Identification of the factors that modulate neutrophil response towards NET formation during normal pregnancy and in gestational diabetes mellitus

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Summary

Circulatory neutrophils exhibit an increased basal activation state in human pregnancy, which is overt in preeclampsia, a severe pregnancy specific disorder. Neutrophils play a defining role in combating infection either by phagocytosis or by degranulation; moreover, there have recently been shown to form neutrophil extracellular traps (NETs), containing extruded DNA, histones and granular proteins. The main activity of the NETs is the entrapment of bacteria, viruses and eukaryotic microorganisms, but more interestingly they seem to play a central role in the regulation of the overall inflammatory response.

Pregnancy presents an extraordinary immunological challenge to the maternal immune system. Successful gestation to term, birth and postpartum survival require tolerance to the semi-allogeneic fetus while maintaining the potential to mount a vigorous response against infection. During normal pregnancy insulin sensitivity also declines with advancing gestation, while a compensatory increase in insulin maintains the normal glucose homeostasis. Gestational diabetes mellitus (GDM) is a condition of glucose intolerance first recognized during pregnancy resulting in hyperglycemia of variable severity. GDM develops when the pancreatic beta cells are unable to regulate the increased insulin demand. During pregnancy there is increased degranulation and phagocytic activity of circulating neutrophils indicates that these immune cells play a key role in pregnancy adaptations. The mechanisms remain unknown, though sex hormones provide likely candidates. We therefore investigated and characterized the neutrophil activity during normal pregnancies and in pregnancies affected with gestational diabetes.

For this purpose, first we explored neutrophil activity during each trimester of normal pregnancy and in addition we studied the neutrophil response in pregnancies during and after the diagnosis of GDM. Neutrophils were isolated from EDTA blood, while products of NETosis were examined in serum and plasma. Cell-free circulating nucleosomes, myeloperoxidase (MPO), neutrophil elastase (NE), granulocyte-colony circulating factor (G-CSF) and tumor necrosis factor α (TNFα) were determined by ELISA. NET formation was detected by Sytox Green, a fluorescent non-permeable DNA

dye and verified by immunofluorescence staining and morphometric analysis. The activated state of neutrophils was also evaluated by monitoring the reactive oxygen species generation. NET associated protein levels were analyzed by Western blots.

Our primary results during normal pregnancy show that neutrophil numbers in the periphery increase during gestation, in parallel with circulatory G-CSF. G-CSF induced NET formation and primed neutrophils toward an intense pro-NETotic response at concentrations prevalent in pregnancy. Moreover, detailed investigation of signaling related to the neutrophil activation revealed that the propensity to form NETs was advanced by both chorionic gonadotropin and estrogen/estriol. In contrast, progesterone acted by retaining cells in a primed pro-NETotic state, but inhibited their progress to formation of NETs. This coincided with the prevention of neutrophil elastase translocation from the cytoplasm to the nucleus, an indispensible step for NET release.

Neutrophils isolated from pregnant women with GDM were determined to be highly reactive and formed NETs more vigorously when compared to neutrophils isolated from healthy pregnant women, as observed by fluorimetry, immunocytochemistry and morphometric analysis. Moreover, high glucose and TNFα, which are increased in gestational diabetic individuals, primed neutrophils towards NET formation. The infiltration to the placenta of these primed neutrophils led to excessive NE release and the degradation of insulin receptor substrate 1 (IRS1).

Our data provide evidence that the increased neutrophil priming and NET formation observed during normal pregnancy is well modulated by the sex steroid hormones and G-CSF. Moreover we propose that neutrophils participate to the pathobiology of GDM. Our data demonstrate that the TNFα driven inflammation, resulting from high glucose levels in the circulation, enhances neutrophil priming and NET formation. The released NE can locally modify glucose tolerance and metabolism in the placenta, which probably results to the anatomical, physiological and functional changes observed in placenta of pregnant women suffering from GDM.

Chapter 1

Innate immune response

The immune function has been divided into innate and adaptive immunity. The innate immune cells are the first line of defense against invading pathogens. They are also required to initiate specific adaptive immune responses.

Innate immune responses are not specific to a particular pathogen in the way that adaptive immune responses are. They depend on specialized cells and soluble molecules that recognize conserved features of pathogens and are quickly activated irrespective of previous contact with the invading agents. The main effector cells of innate immunity are macrophages, neutrophils, dendritic cells (DC), natural killer cells (NK) and innate lymphoid cells (ILC).

What triggers the innate immune response?

The recognition of damaged or dying cells either in the presence or absence of infection initiates the innate responses. After a pathogen invasion, molecules commonly found on the surface of microorganisms, or on the surface of the infected cells are called pathogen-associated molecular patterns (PAMPs). In contrast, damaged cells release damage-associated molecular patterns (DAMPs) as endogenous danger signals to alert the innate immune system. Cell damage or death in the absence of pathogen gives rise to DAMPs only, but when a pathogen attacks, both DAMPs and PAMPs will be present. The recognition of DAMPs and PAMPs activate the innate immune response by interaction with different receptors known as pattern recognition molecules (PRM), most of which are expressed by innate leukocytes and are called pattern recognition receptors (PRR). There is a limited repertoire of molecular patterns that can be recognized, however, each PRR recognizes a DAMP or PAMP that is shared by many different damaged cells or pathogens. Thus, PRRs give the innate immune response the

property of *broad recognition*. Furthermore, because the innate leukocytes expressing PRRs are present in large numbers, these cells do not need to multiply to work effectively and the response is *immediate*.

PRRs can be located in the plasma membrane of an innate leukocyte, or can be soluble molecules free in the leukocyte's cytoplasm, or fixed in the membranes of the endosomes. The interaction between PRRs with a DAMP or PAMP stimulates the inflammatory response and the phagocytosis by cells such as neutrophils and macrophages. Many of the mammalian cell-surface pattern recognition receptors responsible for triggering host cell gene expression in response to pathogens are members of the Toll-like receptor (TLR) family. TLRs are abundant on the surface of macrophages and neutrophils and act as an alarm to the immune system whenever an infection occurs.

General features of the innate immune response- Inflammation

The inflammatory response is characterized by pain, redness, heat and swelling at the site of infection. The local blood vessels dilate and become permeable to fluid and proteins, leading to local swelling and an accumulation of proteins, including the components of the complement cascade. Inflammation induces new gene transcription and synthesis of pro-inflammatory cytokines. Especially activation of TLRs results in the production of lipid signaling molecules such as prostaglandins and peptide signaling molecules such as cytokines. Concurrently, the endothelial cells express cell adhesion proteins that facilitate the attachment of neutrophils, lymphocytes and monocytes. The activated macrophages produce chemokines that attract other cells, especially neutrophils and dendritic cells (DCs). Neutrophils are the first cells recruited in large numbers to the site of infection. The dendritic cells take antigens from the invading pathogens and carry them to the nearby lymph nodes.

Once the threat is eliminated, the inflammation resolves naturally. However, if the inflammation fails to resolve and becomes chronic it can cause tissue damage and impair immune system function, as in sepsis and asthma.

Neutrophils

Polymorphonuclear neutrophils (PMNs), also known as granulocytes, are the most abundant leukocytes in the human circulation with an important role in the early stages of inflammatory reaction. They constitute the 50-70% of the human circulating leukocytes (Fig.1). Granulocytes are produced in the bone marrow as terminally differentiated cells and they have a half-life of 6 to 8 hours [3] which can be extended after exposure to certain stimuli [4].

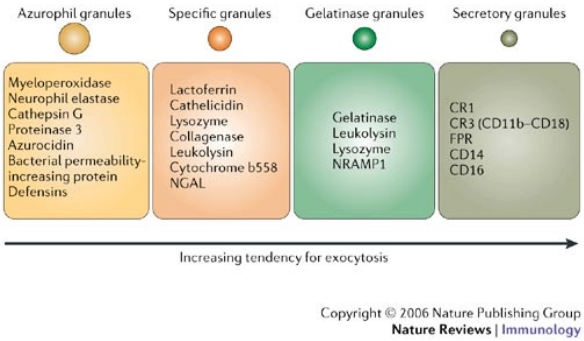


Figure 1.:Neutrophil. Modified from Wikipedia.

Neutrophils are sensitive to chemotactic agents, such as cleavage products of complement fractions (C3a and C5a) and substances released by mast cells and basophils. They are among the first cells to migrate from vessels to tissues attracted by chemokines, such as IL-8, and are activated by various stimuli, such as bacterial products, complement proteins (C5a), immune complex (IC), chemokines, cytokines. In response to different signals, neutrophils express a broad range of molecules that are crucial to the development of innate and adaptive immune responses against several pathogens [5]. Activated neutrophils, for example, release several cytokines that mediate the induction and recruitment of CD4+ Th1 cells, T helper 17 (TH-17) cells and CD8+ T cells [6]. They can also act as antigen-presenting cells, as they can cross-present exogenous antigens to CD8+ T cells and provide co-stimulatory signals to activate T cells [7].

Neutrophil morphology

In the circulation mature neutrophils have an average diameter of 7-10μm, their nucleus is segmented and their cytoplasm is enriched with granules [8]. Neutrophils contain four types of granules, which are formed consecutively during their maturation, and they are filled with pro-inflammatory

proteins. The azurophilic or primary granules among other factors contain myeloperoxidase (MPO), neutrophil elastase (NE), cathepsin G, lysozyme and defensins. The specific or secondary granules contain mainly antimicrobial proteins such as lactoferrin and lysozyme. The gelatinase or tertiary granules store mainly metalloproteases such as gelatinase and a few antimicrobials. The secretory granules contain cytokines and important phagocytic receptors that are exposed to the neutrophil surface upon priming and fusion of these granules with the plasma membrane [9, 10]. The granules are classified according to their protein content and their differential ability to be exocytosed after neutrophil stimulation (Fig. 2). The azurophilic granules lack the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) that would direct them to fuse with the plasma membrane [11]. This means that the contents of these granules are either deployed inside the phagosome or are released extracellularly via the formation of NETs.

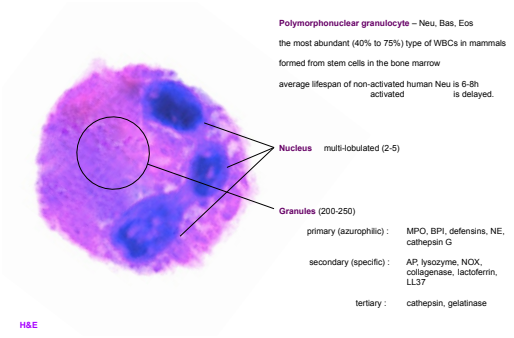


Figure 2.:Neutrophil serine proteases, adapted from[2].

Neutrophil activation

Neutrophil activation is usually a multistep process. It begins with the partial activation of cells as they transit through the vascular endothelium during the recruitment process (Fig. 3). After entry into the inflammatory tissue site, in response to pro-inflammatory stimuli in the tissue, neutrophils become fully activated, a state characterized by release of granule proteins, phagocytic activity, and the production of NETs. Neutrophils are relatively nonresponsive to a single stimulus, but exposure to one stimulus (e.g., lipopolysaccharide, tumor necrosis factor, chemokines, growth factors, adhesion) enhances the ability of the cell to mount an enhanced activation response to a second individual stimulus [12]. This effect, referred to as

neutrophil priming, allows rapid and maximum neutrophil activation, including enhanced phagocytosis and radical oxygen generation [13].

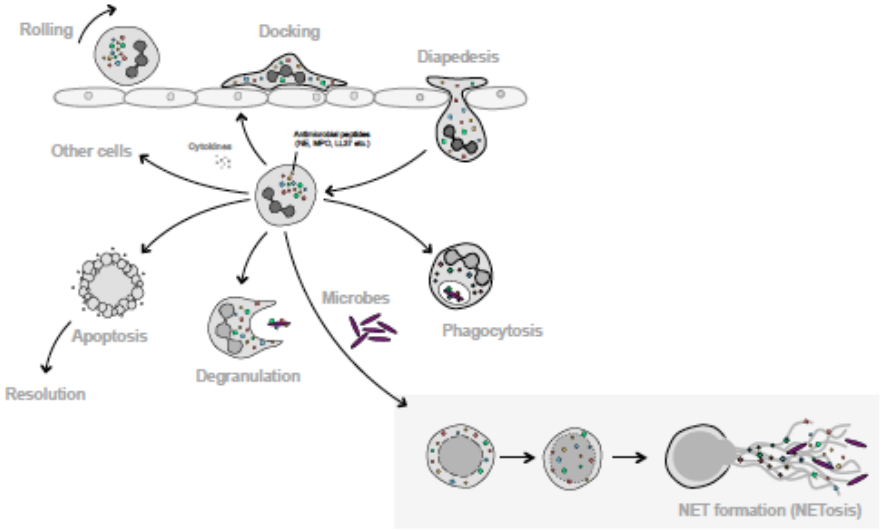


Figure 3.: Neutrophil functions. Modified from [14].

Neutrophil activation by PAMPs or DAMPs

Neutrophils are able to recognize pathogens through cell surface and intracellular receptors that bind to microbe-specific molecules and host proteins, such as IgG and complement, that are opsonizing the microbe. These receptors induce intracellular signals that lead to full pathogen-killing capacity.

The neutrophil pattern-recognition receptors (PRRs) recognize Pathogen associated molecular patterns (PAMPs), like lipopolysaccharide, peptidoglycan and lipoteichoic acids, double-stranded viral RNA and bacterial DNA. During sterile inflammation such as burns or hypoxia, necrotic cells release damage-associated molecular patterns (DAMPs), like high mobility group protein B1, mitochondrial formyl peptides, mitochondrial DNA, that are also recognizable by PRRs.

In neutrophils, the primary endocytic PRRs are the C-type lectin receptors, the most important of which is Dectin-1 that recognizes fungal β-glucan. Dectin-1, together with the integrin Mac-1, internalizes and eliminates fungal pathogens [15]. Another endocytic receptor is TREM-1 that binds various pathogens [16].

The major type of PRRs on neutrophils is the TLRs, which recognize lipids, carbohydrates, peptides, DNA, and single- and double-stranded RNA [17]. At the RNA level, neutrophils express TLR-1, -2, -4, -5, -6, -8, and -10, and after GM-CSF treatment, TLR-9 [18]. TLR engagement primes neutrophils for enhanced responses to other stimuli, thus augmenting their phagocytic capacity, stimulating increased cytokine release and slowing neutrophil apoptosis [19].

Other signaling PRRs include the cytosolic microbial sensors NOD1 and NOD2, which recognize peptidoglycan-related molecules of gram-negative and gram-positive bacteria respectively [20].

Neutrophils express opsonic receptors and the engagement of the complement or the IgG-opsonized pathogens lead to a strong stimulation of neutrophil killing mechanisms. Resting neutrophils express two types of complement receptors, CR3 (also known as Mac-1) and CR4, which recognize targets opsonized by the complement activation product C3bi [21].

Neutrophils express both low and high affinity receptors for the Fc portion of IgG, termed FcγRs. Resting human neutrophils express the low affinity activating FCγRIIA and FCγRIIB, which have low affinity for monomeric IgG, but high affinity for immune complexes, whereas activated neutrophils upregulate expression of the high-affinity FcγRI for IgG [22].

A third major type of opsonin that engages both FcγRs and CRs are the pentaxin molecules. The best-studied pentraxins are the C-reactive protein (CRP) and serum amyloid P component (SAP), both of which are produced in the liver during inflammation. CRP and SAP can opsonize microbial pathogens through recognition of PAMPs. Association of CRP or SAP with a microbe induces rapid complement activation on the pathogen surface, facilitating recognition of the pathogen by neutrophil CRs. Additionally, both CRP and SAP are directly recognized by FcγRs to induce neutrophil activation [23].

Neutrophils express a large repertoire of G protein-coupled receptors (GPCRs) that recognize bacterial products, like formyl peptides, as well as endogenous molecules released during inflammation, such as leukotrienes, chemokines (e.g., IL-8), C5a and adenosine. These GPCRs are involved

mainly in guiding neutrophil migration, but can promote neutrophil priming that in response to a second stimulus lead to full cellular activation [24].

Neutrophil killing mechanisms

Phagocytosis

Neutrophils are efficient phagocytes that engulf and degrade microorganisms using a combination of oxidative and non-oxidative mechanisms. Once inside the neutrophils, microorganisms are sequestered in a specialized compartment called phagolysosome. During the oxidative arm, phagocytosis activates the membrane-bound NADPH oxidase system and this generates large quantities of reactive oxygen species (ROS), such as superoxide ($O2^-$), hydrochlorite (HOCl), hydrogen peroxide, hydroxyl redicals, and nitric oxide (NO) that are released into the phagolysosome (Fig. 4). The non-oxidative arm is constituted by fusion of neutrophil granules containing all the peptides and proteases with the phagolysosome leading to efficient killing of the microbes [2].

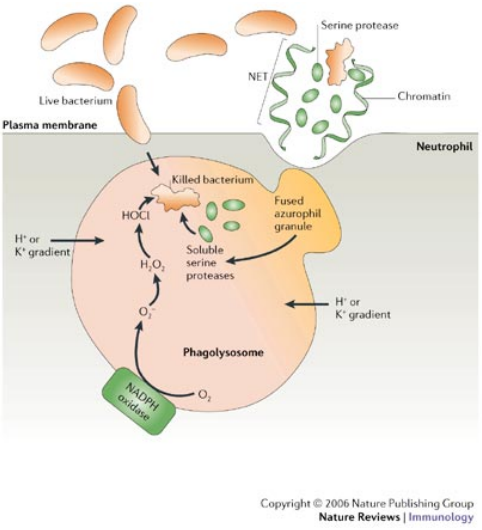


Figure 4. : Intracellular and extracellular bacterial killing. Adapted from [2]

Generation of reactive oxygen species

Together with phagocytosis the respiratory burst is associated with the activation of NADPH oxidase. The assembly of a functional NADPH oxidase requires the inducible translocation of the cytosolic NADPH oxidase components $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ to the membrane, where the $gp91^{phox}$ (NOX2), $gp22^{phox}$, and the GTPase Rac2 reside [25]. Its importance is revealed in patients with chronic granulomatous disease (CGD), who lack any one of the oxidase subunits.

The primary granule protein MPO catalyzes the formation of hypochlorous acid through reaction of hydrogen peroxide with chloride [26]. Nitric oxide production also complements ROS production by neutrophils. Nitric oxide (NO) is a short-lived (half-life of a few seconds), highly reactive molecule, which is produced by inducible nitric oxide synthase (iNOS). NOS is present in primary granules and is induced upon neutrophil priming (via TNF, IL-1, INF-γ) and during bacterial infection [27].

Degranulation

Degranulation involves the fusion of granules with the plasma membrane and the release of cytokines and antimicrobial contents into the extracellular space. All the types of granules have the capacity to degranulate extracellularly except from the primary granules. This is probably because they lack the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) that would direct them to fuse with the plasma membrane [11]. The primary granules are largely degranulated into the phagosome thereby exposing ingested microorganisms to high concentrations of granule contents or are released extracellularly through the formation of neutrophil extracellular traps (NETs).

NETosis

Another antimicrobial strategy of neutrophils is the formation of NETs. NETs contain extruded DNA in the extracellular environment, decorated with histones and granular proteins. Their main activity is the entrapment of bacteria, viruses and eukaryotic microorganisms, but more interestingly they seem to play an important role in various autoinflammatory autoimmune diseases.

NETosis is activated not only by pathogens and their components, but also by activated platelets through many mechanisms such as TLR4 [28] or P-selectin/PSGL-1 [29], the release of thromboxane A2 [30] and β-defensin-1 [31]. In addition, both soluble and insoluble immune complexes (ICs) induce neutrophil extracellular trap release via FcγRIIIA and FcγRIIIB [32, 33].

According to preliminary studies, anti-neutrophil antibodies that can directly induce NETosis NETosis have been isolated from patients suffering from the autoimmune disease Small Vessel Vasculitis (SVV) [34], Rheumatoid Arthritis (RA) [35] and Systemic Lupus Erythematosus (SLE) [36].

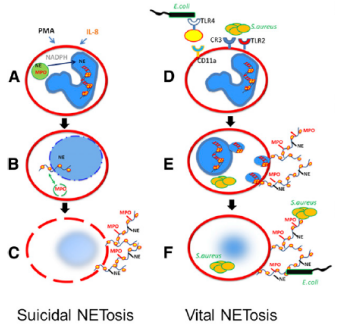


Figure 5. : Suicidal vs Vital NETosis. Adapted from [1]

At present, two NET formation mechanisms have been described (Fig. 5.), the NETs via a slow lytic cell death mechanism (120-240 min) and the rapid (5-60 min) release of NETs from live cells [1].

Molecular mechanisms of NETosis

NET formation is a three-step process that begins with a change in the characteristic morphology of the nucleus. Accordingly, nuclear membranes disassemble and chromatin decondenses into the cytoplasm. Finally, the plasma membrane bursts and the NETs are released. The process is irreversible and is dependent on reactive oxygen species (ROS) such as superoxide, whish is generated by the NADPH oxidase Nox2. Several enzymes that regulate Nox2 activity such as protein kinase C (PKC) isoforms and MAPK kinases have been implicated in NET formation.

Interestingly, MPO appears to drive NETosis independently from its enzymatic activity in a mechanism that involves synergy with NE. In response to ROS, the neutrophil-specific protease, NE escapes from the azurophilic granules into the cytoplasm and translocates to the nucleus where it partially degrades histones. Subsequently, MPO associates with chromatin and promotes chromatin relaxation. MPO and NE synergize to drive massive chromatin decondensation [37] (Fig. 6).

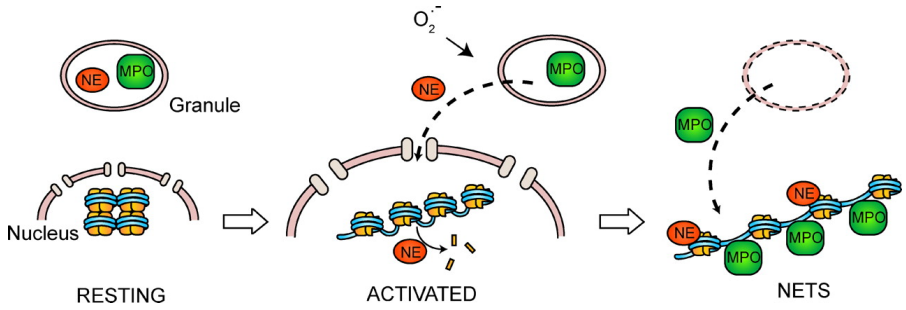


Figure 6. :Chromatin decondensation by granule proteins. Modified from [37].

Another prominent hallmark of NETosis that leads to chromatin the decondensation is the citrullination of the histone proteins by the peptidylarginine deiminase (PAD) enzymes. Citrullination, also known as deimination, is the conversion of positively charged arginine side chains into polar but uncharged citrulline side chains by deamination (Fig. 7).

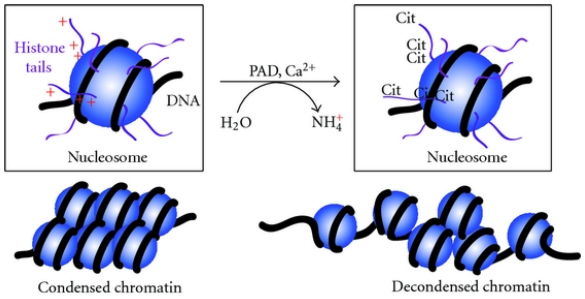


Figure 7. : Histone citrullination.Adapted from Mohanan S et al. . Hindawi Publishing Corporation Biochemistry Research International Volume 2012

Especially the enzyme PAD4, the only of the five known peptidylarginine deiminases in human that has a classical nuclear localization signal, targets histone arginine and mono-methylarginine residues for citrullination in a calcium dependent reaction [38] [39]. Consequently, histones H3 and H4 and linker H1 histone lose their positive charge and their heterochromatin structure resulting in the disassembly of the histone-DNA complex [40].

NETosis is a novel cell death mechanism

NETosis dinstinct from necrosis and apoptosis was first described following chemical stimulation with phorbol 12-myristate 13-acetate (PMA) [41]. Neutrophil stimulation by PMA activates protein kinase C and the raf-mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase

(ERK) pathway. In comparison to apoptotic cells, cells that undergo NETosis do not display eat-me signals to the phagocytes, such as phosphatidylserine (PS) before plasma membrane disruption. In contrast to apoptosis or programmed necrosis (necroptosis), during NETosis both the nuclear and granular membranes disintegrate, but plasma integrity is maintained. During this stage the antimicrobial granular proteins mix with the nuclear content. No morphologic signs of apoptosis are observed, such as membrane blebbing, nuclear chromatin condensation, PS exposure before plasma membrane rupture and internucleosomal DNA cleavage. Caspase activity is only detected during spontaneous apoptosis, but not during PMA-induced NETosis.

The presence of histones in NETs further indicates that nuclear and not mitochondrial chromatin is the major constituent of NETs. However, Simon's group displayed that following priming with granulocyte/macrophage colony-stimulating factor (GM-CSF) and subsequent short-term toll-like receptor 4 (TLR4) or complement factor 5a (C5a) receptor stimulation, viable neutrophils are able to generate NETs. These forms of NETs contained only mitochondrial and not nuclear DNA and did not require neutrophil death [42].

Antimicrobial activity of NETs

The main role of neutrophils is to eliminate microbes. It is probable that NETs evolved to restrain infection by trapping, preventing dissemination, inactivating virulence factors and eliminating microbes.

Trapping microbes prevents their dissemination from the initial infection site. Microbes most likely stick to NETs through charge interaction [43]. Nonetheless, pathogens to prevent binding to NETs, mask themselves with a capsule or change their surface charge [44]. Bacteria also attach nucleases to their surfaces to disengage themselves from NETs [45]. For example, group A *S. pyogenes*, pneumonococcus and *Staphylococcus aureus* encode endonucleases that detach them from NETs [46-48].

The antimicrobial activity of NETs depends on their structure, which provides a high local concentration of antimicrobials in direct proximity to trapped microorganisms. This structure is lost after DNase digestion.

Accordingly, the expression of DNases by the bacteria is essential for their pathogenicity [46].

NETs contain several proteins that kill or inhibit microbes. These include enzymes (lysozyme, proteases), antimicrobial peptides (BPI, defensins), ion chelators (calgranulin) and interestingly, histones. For instance, NE and other proteinases like Cathepsin G and Proteinase 3 (PR3) on the NETs specifically are able to cleave virulence factors [49].

NETs in infection

NETs appear to be a form of innate immune response that kill a wide variety of microorganisms ranging from gram-positive to gram-negative bacteria, fungi, parasites and viruses [50, 51].

Several studies report that gram-negative bacteria such as *S. flexneri* and *E.coli* are able to trigger NET release and NET formation is important for the effectiveness against *K. pneumonia* [51]. Likewise, the gram-positive bacteria *S. pneumoniae*, *S. pyogenes* and *S. aureus* are good inducers of NETosis although many observations suggest that these bacteria may have evolved mechanisms to escape NETs [51]. Notable, although neutrophils and macrophages cooperate in order to confront M. tuberculosis, the bacteria are trapped but not killed [50]. These results suggest that NETs may be important to restrict physically these bacteria, preventing their spreading from the site of infection systemically.

NETs could also been induced by eukaryotic pathogens such as *C. albicans* and are sufficient to kill either the yeast or the hyphal form. A major antifungal component of NETs is calprotectin, a cytoplasmic protein, with calcium- and zinc-binding properties that when released bound to NETs leads to the killing of the fungus [43]. In addition to C. albicans, NETs have been described effective against *A. nidulans* and *A. fumigatus* [52].

Little is known regarding the role of NETs in viral infections. Infection with an oncolytic poxvirus in a murine model resulted in an immune-thrombotic response and generation of NETs. Presumably, NETs appeared to be useful for the clearance of the virus [53]. In contrast, viral infections appear to modulate host response in order to suppress NETosis. For instance, in

Feline leukemia virus (FeLV), neutrophil activation is inhibited through the inhibition of PKC and reduced ROS production [54]. On the other hand, secondary stimulation by *Leishmania* promastigotes induced NET formation [54]. In the case of HIV infection, although NETs are able to capture and neutralize the negatively charged HIV virions, engagement of DC-SIGN on dentritic cells (DCs) leads to the production of IL-10 by DCs, which suppresses NET formation [55].

The role of NETs has also been explored in parasitic infections. *Leishmania* parasites have been reported to trigger rapid NETosis in a manner dependent on NE and independent of ROS production [56]. NETs have also been detected in blood smears of pediatric patients infected with *P. falciparum*, one of the protozoa that cause Malaria [57]. During pregnancy malaria infection is associated with anemia and increased risk of spontaneous abortion, prematurity, low birth weight or stillbirth [50]. Another parasite that increases the risk for complications in pregnancy is the T.gondii [58].

Neutrophils influence the resolution of inflammation

The inhibition of neutrophil influx is required to prevent the perpetuity of the inflammatory reaction, as these cells harbor the most destructive potency for tissue damage. Recent evidence suggests that neutrophils are involved in active induction of resolution of inflammation through dismantling chemokine and cytokine gradients [59], through pro-inflammatory lipid mediators that are switched to pro-resolving mediators [60] and by the inhibition of the activation and recruitment of the circulating neutrophils to the site of inflammation.

During the late, final phases of acute inflammatory responses, neutrophils switch their eicosanoid biosynthesis from leukotriene B4 (LTB4) to lipoxin A4 (LXA4), which can inhibit neutrophil recruitment through its interaction with its G protein coupled receptor LXA4R. Neutrophils can also contribute to the biosynthesis of resolvins (such as resolvin E1, resolvin E2, resolvin D1 and resolvin D2) and protectin D1, which are derived from omega-3 essential polyunsaturated fatty acids. These pro-resolving lipid mediators inhibit neutrophil transendothelial migration and tissue infiltration [60].

The pro-resolving lipid mediators increase the expression of CC-

chemokine receptor 5 (CCR5) by apoptotic neutrophils, and this, in turn, promotes the sequestration and clearance of CC-chemokine ligand 3 and CCL5 [59].

Other mechanisms are oriented to limit the pro-inflammatory effects of IL-1. Neutrophils stimulated with the anti-inflammatory cytokine IL-10 express IL-1 receptor antagonist (IL-1RA), a soluble molecule that binds to and blocks IL-1R1 [61]. Moreover, the expression of type 2 IL-1 receptor (IL-1R2) binds IL-1 and prevents its interaction with the signal-transducing receptor IL-1R1 [62].

The dark side of NETosis

Excessive NET formation is linked to tissue injury and is related to various neutrophil mediated pathologies. These include inflammatory diseases as Psoriasis [63] and Cystic fibrosis (CF) [51]. NETs are also a rich source of pro-inflammatory molecules and autoantigens and may be implicated in systemic autoimmune disorders as Systemic Lupus Erythematosus (SLE) [64], Small-vessel vasculites [34] and Rheumatoid Arthritis (RA) [65]. Excessive NET formation and endothelial cell activation are also associated with preeclampsia of pregnancy [66].

NETs in inflammation

Psoriasis is a common inflammatory disorder of the skin and other organs. Psoriasis pathogenesis has been attributed to the externalization of IL-17 in the extracellular traps of neutrophils and mast cells [63].

Cystic fibrosis (CF) is a debilitating hereditary disease, caused by a mutation in the CF transmembrane conductance regulator (CFTR) ion channel. When CFTR is not functional the viscosity of the sputum is very thick and is difficult to clear from the airways. The lack of sputum clearance promotes bacterial colonization leading to chronic airway inflammation and low life expectancy of the affected individuals [67]. Several studies have confirmed the abundance of NETs in CF sputum, where high levels of decondensed extracellular DNA, MPO and NE are detected [51, 68].

Periodontitis is a chronic inflammation of the periodontium caused by bacteria in the gingival crevice. During this inflammatory condition, neutrophil influx leads to the formation of a purulent crevicular exudate that contains phagocytosing and Netting neutrophils [69].

Gout is characterized by acute joint inflammation triggered by inflammatory responses to uric acid crystals. Neutrophilia and neutrophil activation in gout is associated with the formation of proinflammatory NETs [70].

Preeclampsia is a severe inflammatory and vasculopathic condition that affects 1-2% of pregnant women and is characterized by proteinuria, oedema and hypertension. Untreated preeclampsia can develop into eclampsia accompanied by seizures, which can be life threatening for the mother and the fetus. Preeclampsia is associated with abundance of microdebris in the maternal blood circulation. Furthermore, massive numbers of NETs have been reported in the intervillus space of the affected placentae, apparently triggered by trophoblat microdebris and IL-8 [71]. This microdebris can activate neutrophils and stimulate the release of NETs in a dose-dependent manner. Moreover, the increased IL-8 release by the placenta is able to recruit neutrophils and seem to be important for priming them to respond to the microdebris [72] [73].

NETs in autoimmune diseases

Systemic lupus erythematosus (SLE) is a diverse autoimmune disease, which mainly affects internal organs of the body as skin, heart, joints, lungs, liver, and kidneys. SLE patients exhibit elevated levels of antineutrophil cytoplasmic antibodies (ANCAs), antibodies against histones, DNA (ANAs) and ribonucleoproteins (RNP) that target host tissues [74]. NETs in lupus complexes contain LL37, which triggers TLR9 in plasmacytoid DCs, and subsequently secrete IFN-α. IFN-α has been shown to prime neutrophils to respond to autoimmune complexes by releasing NETs, which in turn activate pDCs further [75]. In a subset of SLE patients there is decreased NET degradation resulting in an imbalance between NET formation and NET clearance [76]. Moreover, low-density granulocytes isolated from the blood of

SLE patients tend to undergo spontaneous NET formation, while high density SLE neutrophils do not form NETs spontaneously [64].

Small-vessel vasculitis (SVV) is a chronic autoinflammatory condition leading to necrotic inflammation of small-sized blood vessels and capillaries. Neutrophils play a central role in the pathophysiological process of SVV since they are responsible for endothelial damage and are the main target for antineutrophil cytoplasm autoantibodies (ANCAs) directed against granule proteins as MPO and PR3. These autoantigens are able to activate pDCs and B cells in a TLR-9 dependent manner, thus maintaining the autoimmune reaction and the production of NETs [34]. It has recently been described that the ANCA autoimmune response is facilitated as well, by insufficient T-cell and B-cell regulation and by the involution of an alternative complement pathway [77].

Rheumatoid arthritis (RA) is a systemic autoimmune diseaseassociated with chronic inflammation, primarily in the synovial joints. NETs are abundant in synovial fluids (SF), rheumatoid nodules, and skin of RA patients. In RA patients, NETs are decorated with citrullinated proteins that induce the generation of anti-citrullinated protein antibody (ACPA) autoantigens, which in turn, augment further NET release [35]. Interestingly, it seems that each different stimulus is able to determine the protein composition of the NETs [35]. RA patients show elevated levels of proinflammatory cytokines such as IL-17A and TNF-α that under LPS stimulation induce enhanced NET release. This NET release could be inhibited by blocking NADPH oxidase or PAD4, suggesting a possible NETosis mechanism [35, 78].

Type 1 diabetes (T1D) is an autoimmune disease resulting from the self-destruction of insulin-producing β-cells. Although reduced neutrophil counts have been observed in patients with T1D, the enzymatic activities of the neutrophil serine proteases NE and PR3 are increased and highly associated with the rise of NET formation. Notably, there is a positive correlation between the circulating levels of NE and PR3 and the titers of the autoantibodies against β-cell antigens. Moreover, the levels of the alpha1-antitrypsin, the endogenous inhibitor of serine proteases, are decreased in these patients [79].

NETs and thrombosis

Deep vein thrombosis (DVT) is caused by disturbances and stagnation in venous blood flow. Inflammatory conditions such as infection, pregnancy, obesity, trauma, cancer and autoimmune diseases are risk factors for the development of Deep vein thrombosis (DVT) [80, 81].

Experimental and clinical studies have shown high neutrophil counts and citrullinated histone H3 in venous thrombosis supporting that neutrophils and especially NETs contribute to thrombus formation. In addition, Fibrin and von Willebrand factor (vWF) have high affinity for histones hence they easily bind to NETs [81]. The interaction with the platelets seems also important, as the proteolytic ennzymes NE and cathepsin G activate platelet receptors resulting in the formation of aggregates on the NETs [82].

NETs in cancer

Neutrophils in cancer have been shown to have both protumor and antitumor activities [83].

On the one hand, tumors produce various soluble factors such as IL8, CXCL5 and migration inhibitory factor (MIF), which activate and recruit neutrophils from the peripheral blood into the tumor tissue [84-86]. Neutrophils, on the other hand, release several mediators with protumor functions. For example, neutrophils release MMP9 and VEGF, which promote angiogenesis and tumor growth, MMPs, serine proteases, HGF and oncostatin promoting tumor migration and invasion and are also able to suppress the function of T cells by the expression of arginase 1 [87, 88]. Furthermore, neutrophils enhance the metastatic potential of the tumor cells by facilitating their adhesion to sinusoids, via Mac-1/ICAM1 interaction [89]. G-CSF secreted by tumor cells predispose neutrophils to generate NETs, allowing distant cancer cells to metastasize and contributing to cancer associated thrombosis [90, 91].

Neutrophil response, however, may prove to have antitumor effects. It is important that neutrophils are the most abundant population of circulating white blood cells that express FcγR and FcαR [83]. Neutrophils also recruit other effector cells of the innate and adaptive immune system and respond to

pathogen-derived biologics through the pattern recognition receptors. All the above make neutrophils very beneficial cytotoxic cells that promote the effectiveness of cancer immunothetrapy. Moreover, the conversion from protumor to antitumor neutrophil response has been achieved by pharmacologic immunomodulation, for example after the inhibition of TGF-b in the tumor host, the antitumor activity of neutrophils is enhanced [83].

Chapter 2

Pregnancy

Pregnancy is a unique condition, in which a genetically and immunologically semi-allogeneic fetus develops and survives to birth without rejection by the maternal immune system [92]. Fetal survival depends on profound changes in the mother's host defense strategies. Tight immune regulation is essential, and involves maternal and fetal factors, which influence the immune system to allow the development and maintenance of immune tolerance and immune privilege [93, 94]. In this context, a variety of immune cells increase locally and modify their activities under the pregnancy specific hormonal milieu [95].

During pregnancy neutrophil numbers increase and alter their activities. Neutrophils exhibit an increased ability to migrate, their phagocytic activity is augmented and their oxidant release is greater compared to neutrophils from non-pregnant women [96, 97] In inflammatory pregnancy disorders such as preeclampsia, neutrophils exhibit a more pronounced activation state, which is accompanied by increased formation of NETs [71, 98].

Initiation of Pregnancy

The first step towards pregnancy is fertilization. Fertilization is the merging of two specialized cells "the gametes" to form a new organism. At a cellular level, the fusion between the sperm and the egg requires a series of cell-matrix and cell-cell interactions [99]. The events of fertilization include a synaptic region, which is formed between the sperm and the egg via adhesion molecules, such as the IZUMO and the JUNO [100], the stabilization of the interaction and the recruitment of proteins that assist the fusion of the gametes [101], and the PLCζ that triggers calcium oscillations in the fertilized egg and initiates the downstream signaling pathways of egg activation and embryogenesis [102].

A few hours after the fertilization the dividing cell mass (morula) travels through the fallopian tube to the bottom part of the uterus. Seven days after the fertilization the embryo consists of about 100 cells that form a sphere filled

with fluid, called blastocyst. The implantation of the blastocyst to the endometrium of the uterus initiates pregnancy. The blastocyst is characterized by an inner cell mass, the embryoblast, which will subsequently form the embryo and a surrounding layer of trophoblast cells, which invade into the endometrium. The outer trophoblasts of the blastocyst differentiate into syncytiotrophoblasts and cytotrophoblasts [103]. These trophoblast cells compose the villi structures filled with maternal blood and form the umbilical cord. The placenta grows as an organ consisting of a fetal (decidua) part and a maternal part. Concurrently, the epiblast of the inner cell mass of the blastocyst differentiates into three primary germ layers, the ectoderm, the mesoderm and the endoderm from which the organs and tissues of the body develop during embryogenesis [103]. These fetal surrounding trophoblast cells play very important roles for a successful pregnancy. They provide a structural and biochemical barrier between the maternal and fetal compartments and also serve as an endocrine organ, which produces numerous growth factors and hormones that support and regulate the placental and fetal development and the maternal immune system [104].

The immune system during pregnancy

The main role of the immune system during pregnancy is to protect the mother from infections. To achieve this the innate immune system is very efficient in cell migration in order to observe, to recognize and respond to invading microorganisms. As pregnancy proceeds, a massive influx of different subtypes of leukocytes occurs mediating various immune regulatory functions [105].

Pregnancy has three distinct immunological phases that are characterized by distinct biological processes. The first stage, the implantation, the placentation and the early second trimester, resemble an open wound that requires a strong inflammatory response. During this first stage, the blastocyst in order to implant has to break through the epithelial lining of the uterus, has to damage the endometrial tissue to invade, and by the trophoblast replacement of the endometrium and vascular smooth muscle of the maternal blood vessels, to secure and adequate placental-fetal blood

supply [106]. All these activities create a variable battleground of invading cells, dying cells and repairing cells. An inflammatory environment is required to secure the adequate repair of the uterine epithelium and the removal of cellular debris. In addition, there are hormonal changes and other factors that characterize the first trimester of pregnancy as a proinflammatory phase.

The second immunological phase of pregnancy is a period of rapid fetal growth and development. The mother, placenta and fetus are symbiotic and the predominant immunological feature is induction of an anti-inflammatory state.

Finally, during the last immunological phase of pregnancy, the fetus has completed its development and the mother experiences a renew inflammation probably to prepare to deliver the baby. Parturition is characterized by an influx of immune cells into the myometrium to promote revival of an inflammatory process. This pro-inflammatory environment promotes the contraction of the uterus, expulsion of the baby and rejection of the placenta. In conclusion, pregnancy is a pro-inflammatory and anti-inflammatory condition, depending upon the stage of gestation [107].

NK cells

During the first trimester the most abundant leukocyte population in the decidua are the NK cells that constitute up to 70% [108]. They probable interact with the invading trophoblasts, which express a large repertoire of class I HLA-C and non-classical HLAC-G and HLA-E antigens and lack the expression of the classical HLA-A and –B antigens [109]. Their possible roles include the control of extravillous invasion, the control of uterine vascular remodeling and the local antiviral activity [110]. For a successful pregnancy, there is probably a balance between the killer and regulatory NK cells, since in normal pregnancy the NK cells mainly express TGF-β and IL-10, while in miscarriage they express INF-γ and kill extravillous trophoblasts [104].

Monocytes, macrophages and DCs

In humans the 20-25% of the decidual leukocytes are macrophages and their amount remains high until the onset of labor at term [111]. In the uterine decidual the macrophages can develop an inflammatory phenotype (M1 macrophages) that is characterized by elevated secretion of the inflammatory cytokines, such as IL-12 and TNF-a. Macrophages that differentiate in an environment that is dominated by Th2-biased cytokines, such as IL-4, IL-10, IL-13 or high glucocorticoid concentrations, develop an anti-inflammatory phenotype (M2 macrophages), which is characterized by arginase activity, scavenger receptor expression and secretion of IL-1 receptor antagonist. In women with healthy, full-term pregnancies, there is increased M2 polarization of decidual macrophages as compared with women with preterm pregnancies [112].

At the fetomaternal interface, monocytes contribute to spiral artery remodeling and fetal tolerance. They can differentiate into dendritic cells (DCs), which on the one hand, can promote immune tolerance by inducing effector T cell apoptosis and expansion of $CD4^+$Treg cells and on the other hand can differentiate to mature APCs and prime effector T cells in draining lymph nodes [104].

T cells and T regs

From the adaptive immune system, T lymphocytes constitute about 3-10% of the decidual immune cells, suggesting that may not be crucial to trophoblast invasion [113].

Although during pregnancy there is an increased release of microparticles and exosomes by the growing placenta, the APCs can effectively capture them and then prime the fetal antigen-reactive T cells [114].

Immune tolerance during pregnancy can be explained by the expansion of the $CD4^+$ $CD25^+$ Tregs. $CD4^+$ Treg cells can be generated in the thymus or induced peripherally from naïve $CD4^+$ T cells and the expression of transcription factor forkhead box P3 (FoxP3) accounts for T cell functions in

suppressing autoimmunity or dampening inflammation at mucosal surfaces [115]. Interestingly, phylogenetic analyses identified a conserved noncoding DNA sequence 1 exclusively in the Foxp3 locus of placental mammals serving as a Foxp3 enhancer [116]. Many studies have indicated that the frequency of Treg cells increases during pregnancy in the decidua, which in turn suppresses effector T cell proliferation and immune responses against the embryo [117]. Moreover the hormones progesterone and 17beta-estradiol during the second trimester of human pregnancy have been shown to induce the systemic reduction of functionally suppressive CD4dimCD25highFoxp3+ Tregs [118].

CD8+ T cells and B cells

As pregnancy proceeds there is also a proliferation of CD8+ Tcell, which coincides with the increased detection of fetal antigens systemically [119]. These CD8+ T regulatory or suppressor cells along with the estrogens may dampen antibody production by B cells contributing to fetal tolerance [120].

Neutrophil granulocytes

Neutrophils are a rich source of inflammatory mediators during delivery, such as plasminogen activators, eicosanoids, collagenase, elastase, and proinflammatory cytokines such as IL-1 and TNF [121]. Before labor, along with the cervical dilation, neutrophils accumulate in the cervical capillaries where they degranulate releasing collagen fibrils in the extracellular connective tissue matrix [122]. Furthermore, it has been suggested that MMPs derived from an influx of decidual neutrophils participate in the placental abruption and are associated with human preterm, premature rupture of the membranes [123]. In addition, it has been demonstrated that before the onset of labor neutrophils infiltration in the myometrium is low and rises during labor and that the neutrophil density is significantly greater in the lower than in the upper uterine segment [111].

Several chemokines participate in neutrophil recruitment into the reproductive tissues, including CXCL8, CXCL2 and CXCL3 [124] [125] [126].

A diverse body of evidence currently serves to link overt or aberrant

PMN activation with the development of preeclampia (PE) [127]. These range from the original observations made by the Redman and Sargent group on excessive neutrophil activation in cases with PE [128], which was greater than in matching cases with sepsis, to our own observations on the presence of NETs in affected placentae [71]. In addition, deficient PP13 production may inadequately subvert neutrophil activity, thereby leading to inadequate modification of the maternal spiral arteries [129] [130].

Of considerable interest is the translation of animal model data suggesting that the interplay between the complement system and neutrophils may play a key role in the development of both PE and recurrent fetal loss (RFL) [131] [132]. This has paved the way for the use of novel biologics targeting complement or TNFa activity as therapies [133] [131]. As such, the treatment of these disorders may finally enter the 21st century, making full use of cutting edge innovations [134] [135].

The recent finding that antiphospholipid antibodies (aPL) can induce NETosis begs the question whether this mechanism is active in RFL or in lupus induced PE-like conditions [136]. This finding also suggests that PMN activation by aPL may involve both the complement system, as well as direct interaction by neutrophils with the aPL antibodies.

In summary, the neutrophil is rapidly emerging as a key player in reproductive biology, on the one hand promoting implantation, spiral artery modification and even assisting with the process of parturition. On the other hand, aberrant or overt activation may play a key role in the development of complex pregnancy related disorders such as RFL or PE.

Neutrophil transmigration to the placenta

Neutrophil migration into tissues includes the following steps: tethering, rolling, adhesion, crawling and transmigration. It is initiated by the stimulation of the endothelium by other activated leukocytes or pattern recognition receptor (PRR)-mediated detection of pathogens. The activated endothelium expresses high levels of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) as well as P- and E-selectins on its

surface [137] [138]. Neutrophil recruitment is mainly mediated through the linkage of P selectin glycoprotein ligand 1 (PSGL1), ESL1, CD44 and L-selectin [139, 140]. The interactions of selectins with their glycosylated ligands mediate rolling and the expression of L-selectin is especially indicative of rolling neutrophils [141]. Neutrophil adhesion can be facilitated through activation by proinflammatory cytokines, chemoattractants or growth factors. Moreover, the stabilization of neutrophils to the endothelium is mediated by the interaction of chemokines with the endothelial cell heparan sulfates. Neutrophils express high levels of the integrins CD11a-CD18 (LFA1 / lymphocyte function associated antigen 1) and CD11b-CD18 (MAC1 / macrophage-1 antigen), which bind to endothelial cell surface molecules such as intracellular adhesion molecules 1 and 2 (ICAM1 and ICAM2) [142, 143]. The expression of CD11b-CD18 is important for the crawling of neutrophils [144]. Neutrophil transmigration requires integrins and cellular adhesion molecules (CAMS) such as ICAM1, ICAM2 and VCAM1, as well as platelet endothelial cell adhesion molecule 1 (PECAM1, also termed CD31), CD99, junctional adhesion molecules (JAMs), epithelial cell adhesion molecule (ECAM) and other endothelial cell mole- cules (Fig. 1) [145]. Transmigration occurs between (paracellu- larly) or through (trancellularly) endothelial cells and in order to pass across the membranes, neutrophils release specific proteases such as matrix metalloproei- nases (MMPs) and serine proteases. These enzymes are able to affect neutrophil migration by the degradation of elastin and collagen, thereby increasing the vascular permeability [146, 147]. Interestingly these proteins are under hormonal regulation during pregnancy [148]. On the other hand, neutrophils are able to recruit other neutrophils through the expression of interleukin-17 (IL-17), which induces the release of chemokines and cytokines such as interleukin-6 (IL- 6) and macrophage inflammatory protein – 2 (MIP- 2) by other cells that recruit neutrophils [149].

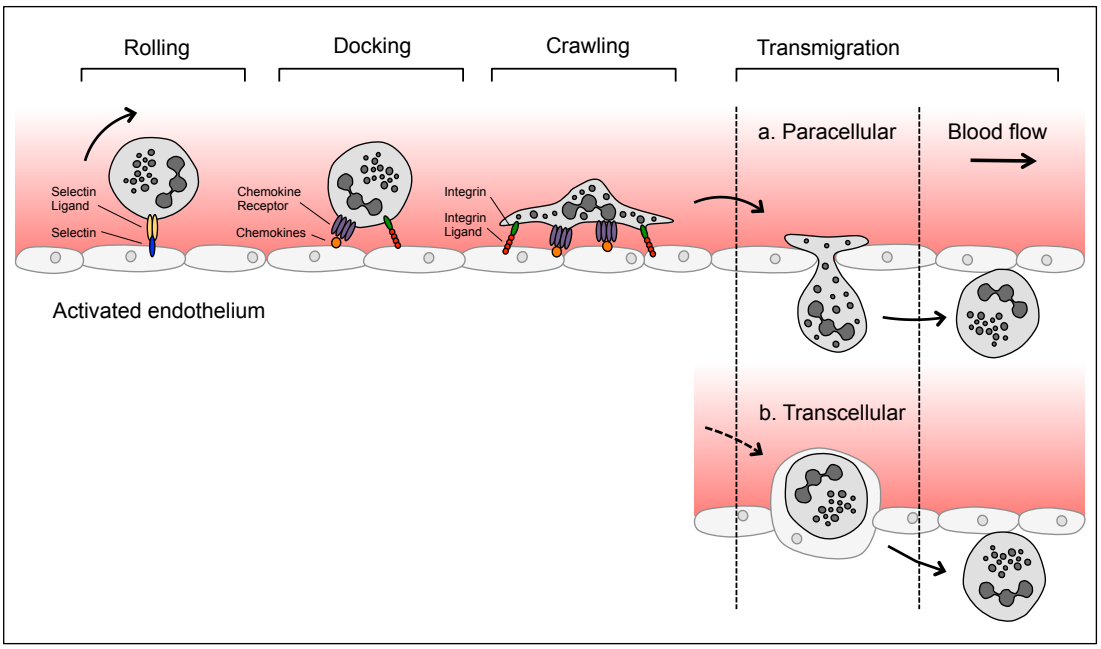


Figure 1. Sequential steps of neutrophil recruitment from the vasculature to the tissue. Two possible mechanisms of transmigration are described: (a.) paracellular - between endothelial cells; and (b.) transcellular - through endothelial cells. Major groups of adhesion molecules are marked. Rolling depends mostly on selectins, whereas adhesion, crawling and transmigration depend on integrin interactions. Chemokines lining the lumen of the vascular endothelium activate rolling neutrophils, thus inducing conformational changes of the integrins on the surface of the neutrophils and facilitating the subsequent events. Crawling neutrophils follow the chemokine gradient along the endothelium, which leads them to the preferential sites of transmigration. Figure adapted from [145].

Immune modulation by pregnancy-associated hormones

Concentrations of steroid hormones, including estrogens and progesterone (P4) are considerably higher during pregnancy than during other times in the female reproductive cycle and increase over the course of pregnancy, with highest levels achieved during the third trimester. Hormonal changes that occur during pregnancy underlie some of the distinct immunological changes associated with pregnancy.

Estrogen

Estrogens belong to the steroid hormones. Three major naturally occurring estrogens have been described in women namely estrone (E1), estradiol (E2) and estriol (E3). Within those E2 is the predominant estrogen produced during the reproductive years. The ovary produces high levels of E2, while smaller amounts are also produced by the adrenal cortex and from E2 precursors in fatty tissues [150]. In the normal menstrual cycle, E2 levels rise with follicular development, drop briefly at ovulation, and rise again during the luteal phase for a second peak. At the end of the luteal phase, E2 levels drop to their menstrual levels unless there is pregnancy [151] (Fig. 2). During pregnancy, E2 levels increase continuously until term due to the production by the growing placenta [152].

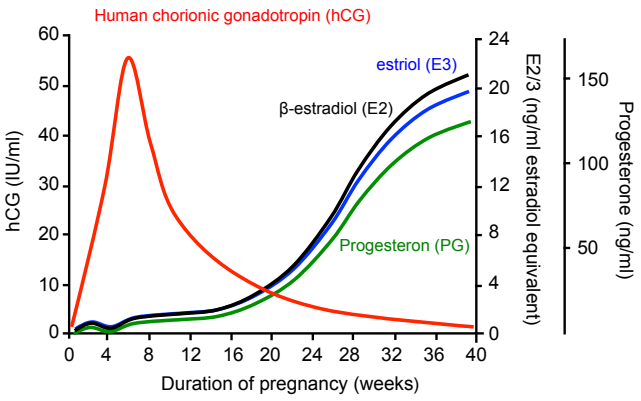


Figure 2.: Hormones concentrations during pregnancy.
Modified from Vitam Horm. 1977;35:109-47,
Am J Obstet. Gynecol. 1796 Nov 15;126(6):678-81,
Acta Endocrinol, August 1969, 61:607-617

Estrogen signaling is primary mediated through Estrogen Receptors (ERs). ERs not only are cytoplasmic but also appear within the nuclei of cells,

where ERs exist as inactive complexes with heat shock proteins and immunophilins. Each ER is a zinc finger-binding protein that, after activation, loses its cytoplasmic receptor-associated proteins and migrates as a TF to bind to the ERE (estrogen response element) of the DNA. Although both types of ERs transduce estrogen signals into a large variety of physiological responses in various organs, the biological events mediated by nuclear ERs occur slowly over hours or even days, whereas the intracellular signaling cascades triggered by cell-membrane ERs respond much faster, even within seconds. Estrogen receptors (ERs) are expressed in various lymphoid tissue cells as well as in lymphocytes, macrophages and dendritic cells. There are two subtypes of the receptor for estrogens, ERα and ERβ that exhibit differential expression in subsets of immune cells, with ERα being highly expressed in T cells and ERβ being upregulated in B cells [153]. The differential effects of estrogens on parameters of immune function may reflect not only the concentration of estrogen but also density, distribution and type of E receptors in immune cells.

Estrogen can upregulate endothelial Nitric Oxide Synthase (eNOS) expression and can cause short-term coronary vasodilatory effects in humans by increasing NO production [154]. Especially in neutrophils, it has been shown that the neurons NOS (nNOS) protein expression is associated with the *in vivo* levels of circulating estrogen [155]. Estradiol along with P4 mediate delayed neutrophil apoptosis in both sexes and enhance female intracellular production of ROIs [156]. In addition, estadiol may act as a pro oxidant, promoting neutrophil degranulation such as MPO and NE release and oxidative stress by superoxide release [157]. Moreover, estrogens may regulate neutrophil activation indirectly as there have been defined estrogen response elements (EREs) in the PAD1, PAD3 and PAD4 gene promoters [158]. It was also shown that steroid hormones such as estrogen and progesterone are modulating the PAD activity [159] [160].

Estriol

Estriol (E3) is produced in high concentrations by the fetoplacental unit during pregnancy and accounts for almost 90% of all estrogens produced

during pregnancy. Estriol is not present in non-pregnant females. The immunological effects of E3 have not been well characterized and it is assumed that the effects of E3 are broadly the same as E2 because both estrogens signal through the same ERs [161].

Progesterone

Progesterone (P4) is a steroid hormone primarily produced by the granulosa cells and the corpus luteum in the ovaries in non-pregnant females and during pregnancy by the placenta. Males produce progesterone in the adrenal glans and testes and this progesterone is the precursor of testosterone.

In females P4 plays a critical role in reproduction and immune function at it plays a crucial role in mammary gland development [162], ovulation [163], embryo implantation and maintenance of pregnancy [164]. Pregnancy is associated with inhibition of Th1 signaling by peripheral T lymphocytes [165] and it has been shown that progesterone inhibits T cell, macrophage and NK cell activity [166]. Specifically, elevated levels of P4 stimulate the synthesis of progesterone-induced binding factor (PIBF) by lymphocytes [167]. In humans, PIBF increases over the course of pregnancy and drops significantly after birth, but in pathological pregnancies that result in preterm labor, abortion, or hypertension, concentrations of PIBF are low [168]. High concentrations of PIBF promote differentiation of $CD4^+$ T cells into helper T cell type 2 (Th2) cells that secrete high concentrations of anti-inflammatory cytokines, including IL-4, IL-5, and IL-10 [169]. The Th2 bias that occurs during pregnancy corresponds with a reduction in inflammatory Th1 responses (e.g., production of INF-γ), both at the maternal-fetal interface and systemically in humans and animal models [170]. Successful pregnancies in humans are associated with elevated IL-4 and IL-10 and reduced IL-2 and INF-γ production by peripheral blood mononuclear cells (PBMCs), with differences in cytokine production being greatest during the third trimester of pregnancy [171]. Moreover in mouse models, it was noted that progesterone withdrawal recruits immune cells, such as neutrophils and macrophages into the cervix, and it is

suggested that this process might be contributing to the remodeling of the cervix before labor [172].

The action of progesterone is mediated through cytosolic Progesterone Receptors (PRs) and also membrane-associated PRs (mPRs) [173]. They belong to the hormone receptor superfamily transcription factors, where ligand binding regulates gene expression thorough direct binding to promoter elements [174] or through interaction with other TFs [175]. Expression of PRs has been identified in epithelial cells as well as in mast cells, eosinophils, macrophages, DCs lymphocytes and neutrophils [176].

Human Chorionic Gonadotropin (hCG)

Human chorionic gonadotropin (hCG) is a glycoprotein hormone that is produced by the syncytiotrophoblast cells of the placenta [177]. Its structure is homologous with the human luteinizing hormone (hLH). However the action of hLH in women is to act synergistically with FSH to induce ovulation and initiate the development of the corpus luteum, whereas the function of hCG is the stimulation of estrogen and progestins secretion by the corpus luteum, in the first few days after fertilization [178].

In women, migration of regulatory T cells to the pregnant uterus is mediated by the chemoattractant activity of hCG [179]. hCG has also been shown to promote the recruitment of regulatory T cells (Tregs) to the placenta, and thereby to play a key role in the induction of feto- maternal tolerance [180].

G-CSF

Granulocyte colony stimulating factor (G-CSF), also known as colony stimulating factor 3 (CSF 3), is a glycoprotein that stimulates the survival, proliferation, differentiation and function of neutrophil precursors and mature neutrophils. G-CSF is a 18kDa, labile protein that act through specific membrane receptors, via JAK-STAT signaling pathways in an endocrine, paracrine or autocrine model [181].

Especially in reproduction, G-CSF and its receptor are localized in the granulosa cells, with concentrations being higher in the follicular fluid than in the serum [182]. G-CSF expression is also elevated during the menstrual cycle [183].

During pregnancy, G-CSF has been detected in the human cytotrophoblast and syncytiotrophoblast on the placental side, and also on the maternal side, in the decidual stromal cells, endometrial glands and epithelium [184]. G-CSF might plays a major role in ovulation, as human ovarian surface epithelium secretes G-SCF [185] and serum concentration after ovarian hyperstimulation [182] is getting higher, as well as the follicular concentrations [183]. G-CSF might be implicated in local follicular inflammation [186] by attracting and activating leucocytes, leading to ovulation [187]. During embryogenesis, the role of G-CSF in immune tolerance in pregnancy can be attributed to the induction of IL-10 production [188]. Moreover, the local uterine production of G-CSF may contribute to the modulation of cytotoxicity of uterine NK cells, probably by suppressing the IFN-γ production [189].

Aim

Since the particular effects of pregnancy on neutrophil physiology relating to formation of NET are unknown, our first aim was to explore neutrophil activity during normal pregnancy. For this purpose we used peripheral blood at discrete time-points during all three trimesters of pregnancy and we studied neutrophil priming towards the formation of NETs. Healthy non-pregnant female blood donors served as controls. In order to understand the factors that modulate neutrophil response during that condition we investigated the impact of prominent sex hormones expressed during gestation, specifically hCG, E2 and P4, and in parallel with G-CSF on neutrophil signaling towards NET formation.

Materials and Methods

Human Subjects

Pregnant women were recruited at the time of their routine examination at the end of the first (median gestational age: 12 weeks and 4 days - n=15; median age: 34.1 years) and second trimesters (median gestational age: 24 weeks and 3 days - n=25; median age: 34.1 years) and at the time of elective caesarean section towards the end of the third trimester (median gestational age at delivery: 38 weeks and 4 days - n=35; median age: 34.1 years) (Table 1). Healthy non-pregnant controls matched for age (n=45; median age: 33.5 years), were recruited at the Blood Bank of the Swiss Red Cross, Basel (Table 1). Inclusion criteria for non-pregnant controls were fair general condition, female sex, age ≥ 25 and ≤ 45 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. Exclusion criteria for pregnant subjects included any major complication of pregnancy or coincident disease, such as preeclampsia, pre- or post-term labor (<37 weeks or >42 weeks), intra-uterine growth retardation and viral, bacterial or parasitic infections. Informed, written consent was obtained from all subjects prior inclusion in the study, which was approved by the Ethical Review Board of Basel/Basel-Land, Switzerland.

Table 1. Demographic characteristics of the study cohort, i.e. women during the three trimesters of pregnancy and non-pregnant healthy controls.

Table 1.

	Non-pregnant donors	Pregnant donors	P
n	40	Total: 45	na
		IT: 15	
		IIT: 25	
		IIIT: 35	
Maternal age (years)	32.7 (23-46)	33.9 (26-41)	ns
Gestational age (weeks)	na	IT: 12.5 (12-13)	na
		IIT: 24.7 (22-26)	
		IIIT: 39.1 (37-41)	

Values expressed as mean ± SE with minimum-maximum, where applicable; IT, first trimester; IIT, second trimester, IIIT, third trimester ns, not significant; na, not applicable.

Blood cell count and preparation of plasma and serum

Whole blood was collected into EDTA- and silicone-coated tubes (Sarstedt) and 25 µl of blood was analyzed by a Hemavet 950FS (Drew Scientific) for complete blood cell counts. Plasma and serum was collected and processed as described previously [190]. Samples were studied immediately or stored at -80°C until analyzed.

Human neutrophil isolation

Neutrophils were isolated by Dextran-Ficoll density centrifugation [65]. Cell viability was assessed by trypan blue dye exclusion in a haemocytometer and was routinely 96–98% with a purity of over 95%. Neutrophils were directly seeded in 24-well or 96-well plates and allowed to settle for 15 minutes at 37°C under 5% CO_2 prior to further experimentation. Time-points of measurements are given in the figure legends.

Cell free DNA isolation and quantification

Cell free DNA was extracted from 850µl serum using the QIAamp Circulating Nucleic Acid Kit (Qiagen) and was quantified by TaqMan Real-time PCR (StepOneTM Plus Real-Time PCR System, Applied Biosystems) specific for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene [190].

Stimulation and neutralization studies

For *in vitro* incubation studies 2.5 x 10^4 neutrophils from healthy women were treated with 3% serum or 6% plasma derived from non-pregnant controls and pregnant donors during the first, second and third trimesters of gestation. All experiments were carried out over 3 hours in 4 to 6 replicates.

Neutrophils from healthy controls were incubated with ascending concentrations of hCG, E2, E3, P4 or G-CSF, which covered the physiological plasma concentrations during gestation, individually or in different combinations.

For the two-step stimulation *in vitro* experiments, neutrophils were pretreated with hormones or G-CSF as a primary stimulus for 60 min, and then exposed to the secondary stimulus (PMA or G-CSF) for another 120 min, for a total time of 3 hours.

To neutralize sex hormones, pooled sera or plasma from the study groups of interest were pretreated for 30 minutes with fulvestrant (10 µg/ml, Sigma) and mifepristone (10 µg/ml, Sigma) for the neutralization of estrogens and progestins activity, respectively. To neutralize G-CSF, pooled sera or plasma from the study groups of interest were pretreated with anti-G-CSF antibody (0.2 µg/ml, Peprotech) for 30 minutes.

Fluorimetric quantification and fluorescence microscopy

NETs were quantified by SytoxGreen fluorimetry. 2.5x10^4 freshly isolated neutrophils were cultured in the presence of 0.2 µM SytoxGreen

(Invitrogen, Life Technologies) in a 96-well dark microtiter plate at 37°C under 5% CO_2 and left untreated or stimulated with the aforementioned agents over 3 hours. PMA was used as the positive control. Fluorescence (excitation 485 nm, emission 535 nm) was measured in a Biotek Synergy H1 Hybrid Reader (Biotek) and results given as DNA fluorescence (MFI). Photomicrographs in bright field and green fluorescence spectra were assessed with an Olympus IX50 inverted fluorescence microscope coupled to an Olympus XM10 monochromatic CCD camera and analyzed with the Olympus CellSens Dimension software (Olympus).

Neutrophil viability

Apoptosis was detected by Annexin V/ 7-aminoactinomycin D (7-AAD) staining (BD BioSciences) according to the manufacturer's instructions. 10^4 cells were counted by flow cytometry using an BD Accuri™ C6 flow cytometer (Becton-Dickinson). The data were analyzed using Flowjo v10 software (Treestar).

Cytokine proteome array

Cytokines, chemokines and acute phase proteins were detected with the Human Cytokine Array Kit (R&D Systems) according to the manufacturer's instructions. Pooled sera collected from control non-pregnant individuals and pregnant donors during the first, second and third trimesters of gestation were centrifuged and incubated with the pre-coated nitrocellulose membranes. After washing and addition of the detection antibody streptavidin-HRP conjugates, the membranes were exposed to X-ray film (Fuji). The cytokine proteomic array comprised 36 targets spotted in duplicate on the membranes. The intensity of each spot in the captured images was analyzed with ImageJ analysis software (NIH Image Processing).

NE, MPO, cell-free histone/DNA complex, MPO/DNA complex and G-CSF protein analysis

The concentrations of NE and MPO were measured in sera and plasma by sandwich ELISA, utilizing respectively the Elastase/a1-PI Complex ELISA Kit (Calbiochem) and the human MPO ELISA Kit. Histone/DNA complexes in sera and plasma were measured using the Human Cell Death Detection ELISA^{PLUS} (Roche Diagnostics); nucleosomes in cell culture supernatants were detected similarly after incubation with DNase I (10U for 5min) (Roche Diagnostics). To identify NET-associated MPO/DNA complexes, a modified capture ELISA was utilized [34]. NET associated MPO in culture supernatant was captured using the coated 96 well plate of the human MPO ELISA Kit, (Hycult Biotech), and the NET associated DNA backbone was detected using the anti-DNA-POD antibody of the Human Cell Death Detection ELISA^{PLUS} (Roche Diagnostics). G-CSF serum and plasma protein concentrations were assessed with the Human G-CSF Quantikine ELISA Kit (R&D Systems).

Oxidative burst analysis

NADPH oxidase mediated ROS production was measured either by a 2′,7′-dichloro dihydrofluorescein diacetate (DCFH-DA) plate assay [191] or a luminol-based chemiluminescence microtitre plate assay [192] [193]. 2.5×10^4 neutrophils per well were incubated without or with stimulants mentioned above in dark 96-well microtitre plates with 25 µ*M* DCFH-DA (Sigma-Aldrich), which reacts with ROS species produced in intracellular compartments (granules or phagosomes). Fluorescence was recorded immediately in a Biotek Synergy H1 Hybrid plate Reader (Biotek) and for 30 minutes. The response was expressed as relative fluorescence units (RFU). Similarly, 2.5×10^4 neutrophils per well were incubated without or with the aforementioned stimulants in white 96-well microtitre plates with 60µM luminol (5-amino-2,3-dihydro 1,4-phthalazinedione)**.** Chemiluminescence was recorded every 5 minutes over a period of 30 minutes in a Biotek Synergy H1 Hybrid plate Reader (Biotek), and the response was expressed as relative luminescence units (RLU).

Immunohistochemistry, morphometric analysis and confocal microscopy

$1x10^5$ neutrophils were seeded on poly-L-lysine-coated glass coverslips (BD Biosciences) in 24-well tissue-culture plates 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% FBS, 0.1% Tween20, and 2mM EDTA) at 4°C. NETs were detected with rabbit anti-NE (Abcam), rabbit anti-MPO (Dako) and rabbit anti-citrullinated histone H3 (citH3, Abcam). Secondary antibodies were goat anti-rabbit IgG AF555, goat anti-rabbit IgG AF488 (Invitrogen Life Technologies), and goat anti-mouse IgG AF647. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). 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 neutrophils) per case were 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.

In another set-up, NETs were quantified by IHC staining of 2.5 x 10^4 neutrophils per well in a 96-well plate 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 Life Technologies). DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). 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 neutrophils) per sample were evaluated for MPO/citH3 and DNA co-staining through ImageJ analysis software (NIH); nuclear phenotypes and NETs were determined, counted, and expressed as percentage of the total area of cells in the fields [194].

Images were captured on a Nikon A1R inverted microscope (Nikon) coupled to a Visitron CSU-W1 spinning disc confocal microscopy module (Visitron) and a Thor ablation laser (Thor Labs) using an UPL APO 60x/1.40 oil objective lens with the Visiview Cell Analyser software (Visitron Systems, Version 3.1.2.2).

Phagocytosis activity

Neutrophil phagocytic activity was examined by uptake of latex beads coated with FITC-labeled rabbit IgG into cells (Cayman Chemical) according to the instruction manual. 1x10^5 untreated neutrophils exposed to various stimulants were resuspended in 200µl phagocytosis buffer to which FITC-labeled beads (1:100) were added and incubated for 2 hrs at 37ºC. The amount of phagocytosis was determined by flow cytometry utilizing the BD Accuri™ C6 flow cytometer (Becton-Dickinson) and analyzed by Flowjo v10 software (Treestar). The uptake of the beads into neutrophils was additionally captured with an Olympus IX50 inverted fluorescence microscope

and phagocytosis quantified as described above with ImageJ analysis software.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from $3x10^6$ neutrophils by using the RNeasy Mini Kit (Qiagen). TaqMan real-time quantitative RT-PCR was performed utilizing the Applied Biosystems StepOne Plus cycler (Applied Biosystems) and TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) for *ELANE* (HS00236952_m1), *MPO* (HS00924296_m1), *PAD4* (HS00202612_m1), IL6 (HS00202612_m1), IL8 (HS00202612_m1), and *TNF (*HS00202612_m1). Data were normalized to the housekeeping gene *B2M* (HS99999907_m1), after a selection procedure from six different endogenous reference genes, as suggested in the MIQE guidelines [195]. Relative values were calculated with 2^{-DDCt} analysis [196].

Protein isolation and western blot analysis for PAD4 and citrullinated histone H3 (citH3)

Total protein was isolated by NucleoSpin TriPrep kit (Macherey-Nagel) from $5x10^6$ neutrophils. All protein concentrations were determined with the MN Protein Quantification Assay (Macherey-Nagel). Western blotting was performed utilizing AnykD Mini-PROTEAN TGX Gels (Biorad) and nylon/nitrocellulose membranes (Biorad). Primary and secondary antibodies utilized were rabbit anti-MPO (Cell Signalling Technologies), rabbit anti-PAD4 (Abcam), rabbit anti-citH3 (Abcam), mouse anti-β-Actin (Sigma), anti-rabbit HRP (Santa Cruz), anti-mouse HRP (Santa Cruz). HRP activity was detected by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Equal loading was verified using beta-actin. Western blots of citrullinated H3 (citH3) protein were prepared as described previously [197]. Gel documentation, densitometric analysis and protein quantification of the western blots was performed using the ChemiDoc XRS+ maging system (Biorad) with the ImageLab 4.1 image analysis software (Biorad).

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 carried out 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 values under < 0.05 were considered statistically significant. Data were processed in GraphPad Prism version 6.0 for MacOSX (GraphPad Software Inc., www.graphpad.com). Professional statistical assistance was provided by Andreas Schoetzau (www.eudox.ch).

Results

Neutrophil activity increases with progression of pregnancy

We observed a substantial rise in neutrophil numbers during pregnancy (Fig. 1a). Using staining by SytoxGreen, a fluorescent cell-impermeable dye, we noted a marked increase in NET formation by neutrophils from pregnant donors versus baseline and non-pregnant controls, which at 3 hours was highly significant (Fig. 1b). This was reflected in increased MPO/cell free DNA complexes, which confirm the identity of the structures as NETs in culture supernatants (Fig. 1c). Since the morphology of the neutrophil nucleus evolves from a lobulated to a delobulated diffused and then to a decondensed NETotic phenotype prior to NET release [37], we measured the fraction of neutrophils with delobulated nuclei over time by morphometric analysis. As shown in Fig. 1d, upper panel and Fig. 1e, left panel, the baseline value of delobulated cells in the first trimester was significantly higher than in the non-pregnant controls, with the neutrophil fraction generating NETs increasing further throughout pregnancy (Figure 1d, lower panel).

Generation of ROS, a product of NADPH oxidase and crucial signalling

step for NET formation, rose significantly as pregnancy progressed (Supplementary Fig. 1a and 1b). Other key elements of signalling leading to NET formation were also increased in neutrophils from pregnant subjects: mRNA and protein levels of MPO, NE, and of PAD4 (Supplementary Fig. 1c, 1d and 1e) as well as PAD4-mediated citrullination of H3 (Fig. 1e, right panel and Supplementary Fig. 1d, middle panel). In combination, these findings are highly suggestive of neutrophil priming towards NET formation.

MPO and NE serum levels increased in a similar manner (Supplementary Fig. 1f), reflecting an elevated propensity for degranulation during pregnancy as reported previously [198]. The increase in NETs paralleled the increase in phagocytic activity (Supplementary Fig. 1g), also described previously [199]. Concomitantly, increases in IL-6 and IL-8 mRNA expression by neutrophils with the advancement of pregnancy were also observed (Supplementary Fig. 1h), providing further evidence of increasing overall activity of neutrophils during pregnancy.

A primed, pro-NETotic state may also indicate an enhanced response to an additional stimulus [65]. We therefore assessed the effect of PMA on NET formation by neutrophils from pregnant women *in vitro* by immunofluorescence staining (Fig. 1g), SytoxGreen fluorimetry (Fig. 1f), morphometric analysis (Fig. 1h) and ROS production (Supplementary Fig. 1i and 1j). The results demonstrated that pregnancy-derived neutrophils generated NETs more vigorously following an additional stimulus compared to the controls.

To summarize, peripheral blood neutrophils show a pro-NETotic phenotype that increases with advancing gestation. They are also progressively more responsive to a secondary stimulus in the course of pregnancy. Together with increased phagocytosis and degranulation, the adaptations of NET formation could amount to a more vigorous anti-microbial response in pregnancy.

Figure 1.

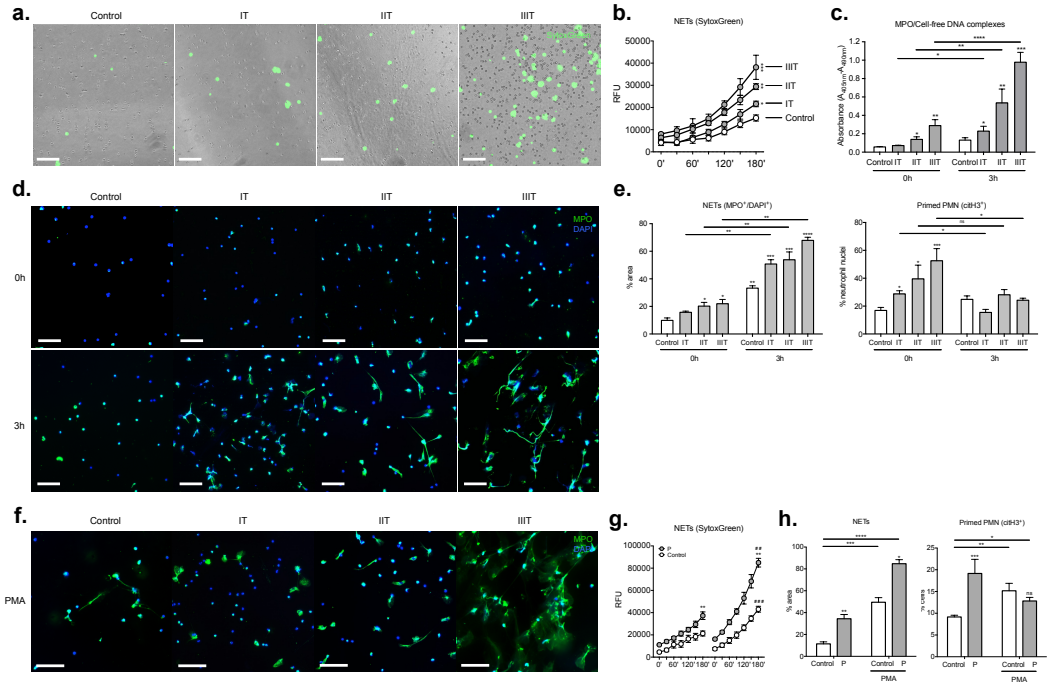
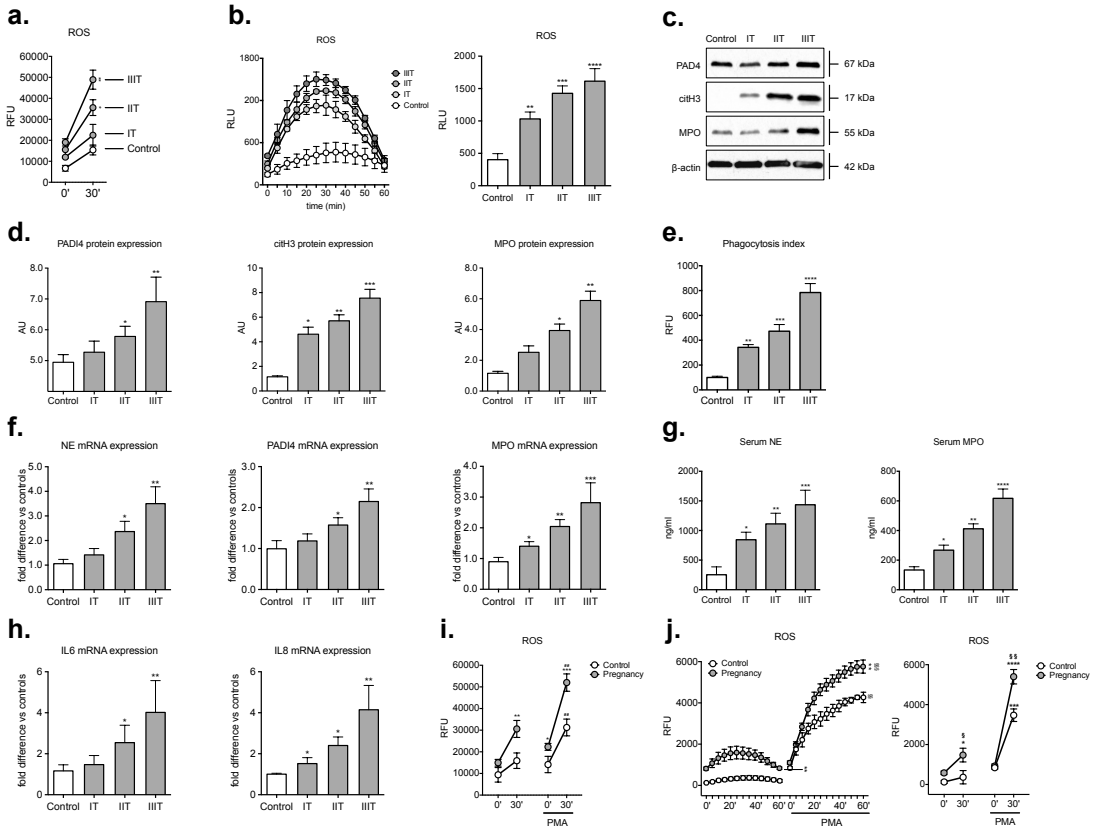


Figure 1. NET formation and neutrophil pro-NETotic priming are augmented during pregnancy. (**a**) *In vitro* spontaneous NET formation by neutrophils from healthy controls pregnant donors in a 3 hour timecourse by fluorescent microscopy using dsDNA-binding fluorescent SytoxGreen dye. Magnification: 10x. Scale bars: 100 µm. (**b**) *In vitro* spontaneous NET formation in a 3 hour timecourse by SytoxGreen quantification. (**c**) Quantification of NET-associated MPO/DNA complex levels in culture supernatants of neutrophils from healthy controls and pregnant donors at baseline (0h) and at 3 hours (3h). (**d**) Immunofluorescence staining for MPO (green) and DNA counterstain with DAPI (blue) at baseline (0h) and after 3 hours of culture (3h). Magnification: 20x; Scale bars: 50 µm. (**e**) Morphometric analysis of the NETotic (MPO+) and proNETotic primed (citH3+) neutrophils from healthy donor controls, donors during pregnancy and post partum, at the baseline steady state (0h) and after a 3 hour culture (3h). (**f**) Fluorescent immunocytochemistry for MPO (green) and DNA counterstain with DAPI (blue) after a 2 hour culture after incubation with the NET-inducing agent phorbol-12-myristate-13-acetate (PMA). Magnification: 20x; Scale bars: 50 µm. (**g**) *In vitro* spontaneous NET release monitored in a 3 hour time course by dsDNA-binding fluorescent SytoxGreen dye after application of PMA as secondary stimulus. Magnification: 10x. Scale bars: 100 µm. (**h**) Morphometric analysis of the NETotic (MPO+) and pro-NETotic primed (citH3+) neutrophils from healthy donors and donors during pregnancy after application of PMA as secondary stimulus. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 6 times with consistent results. IT, first trimester; IIT, second trimester; IIIT, third trimester; P, pregnancy; RFU, relative fluorescence units.

Supplementary Figure 1



Supplementary figure 1. Signaling molecules involved in NET formation and neutrophil pro-NETotic priming are upregulated during pregnancy. Intracellular (**a**) and overall (**b**) oxidative burst in neutrophils from healthy control donors and donors during pregnancy by DCFH-DA and luminol detection, respectively. Western blot (**c**) and densitometric analysis (**d**) of PAD4, citH3, MPO and beta-actin protein expression levels in neutrophil lysates from healthy female controls and donors during pregnancy. (**e**) Phagocytic activity of neutrophils obtained from healthy female controls and donors during pregnancy. (**f**) NE, PAD4 and MPO gene expression analysis by Taqman qRT-PCR in RNA samples obtained from healthy female controls and donors during pregnancy. (**g**) Detection of serum NE and MPO levels by ELISA. (**h**) IL6 and IL8 gene expression analysis by Taqman qRT-PCR in RNA samples obtained from healthy female controls and donors during pregnancy. Intracellular (**i**) and overall (**j**) oxidative burst in neutrophils from healthy control donors and donors during pregnancy after treatment with PMA by DCFH-DA and luminol detection, respectively. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 6 times with consistent results. IT, first trimester; IIT, second trimester; IIIT, third trimester; RFU, relative fluorescence units; RLU, relative luminescence units; AU, arbitrary units.

G-CSF effects on neutrophil priming and NET formation during pregnancy

Pooled sera from pregnant women augmented NET formation by neutrophils from non-pregnant women *in vitro* (Fig. 2a and Fig. 2b). To identify the responsible factors, we performed a cytokine proteome array. There were considerable changes in cytokines and chemokines with a strong pro-inflammatory signature in the pregnant as compared to the non-pregnant state (Supplementary Fig. 2a). Of the factors assessed, G-CSF showed a prominent signal increasing from the first through the third trimesters (Fig. 2c), which correlated with elevated numbers of neutrophils and lower numbers of monocytes and platelets (Supplementary Fig. 2b, 2c and 2d). Since G-CSF has been reported to elicit a pro-NETotic response in a mouse tumor model [91], we decided to study it in more detail.

In vitro incubation of neutrophils from non-pregnant women with a range of concentrations of recombinant human G-CSF, including those present in normal pregnancy, increased the proportions of pro-NETotic priming and NETosis as detected by SytoxGreen fluorimetry (Fig. 2d, upper panel and Fig. 2e), ROS production (Supplementary Fig. 3a and 3b), immunofluorescence microscopy (Figure 2d, lower panel) and morphometry (Fig. 2g). G-CSF at physiological pregrancy concentrations led to the highest pro-NETotic priming ($citH3^+$ status), but low-level NET formation (Fig. 2g). G-CSF reduced phagocytic activity in favour of the increased pro-NETotic responses (Fig. 2h). Immunoblotting confirmed this finding, while real-time PCR demonstrated marked elevations of MPO, NE, PAD4 and citrullinated histone H3 (citH3) due to response to G-CSF treatment (Supplementary Fig. 3c, 3d and 3e).

Figure 2.

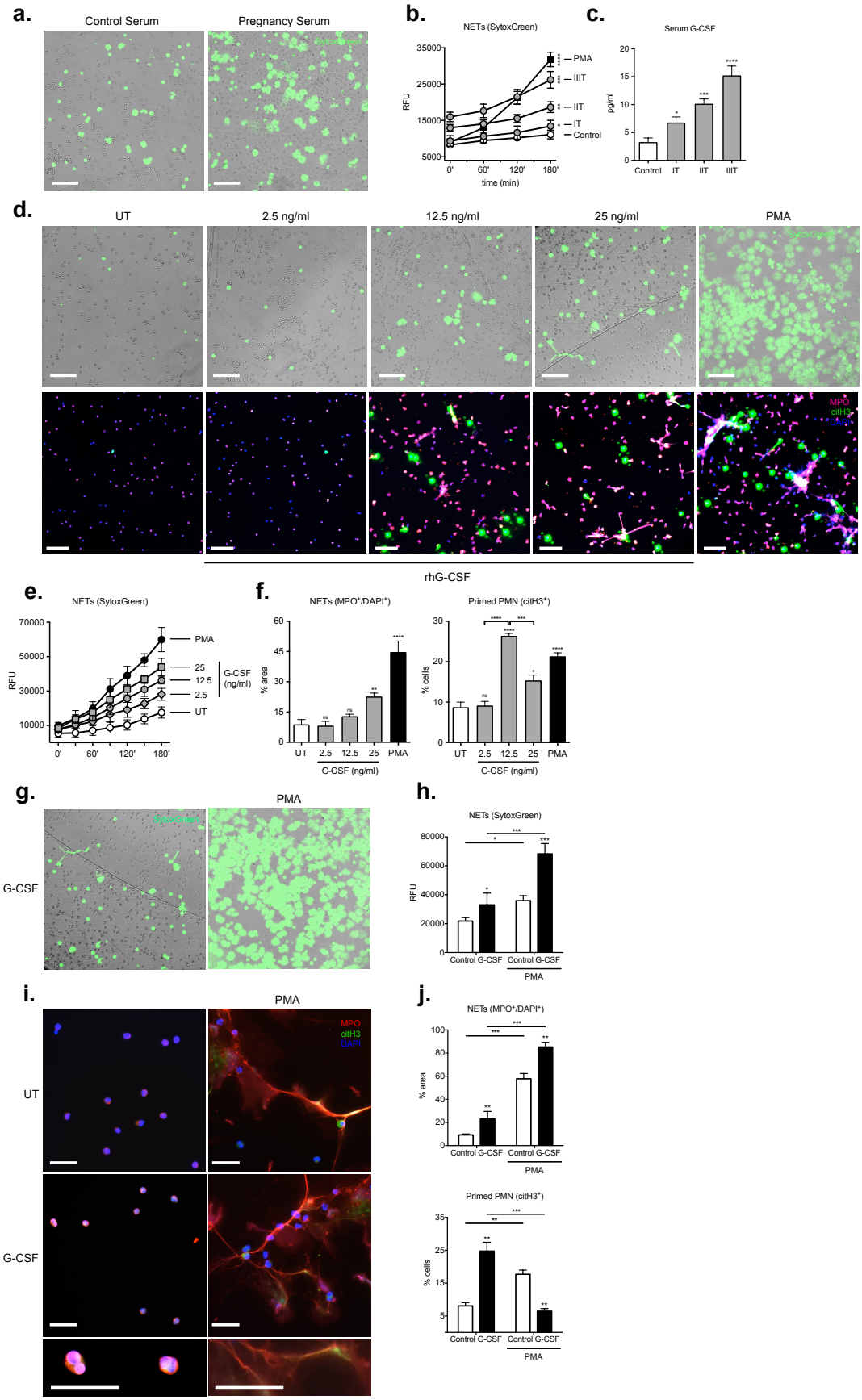
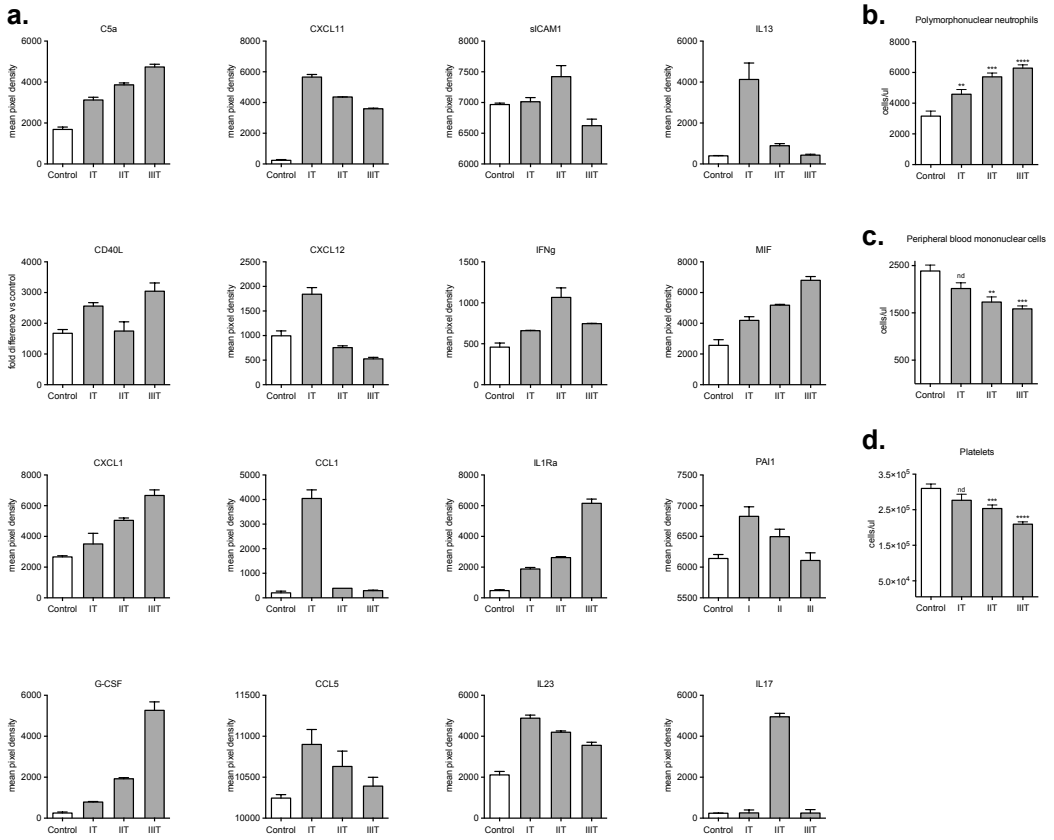

a.
Control Serum
Pregnancy Serum
b.
NETs (SytoxGreen)
RFU
time (min)
PMA
IIIT
IIT
IT
Control
c.
Serum G-CSF
pg/ml
Control
IT
IIT
IIIT
d.
UT
2.5 ng/ml
12.5 ng/ml
25 ng/ml
PMA
MPO
citH3
DAPI
rhG-CSF
e.
NETs (SytoxGreen)
RFU
PMA
25
12.5
2.5
UT
G-CSF (ng/ml)
f.
NETs (MPO+/DAPI+)
% area
Primed PMN (citH3+)
% cells
UT
2.5
12.5
25
PMA
G-CSF (ng/ml)
g.
PMA
G-CSF
h.
NETs (SytoxGreen)
RFU
Control
G-CSF
PMA
i.
PMA
UT
G-CSF
MPO
citH3
DAPI
j.
NETs (MPO+/DAPI+)
% area
Primed PMN (citH3+)
% cells

Figure 2. G-CSF leads to neutrophil pro-NETotic priming during pregnancy. (**a**) *In vitro* spontaneous extracellular DNA release monitored in a 3 hour time course by dsDNA-binding fluorescent SytoxGreen dye microscopy after treatment of control neutrophils with pregnancy serum. Magnification: 10x. Scale bars: 100 µm. (**b**) Fluorimetric quantification of extracellular DNA release from neutrophils treated with pregnancy serum. (**c**) Detection of serum G-CSF levels by ELISA. (**d**) Evaluation of rhG-CSF activity by *in vitro* titration experiments using fluorescent SytoxGreen staining DNA and microscopy (upper panel) and immunofluorescence of peripheral neutrophils after incubation with rhG-CSF at the indicated concentrations for MPO (red), citH3 (green) and DNA counterstain with DAPI (blue) after a 2 hour culture (lower panel), compared to untreated (UT) and PMA-treated neutrophils. Magnification: 10x. Scale bars: 100 µm. (**e**) Fluorimetric analysis of peripheral neutrophils after incubation with rhG-CSF at the indicated concentrations compared to untreated (UT) and PMA-treated neutrophils. (**f**) Morphometric analysis of the NETotic (MPO+/DNA+) and pro-NETotic primed (citH3 positive) neutrophils from healthy control donors under hormonal treatment. (**g**) Phagocytic activity of differentially rhG-CSF-treated neutrophils obtained from healthy controls, 30 minutes and 2 hours after stimulation. (**h**) NET formation *in vitro* monitored microscopically in a 3 hour time course with dsDNA-binding fluorescent SytoxGreen dye after a 1 hour pretreatment with physiological concentrations of rhG-CSF and application of the NET-inducing agents PMA as a second hit for additional 2 hours. Magnification: 10x. Scale bars: 100 µm. (**i**) Fluorimetry of extracellular DNA release of rhG-CSF-treated control neutrophils after addition of PMA. (**j**) Immunostaining for MPO (red), citH3 (green) and DNA (blue) after a 3 hour *in vitro* co-culture of healthy neutrophils with rhG-CSF and addition of PMA. Magnification: 20x; Scale bars: 50 µm. (**k**) Morphometric analysis of the NETotic MPO positive and pro-NETotic primed citH3 positive neutrophils from healthy non-pregnant donors under the aforementioned experimental setup. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one or two way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 6 times with consistent results. IT, first trimester; IIT, second trimester; IIIT, third trimester; UT, untreated; RFU, relative fluorescence units.

Supplementary Figure 2



Supplementary figure 2. Pregnancy changes in a broad series of pro- and anti-inflammatory cytokines and chemokines and cell populations. (**a**) Detection of serum levels of a C5a, CD40L, CXCL1, G-CSF, CXCL11, CXCL12, CCL1, CCL5, cICAM1, IFNg, IL1Ra, IL13, IL17, IL23, MIF,and PAI1 by Human Cytokine Array analysis. Bar graphs depicting the differential counts of neutrophils (**b**), PBMCs (**c**) and platelets (**d**) in blood samples obtained from healthy female controls and donors during pregnancy. Data are presented as mean ± SEM. **P < 0.01, ***P < 0.001, ****P < 0.0001 (one or two way ANOVA followed by Bonferroni's multiple comparison post-test).

In addition, we observed that treatment of normal neutrophils with concentrations of G-CSF detected in normal pregnancy (see above) rendered these cells highly responsive with NETosis to further stimulation with PMA as assessed by *in vitro* cell free DNA release (Fig. 2k and Fig 2l), ROS production (Supplementary Fig. 3f and 3g), immunofluorescence microscopy (Fig. 2m) and morphometry (Fig. 2n).

To evaluate whether plasma G-CSF could drive the neutrophils to priming and to form NETs, endogenous G-CSF in plasma from pregnant women was sequestered by the use of an anti-G-CSF antibody. Neutralization

of G-CSF prevented sensitization of neutrophils to generate NETs (Fig. 3a and Fig. 3b) and reduced their capacity to generate ROS (Supplementary Fig. 4a and 4b). Immunostaining revealed hypercitrullination of H3, consistent with a predisposition of neutrophils exposed to G-CSF to form NETs (Fig. 3c and Fig. 3d).

These data suggest that G-CSF plays a vital role in promoting an enhanced pro-NETotic phenotype in pregnancy.

Figure 3.

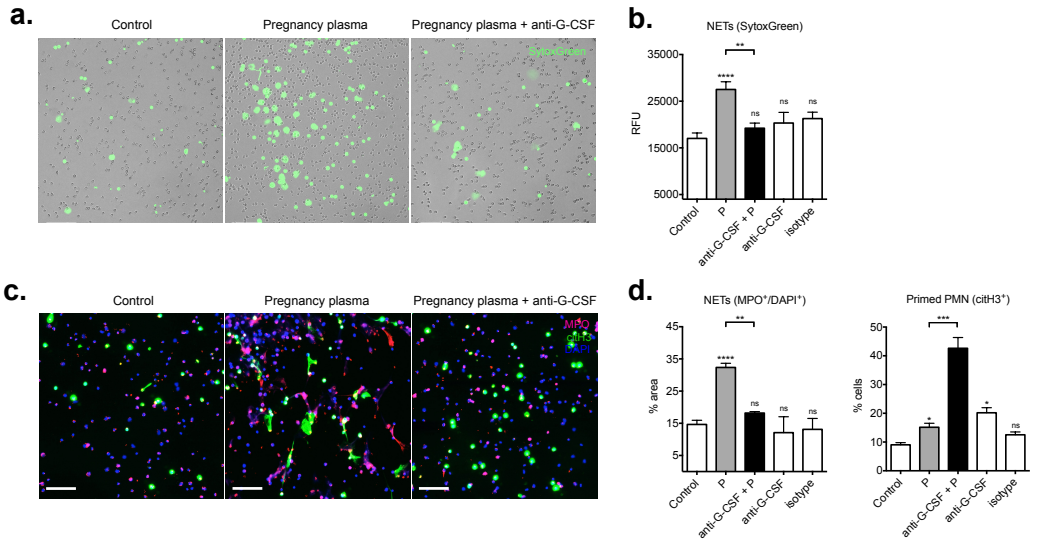
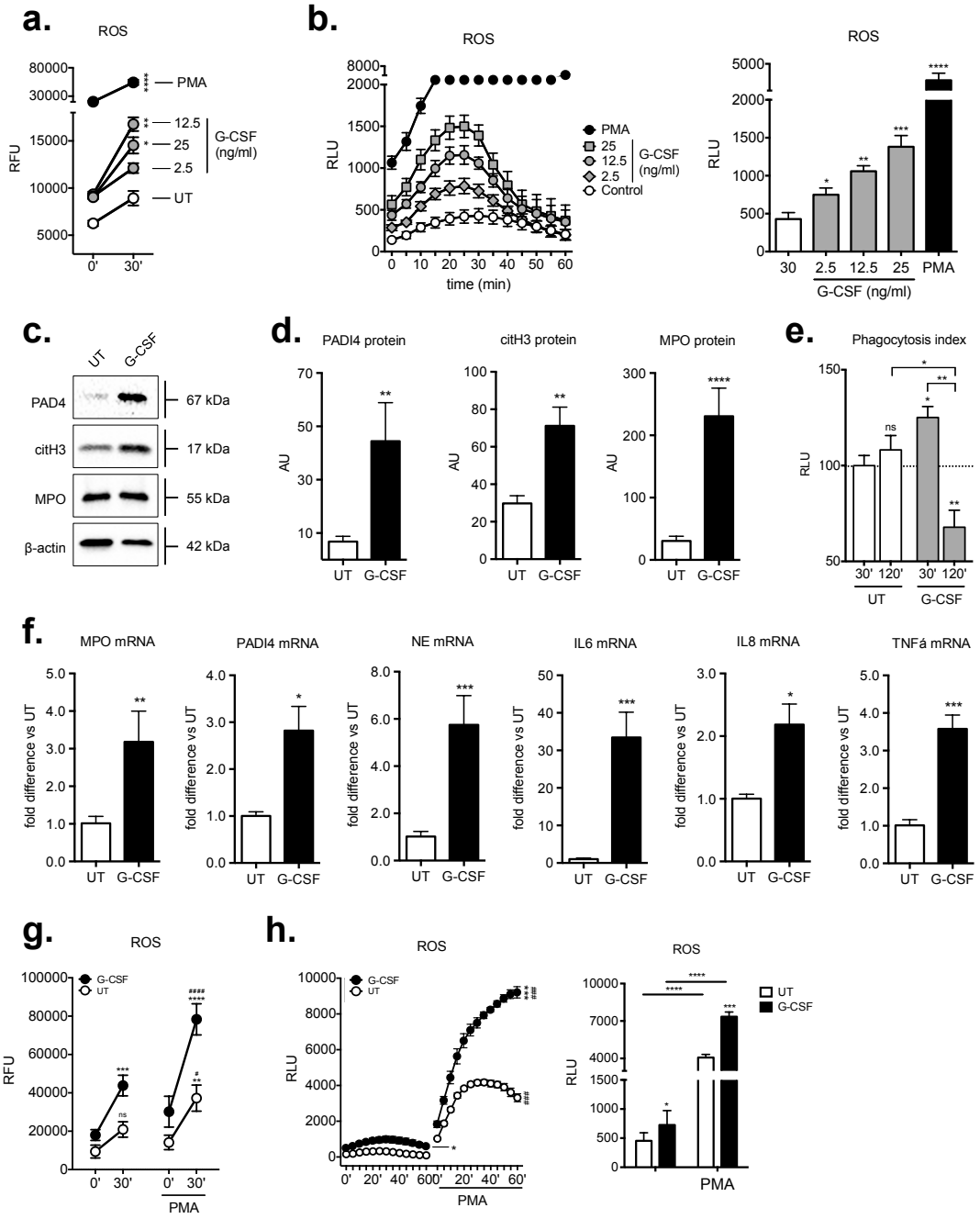


Figure 3. Pregnancy plasma G-CSF determines neutrophil pro-NETotic priming during gestation. (**a**) NET formation assessed microscopically in a 3 hour time course with SytoxGreen DNA binding dye after *in vitro* incubation of control neutrophils with pregnancy plasma with and without pretreatment with anti-G-CSF neutralizing antibody. Magnification: 10x. Scale bars: 100 µm. (**b**) Fluorimetric evaluation of extracellular DNA release in control neutrophils treated with pregnancy plasma in the presence of anti-G-CSF neutralizing antibody. (**c**,**d**) Fluorescent immunostainings for MPO (red), citH3 (green) and DNA (blue) (**c**) and morphometric analysis (**d**) of the NETotic MPO positive and pro-NETotic primed citH3 positive neutrophils from healthy non-pregnant donors under the aforementioned experimental setup. Magnification: 20x; Scale bars: 50 µm. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 6 times with consistent results. RFU, relative fluorescence units; P, pregnancy plasma.

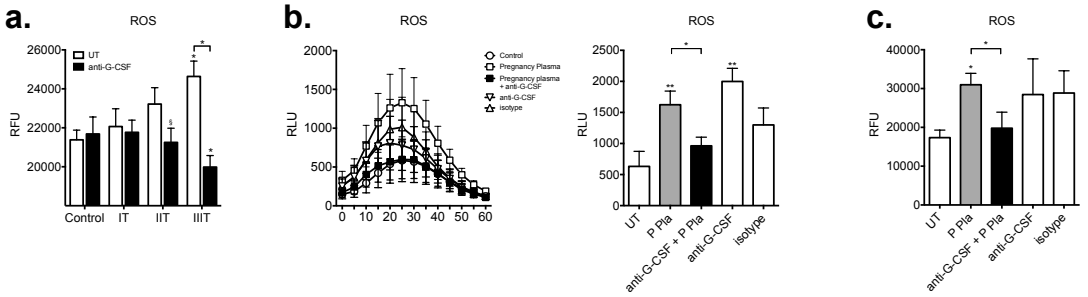
Supplementary Figure 3



Supplementary figure 3. Plasma G-CSF determines neutrophil pro-NETotic priming during pregnancy. Intracellular (**a**) and overall (**b**) oxidative burst in neutrophils from healthy control donors treated with 2.5, 12.5 and 25 ng/ml recombinant human (rh)G-CSF by DCFH-DA and luminol detection, respectively. PMA was used as a positive control for NET formation related ROS generation. Western blot (**c**) and densitometric analysis (**d**) of PAD4, citH3, MPO and beta-actin protein expression in neutrophil lysates from healthy female controls treated with the aforementioned concentrations of rhG-CSF. (**e**) Phagocytic activity of neutrophils obtained from healthy female controls under the above described experimental conditions. (**f**) *MPO*, *PAD4*, *NE*, *IL6*, *IL8* and *TNF* gene expression analysis by Taqman qRT-PCR in RNA samples obtained from healthy female controls treated *in*

vitro with the aforementioned concentrations of rhG-CSF. Intracellular (**g**) and overall (**h**) oxidative burst measured in PMA-treated control donors' neutrophils preincubated for 1 hour with 12.5 ng/ml rhG-CSF by DCFH-DA and luminol detection, respectively. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 6 times with consistent results. RFU, relative fluorescence units; RLU, relative luminescence units; AU, arbitrary units; UT, untreated.

Supplementary Figure 4



Supplementary figure 4. Neutralization of plasma G-CSF diminishes the release of NETs but not neutrophil priming towards NET formation. (**a**) Intracellular ROS generation from neutrophils co-cultured with 6% plasma derived from donors during the three trimesters of pregnancy and 50 ng/ml anti-GCSF neutralizing antibody monitored with DCFH-DA. Intracellular (**b**) and overall (**c**) oxidative burst in neutrophils from healthy control donors treated with 6% plasma derived from donors during pregnancy and 50 ng/ml anti-GCSF neutralizing antibody monitored by DCFH-DA and luminol detection, respectively. PMA was used as a positive control for NET formation related ROS generation. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one or two way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 3 times with consistent results. RFU, relative fluorescence units; RLU, relative luminescence units; IT, first trimester; IIT, second trimester; IIIT, third trimester.

Regulation of NET formation by the gestational hormonal milieu

Pregnancy involves profound changes in the production of gestational hormones, namely hCG, E2, E3 and P4. In neutrophils from non-pregnant controls, incubation with these four sex hormones indicated that hCG and E2 or E3 lead to increased NET formation, a feature not observed in P4 treated cells, or those treated with a combination of E2 and P4. On the other hand, phagocytic activity was greatest in P4 treated cells, (Fig. 4a-e). This facet was reflected in the measurement of ROS production, which was greatest in E2

treated cells and lowest for P4 treated cells, with the combination E2 and P4 at the level of P4 alone (Supplementary Fig. 5a and 5b). hCG, E2 and P4 influenced the levels of molecules required for NETosis in parallel with their effects on NET induction (Supplementary Fig. 5c, 5d and 5e). These were comparable to those of the neutrophils from pregnant women described above.

The effects of the hormones on neutrophils from non-pregnant donors were also studied in various concentrations and combinations of hCG, E2, E3 and P4 using the physiologic levels in pregnancy as a guide. The results obtained were assigned to categories for display in infograms. hCG, E2 and E3 induced vigorous NET formation as determined by SytoxGreen dye staining, while that induced by P4 was distinctly lower (Fig. 4f, first row and Supplementary Fig. 6a). E2 and E3 synergized with each other, while either combined with P4 showed less NET induction than when used alone. The combination of E2 and E3 with P4 led to lower levels of NET formation than with either estrogens alone (Fig. 4f and Supplementary Fig. 6a).

Similar patterns were seen when NETs were determined morphometrically by DNA-bound MPO (Fig. 4g, left panel and Supplementary Fig. 6b) and by citH3+ nuclei (Fig. 4g, right panel and Supplementary Fig. 6c) indicated that neutrophils were primed for NETosis. High concentrations of hCG, E2 and E3 led to high cell free DNA release *in vitro*, while physiological concentrations resulted in a milder effect. (Fig. 4e and Supplementary Fig. 6a). In all cases, P4 reduced NET release significantly, at physiologic concentrations as well as when co-incubated with E2 and E3 (Fig. 4e, Supplementary Fig. 5a to 5c and Supplementary Fig. 6a to 6c). Surprisingly, extensive citH3 immunostaining was also evident in P4 treated cells indicating priming for NETosis, but inability to progress to NETosis (Fig. 4g, right panel).

Cells treated with either hCG or E2 displayed an enhanced pro-NETotic response upon subsequent stimulation with PMA. While P4 reduced PMA induced NETs, this effect was less pronounced in cells treated with a combination of E2 and P4 upon secondary stimulation with PMA (Fig. 4h and 4i). ROS production (Supplementary Fig. 6a and 6b) and immunofluorescence quantitation of MPO showed similar patterns, while terminal decondensation

of the nuclei was inhibited by combining E2 with P4 (Fig. 4j). At the same time, nuclear delobulation and hypercitrullination of H3 pointed to an enhanced primed state under the presence of P4 (Fig. 4j).

To evaluate whether gestational hormone levels increase priming of peripheral blood neutrophils and formation of NETs, we pre-treated plasma isolated during gestation with the estrogen antagonist fulvestrant and the progestin agonist mifepristone *in vitro*. Fulvestrant was preferred to tamoxifen, because it does not induce NET formation [200]. Fulvestrant treatment of plasma from pregnant women partially prevented NET formation (Fig. 4k and 4l). On the other hand, NET extrusion was distinctly increased after pre-treatment of pregnancy plasma with the P4 receptor antagonist mifepristone (Fig. 4k, 4l and 4 m, left panel). Immunofluorescence of neutrophils exposed to mifepristone showed hypercitrullination of H3, consistent with a predisposition of neutrophils exposed to mifepristone-treated plasma to form NETs (Fig. 4m, right panel).

Therefore, our data show that hCG, E2 and E3 stimulate NET formation and that PMA, as an additional stimulus, induces distinctly higher levels of NETosis than either of these agents alone. P4 does not induce formation of NETs, but rather diminishes the NETotic response to these agents either alone or in combination.

Figure 4.

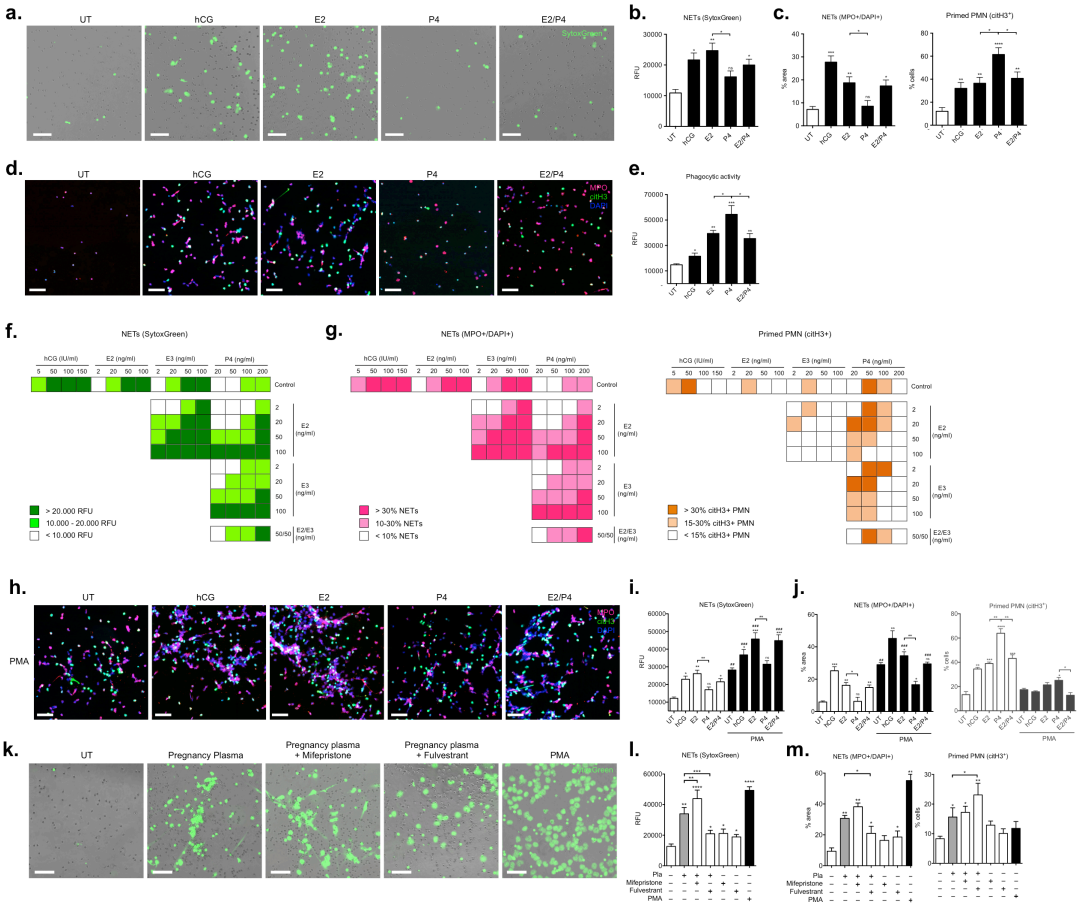
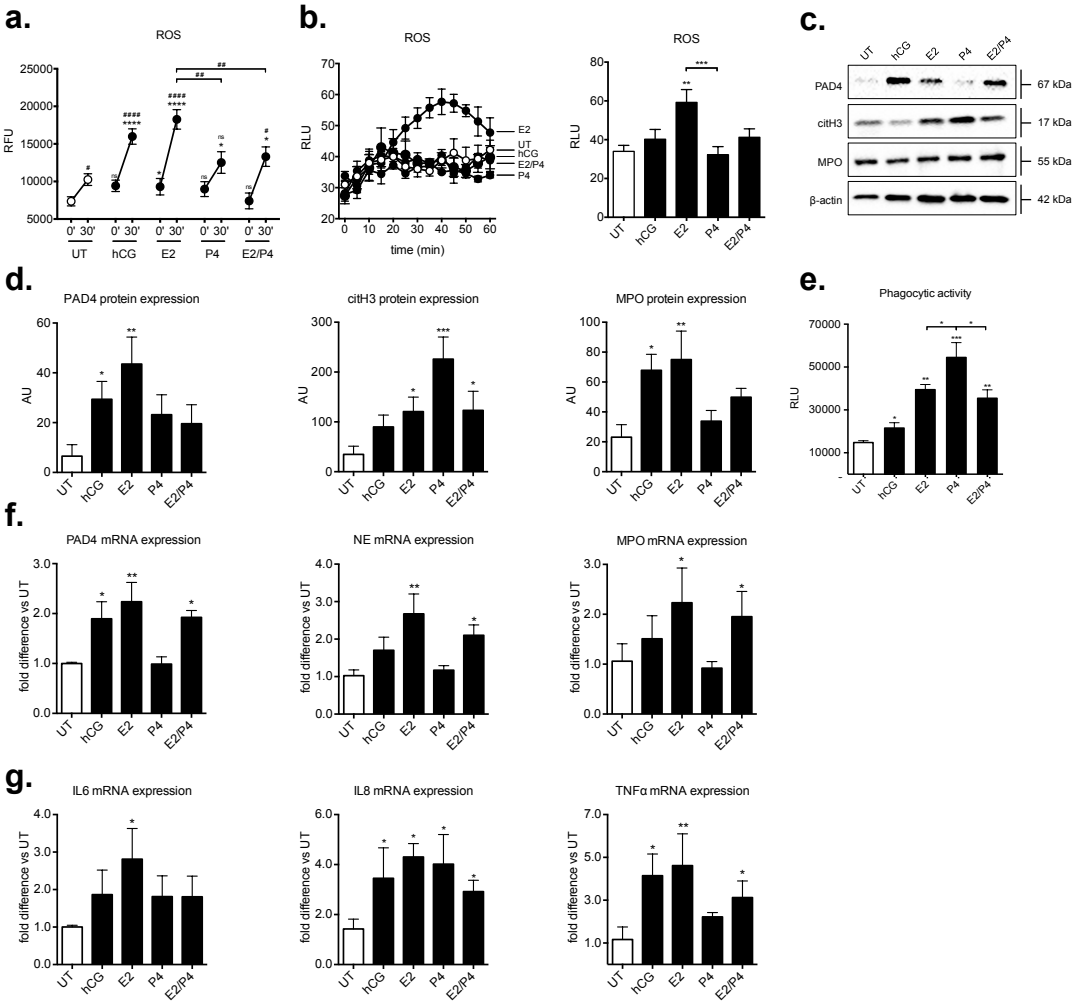


Figure 4. Neutrophil *in vitro* pro-NETotic priming is regulated by pregnancy hormones. (**a**) NET formation assessed microscopically in a 3 hour timecourse with SytoxGreen DNA binding dye after *in vitro* incubation of control neutrophils with physiological concentrations of the sex hormones hCG, E2 and P4. Magnification: 10x. Scale bars: 100 µm. (**b**) Fluorimetric quantification of extracellular DNA release of gestational hormone treated control neutrophils. (**c**) Morphometric analysis of the NETotic (MPO+) and proNETotic primed (citH3 positive) neutrophils from healthy control donors under hormonal treatment. (**d**) Fluorescent immunocytochemistry of peripheral neutrophils after incubation with pregnancy hormones for MPO (red), citH3 (green) and DNA counterstain with DAPI (blue) after a 2 hour culture. (**e**) Neutrophil phagocytic activity after a 2 hour incubation with pregnancy-specific hormones. (**f**) Information graphic summarizing *in vitro* spontaneous NET release monitored in a 3 hour timecourse by dsDNA-binding fluorescent SytoxGreen dye after treatment with four ascending concentrations of each of the gestational hormones hCG, E2, E3 and P4, separately or in crossing combinations. (**g**) Information graphics summarizing *in vitro* spontaneous NET release and H3 hypercitrullination in a 3 hour timecourse after hormonal treatment as in (**a**), assessed by MPO (left panel) and citH3 (right panel) immunofluorescence microscopy and subsequent morphometric analysis, respectively. (**h**) *In vitro* spontaneous NET release monitored microscopically in a 3 hour time course by fluorescent immunostaining for MPO (red), citH3 (green) and DNA (blue) after 1 hour pretreatment with physiologic concentrations of hCG, E2, P4 and E2/P4 and stimulation with the NET-inducing agent PMA as a second hit for

additional 2 hours. Magnification: 20x; Scale bars: 50 µm. (**i**) Quantification of extracellular DNA release of gestational hormone treated control neutrophils after addition of PMA by fluorimetry. (**j**) Morphometric analysis of the NETotic MPO positive and pro-NETotic primed citH3 positive neutrophils from healthy non-pregnant donors under the aforementioned experimental setup. (**k**) Detection of *in vitro* spontaneous NET formation of neutrophils from healthy controls co-cultured with pregnancy plasma pretreated with mifepristone and fulvestrant in a 3 hour time course by fluorescent microscopy using dsDNA-binding fluorescent SytoxGreen dye. Magnification: 10x. Scale bars: 100 µm. (**l**) Fluorimetric quantification of extracellular DNA release of pregnancy plasma treated control neutrophils and preincubation with the sex hormone inhibitors mifepristone and fulvestrant. (**m**) Morphometric analysis of the NETotic (MPO+) and pro-NETotic primed (citH3 positive) neutrophils from healthy control donors after hormonal inhibition. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one or two way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 6 times with consistent results. RFU, relative fluorescence units; Pla, pregnancy plasma.

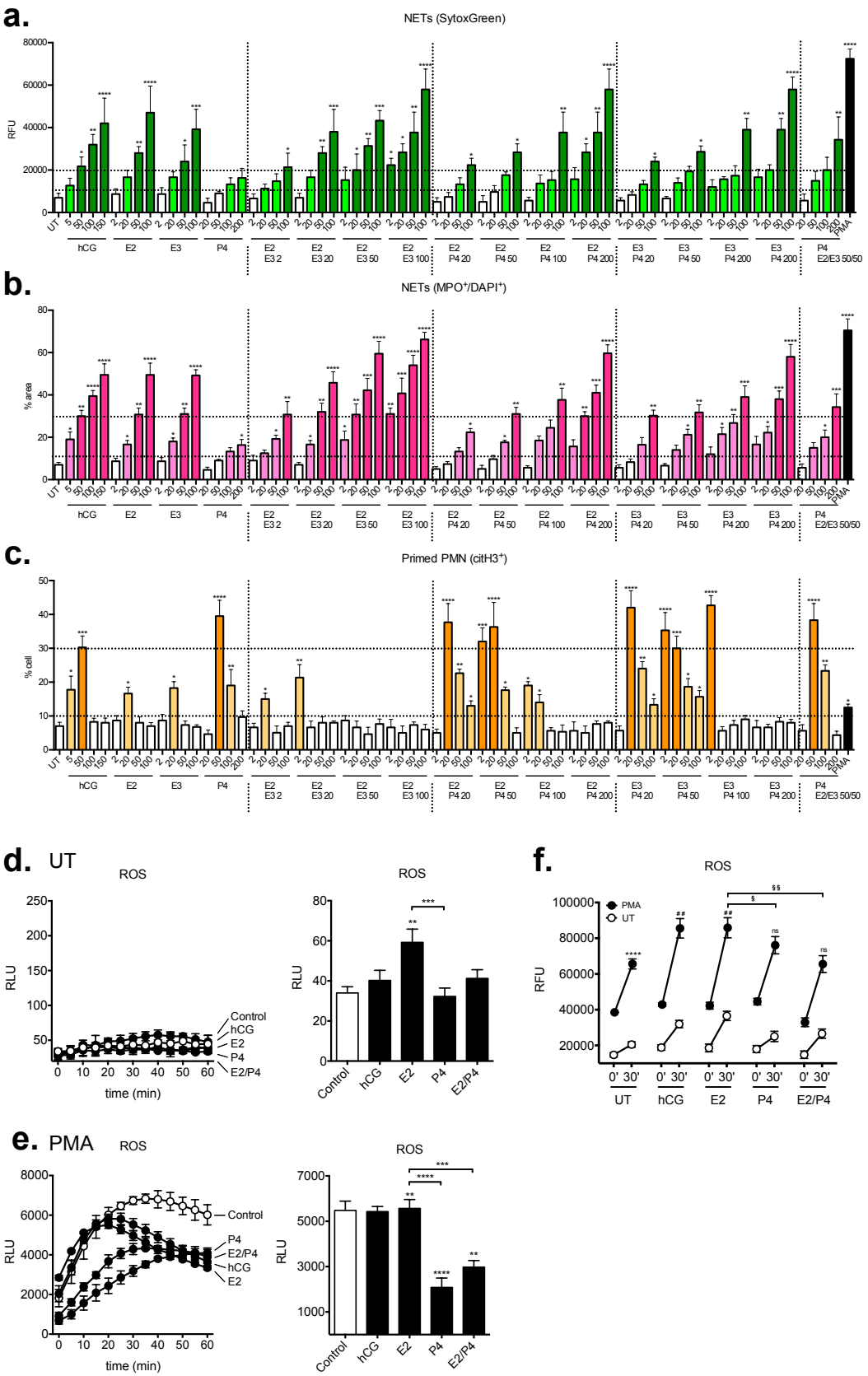
Supplementary Figure 5



Supplementary figure 5. Sex hormones regulate neutrophil pro-NETotic

priming. Intracellular (**a**) and overall (**b**) oxidative burst in neutrophils from healthy control donors treated with physiologic pregnancy concentrations of hCG (50 IU/ml), E2 (50 ng/ml), P4 (50 ng/ml) and the 1:1 combination of E2 and P4 for 3 hours by DCFH-DA and luminol detection, respectively. Western blot (**c**) and densitometric analysis (**d**) of PAD4, citH3, MPO and beta-actin protein expression levels in lysates from healthy female control neutrophils treated with the aforementioned concentrations of gestational hormones. (**e**) Phagocytic activity of neutrophils obtained from healthy female controls during pregnancy. (**f**) *PAD4*, *NE* and *MPO* gene expression analysis by Taqman qRT-PCR in RNA samples obtained from healthy female controls during pregnancy. (**g**) *IL6*, *IL8* and *TNF* gene expression analysis by Taqman qRT-PCR in RNA samples obtained from healthy female controls during pregnancy. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 6 times with consistent results. RFU, relative fluorescence units; RLU, relative luminescence units; AU, arbitrary units; UT, untreated.

Supplementary Figure 6



Supplementary figure 6. Sex hormones modulate neutrophil pro-NETotic priming and NET formation *in vitro*. (**a**) Fluorimetric quantification of extracellular DNA release of sex hormone-treated control neutrophils and in vitro culture for 3 hours. (**b**) Morphometric analysis of the NETotic MPO positive and (**c**) pro-NETotic primed citH3 positive neutrophils from healthy non-pregnant donors under the aforementioned experimental setup. (**d, e**) Intracellular and (**f**) overall oxidative burst in neutrophils from healthy controls treated with physiologic pregnancy concentrations of sex hormones for 1 hour and addition of PMA as a secondary hit for 2 hours (right panels) by DCFH-DA and luminol detection, respectively. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 3 times with consistent results. UT, untreated; RFU, relative fluorescence units; RLU, relative luminescence units; AU, arbitrary units.

P4 suppresses NET formation while maintaining a stable primed neutrophil pro-NETotic state

Since physiological concentrations of G-CSF fostered a pro-NETotic phenotype and gestational hormones modulate this process, we investigated whether pregnancy hormones influence NET formation when applied after G-CSF treatment. SytoxGreen fluorimetric analysis revealed that hCG and E2 augmented NET formation induced by pre-treatment with G-CSF (Fig. 5a and Fig. 5b). Strikingly, P4 promoted increased ROS generation by neutrophils (Supplementary Fig. 7a an Fig. 7b), yet retained a delobulated, primed state, retarding their progression to NET formation (Fig. 5a and Fig. 5b). Immunohistochemistry (Fig. 5c) and morphometry (Fig. 5d, left panel) were consistent with the cell-free DNA measurements of culture supernatants, while, as expected, low grade NETosis, yet high levels of primed citH3+ neutrophils were observed in the presence of P4 (Fig. 5d, right panel). Hence, P4 suppressed NET formation, maintaining a stable primed pro-NETotic state of neutrophils, and preventing or delaying active NET release despite advanced H3 citrullination.

To investigate the possible mechanism, we resorted to immunofluorescent confocal microscopy, which showed that the nucleus of P4 treated cells retained its lobulated state, despite high levels of H3 citrullination. Furthermore, unlike in highly pro-NETotic G-CSF treated cells, there was no intermingling of NE with nuclear contents. It thus appears that

P4 hinders either the release or transfer of NE from the cytoplasmic granules to the nucleus, where it might assist in initiating the NETotic cascade (Fig. 5e).

Figure 5.

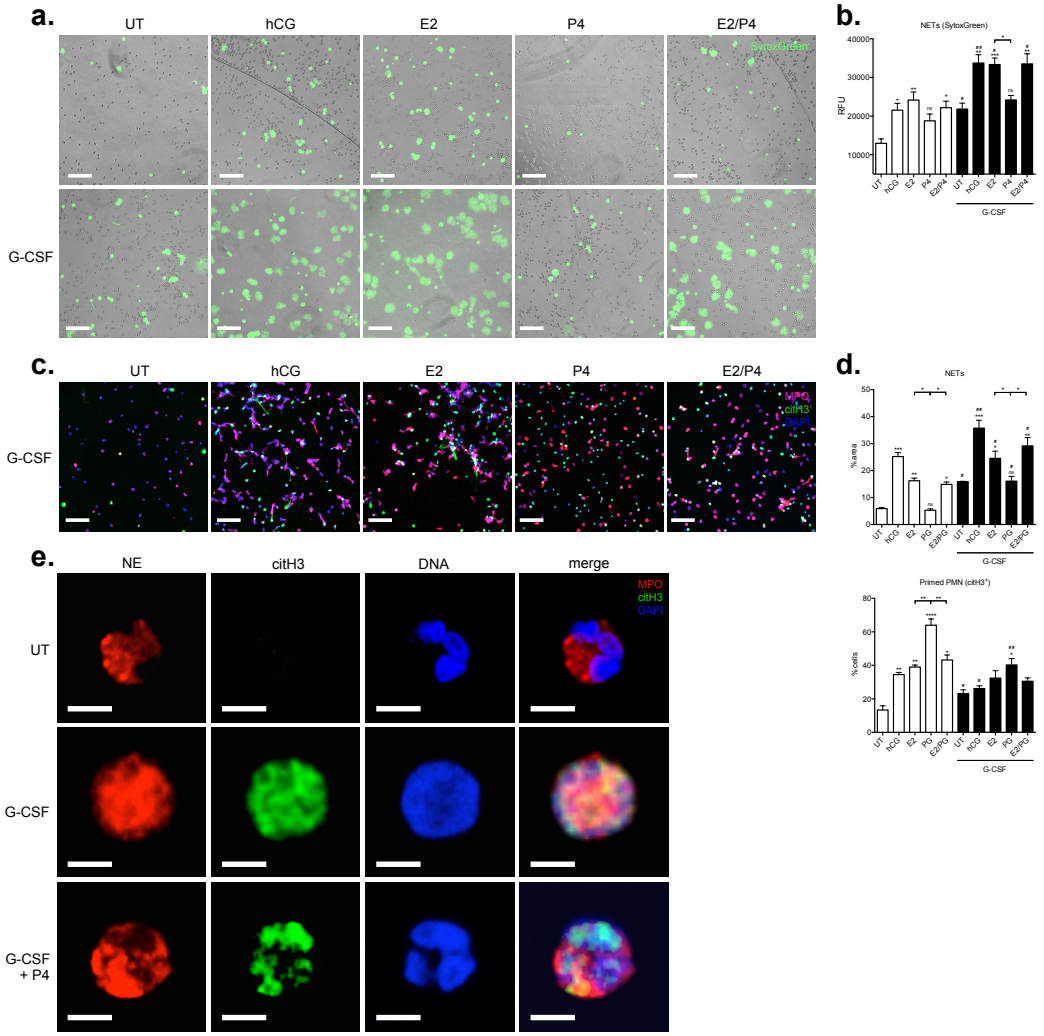
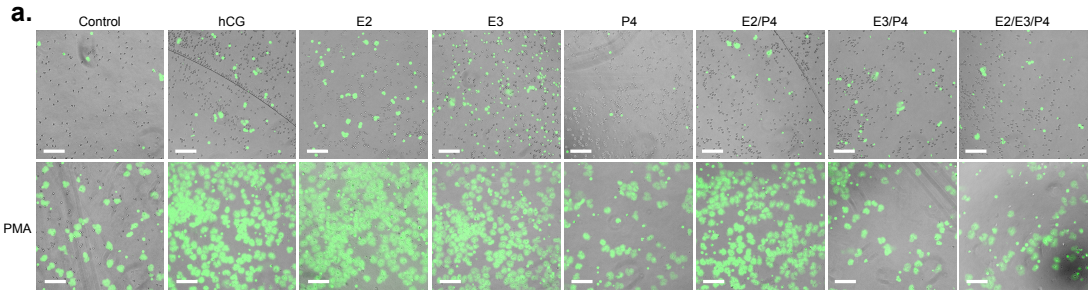


Figure 5. Sex hormones differentially modulate G-CSF-driven neutrophil pro-NETotic priming. (**a**) *In vitro* NET formation assessed microscopically in a 3 hour timecourse with SytoxGreen DNA binding dye after without (upper panel) and with a 1 hour pretreatment with 12.5 ng/ml rhG-CSF (lower panel) and stimulation with the gestational hormones hCG (50 IU/ml), E2 (50 ng/ml), P4 (50 ng/ml) and the 1:1 combination of E2 and P4 for additional 2 hours. Magnification: 10x. Scale bars: 100 µm. (**b**) Fluorimetric quantification of extracellular DNA release of rhG-CSF-treated control neutrophils after addition of pregnancy hormones at concentrations given under (**a**). (**c**) Fluorescent immunostaining for MPO (red), citH3 (green) and DNA (blue) after a 3 hour *in vitro* co-culture of control neutrophils with rhG-CSF and addition of hormones at concentrations given under (**a**). Magnification: 20x; Scale bars: 50 µm. (**d**) Morphometric analysis of the NETotic MPO positive and pro-

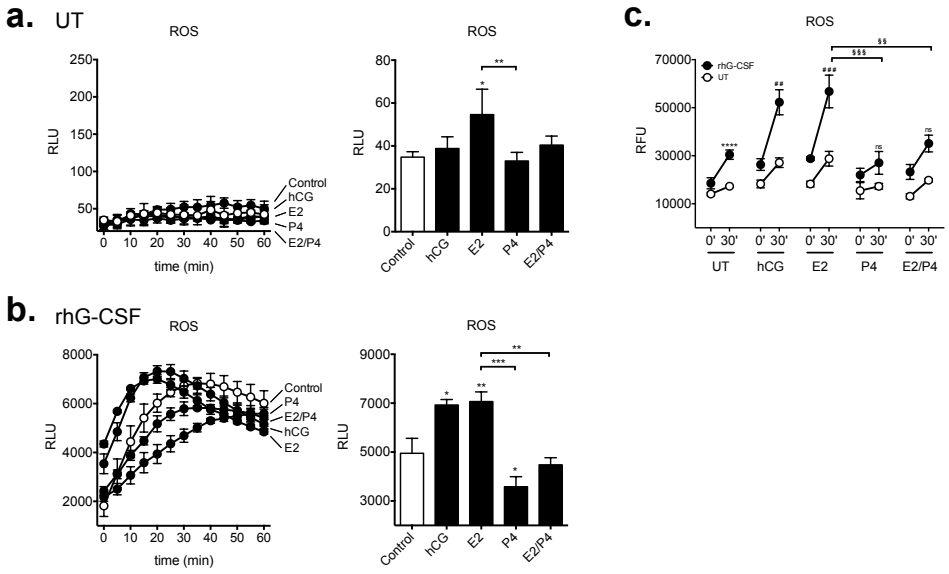
NETotic primed citH3 positive neutrophils from healthy non-pregnant donors under the aforementioned experimental setup. (**e**) Fluorescent immunostaining and confocal microscopy for MPO (red), citH3 (green) and DNA (blue) after a 3 hour *in vitro* co-culture of control neutrophils with rhG-CSF and addition of P4. Magnification: 63x; Scale bars: 10 µm. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 3 times with consistent results.

Supplementary Figure 7



Supplementary figure 7. Neutrophil *in vitro* pro-NETotic priming and NET formation is regulated by pregnancy hormones. (**a**) NET formation assessed microscopically at 3 hours with SytoxGreen DNA binding dye after *in vitro* incubation of control neutrophils with physiologic concentrations of the sex hormones hCG, E2, E3 and P4, and the combinations of E2, E3 and E2/E3 with P4. Magnification: 10x. Scale bars: 100 µm.

Supplementary Figure 8



Supplementary figure 8. G-CSF-driven neutrophil pro-NETotic priming is differentially modulated by sex hormones. Intracellular (**a**) and overall (**b**) oxidative burst in neutrophils from healthy control donors pre-treated for 1 hour with

12.5 ng/ml rhG-CSF and additionally co-cultured with physiologic pregnancy concentrations of hCG (50 IU/ml), E2 (50 ng/ml), P4, (50 ng/ml) and the 1:1 combination of E2 and P4 for extra 2 hours by DCFH-DA and luminol detection, respectively. UT, untreated; RFU, relative fluorescence units; RLU, relative luminescence units.

Discussion

In the present study we demonstrate that neutrophils exhibit an activated pro-NETotic state and enhanced propensity to release NETs during normal pregnancy. This activity is modulated at several key levels. First, G-CSF seems to be a major signal inducing neutrophil release from the bone marrow throughout gestation, providing an important mechanism by which the number of circulating neutrophils and activation state is increased. Second, the degree of activation seems to be finely tuned by the unique stoichiometric balance of the sex steroid hormones, which are produced solely by the placenta and reach their peak concentrations towards term. Finally, P4 suppresses NET formation while maintaining a stable primed neutrophil pro-NETotic state (Fig. 7). This is the first time such a regulatory mechanism of neutrophil activation is described in pregnancy, including the unique inhibitory role of P4 in NET formation.

Our experiments revealed an unexpectedly large increase in the propensity of neutrophils to form NETs in normal pregnancy. This tendency increased with the advancement of gestation and concurred with activation of the major signaling steps leading to NETosis including ROS production, nuclear delobulation, translocation of cytoplasmic granule NE and MPO to the nucleus and the citrullination of H3. Expression of mRNA and protein of NE, MPO and PAD4 were also increased. These findings justify the addition of NET formation and the underlying signaling events to the previously described increased phagocytosis and degranulation as functional alterations of neutrophils in pregnancy [96] [201] [202].

Elevations of G-CSF in murine cancer models have been noted to promote neutrophilia and priming in conjunction with moderate NET formation, and to induce thrombosis under addition of low doses of LPS [91]. This raises the prospect that G-CSF could assume a similar role in neutrophil responses in pregnancy. Plasma from pregnant women increased priming and moderated NET formation, though specific neutralization of G-CSF abrogated NET formation, but not priming. Akin to G-CSF, hCG, E2 and E3 increased NET formation, yet did not promote pro-NETotic priming. The extensive analysis of various combinations of hCG, E2, E3 and P4 in promoting neutrophil priming demonstrated a crucial role of P4 as an antagonist of the pro-NETotic effects of hCG, E2 and E3. In contrast, G-CSF conditioned neutrophils treated with hCG, E2 and E3 showed enhanced NETosis. Furthermore, neutrophils exposed to P4 did not appear to readily progress to NETosis even though their nuclei were citH3+, indicating an arrest in the primed, pro-NETotic state. These findings mirror these observed in murine cancer models, as published for breast cancer [91]. Steroid sex hormones are involved in breast cancer, and neutrophils have been implicated in promoting or inhibiting breast cancer growth and metastasis depending on the expression of TGFβ and TNFα [203, 204]. Recent data from a different murine model of breast cancer support a fundamental role of neutrophils in fostering metastasis of the neoplasia to the lung [203]. Our data suggest that P4 or similarly acting agents could exert a beneficial inhibitory effect on neutrophil behaviour in the setting of mammary neoplasia.

Since signalling up to the step of H3 citrullination, encompassing the activation and transfer of PAD4 from the cytoplasm to the nucleus, was unaffected by P4, we hypothesized it must utilize a different pathway. Given the essential role of translocation of NE from the cytoplasm to the nucleus for the extrusion of nuclear contents [37], we studied the influence of P4 on NE translocation. P4 completely prevented NE translocation to the nucleus *in vitro*, while the nucleus maintained its lobulation despite citrullination of H3. The specificity of P4 was confirmed by its inhibition with mifepristone, which abrogates the binding of P4 to its intracellular receptor [205]. These results concur with the opposing effects of estrogens and progestins on immune cell

function seen in other studies [206] [207].

The adaptation of neutrophils from peripheral blood could complement the alterations of Tregs and placental NK cells in balancing the reduced local neutrophil reactivity in the placenta by priming for a response to a second stimulus by infectious agents encountered in the systemic periphery. The potency of NET formation to mount effective responses against infectious agents has been shown in several studies [208], and increased preparedness to produce NETs could help ensure the protection of mother and fetus against infections during pregnancy.

As with other states of increased immune system activity, inappropriate NET formation conceivably poses a threat to tissue integrity in pregnancy. In preeclampsia, for instance, increased NETosis coincides with inappropriate NET formation in the sinusoids of the trophoblast and the intervillous space. The interaction of excessive NETs with endothelial cells [71] or the trophoblast might provide the basis for the release of Flt-1 into the maternal circulation and the peripheral inflammatory activity, which underlies the elevated blood pressure and increased procoagulant activity, the hallmarks of PE [209]. Another relevant condition is the anti-phospholipid syndrome, in which anti-cardiolipin antibodies interact with both neutrophils and the endothelium [93] [210] [211].

During pregnancy, the increased propensity to form NETs could at least partly explain the raised frequency of thrombosis [212]. In this case, it is possible that signaling elements along the PKC pathway substitute for NE signaling, as has recently been reported in a mouse model of deep vein thrombosis [213]. The effects of NET formation on the endothelium and on thrombosis related to pregnancy conditions find counterparts in tumor-associated thrombosis, inflammatory blood vessel diseases and atherosclerosis [214] [215] [216] [91].

Figure 6.

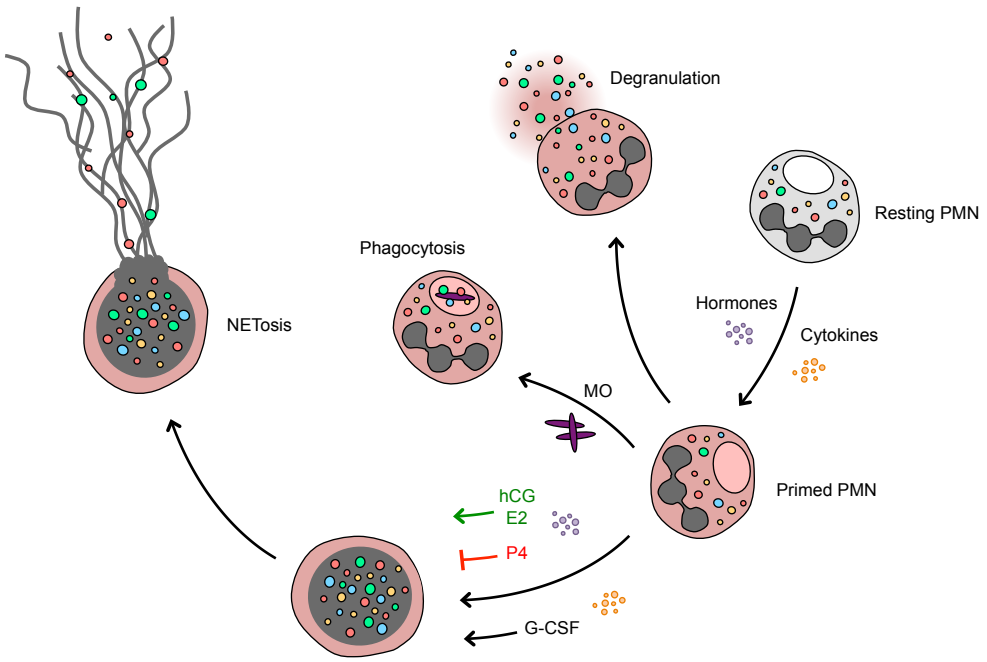


Figure 6. Progesterone antagonizes the estrogen and G-CSF driven neutrophil extracellular trap formation during pregnancy. Neutrophils during pregnancy lie under the increased influence of cytokines, e.g. G-CSF, and sex hormones. This specific milieu appears to poise the neutrophils in a stable pro-NETotic primed state. Depending on the stimulus, for instance microorganisms (MO), neutrophils react by phagocytosis or degranulation. When a different NETotic stimulus is present, such as excessive placentally derived plasma microparticles (MP) in preeclampsia, primed neutrophils react with overt NET release. Pro-NETotic combinations of hormones and cytokines are given in green, the most potent in bold green. Inhibitory combinations are given in red, the most potent in bold red.

In summary, we show that E2 and E3 lead to pronounced neutrophil activation and NET release *in vitro*, which was reflected by increased NETosis during pregnancy (Fig. 6). Remarkably, treatment with P4 significantly reduced NETosis and antagonized the pro-NETotic effects of E2 and E3. Strikingly, P4 promoted a pro-NETotic citH3 positive primed state of neutrophils, akin to that induced *in vitro* by G-CSF in our experiments and previously reported in murine models of several cancers [91]. These observations are corroborated with a detailed analysis of signaling pathways

and extended by the finding that P4 inhibited the translocation of NE from the cytoplasm to the nucleus, which is required for the progression of NETosis.

Our analysis of ex vivo and *in vitro* effects of sex steroids and G-CSF during gestation provide conclusive evidence that increased neutrophil pro-NETotic priming and NET formation form a significant part of the adaptations of the immune system in pregnancy. Furthermore, our observations concerning P4 provide a basis for further analysis of the physiologic sex hormone influences on innate immunity and neutrophil functions in members of the female sex, as well as the implications and consequences of disturbed function on inappropriate inflammation and pathogenesis. Our findings regarding neutrophil responses during normal pregnancy provide new insight concerning gestational and hormone-driven pathologies, since neutrophil recruitment, activation and NET release could be associated with excessive endothelial and placental injury.

Chapter 3

Gestational Diabetes Mellitus

Gestational Diabetes Mellitus (GDM) is defined as glucose intolerance resulting in hyperglycaemia of variable severity with onset or first recognition during pregnancy [217]. Prevalence is most often reported as 2-6% of pregnancies in Europe [218].

In humans, the balance between adequate insulin secretion and insulin sensitivity maintains the normal glucose tolerance. The ability of insulin to dispose of carbohydrates is based on the pancreatic beta-cell response to glucose by secreting insulin and the sensitivity of the glucose utilizing tissues to insulin [219]. During normal pregnancy, insulin sensitivity declines with advancing gestation. In physiological conditions, a compensatory increase in insulin maintains the normal glucose homeostasis. GDM occurs when pancreatic beta cells are unable to face the increased insulin demand during pregnancy [220].

Glucose regulation during normal pregnancy

From the beginning of pregnancy fasting blood glucose moderates and proceeds with advancing gestation [221]. Insulin sensitivity declines during gestation to reach at late gestation (34-36 weeks) 50-60% of the non-pregnant condition [222]. As a consequence to insulin resistance, fasting insulin concentrations increase. These changes in insulin sensitivity are inversely related to changes in the maternal body fat mass [223]. These alterations in the maternal metabolism have been attributed to placental factors, such as progesterone and estrogen [224]. In addition, changes in inflammatory factors such as TNFα might also be involved in the insulin resistance during pregnancy [225]. It has also been reported that the level of placental TNFα determines the insulin resistance in pregnancy independently from the fat mass changes [226].

Pathogenesis of GDM

It is suggested that women who develop GDM are before pregnancy insulin resistant compared to women who did not develop GDM [227]. The significant decrease in insulin sensitivity in late gestation is correlated with the decreased insulin sensitivity that pre-exists prior to pregnancy [228]. The circulating factors TNFα and IL-6 have also been inversely correlated with insulin sensitivity and the pathogenesis of GDM [225] [229]. From the other factors that are elevated in GDM, leptin is mainly associated with the prior to pregnancy maternal weight [230].

Maternal and fetal complications of GDM

The most common maternal outcome of GDM in the clinic is Caesarean section. Furthermore, gestational diabetes raises the risk for preeclampsia, which combines gestational hypertension and proteinuria. Adverse fetal complications of GDM are mainly related to macrosomia caused by fetal hyperinsulinism in response to high glucose levels coming from maternal hyperglycemia. Moreover, the developing fetus has higher risk of experiencing respiratory distress syndrome, developing hypoglycemia or Type 2 diabetes later in life and having higher morbidity and mortality incidence. High-risk patients are screened as early as possible using fasting plasma glucose and if normal, they proceed with a 75g Oral Glucose Tolerance Test (OGTT) at 24-28 weeks of gestation [231].

Neutrophils in diabetes

Traditionally neutrophils are not regarded as key factors in the aetiology of diabetes, however, recent data have suggested that they could contribute to the autoimmune condition underlying Type 1 Diabetes (T1D) [232] or the inflammatory condition occurring in Type 2 Diabetes (T2D) [14] [233]. Recently it was suggested that NETosis is reduced in T2D affected individuals and that this in part is mediated by elevated glucose levels [234]. Patients with diabetes mellitus (type 1 or type 2) exhibit impaired wound healing and it has

been recently described that this defect could stem from enhanced NETosis [235] [236].

Aim

In an attempt to explore whether hyperglycemia affects neutrophils activation during pregnancy, we investigated the biological response of neutrophils in pregnant women with gestational diabetes. Moreover we explored the effect of primed neutrophils in the placenta.

Methods

Human Subjects

Pregnant women (n=20) were recruited at the time of their routine examination for glucose intolerance at the 24th to 28th weeks of pregnancy and had no preexisting diseases such as diabetes mellitus or high blood pressure (median gestational age: 26 weeks and 2 days; median age: 33.7 years). 12.5ml of blood was collected before, 60 and 120 minutes after the oral glucose tolerance test, at the 24th to 28th weeks of gestation and 48 hours after delivery. At the time of caesarean section placenta tissue was also collected. Healthy non-pregnant controls matched for age (n=10) were recruited at the Blood Bank of the Swiss Red Cross, Basel.

Inclusion criteria for the pregnant subjects included healthy singleton pregnancies. Exclusion criteria included maternal diseases like hypertension, diabetes mellitus and chronic disease, known infection like hepatitis or human immunodeficiency virus, maternal history of hypertensive diseases in previous pregnancy, fetal genetic, chromosomal or intervention-requiring morphologic abnormalities. Informed, written consent was obtained from all subjects prior inclusion in the study, which was approved by the Ethical Review Board of Basel/Basel-Land, Switzerland. Inclusion criteria for non-pregnant controls were fair general condition, female sex, age ≥ 25 and ≤ 45 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.

Women with GDM were diagnosed according to the new recommendations of European Association for the Study of Diabetes and World Health Organization (WHO) for the 2-h 75-g OGTT as at least two

values greater than a fasting glucose of 5.1 mmol/l, a 1-h glucose of 10 mmol/l, or a 2-h glucose of 8.5 mmol/l.

Human placentae were obtained from pregnant women who delivered healthy, singleton infants at term (> 37 weeks' of gestation) undergoing elective Caesarean section. After written informed consent, 5 placentae samples for each group were collected and dissected from the middle cross-section of villus tree within 15 min of the delivery. To compensate for intra-placental variability, we collected 3 independent samples per placenta giving totals of 15 GDM and 15 control samples. Tissue was bluntly dissected to remove visible connective tissue, blotted dry on filter paper, snap frozen in liquid nitrogen and stored at -80 °C. Small pieces of tissues are embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek USA) and kept at −80 °C until cryosection.

Oral glucose tolerance test

Pregnant women at the 24th to 28th weeks of gestation, after 10 - 16 hours of fasting give blood and the fasting glucose levels were tested. They drink the 300ml of Accu-Chek Dextrose O.G.-T. (Roche) that contains 75 gram of glucose and give blood after 60 min and after 120 min, according to manufacturer's instructions.

Blood cell count and preparation of plasma and serum

Whole blood was collected into EDTA- and silicone-coated tubes (Sarstedt) and 25 µl of blood was analyzed by a Hemavet 950FS (Drew Scientific) for complete blood cell counts. Plasma and serum was collected and processed as described previously [190]. Samples were studied immediately or stored at -80°C until analyzed.

Human neutrophil isolation

Neutrophils were isolated by Dextran-Ficoll density centrifugation [65]. Cell viability was assessed by trypan blue dye exclusion in a haemocytometer

and was routinely 96–98% with a purity of over 95%. Neutrophils were directly seeded in 24-well or 96-well plates and allowed to settle for 15 minutes at 37°C under 5% CO_2 prior to further experimentation.

BeWo cells and co-culture conditions

BeWo cells (ATCC CCL-98) were grown at 37°C under a humidified 5% CO_2/95% air atmosphere in F-12K Medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Experiments were performed in serum-starved conditions using cells at 80% confluence between passages 30 and 40. BeWo cells were grown under low glucose (LG: 7 mM) or high glucose (HG: 25 mM) conditions. Supernatants were harvested, submitted to centrifugation, aliquoted, and frozen at −80°C until use. The co-culture experiments were performed in 6-well cell culture plates with 12 mm coverslips (Corning® BioCoat™ Coverslips). When indicated, neutrophils were directly added to the BeWo cells at a density of 0.5×10^6 neutrophil/ml for 3 hours. In neutralization studies, 5 ug/ml of chimeric anti-TNF IgG1 antibody infliximab (Remicade, MSD) was given simultaneously to neutrophil addition. Dr. Franco Grimolizzi performed all the experiments with BeWo cells.

Fluorimetric quantification and fluorescence microscopy

NETs were quantified by SytoxGreen fluorimetry. 2.5×10^4 freshly isolated neutrophils were cultured in the presence of 0.2 μM SytoxGreen (Invitrogen, Life Technologies) in a 96-well dark microtiter plate at 37°C under 5% CO_2 and left untreated or stimulated with the aforementioned agents over 3 hours. PMA was used as the positive control. Fluorescence (excitation 485 nm, emission 535 nm) was measured in a Biotek Synergy H1 Hybrid Reader (Biotek) and results given as DNA fluorescence (MFI). Photomicrographs in bright field and green fluorescence spectra were assessed with an Olympus IX50 inverted fluorescence microscope coupled to an Olympus XM10 monochromatic CCD camera and analyzed with the Olympus CellSens Dimension software (Olympus).

Immunohistochemistry and morphometric analysis

$1x10^5$ neutrophils were seeded on poly-L-lysine-coated glass coverslips (BD Biosciences) in 24-well tissue-culture plates 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% FBS, 0.1% Tween20, and 2mM EDTA) at 4°C. NETs were detected with rabbit anti-NE (Abcam), rabbit anti-MPO (Dako) or mouse anti-MPO (Abcam) and rabbit anti-citrullinated histone H3 (citH3, Abcam). Secondary antibodies were goat anti-rabbit IgG AF555, goat anti-rabbit IgG AF488 (Invitrogen Life Technologies), and goat anti-mouse IgG AF647. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). 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 neutrophils) per case were 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.

In another set-up, NETs were quantified by IHC staining of 2.5×10^4 neutrophils per well in a 96-well plate with rabbit anti-NE (Abcam), 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 Life Technologies). DNA was counterstained with 4′,6- diamidino-2-phenylindole (DAPI, Sigma-Aldrich). 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 10X magnification (at least 500 to 1,000 neutrophils) per sample were evaluated for MPO/citH3 and DNA co-staining through ImageJ analysis software (NIH); nuclear phenotypes and NETs were determined, counted, and

expressed as percentage of the total area of cells in the fields [194]. Images were captured on a Nikon A1R inverted microscope (Nikon) coupled to a Visitron CSU-W1 spinning disc confocal microscopy module (Visitron) and a Thor ablation laser (Thor Labs) using an UPL APO 60x/1.40 oil objective lens with the Visiview Cell Analyser software (Visitron Systems, Version 3.1.2.2).

For double immunohistochemistry of the human placentas, 8 µm thick placental sections were fixed in 4% paraformaldehyde/PBS for 10 min, permeabilized by incubation with 0.2% Triton X-100 in PBS for 5 min and blocked in with 3% BSA/PBS for 60 min. Then, the slides were probed with first antibodies diluted 1:150 in PBS with 3% BSA and 0.05 % v/v Tween 20 (staining buffer) for 1 hour at room temperature (RT). Primary antibodies used were: rabbit anti-citH3 (Abcam), rabbit anti-MPO (Abcam), NE (Hycult Biotech) and rabbit anti-IRS1 (Santa Cruz). Negative controls were obtained by incubating with isotype-specific mouse/rabbit IgGs (Santa Cruz) instead of the specific primary antibodies. Sections were then incubated for 1h at RT in a light-protected humidified chamber with Alexa 488-conjugated anti-rabbit (Life Technologies) and Alexa 647-conjugated anti-mouse (Life Technologies) secondary antibodies diluted 1:1000 in staining buffer. Sections were stained with DAPI (DAPI, Sigma-Aldrich), mounted in Mowiol (Sigma-Aldrich) and visualized using an Olympus BX61 Diana fluorescent microscope. In addition, a further consecutive section was stained using H&E for comparison against immunostained tissues.

ROS generation analysis

BeWo cells were incubated overnight with different concentrations of NE (20-320 nM). The cells were washed twice with pre-warmed PBS, incubated with 10 µM 2′,7′-dichloro dihydrofluorescein diacetate (DCFH-DA) in PBS for 1 h at 37°C, and then resuspend in PBS. Fluorescence was measured with a multiplate reader (Synergy H4 Hybrid Multi-Mode Reader, BioTek, Germany) at 535 nm with excitation at 480 nm.

Cell-free nucleosome, NE and TNFα ELISA

Histone/DNA complexes in sera and plasma were measured using the Human Cell Death Detection ELISA^{PLUS} (Roche Diagnostics). The concentrations of NE in sera and plasma were measured by sandwich ELISA, utilizing the Elastase/a1-PI Complex ELISA Kit (Calbiochem). TNFα concentrations in sera and plasma were assessed by sandwich ELISA with Human TNF-alpha DuoSet ELISA Kit (R&D Systems).

Stimulation and neutralization studies

For *in vitro* incubation studies 2.5 x 10^4 neutrophils from healthy women were treated with 6% plasma derived from non-pregnant controls, pregnant donors and donors with GDM during second and third trimesters of gestation. All experiments were carried out over 3 hours in 4 to 6 replicates.

For similar *in vitro* incubation studies 2.5 x 10^4 neutrophils from healthy women were treated with supernatants from BeWo cells after exposure to normal or high glucose for 12h.

To neutralize TNFα, pooled plasma from the study groups of interest or BeWo supernatants were pretreated for 30 minutes with Infliximab (5ug/ml).

For the two-step stimulation *in vitro* experiments, neutrophils from healthy controls were pretreated or not with TNFα (50ng/ml) for 30min and then exposed to high glucose (25mM) for 150min, for a total time of 3 hours.

Protein isolation and western blotting

Total cellular protein was extracted using Pierce RIPA Buffer (Thermo Scientific) supplemented with PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche Life Science). For the placenta, frozen tissues were thawed for a few minutes in pre-chilled RIPA buffer and homogenized with a Polytron (Kinematica). Western blotting was performed by using AnykD Mini-PROTEAN TGX Gels (Biorad, Hercules, CA, USA) and nylon/nitrocellulose membranes (Biorad). Primary and secondary antibodies used were: rabbit anti-IRS1 (Santa Cruz Biotechnology), rabbit anti-GLUT4 (Millipore) mouse anti-β-Actin (Sigma-Aldrich), goat anti-Mouse and/or anti-Rabbit, human anti-

HRP (Santa Cruz Biotechnology). HRP activity was detected by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Densitometric analysis and protein quantification of the Western blots was performed by using Image Lab Software (version 5.2.1). Blots were stripped briefly in 0.2 M sodium hydroxide and reprobed for mouse anti-β-Actin (Sigma-Aldrich) as a loading control.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from $3x10^6$ neutrophils by using the RNeasy Mini Kit (Qiagen). TaqMan real-time quantitative RT-PCR was performed utilizing the Applied Biosystems StepOne Plus cycler (Applied Biosystems) and TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) for *ELANE* (HS00236952_m1), TNF (HS01113624_g1) and RPLP0 (HS99999902_m1). Data were normalized to the housekeeping gene *B2M* (HS99999907_m1), after a selection procedure from six different endogenous reference genes, as suggested in the MIQE guidelines [195]. Relative values were calculated with 2^{-DDCt} analysis [196].

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 carried out 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 values under < 0.05 were considered statistically significant. Data were processed in GraphPad Prism version 6.0 for MacOSX (GraphPad Software Inc., www.graphpad.com). Professional statistical assistance was provided by Andreas Schoetzau (www.eudox.ch).

Results

GDM is characterized by primed circulating neutrophils which undergo excessive NET formation

We observed a marked increase in NET formation *in vitro* by neutrophils from GDM donors versus pregnant and non-pregnant controls, which at 3 hours was highly significant, as assessed by immunocytochemical fluorescent staining (Fig. 1a). This was reflected in augmented cell free nucleosomes in culture supernatants, which corresponds to the presence of NETs (Fig. 1b). The morphology of the neutrophil nucleus evolves from a lobulated to a primed phenotype, identified by citrullinated histone H3 staining, and then to excessive NETs formation, having a spread phenotype [37]. After assessing the fraction of primed neutrophils (citH3+) and those with the spread NETotic phenotype, it was evident that NET formation was elevated in patients with GDM (Fig. 1c, left panel), especially after incubation with PMA as an additional stimulus. High levels of primed citH3+ neutrophils were also observed (Fig. 1c, right panel). Although a substantial rise in neutrophil numbers was observed in pregnancies with GDM (Fig. 1d), this could, however, not solely account for the increase in neutrophil activation and NETs formation. Since serum and plasma cell free nucleosomes levels were also elevated in GDM (Fig. 1f), this is further suggestive of increased neutrophil NETotic activity in affected pregnancies. This activity was not restricted to NETs formation, as our analysis indicated that serum NE levels were also elevated when compared to healthy pregnant controls (Fig. 1e). Of interest is that NE levels were also greater in cases with GDM than those detected T1D and T2D patients (Fig. 1e), thereby clearly illustrating that GDM may have distinct clinical features different from other forms of diabetes.

To summarize, peripheral blood neutrophils exhibit a primed pro-NETotic phenotype, which increases during the course of pregnancies with GDM, and are hyper-responsive to a secondary stimulus. This activation is associated with an increase release of NE.

Figure 1.

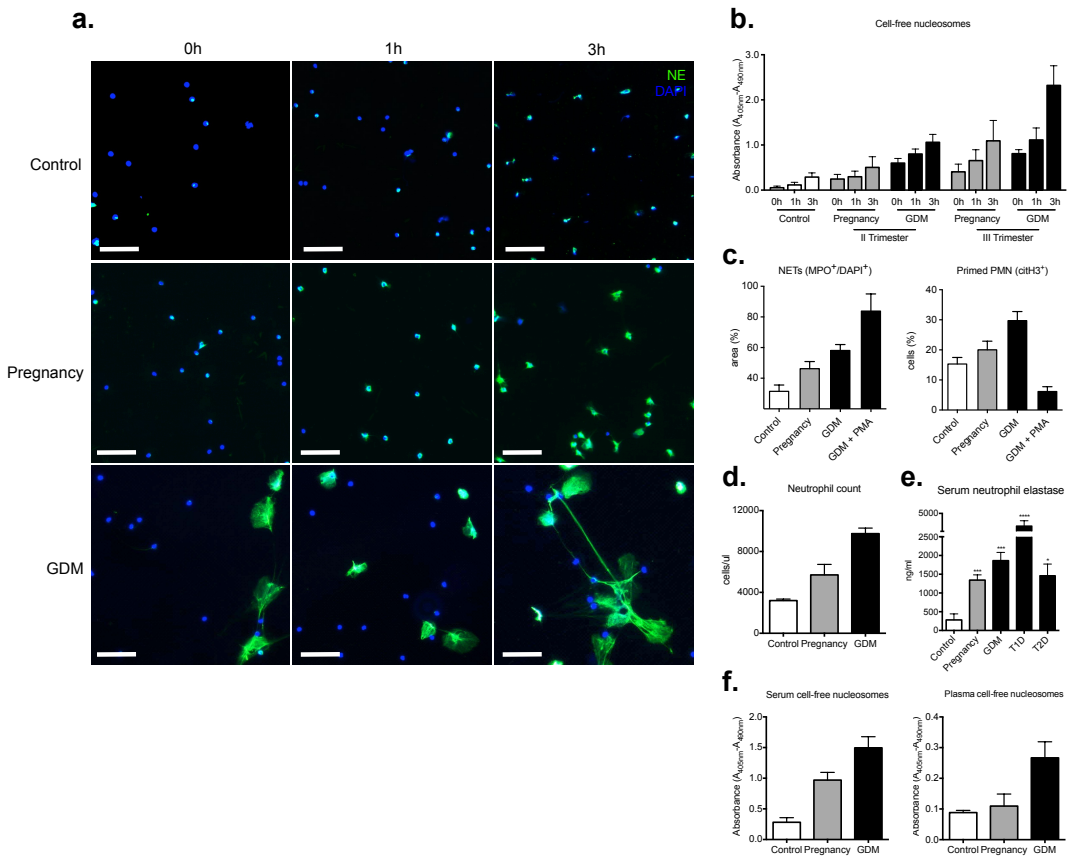


Figure 1. NET formation and neutrophil pro-NETotic priming are augmented during GDM pregnancy. (**a**) *In vitro* spontaneous NET formation by neutrophils from non-pregnant controls, healthy pregnant donors and GDM patients in a 3 hour timecourse by Immunofluorescence staining for NE (green) and DNA counterstain with DAPI (blue) at baseline (0h), in 1 hour (1h) and after 3 hours of culture (3h). Magnification: 20x; Scale bars: 50 µm. (**b**) Quantification of cell free nucleosome levels in culture supernatants of neutrophils from healthy controls and pregnant donors at baseline (0h), after 1 hour (1h) and at 3 hours (3h) of culture. (**c**) Morphometric analysis of NETs (MPO+/DAPI+) and pro-NETotic primed neutrophils (citH3+) from healthy controls, donors during pregnancy, patients diagnosed with GDM, the latter treated with PMA as an secondary stimulus, all after a 3 hour culture (3h). (**d**) Neutrophil count in EDTA blood samples obtained from the enrolled healthy controls, pregnant donors and GDM patients. (**e**) Detection of serum NE levels by ELISA. (**f**) Detection of serum (left panel) and plasma (left panel) cell-free nucleosome levels by ELISA. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 6 times with consistent results. GDM, gestational diabetes mellitus.

High glucose primes human neutrophils towards NET formation in GDM

Since hyperglycaemia is frequently manifest in gestational diabetes mellitus, as indicated by the higher glucose levels compared to the healthy controls, we postulated that high glucose might contribute to neutrophil priming. Isolated neutrophils from healthy donors were cultured in media with normal (5.5 mM) or high (25 mM) glucose concentrations before stimulation with phorbol myristate acetate (PMA). SytoxGreen fluorimetry (Fig 2a, left panel and Fig. 2b) and immunofluorescence microscopy for NE, MPO and citH3 (Fig 2a, right panel, Fig. 2c and Fig. 2d) revealed that neutrophils exposed to high glucose became primed and generated more NETs compared to what we observed when incubated with normal glucose. Thus, the increased tendency of neutrophils from women with GDM towards NET formation is at least partially due to elevations in blood glucose. To further explore whether hyperglycaemia in patients with GDM predisposes neutrophils to increased NET formation, we isolated neutrophils from fresh whole blood obtained from individuals at the time of the diagnostic oral glucose tolerance test for GDM (OGTT) (Fig. 2f). Our analysis of circulatory neutrophils following exposure to high glucose during the OGTT test permitted a clear distinction between patients with GDM, normal pregnant controls and non-pregnant controls (Fig 2e, Fig. 2h, Fig. 2i), in that these cells were highly primed and pro-NETotic. This was confirmed by an examination of serum cell free nucleosomes (Fig. 2j) and NE mRNA expression levels (Fig. 2k), which were similarly elevated in cases with GDM, when compared to the 2 control groups.

Taken together, these data indicate that hyperglycaemic conditions in GDM have profound effects on neutrophil activity.

Figure 2.

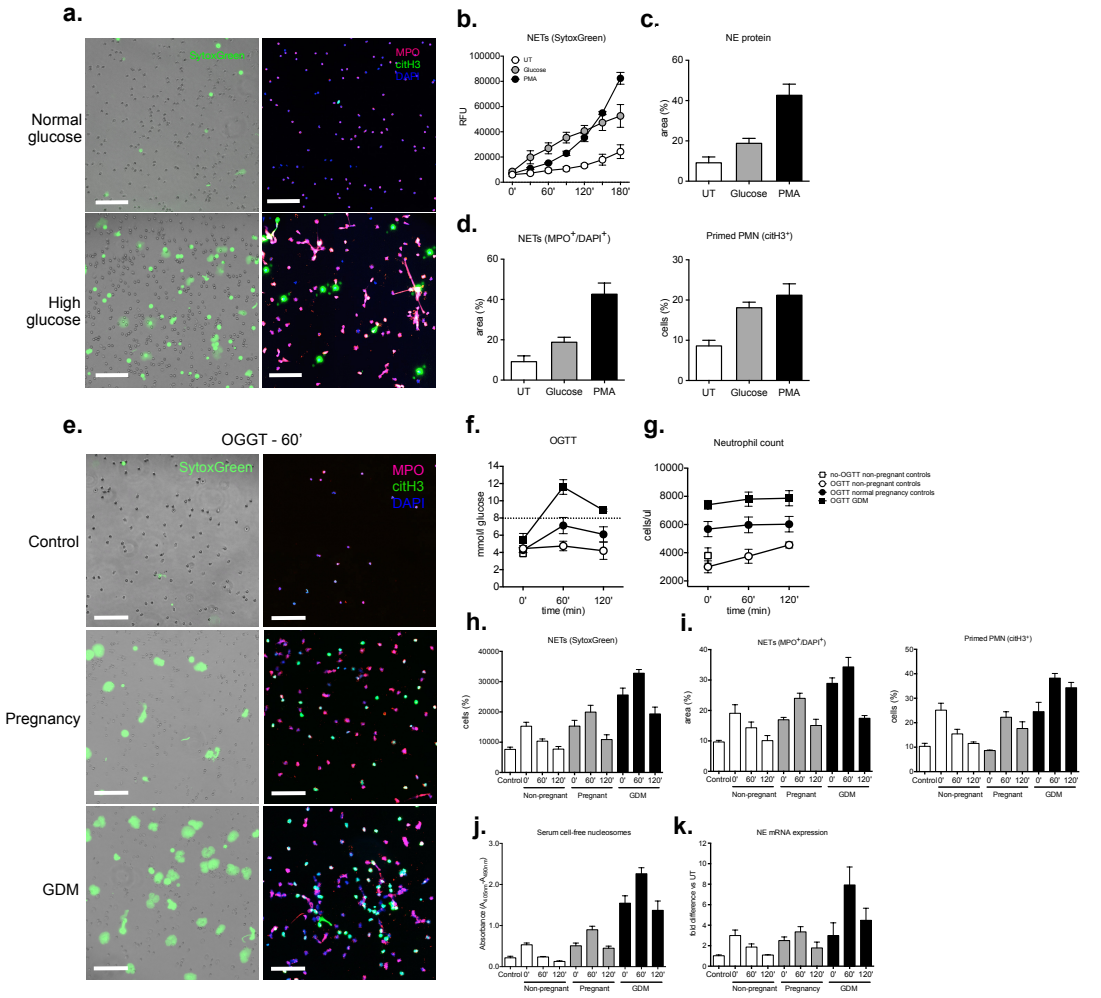


Figure 2. High glucose primes human neutrophils towards NET formation in GDM. (**a**) *In vitro* spontaneous extracellular DNA release monitored in a 3 hour time course by dsDNA-binding fluorescent SytoxGreen dye microscopy (left panel) and fluorescent immunocytochemistry for MPO, citH3 and DAPI counterstain (right panel) after treatment of control neutrophils with high concentrations of glucose. Magnification: 10x. Scale bars: 100 μm. (**b**) Three hour time course fluorimetric evaluation of SytoxGreen-bound extracellular DNA release from neutrophils treated high glucose concentrations. (**c**) NE protein expression morphometric evaluation of control neutrophils treated with high concentrations of glucose for 3 hours. (**d**) Morphometric analysis of the NETotic (MPO+/DAPI+, left panel) and pro-NETotic primed (citH3+, left panel) neutrophils from healthy control donors under high glucose treatment. (**e**) Evaluation of the effect of glucose during the diagnostic for GDM OGTT in neutrophils isolated from non-pregnant controls, healthy pregnant donors and GDM patients using fluorescent SytoxGreen staining DNA and microscopy (left panel) and immunofluorescence for MPO (red), citH3 (green) and DNA counterstain with DAPI (blue) after a 3 hour culture (right panel). Magnification: 10x. Scale bars: 100 μm. (**f**) Glucose levels monitored during the OGTT utilized for the differential diagnosis of GDM. (**g**) Neutrophil count in EDTA blood samples

obtained from the enrolled healthy controls, pregnant donors and GDM patients during the diagnostic OGTT. (**h**) Fluorimetric analysis of peripheral neutrophils isolated from healthy controls, donors during healthy pregnancy and patients suffering from GDM enrolled for the diagnostic OGTT. (**i**) Morphometric analysis of the NETotic MPO positive (left panel) and pro-NETotic primed citH3 positive neutrophils (right panel) of neutrophils from the aforementioned diagnostic setup. (**j**) Detection of cell-free nucleosome levels by ELISA in sera from healthy controls, pregnant donors and GDM patients during the diagnostic OGTT. (**k**) Detection of NE levels in the aforementioned sera by ELISA. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one or two way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 6 times with consistent results. GDM, gestational diabetes mellitus; OGTT, oral glucose tolerance test; RFU, relative fluorescence units.

TNFα is relevant for neutrophil activation and NET formation in GDM

Our data confirm previous reports that TNFα levels are elevated in cases with GDM (Fig. 3a). This is important in the context of our study as TNFα has been shown to promote neutrophil activation and pro-NETotic priming [226]. Therefore, TNFα expression due to a hyperglycaemic microenvironment in GDM may facilitate enhanced NET formation or degranulation.

Our data support such a hypothesis since treatment of plasma from pregnant women with GDM with the TNFα receptor antagonist infliximab reduced the degree of NETosis when compared to untreated plasma samples applied on neutrophils (Fig. 3b and Fig. 3c). We further determined that the combination of high glucose and TNFα lead to high degrees of neutrophil priming, as detected by histone H3 citrullination and increased propensity for subsequent NETosis (Fig. 3d to 3f).

Metabolic dysfunctions in GDM, such as hyperinsulinemia, hyperglycaemia, and dyslipidemia can induce functional abnormalities of the placenta and trophoblast cells in particular [237]. It has been also suggested that TNFα is a predictor of insulin resistance in human pregnancy [226] and that neutralization of TNFα signaling leads to sufficient improvements in insulin sensitivity [238]. Since circulating neutrophils exert a pro-NETotic primed state in GDM, we hypothesized that TNFα production by trophoblast cells could lead to a neutrophil infiltration and activation. To explore this hypothesis, we employed BeWo choriocarcinoma cells, commonly used as a model system

for placental syncytiotrophoblast cells. Treatment of BeWo cultures with high concentrations of glucose *in vitro*, lead to increased TNFa production as assessed by both qRT-PCR of cell lysates and EIA of protein levels in the supernatants (Fig. 3h). When normal healthy control neutrophils were treated with these BeWo culture supernatants, they exhibited an increased NETotic potential, which was inhibited by addition of infliximab (Fig. 3h to 3j). In addition, when BeWo cells were co-cultured with control neutrophils under high glucose conditions, increased numbers of NETs were observed, which once again was reduced by inclusion of infliximab (Fig. 4a and Fig. 4b).

Collectively, these data show that TNFα, which is produced by BeWo cells after exposure to hyperglycaemic conditions *in vitro*, stimulates NET formation. It is hence plausible that such a mechanism may be operative *in vivo* in GDM.

Figure 3.

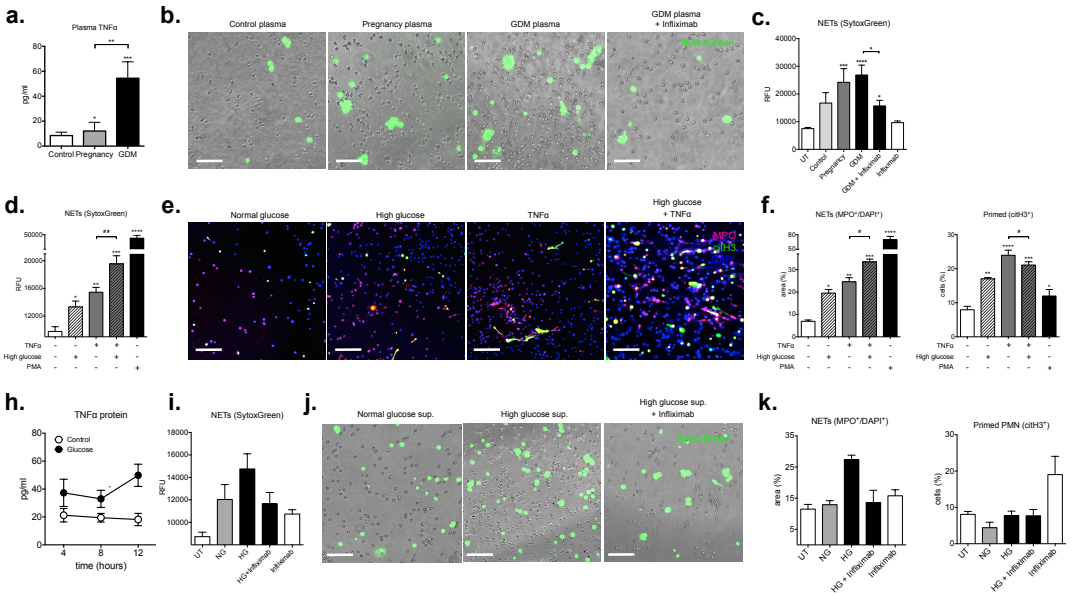


Figure 3. TNFα determines neutrophil activation and NET formation in GDM pregnancy. (**a**) Determination of TNFα levels in plasma samples from healthy non-pregnant donors, women during their healthy pregnancies and pregnant women diagnosed with GDM. (**b**) NET formation assessed microscopically in a 3 hour time course with SytoxGreen DNA binding dye after *in vitro* incubation of control neutrophils with control plasma, pregnancy plasma and GDM plasma with and without pretreatment with the TNFα receptor antagonistic antibody infliximab. Magnification: 10x. Scale bars: 100 µm. (**c**) Fluorimetric evaluation of extracellular

DNA release in control neutrophils treated with the aforementioned series of plasma in the presence of anti-TNFR neutralizing antibody. (**d**) Fluorimetry of peripheral neutrophils isolated from healthy non-pregnant controls treated with high glucose concentrations, TNFα and both agents for a 3 hour time course, compared to treatment with PMA. (**e,f**) Fluorescent immunostainings for MPO (red), citH3 (green) and DNA (blue) (**e**) and morphometric analysis (**f**) of the NETotic MPO positive and pro-NETotic primed citH3 positive neutrophils from healthy non-pregnant donors under the aforementioned experimental setup. Magnification: 20x; Scale bars: 50 µm. (**h**) ELISA determination of TNFα levels in the supernatants from BeWo cells treated with high concentration of glucose. (**i**) Fluorimetric quantification of extracellular DNA release of control neutrophils cultured with supernatants from BeWo cells treated with high glucose concentration with and without the addition of infliximab. (**j,k**) Fluorescent immunostainings for MPO (red), citH3 (green) and DNA (blue) (**j**) and morphometric analysis (**k**) of the NETotic MPO+ and pro-NETotic primed citH3 positive neutrophils from healthy non-pregnant donors under the aforementioned experimental setup. Magnification: 20x; Scale bars: 50 µm. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 6 times with consistent results. GDM, gestational diabetes mellitus; RFU, relative fluorescence units; PMA, phorbol myristate acetate; NG, normal glucose; HG, high glucose; sup., supernatant.

Figure 4.

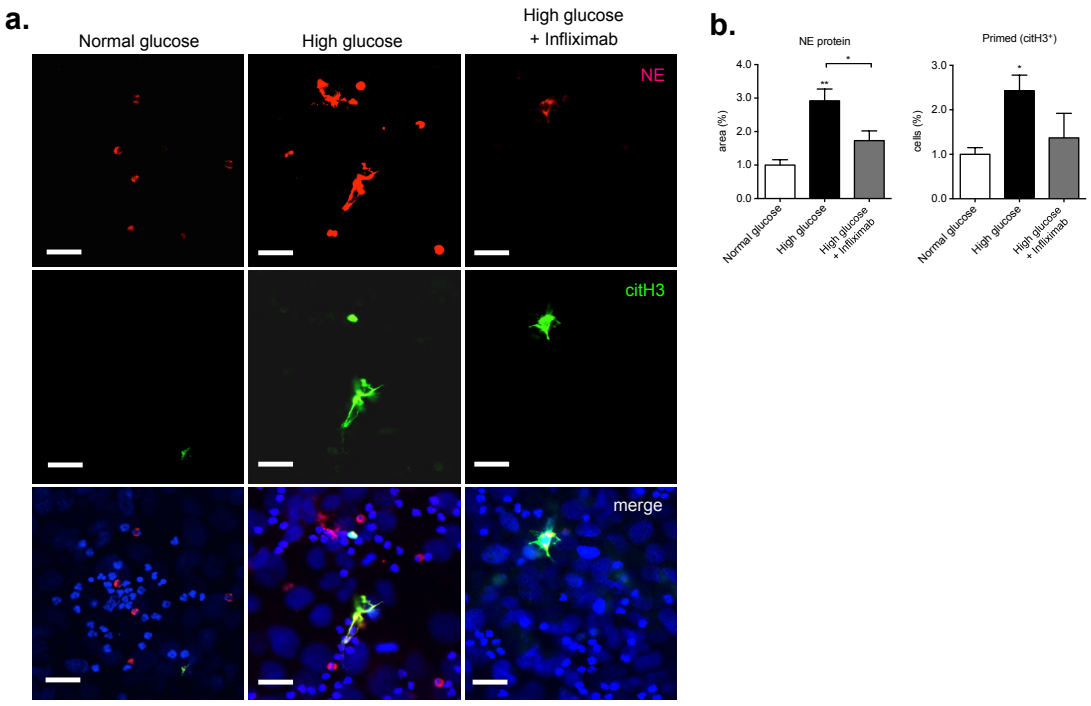


Figure 4. TNFα originating from BeWo cells induces neutrophil activation which leads to excessive NET formation and NE release. (**a**) NET formation in a 3 hour timecourse assessed microscopically by immunostaining for NE (red) and citH3 (green), counterstained with DAPI. Magnification: 20x; Scale bars: 50 µm. (**b**)

Morphometric analysis of the NETotic (MPO+) and proNETotic primed (citH3+) neutrophils from healthy control donors cultured under high concentrations of glucose, with or without pretreatment with the TNFα receptor antagonising antibody infliximab. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one or two way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least 3 times with consistent results.
The experiments were performed by Dr. Franco Grimolizzi.

Placental infiltration of primed neutrophils leads to excessive NE release and IRS1 degradation

Since BeWo cells under hyperglycemic conditions express TNFα, which promotes neutrophil infiltration, activation and NETosis, we investigated whether these findings are valid *in vivo* in placentae from pregnant women diagnosed with GDM. Indeed, an intense inflammatory cell infiltration was observed in GDM placentas, as evaluated by H&E stainings (Fig. 5a), which was predominantly of neutrophilic content, as confirmed by MPO immunostaining (Fig. 5b). Strikingly, numerous of these cells are in a primed citH3+ state, while a proportion of these infiltrating neutrophils appear to be undergoing NETosis (Fig. 5c). Neutrophil activity is, however, not restricted to NETosis, since placental lysates from GDM patients contained increased levels of NE mRNA and protein levels when compared to normal pregnancies (Fig. 5d).

Recent reports have described a significant neutrophil infiltration of adipose tissue of diabetic patients, accompanied by elevated release of neutrophil elastase (NE) and activity [233]. It was also observed that inhibition of NE led to a remarkable improvement of glucose tolerance and insulin sensitivity *in vivo*. The mechanism leading to altered glucose metabolism was shown to involve the degradation of insulin receptor substrate 1 (IRS1) in hepatocytes by exogenous NE, thereby inhibiting insulin-driven signal transduction, akin to a mechanism previously described in lung cancer cells [239].

In order to investigate if such a mechanism is operative in the case of GDM, placental sections were examined by fluorescent immunohistochemistry for NE and IRS1. This revealed more pronounced NE positive areas in cases with GDM when compared to controls (Fig. 5d).

Interestingly, staining for IRS1 was reduced in GDM placentae compared to healthy pregnancy controls (Fig. 5e). This reduction in IRS1 protein levels was confirmed by immunoblot analysis of placental lysates from the same GDM patients.

Treatment of BeWo cells with recombinant NE lead to a 10 fold reduction of IRS1 protein levels and a 2 fold reduction in the glucose transporter type 4 (GLUT4) protein levels (Fig. 5g). These changes indeed affected glucose metabolism by BeWo cells, as assessed by the uptake of tritiated glucose, which indicated this was significantly reduced in BeWo cells subjected to exogenous recombinant NE (Fig. 5h). This effect was largely neutralized by addition of insulin (Fig. 5g and Fig. 5h).

These data underline the NE, and possibly NETs, in dysregulating trophoblast glucose uptake and tolerance.

Figure 5.

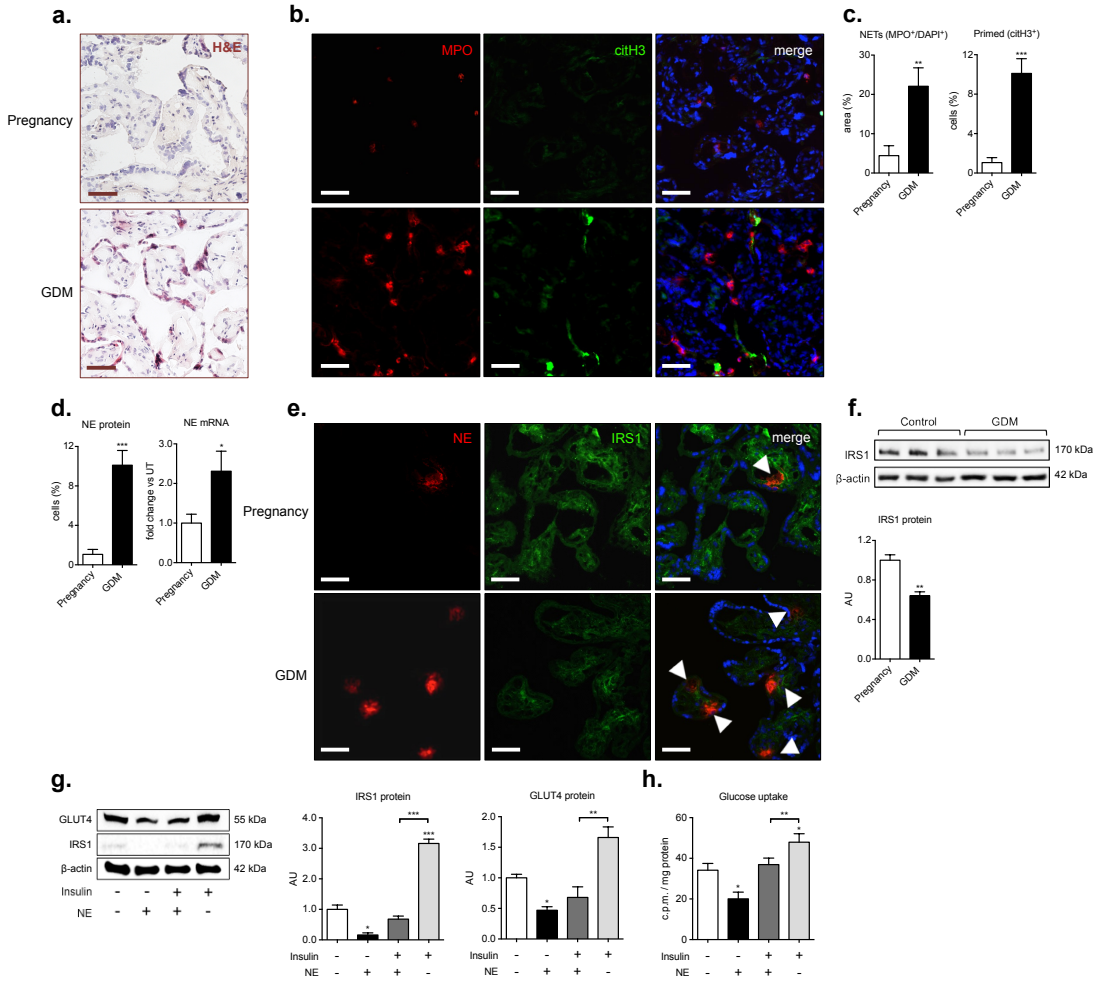


Figure 5. Primed neutrophils infiltrate in the GDM placenta leading to excessive NE release and IRS1 degradation. (**a**) H&E staining of tissue sections from term placentas of normal pregnancy and a patient diagnosed with GDM. Magnification: 20x; Scale bars: 50 µm. (**b,c**) Fluorescent immunostaining for MPO (red), citH3 (green) and DNA counterstain (blue) (**b**) and morphometric analysis of the NETotic MPO positive and pro-NETotic primed citH3 positive neutrophils (**c**) in tissue sections from term placentas of normal pregnancy and a patient diagnosed with GDM. Magnification: 20x; Scale bars: 50 µm. (**d**) NE protein (left panel) and mRNA expression levels (right panel) determined by morphometric analysis and qPCR, respectively in placental tissue lysates from healthy placentas and GDM patients. (**e**) Fluorescent immunostaining and confocal microscopy of tissue sections from term placentas of normal pregnancy and a patient diagnosed with GDM. Magnification: 20x; Scale bars: 50 µm. (**f**) Western blot analysis (upper panel) and densitometry for IRS1 protein expression (lower panel) in placental tissue lysates from healthy placentas and GDM patients. (**g**) Immunoblot analysis for IRS1 and GLUT4 protein expression in BeWo trophoblast cell lysates after treatment with NE, with insulin and both agents (left panel) and densitomentry for IRS1 (middle panel) and GLUT 4 protein expression (right panel). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one way ANOVA followed by Bonferroni's

multiple comparison post-test). All experiments were performed at least 3 times with consistent results. GDM, gestational diabetes mellitus; AU, arbitrary units.
Dr. Franco Grimolizzi performed these experiments.

Discussion

In GDM the metabolic and circulatory adaptations to pregnancy are impaired, leading to hypertension, poor placental blood perfusion and failure to respond properly to crucially relevant hormones such as insulin. The fetal, neonatal and maternal adverse outcomes associated with GDM result from the altered metabolic milieu, which are projected on the fetus via the placental interface. These effects have immediate consequences for the health of both the mother and baby, but may also affect their future health.

Here we show that GDM is characterized by peripheral blood neutrophils spontaneously undergoing NETosis. This process is directly related to the high glucose levels present in the blood of the mother, which primes neutrophils towards NET formation.

The inflammatory milieu resulting from high glucose levels, similar to T1D and T2D, additionally stimulate the production and release of TNFα, that in turn increases spontaneous NETosis of circulating neutrophils thus regulating the release of NE. In the placenta, infiltration of primed overreactive neutrophils leads to forced NE release, which induces the degradation of IRS1, possibly resulting into organ specific metabolic changes, contributing to the placental pathology of GDM.

Several studies during the last years attempted unsuccessfully to correlate the incidence of GDM to the ratio of neutrophils to lymphocytes and other blood cells. Herein we observe a marked increase of the neutrophil total number in GDM pregnancy compared to healthy gestation that is already higher than in non-pregnant healthy women. Neutrophils from pregnant women with GDM massively increased their tendency for spontaneous release of NETs, when compared to healthy pregnancy. In serum and plasma the amount of free nucleosomes is extremely high, which cannot be justified solely by the increased number of circulatory neutrophils. Since freshly

isolated neutrophils from patients diagnosed with GDM showed increased expression of MPO and citH3, overt NETosis results from this enhanced primed phenotype.

It was previously shown that hyperglycaemia increases NETs formation *in vitro* by priming neutrophils and constitutively activates NETosis, leading to reduced responses to subsequent external stimuli [234]. In our case, addition of a secondary stimulus like PMA exacerbated NET formation. Kummer et al., recently described utilizing an *in vitro* model that elevated glucose concentrations in the range of glucose levels found in the peripheral blood of uncontrolled diabetics, were able to activate neutrophils altering their metabolic oscillation response [240]. To mimic the initial exposure of neutrophils to high glucose levels like in the insurgence of GDM we directly stimulated neutrophils from healthy non-pregnant donors with glucose and we confirmed the stimulatory properties of glucose directing NETs formation. Since neutrophils during pregnancy exhibit already a primed NETotic status, we took advantage of the diagnostic oral tolerance test for GDM to study the direct exposure of neutrophils to high glucose *in vivo*. For diagnostic purposes, pregnant women ingest 75 g (Check) glucose after 8 to 12 hours of fasting and their ability to assimilate blood glucose levels during a 2 hours period is measured. Also in this case we observed neutrophils primed towards formation of NETs, presenting elevated citrullination of histone 3 and MPO levels. Interestingly, priming of neutrophils is not correlated with a parallel increase neutrophil numbers in this short time, excluding a possible direct effect of high levels of glucose on the mobilization of neutrophils from the bone marrow during pregnancy. We concluded that glucose is a direct activator of neutrophils towards NETosis. This hypothesis was also confirmed by Rodriquez-Espinosa et al., which studied the metabolic requirement to produce NET. They were able to show that NETs formation is dependent on external glucose and to a lesser extent on glutamine [241].

The next issue arising concerning the role of glucose on proNETotic priming neutrophils and active NET formation implies an additional cytokine mediated effect. Since it was shown that TNFα levels in GDM are elevated compared to normal pregnancy [242] we studied the role of TNFα in presence of high glucose and the TNFα inhibitor Infliximab. First, we confirmed the

elevated level of TNFα in the plasma of pregnant women with GDM. Moreover when we blocked TNFα in GDM derived plasma, NET formation was also inhibited, although glucose levels in the plasma were still high. This suggests that glucose induces TNFα and together they synergize towards neutrophils proNETotic priming leading to excessive NET formation. Interestingly we could measure increased TNFα release upon stimulation of BeWo cells with high glucose concentrations, BeWo being an endocrine active cell line of trophoblastic origin used as an *in vitro* model for placental function. Co-culture of BeWo with control untreated neutrophils from non-pregnant female donors resulted in increased priming and NET formation. This effect could be reversed by addition of infliximab, stating the importance of TNFα in this process.

Increased NET release correlated with the augmented local exposure of the underlining tissue to neutrophil-derived serine proteases, such as NE. In addition to host defence, NE has also been implicated in sterile inflammation [2], while NE directly induces tumor cell proliferation in both human and mouse lung adenocarcinomas by directly degrading insulin receptor substrate-1 (IRS-1) [239]. Furthermore, treatment of hepatocytes or adipocytes with NE resulted in insulin resistance, and, in the context of diabetes, NE knock out mice were protected from inflammation of liver and adipose tissue [233]. Indeed, we first observed excessive inflammatory cell infiltration directly in biopsies from GDM placentae. This infiltration was primarily comprised by primed PMN. Strikingly, we also observed excessive local abundance of NETs, which is accompanied by extensive release of NE. Moreover, immunohistological staining and immunoblot revealed a simultaneous decrease of IRS-1 expression in placentae from GDM patients. To confirm the mechanistic aspects of this observation, BeWo cells were treated with recombinant elastase and insulin. Elastase treatment leads to downregulation of IRS-1 expression *in vitro*. Glucose intolerance was also evident, as shown by the downregulation of GLUT4 glucose receptor expression and the capacity of glucose uptake in BeWo cells, while insulin could partially reverse these effects. These data suggest a possible role of NE and IRS1 to the aetiology of inflammation-induced insulin resistance and the altered placental physiology during GDM.

Collectively, we propose that neutrophils participate directly to the pathobiology of GDM. Our data show that the TNFα driven inflammation, resulting from high glucose levels in the circulation, augments neutrophil priming and NET formation. The released NE can locally modify glucose tolerance and metabolism in the placenta, which probably results to the anatomical, physiological and functional changes observed in placenta of pregnant women suffering from GDM (Fig. 6). Our findings underline the competence of NETs as a highly relevant diagnostic biomarker for GDM, and NE and TNFα as potential prophylactic and therapeutic targets in GDM.

Figure 6.

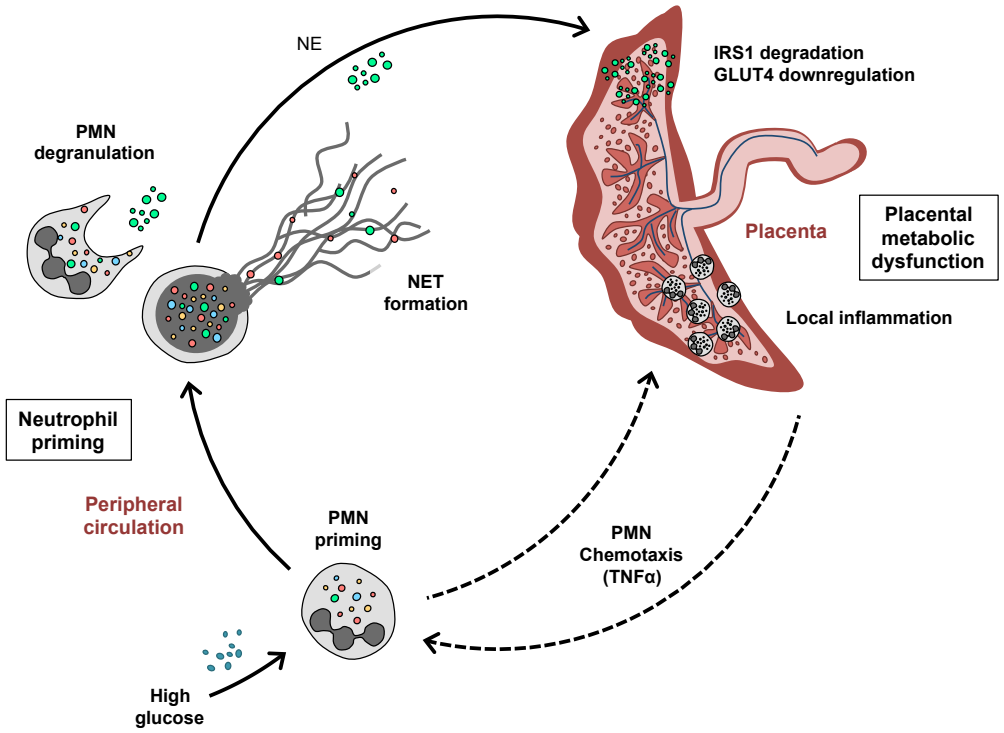


Figure 6. Neutrophils are involved in the pathogenesis of gestational diabetes mellitus via TNFα mediated NE release and excessive IRS1 degradation. Circulatory neutrophils during GDM pregnancy lie under the increased influence of glucose. This appears to poise the neutrophils in a stable pro-NETotic primed state. Depending on the stimulus, for instance TNFα, primed neutrophils transmigrate to the placenta and react with overt degranulation and extrusion of NETs. This excessive NE release modulates the tolerance of the placental trophoblast to glucose via controlling the IRS1-dependent glucose uptake. Dysregulation of these pathways may cause serious pathogenetic consequences as the ones appreciated in the GDM placenta.

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Highly motivated, well organized, creative and flexible. Ready to take responsibilities and face new challenges.

// Educational Qualifications

2013 - 2016	**PhD Candidate in Biomedicine** Focal Area Immunology, Laboratory for Prenatal Medicine, Department of Biomedicine, University Hospital of Basel
2008 - 2010	**MSc in Pharmacy** Specialization in Biotechnology - Molecular Diagnostics School of Pharmacy, Aristotle University of Thessaloniki
2002 - 2007	**BSc in Molecular Biology and Genetics** Health Sciences School, Democritus University of Thrace, Greece

// Professional Experience

10.2013- 09.2016	**PhD Candidate** Laboratory for Prenatal Medicine, Focal Area Immunology, Department of Biomedicine, University Hospital of Basel, Switzerland
	Responsible for three projects: • Identification of the factors that modulate neutrophil response towards formation of NETs during normal pregnancy. • Study of the mechanisms of NET formation in gestational diabetes mellitus. • Pharmacologic modulation of neutrophil extracellular trap formation. Outlining and planning research project objectives and scheduling activities, analysis and interpretation of results, and identifying consequent resource needs Coordinating, writing and submitting two research applications to the Swiss Ethics Committee: Ethics Committee northwest/central Switzerland EKNZ. Laboratory technologies: • Leukocyte isolation from whole blood • RNA isolation, Real-Time qRT-PCR • Protein isolation, Western Blotting • Flow Cytometry • Immunohistochemistry, Live cell fluorescence assays • Fluorescence and Confocal microscopy • ELISAs
06.2012- 09.2013	**Product Development Manager** Marie & Menel Cosmetics, Greece
	• Product development of skin care products • Quality control of product development

	• Procurement management of laboratory material
12.2011-06.2012	**Laboratory assistant** Laboratory of Embryology, Department of Assisted Reproduction, University Hospital of Alexandroupolis, Greece
	• Maintaining and supporting in-vitro fertilization procedures • Collecting and handling biological samples
01.2008-10.2010	**Research practice for the purposes of the MSc Thesis** Aristotle University of Thessaloniki, School of Pharmacy, Molecular Biology Laboratory, Greece
	MSc Thesis: Physical interactions between ICP0, a viral transactivator, and architectural proteins of the HMGA family. • Cloning of PCR products into expression vectors • Bacterial transformation and protein expression in bacteria • Purification of His-tagged and GST-tagged recombinant proteins • Protein interaction studies, pull-down assays, • Cell Line culture, Transfection, Immunoprecipitation
05.2005-05.2006	**Research practice for the purposes of the diploma Thesis** Molecular and Cellular Biology Laboratory**,** Bone Marrow Transplantation Unit of the *Hematology Department,* "G. *Papanikolaou" General Hospital*, Greece
	BSc Thesis: Molecular diagnosis of Epstein-Barr virus in patients with hematologic malignancies. • DNA purification from leukocytes • Real-Time PCR
09.2002-09.2013	**Biology Tutor** Self-employed, Greece
	Over 10 years of experience in teaching Biology to high school (Lyceum) students and training preparation for their University entrance examinations

// Professional Training Courses

02.2016-11.2016	**Antelope@Novartis,** Mentoring program for female PhD students, University of Basel and Novartis
2015	**Leadership Management,** (15h)**,** University of Basel
2015	**Presentation Training,** 1 ECTS (25h), University of Basel
2014	**Scientific Writing,** 3 ECTS (75h), University of Basel
2015	**Intellectual property rights in life sciences,** 2 ECTS (50h) Law Faculty, University of Basel

// Languages

English: Business fluent
German: Good knowledge (B1), currently taking intensive courses
Greek: Mother language

// Computer literacy

Office applications, Endnote, Adobe, statistic software Prism, FlowJo flow cytometry analysis software, sequence alignment tools (DNASIS, BLAST).

// Publications

- *Neutrophil migration into the placenta: Good, bad or deadly?* Giaglis Stavros, **Stoikou Maria**, Grimolizzi Franco, Subramanian Bibin Y., van Breda Shane V., Than Nándor Gábor, Hahn Sinuhe. Cell Adh Migr. 2016 Mar 3;10(1-2):208-25. doi: 10.1080/19336918.2016.1148866. Epub 2016 Mar 2
- Accepted: *Multimodal regulation of NET formation in pregnancy: progesterone antagonizes the pro-NETotic effect of estrogen and G-CSF.* Stavros Giaglis, **Maria Stoikou**, Chanchal Sur Chowdhury, Franco Grimmolizzi, Guenther Schaefer, Irene Hoesli, Olav Lapaire, Simona Rossi, Paul Hasler, Sinuhe Hahn. Frontiers in Immunology
- In preparation: *Neutrophils are involved in the pathogenesis of Gestational Diabetes Mellitus via TNFα mediated NE release and excessive IRS1 degradation.* **Maria Stoikou***, Franco Grimolizzi*, Stavros Giaglis, Sinuhe Hahn, Paul Hasler, Simona Rossi, Olav Lapaire, Irene Hösli, Evi Huhn, Marc Donath. Nature Communications