Neutrophil Extracellular Traps in Inflammatory Disorders

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Table of Contents

Abbreviations

ACPA	Anti-citrullinated protein antibody
Ad-Sflt	Adenovirus vector containig Sflt-1 gene
AFU	arbitrary fluorescent units
ANCA	Antineutrophil cytoplasm autoantibody
APCs	antigen presenting cells
aPL	Anti Phospholipid
CaI	Calcium Ionophore
CCP	Cyclic Citrullinated Peptide
cDNA	Complementary DNA
CGD	Cronic granulomatous disease
CXCL	Chemokine (C-X-C motif) ligand
DAMPs	Damage associated molecular patterns
DAS	Disease Activity Score
DCs	Dendritic Cells
DMARD	Disease-modifying antirheumatic drug
DNase	deoxyribonuclease
DPI	diphenylene iodonium
DVT	Deep vein thrombosis
EULAR	European League Against Rheumatism
FCS	Fetal Calf Serum
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GM-CSF	Granulocyte-macrophage colony stimulating facto
H3Cit	Citrullinated Histone H3
HIV	Human immunodeficiency virus
HLA-DRB1	HLA class II histocompatibility antigen, DRB1-9 beta chain
IC	Immune Complex
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IUGR	Intrauterine growth restriction
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MSU	Monosodium urate
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase

NETs	Neutrophil extracellular traps
NOX2	NADPH) oxidase complex Nox2
PAD	Peptidylarginine deiminase
PAF	Platelet Activating Factor
PAMP	Pathogen AssociatedMolecular Patterns
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
pDCs	Plasmacytoid Dendritic Cells
PIGF	Placental growth factor
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear (cells)
PR3	Proteinase 3
PRR	Pattern Recognition Receptors
PRRs	pattern recognition receptors
PS	Phosphotidylserine
RA	Rheumatoid Arthritis
RBC	Red Blood Cells
RF	Rheumatoid Factor
ROS	Reactive oxygen species
SE	Standard error
Sflt-1	Soluble fms-like tyrosine kinase-1
SLE	Systemic Lupus Erythematosus
SLE	Systemic lupus erythematosus
TF	Tissue Factor
TGF-β	Transforming Growth Factor-Beta TLR Toll-like Receptor
TGF-β1	Transforming growth factor
TLR	Toll like receptor
TNF-α	Tumor Necrosis Factor Alpha TSH Thyroid Stimulating Harmone
VEGF	Vascular endothelial growth factor
Vwf	von Willebrand factor

CHAPTER 1: Introduction

1.1 Innate Immunity

During evolution different components of the immune system have evolved to protect against a wide array of pathogens. One of such key event was the ability to distinguish between self and non-self, which is necessary to protect the organism from invading pathogens and to eliminate, modified or altered cells. In response to the initial infection, three phases of immune reactions can be seen **(Figure 1)**. These are characterized by the innate phase, the early induced innate response, and the adaptive immune response [1]. The first two phases rely on the recognition of pathogens by germline-encoded pattern recognition receptors on the innate cells like NK cells, macrophages and Neutrophils, whereas adaptive immunity uses variable antigen-specific receptors [2]. Adaptive immunity is a delayed response, because of the involvement of B cells and T cells that must first undergo clonal expansion before initiating its effector functions [3]. The containment of infection until the adaptive arm of the immune system gets recruited to local site has long been considered the primary function of innate immunity. Once a pathogen gains access to the host tissue, contribution from both innate and adaptive immune responses becomes crucial. Overtime, this view has changed in the light of more important and fundamental role of host defense. Innate immunity provides a non-specific response against any pathogen via a variety of components and processes. These include barrier functions, complement, innate cells, antimicrobial peptides, mucosal secretions, pattern recognition receptors (PRRs) and the commensal micro-organisms[2]. PRRs function as molecular sensors of infection and are predominantly found on critical immune cells such as macrophages and dendritic cells (DC). PRR activation, results in changes in the protein conformation. Further, it activates intracellular signaling pathways to amplify the signal and initiates the innate response [2] [4].

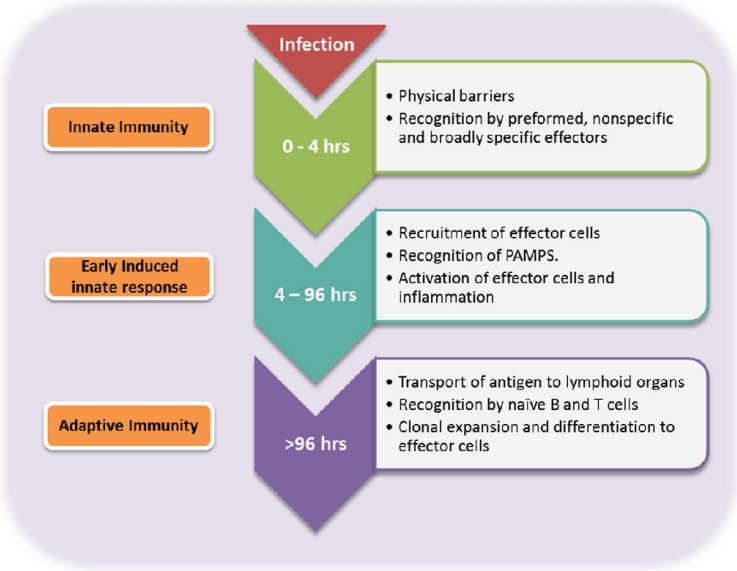

Figure 1. The response to the initial infection occurs in three phases. Modified from [1,2]

1.1.1 Innate immune recognition mechanism

In response to endogenous stimuli (cell death) or exogenous stimuli (pathogen invasion), damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) are released respectively. These molecules get identified by genetically inherited PRRs on the innate cells and further activate them. Activation of PRR and signaling is a complex process that results in upregulated expression of pro-inflammatory chemokines, cytokines and anti-viral proteins [4]. Intracellular molecules such as ATP and heat shock proteins can activate PRRs by acting as a ligands. These endogenous ligands are collectively known as damage associated molecular pattern (DAMPs) [5]. PRR activation results in conformational changes that amplify the signal and initiates the innate response **(Figure 2).** PRRs use specific adaptors and different adaptor proteins result in the activation of different signaling pathways which activates downstream signaling complex [4]. The immune cells that participate in these processes include, for example, APC, such as dendritic cells and macrophages, as well as T cells and neutrophils (PMN). PRRs can further stimulate the adaptive response, resulting in autoimmune responses and tissue repair [5].

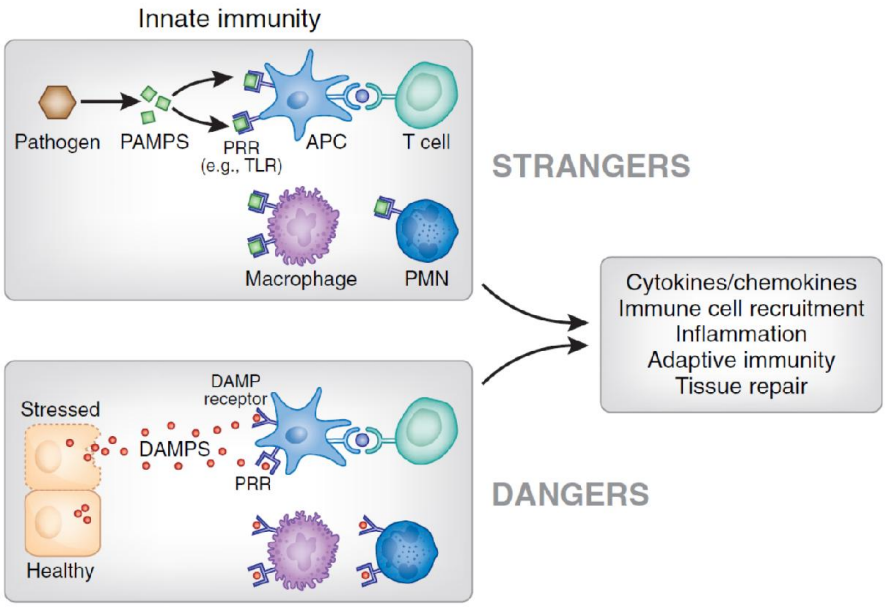

Figure 2.Danger and stranger models of innate recognition system. Infections of pathogenic bacteria or viruses cause release of PAMPs that bind to pattern recognition receptors (PRRs) on immune cells and stimulate an innate immune response that is accompanied by inflammation, activation of adaptive immunity, and eventually processes to resolve the infection and allow for tissue repair. The danger model recognizes similar events that occurs when cells are stressed or injured and that necrotic cells release molecules that are normally hidden within the cell. In the extracellular space these DAMPs can bind to PRRs or to specialized DAMP receptors to elicit an immune response by promoting release of pro-inflammatory mediators and recruiting immune cells to infiltrate the tissue. Adapted from [5].

1.1.2 Outcome of innate immune activation

The cellular and molecular changes associated with PRR activation are both complex and subtle. However, they create a response that can be shaped to deal with the specific nature of the infection. As a result of PRR stimulation are proinflammatory effectors, an array of cytokine, most important of which such as tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6 are produced [4]. These cytokines increases the permeability of the vasculature around a site and which helps in the recruitment of specialized immune cells, such as neutrophils, monocytes and macrophages [6]. The consequence of such infiltration is evidenced by the appearance of common symptoms, which include redness, heat, swelling and pain. Cellular death in localized areas of infection along with coordinate events in the whole body may lead to activation of the acute-phase response [1]. For example, in response to viral infection, type I IFNs production is triggered which further induces apoptosis in infected cells, thereby

removing the virus from the system. At the same time it triggers resistance to viral infection in neighbouring cells and so helps restrict the spread of infection.

1.1.3 Interplay of Innate and Adaptive Immunity

After being recognized by the innate immune system, the adaptive immune system comes into play [7]. Recognition of the PAMPs by PRRs, such as the TLR, generates signals that activate the adaptive immune system [8]. Following activation, endocytic pattern-recognition receptors, can bind to microbial components and mediate internalization and phagocytosis of pathogens by antigen-presenting cells such as dendritic cell or macrophage [8]. The internalized poteins are then processed in the lysosomes to generate antigenic peptides, which form a complex with major-histocompatibility-complex (MHC) class II molecules and presented on the surface of the antigen presenting cell **(Figure 3).** These processed peptides are then identified by T-cell receptors [3]. In the case of the signaling class of PRRs, the recognition of PAMPs by TLRs leads to the activation of signaling pathways that induce the expression of cytokines, chemokines, and costimulatory molecules. Thus, pattern-recognition receptors act as a bridge between innate and adaptive system by generating both the peptide–MHC-molecule complex and the costimulation required for the activation of T cells [3].

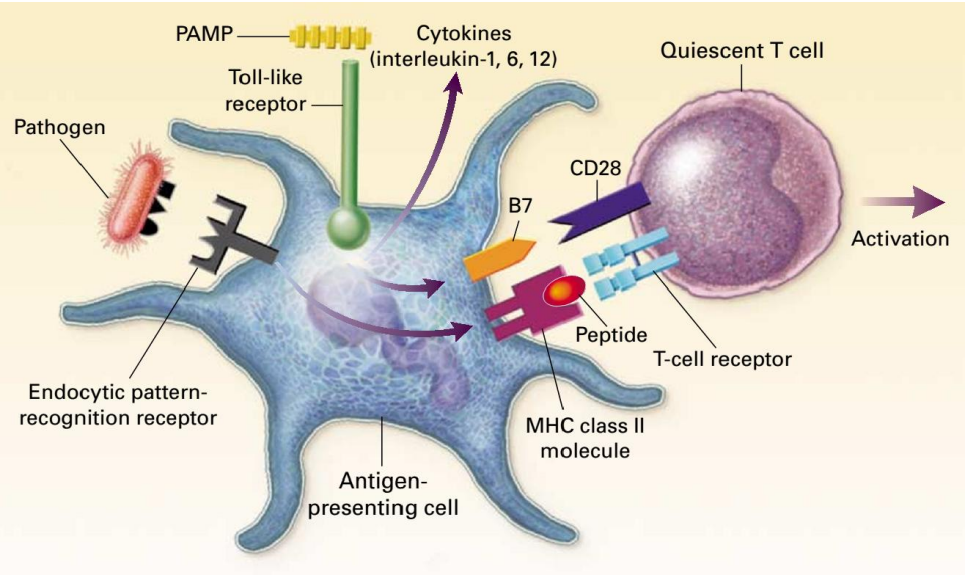

Figure 3.The Receptors Involved in the Interplay of the Innate and Adaptive Immune Systems. Adapted from [3].

1.2 Neutrophils: First Line of Defense against Infections

Neutrophils are the major antimicrobial phagocytes of the innate immune system [9]. They originate from common myeloid progenitor cells in the bone marrow. After being released from bone marrow, neutrophils circulate in vessels until being attracted to tissues by chemotactic signals (e.g. formyl peptides, lipid mediators and chemokines) [6]. They are the most abundant leukocytes in the peripheral blood, comprising up to 50% of white blood cells and further make up the majority of infiltrating cells found at the sites of infection or tissue injury [10]. Through interactions with various PPRs, neutrophils can recognize a large variety of stimuli, including immune complexes (IC), complement, and PAMPs. Thus, it is capable of shaping the immune response by affecting the early inflammatory milieu [6]. Although, neutrophils are known for their critical role in innate responses, recent discoveries have greatly broadened our knowledge about the functional role of this cell type in modulating secondary immune functions. Different types of granules are released depending on the strength and type of signal, allowing neutrophils to modulate their responses. Although, they are the first of defense against infection, under certain conditions, neutrophils known to be responsible for much of the damage to host tissues in some types of autoimmune disorders, such as rheumatoid arthritis. Depending upon the type of stimulation neutrophils switches between its multiple effector functions. Phagocytosis, oxidative damage, degranulation and newly identified NETosis plays prime function in host defense [10].

1.3 Stages of Neutrophil activation

The triggering of neutrophil towards effector function is a two-stage process involving an initial prerequisite “priming” step and a second “activation” step. Resting neutrophils, undergoing apoptosis within 12–18 hr. While, primed and activated neutrophils undergone molecular changes that extend their life span, probably by delaying apoptosis and alter their molecular properties, thereby allowing them to carry out multiple functions [11].

1.3.1 Neutrophil Priming

The priming of neutrophils can be defined as the process in which neutrophil responsiveness is significantly amplified to subsequent external stimuli, which may serve to augment the inflammatory response, but itself does not result in the desired response [9,12]. For example, exposure of neutrophils to high concentrations of LPS does not induce the oxidative burst, but will greatly potentiate the oxidative burst in response to another stimulus such as fMLP, C5a or PAF[13]. Later it was proposed that priming could serve as an essential "check point" which assures that the effector functions of neutrophil is localized to the site of infection[14]. Deregulation of such checkpoint could potentially contribute to disease pathogenesis. Alternately, inhibition could serve as potential drug targets against inflammation.

Primed PMNs display altered structural organization of the NADPH oxidase, depending upon the type of stimuli which leads to phosphorylation of the oxidase subunits and/or translocation from the cytosol to the plasma or granular membrane [15]. Studies have suggested that priming of neutrophils by TNFα and GM-CSF induces an increase in fMLP-receptor plasma membrane expression and triggers heterotrimeric G-protein activation or reorganization. In addition to partial p47phox phosphorylation and cytochrome b558 translocation, other mechanisms may be at play but remain to be identified [13]. Priming occurs via two separate mechanisms. Rapid priming (within minutes of the cell receiving a signal) results from the mobilization of intracellular granules that possess pre-formed receptors **(Figure 4)** to the plasma membrane. This process increases the number (and sometimes the affinity) of surface-expressed plasma membrane receptors by mechanisms that do not involve protein biosynthesis. Often, however, the priming agent will also result in activation of transcription factors that trigger the de novo expression of molecules (e.g. receptors and cytokines), which enhance neutrophil function or lifespan. Thus, the molecular properties and hence functions of resting blood neutrophils and primed neutrophils are very different. For this reason, in vitro experiments using freshly isolated blood neutrophils often fail to recognize the full functional repertoire and capability of neutrophils.

1.3.2 Neutrophil De-priming

Neutrophil priming is not an irreversible event [16]. Work from Kitchen et.al., using platelet activation factor (PAF), has demonstrated a complete cycle of priming, depriming, and repriming [16]. This property of neutrophil is of prime importance, as it offers the potential for functional recycling of neutrophils at sites of inflammation. This is further evidenced by work form Singh et.al., has shown that neutrophils migrating into the healthy pulmonary vasculature, can be de-primed and released back into the circulation in a quiescent state, in the absence of further stimuli. However, if this pulmonary 'de-priming' mechanism fails, or a second insult occurs, such as trauma, primed neutrophils migrate from the pulmonary vasculature into the interstitial space with resultant lung injury [14]. Combining mathematical approach to inflammatory bowel disease (IBD), it was recently concluded that in vivo de-priming must take place to limit the numbers of primed neutrophils in the circulation.

1.3.3 Neutrophil Activation

Neutrophil activation refers to processes that lead to recognizable (i.e. measureable) alterations in cells. Following priming state neutrophils could enter into a fully activated stage, by activation of transcription factors that trigger the de novo expression of molecules (e.g. receptors and cytokines) which increases the lifespan, followed by enhancement of neutrophil function such as phagocytosis, production of reactive oxygen species (ROS), degranulation, and generation of neutrophil extracellular traps (NETs) [13]. During this process complete assembly of the membrane-linked and cytosolic NADPH oxidase components occur on a PMN membrane, the plasma or granular membrane [15]. Thus, the molecular properties and hence functions of resting blood neutrophils and primed neutrophils are very different. This is probably why, in vitro experiments using freshly isolated blood neutrophils often fail to recognize the full functional repertoire and capability of neutrophils.

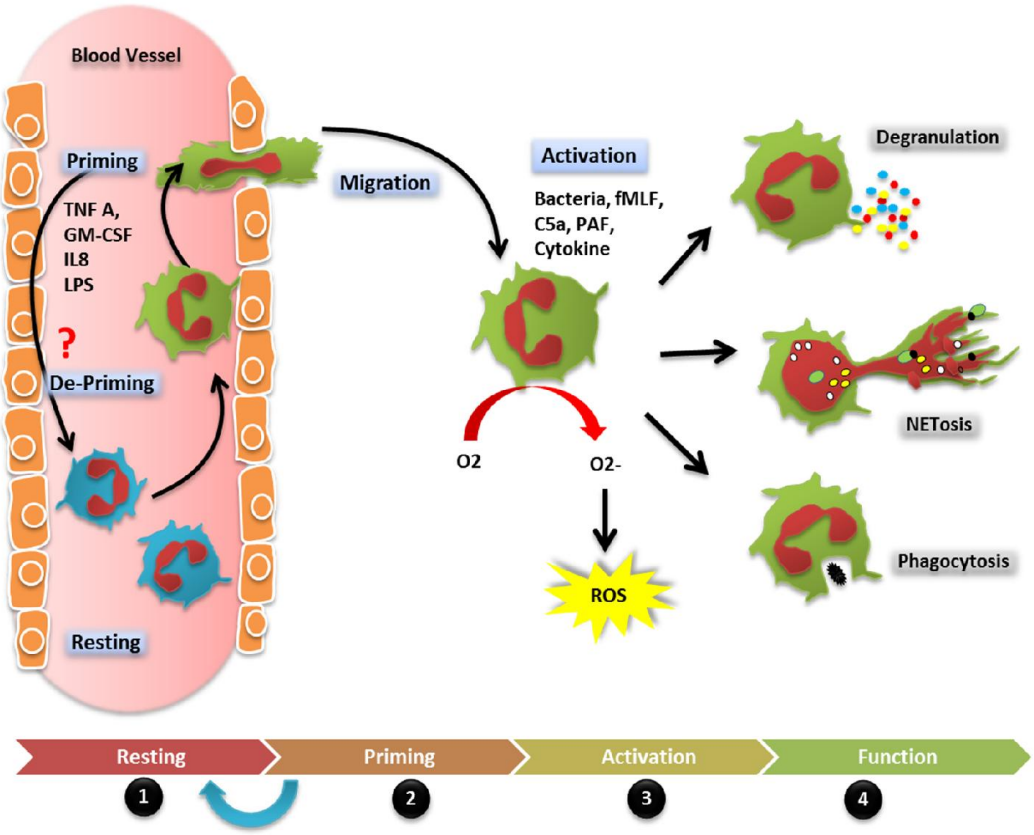

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Figure 4. Schematic overview of the stages of neutrophil activation. Circulating neutrophils are freely flowing in a dormant state (resting). Upon infection and inflammation, pro-inflammatory mediators (ex-TNFα, GM-CSF, IL-8, LPS) induce changes in the vascular endothelium that signal to circulating neutrophils to roll, adhere, and cross the endothelium. These pro-inflammatory mediators also prime neutrophils (priming). Primed neutrophil migrate to the inflammatory or infection site, where they get activated by a secondary stimuli, which may include the pathogen itself, its components or unknown factors (activation). In the absence of secondary simulation, neutrophil may get de-primed and migrate back into the blood vessel by an unknown mechanism. Activation of neutrophil is known to increases intracellular ROS level. The effector function of neutrophil is dependent on the specific type of secondary stimuli, which may results in degranulation, phagocytosis or NETosis. Modified from [13].

1.4 Effector Mechanisms of Neutrophil

1.4.1 Phagocytosis

Elie Metchnikoff in the year 1880s observed specialized phagocytic cells ingesting bacteria and called it “Phagocytosis “. In this process neutrophils internalize or take up microbes into specialized compartments known as phagosomes. This process of phagocytosis is a receptor-mediated, clathrin-independent process [17]. Fusion of neutrophil granules with the phagosome results in the formation of a phagolysosome, allowing for the assembly of the

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex Nox2. The NADPH transfers electrons to molecular oxygen, to generate superoxide anions into the lumen of the phagolysosome [7] causing an oxidative environment that along with antimicrobial factors leads to the inactivation and killing of ingested microbes [18].

1.4.2 Degranulation

Neutrophils are known to secrete 4 different types of granules classified as (1) primary granules, also known as azurophilic granules; (2) secondary granules, also known as specific granules; (3) tertiary granules; and (4) secretory vesicles **(Figure 5)**. Degranulation from neutrophils has been has been associated with pulmonary disorders, including severe asphyxic episodes of asthma [19]. However, not much is known about the mechanisms that control neutrophil degranulation. Degranulation involves the fusion of granules with the plasma membrane and the release of cytokines and antimicrobial contents into the extracellular space. These molecules help coordinate the immune response and control pathogens extracellularly. Azurophilic granules lack the soluble ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) that would direct them to fuse with the plasma membrane. The contents of these granules are either deployed inside the phagosome or are released extracellularly via the third antimicrobial strategy in the neutrophil repertoire: the formation of NETs.

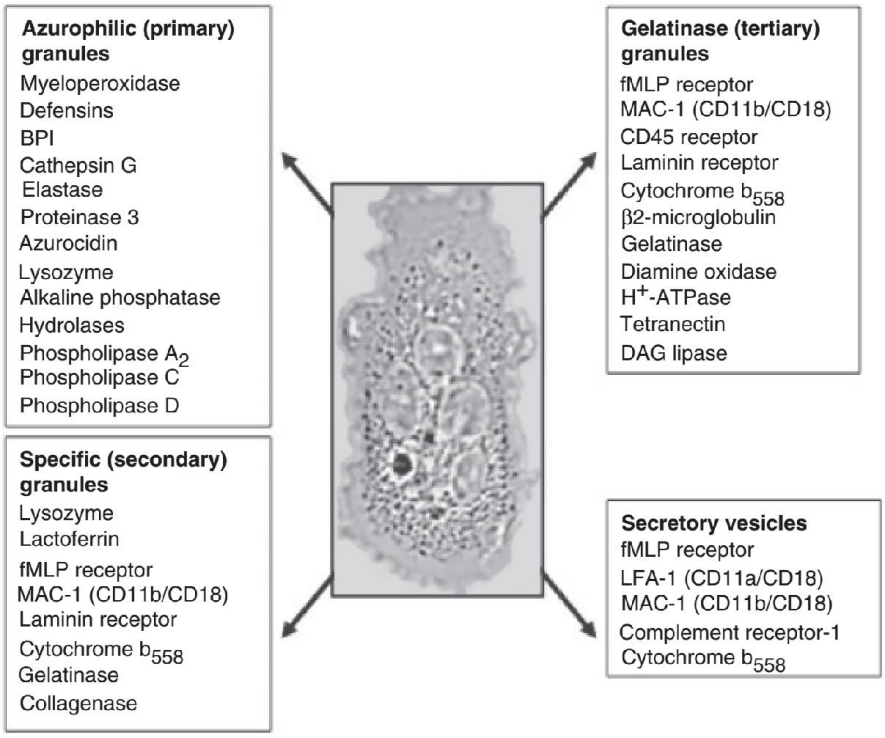


Figure 5. Summary of neutrophil granules and their contents. Neutrophil granules are mobilized upon priming of the cell:secretory vesicles are mobilized first, followed by gelatinase granules, specific granules and finally azurophilic granules. Adapted from [9]

1.4.3 Neutrophil Extracellular Traps

NETs are web-like structures that are composed of decondensed chromatin in complex with over 30 different neutrophil proteins that can capture, neutralize, and kill a variety of microbes. Several studies have shown that extracellular chromatin traps are not exclusively released by neutrophils. Eosinophils and mast cells have also been reported to release ETs. These large extracellular structures provide a physical barrier to prevent microbial dissemination and increase the local concentration of antimicrobial effectors [13–15]. Aside from infection, NETs have been recently found to regulate B cell function in the spleen [16] and to play a role in various sterile diseases, such as auto inflammation or autoimmune disease. An increasing number of bacteria, fungi, viruses, and protozoan parasites have been shown to induce NETs. Deficiencies leading to impaired NET formation result in high susceptibility to opportunistic infections in humans and mouse models and imply a significant contribution of NET formation in antimicrobial defense. NETosis appears to be tightly

regulated and dysregulation has been implicated in severe autoimmune and autoinflammatory disease. Below, we discuss the molecular mechanisms that lead to release of NETs, taking into consideration the differences between different physiological stimuli in infection and highlighting the importance of tight regulation of NET formation in autoimmunity and sterile inflammation. In response to pro-inflammatory stimuli, neutrophil adopts a primed phenotype, which stimulates migration into the inflammatory tissue site. In the tissue, neutrophils exposed to secondary stimuli gets "activated", a state characterized by release of granule proteins and acquisition of phagocytic capabilities [10]. However, in 2004, Brinkmann et al. through an elegant series of experiments documented a powerful method of neutrophil-mediated microbial killing through release of extracellular fiber-like structures **(Figure 6)**, and termed it as neutrophil extracellular traps (NETs) [20].

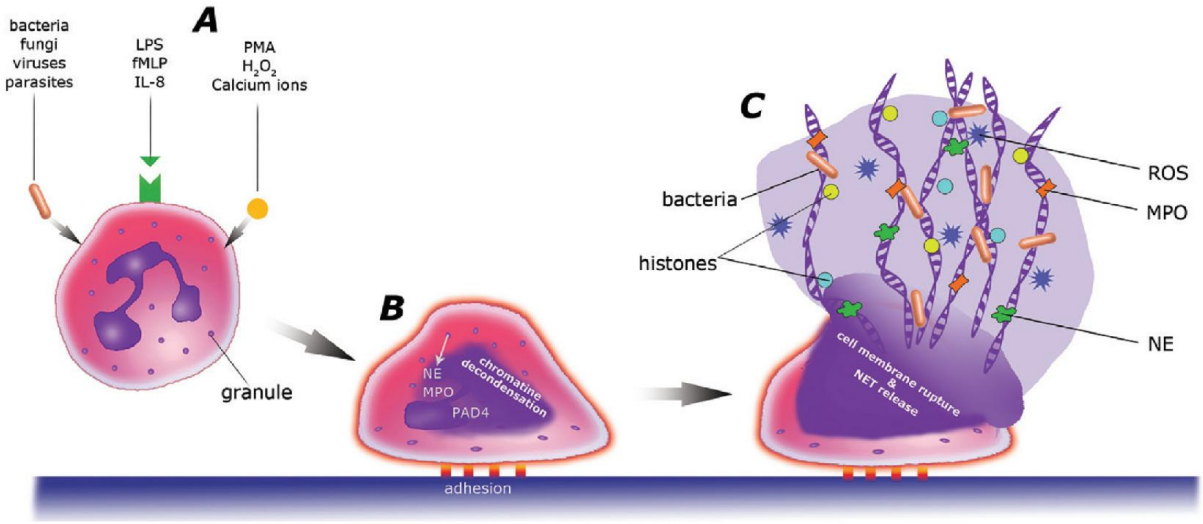


Figure 6. Mechanism of NET release. Stimulation of receptors (A) by triggers (e.g. bacteria, fungi, viruses, parasites, chemical factors like PMA or LPS) leads to the adherence of neutrophils to endothelium. Activation of signalling components leads to chromatin decondensation mediated by PAD4, NE and MPO (B). In the final phase, the cytoplasmic membrane ruptures and NETs are released into the surrounding (C). Adapted from [21]

1.4.3.1 NETosis Different from Necrosis and Apoptosis

NETosis is a novel cell death mechanism, shown to be different from necrosis and apoptosis **(Table 1)**. Initial in vitro studies using phorbol-12-myristate-13-acetate (PMA), demonstrated that NET formation is a cell death dependent process. However, physiological stimuli such as, GM-CSF in combination with C5a, could show that NETs are generated by viable cells [22]. Whether the anuclear neutrophil should or should not be considered dead is questionable, and it remains unclear if these cells retain the capacity to activate other death programs. In this regard, it is important to note that similar to erythrocytes and platelets, cytoplasts (anuclear neutrophils generated in vitro) retain full capacity to die by apoptosis [22,23].

Table 1- Differences between NETosis, apoptosis, and necrosis. Updated from [24]

Necrosis	Apoptosis	NETosis
Membrane and organelle disintegration	Membrane blebbing	Vacuolization
Phosphatidylserine exposure during early steps of necrosis	Phosphatidylserine exposure	No exposure to Phosphatidylserine
Cellular swelling and bursting	Nuclear chromatin condensation without disintegration of the nuclear membrane	Nuclear chromatin decondensation with disintegration of the nuclear membrane
Cell damage releasing the intracellular contents	Programmed cell death	Programmed cell death
DNA fragmentation	DNA fragmentation	No DNA fragmentation
Dependent of caspases and RIP-1 kinases	Dependent of caspases and RIP-1 kinases	Independent of caspases and RIP-1 kinases
Process require more than 10 minutes	Process require more than 10 minutes	NETs were formed as early as ten minutes after activation

1.4.3.2 Slow vs Rapid mechanisms of NETosis

To date, two major NET release mechanisms have been described. In the first mechanism, neutrophils release NETs via a slow lytic cell death mechanism. This appears to be a major

route for NET release. In addition, Pilsczek et al. have described that a small number of neutrophils rapidly expulse their nuclear content via vesicular secretion, yielding NETs and live intact cytoplasts that continue to crawl and digest microbes [25]. Both NET formation strategies are dependent on TLR2 and complement factor 3 (C3). NET formation is deficient in mice lacking either of these molecules. However, when added alone they were not sufficient to induce NET release in isolated neutrophils [23], suggesting that additional mediators or more complex mechanisms of activation are involved.

Lytic or Slow Mechanism of NETosis

The majority of neutrophils undertake a cell death-mediated NETosis program that lasts from 2 to 4 hr. The initiating event generally occurs through engagement of cell surface receptors in the presence of a specific ligand through poorly understood mechanism. Evidence indicates involvement of the Raf–MEK–ERK pathway during NET. In most cases, NADPH oxidase is also involved. Signaling to the nucleus results in chromatin modification. Histone citrullination mediated by peptidylarginine deiminase (PAD) appears to be a prerequisite for NET release. Concurrent with chromatin decondensation, the nuclear membrane disintegrates. Alterations of nuclear shape with chromatin decondensation, swollen and fragmentation of the nuclear membrane, which allow the association of granules and cytoplasmic proteins with the chromatin. Finally, the plasma membrane ruptures and DNA with associated histones and granule molecules are released into the extracellular environment.

Non-Lytic or Rapid Mechanism of NETosis

An alternative rapid mechanism for NET release has recently been described, that takes 5–60 min after stimulation with *S.aureus* or LPS in the presence of platelets [25]. This was shown to be undertaken by a small subset of neutrophils, yielding NETs and live cytoplasts that continue to phagocytose. Since neutrophils are terminally differentiated cells with low transcriptional activity, loss of the nucleus does not incapacitate these cells. On the contrary, this alternative mechanism of NET release provides a multitasking means for rapid extracellular antimicrobial action while maintaining the capacity for phagocytosis. In this mechanism, the nuclear membranes separate and decondensed chromatin is seen in the inter-membrane space.

Chromatin-containing vesicles bud from the nucleus and accumulate below the plasma membrane **(Figure 7)**. Finally, NETs are formed through the degranulation of vesicle content into the extracellular space and their assembly with decondensed chromatin. Rapid NET release was also observed by intravital microscopy in mice that were treated with MIP-2 (CXCL2) and intradermal infection of *S. Aureusor* or *S. pyogenes.* These cells were highly motile and were phagocytosing bacteria. In addition, a minority of anuclear neutrophils had already released their nuclear material and were crawling slowly, still able to digest microbes [23].

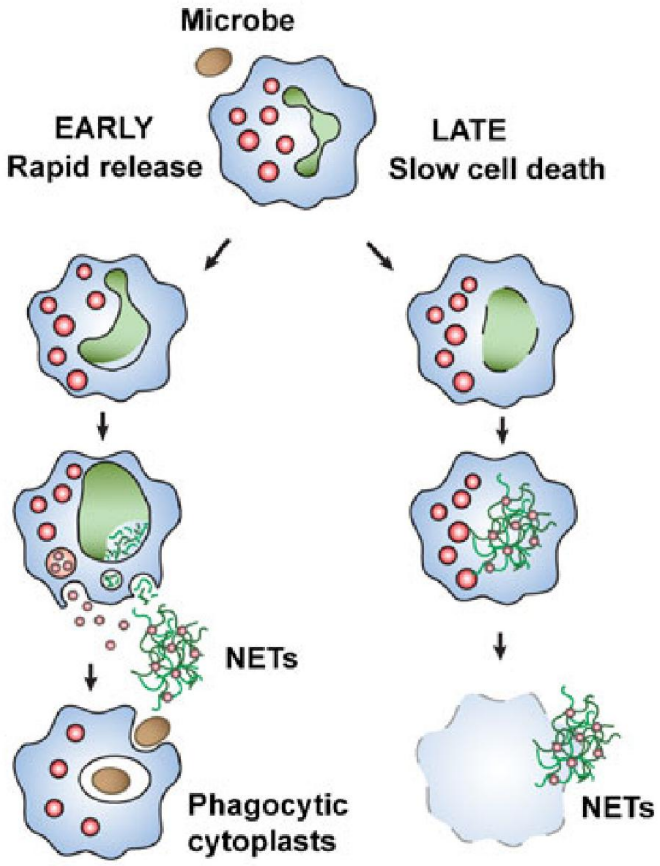


Figure 7. Lytic and non-lytic mechanism of NETosis: Adapted from [17]

1.4.3.3 Molecular basis of NETosis

The signalling mechanism NETosis is poorly understood and involves a complex interaction of proteins and factors, which ultimately leads to the release of the chromatin in the extracellular medium. Nevertheless, understanding of some of the fundamental steps has laid the primary foundation. The initiating process can be triggered by a number of stimuli including, PMA, LPS,

C5a +GM-CSF, IFNα/γ. LPS, bacteria, and viruses. IL-8 is also able to trigger ET release by interacting with the CXCL2/8 receptor[26]. Following activation, neutrophils are known to produce large amounts of ROS through the action of NADPH oxidase **(Figure 8)**. This observation was further supported by observations using different stimuli in both human and mouse primary neutrophils. After being stimulated, it was shown that enzymes stored in the azurophilic granules, neutrophil elastase (NE) and myeloperoxidase (MPO), could get relocated into the nucleus.

It was hypothesized that in the nucleus, NE degrades the linker histone H1 and the core histones, leading to chromatin decondensation. Further decondition is enhanced by binding of MPO [27]. Later importance of another enzyme called PAD4enzyme was identified as mouse knockout for PAD4 losses their ability to release NET and histone hypercitrullination was not detectable [28]. Histone citrullination is a hallmark of NETosis and PAD4 is the only known neutrophil enzyme capable of translocation into to nucleus [29].

Histone citrullination catalyzed by peptidylarginine deiminase 4 (PAD4) during NETosis, results in loss of negative charge. Following uncoiling of the heterochromatin, the nuclear membrane gets ruptured. Further, the expanding chromatin gets mixed with granular antimicrobial factors. Finally, the cell membrane breaks, releasing NETs. Interestingly in a recent observation it was shown that, NETs can be also released within minutes from living neutrophil cells through an oxidant-independent mechanism as it was demonstrated in *S. aureus* infection. The signalling mechanism of such a process remains a mystery.

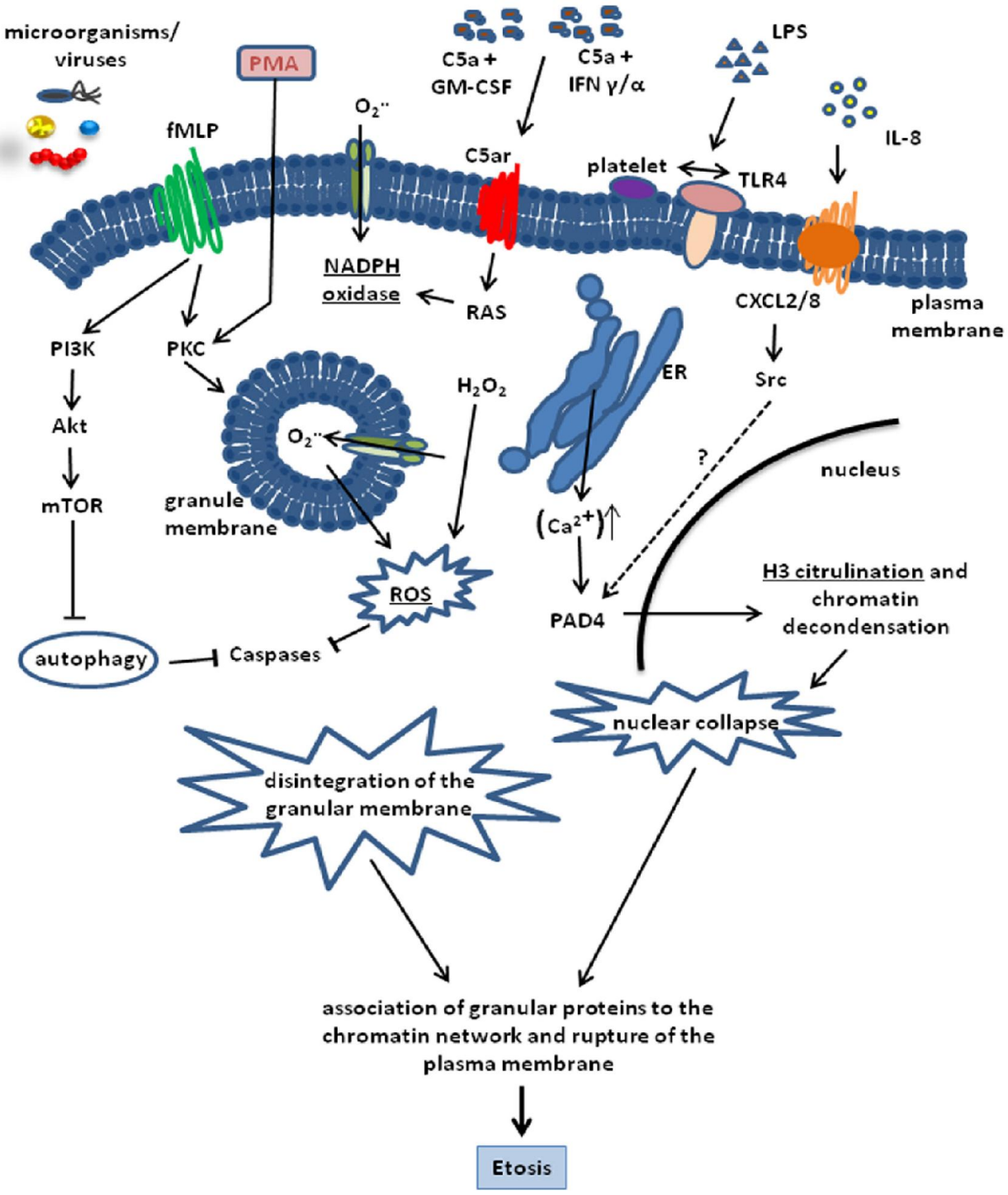


Figure 8. Schematic representation of the cellular processes involved in the formation of NETs. Adapted from [26]

1.4.3.4 Activation of PAD4 during NET formation

Out of five PAD enzyme isotype expressed in humans and mice [30], PAD4 and PAD2 is reported in granulocytes. PAD4 is of potential interest, as it is known to be associated with a number of pathogenic states, including autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), sepsis and thrombosis [31]. Although the activation of neutrophil towards NETosis by a number of stimuli has been shown as PAD4 dependent, however the downstream signaling pathways required for PAD4 activation in neutrophils is not clear (Table- 2). It was shown that pretreatment of cells with nocodazole or cytochalasin D, which inhibit microtubule polymerization, prior to LPS stimulation leads to a reduction of histone citrullination and NET formation. Additionally, blockade of integrin signaling through Mac-1 and cytohesin-1 impeded PAD4 activity and NET formation. How cytoskeletal signaling impacts PAD4, is unknown; however, it has been proposed that the same receptors establish whether a cell will undergo phagocytosis or NET formation Indeed, studies have indicated that neutrophils initiate NET formation when phagocytosis of a large particle fails [32]. Perhaps cytoskeletal activity and PAD4-mediated citrullination are linked because the initiation of NET formation represents a back-up killing mechanism following unsuccessful phagocytosis. Subsequent studies have demonstrated that ROS generation is upstream of chromatin decondensation [33], suggesting that NADPH oxidase activation may also be a prerequisite for PAD4 activation (Figure 9).

At the protein level, calcium binding, dimerization, and autocitrullination may help regulate its activity. Catalysis by all of these enzymes is calcium dependent, and, at least in vitro, requires calcium concentrations that are higher than that available in homeostatic cytoplasm, indicating calcium flux or a calcium-producing event is necessary to induce activity [34]. The generation of reactive oxygen species (ROS) is initiated by a wide variety of neutrophil stimuli, including phagocytosis of pathogens and signaling by LPS and TNF [35]. Interestingly, the addition of H2O2 to primary murine or human neutrophils induces PAD4-dependent histone citrullination [34]. The link between ROS and NET formation was first recognized by the fact that patients with chronic granulomas disease (CGD), who are missing the Nox2 protein essential for NADPH assembly and, thus, cannot form ROS. Neutrophils isolated from CGD

patients do not make NETs in response to S. aureus or phorbol myristate acetate [36]. This phenotype is rescued by addition of H_2O_2 or exogenous glucose oxidase, which generates H_2O_2, indicating that the ROS production facilitated by Nox2 is necessary for NETs [36].

Table 2 NETotic stimuli and PAD activation. Adapted from [34]

NET Stimuli	Activation of PAD4
Activated endothelial cells	n.d
Aspergillus fumigatus	n.d
Candida albicans	n.d
OpsonizedCandida albicans	n.d
Cryptococcusspecies	n.d
Escherichia coli	Yes
f-MLP	Yes
H_2O_2	Yes
Haemophilus influenzae	n.d
IL-8+Shigella flexneri	n.d
IL-8	Yes
Calcium ionophore	Yes
Klebsiella pneumoniae	n.d
Leishmaniaspecies	n.d
Listeria monocytogenes	n.d
LPS	Yes
Lipoteichoic acid	n.d
Mycobacteriumspecies	n.d
Nitric Oxide	n.d
Platelet activating factor	n.d
Platelet TLR-4	n.d
Phorbol-12-myristrate-13-acetate	Yes
Pseudomonas aeruginosa	n.d
Salmonella typhimurium	n.d
Shigella flexneri	Yes
Staphylococcus aureus	n.d
OpsonizedStaphylococcus aureus	n.d
Staphylococcus epidermidisδ-toxin	n.d
Streptococcusspecies	Yes
Streptococcus pneumoniae	n.d
α-Enolase	n.d
TNFα	Yes
Toxoplasma gondii	n.d
Yersinia enterocolitica	n.d
Zymosan	Yes

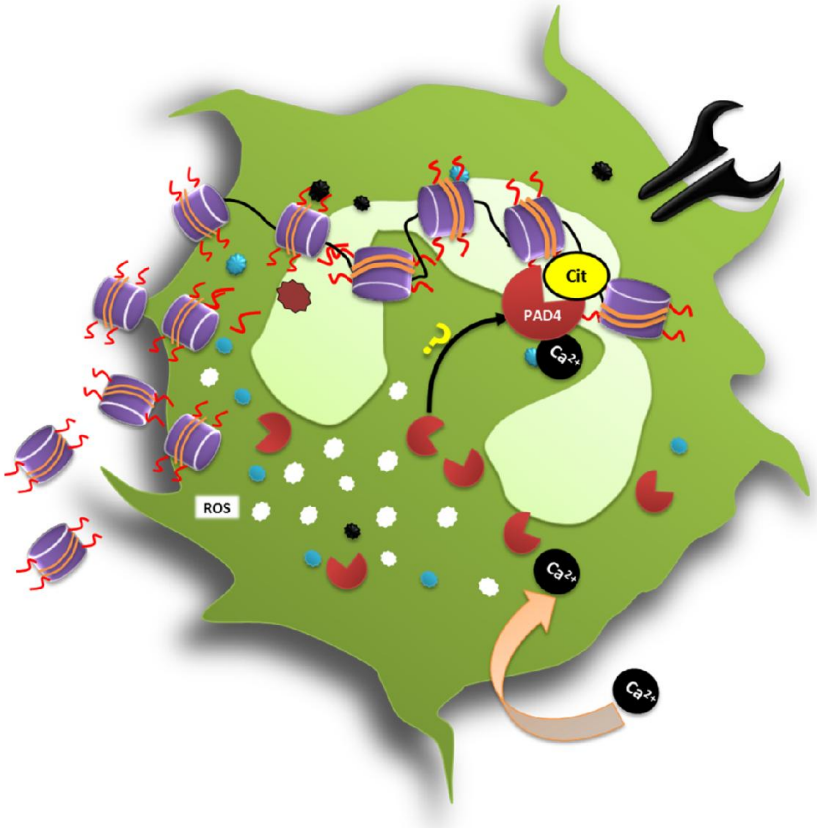


Figure 9. Model of PAD4 activation in NET formation. Pathways that activate NET formation are less defined than phagocytic pathways, but are known to require NADPH oxidase activity and the activation of PAD4 and subsequent histone citrullination. PAD enzymes are Ca2+-dependent. Since PAD4-mediated histone citrullination is abrogated by the NADPH inhibitor apocyanin we speculate that NADPH regulated ROS generation and increase Ca2+ levels may converge to activate PAD4 in neutrophils [34].

1.4.3.5 Citrullination- A key event during NETosis

Conversion of arginine residues to the non-ribosomally encoded amino acid citrulline by the action of PAD enzymes is known as "Citrullination". During NETosis PAD4 has been shown to citrullinates a number of nuclear proteins, including the histones and protein arginine methyltransferase 1 [28,37]The loss of charge following citrullination of cytokeratin causes disassembly of the histone DNA complex and results into decondensation of the chromatin **(Figure 10)** which is a key event of NETosis [38]. Citrullination is capable modifying biochemical pathways by altering the structure and function of target proteins. For example the proteomic analysis of NETotic material contained several citrullinated protein including vimentin that are

important RA autoantigens[24]. Citrullination of vimentin is known to correlated with the proliferation of fibroblast-like synoviocytes which can further stimulates TNF-α and IL-1 production in these cells[39].

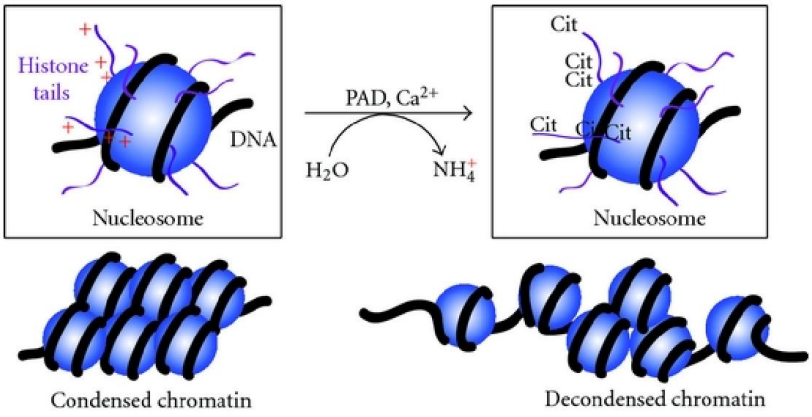


Figure 10: PAD-mediated histone tail citrullination leads to chromatin decondensation. Adapted from [38].

1.4.3.6 Composition of NETs

The protein cargo extruded on NETs varies depending on the specific type of stimulant used to induce these structures. For example, Matrixmetalloproteinase-8 (MMP-8), histone 3, and vasodilator-stimulated phosphoprotein were only detected in RA IgG–induced NETs, whereas catalase, moesin, transaldolase, phosphoglyceratemutase, and olfactomedin-4 were only found in IgMRF–induced NETs. In a recent experiment when neutrophils were stimulated with IgM RF or RA IgG enriched in ACPA, as many as 36 proteins were identified in the NETs, whereas TNF-a stimulation led to the identification of 28 proteins **(Table-3)** [24].

Table 3. Proteins expressed in control NETs upon TNF-α stimulation [24]			
1	TNF	15	Myosin 9
2	β-Actin	16	Neutrophil defensin 2
3	α-Actinin-1	17	Neutrophil elastase
4	Actin-related protein	18	Lysozyme
5	α-enolase	19	Plastin-2
6	Filamin-A	20	Profilin-1
7	Glucose-6-phosphate dehydrogen	21	ProteinS100-A8
8	Glyceraldehyde-3 phosphate isomerase	22	Protein S100-A9
9	Histone H2A	23	Protein S100-P
10	Histone H2B	24	Resistin
11	Histone H4	25	Transketolase
12	Lactoferrin	26	Tropomyosin 3
13	Myeloperoxidase heavy chain	27	Vimentin
14	Neutrophil gelatinase associated lipocalin	28	Calmodulin

1.5 NETs in infection

NETs possess a broad range of antimicrobial activities, which can kill or restrict the invading pathogen. They are known to can act upon different species of gram-negative and gram-positive bacteria, fungi, parasites and viruses[40] [41]. For example, S. flexneriis trapped and killed by NETs, which contain NE that degrades virulence factors such as IcsA and IpaB. NE-deficient mice, lacking the capacity to form NETs, are more susceptible to infection with K.pneumoniae [42], but this may be due to inefficient phagocytic killing as well as the absence

of NETs. Neutrophils play a crucial role in containing fungal infections and NETs appear to be an important part of the neutrophil antifungal arsenal. Hyphae of C. albicans are too large to be phagocytosed. Extracellular killing by release of NETs is an ideal strategy to contain the hyphal form and a number of studies have demonstrated that NETs are sufficient to kill such yeast and its hyphae **(Figure-11)** [43]. Calprotectin has also been shown to be an important NET component in the defense against *Aspergillus nidulansas.* Calprotectin deficient mice are more susceptible to aspergillosis. Neutrophils are not regarded as important effector cells against viruses and few studies have examined the role of NET formation in response to viral infection. NETs are able to capture and neutralize the negatively charged HIV virions, significantly decreasing HIV infectivity [44]. Several studies have explored the potential role of NETs in the immune response against protozoan parasites. Circulating NET structures have been detected in the blood of *Plasmodium falciparum* infected children with uncomplicated malaria [45]. Entrapment in NETs leads to decreased viability of the parasites, although authors of different studies conclude that the main function of NETs in Leishmania infection is the immobilization of the parasite and containment of the infection [46]. Of interest is Induction of NETs by Leishmania spp has been reported to be independent of NADPH oxidase activity and ROS production. Interestingly, some pathogenic organisms have evolved mechanisms to escape NETs mediated killing and some are completely resistant to it. For example, *S. pneumoniaehas* evolved strategies to escape NETs. In a passive manner, the polysaccharide capsule reduces NET binding [47]. The invasive pneumococcus type TIGR4 expresses the DNase endA, which enables escape from NETs, leading to increased virulence in vivo. Saitoh *et. al.* demonstrated that HIV engages CD209 on dendritic cells (DCs) with its envelope glycoprotein gp120. Engagement of DC-SIGN leads to production of IL-10 by DCs, which suppresses NET formation [44].

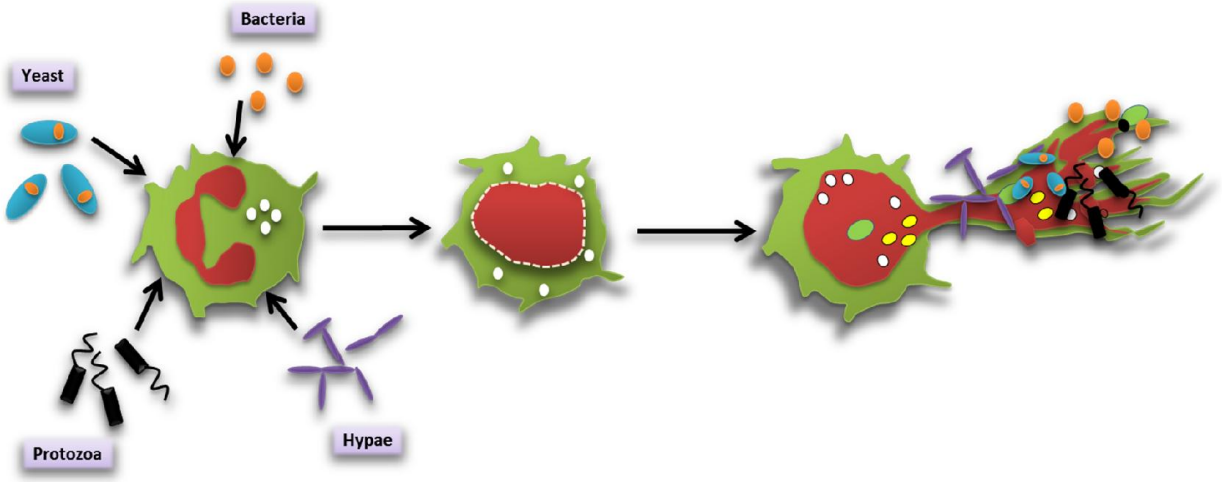


Figure 11. Mechanism of NETosis under infection. Neutrophils are stimulated by contact with bacteria, protozoan, fungi (yeast and hyphae forms) or their products (not shown), leading to: (a) ultrastructural alterations of nuclear shape with chromatin decondensation, swollen and fragmentation of the nuclear membrane, which allow the association of granules and cytoplasmic proteins with the chromatin, and (b) release of extracellular structures consisting of a DNA-backbone, decorated with histones, neutrophil granular and cytoplasmatic proteins (NETs), which ensnare and kill microorganisms. Modified from [11]

1.6 NETs in Inflammation

In the development of Deep vein thrombosis (DVT), NETs have been shown to form scaffolds in circulation that promote thrombus formation by interacting with the endothelium, platelets, coagulation factors and red blood cells. IL-8 and ROS released from endothelial cells can recruit and trigger neutrophils to form NETs, which in return activate and damage the endothelium by binding of histones to endothelial membranes. The release of Weibel–Palade bodies from the endothelium and deposition of fibrin and von Willebrand factor (vWF) promotes blood coagulation by formation of thrombus scaffolds. vWF and fibrin have a high affinity for histones and therefore readily bind to NETs [17]. Furthermore, histones have been shown to inhibit anticoagulants in the plasma, thereby further promoting thrombus formation[48]. Depletion of neutrophils or injection of exogenous DNase I have been shown to prevent thrombus formation in mouse models [49]. In Periodontitis, which is a chronic inflammation of the periodontium, neutrophil influx followed by NETosis in the gingival crevice leads to formation of a purulent crevicular exudate, which may prevent bacterial spread to the gingival surface [50]. Previously it was shown that placental micro-debris has been shown

to activate neutrophils and stimulate the release of NETs in a dose-dependent manner. Excessive release of these micodebris in the maternal blood circulation might be associated with the pathology of Preeclampsia [51,52]. Direct contact of neutrophil elastase is known to injure epithelial cells, altars mucus secretion and upregulates expression of pro-inflammatory cytokines [53]. Therefore, extracellular NE release via NETosis may be an important cause of lung tissue damage and disease progression in CF although there is no published evidence of this finding.

1.7 NETs in Autoimmune disease

Autoimmune diseases are characterized by defective discrimination of self and non-self molecules, leading to inappropriate recognition of host tissues as foreign structures, and concomitant immune attack against host organs. Current research appears to validate the view that NETs could be the key player in the aetiology of a number of inflammatory conditions, including preeclampsia, and most recently in rheumatoid arthritis (RA) [51] [54] [55]. In a recent investigation, 84% of NET components have been identified as autoantigens in patients with autoimmunity, cancer, or both. Out of these 74% have been reported to be autoantigens in SLE, RA, and vasculitis (**Figure- 12)**.

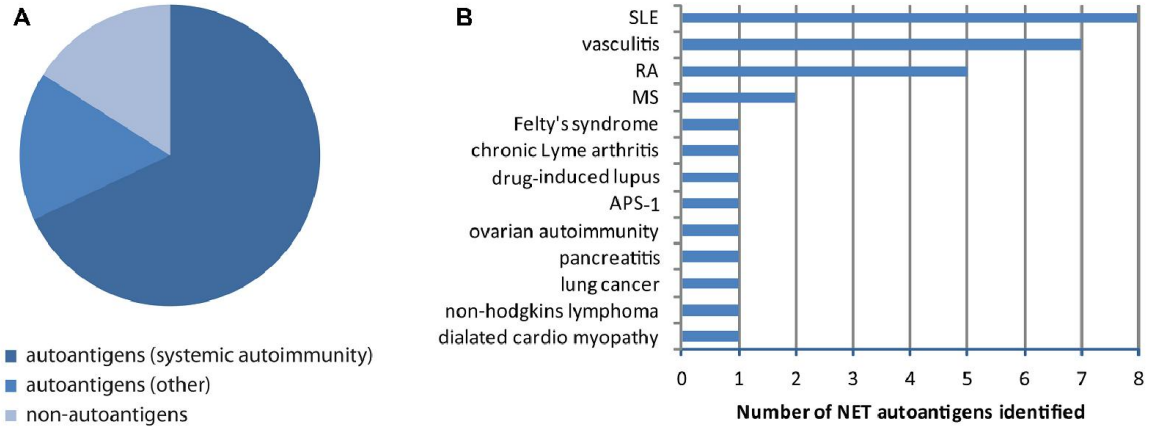


Figure 12. NETs as autoantigens. (A) Of the 25 NET components identified, 84% have been reported as autoantigens in cancer, autoimmunity, or other disorders. 74% of these proteins have been reported to be the target of autoantibodies in systemic autoimmune diseases. (B)The number of NET proteins reported to be autoantigens in various diseases is quantified and reveals that NET autoimmunity is most common in patients with vasculitis, SLE, and RA. Adapted from [56].

This observation suggests that redistribution into NETs may be a previously unappreciated unifying property of several autoantigens [56]. For example in Gouty arthritis which is the most common form of arthritis, deposition of monosodium urate (MSU) needles in the SF leads to release of IL-1βby monocytes and induction of IL-8,with subsequent neutrophil recruitment. MSU needles induce release of NETs that stimulate a feedback loop of IL-1β production by monocytes [57]. In Systemic lupus erythematosus (SLE) a subset of low-density granulocytes has been shown to spontaneously release NETs in SLE patients. The NET component LL-37 enhances the ability of naked DNA to activate plasmacytoid dendritic cells (pDC) via toll-like receptor 9 (TLR9). pDCs subsequently secrete IFN-αthat primes neutrophils to recognize autoimmune complexes of antinuclear

1.8 Study Objective

The main objective of this thesis is to investigate whether PMN derived from inflammatory disorders are more prone to undergo NETosis. In the chapters to follow we provide evidence in support of the notion that NETosis is implicated in autoinflammatory disorders like rheumatoid arthritis and preeclampsia. Next, we identity PAD4 translocation as a key event during NETosis signalling, which could contribute towards the generation of auto-antigens or be the target of auto-antibodies (ACPA) (Chapter 2). Further we identify that neutrophil derived from RA patients exhibit an increased propensity to undergo NETosis or degranulation during the serum clotting process, products of which may have diagnostic implications (Chapter 3). Finally, we provide evidences of profound NETosis in the feto-placental junction of Ad-Sflt-1 treated mouse model of preeclampsia (Chapter 4)

CHAPTER 2: Enhanced neutrophil extracellular trap formation in RA is characterized by increased nuclear translocation of PAD4 and augmented histone H3 citrullination

2.1 Summary

Rheumatoid Arthritis (RA) is a progressive chronic disease causing inflammation in the joints and resulting in painful deformity and immobility. Despite intensive work, the cause of RA remains unknown. Citrullinating enzymes and citrullinated proteins are known to be involved in the pathogenesis of the RA. Immune response against citrullinated proteins have been the basis for the early recognition of the disease and provided a better understanding of its pathophysiology. Innate immune mechanisms are indispensable for the onset and course of synovitis. As part of the first line of defense, neutrophils are known to play a pivotal role by regulating its effector functions such as phagocytosis, degranulation or recently reported Neutrophil Extracellular Trap (NETs) formation. Interestingly, many studies have shown exuberant NETosis, and its impaired clearance could implicate in various, autoimmune diseases, such as preeclampsia, psoriasis, systemic lupus erythematosus (SLE), and most recently in rheumatoid arthritis (RA). In the latter instance, it has been suggested that NETs could serve as the source of citrullinated autoantigens, thereby stimulating an auto-inflammatory condition. Since peptidylarginine deiminase 4 (PAD4) play a key role in the NETotic process and is implicated in the production of citrullinated autoantigens, we have examined its behavior in neutrophils isolated from RA patients. Our data confirm that neutrophils from RA patients display an increased propensity to undergo NETosis, a feature that correlated with increased levels of key components of the underlying signal transducing cascade such as reactive oxygen, myeloperoxidase and neutrophil elastase. Most noteworthy,

however, was a significant increase of PAD4 in the nucleus of RA neutrophils and associated elevation in citrullinated histone H3 levels. Our data, furthermore, suggest that PAD4 may be extruded into the extracellular environment during NETosis, as cell-free DNA associated complexes could be detected in culture supernatants. Since anti-PAD4 antibodies precede RA and enhance the citrullinating activity of PAD4 in an extracellular milieu by reducing their calcium requirement, our findings provide a mechanism by which such autoantigens could be generated to form auto-antibodies, as well as a source of auto-antigens in their presence.

2.2 Introduction

Rheumatoid arthritis (RA) is a disease of multifactorial aetiology, resulting in progressive disability and systemic complication by affecting the anatomical components of articular and juxta-articular tissues of diarthrodial joints [58]. After being hit by an unknown trigger, the process involves infiltration of the tissue by inflammatory cells, such as neutrophils, macrophages and plasma cells, which characterize the early events in the synovium. A secondary joint specific hit has been shown to increase vascularization, proliferation of the synovial lining cells [59,60]. This leads to inflammation of the capsule around the joints causing swelling of synovial cells, excess accumulation of synovial fluid, and the development of fibrous tissue called as “pannus” in the synovium [61]. Symptoms of RA include joint pain, swelling, stiffness, and fatigue. Some individuals may have mild and moderate forms of the disease while others can experience severe disease involvement characterized by acute episodes of pain and inflammation, known as “flares” followed by phases of reduced or no symptoms called as “remissions”. RA is a multi-joint disorder. In a quick frame of time, inflammation could progress from few joints to many other joints. Further it known to be associated with increased rates of cardiovascular illness, including myocardial infarction, cerebrovascular events, and heart failure. RA affects between 0.5 and 1% of adults in the developed world with between 5 and 50 per 100,000 people newly developing the condition each year. Women and elderly people are highly susceptible to this disease [62]. In fact, 70% of the patients with rheumatoid arthritis are women, with 80% of the total cases range

between 35 and 50 years of age[63]. Compared to healthy persons, the mortality rate is higher among patients with RA.

2.2.1 Normal vs RA Joint

The normal synovial joint, also referred as a “diarthrosis”, are the most movable joint in the body found in the bones of the limbs. The complex structure of a synovial joint consists of two opposing bone surfaces that are protected with the cover of specialized, fibrous hyaline cartilage (composed of fibrous connective tissue), which provides an articulating interface with reduced friction. The interface of the synovial joint is filled with synovial fluid and has special ligaments which hold the bones together [64]. The “synovium” is the clear, viscous, lubricating fluid secreted by synovial membrane which provides essential nutrition for the articular cartilage and lubricates the top layer of the cartilage. It surrounds the joint cavity and is the site of production of synovial fluid [58]. The synovial membrane is usually less than 100 µm in thick. The synovial lining, facing towards the bone and cartilage consists of a thin layer of synoviocytes (1–3 cells). Synovial joint can be differentiated from fibrous joint by the presence of a protective capsule around the articulating surface and the synovial joint and secondly by the lubricating synovial fluid within those capsules. Only a few mononuclear cells are distributed in the sublining connective tissue layer [58].

The RA synovial joint is characterized by a transendothelial migration of a variety of inflammatory cells such as T cells, B cells, dendritic cells, neutrophils, mast cells, plasma cells and macrophages followed by a phase of angiogenesis [62]. Invasion of inflammatory cells results in thickening of the inner lining layer, which gains a thickness of more than 20 cells. However recent reports suggest; change in histological appearance of RA is independent of the phase of the clinical signs [65]. While the synovial linings grows and expands, the inflammatory mass of tissue adjacent to the articular cartilage is eventually extended **(Figure 13),** and further leads to villus like projections [66]. The progressive overgrowth of the articular surface causes formation of the so-called 'pannus', which means 'cloth' in Latin and ‘web’ in Greek [58]. The pannus containing osteoclast is the primary destructive cellular element, whereas the repair function of osteoblasts is inhibited in RA, resulting in alteration of bone

resorption machinery [67]. The destruction of bone is often initiated at the cartilage-bone-synovial membrane junction. Although joint fluids are rich in polymorphonuclear leukocytes, very rarely they are seen in the synovial membrane[68]. A set of proteolytic enzymes released from neutrophils along with enzymes secreted by chondrocytes and synoviocytes causes degradation of the cartilage around the synovium [66]. Important to mention that, although the association between inflammation and progression of joint damage is clear from the literature, the destruction of bone, may still progress even under suppressed inflammatory condition [66].

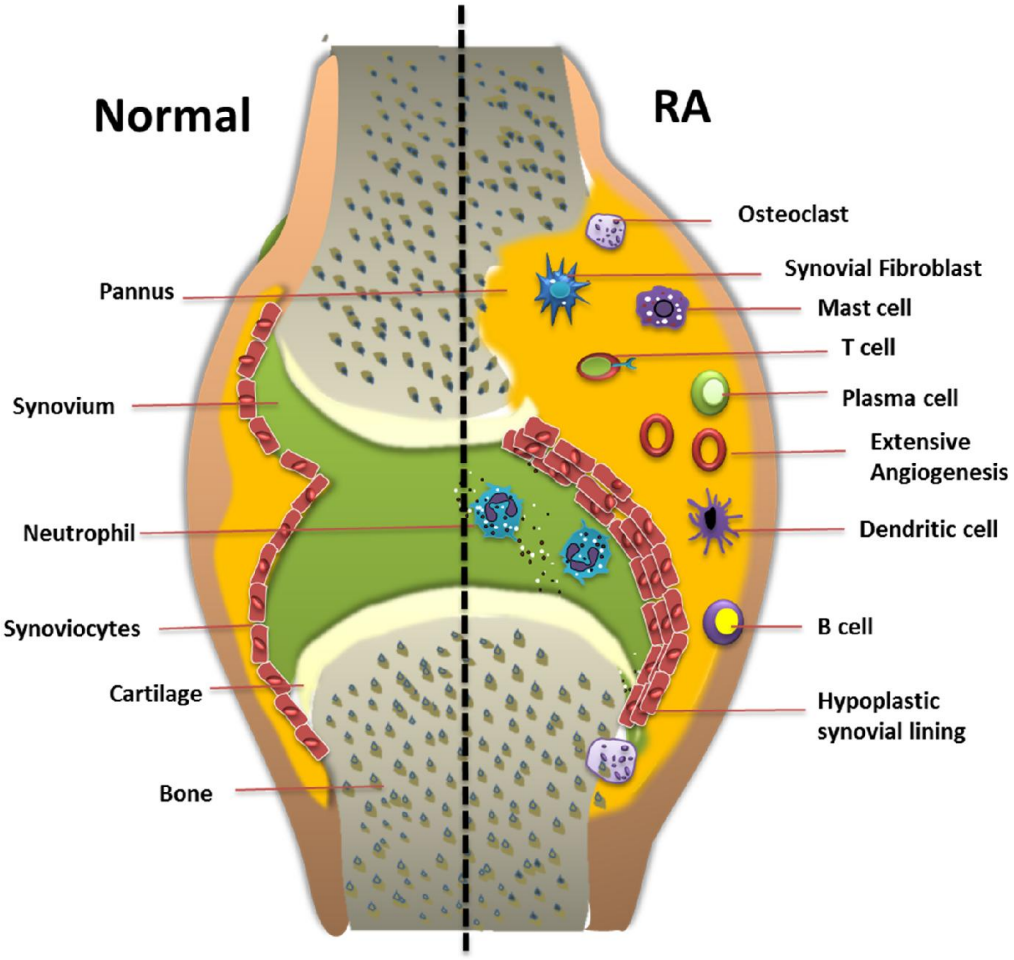


Figure 13- Schematic view of normal joint and rheumatoid arthritis joint. In the healthy joint (Left) the thin synovial membrane lines the non-weight-bearing aspects of the joint. In rheumatoid arthritis (b) the synovial membrane becomes hyperplastic and infiltrated by chronic inflammatory cells. Ultimately it develops into 'pannus', which migrates onto and into the articular cartilage and underlying bone. Reproduced from [69]

2.2.2 Clinical Phases in Rheumatoid Arthritis

Six phases (phases A–F) of RA development were formulated by the European League Against Rheumatism (EULAR) Standing Committee on Investigative Rheumatology in the year 2011 [70].

Phases A-B: Genetic and environmental trigger

Risk factors for developing RA are both genetic and environmental [61]. Family history of RA and tobacco smoking are frequently being associated as risk factors for RA. A change in lifestyle such as, to avoid tobacco smoking and consuming foods rich in Omega 3 [71]and awareness of family history can help to prevent the risk of RA **(Figure 14).**

Phase C: Systemic autoimmunity associated with RA

Systemic autoimmunity without synovial inflammation followed by a short period of asymptomatic synovitis characteristics the initial phase of RA. During this period, abnormalities in different body parts can be found preceding the clinical onset of RA. Although, synovium is known as the primary site of clinical pathogenesis, it may not be the place where the disease initiates. Lungs, lymph nodes, bone marrow, periodontal tissue, gut and neuroendocrine system could be the site where the initiating stimuli might have originated [70].

Phase D: Symptoms without clinical arthritis

This phase is characterized by symptoms such as joint pain and morning stiffness. Synovial and bone abnormalities can be detected by imaging tools like ultrasound and MRI [72]. Other abnormalities, for example, increased synovial vascularity, thickening of the synovial membrane and bone marrow edema are seldom present.

Phase E: Unclassified arthritis

Although, in this phase, inflammatory joint swelling is clinically apparent, patients still do not fulfil the characteristic criteria of a recognized RA case.

Phase F: RA

In this phase, patients fulfil the classification criteria for RA. Many patients will develop damage to cartilage bone as a result of acute synovial inflammation.

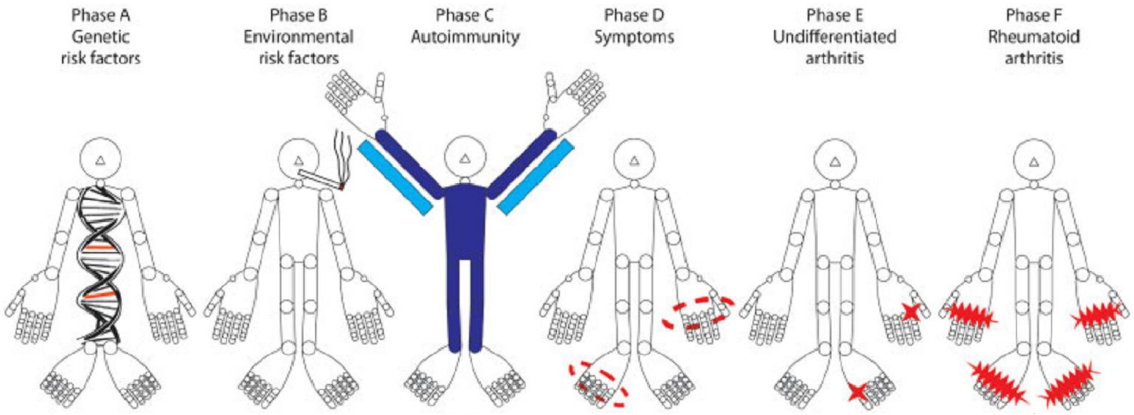


Figure 14.Overview of the six preclinical phases of rheumatoid arthritis (RA). Modified from [73].

2.2.3 Risk Factors

In recent years, a number risk factors have been identified for the development of RA. It is now known that a random set of genetic, physiological or environmental factors is involved, and their interaction may be decisive in the development and progression of the disease.

Genetic Risk Factors

The most compelling evidence for a genetic component was in monozygotic twins, in whom the concordance rate is 12% to 15% when one twin is affected compared to 1% for the general population. In the predetermined aspect of the genetic component, the human leukocyte antigen (HLA) and major histocompatibility (MHC) genes are the most important. (HLA)–DRB1 locus is known to be strongly associated with rheumatoid factor and/or ACPA positive RA cases [74]. Some of the HLA-DRB1 alleles contain a common amino acid motif (QKRAA) known as "shared epitope" or SE, which has been shown to confer particular susceptibility [74]. Additionally, it was shown that, in individuals with the shared epitope (SE), arginine to citrulline conversion may increase peptide binding affinity to MHC class II molecules specifically in the P4 binding pocket [75]. Thus, it plays a role in determining which ACPA-positive individuals will ultimately develop arthritis [76].

Non genetic - Risk Factors

Recently it was estimated that the heritability for ACPA negative RA is approximately 20%, which indicates that the significant risk factors for this subset of disease is dependent on non-genetic factors [77]. However it is noteworthy that, a recent finding has reported stochastic factors as the main contributors to ACPA development of RA [78]. Although no specific exposure has been identified as a pivotal agent, numerous environmental or non-genetic factors certainly contribute to RA susceptibility **(Table-4).** Among the known environmental risk factors, smoking is the best defined for seropositive RA in certain populations. A recent finding implicates that ACPA positivity, in unaffected first-degree relative RA patients; with a high prevalence of smoking and SE was 48%, whereas, in the healthy controls, ACPAs were hardly evident [79]. Apart from smoking, citrullinated proteins such as citrullinated enolase can be produced by bacterial infection that may cross-react with endogenous enolase peptides to produce anti-citrulllinated antibodies [80]. It was hypothesized that activation of innate immunity, especially in an individual with underlying genetically determined autoreactivity, potentially could contribute to the autoreactivity and the initiation of RA.

Table 4- Non Genetic Risk factors for RA. Data Source [81]. *Increase disease severity (+), Decrease disease severity (-)

Non-genetic Risk Factors	Determent component	**Final Effect on RA**
Socioeconomic factors	Without a university degree	+
Hormones	Oral contraceptives	+ –
	Extended breastfeeding	+ –
	Early menopause	+
	Low testosterone	+
Dietary factors	More intake of Omega 3 rich food	–
Vitamin D	Lower serum levels of vitamin D	+ –
Alcohol	More alcohol consumption	–
Coffee	More than 10 cups a day	+
Bacterial Infections	*Porphyromonas gingivalis*	+
	Periodontitis	+

Silica	Crystalline silica	+
Tobacco/Smoking	Cyanate	+
	Free radical	+
Viral infection	Parvovirus	+
	Retroviruses	

2.2.4 Cellular Mediators of joint inflammation

The inflammatory process of RA is coordinated event, involving cellular mediators of both innate and adaptive immune system **(Table-5)**, which not only play a critical role in driving and maintaining inflammation, but also has a protective function to suppress or shut the inflammation of the synovitis. In patients with RA the synovial membrane is characterized by infiltration of inflammatory cells, hyperplasia and increased vascularity [68]. After invasion into the synovium, dendritic cells process the local antigens and present them to germinal centers of the synovium. Thus, through interactions with native T they via surface receptors, they help B cells to produce pathogenic antibodies [82]. Alternately, they can also migrate to specific sites in the joints where they produce an array of chemokines with destructive properties such as IL-17 on the cartilage [82]. Furthermore they can stimulate other cells like osteoclast resulting in bone erosion and damage. The detailed mechanisms of these complex cellular interactions remain elusive.

Table 5.Key Cellular Mediators Implicated in the Pathogenesis of Rheumatoid Arthritis. Source [83,84]

Cellular Mediators	Key Disease-Relevant Functions
Monocytes and macrophages	Produced GM-CSF that activates expression of human leukocyte antigen (HLA)-DR molecules on antigen-presenting cells (APCs). Plays a major role in osteoclast development. Major source of IL-7 in RA synovium.
Dendritic cells	Presenting arthritogenic antigens to T cells. Produce cytokines that can influence T cell differentiation in the joint.
Synovial T lymphocytes	Th1 and Th17 subset dependent antibody production. Express RANKL and other effector cytokines with either stimulatory or inhibitory effects on osteoclastogenesis
Synovial B cells	Production of autoantibodies such as rheumatoid factors (RFs) and ACPA and immune complexes.

Mast cells	Activate chondrocytes, synovial fibroblasts, and macrophages to produce metalloproteinases, cytokines, and prostaglandins that all contribute to joint destruction and induce localized tissue edema. Mast cell derived heparin has significant effects on connective tissue
Natural killer cells	Can stimulate B cells to produce RFs, produce cytokines or enhance proinflammatory cytokine production by T lymphocytes and Macrophages.
Polymorphonuclear neutrophils	Release various proteases that can adversely affect the lubricating properties of synovial fluid and the integrity of the cartilage
Osteoclast	Bone resorption through the elaboration of MMPs and cathepsin K
Chondrocytes	Remodeling the cartilage matrix in the RA joint.
Fibroblast-like synoviocytes	Production of proteases that degrade the extracellular matrix, and invasion into cartilage. Produce a variety of molecules that modulate the growth, inflammation, angiogenesis, and cell recruitment, and induce activation of and cytokine production by immune cells.
Bone marrow cells	Contribute to synovial inflammatory responses

2.2.5 Molecular Mediators of joint inflammation

Cytokines and chemokines are at mediators of cell-to-cell communications. They stimulate the cells involved in innate and adaptive immunity to enhance or inhibit inflammatory responses [85].The transendothelial influx of inflammatory cells from the circulation following synovial cell hyperplasia, stimulate the secretion of an array of cytokines with a broad range of functions **(Table-6)** which leads to induction of acute phase response and appearance of systemic features (e.g. fever, fatigue, cachexia) [59]. The formation of ectopic lymphoid structures in the synovium which are found in ~25% of RA synovial tissue samples, involve the expression of several key cytokines and chemokines [68]. In the synovium, they can carry out a broad range of activity such as activation of B cells and osteoclast, regulate expression of adhesion molecules, upregulated expression of cartilage-degrading enzymes such as matrix metalloproteinase (MMP) at the cartilage–pannus junction and promote inflammation and tissue catabolism.

Table 6 .Key Molecules and Signal Mediators Implicated in the Pathogenesis of Rheumatoid Arthritis. Updated from **[61,83,84]**

Molecule or Signal Mediator Cytokines	Key Disease-Relevant Functions
Cytokine	
TNF-α	Activates leukocytes, endothelial cells, and synovial fibroblasts, inducing production of cytokines, chemokines, adhesion molecules, and matrix enzymes; suppression of regulatory T-cell function; activation of osteoclasts; and resorption of cartilage and bone; mediates metabolic and cognitive dysfunction
Interleukin-1α and 1β	Activate leukocytes, endothelial cells, and synovial fibroblasts; induce matrix-enzyme production by chondrocytes; activate osteoclasts; mediate fever; enhance glucose metabolism; and reduce cognitive function
IL-2 and IFN-gamma	
Interleukin-6	Activates leukocytes and osteoclasts; is involved in B-lymphocyte differentiation; regulates lipid metabolism, acute-phase response, and anemia of chronic disease; and is implicated in hypothalamic–pituitary–adrenal axis dysfunction and fatigue
Interleukin-7 and 15	Promote and maintain T-cell and natural killer–cell activation and T-cell memory, block apoptosis, and maintain T-cell–macrophage cognate interactions
Interleukin-12	key role in the differentiation of T cells and inflammation
Interleukin-15	T cell chemotaxis and proliferation, production of immunoglobulins by B cells, and the generation of natural killer cells
Interleukin-17A and 17F	Act synergistically to enhance activation of synovial fibroblasts, chondrocytes, and osteoclasts.
Interleukin-18	Promotes activation of Th1, neutrophils, and natural killer cells
Interleukin-21	Activates Th17 and B-cell subsets
Interleukin-23	Expands Th17
Interleukin-32	Activates cytokine production by several leukocytes and promotes osteoclast differentiation
Interleukin-33	Activates mast cells and neutrophils
TGF-beta	Inhibits T cell activation and proliferation, downregulates B cell proliferation and differentiation, inhibits biosynthesis of metalloproteinases, protects articular cartilage from the degradative influences of IL-1, protects articular cartilage from the degradative influences of IL-1
Growth and differentiation factors	
BLyS and APRIL	Activate B cells and have a role in the maturation of B cells and enhancement of autoantibody production
GM-CSF and M-CSF	Enhance differentiation of granulocyte and myeloid-lineage cells in the bone marrow and synovium
RANKL	Promotes maturation and activation of osteoclasts

Intracellular signaling molecules and transcription factors	
JAK	Tyrosine kinase that regulates cytokine-mediated leukocyte maturation and activation, cytokine production, and immunoglobulin production
Syk	Tyrosine kinase that regulates immune-complex–mediated and antigen-mediated activation of B and T cells and other Fc receptor–bearing leukocytes
PI3K	Mediates signals that drive proliferation and cell survival
BTK	Plays important role in the activation of B cells, macrophages, mast cells, and neutrophils, through regulation of B-cell receptor and Fc receptor signaling as appropriate
NF-κB	Helps integrate inflammatory signaling and is important for cell survival

2.2.6 Innate immune system in RA

Depletion of neutrophils and mice lacking mast cells are both resistant to onset arthritis, indicating a clear role of innate immune system in RA pathogenesis. Further, environmental triggers such as smoking, bacterial products, viral elements, are known to be involved in RA pathogenesis, suggesting a possible involvement of the innate arm as a key event [86]. Innate immune cells through their primitive pattern-recognition system, are known to cause rapid inflammatory responses [87]. The pattern recognition receptors recognize preserved structures in bacteria and other infectious agents and permit rapid release of inflammatory mediators, activation of antigen presenting cells [88]. Repeated activation of innate immunity, especially in an individual with underlying genetically determined autoreactivity, potentially could contribute promoting the development of adaptive immunity in RA [84]. Further clinical phases of RA were shown to be driven by a cascade of inflammatory events in the synovium, defined by intercommunication between cells of the innate immune system, including NK cells, macrophages, dendritic cells and mast cells with fibroblast-like synoviocytes, along with cells of the adaptive immune system [88]. The central role of innate cells in RA pathogenesis is to produce proinflammatory cytokines, chemokines and matrix-degrading enzymes that drive chronic inflammation [84]. The activation of the innate response can further, activate the complement proteins, which by the engagement of C5a can producing membrane attacking complexes (MAC) which can cause tissue damage in RA [89].

2.2.7 Adaptive immunity in RA

Over last few years, a repertoire of antibodies has been identified that are associated with the pathology of RA. To understand how and why such autoimmune system gets activated to drive pathogenetic consequences is the recent focus in arthritis research. The autoimmune mechanism in RA begins with the recognition of endogenous and exogenous citrullinated autoantigens by antigen presenting cells (APCs). After antigen processing, they present them to T cells with the help of MHCII molecules on its surface. T cells then differentiate into TH1 and TH17 cells that stimulate macrophages to release proinflammatory cytokines that stimulate B cells to produce antibodies. These autoantibodies bind to target antigen to form immune complexes. Binding of complement and to immune complexes can augment secretion of TNF, IL-6 and IL-1 from macrophages **(Figure 15)**. These cytokines by virtue of osteoclast activation and condrocyte causes damage to cartilage and bone and thus establishes the clinical onset of the disease [90]. However, there is growing data to support the claim that both T-cell-dependent and independent mouse models of arthritis requires innate immunity in the initiation phase for the activation of adaptive immunity.

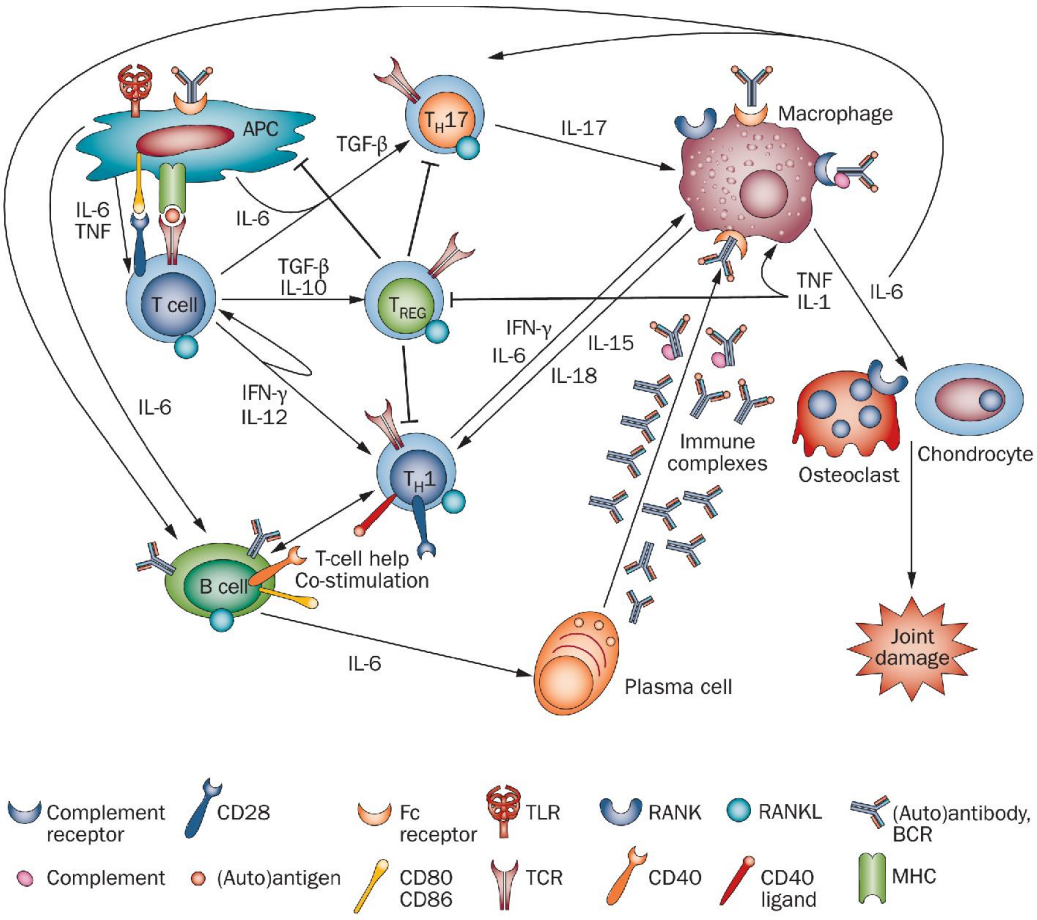


Figure 15- Adaptive mechanism of RA pathogenesis. Adapted from [90]

2.2.8 ACPA/anti-CCP hallmark of RA

Anticitrullinated protein antibodies (ACPA) are a group of (partly) cross-reactive antibodies, which are capable of identifying citrulline-containing proteins and peptides [91]. Extensive research over a decade has confirmed the notion that the pathophysiology of RA is strongly associated with autoantibodies to citrullinated protein antigens (ACPAs), which can further characterize the heterogeneous phenotype of RA with respect to outcome and therapeutic intervention. These auto-antibodies bind to the Fc domain of IgG molecules (IgM rheumatoid factor, IgM-RF) and against citrullinated autoantigens to form immune complexes which can initiate a cascade of events resulting in joint inflammation. PAD2 and PAD4 enzymes as well as citrullinated proteins can be identified in the inflamed synovium during all stages of the

disease and correlate with local inflammation [92]. Direct transfer of serum anticitrullinated antibodies from RA patient, has failed to induce arthritis in mice [93]. Similarly, direct transfer of mouse monoclonal antibodies against citrullinated fibrinogen to DBA/1J mice, also failed to induce arthritis. However, in the presence of mild synovitis with anticollagen II antibodies, could successfully induce the disease [94]. Moreover ACPA-positive patients with RA seem to be ACPA positive years before the onset of disease [95], therefore suggesting indirect involvement of ACPA in the pathogenesis of RA. The efficacy of selective B-cell depletion in the treatment of RA provides evidence for the involvement of B cells and possibly ACPA in its pathogenesis [96]. In addition to immune complex formation, ACPAs have been suggested to mediate a number of effector function such as activation of macrophages by binding to toll-like receptors, activation of osteoclast which may induce bone loss, mast cells degranulation which contribute to the development of synovitis, activation of neutrophils to produce NETs which may promote aberrant adaptive and innate immune responses in the joint, activation of complement proteins to form membrane attacking complex (MAC) [93] **(Figure-16).** Majority of individuals with RA (50–80%) have serum positive titers for IgM-RF and/or ACPA. However, in comparison to IgM-RF, ACPA seems to be more reliable diagnostic marker predictors of poor prognosis of RA as it has higher specificity (98%) and sensitivity (up to 80%) for diagnosis of RA than IgM-RF.

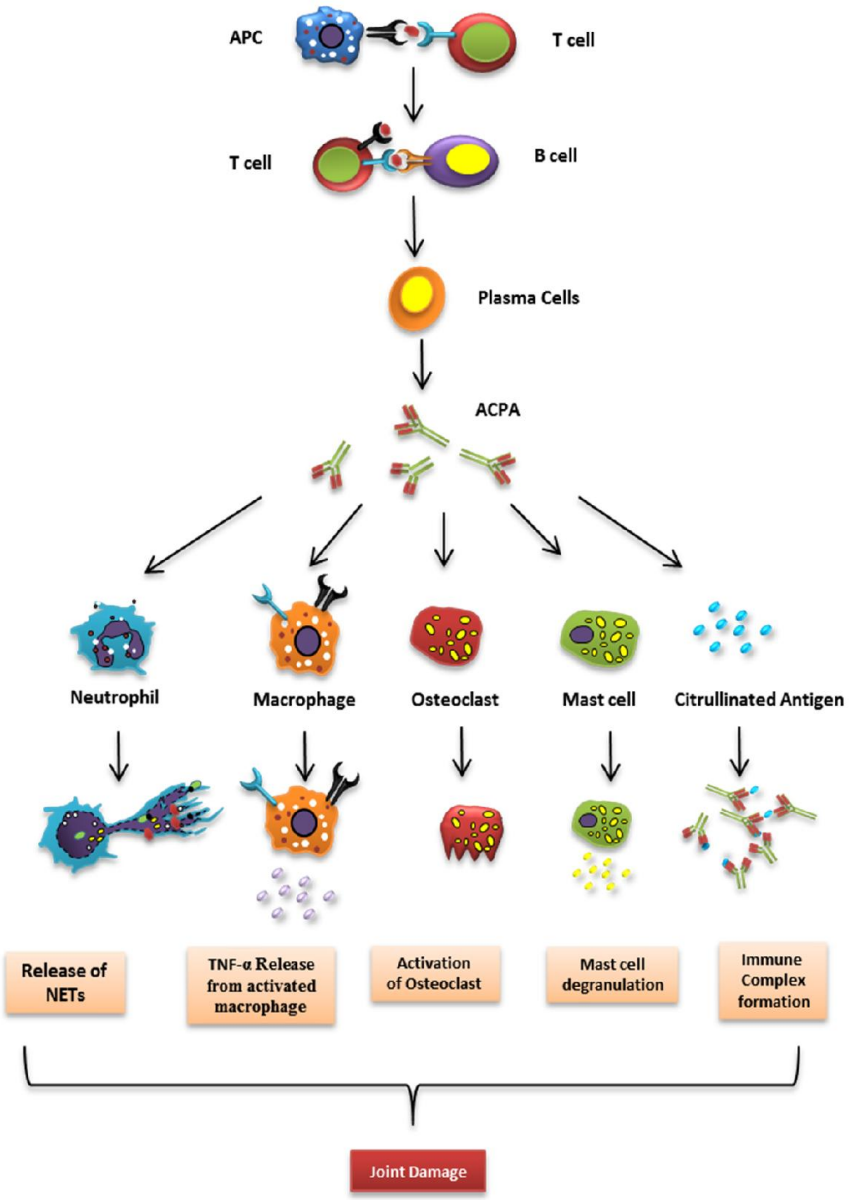


Figure 16. Model of ACPA generation and its tentative effector functions [93].

2.2.9 PAD Enzymes- key regulator of RA pathogenesis

It is now well known that PAD enzymes are activated during the inflammation process. Some of its isotypes (PAD2 and PAD4) are in abundance in plasma and synovial biopsy specimen from patients with RA[97]. Calcium ions catalyze PAD dependent enzymatic reactions. However, under physiological conditions the levels of Ca2+ is about 100 fold less than the required amount for its activity[98]. In the synovium, neutrophils, macrophages and mast cells could be the possible source of PAD4 and PAD2 [99]. PAD enzymes are known to generate

self-antigen, which can as the fuel for ACPA generation. However, they are also known to play a role in the recruitment of immune cells and destruction of joint during RA pathogenesis [100]. In the K/B×N model Severity of inflammation including infiltration, synovial hyperplasia and hypervascularization correlate significantly with the expression levels of PAD2 and PAD4 in the vicinity of citrullinated fibrin deposits [97]. However, induction of arthritis was found to be independent of PAD4 in K/B×N serum transfer model, using PAD4 deficient mice. Notably, Willis et al. showed that the PAD inhibitor Cl-amidine could rescue joint damage in collagen-induced arthritis model although this theory did not hold grounds when the disease was induced by the administration of anti-collagen antibodies. Further studies are essential to fully characterize the role of the PAD enzymes in various phases of the disease.

2.2.10 Why Joints of RA are affected when citrullination is non-specific?

The process of citrullination is not specific to the synovium RA patients [101]. It can also be detected in non-rheumatoid arthritis inflammatory synovitis as well [91]. It is a part of normal biological processes of the body, such as epidermal differentiation, formation of the hair follicle and during development of the central nervous system[99]. Even in their presence, it may not elicit an anticitrullinated protein response. Now the question is, if citrullination is not a unique feature, then why particularly the joints are affected in RA? A possible explanation could be that, under normal circumstances dying cells will be removed by phagocytes. As a consequence, the immune system would not respond to citrullinated proteins. However, when the clearance system is impaired or when too many cells are dying, the dying cell can release citrullinated proteins into the extracellular space [102]. PAD enzymes released in this process will citrullinate extracellular synovial proteins, such as fibrin. The presence of ACPA and citrullinated proteins in inflamed joints will lead to the formation of immune complexes, which will result in complement and inflammatory cell activation. Smoking and possession of HLA-DR shared epitope (SE) alleles are established risk factors for the development of RA, which may trigger HLA-DR restricted immune reactions [99]. Further, it is known that individuals having these SE alleles, will present citrullinated peptides more efficiently (to CD4 + T lymphocytes) than the corresponding arginine-containing peptides, as the binding pocket on MHC classII prefers negatively or neutral charged peptides [34]. Therefore, it can be

concluded that only in some individuals (depending on their hormonal and genetic backgrounds) ACPA can be produced which may lead to the development of chronic inflammation of joints leading to RA [84].

2.2.11 Mechanism of Rheumatoid Arthritis

2.2.11.1 The Frist Hit - Environment and Genetic trigger

A combination of predetermined (genetic) and stochastic (random/ environmental or non-genetic) events is known to initiate the cascade of events in RA. Genetic risk factors such as HLA-DRB1 alleles and shared epitope account for approximately 36% of the heritability whereas 45 non-HLA variants explain approximately 15% of heritability [103]. But many other genes are also involved and contribute to susceptibility and severity [103]. Environmental factors such as tobacco, silica, hormones, bacterial infections, alcohol, coffee, vitamin D and dietary factors are designated as a risk factor for RA, although the scientific evidence on their exact involvement is inconclusive in many cases [81](Table 1).

Heavy cigarette smoking was shown to stimulate an influx of cells into the lungs. In the presence of toxic components of the smoke, the inflated cells can get activated which render more prone to cell death [104].

2.2.11.2 Activation of Innate Immune System to create autoantigen

Activation of the innate immune system (particularly mast cells, macrophages, neutrophils, and dendritic cells) might provide the foundation, or lower the threshold, for disease initiation through other immune mechanisms [88]. Innate immune cells through its primitive pattern-recognition system can lead to rapid inflammatory responses by engaging of Fc receptors by immune complexes and perhaps Toll-like receptors (TLRs) by bacterial products [87].

2.2.11.3 Autoantigen processed to produce ACPA Formation

By virtue of cell death mechanisms, PAD enzymes comes out of the cell and may citrullinate native proteins in the local organ. Presentation of citrullinated peptides or other neo-epitopes from citrullinated proteins, in genetically predisposed individuals, such as those carrying the

HLA-DR, SE alleles could activate autoreactive T cells, which via B cells stimulate the production of ACPA. Research indicates that IgM rheumatoid factor (IgM-RF) and anticitrullinated protein antibodies (ACPA) can be detected in patient serum samples up to 14 years before the first clinical signs and symptoms [105,106]**(Figure- 17).**

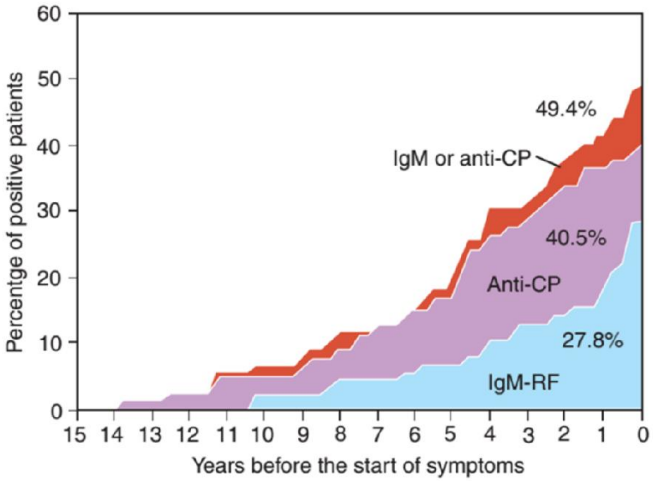


Figure 17- Autoantibody production in rheumatoid arthritis. Rheumatoid factors and anticitrullinated peptide (CP) antibodies are detected in the blood long before the onset of clinical arthritis in many patients. Adapted from [76]

2.2.11.4 Inflammation of Synovium- Second Hit

ACPA positive individuals remain unaffected for years and majority of them never get affected, suggest a second hit or stimulus in the joint is required for the induction of RA [84]. A non-identified second hit, could be a minor trauma or viral infection that might lead to synovial citrullination with local emergence of identical citrullinated epitopes as presented previously during the first hit, resulting in a “transformed phenotype” of synovial lining cells. In the G6PI (KBxN) serum transfer mouse model of RA, it was shown that the production of immune complexes may act as a critical initial step which would allow pathogenic autoantibodies to access the synovium [107]. Circulating ACPA enter the joint, bind to the citrullinated proteins, and form immune complexes (IC). Circulating immune complexes containing citrullinated fibrinogen, was found in a significant proportion, in ACPA positive RA patients. These immune complexes and citrullinated fibrinogen co-localize with complement component C3 could

result in immune complex deposition and complement activation causing ongoing synovitis [76].

2.2.11.5 Angiogenesis and immune cell infiltration

One of the earliest histopathologic responses in RA joint is the generation of new synovial blood vessels. Morphometric studies reveal the presence of relatively small number of blood vessels, adjacent to the expanded lining layer. These vessels in combination with an increasing metabolic demand of the highly cellular structure could drive the onset of hypoxic environment [108]. Hypoxia is a strong stimulus for the expression of vascular endothelial growth factor (VEGF) and other angiogenesis mediators, which are highly expressed in inflamed RA synovium. As the new vessels develop, inflammatory mediators such as IL-17A, TNF, IL-1, IL-6, IL-18, VEGF, IL-33 and HMGB1 are produced in the synovium[68]. This may activate endothelial cells to produce adhesion molecules, which expedite activation-dependent sticking of leukocytes, thereby facilitating diapedesis and extravasation of both lymphocytes and polymorphonuclear leukocytes into the synovial fluid. Mast cell products may also have an important role[84]. Cadherin-11, a synovial fibroblast membrane protein, mediates the organization and invasion of fibroblast-like synoviocytes (FLS) into synovial tissue [109]

2.2.11.6 Pannus formation

Positive feedback loops mediated by the interactions among leukocytes, synovial fibroblasts, chondrocytes, and osteoclasts, together with the molecular products of damage, drive the chronic phase in the pathogenesis of RA **(Figure 18)** [110]. In the chronic phase of inflammation the synovial tissue lining the joint, leads to tissue proliferation, also known as “pannus” formation. Pannus is defined as thickening of the synovial tissue that covers articular cartilage. A progressive pannus can invade the bone marrow and destroy structures such as joint capsule and tendons of the surrounding leading to loss of joint motion, loss of joint space, bony fusion (ankylosis), joint subluxation, tendon contractures, and chronic deformity [86].

2.2.11.7 Cartilage and destruction

Activated rheumatoid synovium eventually destroys cartilage at the cartilage-pannus junction [84]. Osteoclasts cause subsequent bone erosions, whereas cartilage dissolution results from proteolytic enzymes produced by synoviocytes in the pannus or synovial fluid neutrophils. The destruction of cartilage, bone, and tendons is initiated mainly by metalloproteinases specially metalloproteinase-3 (MMP-3), as it is known to degrade cartilage proteoglycans, fibronectin, and type IV collagen in the basement membrane, and activates collagenase [86]. At sites of active RA, there is a dramatic imbalance of bone turnover in which local bone resorption outweighs bone formation [111].

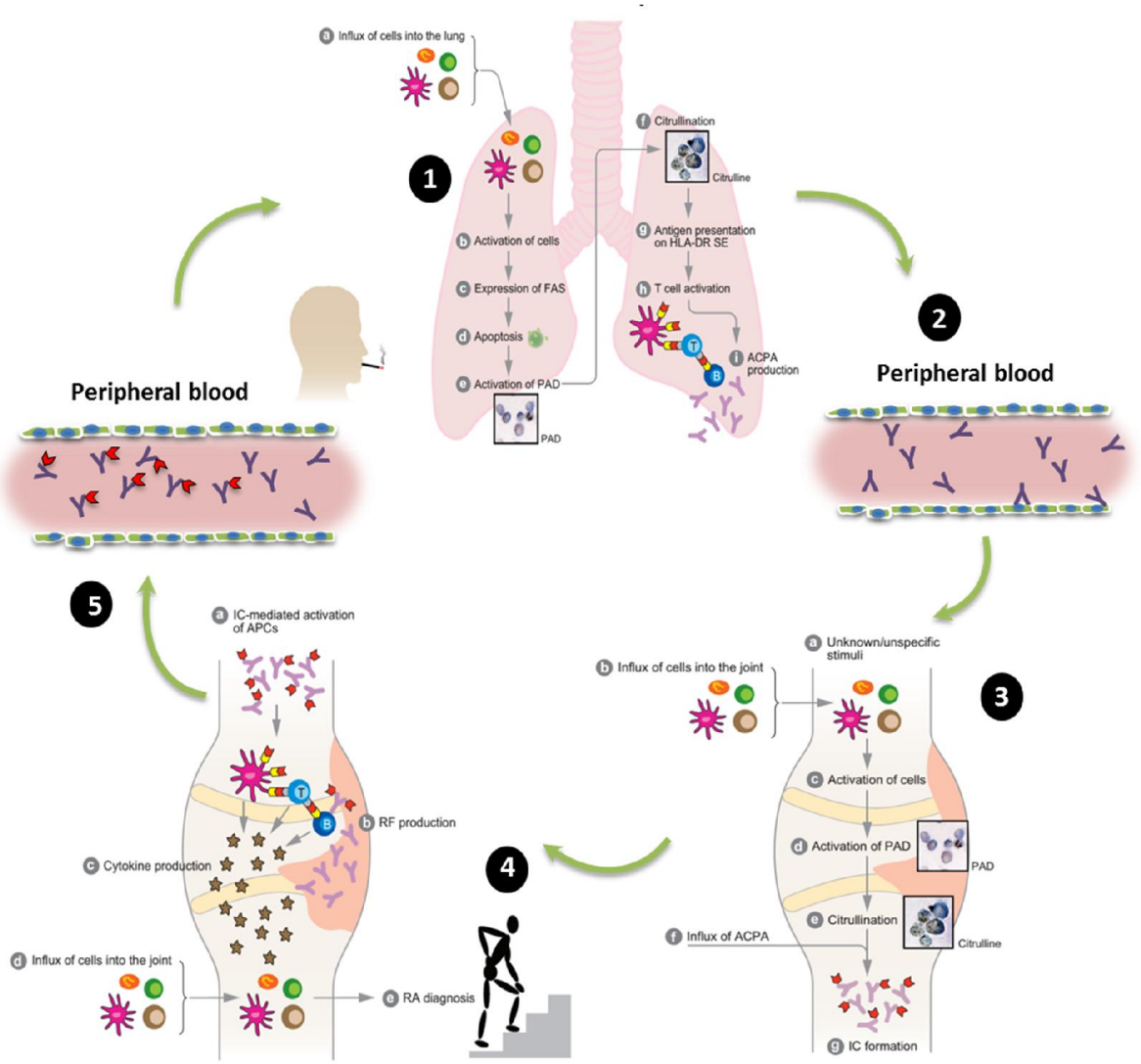


Figure 18- Model of Mechanism of Joint Inflammation in RA. 1) A random set of intrinsic or extrinsic factors like smoking or hormones can induces citrullination of proteins in local organ. In the presence of specific susceptible gene, the newly generated epitopes are more efficiently presented to the immune system that breaks tolerance resulting in the generation of ACPA long before disease onset. 2) In the following years, isotype repertoire expansion and epitope spreading lead to the appearance of multiple ACPA specificities with the individual ACPA phenotype already present when the diagnosis of RA is finally, made. 3) A non-identified second hit, possibly minor trauma or viral infection might lead to synovial citrullination with local emergence of identical citrullinated epitopes as presented previously during the first hit. 4) This will promote localization of ACPAs in the joint that will initiate a cascade of antibody and immune complex mediated events, leading to chronic inflammation. 5) Local synovial presence of citrullinated antigens might facilitate local autoantigen-driven differentiation of B cells into ACPA-producing plasma cells, further contributing to the vicious circle of chronic inflammation and tissue destruction. Modified from [112]

2.2.12 Current treatments and new targets in RA

The pathogenesis of RA is complex and thought to involve a number of different interacting cells and molecules that provide numerous suitable targets for therapy [90]. Treatment of early rheumatoid arthritis by Methotrexate is usually the first DMARD administered to people with rheumatoid arthritis. When methotrexate is contraindicated, sulfasalazine or leflunomide are alternatives. The key aim of treatment for established rheumatoid arthritis is minimization of disease activity. This goal can be achieved with DMARDs and biological agents singly or in combination, with or without glucocorticoids. Growing knowledge of inflammatory pathways in RA has led the foundation for new drug developments that target inflammatory cytokines, B cells and T-cell co-stimulation. T-cell directed approaches target co-stimulation, as well as molecules involved whereas B-cell, directed therapies comprise anti--CD20 therapies, CD19-directed and CD52-directed depletion strategies; alternative approaches include CD22 mediated ligation of inhibitory B-cell receptor and modification of adhesion molecule expression by B cells in T-cell activation and regulation (such as CD4 antigen). Cytokines such as IL-20 and IL-21 contribute to the pathogenesis of RA and have a promise as potential targets for treatment [113].

Despite remarkable success of such approaches in a proportion of patients with RA, many individuals do not derive sufficient benefit from these treatment modalities and new approaches are still necessary. Biological therapies that target pathogenic cytokines such as TNF, IL-1b or IL-6 have heralded a so-called therapeutic revolution, transforming the outlook for patients with RA [87]. However, about 20% to 40% of patients treated with biologics such as TNF inhibitor do not respond to the treatment and further 20% and more lose response

over time [114]. Additionally biological agents such as anti TNF-α are associated with an increased risk of serious infections, including tuberculosis, suggesting alternative drivers of RA pathogenesis that might serve as promising therapeutic targets [108]. Future targets will include target specific cytokines such as IL23, IL37, IL36 and Il38, which will be capable of modulating synovial fibroblast and proteases involved in joint destruction. Transcription factor like FOXO3 is known to be involved in the biology of neutrophils and lymphocytes. Its overexpression in patients with RA seemed to contribute to neutrophil activation and increased lifespan [115]. Further, it was shown that FOX03 inhibition could reduce production of proinflammatory cytokines, including TNFα, and increase production of anti- inflammatory cytokines such as including IL-10. Recent work from Nie et al. reported defect in T_{REG} cell function in RA as a consequence of abnormal phosphorylation of forkhead box protein P3 (FOXP3), which is a key transcription factor for T_{REG} cell function. By inducing the dephosphorylation of FOXP3, it would be possible to reestablish the balance between TNF, T_{REG} cells and pathogenic T_H17 and T_H 1 cells in the joints of patients with RA [116].

2.2.13 Polymorphonuclear Neutrophils in RA

The presence of PMNs remains one of the most consistent indices, which contribute to the perpetuation of the inflammation within joints. In very active disease, up to one billion cells may gain access to a rheumatoid knee joint each day[84]. Although, in the setting of RA, neutrophils have been subjected to less intensive scrutiny than have, T and B lymphocytes[90,113] they are the most abundant cells in the synovial fluid (SF) of the rheumatoid joint, comprising as much as 80% of all infiltrating cells [117]. They are also present in high numbers at sites of bone erosion [118,119].

Upon entry to the synovial fluid via the postcapillary venules of the synovium, they adhere to activated synovial microvasculature by interacting with selectins and integrins [9]. Inside the synovium, they move rapidly to the synovial fluid, drawn by the activated component of cleavage of C5a, LTB 4, platelet-activating factor, and chemokines. The CXC family of chemokines, including ENA-78 and IL-8, are especially abundant in synovial fluid and

can attract neutrophils into the intra-articular space. In addition, soluble immune complexes in the circulation can bind neutrophils in the synovial microvasculature and induce increased vascular permeability. Expression of the transcription factor, Foxo3a, ensures neutrophil survival during inflammation by suppressing the induction of Fas ligand (FasL), a molecule that promotes programmed cell death. Foxo3a inhibition in mice can potentially limit the acute inflammatory response in certain diseases where neutrophils play a prominent role.

Neutrophils, although detected in the synovial membrane, are found mainly in the synovial fluid compartment. The cells in the fluid region seem to be primed **(Table 7)** and activated, but their precise contribution to pathogenesis is disputed.

Table 7 - Neutrophil-activating factors found within SF [9].

	Priming	Adhesion and chemotaxis	ROS and granule enzyme release	Apoptosis delay	MHCII	Production of inflam-matory mediators
C5a		Yes				
G-CSF	Yes	Yes	Yes	Yes		Yes
GM-CSF	Yes		Yes	Yes	Yes	Yes
IFNγ	Yes			Yes	Yes	Yes
IgG/IgM			Yes			
IL-1β	Yes	Yes	Yes	Yes		
IL-6		Yes				
IL-8	Yes	Yes	Yes			
IL-15				Yes		
TNF-α	Yes	Yes	Yes	Yes		Yes

In the joint, neutrophils engage immune complexes through Fc receptors and other activating signals. This engagement leads to cytoskeletal reorganization, release of granule content, generation of reactive oxygen and nitrogen species by enhanced phagocytosis, as well as recently reported neutrophil activity called as NETosis [120]. These reactive oxygen intermediates, prostaglandins, proteases secreted by neutrophils contribute to synovitis [61]. Adhesion of platelets-neutrophil complexes and release of chemokines in vivo is known to contribute towards acute inflammatory responses as well as enhance migration of new cells into the joint space. These cells also release numerous proteases that can adversely affect the lubricating properties of synovial fluid and the integrity of the cartilage, including elastase,

trypsin, and neutrophil collagenase. They also contain immune complexes within phagosomes that include IgG and IgM along with complement proteins such as C1q, C3, and C4.

PMNs from synovial fluid in RA release de novo synthesized proteins, including matrix proteins such as fibronectin, neutral proteinases, and IL-1. Neutrophils also secrete IL-1Ra as a major product. Although the amount of IL-1Ra each neutrophil produces is low compared with that produced by macrophages, the sheer number of PMNs allows them to produce large amounts in synovial effusions. Oncostatin M, known as a member of the IL-6 family, is released by synovial fluid neutrophils **(Figure-20).**

In animal models, a variable role for neutrophils in the inflammatory and destructive processes is seen. The K/BxN and collagen-induced arthritis models, depleting neutrophils with antibodies almost completely prevents synovial inflammation. In the K/BxN model, neutrophils initiate vascular permeability, which permits pathogenic antibodies to gain access to the joint space. In serum transfer model of arthritis, Sky deletion from neutrophils was sufficient to block the initiating event of the disease [121]. Similar findings in immune complex nephritis [122], suggest neutrophils being the key dominant pathogenic cell in most immune complex-mediated diseases. .

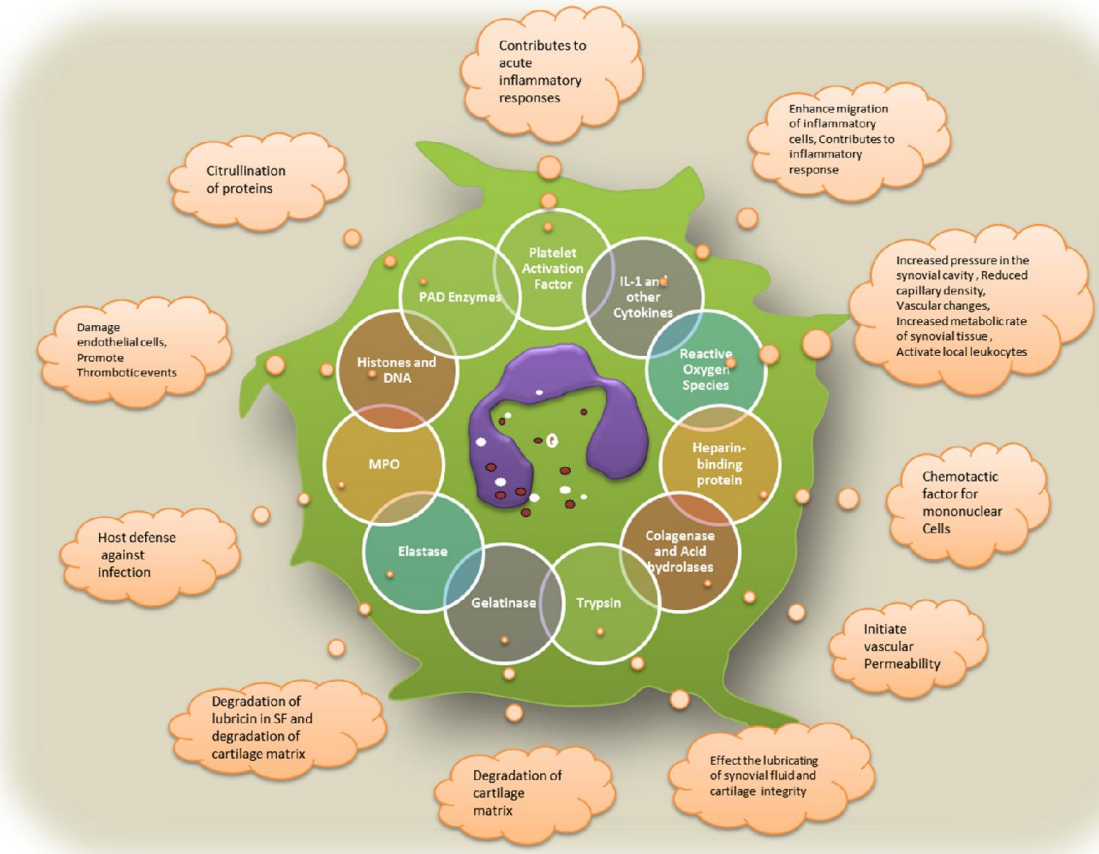


Figure20. Neutrophil activity in the RA joint. Data source [9,119]

2.2.14 Neutrophil extracellular traps in RA

In response to pro-inflammatory stimuli, neutrophil adopts a primed phenotype, which stimulates migration into the inflammatory tissue site. In the tissue, neutrophils get exposed to secondary stimuli and gets "activated", a state characterized by release of granule proteins and acquisition of phagocytic capabilities. [10] However, in 2004, Brinkmann et al. through an elegant series of experiments documented a powerful method of neutrophil-mediated microbial killing through release of extracellular fiber-like structures and termed it as neutrophil extracellular traps (NETs) [20]. NETs consist of chromosomal extruded DNA decorated with granular components that include antimicrobial peptides and proteases. The molecular pathways leading to NETosis involve generation of reactive oxygen species (ROS) by NADPH-oxidase, calcium mobilization, nuclear delobulation involving the enzymatic activities

of myeloperoxidase (MPO) and neutrophil elastase (NE), and chromatin modification via the citrullination of histones by peptidyl arginine deiminase 4 (PAD4) [27,36,123,124].

A number of studies have implicated NETs in the etiology of auto-inflammatory or autoimmune conditions such as preeclampsia, Felty's syndrome, systemic lupus erythematosus (SLE), multiple sclerosis (MS), and most recently, rheumatoid arthritis (RA) [24,125-128]. In the context of RA these findings are especially interesting, as NETs have been proposed to contribute to the generation of anti-citrullinated protein antibody (ACPA) auto-antigens [24,129,130]. These findings indicated that neutrophils isolated from RA cases exhibited an increased propensity to undergo spontaneous NETosis in-vitro; a feature was enhanced when triggered with a second stimulus such as lipopolysaccharide (LPS) [24]. This activity could be transferred to normal PMN by the addition of either RA serum or synovial fluid (SF) to in-vitro cultures, and appears to be in-part mediated via inflammatory cytokines such as tumour necrosis factor alpha (TNF-□□ or interleukin-17 (IL-17), as it could be diminished by the addition of corresponding antibodies [24]. Of interest is that this NETs promoting activity could be hindered by the addition of agents that inhibited NADPH-oxidase or PAD4 activity, implying that enhanced NETosis induced by RA serum or SF was evoked by changes in the underlying signal transduction cascade. These authors further demonstrated the presence of citrullinated autoantigens, specifically enolase and vimentin, directly on NETs, thereby providing insight into a possible mechanism whereby ACPA are generated. Their data also provide a basis for the targeting of NETs by such auto-antibodies [24]. As the presence of ACPA has been shown to precede the onset of RA symptoms [131], and have high predictive value for the development of this disorder [61], these data suggest that the erroneous NETosis could contribute directly to the underlying aetiology of RA [24,129,130].

In the context of these data, it is apparent that PAD4 could play a duel role in the development of RA: on the one hand by being intimately entwined with the NETotic process, and on the other hand by contributing to the production of citrullinated auto-antigens. This hypothesis is supported by recent studies suggesting that a pharmacological modulation of PAD4 activity may ameliorate RA symptoms, albeit in model systems [132].

The citrullination of a broad spectrum of auto-antigens as is evident in RA would, however, require an extracellular presence of PAD4, and probably other members of this family, with different substrate specificities [133]. Indirect evidence for such an event is provided by the detection of anti-PAD4 antibodies [30], a phenomenon that frequently precedes the onset of RA symptoms, and the finding that such antibodies enhance enzymatic activity of PAD4 by reducing its extracellular calcium requirement [134]. Our data indicate that the enhanced propensity of RA-derived PMN to undergo NETosis is characterized by a significantly increased nuclear presence of PAD4 and associated levels of citrullinated histone H3. Our data, furthermore, suggest that PAD4 may be extruded into the extracellular environment on extruded NETs, as we could readily detect cell-free DNA/PAD4 complexes in culture supernatants. Consequently, these findings provide a possible mechanism for anti-PAD4 auto-antibody production, and enhanced peptide citrullination in their presence.

2.3 Materials and Methods

2.3.1 Human Subjects

All patients fulfilled the American College of Rheumatology classification criteria for RA. Healthy volunteers, matched for gender and age, were recruited at the Blood Bank of the Swiss Red Cross, Basel. Inclusion criteria for healthy controls were fair general condition, age ≥ 28 and ≤ 70 years and for blood donors fulfilling national criteria for blood donation. Exclusion criteria were current or previous systemic autoimmune disease, asthma, reconvalescence after major illness, surgery, current medication with corticosteroids, immunosuppressive agents and malignant neoplasia or chemotherapy within 5 years before recruitment for the study. RA cases had a DAS ≤ 3.0, were from age ≥ 27 to ≤ 70 years and had no other systemic autoimmune disease, including ankylosing spondylitis and psoriatic arthritis. Exclusion criteria were corticosteroids ≥ 40 mg equivalent of prednisone daily, and

those mentioned above for healthy controls. Informed, written consent, was obtained from all subjects in the study, which was approved by the Ethical Review Boards of the Cantons of Aargau-Solothurn and Basel/Basel-Land, Switzerland.

2.3.2 Neutrophil isolation

PMNs were isolated by Dextran-Ficoll density centrifugation [125]. Briefly, peripheral blood was obtained by venipuncture and collected in EDTA-containing BD vacutainer tubes. PB was fractionated via density gradient centrifugation using Ficoll-Paque Plus (GE) at 1800rpm for 20min without breaks. After carefully removing the PBMC layer, the pellete was resuspended in 1X HBSS solution,-without Ca or Mg (Gibco). Neutrophils were sedemented by dextran sedimentation of the RBC layer. RBC lysis was performed with hypotonic salt solution for 5 mins. After two cycles of washes using 1X HBSS, cells were resuspended in RPMI without phenol red(Gibco), containing 2% FCS. Isolated neutrophils were stained with trypan blue (MP Biomedical), to confirm >95% viability.

2.3.3 ELISA of NETotic Complexes

2.3.3.1 Neutrophil elastase (NE), myeloperoxidase (MPO), peptidyl arginine deiminase 4 (PAD-4) and cell-free histone/DNA complex expression analysis

The concentration of neutrophil elastase (NE), myeloperoxidase (MPO), peptidyl arginine deiminase 4 (PAD4) were measured by sandwich ELISA (Elastase/a1-PI Complex ELISA Kit, Calbiochem), the human MPO ELISA Kit, Hycult Biotech; the human PAD4 ELISA Kit, (USCN Life Science). Nucleosomes were measured using the Human Cell Death Detection ELISA^{PLUS} (Roche Diagnostics) and histone/DNA complexes in cell culture supernatants by incubation with DNaseI (10U for 5min) (Roche Diagnostics).

2.3.3.2 MPO/DNA complex detection

MPO is present on extruded NETs. To detect such structures, NETs associated MPO/DNA complexes were quantified utilizing a modified capture ELISA [54]. In brief, NETs associated MPO in serum or culture supernatant was captured using the coated 96 well plate of the human MPO ELISA Kit, (Hycult Biotech), following which the NETs associated DNA backbone

was detected using the detection antibody of the Human Cell Death Detection ELISA^{PLUS} (Roche Diagnostics).

2.3.3.3 PAD4/DNA complex detection

To detect the presence of PAD4 on extruded NETs in culture supernatants following spontaneous NETosis, cell-free PAD4/DNA complexes were quantified utilizing a modified capture ELISA, akin to that described for MPO above. In brief, cell-free PAD4 were captured using the coated 96 well plate of a commercial human PAD4 ELISA (USCN Life Science Inc) and associated DNA was detected using Human Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics).

2.3.4 ROS generation analysis

ROS was measured using a 2', 7'-dichloro dihydro fluorescein diacetate (DCFH-DA) assay [135]. 5 x 10^5 cells in a final volume of 500 µl were incubated for 30 min with 25 µM DCFH-DA. Fluorescence was measured by flow cytometry (FACSCalibur; BD Biosciences).

2.3.5 Fluorescence and scanning electron microscopy

5 x 10^4 cells isolated PMN seeded on poly-L-lysine coated coverslips (BD Biosciences) were stimulated with PMA for 90 minutes and dehydrated with a graded ethanol series (30%, 50%, 70%, 100%), coated with 2nm platinum and analysed with a Nova NanoSEM 230 scanning electron microscope (FEI) [125]. PMNs were incubated for 10min with 5µM Sytox Green dye (Invitrogen Life Technologies) for assessment of NETs with an Axiovert fluorescence microscope coupled to a Zeiss AxioCam colour CCD camera (Carl Zeiss) [125].

2.3.6 Immunohistochemical staining and quantification of NETs

5 x 10^4 isolated PMNs seeded on poly-L-lysine-coated glass coverslips (BD Biosciences) in tissue-culture wells and allowed to settle prior to stimulation as described above. Coverslips were rinsed with ice-cold HBSS and the cells fixed with 4% paraformaldehyde and blocked overnight (HBSS with 10% goat serum, 1% BSA, 0.1% Tween20, and 2 mM EDTA) at 4°C. NETs were detected with rabbit anti-NE (Abcam), rabbit anti-MPO (Dako), rabbit anti-PAD 4 (Abcam), mouse anti-histone H1+core proteins (Millipore) and rabbit anti-citrullinated histone

H3 (citH3, Abcam). Secondary antibodies were goat anti-rabbit IgG AF555 and goat anti-rabbit IgG AF488 (Invitrogen). DNA was stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) and NETs were visualized using a Zeiss Axioplan 2 Imaging fluorescence microscope in conjunction with a Zeiss AxioCam MRm monochromatic CCD camera and analyzed with Axiovision 4.8.2 software (Carl Zeiss). A minimum of 20 fields (at least 500 PMNs) per case was evaluated for MPO/NE and DNA co-staining; nuclear phenotypes and NETs were counted and expressed as a percentage of the total number of cells in the fields.

2.3.7 Protein isolation and western blot analysis

Total protein was isolated by NucleoSpin® TriPrep kit (Macherey-Nagel) from 3 x 10^6 PMNs. Proteins from the nuclear and cytoplasmic fractions were isolated using the Nuclear and Cytoplasmic Protein Extraction Kit (Thermo Scientific). Western blotting was performed using AnykD™ Mini-PROTEAN® TGX Gels (Biorad) and nylon/nitrocellulose membranes (Biorad). Primary and secondary antibodies utilized were: rabbit anti-PAD4 (Abcam), rabbit anti-MPO (Cell Signalling), mouse anti-β-Actin (Sigma), goat anti-Mouse and/or anti-Rabbit, human anti-HRP (Southern Biotech). HRP activity was detected by using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific). Equal loading was verified using beta-actin or histone H3, where appropriate. Western blots of citrullinated H3 (citH3) protein as described previously [136]. Densitometric analysis and protein quantification of the western blots was performed using the ImageJ software.

2.3.8 RNA isolation and quantitative real-time PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen). TaqMan real-time quantitative RT-PCR was performed using the Applied Biosystems StepOne Plus™ cycler (Applied Biosystems) and TaqMan Gene Expression Assay primer/probe sets (Applied Biosystems) for NE (HS00236952_m1), MPO (HS00924296_m1), PAD4 (HS00202612_m1) and β2-microglobulin (HS99999907_m1). Data were normalized using the housekeeping gene B2M, after a selection procedure involving 6 different endogenous reference genes as suggested in the MIQE guidelines [137]. Relative values were calculated by 2^{-DDCt} analysis.

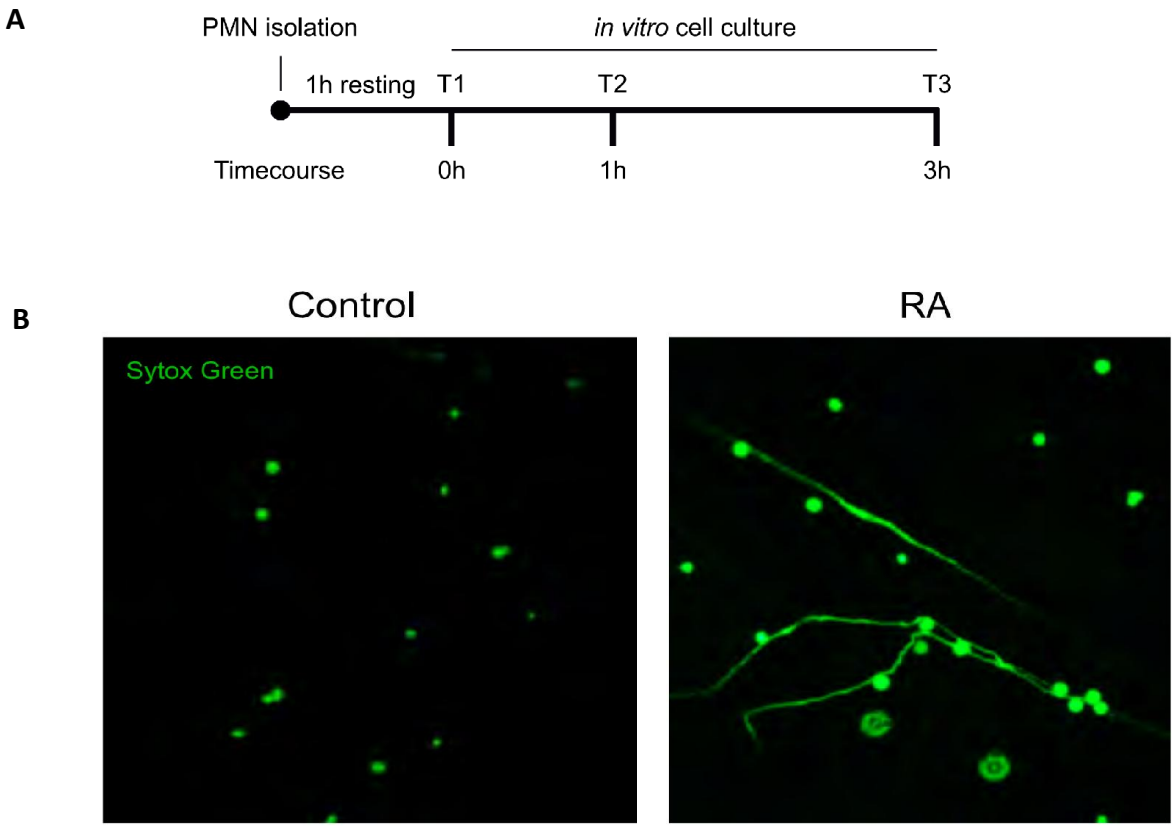
2.3.9 Statistical analysis

All data are presented as mean ± SE. Descriptive statistics for continuous parameters consisted of median and range and categorical variables were expressed as percentages. Comparisons between patients and healthy controls were by the Mann-Whitney U test with a Welch post-test correction. Statistical significance in multiple comparisons was by one-way analysis of variance (ANOVA) with a Dunn's post-test correction. $P < 0.05$ was considered statistically significant. Data were processed in GraphPad Prism version 5.0b for MacOSX (GraphPad software Inc., www.graphpad.com). Additional professional statistical assistance was provided by A. Schoetzau (www.eudox.ch).

2.4 Results

2.4.1 RA-derived PMN exhibit increased spontaneous NETosis

Recent observations have described greater degrees of spontaneous NETosis in RA-derived PMN than control PMN in vitro culture [24]. In order to study this facet in more detail, we examined the kinetics of spontaneous NET extrusion, for which purpose PMN were isolated from peripheral blood samples, allowed to settle for 1 hour and then cultured for a period of up to three hours in vitro (Figure-21A). Akin to very recent observations [24], we observed that RA-derived PMN underwent greater degrees of NETosis than control PMN in vitro, as detected by fluorescence microscopy for Sytox Green (Figure- 21B) and scanning electron microscopy (SEM) (Figure-21C). In addition NETs being detected by immunohistochemistry for neutrophil elastase (NE) and DAPI (4',6-diamidino-2-phenylindole) (Figure-21D). These results suggest that the peripheral blood neutrophils from RA patients has a tendency of spontaneous NETosis under unstimulated in vitro conditions.



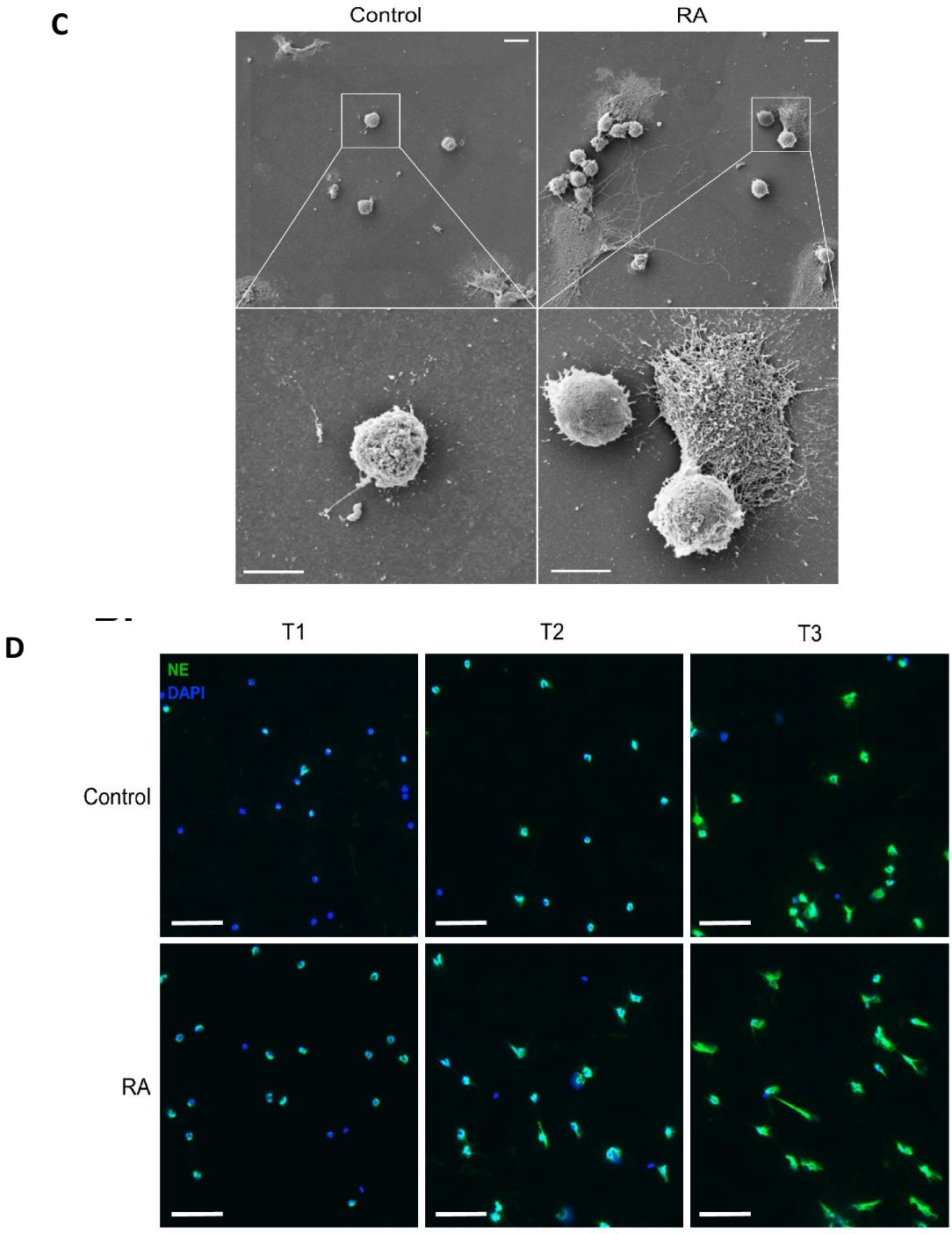


Figure 21 . RA-derived PMNs exhibited increased spontaneous NETosis and elevated levels of NET component release. (A) Schematic representation of the time course design for studying in-vitro spontaneous NET release. (B) Fluorescent microscopy using Sytox Green DNA stainings, (C) Scanning electron micrographs, Magnification: 10x; scale bars: 10 µm and (D) in vitro NETosis by immunohistochemistry for neutrophil elastase (NE) (green) and DAPI (blue). Magnification: 20x; scale bar: 50 nm indicative of increased spontaneous NETosis observed in PMN isolated from representative patients with RA compared to healthy donors. **IF Data provided by S. Giaglis.**

2.4.2 NETotic spread in RA PMN is more pronounced than in normal PMN

During NETosis, the morphology of the PMN nucleus changes from the familiar lobulated to a diffused and then to a spread phenotype (Figure-22A) [36,138]. By examining and enumerating these features, it was observed that at baseline (T1) nuclei from healthy control PMN were predominantly lobulated, while the majority of RA-derived PMN nuclei exhibited a delobulated or diffused nuclear phenotype (Figure-22B). In RA derived PMN this delobulated population decreased over time, giving rise to NETotic cells with a spread phenotype (Figure-22C). In contrast, in normal PMN there was a steady progression in the proportion of delobulated cells (Figure-22D). The spontaneous progression of nuclei to the NETotic spread phenotype was more pronounced in RA than in normal PMN, a feature most evident after 3 hours (T3).

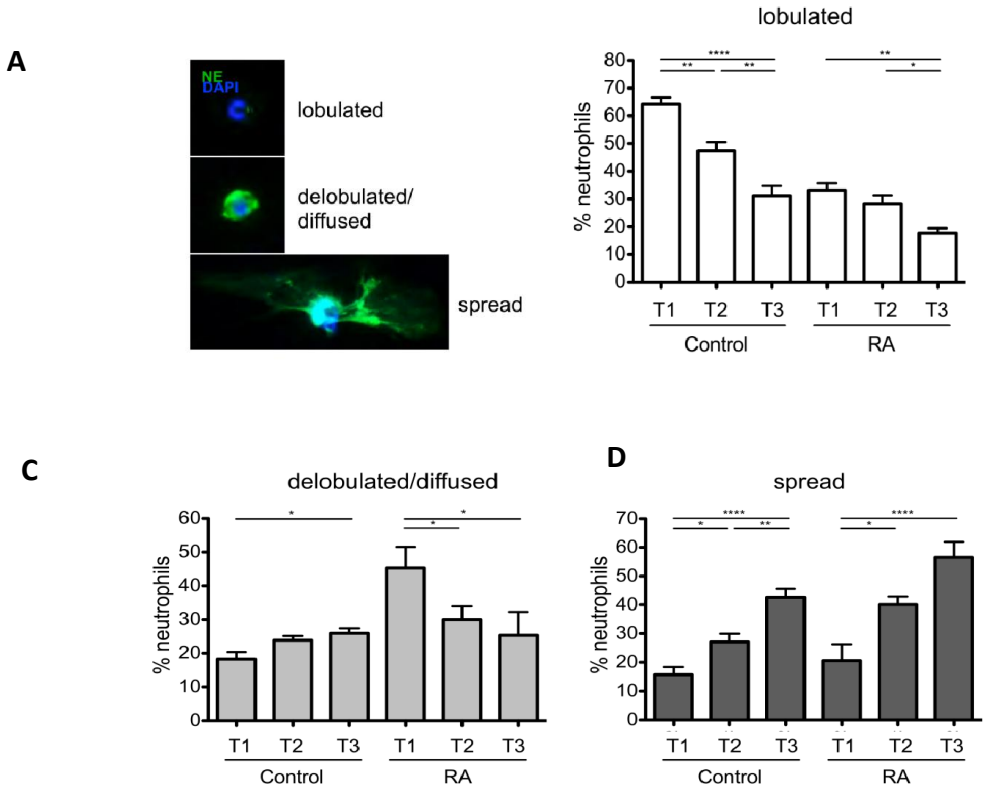


Figure 22. Changes in PMN nuclear morphology during NETosis. (A) Changes in PMN nuclear morphology during NETosis detected by immunohistochemistry for NE and DAPI. (B) Steady state (T1) RA-derived PMNs exhibited a greater proportion of delobulated/diffused cells and progressed rapidly to a NETotic spread phenotype during *in vitro* culture. Data are represented as mean ± SE. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All data are representative of at least six independent experiments. **IF Data provided by S. Giaglis**

2.4.3 RA derived PMN generates more extracellular complexes of NETotic origin

During the process of NETosis the released DNA forms complex with several cellular proteins like MPO, histones and LL337, which could be considered specific products of NETosis. Here we quantitatively assessed the degree of *in vitro* NETosis in culture supernatants by determining the concentration of cell-free nucleosomes in the respective supernatants (Figure-23A), MPO-DNA complex (Figure-23B) and MPO-histone complexes (Figure- 23C), indicative of the NETotic origin of this material. These experiments clearly indicated that RA-derived PMNs generated NETs more rapidly, to a greater magnitude and more extensively than control healthy PMNs, a feature particularly evident at the 3-hour stage of in vitro culture.

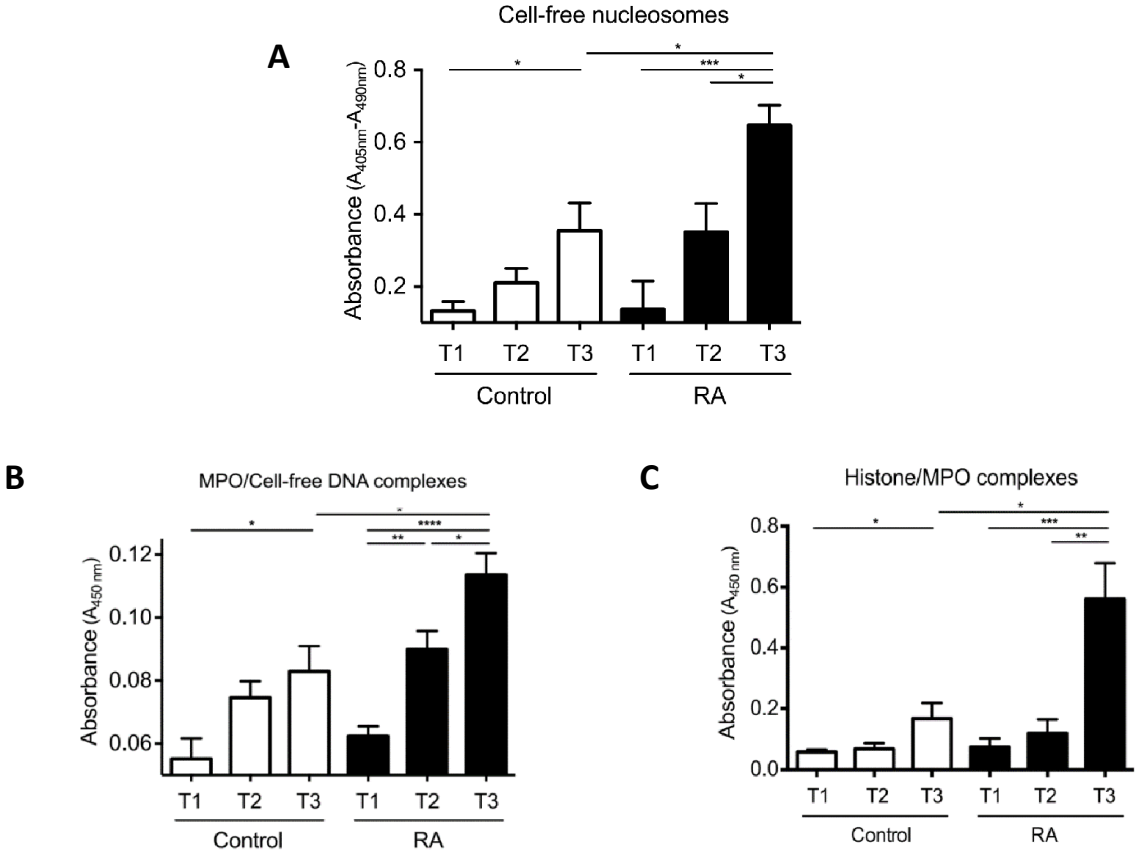


Figure 23. Quantification of complexes of NETotic origin. (A) Concentration of cell-free nucleosomes in PMN culture supernatant by ELISA. (B) Quantification of NETs associated MPO/DNA complexes and (C) Histone-MPO complexes. These assays indicate that, more rapid and extensive progression of NET formation is observed in RA versus control PMNs.

2.4.4 Increased expression of NET-associated signaling elements

NETosis has been shown to depend on a number of biochemical signaling elements, among which are the generation of reactive oxygen species (ROS) by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the action of NE in combination with MPO, and histone citrullination by PAD4 [20,27,29,36,123,124]. RA-derived PMN exhibited increased basal intracellular ROS levels (Figure 25A), as well as increased levels of NE (Figure 24B) or MPO (Figures 24C and 24D), as determined by real-time PCR and/or western blotting.

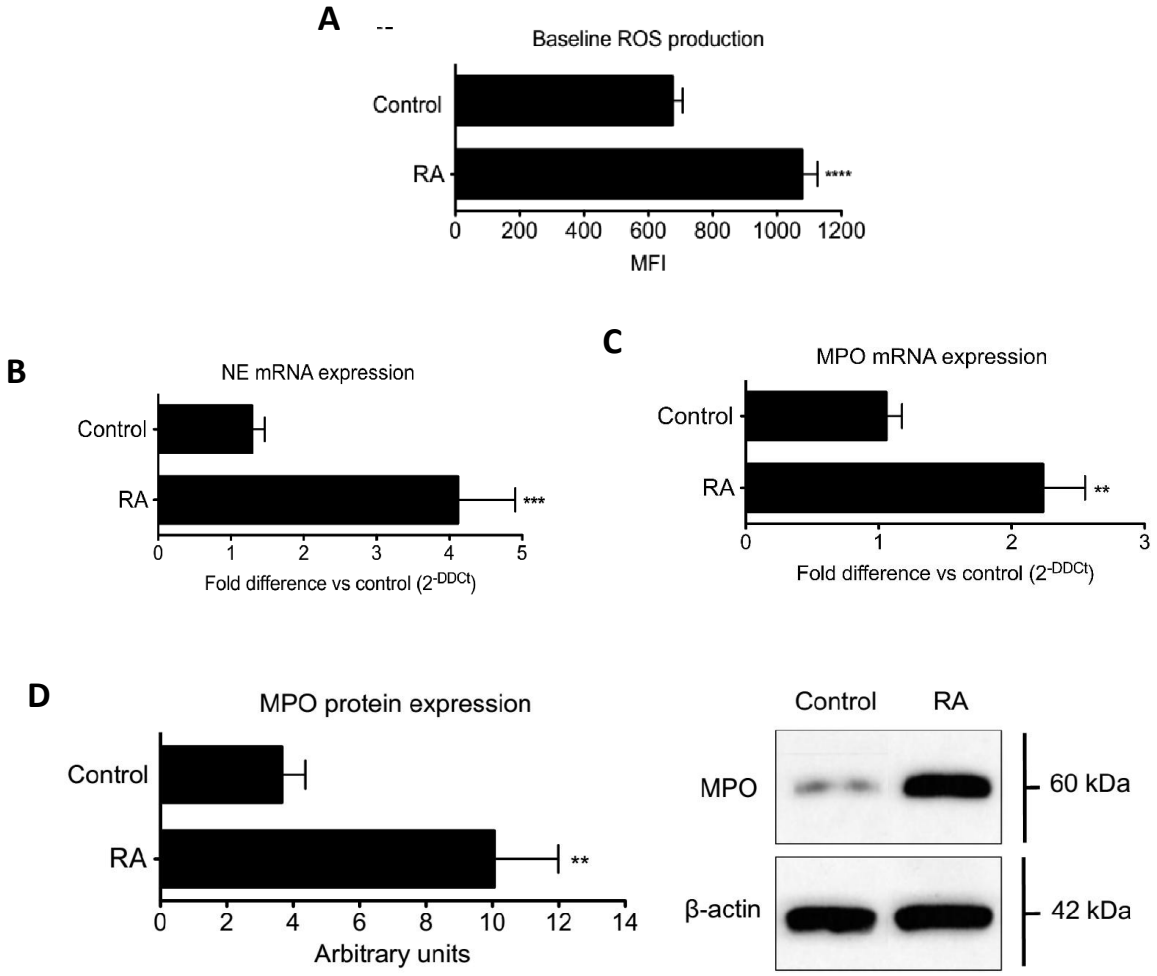
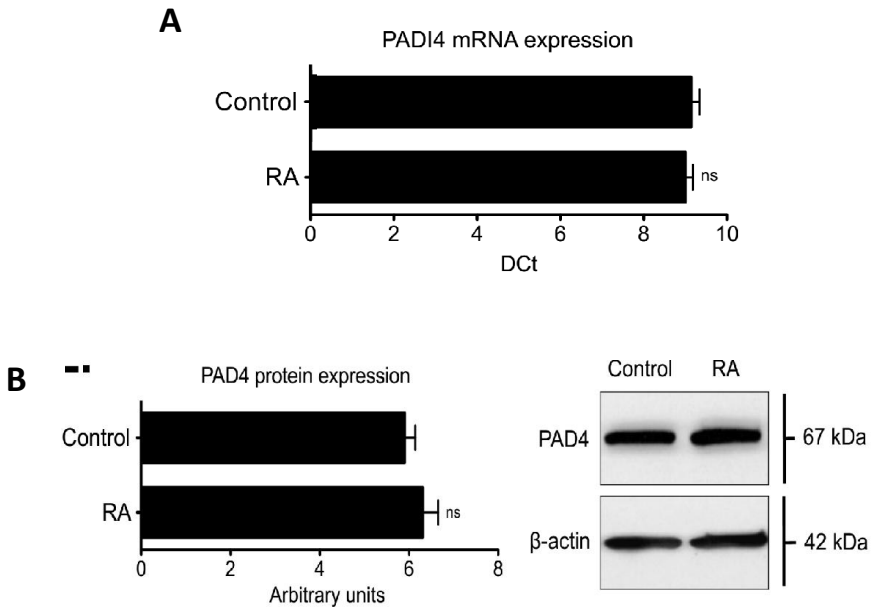
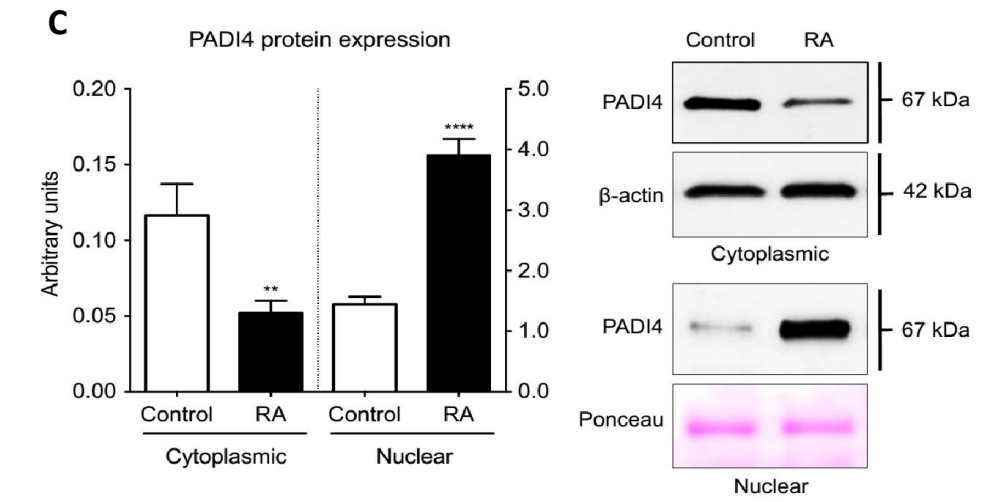


Figure 24. Increased expression of NET-associated signaling elements (A) Baseline ROS levels, measured by flow cytometry, were higher in RA-derived PMN than control PMN. (B) Quantitative real-time PCR analysis of NE mRNA and (C) MPO mRNA expression as well as (D) western blot analysis of MPO protein levels indicated that the levels of these two components required for NETosis were elevated in RA-derived PMN.

2.4.5 Nuclear localization of PAD4 and augmented H3 citrullination in RA derived PMN

Work from Foulquier et. al., has shown that PADI2 and PAD4 are the only PAD isotypes expressed in the synovial tissue of patients with RA. Further it was shown that inflammatory cells are a major source of these enzymes [97]. Surprisingly, neither PAD4 mRNA expression nor PAD4 levels in total cellular protein showed any discernible difference between RA PMN and controls (Figures 25A and 25B, respectively). Since PAD4 translocates to the nucleus upon activation where it citrullinates histone proteins [37,139,140], such as H3, we examined nuclear and cytoplasmic PMN fractions for its presence. When compared to control PMNs, we found that PAD4 was preferentially located in the nucleus of RA-derived PMN (Figure 25C). The nuclear presence of PAD4 was associated with increased citrullinated histone H3 (citH3) levels by western blot analysis in PMN from RA cases compared to control (Figure 25D). Furthermore, citrullinated histone H3 could be readily detected on NETs structures (Figure2 5E).

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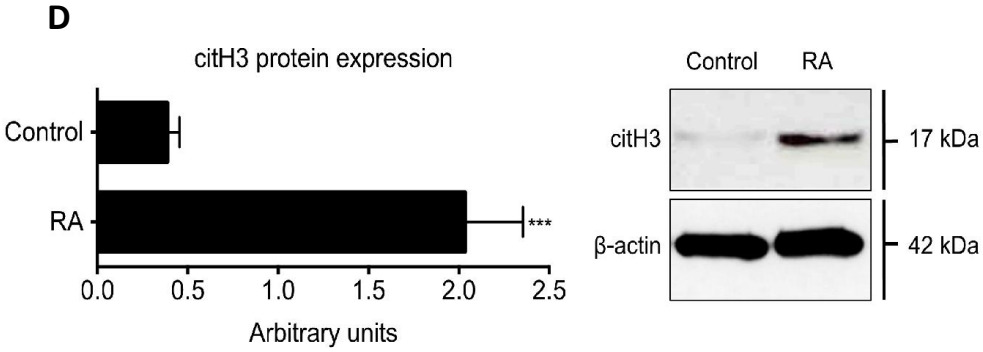
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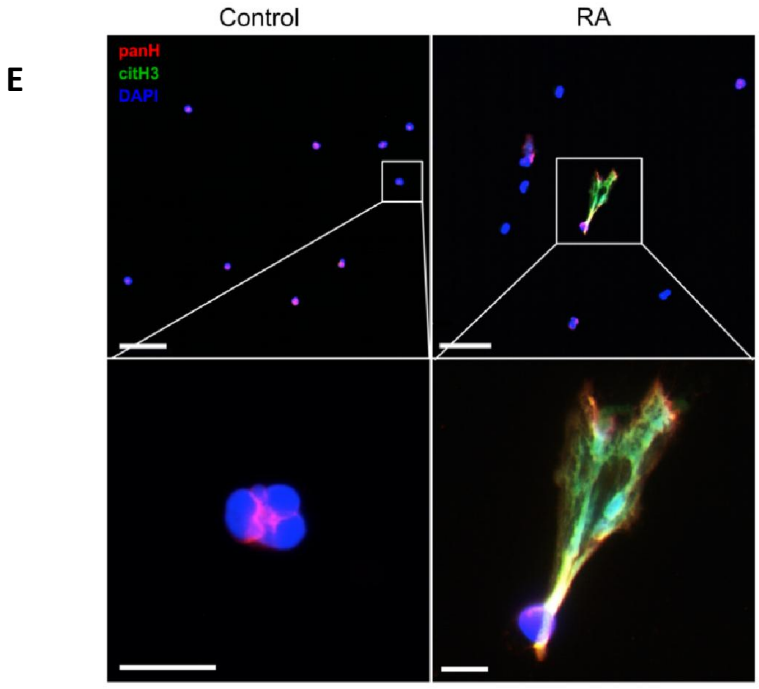
E
Control
RA
panH
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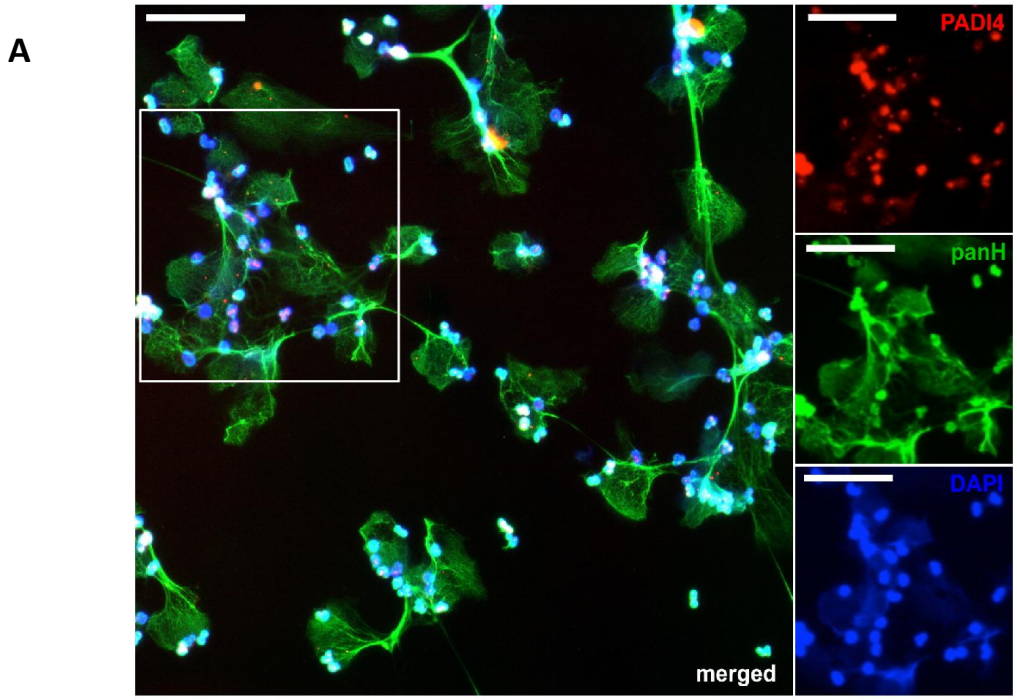
Figure 25- Nuclear localization of PAD4, a key fetcher of RA PMN. (A) Total PAD4 protein or (B) PAD4 mRNA expression levels did not indicate any significant difference between control and RA-derived PMN. (C) Quantification of PAD4 protein levels in cytoplasmic and nuclear fractions of PMNs from healthy controls and RA patients. Nuclear levels of PAD4 were significantly increased in RA patients, whereas the cytoplasmic levels were lower compared to the healthy control PMN. (D) Elevated citrullinated histone H3 levels in RA PMN extracts detected by western blot. (E) Co-localization of citrullinated histone H3 (green) with histone components detected with a pan-histone antibody (red) spread over the entire NETs surface (blue). Magnification: upper panel 20x, scale bar: 50 nm; lower panel 63x, scale bar: 10 nm. IF Data provided by S. Giaglis

2.4.6 Potential extracellular localization of PAD4 on NETs

Since we observed elevated nuclear translocation of PAD4 in RA PMN, we examined whether this enzyme is extruded into the extracellular environment during NETosis. Unfortunately, the visualization of such an event by fluorescent immunohistochemistry proved to be difficult using a variety of commercially available antibodies, and we only obtained rudimentary evidence for the presence of PAD4 on NETs by this means (Figure 26A). We were, however, able to detect PAD4/cell-free DNA complexes in culture supernatants from isolated PMN, the levels of which were increased in cases with RA (Figure 26B). It is, therefore, quite probable that PAD4 is associated with NETs structures following aberrant NETosis in RA.



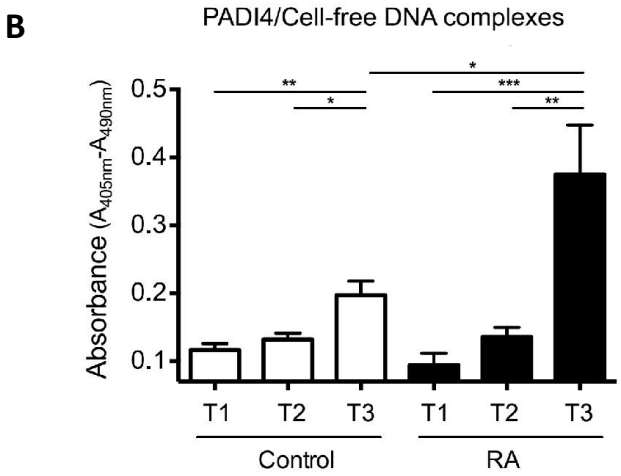
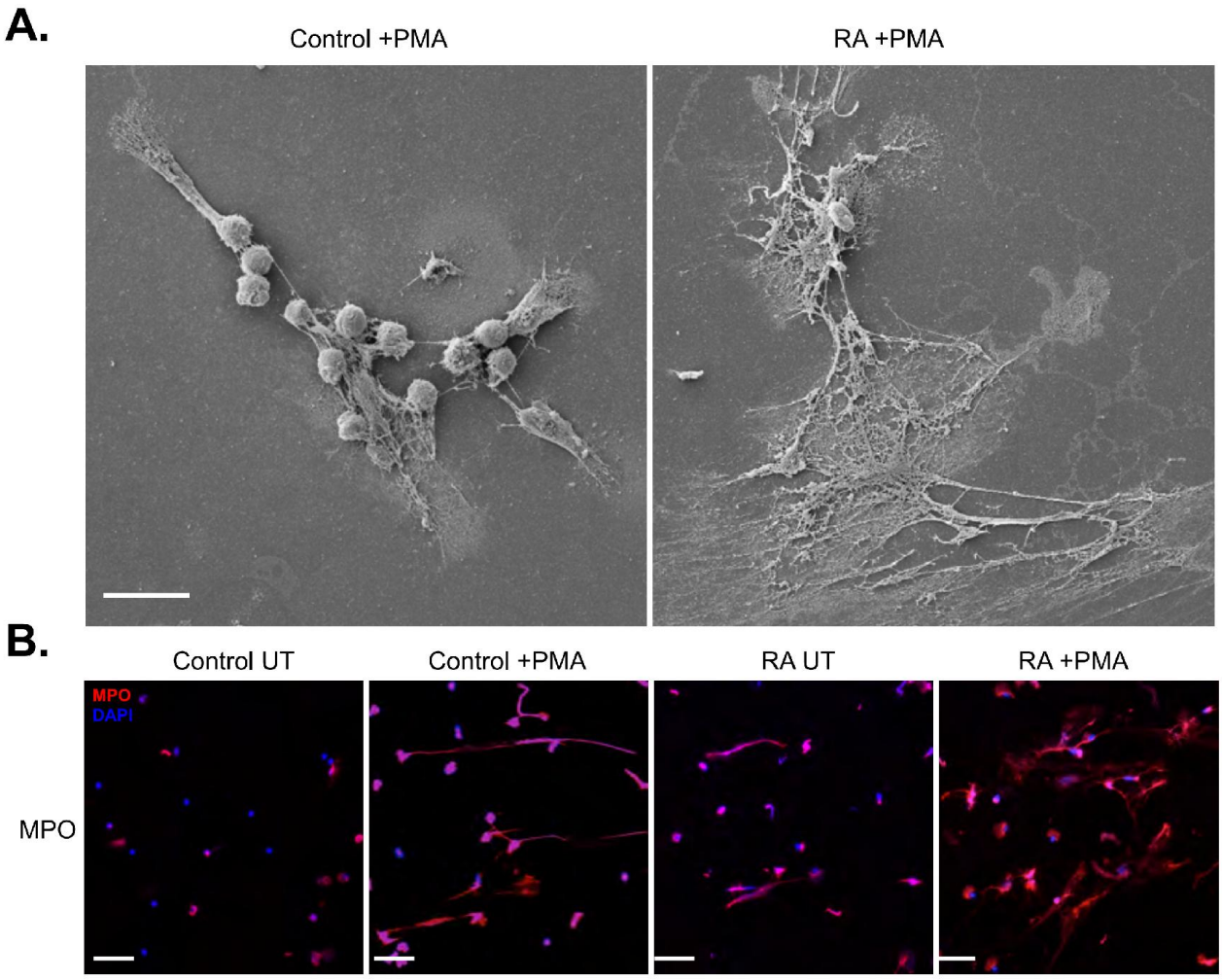


Figure 26- Extracellular localization of PAD4. (A) Immunofluorescence staining of PAD4 (red) on extruded NETs by multi-colour fluorescent immunohistochemistry. NET DNA is stained blue (DAPI) and histones (panH) are stained green. Magnification: 20x, scale bar: 50 nm. (B) Detection of PAD4/cell-free DNA complexes in the culture supernatants of isolated PMN undergoing spontaneous NETosis. Higher levels of these complexes were detected in RA derived PMN cultures. Data are represented as mean ± SE. **P < 0.01, ***P < 0.001, ****P < 0.0001, n.s.: statistically not significant. All data are representative of at least six independent experiments. **IF Data provided by S. Giaglis**

2.4.7 RA neutrophils are subject to increased NETosis following stimulation by a secondary signal

In certain auto-inflammatory or malignant conditions, such as SLE or cancer, an elevated NETotic response of PMNs to an external activation signal has been shown [127,128,141]. A similar phenomenon was recently observed in RA-derived PMN [24]. In addition, RA serum and SF were determined to enhance the NETotic response of normal PMN [24]. In our experiments, it was noted that when treated with PMA, RA-derived PMN responded far more vigorously with regard to NETosis than controls, as detected by SEM and fluorescence microscopy (Figures 27A and 27B respectively). In addition, this could be assessed morphologically, which indicated that RA-derived PMN exhibited a larger decrease in cells with a delobulated phenotype and a greater progression towards a NETotic spread nuclear phenotype than control PMN (Figure 27C), a feature accompanied by excessive release of cell-free nucleosomes in culture supernatants (Figure 27D). PMA appears to activate PAD4, as it enhanced translocation from the cytoplasm to the nucleus (Figure 27E). The stimulatory effect of PMA on the release of nucleosomes into the supernatant was abrogated by chloramidine,

a chemical inhibitor of PAD4, indicating that PAD4 signaling is necessary for NETosis induced by PMA (Figure 7D). These data confirm that PMN in RA is subject to increased NETosis following stimulation by a secondary signal, such as that mediated by PMA, and indicate that nuclear translocation of PAD4 (Figure 27E) and its enzyme activity are involved in this process. We examined these features with regard to PAD4 nuclear translocation and extrusion on NETs. As an additional stimulus, we used phorbol-12-myristate-13-acetate (PMA), while chloramidine was used to inhibit PAD4 activity.



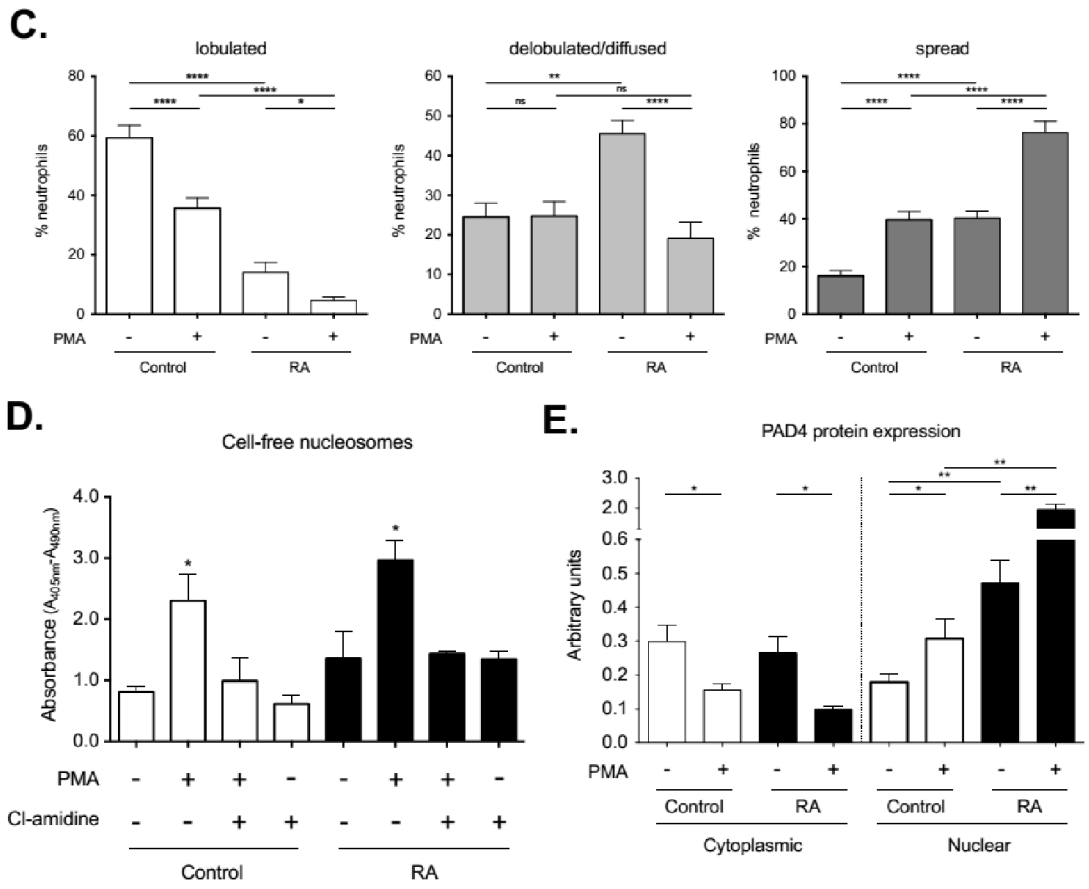
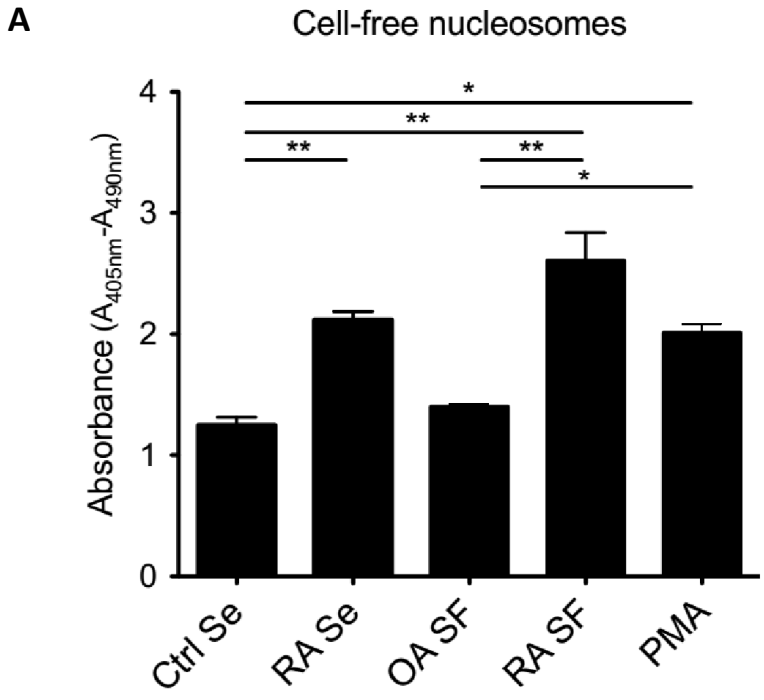


Figure 27. Increased NETotic response of RA-derived PMNs to PMA. (A) Scanning electron micrographs of NETs induced by PMA (25 nM) indicate the excessive NETotic response of RA-derived PMN. Scale bar: 20 µm. (B) Assessment of NETs induced by PMA treatment by fluorescent immunohistochemistry for MPO (red) and DAPI (blue) indicating the increased response of RA PMN to PMA (25 nM). Magnification: 20x, scale bar: 50 nm. (C) Analysis of the nuclear phenotype indicated that a vast decrease in delobulated/diffused RA PMN nuclei after treatment with PMA and rapid increase in the NETotic spread phenotype. (D) Release of cell-free nucleosomes following PMA treatment is abrogated by the PAD4 inhibitor chloramidine. (E) Increased nuclear localization and a concomitant decrease in cytoplasmic PAD4 protein levels following PMA treatment, with clear tendency for increased responsiveness to the PMA stimulus by RA PMN. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n.s.: statistically not significant. All data are representative of at least four independent experiments. **IF Data provided by S. Giaglis**

2.4.8 RA serum and synovial fluid stimulate enhanced NETosis to normal PMN.

Sera from auto-inflammatory conditions such as SLE have previously had been shown to confer an increased NETotic response on normal PMN [127,128]. Since this feature was also recently observed with RA serum and synovial fluid [24], we examined the influence of these factors on PAD4 activity. As a non-inflammatory control, we used healthy serum or osteoarthritis synovial fluid. Both RA sera and synovial fluid induced a pronounced increase in ROS production (data not shown), as well as in NETosis (Figures 28A and B) when compared to healthy serum or osteoarthritis synovial fluid, respectively. The activity of RA synovial fluid appeared to be more pronounced than RA serum (Figure 28A), confirming previous observations [24].



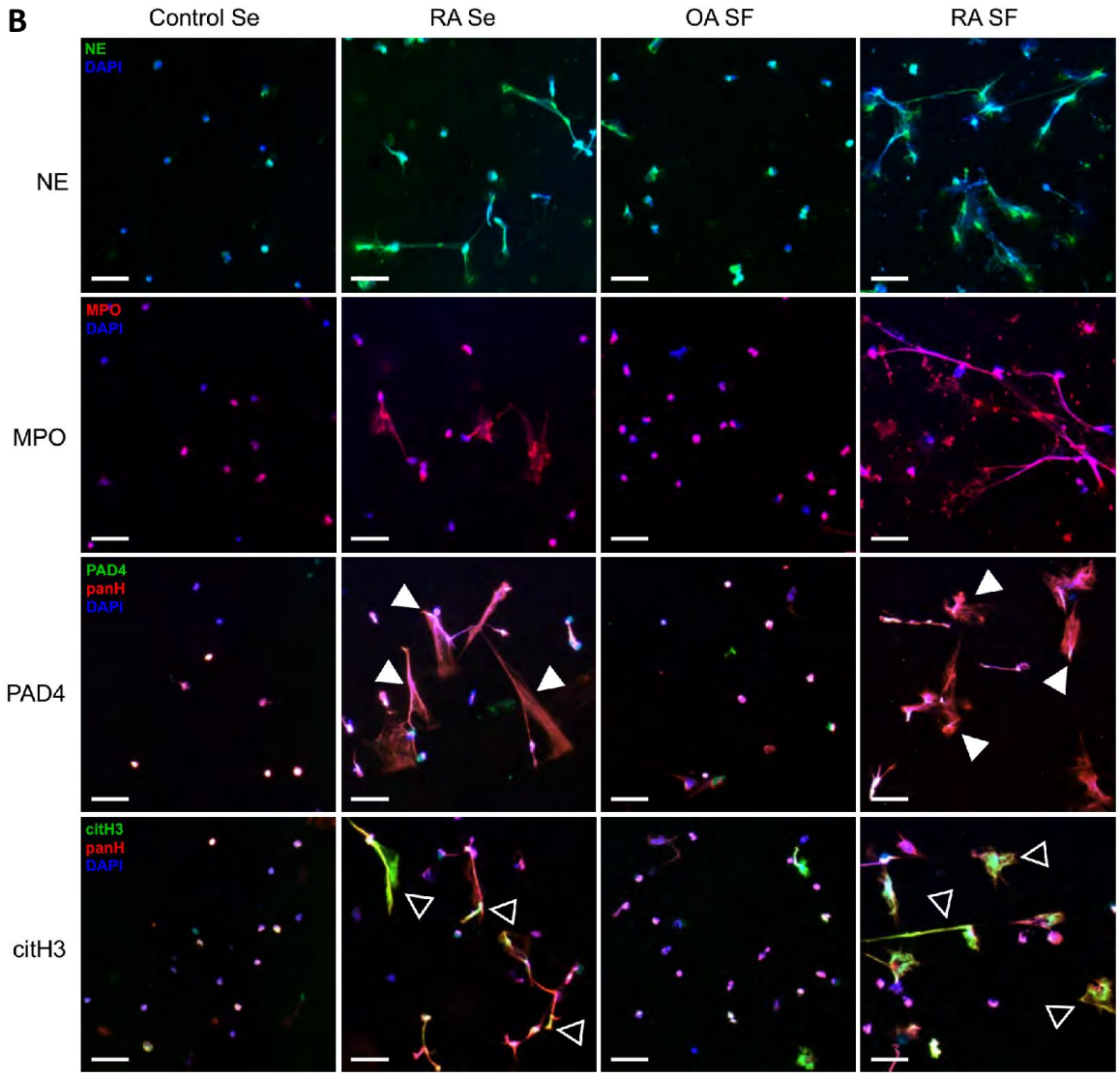


Figure 28. Influence of RA serum and synovial fluid on normal PMNs. (A) Incubation of healthy donor PMN with serum (Se) from healthy donors orRA patients, synovial fluid (SF) from patients with non-inflammatory osteoarthritis (OA) or synovial fluid from RA patients. Immunohistochemical analysis of three main components of NETs (NE, MPO and citH3) revealed that RA-derived serum and SF enhanced NETosis in normal PMN compared to healthy control serum or non-inflammatory OA SF. CitH3 (empty arrowheads) co-localizes with unmodified histones on NETs. Magnification: 20x; Scale bars: 50 µm. (B) Release of cell-free nucleosomes during in vitro culture by PMNs from healthy controls incubated with control serum, RA serum, OA SF or RA SF or PMA. Data are represented as mean ± SE. *P < 0.05, **P < 0.01. All data are representative of at least four independent experiments. **IF Data provided by S. Giaglis**

2.5 Discussion

Although PMN figures prominently in the joint effusions and inflamed synovial tissue of RA patients [61], the potential roles of NETotic events in the pathophysiology of this disorder have only recently been become the focus of attention [24,129,130]. These studies indicated that RA-derived PMN were more prone to undergo NETosis and that NETs themselves could contribute to the generation of auto-antigens (ACPA) or be the target of auto-antibodies **(Figure-29)** [24,130].

In our studies, which were performed independently at a similar time as these current reports, it is noteworthy that our data confirm that NETosis is enhanced in RA, thereby suggesting a possible fundamental role of this phenomenon in the underlying aetiology of RA. More importantly, we extended upon these observations by detecting changes in the underlying signalling elements required for the induction of NETosis. These findings indicate that the propensity of circulatory PMN in RA patients to undergo NETosis is associated with elevations in elements of this cascade including increased intracellular ROS production, enhanced expression of NE and MPO, increased nuclear translocation of PAD4 and citrullination of histones, notably H3. Consequently, these key NETotic pathway members could serve as potential therapeutic targets for intervention strategies, a hypothesis which is supported by recent data using PAD4 inhibitors in model systems [132].

Furthermore, by examining kinetic changes during extended in vitro culture it was observed that PMN from RA cases exhibited different nuclear morphometric characteristics, having a lower proportion of the classical lobulated phenotype, coupled with a much higher proportion of delobulated cells at the initial time-point. Unlike controls, in which an increase in this population was noted over time, this latter population decreased during in vitro culture of RA PMN. RA PMN also progressed more rapidly and extensively to a NETotic spread phenotype than controls, a finding confirmed by analysis of culture supernatants for the products of NETosis. Akin to what has been observed in an array of other pathological conditions ranging from SLE to cancer [24,127,128,141], PMN from RA patients exhibited an increased response to further stimulation, for instance by treatment with the phorbol ester

PMA. This response is in part mediated via the action of PAD4, as the effect of PMA could be significantly reduced by treatment with chloramidine, an inhibitor of PAD4. In addition, PMA treatment lead to increased nuclear presence of this enzyme, where it presumably could be able to carry out more extensive citrullination of histone proteins, thereby speeding up the induction of NETosis.

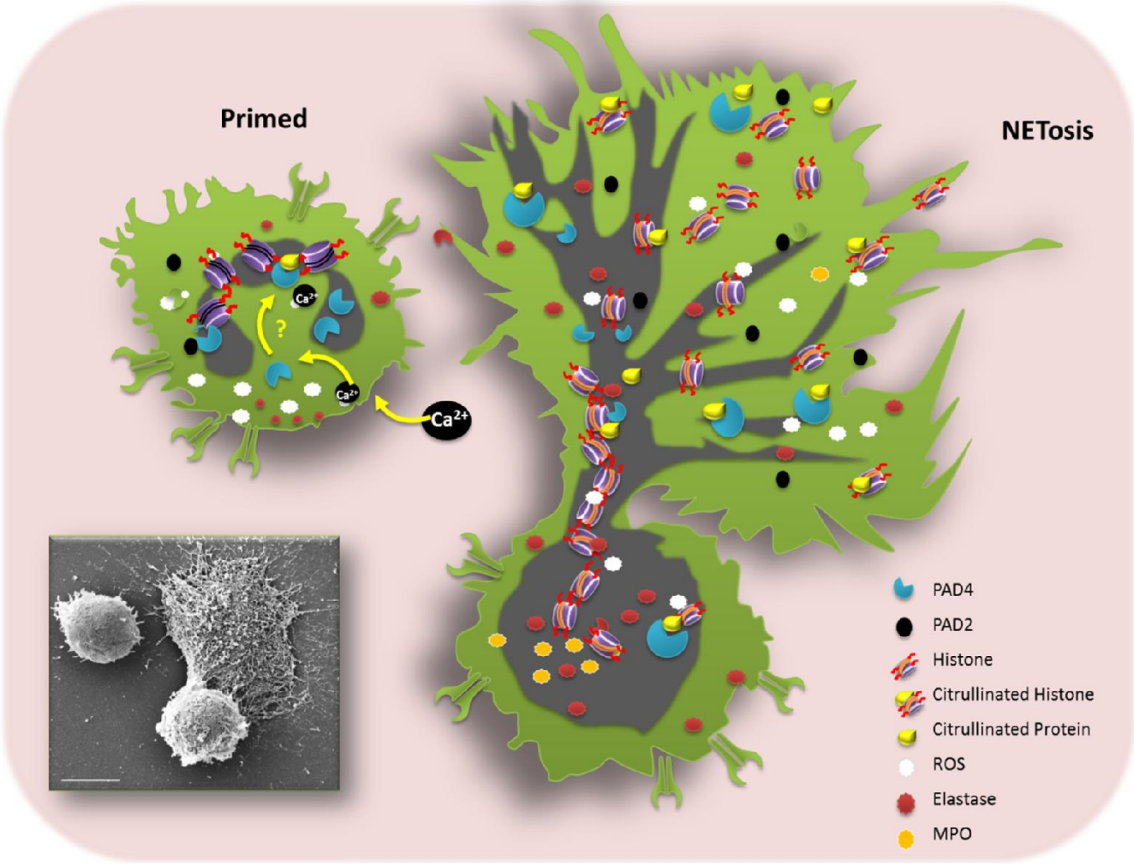


Figure 29. Model of NETosis in RA PMN

Although our data are very preliminary, they do suggest that PAD4 is extruded onto the NETs during NETosis, as detected by ELISA technology and to a lesser extent by fluorescence microscopy. Such an occurrence would have important implications for the development of anti-PAD4 auto-antibodies observed in cases with RA [30]. Since the presence of such antibodies precedes the development of RA, our data provide further support that NETs may contribute to the underlying aetiology of RA, and may be a relatively early event. As the presence of such anti-PAD4 antibodies has been shown to enhance the enzymatic activity of

PAD4 in an extracellular environment by reducing the calcium requirement [134], their combination with NETs associated PAD4 could lead to prodigious quantities of citrullinated autoantigens. In addition, the extracellular presence of PAD4 on NETs may further promote the prodigious generation of citrullinated antigens, since molecular structures involving the attachment of enzymes to DNA lattices have been shown to increase their catalytic activity enormously, and thereby form the basis of nano-machines or nano-factories, generating such autoantigens [142].

Although these findings will need to be verified, and it remains to be ascertained whether extracellular NETs associated PAD4 is active, these data do support and extend upon recent reports indicating that NETs can be a source for citrullinated autoantigens and that they react with ACPA or anti-PAD4 antibodies [24,129,130]. Taken together, these data provide further evidence concerning a key role for PAD4 in the underlying aetiology of RA, and offer a potential explanation for the efficacy of PAD4 inhibitor chloramidine in reducing disease symptoms, in collagen-induced rat and murine models for RA [132]. In summary, our data reaffirm an intricate relationship between NETosis and the aetiology of RA, since the signalling elements associated with NETs extrusion are significantly enhanced to promote NETosis in RA compared to healthy controls. Furthermore, by implying the extracellular presence of PAD4 on NETs, these data provide a potential link with the generation of anti-PAD4 autoantibodies and ACPA in the development of RA.

2.5.1 Hypothetical model of pathophysiology of Rheumatoid Arthritis

A number of exogenous or endogenous trigger may cause heavy influx of cells into the lungs and may prime the circulating neutrophils or macrophages. A simultaneous second hit may activate neutrophils and render the RA neutrophils more prone to NETosis compared to neutrophils from normal subjects. As a consequence of which citrullinated proteins along with native intracellular proteins including PAD enzymes can be released into the local site. The extracellular PAD enzymes tangled to the NET structure or its free form, could further citrullinate proteins present in the local site and thus generate an array of citrullinated proteins some of which act as a potential autoantigen for the onset of the disease. These

autoantigens are then engulfed by resident macrophages or dendritic cells which flags the autoantigen at its cell surface in combination with class II MHC. This processing and presentation of unspecified but not necessarily rheumatoid-specific antigen, likely occurs in central lymphoid organs and initiates the adaptive immune response. In genetically predisposed individuals, such as those carrying the HLA-DR SE alleles, presentation of citrullinated peptides or other neo-epitopes from citrullinated proteins could activate autoreactive T cells, which in turn could induce B cell help and stimulate the production of ACPA. A second, joint-specific inflammatory event is initiated by an unknown and unspecific stimulus such as, infection or trauma resulting in the release of angiogenic factors such as vascular endothelial growth factor (VEGF). The inflammatory cytokine milieu, along angiogenic factors, encourages angiogenesis, endothelial activation, cell migration and hypoxia. Activate endothelial cells produce adhesion molecules, which expedite activation-dependent sticking of inflammatory leukocytes, thereby facilitating diapedesis and extravasation into the synovium. Inside the synovium, a number of pro-inflammatory cytokines such as TNFα, IL8, IL17a along with HIF gene products, could potentially activate neutrophils towards extracellular trap formation as a result of which PAD enzymes are released in the synovial fluid which produce a repertoire of citrullinated proteins. The resulting anti-citrullinated protein/peptide antibodies (ACPAs) are distributed through the circulation and may form immune complexes with citrullinated proteins produced in an inflamed synovium. Immune complexes, containing autoantibodies such as RFs or anticitrullinated protein antibodies can fix complement, leading to the generation of chemoattractants. Further, it can stimulate antigen-presenting cells (APCs) such as dendritic cells, by binding to complement and Fc receptors. Activated APCs present more citrullinated antigens activate more T and B cells and thus increase the ACPA production along with RF production. Increased production of proinflammatory cytokines, including TNF, IL-1, and IL-6, in turn recruits more immune cells into the joint, perpetuating the inflammatory process. Activation of neutrophils towards NETosis will release more PAD enzymes which generate more citrullinated proteins, establishing a vicious cycle that ultimately leads to the development of chronic RA (**Figure 30)**.

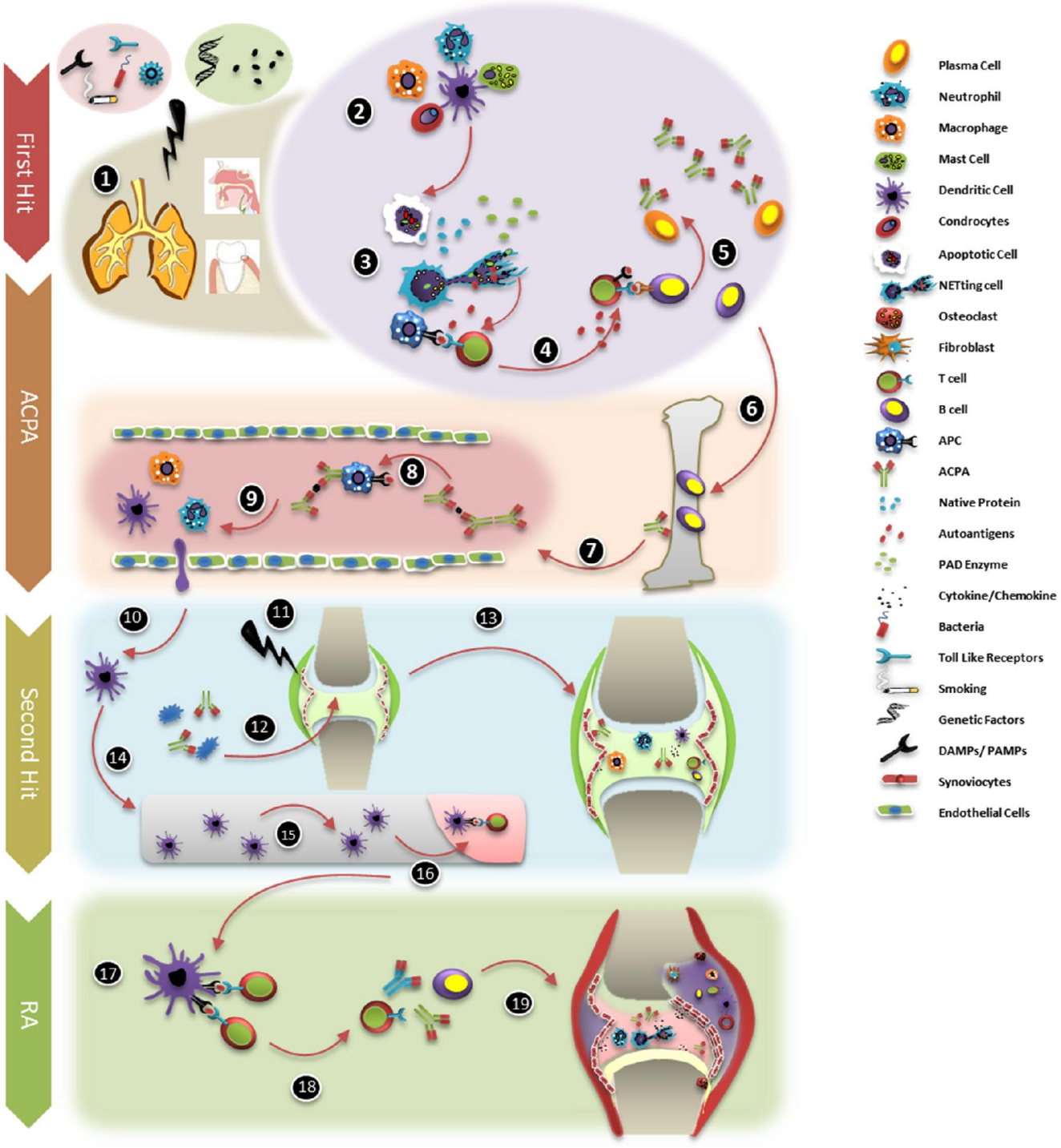


Figure 30. Hypothetical model of the immune response in the development of RA. Multiple preclinical immune and inflammatory events eventually exceed a threshold after which clinical disease is initiated. 1) Repeated episodes of stimulation of the innate immune system, leads to 2) activation of myeloid cells, and possibly chondrocytes. 3) Local inflammation in mucosal-lined organs leads NETosis by activated neutrophils causing release of citrullinated autoantigens and PAD enzymes.4) Autoantigens are presented by antigen presenting cells to T-cells via MHC II. 5) Interaction of T-cell with B cell leads to differentiation

into plasma cells leading to ACPA production. 6) Memory B cells migrates to bone marrow. 7) ACPA antibodies released into the circulation 8) ACPAs bind to exogenous or modified endogenous antigens to form circulating immune complexes that 9) interact with myeloid cells in the synovial microvasculature and tissue. 10) Increased vascular permeability results, with diffusion of autoantibodies into the joint. 11) Second unknown hit leads to citrullination of proteins in the cartilage 12) ACPAs bind to specific citrullinated epitopes in the cartilage 13) leading to more influx of inflammatory cells and damage to cartilage components. ACPAs might also bind to citrullinated epitopes in the synovium. In both sites, the classical and alternative pathways of complement are activated. 14) Synovium inflammation is induced with infiltration of macrophages leading to further citrullination, enzymatic and oxidative damage to structural proteins and creation of neoepitopes. 15) DCs loaded with joint-specific antigens are present in the synovium and 16) process the altered self-peptides, and then 17) migrate to regional lymph nodes where 18) T-cell activation initially occurs. 19) Epitope spreading follows over time with the development of a true autoimmunity. Modified from [82].

2.6 Future Directions

2.6.1 NETosis could be a link between RA and associated cardiovascular disease.

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease with leading cause of mortality being coronary artery disease (CAD), accounting for nearly 40–50% of deaths [143]. In a recent cohort study from Taiwan consisting 29238 RA patients and 116952 controls, it was found that the risk of developing deep vein thrombosis (DVT) and pulmonary thromboembolism (PE) was 3.36-fold and 2.07-fold, respectively, in patients with RA compared with patients without RA [144]. RA is associated with increased plasma levels of fibrinogen, von Willebrand factor, and plasminogen activator inhibitor 1, producing a prothrombotic state [145]. Recent evidence from Fuchs et al., implicates the release of neutrophil extracellular traps (NETs) can stimulate thrombus formation and coagulation and are abundant in thrombi in animal models of DVT by representing a third thrombus scaffold of extracellular DNA, in addition to fibrin and von, Willebrand factor [146]. Our results and recent investigations reported that neutrophils in RA are primed to make NETs [24,130]. Low-dose aspirin has been shown to be beneficial for the prevention of myocardial infarction, and ischemic stroke, and increases hemorrhagic stroke and major bleeding associated with cardiovascular disease[147] and thus reduce the risk of mortality [148]. However, recent observation reported that treatment of neutrophils with aspirin inhibited NET formation by inhibiting NF-kB signaling in both in vitro and vivo condition [149]. Additionally, in the

experimental TRALI model, treatment with aspirin decreased NET formation and lung injury [150]. Thus monitoring cardiovascular risk and damage in a NET deficient PAD4$^{-/-}$ mouse model which is injected with serum or IgG from arthritic miceK/BxN [151] will provide useful information about the contribution of NETotic DNA towards cardiovascular disease in rheumatoid arthritis **(Figure-31).** Future studies should focus on investigating whether the extracellular DNA of NETotic origin could contribute towards enhanced thrombotic events in RA and the underlying mechanism.

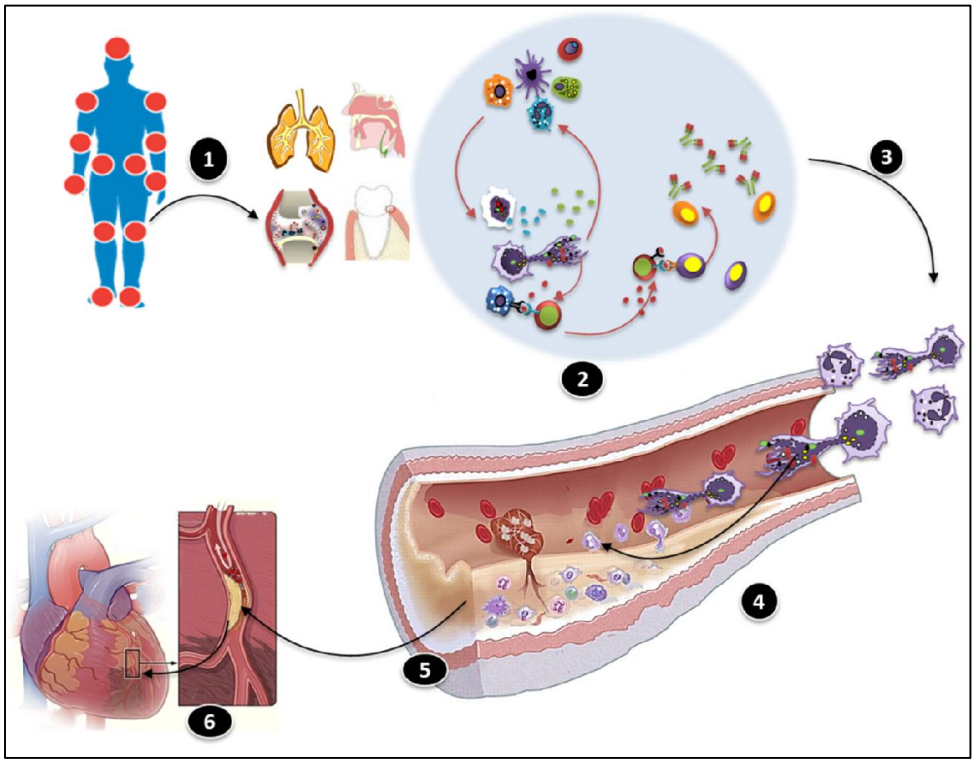


Figure 31- Model of NETs mediated cardiovascular damage in RA

2.6.2 Can hypoxia regulate NETosis in RA?

One of the most important characteristic fetcher of RA is synovial tissue hypoxia of the inflamed joints which was first revealed in 1970. This was further confirmed by other studies using nuclear magnetic resonance spectroscopy, pimonidazole, as well as video arthroscopy. [152] Hypoxia-inducible factor 1a (HIF-1a), the key transcriptional factor in the hypoxic response, shows unregulated expression in RA. [153] Hypoxia and HIF-1α could regulate many

pathways in RA, such as inflammation, angiogenesis, migration and cell survival. Recent findings from Fanlei Hu, et al. suggest that hypoxia and HIF-1α may function in conjunction with TLR-stimulated innate immune responses to, drive inflammation in RA [154]. Deletion of HIF-1α in conditional murine model resulted in impaired myeloid leukocyte aggregation, motility, extravasation, and microbial killing. HIF-1α thus been termed a "master regulator" of innate host defense responses. [91] Nevertheless, the activities of HIF-1α in human neutrophils remain largely uncharacterized. Pharmacologic and genetic inhibition of mTOR and HIF-1α signaling inhibit NET-mediated extracellular bacterial killing. [16] Recent findings from Fanlei Hu, et al., suggest that HIF-1α may function in conjunction with TLR-stimulated innate immune responses to, drive inflammation in RA [154]. In another study, it was shown that pharmacological inhibition of HIF-1α signaling could inhibit NET-mediated extracellular bacterial killing. They further describe that HIF-1α mediated bacterial killing is dependent on mTOR signaling cascade [155]. Based on preliminary data from our lab which indicates that exposure of native PMNs to hypoxic conditions in-vitro, leading to the higher degree of NETosis, it can be postulated that upregulation of HIF signaling could regulate signaling during NETosis via PI3K/Akt/mTOR signaling pathway. Further inhibition of PI3K/Akt/mTOR signaling pathway in vivo arthritis model and in an isolated cell from clinical samples could reveal novel drug targets to rescue damage in RA (**Figure 32)**.

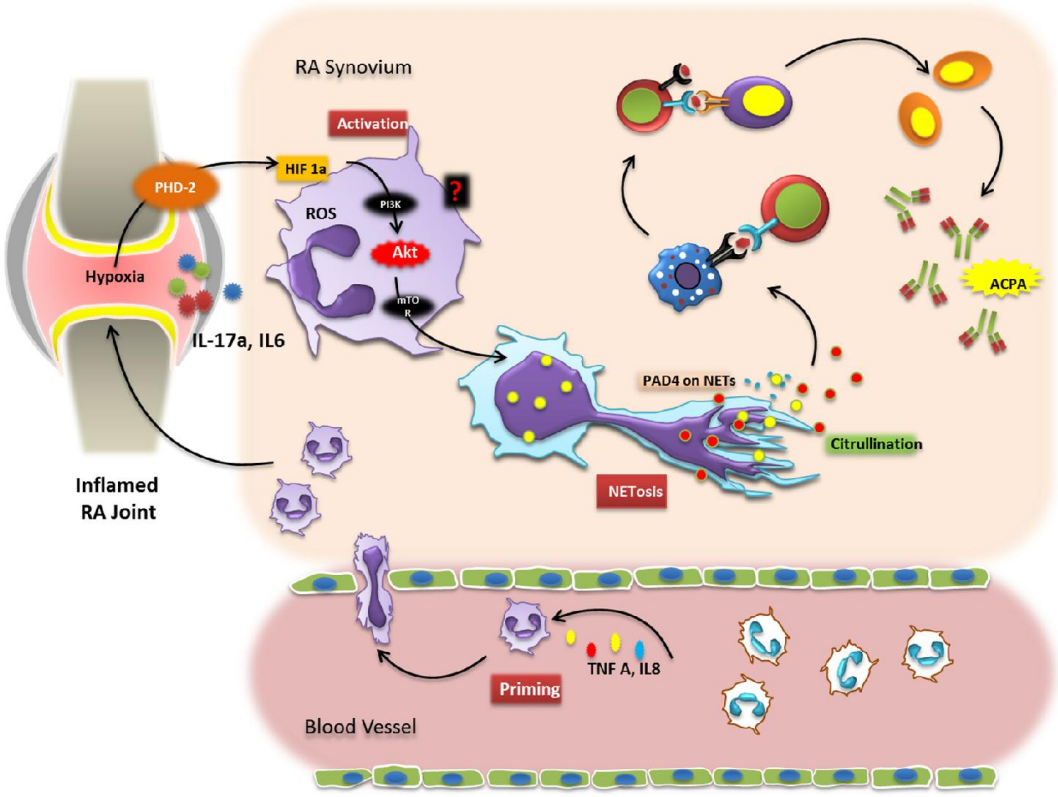


Figure 32- Model of regulation of NETois by hypoxia of Joint.

2.6.3 Switching neutrophils from NETosis to Apoptosis

NETosis tends to be pro-inflammatory process, leading to the release of novel self-antigens, such as deiminated proteins, which stimulate auto-inflammatory response. By contrast, apoptosis is anti-inflammatory, in that it leads to the promotion of M2 macrophage development associated with wound healing and tissue repair processes [6]. On the basis of the evidence currently available, suggesting NETosis as a probable regulator of RA pathogenesis, it seems fair to meditate a possible mechanism, which could switch the cells from NETosis to apoptosis. In a recently published data from Douda *et. al,.* propose that the inhibition of Akt, a regulator of mTOR, switches PMA treated neutrophil death, from NETosis to apoptosis and thus acts as a "bona fide molecular switch" that regulates the NETosis-apoptosis axis [156]. This finding is further supported by another study showing, mammalian target of rapamycin (mTOR) signaling is crucial for joint destruction in experimental arthritis and is activated in osteoclasts from patients with rheumatoid arthritis [155]. Interestingly generation of intracellular reactive oxygen species (ROS), which is a key event behind the

mechanism of NETosis, was found in synovial fluid neutrophils of RA patients, but not with other arthritis [157]. ROS, which is overexpressed in RA neutrophils, may inactivate intracellular caspases to inhibit apoptosis and induce autophagy, which would promote the breakdown of cellular membranes during NETosis [28]. However, the identities of other key kinases that regulate NETosis apoptosis pathways remain elusive. Inhibitors of Akt inhibit DNA release by activated neutrophils in a dose-dependent manner and, therefore, activation of Akt is essential for NADPH oxidase mediated NETosis (**Figure-33).** Additionally it demonstrates that the cartilage-degrading activity of blood- and synovial fluid-derived neutrophils is regulated, at least partially, by PI3-K [158]. Further, it was proposed that ROS could inactivate intracellular caspases to inhibit apoptosis and induce autophagy, which would promote the breakdown of cellular membranes during NETosis [28]. Based on current evidences suggesting NETosis as a probable regulator of RA pathogenesis [21, 22], it seems fair to hypothesize that inhibition of Akt could switch primed neutrophils in RA from NETosis towards apoptotic clearance (Figure 2).

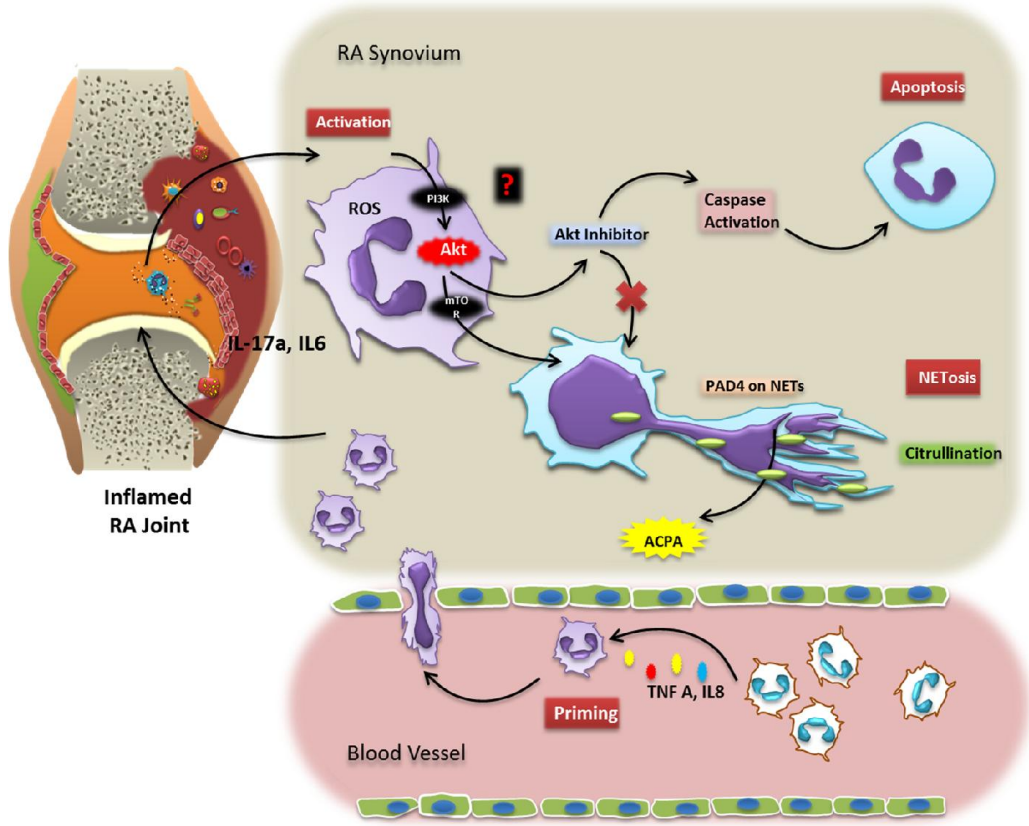


Figure 33. Model of HIF regulation of NETosis signaling and Inhibition of NETosis by inhibitor of AKT signaling pathway.

2.6.4 Role of Neutrophil Hybrids in RA

The initial perception of the neutrophil playing a passive role and merely responding to external signals has now been replaced by appreciation that activated neutrophil can respond to factors within their local environment to change their molecular properties and hence acquire the capacity to perform new cellular functions [9]. An interesting observation by Cross, A., et al, has shown that, peripheral blood neutrophils from patients with RA but not healthy controls express class II MHC mRNA, but the SF neutrophils in RA can synthesize and express large amounts of class II MHC [159]. This finding was further supported by a recent observation suggesting neutrophils that extravagate at sites of inflammation or infection, differentiate into a unique hybrid population that expresses surface markers of both neutrophils (Ly6G, 7/4, CXCR2, and CD62L) and DCs (CD11c,MHC II, and costimulatory

molecules) [160], underlining a novel interaction of neutrophils with T cell in RA disease pathology. In the K/B x N mouse model of rheumatoid arthritis, anti-Ly6G mAb administration (which depletes both neutrophils and hybrids) not only completely prevented the disease onset but also reversed disease progression [161]. At this point, it is tempting to speculate that neutrophil-DC hybrids may contribute to the pathophysiology of autoimmune inflammatory diseases like RA although their pathophysiology of inflammatory diseases remain totally unknown.

2.6.5 Pregnancy and RA

Pregnancy results in altered immune state, which contributes to a change in the course of autoimmune illness, include RA. Hormonal factors linked to age, gender, and reproductive status are undoubtedly involved in regulating the onset of numerous autoimmune diseases. It does not appear to adversely affect the fetal outcome. Pregnancy loss rate in RA has been recorded (17%), which is similar to a control population of (16%) [162]. Pregnancy often is associated with remission of the disease in the last trimester. More than three quarters of pregnant patients with RA improve in the first or second trimester, but 90% of these experience a flare of disease associated with an increase in RF titers in the weeks or months after delivery. The increased risk of ACPA-negative RA in parous women of reproductive age seemed to be associated with an increased postpartum risk and young age at first birth[163]. Infants of women with JIA did not have an increased risk of adverse neonatal outcomes HOWEVER compared with other women, those with JIA had significantly higher rates of pre-eclampsia, postpartum hemorrhage, premature birth and severe maternal morbidity [164].

2.7 Conclusion

It is evident by now that the study of citrullination, ACPAs, and citrullinating enzymes becomes more and more important to understand the pathophysiology of RA. It is likely that other modifications of cellular proteins may be intrinsic factors of other autoimmune diseases. Future studies have to prove this idea that both predetermined and random events contribute to the initiation of the disease. However, much remains to be resolved. It is widely accepted that RA must be treated early with effective therapy in order to prevent unfavourable outcome.

At the present no univocal, effective and safe pharmacological treatment is available even if growing advancement has been accomplished in recent years by using biological drugs. There is a growing interest of better understanding of the factors and cascade of events that lead to loss of tolerance, and causes localization of inflammation in the joint. It is still uncertain whether arthritis commences as a principal problem in the bone and subsequently moves to the joint, or the other way around. Extensive research over decades has made progress in understanding the complexities of RA. Questions about the origin, pathophysiology and disease specificity still require further extensive investigations. Results from research are having an impact today, enabling people with rheumatoid arthritis to remain active in life, family, and work far longer than was possible 20 years ago. There is also hope for tomorrow, as researchers continue to explore ways of stopping the disease process early, before it becomes destructive, or even preventing rheumatoid arthritis altogether. Early treatment in RA is important as it can prevent irreversible damage of the joints. The mechanisms underlying the activation of PADs, which generate citrullinated autoantigens in RA, remain unclear. Being able to predict who will develop RA would allow researchers to look at ways to prevent it.

During the last decade, the involvement of citrullinated proteins and antibodies reactive with these proteins in a citrulline-dependent manner in the pathophysiology of RA has been well established. Only a minority of those individuals who develop anticitrulline immunity will develop arthritis, and that a large proportion also of those ACPA positive individuals never develops RA. Thus, we need to understand what determines the emergence of the autoimmunity to citrullinated and other autoantigens; which factors determine who of

the autoantibody-positive individuals will develop arthritis; and who may eventually actively be protected against arthritis.

The initial trigger for the immune system to respond to citrullinated epitopes is still enigmatic, although increasing evidence suggests that environmental factors, such as smoking, are involved. Today, the use of biologicals has revolutionized the treatment of RA patients. In recent years, use of biologicals has brought a revolution in the treatment of patients with RA. However subclinical inflammation along with flares is evident despite clinical remission. Therefore, the need for development of targeted drugs that could abolish or further, reduce the remaining inflammation is highly indispensable. Drug targets, which could abrogate ACPA signalling cascade or could specifically neutralize such antibodies could indeed be a way of terminating the subclinical inflammation.

2.8 Tables 8. Demographics and patient population characteristics versus healthy blood donors.

	Controls	RA	Statistics
Age	50.34 ± 1.5	53.03 ± 1.5	P = 0.214
Gender (F / M)	24 / 32	24 / 8	-
DAS28	n.a.	3.07 ± 1.12	-
Bone erosion (pos / neg)	n.a.	22 / 10	-
Serum ACPA (pos / neg)	n.a.	20 / 12	-
Serum RF (pos / neg)	n.a.	19 / 13	-
Serum ANA (pos / neg)	n.a.	21 / 11	-
ESR*	n.a.	16.8 ± 13.1	-
CRP**	n.d.	6.9 ± 5.2	-
PBMC***	1961 ± 81.69	1513 ± 75.90	P < 0.0001
PMN***	3641 ± 149.7	4575 ± 546.0	P = 0.021
Therapy (yes / no)	n.a.	31 / 1	-
DMARDs (yes / no)	n.u.	27 / 5	-
Biologics (yes / no)	n.a.	30 / 2	-

F: female; M: male; DAS28: disease activity score; n.a.: not applicable; pos: positive; neg: negative; *: mm/h;**: mg/l;***: cells/µl; ACPA: anti-citrullinated protein antibodies; RF: rheumatoid factor; ANA: antinuclear antibodies; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; n.d.: not determined; PBMC: peripheral blood mononuclear cells; PMN: polymorphonuclear leukocytes; DMARDs: disease-modifying anti-rheumatic drugs; n.u.: not utilized.

CHAPTER 3: Can neutrophil extracellular trap products in serum assist with the detection of RA?

3.1 Summary

A number of studies have suggested that elevations in cell-free DNA in serum or plasma can serve as surrogate markers for aberrant neutrophil extracellular traps (NETs) generation. These are based on observations made in variety of conditions including preeclampsia, sepsis and cancer. Deregulated NETosis been recently been implicated in rheumatoid arthritis. As we had previously observed that rheumatoid arthritis is associated with elevated concentrations of serum cell-free DNA, we did a more extensive analysis of neutrophil derived or NETosis associated products in RA serum. For this purpose we examined serum samples from 32 cases with RA and 34 matching control samples. Our data indicate that serum cell-free DNA concentrations are indeed significantly elevated in RA samples when compared to controls. This was paralleled by similar elevations in cell-free nucleosomes, and mirrored by equivalent increases in neutrophil elastase and myeloperoxidase. To confirm the NETotic origin of the cell-free DNA in RA serum, we assessed MPO/cell-free DNA complexes. As these were similarly elevated in RA serum, these data suggest that RA neutrophil exhibit an increased propensity to undergo NETosis or degranulation during the serum clotting process. Thereby, these data provide independent evidence of aberrant NETosis in RA. These findings may have clinical implications, as suggested by a ROC (receiver operator curve) analysis of the individual factors examined, particularly that of cell-free nucleosomes.

3.2 Introduction

"Arthritis", also known as inflammation of one or more joints, may be a symptom of a more serious condition such as rheumatoid arthritis, lupus, infection or malignancy [84]. In such a scenario, an accurate diagnosis becomes indispensable to lead the way for proper treatment. A number of serological test are globally used for the diagnosis of RA, apart from disease characteristics and specific symptoms (Figure 1). Additionally, medical history, genetic background and imaging studies are also considered as vital information[165]. Increased risk of organ damage is often associated with certain medication. For example, kidney or lung failure is often associated with non- steroidal anti-inflammatory drug such as Ibuprofen. Commonly use drug Methotrexate, can be associated with liver damage and low white blood count. Thus, monitoring blood parameters for abnormalities could help to avoid long-term health problems and at the same time, help doctors to track response to certain therapy.

3.2.1 Serological diagnosis of RA

A number of blood test are available to help, diagnose arthritis, monitor treatments, and track disease activity of patient having signs or symptoms of arthritis of over two weeks (**Figure-34)** [166]. Rheumatoid factor (RF), which is known as the immunologic hallmark of RA, was later found to be non-specific for RA, as could be elevated during chronic hepatitis, primary biliary cirrhosis, any chronic viral infection, bacterial endocarditis, leukemia, dermatomyositis, infectious mononucleosis, systemic sclerosis, and systemic lupus erythematosus (SLE) [99]. Moreover, 5% of healthy individuals and in 10–20% of those over the age of 65 years are also positive for RF. However, up to 30% of RA patients remain negative for RF throughout the course of their disease [167]. The discovery of citrullinated proteins as autoantigens and the development of new assays detecting antibody against citrullinated proteins, has given a major breakthrough in the laboratory diagnostics of RA **(Table 9)** [168]. Anti-cyclic citrullinated protein antibodies (ACPA) test is currently the best-known biomarker of RA [169]. ACPA can be detected up to 10 years before RA patients first present to a clinician, predicting the future development of RA [84]. In both early and fully established disease anti CCP test is more specific and sensitive than, IgM rheumatoid factors [167].

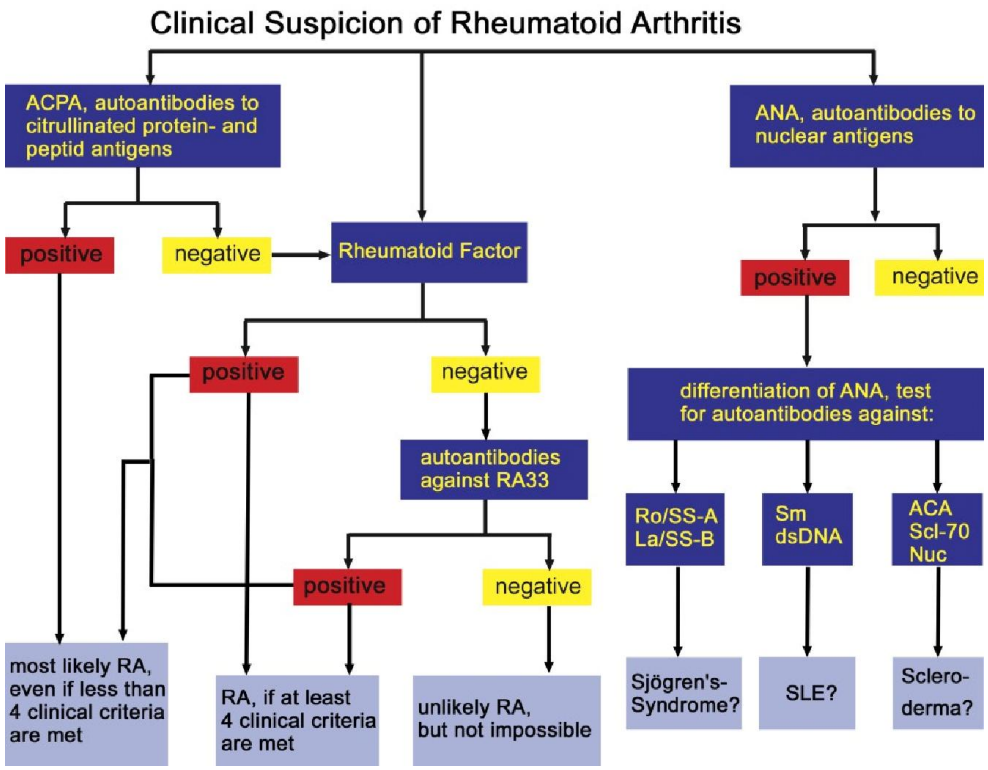


Figure 34. Schematic representation of the sequence of different blood tests for differential diagnosis of RA. Adapted from [165].

By comparing different ACPA assays it was shown that, compared to protein- based assays, peptide based assay can further improve both sensitivity and specificity [170]. However, other investigation has contradicted this observation [39]. Following the success of the CCP test, a number of optional methods for detecting ACPA have been developed such as, mutated citrullinated vimentin (MCV) and viral citrullinated peptide (VCP; VCP1 and VCP2) [171]. Simultaneously, several other autoantigens have been suggested as a target of autoantibodies in RA such as fibrinogen, immunoglobulin binding protein (BiP), fibronectin, alpha-enolase, type II collagen, Ra33. However due to limited data and contradictory results the data remains inconclusive with respect to the sensitivity and specificity of these assays and hence, none of these markers are currently widely used in routine diagnosis of RA [172]. In addition to ant-CCP, Autoantibodies recognizing carbamylated proteins has been reported in sera of patients with rheumatoid arthritis which can further predict joint damage [173].

Table 9- Specificity and sensitivity of serological test for the diagnosis of RA . Modified from [169] .

Test	Target	Specificity %	Sensitivity%
IgM-RF	Fc portion of IgG	96.7	45
APF (Anti-perinuclear facto)	Keratohyalin granules in the cytoplasm, profilaggrin	90	50-70
AKA (Antikeratin antibodies)	Filaggrin in keratin	94	45
AFA (Anti-citrullinated filaggrin antibody)	Citrullinated fibrin	>90	60
ACF (Anti-citrullinated fibronectin)	Citrullinated Fibrinogen	>90	55
Anti CCP (anti-cyclic citrullinated Peptide antibodies)	Synthetic peptides	>98	~80
Anti-MCV (Recombinant mutated citrullinated vimentin)	Vimentin, Recombinant MCV	99	40

3.2.2 Serum as diagnostic sample for RA

Serological diagnostic testing is an essential tool for the growing importance in the early detection and differentiation of rheumatoid arthritis. Cell free DNA from serum and plasma has been reported to be a valuable biomarker in a number of diseases including cancer [174]. However the amount of cell-free DNA in the serum samples is reported to be higher than in plasma [175]. Based on the observations suggesting that serum DNA concentrations correlate with leukocyte counts, it was hypothesized that rupture of leukocytes during serum separation might release DNA into the serum that accounts for 4-6 times more abundance of DNA in the serum compared to plasma. By quantifying DNA from serum and plasma without purification, using PCR based detection of ALU repeats, Umetani et al., found that the contribution of extraneous DNA, was 8.2% of total DNA, which is minor for explaining the 4-6 times difference between serum and plasma DNA levels [176]. Unequal distribution of DNA during separation of serum or plasma from whole blood could be a possible explanation for such difference.

3.2.3 Blood coagulation in serum tubes

BD Vacutainer® Plus Plastic Serum and SST™ Tubes are coated with silicone and micronized silica particles which promote and accelerate the clot formation, silicone coating reduces adherence of red cells to tube walls[177]. Work form Margolis. J has shown that, colloidal silica can accelerate blood coagulation by adsorption and partial denaturation of specific plasma protein, the Hageman factor [178].

3.2.4 Neutrophil as source of Cf-DNA

Cell-free DNA (cfDNA) in body tissues or fluids is extensively investigated in clinical medicine and other research fields. However, due to lack of understanding, the initial discovery of cell-free nucleic acids in patient plasma made in 1948 remained largely overlooked, until the 1990s when the presence of tumor-derived oncogenic DNA was observed in the plasma of patients with cancer and DNA of fetal origin was detected in the maternal circulation [179]. Subsequently it was discovered that cfDNA levels were significantly increased in patients with trauma, stroke, autoimmune disorders and sepsis. The entire mechanism of cfDNA increases has not been elucidated. However, the origin of these cf-DNA was formerly thought from necrotic and/or apoptotic cells [180,181], although the degree of the input of the other cell death type over the other has been a subject of controversy[181]. Recent investigations explain more spontaneously occurring accumulations of cfDNA might be an active cfDNA release of extracellular or intracellular DNA [182], leukocyte oxidative burst or extracellular trap formation [52]. Neutrophils are known to form extracellular traps under inflammation [183] along with significant change in turnover. Impairment in clearing this DNA may play a subsequent role in the appearance of increased amounts of circulating DNA in the blood of individuals with different ailments [184,185]. Previously it was reported that predominant portion of cfDNA in hemodylisis patients originates from apoptotic leukocytes. Although, neutrophils die by apoptosis under physiological conditions, after their activation, they are able to switch to different types of cell death like autophagy or NETosis[186]. Recent investigations have suggested that under certain conditions NETs could be a major factor for increase in cf-DNA [127,141,146,187]. More interestingly, in an recent report it was shown that Akt, also known as Protein Kinase B, can act as a bona fide molecular switch which could regulate the NETosis-apoptosis axis [156]. During inflammation neutrophils become activated and their longevity increases by several fold. It was therefore concluded that the concentration of cf-DNA could serve as a non-invasive blood biomarker to reflect the rate of tissue damage, cellular death and turnover [188].

3.2.5 Working Hypothesis

Although the underlying etiology is currently unknown, several lines of evidence suggest that PMN may play an important role in the development of RA. These include the observation that PMN form a large proportion part the primary leukocyte infiltrate in RA joints and that their depletion in animal model systems can reduce RA symptoms [189,190]. Recent studies have suggested that neutrophil NETs are implicated in rheumatoid arthritis (RA), in that they exhibit an increased NETotic response and interact with auto-antibodies frequently observed in RA, termed anti- citrullinated protein antibodies (ACPA) [24,130]. Subsequently, a number of studies ranging from sepsis to patients at risk for thrombosis have suggested that the analysis of cell-free DNA, or cell-free nucleosomes may be used as a surrogate marker for NETosis[144,146]. As we had previously shown that RA is associated with significantly elevated concentrations of cell-free DNA[191], we wished to determine whether this material was of NETotic origin.

3.1 Materials and Methods

3.3.1 Human Subjects

All patients fulfilled the American College of Rheumatology classification criteria for RA. Healthy volunteers, matched for gender and age, were recruited at the Blood Bank of the Swiss Red Cross, Basel. Inclusion criteria for healthy controls were fair general condition, age

≥ 28 and ≤ 70 years and for blood donors fulfilling national criteria for blood donation. Exclusion criteria were current or previous systemic autoimmune disease, asthma, reconvalescence after major illness, surgery, current medication with corticosteroids, immunosuppressive agents and malignant neoplasia or chemotherapy within 5 years before recruitment for the study. RA cases had a DAS ≥ 3.2, were from age ≥ 27 to ≤ 70 years and had no other systemic autoimmune disease, including ankylosing spondylitis and psoriatic arthritis. Exclusion criteria were corticosteroids ≥ 40 mg equivalent of prednisone daily and those mentioned above for healthy controls. Informed, written consent was obtained from all subjects in the study, which was approved by the Cantonal Ethical Review Boards of Aargau-Solothurn and Basel/Basel-Land, Switzerland. Since this study was conducted in a blinded manner, it was not possible to correct for any potential gender imbalance, evident by the large number of female cases with RA (N=24). Undue skewing is countered by the inclusion of an equivalent number of female control samples.

3.3.2 Preparation of plasma and serum

Plasma and serum was collected and processed as described previously. Care was taken to process the plasma samples rapidly after phlebotomy to avoid any artefacts. Serum samples were allowed to coagulate, without the addition of an accelerator, at room temperature for at least 1 hour. Samples were studied immediately or stored in aliquots at -70°C until analysis.

3.3.3 Cell free DNA isolation and quantification

Cell free DNA extracted from 850 µl plasma or serum using the QIAamp Circulating Nucleic Acid Kit (Qiagen) was quantified by TaqMan® real-time PCR (StepOne™ Plus Real-Time PCR System, Applied Biosystems) specific for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene[191].

3.3.4 NETotic complex detection analysis

The concentration of neutrophil elastase (NE) and myeloperoxidase (MPO) were measured by sandwich ELISA (Elastase/a1-PI Complex ELISA Kit, Calbiochem) and the human MPO ELISA Kit, Hycult Biotech. Nucleosomes were measured using the Human Cell Death Detection ELISA^{PLUS} (Roche Diagnostics).MPO is present on extruded NETs. To detect such structures, NETs

associated MPO/DNA complexes were quantified utilizing a modified capture ELISA. In brief, NETs associated MPO in serum or culture supernatant was captured using the coated 96 well plate of the human MPO ELISA Kit, (Hycult Biotech), following which the NETs associated DNA backbone was detected using the detection antibody of the Human Cell Death Detection ELISA^{PLUS} (Roche Diagnostics).

3.3.5 Statistical analysis

All data are presented as mean ± SE. Descriptive statistics for continuous parameters consisted of median and range, and categorical variables were expressed as percentages. Comparisons between patients and healthy controls were by the Mann-Whitney U test with a Welch post-test correction. Statistical significance in multiple comparisons was by one-way analysis of variance (ANOVA) with a Dunn's post-test correction. $P < 0.05$ was considered statistically significant. Data were processed in GraphPad Prism version 5.0b for MacOSX (GraphPad Software Inc., www.graphpad.com). Additional professional statistical assistance was provided by A. Schoetzau (www.eudox.ch).

3.4 Results

3.4.1 Peripheral blood samples of RA has more PMN and less PBMC than healthy controls

Details of the RA study group and control group are described in Table 11 and Figure 35. These data confirm that RA is associated with an increase in the number of circulating PMN and a decrease in PBMC (peripheral blood mononuclear cell) counts. They also confirm the gender disparity regarding RA incidence, in that in our study more women were affected by RA, than men. To counter this aspect, we examined samples from an equivalent number of age-matched healthy female blood donors.

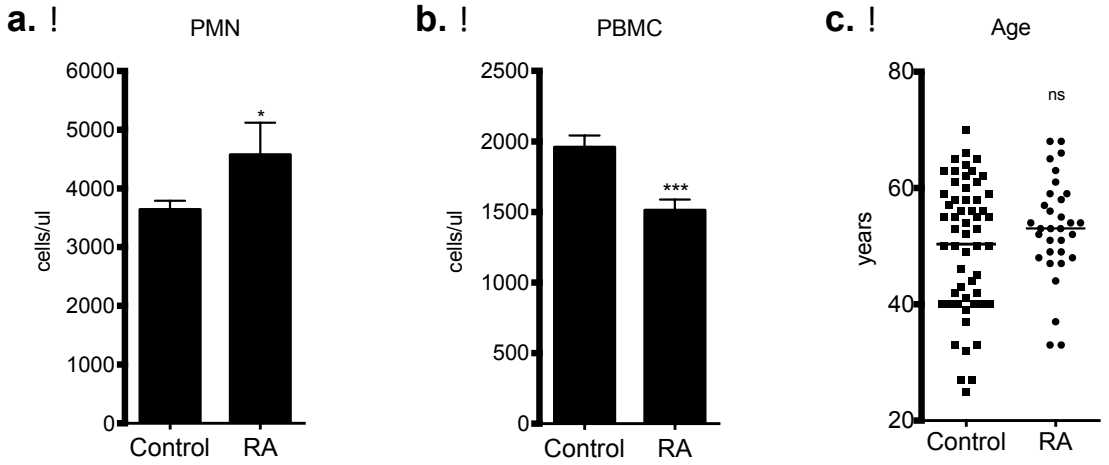


Figure 35. Neutrophil, peripheral blood leucocyte counts and age distribution in RA cases and control cohorts. (A) Neutrophil levels in cases with RA (n = 32) and matched healthy blood donors (n = 56). (B) Peripheral blood mononuclear cell count in RA cases and healthy control donors. (C) Age distribution of RA cases and matched healthy blood donors. *P < 0.05, ***P < 0.001, n.s.: statistically not significant, Mann-Whitney U test; PMN: polymorphonuclear leukocytes; PBMC: peripheral blood mononuclear cells.

3.4.2 Histone associated DNA fragments significantly elevated in RA serum samples

By using real-time PCR we confirmed that cell-free DNA levels were indeed significantly elevated in RA serum samples when compared to controls (Figure 36A). No elevation was observed in rapidly processed plasma samples obtained from RA patients. As the analysis of cell-free DNA by real-time PCR is costly and requires large sample volumes, we examined the

use of a commercial EIA kit to detect cell-free nucleosomes. This analysis confirmed that histone associated DNA fragments were highly elevated in RA serum samples (Figure 36B).

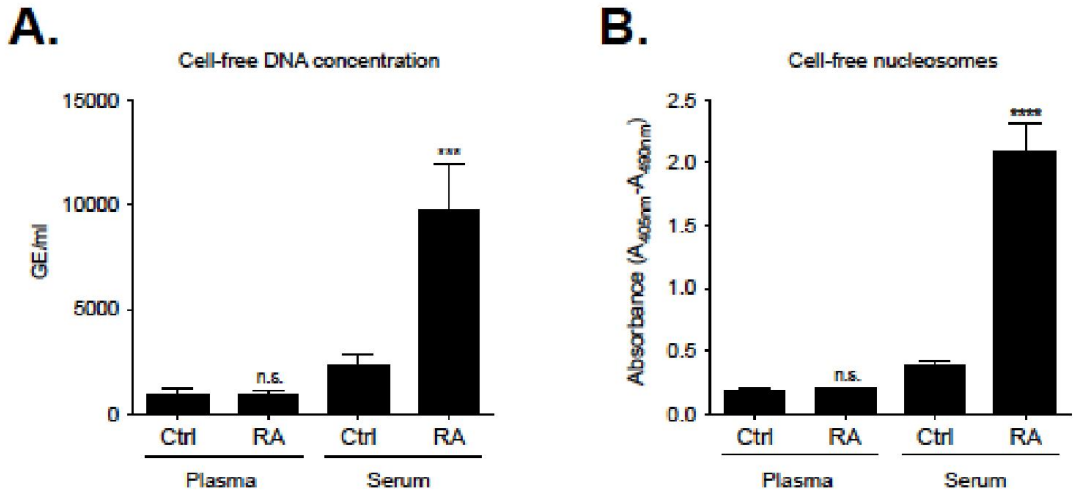


Figure 36. **Elevated serum levels of NETs components, in RA patients have potential clinical utility.** (**A**) Cell-free DNA levels in plasma and serum from healthy matched blood donors (n=41) and patients with RA (n=32) determined by real-time PCR. (**B**) Cell-free nucleosome levels in plasma and serum from healthy donor controls and patients with RA, determined by ELISA. In contrast to the serum levels, none of the plasma levels of these NET components attained statistical significance (**Fig. 2A-2B**).

3.4.3 Enhanced PMN degranulation or NETosis in serum samples of RA

To determine the contribution by PMN to this material, we examined the levels of azurophilic granular proteins, myeloperoxidase (MPO) and neutrophil elastase (NE), as these have been implicated in the NETotic process, and are associated with externalised NETs structures. These analyses indicated a parallel elevation of these enzymes in RA serum (Figure 37A and B). A similar increase in MPO/cell-free DNA complexes in RA serum suggests that a large proportion of this material is derived via NETosis (Figure 37C). In all instances no significant elevations were noted in rapidly processed plasma samples. From these experiments it was however not clear whether these elevations were merely attributable to the increased number of PMN in RA cases or whether they were reflective of a propensity of these cells to undergo increased NETosis during clotting. In this manner, these data do provide additional independent support that RA PMN are more prone to undergo NETosis than those from healthy counterparts.

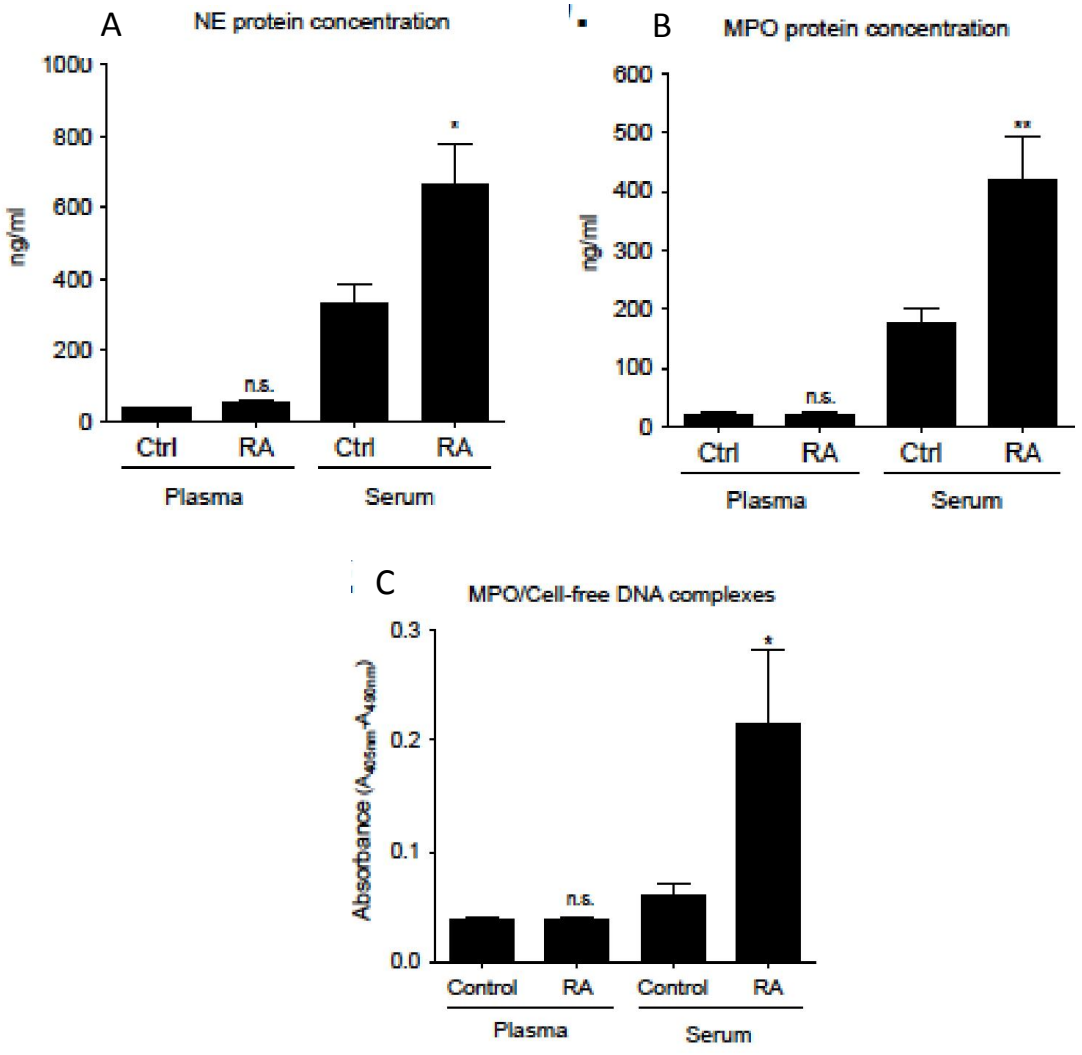


Figure 37. Enhanced PMN degranulation or NETosis in serum samples of RA (**A**) Determination of NE protein concentrations in plasma and serum from healthy donors and patients with RA as assessed by sandwich ELISA. (**B**) MPO concentrations in plasma and serum from healthy donors and patients with RA as determined by sandwich ELISA. (**C**) NETs-associated MPO/DNA complexes quantified utilizing a modified capture ELISA. In contrast to the serum levels, none of the plasma levels of these NET components attained statistical significance (**Fig. 2A-C**).

3.4.4 Influence of clotting accelerator on cell-free nucleosome quantification

A crucial finding made during these examinations is that the results were considerably skewed if the clotting of the serum sample was sped up by the addition of accelerators, as is evident from an analysis of comparable serum samples obtained with or without such agents (Figure 38A).

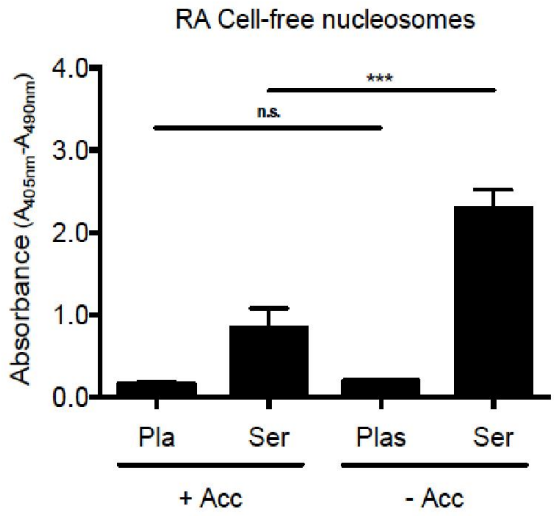


Figure 38. Influence of clotting accelerator on levels on cell-free nucleosomes in RA serum. The use of agents to speed up blood clotting in serum preparation was determined to significantly reduce the levels of cell-free nucleosomes in comparable RA serum samples.

3.4.5 Elevated serum levels of NETs components, in RA patients have potential clinical utility.

As the significance attained by cell-free nucleosomes in RA serum was very striking, we ascertained whether an analysis of these serum products could be diagnostically useful, for which purpose we performed receiver operating characteristics (ROC) analyses. These analyses indicated that the ROC analysis of serum cell-free nucleosome determinations yielded a surprisingly large area under the curve (AUC) value of 0.97 (Table 12 and Figure 39A). Of interest is that there was little difference in this value regardless of whether the RA cases were ACPA positive or not (Figure 39B), although there was a trend for serum nucleosome levels to be higher in ACPA positive cases than ACPA negative cases (Figure 39C). The AUC for serum nucleosomes was significantly higher than for any of the other parameters examined (Figures 39D to F). With the cut-off set at 0.78 the ROC AUC translates into a sensitivity of 91% with a specificity of 92% for differentiating between RA cases and healthy controls.

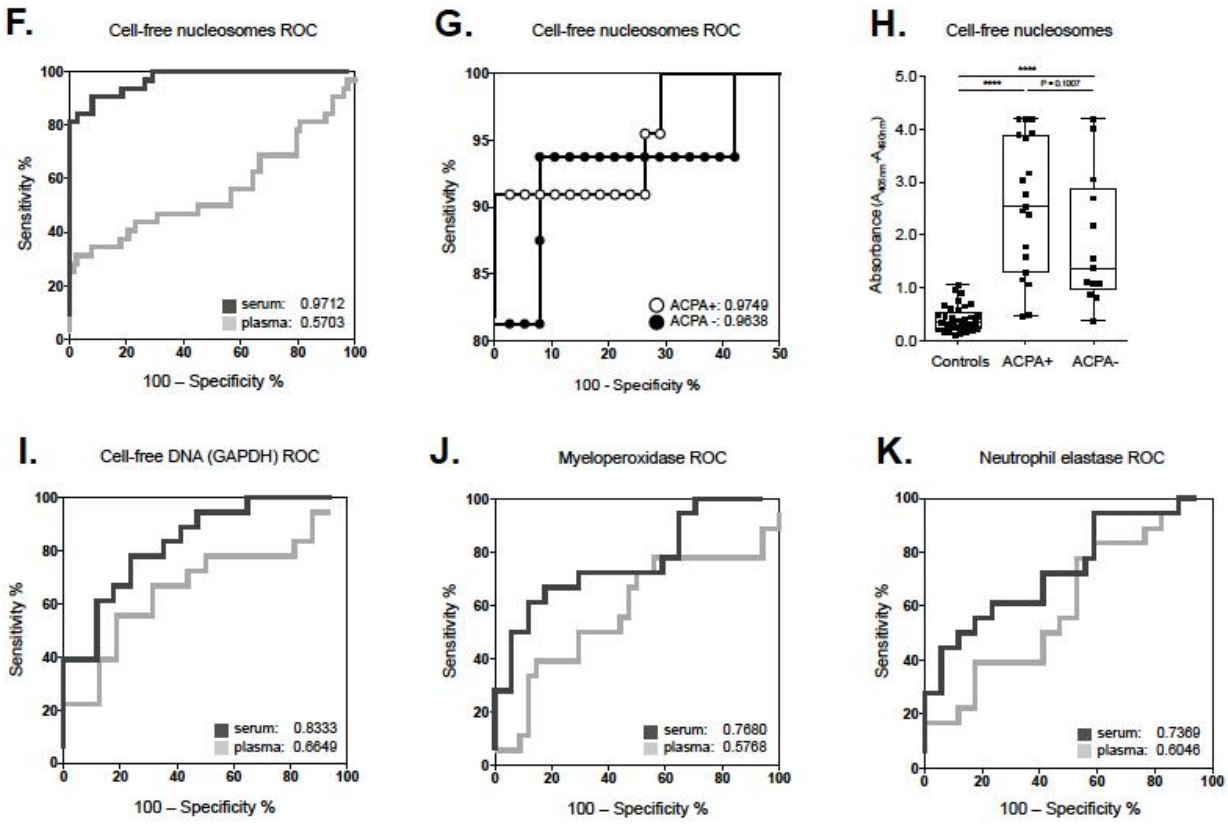


Figure 39. ROC analysis of cell-free nucleosomes in serum of patients with RA and healthy controls. (**A**) ROC analysis of cell-free nucleosomes in RA serum. (**B**) Detail of cell-free nucleosome ROC curve with groups of ACPA+ and ACPA- RA cases and (**C**) scatter box and whisker plots with individual values for control, ACPA+ and ACPA- groups. The ROC curve analysis of other NET components, cell-free DNA (**D**), NE (**E**) and MPO (**F**), was not as conclusive as that for cell-free nucleosomes. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n.s.: statistically not significant.

3.5 Discussion

A number of different studies have indicated that neutrophil NETs may contribute to human pathologies ranging from preeclampsia, SLE, sepsis to coagulopathies [26]. As many of these are associated with elevations in circulatory cell-free DNA, it has proposed that the quantitation of this material may serve as a surrogate marker for NETotic events [187,191]. Most recently, such an approach has been used to assess patients at risk for thrombosis, or to detect NETosis in stored blood cell products[192].

As we had previously observed significant elevations in the concentration of cell-free DNA in RA serum, we were intrigued by recent reports indicating that RA PMN were more prone to undergo NETosis. The presented analysis of cell-free DNA and cell-free nucleosomes confirm previous observations, while that of neutrophil granular enzymes (MPO and NE), as well as combinations thereof (MPO/cell-free DNA complexes) suggest that RA PMN are more prone to degranulate or generate NETs during serum clot formation. In this manner, these data would corroborate previous studies on enhanced NETosis in RA by alternate means.

It is of considerable interest that the more pronounced elevations detectable in RA were only discernible in serum, and not in rapidly processed plasma. Although it is well established that cell-free DNA levels are higher in serum samples than matching plasma samples, this difference was largely attributed to lysis of leucocytes during coagulation. The presented data, however, indicate that the coagulation process appears to trigger an enhanced response by RA PMN. The mechanistic basis for this feature is unclear and will need to be resolved. The data do, however, imply that serum cell-free DNA or nucleosome levels may be a better surrogate marker for NETosis than corresponding plasma evaluations.

A further striking feature is that our observations were made in a patient cohort deemed to be responsive to DMARD or biologics therapy, having a low DAS score of 3.2. Consequently, these data would seem to suggest that while pharmacological intervention has dampened the clinical symptoms, it has not effectively down-modulated the underlying systemic inflammation inherent in RA. Akin to other studies on sepsis or thrombosis, a possibly useful side feature of the presented data are that they may have a clinical bearing by assisting

with the detection of a patients with suspected RA, as is evident from the ROC analysis. Of interest is that no significant difference was noted between ACPA positive and negative patients. While this biomarker does have high prognostic value in detecting cases at risk of developing RA, their use is diminished in patients not expression the prerequisite HLA-DRB1 'shared epitope'. Consequently, cell-free nucleosome analysis may assist in detecting RA in such ACPA negative cases. The clinical validity of such an approach would, however, require extensive validation, especially to discern how useful these assays would be in distinguishing cases with RA from other auto-inflammatory conditions.

3.6 Conclusion

Our data suggest that PMN in RA exhibit an increased propensity for the release of granular proteins and NETs during the serum clotting process, which could have clinical implications. Of interest is that the levels of circulating nucleosomes are known to be elevated in plasma and serum in various non-malignant and malignant diseases [193]. However, they may be clinically valuable in the detection of disease during early phase. In addition, nucleosomes may be valuable surrogate markers for the evaluation of new drugs in preclinical studies. To be valuable in the clinical setting, nucleosome assays will have to be standardized, automated, and certified in order to enable rapid and reliable quantification in routine laboratories. The use of a universal set of RA patients and control sera will further allow a direct comparison of the diagnostic performance of current tests and those yet to be developed.

3.7 Tables

Table 10. Demographics and patient population characteristics versus healthy blood donors.

	Controls	RA	Statistics
Age	50.34 ± 1.5	53.03 ± 1.5	P = 0.214
Gender (F / M)	24 / 32	24 / 8	-
DAS28	n.a.	3.07 ± 1.12	-
Bone erosion (pos / neg)	n.a.	22 / 10	-
Serum ACPA (pos / neg)	n.a.	20 / 12	-
Serum RF (pos / neg)	n.a.	19 / 13	-
Serum ANA (pos / neg)	n.a.	21 / 11	-
ESR*	n.a.	16.8 ± 13.1	-
CRP**	n.d.	6.9 ± 5.2	-
PBMC***	1961 ± 81.69	1513 ± 75.90	P < 0.0001
PMN***	3641 ± 149.7	4575 ± 546.0	P = 0.021
Therapy (yes / no)	n.a.	31 / 1	-
DMARDs (yes / no)	n.u.	27 / 5	-
Biologics (yes / no)	n.a.	30 / 2	-

F: female; M: male; DAS28: disease activity score; n.a.: not applicable; pos: positive; neg: negative; *: mm/h; **: mg/l; ***: cells/µl; ACPA: anti-citrullinated protein antibodies; RF: rheumatoid factor; ANA: antinuclear antibodies; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; n.d.: not determined; PBMC: peripheral blood mononuclear cells; PMN: polymorphonuclear leukocytes; DMARDs: disease-modifying anti-rheumatic drugs; n.u.: not utilized.

Table 11. AUC values with corresponding 95% confidence intervals, P values and standard errors for serum cell free nucleosomes and the 3 different parameters, which were analyzed individually by logistic regression.

Parameter	Sample	AUC	95% CI	S.E.	P value
Cell-free nucleosomes	Serum	0.97	0.94 to 1.00	0.016	< 0.0001
	Plasma	0.57	0.43 to 0.71	0.072	0.31
Cell-free DNA (GAPDH)	Serum	0.83	0.70 to 0.97	0.067	0.000
	Plasma	0.67	0.48 to 0.85	0.095	0.10
Myeloperoxidase	Serum	0.77	0.61 to 0.93	0.081	0.007
	Plasma	0.58	0.38 to 0.77	0.099	0.44
Neutrophil elastase	Serum	0.74	0.57 to 0.90	0.084	0.017
	Plasma	0.61	0.42 to 0.79	0.097	0.29

AUC: Area under the curve; 95% CI: 95% confidence interval; S.E.: standard error; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

CHAPTER 4: Abnormalities in the feto-placenta junction in PE could be a result of thrombotic event, driven by extracellular DNA of NETotic origin

4.1 Summary

Preeclampsia (PE) is a major cause of maternal and neonatal morbidity and mortality. Anomalies in the spiral arteries have been associated with abnormal placental development, miscarriage and fetal injury in PE. Previously, we have reported the presence of NETs in the intervillous space of preeclamptic placenta. Using in vitro experiments, we have shown that trophoblast micro-debris could stimulate neutrophils to form neutrophil extracellular traps (NETs). However the effect of such phenomena remained elusive. Recent data from Wagner lab suggest, NETs could act as a lattice, to stimulate platelet adhesion, promotes coagulation and thrombotic events. Given that PE, IUGR and even fetal loss are broadly related to elevated thrombotic events causing dysfunctions at the interface between innate immunity and haemostasis, it is of cardinal importance to investigate whether NETs could elucidate such a response. In this study, we adopted mice sFLT-1 overexpression model, to elucidate the impact of NETosis in murine pregnancy outcome. Preliminary results indicates 50% drop in pregnancies after sFLT-1 overexpression although there was only a partial induction of PE phenotype characterized by minor elevation in blood pressure and proteinuria. Staining of placentas from mice that retained pregnancy after sFLT-1 overexpression indicates heavy neutrophil infiltration in the feto-placental junction, with zones positive for NET specific markers. Plasma isolated from sFLT-1 treated mouse also indicates elevation of NET specific markers compared to healthy pregnant controls. Minor increase in plasma TAT complexes (although not significant), along with a drop in platelet count, indicates elevated thrombotic events. Further ongoing experiments will compare the impact of sFLT-1 overexpression in normal WT mice to those that are unable to form NETs. If NETs are implicated in the etiology

of preeclampsia, PAD4 inhibitors may provide a novel therapeutic approach for the treatment of this disease.

4.2 Introduction

PE is a syndrome that is usually defined as the onset of hypertension and proteinuria after 20 weeks of gestation in previously normotensive non-proteinuric pregnant women. A worldwide incidence of 8,370,000 cases per year has been estimated [194]. PE complicates 6%–10% of all pregnancies in the United States, and the number goes even higher in underdeveloped countries. Recent evidence suggests that PE is a significant cause of perinatal morbidity and death a as it accounts for approximately 15.9% of all maternal deaths in the United States [195]. PE is a multisystem disorder of human pregnancy that may be explained by two generalized processes occurring within the maternal and fetal compartments, vasospasm and endothelial dysfunction (Figure)[196]. It is widely accepted that circulating factors, most likely placental in origin, are responsible for these systemic disturbances. The precise nature of these factors and the mechanism by which a placental disorder may induce these pathophysiologic changes remain unknown. It has been thought to result from reduced placental perfusion due to abnormal trophoblast differentiation [197]. Placental Infraction is frequently detected in PE and further characterised by rapid onset of hypertension and oedema in previously normotensive pregnant women. The underlying aetiology appears to involve a defect in trophoblast differentiation, resulting in failure to modify the maternal spiral arteries. This leads to a condition of placental hypoxia (oxidative stress), due to an inadequate supply of maternal blood to the fetal tissues. There is some data suggesting that the severity of clinical symptoms of PE correlates with the placental infraction. Furthermore, this may serve to distinguish PE from the associated HELLP (Haemolysis, Elevated Liver Enzymes, Low platelet Count) syndrome. When remains untreated, it moves towards more severe consequences such as eclampsia, defined by the presence of seizures, IUGR (Intra Uterine Growth Restrictions). It takes place only in the presence of placenta even without fetus (hydatidiform mole), and typically improves postpartum.

4.2.1 How PE complicates pregnancy?

With the classical presentation, women typically develop PE after 20 weeks gestation and prior to 48hr postpartum. Current clinical guidelines support the differentiation of PE into mild and severe categories; these entities are treated differently, particularly at preterm gestations. Typically, eclampsia occurs after the onset of hypertension and proteinuria. A severe headache or visual blurring often heralds its onset. The cardinal features of PE are new-onset hypertension (defined as systolic blood pressure⩾140 mm Hg or diastolic blood pressure⩾ 90 mm Hg) and proteinuria (300 mg or higher in a 24-h urine specimen) **(Figure-40)** [198]. The degree of proteinuria in PE may vary from minimal to nephrotic; however, the amount of proteinuria does not seem to affect maternal or fetal outcomes [199]. A percentage of women present atypically without one of these cardinal signs, making the diagnosis difficult to confirm or exclude. Up to 20% of women with atypical PE have minimal or no proteinuria [200].

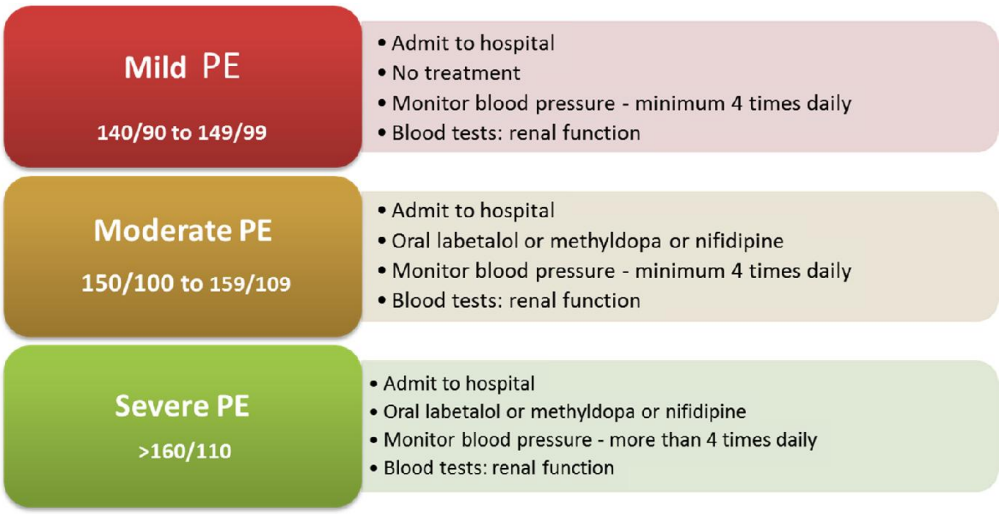


Figure 40- Classification and management of PE [201].

Early-Onset PE could get complicated with termination of pregnancy, intrauterine deaths and neonatal deaths [202] . However, in severe disease, women may develop severe headaches or visual changes, right upper quadrant pain from acute liver injury, pulmonary edema, oliguria from acute renal failure, hemolysis and/or thrombocytopenia, and/or grand mal

seizures or eclampsia. However, 20% of women who develop eclampsia do not have proteinuria [203]. Eclamptic seizures can occur in the immediate puerperium and, infrequently, 48 h to one month postpartum, in which case the condition is described as late postpartum eclampsia. Historically, edema was part of the diagnostic triad of PE (i.e., hypertension, proteinuria, and edema); however, edema is too nonspecific to be used for diagnostic purposes because a majority of pregnant women without PE develop edema toward the end of their pregnancies.

Along with maternal complications, the developing fetus can also be affected **(Figure-41)**. These include iatrogenic prematurity, fetal growth restriction oligohydramnios, and increased risk of perinatal death [204]. Pathogenesis of these fetal complications is not clear, yet impaired uteroplacental blood flow, placental abruption and infarction probably contributes.

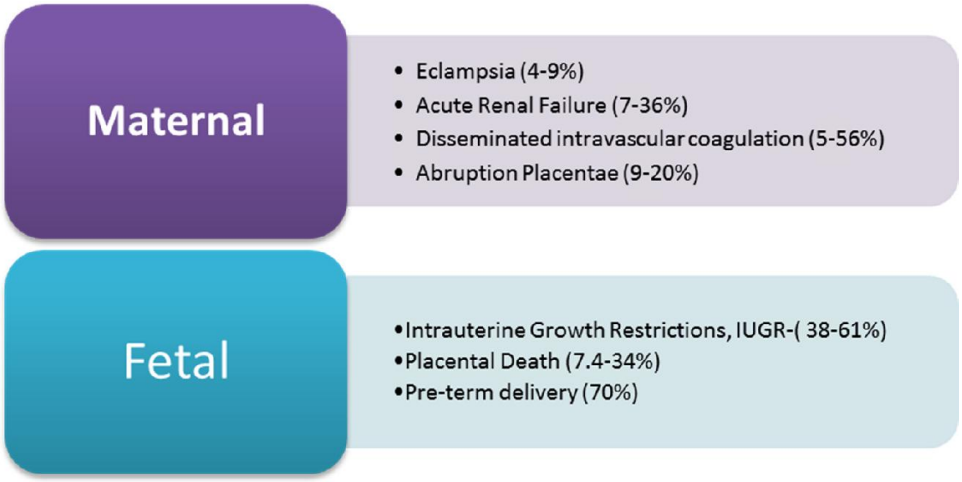


Figure 41- Maternal and fetal complications in PE. Data source [201]

4.2.2 What are the risk factors?

Risk factors for PE are quite diversified **(Table-12)**. Broadly, they can be classified into maternal constitutional factors, pregnancy related factors and maternal environmental factors [205]. Genetic factors, bacterial infections, thrombotic diseases, diabetes, rheumatic disease has been linked to the onset of this condition. For example, past history of PE could increase the risk to seven-fold. Genetic factors are at least partially responsible, because both a maternal and a paternal family history of the disease predispose to PE [206]. Multiple gestation and

triplet gestation carries a higher risk than twin, suggesting that increased placental mass plays some role. Interestingly, smoking during pregnancy was shown to reduce the risk of PE [207].

Table 12- Risk factors for PE. Data source [205].

Maternal constitutional factors	Pregnancy related factors
Chronic hypertension	Nulliparity
Extreme age	Paternity
Ethnicity	Previous history of abortion (spontaneous and/or induced)
Renal disease	Previous history of abortion (spontaneous and/or induced)
Obesity	Assisted reproduction
Urinary tract infection	Molar pregnancy and fetal hydrops
Glucose intolerance, insulin resistance, gestational diabetes mellitus	Twin pregnancy
Diabetes mellitus	
Systemic lupus erythomatosis and antiphospholipid syndrome	
Hyperthyroidism/hypothyroidism	Maternal environmental factors
Epilepsy	Working activity
Thrombophilia	Smoking
Migraine	
Polycystic ovary syndrome	
Family history of PE	
Family history of hypertension	

Rheumatic Disease	

4.2.3 Abnormal placental development in PE

PE is a condition characterized by systemic vascular endothelial dysfunction. A number of mechanisms have been shown to contribute towards the pathogenesis of PE. However, it is unclear whether the elucidated pathways have direct or indirect effects. Placenta is central to the pathogenesis of PE, as a number of researches have shown association between abnormal placental vascular developments, as the key event in the development of this disease [208]. The placenta plays a vital organ for the exchange of nutrients, oxygen and waste between the mother and developing fetus **(Figure-42A)**.

For proper development of placenta, a coordinated vascularization of the placenta is essential which involves the process of formation and growth of blood vessels [209]. Normally trophoblast cells transform from an epithelial phenotype to an endothelial phenotype as they invade the maternal decidua and myometrium in a process termed pseudovasculogenesis [210]. They express markers such as vascular endothelial-cadherin (VE cadherin), and alphavbeta3 integrin.

These migrating trophoblasts transform the maternal spiral arterioles that supply maternal blood to the placenta from small caliber resistance vessels to large caliber capacitance vessels allowing adequate maternal blood flow to the placenta **(Figure- 42B).** In PE this process is disordered and the fetal trophoblasts fail to properly invade the maternal myometrium and spiral arterioles causing placental ischemia [196].

This placental ischaemia stimulates a release of factors into the maternal vascular system resulting in systemic endothelial dysfunction which leads to hypertension, oedema and proteinuria [211]. Such a defective trophoblast invasion and inadequate maternal spiral artery remodelling are common to both intrauterine growth restriction and PE [212].

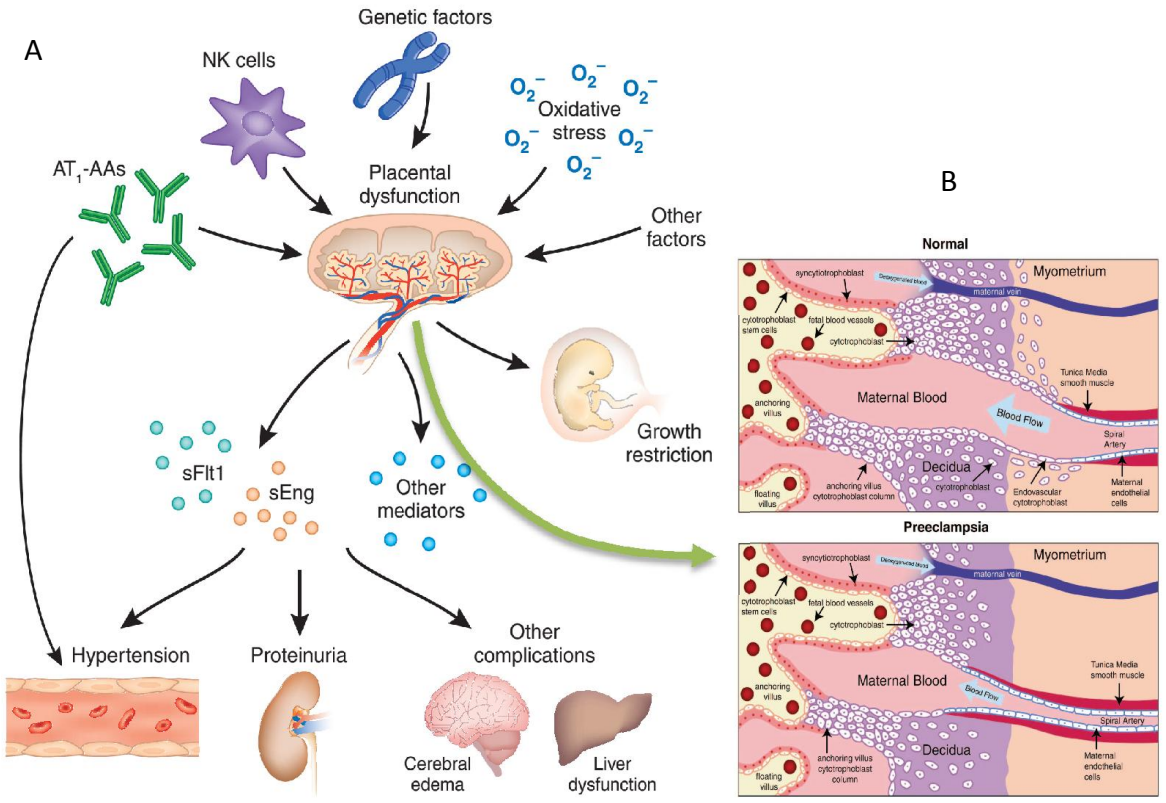


Figure 42- Pathogenesis of pre-eclampsia Immunologic, genetic and environmental risk factors lay the foreground for placental dysfunction by unknown mechanism. As a result of which cytotrophoblasts fail to adopt an invasive endothelial which alters small-caliber resistance vessels to high-caliber capacitance vessels, required for providing placental perfusion sufficient to sustain the growing fetus. This, in turn, leads to the release of antiangiogenic factors (such as sFlt1 and sEng) and other inflammatory mediators to induce hypertension, proteinuria (glomerular endotheliosis), seizures (cerebral edema and/or vasospasm), and the hemolysis, elevated liver function tests, and low platelet count (HELLP) syndrome can be attributed to vascular and endothelial effects. Adapted from [196] [213].

4.2.4 Role of anti-angiogenic factors

VEGF and TGF- β1 signalling in the vasculature plays a key role during pregnancy by maintaining vascular homeostasis. High quantities of sFLT-1, a soluble version of the VEGF receptor 1, is known to be present in the serum of preeclamptic women, which further correlate with low levels of free VEGF and PlGF [196]. sFlt1 binds to and neutralizes the proangiogenic actions of VEGF and placental growth factor (PlGF) [214]. During normal pregnancy, PlGF increases during the first and the second trimesters and decreases as pregnancy progresses to term. In contrast, levels of the anti-angiogenic factor sFLT-1 don't

change during the early and middle stages of gestation and increase steadily until term. When sFLT-1 was overexpressed in rats, it reproduces phenotypes similar to human PE. Later, another anti-angiogenic protein, soluble endoglin (sEng) was found to be raised in maternal serum. Rats injected with sEng along with sFLT-1 could produce more severe condition, which is similar to HELLP syndrome. Although, it is not yet clear whether the abnormal sFLT-1 production is a cause or consequence of abnormal placentation. In PE, excessive placental secretion of sFlt1 and sEng (2 endogenous circulating antiangiogenic proteins) is triggered by some unknown mechanism. These circulating sFLT-1 and sEng acts as a scavenger for VEGF and TGF-β1 and thus inhibits their signalling respectively, in the vasculature. This results in endothelial cell dysfunction, nitric oxide production, including decreased prostacyclin and release of procoagulant proteins (**Figure 43)**. In preeclamptic women this results in hypertension, proteinuria, as well as seizures from cerebral edema [211].

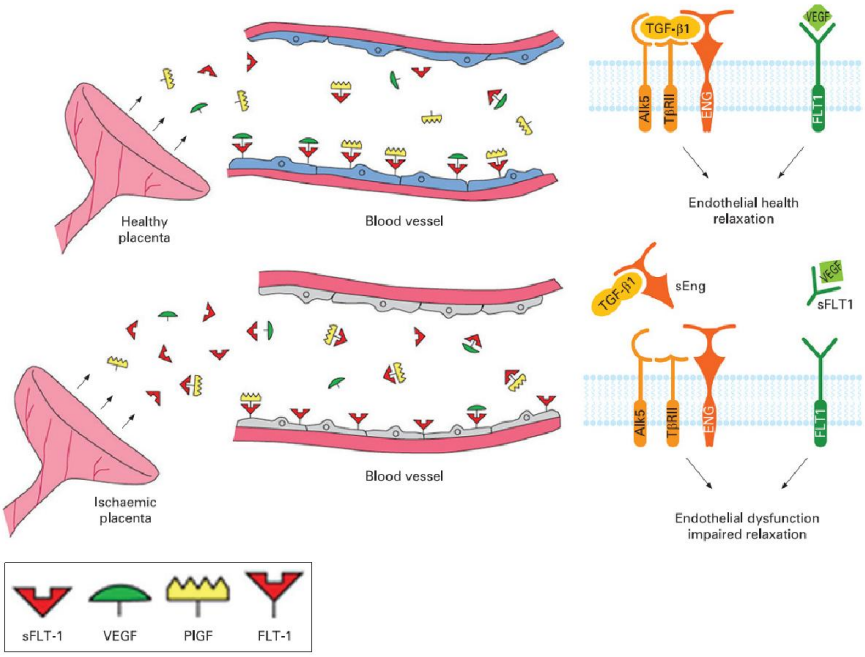


Figure 43. Molecular mechanism of PE. Healthy placenta secretes a balanced amount of soluble fms-like tyrosine kinase (sFLT) leading to normal levels of provasodilatory and anticoagulant factors available for binding to fms-like tyrosine kinase 1 (FLT1) on endothelial cells systemically, leaving healthy and responsive endothelium. However PE placenta secretes increased amount of sFLT, which scavenge VEGF from circulation thus depleting their availability to FLT1 binding. The result is a dysfunctional endothelial cell leading to maternal systemic vasculopathy. Adapted from [211].

4.2.5 Neutrophil NETs and thrombosis in PE

Preeclampsia is associated with an increased risk for venous thrombosis, an event that can lead to pregnancy complications. Preeclamptic women show an activation of their innate immune system, including the production of neutrophil extracellular traps (NETs). These fibers of chromatin were initially described as a part of antimicrobial defence, but accumulating evidence from several laboratories shows that they are implicated in a wide range of inflammatory and thrombotic diseases. While NETs can be found in placental tissue sections and their biomarkers in the blood of preeclamptic women, their role in the pathogenesis of this disease is completely unknown. In all cases, treatment with DNaseI prevents thrombus formation, underscoring the importance of NETs in triggering DVT. NETs bind Factor XII and stimulate fibrin and thrombin formation via the intrinsic coagulation pathway[215] .NE, a major component of NETs, is also known to enhance Factor Xa (FX) activity and proteolytically cleave tissue factor pathway inhibitor (TFPI), a major down-regulator of the coagulation cascade[216]. Furthermore, both NE and cathepsin G serine proteases are present in the NETs, and degrade a broad range of coagulation mediators [217]. *In vitro*, NETs stimulate fibrin formation and deposition, and fibrin colocalize with NETs in blood clots. NETs may also regulate haemostasis through its direct involvement in the resolution of the clots. *In vitro* studies have shown that the proteases NE and cathepsin G can degrade fibrin[218], and since they are present on NETs they could potentially enhance fibrinolysis. In addition, NETs could also recruit plasminogen from the plasma. Histones are shown to serve a receptors for plasminogen on the surface of human monocytes/macrophages[219] and could eventually act in the same capacity in NETs, since they represent their main structural component. In this context, neutrophils are most probably stimulated by activated platelets via TLR-4 in order to form NETs. Activated platelets not only stimulate NET formation, but the NETs that are generated trigger *de novo* platelet activation, red cell accumulation and thrombosis[220,221]. On the other hand, PMN co-cultures with activated endothelial cells have been shown to promote the formation of NETs, which is dependent on platelets or endothelium released IL-8 and ROS[125]. NETs in turn induce endothelial cell death, an effect mediated by the proteases mounted on the NETs or related cationic proteins, such as defensins and histones.

Interestingly, platelets from patients with systemic lupus erythematosus (SLE) carry anti-DNA antibodies and DNA immune complexes on their surface, which can be released in the circulation after treatment with DNase[222].

4.2.6 Hypothesis

In a series of observations we previously reported tha PE is associated with increased presence of both fetal and maternal cell-free DNA [223]. Elevated levels of circulating cell-free DNA are present in patients with severe preeclampsia and HELLP syndrome, which further correlate with severity of the disease [224]. Further we reported prodigious amounts of NETs directly in the intervillous space of affected placentae [125], which could be a result of simulation of neutrophils by placental micro-particles, which are released in elevated amounts in PE [125]. Although these studies were supported by additional evidence for neutrophil involvement in PE, such as the detection of elevated amounts of cell-free nucleosomes [225], or neutrophil elastase in patient sera, our interest grew as to whether NETs could contribute to placental abnormalities which is a hallmark PE and effect pregnancy outcome [51].

A higher prevalence of risk factors for venous thromboembolism (VTE) has been found in women with preeclampsia and fetal loss [226]. For this reason we were intrigued by the findings of the Wagner Lab, which indicated that NETs could act as a lattice to promote coagulation and thrombotic events [141,227,228]. By utilizing a sFLT-1 induced murine model for PE [229], our intention is to investigate whether there is an impact of NETosis in placental dysfunction and modulating pregnancy outcome. The goal of our study is to determine the role of NETs in a murine model of sFLT-1 induced PE by comparing severity of the disease in wild type mice and mice lacking the chromatin-modifying enzyme peptidyl arginine deiminase 4 (PAD4), as PAD4 deficient mice are unable to form NETs[123]. Thus, broadly it can be hypothesized that, abnormalities in the placenta of sFLT-1 treated mice, could be a result of thrombotic event, driven by extracellular DNA of NETotic origin.

4.3 Materials and Methods

4.3.1 Mouse model of Preeclampsia

All animal procedures were performed using 8- to 12-wk old C57BL/6 female mice (Jackson Laboratory). The Animal Care and Use Committee of the Immune Disease Institute approved all experimental procedures involving mice. All transplantation experiments were approved by the Institutional Animal Use and Care Committee of Massachusetts General Hospital. Beds from breeder male cages were transferred to induce estrous cycle in 8-week-old virgins C57BL/6 female mice (Jackson Laboratory). Mice with confirmed estrous were paired with proven breeder. After overnight mating (E=0.5), females were removed from the breeding cage, checked for postcopulatory plugs and weight was recorded. After 7.5 days of mating (E=7.5), those, which gained minimum of 2 gm of weight, were considered pregnant. On day 9.5, weight was recorded, followed by tail vein injection of 5 X 10^{10} PFU of adenovirus to overexpress sFLT-1 (Ad sFLT-1) or adenovirus encoding murine Fc protein (Ad Fc) at equivalent doses or left untreated. Weight was recorded again on E=15.5 and E=17.5. Early morning urine was collected on E=17.5 and all of them were sacrificed. Litter was cut open. Weight and number of foetus and placenta was recorded. Heart, lung, liver, placenta, spleen, brain were isolated and fixed in formalin, OCT, zinc-fix or glutaraldehyde.

4.3.2 Mouse Plasma Preparation and Analysis

Blood was collected from the retro-orbital sinus (49:1 vol/vol of blood:0.5 mol/L EDTA). Plasma was prepared by centrifuging anticoagulated whole blood for 5 minutes at 2300g. Plasma supernatant was carefully removed and centrifuged again for 5 minutes at 2300g to remove any remaining blood cells [230]. Plasma was stored at −80°C until analysis. Nucleosome levels were measured using the Cell Death Detection ELISA or Cell Death Detection ELISAPLUS (Roche, Indianapolis, IN). This assay allows for the relative quantification of histone-complexed DNA fragments (mono- and oligonucleosomes). sFLT-1 was measured in 1:500 diluted plasma using ELISA kit (R&D Systems Inc, Minneapolis, MN). Human TAT and DNA was measured using ELISA (Affinity Bio-logicals), and Quant-iT Picogreen assay (Invitrogen) according to the instructions of the manufacturers

4.3.3 Differential Counts

Whole blood was collected via the retroorbital sinus into EDTA-coated capillary tubes. Twenty-five µL of blood was analysed by a Hemavet 950FS (Drew Scientific) for complete blood counts.

4.3.4 Blood Pressure measurement by tail cuff method

Tail cuff systolic blood pressure was measured by tail cuff method using (IITC, Inc.; Woodland Hills, CA) [231]. Mice were trained by placing them for 20mins inside the BP machine twice before taking the final measurement.

4.3.5 Mouse peripheral blood neutrophil isolation

Peripheral blood was collected via the retroorbital venous plexus, as described previously [141]. Briefly, mice were exsanguinated into PBS containing 1% (wt/vol) BSA and 15 mM EDTA. After centrifugation, blood cells were resuspended and layered onto a Percoll gradient of 78%, 69%, and 52% in PBS (vol/vol), centrifuged and cells at the 69%/78% interface were collected. Red blood cell contamination was eliminated by hypotonic lysis, and final cell concentration was determined by hemacytometer. Neutrophil purity was established to be routinely>90%, as assessed by Wright–Giemsa staining on cytospin.

4.3.6 NET induction and quantification

Immunostaining of fixed cells was performed using anti–Ly6G and anti–H3cit antibodies [141]. Isolated neutrophils were plated on a 96 well cell culture dish and stimulated with PMA. 10^5 cells suspended in RPMI medium, was plated in each well of a 96 well plate and stimulated with Calcium Ionophore or left untreated for 3 hours. After stimulation, cells were fixed in 2% paraformaldehyde and permeabilized (0.1% Triton X-100, 0.1% sodium citrate) for 10 min at 4 °C. Samples were blocked with 3% (wt/vol) BSA for 90min at 37 °C, rinsed, and then incubated overnight at 4 °C or for 1 h at 37 °C in antibody dilution buffer containing 0.3% BSA, 0.1% Tween-20, and either rabbit antihistone H3 (citrulline 2, 8, 17) (0.3µg/mL, ab5103; Abcam) and rat anti-Ly6G (0.5µg/mL, clone 1A8; Biolegend). After several washes, samples were incubated for 2 h at room temperature in antibody dilution buffer containing Alexa Fluor-conjugated secondary antibodies in 0.3% BSA in PBS. DNA was counterstained with 1 µg/mL

Hoechst 33342 and slides were cover slipped with Fluoromount gel (Electron Microscopy Sciences). Fluorescent images were acquired using an Axiovert 200 wide field fluorescence microscope (Zeiss) in conjunction with an Axiocam MRm monochromatic CCD camera (Zeiss) and analyzed with Zeiss Axiovision software. All channels were acquired using Zeiss Axiovision. Composite images were generated with the MosaicJ plugin (36) for ImageJ software. NETs were counted from six different fields in triplicate wells and expressed as percentage of NET-forming cells per total number of cells in the field.

4.3.7 Placental Histology and Immunostaining

Placentas were harvested from killed animals and fixed in zinc fixative (100 mM Tris-HCl containing 37 mM zinc chloride, 23 mM zinc acetate, and 3.2 mM calcium acetate). Paraffin-embedded sections were deparaffinized in xylenes and rehydrated through a graded alcohol series. Sections were stained with hematoxylin and eosin and observed by light microscopy. For fibrinogen/fibrin and VWF staining, the sections were stained with a sheep anti-fibrinogen antibody (ABD Bio-logicals) and rabbit anti–human VWF antibody (Dako) and incubated with anti-sheep Alexa-555 and anti-rabbit Alexa-488 (Invitrogen) as secondary antibodies. Sections were counter-stained with Hoechst-33342 to visualize all nuclei, mounted with Fluoro-gel (Electron Microscopy Sciences), and observed under an epifluorescent Axiovert microscope (Zeiss).

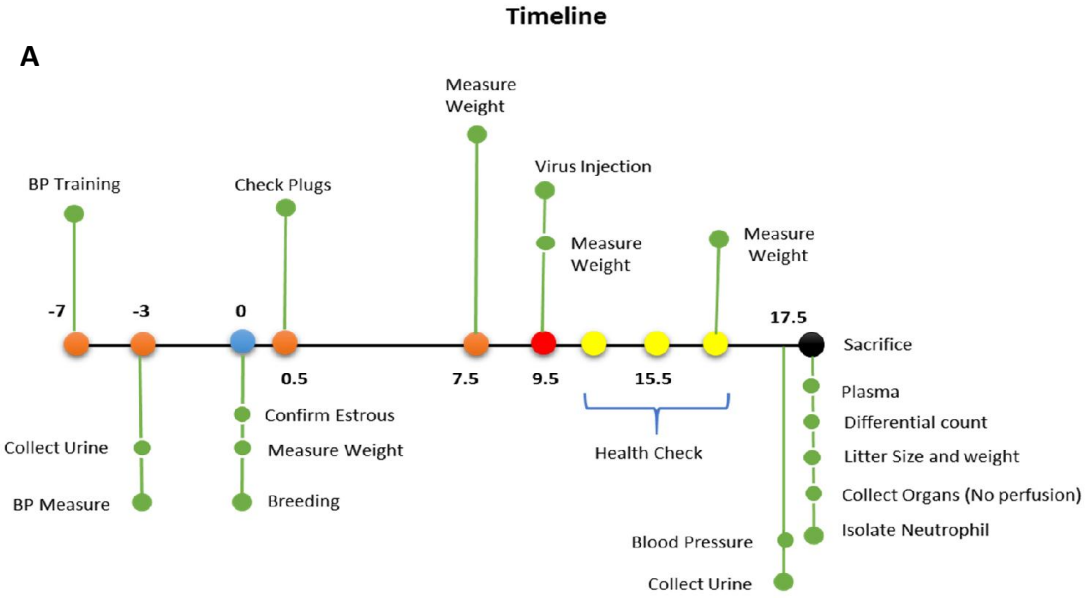
4.3.8 Statistics

Data are presented as means±SEM unless otherwise noted and were analyzed using a two-sided Student t-test or Mann–Whitney Utest. All analyses were performed using GraphPad Prism software (Version 5.0). Results were considered significant at $P<0.05$ (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

4.4 Results

4.4.1 Overexpression of sFLT-1 in mice is associated with high risk of fetal loss

Administration of adenovirus carrying sFLT-1 to pregnant rats has been shown by others to induce PE phenotype [214] as well as fetal growth restriction in pregnant mice[232]. Adenovirus (Ad sFLT-1 or Ad Fc) was injected intravenously into wild-type (WT) mice pregnant female at E=9.5 (Figure 44A). A decline in body weight along with loss of litter by E=17.5 was found in 50% of the mice injected with sFLT-1(Figure 44B). Plasma levels of sFLT-1 was found significantly increased in the sFLT-1 mice and was significantly higher than the adeno control group, indicate the overexpression of the sFLT-1 protein by the Ad sFLT-1 vector. Although, equal amount of Ad sFLT-1 was injected into the pregnant mice, it was found that those which lost their pregnancies after Ad sFLT-1 injection, expressed significantly higher amount of sFLT-1, compared to those which retained their pregnancy (Figure 44C). Thus, we conclude that overexpression of sFLT-1 after a certain threshold, could induce a phenotype similar to sever form of PE, which could be responsible for fetal death and pregnancy loss.



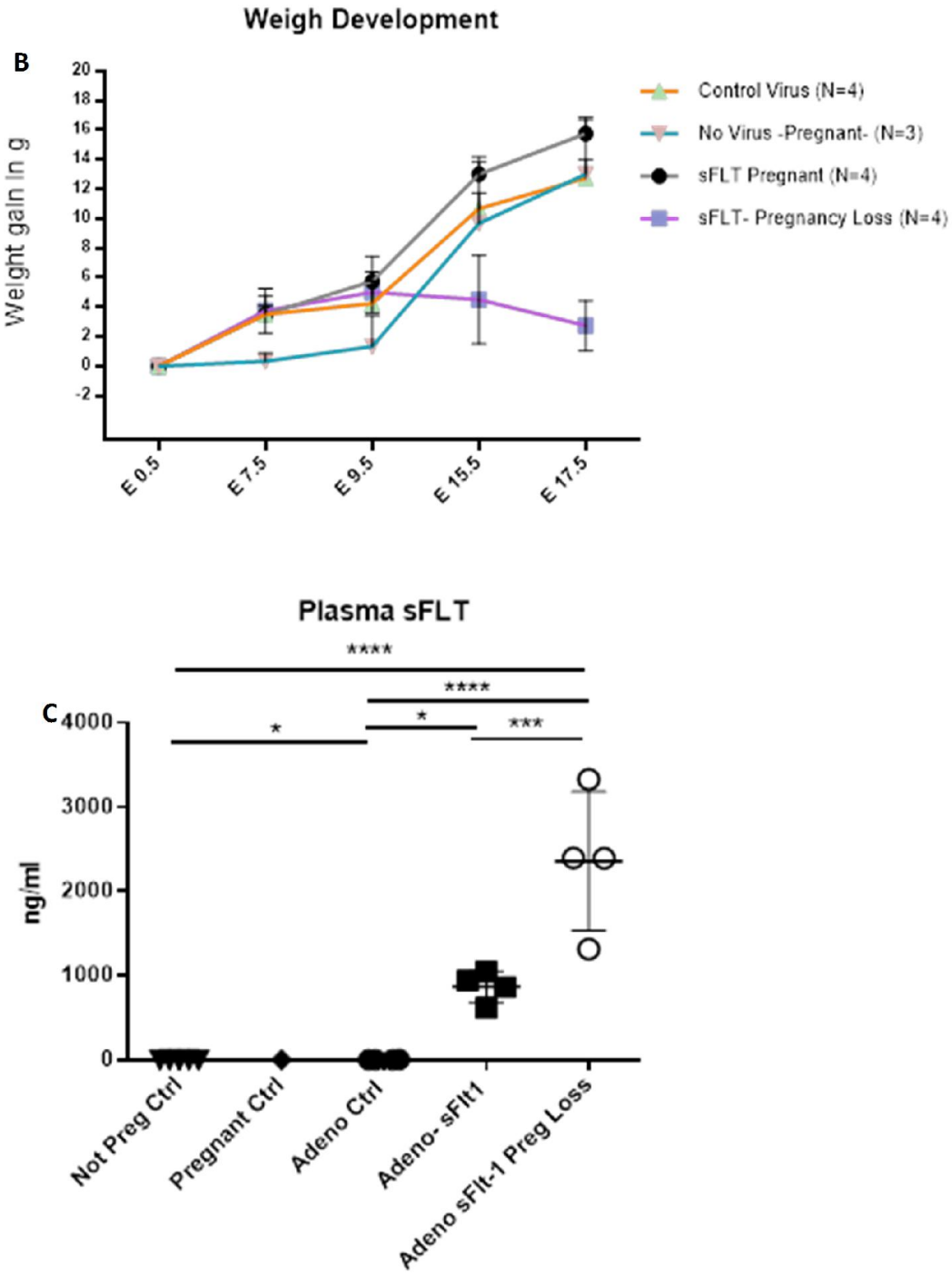


Figure 44- sFLT-1 overexpression may lead to fetal loss. Treatment of pregnant wild type mice with an adenoviral vector on E=9.5. Body weight was record on five different time points and plasma sFLT-1 was measured to check the expression of the virus. (A) Administration of Ad sFLT-1, resulted into decrease of body weight and loss of pregnancy in 50% of them (B). More than 2.5 fold increase in plasma sFLT-1 expression was evident in the fetal loss group of mice, compared to those which retained the pregnancy at the same dose of virus (C). Data are expressed as mean±SD in this and all subsequent figures, unless indicated otherwise. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n.s.: statistically not significant.

4.4.2 Elevated systolic pressure and markers of kidney dysfunction after sFLT-1 treatment

PE is characterized by the onset of hypertension and proteinuria [204]**.** To induce the clinical features of PE in pregnant mice, we used adenoviral expression sFlt1 on E=9.5 day pregnant mice. High albumin and low creatinine concentration is a hallmark of kidney dysfunction, was evident in the group of mice treated with Ad sFLT-1 and attend significance in the group with pregnancy loss (Figure 45A and 45B). Further, the ratio of albumin to creatinine is significantly increased in the sFlt1-treated group and attained significance in the pregnancy loss group, when compared with the Adeno control group (45C). The overexpression of sFLT-1 induced a change in mean systolic blood pressure compared to non-pregnant (45D).

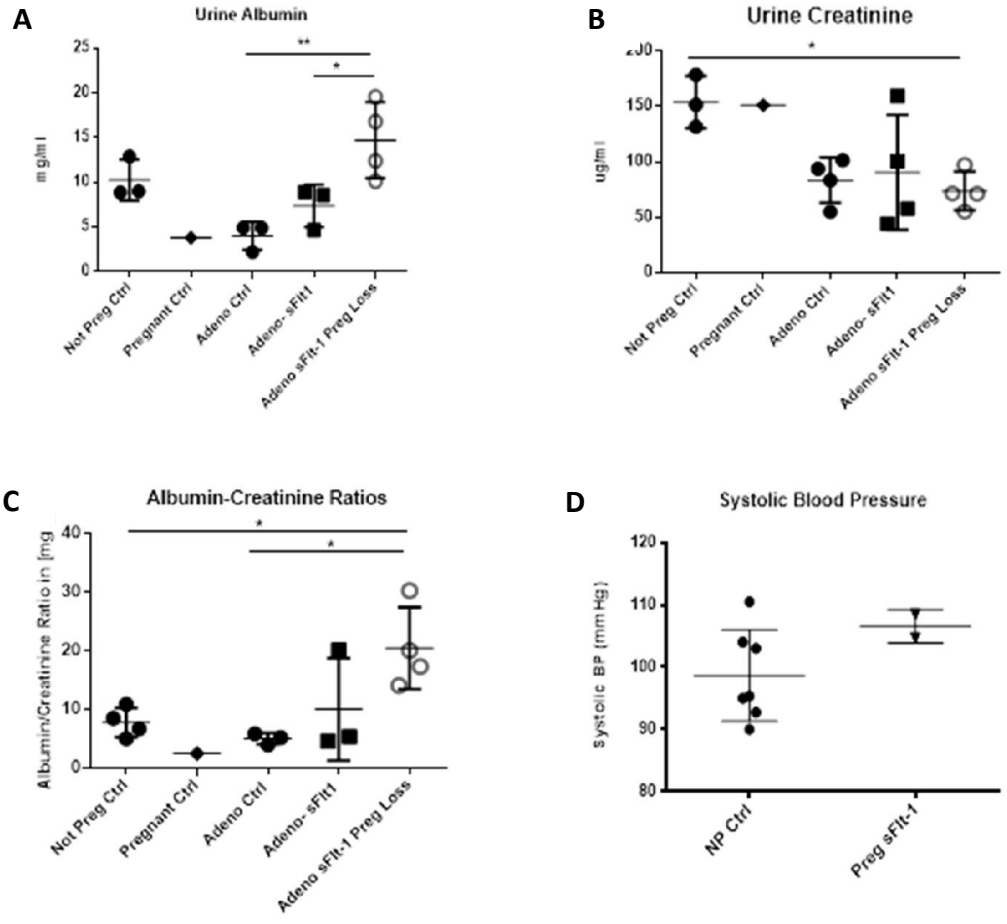


Figure 45- Overexpression of sFLT-1 in pregnant mice causes PE like symptoms. Pregnant mice on day E=9.5 were injected with Ad sFLT-1. Urine samples were collected on day 17.5. Concentration of Urine albumin and (B) creatinine was measured by commercial ELISA kit. (C) Ratio of albumin to creatinine is plotted. (D)Systolic blood pressure was measured by tail cuff method on E=16.5.

4.4.3 Effect on sFLT-1-1 overexpression on fetus, placenta and spleen

SFLT-1 overexpression during pregnancy on fetal and placental development had no significant effects on average fetal weight which were, ~1 g in all 3 groups (Figure 46A) or on the average placenta weight, although the sFLT-1 treated placenta has as tendency to be smaller than the control group (Figure 46B). Splenomegaly (enlarged spleen) is also commonly found in PE. In TLR activation model of PE enlarged spleen was previously reported [231]. In our experiments we found over expression of SFLT-1 significantly increased the size of the spleen compared tountreated pregnant mice (Figure 46C and 46D).

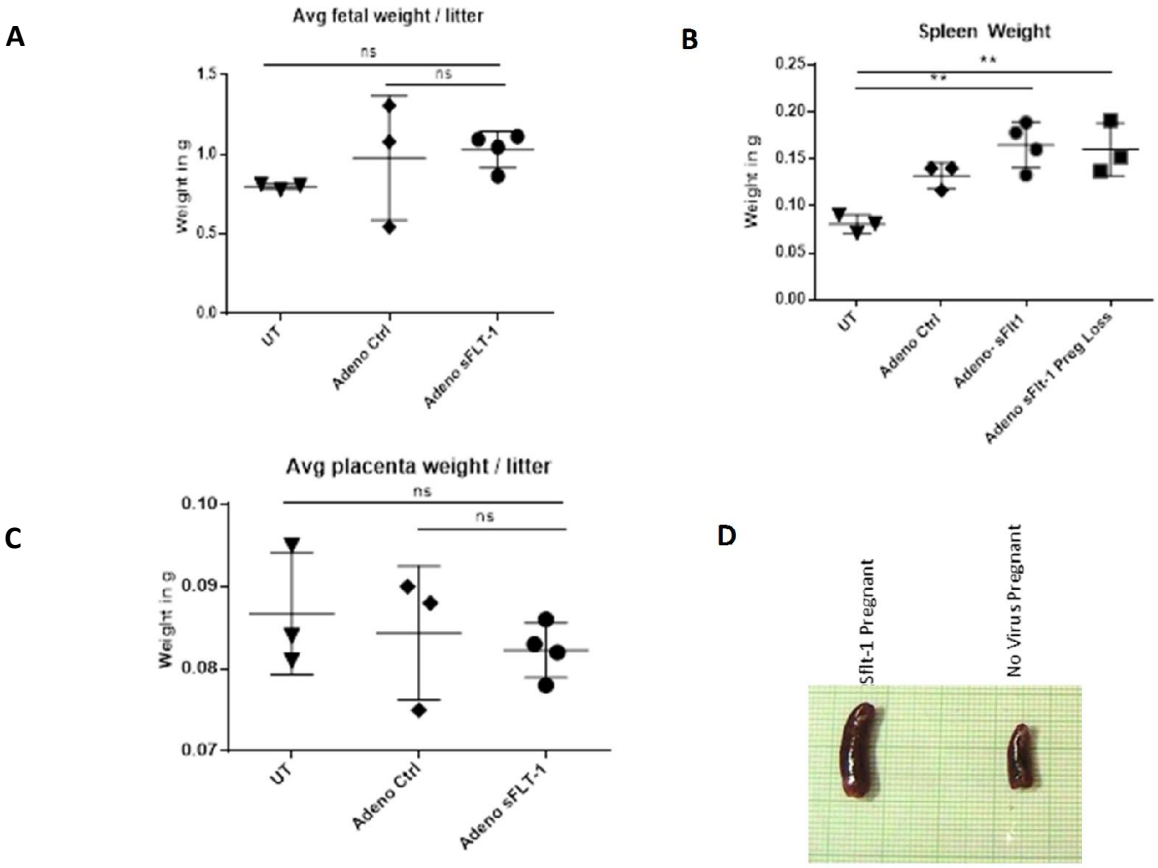


Figure 46. Effect of overexpression on sFLT on pregnancy and spleen Measures of (A) Fetal weight, (B) Placental weight (C) Spleen weight (D) Spleen size in pregnant mice treated with Ad SFLT-1.

4.4.4 Overexpression of SFLT-1 does not alter TAT complexes or platelet count

VTE is one of the leading causes of morbidity and mortality during pregnancy [233]. A higher prevalence of risk factors for VTE has been found in women with PE and fetal loss [234]. The measurement of thrombin-antithrombin complexes (TAT) is a parameter for coagulation and fibrinolysis, used for the diagnosis of thrombotic events. In preeclampsia elevated concentrations of TAT and decrease in platelet count has been reported previously [235]. However in our experimental setup we did not see any significant increase in TAT complexes between sFLT-1 treated and control groups, although there is a slight tendency to be higher in sFLT-1 treated group (Figure 47A). Further analysis of platelet count was also not statistically significant between control and treated group both, although there is a tendency of reduced platelet count in sFLT-1 treat group (Figure 47B).

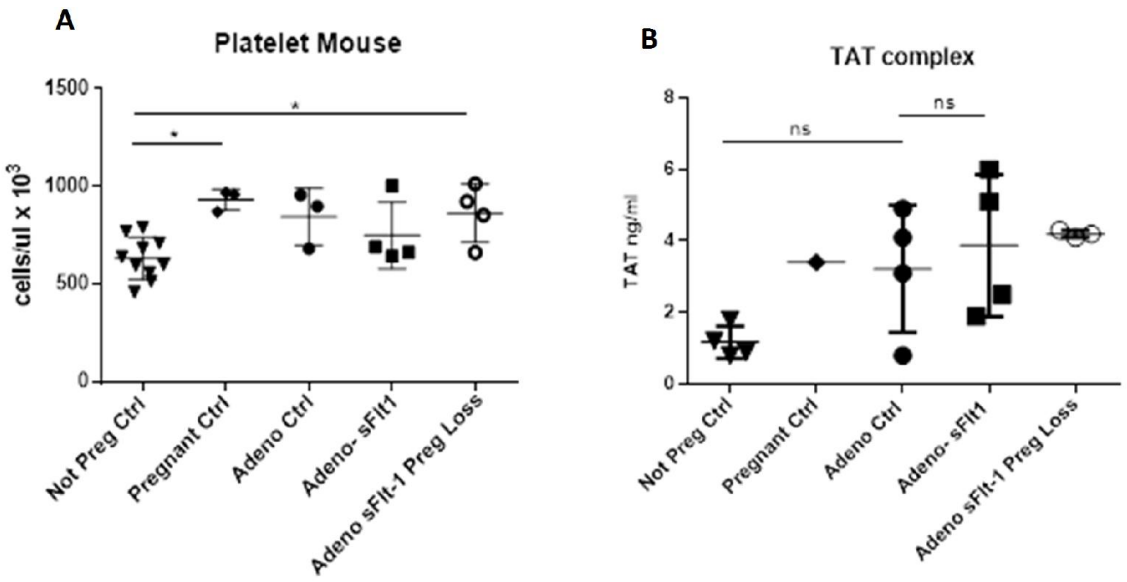


Figure 47. Effect of sFLT-1 expression on thrombotic events (A) Plasma TAT complex concentration was measured by commercial ELISA. (B) Platelet count was measured by automated differential count of peripheral blood.

4.4.5 Circulating extracellular DNA is significantly elevated in sFLT-1 mouse plasma

Circulating DNA is known to be elevated in a number of autoimmune diseases including rheumatoid arthritis, SLE. Recently, it has been reported to be increased in PE and HELLP syndrome [224]. To test whether sFLT-1 overexpression could promote extracellular DNA release, we measured the levels of circulating DNA in the plasma of our mouse model. Cell-free DNA levels were indeed significantly elevated in plasma of sFLT-1 treated group compared to controls (Figure 48A).

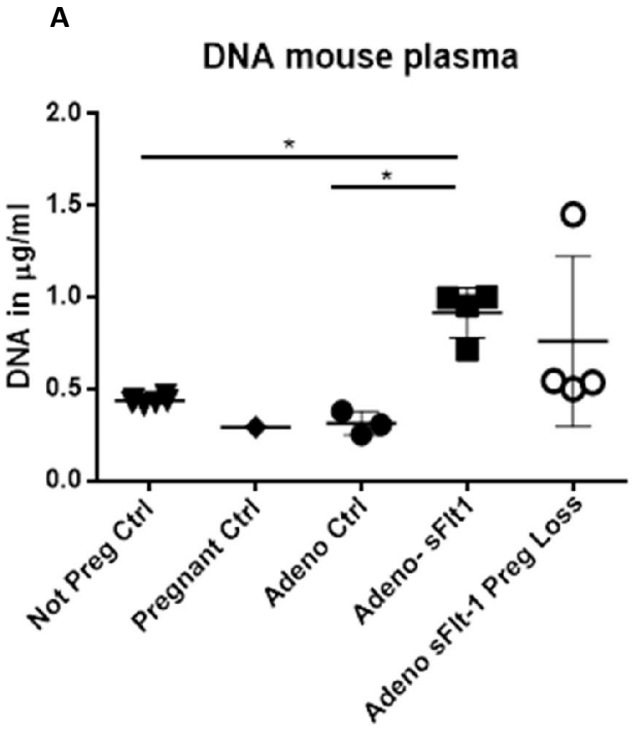
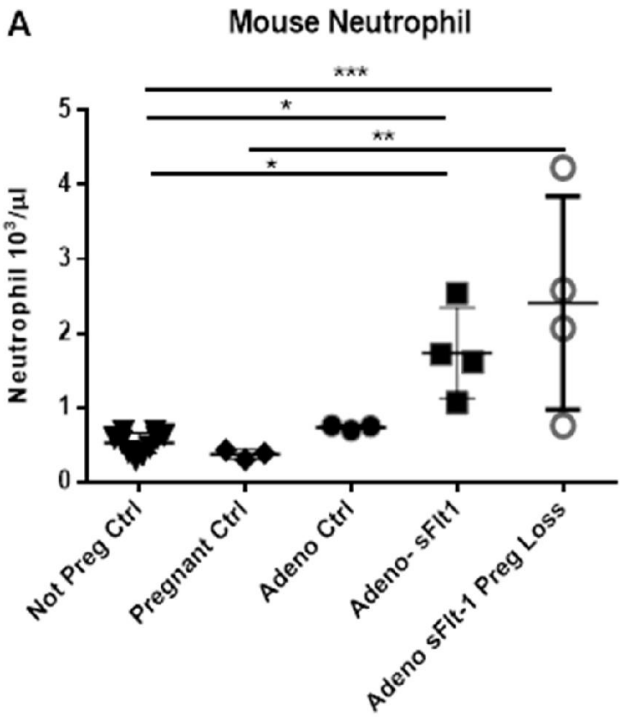
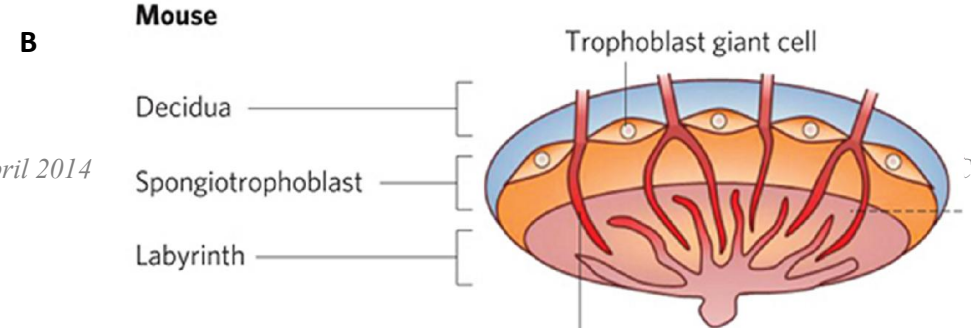


Figure 48 Overexpression of sFLT-1 induces extracellular DNA release. Quantification of extracellular DNA in the (A) plasma and in (B) serum of healthy donors and patients with PE. (C) Determination of extracellular DNA concentrations in mouse plasma. Data provided by L.Erpenbeck

4.4.6 sFLT-1 overexpression is associated with granulocyte aggregation and tissue damage

The placenta has a central role in PE, as evidenced by rapid disappearance of disease symptoms after delivery. Because abnormalities in the placenta such as failure of modification of spiral arteries at the feto-placental junction, has been proposed to be a key pathogenic event [213]. Therefore we investigate the effect of sFLT-1 treatment in mouse placenta. In our PE model when we expressed sFLT-1 by intravenous injection, extensive granulocyte infiltration including infarction at the feto-placental junction was evident (Figure 49E). Although, necrotic regions are seen also in control groups (Figure 49D), but no granulocyte infiltration was evident (Figure 49C and D). Further, the number of circulatory neutrophils in sFLT-1 treated or control groups attain no significant difference (Figure 49A), which further provides evidence of specific and enhanced neutrophil migration into the PE placenta.





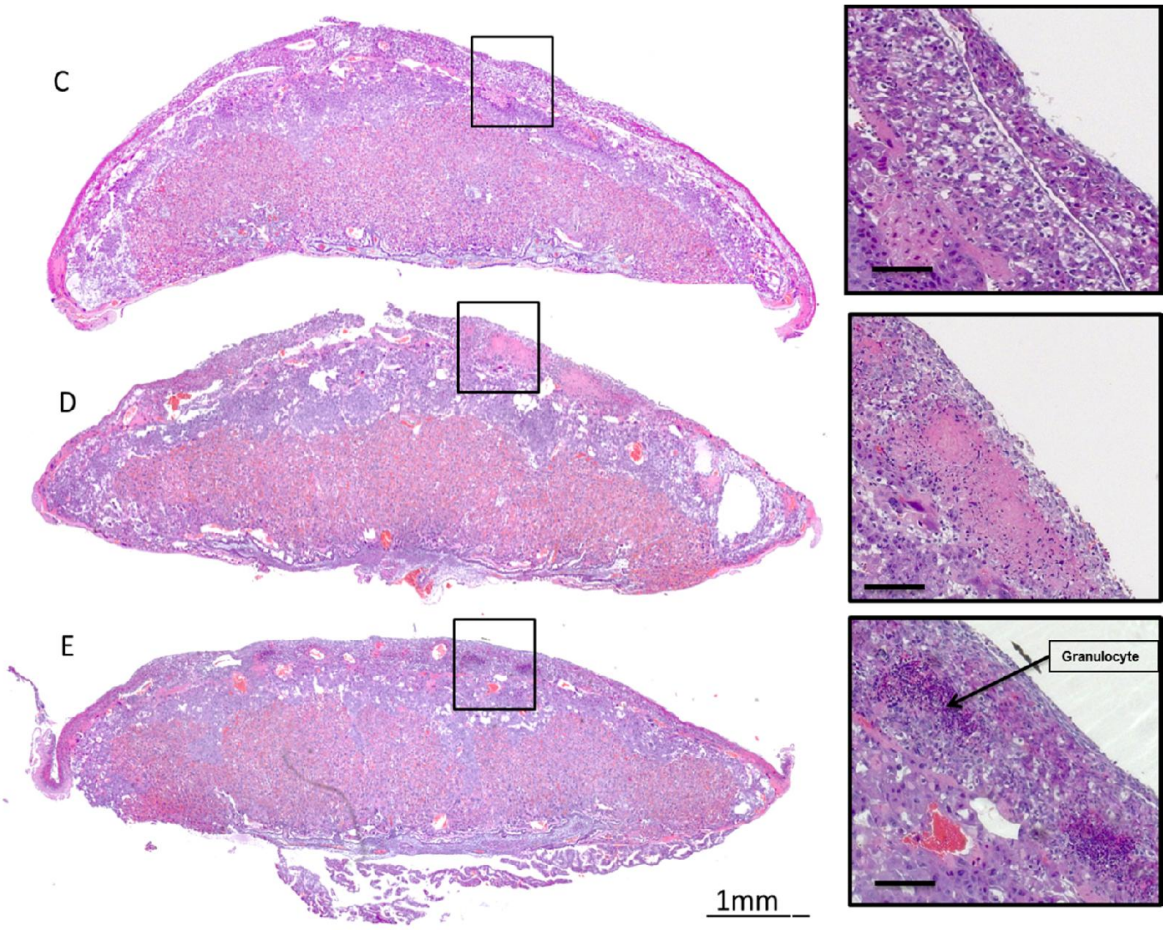


Figure 49. Placental histology (H&E stain) of control and sFlt1 groups. (A) Schematic diagram of mouse placenta showing different layers, modified from. (B) Neutrophil count was not significant between the control and the SFLT-1 treated group. (E) SFlt-1treated mouse showed diffuse inflammation along with granulocyte aggregation (arrowheads) and fibrinoid necrosis at the feto-placental junction, which was not seen in control groups (C and D). Scale bar 1mm and 0.2mm.

4.4.7 Enhanced NETosis in feto-placental junction of preeclamptic placenta

A significantly greater number of neutrophils but not lymphocytes or monocytes, have been shown to adhere to endothelium and infiltrated into the intimal space in the maternal systemic vasculature of preeclamptic women than in that of normal pregnant women or normal non-pregnant women [236]. Previously we have demonstrated the presence of diffused DNA staining, colocalizing with neutrophil specific elastase staining, in placenta of preeclamptic patients [125]. However, in the absence of NETs specific marker, it was not clear whether the diffused DNA staining was of NETotic origin. Further, location specific effect of these NETs remained unidentified. To understand whether sFLT-1 overexpression is sufficient to induce formation of NETs in the placenta causing impairment of the feto-placental junction, we combined immunofluroscence staining for DNA, neutrophil specific anti-Ly6G and NETs specific anti-H3Cit. Results indicate negligiable or quite small amount of NETs in untreated or control placenta (Figure 50B and 50C). However, a large area in the decudal region spanning the feto-placental junction of the sFLT treated placenta was covered with NETs specific sataining (Figure 50D). Knowing the fact that NETotic DNA could potentially contribute towards elevated thrombotic events [124], which is a featcher in pre eclemptic placenta, it can hypothesised that NETotic neutrophils, could impair the exchange of oxygen from materal circulaiton to the growing placenta, causing hypoxic condition which may lead to abnormalities in the placenta.

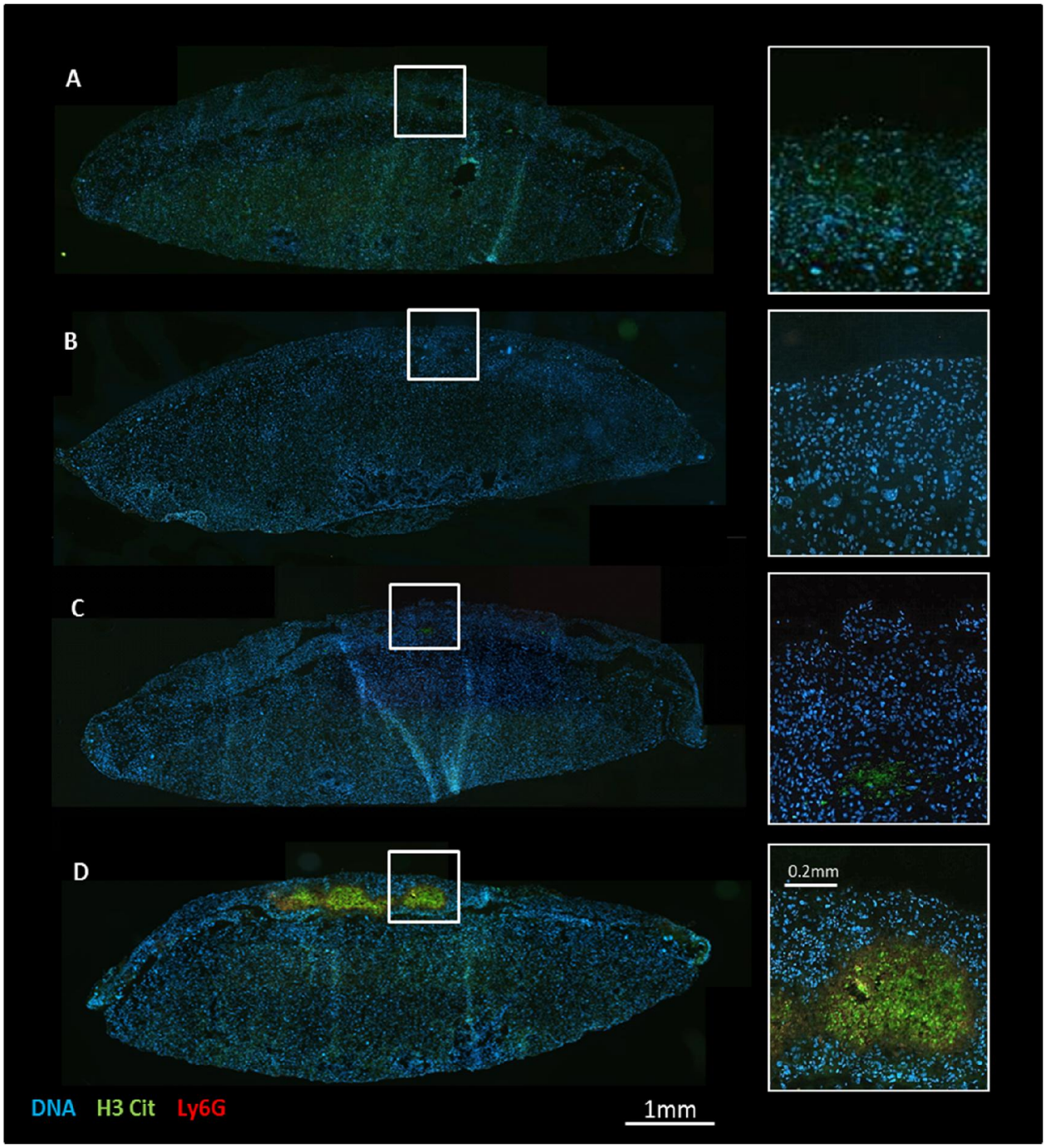
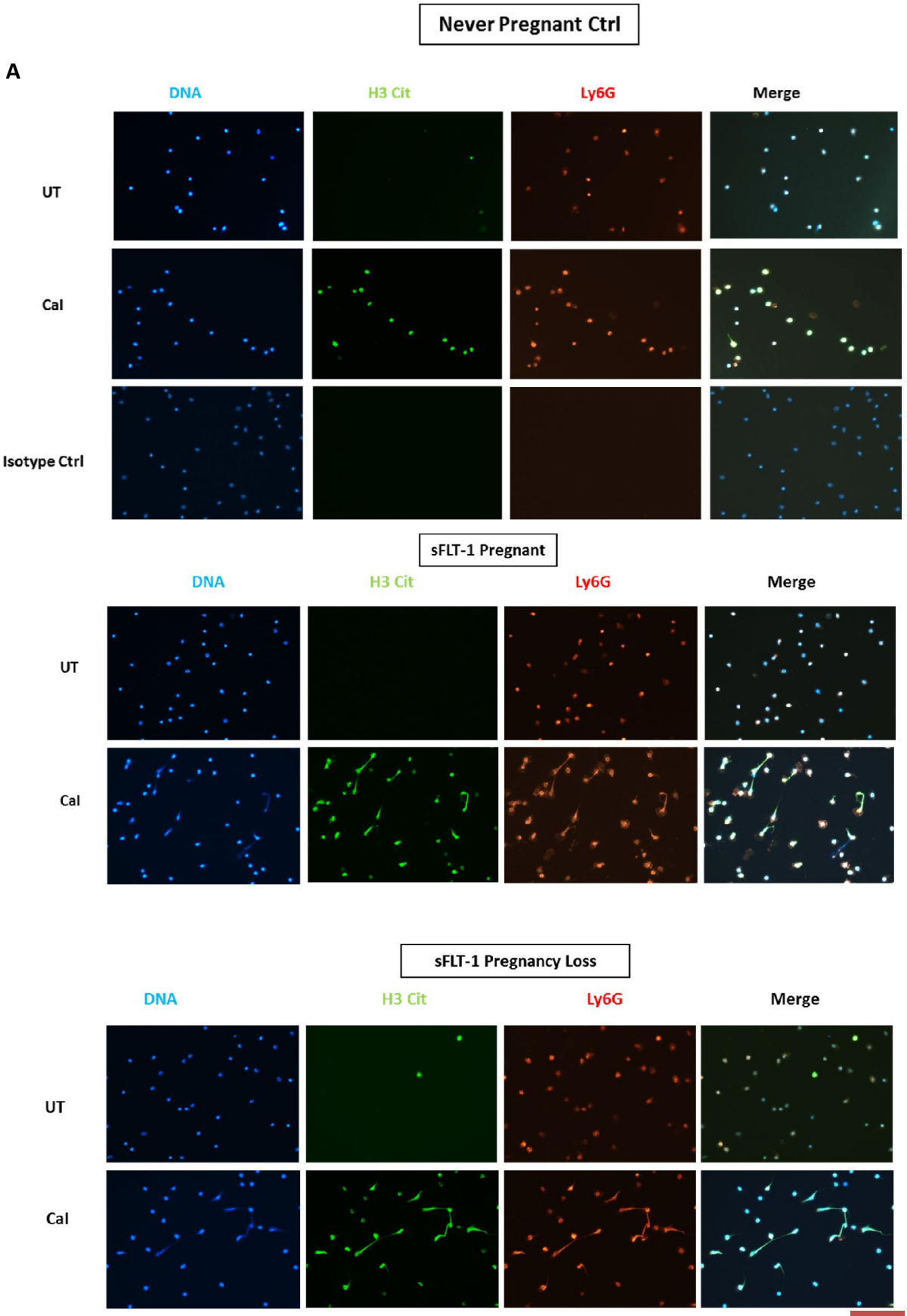


Figure 50. Immunofluroscent staining of mouse placenta Placent from mouse on 17.5 day of pregnancy was isolated and transverse sections were statined with DAPI (Blue), anti-H3Cit (Green) and anti-Ly6g (Red) antibodies (Neutrophil) . A) Isotype contorl B) Untreated control C) Adeno control D) Treated with adeno SFLT-1. H3cit staining colocalizes with neutrophil specific Ly6G staining at the feto-placental junction in the decudal region can be seen only in the PE placenta (D). Bar in the figures are 1mm and 0.2mm. **Data provided by L.Erpenbeck**

4.4.8 Neutrophils from Ad sFLT-1 mice show enhanced NETosis upon stimulation.

Formation of NETs is known as a two-step mechanism involving priming followed by activation resulting into NETotic spread [10]. Further neutrophils from SLE patients and mouse model were shown to be primed for NET formation [127,237]. To determine whether sFLT-1 overexpression could promotes NET formation, we isolated the peripheral blood neutrophils form sFLT-1 treated pregnant mice (N=1), sFLT-1 treated mice with pregnancy loss (N=1) and a control non pregnant mice (N=1), all at the same time. The isolated neutrophils were stimulated with calcium ionophone (CaI), which is a known stimulator for NETosis. After 3hrs of stimulation, the cells were fixed and stained for NET specific anti- H3cit and neutrophil specific Ly6G antibodies. DNA was stained with DAPI and visualized under fluorescent microscope. Enhanced NET formation was observed in sFLT-1 treated group compared to the non-pregnant control mice (Figure 50A). Morphometric analysis indicate more spread phenotype in the sFLT-1 treated pregnant mice compared to the mice which with pregnancy loss (Figure 50C). Further, the percentage of H3cit positive cells was lowest in the sFLT-1 treated pregnant mice indicating the more number of neutrophils has attained the NETotic phenotype (Figure 50B). This indicates towards a faster kinetics of NETosis in neutrophils from sFLT-1 pregnant group, which suggest that the peripheral blood neutrophils that are treated with Ad sFLT-1 are more sensitive towards NET formation following a secondary stimulation.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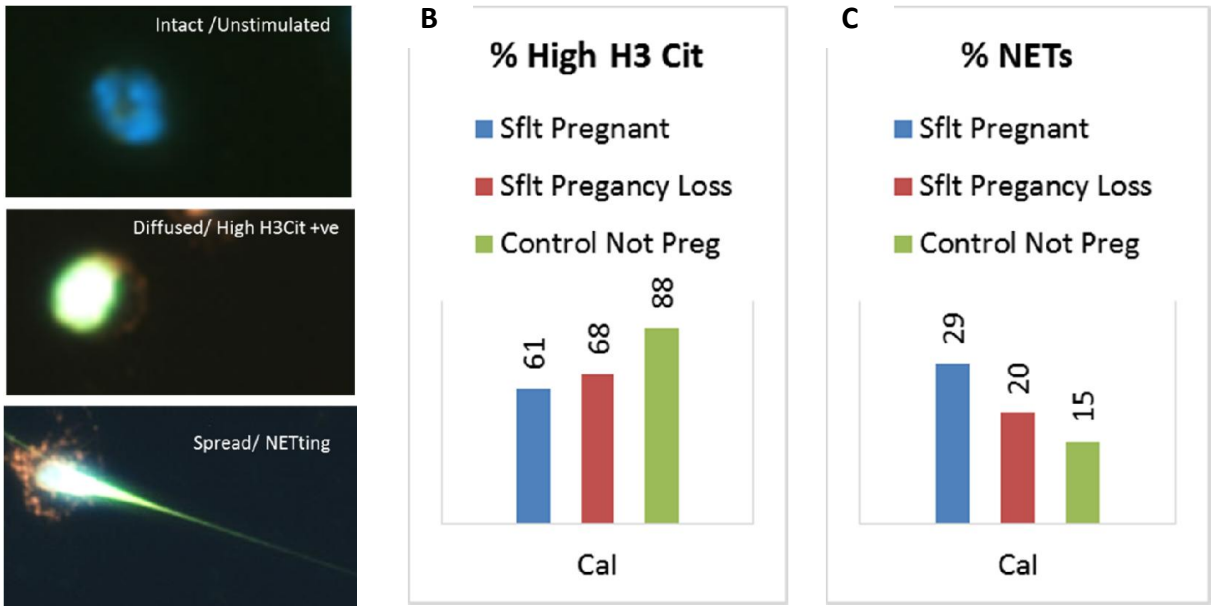

Figure 51. Increased NETotic response to CaI in neutrophils derived from sFLT-1- treated mice. (A) Immunostaining of neutrophils from sFLT-1 and control groups, after 3hrs of stimulation with CaI. Following fixation cells were stained for DNA (Blue), H3Cit(Green) and Ly6G(Red) (B) Morphological changes were divided into unstimulated/intact, diffused/highH3cit and Spread/NETting phenotypes. Morphometric quantification was done after counting six different fields from each well and represented as percentage change.

4.5 Discussion

For a long time, abnormalities of blood clotting in PE have been known [238]. But recent investigations suggests blood coagulation is not directly involved, as fetal loss could not be hindered by anti-clotting agents such as hirudin [239]. Nevertheless, NETs could eventually provide the necessary stimulus and scaffold for clot formation by promoting platelet and RBC adhesion and by concentrating effector proteins and coagulation factors involved in hemostasis[240].

PE, was reported to be associated with increased presence of both fetal and maternal cell-free DNA [223], prompt us to investigate whether neutrophil NETs could be a contributing factor [52] to the disease's pathobiology and progression. This study indicated that placental micro-particles, which are released in elevated amounts in PE, could trigger NETosis in isolated neutrophils *in vitro* [125]. We furthermore observed that there were prodigious amounts of NETs directly in the intervillous space of affected placentae [125]. Although these studies were supported by additional evidence for neutrophil involvement in PE, such as the detection of elevated amounts of cell-free nucleosomes [225], or neutrophil elastase in patient sera, our interest grew as to whether NETs could contribute to placental hypoxia evident in PE [51].

Our results reveal that treatment of pregnant wild type mice with an adenoviral vector carrying sFLT-1 cDNA leads to a significant elevation of serum sFLT-1 levels, although the mouse model did not completely mirror the human condition evidenced by low albumin to creatinine ratio and mild elevation in blood pressure. These sFLT-1 overexpressing wild type mice also showed an elevation of DNA in their plasma, indicative of NETosis. While no significant NETosis were evident in lung, liver or kidney sections of these mice. However, elevated numbers of neutrophils infiltrating the placenta of sFLT-1 treated mouse was clearly evident which further co-localize with NET specific anti-H3Cit staining. Further, the presence of NETotic areas in the feto-placental junction, suggest a specific inflammatory response in the placenta of these mice

Circulating levels of VEGF and sFlt-1 are known to be increased in women during early pregnancy compared women that are not pregnant, indicating that VEGF and sFlt-1 are both associated with pregnancy [241]. In a recent study involving patients from recurrent spontaneous abortion (RSA), significant increase in sFlt1 and VEGF levels in serum was reported, suggesting over expression of sFlt-1 and VEGF might be associated with the pathogenesis of RSA [242]. Since 50% of the our mice treated with sFLT-1 lost their pregnancies within few days of the intravenous injection, it can be broadly postulated that uncontrolled NETosis production leads to impairment of maternal exchange with the growing fetus, resulting in placental injury and subsequent fetal loss. A possible trigger for neutrophil activation towards NETosis in PE could be interaction of neutrophils with activated cells, such

as platelets or endothelium [243,244]. Alternatively, hypoxia, ROS, cytokines, possibly coagulation proteases or even other environmental factors could additionally induce NETosis [36].

As the production of ROS is a vital component of the pathway triggering NETosis and the release of DNA into the extracellular environment [245], it is enticing to speculate that NETs may occur in aPL induced fetal loss. Furthermore, as the presence of NETs can be cytotoxic to closely adjacent cells [243], it is possible that the occurrence of such entities can contribute to trophoblast injury apparent in this disorder. Recent data suggest that infections with *Brucella abortis[246]* or *Listeria monocytogenes*[247] leads to PMN recruitment and activation, including release of IL-8 and ROS production. As *brucellosis* in cattle or *listeriosis* in humans can be directly associated with spontaneous abortion[248], it is open to speculation whether NETs occur in infected placentae in these conditions, and thereby contribute to the process of fetal loss . Although there is no direct evidence that NETs may be implicated in fetal loss, induced either via the presence of autoantibodies or infectious agents, there is accruing evidence that PMN activation may play a crucial part in these events [249-251].

4.6 Future Directions

4.6.1 Role of NETs in Recurrent Fetal Loss (RFL)

A recent finding suggest, high levels and over expression of sFlt-1 and VEGF might be associated with the pathogenesis of RFL, as *significant ($p<0.05$) increase in sFlt1 and VEGF expression can be seen in patients who suffered subsequent miscarriages compare to controls [242].* Considering that we also observed significant loss in mice pregnancies after sFLT-1 overexpression, suggest a possible role of NETosis in the underlying pathogenesis. However it is noteworthy that the presence of PMN is required for aPL induced fetal loss, as this effect was blocked via the depletion of PMN using appropriate antibodies[252]. When aPL were

infused into pregnant mice, fetal demise was not associated with a deposition of fibrin or increased presence of thrombi [253,254]. It was furthermore determined that aPL induced fetal loss involved the activation of the complement system, in particular components C3 and C5, and the repressive activity of Crry[253,254]. The activation of the complement implicated in the innate arm of the immune system, as the decidua of treated mice exhibited considerable PMN infiltration and elevated tissue factor (TF) expression[255].

4.6.3 What is the possible role of hypoxia in the pathogenesis of PE?

The role of hypoxia in triggering NETosis is not clear. Preliminary data from Basel indicates that exposure of native PMNs to hypoxia in-vitro leads to higher degrees of NETosis. This may play a role in pregnancy, since the placenta is a relatively hypoxic environment, a condition exacerbated in PE or intra-uterine growth restriction (IUGR) [211] . Moreover, PE occurs more frequently in pregnancies at high altitude suggesting reduced oxygen tension might trigger increased NETosis. This is supported by our previous observation of significant increases in cell-free DNA in pregnancies in Tibet.

[256].

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Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility

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Abstract

Introduction: Neutrophil extracellular traps (NETs) have recently been implicated in a number of autoimmune conditions, including rheumatoid arthritis (RA). We examined the underlying signaling pathways triggering enhanced NETosis in RA and ascertained whether the products of NETosis had diagnostic implications or usefulness.

Methods: Neutrophils were isolated from RA patients with active disease and from controls. Spontaneous NET formation from RA and control neutrophils was assessed *in vitro* with microscopy and enzyme-linked immunosorbent assay (ELISA) for NETosis-derived products. The analysis of the signal-transduction cascade included reactive oxygen species (ROS) production, myeloperoxidase (MPO), neutrophil elastase (NE), peptidyl arginine deiminase 4 (PAD4), and citrullinated histone 3 (citH3). NET formation was studied in response to serum and synovial fluid and immunoglobulin G (IgG) depleted and reconstituted serum. Serum was analyzed for NETosis-derived products, for which receiver operator characteristic (ROC) curves were calculated.

Results: Neutrophils from RA cases exhibited increased spontaneous NET formation *in vitro*, associated with elevated ROS production, enhanced NE and MPO expression, nuclear translocation of PAD4, PAD4-mediated citrullination of H3, and altered nuclear morphology. NET formation in both anti-citrullinated peptide antibody (ACPA)-positive and -negative RA was abolished by IgG depletion, but restored only with ACPA-positive IgG. NETosis-derived products in RA serum demonstrated diagnostic potential, the ROC area under the curve for cell-free nucleosomes being >97%, with a sensitivity of 91% and a specificity of 92%. No significant difference was observed between ACPA-positive and -negative cases.

Conclusions: Signaling elements associated with the extrusion of NETs are significantly enhanced to promote NETosis in RA compared with healthy controls. NETosis depended on the presence of ACPA in ACPA-positive RA serum. The quantitation of NETosis-derived products, such as cell-free nucleosomes in serum, may be a useful complementary tool to discriminate between healthy controls and RA cases.

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Introduction

A novel feature of the biology of polymorphonuclear granulocytes (PMNs) is their ability to generate neutrophil extracellular traps (NETs) [1] via a distinct process of cell death termed NETosis [2]. NETs consist of extruded chromosomal DNA decorated with granular components that include antimicrobial peptides and proteases. The molecular pathways leading to NETosis encompass calcium mobilization, generation of reactive oxygen species (ROS), nuclear delobulation involving the enzymatic activities of myeloperoxidase (MPO) and neutrophil elastase (NE), and chromatin modification via the citrullination of histones by peptidyl arginine deiminase 4 (PAD4) [2-6].

A number of studies have implicated NETs in the etiology of autoinflammatory or autoimmune conditions such as preeclampsia, Felty syndrome, systemic lupus erythematosus (SLE), multiple sclerosis, and, most recently, rheumatoid arthritis (RA) [7-13]. In the context of RA, these findings are especially interesting, as NETs have been proposed to contribute to the generation of anti-citrullinated protein antibody (ACPA) autoantigens, and may also be a target for autoantibodies [13,14]. PMNs isolated from RA patients showed an increased propensity to undergo spontaneous and LPS-induced NETosis, which was in part mediated by TNF and IL-17 and could be inhibited by blocking NADPH oxidase or PAD4. Whereas the citrullinated autoantigens vimentin and α-enolase were expressed on NETs from RA PMNs, antibodies to the former were able to induce NET formation by healthy control PMNs [13,14].

As we had previously detected significantly increased concentrations of cell-free DNA in the sera of RA patients compared with healthy controls, we were intrigued whether the provenance of this material involved NETosis [15]. The premise for the current investigation was that a link between circulating cell-free DNA levels and NETs has previously been made in a number of conditions, including preeclampsia, sepsis, cancer, thrombosis, or even storage of blood-transfusion products [16-19].

In view of these findings and reports on the complex involvement of neutrophil NETs in autoimmunity, we sought to investigate the NETotic response of PMNs in RA, with particular regard to the underlying signal-transduction cascade, and whether the products of overt NETosis could be diagnostically useful.

Materials and methods

Human subjects

All patients fulfilled the American College of Rheumatology classification criteria for RA, or for systemic lupus erythematosus, respectively. Healthy volunteers, matched for gender and age, were recruited at the hospitals or at the Blood Bank of the Swiss Red Cross, Basel. Inclusion criteria for healthy controls were fair general condition, age ≥28 and ≤70 years, and for blood donors fulfilling national criteria for blood donation. Exclusion criteria were current or previous systemic autoimmune disease, asthma and reconvalescence after major illness, surgery, current medication with corticosteroids, immunosuppressive agents, and malignant neoplasia or chemotherapy within 5 years before recruitment for the study. RA cases had a DAS ≥2.6, were from age ≥27 to ≤70 years, and had no other systemic autoimmune disease, including ankylosing spondylitis and psoriatic arthritis.

Exclusion criteria were corticosteroids ≥40 mg equivalent of prednisone daily, and those mentioned earlier for healthy controls. Informed, written consent was obtained from all subjects in the study, which was approved by the Cantonal Ethical Review Boards of Aargau-Solothurn and Basel/Basel-Land, Switzerland.

Preparation of plasma and serum

Plasma and serum were collected and processed as described previously [15]. Samples were studied immediately or stored at −80°C until analysis.

Cell isolation

PMNs were isolated with Dextran-Ficoll density centrifugation [8]. Cell viability was 96% to 98%, with a purity of >95% PMNs. Neutrophils seeded in 24-well plates were allowed to settle for 1 hour at 37°C under 5% CO_2 before further experimentation.

Cell-free DNA isolation and quantification

Cell-free DNA extracted from 850 μl plasma or serum by using the QIAamp Circulating Nucleic Acid Kit (Qiagen) was quantified by TaqMan real-time PCR (StepOne Plus Real-Time PCR System, Applied Biosystems) specific for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene [15].

Detection of neutrophil elastase (NE), myeloperoxidase (MPO), and cell-free nucleosomes

The concentrations of neutrophil elastase (NE) and myeloperoxidase (MPO) were measured with sandwich ELISA (Elastase/a1-PI Complex ELISA Kit, Calbiochem) and the human MPO ELISA Kit, Hycult Biotech, respectively. Nucleosomes were measured by using the Human Cell Death Detection ELISA^{PLUS} (Roche Diagnostics). Cell-culture supernatants were incubated with DNaseI (10 U for 5 minutes) (Roche Diagnostics) before analysis [20].

MPO/DNA complex detection

MPO is present on extruded NETs. To detect such structures, NET-associated MPO/DNA complexes were quantified by using a modified capture ELISA [21]. In brief, NET-associated MPO in serum or culture supernatant

was captured by using the coated 96-well plate of the human MPO ELISA Kit, (Hycult Biotech), after which the NET-associated DNA backbone was detected by using the detection antibody of the Human Cell Death Detection ELISA^{PLUS} (Roche Diagnostics).

PAD4/DNA-complex detection

To detect the presence of PAD4 on extruded NETs in culture supernatants after spontaneous NETosis, cell-free PAD4/DNA complexes were quantified by using a modified capture ELISA, akin to that described for MPO earlier. In brief, cell-free PAD4 was captured by using the coated 96-well plate of a commercial human PAD4 ELISA (USCN Life Science, Inc.), and associated DNA was detected by using Human Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics).

ROS generation analysis

ROS was measured by using a 2′,7′-dichloro dihydrofluorescein diacetate (DCFH-DA) assay [22]. The 5×10^5 cells in a final volume of 500 μl were incubated for 30 minutes with 25 μ*M* DCFH-DA. Fluorescence was measured with flow cytometry (FACSCalibur; BD Biosciences).

Fluorescence and scanning electron microscopy

The 5×10^4 cells isolated PMNs seeded on poly-L-lysine-coated coverslips (BD Biosciences) were stimulated with phorbol-12-myristate-13-acetate (PMA) for 90 minutes and dehydrated with a graded ethanol series (30%, 50%, 70%, 100%) [8], coated with 2-nm platinum, and analyzed with a Nova NanoSEM 230 scanning electron microscope (FEI). PMNs were incubated for 10 minutes with 5 μ*M* Sytox Green dye (Invitrogen Life Technologies) for assessment of NETs with an Axiovert fluorescence microscope (Carl Zeiss) coupled to a Zeiss AxioCam color CCD camera (Carl Zeiss) [8,23].

Immunohistochemical staining and quantification of NETs

The 5×10^4 isolated PMNs were seeded on poly-L-lysine-coated glass coverslips (BD Biosciences) in tissue-culture wells and allowed to settle before stimulation, as described earlier. Coverslips were rinsed with ice-cold HBSS and the cells fixed with 4% paraformaldehyde and blocked overnight (HBSS with 10% goat serum, 1% BSA, 0.1% Tween20, and 2 m*M* EDTA) at 4°C. NETs were detected with rabbit anti-NE (Abcam), rabbit anti-MPO (Dako), two different rabbit anti-PAD 4 (Abcam), mouse anti-PAD4 (Abcam), mouse anti-histone H1 + core proteins (Millipore), and rabbit anti-citrullinated histone H3 (citH3, Abcam). Secondary antibodies were goat anti-rabbit IgG AF555, goat anti-rabbit IgG AF488 (Invitrogen), and goat anti-mouse IgG AF647. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma), and NETs were visualized by using a Zeiss Axioplan 2 Imaging fluorescence microscope in conjunction with a Zeiss AxioCam MRm monochromatic CCD camera and analyzed with Axiovision 4.8.2 software (Carl Zeiss). A minimum of 20 fields (at least 1,000 PMNs) per case was evaluated for MPO/NE and DNA co-staining; nuclear phenotypes and NETs were counted and expressed as percentage of the total number of cells in the fields.

RA serum depletion, IgG purification, and quantification of NETs

After three washes with PBS, 200 μl protein G agarose (Pierce Biotechnology Inc.) was incubated with 200 μl ACPA + and ACPA- RA or control serum diluted in an equal volume of phenol red-free RPMI 1640 medium overnight at 4°C. The serum/protein G agarose mixture was centrifuged at 2,500 *g* for 5 minutes, and the supernatant (IgG-depleted serum) was carefully transferred into a new Eppendorf microcentrifuge tube. The protein G agarose pellet was gently washed 3 times with 500 μl ddH_2O, and the bound antibody was released by the addition of 50 μl 0.1 *M* glycine pH 2–3, and immediately equilibrated with 10 μl of 1 *M* Tris pH 7.5-9. All protein concentrations were determined with the MN Protein Quantification Assay (Macherey Nagel), and isolation of IgG was verified with Coomassie staining of SDS-PAGE.

Neutrophils from healthy donors ($n = 3$) were isolated and cultured for 2 hours in 96-well culture dishes (Thermo Fischer), supplemented with: serum, depleted serum, and purified IgG from ACPA-positive RA patients ($n = 3$),

Table 1 Demographics and patient population characteristics versus healthy blood donors

	Controls	RA	Statistics
Age	50.34 ± 1.5	53.03 ± 1.5	$P = 0.214$
Gender (F/M)	24/32	24/8	-
DAS28	n.a.	3.07 ± 1.12	-
Bone erosion (pos/neg)	n.a.	22/10	-
Serum ACPA (pos/neg)	n.a.	20/12	-
Serum RF (pos/neg)	n.a.	19/13	-
Serum ANA (pos/neg)	n.a.	21/11	-
ESR (mm/h)	n.a.	16.8 ± 13.1	-
CRP (mg/L)	n.d.	6.9 ± 5.2	-
PBMC (cells/μl)	1,961 ± 81.69	1513 ± 75.90	$P < 0.0001$
PMN (cells/μl)	3,641 ± 149.7	4575 ± 546.0	$P = 0.021$
Therapy (yes/no)	n.a.	31/1	-
DMARDs (yes/no)	n.u.	27/5	-
Biologics (yes/no)	n.a.	30/2	-

F, female; M, male; DAS28, disease activity score; n.a., not applicable; pos, positive; neg, negative; ACPA: anti-citrullinated protein antibodies; RF, rheumatoid factor; ANA, antinuclear antibody; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; n.d., not determined; PBMC, peripheral blood mononuclear cell; PMN, polymorphonuclear leukocyte; DMARD, disease-modifying antirheumatic drug; n.u., not used.

ACPA-negative RA patients ($n = 3$), and healthy individuals ($n = 3$) to a final concentration of 100 μg/ml.

NETs were quantified after IHC staining with mouse anti-human MPO antibody (Abcam) and rabbit anti-human citH3 antibody (Abcam), or the respective isotype controls, followed by incubation with goat anti-mouse IgG AF555 and goat anti-rabbit IgG AF488 (Invitrogen). DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma). NETs were visualized by using an Olympus IX81 motorized epifluorescence microscope (Olympus) in conjunction with an Olympus XM10 monochromatic CCD camera (Olympus) and analyzed with the Olympus CellSens Dimension software (Olympus). A minimum of 20 fields at 10× magnification (at least 500 to 1,000 PMNs) per case was evaluated for MPO/citH3 and DNA co-staining through ImageJ analysis software (NIH Image Processing); nuclear phenotypes and NETs were determined, counted, and expressed as percentage of the total area of cells in the fields [24].

Protein isolation and Western blot analysis

Total protein was isolated with NucleoSpin TriPrep kit (Macherey-Nagel) from 3×10^6 PMNs. Proteins from the nuclear and cytoplasmic fractions were isolated by using

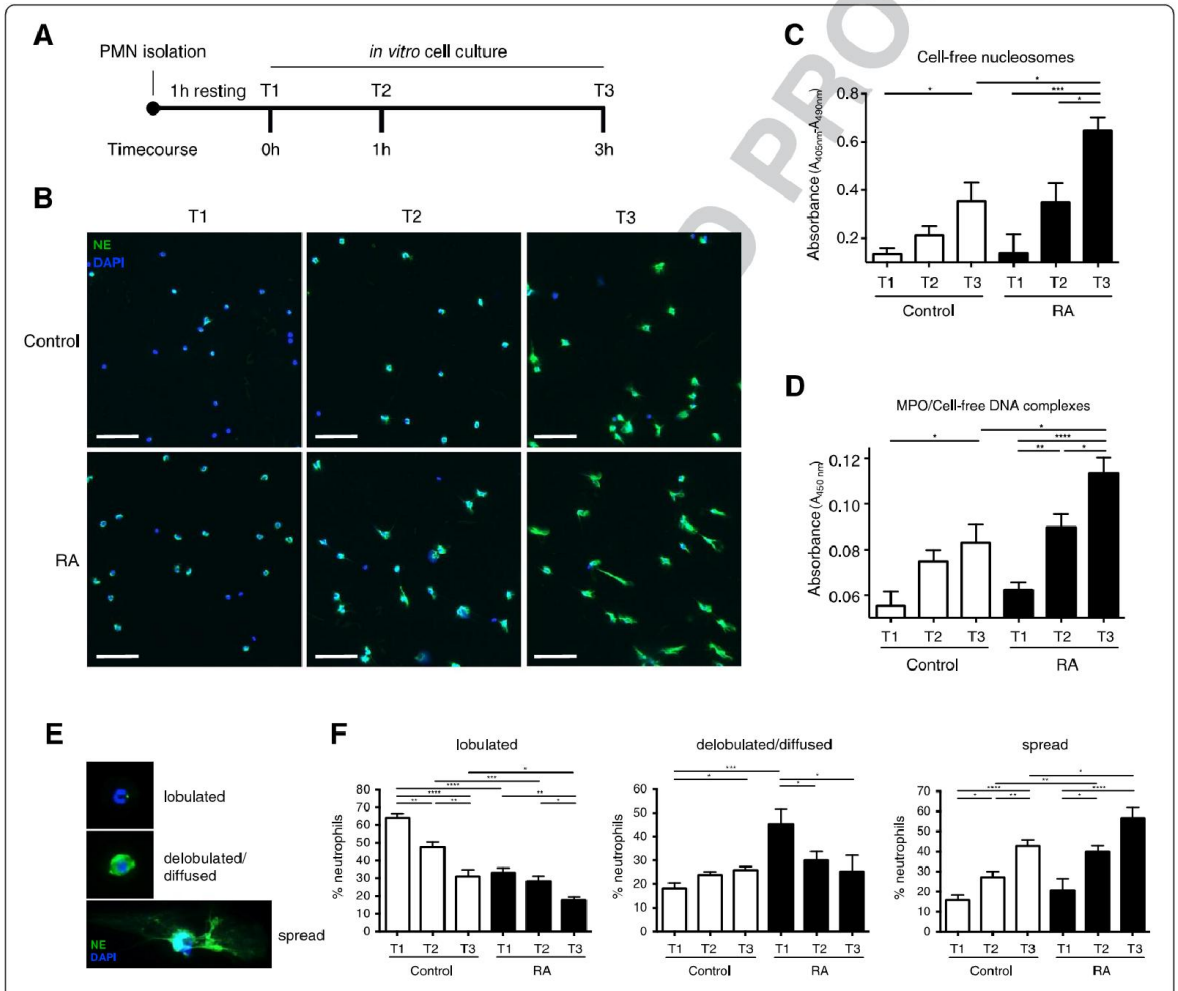

Figure 1 RA-derived PMNs exhibited increased spontaneous NETosis and elevated levels of NET-component release. (A) Schematic representation of the time-course design for studying *in vitro* spontaneous NET release. **(B)** Detection of *in vitro* NETosis by immunohistochemistry for neutrophil elastase (NE) (green) and DAPI (blue). Magnification: 20×; scale bar, 50 nm. **(C)** Concentration of cell-free nucleosomes in PMN culture supernatant by ELISA. **(D)** Quantification of NET-associated MPO/DNA complexes. These assays indicate that more-rapid and extensive progression of NET formation is observed in RA versus control PMNs. **(E)** Changes in PMN nuclear morphology during NETosis detected with immunohistochemistry for NE and DAPI. **(F)** Steady-state (T1) RA-derived PMNs exhibited a greater proportion of delobulated/diffused cells, and progressed rapidly to a NETotic spread phenotype during *in vitro* culture. Data are presented as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. All data are representative of at least six independent experiments.

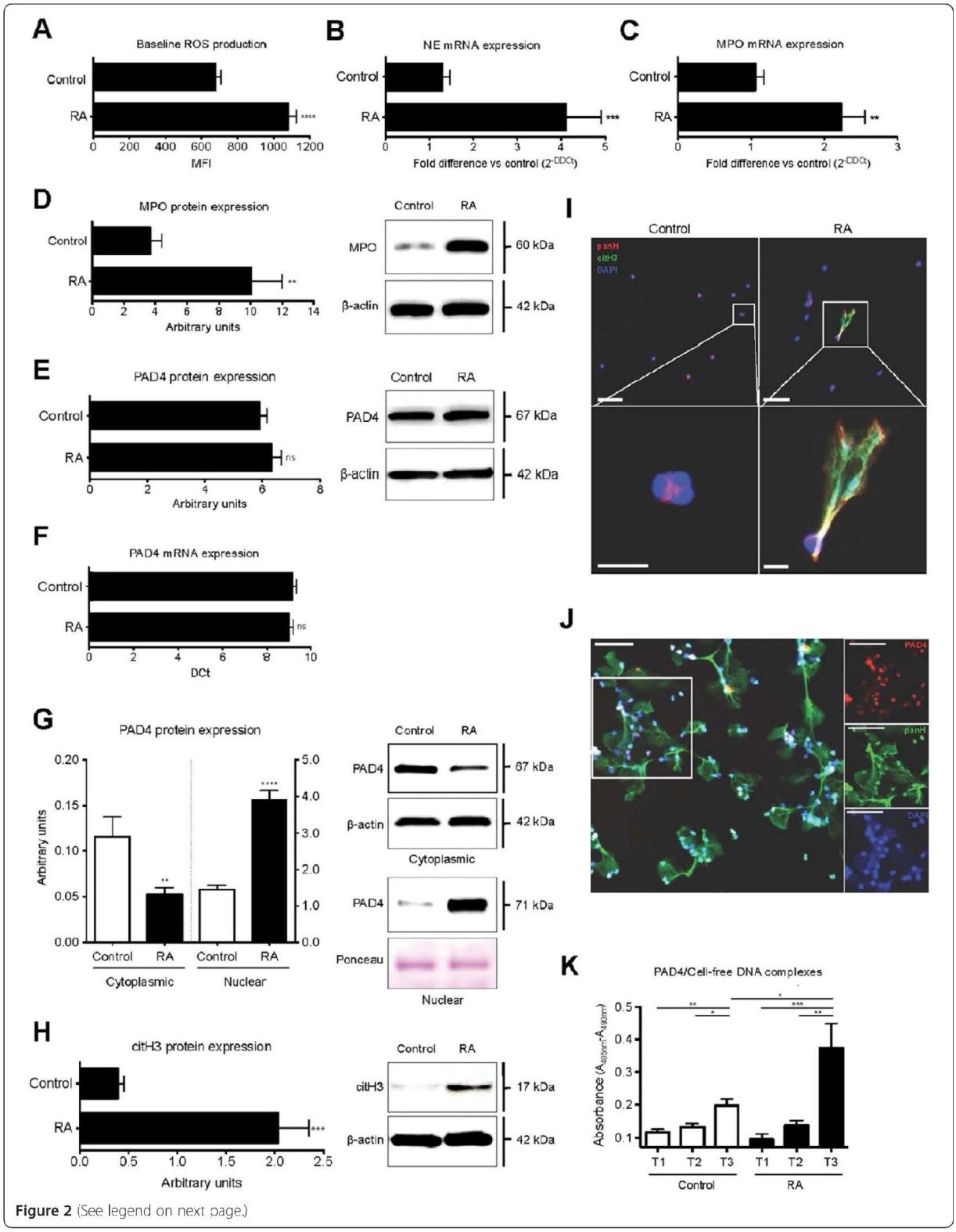

Figure 2 (See legend on next page.)

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Figure 2 Increased expression of NET-associated signaling elements and nuclear localization of PAD4, citrullination of histone H3 in RA-derived PMN, and possible extrusion of PAD4 on NETs. Baseline ROS levels, measured with flow cytometry, were higher in RA-derived PMNs than in control PMNs **(A)**. Quantitative real-time PCR analysis of NE mRNA **(B)** and MPO mRNA expression **(C)**, as well as Western blot analysis of MPO protein levels **(D)**, indicated that the levels of these two components required for NETosis were elevated in RA-derived PMNs. Total PAD4 protein **(E)** or PAD4 mRNA expression levels **(F)** did not indicate any significant difference between control and RA-derived PMNs. **(G)** Quantification of PAD4 protein levels in cytoplasmic and nuclear fractions of PMNs from healthy controls and RA patients. Nuclear levels of PAD4 were significantly increased in RA patients, whereas the cytoplasmic levels were lower compared with the healthy control PMNs. **(H)** Elevated citrullinated histone H3 levels in RA PMN extracts detected with Western blot. **(I)** Co-localization of citrullinated histone H3 (green) with histone components detected with a pan-histone antibody (red), spread over the entire NET surface (blue). Magnification: upper panel 20×, scale bar, 50 nm; lower panel 63× scale bar, 10 nm. **(J)** Extracellular localization of PAD4 (red) on extruded NETs by multicolor fluorescent immunohistochemistry. NET DNA is stained blue (DAPI), and histones (panH) are stained green. Magnification, 20×; scale bar, 50 nm **(K)**. Detection of PAD4/cell-free DNA complexes in the culture supernatants of isolated PMN undergoing spontaneous NETosis. Higher levels of these complexes were detected in RA-derived PMN cultures. Time points correspond to those of Figure 1A. Data are represented as mean ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; n.s., statistically not significant. All data are representative of at least six independent experiments.

the Nuclear and Cytoplasmic Protein Extraction Kit (Thermo Scientific). Western blotting was performed by using AnykD Mini-PROTEAN TGX Gels (Biorad) and nylon/nitrocellulose membranes (Biorad). Primary and secondary antibodies used were: rabbit anti-PAD4 (Abcam), rabbit anti-MPO (Cell Signalling), mouse anti-β-Actin (Sigma), goat anti-Mouse and/or anti-Rabbit, human anti-HRP (Southern Biotech). HRP activity was detected by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Equal loading was verified by using beta-actin or histone H3, when appropriate. Western blots of citrullinated H3 (citH3) protein were performed according to Shechter *et al.* [25]. Densitometric analysis and protein quantification of the Western blots was performed by using the ImageJ software.

RNA isolation and quantitative real-time PCR

Total RNA was isolated by using RNeasy Mini Kit (Qiagen). TaqMan real-time quantitative RT-PCR was performed by using the Applied Biosystems StepOne Plus cycler (Applied Biosystems) and TaqMan Gene Expression Assay primer/probe sets (Applied Biosystems) for NE (HS00236952_m1), MPO (HS00924296_m1), PAD4 (HS00202612_m1), and β_2-microglobulin (HS99999907_m1). Data were normalized by using the housekeeping gene B2M, after a selection procedure involving six different endogenous reference genes, as suggested in the MIQE guidelines [26]. Relative values were calculated with 2^{-DDCt} analysis [27].

Statistical analysis

All data are presented as mean ± SEM. Descriptive statistics for continuous parameters consisted of median and range, and categoric variables were expressed as percentages. Comparisons between patients and healthy controls were by the Mann–Whitney *U* test with a Welch posttest correction. Statistical significance in multiple comparisons was by one-way analysis of variance (ANOVA) with a Dunn posttest correction. $P < 0.05$ was considered statistically significant.

Receiver operating characteristic (ROC) curves were calculated, and the area under the curve (AUC) with corresponding standard errors of means was calculated. Data were processed in GraphPad Prism version 5.0b for MacOSX (GraphPad Software Inc.). Analysis of covariance (ANCOVA) was conducted with SPSS version 21.0 statistical software (IBM). Additional professional statistical assistance was provided by A. Schoetzau, Basel.

Results

RA-derived PMN exhibit increased spontaneous NETosis

Details of the RA study group and healthy control group are described in Table 1 and Additional file 1. T1

As in very recent observations [13], we observed that RA-derived PMNs underwent greater degrees of NETosis than did control PMNs *in vitro* (data not shown). To study this facet in more detail, we examined the kinetics of spontaneous NET extrusion, for which purpose, PMNs isolated from peripheral blood samples were allowed to settle for 1 hour and then cultured for a period of up to 3 hours *in vitro* (Figure 1A), NETs being detected by immunohistochemistry for neutrophil elastase (NE) and DAPI (4′,6-diamidino-2-phenylindole) (Figure 1B). In addition, we quantitatively assessed the degree of *in vitro* NETosis in these cultures by determining the concentration of cell-free nucleosomes in the respective supernatants (Figure 1C), specifically their association with myeloperoxidase (MPO), indicative of the NETotic origin of this material [2,21] (Figure 1D). We also measured NET-associated MPO enzymatic activity by using tetramethylbenzidine (TMB) as a substrate (data not shown). The results clearly indicate that RA-derived PMNs generated NETs more rapidly, to a greater magnitude, and more extensively than did control healthy PMNs, which was particularly evident at the 3-hour stage of *in vitro* culture (Figure 1B to D). Accounting for variances in PMN counts, the difference between the healthy control and RA subjects remained highly significant F1

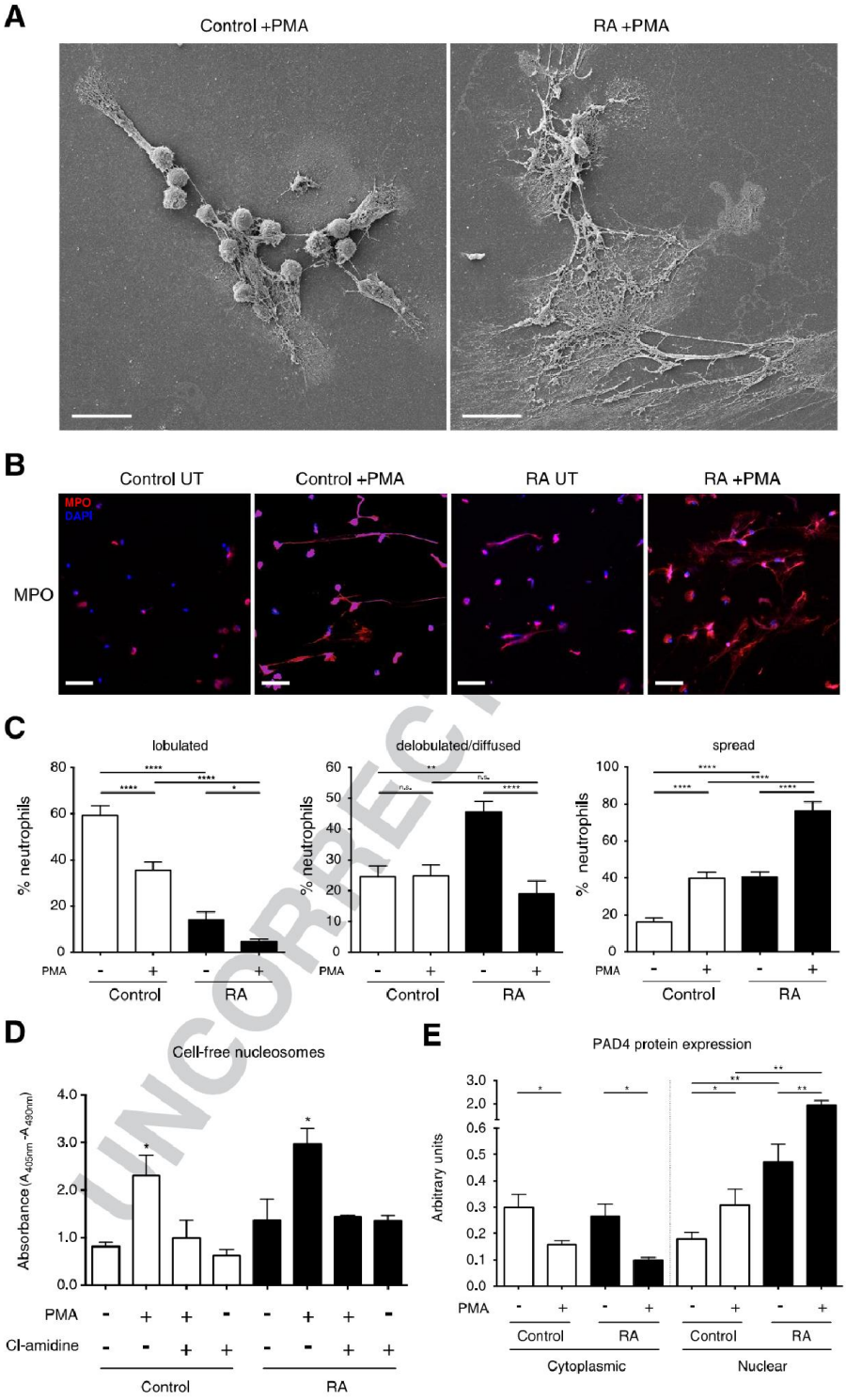

Figure 3 (See legend on next page.)

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Figure 3 Increased NETotic response of RA-derived PMNs to PMA. (A) Scanning electron micrographs of NETs induced by PMA (25 n*M*) indicate the excessive NETotic response of RA-derived PMN. Scale bar, 20 nm. **(B)** Assessment of NETs induced by PMA treatment by fluorescent immunohistochemistry for MPO (red) and DAPI (blue), indicating the increased response of RA PMNs to PMA (25 n*M*). Magnification, 20x; scale bar, 50 nm. **(C)** Analysis of the nuclear phenotype indicated a vast decrease in delobulated/diffused RA PMN nuclei after treatment with PMA and rapid increase in the NETotic-spread phenotype. **(D)** Release of cell-free nucleosomes after PMA treatment is abrogated by chloramidine, a PAD4 inhibitor. **(E)** Increased nuclear localization and concomitant decrease in cytoplasmic PAD4 protein levels after PMA treatment, with a clear tendency for an increased responsiveness to the PMA stimulus by RA PMN. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; n.s., statistically not significant. All data are representative of at least four independent experiments.

in an analysis of covariance (ANCOVA) of the nucleosome assay ($P < 0.001$).

During NETosis, the morphology of the PMN nucleus changes from the familiar lobulated to a diffused and then to a spread phenotype (Figure 1E) [28]. By examining and enumerating these features, it was observed that at baseline (T1), the nuclei from healthy control PMNs were predominantly lobulated, whereas in this instance, the majority of RA-derived PMN nuclei exhibited a delobulated or diffused nuclear phenotype (Figure 1F). In RA-derived PMNs, this delobulated population decreased over time, giving rise to NETotic cells with a spread phenotype (Figure 1F). In contrast, in normal PMNs, we noted a steady progression in the proportion of delobulated cells (Figure 1F). The spontaneous progression of nuclei to the NETotic-spread phenotype was more pronounced in RA than in normal PMNs, a feature most evident after 3 hours (T3) (Figure 1F).

RA-derived PMNs demonstrate increased expression of NET-associated signaling elements, nuclear localization of PAD4, and augmented H3 citrullination

NETosis has been shown to depend on a number of biochemical signaling elements, among which are the generation of ROS by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the action of NE in combination with MPO, and histone citrullination by PAD4 [2,3,5]. RA-derived PMNs exhibited increased basal intracellular ROS levels (Figure 2A), as well as increased levels of NE (Figure 2B) and MPO (Figure 2C and D), as determined with real-time PCR and/or Western blotting.

Surprisingly, neither PAD4 mRNA expression nor PAD4 levels in total cellular protein showed any discernible difference between RA and control PMNs (Figure 2E and F, respectively). Because PAD4 translocates to the nucleus on PMN activation [4,29], where it citrullinates histone proteins, such as H3, we examined its presence in nuclear and cytoplasmic PMN fractions. Compared with control PMNs, PAD4 was preferentially located in the nucleus of RA-derived PMNs (Figure 2G). The nuclear presence of PAD4 was associated with increased citrullinated histone H3 (citH3) levels with Western blot analysis in PMNs from RA cases compared with controls (Figure 2H). Furthermore, citrullinated histone H3 could be readily detected on NET structures (Figure 2I).

Potential extracellular localization of PAD4 on NETs

Because we observed elevated nuclear translocation of PAD4 in RA PMNs, we examined whether this enzyme is extruded into the extracellular environment during NETosis. Unfortunately, the visualization of such an event by fluorescence immunohistochemistry proved to be difficult with a variety of commercially available antibodies, and we obtained only rudimentary evidence for the presence of PAD4 on NETs by this means (refer to Figure 2J).

We were, however, able to detect PAD4/cell-free DNA complexes readily in culture supernatants from isolated PMNs, the levels of which were increased in cases with RA (Figure 2K). It is, therefore, quite probable that PAD4 is associated with NETs structures after aberrant NETosis in RA.

PMNs from RA patients present an enhanced NETotic response to PMA, and normal PMNs are strongly affected by RA serum and synovial fluid

In autoinflammatory or malignant conditions, such as SLE or cancer, an elevated NETotic response of PMNs to an external activation signal has been shown [9,13]. In our experiments, we noted that when RA-derived PMNs were treated with PMA, they responded far more vigorously with regard to NETosis than did controls, as detected by SEM and fluorescence microscopy (Figure 3A and B, respectively).

In addition, morphometric assessment indicated that RA-derived PMNs exhibited a larger decrease in cells with a delobulated phenotype and a greater progression toward a NETotic-spread nuclear phenotype than control PMNs (Figure 3C), a feature accompanied by excessive release of cell-free nucleosomes in culture supernatants (Figure 3D). PMA appears to activate PAD4, as it enhanced translocation from the cytoplasm to the nucleus (Figure 3E). The stimulatory effect of PMA on the release of nucleosomes into the supernatant was abrogated by Cl-amidine, a chemical inhibitor of PAD4, indicating that PAD4 signaling is necessary for NETosis induced by PMA [4,30] (Figure 3D). These data indicate that PMNs in RA are susceptible to increased NETosis after stimulation by a secondary signal, such as that mediated by PMA.

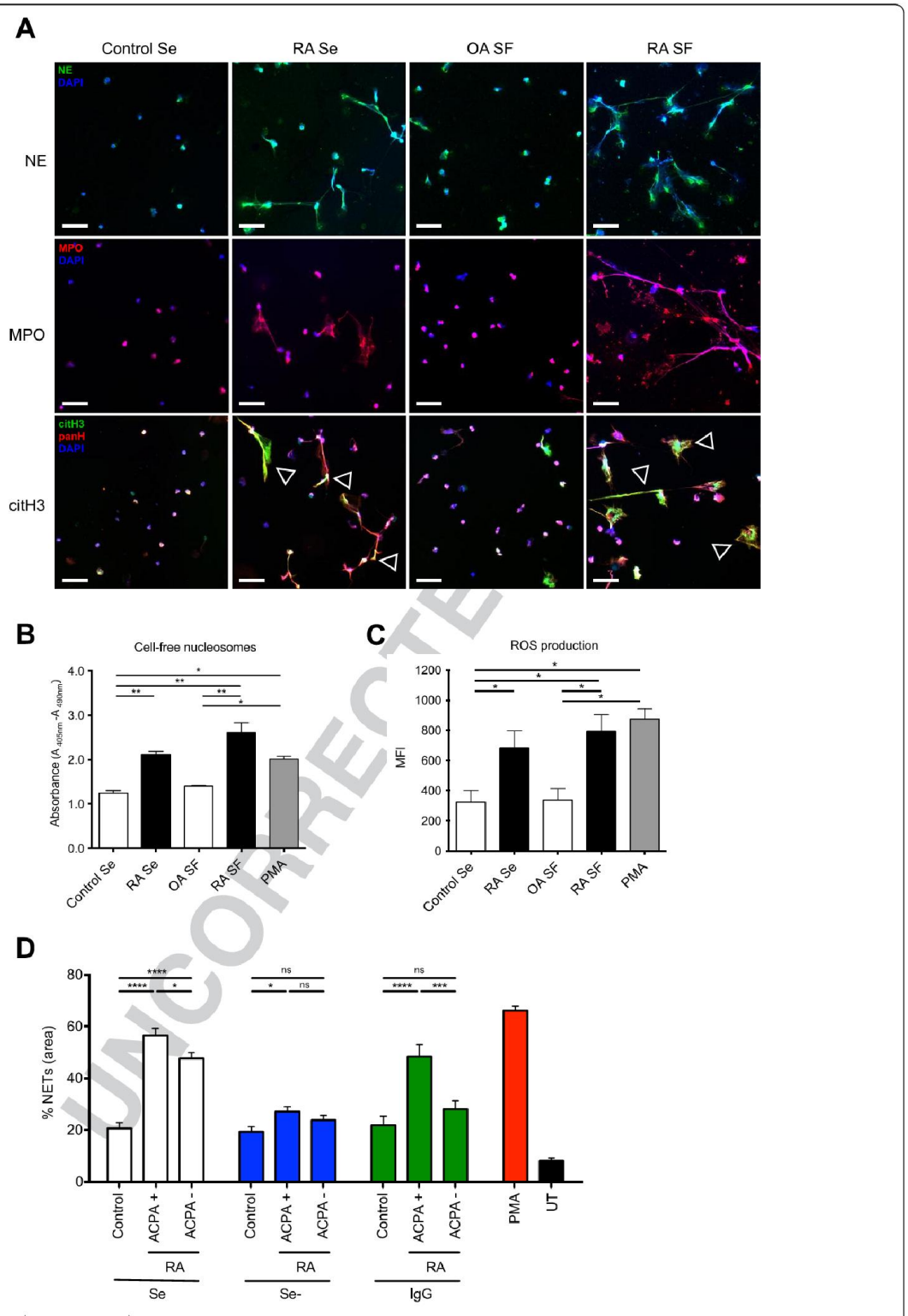

Figure 4 (See legend on next page.)

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Figure 4 Influence of RA serum and synovial fluid on normal PMNs. (A) Incubation of healthy donor PMNs with serum (Se) from healthy donors or from RA patients, synovial fluid (SF) from patients with noninflammatory osteoarthritis (OA) or synovial fluid from RA patients. Immunohistochemical analysis of four main components of NETs (NE, MPO, PAD4, and citH3) revealed that RA-derived serum and SF enhanced NETosis in normal PMN compared to healthy control serum or noninflammatory OA SF. PAD4 (white arrowheads) and citH3 (empty arrowheads) colocalize with unmodified histones on NETs. Magnification, 20×; scale bars, 50 nm. **(B)** Release of cell-free nucleosomes during *in vitro* culture by PMNs from healthy controls incubated with control serum, RA serum, OA SF or RA SF, or PMA. Data are represented as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$. **(C)** Increased ROS generation during *in vitro* culture by PMNs from healthy controls incubated with control serum, RA serum, OA SF or RA SF, or PMA. Data are presented as mean ± SEM. **(D)** Release of cell-free nucleosomes during *in vitro* culture by PMNs from healthy controls, ACPA-positive and ACPA-negative RA patients incubated with serum (Se +), IgG-depleted serum (Se -), or serum reconstituted with their respective eluted IgG, or with PMA. Data are presented as mean ± SEM. $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$; n.s., statistically not significant. All data are representative of at least four independent experiments in Figures 4A–C, and 3 in 4D.

Because SLE sera and RA sera and synovial fluid (SF) have been shown to confer an increased NETotic response [9,13], we examined whether RA-derived sera or SF exerted similar effects on normal PMNs. As noninflammatory controls, we used healthy serum or osteoarthritis SF. Both RA sera and SF induced a pronounced increase in NETosis, which was paralleled by an increase in the nucleosome content of the supernatant (Figure 4A,B), as well as in ROS production (Figure 4C) when compared with healthy serum or osteoarthritis SF, respectively.

To assess whether antibodies participate in the effects of RA serum on normal PMNs, we depleted IgG from serum of ACPA-positive and -negative RA patients and healthy controls. Compared with nondepleted sera, IgG depletion of both ACPA-positive and -negative sera markedly reduced NET induction to levels of normal serum (Figure 4D). Whereas the reconstitution of ACPA-negative IgG to serum did not increase NET formation significantly compared with controls, it was practically reversed to the original value in the ACPA-positive cases. This indicates a prominent role for ACPA in the induction of NETs in ACPA-positive RA, while suggesting that an alternative mechanism is responsible for the increased NETosis in ACPA-negative RA patients.

These data are in accordance with recent findings that RA-derived serum and SF induce NETosis in normal PMNs, and that ACPA and also IgM RF are to a large part responsible for this effect [13,14].

Serum from RA patients shows elevated levels of the principal components of NETs, indicating enhanced NET extrusion during clot formation, which has potential clinical utility

As we had previously observed increased levels of cell-free DNA (cfDNA) in RA sera [15], we determined whether this resulted from enhanced NETosis, and whether this could have diagnostic applications. cfDNA concentrations were indeed significantly higher in serum samples from RA cases compared with age-matched healthy control sera (Figure 5A). In parallel, the concentrations of cell-free nucleosomes, NE, and MPO were significantly elevated in RA serum compared with control sera (Figure 5B to D). The association of a significant fraction of MPO with markedly elevated cell-free nucleosomes in RA serum, which constitute a main component of NETs (Figure 5E), clearly suggests that NETosis is indeed the source of nucleosome material present in RA serum [21].

Since there was no significant elevation of these parameters in simultaneously obtained plasma samples that were processed immediately, these data demonstrate a propensity for RA PMN to undergo increased NETosis during coagulation (Figure 5A to E).

To ascertain whether NET-associated serum components could be diagnostically useful, we conducted ROC analyses. For serum cell-free nucleosomes, this yielded the surprisingly high AUC value of 0.97 (see Additional file 2 and Figure 5F). Of interest is that no significant difference was found in this value regardless of whether the RA cases were ACPA positive or not (Figure 5G), although a slight trend for serum nucleosome levels was higher in ACPA-positive cases than in ACPA-negative cases (Figure 5H). The AUC for serum nucleosomes was significantly higher than for any of the other parameters examined (Figure 5I to K). With the cut-off set at 0.78, the ROC AUC translates into a sensitivity of 91%, with a specificity of 92% for differentiating between RA cases and healthy controls.

In contrast to RA cases, cell-free nucleosome serum values of 14 cases with SLE showed a slight, but statistically significant increase (Additional file 2). This translated into an ROC AUC of 0.7639 (see Additional file 3), which is below the clinically useful value for diagnostic purposes.

Discussion

Although PMNs figure prominently in the joint effusions and inflamed synovial tissue of RA patients [31], the potential roles of NETotic events in the pathophysiology of this disorder have only recently become a focus of attention [13,14]. These studies indicated that RA-derived PMNs were more prone to undergo NETosis, and that NETs themselves could contribute to the generation of auto-antigens (ACPAs) or be the target of auto-antibodies [13,14].

Our studies, performed independently at a time similar time to these, corroborate that NETosis is enhanced in

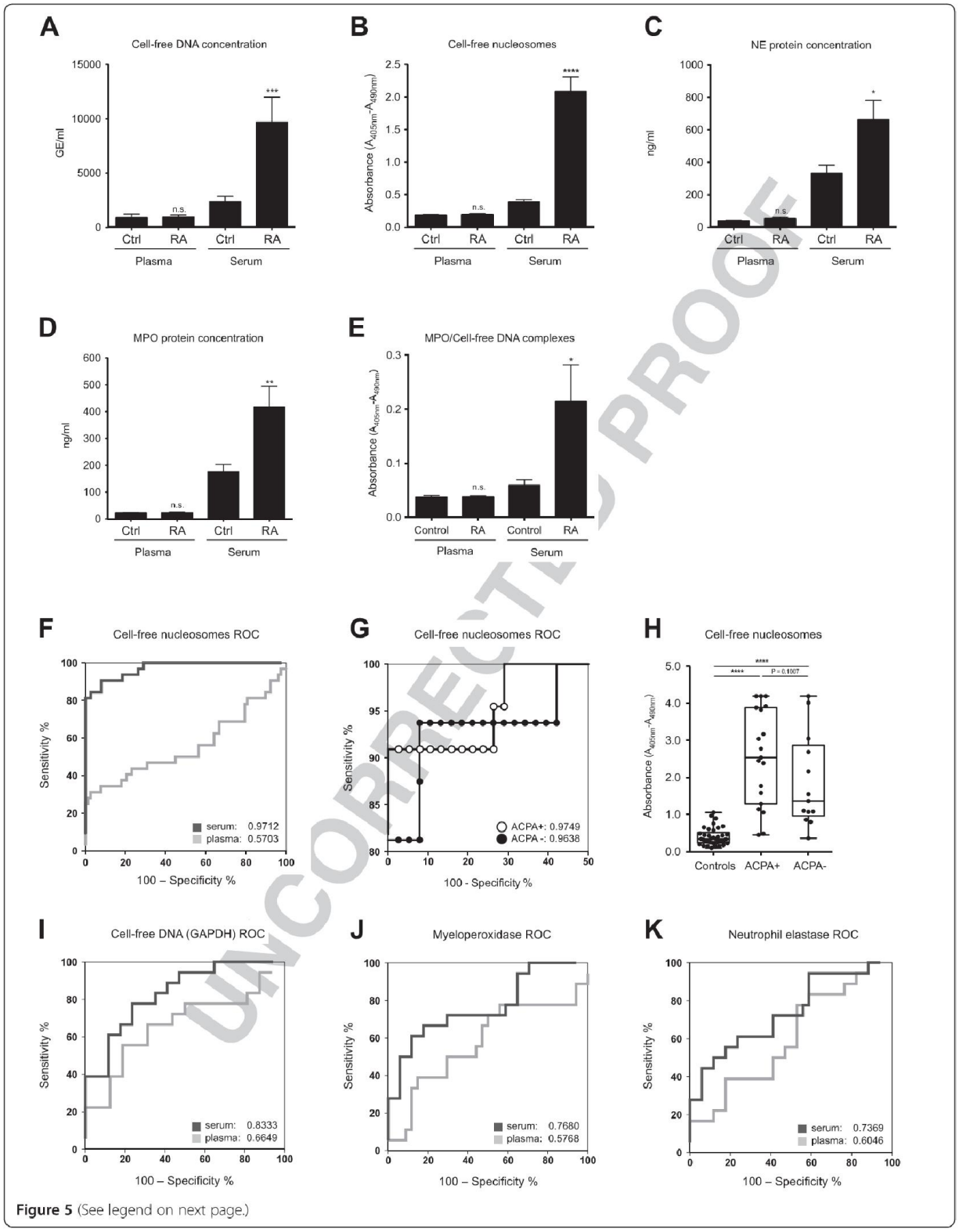

Figure 5 (See legend on next page.)

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Figure 5 Elevated serum levels of NET components in RA patients have potential clinical utility. (A) Cell-free DNA levels in plasma and serum from healthy matched blood donors ($n = 41$) and patients with RA ($n = 32$) determined with real-time PCR. **(B)** Cell-free nucleosome levels in plasma and serum from healthy donor controls and patients with RA, determined with ELISA. **(C)** Determination of NE protein concentrations in plasma and serum from healthy donors and patients with RA, as assessed with sandwich ELISA. **(D)** MPO concentrations in plasma and serum from healthy donors and patients with RA, as determined with sandwich ELISA. **(E)** NET-associated MPO/DNA complexes quantified by using a modified capture ELISA. In contrast to the serum levels, none of the plasma levels of these NET components attained statistical significance (Figure 5A to E). **(F)** ROC analysis of cell-free nucleosomes in serum of patients with RA and healthy controls. **(G)** Detail of cell-free nucleosome ROC curve with groups of ACPA + and ACPA- RA cases and **(H)** scatterbox and whisker plots with individual values for control, ACPA + and ACPA- groups. The ROC curve analysis of other NET components, cell-free DNA **(I)**, NE **(J)**, and MPO **(K)**, was not as conclusive as that for cell-free nucleosomes. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; n.s., statistically not significant.

RA, confirming a possible fundamental role of this phenomenon in the underlying etiology of RA. In addition, we extended these observations by examining for changes in the underlying signal-transduction cascade required for the induction of NETosis. The results show that the propensity of circulatory PMNs in RA patients to undergo NETosis is associated with elevations in members of this cascade, including increased intracellular ROS production, enhanced expression of NE and MPO, increased nuclear translocation of PAD4, and citrullination of histones, notably H3. Consequently, these and other key NETotic pathway elements [6] could serve as potential therapeutic targets for interventional strategies.

Furthermore, by examining kinetic changes during extended *in vitro* culture, different nuclear morphometric characteristics were observed in PMNs from RA cases, with a lower proportion of the classic lobulated phenotype, coupled with a much higher proportion of delobulated cells at the initial time point. Unlike in controls, in which an increase in this population was noted over time, this latter population decreased during *in vitro* culture in RA PMNs. RA PMNs also progressed more rapidly and extensively to a NETotic-spread phenotype than controls, a finding confirmed by analysis of culture supernatants for the products of NETosis.

Akin to that observed in an array of other pathologic conditions ranging from preeclampsia and SLE to cancer and RA [8,9,12,13,17], PMNs from RA patients exhibited an increased response to further stimulation (for instance, by treatment with IL-8, the phorbol ester PMA, or with LPS). This response is in part mediated via the action of PAD4, as the effect of PMA could be significantly reduced by treatment with Cl-amidine, an inhibitor of PAD4 [30]. In addition, PMA treatment led to an increased nuclear localization of this enzyme, where it presumably could catalyze a more-extensive citrullination of histone proteins, thereby speeding the induction of NETosis.

Although our data are preliminary, they do suggest that PAD4 is extruded onto the NETs during NETosis, as detected with ELISA technology and, to a lesser extent, by fluorescence microscopy. Such an occurrence would have important implications for the development of anti-PAD4 autoantibodies observed in cases with RA [32]. Because the presence of such antibodies precedes the development of RA, our data provide further support that NETs may contribute to the underlying etiology of RA, and may be a relatively early event. As the presence of such anti-PAD4 antibodies has been shown to enhance the enzymatic activity of PAD4 in an extracellular environment by reducing the calcium requirement [33], their combination with NETs-associated PAD4 could lead to prodigious quantities of citrullinated autoantigens. In addition, the extracellular presence of PAD4 on NETs may further promote the prodigious generation of citrullinated antigens, because molecular structures involving the attachment of enzymes to DNA lattices have been shown to increase their catalytic activity enormously, and thereby form the basis of nano-machines or nano-factories, generating such autoantigens [34].

Although these findings must be verified, and it remains to be ascertained whether extracellular NETs-associated PAD4 is active, these data do support and extend recent reports indicating that NETs can be a source for citrullinated autoantigens, and that they react with ACPA or anti-PAD4 antibodies [13,14,35]. Taken together, these data provide further evidence concerning a key role for PAD4 in the underlying etiology of RA, and offer a potential explanation for the efficacy of PAD4 inhibitor chloramidine in reducing disease symptoms in collagen-induced rat and murine models for RA [36].

It recently was reported that ACPA or IgM RF led to potent increases in NET formation compared with control IgG [13]. In our IgG-depletion experiments on ACPA-positive and -negative RA cases, we observed a marked reduction of NET induction to control levels in both cases, whereas reconstitution of serum with IgG gained from depletion almost completely restored NET induction in the ACPA-positive cases. However, in the ACPA-negative cases, no significant increase followed reconstitution. These results support the notion that ACPAs are important inducers of NET formation in ACPA-positive RA cases, and indicate that other mechanisms, such as IgG complexes similar to those involved in NET induction in SLE [8], are operative in ACPA-negative RA. Both mechanisms could lead to a common distal mechanism of induction of arthritis.

The observation that the coagulation of blood samples from RA patients during serum preparation triggers extensive NETosis, evident by increased concentrations of cell-free DNA, nucleosomes, or nucleosome/MPO complexes, may have unexpected clinical ramifications. With a sensitivity of 91% and a specificity of 92%, it is possible that the assessment of serum cell-free nucleosomes may serve to distinguish suspected RA patients from healthy controls with a high degree of specificity. It is of interest that this aspect was not significantly influenced by the ACPA status of the RA patients. As such, this assay may be a useful complementary test to perform in conjunction with current ACPA or RF assays, not only to extend diagnostic accuracy, but also to assist in detecting RA in cases that are either ACPA or RF negative. Similar NET induction by ACPA-positive and -negative RA sera and its abrogation by IgG depletion, as discussed earlier, supports the functional aspect of the nucleosome measurement in RA serum.

In a preliminary series of SLE sera, a small and not statistically significant increase of cell-free nucleosomes over normal controls was observed, indicating a slightly elevated propensity for PMNs from SLE patients to undergo NETosis. This was, however, nowhere near the range seen in RA cases, and failed to reach an ROC AUC considered to be clinically relevant. These aspects must be validated in larger multicenter studies.

Conclusions

In summary, our data reaffirm an intricate relation between NETosis and the etiology of RA, because the signaling elements associated with NET extrusion are significantly enhanced to promote NETosis in RA patients compared with healthy controls. Both ACPA-positive and -negative serum lost the ability to induce NETosis on depletion of IgG molecules, but reconstitution of NET induction was seen only with IgG molecules obtained from ACPA-positive serum. The assessment of NETosis-derived products in the sera of suspected RA cases may offer a novel complementary diagnostic tool.

Additional files

Additional file 1: Figure S1. Neutrophil, peripheral blood leukocyte counts, and age distribution in RA cases and control cohorts.

Additional file 2: Table S1. AUC values with corresponding 95% confidence intervals, *P* values and standard errors for serum cell-free nucleosomes and the three different parameters, which were analyzed individually by logistic regression.

Additional file 3: Figure S2. Elevated serum levels of NET components, in RA patients, have potential clinical utility.

Abbreviations

ACPA: anti-citrullinated peptide antibody; AUC: area under the curve; citH3: citrullinated histone; DAPI: 4′,6-diamidino-2-phenylindole; DCFH-DA: 2′,7′-dichlorodihydro fluorescein diacetate; ELISA: enzyme-linked immunosorbent assay; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HBSS: Hanks balanced salt solution; MPO: myeloperoxidase; NADPH: nicotinamide adenine dinucleotide phosphate; NE: neutrophil elastase; NET: neutrophil extracellular trap; PAD4: peptidyl arginine deiminase 4; PMA: phorbol-12-myristate-13-acetate; PMN: polymorphonuclear granulocyte; RA: rheumatoid arthritis; RF: rheumatoid factor; ROC: receiver operator characteristic; ROS: reactive oxygen species; SLE: systemic lupus erythematosus.

Competing interests

A patent filing has been submitted by the University of Basel and the Cantonal Hospital Aarau for tests developed during this research. We have no other interests to declare.

Authors' contributions

CSC and SG carried out molecular, cellular studies, immunoassays, and assisted with the manuscript draft. SG conducted *in vitro* depletion experiments, performed immune histochemical analyses, morphometric analyses, statistical analysis and contributed to writing the manuscript. UW and AB participated in the design of the study, assisted with stratification of patients and healthy donor controls, and assisted with the manuscript draft. SH and PH conceived the study, participated in its design, coordination and wrote the manuscript. All authors read and approved the final manuscript.

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REVIEW

Modulation of neutrophil NETosis: interplay between infectious agents and underlying host physiology

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Abstract The ability of neutrophils and other leucocyte members of the innate immune system to expel their DNA into the extracellular environment in a controlled manner in order to trap and kill pathogenic microorganisms lead to a paradigm shift in our understanding of host microbe interactions. Surprisingly, the neutrophil extracellular trap (NET) cast by neutrophils is very wide and extends to the entrapment of viruses as well as multicellular eukaryotic parasites. Not unexpectedly, it has emerged that pathogenic microorganisms can employ a wide array of strategies to avoid ensnarement, including expression of DNAse enzymes that destroy the lattice backbone of NETs. Alternatively, they may use molecular mimicry to avoid detection or trigger events leading to the expression of immune modulatory cytokines such as IL-10, which dampen the NETotic response of neutrophils. In addition, the host microenvironment may contribute to the innate immune response by the production of lectin-like molecules that bind to bacteria and promote their entrapment on NETs. An example of this is the production of surfactant protein D by the lung epithelium. In addition, pregnancy provides a different challenge, as the mother needs to mount an effective response against pathogens, without harming her unborn child. An examination of these decoy and host response mechanisms may open the path for new therapies to treat pathologies mediated by overt NETosis.

Keywords Neutrophil extracellular traps (NETs) · Infection · Lung · Pregnancy · Modulation

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Introduction

Neutrophil granulocytes, also frequently termed polymorphonuclear neutrophils (PMN), are the most abundant members of the leucocyte population, comprising between 50 and 70 % of total white blood cells in normal healthy individuals [1, 2]. PMNs are a vital component of the innate immune system and form the first line of defense against invading pathogenic microorganisms [1, 2]. Traditionally, this interaction has been considered to involve phagocytosis and lytic destruction of pathogens by toxic neutrophil granular proteins and reactive oxygen species (ROS) in the phagolysosome or the release of these agents directly into the extracellular environment by degranulation [1, 2].

This paradigm was altered by the discovery that PMNs could release their genomic DNA into the extracellular environment in the form of neutrophil extracellular traps (NETs) that could ensnare invading microorganisms [3]. Analysis of these novel NET structures showed that they were decorated by proteins from all three PMN granule groups (azurophilic, secondary, and tertiary), including neutrophil elastase (NE), myeloperoxidase (MPO), lactoferrin, and gelatinase [3]. It was proposed that by being tightly

associated with the DNA lattice structure of NETs, the presence of bactericidal granular proteins in these molecular traps would be able to kill pathogenic invaders in a highly efficient manner with minimal damage to the surrounding tissue [3].

In the interim, it has emerged that the interaction between hostile microorganisms and neutrophil NETs is more complex than originally thought. In this review, we will highlight some of these developments.

Interaction with bacteria and fungi

In their seminal publication, Brinkmann et al. demonstrated that NETs could be induced by both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Salmonella typhimurium* and *Shigella flexneri*) bacteria and displayed signs of incapacitating the virulence of these agents by the action of NET-associated extracellular proteases [3].

This finding was rapidly extended to show that neutrophil NETs could also be induced by eukaryotic pathogens such as the ascomycetous yeast, *Candida albicans* [4]. In this study, it was demonstrated that NETs interact both with the single cell yeast form as well as the multicellular hyphal form and incapacitate these via action of granular components. The generation of NETs was more pronounced in cases in which the yeast cells, either yeast or hyphal form, were opsonized. Opsonization was, however, not a prerequisite for NETosis.

The fungicidal activity of NETs was subsequently shown to be largely due to the presence of calprotectin, an antimicrobial heterodimer with calcium- and zinc-binding properties [5]. The absence of calprotectin in S100A9 knockout mice rendered these animals highly susceptible to infections with *C. albicans*.

Evidence that NETs may play a role in the human innate immune response was provided by patients with chronic granulomatous disease (CGD) [6]. In such patients, NETosisis abrogated due to a deficiency in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase genes that prevent efficient ROS generation. This genetic deficiency renders this patient group particularly prone to pulmonary infections with *Aspergillus* fungi.

To correct for this deficiency, a gene therapy approach was investigated by which the defective $gp91^{phox}$ gene was replaced by a functional gene encoded from a retroviral vector. By this means, it was possible to cure a child from refractory aspergillosis and to enable a return to a normal life free from the restrictions of sterile confinement [6]. Although functional NADPH oxidase was detectable in only 16 % of PMN for a period of 3 months postgene therapy, this low level was sufficient for efficient clearing of the fungal infection. Furthermore, in in vitro examinations of the gene-modified cells, it was demonstrated that they underwent NETosis following interaction with *A. nidulans* isolated from the patient's lungs and that such NETs effectively inhibited fungal growth.

Direct real-time in vivo evidence of the interaction between neutrophil NETs and invading fungal pathogens was obtained in murine lungs using two-photon microscopy [7]. In this study, it was shown that neutrophils generate NETs in response to interaction with both *Aspergillus fumigatus* morphotypes, namely conidia and hyphae. NETs detected in the lungs of infected animals formed with rapid kinetics, occurring within 3–4 h of PMN exposure to the fungi. A discrepant finding of this study was that NETs did not appear to be the major contributor to the killing of *A. fumigatus*, unlike what was previously clearly demonstrated to be the case for *C. albicans*, but may, in this instance, contribute to fungal demise by binding of secreted proteins or membrane structures and thereby preventing fungal spread.

Mechanisms to evade NET entrapment by bacteria or fungi

Similar to "spy vs. spy" scenarios, it is obvious that in biology, attacker and evader will develop different strategies to counteract each other. In the case of NETs, several systems appear to be employed by bacteria or fungi to render them ineffective. Not unexpectedly, these strategies rely on the use of nucleases that attack the DNA backbone of the NET lattice structure [8, 9].

The first report of such an evasive strategy was made in strains of Group A streptococcus, which, by the expression of a DNAse molecule Sda1, were determined to be more virulent than strains lacking this enzyme. In addition, the inhibition of this enzyme reduced infectivity and promoted clearance by PMN [10] (Fig. 1a).

In a similar manner, endonuclease expression by *Streptococcus pneumoniae* [11] or *S. aureus* [12] was shown to permit these organisms to evade ensnarement by NETs, thereby boosting their virulence (Fig. 1a). Consequently, these nucleases are currently being evaluated as targets for novel antibacterial therapies [13].

Recently, evidence of more complex evasive strategies employing elements of molecular mimicry has emerged. In the instance of *Pseudomonas aeruginosa*, it appears that this opportunistic pathogen exploits the acquisition of sialic acid motifs form host cells [14]. Sialic acids, also termed Sias, are unusual monosaccharides characterized by a shared nine-carbon backbone [15]. They are widely expressed on the cell surface of most mammalian cells. In order to evade detection and destruction by the host immune system, a number of bacteria have been shown to acquire and coat

Semin Immunopathol (2013) 35:439–453

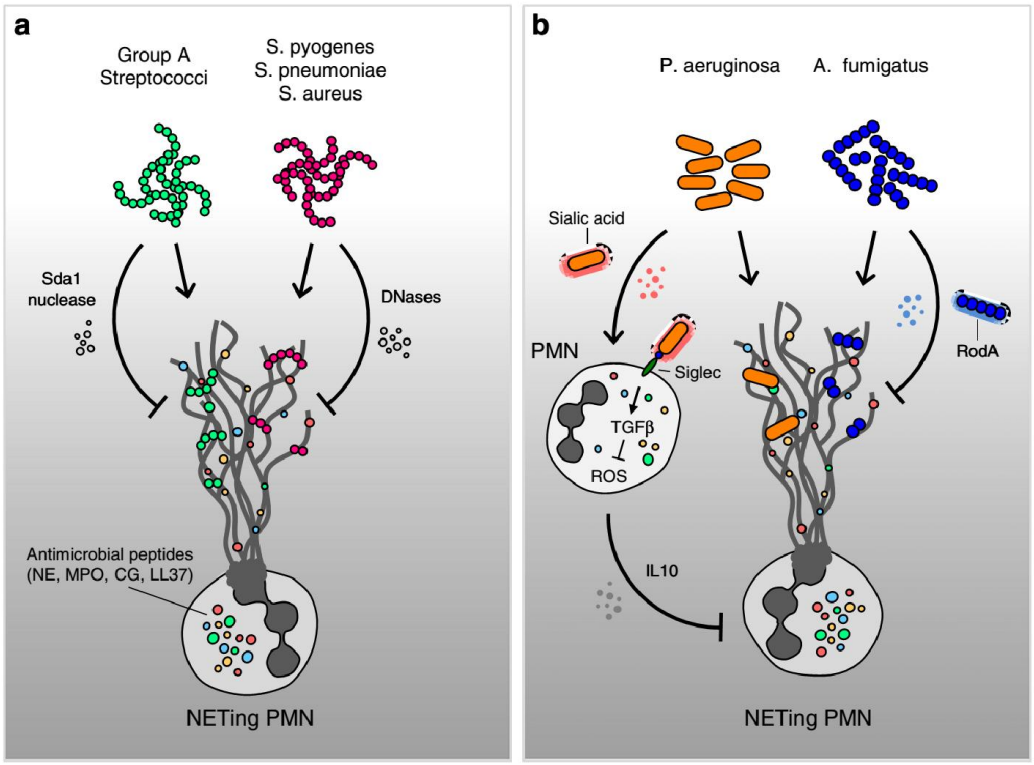

Fig. 1 Mechanisms of defense against bacteria and fungi and strategies of evasion of NET entrapment. NETs can be induced by both Gram-positive or Gram-negative bacteria (**a**). Evasion of the antimicrobial activity of NETs may include the use of nucleases that attack the DNA backbone of the NETs' lattice structure (**a**) or elements of molecular mimicry that stimulate the production of the potent immune-modulating cytokines (**b**). *NE* neutrophil elastase, *PMN* polymorphonuclear neutrophil, *MPO* myeloperoxidase, *LL37* cathelicidin, *CG* cathepsin G, *IL10* interleukin 10, *TGF*β transforming growth factor beta

themselves with host Sias [16]. In this manner, such pathogens will not only immunologically appear as "self" rather than as "foreign," but by binding to specific Sias, lectin-like receptors (siglecs) on host cells dampen the host immune response against them [16].

By absorbing host serum Sias, *P. aeruginosa* was able to bind directly to PMN via siglec-9 and, thereby, stimulate the production of the potent immune-modulating cytokines IL-10 and TGF-β. Furthermore, this interaction attenuated the generation of ROS and the release of neutrophil elastase (NE) by PMN engaged in this manner. Since the generation of ROS is a pivotal step required for NETosis, it comes as no surprise that PMN exhibited a reduced ability to generate NETs when confronted with Sias-coated *P. aeruginosa* bacteria [14] (Fig. 1b). It is not clear if the IL-10 present in this system is produced by neutrophils or by bystander cells, as it has been suggested that human neutrophils have an inactive IL-10 gene locus [17].

Fungi have also developed analogous evasive countermeasure. In the interaction between PMN and with *Aspergillus* species, it was determined that the presence of the hydrophobin RodA protein on the cell surface of conidia leads to a reduction in NETosis [7]. The presence of this hydrophobic protein that coats resting conidia, but not hyphae, has previously been shown to largely negate an antifungal innate immune response [7] (Fig. 1b). A summary of the reported mechanisms of pathogen evasion from NETs is given in Table 1.

Tuberculosis: interaction between macrophages and neutrophils

The elimination of invasive pathogenic microorganisms involves a delicately orchestrated interplay between them and among members of the innate and the adaptive immune systems [18, 19]. A prime example of this system breaking down, permitting the virulent bacterium to thrive and eventually kill the host, is provided by *Mycobacterium tuberculosis* [20].

Even though the tubercle bacillus was described by Robert Koch back in 1882, tuberculosis (TB) remains a leading cause of worldwide mortality (1.4 million deaths in 2011) and morbidity (8.7 million new infections in 2011),

Table 1 Strategies of NET evasion by various pathogens

Pathogen	Mediator	Mechanism of action	Refs.
Group A *Streptococci*	Sda1	NET degradation	[8–10]
	Streptokinase	Fibrinolysis	[76, 77]
Streptococcus pneumoniae	Endonuclease	NET degradation	[11]
Staphylococcus aureus	Endonucleases	NET degradation	[12, 13]
	Staphylocoagulase	NET degradation	[78, 79]
	vWF-binding protein	Exploitation of coagulation	[78, 79]
	CLFA and CLFB	Platelet aggregation	[74]
Pseudomonas aeruginosa	Sialic acid	Molecular mimicry	[14, 16]
Aspergillus fumigatus, *A. nidulans*	RodA	Molecular mimicry	[7]
Mycobacterium tuberculosis H37Rv	High lipid cell wall content	Molecular mimicry	[19, 23]
Mycobacterium canetti	High lipid cell wall content	Molecular mimicry	[23]
Mycobacterium bovis BCG	Mφ-ingested PMN CG and NE	Modulation of NETosis	[25, 26]
Mycobacterium smegmatis	Microparticles and ectosomes	Modulation of NETosis	[27, 28]
	IFNγ and ESX-1 system	Modulation of NETosis	[29]
Leishmania amazonensis	Coinfection with FeLV	Modulation of NETosis	[84]
Human immmunodeficiency virus	CD209 binding	Modulation of NETosis	[86, 87]

NET neutrophil extracellular trap, *vWF* von Willebrand factor, *CLFA* clumping factor A, *CLFB* clumping factor B, *RodA* hydrophobin RodA protein, *BCG* bacillus Calmette-Guérin, *Mφ* macrophage, *PMN* polymorphonuclear neutrophil, *CG* cathepsin-G, *NE* neutrophil elastase, *IFNγ* interferon gamma, *ESX-1* mycobacterial ESX-1 secretion system, *FeLV* feline leukemia virus

second only to human immunodeficiency virus (HIV) [20]. Despite this large health care burden, surprisingly little has been undertaken to investigate the potential role of neutrophils or their NETs in combating this insidious infection [21].

This may be largely due to the intrinsic properties of PMN, in that they are short lived and cannot be cryopreserved, necessitating the constant use of fresh primary cells, which restrict their availability and utilization [21]. Furthermore, macrophages are considered to be the prime target of this bacterium and to be crucial for its removal and containment in granulomas.

Nonetheless, the role played by PMNs in the initial combating and subsequent harboring of the *M. tuberculosis* bacilli is gaining recognition, since a large influx of PMNs is evident at the site of infection and PMNs may account for the predominant phagocytic cells infected by *M. tuberculosis* bacilli [22]. In addition, a transcriptomic analysis of human blood samples from TB patients suggests that tuberculosis is associated with a neutrophil-driven interferon-induced profile [23]. Furthermore, PMNs are required for granuloma formation in chronic infection. Experiments in mice suggest that the recruitment of PMNs to the site of infection reduces the number of infective *M. tuberculosis* bacilli from the lungs and prevents their spread to secondary organs, such as the spleen [21]. This is consistent with reports in humans, where the risk of infection is inversely proportional to peripheral PMN cell counts [21].

That *M. tuberculosis* bacilli induce NETosis has become evident from the study by Ramos-Kichik et al. [24]. In examining the influence of two strains of *M. tuberculosis* with high or low (*M. tuberculosis* H37Rv and *M. canetti*) virulence on isolated PMNs in vitro, it was observed that both strains lead to the formation of leucocyte clusters or aggregates [24]. Remarkably, both *M. tuberculosis* strains induced the formation of neutrophil NETs, as detected by scanning and transmission electron microscopy. This feature was confirmed by fluorescence immunohistochemical staining for DNA, histones, and neutrophil elastase [24]. NETosis was found to be associated with the demise of PMNs and the release of intracellular constituents (LDH), as originally reported by Brinkmann et al. [3]. No significant difference in the degree of NETosis induction by either the virulent N37Rv or the nonvirulent *M. canetti* strains could be discerned. Of particular interest was the observation that, although the PMN NETs were induced by *M. tuberculosis* bacilli and could ensnare these microorganisms, they were incapable of killing these bacilli. This was in strong contrast to what was observed in the case of *Listeria* monocytogenes under the same conditions, in which the entrapment by NETs lead to a significant reduction in bacterial survival and infectivity. This effect was not due to the *M. tuberculosis* bacilli rendering the NETs inert, as the addition of such NETs to *L. monocytogenes* cultures was capable of killing the latter. This effect was abolished if the DNA lattice of the NETs was destroyed by addition of nuclease. Consequently, the inability of NETs to kill *M. tuberculosis* bacilli must be a reflection upon the unique nature of the cell wall of these bacilli, its high lipid content rendering them impervious to the action of immune cell attack, be it by PMNs or macrophages.

Semin Immunopathol (2013) 35:439–453

The interplay between *M. tuberculosis* bacilli and members of the innate immune system may, however, be more complex than previously thought, a change in concept supported by a spate of recent publications [18, 19, 25]. In this context, it has been observed that macrophages are able to ingest neutrophil azurophil granular proteins and use them to kill invasive mycobacteria [26, 27]. In the first of these reports, Steinwede et al. examined the role of cathepsin G (CG) and neutrophil elastase (NE) in the alveolar clearance of mycobacterial infections, in particular *M. bovis* bacillus Calmette-Guérin (*M. bovis-BCG*). They observed that genetically altered knockout mice for CG, and especially CG/NE, exhibited a reduced capacity for clearing bacterial infections, resulting in increased bronchoalveolar pathology. This could be overcome by aerosol therapy with CG/NE-loaded liposomes. Of interest is that these enzymes were taken up by alveolar macrophages and used by these to expedite bacterial elimination.

In a subsequent study, Jena et al. observed that macrophages can endocytose neutrophil-derived azurophilic enzymes and employ these for bacillus destruction [27]. They included *M. smegmatis*, *M. bovis-BCG* as well as the virulent *M. tuberculosis* H37Rv strain. Their data indicate that the acquisition of azurophilic enzymes and their presence within macrophages leads to the disintegration of the bacterial cell membrane and to bacterial cell lysis (Fig. 2).

As these two studies report on very similar phenomena, this would suggest that the uptake of PMN-derived granules by macrophages could be a relatively common cooperative mechanism utilized by the innate immune system to eliminate certain bacteria. It also opens a potential pathway for novel therapeutic strategies.

Such cooperation may include other factors such as microparticles (MP) released by infected macrophages or PMN. In the instance of macrophage-derived MPs, these appear to be proinflammatory and attract PMN and other immune effector cells to sites of application [28]. These MPs were shown to contain mycobacterial antigens and promote specific $CD4^+$ T cell activation both in vitro and in vivo. In contrast to these proinflammatory observations, it has been suggested that ectosomes derived from *M. tuberculosis* bacilli-infected PMN reduced the antimycobacterial activity of accessory macrophages [29]. Clearly, these disparities will need to be resolved.

A very intriguing recent observation in this context was made by Wong and Jacobs, in which they report on the generation of ETs (extracellular traps) by macrophages heavily infected with *M. tuberculosis* bacilli [30]. This effect was dependent on the presence of IFN-γ and the mycobacterial ESX-1 system, which mediated phagosomal rupture and subsequent bacterial escape. The macrophage ETs were determined to be remarkably similar to neutrophil NETs by having a DNA backbone readily destroyed by DNAse action and the occurrence of citrullinated histones. Furthermore, akin to the

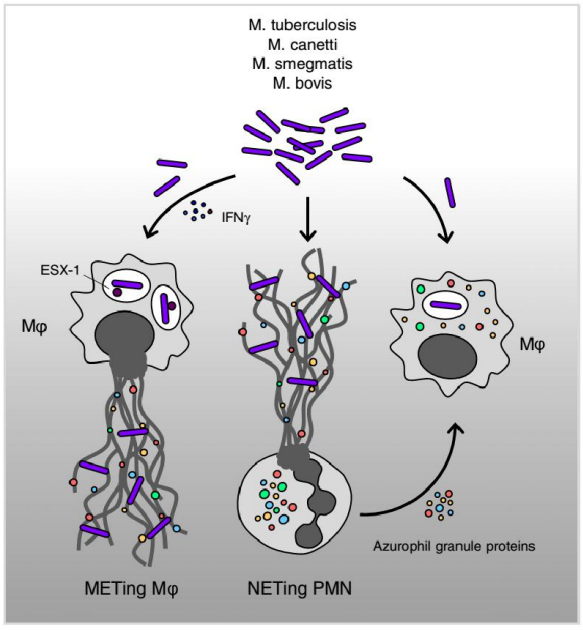

Fig. 2 Neutrophils and macrophages cooperate in order to confront mycobacterial infections. *Mycobacterium* bacilli induce leucocyte clustering or aggregation and, subsequently, NETosis, but not killing of these pathogens. Macrophages are able to ingest neutrophil azurophil granular proteins and dispatch invasive mycobacteria. The generation of ETs (extracellular traps) by macrophages is dependent on the presence of IFN-γ and the mycobacterial ESX-1 system, which mediate phagosomal rupture and subsequent bacterial escape

neutrophil NETotic process, the generation of macrophage ETs was also dependent on an elastase activity, as the process could be blocked by treatment with an elastase inhibitor, AAPV. The ability to generate traps was strongly dependent on whether the primary human macrophages had been differentiated with the aid of G-CSF but not GM-CSF. In addition, priming with IFN-γ was an essential requirement for ET triggering by *M. tuberculosis* bacilli. This feature was observable both under physiological oxygen tensions (5–10 %) as well as under normal tissue culture conditions (20 %).

The triggering of ETs by *M. tuberculosis* bacilli was strongly dependent on the bacteria possessing an intact ESX-1 system. It has previously been shown that the expression of these factors leads to caspase-1-independent cell death of infected macrophages. ESX-1 also leads to the activation of the inflammasome and release of IL-1 beta [31]. The role of this proinflammatory cytokine in macrophage ETosis is currently unclear.

On the other hand, the action of IFN-γ appears to promote the aggregation of *M. tuberculosis* bacilli in macrophages, thereby providing the necessary impetus for ETosis. This action was also accompanied by widespread necrosis of infected macrophages. It, therefore, appears that the ESX-1 system subverts the action of IFN-γ to enhance or amplify

the aggregation of bacilli, macrophage ETosis, and necrosis (Fig. 2).

Role of cathelicidin in combating microorganisms

Antimicrobial peptides play a fundamental role in the protection against microbial attack in a number of systems, evident by their widespread distribution throughout the animal and plant kingdoms [32]. In humans, two major families of antimicrobial peptides have been identified as defensins and cathelicidin. Cathelicidin family of antimicrobial polypeptides are characterized by a highly conserved region (cathelin domain) and a highly variable cathelicidin peptide domain [33]. Human Cathelicidin(LL-37) is a cationic, host defense peptide, mainly expressed by neutrophils and epithelial cells during acute inflammation. LL-37 corresponds to amino acid 134–170 of Human cationic Antimicrobial Protein 18 (hCAP-18), which is present in specific granules of human neutrophils and produced after the C terminal cleavage of hCAP 18 by caspase 3 [34].

LL-37 is known to exhibit broad-spectrum antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria species [35–37], including tuberculosis, where in combination with lipocalin 2, it restricts the growth of this microorganism in an iron-dependent manner [38]. It also exhibits inhibitory activity against certain fungi and enveloped viruses [39]. This capability can be transferred to other species, including plants, as demonstrated by a recent experiment in which expression of LL-37 in transgenic plants lead to resistance to bacterial and fungal pathogens [40]. The mechanism of bacterial killing by LL-37 is rapid and mostly involves intercalation and assembly of peptides within the bacterial membrane to disrupt membrane integrity [41]. By the use of time-lapse fluorescence microscopy, it was shown that LL-37 adopted alternate mechanisms for membrane disruption depending upon the concentration of the peptide [42]. In addition to antimicrobial activity, LL-37 has been shown to be associated with a wide range of biological activities including the inhibition of biofilm formation, chemotaxis and mast cell degranulation, induction of chemokines, enhancement of keratinocyte migration and proliferation, and enhancement of vascularization [43, 44].

Inappropriate activity by LL-37 can lead to considerable tissue damage, especially in infections of the respiratory tract by pathogens such as *S. aureus*, which can lead to excessive fibrinolysis [45]. In addition, aberrant expression of cathelicidin has been linked to increased susceptibility to skin infections, frequent oral bacterial infections, severe periodontal disease, and cutaneous infections [46]. For example, histological examination of a number of chronic inflammatory skin diseases such as atopic dermatitis, rosacea, and psoriasis have shown that the proinflammatory peptide is dysregulated in these disorders [39].

This facet may be due to an unexpected interaction between LL-37 and extracellular host DNA derived from dying cells [47, 48]. In these studies, it was shown that LL-37 binds very stably to DNA and then acts as a transfecting agent, dragging this macromolecular complex into adjacent cells, where it can interact with toll-like pattern recognition receptors. In psoriasis, these bystander cells happen to be plasmacytoid dendritic cells (pDCs), which can elicit a potent immune response via the production of interferons [47].

Since cathelicidin is intimately associated with NETs, its presence may contribute to inflammatory conditions associated with aberrant NETosis such as occurs in autoantibody (ANCA) small vessel vasculitis [49]. This may involve further autoinflammatory conditions such as rheumatoid arthritis [50].

Although LL-37 constitutes a formidable innate immunity defense barrier against infections, some bacterial species have developed various mechanisms to circumvent its action. An example is the oral pathogen *Porphyromonas gingivalis* associated with chronic periodontal disease, which can degrade LL-37 by utilizing its arginine-specific gingipains enzyme [51]. In addition, *S. aureus* can cleave and inactivate LL-37 by the action of its metalloproteinase called "aureolysin," in a time- and concentration-dependent manner [52]. Analogously, M1 protein promotes the survival of Group A streptococcus through the inhibition of cathelicidin [53]. On the other hand, the action of cathelicidin may be affected by pathological conditions such as cystic fibrosis, where its interaction with glycosaminoglycans can inhibit its antimicrobial activity, a facet that can be restored by treatment with hypertonic saline [54].

LL-37 may also influence neutrophil behavior, inducing secondary necrosis in these cells, leading to the release of IL-8, IL-1R antagonist, and granules [55]. Furthermore, LL-37 seemed to enhance the production of IL-8 and ROS production. It is currently unclear if such action by LL-37 leads to the induction of NETosis. Apart from powerful chemotactic activities, which appear to be, in part, mediated via interaction with the formyl peptide receptors [56], LL-37 can also promote monocyte adhesion, thereby illustrating a new mode of neutrophil interaction with these cells [57].

The induction of IL-8 by LL-37 may be pivotal in the combat of respiratory infections. Of particular interest is that this is not restricted to neutrophils but may include airway smooth muscle cells [58].

Role of immunothrombosis in combating microbial infections

In a recent review by Engelmann and Massberg [59], recapitulating the recent progress in the field of hemostasis and thrombosis activated by circulating microorganisms, the

Semin Immunopathol (2013) 35:439–453

concept of immunothrombosis was coined. Immunothrombosis is defined as an innate immune response led by specific cells and molecules, which interact to form thrombi inside the blood vessels. When uncontrolled, it represents a major biological process fostering thrombosis-associated pathologies.

NETs appear to play a central role in immunothrombosis, supporting the above-described process through several means. NETs can directly activate factor XII (the contact pathway of coagulation), probably due to their electrochemical properties [60]. It has been shown that NETs bind directly to von Willebrand factor (vWF) and enhance the process of platelet recruitment [61]. Moreover, histones H3 and H4, present in the NETs, can trigger the activation of platelets [62] and stimulate platelet adhesion [63]. Other indispensable components of NETs, such as the proteolytic antimicrobial enzymes NE and MPO, can actively regulate the clotting cascade, on the one hand, by cleaving or oxidizing major natural anticoagulant substances, including tissue factor pathway inhibitor (TFPI) [64] and thrombomodulin [65], which propagates coagulation. On the other hand, in vitro studies have shown that the aforementioned proteases can also degrade fibrin [66], and since they are present on NETs, they could be potentially involved in the resolution of the clots [67, 68]. In all cases, treatment with DNase1 prevents thrombus formation, underscoring the importance of NETs for the process. Additionally, NETs can bind to TF and promote the activation of the extrinsic pathway of coagulation [69]. In vitro, NETs stimulate fibrin formation and deposition, and fibrin colocalizes with NETs in blood clots [67, 70].

In this context, the engaged neutrophils and monocytes are most probably stimulated by activated platelets via PRRs in order to form NETs [60, 71]. In turn, activated platelets not only stimulate NET formation but also the NETs that are generated trigger de novo platelet activation, red cell accumulation, and thrombosis [72, 73].

In the framework of the organism's defense tactics against pathogens, immunothrombosis is thought to perform a broad series of physiological functions. First, it facilitates capture and tanglement of blood-borne pathogens, limiting their spread by retaining the microorganisms within the fibrin clot. In this way, tissue invasion by pathogens is prevented through microthrombus formation [68]. Further, these intravascular clots generate a separate compartment that focuses all antimicrobial strategies to distinct loci favoring pathogen killing. This involves both innate immune cells and the antimicrobial substances generated during their activation [74, 75]. Finally, fibrinogen or fibrin accumulation and deposition in the small vessels enhances the additional recruitment of reactive cells to the damaged site of tissue infection, supporting the pathogen recognition and directing the immune response [76]. The basic features of immunothrombosis are depicted in Fig. 3.

The broad range of sophisticated strategies that pathogenic microorganisms have evolved in in order to subvert, additionally, immunothrombosis, apart from the herein described NETosis, underlines the crucial role of this process against their propagation [75, 77–80]. These strategies are also summarized in Table 1.

Viral interactions with neutrophil NETs

The interaction of neutrophils and viruses is less well characterized than those with bacteria or fungi, especially with regard to NETosis. Indeed, it was unclear if PMN NETs were beneficial or detrimental in cases of viral infections, as PMNs have been associated with increased pulmonary damage in murine influenza model systems [81–83].

This enigma was clearly addressed in a recent publication within travital microscopy to track the murine PMN response to infection with an oncolytic poxvirus in vivo [84]. In their experiments, the authors determined that PMN were recruited to the liver following administration with poly I:C (polyinosinic:polyicitidylic acid), a double-strand nucleotide viral analog, lypopolysaccheride (LPS), or challenge with the pox virus [84]. The activation of PMNs by poly I:C or pox virus involved toll-like receptor 3 (TLR3) of the innate immune pattern recognition receptors (PRR). This treatment increased the expression of the cell surface integrin molecule CD11b, which facilitates attachment of PMN on host endothelium. Furthermore, viral challenge induced thrombocytopenia, platelet accumulation in the liver, and generation of PMN NETs. In this manner, it is possible that infection with pox virus mobilizes an immune–thrombotic response [59], which would serve to immobilize the virus for efficient clearance by macrophages. The generation of NETs appears to be useful in the clearance of virus, as their induction by LPS treatment promoted viral clearance, whereas their dissolution by DNAse treatment facilitated viral persistence [84].

Viral modulation of NETosis: feline leukemia virus

Viral infections appear to be able to modulate the host NETotic response. The first indication of such an occurrence was in cases with feline leukemia virus (FeLV) [85]. Feline infections with this gammaretrovirus generally result in a mild viremia with high titers of neutralizing antibodies, while about a third of cases fail to mount an effective humoral response and succumb to complications arising from virally induced immunosuppression. For this purpose, FeLV has been suggested to serve as a model system for human infections with HIV (human immunodeficiency virus-1) [86].

Since immunosuppression in FeLV cases has been associated with reduced PMN function, Wardini et al. addressed this issue in more detail, including an analysis of the host NETotic response [85]. Their results indicated that feline

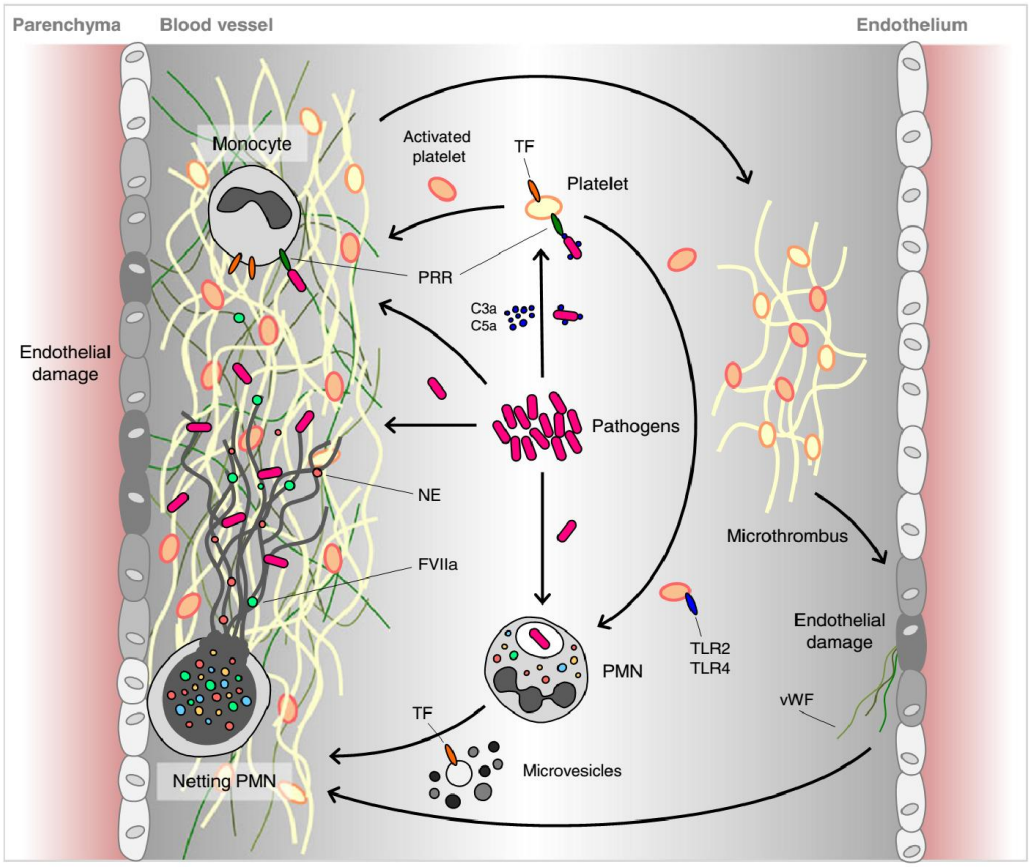

Fig. 3 Immunothrombosis supports the innate immune defense against pathogens. Immunothrombosis involves an intricate interplay between complement, coagulation, and innate immune effector cells. By assembly of a thrombus around the pathogenic microorganisms, their systemic spread is prevented and the damage to the host is minimized. *PRR* pattern recognition receptor, *TLR* toll-like receptor, *TF* tissue factor, *vWF* von Willebrand factor, *NE* neutrophil elastase, *PMN* polymorphonuclear neutrophil. Adapted and modified from Engelmann et al. [59]

PMNs underwent NETosis when exposed to *Leishmania* promastigotes. The degree of NETosis was elevated in asymptomatic FeLV-infected cats and was dramatically elevated in symptomatic FeLV-infected cats. These data suggest that FeLV alters the NETotic response of PMNs and that the measure of this index can be used as a prognostic marker of disease progression [85]. It is currently unclear how virally enhanced NETosis can lead to immunosuppression, but it is possible that overt stimulation of the NETotic response may lead to PMN depletion and exhaustion of the underlying progenitors.

Downmodulation of NETosis by HIV: paving the way for secondary infections?

In a recent report by Saitoh et al., it was shown that HIV triggers NETosis via a process that involved the recognition of viral nucleic acids via engagement with TLR7 and TLR8 [87, 88]. A unique feature of this study is that by the use of super-resolution structured illumination microscopy (SR-SIM) [89], they were able to directly visualize individual HIV virions trapped in the NET lattice. They were, furthermore, able to determine that PMN NETs not only immobilized HIV virions but were able to reduce their infectivity via the action of MPO (myeloperoxidase) and α-defensin. This appears to involve intact NETs, as it was reduced if the DNA lattice backbone was digested by DNAse treatment. These data, therefore, suggest that PMN NETs could play an important part in the initial innate response to HIV infection by facilitating viral trapping, inactivation, and clearance.

Unfortunately, HIV is a devious pathogen and will use a whole range of mechanisms to modulate and manage the host immune system in order to foster its propagation. One such means is by binding to Dendritic Cell-Specific

Semin Immunopathol (2013) 35:439–453

Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), also termed CD209. This mannose-binding C-type lectin receptor can serve as an anchor for HIV virions on dendritic cells (DC) and, thereby, promote the efficient infection of $CD4^+$ T cells that engage with this antigen-presenting cell [90].

By examining this interaction, it was determined that the binding of HIV virions to isolated DCs leads to the production of a factor that inhibited PMN NETosis. This feature was dependent on the gp120 membrane glycoprotein of HIV, as it could be mimicked by addition of recombinant gp120 to DCs. (Fig. 4). Further analysis indicated that the factor released by gp120/CD-SIGN-primed DCs was the immune-modulating cytokine IL-10. In this manner, addition of recombinant IL-10 to PMN cultures lead to a reduction in their NETotic capability and prevented these cells from effectively engaging and debilitating HIV.

Individuals with HIV are highly prone to secondary infections, especially those of the pulmonary system with pathogens such as *M. tuberculosis* or *S. pneumoniae*, which result in high degrees of mortality. Consequently, these findings have considerable implications for anti-HIV therapeutic strategies in order to counter AIDS-associated neutropenia and to ensure an effective innate immune response. In this context, it is worth noting that coinfection with *M. tuberculosis* bacilli can accelerate AIDS progression, since this bacterium can promote the replication of HIV and infection of $CD4^+$ T cells [91].

Malaria: a possible autoimmune component?

A number of previous studies have shown that PMNs can generate NETs against multicellular eukaryotic parasites, including the use of *Leishmania* promastigotes in the study of NETosis in FeLV-infected cats [85, 92]. In human pathology, among the most important parasites are *Plasmodium falciparum* and *Plasmodium vivax*, the agents responsible for malaria with lethal outcome [93].

In pediatric patients infected with *P. falciparum*, Baker et al. made the striking observation that NETting PMNs could be readily detected directly in blood smears [94]. An examination of total PMNs in the smears indicated that *P. falciparum* infection was associated with a high proportion of segmented PMN, which decreased following treatment with Fansidar® (sulphadoxine–pyrimethamine). Of interest is that drug therapy leads to an increase in immature PMNs (metamyelocytes) and a significant decrease in bands. The degree of NETosis did

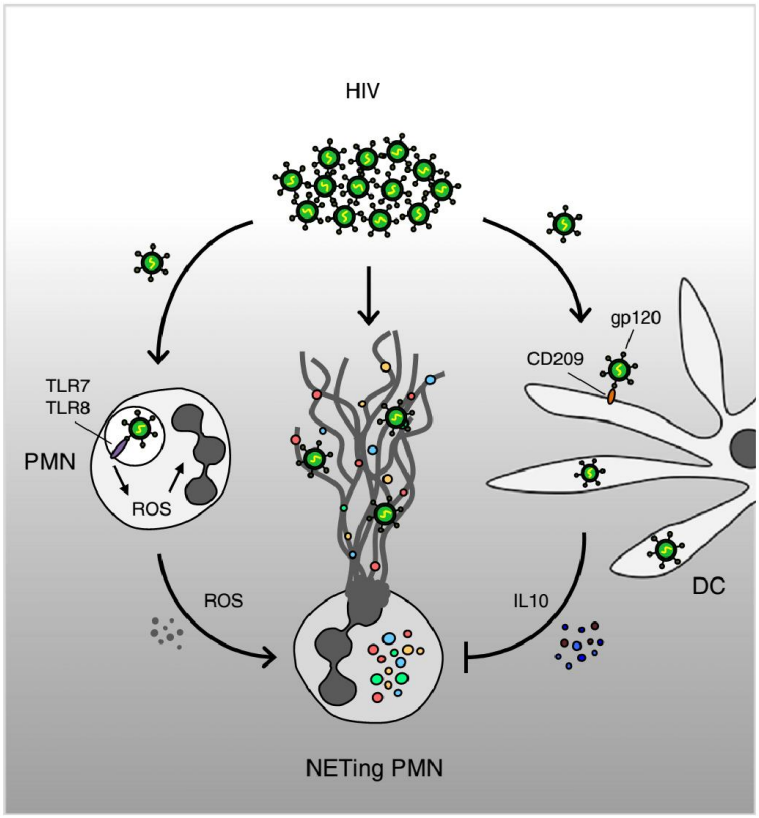

Fig. 4 HIV downmodulates NETosis via dendritic cells. HIV triggers NETosis via a process that involves TLR7 and TLR8, trapping individual HIV virions in the NET lattice. Viral binding to CD209 anchors the virions on dendritic cells (DC), thereby promoting the efficient infection of $CD4^+$ T cells. The gp120/CD209-primed DC cells produce the immune-modulating cytokine IL-10, leading to a reduction in NETotic capability, thereby preventing PMN from effectively engaging and eliminating HIV

not appear to be significantly influenced by therapy, although there was an increase in the number of cases observed post therapy (100 vs. 86 %). NETosis did, however, appear to correlate with plasma TNFα levels, indicating that a strong proinflammatory component was driving this response.

Of additional interest is that the occurrence of NETs appeared to correlate with the presence of antinuclear antibodies (ANA), with high levels of antibodies reactive with dsDNA, predictive of autoimmunity. Such autoantibodies could contribute to other malaria-related features such as renal dysfunction and uremia. These data may provide additional insight into the possible link between lupus susceptibility genetic loci and resistance to malaria [95], or into the possible mechanism of action of antimalarial drugs such as chloroquine and hydroxychloroquine used for the treatment of autoimmune conditions [96].

Influence of the host: pulmonary system

The ability of PMNs to form NETs is not only dependent on the external stimulus but also depends on the physiological status of the PMNs and the host microenvironment. One of the best characterized host microenvironments is the pulmonary system.

The alveolar epithelium synthesizes proteins such as the surfactant protein A and D (SP-A, SP-D), members of the collectin family of PPRs, which bind carbohydrate moieties on pulmonary pathogens such as *M. tuberculosis* or *S. pneumonia* as well as fungi. Absence of these factors in genetically manipulated knockout mice diminished their capability to clear *P. aeruginosa* infections [97]. That these factors can interact with members of the innate immune system to promote an antibacterial response is underscored by the recent finding that SP-D can bind pathogens as well as NETs in order to augment NET-mediated bacterial trapping and eradication [98] (Fig. 5).

On the other hand, numerous observations suggest that aberrant NETosis can lead to damage of the fragile alveoli [81]. Such NETosis-associated damage may be triggered by host trauma in conditions such as transfusion-related acute lung injury (TRALI) following blood transfusions [99, 100] or be the result of viral infection [82]. Indeed, under such conditions of influenza infection, NETs appear to be incapable of clearing any secondary infections with pneumococci but rather contribute to increasingly compromised lung function [101].

It has, furthermore, been shown that an underlying disturbance of the alveolar tissue, as in the instance in cystic fibrosis (CF), can trigger NETosis, thereby contributing to the bronchial pool of cell-free DNA [102]. In order to clear this thick and sticky sputum, exogenous DNAse is used as a therapeutic agent [102]. In examining the contribution of PMN NETs to this phenomenon, it was observed that neutrophil elastase (NE) promoted the solubilisation of CF sputum by assisting with the degradation of histones.

This PMN-derived enzyme, however, does not only play a role in the clearance of excess NET debris but also, in combination with protease 3 and cathepsin G, may play a significant role in the damage caused to the underlying alveolar epithelium [103]. In a further study using atomic force and scanning electron microscopy, it was determined that the major portion of DNA in CF sputum resulted from excessive NETosis [104]. Consequently, it will be necessary to refocus therapeutic strategies to tackle NETs as a whole rather than focus on the sole dissolution of individual DNA fibers.

The occurrence of this highly gelatinous mucous in the lungs may, however, render this microenvironment more amenable to infections, such as those with *P. aeruginosa*, which frequently occur in patients with CF [105]. In this scenario, it appears that the highly dense DNA-based biofilm permits *P. aeruginosa* to adopt a NET resistance phenotype, thereby evading efficacious removal by this arm of the innate immune system. This phenomenon appears to be specific for *P. aeruginosa* isolates from affected CF lungs, as PMN isolated from CF patients were quite capable of generating NETs against normal *P. aeruginosa* isolates and killing these efficiently [105].

Influence of the host: pregnancy

Pregnancy poses a unique immune challenge as the mother has to maintain an effective immune response against pathogens deleterious to her own health and yet not mount a response that be deleterious to her unborn child [106]. Although most studies have largely focussed on how the maternal T cell response is modulated to avoid rejection of the semiallogeneic components of her fetus via regulatory T cells [107] or a shift in T cell cytokine profiles (Th1 vs. Th2) [108], it has become evident that pregnancy involves considerable changes in the activity of the innate immune system, specifically in that of neutrophils [109]. In general, pregnancy is associated with neutrophil leucoytosis, with numbers increasing from the second month of pregnancy and plateauing at term. Neutrophil action in the reproductive cycle is quite diverse and may range from the trapping and elimination of excess spermatozoa directly in the reproductive tract during fertilization, the rendering of the myometrium more receptive to implantation, or the contribution to the etiology of preeclampsia via overt NETosis [109, 110].

The modulation of the immune system, both innate and adaptive, during pregnancy may, however, be more extensive that initially thought. In this manner, due to elevated

Semin Immunopathol (2013) 35:439–453

oestrogen levels, the upper respiratory tract in pregnancy is associated with increased mucous secretion, capillary congestion, and fragility [81]. This may render pregnant women more susceptible to airborne noxins or pathogens. Such respiratory infections can have severe consequences for the fetus, as they can lead to secondary placental infections, which may even result in stillbirth or septic abortion [111].

While it is well appreciated that prenatal exposure to maternal smoking or airborne particulate matter is associated with higher rates of pediatric asthma [112], new data indicate that these events may debilitate the neonatal immune response [113], rendering the developing child more susceptible to infections [114].

With regard to the pathogenic agents covered in this review in the context of neutrophil interactions, two points are worth noting:

1. Low maternal and neonatal neutrophil counts are associated with an increased risk of HIV transmission in utero in African populations [115]. This suggests that anti-HIV therapy in women with low neutrophil counts

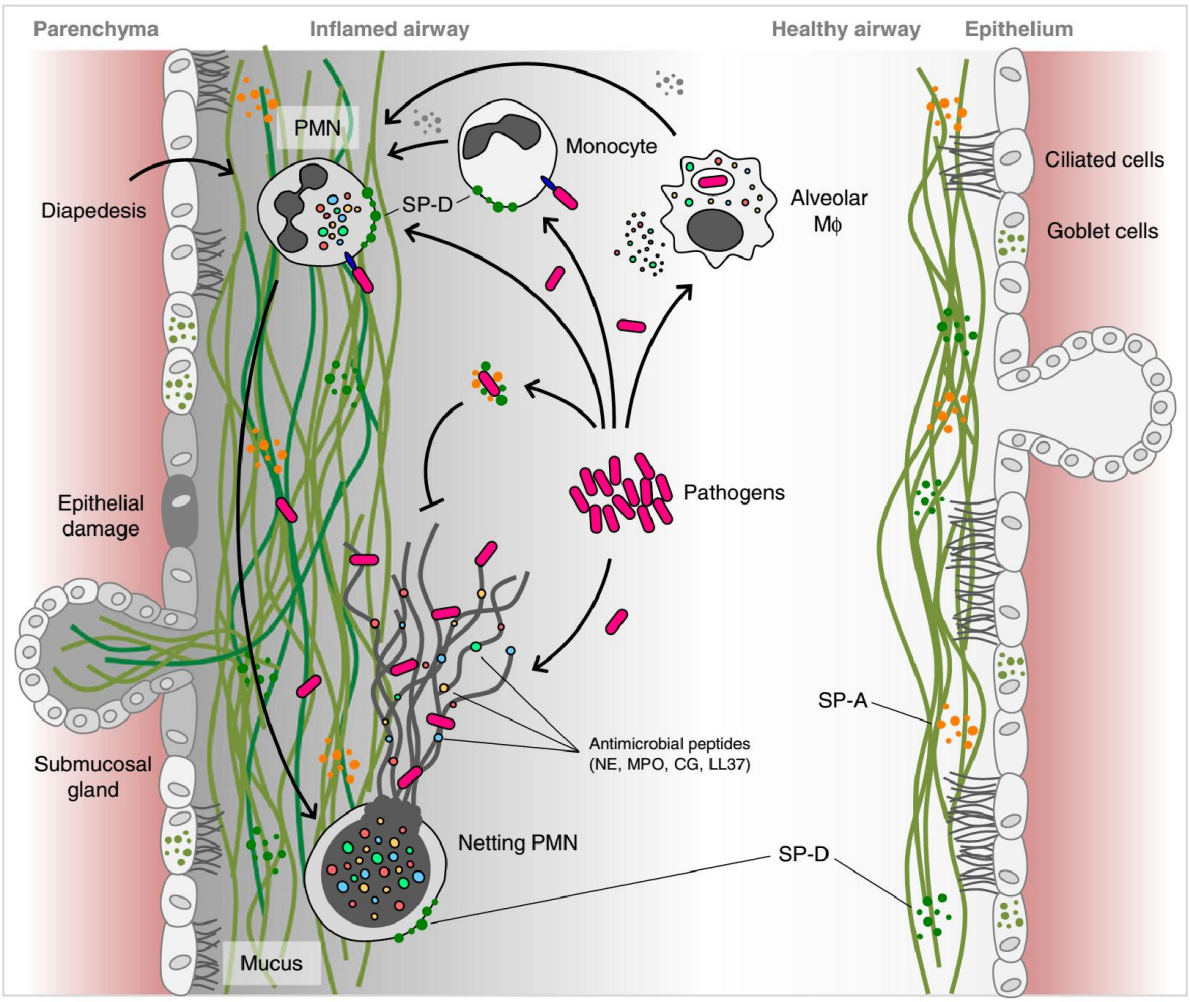

Fig. 5 NETosis—modulation by the respiratory system. The response of the respiratory system to infection is complex involving chemokine recruitment of neutrophils and monocytes into the lumen of the alveoli. The NETotic release of cytotoxic DNA–protein complexes with NE, MPO, LL37, and other neutrophil proteases increases mucus viscosity and contributes to lung epithelial damage, thereby perpetuating a vicious cycle of injury and inflammation. Innate immune surfactant proteins (also termed collectins) contribute in maintaining the lungs' homeostasis with minimal inflammation. NETs are removed by DNAse degradation and macrophage ingestion. A delicate equilibrium between NETosis and NET clearance is fundamental for the successful combating of infectious agents with minimum tissue damage. *NE* neutrophil elastase, *MPO* myeloperoxidase, *LL37* cathelicidin, *SP-A* surfactant protein A, *SP-D* surfactant protein D, *PMN* polymorphonuclear neutrophil, *Mφ* macrophages. Adapted and modified from Chen et al. [81]

may need to be started earlier in pregnancy to prevent viral transmission to the fetus.
2. Pregnant women are more susceptible to infections with influenza, resulting in high degrees of maternal or fetal morbidity and mortality [116].
3. In affected populations, pregnant women generally have a higher risk for malaria than nonpregnant women [117]. Since the malaria parasite is sequestered to the placenta, this leads to severe complications such as fetal growth restriction and preterm delivery [117, 118].

It is not clear why pregnant women should be more susceptible to malaria infection, especially if they are primigravid, but it may be linked to a reduction in neutrophil counts [119] and dysfunction of residual neutrophils due to impaired NADPH oxidase activity [120].

These findings suggest that pregnant women should be regarded as a separate entity at high risk for infection with a diverse array of pathogens. This may result from an altered immune response and, consequently, implies that pregnant women will require tailored therapeutic strategies.

Discussion and conclusions

In this review, the emphasis has been placed on mechanisms used by pathogenic microorganisms to evade entrapment and killing by PMN NETs. These range from the expression of DNAse molecules that degrade the nucleic acid lattice backbone [10] to the induction of immune-modulatory molecules such as IL-10 that dampen the NETotic response of PMN. The latter is employed by both HIV [88] or Sias-coated *P. aeruginosa* bacteria [14]. In the context of HIV, it is possible that IL-10-mediated dampening of the innate immune response may contribute to the secondary infection of affected individuals with tuberculosis or pneumococci.

By studying such interactions in more detail, it may not only be possible to devise strategies to optimize the host immune response but also develop therapies to counter pathologies associated with overt NETosis. In this manner, by antagonising the effect of IL-10 in cases with AIDS, it may be possible to reduce concomitant secondary infections. On the other hand, the use of IL-10 may be of interest in autoimmune conditions associated with increased NETosis such as systemic lupus erythematosus [121, 122].

In this regard, it is ironic that the first therapy used to alleviate and facilitate clearing of the dense mucus, which largely consists of NETs, from the lungs of CF patients was by the application of rhDNAse [123], thereby mimicking a strategy first explored by streptococcal bacteria [10, 11].

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