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PREECLAMPSIA (V GAROVIC, SECTION EDITOR)

The Role of Neutrophils and Their Extracellular Traps in the Synergy of Pre-eclampsia and HIV Infection

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Abstract

Purpose of the Review In our innate immune system, neutrophils are the first cells to sense signals of infection and to proceed to kill the invading pathogen. This is mediated by their production of neutrophil extracellular traps (NETS) to entrap pathogenic micro-organisms, preventing their amplification and dissemination. Pre-eclampsia (PE) is the leading cause of global maternal mortality, yet to date, there is no cure nor a gold-standard diagnostic strategy. The purpose of this review is to discover the role of neutrophils in PE as early identification markers. Additionally, this review aims to explore the role of neutrophils in HIV-infected pregnancies with PE as a source of synergy.

Recent Findings Recent findings demonstrate an elevation of neutrophils and neutrophil extracellular traps (NETs) in PE placentae. This is due to their activation by excessive release of syncytiotrophoblast microparticles (STBM). There is also an elevation of NETs in HIV-infected placentae—where histone H3 entraps HIV by binding to its glycoprotein envelope. Additionally, histones H1 and H2A inhibit HIV infection. It is interesting to note that women with both PE and HIV infection have supressed NETs.

Summary This review focuses on the role of neutrophils in the synergy of PE and HIV infection. It is plausible that the deregulation of NETs in the synergy of pre-eclamptic HIV-infected women is strategic for the entrapment of the HIV-1 virus. Finally, it is plausible that neutrophils and NETS may act as early biomarkers of PE development.

Keywords Neutrophils · Neutrophil extracellular traps · Pre-eclampsia · Human immunodeficiency virus

Introduction

Neutrophils are derived from the myeloid lineage of haematopoietic stem cells in the bone marrow [1]. They have a spherical neutrally stained nucleus with three to five lobes which increase in number with maturity. Nucleosomes are located within the nucleus, containing DNA molecules wrapped around histones. The core histone components are: histones H2A, H2B, H3 and H4 [2, 3] (Fig. 1). Neutrophils are the first cells recruited to the infected inflammatory site upon activation by pro-inflammatory chemotactic signals [4]. Upon activation, they express CD69 and exert a potent bactericidal effect [5]. Neutrophils form an integral component of granulocytes and contribute to the bacterial phagocytic arm of the innate immune system [5]. Their short half-life of just 6 to 8 h in the blood circulation is dependent on the macrophage-mediated clearance of activated neutrophils from the site of infection and is designed to prevent physiological

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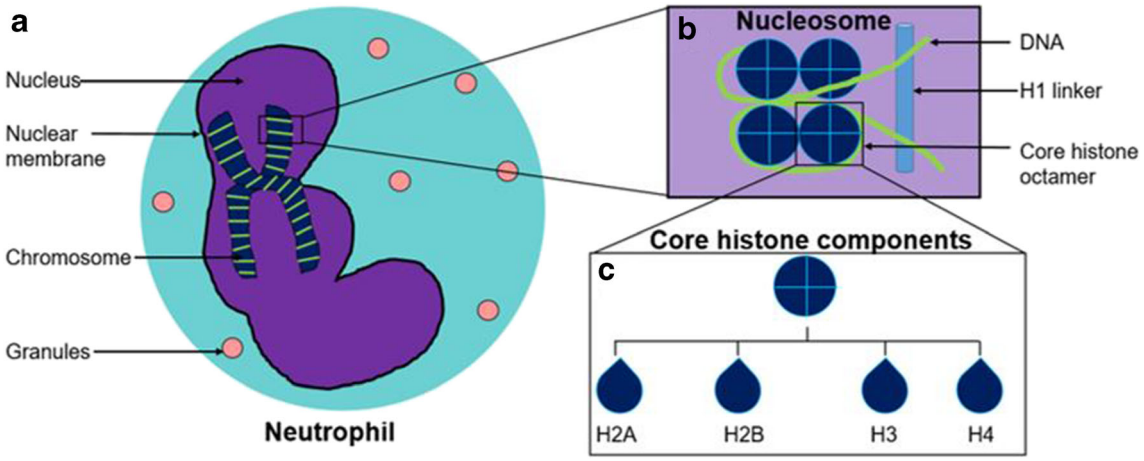

Fig. 1 Neutrophil structure. **A** Neutrophils are granular leukocytes. A nuclear membrane surrounds the tri-lobed nucleus of neutrophils which contains chromosomes. **B** Chromosomes are composed of nucleosomes linked by linker histones (e.g. H1) and formed when deoxyribonucleic acid (DNA) coils around core histone octamers **C** which has two molecules of each of the core histones (H2A, H2B, H3 and H4)

tissue damage [2, 6]. However, in vivo labelling shows a human neutrophil lifespan to be 5.4 days [7]. This lifespan is > 16-fold longer than originally perceived, making these 'short-lived' cells into long lasting potential targets.

Neutrophil Migration: Forward and Reverse

During stimulation by pro-inflammatory stimuli, the primary goal of the initial arms of the innate immune system is to activate vascular neutrophils by chemotactic signals such as C5a of the complement system [8] or tumour necrosis factor (TNF-alpha) from resident tissue macrophages [9]. Capillary endothelium is simultaneously activated by pro-inflammatory cytokine signalling. This dual activation of neutrophils and endothelial cells facilitate the subsequent steps in neutrophil forward trans-epithelial migration [10] (Fig. 2).

Activation of neutrophils results in a phenotypic switch where pseudopodia facilitate movement. Activation also triggers the expression of specialized receptors on endothelial cells, as well as their complementary glycoprotein ligands. This is designed to traffic neutrophils to adhere to the endothelium by ligand-receptor binding. Thereafter, diapedesis occurs whereby neutrophils will squeeze through a pore formed by the slight opening of tight junctions between the endothelium into the infected tissue [5, 11] (Fig. 2).

Recently, reverse transmigration of neutrophils from the tissue back into the circulation was observed thereby enhancing the function of this cell [12, 13]. Activated neutrophils are transcriptionally and translationally active with the expression

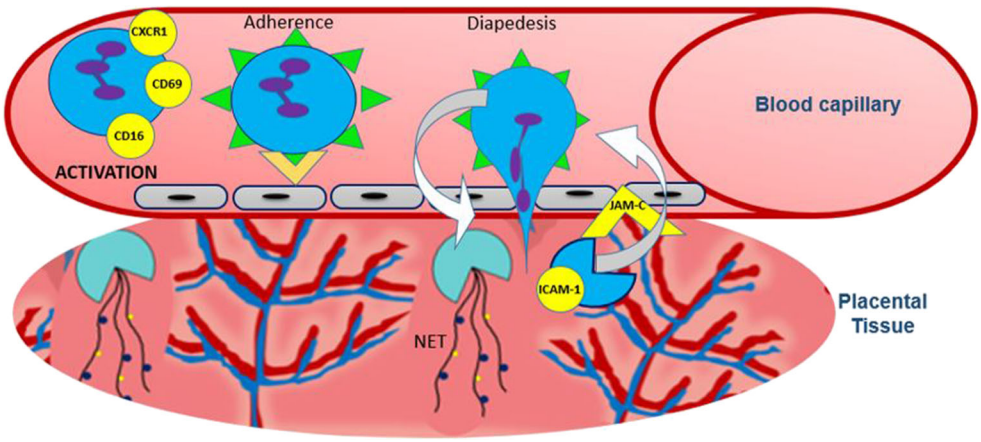

Fig. 2 Neutrophil markers and transmigration. Mature neutrophils circulate in the blood stream and express the CD16 identification marker and CXCR1. Once activated, neutrophils then express CD69 and are programmed to undergo forward trans-migration from the capillary into the placental tissue. During forward migration, neutrophils first adhere to the endothelial cells of the capillary and undergo diapedesis to enter the placenta: where they form NETs upon contact with pro-inflammatory stimuli. Additionally, activated placental NETs may undergo reverse trans-migration back into the maternal circulation. These neutrophils display ICAM-1 and the endothelial cells display JAM-C to regulate reverse-migration

of specific markers: ICAM1^{hi} and CXCR1^{low104,110} [12, 14]. Their reverse migration ability is believed to be driven by the release of CXCL8 (IL-8) from tissue macrophages [13] and regulated by JAM-C on endothelial cells [15]. Neutrophils are also less susceptible to apoptosis and following reverse migration produce reactive oxygen species (ROS) [12].

Neutrophil Extracellular Traps

Neutrophil extracellular traps (NETS), as their name suggests, trap pathogenic micro-organisms thereby preventing their amplification and dissemination [16]. Structurally, NETs consist of nuclear material that are expelled extracellularly in a chromatin mesh-like network in which histones and granular enzymes such as elastase are embedded (Fig. 3). Furthermore, NETs are made up of smooth fibres, 15–17 nm in diameter with globular domains of 25–28 nm in diameter [16].

Phorbol myristate acetate (PMA) is an inducer of NET formation, a process which is mediated by ROS [16] and mitogen-activated protein kinase (MAPK). Phorbol myristate acetate stimulates the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase by MAPK promoting the generation of ROS [17–19] (Fig. 3A). Reactive oxygen species cause neutrophil membrane damage including azurophilic granules thereby enabling the release of elastase which then translocate to the nucleus. Elastase functions in histone cleavage to release chromatin into the cytosol, this mixes with neutrophil granules such as myeloperoxidase and histones (Fig. 3B) and are then expelled extracellularly to form NETs (Fig. 3C).

A major source of extracellular histones are NETs that occur as a controlled form of necrosis called NETosis. In the presence of a pathogen, mature neutrophils receive pro-inflammatory activation signals from IL-8 and tumour necrosis factor alpha (TNF-α) to undergo NETosis [16]. During this process, the cell membrane of neutrophils rupture [20] and their nuclear chromatin, along with their granular enzymes and histones are released in a net-like structure to ensnare the pathogen [16]. The NETs then kill the pathogen via their pre-released granular content and histones [16, 21].

The Physiological Placenta

The placenta is a dynamic foetal-maternal interface which requires a fully functioning perfusion system to optimally perform its physiological function [22]. This unique organ functions as a bi-directional exchange unit for oxygen, carbon dioxide, nutrients, waste products and immune cells between the mother and the foetus [23, 24]. Unique to the human discoidal haemochorial placenta is the ingenious multi-villous flow system [25]. This is structured with the interdigitating of maternal and foetal villous structures arising from the basal and chorionic plate, respectively [25]. Optimization of maternal blood flow is achieved through trophoblast-mediated remodelling of spiral arteries into dilated sinusoidal conduits [26]. These spiral arteries then transport blood containing oxygen, cytokines and immune cells to the inter-villous space, where it enters and interacts with chorionic villi.

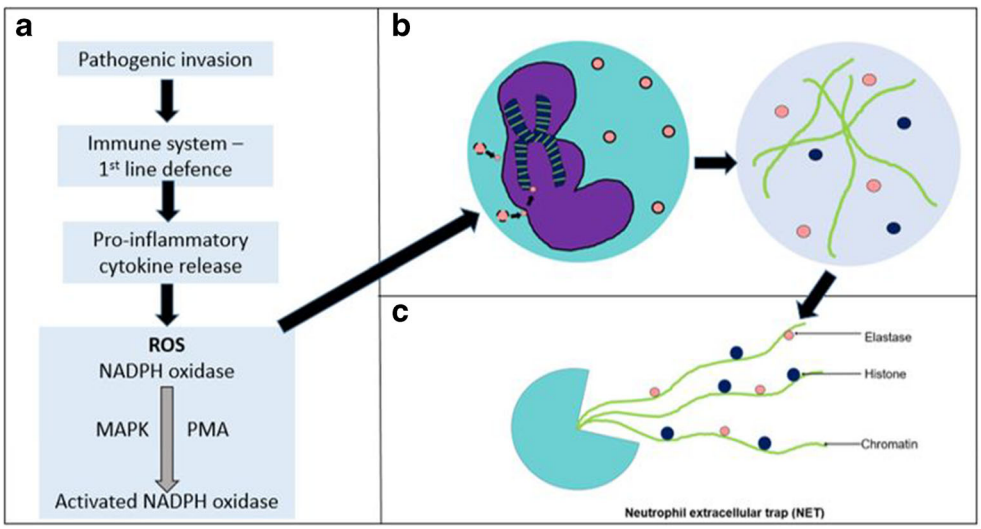

Fig. 3 The production of neutrophil extracellular traps (NETs). **A** A pathogenic invasion stimulates the first-line defence of the immune system to release pro-inflammatory cytokines which facilitates reactive oxygen species (ROS) production. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is activated by phorbol myristate acetate (PMA) and facilitated by mitogen-activated protein kinase (MAPK). **B** Elastase is released from azurophilic granules and trans-locates to the nucleus where it cleaves histones. The nuclear and cellular membranes disintegrate resulting in the release of widespread genetic material such as histones and DNA into the cytoplasm. **C** The intracellular and nuclear contents are then released to form a neutrophil extracellular trap (NET), with a web-like chromatin mesh embedded with histones and elastase

Chorionic villi encompass the foetal villous tree and consists of a centrally located conducting villous which forms intermediate branches that terminate in exchange villi [23]. Cytotrophoblasts (CTB) are villous trophoblasts which proliferate, differentiate and fuse to form a continuous multinucleated layer of syncytiotrophoblasts (STB) [27, 28]. Sixty billion STBs line the outermost covering of the chorionic villi at term with a surface area of 12 m^2 [29]. These STB are the site of protein synthesis and function as core transport units of maternal and foetal blood [30]. However, placental pathologies leading to or arising from abnormal placentation may impair STB function and subsequently result in complications to the mother and foetus [30, 31].

Immune Reactions in Pregnancy

Successful pregnancies thrive on a T helper 2 polarized immune response [32]. Gestation is a complex phenomenon for the maternal immune system that adapts to accept the semi-allogenic foetus whilst still protecting the mother against infections. However, an immune imbalance may lead to pregnancy-specific complications such as pre-eclampsia (PE).

The decidua hosts a rich maternal niche of decidual immune cells (DICs) that consists of both innate and adaptive immune cells [33]. Decidual immune cells are the front-line defence force and lie in close proximity to the inter-villous space to ensure rapid feto-maternal exchange [34]. The mechanisms by which the invading foetus escapes maternal immune rejection is a fascinating concept. The ultimate goal of the foetus is to breach the 'ready-to-fight resident DICs' and suppress the maternal immune system. This is achieved by the mass recruitment of immunosuppressive regulatory T lymphocytes (T-regs) and the polarization to a Th2 response to prevent the overt activation of the adaptive immune system. However, innate immune surveillance by a mild neutrophilia in normal pregnancies ensures a quick-acting first respondent to the inflammatory scene [35]. With increasing gestational age, there is a further increase in neutrophil and white blood cell count with a concurrent decline in the numbers of lymphocytes, suggesting that the acute innate immune system is a vital front-runner in maintaining immune tolerance [36]. Granulocytic myeloid–derived suppressor cells (MDSC), namely NK cells are noted as promising targets for pregnancy complications [37] due to their ability to suppress T cell proliferation, thus relieving the chronic inflammatory state to some extent. Recently, CD15+ neutrophils have been shown to elicit the effects of MDSC [38].

First-trimester Immune Cells

In addition to trophoblast dysregulation during placentation [39], immune cells also play a pivotal role during the first trimester. In the first trimester, natural killer cells form 70% of DICs and function to regulate angiogenesis, invasion of trophoblasts and spiral artery remodelling [40]. Other resident DICs are predominantly macrophages, dendritic cells, B cells and Tc cells which target 'foreign' threats [40]. These immune cells also have angiogenic properties and are believed to influence the initial stages of spiral artery transformation [41–43].

Second Trimester Neutrophil-placental Interactions

In addition to the role of immune cells in spiral artery transformation in the first trimester, Amsalem et al. identified a novel mature pro-angiogenic immunosuppressive neutrophil population (N2 phenotype) within the decidua during the second trimester of pregnancy [44]. During gestational weeks 6–20, neutrophils migrate from the maternal venous circulation into the decidua. During this period, the vascular adhesion molecule CD66 on neutrophils is upregulated to facilitate this trans-epithelial migratory process and it also aids in neutrophil activation. Placental interleukin 8 (IL-8), also called CXCL8, stimulates neutrophil activation [44]. Furthermore, these decidual neutrophils are increased in number during 11–15 weeks gestation and correlate with the opening of the inter-villous space to accommodate the maternal blood flow [44]. Classical neutrophils (N1 phenotype) migrate to decidual areas of inflammation [45]. It is only in trimester three that neutrophils take on the activated pro-inflammatory phenotype [46] Neutrophils also mediate immune tolerance in the adaptive immune system during pregnancy. When exposed to the pregnancy hormones: progesterone and estriol, neutrophils induce a subpopulation of T cells to produce cytokines which favour vessel growth [47].

Pre-eclampsia and HIV infection

Pre-eclampsia, a complex human pregnancy disorder which complicates approximately 10% of all pregnancies, is the leading direct cause of maternal mortality in South Africa (SA) [48]. However, women with the duality of both PE and human immunodeficiency virus (HIV) infection have a lower maternal mortality ratio compared to women individually exposed to each disorder, indicating a synergy response between these opposing inflammatory conditions [49]. Pinpointing the synergy source is vital in exploiting attempts to attain the Sustainable Development Goals (SDGs) of saving mothers and babies where: 'SDG 3.1 aims to reduce the global MMR to < 70 per 100,000 live births by 2030' and 'SDG 3.2 seeking to end preventable deaths of new-borns and children under five' [50].

The Role of Neutrophils in Pre-eclampsia

Given the early interaction between trophoblasts and neutrophils in pregnancy, it is plausible to explore the role of neutrophils further in PE pathogenesis. Pre-eclamptic pregnancies have enhanced activation of leukocytes such as neutrophils [51, 52] as a result of elevated STBM release from placental villi [53]. Additionally, a subset of women with PE favours placenta-specific bacterial microbiome generation which enhances leukocyte count (> 11,000 k/uL) and the percentage of neutrophil expression [54]. Neutrophil activation results in ROS production and pro-inflammatory cytokine release, subsequently leading to endothelial damage [55]. In addition to enhanced leukocyte activation, PE also features enhanced leukocyte trans-placental migration [56] (Fig. 4).

Moreover, elevated levels of maternal cell-free DNA occur as a result of PE [57–59] and correlate with disease severity [58]. The source of elevated levels of maternal cell-free DNA was unclear until Gupta et al. performed an ex vivo analysis to determine the effect of STBM on neutrophil activation, and NET generation in association to PE [60••]. Their results revealed that placental STBM and IL-8 activate neutrophils which subsequently leads to NET production in a ROS-mediated pathway [53] (Fig. 4). Despite the general nature of NETs to trap large bacterial micro-organisms, NETs can also trap microparticles smaller than 200 nm in diameter such as STBMs [60••]. Pre-eclamptic placental analysis reveals an enhanced presence of NETs in the inter-villous space [61••] in close proximity to STB, the source of STBM and placental IL-8 [60••].

Trophoblast cells which line the chorionic villi interact with NETs in a ying-yang way: (1) NETs hinder trophoblast migration and (2) factors released from trophoblasts prime neutrophils to a pro-angiogenic state to release increased vascular endothelial growth factor for vessel formation [62].

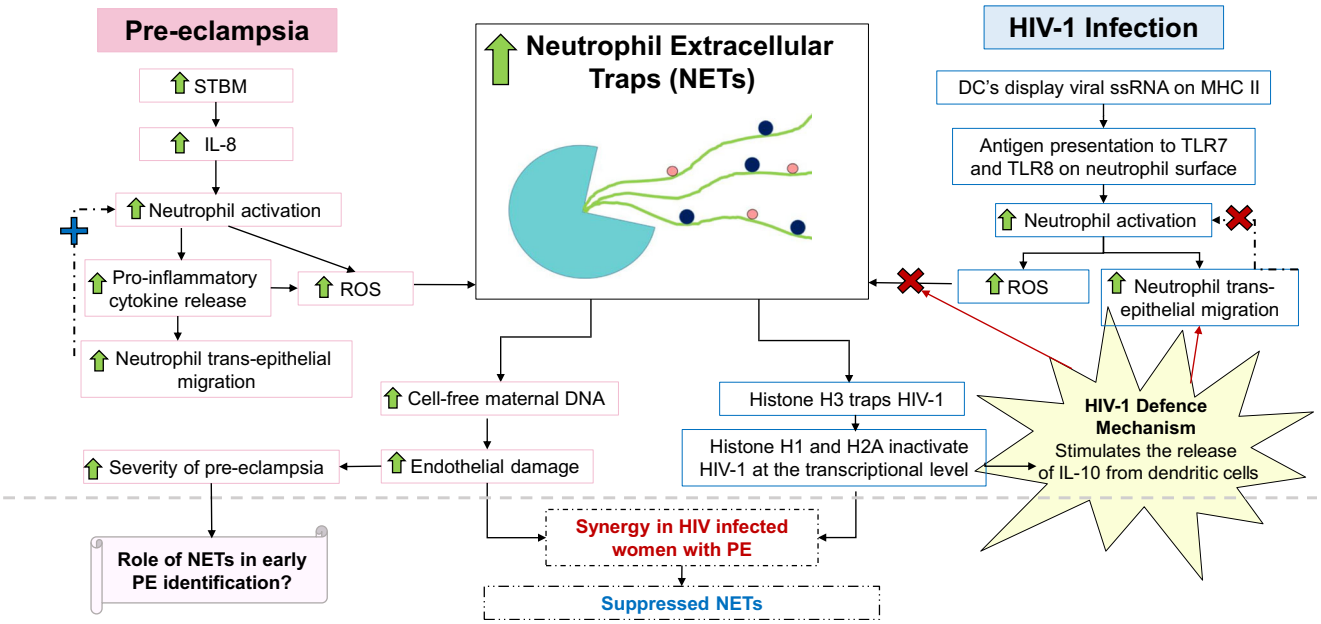

Fig. 4 Literature map and gap. The literature map of the effect of pre-eclampsia and HIV-1 infection on the production of neutrophil extracellular traps (NETs). In PE, the syncytiotrophoblast releases elevated amounts of syncytiotrophoblast microparticles (STBM) and the pro-inflammatory cytokine interleukin 8 (IL-8) which activates placental neutrophils. These activated neutrophils then release pro-inflammatory cytokines within the placenta which travel to the bloodstream and stimulate neutrophil trans-epithelial migration. The trans-migrated neutrophils serve as an elevated source of neutrophils which then add to the activated placental neutrophil pool in a positive feedback loop. Activated neutrophils also secrete reactive oxygen species (ROS) which trigger the release of neutrophil extracellular traps (NETs). These NETs serve as a source of elevated levels of cell-free maternal DNA observed in PE, leading to endothelial damage which enhances the severity of PE. During an HIV-1 infection, dendritic cells (DCs), which are antigen presenting cells (APCs), display viral single-stranded ribonucleic acid (ssRNA) on their major histocompatibility class two (MHC II) molecule. The viral ssRNA then interacts with toll-like receptors (TLR7 and TLR8) on the surface of neutrophils leading to neutrophil activation. Activated neutrophils stimulate neutrophil trans-epithelial migration in a positive feedback loop and activated neutrophils also secrete ROS which stimulates the production of NETs. Histone H3 within the NETs traps the HIV-1 virus, while histones H1 and H2A inactivate the HIV-1 virus at the transcriptional level. However, as a defence mechanism, HIV-1 counteracts this response by stimulating the release of anti-inflammatory interleukin 10 (IL-10) from DCs. This then inhibits the positive feedback loop of neutrophil activation by inhibiting neutrophil trans-epithelial migration and hinders the production of NETs. Although much knowledge exists on the effects of PE and HIV-1 infection on the production of NETs, combined effect of pre-eclampsia and HIV-1 infection on the production of NETs has not been investigated to date. Therefore, a literature gap exists in investigating the combined effect of pre-eclampsia and HIV-1 infection on placental NETosis in order to confirm if a balancing act of immune responses occurs which could possibly alleviate the NET-associated endothelial damage associated with PE or possibly reduce the rates of HIV-1 vertical transmission. Additionally, there is also a gap pertaining to the role of NETs as early identification markers of PE

Additionally, first-trimester trophoblast cells cultured with NETs become pro-inflammatory [62]: making it plausible to link the role of neutrophils and their NETs as early underlying pathogenic factors of PE. Supporting this is the ability of NETs to directly induce epithelial and endothelial cell death [63].

Current detection methods of PE reported by the International Society of Hypertension in Pregnancy (ISSHP) include identifying risk factors and monitoring all pregnant women for new-onset hypertension and/or proteinuria [64]. However, as the new-onset hypertension usually only presents at or after 20 weeks of gestation, no effective first- or early second-trimester tests are available, particularly in low-resource settings [64]. Latest improvements have been made in the detection strategy by the incorporation of the soluble FMS-like tyrosine kinase 1 (sFLT) and placental growth factor (PGF) ratio [65, 66]. A high sFLT/PGF ratio is indicative of PE development [65] and furthermore, Krysiak et al. describe a relationship between neutrophils and sFLT [67]. They demonstrated that although women with PE have high serum sFLT levels, neutrophils also express FLT (in their cytoplasm and cell membranes) and have low FLT levels in PE. This contrasting phenomenon is explained as follows: the migration of neutrophils is promoted when vascular endothelial growth factor (VEGF) binds to FLT-1 on neutrophils, and there is a neutralization effect to supress FLT-1 neutrophil expression by the elevated serum sFLT levels in PE [67]. Although this effect is intended to decrease neutrophil chemotaxis and migration, neutrophils are still activated and migrate through an alternate pathway and neutrophil FLT-1 expression is related to gestational age [67]. Although the sFLT/PGF ratio is being used as marker of the onset of PE, some experts in the field still claim that currently, there is no gold standard for the diagnosis of PE [68]. Given the interaction between neutrophils and sFLT, is it really the sFLT that orchestrates neutrophil FLT expression with subsequent function? Do neutrophils also exert modulation of sFLT and moreover, do neutrophils have the potential to be early identification markers of PE independently of sFLT orchestration?

The neutrophil-to-lymphocyte ratio (NLR) has been shown to be elevated in term PE pregnancies compared to normotensive pregnancies [69], and a first-trimester NLR > 5.8 is associated with miscarriages [70]. Neutrophil gelatinase-associated lipocalin concentrations are elevated in second trimester PE serum samples and positively correlated to proteinuria and blood pressure [71].

Moodley et al. have recently reported an elevation of NETs in the placental inter-villous space of PE pregnancies [61••]. There is also increased neutrophil activation and production of NETs in the PE maternal circulation [72]. In in vitro analysis of placental villous culture, CD45 (the pan-leukocyte marker) was shown to be reduced over time—indicating that these cells were possibly of maternal origin [73]. Since neutrophils are the most abundant leukocytes, maternal circulatory neutrophil signalling into the placenta is possibly an early identifiable event in PE. Supporting this is the phagocytic role of neutrophils in engulfing the aged trophoblast cells [74] shed as remnants of PE pathology [75]. Given these findings, it is plausible that neutrophil markers and/ or NETs have the potential to be early markers of PE identification.

Neutrophils, Neutrophil Extracellular Traps and Human Immunodeficiency Virus Infection

From the initial discovery of NETs by Brinkmann et al., the mechanism of NETosis on bacterial and fungal infections has been widely investigated [16]. Despite this, information on the role of NETs in viral infections is scanty, particularly in HIV infection. Recently, Saitoh et al. reported on the ability of NETs to trap, inactivate and clear HIV-1 [76••].

Antigen-presenting cells (APCs) such as DCs initially phagocytose the invading HIV-1 virus. These APCs then display viral single-stranded ribose nucleic acid (ssRNA) on major histocompatibility complex class 2 (MHC-II) molecules on their surface. The neutrophil–HIV-1 interaction begins when toll-like receptors (TLR) TLR7 and TLR8 on neutrophils interact with the ssRNA on MHC-II on APCs. This interaction activates neutrophils and induces ROS formation, subsequently leading to the formation of NETs in the extracellular space to trap and inhibit HIV-1 infection [76••] (Fig. 4).

During inflammation, histone H3 is a site for HIV-1 entrapment within the NETs [76••]. This may be attributed to the electrostatic forces between the opposing charges of histones and the HIV-1 envelope glycoprotein [76••]. Additionally, Kozlowski et al. found that extracellular H1 and H2A inhibit HIV-1 infection at a transcriptional level. Supporting this, a downregulation of H1 and H2A in HIV-negative individuals [77] strengthens the role of HIV-1 in the stimulation of extracellular histone production in NETs. Human recombinant H2A is in fact a potent inhibitor of HIV-1 [78••]. In addition to inactivating virions, NETs also inhibit HIV-infected CD4+ T cells [76••].

Human immunodeficiency virus infection is renowned for its evasion mechanisms that counteract the threat of both mature neutrophils and NETs. The HIV-1 infection is associated with a gradual decline in neutrophil count termed 'neutropenia' [79]. In addition, abnormal chemotaxis, phagocytosis, oxidative metabolism and pathogen-killing activities of neutrophils occur [80]. Neutrophils from HIV-infected individuals have elevated interleukin 12 (IL-12) expression, exacerbating the chronic inflammatory process [81]. As a defence mechanism, HIV triggers the release of the anti-inflammatory interleukin 10 (IL-10) from dendritic cells that neutralize NETs [76••] (Fig. 4). However, it is unknown whether this occurs within the placenta.

NETosis in HIV-infected Pre-eclampsia

Neutrophils are the abundant front runners of the immune system, being the first cells recruited to the inflammatory site. In addition to their phagocytic role, neutrophils undergo reverse trans-epithelial migration, stimulate T and B lymphocytes and have an increased lifespan, and the ability to project NETs has enhanced the credibility of this innate immune cell [12]. Furthermore, the ability of neutrophils to be unaffected by HIV enhances their credibility as effective biomarkers of inflammation. Furthermore, Moodley et al. have demonstrated that NETs are suppressed in term placenta in mothers that are both PE and HIV-infected [61••]. This indicates that NETs are the synergy source in the placentae of HIV-infected women with PE. The viral evasion mechanism elicited by HIV is unable to suppress NETs in normotensive HIV-infected women. However, in women with PE and HIV infection, there is a significant immune-suppression of NETs in the placental intervillous space—this may be due to the reservoir of dendritic cells from PE which are vital components of the HIV-mediated suppression strategy of NETs [61••].

Conclusion

Neutrophils are the front-runners of the immune system, with emerging evidence outlining that they are more than just abundant phagocytes. The ability of neutrophils to produce NETs and the role of NETs in the synergy of pre-eclamptic HIV-positive women are strategic for the entrapment of HIV. This displays an immuno-protective role of PE in HIV+ pregnancies since PE elevates NETs [60••, 76••]. There is currently no 'gold standard' for the diagnosis of PE and the following attributes of neutrophils potentiate the investigation of neutrophils as early markers of PE: (1) PE is an exaggerated immune response; (2) neutrophils are the most abundant immune leukocytes, making them easy to locate; (3) NETs are elevated in the PE placenta and (4) the ability of neutrophils to undergo reverse migration potentiates their ability to travel back to distal maternal sites from the placenta whilst carrying their pro-inflammatory signals as seen by NETs. This may be beneficial as an early identification marker of PE in the maternal peripheral blood.

Future Research

Future research requires investigations that outline the explicit role that neutrophils and NETs have as sources of synergy in HIV-infected PE pregnancies. Maternal blood should be collected longitudinally and neutrophil forward and reverse migratory markers should be investigated along with NETs.

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Compliance with Ethical Standards

Conflict of Interest The authors report no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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