UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Zentrum für Diagnostik, Institut für Klinische Chemie und Laboratoriumsmedizin

Institutsdirektor

Professor Dr. Dr. Thomas Renné

Characterization of NETotic neutrophils as a novel tool for autoimmune disease diagnostics.

Dissertation

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Luke Sydney Hopf
aus Hamburg

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Prüfungsausschuss, zweite Gutachterin: Prof. Dr. Ina Kötter

1. Introduction

1.1 Neutrophil granulocytes are the front line of the immune system

Neutrophil granulocytes are the most common and arguably the most important front-line immune cell of the human body. They represent a crucial part of the innate immune system capable of important functions in protecting the body from foreign intruders.
Neutrophil granulocytes (PMN, neutrophils) form in the bone marrow under the influence of a vast number of cytokines and growth factors from pluripotent hematopoietic stem cells, which differentiate into myeloblasts (Borregaard 2010, Dexter 1990). This cell population grows on to be differentiated either into monocytes or granulocytes (Akashi et al. 2000). Production of neutrophils is quantitatively speaking the major activity of the marrow, devoting nearly two–thirds of the blood cell forming capacity into myelopoesis, resulting in a production of 1-2x10^{11} neutrophils every day in an adult (Borregaard 2010). However, neutrophils only circulate for approximately 8 hours and are among the shortest-lived cells in the human body (Kolaczkowska and Kubes 2013), although studies have shown that neutrophils can live up to 5.4 days in humans (Pillay et al. 2010).
The production of neutrophils however can be regulated upwards considerably if the organism is presented with an infection or stress. Important factors for this stimulation include granulocyte-colony stimulating factor (GCSF) (Lieschke et al. 1994), interleukin 23 (IL-23) and interleukin 17 (IL-17), which is regulated by the rate of apoptosis of neutrophils in tissues (Stark et al. 2005). As the maturing cell changes its transcriptional program during its differentiation, granule content also changes (Amulic et al. 2012).

Three types of granulocytes can be differentiated depending on their specific dye. Neutrophilic, eosinophil and basophil granulocytes are differentiated in an advanced blood count. Eosinophil granulocytes are mostly associated with parasite infections and allergic response (Eng and Defelice 2016), basophil granulocytes are, together with mast cells, typical innate effector cells of allergen-induced immunoglobulin E (IgE) dependent allergy disease (Sarfati et al. 2015). However, compared to neutrophil granulocytes, which make up to 50-70 % of leucocytes in humans (Mestas and Hughes

2004), both of the other cell types are very rare in the peripheral blood and only make up a small amount of the entire granulocytes of the human body.

The differentiation from myeloblast to neutrophil granulocyte is accompanied by the synthesis of proteins which are sorted into different granules. Traditionally, granules are divided into three different categories based on cargo molecules: azurophilic, specific and gelatinase granules, also referred to as primary, secondary and tertiary granules (Borregaard 1997). The content of the granules is influenced by the transcriptional program of the cell (Borregaard and Cowland 1997). The release of neutrophils from the bone marrow is highly regulated by chemokines, like stromal cell-derived factor 1 (SDF1) (Eash et al. 2010).

Mature neutrophils play a vital role in immunity against bacterial and fungal pathogens (Mocsai 2013). Also, recent studies have indicated that neutrophils play a role in the immunity against viral infections such as human immunodeficiency virus-1 (HIV-1) infection (Saitoh et al. 2012).

For all of these purposes, neutrophils have a wide array of weapons such as phagocytosis (Nordenfelt and Tapper 2011), degranulation which releases bactericidal cargo (Borregaard et al. 2007), antimicrobial proteins and reactive oxygen species (ROS) (Leto and Geiszt 2006) and the formation of neutrophil extracellular traps (NETs), a special form of apoptosis which creates a net-like structure trapping and destroying pathogens (Fuchs et al. 2007). However, these weapons can be just as harmful to the host cells as to the invading bacteria. Therefore, the release of neutrophil granular content is strictly regulated (Sengelov et al. 1995, Sengelov et al. 1993).

Neutrophil activation is triggered through stimulants such as lipopolysaccharide (LPS) and cytokines like tumor necrosis factor α (TNF-α), interleukin (IL)-1β and IL-17 (Amulic et al. 2012). These mediators prompt endothelial cells to produce adhesion molecules which circulating neutrophils recognize and signals them to a site of infection (Vestweber 2007). Through the engagement of P-selectin glycoprotein ligand (PSGL)-1 and L-selectin a cascade of kinases are activated, leading to the selectin-mediated rolling of neutrophils on the endothelial cells (Yago et al. 2010, Mueller et al. 2010, Ley et al. 2007). Once the neutrophil is at the sight of infection, following the chemokine

gradient, it activates its arsenal of antimicrobial weapons. This neutrophil activation is initiated by an oxidative burst, which results in degranulation and, for example, the formation of neutrophil extracellular traps (Goldfinger et al. 2003, Xu et al. 2003).

1.1.1 Neutrophils are linked to autoimmune diseases

Increasing evidence seems to suggest, neutrophils are both targets and effectors of inflammation in autoimmune diseases (Hu et al. 2011). While some studies have shown that myeloperoxidase (MPO) and proteinase 3 (PR3) titers correlate with disease activity (Stegeman 2002), it still remains an open debate, with other studies presenting contrary findings (Finkielman et al. 2007).

The pathogenesis of anti-neutrophil cytoplasmic antibodies (ANCA), associated vasculitis (AAV) and the role of ANCA in it remain unclear. Studies show that ANCA induces glomerulonephritis and vasculitis in mice (Xiao et al. 2002). Also, ANCA activate neutrophils by inducing a respiratory burst, degranulation as well as NETosis (Falk et al. 1990, Kessenbrock et al. 2009). Importantly, the effectiveness of anti-CD20 B-cell targeting treatment using rituximab in treating Granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA) has shown non-inferior to other treatments in induction and maintenance therapy (Stone et al. 2010, Guillevin et al. 2014). This indicates that the associated antibodies are a major contributor to the disease activity.

The loss of tolerance which leads to AAV is still very poorly understood. There are several models being discussed currently. The “Complementary Peptide Model” postulates that there is an immune response against peptides derived from transcription and translation of the antisense DNA strand encoding for PR3 and MPO (Jennette and Falk 2014, Jennette et al. 2011). Recently, this model has been expanded towards dysregulation in the post-transcriptional process of ANCA related mRNA (Mcinnis et al. 2015). Another model being discussed is the molecular mimicry model, proposing that ANCA production occurs after an infection with for example Staphylococcus aureus (Zycinska et al. 2008, Stegeman et al. 1994).

Also, NETs and NETosis have also been discussed as a potential source of ANCA generation. Dendritic cells take up MPO presented on NETs which was followed by induction of anti MPO-ANCA (Sangaletti et al. 2012). However, the major histocompatibility complex (MHC) II-presentation of MPO or PR3 on antigen presenting cells remains unconfirmed.

1.2 Neutrophils produce neutrophil extracellular traps through a cell death program called NETosis

In 2004 the generation of NETs (NETosis) was discovered and classified as an additional form of cell death next to apoptosis and necrosis (Fuchs et al. 2007, Brinkmann et al. 2004). The neutrophil undergoes major morphological changes in the process of NETosis (Brinkmann et al. 2004). The major necessity for the induction of NETosis is the production of reactive oxygen species (ROS) (Fuchs et al. 2007). In neutrophils, ROS are created by a Nicotinamide adenine dinucleotide phosphate-oxidase complex (NADPH), creating a so-called oxidative burst (Fuchs et al. 2007, Steinberg and Grinstein 2007). An additional enzyme which is required for the formation of NETs is myeloperoxidase (MPO) (Metzler et al. 2011). If these mandatory enzymes are defective, NETosis can be diminished, which results in immune defect syndromes like chronic granulomatosis disease (Segal et al. 2000) or severe aspergillosis (Bianchi et al. 2009).

NETosis is proposed to be induced by microbes (such as bacteria, viruses and fungi) interacting with cell surface receptors of neutrophils (Brinkmann et al. 2004, Urban et al. 2006). Toll-like receptors are activated by bacterial molecules such as lipopolysaccharide (LPS) which then could initiate a signal cascade downstream inducing NETosis (Clark et al. 2007). This stimulus leads to the activation of the Raf/MERK/ERK pathway, including the activation of protein kinase C (PKC), NADPH-oxidase complex and extracellular signal activated kinase 1/2 (ERK 1/2) (Hakkim et al. 2011). ERK 1/2 leads to the activation of the NADPH-oxidase complex which in itself results in a so-called oxidative burst (Hakkim et al. 2011). Chromatin decondensation, nuclear envelope breakdown and the mixing of chromatin with granular und cytoplasmic content follows. Finally, the cell membrane breaks, and the extracellular structures composed of chromatin and granule proteins that bind and kill microorganisms are released (Fuchs et al. 2007).

The ultrastructure of NETs consists of a framework of chromatin filaments about 15-17nm in diameter (Brinkmann et al. 2004). Chromatin filaments are dotted with globular structures of about 50nm in diameter (Brinkmann and Zychlinsky 2007). The composition of these chromatin filaments consists of mainly Deoxyribonucleic acid

(DNA) and histones, with histones making up to 70 % of the total proteins of the traps (Pinegin et al. 2015). The globular structures consist of primary and secondary granules of neutrophils, e.g. neutrophil elastase (NE), MPO, cathepsin G and bactericidal proteins (Averhoff et al. 2008, Parker et al. 2012, Bianchi et al. 2011).

NETs are vacated by serum Desoxyribunuclease I (DNase1) (Hakkim et al. 2010). This is a calcium dependent ribonuclease which preferably attacks double stranded DNA, producing oligonucleotides (Fujihara et al. 2012). If the activity of this enzyme is reduced, associations with autoimmune diseases such as Systemic lupus erythematosus (SLE) were observed (Hakkim et al. 2010).

Functionally, NETs are designed as an extracellular killing mechanism for foreign pathogens (Brinkmann et al. 2004). Both gram-positive (e.g. Staphylococcus aureus (Brinkmann et al. 2004), group A Streptococcus (Buchanan et al. 2006), Streptococcus pneumoniae (Wartha et al. 2007)) and gram-negative pathogens (e.g. Shigella flexneri, Salmonella typhimurium (Brinkmann et al. 2004)) as well as fungi (e.g. Candida albicans and yeast (Urban et al. 2006)) and viruses (Saitoh et al. 2012) are susceptible to NETs. One of the main differences in antimicrobial mechanisms between phagocytosis and NETosis is the addition of nuclear components in NETs. DNA binding proteins like histones and histone fragments have very potent antimicrobial activity (Park et al. 2000, Kim et al. 2000).

1.3 NETs can be linked to autoimmune diseases

NETs have recently been associated with autoimmune diseases (Pinegin et al. 2015, Lee et al. 2017). The linkage between NETs and autoimmunity is twofold: firstly, NETosis exposes nuclear components to the extracellular space combined with the possible generation of neo-autoantigens by protein-modifications due to, for example, ROS (NADPH-oxidase/MPO) or histone deamination through Protein Arginine Deiminase-4 (PAD-4) (Dwivedi and Radic 2014); secondly, the degradation of NETs by plasma DNase1 is reduced in autoimmune patients (Hakkim et al. 2010).

Publications are increasingly linking NETs to autoimmune conditions. NETting neutrophils induce plasmacytoid dendritic cells (Lande et al. 2011) and have been shown to induce endothelial damage (Villanueva et al. 2011).

Increasing associations between NETosis and SLE have been made recently. The combination of NETosis and reduced NET degradation results in elevated interferon-1 (IF-1) production and endothelial damage (Lood et al. 2016). Patients who suffer from SLE have a decreased NET degradation due to antibodies, which block the binding sites of the degrading enzyme which is DNase1 and additionally, SLE patients show a decreased activity of DNase1 in general (Hakkim et al. 2010). This results in the externalization of autoantigens, which supposedly triggers autoimmunity (Knight et al. 2015). Circulating chromatin-containing apoptotic material, which is elevated in SLE patients, also contributes to the activation of dendritic cells, which induce NETosis in neutrophils (Dieker et al. 2016), supposedly adding to the vicious cycle in the pathogenesis of the disease. NETs are a potent stimulus for the release of IFN-α, which is believed to lead to the lupus phenotype, and that endothelial damage in lupus patients is mainly caused by uncontrolled NETosis (Knight and Kaplan 2012).

NETs have also been associated with autoimmune small vessel vasculitis (SVV). SVV is a group of systemic autoimmune diseases of unknown etiology which result in necrotizing flares of inflammation of the small blood vessels. The majority of SVV patients have antibodies against neutrophil components, termed anti-neutrophil cytoplasmic antibodies (ANCA). ANCA can induce NETosis effectively, which could explain the pathogenesis of these diseases, though this is currently still unclear (Chen and Kallenberg 2009). Also, myeloid dendritic cells loaded with NET components induce ANCA and autoimmunity (Sangaletti et al. 2012). Patients with SVV have been shown to have increased DNA-MPO complexes in their circulation, which could presumably originate from NETosis (Kessenbrock et al. 2009). Furthermore, a link between the adaptive immune system and NETs has been established. NET proteins were preferentially uploaded into myeloid dendritic cells in vitro. This could be prevented with the addition of DNase, the enzyme, which degrades NETs (Sangaletti et al. 2012).

Rheumatoid arthritis (RA) and Felty's Syndrome patients also display several associations regarding NETs. Autoantibodies against citrullinated proteins play a key role in the pathogenesis of the disease (Valesini et al. 2015). These citrullinated proteins are often found in NETs (e.g. nuclear proteins like histones) (Khandpur et al. 2013). In addition, these patients show an increased number of circulating antibodies against PAD-4 deiminated histones, which are also components of NETs (Spengler et al. 2015). These PAD-4 autoantibodies bind to activated neutrophils and NETs (Dwivedi et al. 2012). Inhibiting PAD-4 disrupts NET formation and leads to decreased kidney, skin and vascular disease in lupus prone mice (Knight et al. 2014).

Furthermore, NETs are considered to play a role in the pathogenesis of psoriasis (Hu et al. 2016), antiphospholipid syndrome (Yalavarthi et al. 2015), gout (Mitroulis et al. 2011), dermatomyositis and polymyositis (Zhang et al. 2014).

Considering that the pathogenesis of many autoimmune diseases is still poorly understood, NETs and NETosis could help us understand diseases like SVV, SLE, RA and many other autoimmune and inflammatory diseases.

It is often postulated that neutrophils, and especially NETs, present modified proteins externally, which may trigger loss of immune tolerance and promotion of organ damage.

The diagnostic value of these findings has not been tested in prospective study. Furthermore, no diagnostic assay based on NETs is available. Instead, quantification of anti-nuclear antibodies (ANA) or ANCA uses fixed cells including unstimulated neutrophils as a substrate.

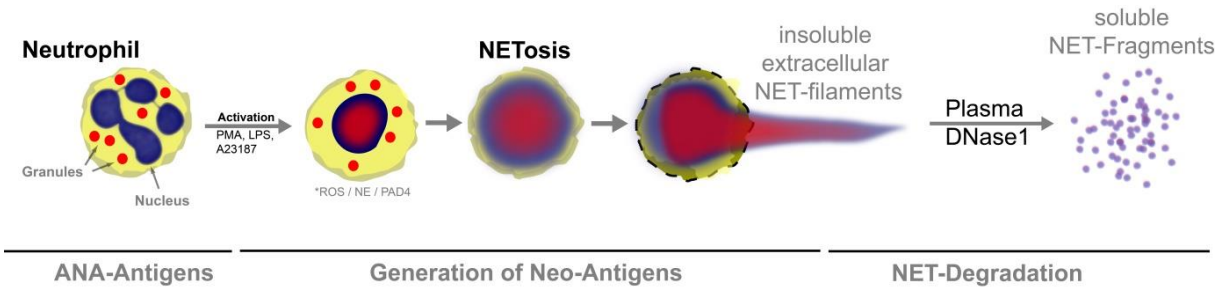

Figure 1: Exemplary illustration of a neutrophil undergoing NETosis. After activation through for example Phorbol 12-myristate 13-acetate (PMA) the nucleus decondensates and reactive oxygen species form. The granules and nuclear structures merge and finally the insoluble NET-filaments are released into the extracellular space after membrane disintegration. Afterwards plasma DNase1 degrades NETs to soluble fragments.

1.3.1 Neo-epitopes are generated through protein modification and post-translational modification which are present in NETosis

The stimuli for the generation of autoantibodies is still poorly understood in systemic autoimmune diseases (Radic et al. 2011). However, post-translational modification has been a target for research in understanding the loss of immune tolerance and the origin of autoimmunity (Doyle and Mamula 2005, 2002). It is mainly believed that post-translational modification leads to the generation of neo-epitopes, which are new binding sites for B- or T-cell antigen receptors, which leads to alteration in antigen processing and presentation (Ohmori and Kanayama 2005). Histones have been shown to be the target of certain autoantibodies in specific autoimmune diseases such as SLE and RA (Dieker and Muller 2010). The citrullination (also called deimination) of histones has increasingly been shown to be linked to autoimmune processes (Dwivedi et al. 2012). Anticitrullinated protein antibodies (ACPA) have a specificity of 90 % and a sensitivity of 75 % for rheumatoid arthritis. Therefore, depending on the specific target of the antibody, ACPA can be used as an indicator for arthritis (Sebbag et al. 2004).

The enzyme responsible for the citrullination of histones is Protein Arginine Deiminase, which has 5 isoforms in all vertebrates (Vossenaar et al. 2004). PAD-4 is the only one that has a nuclear signal and therefore is the main enzyme to deiminate histones H2A, H3 and H4 (Hagiwara et al. 2002, Nakashima et al. 2002). PAD-4 is mostly expressed

in granulocytes, monocytes and mast cells (Asaga et al. 2001, Vossenaar et al. 2004, Von Kockritz-Blickwede et al. 2008). The deimination of histones results in the loss of positive charge and ultimately leaves the cell highly sensitive to inflammatory stimuli through, for example, LPS and TNF (Neeli et al. 2008). Certain Alleles of PAD-4 are linked to a higher risk in RA. These haplotypes show a more stable PAD-4 mRNA which could lead to increased expression of it (Suzuki et al. 2003), thus linking higher PAD-4 levels to increased susceptibility to autoimmune processes.

1.4 Specific autoantibodies are used to diagnose autoimmune diseases

1.4.1 Anti-neutrophil cytoplasmic antibodies (ANCA) are used to diagnose small vessel vasculitis

ANCA are antibodies directed against cellular components of neutrophil granular components and monocyte lysosomes (Van Der Woude et al. 1985). They are associated with various inflammatory and autoimmune diseases, with specific antibodies targeting neutrophil granulocytes which are crucial cells in inflammatory processes (Lindgren et al. 2000, Stoffel et al. 1996). A subset of diseases linked with ANCA are termed ANCA associated vasculitis (AAV). These diseases are categorized as autoimmune diseases of the small and medium vessels of the body. Granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic polyangiitis (EGPA) are the three main forms of AAV (Jennette et al. 1994, Jennette 2013). Approximately 80-90 % of GPA and MPA patients and about 40 % of EPA patients express ANCA (Bosch et al. 2006, Pagnoux et al. 2007).

ANCA were first detected though IIF in patients with chronic inflammatory disease in 1959 (Calabresi et al. 1959). However, they were first used as a diagnostic marker in GPA, published in 1985 by Van der Woude et al. (Van Der Woude et al. 1985). Initially, the evidence was only based on immunofluorescence patterns in the sera of patients with GPA (formerly known as Wegner's granulomatosis). However, with the development of an additional technique for the detection of ANCA through Enzyme-linked immunosorbent assay (ELISA), the diagnostic value of ANCAs grew (Nolle et al. 1989, Tervaert et al. 1989). Soon after that, two different fluorescence patterns were

discovered in the first ANCA workshop in 1989, leading to the differentiation of P-ANCA and C-ANCA. At the second ANCA workshop, PR3 was introduced as a target for C-ANCA (Ludemann et al. 1990).

The components of peripheral blood neutrophils which ANCA target are enzymes stored in cytoplasmic granules such as proteinase 3 (PR3) and myeloperoxidase (MPO) (Savige et al. 2003). Quantification of ANCA is used to diagnose and monitor disease activity in patients (Aggarwal 2014). In addition to the above mentioned, SVV ANCA are also used in non-vasculitic conditions, for example, inflammatory bowel disease (Saxon et al. 1990), primary sclerosing cholangitis (PSC), autoimmune hepatitis type I (AIH) (Vidrich et al. 1995), and Felty's syndrome (Coremans et al. 1993). The frequency of ANCA-positive sera varies from 10-90 % depending on the disease type (Savige et al. 2005). Consequently, ANCA are not detected in all patients with ANCA-associated diseases, limiting the sensitivity of ANCA as a diagnostic parameter.

The demand for better biomarkers in vasculitis and inflammatory diseases has recently been getting stronger, however, none of the new diagnostic tests have been very promising (Monach 2014).

ANCA are quantified by indirect immunofluorescence (IIF) or ELISA (Savige et al. 2003). The ELISA is based on immobilized antigens of ANCA (e.g. PR3, MPO) (Rasmussen et al. 1990). After incubation with patient serum, binding of anti-MPO or anti-PR3 antibodies is detected by fluorochrome-labeled anti-human immunoglobulin (Ig) conjugates. ANCA detection by IIF uses purified blood neutrophils, which are fixed with ethanol onto slides. The ethanol fixation disrupts the granules, which causes the translocation of MPO to the periphery of the nucleus, while PR3 remains in the cytoplasm. After incubation with patient sera, autoantibody binding to neutrophils is also detected by fluorochrome-labeled anti-human immunoglobulin antibodies. Depending on the staining pattern and localization, patients are diagnosed with C-ANCA (cytoplasm), P-ANCA (perinuclear) or atypical ANCA (rim-like pattern). Due to the ethanol fixation, C-ANCA are mostly associated with anti-PR3-autoantibodies, whereas P-ANCA are linked to anti-MPO-antibodies (Csernok and Moosig 2014).

However, the international consensus statement on testing and reporting ANCA states that ANCA are verified most reliably by using a combination of IIF and ELISA that detect ANCA specific for PR3 or MPO. A well standardized test and an adherence to published guidelines must be closely followed, as well as restricting the test to clinical situations with a high pretest probability for ANCA-vasculitis (Savige et al. 2003). A treatment decision should never be based on an ANCA test alone but should always consider the clinical and histologic findings (Schmitt and Van Der Woude 2004).

1.4.2 Anti-nuclear antibodies (ANA) are used to screen for a variety of autoimmune diseases

ANA are associated with a variety of autoimmune diseases, including SLE, glomerulonephritis and unexplained multisystem disease (Aggarwal 2014). Once ANA is found to be positive, diverse sub-types are identified (Damoiseaux et al. 2014) to narrow the field of possible ANA associated diseases. ANA are commonly detected by IIF of a Hep-2 cell or variants (Copple et al. 2012). ANA target nuclear components such as DNA, histones and ribonuclear proteins (Aggarwal 2014). Consequently, ANA-positive sera may also bind to nuclei of neutrophils leading to a staining pattern comparable to P-ANCA.

Formalin fixed neutrophils are used to eliminate a potential false-positive diagnosis for P-ANCA due to ANA. (Schulte-Pelkum et al. 2014). Formalin does not disturb the integrity of neutrophil granules and therefore MPO does not translocate to the nucleus (Pollock et al. 2008). On formalin fixed neutrophils, P-ANCA display a cytoplasmic staining pattern and can therefore be distinguished from the anti-nuclear staining pattern of ANA (Savige et al. 2005).

2. Work hypothesis and specific aims

2.1 Hypothesis

NETosis generates neo-autoantigens, which bind auto-antibodies and therefore provide a novel diagnostic tool for the stratification of distinct autoimmune diseases.

2.2 Specific aims

Aim 1: Identification of staining patterns in NETotic neutrophils and characterization of antigen generation during NETosis.

Approach: Pre-diagnosed patient sera are screened on stimulated neutrophils and the results of conventional tests and tests on stimulated neutrophils are compared.

Aim 2: Identification of neo-autoantigens in stimulated neutrophils which cannot be detected with conventional ANCA-screening.

Approach: Screening of patient sera with diagnosed AAV, which were previously screened ANCA-negative on stimulated neutrophils.

Aim 3: Identification of neo-autoantigens in sera with suspected autoimmune disease and stratification of neo-autoantibodies staining patterns.

Approach: Collection and screening for neo-autoantibodies on stimulated neutrophils of anonymized residual patient sera from the diagnostic laboratory of the Institute of Immunology with a request for quantification of ANA or ANCA.

3. Materials and methods

3.1 Blood cells used

3.1.1 Acquiring blood for experiments through blood donors

Unless otherwise stated, neutrophils were isolated from Ethylenediaminetetraacetic acid (EDTA) blood of healthy donors from the University Medical Center Hamburg-Eppendorf (UKE) Institute of Transfusion Medicine.
Healthy sera were also donated by the UKE Institute of Transfusion Medicine.
All blood donors signed a consent to allow the blood to be used for research purposes.

3.1.2 Isolating PMNs through Percoll density separation

Falcon tubes (15 ml and 50 ml) were used for all steps. Solution transfer was achieved with sterile transfer pipettes (Sarstedt). To avoid contamination, all cell isolation steps were completed under a clean hood environment.
One volume of blood was layered on one volume of “Histopaque 1119” (life technologies) and centrifuged at 800 g for 20 minutes at room temperature (RT). This step separates the blood into 4 different layers. The top layer consists of plasma, the interphase consists of mostly peripheral blood mononuclear cells (PBMCs), and the bottom phase consists of neutrophils contaminated with red blood cells (RBCs). The bottom pellet is entirely made up of RBCs. The plasma was removed and discarded, and the bottom neutrophil rich layer was collected with a sterile transfer pipette.
A washing step with Hank’s balanced salt solution (HBSS (-)) (life technologies) followed, containing 0.1 % bovine serum albumin (BSA) in phosphor buffered saline (PBS) and 5 mM EDTA at a centrifugal speed of 300 g for 10 minutes at RT. At least 2 ml of re-suspended pellet (in HBSS(-)) was layered upon a “Percoll plus” (GE Healthcare sciences) gradient. 36 ml of “Percoll plus” was mixed with 4 ml of 10x HBSS (-). This is considered 100 % “Percoll solution”. 8.5 ml of the “Percoll solution” was mixed with 1.5 ml of 1x HBSS (-) for an 85 % diluted “Percoll solution”. This was repeated with 80 %, 75 %, 70 % and 65 % “Percoll solution” diluted with HBSS (-). These solutions were carefully layered on each other in a 15 ml falcon tube in a thickness of 2 ml, so that a pillar of liquid with increasing density from the top to the bottom was created. This gradient consists of 5 densities from 85 % until 65 % diluted

in 5 % steps. Then, at least 2 ml of re-suspended pellet was layered on top of the “Percoll gradient”. In this step, different blood samples from the donors could be pooled if desired. Unless otherwise stated, at least 5 different donors were pooled and used for experiments, to ensure a mixed population of neutrophils.
After centrifugation at 800 g for 20 minutes at RT, the neutrophil rich middle layer was collected and washed once more with HBSS (-) + 0,1 % BSA at a centrifugal speed of 300 g for 10 minutes at RT. The resulting pellet was re-suspended in 1 ml of HBSS (-) and cells were counted using a hemocytometer.
The cells were then diluted in the desired concentration with cold Dulbecco's modified eagle medium (DMEM, ThermoFisher scientific) and kept at room temperature until used.

3.2 Types of serum used in experiments

3.2.1 Collecting patient samples with suspected autoimmune disease

Anonymized sera were collected from 400 patients who were referred to the Diagnostic Center of the UKE for ANA or ANCA testing. Only sera which would have been discarded otherwise were collected.
Serum tubes were labeled with an ID-code to later identify clinical parameters. Also, tubes were labeled with number 1-400 as to identify them easier in experiments.
Serum was then aliquoted on non-binding 96-wellplates (Greiner bio one) and original tubes were kept at -80 °C. Aliquot plates were kept at -20 °C or, if experiments would take place in high frequency, kept at 4 °C for a maximum of 5 days. After approximately 10 thawing processes, new aliquot plates were prepared.

3.2.2 Acquiring seronegative SVV samples

Serum was kindly donated by the III. Medical Clinic and Polyclinic of the UKE by Dr med. Silke Brix.

3.3 Acquiring data

3.3.1 Image generation through Indirect immunofluorescence (IIF):

A standard operating procedure (SOP) was established for IIF using previously tested ANA and ANCA positive sera as controls. The steps of IIF are well established and

commercially available in many diagnostic tests. This SOP was based on an IIF-ANCA-kit by Euroimmun, product number FA1201-1005-22 as used in routine diagnostics at the UKE.

3.3.1.1 Preparation of optical plates with Poly-lysine

All experiments were conducted in an optical 96 well plate (greiner bio one), allowing images using the 20x- and 40x-magnifiation objective. Wells were coated with 0,0001 % Poly-lysine (sigma) in PBS for 20 minutes and washed off with distilled H_2O and dried under the hood.

3.3.1.2 Seeding and activation of neutrophils with PMA

After isolation, cells were seeded at a concentration of $2x10^5$ cells per well and activated depending on the experiment with either Phorbol 12-myristate 13-acetate (PMA) (0,1 µM) or Calcium-Ionophore A23187 (Ca-I) (4 µM) at 37 °C. Cells were then fixed with 2 % paraformaldehyde (PFA) (Sigma Aldrich) and left at 4 °C for at least 30 minutes and up to one night.

3.3.1.3 Washing steps between IIF steps with PBST

The cells were washed using the Tecan-plate washer with PBS + 0,05 % tween (Sigma) (PBST) as washing buffer. The plate-washer was set up to always leave 50 µl of residual volume in each well. This prevented the NETs from drying out or being damaged by aspiration or the washing solution. The routine washing procedure between each step of the IIF included three cycles. Firstly, fluid is aspirated from the wells leaving 50 µl of residual volume, then 200 µl of washing buffer is added slowly. This process is repeated 3 times to ensure a sufficient dilution of the initial medium. After the final washing cycle fluid is aspirated, leaving 50 µl residual volume of washing buffer in the wells. Between each of the steps during the IIF, a washing step was included.

3.3.1.4 Permeabilization and protein blocking to reduce background noise

After the permeabilization of the cell membrane with PBST for 15 minutes at room temperature, protein blocking was conducted with a blocking solution consisting of 10

% normal goat serum (Invitrogen), 5 % cold water fish gelatin (sigma) and 1 % BSA (Sigma) for 30 minutes at 21 °C.

3.3.1.5 Adding patient sera and secondary fluorescent antibodies

If not otherwise stated, patient sera were added at a dilution (in PBST) of 1:80 and incubated at 37 °C for one hour. After incubation and an additional washing step, the goat-anti human-antibody Alexa Fluor 555 (Thermo Fischer scientific) was added at a concentration of at 7,5 µg/ml in a dilution buffer of PBST and 0,1 % BSA. This antibody incubated for one hour at 37 °C. After an additional washing step, DAPI was added in a final concentration of 2 µM and incubated for 7 minutes. After this, a final washing step concluded the staining.

3.3.2 Image acquisition using the microscope

Images were acquired with an AxioVision 200 from the UKE microscopy imaging facility (umif) with the 20x- or 40x-magnification objectives. Acquisition was established in an automated protocol in which the microscope automatically took pictures of the centre of each well, using an autofocus function without user intervention. In consideration of the manufacturer's instructions, our secondary antibody emitted its excitation maximum at a wavelength of 555 nm and the emission maximum at a wavelength of 580 nm. To acquire the secondary antibody’s best signal, the Cyanin 3 microscope filter was used with an excitation peak at 550 nm and emission peak of 570 nm. The DNA signal was established using a DAPI based DNA dye. Therefore, the blue DAPI microscope filter was used to acquire the DNA-based images. Unless otherwise stated, exposure time of the Cyanin 3 channel was set to 250 µs and exposure time of the DAPI channel was set to 10 µs. For better image- and displayquality we set the blue signal from DAPI to appear in green in the images through the AxioVision-software.

3.3.3 Image quantification with ImageJ

The signal intensity was measured by exporting the previously acquired image to a Joint Photographic Experts Group (JPEG) format. Then, images were processed using the open source program ImageJ. The image was converted to 8 bit and the global threshold setting was changed to 20/225 which promotes the pixels generated by the fluorescence of the secondary antibody in the images from the Cyanine 3 channel while reducing background signals. This has shown to be the optimal setting in numerous

tests, ensuring the best acquisition of the signal without generating high background signals.

The total area, the average size, the percentage area and the count of structures with a positive signal were analyzed and the numeric value of each parameter was copied in an Excel sheet. This entire process was automated by a macro, written with the ImageJ macro recorder. A manual data analysis was also conducted by exporting the images of the Cy3 channel and observing these pictures individually.

Statistical analysis was performed using Prism Software (GraphPad, USA) and by a Man-Whitney test, if not otherwise stated. Results were considered significant at $p < 0.05$.

3.4 Technical aids

3.4.1 Tecan plate washer

The Tecan plate washer was used to wash 96 well plates after fixation. Aspiration speed was set to 3 mm/s, which is the slowest setting. The pump speed, which is responsible for the pressure of the fluid when dispersed, was also set to the slowest possible setting to prevent damaging the cells and NETs. As previously mentioned, the washing program included 3 cycles. With 50 µl residual volume from the previous cycle being left, the washer added 200 µl in each washing step. This led to a dilution of 1:5 for each washing cycle. Therefore, after three cycles, a dilution of <1 % of the initial volume was achieved.

The washer was primed in the morning and left in a “Rinse: Soak” program, ensuring the needles were immersed in fluid and could not dry out. At the end of the day, the washer was rinsed with distilled H_2O to prevent crystals from forming in the tubes.

The washer was cleaned with a disinfectant solution (Antifect) according to the manufacturer’s instructions and left for at least 2 hours every 4 weeks.

Before conducting all experiments, the washer was sent to Tecan for general maintenance and cleaning.

3.4.2 12-channel pipette used in experiments

For all manual pipetting during the experiments Eppendorf Research Pro ® electronic pipette, multichannel, with charging adapter, size 20-300 µL. P9360 SIGMA, mrf Number: Eppendorf®, 4860 000.569 (European) was used at speed level 3/10 while aspirating and dispersing.

3.5 Microscopy

3.5.1 Zeiss Axiovert 200M with ApoTome microscope

The Axiovert 200M with ApoTome was used to acquire all images.
Images were taken with the 20x LD Plan-Neofluar lens from Zeiss, henceforth referred to as “20x-magnification objective”. Also, high resolution images were taken with the 40x EC Plan-Neofluar lens, which will be referred to as “40x-magnification objective” throughout this dissertation.

The cameras in use were CCD AxioCam HRm 1.4MP monochrome and CCD AxioCam MRc 1.4MP color.

The UV-Lamp which was used in all experiments was a HBO 100W with Hg lamp
The technical data of the filters used is for DAPI: 49 (blue; DAPI): G 365 | FT (=”Farbteiler”) 395 | BP (“Bandpass”) 445/50 and for the Cy3-filter: 43 (red; AF568, mCherry): BP 545/25 | FT 570 | BP 605/70.

The Software used for all images and the programming of the automated protocol was kindly provided by Zeiss (Software: Zeiss Axiovision v4.8.2).
Additional components of the microscope were the ApoTome for structured Illumination (HL and VL grids) as well as an anti-vibrational plate.

All information on the microscope can be found online on the umif website (http://www.umif.de/index.php/systems).

3.5.2 Automated image acquisition protocol

An automated protocol was written for the image acquisition to take one picture of each well in the DAPI and Cy3 channel. Coordinates for each of the 96 wells were pre-programmed and a heavy aluminum plate was used to ensure that the 96 well-plate was in the same position each time by putting it on top of the plate before image acquisition. Minimal changes in position were corrected by the autofocus of the ApoTome.

Acquiring 96 pictures in 2 channels for an entire 96 well plate took about 45 minutes and required the precise calibration of the microscope before use.

3.6 Experiment protocols

3.6.1 Stimulating neutrophils and recording time points

After the isolation of neutrophils, as previously described, neutrophils were activated with PMA or Ca-I and if not otherwise stated, activated for 180 or 90 minutes respectively. Cells were fixed with PFA 2 % or with a formalin fixation (3.6.2) adopted from an SOP from the German association of vasculitis diagnostic (Deutschen Referenzzentrum für Vasculitisdiagnostik). PFA was added in a concentration of 2 % and kept for 30 minutes at 4 °C and then washed with PBST. If different time points were measured in the same 96 well plate, the wells which were already fixed were sealed with plastic or aluminum foil before being returned to the incubator so the PFA fumes could not interfere with the stimulation of other cells.

3.6.2 Fixating neutrophils with formalin

After the seeding and activation of neutrophils as described above, the supernatant was aspirated, then 100 µl of fixation medium was added containing 45 % acetone, 9 % formalin, 46 % PBS, for 50 seconds. Next, the supernatant was carefully aspirated 100 µl of pure ethanol (4 °C) was added and left at 4°C for 15 minutes. After this, the ethanol was aspirated carefully, 200 µl of PBST was added and the 96 wellplate was left at 4 °C overnight.

3.6.3 Inhibiting key enzymes of NETosis

In the following experiments, three enzymes were inhibited during neutrophil activation. All these enzymes play a role in NETosis. Inhibitors are well established and used in other publications.

Diphenyleneiodium Chloride (DPI) (Sigma) was added at a concentration of 25 µM before activation of neutrophils, to block the NADPH-Oxidase (Fuchs et al. 2007). Diisopropyl Fluorophosphate (DFP) (Sigma) was added at a concentration of 20 mM to block the Serine-Protease (Oda et al. 1998) and N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-l-ornithine amide (Cl-amidine) was added at a concentration of 200 µM to

block PAD-4 (Luo et al. 2006). All inhibitors were mixed well with a pipette and added diluted in 50 µl of DMEM.

3.6.4 Dilution titer determination

The titer of activated stimulated neutrophil antibody (ASNA) suspected probes were established through 4-fold dilution in a 96 well plate. Each sample was stained at 1:80, 1:320, 1:1280 and 1:5120 serum concentration. Depending on the intensity of the staining in each well, the titer was established. Positive samples were identified by microscopic evaluation of the image taken with the protocol described above. Each 96 well plate had one pANCA positive sample used as a positive control and one healthy control as negative control. If a sample was positive in the 1:80 dilution but negative in the 1:320 dilution, the sample was classified as 1:160. If the sample only had a weak signal in the 1:80 dilution but no signal in the 1:320 dilution it was classified as 1:80.

4. Results

4.1 NETosis has three distinct stages

As portrayed in Figure 1 (p. 8), NETosis contains three major phases, which result in significant intracellular changes. Firstly, after stimulation, neutrophil enzymes like NE, MPO and NADPH-oxidase are activated and produce reactive oxygen species resulting, for example in the oxidative burst, citrullination of histones and chromatin decondensation. In the second phase, granular and nuclear content mix and the nuclear envelope breaks down. In the third phase, the cell membrane ruptures, and the NETs are released into the extracellular space.

Different phases of NETosis were identified after PMA activation to later correlate the stages of NETosis to the generation of autoantigens. These three stages were identified and defined (Figure 2).

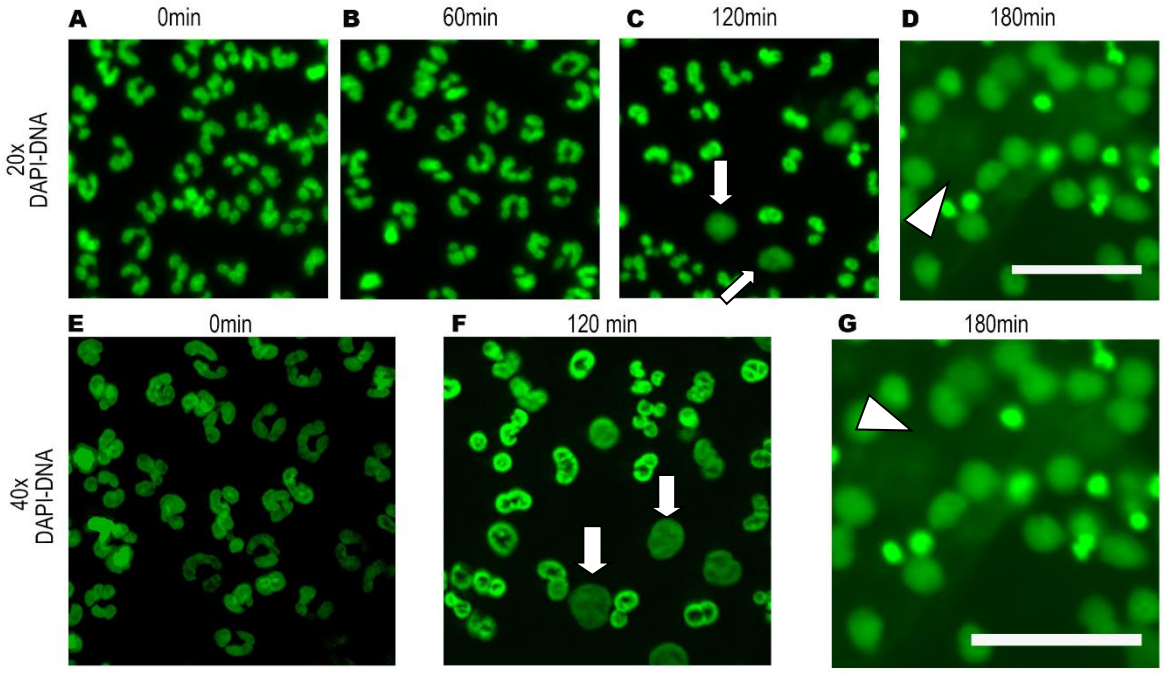

Figure 2: Stages of NETosis after PMA stimulation. Purified neutrophils were stimulated with PMA and stained after different timepoints with DAPI-DNA dye. (A) Untreated neutrophils, (B-D) Neutrophils stimulated for 60 minutes (B), 120 minutes (C), 180 minutes (D). Images acquired with 20x magnification (A-E) and selected timepoints with 40x magnification (E-G). White bar represents 50 µm. White arrows indicate neutrophils with merged nuclear and cellular components. Arrowheads indicate released NETs.

Neutrophils were isolated and activated with PMA as described in *Materials and methods*. After fixation and staining with DAPI-DNA dye, images were acquired in a 20x-magnification at selected timepoints (Figure 2 A-D). In addition, to better identify intracellular morphologic changes, 40x-magnification images were acquired at selected timepoints of NETosis (Figure 2 E-G).

Unstimulated neutrophils (Figure 2 A, E) display their characteristic segmented nucleus and condensed nuclear envelope. This represents the natural state of neutrophils in the human body. This also demonstrates that the isolation process did not artificially stimulate the neutrophils. After 60 minutes of stimulation (Figure 2 B) the first stage of NETosis has been engaged in most neutrophils. Morphological changes cannot be observed yet, but intracellular enzymes are active, and the merging of nuclear and granular components is being prepared. After 120 minutes of stimulation (Figure 2 C, F) neutrophils lose their segmented nuclear structure. Compared to the previous nuclear staining, the segments which are typical for neutrophils cannot be observed and the nuclear envelope appears to be swelling and the outline of the nucleus seems blurred. Additionally, it can be observed that singular neutrophils have already entered phase two of NETosis, where nuclear and granular components mixed, but the membrane is still intact. These cells appear as big round cells (Figure 2 C, F; white arrow) because the DNA fills the entire cellular body after the components mix. After 180 minutes of stimulation with PMA, most neutrophils have mixed nuclear and granular contents (Figure 2 D, G). In addition, some cells have already entered phase three of NETosis, releasing the NETs into the extracellular space which appears as a lighter green then the still intact cells (Figure 2 D, G; arrowhead) which completes NETosis.

In conclusion, NETosis can be divided into three major phases with distinct morphological patterns. Phase I consists of neutrophil enzyme activation and lasts for about 60 minutes in which the nuclear envelope appears blurry and swollen. Phase II involves nuclear decondensation and mixing of granular and nuclear contents, which can be observed between 90 and 180 minutes of stimulation in which the cell appears as a big round structure. Phase III concludes NETosis with the release of NETs into the extracellular space. We can observe this stage at about 180 minutes of stimulation with PMA.

4.2 NETotic neutrophils can be used as substrate for antibody testing

ANCA-diagnostics use naïve neutrophils as a substrate which is fixed on ethanol or formalin. However, considering that ANCA usually target PR3 or MPO which are both still prevalent in stimulated neutrophils, it can be hypothesized that activated neutrophils would also be a suitable substrate for antibody testing. Additionally, it was considered if ANA can also be screened on activated neutrophils. ANA target nuclear components and are usually screened on Hep2-cells. However, considering that NETs mainly contain nuclear components, it was hypothesized that stimulated neutrophils could be used as a substrate for this as well.

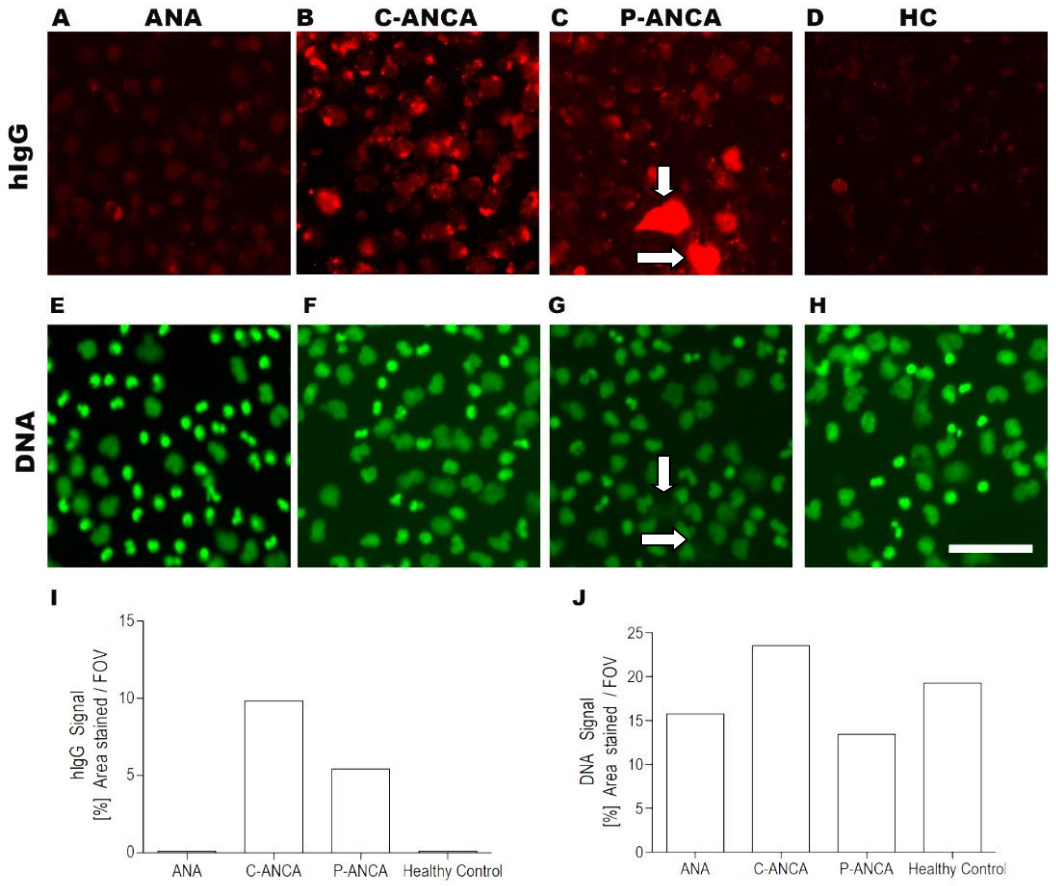

Figure 3 NETotic neutrophils as a substrate for antibody testing. Purified neutrophils were stimulated with PMA for 120 minutes, incubated with patient sera containing ANA (A), C-ANCA (B), P-ANCA (C) and a healthy control serum (HC) (D), and analysed by IIF. (A-D) IIF of human IgG (hIgG); (E-H) DNA staining by DAPI; Bar equals 50 µm. White arrow indicates staining on released NETs. Image quantification though ImageJ as described in *Material and methods* showing the percentage of the field of view (FOV) stained by the hIgG Signal (I) and the DNA signal (J).

To test this hypothesis, neutrophils were isolated and stimulated with PMA for 120 minutes as described in *Materials and methods*. After fixation, the cells were incubated with patient sera which had previously been tested positive for ANA (Figure 3 A), C-ANCA (Figure 3 B), P-ANCA (Figure 3 C) as well as a healthy control (Figure 3 D). Human IgG (hIgG)-IIF signals were acquired to identify signal patterns produced by antibodies attached to NETs or neutrophil structures. To compare these signals to the neutrophil components, DNA was additionally dyed with DAPI. In addition, the images were quantified to show the percentage of signal per field of view (FOV) created by the hIgG (I) and the DNA-Dye (J) signal.

The ANA hIgG signal (Figure 3 A) appears very weak which indicates, NETotic neutrophils are a poor substrate for ANA-testing although in theory they contain the nuclear components targeted by ANA. Considering that routine diagnostic uses Hep-2 cells which originate in the liver, this is not surprising. However, this provides the useful information that ANA-positive samples do not generate a false-positive signal, if searching for new autoantibodies.

If incubated with C-ANCA positive serum, a signal can be detected in IIF (Figure 3 B), demonstrating that C-ANCA can be detected on neutrophils which have artificially been activated. Approximately 10% of the image shows a hIgG signal, while about 25% of the FOV is covered by the DNA-dye. The comparing the location of the hIgG signal to the DNA signal (Figure 3 E) indicates, that the hIgG signal is located mostly in the cytoplasm of the still intact cell or in the remaining cell body once the NETs are released. This shows that C-ANCA and therefore most likely PR3 does not join in the merging of nuclear and granular components. This also implies there are considerable comparisons to the classical C-ANCA pattern, meaning all neutrophils, regardless of NETosis stage, can be used for C-ANCA detection.

If incubated with P-ANCA positive serum, the hIgG signal in the IIF shows a different pattern based on the NETosis phase the cell is in (Figure 3 C). Only in later stages of NETosis P-ANCA produces an increasingly strong signal, which is especially strong in NETs which are already released into the extracellular space (Figure 3 C/ white arrows). Therefore, the hIgG signal only covers about 5% of the FOV (Figure 3 I), however these signals appear much more intense than the homogeneous C-ANCA pattern. The DNA-dye signal (Figure 3 G) shows that cells in advanced stages of NETosis are producing the strongest hIgG signals.

The healthy control serum produces no hIgG-signal in the IIF, confirming that neither the isolation method nor the IIF components and procedures produce false-positive signals, indicating the fluorescent secondary antibody only attaches to antigen-bound human antibodies.

In conclusion, the data indicates that activated neutrophils provide a substrate for ANCA diagnostics. Additionally, it can be observed that ANA does not produce an IIF signal on activated neutrophils. The healthy control demonstrates that the antibody specifically targets attached antibodies which are bound to neutrophil and NET structures.

4.3 NETosis generates neo-autoantigens

NETosis leads to multiple radical changes inside neutrophils. It is speculated that these changes can lead to the generation of neo-autoantigens which are targeted by autoantibodies. These antibodies cannot be detected by conventional ANCA-testing, since conventional testing is done on naïve neutrophils.

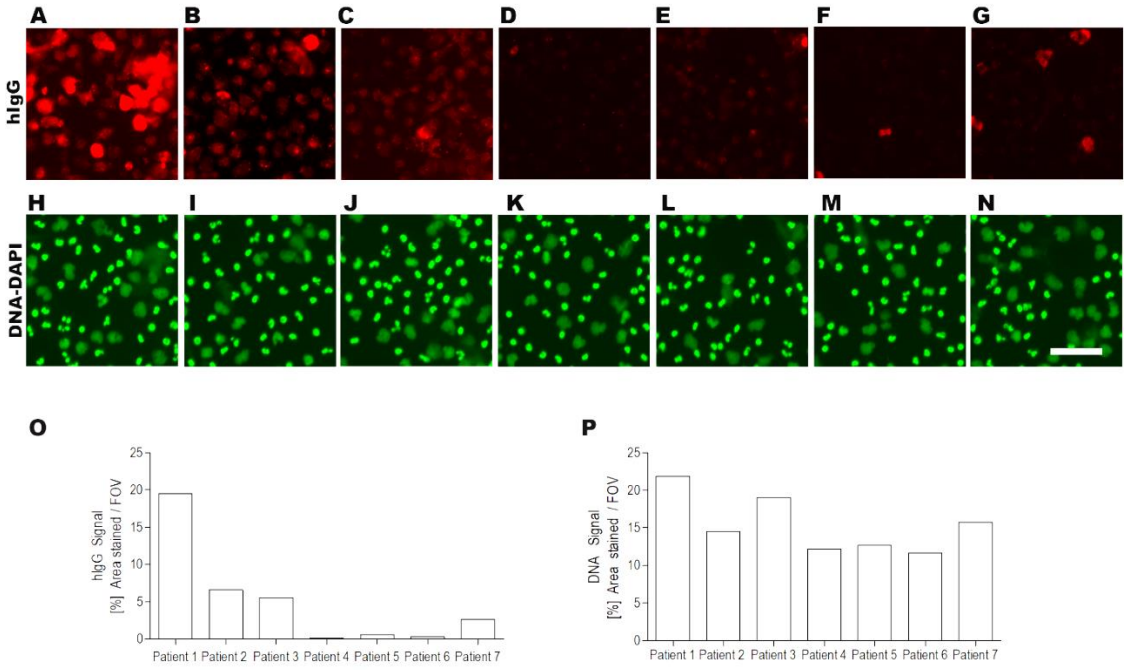

Figure 4 NETosis generates neo-autoantigens. Purified neutrophils were stimulated with PMA for 120 minutes, incubated with patient sera, and analysed by IIF as described in *Materials and methods*. (A-G) IIF of human IgG (hIgG); (H-N) DNA staining by DAPI; Bar equals 50 µm. Quantification of hIgG (O) and DNA-DAPI (P) signal through ImageJ.

Therefore, 7 ACNA-negative samples of patients with confirmed SVV were incubated on stimulated neutrophils. Since ANCA has been ruled out by conventional testing beforehand, it was speculated that a hIgG-signal indicates that preformed antibodies are present in the serum which bind to neo-autoantigens. The hIgG-signal (Figure 4 A-G) was acquired and a DAPI-DNA dye was added to identify the neutrophils and the NETosis stage (Figure 4 H-N).

A strong signal can clearly be observed in at least one serum sample (Figure 4 A), which also presents in image quantification (Figure 4 O). If inspected carefully and compared to the respective DNA signal (Figure 4 H), the signal is concentrated mainly on neutrophils in later stages of NETosis which present in a lobulated shape. The staining pattern in Figure 4 A can most likely be compared to the P-ANCA staining displayed above (Figure 3 C). However, considering that P-ANCA was ruled out by previous testing, this indicates a different antigen which, considering the pattern, might be located in similar cell compartments as MPO during NETosis.

Additionally, a weaker signal can be observed in samples B and C (Figure 4) as well as in the signal intensity by quantification (Figure 4 O, Patient 2 and 3). The staining pattern in these samples can be observed in all neutrophils without it appearing stronger in lobulated cells. Compared to the ANCA patterns observed in Figure 3, the pattern observed in images B and C can be compared to a C-ANCA staining, which indicates the neo-autoantigen could be located in similar parts of the NETotic cell as PR3. Samples displayed in D - G (Figure 4) show no significant hIgG-signal, confirmed by the quantification method (Figure 4 O), indicating no antibody has bound to compounds of the neutrophil which suggests that no autoantibodies are present in these serum samples.

In conclusion, a hIgG signal can be observed in 3 serum samples of patients with ANCA-negative SVV. This indicates that stimulated neutrophils provide a substrate to detect autoantibodies which are not shown on conventional ANCA-testing. Therefore, stimulated neutrophils provide additional information on patients with ANCA-associated diseases and can be considered as a new diagnostic parameter.

4.4 Collecting sera of patients containing autoantibodies

To identify if samples of patients with autoantibodies and autoimmune diseases also contain antibodies against stimulated neutrophils, 80 anonymized residual patient sera from the diagnostic laboratory in the Institute of Immunology of the UKE were collected. The inclusion criteria were a request for quantification of ANA or ANCA. These 80 serum samples were screened on stimulated neutrophils which were activated for 120 minutes with PMA. Here, 11 samples were identified, which showed similar hIgG signals as seen before in Figure 4. These 11 samples were additionally screened for ANCA on unstimulated neutrophils following the protocol mentioned in *Material and methods (3.6.2)*. Five out of the 11 samples displayed an ANCA positive IIF signal, while six samples did not display a signal on unstimulated neutrophils. These 6 samples were considered to contain preformed antibodies against stimulated neutrophils and further experiments were conducted using these 6 samples.

4.5 Neo-autoantigens develop over time in stimulated neutrophils.

Neo-autoantigens develop during NETosis and provide a binding site for preformed autoantibodies. However, to further understand when and how these neo-autoantigens form, the kinetics of antigen generation in stimulated neutrophils was analyzed. Therefore, purified neutrophils were activated for different lengths with PMA (Figure 5). After fixation, stimulated neutrophils were incubated with samples which were previously screened positive for antibodies against stimulated neutrophils and analyzed by IIF.

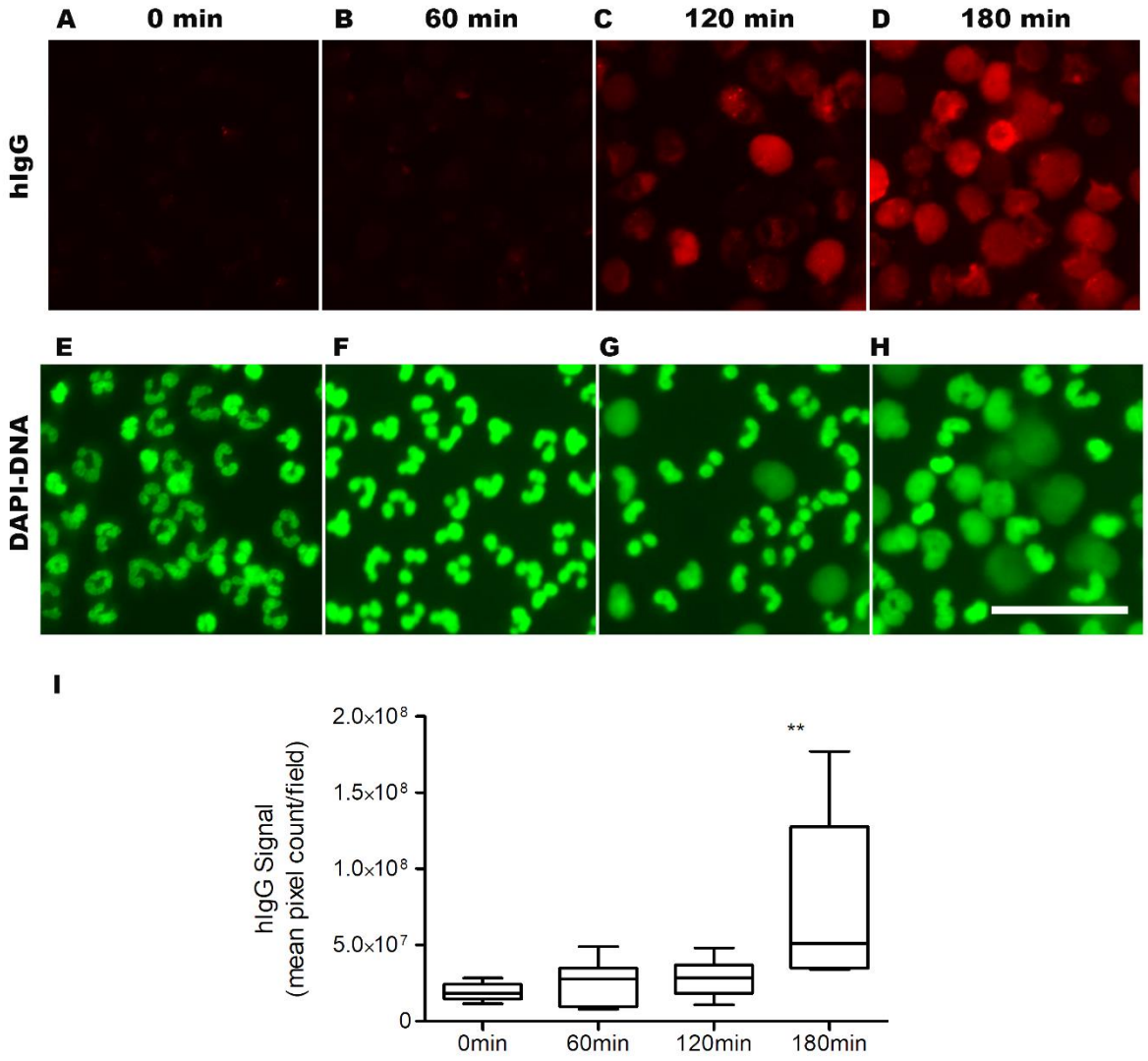

Figure 5 Time course of antigen generation in stimulated neutrophils. Purified neutrophils were stimulated with PMA, incubated with patient serum and analyzed by IIF as described in *Materials and methods*. (**A**) Untreated neutrophils, (**B-D**) Neutrophils stimulated with PMA for 60 minutes (B), 120 minutes (C), and 180 minutes (D). (A-D) IIF of human IgG (hIgG); (E-H) DNA staining by DAPI; Data shown is representative of six serum samples. Bar equals 50 µm. (I) staining was quantified using ImageJ, by pixel counting, selected and scanned in blinded manner. **p<0.05 (Mann-Whitney U test).

Patient hIgG did not bind to untreated neutrophils (Figure 5 A), indicating that naïve neutrophils do not express or expose neo-autoantigens. Neutrophils which were activated for 60 minutes with PMA (Figure 5 B) did not display any hIgG-signal either. However, after 120 minutes of stimulation, a hIgG signal can be observed in

neutrophils (Figure 5 C) which increases after 180 minutes (Figure 5 D). There is no significant signal increase in the quantification method, underlying that this is only a semi-quantitative evaluation of the data. If the hIgG signal is compared to the stage of NETosis, which is derived from the DNA signal (Figure 5 E - H), it can be assumed that, after the neutrophils enter phase 2 of NETosis between 60 and 120 minutes of PMA stimulation (Figure 5 F, G), the hIgG signal is present in most neutrophils. Phase one of NETosis is indicated by the blurry appearance of the nuclear envelope (Figures 5 F) if compared to unstimulated neutrophils (Figure 5 E). The DNA-signal after 120 minutes (Figure 5 G) additionally displays few neutrophils which have already merged nuclear and granular content, indicating phase two of NETosis and that the cell is about to lose membrane integrity and release NETs. These cells display the strongest hIgG signal, supporting the previously made observation. This observation can also be reinforced by objectifying the number of pixels created by the staining (Figure 5 I). Here the statistically significant rise in signal intensity can be observed after 180 minutes of stimulation.

In conclusion, the data suggest that the generation neo-autoantigens are time dependent and only express in later stages of NETosis. The pattern observed indicates that the neo-autoantigen forms outside of the nucleus, however after mixing with the nuclear component the signal intensifies. This suggests that the mixing of cellular compartments is the driving force behind the generation of the discovered neo-autoantigen.

4.6 The generation of neo-autoantigen is affected by different stimuli

In vivo, neutrophils are activated by microbes (such as bacteria, viruses and fungi) interacting with cell surface receptors of neutrophils (Brinkmann et al. 2004, Urban et al. 2006, Brinkmann and Zychlinsky 2007). In vitro, neutrophils can be activated through different pathways, including PMA, lipopolysaccharide (LPS), Calcium-Ionophore A23187 (Ca-I) and other means (Hoppenbrouwers et al. 2017, Pieterse et al. 2016).

To confirm that PMA is not artificially generating autoantigens and to further comprehend the kinetics of antigen-generation, Calcium-Ionophore was used to stimulate neutrophils. Compared to PMA, Calcium-Ionophore has shown to lead to

higher peroxidase activity and higher concentrations in citrullinated histone 3 during NETosis (De Bont et al. 2018). Additionally, Calcium-Ionophore leads to a faster membrane rupture, usually within 90 minutes, compared to PMA which usually exceeds 120 minutes of stimulation before membrane integrity is lost (De Bont et al. 2018). The signal intensity of neutrophils stimulated with Calcium-Ionophore and PMA was compared using the same serum samples which have shown to contain antibodies binding to stimulated neutrophils (Figure 6).

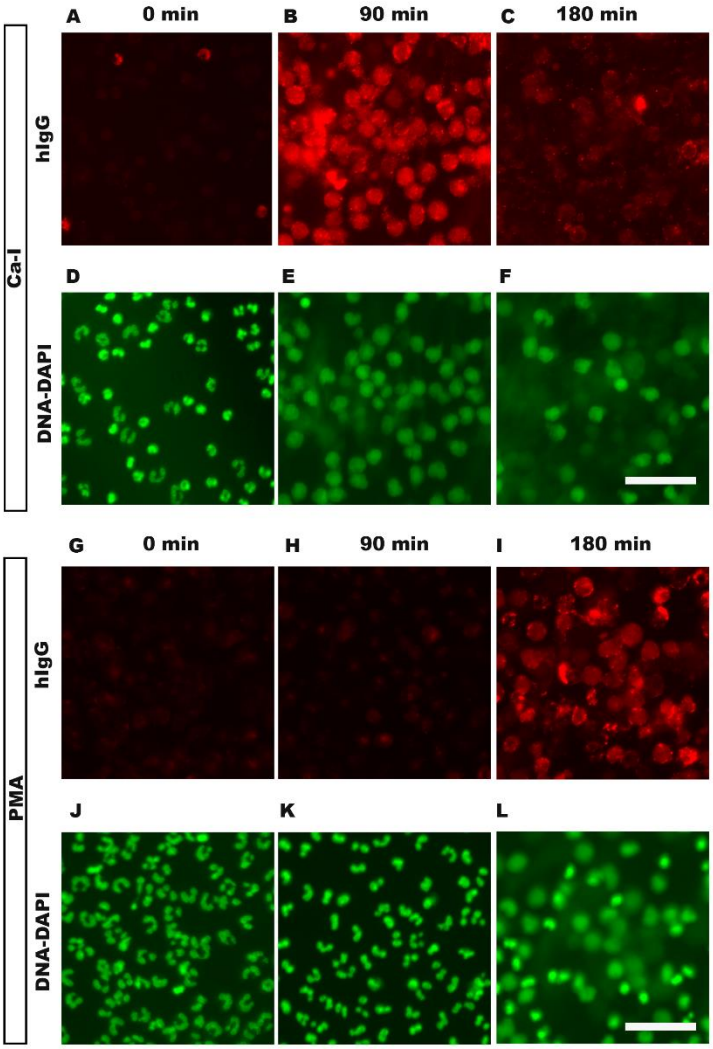

Figure 6. Antigen generation in stimulated neutrophils using different stimulation methods. Purified neutrophils were stimulated with Calcium-Ionophore A23187 (Ca-I) (A-C) and Phorbol 12-myristate 13-acetate (PMA) (G-I) and incubated with the same serum and analyzed by IIF as described in *Materials and methods*. (**A,G**) Untreated neutrophils, (**B-C/H-I**) neutrophils stimulated for 90 minutes (B/H) and 180 minutes (C/I). (A-C/G-I) IIF of human IgG; (D-F/J-L) DNA staining by DAPI; Bar equals 50µm. Data shown representative of 6 serum samples.

Untreated neutrophils, in both Calcium-Ionophore (Figure 6 A) and PMA (Figure 6 G) settings, displayed no hIgG signal, indicating that no antigens are present at this stage. The DNA-signal is identical, showing neutrophils in their naïve state with segmented nuclei (Figure 6 D, J), as to be expected.

However, after 90 minutes of stimulation with Calcium-Ionophore, there are noticeable changes in the cellular morphology (Figure 6 E). Most cells have already transformed to lobulated cells, where nuclear and granular components have mixed, and the DNA signal fills the entire cell body (Figure 6 E). This confirms the previously mentioned faster course of NETosis when using Calcium-Ionophore as stimulus. Interestingly, the hIgG signal in cells stimulated with Ca-I (Figure 6 B) is very prevalent, thus verifying previously made observations, that the merging of granular and nuclear components is the driving force behind the surge in signal intensity. The staining pattern is highly concordant to the pattern observed in neutrophils stimulated with PMA for 120 minutes or longer. After 180 minutes of stimulation with Calcium-Ionophore it can be observed that most cells have released NETs and the integrity of the cells has been lost (Figure 6 F). The hIgG signal after 180 minutes of Calcium-Ionophore stimulation (Figure 6 C) is weaker than before.

The data provided by the PMA stimulated neutrophils confirm previous experiments. Naïve neutrophils (Figure 6 J) and PMA stimulation of 90 minutes (Figure 6 K) show mostly segmented nuclei which have not yet entered phase II of NETosis. However, after 180 minutes of PMA stimulation most neutrophils can be observed as lobulate cells or as released NETs (Figure 6 L). As expected, the hIgG signal is not detectable after 0 minutes (Figure 6 G) or 90 minutes (Figure 6 H) of PMA stimulation respectively. However, after 180 minutes there is a strong hIgG signal (Figure 6 I), which is highly concentrated in the lobulated cells in later stages of NETosis, as well as the extracellular space.

It can be concluded that the increase in the hIgG signal can be linked to phases of NETosis. It was confirmed that Calcium-Ionophore stimulation leads to faster merging of granular and nuclear components. After 90 minutes of stimulation with Calcium-Ionophore most neutrophils already display a lobulated shape and isolated neutrophils

have released NETs already. After 90 minutes of stimulation with PMA, the segmented nucleus is still prevalent in most neutrophils which indicates an earlier stage of NETosis. After 180 minutes of stimulation Calcium-Ionophore stimulated neutrophils have released NETs and both the hIgG and the DNA signal can be observed spread out evenly over the entire field of view. This signal after NET release appears weaker and speckled. This could be attributed to different factors open to discussion. With Calcium-Ionophore stimulation, NETs are released after roughly 120 minutes. NETs release for example enzymes and reactive oxygen species which can alter NET components and the surroundings. The newly generated autoantigens could already be degraded 60 minutes after NET release. The merging of granular and nuclear content has been the trigger for neo-autoantigens in both Calcium-Ionophore and PMA stimulation, further indicating that this phase of NETosis is responsible for the generation of these novel autoantigens.
In conclusion, this data indicates that NETosis itself is creating neo-autoantigens. Specifically, peroxidase activity and citrullinated histones appear to have an impact on signal intensity. Also, the data shows there is a specific timepoint in which the signal intensity is strongest. This seems to be just before the membrane ruptures and granular and nuclear components mix.

4.7 Autoantigen generation is enzyme dependent.

Several enzymes play crucial parts in NETosis and some have been associated with autoimmunity. To identify possible enzymes and reactions which lead to the formation of neo-autoantigens in NETosis it was tested whether the hIgG signal would decrease if crucial enzymes of NETosis were inhibited before stimulation (Figure 7). It was hypothesized that crucial enzymes of NETosis would stop the merging of granular and nuclear content would prevent the generation of neo-autoantigens. Furthermore, it was speculated, that enzymes which are suspected of having an influence on the generation of autoantigens (proteases, deiminases) could lead to the reduction of the hIgG generated signal.

Diphenyleneiodium Chloride (DPI) was used to block the NADPH-oxidase. This enzyme is crucial for NETosis through the generation of ROS. Without this enzyme,

NETosis does not occur through PMA stimulation but can only be achieved through adding H_2O_2 (Fuchs et al. 2007).

Diisopropyl Fluorophosphate (DFP) was used to inhibit the serine protease (Oda et al. 1998). Under the influence of this substance NETosis is reduced, due to the inhibition of neutrophil elastase (NE) which mediates the unfolding of chromatin by cleaving histones and is required for NET formation (Papayannopoulos et al. 2010).

N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-l-ornithine amide (Cl-amidine) was used to inhibit the neutrophil PAD-4 (Knight et al. 2013). This enzyme is responsible for the citrullination of histones during NETosis. This process has often been discussed as a potential cause of the formation of neo-autoantigens and autoimmune disease.

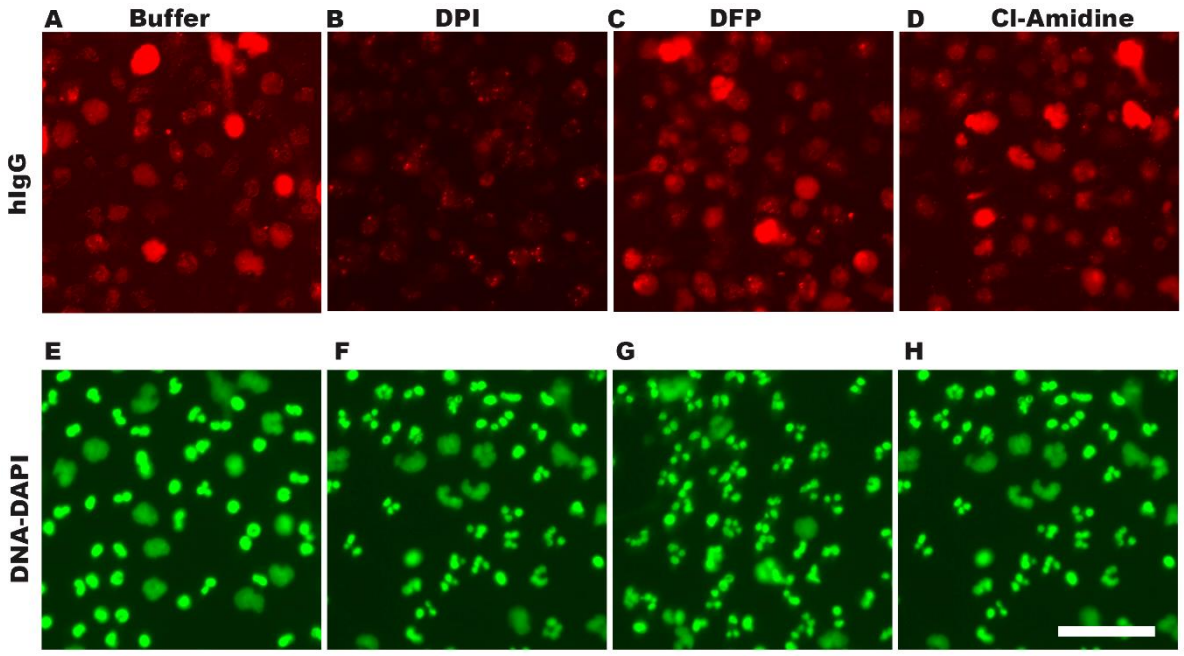

Figure 7 Generation of autoantigens in stimulated neutrophils after inhibiting specific enzymes prevalent in NETosis. Purified neutrophils were stimulated with PMA for 120 minutes and incubated with serum which contained antibodies against stimulated neutrophils. **(A-D)** Reagents were added to block enzymes which are active during NETosis. (A) Buffer, (B) DPI, (C) DFP, (D) Cl-amidine. (A-D) IIF of human IgG; (E-H) DNA staining by DAPI; Bar equals 50µm. Data shown representative of 6 serum samples.

It can be observed that in conventionally stimulated neutrophils (Figure 7 A) the serum sample contained antibodies against stimulated neutrophils and the previously described hIgG signal is prevalent under normal conditions. This hIgG signal is mainly located in lobulated neutrophils after 120 minutes of PMA stimulation (Figure 7 A), as described in previous experiments. This observation is matched by the DNA signal which indicates NETosis has progressed normally (Figure 7 E). Several neutrophils have entered phase two of NETosis indicated by the lobulated shape of the DNA signal.

When examining the cells treated with DPI, it can be concluded, that NETosis was halted, as expected. The neutrophil nuclei still appear as small, segmented cells indicating that the cells are still in a naïve state (Figure 7 F). DPI treated cells do not show any hIgG signal (Figure 7B), indicating that the ROS produced by the NADPH-oxidase is a crucial part of NETosis and the generation of neo-autoantigens.
The DFP treated neutrophils also retain their segmented nuclear structure in most cells (Figure 7 G). However, the hIgG signal can clearly be observed in these cells (Figure 7 C). The signal can be observed in the entire cytoplasm and the cells have not merged granular and nuclear content. This indicates that a process which is not influenced by the NE leads to the formation of autoantigens and the autoantigen is located in the cytoplasm rather than in the nucleus before merging.
The Cl-amidine treated neutrophils also display a hIgG signal (Figure 7 D). This signal is mainly located in the lobulated cells shown in the DNA stain (Figure 7 H), as is the case in the untreated neutrophils. However, the signal in general appears to be weaker than in untreated neutrophils (Figure 7 A).
If the signal is quantified by counting the total pixels of the hIgG signal with an ImageJ macro, this observation can be confirmed. However, the only significant difference in signal intensity, if calculated by Mann-Whitney test, can be seen between the buffer and the DPI treated neutrophils which only display a very weak signal (Figure 8).

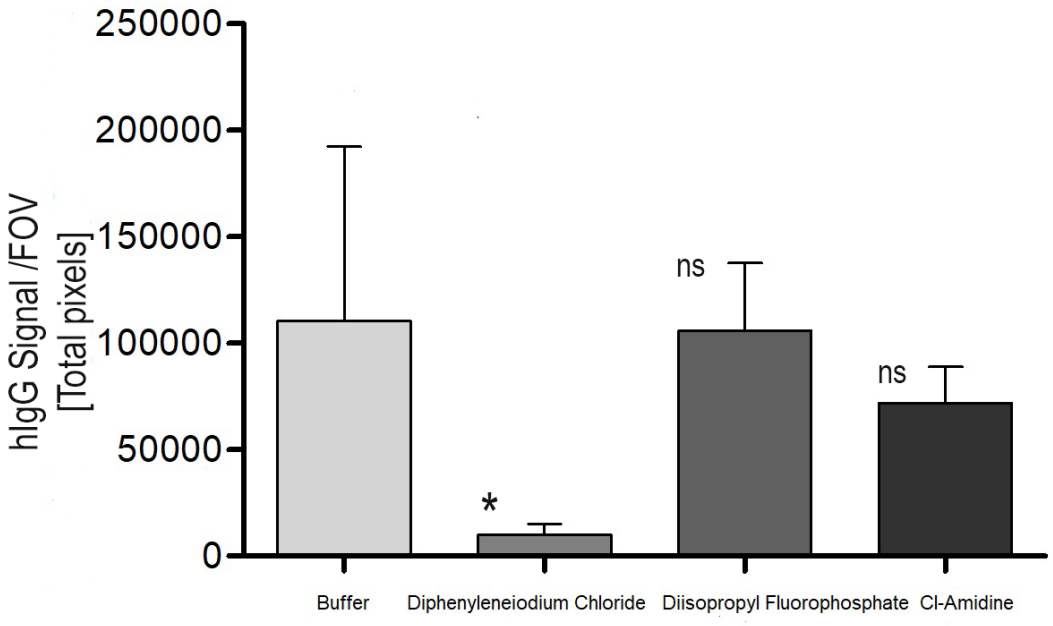

Figure 8 Signal intensity of hIgG signal after enzyme inhibition. X-axis represents the enzyme inhibitor used. Y-axis represents the signal intensity assessed by ImageJ Macro as described in *Material and methods* through analyzing total pixels in the image of the hIgG-channel. Six serum samples were tested for every condition. * p <0,05 (Mann-Whitney test) was performed compared to Buffer, ns = not significant.

To conclude, the data suggests that the oxidative burst created by the NADPH-oxidase is a crucial part for the generation of neo-autoantigens. However, even if the decondensation of the nuclear envelope is stopped by inhibiting the NE, an hIgG signal can still be observed in the cytoplasm, indicating that the generation of these autoantigens are independent of this step and the autoantigens are present in the cytoplasm rather than in the nucleus. When inhibiting PAD4, the signal pattern remains intact, however it appears to be weaker than in untreated neutrophils (Figure 8) which could indicate that the citrullination of histones can contribute to the frequency in the generation of neo-autoantigens.

This indicates, that previously made assumptions that histone citrullination adds to antigen generation is not highly prevalent, could however play a small role. Additionally, this data shows how the oxidative burst is an essential part of NETosis and antigen generation.

4.9 Patients with suspected autoimmune disease contain antibodies against stimulated neutrophils.

After demonstrating that patients with selected ANCA-associated diseases contain antibodies against activated neutrophils (4.3), it was hypothesized that a subset of patients with other autoimmune diseases contain these antibodies as well. Therefore, we collected 320 additional serum samples which were previously tested for ANA or ANCA in the immunodiagnostics department of the UKE and added them to the already collected 80 samples. We termed these samples “UKE-Cohort”. To differentiate between anti-stimulated neutrophil antibodies (ASNA) and conventional ANCA, positive screened samples on stimulated neutrophils were additionally screened on unstimulated neutrophils. The entire cohort was screened three times to ensure the results were reproducible. All positive samples were tested for antibody dilution titer as described in *Material & Methods*. Unfortunately, 24 samples had to be disregarded for the data analysis, because of missing information from the diagnostic laboratory of the Institute of Immunology. Therefore, the UKE-Cohort contained 376 samples in total.

Table 1. Screening for anti-neutrophil antibodies in UKE-Cohort

Laboratory screening *	ANA (N=271)	ANCA (N=10)	Negative (N=95)	Total (N=376)
Titer – number of sera (%)	1:80 – 61 (22) 1:160 – 58 (21) 1:320 – 33 (12) 1:640 – 39 (14) 1:1280 – 22 (8) 1:2560 – 25 (9) 1:5120 – 25 (9) >1:5120 – 8 (9)	1:20 – 6 (60) 1:100 – 3 (30) 1:1000 – 1 (10)		
ASNA screening - number of positive sera (%) **	37 (13)	5 (50)	6 (6)	48 (13)
Laboratory screening titer of ASNA positive samples – number of positive sera (%) ***	1:80 – 6 (16) 1:160 – 4 (10) 1:320 – 1 (3) 1:640 – 6 (16) 1:1280 – 3 (8) 1:2560 – 9 (24) 1:5120 – 6 (16) >1:5120 – 2 (5)	1:20 – 1 (20) 1:100 – 3 (60) 1:1000 – 1 (20)		
ASNA titer - number of positive sera (%) ***	1:80 – 5 (14) 1:160 – 5 (14) 1:320 – 4 (10) 1:640 – 2 (5) 1:1280 – 9 (24) 1:2560 – 9 (24) 1:5120 – 1 (3) >1:5120 – 2 (5)	1:80 – 2 (40) 1:160 – 1 (20) 1:320 – 1 (20) 1:640 – 1 (20)		

Characteristics UKE-Cohort	Age (average Years)	Gender	
Entire UKE-Cohort	48,25	♂ 99 (26%)	♀ 277 (74%)
Anti-neutrophil antibody positive	53,17	♂ 13 (27%)	♀ 35 (73%)

* Screening was performed by the diagnostic laboratory of the Institute of Immunology with a request for quantification of ANA or ANCA

** Screening was performed as described in Material and methods three times

*** Compared to the cohort of ASNA positive samples

The entire UKE-Cohort contained 271 ANA positive samples containing titers from 1:80 up to >1:5120. Additionally, 10 C-ANCA positive samples were identified. No P-ANCA positive samples were screened. In total, 95 samples were screened ANA and ANCA negative by the diagnostic laboratory of the Institute of Immunology. Age and gender, but no clinical information on the patients were acquired. The average age was 48,25 years and the ratio female to male was 74:26 %. Considering that the ratio for lupus erythematosus is 4:1 (female to male) in Germany, this is can be considered an acceptable and semi-representative cohort (Kuhn et al. 2015).

All samples were screened for anti-stimulated neutrophil antibodies (ASNA). In total 48 serum samples showed a positive screening on stimulated neutrophils indicating, that approximately 13 % of the sera of the UKE-Cohort contain antibodies against stimulated neutrophils. Out of the 271 ANA positive samples, 37 (13 %) contained antibodies against stimulated neutrophils.
Out of the 10 ANCA positive samples, ASNA screening only detected antibodies in 5 samples (50 %). Although it had been previously demonstrated, that ASNA screening can detect C-ANCA, these samples were screened negative. The 5 samples which were screened negative on stimulated neutrophils, all had a C-ANCA antibody titers of 1:20. This could imply, that detecting low antibody titer on stimulated neutrophils is less sensitive than on naïve neutrophils. However, low titers such as 1:20 rarely have clinical significance, so that this diagnostic deficiency can be disregarded.
Anti-neutrophil antibodies were detected in 6 (6 %) sera with a negative screening for ANA or ANCA, respectively. This could indicate, that ASNA screening can add diagnostic value to autoantibody screening and detects autoantibodies previously missed by using naïve neutrophils. However, larger cohorts are needed to safely make this assumption. Also, we must consider clinical information when detecting antibodies which in this case was not available.

If we compare the titers of ASNA and ANA positive samples, 24 (64 %) of them correlate, meaning they are equal or only differentiate in one dilution step. This suggests, there is a correlation between antibody titers of ANA and ASNA antibodies. However, antibody titers mostly do not correlate with disease activity or severity, so the implication of antibody titer correlation remains unclear. If we compare the characteristics of the different cohorts, ASNA positive samples are more likely to be

found in older patients (53,17 years). Considering for example that GPA can occur at any age but new-onset commonly occurs in patients over 50, whereas lupus erythematosus usually occurs at a younger age around 20-40 years this supports the assumption, that ASNA are associated to ANCA-associated diseases more than ANA associated diseases (Jennette and Nachman 2017, Kuhn et al. 2015).
Conclusively, antibodies against stimulated neutrophils are prevalent in approximately 13 % of sera with suspected autoimmune disease and ~6 % of sera with negative screening for ANA and ANCA contain them. The clinical indication however is currently unknown and should be subject to further investigation.

4.10 Neutrophil stimulation produces distinct staining patterns.

It was concluded that the novel antibodies against structures of stimulated neutrophils could represent a new diagnostic parameter and an antibody which has not previously been identified. Therefore, these antibodies were termed anti-stimulated neutrophil antibody (ASNA). A detailed microscopic analysis of the staining of ASNA revealed distinct patterns (Figure 10).

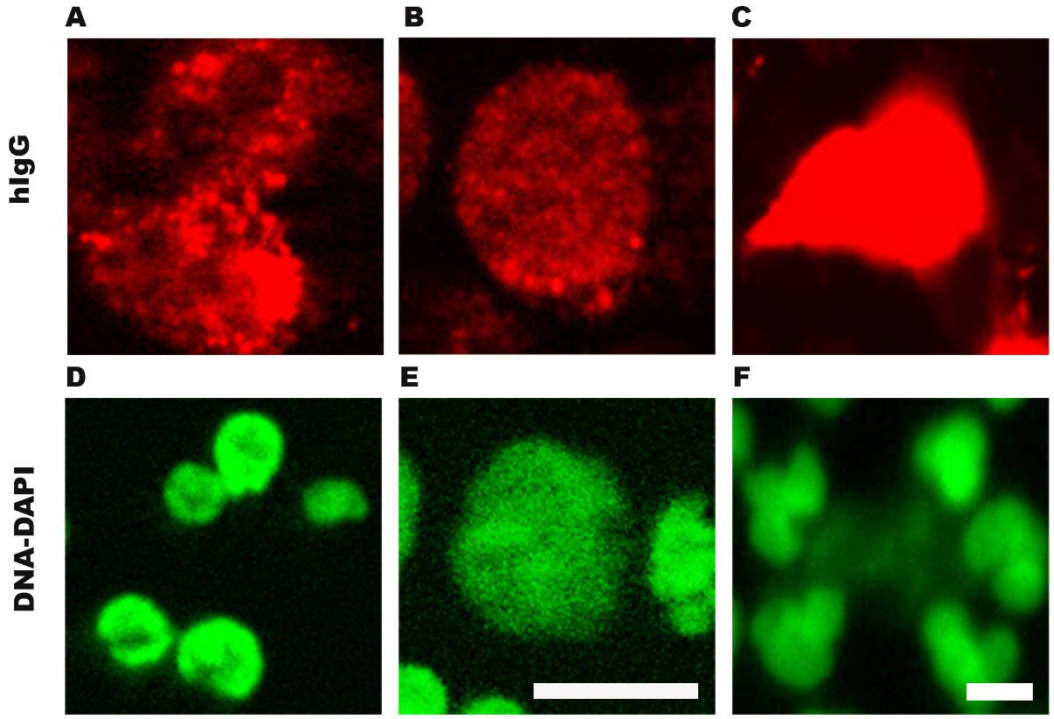

Figure 10 Representative ASNA-staining patterns. Purified neutrophils were stimulated with PMA and incubated with serum which contained antibodies against

stimulated neutrophils. **(A-C)** IIF of hIgG (**A**) Cytoplasmic staining in neutrophils activated with PMA for 60 minutes. (**B**) Whole cell staining in neutrophils activated with PMA for 120 minutes. (**C**) Staining of NETs released by neutrophils after 180 minutes of PMA activation. **(D-F)** DNA staining by DAPI. Bar equals 10 µm.

Few selected sera stained the cytoplasm of neutrophils, which were activated for only 60 minutes with PMA (Figure 11 A). These cells are viable and have intact nuclei. The cytoplasmic staining is punctuated suggesting that the antigen is stored in vesicles. In analogy to the ACNA nomenclature, we term this staining pattern C-ASNA. We also observed a staining of whole cells, when neutrophils were stimulated for at least 120 minutes (Figure 11 B). Under these conditions, nuclei are dissolved, and the nuclear and granular components mix inside viable neutrophils (Fuchs et al. 2007). This pattern is named W-ASNA because of the wholistic staining of the entire neutrophil. We also detected a prominent staining of NETs in selected sera (Figure 11C). NETs are generated by neutrophils, which were stimulated for 180 minutes with PMA. The staining pattern is localized in the extracellular space and was therefore labeled as X-ASNA. Importantly, anti-NET-antibodies (X-ASNA) were mainly detected in patient sera, which were positive of P-ANCA and therefore most likely target MPO or similar proteins.

To conclude, this data suggests that ASNA has a subset of targets which remain to be identified in future experiments. However, the data suggests that these antibodies do not exclusively detect ANCA and therefore NETotic neutrophils provide a novel diagnostic tool for the stratification of autoimmune diseases.

5 Discussion

5.1 Activated neutrophils can be used as a substrate for ANCA testing

ANCA were first detected in chronic inflammatory disease in 1959 (Calabresi et al. 1959) and first described by two Australian groups in necrotizing and crescentic glomerulonephritis or unclassified vasculitis in 1982/84 (Van Der Woude et al. 1985). The international consensus statement agrees, that currently IIF on ethanol-fixed unstimulated neutrophils is still the best assay available for ANCA testing. If the IIF is positive, an ELISA for MPO- and PR3-antibodies should immediately follow. Further requirements are trained and experienced staff, working in an ANCA-reference laboratory (Savige et al. 1999). These guidelines only allow ANCA-screening a very niche usefulness in clinical testing. It is still mainly used for accurate identification of AAV and the avoidance of misdiagnosis (Hagen et al. 1998).
ANCA detection is a major diagnostic clue to secure rapid diagnosis and treatment in SVV, and most patients with MPA and GPA present with ANCA (Lionaki et al. 2012). However, only about half of EGPA patients test positive for them (Kallenberg et al. 2006). Therefore, ANCA associated conditions do not require a positive ANCA screening as a diagnostic criterion. If ANCA is not detected in AAV patients, this is referred to as “seronegative” AAV. Studies suggest that, for example, ceruloplasmin, which is responsible for the copper transportation and is an acute phase protein, can bind to MPO which leads to false negative results in ANCA testing (Roth et al. 2013). This may explain some of the seronegative AAV patients.

A key flaw of the current ANCA technique is, that it relies on an in-vitro caused artifact created through ethanol fixation. In its physiological condition, both MPO and PR3 are present in the primary granules of neutrophils. Ethanol disrupts the granular membrane, resulting in the translocation of MPO to the perinuclear region. Therefore, ethanol fixation imitates NETosis where granular enzymes are released into the cytoplasm and mix with nuclear components. However, our data seems to diverge from previous articles, which have shown that PR3 is present in NETs through mass spectrometry or IIF (Kessenbrock et al. 2009, Urban et al. 2009). We do not reject the hypothesis that low amounts of PR3 can be found in NETs, however the discovery that

PR3 does not translocate to the nucleus during NETosis backs our findings that the amount of PR3 in NETs is comparably small (Papayannopoulos et al. 2010) .

Until now the focus for improving ANCA diagnostics has been mainly through two different approaches. Firstly, the addition of novel, antigen specific immunoassays. Direct ELISA was developed in the late 1990s and since then has been continuously improved, while the second generation, so called “capture ELISA” and third generation, named “anchor ELISA” or “high sensitivity ELISA” (hsELISA), are in use in clinical practice today (Holle et al. 2005, Hellmich et al. 2007). Secondly, through the identification of new staining patterns using computer algorithms with limited success to date (Willitzki et al. 2012, Melegari et al. 2012, Knutter et al. 2012). Also, new antibodies against neutrophils have been found in seronegative AAV patients. Anti-pentraxin 3 (PTX3) antibodies were detected in 50% of MPO and PR3 ANCA negative AAV patients (Simon et al. 2016). This indicates that the potential for new antibodies directed against neutrophils is not fully explored. Therefore, it is feasible that antibodies may target other neutrophil structures which are only expressed in certain cellular compartments and stages (e.g. NETosis).

Activated neutrophils may offer an advantage over unstimulated ethanol-fixed neutrophils. This thesis explores this additional path of detecting antibodies targeting neutrophils by using stimulated neutrophils as a substrate. Activated neutrophils have only recently been used as a substrate for ANCA diagnostics by our team, showing that P-ANCA mainly target NETs and C-ANCA mainly target cell bodies of netting neutrophils (Panda et al. 2017).The data presented in this thesis further confirms that stimulated neutrophils can be used to identify ANCA (Figure 3). While conventional ANCA screening relies on an artifact of the ethanol fixation method to distinguish between P-ANCA and C-ANCA, stimulated neutrophils provide a more expansive surface for antigen binding, making pattern recognition easier which reduces the experience needed by evaluating staff, considering that the differences between the staining patterns is greater than the microscopic identification on a cellular level in conventional ANCA-screening. Additionally, the new assay would make it easier for automated pattern recognition software to differentiate these patterns. However, additional studies using sera from large patient cohorts are required for identifying a

diagnostic benefit of NETs and/or NETotic neutrophils compared to conventional ANCA testing.

In conclusion, conditions closer to the hypothesized physiological formation of antigens, such as NETs and NETotic neutrophils, could provide an important improvement in the evaluation of AAV and other ANCA-associated diseases. Also, our newly developed assay using stimulated neutrophils may reduce labor intensity and lower the possibility of misdiagnosis through human error and may simplify automated pattern recognition through software to advance ANCA diagnostics.

5.2 Stimulated neutrophils create neo-autoantigens

Neutrophils produce NETs to contain and fight infections through external pathogens (Brinkmann and Zychlinsky 2007). They are recruited to infection sites and are present in inflammation but have also been associated with autoimmune diseases and autoantibodies (Jorch and Kubes 2017, Delgado-Rizo et al. 2017). Furthermore, patients with systemic vasculitis often contain autoantibodies against neutrophil proteins (Savige et al. 1991). We propose that defects and deregulation in NETosis leads to autoantigen generation and therefore triggers loss of tolerance and leads to autoimmune diseases (Khandpur et al. 2013, Dwivedi and Radic 2014).

In this thesis we demonstrate, that seronegative-AAV sera contain antibodies against stimulated neutrophils (Figure 4). These findings support the hypothesis that NETosis is linked to the generation of autoantigens and the production of autoantibodies. We showed that autoantigens develop in specific stages of NETosis after the granular content has been released into the cytoplasm (Figure 5 and 6) and may be influenced by enzyme reactions such as histone citrullination (Figure 7). This confirms the hypothesis that mixing of granular and nuclear components may lead to protein alteration and generation of autoantigens and supports the observation that the citrullination of autoantigens implicates NETosis in the induction of autoimmunity (Dwivedi and Radic 2014). NETs are created in reaction to a stimulus such as infections and expose chromatin and neutrophil proteins at locations of inflammation. They require ROS (Fuchs et al. 2007) which modify DNA and proteins, producing more immunogenic particles (Kurien and Scofield 2008, Griffiths 2008). Dendritic cells have

been shown to absorb NET components and further prime the immune system to increase autoimmunity (Dieker et al. 2016). Also, anti-NET antibodies have been shown to impair NET degradation in SLE. This reduced impairment correlates with organ damage in lupus nephritis (Hakkim et al. 2010).

There was no diagnostic information on patients analyzed in this study. The only clinical information given to us was whether they have tested positive for ANA or ANCA. Further prospective studies could analyze the question of which diseases produce ASNA and how they correlate with disease activity.

Interestingly, we observed a correlation between autoimmunity and antibodies against stimulated neutrophils exist. While only about 6 % of ANCA and ANA negative sera contained antibodies against stimulated neutrophils, approximately 13 % of patients containing ANA also produce ASNA. The correlation in ANCA positive samples is even higher (Table 1). We propose, that the overlap is created by faulty and insufficient clearance of NETs through diminished DNase1 degradation, leading to chronic inflammation and later anti-NET-antibodies, blocking the degradation of NETs. If the activity of this nuclease is reduced or obstructed, NETs might persist and create a source of autoantigens. NETs contain nuclear-antigens, neutrophil enzymes and other NETotic components, which are all targets of autoantibodies. The prolonged exposure of these autoantigens may lead to the formation of immune complexes, which have been shown to induce antibody production (Lande et al. 2007, Leadbetter et al. 2002). Immune complex deposition in the kidneys can be observed in SLE as well as SVV (Hakkim et al. 2010, Kessenbrock et al. 2009), leading to organ damage in both diseases.

We suspected that ASNA may not represent one singular neo-autoantigen, therefore, we closely analyzed the ASNA positive samples and identified three distinct patterns which we termed analogous to the ANAC nomenclature.

Our results provide an additional link between NETosis and autoimmunity. This correlation might help in the development of better diagnostics and therapeutic interventions for AAV and other autoimmune conditions such as SLE and RA.

5.3 ASNA may be used as a screening parameter for ANCA-associated diseases

Autoantibodies remain a crucial part of clinical testing in rheumatic and inflammatory diseases. They aid physicians in providing a diagnosis/prognosis or serve as follow-up markers for patients. However, like any laboratory test, the interpretation is dependent on the clinical presentation and the characteristics of the test at hand (Aggarwal 2014). Autoantibodies can be directed against any number of cellular components and are used in a multitude of situations.
ANA are used as a screening test for a multitude of connective tissue diseases. These include SLE, systemic sclerosis, rheumatoid arthritis, primary Sjögren's syndrome, polymyositis and mixed connective tissue disease (MCTD). It identifies antibodies against all nuclear antigens including DNA- and RNA-associated proteins, centromere, nuclear membrane as well as nucleoli (Stinton and Fritzler 2007, Damoiseaux et al. 2014). It can be used so widely because it provides binding sites to several more specialized autoantibodies which all show up positive in ANA screening and are tested if ANA are detected.
To further understand the frequency of ASNA in patients with autoimmune diseases, we collected 400 serum samples in total. We screened these samples both for ANCA and ASNA. Out of 48 ASNA positive samples and 6 of these samples were classified as ANA and ANCA negative (Table 1). This suggests there is a significant amount of serum samples containing antibodies against neutrophils which could not be detected by conventional screening methods. ASNA detects traditional ANCA and therefore has no disadvantage to testing on unstimulated neutrophils (Figure 3), except for a slightly reduced sensitivity on low titer sera (Table 1). Also, ASNA was detected in samples which were negative for ANCA and ANA screening (Figure 4).

Analogous to ANA, ASNA could provide a similar role in the screening for ANCA associated diseases, because stimulated neutrophils provide additional binding sites compared to conventional ANCA testing. Similar to the ANCA patterns, we can identify and describe ASNA patterns (Figure 10). However, further investigations will have to comprehensively determine if there is a clinical advantage to knowing the ASNA status of a patient with an ANCA associated autoimmune disease, yet the data indicates it could provide additional information conventional autoantibody testing does not yet allow.

5.4 Outlook

The main intention of this project was to detect, whether stimulated neutrophils generate neo-autoantigens which could not be detected with conventional ANCA-screening assays. Even if it is currently unclear if a positive ASNA screening provides additional diagnostic information, ANCA screening on stimulated neutrophils has advantages, such as easier interpretation and not relying on an in vitro caused artifact but using pathophysiological relevant substrates. Therefore, using NETs or NETotic neutrophils could be an important contribution to the diagnostic repertoire in the assessment of AAV and other ANCA-associated diseases.

However, the data shown provides a proof of concept, indicating that there is an antibody present which was previously not measured by other tests. This could assist in understanding the linkage between neutrophils, NETs and the associated autoimmune conditions. It still needs to be considered that ASNA may be an artifact of NETosis or provoked by methods used during experiments, even if efforts were made to ensure to prove NETosis is the key trigger for the generation of neo-autoantigens.

However, the data encourages further investigation in the area rather than weakening our hypotheses. Therefore, it can be concluded that ASNA should be investigated more to further strengthen the initial hypothesis that activated neutrophils provide a novel tool for autoimmune disease diagnostics.

6 Summary

Neutrophils granulocytes are the most common and, arguably, the most important front-line immune cell of the human body. They represent a crucial part of the innate immune system, capable of important functions in protecting the body from foreign intruders.

They can also produce NETs, which are formed in a unique way of cell death, discovered in 2004 and classified as an additional form of cell death next to apoptosis and necrosis (Brinkmann et al. 2004, Fuchs et al. 2007). NETs have recently been associated with autoimmune diseases (Pinegin et al. 2015, Lee et al. 2017). However, the role which NETs play in the pathophysiology of these diseases, and the consequences of these findings, largely remains unclear. Neutrophils are already used as a substrate to detect ANCA which are associated with small-vessel vasculitis. However, the detection of ANCA is based on the screening of patient sera using indirect immunofluorescence on ethanol-fixed unstimulated neutrophils.

This thesis shows, that NETosis has three distinct stages and NETotic neutrophils can be used as substrate for ANCA testing. We also demonstrated, that NETosis generates neo-autoantigens. These neo-autoantigens develop over time in stimulated neutrophils and the frequency is affected by different stimuli. Also, the autoantigen generation is dependent on specific neutrophil enzymes. Furthermore, we detected antibodies against stimulated neutrophils in patient sera with suspected autoimmune disease and other preformed antibodies such as ANCA and ANA. These new antibodies stain stimulated neutrophils in distinct patterns analogous to the different staining patterns of ANCA.

In conclusion, these results suggest that antibodies against activated neutrophils could play a role in the previously mentioned linkage between NETs and autoimmunity and are deserving of further investigation in the future. Further studies should investigate serum samples of patients with an already diagnosed autoimmune disease associated to neutrophils (e.g. GPA, MPA, EPA). Furthermore, the molecular target of antibodies against NETs should be identified and an ELISA with the targets of ASNA should be established.

Neutrophile Granulozyten sind die häufigsten und vermutlich eine der wichtigsten Abwehrzellen des menschlichen Körpers. Sie repräsentieren einen der bedeutsamsten Teile des angeborenen Immunsystems, welche eine Vielzahl von Funktionen ausübt, um den Körper vor fremden Eindringlingen zu beschützen.

Sie können NETs produzieren, welche in einer einzigartigen Form des Zelltods entstehen und nach deren Entdeckung im Jahre 2004 als zusätzliche Form des Zelltods neben Apoptose und Nekrose klassifiziert wurde (Fuchs et al. 2007, Brinkmann et al. 2004). NETs werden zunehmend mit Autoimmunerkrankungen assoziiert (Pinegin et al. 2015, Lee et al. 2017). Die genaue Rolle, die NETs in der Pathophysiologie dieser Erkrankungen spielt und die Konsequenzen dieser Assoziationen bleiben größtenteils unklar. Neutrophile werden bereits als Substrat für ANCA Diagnostik genutzt, welche mit Vaskulitiden der kleinen Gefäße assoziiert sind. Die Detektion von ANCA basiert auf indirekter Immunfluoreszenz auf unstimulierten, Ethanol fixierten neutrophilen Granulozyten.

Diese Doktorarbeit zeigt, dass NETose drei separate Phasen aufweist und NETotische Neutrophile als Substrat auf ANCA Diagnostik genutzt werden können. Wir zeigen ebenfalls, dass NETose neo-Autoantigene produziert. Diese neo-Autoantigene entwickeln sich im Verlauf der NETose und die Frequenz dieser Antigene wird durch unterschiedliche Stimulantien beeinflusst. Weiterhin ist die Entstehung von Autoantigenen abhängig von bestimmten neutrophilen Enzymen. Zusätzlich konnten wir Antikörper gegen stimulierte Neutrophile in Patientenseren detektieren, welche den Verdacht auf eine systemische Autoimmunerkrankung hatten und für ANCA oder ANA untersucht wurden. Diese neuen Autoantikörper lassen sich auf stimulierten Neutrophilen nachweisen und weisen ein spezifisches Färbemuster auf, welche analog zu der ANCA Klassifizierung eingeordnet werden kann.

Zusammenfassend deuten diese Ergebnisse an, dass Antikörper gegen stimulierte Neutrophile eine Rolle in dem vorher erwähnten Zusammenhang zwischen NETs und Autoimmunität spielen könnten und dies weiterer Untersuchungen bedarf. Künftige Analysen sollten Seren von Patienten mit bereits diagnostizierten Erkrankungen Untersuchen, die mit ANCA assoziiert sind (bspw. GPA, MPA, EPA). Weiterhin sollten die molekularen Epitope der Antikörper gegen NETs identifiziert werden, um einen entsprechenden ELISA entwickeln zu können.

7 List of abbreviations

ABBREVIATION	ORIGINAL
ACPA	Anti–citrullinated protein antibody
AIH	Autoimmune hepatitis
AMA-M2	Anti-mitochondrial antibody m2
ANA	Anti-nuclear antibody
ANCA	Anti-neutrophil cytoplasmic antibody
ASNA	Anti-stimulated neutrophil antibody
BSA	Bovine serum albumin
Cy3	Cyanin 3
DAPI	4’,6’-diamidino-2-phenylindole
DFP	Diisopropyl Fluorophosphate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodium Chloride
EDTA	Ethylenediaminetetraacetic acid
EGPA	Eosinophilic granulomatosis with polyangeitis
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular-signal-regulated kinases
GCSF	Granulocyte-Colony Stimulating Factor
HBSS	Hanks buffered salt solution
hIgG	Human immunoglobulin G
HIV-1	Human immunodeficiency virus-1
Ig	Immunoglobulin
IgE	Immunoglobulin E
IIF	Indirect Immunofluorescence
IL	Interleukin
LPS	Lipopolysaccharide
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
PAD	Peptidyl arginine deiminase

PBC	Primary biliary cirrhosis
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocytes
PR3	Proteinase 3
PSC	Primary sclerosing cholangitis
PSGL	P-selectin glycoprotein ligand
RBC	Red blood cell
ROS	Reactive oxygen species
RT	Room temperature
SLE	Systemic lupus erythematosus
SMA	Smooth muscle cell antibody
SVV	Small vessel vasculitis
TNF	Tumour necrosis factor
UKE	University Medical Center Hamburg-Eppendorf
Umif	UKE Microscopy Imaging Facility

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10. Resume

Lebenslauf wurde aus datenschutzrechlichen Gründen entfernt

11. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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