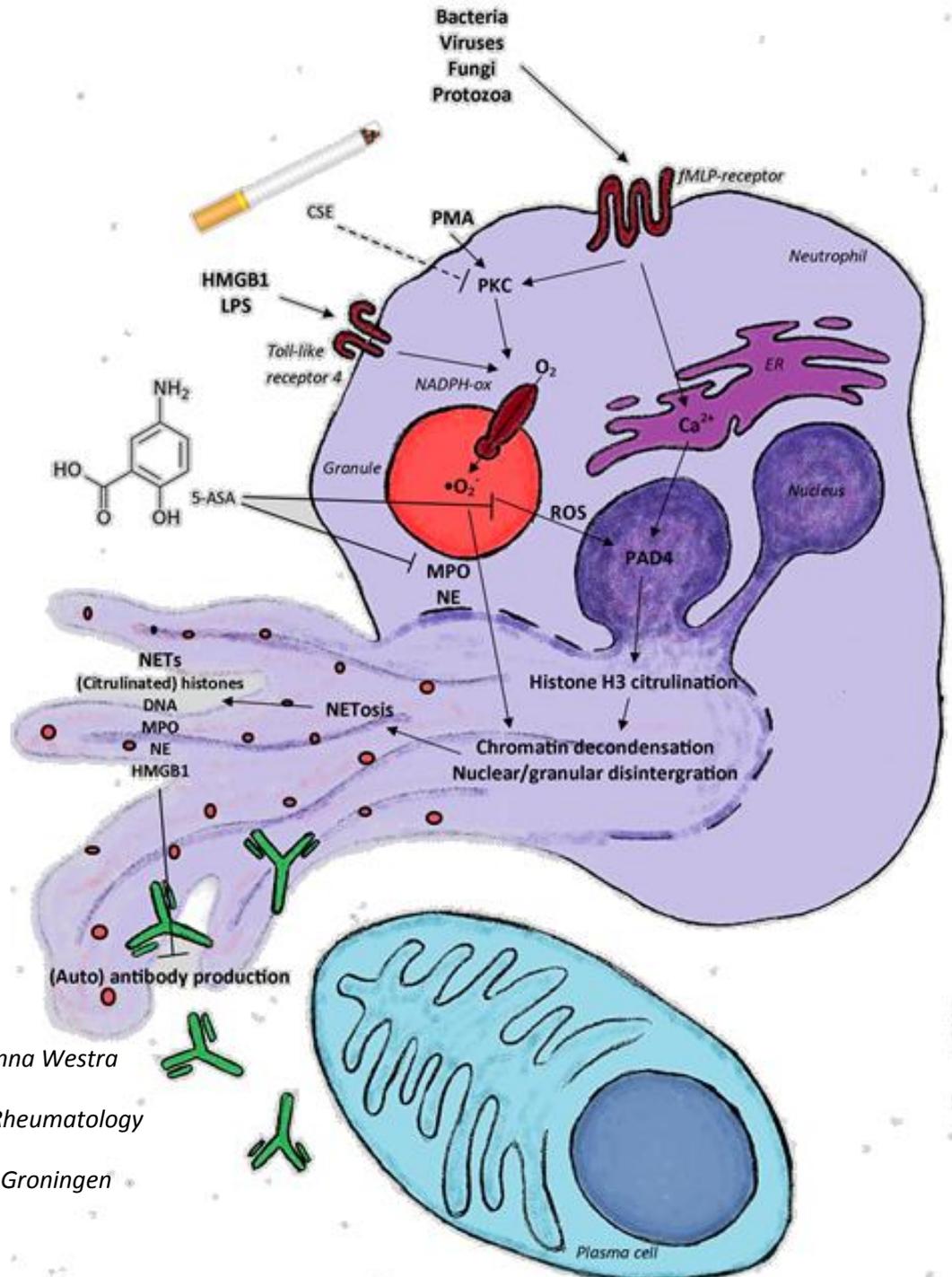


Netting Neutrophils

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IgG production after B-cell stimulation with NETs



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Clinical Immunology and Rheumatology

University Medical Centre Groningen

Groningen, 3 June 2014



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Place and date: Groningen, 3 June 2014

“Equipped with his five senses, man explores the universe around him and calls the adventure Science.”

~Edwin Powell Hubble (1954)

“Imagination is more important than knowledge. For knowledge is limited...”

~Albert Einstein (1879-1955)

Summery

NETosis is a form of cell death used by neutrophils to trap microorganisms. NETosis is induced by microorganisms, but also by inflammatory stimuli like cytokines, antibodies and HMGB1. The formation of NETs is dependent on a correct working of the NADPH-oxidative complex and the formation of ROS. NETosis plays a role in innate immunity, but has also a pathogenic role in auto-immune diseases like SLE and UC. NETs form a pool of auto-antigens because NET components like DNA, histones, and granular proteins are available in the extracellular space. This makes it easy for auto-antigens to bind. A hypothesis is that NETs can directly stimulate B-cell to produce (auto)antibodies. NETosis could play a crucial role in the manifestation of auto-immune diseases, and therefore it is important to study this subject. Therapeutics that can inhibit NETosis might be necessary. From this study can be concluded that NETs are formed after 3 hours, but change in form and composition after 4 hours. After 4 hours of stimulation MPO leaves NETs and the NETs have a more dread-like structure compared to the cloud-like structure of NETs after 3 hours of stimulation. NETosis can be induced by PMA, LPS and HMGB1 in healthy controls. Neutrophils from SLE patients show a higher spontaneous NETosis, but the difference with healthy controls is not significant. This may be caused by the inclusion of quiescent patients in this study. Neutrophils from SLE patients form NETs after stimulation with PMA, but not after stimulation with LPS or HMGB1. This could be caused by the medication used by the patients. NETosis could be inhibited by 5-ASA and Cigarette Smoke Extract (CSE), but not by prednisolone or hydroxychloroquine. After pre-treatment with 5-ASA the amount of NETs is decreased, but neutrophils do show signs of activation. The cells are stretched-out and have a round nucleus. CSE paralyzes neutrophils and inhibits NETosis in an early state of the signal transduction route. Neutrophils pre-treated with CSE maintain their round shape and polymorph nucleus. It is important to find the working compound and the working mechanism behind CSE. NETs do not increase the amount of IgG found in B-cell supernatants. It could be the case that NETs inhibit antibody production of B-cells, but other reasons like the trapping of IgG by NETs should be excluded.

Samenvatting

NETose is een vorm van celdood waarbij neutrofielen micro-organismen kunnen vangen. NETose wordt geïnduceerd door micro-organismen, maar ook door inflammatoire stoffen zoals cytokines, antilichamen en HMGB1. Voor het maken van NETs is de werking van NADPH en de formatie van ROS noodzakelijk. NETose speelt een rol in onze humorale afweer, maar heeft ook een pathologische rol in auto-immuunziekten zoals SLE en UC. NETs vormen een bron van auto-antigenen, omdat NETs bestaan uit DNA, histonen en granulaire eiwitten die in de extracellulaire ruimte liggen. Hierdoor kunnen er gemakkelijk auto-antilichamen aan binden. Een hypothese is bovendien dat NETs direct B-cellen zouden kunnen activeren en aanzetten tot het maken van (auto)antilichamen. NETose zou wel een eens een cruciale rol kunnen spelen in auto-immuun ziekten, en daarom is het belangrijk de rol van NETose in deze ziekte te onderzoeken. Daarnaast is het belangrijk om middelen te vinden die NETose tegengaan. In dit onderzoek blijkt dat NETs na 3 uur gevormd worden, maar na 4 uur van vorm en compositie veranderen. Na 4 uur verdwijnt MPO uit de NETs en zijn NETs meer draderig van structuur. Na 3 uur zijn ze meer wolk-vormig. NETose kan worden geïnduceerd door PMA, LPS en HMGB1 in gezonde controles. Neutrofielen van SLE patiënten laten een wat hogere spontane NETose zien, maar deze is niet significant hoger dan gezonde controles. Waarschijnlijk komt dit omdat er in dit onderzoek bloed is gebruikt van rustige patiënten. Bij neutrofielen van SLE patiënten word NETose geïnduceerd door PMA, maar niet door LPS en HMGB1. Dit zou aan medicatie van de patiënten kunnen liggen. NETose kan worden geremd door 5-ASA en door Cigarette Smoke Extract (CSE), maar niet door prednisolone of hydroxychloroquine. Na een voorbehandeling met 5-ASA is de hoeveelheid NETs verminderd, maar de neutrofielen tonen wel tekenen van activatie. De cellen zijn niet rond en de kernen hebben geen polymorfe vorm. CSE verlamd neutrofielen, en remt NETose al in het begin van de signaaltransductie route. Neutrofielen blijven rond en behouden hun polymorf vormige kern. Het is daarom van belang dat de werkzame stof en het werkingsmechanisme van CSE gevonden wordt. NETs blijken niet in staat om de hoeveelheid IgG in supernatant van een B-cel kweek omhoog te brengen. Het kan zijn dat NETs de antilichaam productie van B-cellen remmen, maar andere oorzaken zoals het vastplakken van IgG in de NETs moet nog uitgesloten worden.

Preface

It is not always easy to choose the right study when you're young. Everyone has to find out what fits them, and that may take a while. The lab is, for now, a nice habitat for me. I'm a turbulent person; I can't sit still all day. I like working with my hands, and as a teenager I was concerned about how I was going to combine that with my intelligence. As a kid I wanted to become a forester, but I doubt the challenge in that job. I want to use both my hands in the practical work, and both my brain hemispheres in designing experiments and interpreting results. My personality is curious and eager to learn, visual, with a lot of fantasy. I want to discover and understand the world around me. The questions *why* and *whereby* emerge every day again. I've never done things just because people tell me to, I always want to know why, I always want to understand the things I'm busy with. This has led to a curious, critical and sometimes a stubborn mind.

Imagination is a key in science and generating knowledge. Where would we be without imagination? We have to imagine how things could work, imagine hypothesis and then try to find out if we can find scientific evidence for it. Imagination is unlimited, as Einstein told us; you can imagine everything you want. Knowledge otherwise, is limited because we can't find scientific evidence for all our hypotheses.

Science has always been a model for me. I don't believe science will bring us the truth about everything around us one day. Maybe we don't even come close, who will say. As long as we are part of the world, and part of our own science, we never can have a real objective view. The rules for scientific evidence are quite clear. We can investigate things and generate more knowledge that fits inside these rules. This system is a useful model; we can investigate more and more, we are solving problems, and cure more and more diseases. But I doubt these findings really cover the truth. Some people feel better because they think they felt the hand of God, others because they get a medicine, even though it is a placebo. We only have our five senses to discover the world around us, nothing more, and nothing less. Science already told us that we can't trust our senses all the time. Quite a paradox I believe! Even though I don't believe science brings us closer to the truth, I really enjoy dissolving the problems, riddles, and mysteries in the medical sciences, enjoy finding new pieces of the puzzle and make them fit.

During this internship I found some new pieces of the puzzle, and I regrettably have no more time to puzzle further right now. I really enjoyed the time spending here. I am thankful for the opportunity to work independent and learn new techniques in the lab. First I want to thank Hannie Westra for her supervision and guidance during my internship and Piet Limburg for his critical view. I also want to express my appreciations to Gerard Dijkstra for his enthusiasm and challenging research proposals. I want to thank Alja Stel and Karina de Leeuw for their effort to collect patient's blood. And last but not least; thanks to all the people on the lab for their alacrity and support, in special Gerda Horst, Johan Bijzet and Tjasso Blokzijl.

List of abbreviations

5-ASA	5-aminosalicylic acid
ANCA	Anti-neutrophil cytoplasmic antibodies
APC	Allophycocyanin
AU	Arbitrary units
BAFF	B-cell activating factor family
BCR	B-cell receptor
BSA	Bovine serum albumine
CpG	-C-phosphate-G-sites
CSE	Cigarette smoke extract
DAPI	Diamidino-2-phenylindole
DNase	Deoxyribonuclease
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
fMLPr	Formyl peptide receptor
HBSS	Hank's balanced salt solution
HCQ	Hydroxychloroquine
HMGB1	High-mobility group protein B1
IBD	Inflammatory bowel disease
IFN	Type I interferon
IL-21	Interleukin-21
LPS	Lipopolysaccharide
MNase	Micrococcal nuclease
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAD4	Peptidylarginine deiminase 4
PBMC	Peripheral blood mononucleated cell
PBS	Phosphate buffered saline 5
pDCs	Plasmacytoid dendritic cells
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PDN	Prednisolone
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
rpm	Revolutions per minute
SLE	Systemische lupus erythematodes
TLR 4/9	Toll like receptor 4/9
UC	Ulcerative colitis

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1. Introduction: Neutrophil Extracellular Traps

1.1 Neutrophils

Neutrophils are polymorphonuclear granulocytes and have a short lifespan of four days in the circulation. Neutrophils have various types of granules, containing hundreds of proteins. This includes anti-bacterial proteins, some of them with important effects on the innate and adaptive immune response of the body (1). Neutrophils form the first defense against microbes in response to chemoattractive factors released by pathogens or host cells, and are rapidly recruited from the bloodstream to the site of infection. Neutrophils leave the blood vessel after attachment to the endothelium and move to the site of infection (2). Neutrophils have three main strategies to eliminate microbes; phagocytosis, generation of reactive oxygen species (ROS) and the release of granules (degranulation). In 2004 a new mechanism was discovered: the formation of neutrophil extracellular traps (NETs), so-called NETosis (2, 3, 4). See [Figure 1](#).

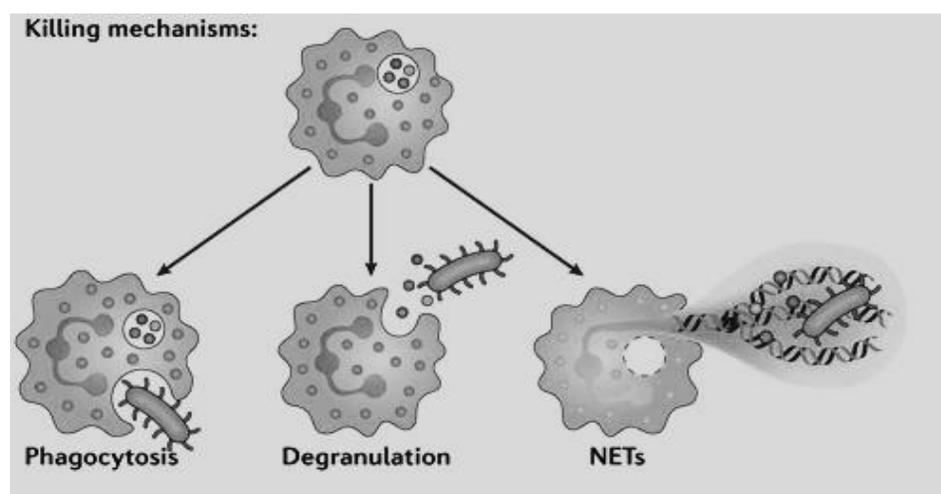


Figure 1, Three main killing mechanisms of neutrophils; phagocytosis, degranulation and NETosis. During phagocytosis the microorganism is absorbed in a granule and killed with enzymes from granules. During degranulation the neutrophil exocytoses granular enzymes that kill the microorganism outside the cell. During NETosis a neutrophil extracellular traps is composed, that traps the microorganism and kills it. Source: (5)

1.2 NETosis

NETosis was first reported by the group of Brinkman and Zychlinsky in 2004. (6)NETosis is a specific type of cell death different from both necrosis and apoptosis. In contrast to apoptotic cells, netting neutrophils do not appear to show "eat-me" signals, and this may prevent their clearance by phagocytes. While phagocytosis and degranulation usually take minutes to occur after stimuli, neutrophils release nets 2-4 hours after stimulation (1, 4). The most important feature specific to NETs is the presence of neutrophil nuclear DNA fibers in the extracellular space. NETs consist of smooth filaments with a diameter of ~17nm composed of DNA and histones, and of a globular domain with a diameter of ~50nm made of granular proteins (6). Other cell types like mast cells, basophils and macrophages are also able to form extracellular traps (7). NET formation is placed in a context of innate immunity, but has also a role in various diseases. Recently is discovered that NETs may also play a role in the stabilization of an inflammation by trapping and degrading pro-inflammatory mediators in

aggregated NETs(8). Several studies suggest a pathophysiological role of NETs and NET components in autoimmune diseases such as small-vessel vasculitis, lupus nephritis, systemic lupus erythematosus (SLE), psoriasis, and rheumatoid arthritis (RA)(9, 10).

1.3 Role of NETosis in Systemic Lupus Erythematosus and Ulcerative Colitis

NETs may function as effective antimicrobial defenses, but may also promote tissue damage and autoimmunity. NETosis is one of the naturally occurring immune responses, and has opened new possibilities of understanding the mechanism that lead to break of tolerance and the promotion of autoimmunity. Several molecules in NETs (like MPO, NE and HMGB1) are auto-antigens in autoimmune diseases like vasculitis and Systemic Lupus Erythematosus (SLE) (1,11-14).

SLE is a systemic autoimmune disease characterized by autoantibody formation against nuclear antigens. Other known auto-antibodies are against neutrophil proteins like lactoferrin, myeloperoxidase (MPO), and neutrophil elastase (NE). Antibodies against these granular proteins are known as anti-neutrophil cytoplasmic antibodies (ANCA) (3, 15, 16). SLE patients display both innate and adaptive immune alterations that affect blood cell composition. They have decreased numbers of neutrophils, dendritic cells (DCs), and lymphocytes. Although plasmacytoid DCs (pDCs) are decreased in the blood of patients, they accumulate at sites of inflammation such as the skin and the kidney, where they secrete type I interferon (IFN)(17). The IFN pathway lowers the threshold for autoreactivity of both antigen-presenting and antibody-producing cells. pDCs are the most important source of IFN α in SLE and are activated by circulating immune complexes (3).

SLE patients have an increased NET production and a decreased ability to degrade NETs. DNase (deoxyribonuclease) I is the most important degrader of NETs in human serum and it is inhibited by globular actin. Globular actin may be increased in SLE patients because it is released by platelets and dying cells during inflammation. Also auto-antibodies against DNA can cross react with DNase leading to inhibition (3,18). During SLE, NETs form a pool of auto-antigens and stimulate auto-antibody production (11). The presence of NETs during SLE and other auto-immune diseases might play a key role, and therefore it is important to investigate the role of NETs more closely. A better understanding of the influence of NETs might lead to new and better possibilities for treatment and diagnosis.

Ulcerative colitis (UC) is a chronic disease characterized by a continuous inflammation of the colon and presents rectal bleeding and lower abdominal pain. Like other autoimmune diseases there are antibodies found against anti-neutrophil cytoplasmic proteins (ANCA), and against goblet cells in the colon (19). During ulcerative colitis neutrophils are present in the crypts abscesses in the colon and are able to format NETs. The role of these NETs is still unknown, but might play a key role in the disease (20).

1.4 Mechanism of NETosis

The last years the mechanism behind NETosis has become more clear. Formation of NETs is dependent of superoxides and ROS produced by the enzymes NADPH-oxidase and myeloperoxidase (MPO) (21, 22). The importance of NADPH oxidase was discovered because patients with Chronic Granulomatous Disease fail to form NETs due to mutations in the NADPH oxidase that disrupt ROS production (23). NETosis starts with the stimulation of various receptors, PKC activation, Ca²⁺ release from the ER,

NADPH oxidase induction and ROS production. Histone H3 becomes citrullinated by peptidylarginine deiminase 4 (PAD4), and the nuclear envelope becomes disassembled. Chromatin decondensates in the cytoplasm and binds to granular and cytoplasmic antimicrobial proteins and leads to the reshape of the nucleus. This process is mediated by the enzymes neutrophil elastase (NE) and myeloperoxidase (MPO). Chromatin decondensation and the association with antimicrobial proteins are two essential steps during NET formation. Finally, the cell membrane ruptures releasing NETs (2,4,23). See [Figure 2](#)

1.5 Induction of NETosis

NETs may serve as an effective antimicrobial defense, and are released when neutrophils have contact with pathogens such as bacteria, fungi, viruses and protozoa, or with a variety of host factors such as activated platelets, inflammatory stimuli or chemical compounds (2). See Table 1. Microorganisms trigger the fMLP receptor, which leads to PKC activation. The most frequently used compound to induce NETosis *in vitro* is Phorbol 12-myristate 13-acetate (PMA), a synthetic activator of the PKC family of enzymes. PKC is directly responsible for activation of NADPH oxidase and ROS production (1). Another receptor involved with NETosis is Toll-like receptor 4 (TLR-4), which also promotes NADPH oxidase activity.

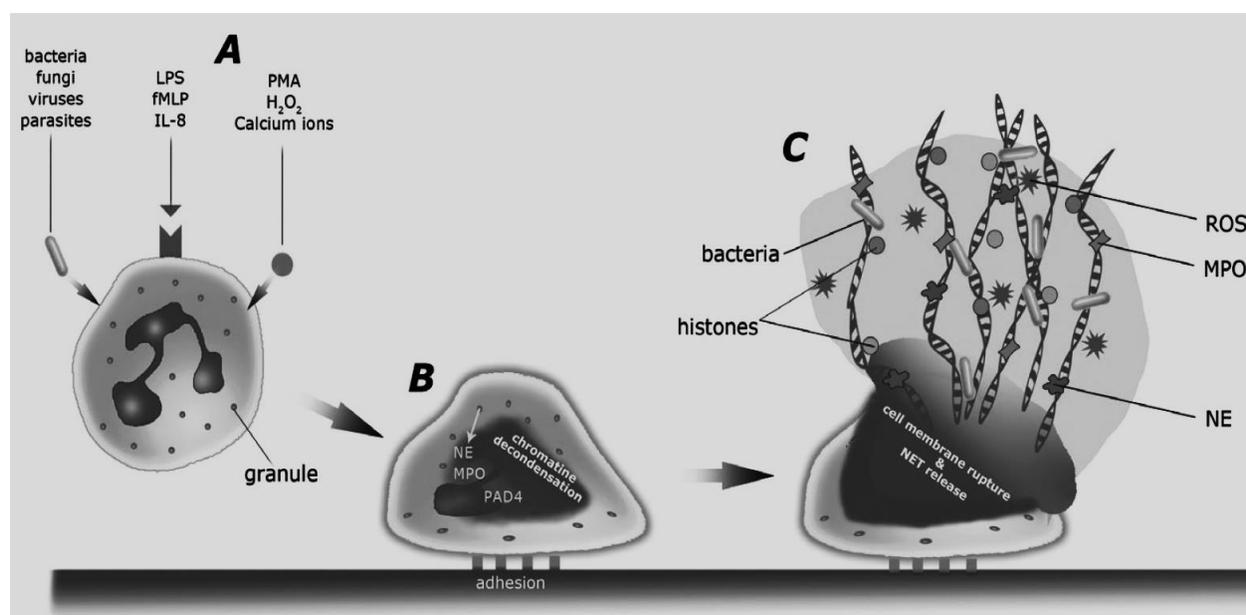


Figure 2. Mechanism of NETosis. Stimulation of receptors (A) by triggers (e.g. bacteria, fungi, viruses, parasites, chemical factors like PMA or LPS) leads to the adherence of neutrophils to endothelium and to chromatin decondensation due to histone cleavage by NE and MPO and histone hypercitrullination by PAD4 (B). In the final phase, NETs are released, trap bacteria and kill them with anti-inflammatory proteins (C). Source: (2)

Table 1. Compounds which stimulate the formation of NETs (2).

NET inducing compounds		
Antibodies	GM-CSF +C5a	Hydrogen peroxide
Calcium ions	GM-CSF + LPS	M1 protein
Glucose oxidase	Interferon- α + C5a	Nitric oxide
Lipopolysaccharide (LPS)	Interleukin 8	Platelet activating factor
Phorbol-12-myristate-13-acetate (PMA)	PMA + ionomycin	
TNF- α	TLR-4	

1.6 Proteins in Nets

The proteins present in NETs are interesting because they could serve as antigens in pathological situations like auto-immune diseases. The protein fraction of NETs classically contains histones, MPO, and NE. The protein constituents clearly linked to NETs are summarized in Table 2 (3). In the following part the most important proteins in NETs are described.

Granular proteins

Myeloperoxidase (MPO) is one of the most abundant proteins in neutrophils, accounting for 5% of the dry weight of the cell. Stored in granules and released when neutrophils are stimulated, MPO catalyzes the oxidation of chloride and other halide ions in the presence of hydrogen peroxide to generate hypochlorous acid and other highly reactive products that mediate efficient antimicrobial action. (24) Neutrophil Elastase (NE) is a neutrophil-specific serine protease that degrades virulence factors and kills bacteria. NE and MPO are essential during NETosis. ROS production leads to the release of NE and subsequently MPO from granules. NE translocates first to the nucleus, where it cleaves histones and promotes chromatin decondensation (23,25). MPO also migrates to the nucleus, where it promotes chromatin decondensation (9, 23).

Table 2. Proteins detected in neutrophil extracellular traps. Source: (3)

Protein	Function	
C1q	Activates complement cascade	Protect NETs against DNase1
Elastase	Serine protease	Organ damage
Histones	Transcriptional quiescence	
HMGB1	Alarmin	Activates antigen-presenting cells
HNP	A-defensin family member	May function similarly as LL37
IL-17	Proinflammatory cytokine	Innate immune activation
LL37	Activates pDCs	DNase1 protection
MPO	Peroxidase activity	Organ damage

HMGB1

High Mobility Group Box 1 (HMGB1) is a chromatin associated nuclear protein and an extracellular damage associated protein (DAMP). HMGB1 binds DNA and has a role in transcription, chromatin remodeling, DNA damage repair and recombination (26). Translocation of HMGB1 to the cytosol and extracellular space occurs during inflammation, trauma, necrosis and oxidative stress. In the extracellular space, HMGB1 is able to induce the production of cytokines, promote chemotaxis, activate immune cells and stimulate the production of auto-antibodies. HMGB1 is also present in NETs. HMGB1 can promote the pathogenesis of inflammatory and autoimmune diseases once it is in an extracellular location – as in NETs. In blood and tissue the levels of HMGB1 are elevated in many inflammatory and autoimmune diseases (27). HMGB1 interacts with TLR-4 and can induce NETosis (28-30). HMGB1 is a redox sensitive molecule, and has therefore different redox states in which it can be active. This leads to three active isoforms of HMGB1. HMGB1 contains three conserved cysteine residues, which can be in different redox states (27). With a disulfide bond and a free thiol, HMGB1 can induce cytokine production. HMGB1 with all free thiols induces chemotaxis. After oxidation of the sulfhydryl groups to sulfonates, HMGB1 loses the ability to induce cytokine production or chemotaxis (27). See [Figure 3](#).

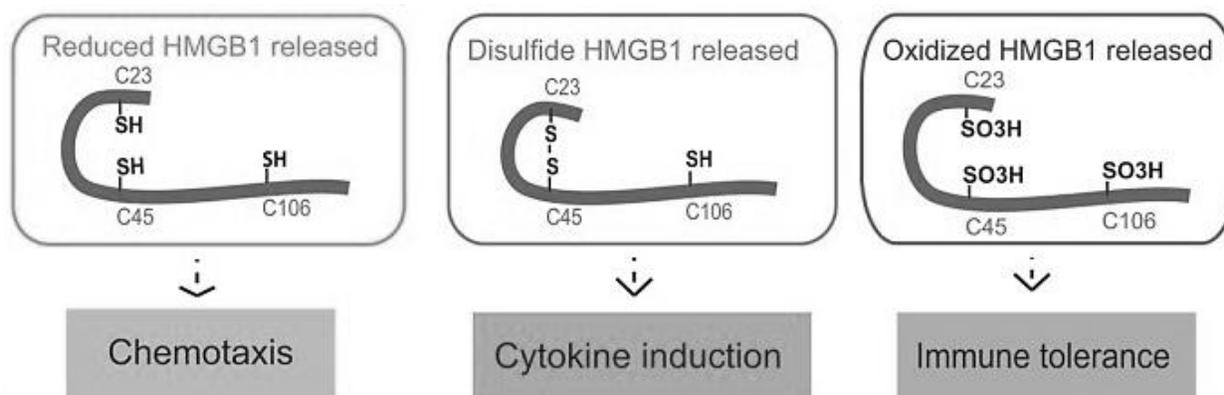


Figure 3, Isoforms of HMGB1 induce chemotaxis, cytokine production or immune tolerance. Figure modified from:(27)

Citrullinated histones

In eukaryotic cells DNA is packed around a core histone octamer composed of two histones H3, H2B, H2A and H4 to form a nucleosome. NETs mainly consist of DNA and histones. During NETosis, histones are citrullinated by the enzyme peptidyl arginine deiminase 4 (PAD4). PAD4 converts arginine residues on histones to citrulline. This process results in the loss of positive charge of the histone and has an effect on protein-DNA interactions (25, 31). PAD4 is of special interest because of its importance in innate immunity, the expression in granulocytes and its essential for the formation of NETs. PAD4 is mainly localized in the nucleus and citrullinates arginines on the histones H3, H2A and H4. Citrullinated histones promote chromatin decondensation during NETosis (25, 31, 32). PAD4 has an important role in various diseases, like ulcerative colitis (UC) and systemic lupus erythematosus (SLE) which all contain auto-antibodies against citrullinated proteins (25). The inhibition of citrullination leads to decreased inflammation in UC (33).

1.7 Influence of NETs on B-cells

There is already discovered that neutrophils are able to form NETs after stimulation with antibodies and ANCAs (34). The hypothesis exists that it also works the other way around: NETs might be able to activate B-cells and promote antibody or ANCA production. In such a system, the auto-immune disease is perpetuating (35). ANCAs stimulate NETosis, and the NETs promote ANCA production. See **Figure 4**. In the presence of B-cell Activating Factor (BAFF), IL-21 and CpG, B-cells differentiate to plasma cells *in vitro*. NETs, which contain large amounts of DNA, might induce the same effect in combination with BAFF and IL-21. B-cells get activated when they bind an antigen with their membrane associated antigen receptors (B-cell receptor, BCR) (36). During for example SLE, a lot of autoantibodies are raised against proteins present in NETs, such as nuclear proteins (histones, dsDNA, HMGB1), lactoferrin, MPO, and NE. NETs can be the source of autoantigens, and may hereby be directly responsible for the break of tolerance and auto-antibody production.

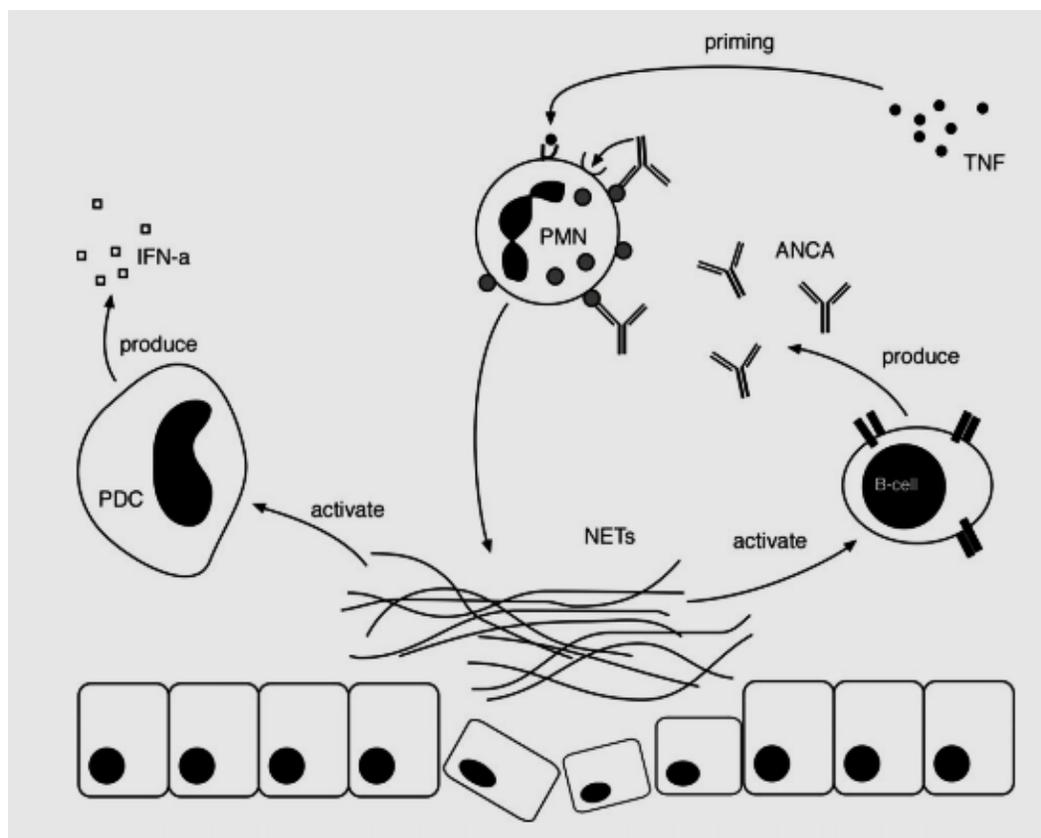


Figure 4: Relation between NETs and ANCA. ANCA can induce NETosis in TNF α primed neutrophils. The proteins in NETs can activate pDCs and induce IFN α production, an important step in the pathogenesis of SLE. NETs may activate autoreactive B cells to the production of ANCA, which results in a vicious circle of NETs and ANCA production. Source: (35)

1.8 Inhibition of NETosis

As mentioned before, NETosis is placed in the context of innate immunity, but also in the context of various auto-immune diseases. Consequently, inhibition of NETosis could result in beneficial therapeutic effects. Because NETosis is ROS and NADPH dependent, any molecule that inhibits the generation of ROS or scavengers ROS should be able to prevent the ROS dependent NETosis (22). There are already various ROS scavengers, NADPH and MPO inhibitors that can reduce NET release, such as diphenyleneiodonium chloride (DPI) or sodium-azide. But these compounds have unspecific or toxic effects on host cells. Molecules lacking such toxic effects could be candidates for medical application, like flavonoids and mesalazine (22, 37). In this study the effects of mesalazine, hydroxychloroquine, cigarette smoke extract (CSE) and prednisolone are investigated.

Mesalazine or 5-aminosalicylic acid (5-ASA) It is a common anti-inflammatory pharmaceutical used in Inflammatory Bowel Disease (IBD) and Ulcerative Colitis (UC)(38). 5-ASA agents are likely to have multiple anti-inflammatory effects. The mechanism of action of 5-ASA is not entirely understood, but several hypotheses have been proposed; 5-ASA may act as a scavenger of ROS and can inhibit MPO (22, 39, 40).

Hydroxychloroquine (HCQ) has been used for the treatment of Rheumatoid Arthritis (RA) and SLE (41). HCQ has many anti-inflammatory actions like interfering with antigen presentation, diminish the release of several cytokines and raising the pH of lysosomes and endosomes (42). HCQ is an inhibitor of TLR

activation pathways, particularly of TLR9 (43). TLR-9 is expressed on the endosomes of neutrophils (44). HCQ is a weak base that can pass through the lipid cell membrane and concentrate in acidic granules, where it elevates the pH. Besides the anti-inflammatory actions, HCQ is also used as an anti-malarial agent (45).

Cigarette smoke contains a highly oxidizing mixture of inorganic and organic reactive oxygen species produced by oxidants like catechol and hydroquinone. Cigarette smoke also contains carcinogenic compounds and nicotine. Cigarette smoke stimulates ROS and NO in neutrophils via the TLR-9, and promotes the NF- κ B pathway (43). The activation of the NF- κ B pathway results in an anti-apoptotic stimulus. Smoking is known to be protective in patients with UC, but the protective mechanism and compound is still unknown (46).

Prednisolone is the active metabolite of prednisone, a synthetic glucocorticoid which is used in a variety of inflammatory and auto-immune diseases such as RA, SLE and UC. Prednisolone is able to pass the cell membrane and bind to glucocorticoid receptors in the cytoplasm. The complex is directly able to interfere with gene expression (47).

1.9 Research aim

The aim of this research is to investigate the inducing role of HMGB1 cytokine, PMA and LPS on NETosis. It is already confirmed that PMA and LPS induce NETosis in 3 to 4 hours, and here these results are compared to the HMGB1 cytokine induced NETosis. HMGB1 is known to induce NETosis, but it remains unclear which redox state of HMGB1 causes this effect. In this study the NET inducing effect of the cytokine inducing variant of HMGB1 is analyzed.

Another question is if neutrophils from SLE patients react different than neutrophils from healthy controls on these stimuli. Neutrophils of SLE patients are known to be more prone to release NETs, and their NETs might differ in composition.

At the other hand, this research focusses on the inhibition of NETs. In this investigation the effect of mesalazine, hydroxychloroquine, prednisolone and cigarette smoke extract on NETosis is analyzed. There is taken a close look at the amount of NETs produced after inhibition, and at the expression of important NET proteins like MPO, NE, HMGB1 and Citr.H3 in the neutrophils.

The last hypothesis tested in this research is the influence of NETs on B-cells. NETs might be able to directly activate B-cells and induce (auto)antibody production.

The results of this investigation will lead to more information about NETs. In the future this could lead to therapeutics that can block or degrade NETs.

2. Materials and methods

2.1 Patients groups

A group of SLE patients (n=22) and a control group (n=35) with healthy volunteers was used in this study. A summary of data from these individuals and in which group they are placed is listed in table 3.

Table 3, data of patients group and healthy controls included in this study.

	SLE patients	Healthy Controls	p	
n	14	15	-	
Gender M:F	2:12	5:10	-	
Mean age (years) [range]	49.6 ± 9.2 [36-62]	37.6 ± 13.1 [22-61]	0,007813	
Mean SLEDAI [range]	1.9 [0-4]	-	-	
	5-ASA	CSE	PDN	HCQ
n (healthy controls)	15	7	11	2
Gender M:F	5:10	0:7	1:10	0:2
Mean age (years) [range]	40.1 ± 14.1 [22-61]	35 ± 14.8 [22-60]	29.7 ± 10.3 [21-52]	28.5 ± 0.7 [28-29]

2.2 Stimulation and inhibition of neutrophils

Neutrophils were obtained from peripheral blood from healthy controls and patients collected by venipuncture using lithium heparin tubes.

Neutrophil isolation: Blood was diluted 1:1 with Hanks' Balanced Salt Solution (HBSS-/-) (Gibco, 14175-053) and put 2:1 on a layer of lymphoprep (Fresenius Kabi Norge AS, LYS 3773). Tubes were centrifuged for 20 minutes at 2400rpm without brake. The erythrocytes from the erythrocyte/neutrophil layer were lysated with ammonium chloride and centrifuged at 1800 rpm for 5 minutes twice. Cell pellets were diluted in HBSS-/- before counting and further diluted in HBSS +CaCl₂+MgCl₂ (Gibco, 14025-050) +0.1% Bovine Serum Albumin (BSA) (Sigma Life Science, A9647-1KG).

Stimulation of neutrophils: A total amount of 1*10⁶cells/ml (or 0.25*10⁶cells/ml for coverslips) was plated out in a 24-wells plate. Neutrophils adhered 30 minutes in an incubator at 37°C. After adhesion stimulation medium with PMA (Sigma-Aldrich: P8139), LPS (Sigma Aldrich, L4516) or Cytokine-HMGB1 (HMGBiotech, HM-122) was added with a final concentration of 20nM, 1µg/ml and 0.3/3.0µg/ml respectively. Neutrophils were stimulated for 3 and 4 hours in an incubator at 37°C in a humidified air atmosphere containing 5% CO₂.

Inhibition of neutrophils: A total amount of 1*10⁶cells/ml (or 0.25*10⁶cells/ml for coverslips) was plated out in a 24-wells plate. Neutrophils were pre-treated 60 minutes in an incubator at 37°C with 1.0mM, 2.5mM and 5.0mM Mesalazine (Pharmacy UMCG), 5µM, 10µM and 20µM Hydroxychloroquine (Sigma Aldrich, H0915), Prednisolon (Pharmacy UMCG) or 5%, 15% and 30% CSE. After pre-treatment the cells were washed once with HBSS and then stimulated with or without 20nM PMA for 3 hours at 37°C in a humidified air atmosphere containing 5% CO₂.

Preparing CSE: 100% CSE was prepared with 2 Reference Cigarettes (Kentucky, 3R4F) in 25ml HBSS+/-+0.1%BSA. To get the smoke through the medium 2 cigarettes were smoked without filter through a peristaltic pump (Watson Marlow 603S) at speed 70-75. Medium was pre-warmed to 37°C.

2.3 Sytox assay

A Sytox Green Assay was used to measure the amount of extracellular DNA (eDNA) in the supernatants of cultured neutrophils. Because NETs consist for a large part of DNA, a sytox assay gives a reliable indication of the amount of NETs produced.

Harvesting NETs: NETs were cut off the neutrophils with 250mU MNase (Worthington, LS004798) for 10min at 37°C. The reaction was stopped with 25µl 0.1M EGTA. Medium was collected and centrifuged for 5 minutes at 3000rpm. Supernatant was stored at -20°C till sytox assay.

Sytox assay: Samples were measured in duplo in a black 96-wells plate (Fluotrac 200) with 5µM Sytox Green (Invitrogen, MP 07020). As a calibration curve the same sample was used in every plate and diluted to form a calibration line in arbitrary units (AU) to compare all samples. The samples were incubated for at least 15min with Sytox Green on a shaker platform and read out in a Victor³ 1420 multilabel counter (Perkin Elmer Precisely) at ex485 em535 (1s) TOP in triplo with Wallac 1420 manager software. The average of this triple is used for further data analysis; the average of the duplo's is used for statistical analysis.

Statistical analysis: Significant difference between effect of stimulating and inhibiting compounds within a group was calculated in GraphPad Prism software 5 with a paired two-tailed Wilcoxon signed rank t-Test. Significant difference between groups was calculated with a non-paired two-tailed Mann Whitney t-Test.

2.4 Immunohistochemistry on coverslips

Fluorescent microscopy was used to visualize NETs and to confirm the results of the Sytox Green Assay. Neutrophils were grown on Ø12 millimeter coverslips (Menzel, CB00120RA1), fixated with 4%formalin and stored in PBS at -20°C until staining. Coverslips were blocked with 2%BSA/PBS. The following primary antibodies are used 1:100: Mouse anti MPO (ICP 155P) or MouseαNE (Dako, M0752) and RabbitαHMGB1 (Abcam, ab18256) or Rabbit pAb to citrulinated H3 (Abcam, ab5103). Primary antibodies are diluted in PBS with 1% BSA, 0.05% Tween-20 and 1µg/ml DAPI (Roche, 10 236 276 001) and incubated for 2 hours at room temperature. As secondary antibodies GoataMouse IgG-APC (Biolegend, 405308) and DonkeyαRabbit-FITC (Jackson ImmunoResearch, 711-096-152) are used 1:50 and incubated for 1 hour at room temperature. Coverslips are mounted upside-down in citifluor (Citifluor Ltd, AF1) and analyzed with a fluorescent microscope (Leica DFC450 C) and Leica Application Suite Version4.2.0 software.

2.5 PBMC isolation and B-cell stimulation

PBMCs were obtained from peripheral blood from healthy controls and patients collected by venipuncture using lithium heparin tubes.

PBMC isolation: Blood was diluted 1:1 with RPMI 1640 (Lonza BioWhittaker, BE12-115F) + 6% Gentamicin (10mg/ml)(Gibco Life Technologies, 15710-049) and put 2:1 on a layer of lymphoprep (Fresenius Kabi Norge AS, LYS 3773). Tubes were centrifuged for 20 minutes at 2400rpm without brake. The white cell layers are mixed with RPMI + 6% gentamicin and centrifuged for 10 minutes at 1800rpm.

Pellets are resuspended in RPMI + 6% gentamicin and centrifuged for 5 minutes at 1200rpm. Cell pellets were diluted in RPMI + 6% gentamicin before counting and further diluted in RPMI + 6% gentamicin + 10% FBS (Lonza BioWhittaker, DE14-801F).

Creating NETs: A total amount of 1×10^6 neutrophils/ml was plated out in a 24-wells plate. Neutrophils adhered 30 minutes in an incubator at 37°C. After adhesion the neutrophils were stimulated with and without 20nM PMA (Sigma-Aldrich: P8139) for 4 hours at 37°C in a humidified air atmosphere containing 5% CO₂. After 4 hours the cells are washed with RPMI.

PBMC stimulation: A total amount of 1×10^6 PBMCs/ml was plated out in the neutrophil/NETs coated plate. PBMCs are stimulated with 3.2 µg/ml CpG (Hycult Biotech, HC4039), 100ng/ml BAFF (Peprotech, 310-13) and/or 100ng/ml IL-21 (Immunotools, 11340215) in RPMI + 6% gentamicin + 10% FBS. The following combinations are used: Control – PBMCs; Negative control – Neutrophils + PBMCs; Conjugate control – Neutrophils + PBMCs + BAFF + IL-21; Positive control – Neutrophils + PBMCs + CpG + BAFF + IL-21; Test 1 – NETs + PBMCs; Test 2 - NETs + PBMCs + BAFF + IL-21; Test 3 - NETs + PBMCs + CpG + BAFF + IL-21. PBMCs were cultured for 12 days at 37°C in a humidified air atmosphere containing 5% CO₂.

Harvesting PBMCs: After 12 days the supernatant of the PBMCs was collected and centrifuged for 5 minutes at 3000rpm. Supernatant was stored at -20°C. The cell pellet was resuspended in 200µl RPMI and used for cytopsin preparation.

2.6 ELISA

In order to measure the amount of IgG produced by plasma cells, a total IgG ELISA is used. A 96 wells EIA/RIA plate (Costar, 9018) was coated overnight with 100µl 1:5000 GoatαHuman-IgG (Jackson; 109-006-088) in PBS. The plate was washed 5x with 0.25M Tris-HCl, 0.15M NaCl, 0.05% Tween-20 in demi water and blocked with 200ul PBS + 2%BSA + 0,05%Tween-20 for 60 minutes. Samples were diluted 1:25 in PBS +1% BSA + 0.05% Tween-20, further diluted 1:4 in the plate and incubated for 90 minutes. As a calibration line standard IgG (Siemens, 08369611) was used with a start concentration of 50ng/ml and diluted further in 2 fold dilutions. After a second wash step the samples were incubated with 100µl 1:2000 MouseαHuman-IgG-HRP (Southern Biotech; 90-40-05) and incubated for 90 minutes. The plate was washed again and the immune complexes were incubated with 100µl 3,3,5,5-Tetramethyl-benzidin dihydrochloride (TAB) (Sigma Aldrich, T3405-100TAB) + 0.006% H₂O₂ (Hydrogen peroxide 30%, Emsure, 1.07209.0250) in acetate buffer (Pharmacy UMCG) for 15-30min. The reaction was stopped with 100µl 1M H₂SO₄ (Pharmacy UMCG) and the plate was read at 450nm in a microplate reader (Tunable versa max, Molecular Devices). Sample analysis and calculation was done with SoftMax Pro 5.4 software.

Statistical analysis: Significant difference between stimulating with neutrophils or NETs was calculated in GraphPad Prism software 5 with a paired two-tailed Wilcoxon signed rank t-Test.

2.7 Cytospins and CD138 immunohistochemistry

A CD138 staining was used to visualize plasma cells that were present in the supernatants of the cultured PBMCs. The staining is also used to amplify the results of the ELISA.

Cytospins: The cell pellets from harvesting PBMC were resuspended in 200 µl RPMI. For the cytopsin 100µl of each sample was used. The cytopsin were made with the Cytospin4 (Thermo Shandon) at 450rpm for 5 minutes and then dried and fixated with 96% ethanol for 15 minutes.

CD138 IHC: The coupes were incubated 10 minutes with 0,3% H₂O₂/PBS to shut off endogen peroxidases and once washed with PBS for 5 minutes. As a primary antibody 1:100 MurineαHuman CD138 (IQP-

153P) in 1% BSA/PBS was used and incubated for 60 minutes. The samples were washed three times with PBS and as a secondary antibody labeled polymer-HRP anti-mouse (DAKO, K4006) was used. The samples were washed three times with PBS again and then incubated for 10-15 minutes with Liquid DAB+ Substrate Chromogen System (DAKO, K3468). Coupes were washed three times with demi water and the nuclei were stained with hematoxillin. Coupes were embedded in Kaiser glycerin, analyzed with a bright field microscope (Olympus BX50) and CellSens Standard software.

3. Results

3.1 Four hours stimulation leads to more NETosis than three hours stimulation

In literature the duration of inducing NETosis varies mostly between 2 and 4 hours. This is an adequate stimulation time for PMA and LPS, but the ideal stimulation time for HMGB1-cytokine was unknown. Neutrophils were stimulated 3 and 4 hours with PMA, LPS and HMGB1-cytokine and analyzed with Sytox Green and fluorescent immunohistochemistry to investigate the differences between the amount of NETs and the composition of NETs between 3 and 4 hours of stimulation.

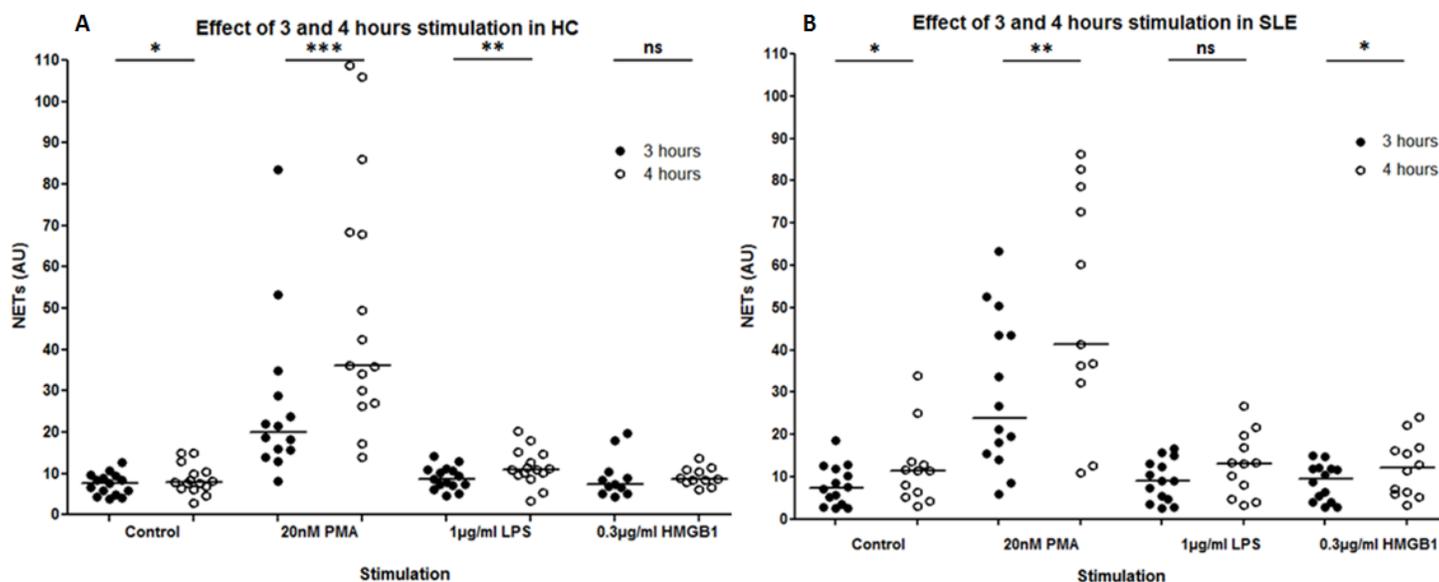


Figure 5, Effect of 3 and 4 hours stimulation with PMA, LPS and HMGB1 Cytokine on NETosis measured with Sytox Green. Neutrophils were incubated with and without 20nM PMA, 1µg/ml LPS and 0.3/3µg/ml HMGB1 Cytokine for 3 and 4 hours. Data were normalized to a calibration sample set on a value of 100 arbitrary units (AU). **A:** Data show median from healthy controls (HC); n=15 (Control, LPS 3+4 hours, PMA 4hours), n=14 (PMA 3hours), n= 11 (HMGB1 Cytokine 3+4 hours) and from SLE patients (**B**); n=14 (Control, PMA, LPS, HMGB1 Cytokine 3 hours), n=12 (Control, LPS, HMGB1 Cytokine 4 hours), n=11 (PMA 4 hours). —=median, *** p < 0.001, ** p < 0.01, * p < 0.05.

In healthy controls the amount of NETs is significantly increased between 3 and 4 hours stimulation in unstimulated (Control) ($p=0.0381$), PMA ($p=0.001$) and LPS (0.0083) stimulated neutrophils. There was no significant difference between 3 and 4 hours HMGB1 cytokine stimulation ($p=0.5195$). See figure 5A. In SLE patients the amount of NETs also increased significantly in unstimulated ($p=0.0341$) and PMA stimulated ($p=0.0029$) neutrophils. There was no significant difference in LPS stimulated ($p=0.1578$) neutrophils, but there was for the HMGB1 cytokine stimulated ($p=0.0210$) neutrophils. See figure 5B.

With fluorescent immunohistochemistry there was no difference between 3 and 4 hours stimulation visible in unstimulated neutrophils. Cells are stained for MPO and NE, two granule proteins, and for HMGB1 and Citrulinated Histone H3 (Citr.H3), two nuclear proteins. All four proteins play an important role in the formation of NETs. In healthy controls the amount of NETs in unstimulated, LPS stimulated and HMGB1 Cytokine stimulated neutrophils is low, and there was no difference seen between these stimulations and the two time points. Results of LPS and HMGB1 Cytokine stimulated neutrophils are shown in the supplementary data I. MPO and NE were located around the nucleus, and HMGB1 and Citr.H3 in the nucleus. HMGB1 is also expressed in the cytoplasm. See figure 6.

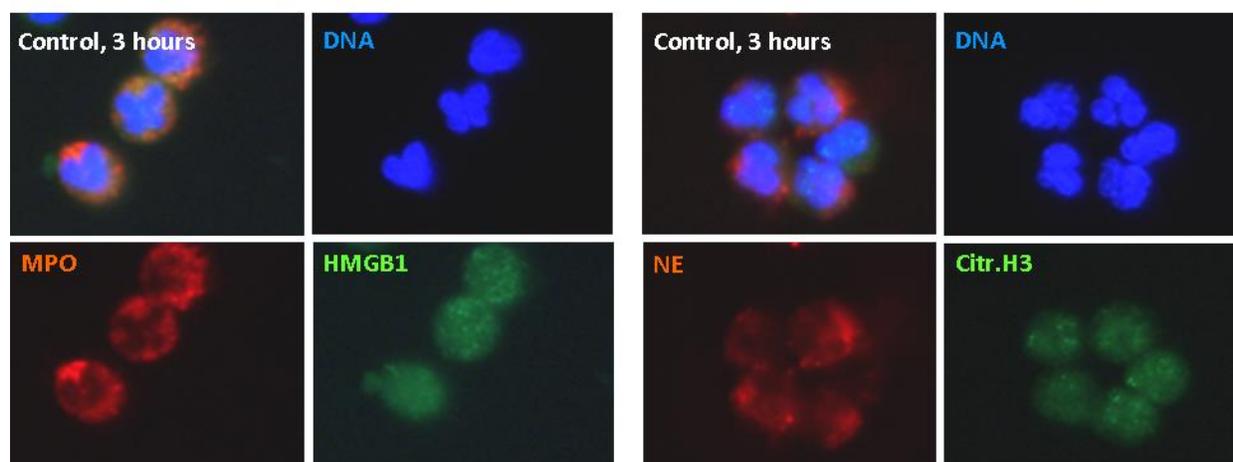


Figure 6, Unstimulated neutrophils after 3 hours of incubation. Neutrophils are stained for DNA (blue), MPO (red) and HMGB1 (green) and for NE (red) and Citr.H3 (green). Exposure time was the same for all pictures in a selected channel. Magnification: x400.

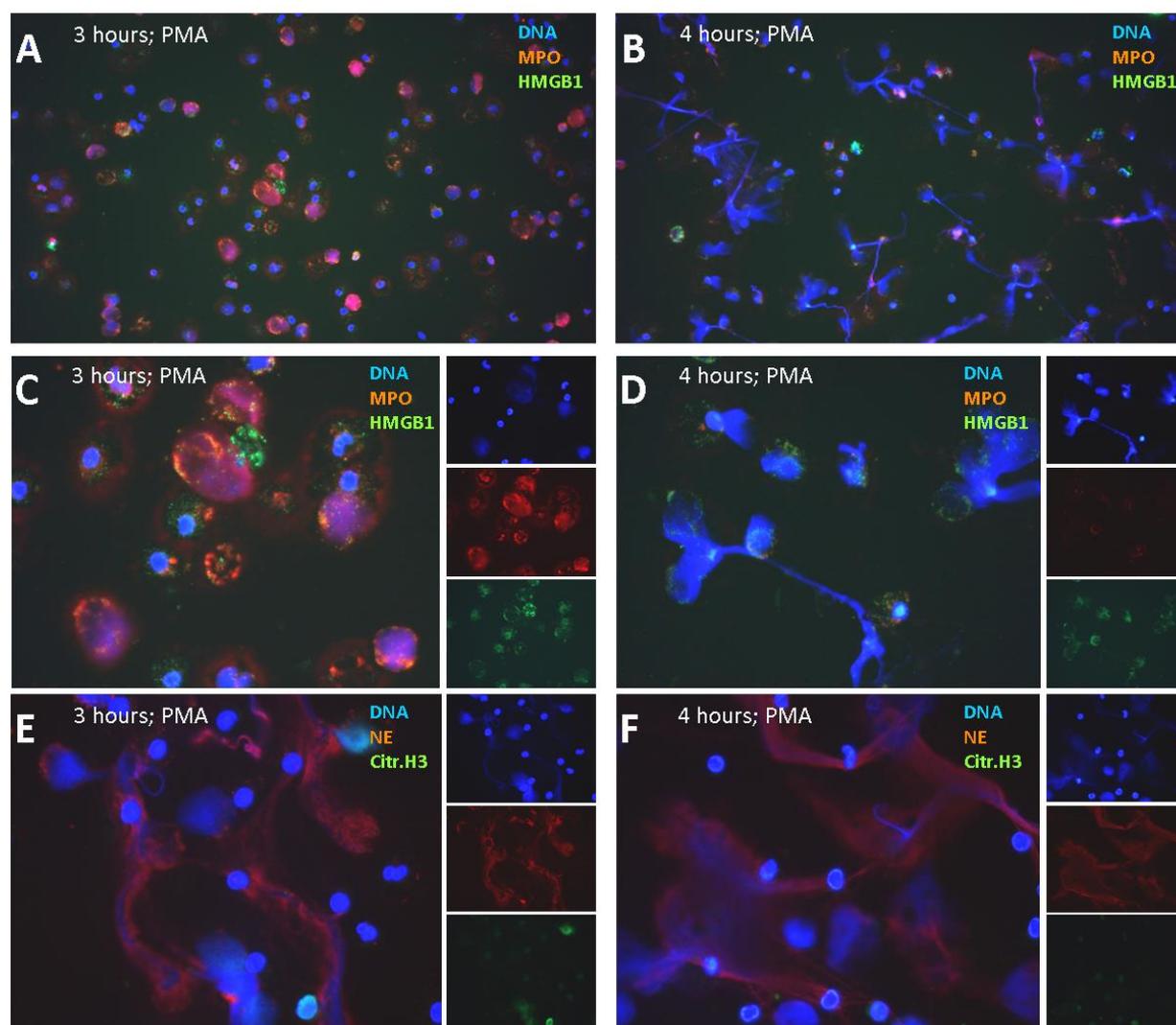


Figure 7, 3 and 4 hours 20nM PMA stimulated neutrophils. Neutrophils are stained for DNA (blue), MPO (red) and HMGB1 (green) and for NE (red) and Citr.H3 (green). Exposure time was the same for all pictures of the same antibody. **A+B**: magnification x200. **C+D+E+F**: magnification x400. **A+C+E**: 3 hours of PMA stimulation. **B+D+F**: 4 hours of PMA stimulation.

After PMA stimulation the neutrophils lose their polymorphologic nucleus and start the formation of NETs. After 3 hours there are NETs present with mostly a cloud-like structure. (Figure 7A) DNA is spread out, together with MPO and HMGB1. (Figure 7C) NE seems to be spread out even more compared to the DNA staining. Citrullinated histone is expressed strongly in the round nucleus of a few neutrophils, probably during a late stage of NETosis. Citrullinated histone H3 is also present in a few NETs, probably just released, and seems to disappear in older NETs. (Figure 7E) After 4 hours of PMA stimulation the NETs present are not cloud-like anymore, but consist of more dread-like structures. (Figure 7B) These dread-like NETs consist mostly of DNA, MPO seems to leave the NETs, and HMGB1 stays in the cloudy part of the NET. (Figure 7D) NE was already widely spread out after 3 hours of stimulation, and there is no difference seen after 4 hours. There are still a few neutrophils that express high levels citrullinated H3 and have not formed NETs yet. In NETs, just like after 3 hours stimulation, citrullinated H3 has disappeared. (Figure 7F)

3.2 Neutrophils from SLE patients do not react different on PMA, LPS or HMGB1 cytokine than healthy controls

Neutrophils from SLE patients during active disease are known to format NETosis effortless. After statistical analysis (Mann-Whitney test) of the data shown in figure 5, there was no significant difference found between stimulation of neutrophils of SLE patients or healthy controls in this study. The p-values are summarized in table 4. With immunohistochemistry the NETs of SLE patients are compared with healthy controls. There was also no difference visible in NET structure and composure. Therefore the immunohistochemic staining of SLE neutrophils are shown in supplementary data II.

Table 4, p values of Mann-Whitney test. Values are calculated between SLE patients and healthy controls for different stimulations.

	3 hours	4 hours
Control	ns; p= 0,9826	ns; p= 0,4205
PMA	ns; p= 0,6792	ns; p= 0,7953
LPS	ns; p= 1,0000	ns; p= 0,6429
HMGB1 cytokine	ns; p= 0,9345	ns; p= 0,4982

3.3 PMA, LPS and HMGB1 cytokine induce NET formation

PMA is a widely accepted method to induce NETosis *in vitro*. Also :LPS is known to induce NETosis on a lower level than PMA. Recently, also HMGB1 is found to induce NETosis. Here these findings are reproduced and there is looked at the inducing effect of the HMGB1 cytokine variant. Neutrophils of healthy controls and SLE patients are stimulated for 4 hours. With Sytox Green the amount of eDNA was measured and the results are shown in figure 8.

After PMA stimulation the amount of NETs significantly increased by healthy controls ($p < 0.0001$) and SLE patients ($p = 0.001$)(figure 8A). Healthy controls release significantly more NETs after stimulation with LPS ($p = 0.0007$), but SLE patients do not ($p = 0.2719$)(figure 8B). HMGB1 Cytokine induces NETosis in neutrophils from healthy controls ($p = 0.0453$), but seem to have no effect on neutrophils from SLE patients ($p = 0.3076$) (figure 8C).

The inducing effect of PMA, LPS and HMGB1 Cytokine is also pointed out with immunohistochemical stainings. The inducing effect of PMA is clearly seen through the microscope, but the differences between unstimulated cells and LPS or HMGB1 stimulated cells cannot be analyzed with this method. A complete view of unstimulated and PMA, LPS and HMGB1 cytokine stimulated neutrophils from healthy controls and SLE patients can be found in supplementary data I and II.

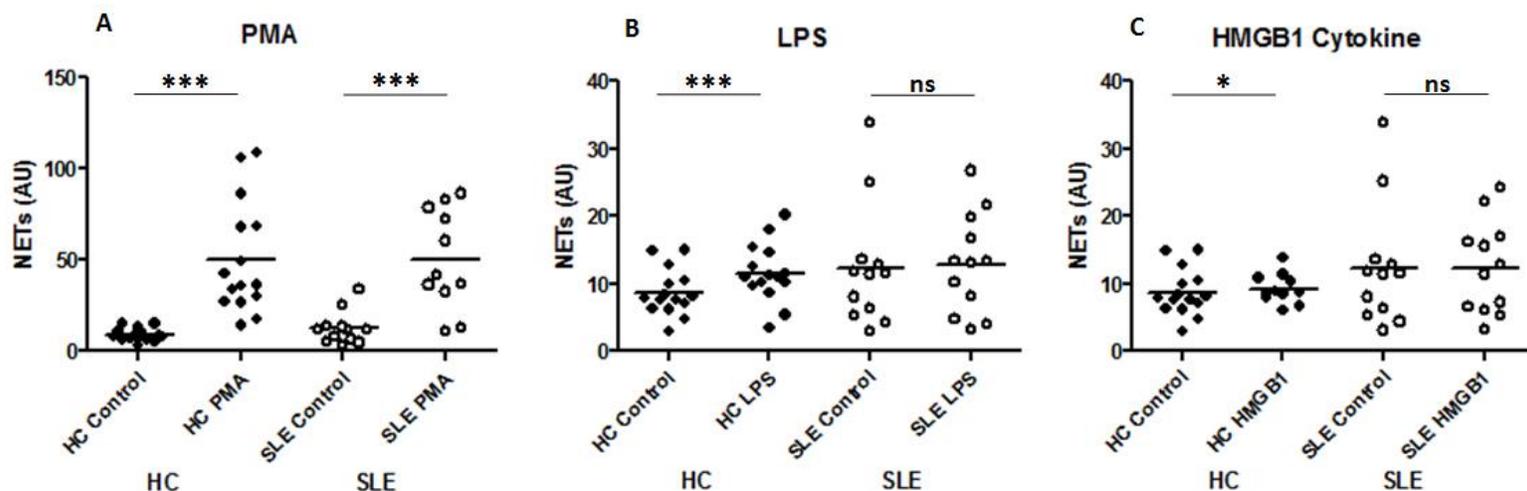


Figure 8, Effect of **A**: PMA, **B**: LPS and **C**: HMGB1 Cytokine on NETosis measured with Sytox Green. Neutrophils were incubated with and without 20nM PMA, 1µg/ml LPS and 0.3/3µg/ml HMGB1 Cytokine for four hours. Data were normalized to a calibration sample set on a value of 100 arbitrary units (AU). Data show median from healthy controls (HC); n=15 (Control, PMA, LPS), n= 11 (HMGB1 Cytokine) and from SLE patients; n=12 (Control, LPS, HMGB1 Cytokine), n=11 (PMA). —=median, *** p < 0.001, ** p < 0.01, * p < 0.05.

3.4 5-ASA and CSE inhibit NETosis

In order to find therapeutics that can inhibit NETosis, the influence of pre-treatment with different concentrations mesalazine (5-aminosalicylic acid (5-ASA)) and different percentages cigarette smoke extract (CSE) was investigated. Smoking is known to be beneficial in patients with UC, a disease where excessive NETs are present in the colon. The influence of mesalazine and CSE is measured in neutrophils from healthy controls that are unstimulated or stimulated with PMA for 3 hours after pre-treatment.

Mesalazine has no effect on the amount of NETs of unstimulated neutrophils, but significantly reduces the amount of NETs in PMA stimulated neutrophils on a dose dependent manner. Results are shown in figure 9A. A concentration of 1.0mM or 2.5mM mesalazine reduces the amount of NETs significantly with a P-value of 0.0093 and 0.0039 respectively. A concentration of 5.0mM mesalazine gives a reduction with a P-value of 0.0117.

Pre-treatment with 5%, 15% and 30% CSE reduces NETosis significantly in both unstimulated and PMA stimulated neutrophils. In unstimulated neutrophils NETosis is inhibited with a P-value of 0.0223 for 5% CSE, 0.0313 for 15% CSE and 0.0156 for 30% CSE. When neutrophils are stimulated with PMA after pre-treatment NETosis is significantly inhibited with P-values of 0.0469, 0.0156 and 0.0156 for respectively 5, 15 and 30% CSE.

The inhibitory effect of mesalazine and CSE is confirmed with immunohistochemistry. The expression of MPO, HMGB1, NE and Citr.H3 is analyzed in both stimulated and unstimulated neutrophils, and for all concentrations and percentages of 5-ASA and CSE. In figure 10 and 11 neutrophils without pre-

treatment, and with a pre-treatment of 30% CSE and 5mM 5-ASA are shown. The expression of MPO, NE, HMGB1 and Citr.H3 at lower concentrations mesalazine and CSE are shown in supplementary data III and IV respectively.

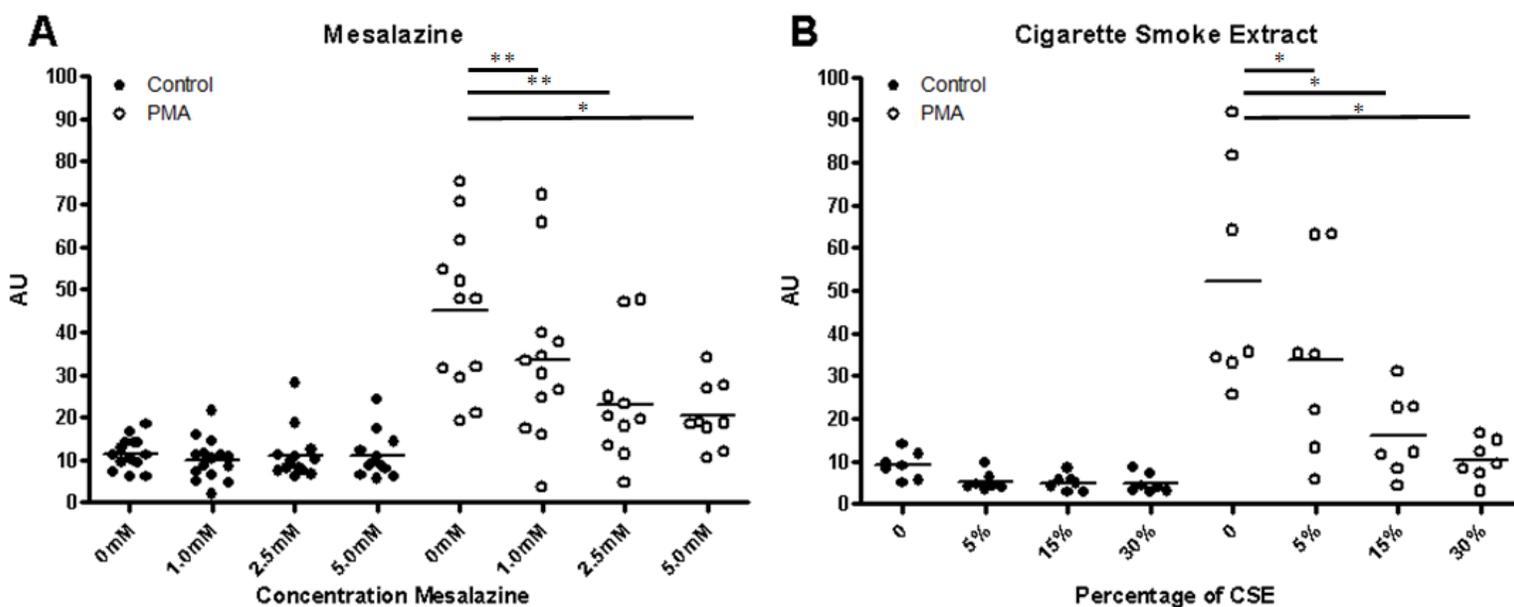


Figure 9, Effect of mesalazine and cigarette smoke extract on NETosis measured with Sytox Green. Neutrophils were pre-treated with 0, 1.0, 2.5 or 5.0mM mesalazine and 0, 5%, 15% or 30% CSE and then stimulated with or without PMA. Data were normalized to a calibration sample set on a value of 100 arbitrary units (AU). **A:**Data show median from healthy controls (HC) without PMA stimulation; n=15 (0mM,1.0mM), n= 14 (2.5mM), n=13 (5.0mM) and with PMA stimulation; n=12 (0mM,1.0mM), n=10 (2.5mM), n=9 (5.0mM).**B:** Data show median from healthy controls (HC) with and without PMA stimulation; n=7 (all groups) —=median, *** p < 0.001, ** p < 0.01, * p < 0.05.

Untreated and unstimulated neutrophils are round and have a polymorph nucleus. MPO and NE are expressed in the cytosol of neutrophils. HMGB1 is primary expressed in the nucleus, but is also present in the cytosol. Citr.H3 is only seen in the nucleus of neutrophils. All proteins are expressed in all neutrophils and in similar amounts, except for Citr.H3, which is expressed unequal in neutrophils. Neutrophils which express more Citr.H3 are probably already in a state of NETosis, but have not released them yet. (figure 10A and 11A). After PMA stimulation most neutrophils have released NETs or are in a state of NETosis. Neutrophils are bigger, nuclei are round-shaped, and MPO and NE are spread out in the living cells and in the NETs. DNA and HMGB1 are cloud-like present in NETs, while MPO and NE are more widely spread out and also form dread-like structures. Citr.H3 is expressed in the nuclei of living neutrophils and in just a few NETs. This NETs are probably just released, and Citr.H3 is likely to disappear in older NETs. (figure 10B and 11B).

A concentration of 5mM 5-ASA has no effect on unstimulated neutrophils and the expression of MPO, NE, HMGB1 and Citr.H3 and morphology of the nuclei stays the same as the control. (figure 10C and 11C). When 5-ASA pre-treated neutrophils are stimulated with PMA, the effect of 5-ASA becomes visible. The amount of NETs are decreased but neutrophils show signs of activation because the nuclei are round-shaped. The neutrophil has lost its round shape, and show an increased expression of MPO in the cytosol. HMGB1 is present in both nucleus and cytosol.(figure 10D) The citrullination of histone 3 seems to be slightly decreased and NE stays in the cytosol of neutrophils(figure 11D).

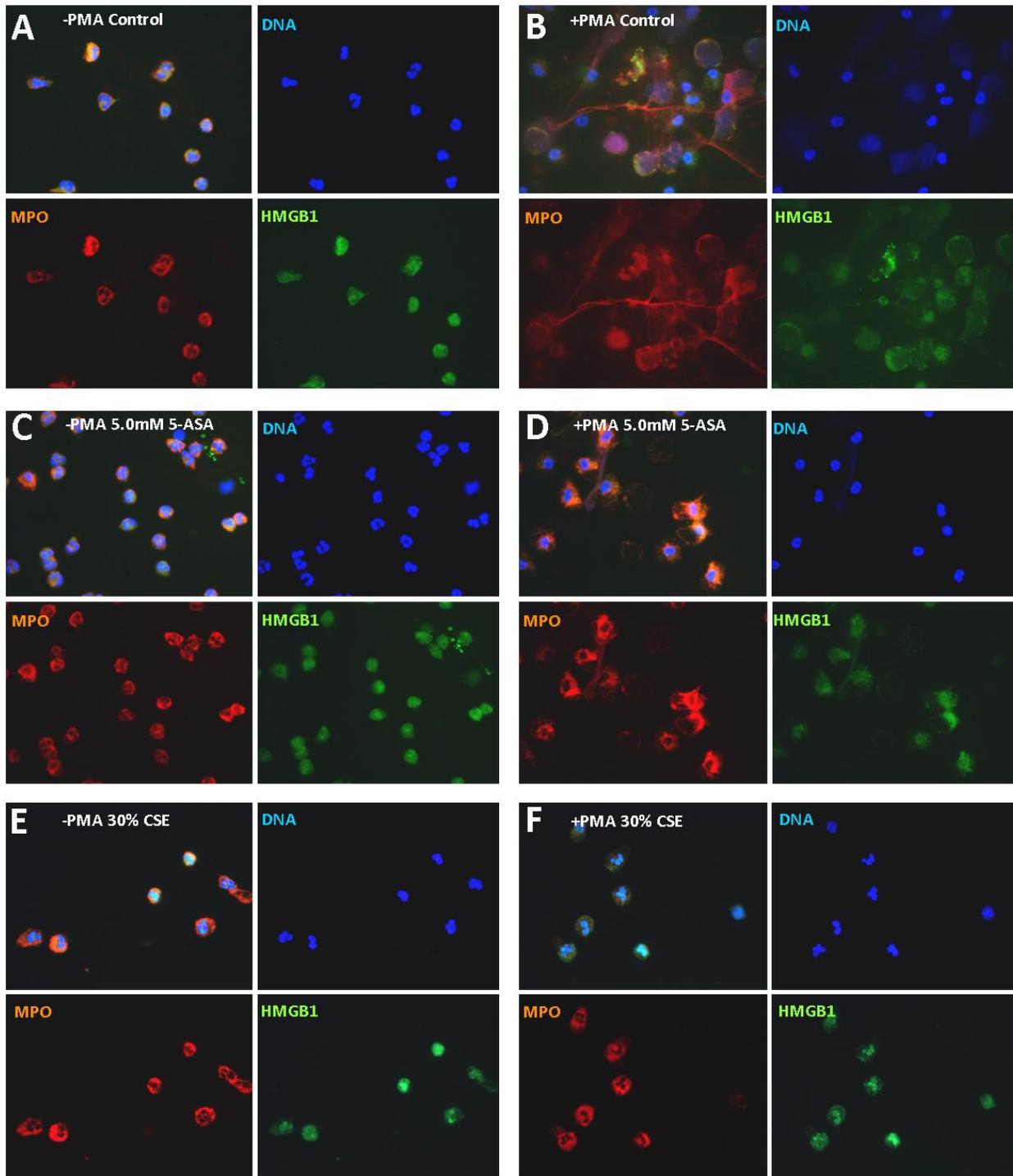


Figure 10, Effect of 60min pre-treatment with 5mM 5-ASA or 30% CSE in unstimulated and PMA stimulated neutrophils. Cells are stained for DNA (blue), MPO (red) and HMGB1 (green). Exposure time was the same for all pictures of the same antibody. Magnification x400. **A+B**: Untreated neutrophils. **C+D**: Pre-treatment with 5mM 5-ASA. **E+F**: Pre-treatment with 30% CSE. **A+C+E**: unstimulated neutrophils. **B+D+F**: PMA stimulated neutrophils.

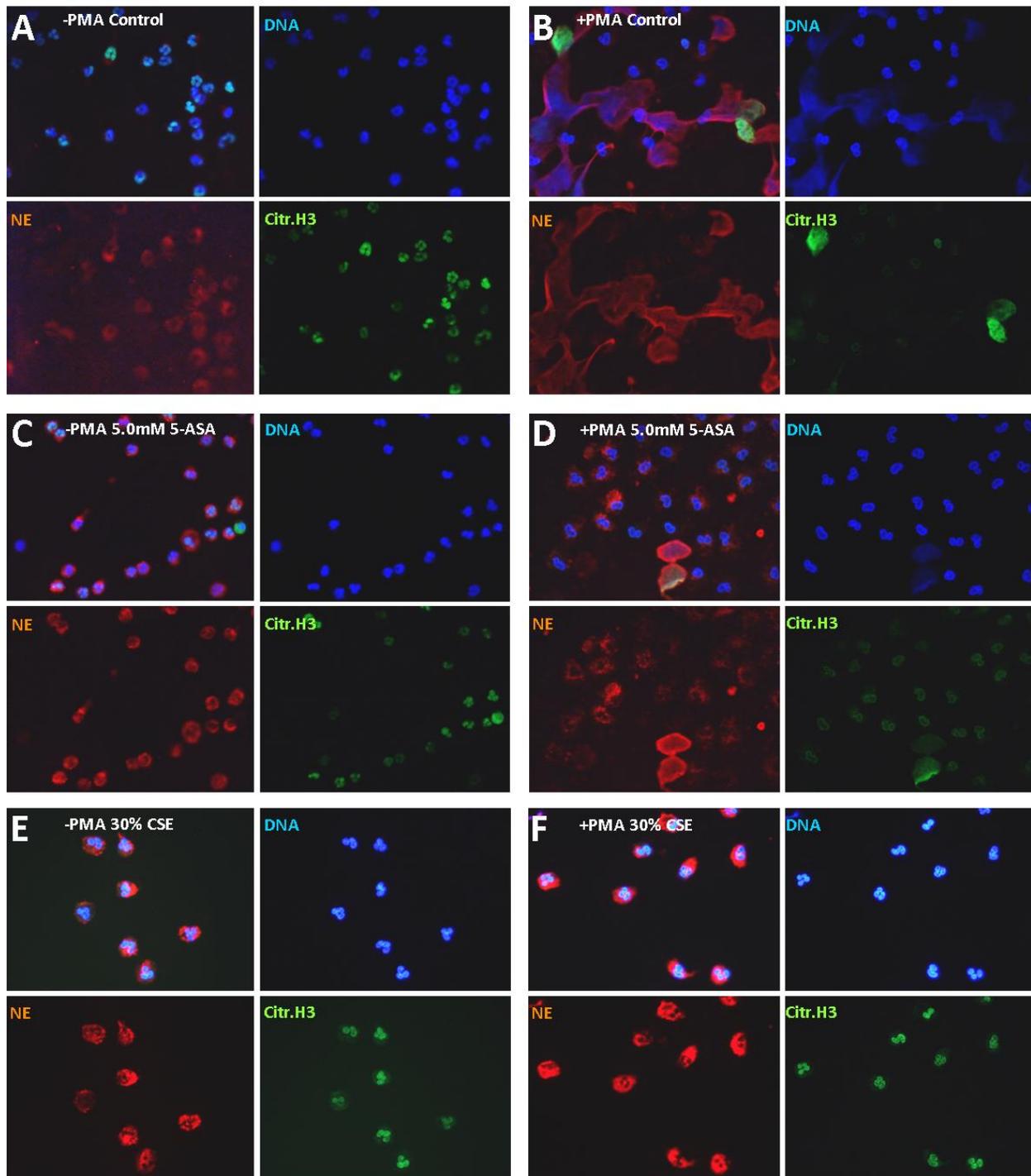


Figure 11, Effect of 60min pre-treatment with 5mM 5-ASA or 30% CSE in unstimulated and PMA stimulated neutrophils. Cells are stained for DNA (blue), NE (red) and Citr.H3 (green). Exposure time was the same for all pictures of the same antibody. Magnification x400. **A+B**: Untreated neutrophils. **C+D**: Pre-treatment with 5mM 5-ASA. **E+F**: Pre-treatment with 30% CSE. **A+C+E**: unstimulated neutrophils. **B+D+F**: PMA stimulated neutrophils.

Cigarette smoke extract has no visible effect on unstimulated neutrophils (figure 10E and 11E), but after PMA stimulation the effect of CSE becomes clear. Not only the amount of NETs is decreased to a level similar as the control neutrophils, the cells have a un-activated morphology too. The neutrophils are

round shaped and have a polymorph nucleus. MPO and NE are present in the cytosol, while HMGB1 and Citr.H3 are expressed only in the nucleus. (figure 10F and 11F).

3.5 Prednisolone and hydroxychloroquine show no effect on NETosis

Prednisolone (PDN) and hydroxychloroquine (HCQ) have different anti-inflammatory effects and are therefore possible NETosis inhibitors. Both medicines are used in different autoimmune diseases like SLE. Their influence on NETosis is measured on neutrophils from healthy volunteers. Neutrophils were pre-treated with PDN or HCQ before stimulation with or without PMA for 3 hours.

The effect of HCQ is tested in only 2 persons, but seems to be not effective as a NETosis inhibitor. Concentrations of 5, 10 and 20 μ M do not affect NETosis. See figure 12A. Pre-treatment with PDN was also not sufficient to reduce the amount of NETs. See figure 12B. A higher concentration of 10 μ g/ml and a lower concentration of 100ng/ml prednisolone were also not effective (data not shown). Because these results implicate that HCQ and PDN have no influence on NETosis, there are no immunohistochemical results obtained to confirm the effect.

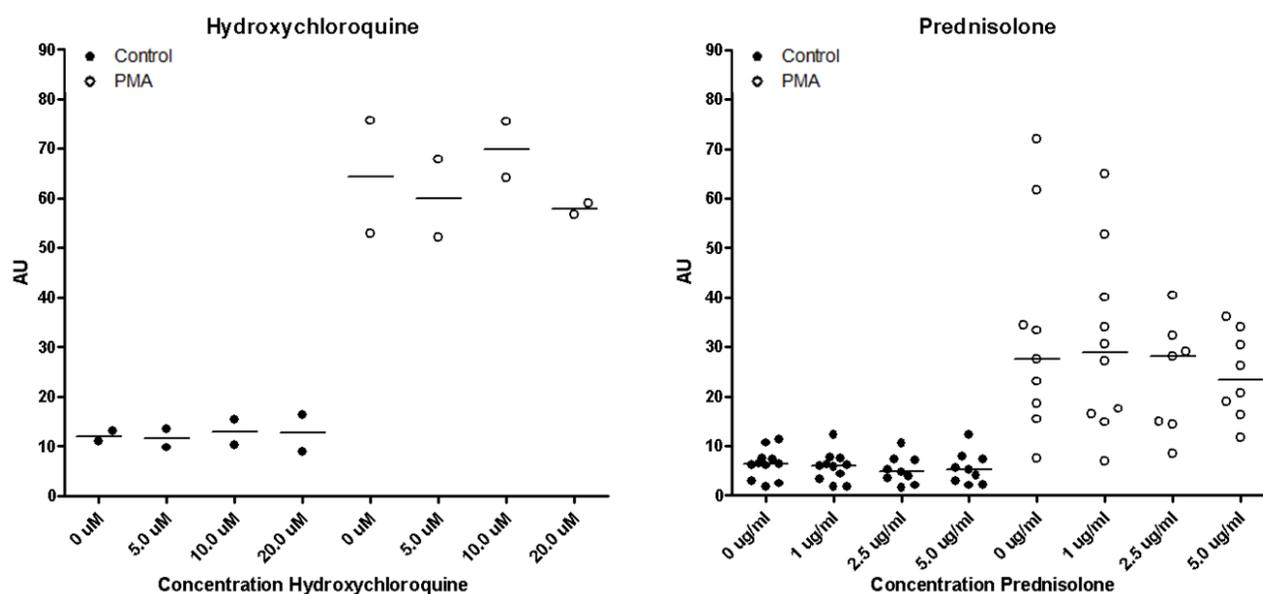


Figure 12, Effect of hydroxychloroquine and prednisolone on NETosis measured with sytox Green. Neutrophils were pre-treated with 0, 5.0, 10.0 or 20.0 μ M hydroxychloroquine and 0, 1.0, 2.5 or 5.0 μ g/ml prednisolone before PMA stimulation. Data were normalized to a calibration sample set on a value of 100 arbitrary units (AU). **A:**Data show median from healthy controls (HC) with and without PMA stimulation; n=2. **B:** Data show median from healthy controls (HC) without PMA stimulation; n=11(0 and 1 μ g/ml), n=9 (2.5 and 5.0 μ g/ml) and with PMA stimulation; n=9(0 μ g/ml), n=10(1 μ g/ml), n=7(2.5 μ g/ml) and n=8 (5.0 μ g/ml). —=median, *** p < 0.001, ** p < 0.01, * p < 0.05.

3.6 NETs inhibit IgG production

NETs play an important role in autoimmune diseases like SLE. Autoantibodies present during autoimmune diseases are able to induce NETosis in TNF-primed neutrophils. The hypothesis is that it also works the other way around; NETs might directly induce antibody production in B-cells. To investigate this hypothesis PBMCs were cultured in the presence of either neutrophils or NETs. The PBMCs were stimulated with BAFF, IL-21 and CpG. A combination of these three stimulating factors is known to induce IgG production. Because both CpG and NETs contain DNA, NETs might have the same

effect as CpG. Therefore a group of PBMCs is stimulated with BAFF and IL-21 without CpG. Another hypothesis is that B-cells derived from SLE patients are more prone to produce IgG after stimulation with NETs compared to B-cells of healthy controls. There is also a possibility that the composition of NETs from SLE patients is different than NETs from healthy controls. This could lead to differences in the ability to induce B-cell proliferation and IgG production. To investigate this hypothesis, different combinations of neutrophils/NETs and PBMCs are made. There are 4 groups analyzed: HC homologous — Neutrophils/NETs and PBMCs are derived from one healthy control; HC heterologous — Neutrophils/NETs are derived from SLE blood, PBMCs are derived from a healthy control; SLE homologous — Neutrophils/NETs and PBMCs are derived from a SLE patient; SLE heterologous — Neutrophils/NETs are derived from a healthy control, PBMCs are derived from a SLE patient.

The total IgG production was measured by ELISA and is shown in figure 13. PBMCs alone or in the presence of neutrophils produce an median of 1.15 μ g/ml and 0.67 μ g/ml IgG respectively, and this is not a significant difference ($p=0.0625$). In the presence of NETs the median amount of IgG drops to 0.34 μ g/ml and this is significantly lower than in the presence of neutrophils ($p= 0.0156$). PBMCs stimulated with BAFF and IL-21 have a median amount IgG production of 5.62 μ g/ml in the presence of neutrophils and lowers in the presence of NETs to 3.15 μ g/ml. After stimulation with BAFF, IL-21 and CpG, IgG production is induced to a median of 73.34 μ g/ml IgG in the presence of neutrophils and to 8.68 μ g/ml in the presence of NETs. The IgG production is significantly lower in the last mentioned group ($p= 0.0156$). These data show that the IgG production is inhibited in the presence of NETs, no matter if it was a homologous or a heterologous stimulation or if the cells were derived from SLE patients or healthy controls.

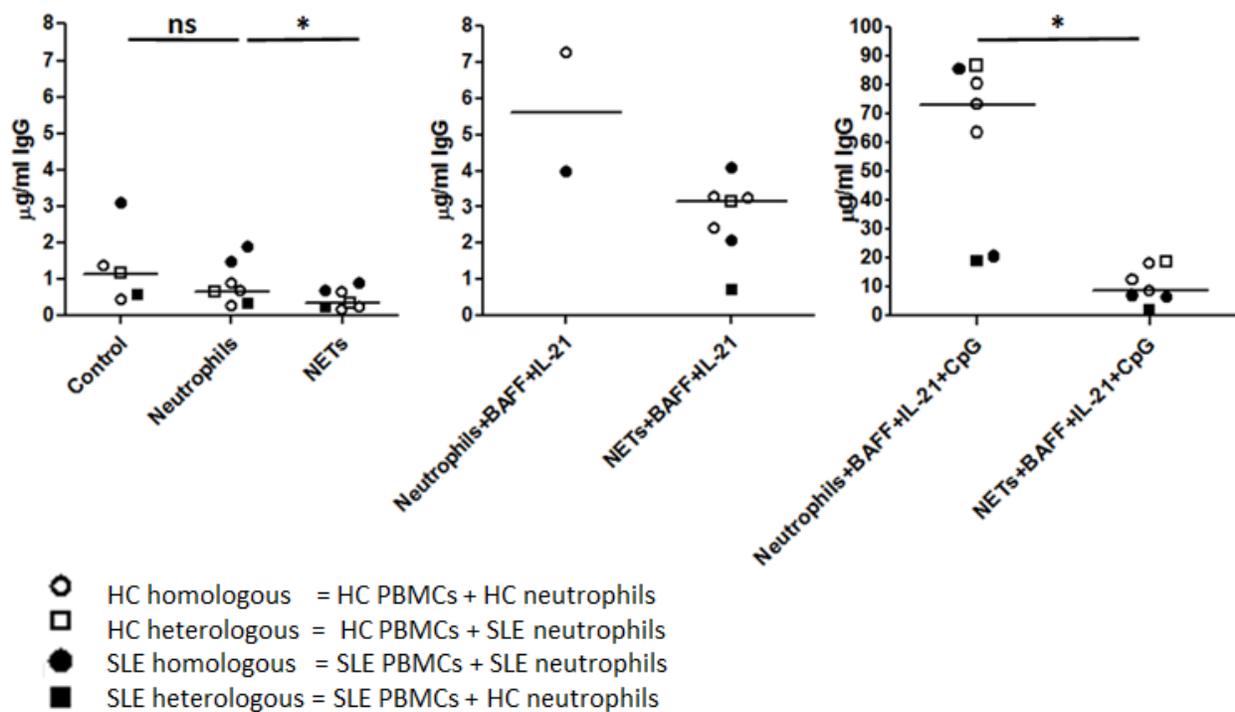


Figure 13, Total IgG production measured by ELISA in supernatants of different groups of PBMC cultures.—=median , * $p < 0.05$.

4. Conclusion and discussion

4.1 Spontaneous and induced NETosis in HC and SLE neutrophils

After 3 hours of culturing neutrophils with or without PMA, LPS and HMGB1-cytokine NET formation is not stable yet. After 4 hours the amount of NETs from unstimulated neutrophils and PMA and LPS stimulated neutrophils is increased compared to the 3 hours stimulation in healthy controls. Only for stimulation with HMGB1-cytokine it makes no difference if the neutrophils are stimulated for 3 or 4 hours. In neutrophils from SLE patients the amount of NETs produced increases also in time for unstimulated neutrophils and PMA and HMGB1-cytokine stimulated neutrophils. There was no significant difference seen in the LPS stimulated neutrophils. So, neutrophils are not ready with releasing NETs after 3 hours, but an increase of NETosis might be not due to the stimulation because unstimulated neutrophils from healthy control also produce more NETs after 4 hours compared to 3 hours. The morphology of NETs also changes over time. After 3 hours the NETs produced by PMA stimulation are cloud-shaped, and after 4 hours there are more dread-like structures present. MPO is present in NETs of 3 hour stimulation, but seem to leave after 4 hour stimulation. MPO might diffuse into the cytoplasm. To analyze this, the amount of MPO can be measured in the supernatant for later studies.

Neutrophils from SLE patients are known to form NETs spontaneously *in vitro*. (3)(18), but in this study these data could not be repeated. There was no significant difference between unstimulated NETosis of SLE patients and healthy controls *in vitro*. This could be due to the small patients group, or the fact that these patients were all quiescent patients. Also the medication used by these patients can influence their spontaneous NETosis. Besides in spontaneous NETosis, there was also no significant difference found in stimulated NETosis between SLE patients and healthy control. Neutrophils from SLE patients do not react differently on PMA, LPS and HMGB1-cytokine than neutrophils from healthy controls.

PMA is able to induce NETosis significantly with a p-value <0.0001 after 4 hours of stimulation. Healthy controls release significantly more NETs after LPS stimulation, but SLE patients do not. SLE patients release more NETs spontaneously, although not significant, and this could be the reason why these neutrophils do not react on LPS anymore. The same counts for HMGB1-cytokine stimulation; neutrophils from healthy controls show increased NETosis, but neutrophils from SLE patients do not. So, it seems that HMGB1-cytokine does induce NETosis, at least in neutrophils from healthy controls. However, the effect of HMGB1-cytokine should be tested in neutrophils from more healthy controls and patients. Besides HMGB1-cytokine, also HMGB1-chemokine and HMGB1 without a redox state should be tested to compare with each other. Another interesting research question for the future would be what the effect of HMGB1-anti-HMGB1 complexes is on NETosis, because these complexes are present during SLE.

4.2 Effect of 5-ASA, CSE, PDN and HCQ on NETosis

Because lots of studies propose a pathogenic role of NETs in various auto-immune diseases, inhibition of NETosis might be beneficial for patients and could act as a new therapeutic target. Any molecule that is able to inhibit the formation of ROS by NADPH, should be able to inhibit NETosis. 5-ASA is a scavenger of ROS, inhibits MPO, and is able to inhibit NETosis on a dose dependent manner. Neutrophils lose their polymorph nucleus, a sign of activation, and seem to be stretched-out. Unless the signs of activation, the amount of NETs made after PMA stimulation by neutrophils pre-treated with 5-ASA is decreased.

MPO and NE stay in the cytoplasm and Citr.H3 is present in the nuclei. The citrullination of histone 3 seems to be slightly decreased after 5-ASA pre-treatment. This could be due to the fact that ROS is scavenged by 5-ASA and ROS normally acts as a PAD4 activator. After treatment with 5-ASA, ROS is inhibited and thereby the activity of PAD4 and the citrullination of Histone 3 are also decreased. From this study it is not clear if neutrophils are permanently unable to form NETs, or if they can recover and form NETs in a larger time span after stimulation.

5-ASA is the working compound of sulfasalazine, a drug used for the treatment of UC. Patients with UC suffer from inflammation of the colon caused by a large infiltration of neutrophils. The fact that 5-ASA is a strong inhibitor of NETosis suggests that neutrophils and NETs might play a major role in this disease. A future plan is to analyze tissue of UC patients for NETs before and during 5-ASA therapy to establish the hypothesis that NETs play a major role in UC. The microbiome of patients might also play a role in the formation of NETs. Interestingly, UC is the only auto-immune disease in which smoking is known to have anti-inflammatory effects. Because of the large neutrophil infiltrations, it is interesting to look for a link between smoking and NETosis.

This study reveals that CSE inhibits NETosis. A pre-treatment with 30% CSE decreases the amount of NETs to a level comparable with unstimulated and untreated neutrophils. Even on unstimulated neutrophils concentration of 5-30% CSE lowers the amount of spontaneous NETosis significantly. This means that CSE does not interact with PMA to achieve this decrease. Neutrophils treated with 30% CSE seem to be paralyzed; even after PMA stimulation neutrophils keep their round shape and polymorph nuclei. MPO and NE are located in the cytoplasm and Citr.H3 is expressed in the nucleus. HMGB1 is expressed in both nucleus and cytoplasm of unstimulated and untreated neutrophils, but after treatment with 30% CSE HMGB1 is only found in the nucleus. Because the PMA stimulated neutrophils show no sign of activation at all, CSE has to inhibit NETosis in an early state. PMA stimulates the PKC pathway directly and does not work with receptors, so the effect of CSE cannot be a consequence of receptor binding. If CSE would interact with ROS or MPO, the same effect as 5-ASA would be seen. These observations lead to the hypothesis that CSE interacts in the PKC pathway, leading to paralyzed neutrophils. These paralyzed neutrophils could also be the reason why smokers suffer more from infections in their upper respiratory tract. Future studies have to unravel the exact working mechanism of CSE and find the working compound.

Prednisolone is not able to inhibit PMA induced NETosis. Prednisolone is a glucocorticoid and is used as an immunosuppressive drug in various diseases. Prednisolone binds to glucocorticoid receptors in the cytoplasm and translocates to the nucleus where it interferes with gene transcription. These actions seem to be unrelated to the mechanisms used for NETosis.

The effect of hydroxychloroquine on NETosis is only investigated with n=2, but no decrease of NETosis is found in these samples. The HCQ used in this study was expired, and this could have induced a false negative effect. The influence of HCQ should be analyzed again with new HCQ and in a greater subset.

4.3 Influence of NETs on IgG production

In the presence of NETs the total amount of IgG in supernatant decreases compared to the IgG level produced in the presence of neutrophils in unstimulated, BAFF+IL-21 stimulated and BAFF+IL-21+CpG stimulated B-cells. There was no control group included with B-cells without neutrophils or NETs and

stimulation with BAFF+IL-21 or BAFF+IL-21+CpG. Therefore it is only possible to compare the influence of neutrophils and NETs on B-cells, but the influence of neutrophils/NETs cannot be compared with stimulated B-cells without neutrophils or NETs. Neutrophils could produce inflammatory stimuli like BAFF or free radicals that could induce IgG production, and that would give a false negative result for the IgG production in the presence of NETs. However, the total IgG level produced in the presence of NETs was decreased in comparison with both PBMCs alone and in the presence of neutrophils. Neutrophils have a short live span and do not live longer than four days in a culture. After these days the neutrophils will die, and this might lead to apoptotic neutrophils in the culture. The consequences of these apoptotic cells is also unknown in this setup. Another possibility for the decreased IgG level in the presence of NETs is that the IgG molecules can stick in the NETs and are as a result not available in the supernatant to be measured with ELISA. For further research also other B-cell activators can be used, like direct BCR-ligands. Besides measuring the total IgG level, also the amount of auto-antibodies like ANAs could be quantified. A biological explanation for the decreased IgG level in the presence of NETs could be the degradation of BAFF, IL-21 and CpG by NETs. NETs contain proteases that can degrade inflammatory mediators. With this property NETs could stabilize an inflammation. (8) (48) When NETs would be able to directly activate B-cells to produce auto-antibodies, and these auto-antibodies are able to induce NETosis, a patient would suffer from a continuously increasing inflammation because of the existence of a vicious circle. From this side of view NETs might play a role in the stabilization of an inflammation.

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Appendix I: Cell isolation

Neutrophil isolation (for 2 10ml tubes lithium-heparin blood)

- Dilute blood 1:1 with Hanks' Balanced Salt Solution (HBSS-/-) in a 50 mL tube
- Add 10ml lymphoprep to 2 50ml tubes
- Overlay with 20ml of the blood solution
- Centrifuge at 2400 rpm, 20 minutes, no brake (prog 6)
- (Put ca. 10 min before centrifuge is ready, a bottle of ammonium chloride at -20°C)
- Aspirate all layers (serum, white band, lymphoprep) but for the red layer (red blood cells and neutrophils)
- Add 30-35mL ice-cold ammonium chloride to the tubes to lyse erythrocytes
- Mix suspension on ice for 10-20 minutes till suspension becomes clear and dark
- Centrifuge at 1800 rpm, 5 minutes (prog 7)
- Aspirate supernatant, repeat lysis and centrifugation steps
- Aspirate supernatant, resuspend pellets in 10 ml HBSS-/-, and collect pellets of the same person to one tube
- Centrifuge at 1800 for 5 min (prog 7)
- Remove supernatant and resuspend in 2 ml of HBSS-/-
- Count cells in Coulter Counter by adding 40µl of cell suspension in 20ml isotone + 2 drops of lyzerglobin
- Dilute neutrophils with HBSS +CaCl₂+MgCl₂ +0.1% BSA

PBMC isolation (for 2 10ml tubes lithium-heparin blood)

- Dilute blood 1:1 with RPMI 1640 + 6% Gentamicin (10mg/ml)
- Add 4ml lymphoprep to 5 12ml tubes
- Overlay carefully with 8ml of the blood solution
- Centrifuge at 2400 rpm, 20 minutes, no brake (prog 6)
- Aspirate supernatant till 0.5cm above white layer
- Set white band to clean sterile 12ml tube with 10ml pipet. Max 2 layers per clean tube.
- Add ca. 10ml RPMI + gentamicin and mix.
- Centrifuge at 1800 rpm, 10 minutes, brake 4 (prog 7)
- Aspirate supernatant, resuspend pellet in 10ml RPMI + gentamicin and collect all pellets in one tube.
- Centrifuge at 1200 for 5 min, brake 4 (prog 8)
- Remove supernatant and resuspend pellet in 2ml RPMI + gentamicin
- Count cells in Coulter counter by adding 40µl of cell suspension in 20ml isotone + 2 drops of lyzerglobin

Appendix II: Induction of NETosis

NET induction with PMA, LPS and HMGB1-Cytokine

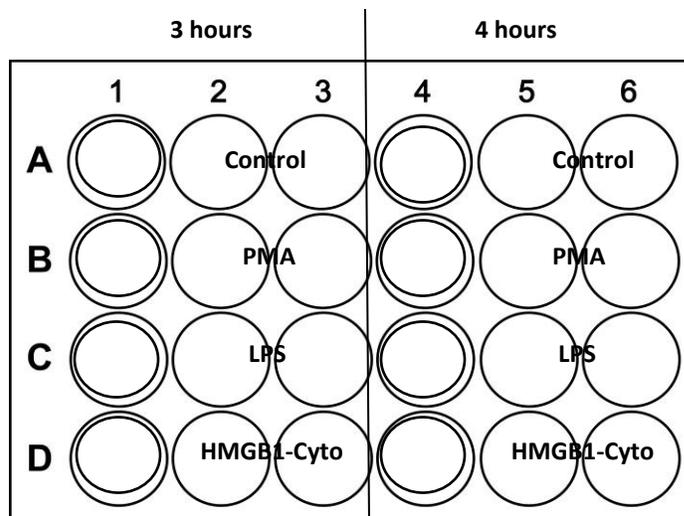
- Dilute cells to 2×10^6 cells/ml in HBSS+/+ + 0.1% BSA or to 0.5×10^6 cells/ml (coverslips)
- Add 500 μ L of cell suspension to wells of 24-wells (1×10^6 cells/ 0.25×10^6 cells)
- Let cells adhere for 30 minutes at 37°C
- Make stimulation dilutions in HBSS+/+ + 0.1% BSA (for 1 plate)
 - Control: 3ml HBSS+/+ +
 - PMA (stock 1,6mM): for 40nM = 1ul in 199ul HBSS+/+ +, from this 15ul in 3ml HBSS+/+ + (final concentration in well=20nM)
 - LPS (stock 0,1mg/ml): for 2ug/ml = 60ul in 3ml HBSS+/+ + (final concentration in well = 1ug/ml)
 - HMGB1 Cytokine (stock 0,1mg/ml): for 6ug/ml = 18ul in 3ml HBSS+/+ + (final concentration in well = 3ug/ml)
- Add 500ul of stimulation medium per well to the 500ul cell suspension (end volume 1ml)
- Stimulate for 3 and 4 hours at 37°C

Coverslips:

- Pipet solution off, add 1ml 4% formalin and incubate overnight at 4°C
- Wash next morning 3x with PBS and store in PBS at -20°C till IHC

Supernatant/sytox

- Add 25ul MNase, 10min at 37°C
 - 250mU MNase per well \rightarrow 1ul at 1ml sterile H₂O \rightarrow 25ul per well
- Block reaction with 25ul 0.1M EGTA (pH 8.0), 3min RT on shaker
- Collect medium, spin at 3000g for 5 min and store supernatant at -20°C



Appendix III: Inhibition of NETosis

Inhibition of NETosis with Mesalazine, CSE, Hydroxychloroquine and Prednisolone

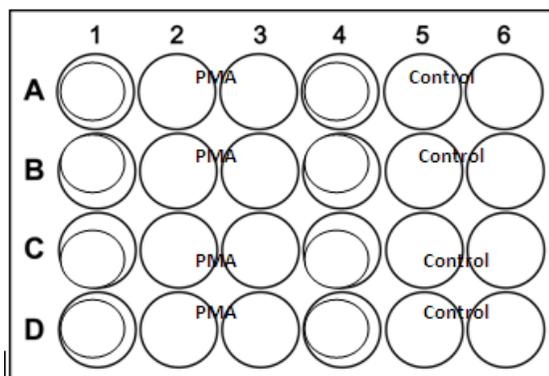
- Dilute cells to 2×10^6 cells/ml in HBSS+/+ + 0.1% BSA or to 0.5×10^6 cells/ml (coverslips)
- Add 500 μ L of cell suspension to wells of 24-wells (1×10^6 cells/ 0.25×10^6 cells per well)
- Dilute mesalazine in HBSS+/+ + 0.1% BSA and add 500ul per well. Mesalazine (5-ASA) stock: 0,10885M
 - Control: 3.3 ml control HBSS+/+ +
 - 2.0mM : 30.3ul in 3.3ml HBSS +/+ + (final concentration per well = 1.0mM)
 - 5.0mM: 85.8ul in 3.3ml HBSS +/+ + (final concentration per well = 2.5mM)
 - 10.0mM: 151.6ul in 3.3ml HBSS +/+ + (final concentration per well = 5.0mM)
- Dilute CSE in HBSS+/+ + 0.1% BSA and add 500ul per well. CSE stock: 100%
 - Control: 3.3 ml control HBSS+/+ + 0.1% BSA
 - 10%: 330ul in 2970ul HBSS +/+ + (final concentration per well = 5%)
 - 30%: 990ul in 2310ul HBSS +/+ + (final concentration per well = 15%)
 - 60%: 1980ul in 1320ul HBSS +/+ + (final concentration per well = 30%)
- Dilute hydroxychloroquine in HBSS+/+ + 0.1% BSA and add 500ul per well. HCQ stock:10mM
 - Control: 3.3ml control HBSS+/+ + 0.1% BSA
 - 10uM: 3.3ul in 3.3ml HBSS+/+ + 0.1% BSA (final concentration per well = 5.0uM)
 - 20uM: 6.6ul in 3.3ml HBSS+/+ + 0.1% BSA (final concentration per well = 10.0uM)
 - 40uM: 13.2ul in 3.3ml HBSS+/+ + 0.1% BSA (final concentration per well = 20.0uM)
- Dilute prednisolone in HBSS+/+ + 0.1% BSA and add 500ul per well. Prednisolone stock: 125ug/ml
 - Control: 3.3ml control HBSS+/+ + 0.1% BSA
 - 2ug/ml: 52.8ul in 3.3ml HBSS+/+ + 0.1% BSA (final concentration per well = 1ug/ml)
 - 5ug/ml: 132ul in 3.3ml HBSS+/+ + 0.1% BSA (final concentration per well = 2.5ug/ml)
 - 10ug/ml: 264ul in 3.3ml HBSS+/+ + 0.1% BSA (final concentration per well = 5.0ug/ml)
- Let cells adhere for 60 minutes at 37°C
- Pipet pre-treatment medium off and wash cells once with HBSS or PBS
- Make PMA stimulation dilution in HBSS+/+ + 0.1% BSA
 - PMA: 20nM \rightarrow 1ul in 199ul \rightarrow 99,0ul in 39,6ml HBSS+/+ +
 - Control \rightarrow 39,6ml HBSS +/+ +
- Add 1ml PMA dilution per well and stimulate for 3 hours at 37°C

Supernatant/sytox

- Add 25ul MNase, 10min at 37°C
 - 250mU MNase per well \rightarrow 3ul at 3ml sterile H₂O \rightarrow 25ul per well
- Block reaction with 25ul 0.1M EGTA (pH 8.0), 3min RT on shaker
- Collect medium, spin at 3000g for 5 min and store supernatant at -20°C

Coverslips

- Pipet solution off, add 1ml 4% formalin and incubate overnight at 4°C
- Wash next morning 3x with PBS and store in PBS at -20°C till IHC



5-ASA	CSE	HCQ	PNS
0	0	0	0
1.0mM	5%	5.0uM	100ng/ml
2.5mM	15%	10.0uM	1ug/ml
5.0mM	30%	20.0uM	10ug/ml

Appendix IV: Sytox Assay

Sytox assay

- Thaw samples on ice or in fridge
- Add 50ul sample per well of a black 96-wells plate (Fluotrac 200), use duplos of each sample
- Add 90ul of calibration curve (duplo)

○ AU	Sample	HBSS+/+
○ AU 90	(200)	0
○ AU 50	110	88
○ AU 20	44	154
○ AU 10	22	176
○ AU 5	11	187
○ AU 1	2.2	195.8
○ Blanc	0	(200)
- Make a sytox solution for the calibration curve: 1.6ul sytox in 160ul HBSS+/+ (concentration: 50uM)
- Make a sytox solution for the samples: 0.1ul sytox in 50ul HBSS+/+ per sample (concentration: 10uM)
- Add 10ul sytox solution to the calibration curve (end concentration: 5uM)(end volume:100ul)
- Add 50ul sytox solution to the samples (end concentration: 5uM)(end volume:100ul) using a multichannel pipet
- Put plate on shaker for at least 15min
- Remove air bubbles
- Read plate at ex485 em535 (1s) TOP in triplo

Appendix V: Immunohistochemistry

Immunohistochemistry on coverslips – General NETs

- Thaw plates
- Transfer coverslips to a parafilm coated surface
- Block with 2%BSA/PBS for 30min
- Pipet solution off
- Add primary antibody solution in PBS+ +DAPI(1:5000) and incubate 2 hours
 - PBS+ = 9.75mlPBS + 250ul 20%BSA + 50ul 10% Tween (+2ul DAPI) (stock solution can be stored at 4°C for a couple of months)
 - Anti-MPO (Ms) (1:100) (ICP 155P)
 - Anti-HMGB1 (Rb) (1:100) (ab18256)
- or
 - Anti-NE (Ms) (1:100) (Dako M0752)
 - Anti-Histone H4 (Rb) (1:100) (sc-10810)
- Wash 3x 5min with PBS 15min, darkness
- Add secondary antibody solution in PBS+ 1 hour, RT, darkness
 - Goat α Mouse-APC (1:50)
 - Goat α Rabbit-FITC (1:50)
- Wash 2x 10min with PBS 20min, darkness
- Mount coverslips upside-down on a slide in citifluor

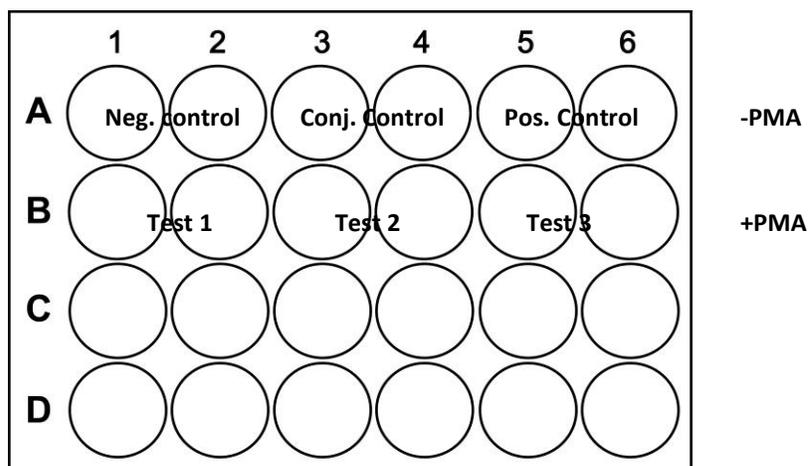
CD138 immunohistochemistry

- Incubate cytopins with 0,3% H₂O₂/PBS (shut off endogen peroxidase) (10min darkness)
 - 10ml PBS + 100ul 30%H₂O₂
- Incubate with 1:100 Murine α Human CD138 (IQP-153P) in 1% BSA/PBS, 50 μ L/coupe. (60min)
- Wash 3x in PBS (3x 5min)
- Incubate with labeled polymer-HRP anti-mouse (DAKO, K4006) (30min)
- Wash 3x in PBS (3x 5min)
- Incubate with Liquid DAB+ Substrate Chromogen System (DAKO, K3468) (10-15min)
 - 1ml substrate buffer + 1drop/20ul chromogen
- Wash 3x with demi water (3x 5min)
- Stain with hematoxillin (30sec)
- Wash with running tap water
- Embed coupes in Kaiser glycerin, cover with a slip

Appendix VI: PBMC stimulation

PBMC stimulation with NETs, CpG, BAFF and IL-21

- Dilute PBMCs to 2×10^6 cells/ml in RPMI+10%FCS
- Store PBMCs on ice till use
- Dilute neutrophils to 2×10^6 cells/ml in HBSS+/+ +0.1%BSA
- Plate out 500ul cell suspension per well of a 24 wells plate (1×10^6 cells)
- let cells adhere for 30min at 37°C
- Add 500ul HBSS+/+ + 0.1%BSA with PMA to induce NET formation (end volume well = 1ml)
 - PMA (stock 1,6mM): for 40nM = 1ul in 199ul \rightarrow 2.75ul in 550ul HBSS+/+ + (final concentration in well=20nM)
 - Control: 500ul HBSS+/+ +0.1%BSA
- Stimulate neutrophils 4 hours at 37°C
- (Harvest 2x +PMA/-PMA NETs from separate plate for sytox analysis to check amount of NETosis)
- Make B-cell stimulation medium (per well):
 - Negative control: (unstimulated neutrophils + 500ul PBMCs) + 500ul RPMI+10%FCS
 - Conjugate control: (unstimulated neutrophils + 500ul PBMCs) + 500ul BAFF+IL-21
 - Dilute in 485ul RPMI+10%FCS:
 - BAFF: 100ng/ml; stock=20ug/ml \rightarrow use 5ul
 - IL-21: 100ng/ml; stock=10ug/ml \rightarrow use 10ul
 - Positive control: (unstimulated neutrophils + 500ul PBMCs) + 500ul CpG+BAFF+IL-21
 - Dilute in 475ul RPMI+10%FCS:
 - CpG: 3.2ug/ml; stock=320ug/ml \rightarrow use 10ul
 - BAFF: 100ng/ml; stock=20ug/ml \rightarrow use 5ul
 - IL-21: 100ng/ml; stock=10ug/ml \rightarrow use 10ul
 - Test 1: (NETs + 500ul PBMCs) + 500ul RPMI+10%FCS
 - Test 2: (NETs + 500ul PBMCs) + 500ul BAFF + IL-21
 - Dilute in 485ul RPMI+10%FCS:
 - BAFF: 100ng/ml; stock=20ug/ml \rightarrow use 5ul
 - IL-21: 100ng/ml; stock=10ug/ml \rightarrow use 10ul
 - Test 3: (NETs + 500ul PBMCs) + 500ul CpG + BAFF + IL-21
 - Dilute in 475ul RPMI+10%FCS:
 - CpG: 3.2ug/ml; stock=320ug/ml \rightarrow use 10ul
 - BAFF: 100ng/ml; stock=20ug/ml \rightarrow use 5ul
 - IL-21: 100ng/ml; stock=10ug/ml \rightarrow use 10ul
- Pipet PMA medium off neutrophils and wash 1x with RPMI. Add 500ul PBMC suspension + 500ul B-cell stimulation medium

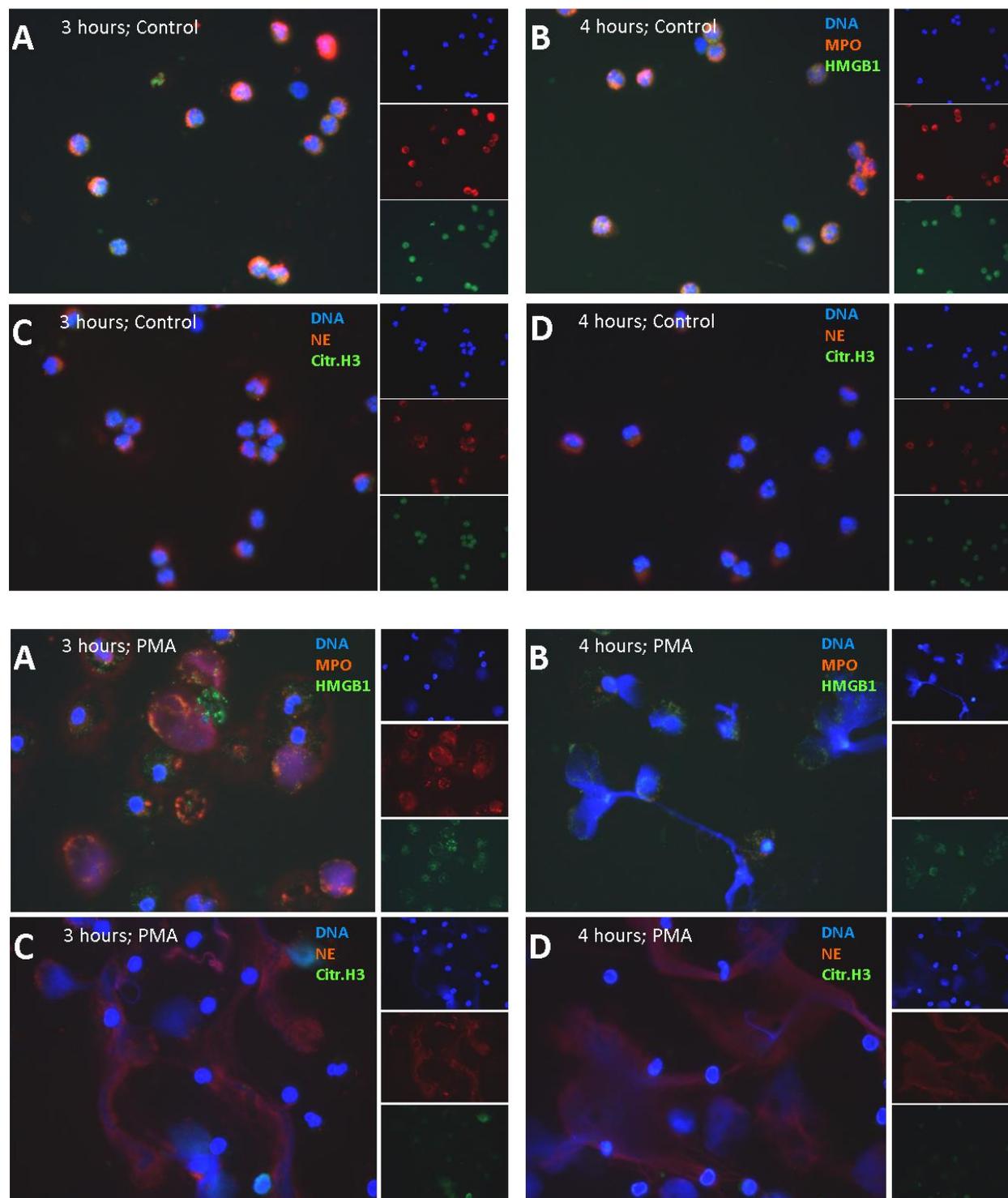


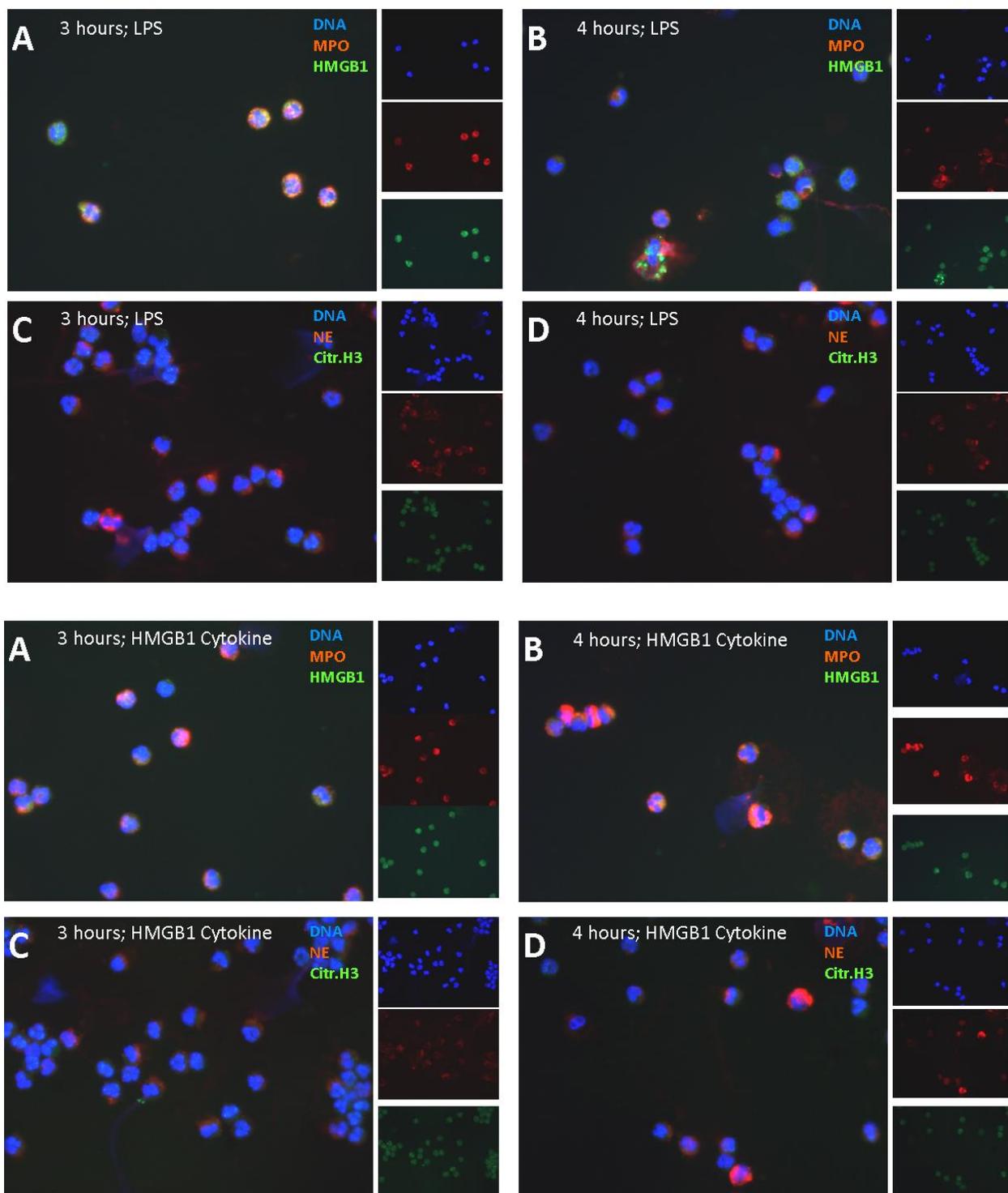
Appendix VII: ELISA

- Coat a 96 wells EIA/RIA plate (Costar, 9018) with 100µl 1:5000 GoataHuman-IgG (Jackson; 109-006-088) in PBS and put overnight on shaker
- Wash plate 5x with wash buffer
 - 0.25M Tris-HCl, 0.15M NaCl, 0.05% Tween-20 in demi water
- Block with 200ul PBS + 2%BSA + 0,05%Tween-20 for 60min on a shaker
 - For one plate: 2.0ml 20%BSA, 1ml 10%Tween-20, 17ml PBS
- Make dilution buffer (PBS +1% BSA + 0.05% Tween-20)
 - For one plate: 2.0ml 20%BSA, 2ml 10%Tween-20, 36ml PBS
- Make sample dilutions: 1:25 (480ul dilution buffer + 20ul sample) and mix well
- Make calibration dilutions with standard IgG sample (Siemens, 08369611), stock = 8.58 mg/ml. Start concentration 50ng/ml, make 2 fold dilutions.
 - 171600x (dilute 2x 100x, and 1x 17,16x) (1ul in 99 → 10ul in 990 → 171ul in 1883 for start concentration)
- Wash plate 5x with wash buffer
- Add 100ul incubation buffer in every well, except for the calibration line
- Add calibration line: Add 100ul calibration dilutions
- Add samples: Add 33ul sample dilution in first sample well and make 1:4 dilutions (33ul from first well to second well and so on)
- Incubate 90min on shaker
- Wash plate 5x with wash buffer
- Incubate with 100ul conjugate 1:2000 MouseαHuman-IgG-HRP (Southern Biotech; 90-40-05) in dilution buffer for 90min on a shaker.
- Wash plate 5x with wash buffer
- Add 100ul Colouring solution for 15-30min
 - 2 tablets 3,3,5,5-Tetramethyl-benzidin dihydrochloride (TAB) (Sigma Aldrich, T3405-100TAB) + 2ul H₂O₂ in 10ml Acetate buffer (Pharmacy UMCG)
- Stop reaction with 100ul H₂SO₄ (Pharmacy UMCG) (1M)
- Read plate at 450nm

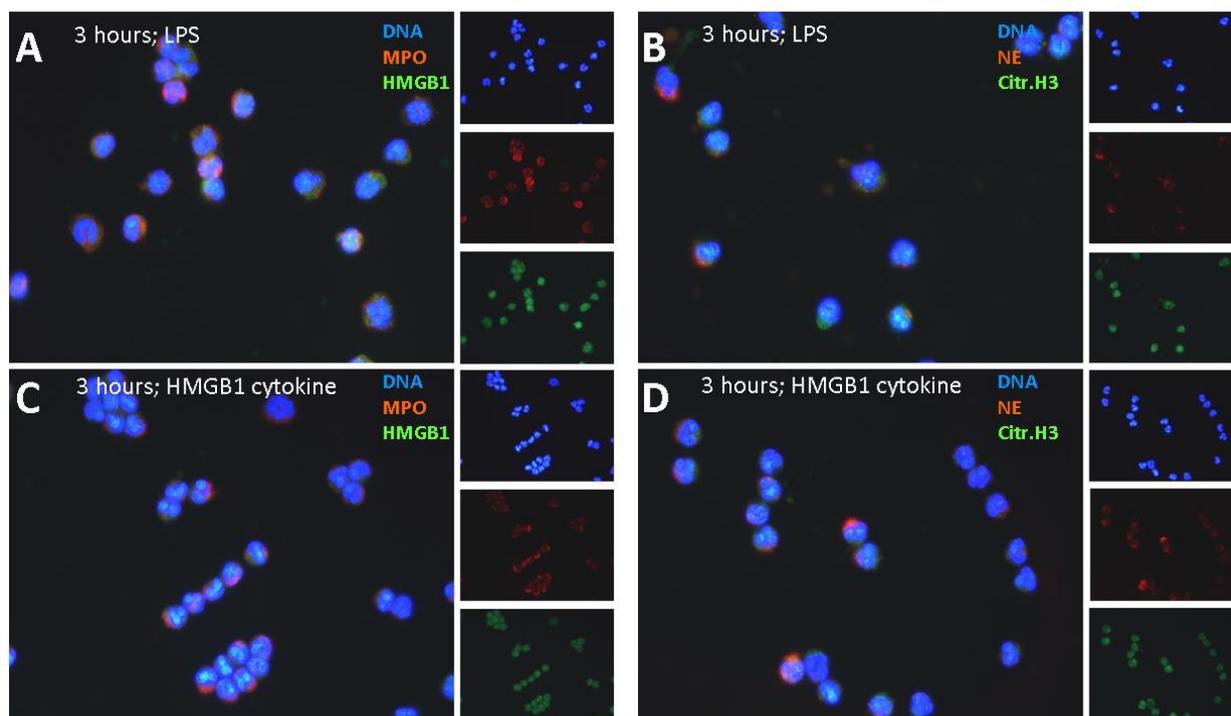
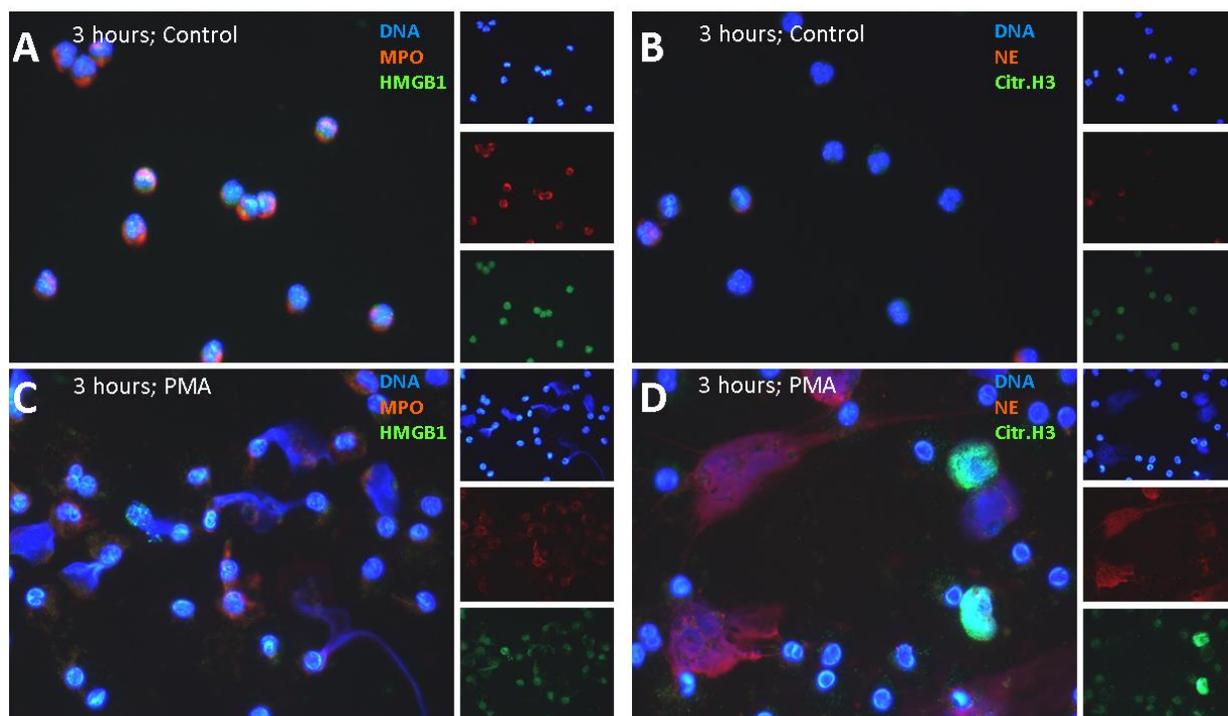
Supplementary Data

I: Control, PMA, LPS and HMGB1 Cytokine stimulated neutrophils from HC

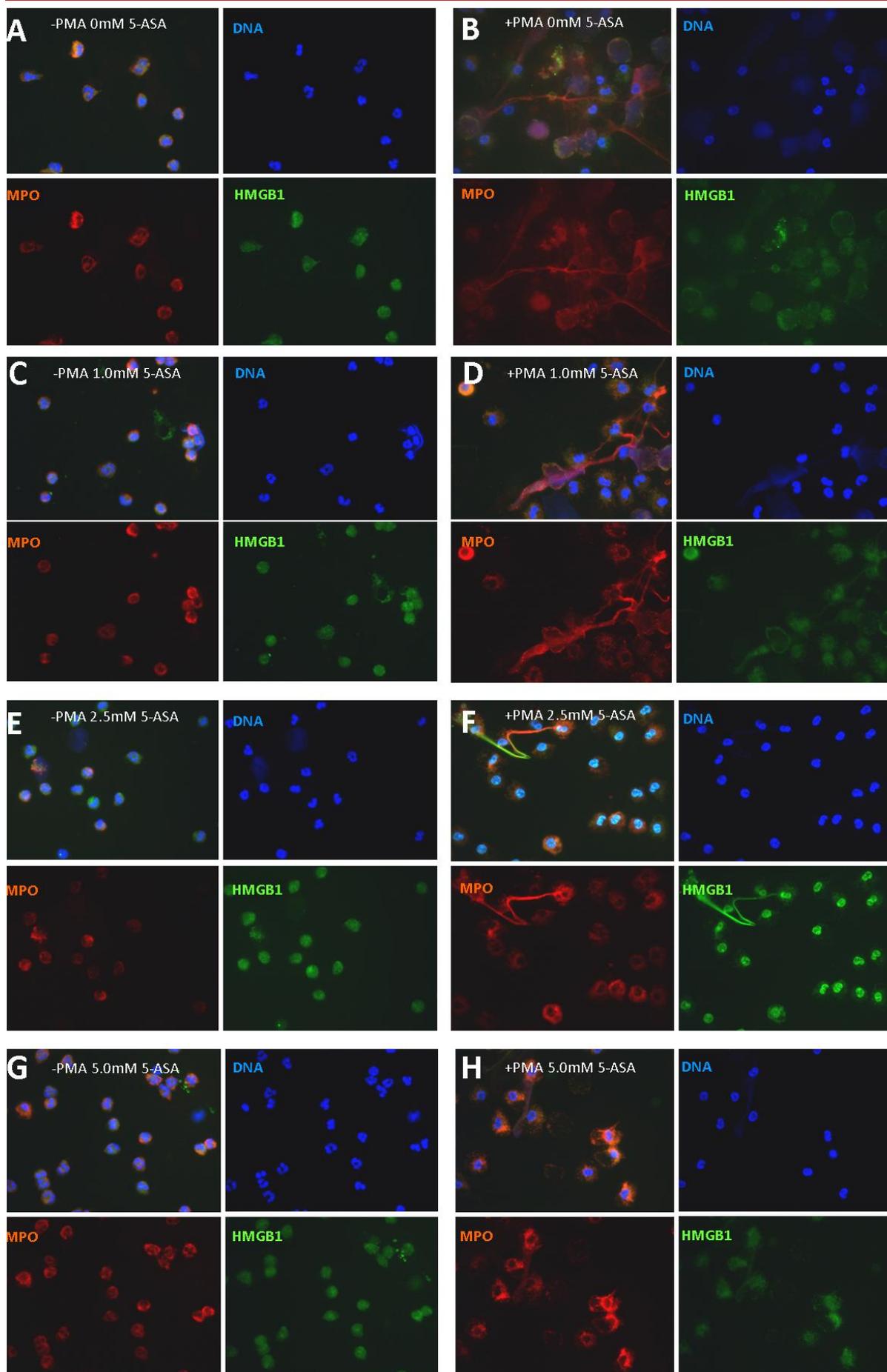


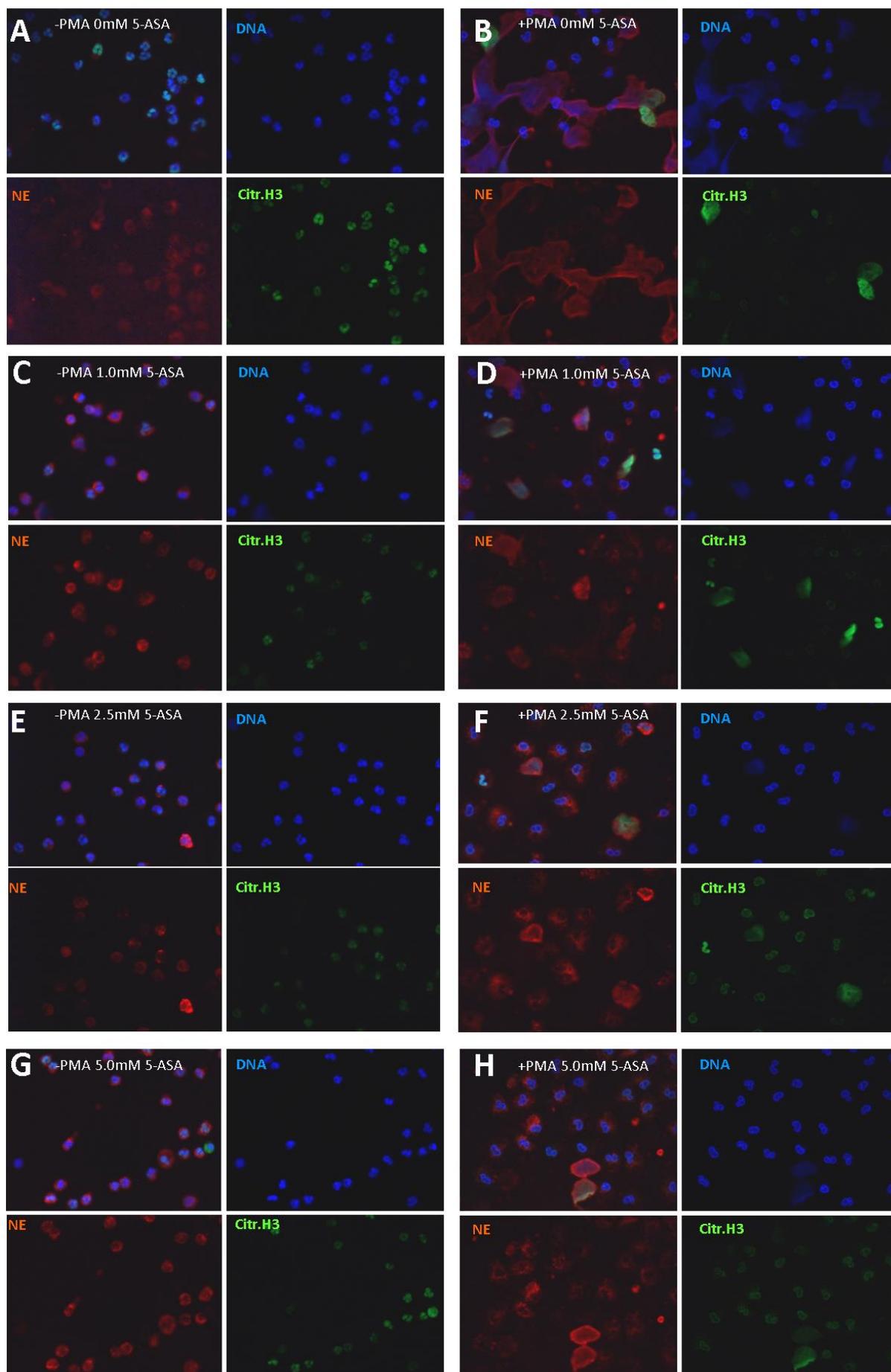


II: Control, PMA, LPS and HMGB1 Cytokine stimulated neutrophils from SLE patients



III: Effect of pre-treatment with 5-ASA on NETosis in HC





IV: Effect of pre-treatment with cigarette smoke extract on NETosis in HC

