems can be affected. The clinical manifestations of the upper respiratory tract include crusting rhinitis, sinusitis, chronic otitis, and epistaxis. In addition, hearing loss and the typical saddle nose deformity may occur. Other signs are dysphonia, inspiratory dyspnea, and subglottic stenosis. In the lungs, bilateral parenchymal nodules, cavitation, alveolar haemorrhage, and infections can be found. Renal involvement is very often reported, with haematuria, proteinuria, and rapidly progressive glomerulonephritis, which may require renal replacement therapy. Necrosis with deposit of immune complexes, note as pauci-immune glomerulonephritis is often a finding of the renal biopsy. The nervous system can be affected by the presence of mononeuritis or cranial nerve involvement. Moreover, cutaneous vasculitis lesions occur, in the form of purpura or necrotic papules and skin ulceration often occur[124]. GPA presents the highest relapse risk among the AAV and PR3-ANCA is predominant[125].

Microscopic polyangiitis is characterized by necrotizing lesions of the small vessels with the kidneys representing the primary target organ[123]. The rapidly progressive glomerulonephritis is one of the main manifestations of the disease, with microscopic haematuria and proteinuria, that quickly can proceed in deterioration of renal function. The lungs are affected with alveolar haemorrhage, haemoptysis,

dyspnea, interstitial lung disease, and bronchiectasis. Cutaneous manifestations may also be found, including purpura and maculopapular lesions[126]and in the gastrointestinal tract may appear abdominal angina, or even haemorrhagic or ischemic events may occur[127]. Moreover, the nervous system could be affected, leading to paraesthesia, mononeuritis, subarachnoid haemorrhage, and cerebrovascular events. Common symptoms such as fever, myalgia, and weight loss may exist. The MPO-ANCA is most frequent in MPA.

Eosinophil granulomatosis with polyangiitis, or previously called Churg-Strauss syndrome, is a granulomatosis disease with hyper eosinophilia[123]. The disease affects both the upper and lower respiratory tracts, with frequent symptoms including pansinusitis, nasal polyposis, asthma, pulmonary infiltrates, and alveolar haemorrhage. Peripheral neuropathy is a common finding, but the central nervous system is rarely involved. The cardiovascular system is affected with manifestations such as pericarditis, myocarditis, arrhythmias, and its involvement is the most critical in EGPA. Other affected organs are the kidneys with necrotizing glomerulonephritis, the skin, and the gastrointestinal track. In EGPA, only a little part of patients is ANCA-positive, and the predominant antibody is directed against MPO[69].

Assessment

The main challenges in ANCA-associated vasculitis are to maintain the organ function, the remission, and prevent the relapse. After the introduction of immunosuppressive therapy, the five year-survival rate is high and the risk for mortality is under 50%[128]. The treatment allows disease control, but comorbidities and adverse events of therapy are frequently observed. Susceptibility to infections, risk of malignancy due to immunosuppressive therapy, hypertension, diabetes, osteoporosis, coronary heart disease, and more, are some of the side effects. Clinicians have tools such as Birmingham Vasculitis Activity Score 3 (BVAS3)[129] and the Vasculitis Damage Index (VDI)[130] to assess the disease.

Treatment

AAV are long life diseases with high mortality rates if left untreated. The treatment is based on immunosuppressive therapies including glucocorticoids and is divided into induction and maintenance therapy. The treatment consists, in most cases, of the combination of glucocorticoids and another drug[131].

Typically, the oral dose of glucocorticoids is 1mg/kg/day for the induction therapy, which can be slowly reduced[67]. Glucocorticoids are an established treatment and have been used for many years but induce severe side effects such as diabetes and

gain of weight, osteoporosis with risk for fractures, avascular necrosis and more, especially when treatment is prolonged.

Different treatments are currently used as methotrexate (MTX), azathioprine (AZA), mycophenolate mofetil (MMF), cyclophosphamide (CYC), rituximab (RTX), Avacopan and more.

The recommended treatment for induction of remission or for relapsing patients with GPA and MPA include rituximab or cyclophosphamide in combination with glucocorticoids[67]. CYC is one of the most effective drugs against AAV, is a cytotoxic agent used in chemotherapy, but increase the risk for malignancy[132]. RTX is a monoclonal chimeric antibody against CD20 protein, expressed on the surface of pre-B, naïve and memory B cells, but is absent from plasmablasts, plasma cells and hematopoietic stem cells. CD8+ effector memory T cells also express CD20. Originally, it was approved for the treatment of hematologic malignancies. The mechanism of action of RTX is the cell depletion, by cell and complement mediated cytotoxicity and is used in the therapy several autoimmune diagnoses. RTX causes adverse events such as headache, skin itchiness and throat irritation, but is also observed high risk for infections, hypogammaglobulinemia and late-onset neutropenia [132, 133].

Most patients have successful disease control after the induction treatment. However, achieving control over relapses, comorbidities and infections, sustaining work ability and maintaining a good quality of life constitute the main treatment goals.

Aims

Study I

We aimed to identify possible correlations between the B cells subsets and the immunoglobulin levels to the disease activity and the tendency to relapse.

Study II

To investigate the relation between granulocytes and monocytes to the disease activity as well as the relapsing disease.

Study III

The aim of this study is to investigate the granulocyte and monocyte function in AAV patients with or without RTX, the susceptibility to infections, and the immune cell response.

Methods

Flow cytometry

Flow cytometry is a qualitative and quantitative method for cellular and particles assessment, providing morphological, functional, physiological or pathological information at single cell level. It is a rapid, powerful, multiparametric technique used both in research as well as an important clinical diagnostic method. In the designated term, the word flow refers to the suspension where the cells or particles are found and, the word cytometry originates from the Greek word *kytos* =cell and *metry* =measurement. The progenitor of the modern flow cytometers goes back to 1954 where an instrument for electronic measurement for cell size and cell count was described by Wallace Coulter[134]. The flow cytometers are made of three different parts, fluidics, optics and detectors. Moreover, a computer is needed for data analysis. Basically, in flow cytometry, the emitted light is measured and translated into data.

Samples of various nature can be analysed by flow cytometry, cell cultures, blood, bacteria, viruses, disaggregated solid tissues, and body fluids. The applications are numerous, including the cell state and the cell activity, such as immunophenotyping, cell cycle, cell identification and differentiation, cytokine secretion, activation of signalling pathways, ROS production, phagocytosis, among others. Clinically, flow cytometry provides information for multiple conditions like leukemias, lymphomas, disease state such as for example HIV-infection, quantification of hematopoietic stem cells, auto-immune diseases, diagnosis of immunodeficiencies and so on[135].

In this thesis, flow cytometry has been used for immunophenotyping and investigation of cell activity, with a focus on immune cells.

Flow cytometers are rapid instruments with a capacity to analyse thousands of cells or particles in each second, permitting measurements as for example size, granularity, receptors, DNA content, and proteins. Accurate control of all parameters, including instrumentation and all stages of analysis (from the sample collection to the data interpretation), is essential to get high quality results[136].

The fluidic system is fundamental as it guarantees the transport of the cells from the reservoir to the flow chamber in a sheath fluid. The studied material is found in an isotonic buffer suspension[137], typically Phosphate-Buffered Saline (PBS), necessary for the cells to flow one by one. The fluidic system focuses hydrodynamically on

the cells on the centre of the flow chamber, achieved by the different pressure between the sample stream and the sheath fluid. Aligned cells in a single file create a laminal flow, which passes through the optical system where a focused light source, e.g. a laser, illuminates them. Each cell that transits the laser beam is called "event". The illumination can be generated by two or more lasers, with different wavelengths, providing specific colours, and creating both fluorescent and nonfluorescent signals. Common lasers in use, translated in colours, are the blue (488nm), violet (405nm), green (532-552nm), green-yellow (561nm), red (640nm), and ultra-violet (355nm)[138].

All materials absorb light in certain wavelengths, and scatter light they do not absorb. Fluorochromes, dyes with which the cells have already been prepared, absorb light of determined wavelength and excite their electrons from the ground state. During the return to a lower energy state, electrons emit light with a longer wavelength. After the first partial release of energy, they dump to the ground state and release the remaining energy in photons. Photons emit energy in the form of fluorescence, which is detected and measured[139].

The emitted light scatter is detected and captured in two directions, forward and at 90º. The forward scatter (FSC) measures the cell size, and the side scatter (SSC) measures the cell granularity or complexity. Two collection lenses (one for the SSC and one for the FSC) capture the scattered light and forward it to optical filters. As distinct fluorochromes are used and as the spectrum of emitted energy is of a certain wavelength for each one, it becomes necessary to add optical filters. They filter the light and are specific for the wavelengths of interest, reflecting other wavelengths. There are three types of optical filters: the dichroic mirrors categorized as long pass (LP) or the short pass (SP), and the bandpass (BP), all of them with specific characteristics. The long pass permits the light with high wavelength to pass through (as for example above 500 nm, like green, yellow, red) and reflect light with low wavelength (like violet or blue) in a specific direction. Exactly the opposite is applied for the short pass filters, where for example light below 500 nm pass the filter and light above 500 nm is reflected. The bandpass filter can be considered as an overlapping of long pass and short pass filters, and it permits the passage of light within a certain range, like 500/50 nm[140].

After the optical filter passage, the light meets the detectors. There are several detectors that convert light energy into electrical signals, but photodiodes (PDs) and photomultiplier tubes (PMTs) constitute often the first choice, because of their sensibility. The photodiodes are more sensible to detect strong signals such as FSC, and the photomultiplier tubes are more suitable for SSC and fluorescence. The photons from the sample are converted primarily in photoelectrons and electrical impulses then in voltage pulses[141] which become digital data. Depending on the number of the considered parameters, the data is presented in histogram (if single-parameter), in dot plots (if dual-parameter, commonly FCS and SSC), in 3D plots or complex plots (if multiple parameters).

The fluorescence and the fluorochromes are essentials for the analysis and interpretation of the data. As mentioned, the particles absorb the light of a certain wavelength, and they re-emit it in a longer wavelength, making it appear brighter, which is the fluorescence concept.

All cells have intrinsic and extrinsic fluorescence. The intrinsic, e.g. the autofluorescence, emerges from certain parts of the cell, such as melanin or chlorophyl. The extrinsic fluorescence is added to the cells by the investigators, called fluorochromes, and include labelled antibodies, dyes, and fluorescent proteins. The size differs between the fluorochromes from big, such as cyclic ring compounds (FITC, Alexa 488, Cyanine 3, Cyanine 5), protein dyes (PE, APC) and tandem dyes (PE-Cy5, PE-Cy5.5 APC-Cy 5.5 and more), to small like nanocrystals, polymer dyes or DNA dyes[138]. Brightness presents also a large variation, specific for each fluorochrome.

Cell sorters are extensions of the flow cytometer and are used for the physical separation of cells subsets from a cell population, providing high purified cell types. An example of a primary sorting set is the electrostatic sorting, where high voltage electrostatic plates are used to separate the cells, after their transition through the optical system. The cells, previously electrically charged, are sorted by their charge characteristics.

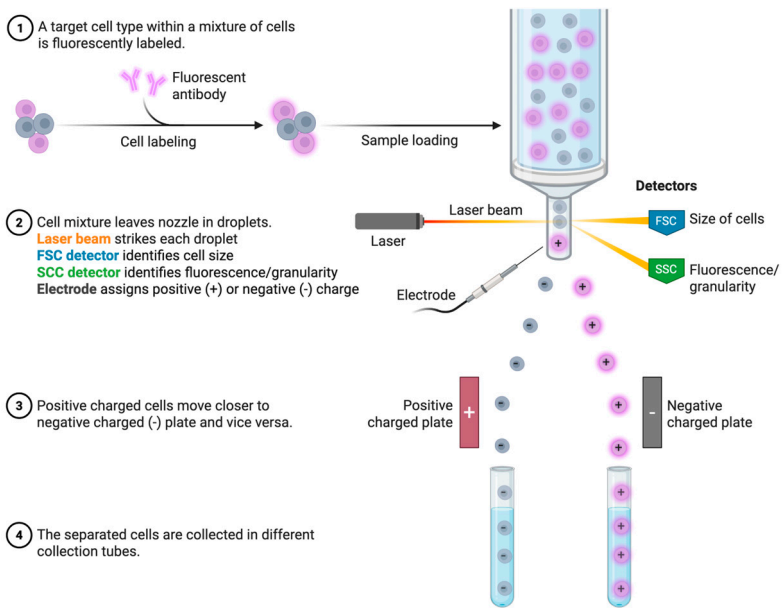

Figure 8. FACS Illustration. Created in https://BioRender.com

In our experiments, we used fluorescence-activated cell sorting (FACS) for the immunophenotyping, phagocytic activity and oxidative burst activity.

FACS is a sort of flow cytometer where a cell sorter separates the cells based on their fluorescence. Cells incubated with dyes, commonly linked to antibodies, pass through the flow cytometer and get analysed based on FSC and SSC.

Immunophenotyping

Immunophenotyping is the process for cell identification using antigens or markers placed on the cell surface[142]. The blood samples are prepared and stained with a cocktail of fluorescent antibodies specific to antigens on the cell surface. More specifically, peripheral heparinized whole blood stored in rum temperature (RT) is processed within 24 hours from the sampling. NH_4Cl 0,84% is added for the erythrocyte lysing. Follows centrifugation and two washings steps with Phosphate Buffered Saline (PBS), before the FACS® buffer is added. The cells get stained with a specific ready mixture of antibodies.

The antigens of interest for the immunophenotyping included in the thesis are briefly shown in table 1 and more detailed in the articles.

Table 1

Article	Target cells	Antigen
Paper I	B Cells	CD19, IgD, CD27, CD38, CD24, CD95 and CD45
Paper II	Granulocytes, Monocytes	CD10, CD14, CD16, CD193, CD177, Siglec-8 and CD88

Oxidative burst- Phagoburst™

Phagoburst™ is a quantitative test used to measure the leucocyte oxidative burst, by calculating the percentage of cells that produce reactive oxidants and their enzymatic activity. Peripheral heparinized whole blood stored in room temperature (RT), get processed within 24 hours from the sampling according to the manufacture´s protocols (Glycotope Biotechnology, GmBH, Germany). The stimulation occurs with unlabelled opsonized bacteria *Escherichia Coli (E.Coli)* and phorbol 12-myristate 13-acetate (PMA) in two separate tubes. Dihydrorhodamine (DHR) 123 is used as a fluorogenic substrate. DHR 123 is a nonfluorescent and membrane-permeative dye, which is oxidated by ROS and converted to green fluorescent compound[143].

Phagocytosis- Phagotest™

Phagotest™ is a quantitative test used for the leukocyte's phagocytosis, which measures the percentage of phagocytes with ingested bacteria and their activity, by the use of FITC-labelled opsonized *E.Coli*. Peripheral heparinized whole blood stored in RT was used here, as well as processed within 24 hours from sampling according to the manufacture´s protocols (Glycotope Biotechnology, GmBH, Germany).

Data Acquisition

FACS Canto II with FACSDiva software (Becton Dickinson, Franklin Lakes, Nj, USA) has been used for the data acquisition of immunophenotyping, oxidative burst and phagocytosis. The data analysis was performed using the Kaluza Analysis Software 2.1 (Beckman Coulter, Brea, CA, USA). Examples of the gating strategies used for the oxidative stress and the phagocytic function are shown in figures 9 and 10. The gating strategies used for immunophenotyping of B cells, granulocytes, and monocytes are reported in the supplementary materials of the two published articles.

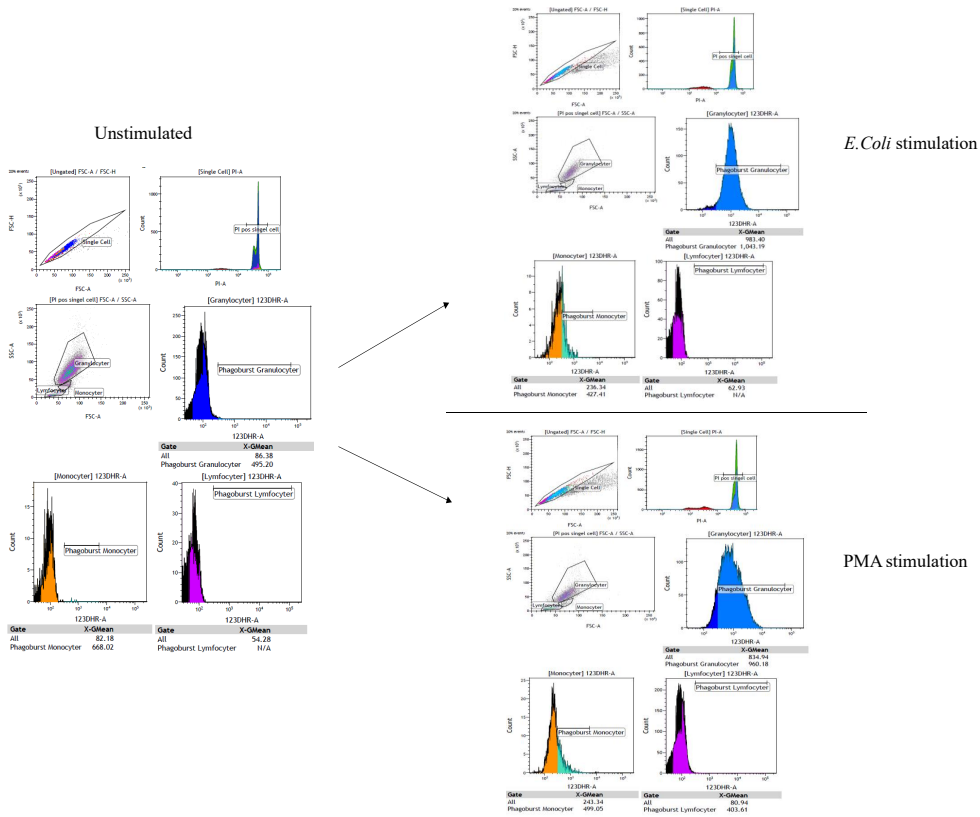

Figure 9. Oxidative burst gating strategy

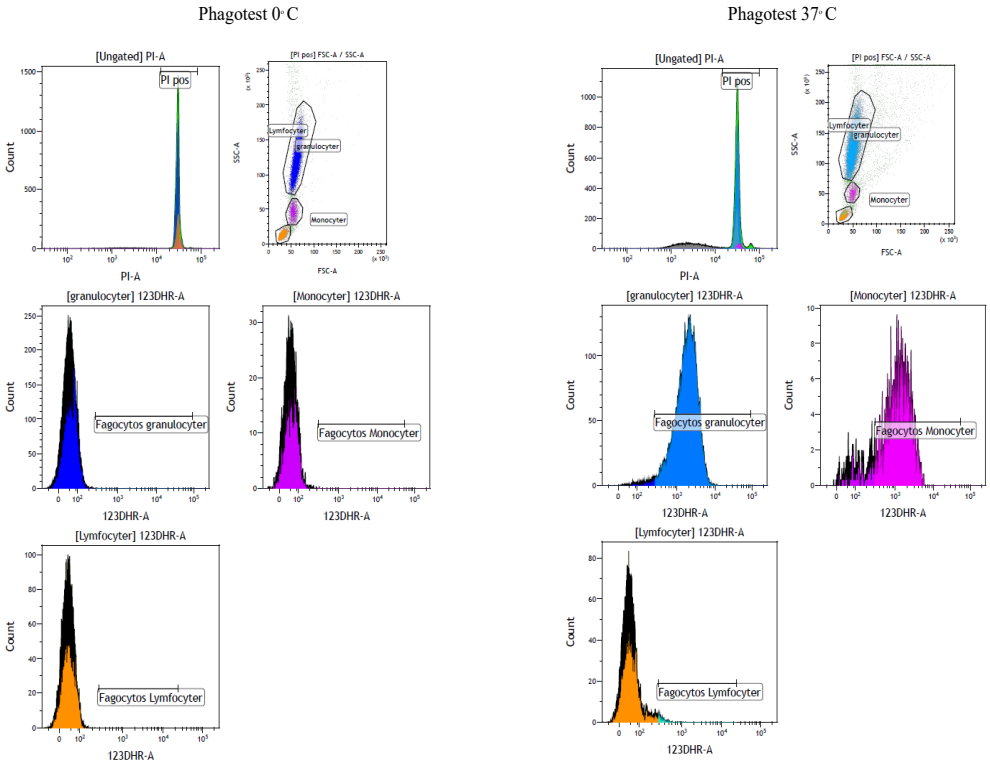

Figure 10. Phagocytic function gating strategy

Statistical analysis

The statistical analyses were performed as below:

Study I: Statistical analyses were performed using GraphPad Prism 8.4.2 software (GraphPad Software, San Diego, CA, USA). The Mann-Whitney *U* test for two groups comparisons and for three or more groups was used the Kruscal-Wallis with Dunn´s multiple comparisons test. For correlations we used the Spearman´s correlation and linear regression analysis.

Study II: Analyses were conducted using GraphPad Prism 9.0.1 software (GraphPad Software, San Diego, CA, USA). The Mann-Whitney *U* test for two groups comparisons and the Wilcoxon signed-rank test for paired data analysis were used.

Study III: Statistical analyses were performed using GraphPad Prism 10.6.1 software (GraphPad Software, San Diego, CA, USA). The Mann-Whitney *U* test for two groups comparisons was used, and row statistics were applied for graphical visualization.

Ethical approval

All three studies included in this thesis were approved by the regional ethical review board, Sweden. Study I: Permit number 2008/110. Study II: Permit number 2008/110 and 2021-04168. Study III: Permit number 2008/110 and 2021-04168.

Prior to inclusion, all participants provided written informed consent.

Artificial Intelligence statement

The three studies have been generated and completed without any use of artificial intelligence. However, during the writing process of this thesis, the artificial intelligence tool ChatGPT has partially been used for grammar and syntax text control.

Results and discussion

Paper I

Increased Frequencies of Switched Memory B cells and Plasmablasts in Peripheral Blood from Patients with ANCA-Associated Vasculitis

Background

B cells have a pivotal role in the pathogenesis of autoimmune diseases including ANCA-associated vasculitis due to antibody-dependent and antibody-independent mechanisms:

i) the production of autoantibodies by auto-reactive B cells induce complement-dependent cytotoxicity and formation of immune complexes that activate inflammation[144]

ii) B cells are antigen-presenting cells (APCs) which activate T cells by co-stimulatory signals and the release of cytokines and growth factors[145]

iii) B cells produce proinflammatory cytokines as IL-17, interferon-γ (INF-γ)[144]

iv) B cells produce IL-10 and have inhibitory effects to T cell proliferation[144].

In addition, it has been shown that patients with ANCA-associated vasculitis have higher percentages of plasma cells and plasmablasts. Moreover, the proportion of activated B cells has been associated with disease activity.

In this study, we included peripheral blood samples from 106 AAV patients (more specifically GPA= 64, MPA=35, and EGPA=7). We performed immunophenotypic characterization of B cells, with the method previously described, to identify the B cells and the B cell subsets, defined as follow: B cells ($CD19^+$), naïve B cells ($CD19^+CD27^-IgD^+$), preswitch memory B cells ($CD19^+CD27^+IgD^+$), switched memory B cells ($CD19^+CD27^+IgD^-$), exhausted memory B cells ($CD19^+CD27^-IgD^-$), transitional B cells ($CD19^+CD27^-IgD^+CD24^+CD38^{++}$), plasmablasts ($CD19^+CD27^+IgD^-CD24^-CD38^{++}$). The activated B cells have been counted as the total activated $CD95^+$ B cells and their subsets. The various subsets of activated B cells are defined as $CD95^+$ naïve B cells ($CD19^+CD27^-IgD^+CD95^+$), $CD95^+$

preswitch memory B cells ($CD19^+CD27^+IgD^+CD95^+$), $CD95^+$ switched memory B cells ($CD19^+CD27^+IgD^-CD95^+$), and $CD95^+$ exhausted memory B cells ($CD19^+CD27^-IgD^-CD95^+$).

Immunoglobulin analyses were performed as part of routine clinical testing at Clinical Immunology and Transfusion Medicine department, Lund, Region Skåne.

Clinical and laboratory parameters such as white blood cell count (WBC), creatinine, C-reactive protein (CRP), PR3- and MPO-ANCA serology, date of diagnosis, disease activity, tendency to relapse, dialysis, date of the last relapse and medication were obtained by the clinical records. The estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI creatinine equation.

As healthy controls (HC) we used 134 healthy blood donors (HBD) where B cell and B cell subsets have been analysed. As the absolute number of lymphocytes in HBD was not available, we used a reference material from the Clinical Immunology and Transfusion Medicine department in Lund as a source to define the range of $CD19^+$B cells. Here, the term HC include the HBD and the reference for the $CD19^+$ B cells. Patients under rituximab treatment have been excluded.

We hypothesized that AAV patients have abnormal frequencies of B cells subsets which are correlated to the disease activity and/or tendency to relapse. We aimed to identify possible correlations between the B cells subsets and the immunoglobulin levels to the disease activity and the tendency to relapse.

Results

We compared the AAV patients to healthy controls (HC) and we noticed that lower frequencies of B cells ($CD19^+$), transitional B cells, and absolute number of B cells were found in the patients with ANCA associated vasculitis. At the same time, we detected higher frequencies of switched memory B cells, plasmablasts and activated B cells referred to HCs.

Immunoglobulin levels of IgA, IgG, and IgM were measured and were predominantly within the reference range. The only deviation observed was in patients with eosinophilic granulomatosis with polyangiitis (EGPA), in whom IgA levels were below the reference range. Within the AAV patients, we found that patients with tendency to relapse and patients on immunomodulating treatment had lower IgG levels compared to patients without tendency to relapse and patients without treatment, respectively.

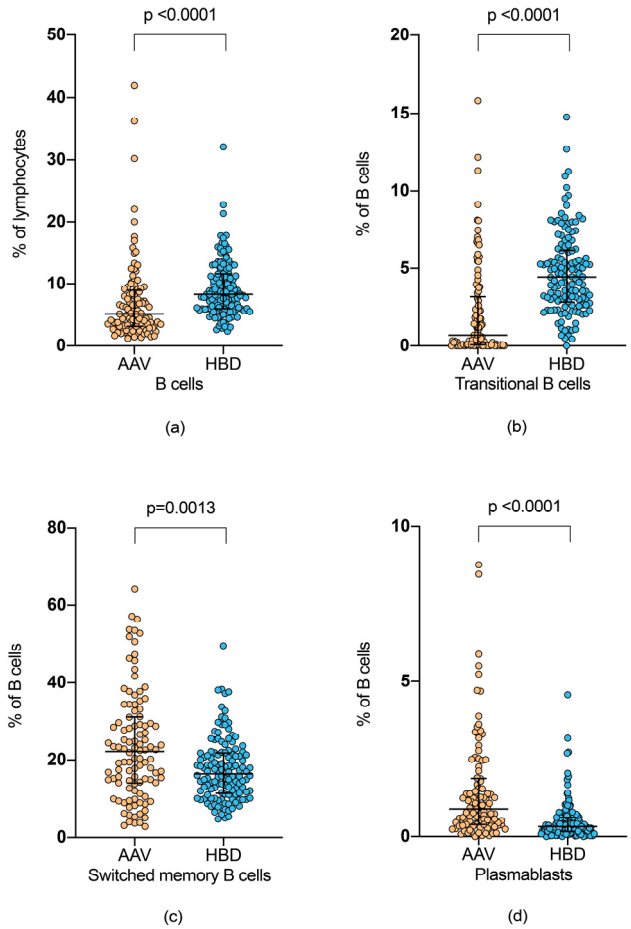

Figure 11. Comparisons of B cells and subsets between vasculitis patients and healthy blood donors. (a) Percentage of $CD19^+$ B cells of lymphocytes, (b) percentage of transitional B cells (of $CD19^+$ B cells), (c) percentage of switched memory B cells (of $CD19^+$ B cells), and (d) percentage of plasmablasts (of $CD19^+$ B cells), in peripheral blood from patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV, *n*=106) and healthy blood donors (HBD, *n*=134). Reprinted from paper I, figure 1.

Grouping the patients regarding the tendency to relapse in two groups (with and without tendency to relapse), we found lower frequencies of B cells ($CD19^+$) and transitional B cells in patients with tendency to relapse. Looking closely, 24% of the individuals within the tendency to relapse group had an active disease at the time of sampling, but all patients without tendency to relapse were in remission. For this reason, we compared patients in remission with or without tendency to relapse and we confirmed the previous results about the lower frequencies of B cells ($CD19^+$) and transitional B cells in patients with tendency to relapse, but we also found higher frequencies of $CD95^+$ exhausted memory B cells in this patient group.

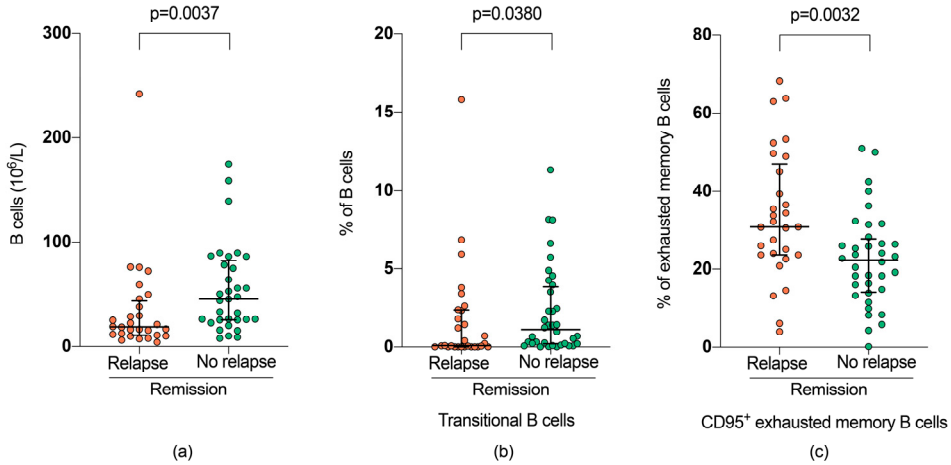

Figure 12. Comparisons of B cells and subsets between patients in remission with and without a tendency to relapse. (a) Concentration of $CD19^+$ B cells in peripheral blood from patients with antineutrophil cytoplasmic antibody (ANCA)- associated vasculitis (AAV) in remission with (*n*=28) and without (*n*=33) tendency to relapse. (b) Percentage of transitional B cells (of $CD19^+$ B cells), (C) percentage of $CD95^+$ exhausted memory B cells (of exhausted memory B cells). Reprinted from paper I, figure 3.

Dividing the AAV patients with or without immunosuppressant treatment, we noticed decreased frequencies in the absolute B cell count and in the B cells subsets in the treatment group.

No differences in B-cell frequencies were observed when comparing the AAV patients with active disease to those in remission.

Discussion

In this study, we investigated the B cells and B cell subsets in patients with diagnosis of GPA, MPA, and EGPA.

B cells, as antibody producing cells, are implicated in auto-immune diseases and likely play a pivotal role in the pathogenesis of ANCA-associated vasculitis. We found that the AAV patients had decreased B cells both in the absolute B cell count and in the percentage of B cells as part of lymphocytes. This is in line with previous studies in GPA patients[146, 147], even if a study conducted by Lepse et al, could not show any difference[148]. This discrepancy may depend on medication regimes or disease activity. In the same study by Lepse at al, patients with active disease showed a lower percentage of transitional B cells compared to patients in remission and healthy controls, which is consistent with our results and with findings reported in multiple sclerosis (MS)[149] and neuromyelitis optica (NMO)[150]. However, a study conducted in GPA patients did not identify differences in transitional B cells among

patients with future relapse, non-relapsing patients, and in healthy controls[147], which contrasts with our findings.

Memory B cells have a central role in the production of antibodies. The autoantibodies play a key role in the chronic and progressive character of the autoimmune diseases[151]. Switched memory B cells reflect normal B cell activation and maturation. We found increased frequencies of switched memory B cells in AAV patients, particularly in those receiving immunosuppressive therapy, compared to patients without medication and healthy controls. Previous studies have demonstrated reduced frequencies of $CD27^+$ memory B cells accompanied by increased proportions of naïve B cells[147, 148]; however, this contrasts with our results, in which naïve B cells were decreased in the patient cohort. This discrepancy may be influenced by medication or disease severity, given that AAV patients without treatment did not show a lower percentage of naïve B cells.

The ability to predict disease activity using biomarkers can be important for clinicians. Previous studies in AAV patients have suggested a correlation between the intermediate monocytes and relapsing disease[152], as well as an association between a low percentage of $CD5^+$ B cells and disease activity and relapse within short time after rituximab treatment[153]. Circulating plasma cells ($CD27^+CD38^{++}$ B cells) and plasmablasts are shown to be higher in GPA patients with future relapse[147]. It has also been shown that in other diseases such as SLE[154] and IgG4-related disease[155], the plasmablast frequency is related to the disease activity. We found an increased percentage of plasmablasts in AAV patients compared to HC; however, no difference was found between the active disease and remission groups or relapse and no relapsing groups.

Activated B cells express the Fas death receptor, so called CD95 and is a marker of B cell activation. We observed an expanded pool of activated $CD95^+$ B cells in AAV patients compared to HC. In a study of GPA patients, B-cell activation was associated with active disease and persistent T cell activation during remission[156]. In contrast to those findings, we did not observe differences of activated $CD95^+$ B cells between patients in active disease and those in remission. However, compared with patients not receiving medication and with healthy controls (HC), patients on medication showed higher percentage of total $CD95^+$ B cells. We observed that patients with tendency to relapse who were in remission had lower B cells frequency compared to the non-relapsing group, which may be attributable to treatment, as a high proportion of relapsing patients were receiving medication. Also, patients under immunosuppressive therapy showed decreased B cells compared to patients without medication or to healthy controls. No difference was found between non medication group and healthy controls. These findings are in line with previous studies, showing that prednisolone or cyclophosphamide therapy was correlated to lower B cell number[157, 158].

Patients with tendency to relapse showed increased percentage of $CD95^+$ exhausted memory B cells. This finding is in line with a study on SLE patients, indicating the impact of autoreactive cells in autoimmune disease[159].

The immunoglobulin levels were within reference range, except for the low IgA level in EGPA patients. The lower IgG level in patients with tendency to relapse compared to the non-relapsing group can been explained by the treatment, it has been reported the negative impact of the AAV treatment to the immunoglobulin level[160].

Paper II

Disease Activity and Tendency to Relapse in ANCA-Associated Vasculitis Are Reflected in Neutrophil and Intermediate Monocyte Frequencies

Background

Neutrophils, like B cells, play an important role in the pathogenesis of AAV, whereas monocytes have been less examined. In this study, we investigated the granulocyte and monocyte subsets in AAV patients and with particular focus on their relationship to disease activity and relapse risk, considering the effects of rituximab treatment.

Neutrophils can cause vessel injury upon ANCA activation. The activated neutrophils degranulate the continent of their granules, generate reactive oxygen species (ROS), activate the complement system, and liberate neutrophil extracellular traps (NETs). We studied three neutrophil subsets; the newly released neutrophils characterized of low expression of CD16 ($CD16^{dim}$), the mature neutrophils that full express CD16 ($CD16^{high}$) and the neutrophils expressing CD177[161], a receptor which binds membrane bound PR3 (mPR3) with high affinity. $CD16^{dim}$ neutrophils can reflect an increased mobilization of neutrophils from the bone marrow. The function of $CD177^+$ neutrophils is still unclear, but studies have shown that $CD177^+$ neutrophil express higher levels of mPR3[85].

Monocytes can infiltrate the vessels and mature in macrophages in the tissues. Monocytes express CD14 on their surface, a lipopolysaccharide receptor, and CD16, the FcγRIII receptor. Based on these two markers, the monocytes are classified in classical ($CD14^{++}CD16^-$), intermediate ($CD14^{++}CD16^+$) and non-classical ($CD14^-CD16^+$). The classical monocytes have antimicrobial and anti-inflammatory functions and phagocytic capacity. The intermediate monocytes have several functions, such as cytokine production, proliferation, and stimulation of T cells, and can act as antigen-presenting cells. The non-classical monocytes have

patrolling functions, can detect viruses and nucleic acid, and maintain the endothelial homeostasis.

In this study, we studied granulocytes and monocytes on the same patient cohort as in study I, including 105 GPA and MPA patients (GPA=68, MPA=37). Blood samples from one hundred and twenty-six healthy blood donors have been used as healthy controls. The clinical and laboratory parameters and the disease activity were obtained by clinical records, including the Birmingham Vasculitis Activity Scare 3, the tendency to relapse, ANCA specificity, white blood cell (WBC) count, C-reactive protein (CRP) and creatinine. The estimated glomerular filtration rate was calculated by the CKD-EPI creatinine equation.

The method for immunophenotyping characterization, including basophils, eosinophils, neutrophils and monocytes, is previously described in the method section and in detail in paper II. The target antigens are CD10, CD14, CD16, CD193, CD177, Siglec-8, and CD88.

Our hypothesis is that the immune system has a pivotal role in the maintenance of chronic inflammation in AAV. The aim of the study is to characterize monocytes and granulocytes in relation to disease activity and tendency to relapse.

Results

We first compared granulocytes and monocytes of AAV patients to healthy controls, founding increased frequencies of all studied neutrophil subsets. Total monocytes and intermediate monocytes also presented increased frequency, but no significant difference was found in eosinophils or basophils. Similar neutrophil results were obtained when subdividing the AAV cohort in MPA and GPA and comparing each one to healthy controls. MPA patients presented increased frequency of intermediate monocytes.

When we compared the GPA cohort to MPA cohort, we noticed a decreased frequency of classical monocytes in MPA patients. No significant difference was found for granulocytes, intermediate monocytes, and nonclassical monocytes. We did not observe any difference in any cell subset when we grouped the patients based on the ANCA serotype.

Samples from 23 repeatedly tested patients, with at least one sample from active disease and one sample from remission, have been used for paired analysis. We found increased concentration of mature $CD16^{high}$ neutrophils and decreased total monocytes and intermediate monocytes in active disease. Similar results were observed when we divided the patients in active disease and remission and compared each group to healthy controls, except for the lower frequency of $CD177^{+}$ neutrophils in patients with active disease.

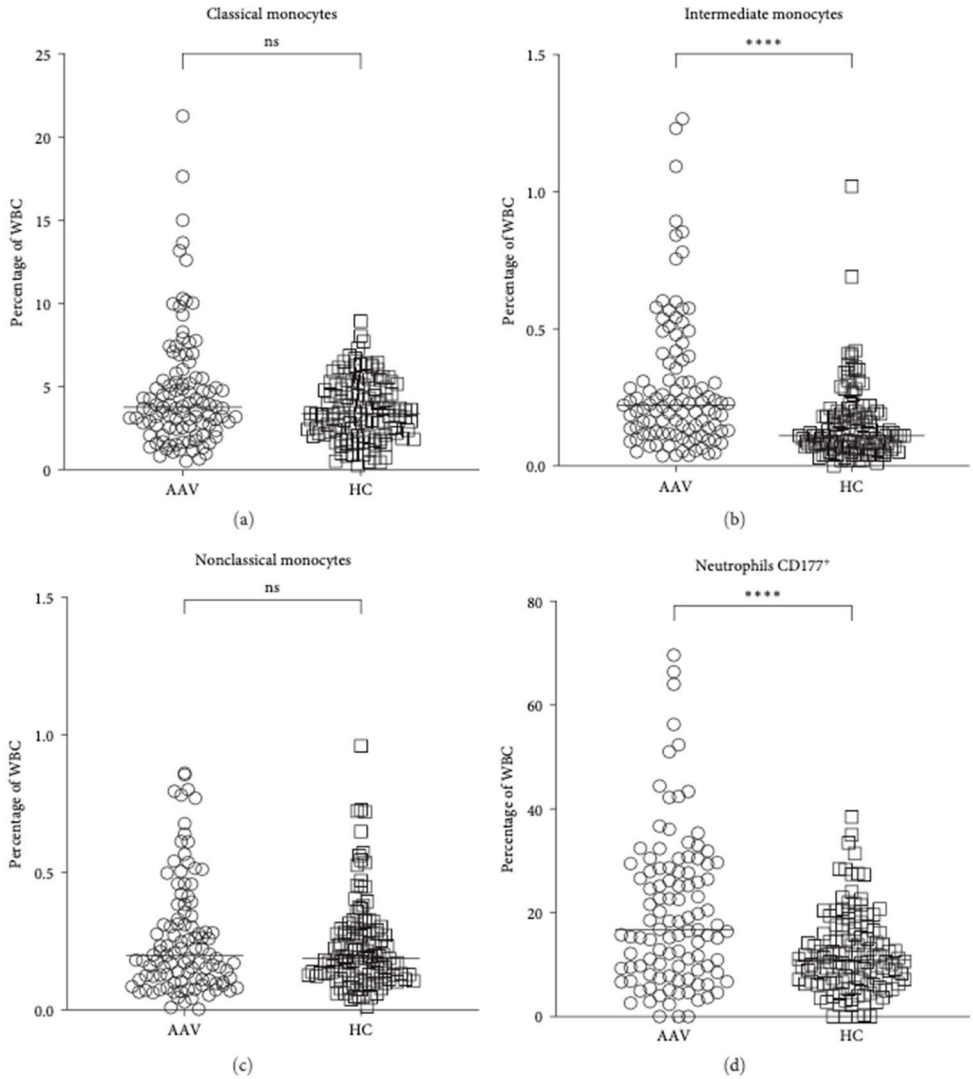

Figure 13. Increased frequency of intermediate monocytes in AAV patients compared to healthy controls. The frequencies of (a) classical monocytes ($CD14^{++}CD16^{-}$), (b) intermediate ($CD14^{++}CD16^{+}$), (c) nonclassical ($CD14^{-}CD16^{-}$) monocytes, and. (d) neutrophils $CD177^{+}$ in AAV patients and HC. AAV patients present a higher frequency of intermediate monocytes and $CD177^{+}$ neutrophils, but not of classical and nonclassical compared to HC **** indicates *p*-value <0,0001; ns, not significant. Reprinted from paper II, figure 1.

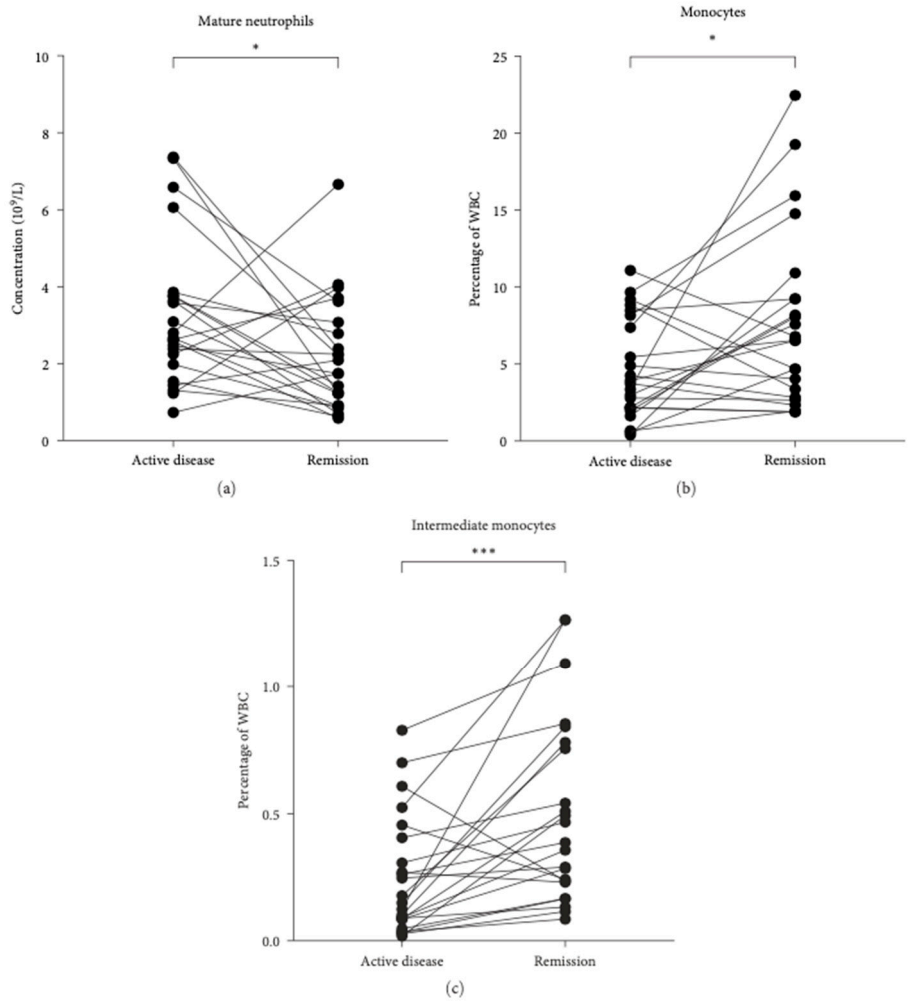

Figure 14. Comparisons of (a) mature ($CD16^{high}$) neutrophils, and frequencies of (b) total monocytes, and (c) intermediate ($CD14^{++}CD16^{+}$) monocytes in 23 patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) in active disease and in remission. Patients with active disease present higher concentrations of mature neutrophils, but lower frequencies of total and intermediate monocytes compared to patients in remission. Reprinted from paper II, figure 3.

Subgrouping the AAV cohort into patients with and without a tendency to relapse, followed by comparative analysis, revealed a lower frequency of eosinophils and higher frequencies of $CD177^{+}$ neutrophils and mature $CD16^{high}$ neutrophils in the relapsing group. Further subdivision of the relapsing group in MPA and GPA highlighted that GPA patients prone to relapse had increased frequency of $CD177^{+}$ neutrophil, while the relapsing MPA patients had reduced frequency of intermediate monocytes.

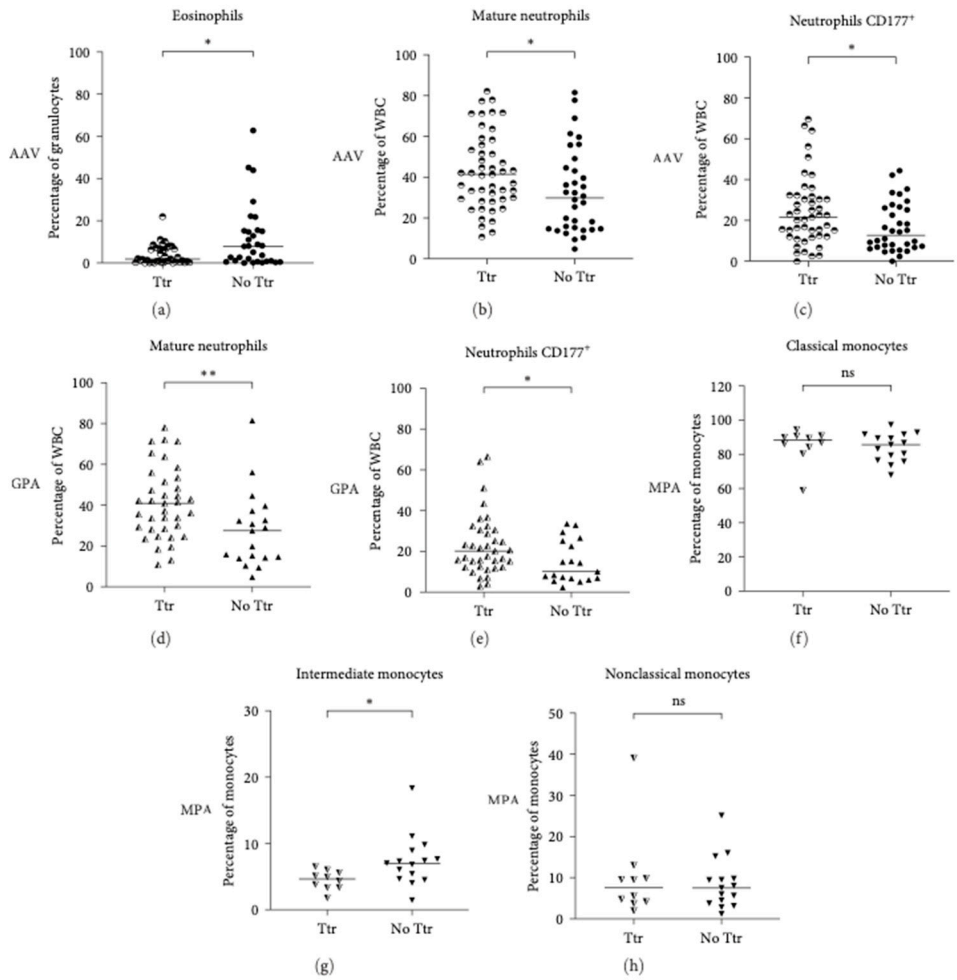

Figure 15. Comparison of granulocyte and monocyte frequencies in patients with and without a tendency to relapse (Ttr). Frequencies of (a) eosinophils, (b) mature $CD16^{high}$ and (c) $CD177^{+}$ neutrophils, in AAV patients with Ttr or No Ttr, where patients with Ttr presented decreased frequency of eosinophils, but increased frequency of $CD177^{+}$ neutrophils. Analysis of cell subsets in GPA patients with and without Ttr demonstrated statistically significant increased frequencies of (d) mature $CD16^{high}$ and (e) $CD177^{+}$ neutrophils. The investigation of the cel subsets in MPA patients with Ttr and without Ttr identified decreased frequency of (g) intermediate ($CD14^{++}CD16^{+}$) monocytes but no statistically significant difference of (f) classical ($CD14^{++}CD16^{-}$), and (h) non-classical ($CD14^{-}CD16^{+}$) monocytes. * and ** indicate *p*-value <0.05 and <0.01, respectively. Reprinted from paper II, figure 4.

Finally, we stratified the AAV cohort into patients treated with rituximab and those who were not receiving the therapy. Comparison between the groups revealed higher frequencies of intermediate and classical monocytes in patients on rituximab.

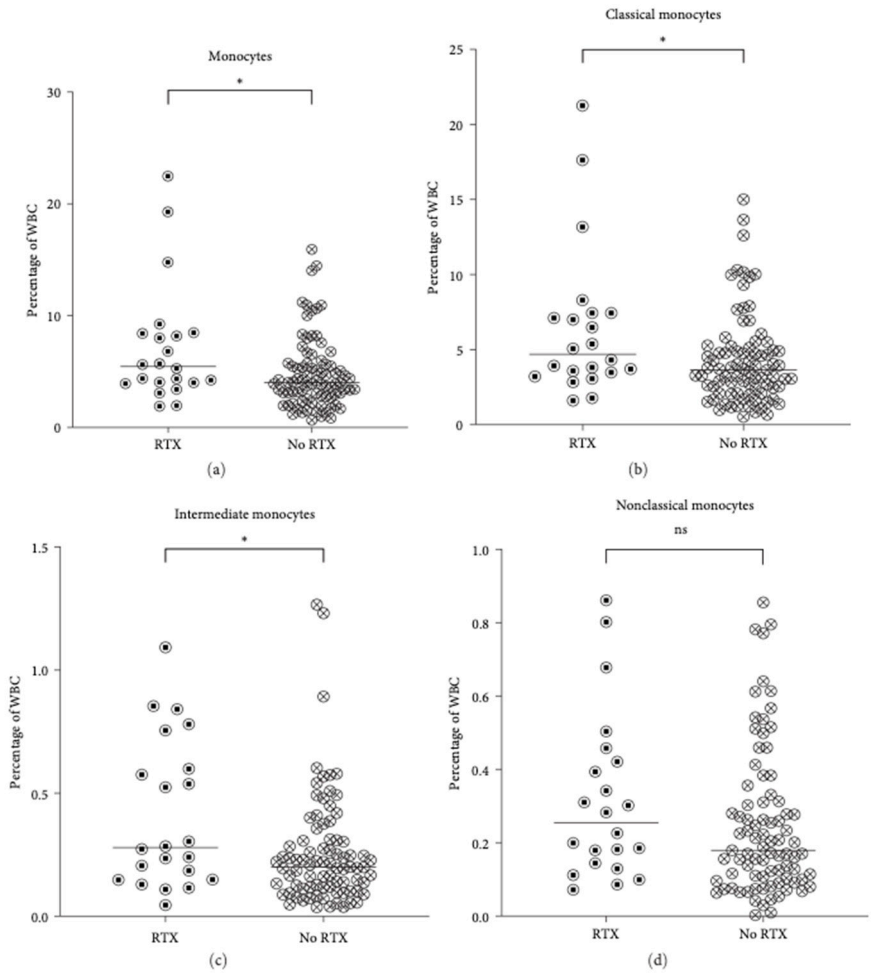

Figure 16. Increased monocyte frequencies in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) patients with rituximab treatment during the past 365 days. The frequencies of (a) total monocytes, (b) classical (CD14++CD16-), (c) intermediate (CD14++CD16+) and (d) non-classical (CD14-CD16+) monocytes, in patients with RTX or No RTX treatment. Total, classical, and intermediate monocytes were presented with higher frequency in patients in RTX treatment. No significant difference was observed in the comparison of nonclassical monocytes between the two groups. * Indicates p-value <0.05, ns, not significant. Reprinted from paper I, figure 5.

Discussion

Neutrophils and their role in the pathogenesis of AAV have been widely studied. Monocytes have attracted interest in the last years, as a distinct monocyte subset- the intermediate- is reported to be associated with inflammatory conditions[13, 162-164]. Here, we report alterations in the repertoire of neutrophils and monocytes

considering disease activity, the disease subtype, the relapse tendency, and the rituximab treatment.

In this study, the AAV patients exhibited higher frequencies of total monocytes and intermediate monocytes compared to healthy controls. Despite this, patients in active disease presented decreased frequencies of total monocytes and intermediate monocytes, which may be caused by the monocyte recruitment into the site of inflammation. Similar results were observed in MPA patients with tendency to relapse suggesting the presence of low-grade inflammation, which is consistent with findings reported in studies of lupus patients[165]. Although the increase of intermediate monocytes has been reported in other studies, changes in total monocyte number, have not been consistently observed, which may reflect differences in study design, treatment, cohort size and relapse propensity[152, 166].

Neutrophils that express CD177 co-express PR3[85] on the cell surface and this has been associated with increased disease activity and poor outcome[167-169]. We observed increased frequency of $CD177^+$ neutrophils and classical monocytes with inflammatory potential in GPA patients with tendency to relapse that could not be explained by elevated disease activity.

No difference was observed between the two ANCA serotypes, MPO and PR3, which are genetically associated with different HLA class II genes[89, 90]. This may be explained by the fact that the clinical phenotype is more dependent on the underlying chronic inflammation.

In this study, we included patients who had received rituximab within the past twelve months prior to sampling. Rituximab, as a monoclonal anti-CD20 antibody, is directed against B cells and lead to cytotoxic cell killing or apoptosis of B cells. Monocytes/macrophages are active actors in the antibody-dependent cytotoxicity which might make changes in their phenotype. The RITUXVAS and RAVE studies[158, 170] demonstrated the superior efficacy of RTX compared with standard therapy for induction of remission. The mechanism of action is not completely understood. B cell depletion may lead to secondary effects, as B cells regulate other cells, have functions as antigen presenting cells, and produce cytokines[171]. It seems that rituximab changes the cytokine profile in patients, such as B-cell activating factor, IL-10[171] and IL-15[172], which may affect monocyte differentiation and phenotype. It has also been suggested that rituximab-IgG complexes formed on the B-cell surface generate immune complexes that attract effector cells as monocytes/macrophages and neutrophils, thereby acting as a decoy. This may lead to a blockade of effector cell recruitment at the site of immune complex deposition with a consequent reduction of inflammation[173]. We showed that patients on RTX treatment had higher frequencies of classical and intermediate monocytes, which might be a secondary effect of B cell depletion.

Paper III

Monocyte and Granulocyte Function and Susceptibility to Infections in AAV Patients Under Rituximab Treatment

Background

Infections in ANCA-associated vasculitis are implicated both in the pathogenesis and as complications, contributing to increased morbidity and mortality. In paper II, we demonstrated the skewed subsets of monocytes and neutrophils in patients with AAV. Our group has previously shown that circulating neutrophil numbers are elevated in AAV patients, particularly during the active disease, while their phagocytic function and ROS production are reduced[174].

Phagocytosis and the reactive oxygen production of neutrophils and monocytes are essential for the first-line defence against various microbes. On the other hand, monocytes and neutrophils are involved in the pathogenesis of AAV and are found in and around inflamed vessel walls[175]. ANCAs that can activate neutrophils and monocytes/macrophages are found in the vascular infiltrates of the affected organs in patients with AAV[176].

Here, we investigated functions of monocytes and neutrophils in GPA and MPA patients considering rituximab treatment status and susceptibility to infections.

We included 50 GPA patients and 32 MPA patients with at least one infection episode during the sampling period. Of these 82 AAV patients, repeated testing was available for 59 patients; 33 individuals experienced at least one relapse, and 16 patients were without any treatment.

Clinical and laboratory parameters were acquired by clinical records and included the disease activity defined by the Birmingham Vasculitis Activity Scare 3 (BVAS3), the tendency to relapse, ANCA specificity, white blood cell (WBC) count, C-reactive protein (CRP) and creatinine. The estimated glomerular filtration (eGFR) rate was calculated by the CKD-EPI creatinine equation.

The infections were classified according to the Common Terminology Criteria for Adverse Events (CTCAE)[177] as follow: grade 1 for the upper respiratory infections, like common cold and grade 2 for urinary tract infections, pneumonia, Herpes Zoster, Erysipelas, nasal cavity infection, dental infection and fungal oral infection. Most infections required local or oral intervention. Twenty-two patients had moderate infection as defined above, and 19 of them did not have any antibacterial treatment at the time of sampling. Patients with severe infections required hospitalization were not included in this study.

The two methods, Phagoburst™ and Phagotest™, used to measure the oxidative burst and phagocytotic capacity, are described in the methods section and more in detail in the manuscript.

The aim of this study is to investigate the granulocyte and monocyte function in AAV patients with or without RTX, the susceptibility to infections, and the immune cell response.

Results

As first we divided the AAV patients based on the ANCA-serotype and then in disease type. We found that PMNs have decreased ROS production in the MPO group compared to the PR3 group after both phorbol 12-myristate 13-acetate (PMA) or *E.coli* stimulation, but no differences in phagocytic capacity were observed. In addition, the percentage of ROS producing monocytes was lower in MPO patients after PMA stimulation. When stratifying by GPA and MPA, we did not observe any differences in ROS production or phagocytic function of PMNs and monocytes between the two groups.

Rituximab is an anti-CD20 monoclonal antibody, commonly used for the treatment of several autoimmune diseases. The mechanism of action is depletion of B-cells with drastic decrease of B-cells, however effects on PMNs like e.g. neutropenia are reported[133, 178-180]. Here, we wanted to investigate if phagocytosis and ROS production are affected by rituximab. We further stratified the AAV patients into those receiving rituximab (RTX) treatment and those not treated with RTX and compared the groups. We found that the amount of ROS production in PMN was decreased in the RTX group after stimulation with PMA or *E.Coli.* No differences in the phagocytosis function were observed between the two groups. In contrast, monocytes from the RTX group exhibited increased phagocytic capacity after activation with opsonized *E. Coli* bacteria, while no differences in ROS production were detected.

To assess whether infections influence phagocytic function and ROS production, we selected patients who had an infection at the time of sampling. Samples during an infection were compared with the latest sample before the infection. ROS production in PMNs tended to be reduced, whereas no consistent pattern was observed in monocytes.

To investigate the potential impact of infections on our results, we excluded patients with infections, grouped the remaining patients by ANCA-serotype, and compared the two groups. MPO patients had decreased capacity of ROS production after stimulation with *E.Coli* and PMA. In addition, the number of monocytes after PMA stimulation decreased in MPO patients. Similar results about monocytes were obtained when dividing non-infection patients in RTX and No RTX groups.

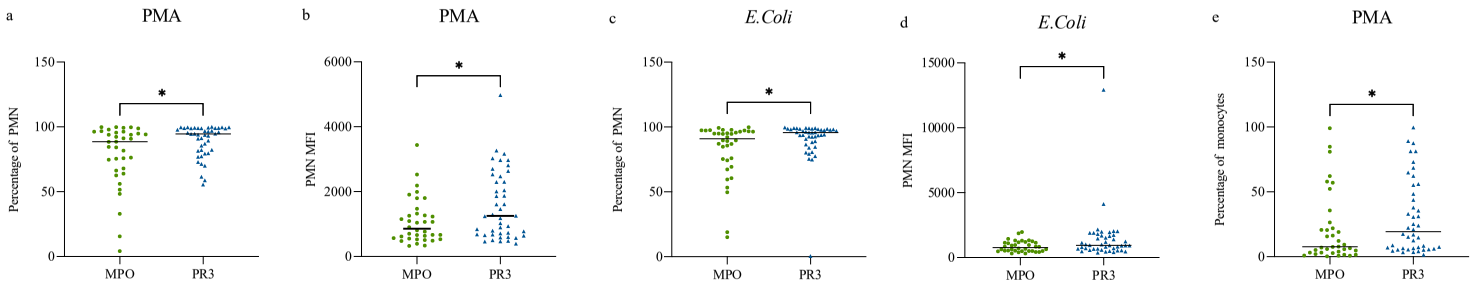

Figure 17. ROS production in MPO and PR3 positive AAV patients. Decreased ROS production in MPO-ANCA positive patients compared to PR3-ANCA positive patients. MPO-ANCA patients present lower frequencies of ROS producing cells, such as PMN and monocytes and decreased ROS production after PMA and *E.Coli* stimulation compared to PR3-ANCA patients. *Indicates *p*-value ≤0,03.

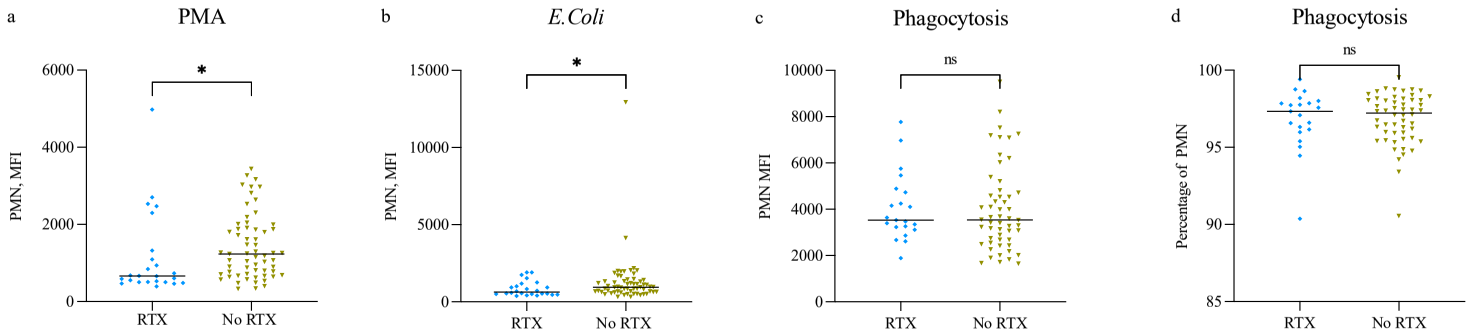

Figure 18. ROS production and phagocytic function in patients on RTX treatment. ROS production in patients with rituximab treatment was reduced in comparison to patients on other treatment, after stimulation of PMNs with PMA (fig. a , $p = 0.02$) and *E. coli* (fig. b, $p < 0.05$). Regarding the phagocyte function, no differences were observed between the two groups (fig. c and d). *Indicates $p>0.05$; ns, not significant.

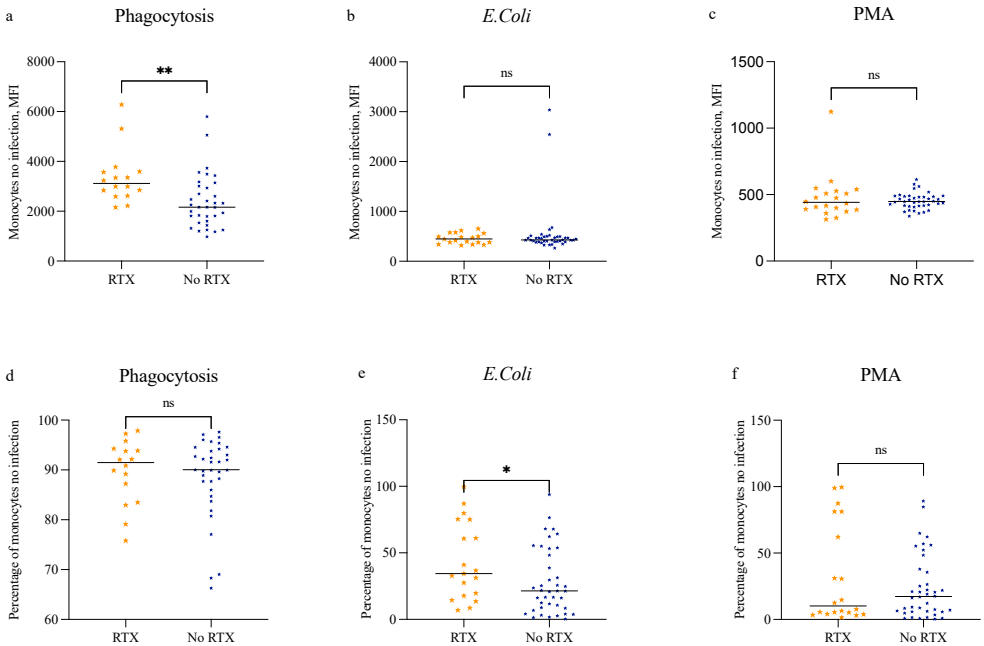

Figure 19. Monocytes in AAV patients without infection, with or without rituximab treatment. Monocytes in AAV patients without ongoing infection and under rituximab treatment presented significantly higher phagocytic function (fig. a, p=0,001) compared to patients on other treatment, but similar number of cells (fig. d). After *E.Coli* stimulation RTX patients exhibited increased ROS producing cells (fig. e, p=0,03), but comparable ROS production (fig. b). Monocytes of the same group stimulated with PMA had no significant difference on ROS production and ROS producing cells (fig. c and f respectively) compared to the patients without rituximab treatment. *Indicates p-value=0,03, ** indicates p-value =0,01; ns, not significant.

Discussion

Several studies have suggested a classification of ANCA vasculitis based on the ANCA-serotype, describing the MPO-positive AAV and the PR3-positive AAV as distinct diseases with different genetic phenotypes, severity grade, organ damage and disease activity[181-185]. We observed lower ROS production in MPO-positive patients compared with PR3-positive patients. This finding contrasts with our previous study, which included patients with EGPA[186]. The inclusion of EGPA patients may have influenced the results, as EGPA is characterized by eosinophilia and MPO-positivity is present in only a small subset of these patients[91].

Rituximab treatment affects directly the B cells by several mechanisms e.g. B-cell apoptosis, complement mediated lysis and cytotoxicity and is also considered to have an indirect anti-inflammatory effect. B cells are antigen-presenting cells (APCs), they produce antibodies and secrete cytokines[187]. Depletion of B cells may

attenuate this immunological effect and potentially modulate PMN and monocyte function.

Patients receiving RTX treatment exhibit enhanced monocyte phagocytic capacity and impaired granulocyte ROS production, compared with patients not treated with RTX. These findings are partially consistent with studies in other diseases, such as multiple sclerosis (MS) and neuromyelitis optica spectrum disorders (NMOSD), treated with anti-CD20 antibodies, in which phagocytosis was restored while ROS production was not affected[179]. The discrepancy in ROS may be attributable to differences in study methodology. The enhanced monocyte phagocytic capacity may be related to findings from our previous study[188], where an increased frequency of classical monocyte was observed in patients receiving RTX treatment.

The granulocyte ROS production appeared to be decreased during infections in AAV patients, while monocytes were not affected. In this study, most infections were classified as grade 2 and severe infection was not included, which may affect the results. The decreased ROS production may contribute to impaired pathogen clearance and increased susceptibility to severe infections, whereas phagocytic capacity was not affected. This impairment may be associated with RTX treatment. We could not find any difference between infected and non-infected AAV patients. However, after excluding samples obtained during infection, impaired ROS production in PMNs became evident, suggesting that active infections may obscure underlying immune dysfunctions.

Conclusions

Paper I

We observed alterations in the total B cell number and in several B cell subsets in individuals with GPA, MPA, and when considering disease activity and relapse stratus, in patients who have not received anti-CD20 treatment. As ANCA-associated vasculitis is a complex and multifactorial disease, we suggest that these alterations may influence the balance between regulatory and effector function of B-cell functions and thereby contribute to the autoantibody production, disease activity and the risk for relapse.

Paper II

In this study, we noticed changes in neutrophil and monocyte subpopulations that were associated with disease phenotype, disease activity, and the tendency to relapse, but not with ANCA subtype. These results suggest a correlation between to the underlying chronic inflammation and clinical features. Rituximab treatment may also contribute to the skewing monocyte subsets. Altogether, these findings may be useful as potential biomarkers of relapse prediction.

Paper III

In this work we report alterations in the capacity of PMNs and monocytes to generate reactive oxygen species and in the phagocytic function, that seems to be associated with ANCA subtype, rituximab treatment, and infections. Our findings indicate that phagocytic dysfunction is related to susceptibility to infections. This dysfunction may be influenced by rituximab treatment, as rituximab has been suggested to indirectly affect myeloid cell function.

Future perspectives

The three studies included in this doctoral thesis have certain limitations. Further investigations aimed at partially or totally addressing these limitations are an important direction for future research. Since the original AAV patient cohort was the same across the three studies, the following joint considerations for future work are reported.

One important parameter is the immunosuppressive treatment. Most AAV patients receive medication at the time of sampling, which differs between the patients and depends on several parameters, such as disease activity or relapsing disease. Our cohort included patients with different types of treatment and in different doses, as well as patients without treatment. More homogenous cohorts, stratified by medication, may provide a clearer understanding of the pharmacological impact of each drug on immune cell subsets and functions, infection risk and disease outcomes.

Our studies were limited by the timing of blood sample collection, which was performed during routine clinical visits. This should be considered in the design of future studies.

Another parameter is the healthy controls, that were not age-and gender- matched to the patient cohort. This is important for minimizing bias related to age and gender. AAV are rare diseases, and the comparison of the AAV cohort with disease controls may be necessary for further and more specific investigations. Disease controls share distinct features with the study cohort, reducing bias from the common characteristics.

It has been widely suggested that rituximab treatment also affects immune cells, other than B cells. The use of rituximab, both as induction therapy and maintenance therapy, is increasing. Further investigations are required to gain a better understanding of the mechanism of action and to explore potential novel interventions involving other immune cells populations.

From the studies included in this thesis and other research, highlights the important role of immune cells in the pathogenesis and the clinical outcome of ANCA-associated vasculitis. At present, we do not have a clear and complete picture of the alterations in immune cells involved in AAV, which warrants further investigations.

ANCA-associated vasculitis encompasses rare diseases with complex and multi-factorial pathogenesis, carrying a high risk for severe morbidity and mortality. Current knowledge remains limited, and further research is needed to improve treatment, enhance patient quality of life, and reduce the social-economic impact on society.

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