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REVIEW

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Neutrophil migration into the placenta: Good, bad or deadly?

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ABSTRACT

Almost 2 decades have passed since the discovery that pregnancy is associated with a basal inflammatory state involving neutrophil activation, and that this is more overt in cases with preeclampsia, than in instances with sepsis. This pivotal observation paved the way for our report, made almost a decade ago, describing the first involvement of neutrophil extracellular traps (NETs) in a non-infectious human pathology, namely preeclampsia, where an abundance of these structures were detected directly in the placental intervillous space.

Despite these remarkable findings, there remains a paucity of interest among reproductive biologists in further exploring the role or involvement of neutrophils in pregnancy and related pathologies. In this review we attempt to redress this deficit by highlighting novel recent findings including the discovery of a novel neutrophil subset in the decidua, the interaction of placental protein 13 (PP13) and neutrophils in modulating spiral artery modification, as well as the use of animal model systems to elucidate neutrophil function in implantation, gestation and parturition. These model systems have been particularly useful in identifying key components implicated in recurrent fetal loss, preeclampsia or new signaling molecules such as sphingolipids. Finally, the recent discovery that anti-phospolipid antibodies can trigger NETosis, supports our hypothesis that these structures may contribute to placental dysfunction in pertinent cases with recurrent fetal loss.

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Introduction

Traditionally, polymorphonuclear neutrophils (PMNs) are viewed as highly abundant, short-lived, terminally differentiated granulocytic leucocytes, characterized by the presence of a multi-lobed nucleus and distinct sets of cytoplasmic granules.[1,2] In this context, PMN are proposed to play a significant role as gate keepers or first line defenders in combatting infection, exploiting an array of biological weapons, including production of reactive oxygen species or hypochlorous acid (HOCl) by the action of myeloperoxidase, and the degranulation of lytic enzymes or peptides, such as neutrophil elastase or cathelicidin (LL37).[1,2] The presence of LL37 on NETs can have a two-fold action. On the one hand this antibiotic peptide can assist with the elimination of pathogenic bacteria.[3] On the other hand, due its amphipathic nature, LL37 can act as a transfecting agent, facilitating the entry of extracellular DNA into adjacent cells, where it can lead to the activation of the Toll-like receptor (TLR) system and consequent production of inflammatory cytokines such as interferon-α (IFN-α).[4] Such a mechanism has been proposed to occur in psoriasis.[4] In addition, the presence of LL37 on NETs has been implicated with the underlying etiology of systemic lupus erythematosus.[5] It is unclear whether this mechanism is active in NETs occurring in placental tissues.[6]

A crack in this rather archaic view of PMN occurred when it was observed that PMN were able to generate neutrophil extracellular traps (NETs) upon stimulation or when encountering bacteria, fungi or even viruses.[7] These lattice like structures with a chromatin backbone function to ensnare microorganisms and kill them via the presence of histones or toxic granular proteins.[8]

Since deregulated or aberrant neutrophil activation is a hallmark of inflammation,[2] resulting in tissue damage, it comes as no big surprise that overt NETosis is

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associated with a number of inflammatory conditions including preeclampsia,[9] systemic lupus erythematosus[10] or rheumatoid arthritis.[11] Furthermore, tissue damage, possibly involving NETs induced apoptosis, is implicated in small vessel vasculitis, cystic fibrosis and transfusion related acute lung injury (TRALI).[12,13]

Further paradigm shifts challenging the view of the PMNs as suicidal mundane uniform foot soldiers, are the observation of distinct subsets with discrete functional differences,[14] the ability of circulatory PMNs to revert to a de-primed state of reduced activity,[15] and surprising longevity under certain conditions.[16] In addition, PMN have been determined to be quite adept at social networking, interacting with numerous other cells, including the ability to modulate the activity of adaptive immune system cells.[17,18]

Neutrophil migration into the placenta – is it really of any relevance?

Sadly, the role of PMNs in reproduction is still a largely neglected topic, despite their possible involvement in various stages, ranging from infertility, preeclampsia to fetal loss.[6] The fact that PMNs may be key players in the development of several pregnancy related perturbations, is underscored by the detection of vast numbers of NETs in preeclamptic placentae,[9] the deleterious action of PMNs in mediating placental damage associated with anti-phospholipid syndrome (APS)[19] or following treatment with the progesterone antagonist (RU-486).[20] In this review we aim to highlight new developments and point to possible new roles of PMNs as immune-modulators promoting efficient placentation.

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.[21,22] Neutrophil recruitment is mainly mediated through the linkage of P selectin glycoprotein ligand 1 (PSGL1), ESL1, CD44 and L-selectin.[23,24] The interaction of selectins with their glycosylated ligands mediate rolling and the expression of L-selectin is especially indicative of rolling neutrophils.[25] Neutrophil adhesion can be facilitated through activation by pro-inflammatory 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 / lacrophage-1 antigen), which bind to endothelial cell surface molecules such as ntracellular adhesion molecules 1 and 2 (ICAM1 and ICAM2).[26,27] The expression of CD11b-CD18 is important for the crawling of neutrophils.[28] 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 molecules.[29] Transmigration occurs between (paracellularly) or through (trancellularly) endothelial cells and in order to pass across the membranes, neutrophils release specific proteases such as matrix metalloproeinases (MMPs) and serine proteases (Fig. 1). These enzymes are able to affect neutrophil migration by the degradation of elastin and collagen, thereby increasing the vascular permeability.[30,31] Interestingly these proteins are under hormonal regulation during pregnancy.[32] 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.[33]

Identification of a novel decidual neutrophil population

Traditionally most studies examining the presence and action of immune cells in the placenta have addressed innate immune effector cells such as uterine or decidual NK cells (dNK), macrophages, dendritic cells or more recently regulatory T cells (Treg).[34-38] In the context of this review, it is gratifying to observe a shift in these tendencies, with more attention being focused on PMN.

In a recent study, Amsalem and colleagues examined leucocytes from 1st and 2nd trimester decidual tissues and matching blood samples.[39] Their data indicated a significant increase in CD45+ and CD15+ neutrophils migrating into the decidua during the period from 6 to 20 weeks of gestation. High levels of CD66b expression further characterized these PMN. CD66b, also termed carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM8), is specifically expressed on neutrophils and eosinophils. It plays an important role in adhesion and activation. Treatment of PMNs with their natural ligand, galectin-3, triggers increased phagocytosis and

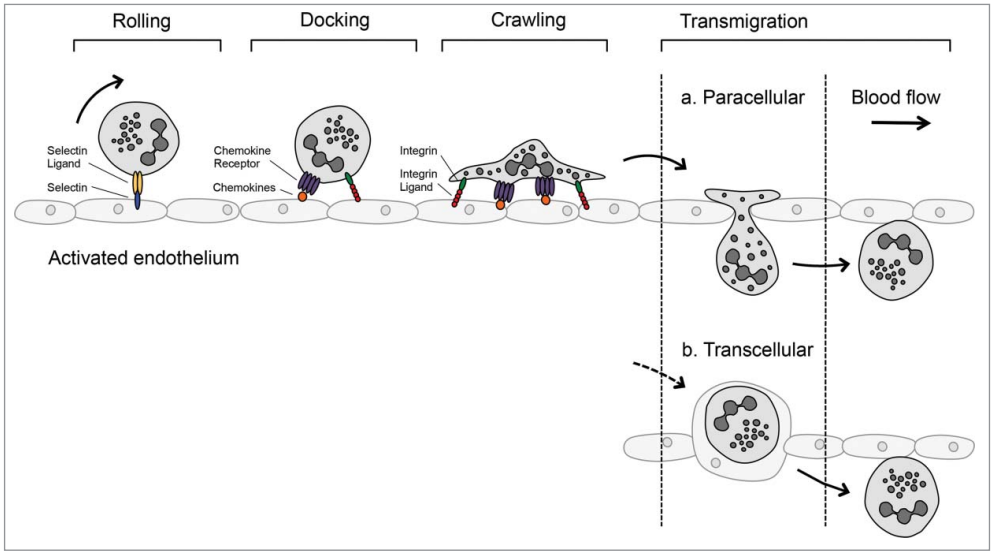

Figure 1. Sequential steps of neutrophil recruitment from the vasculature to the tissue. Two possible mechanisms of transmigration are described: (a) paracellular - between endothelial cells; and (b) transcellular - through endothelial cells. Major groups of adhesion molecules are marked. Rolling depends 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 Kolaczkowska and Kubes.[26]

degranulation. Its effect on migration or induction of NETosis is currently unknown.

Immunostaining for neutrophil elastase (NE) and CD66b revealed that PMNs were indeed physically present in 2nd trimester decidua, specifically the decidua basalis, and that clusters of these cells were frequently located close to spiral arteries. Furthermore, infiltrating PMN could be detected migrating from the venous endothelium into the decidua.

Further characterization of these cells revealed that they expressed reduced levels of the IL-8 (also termed CXCL8 (chemokine (C-X-C motif) ligand 8) receptors CD181 and CD182, and higher levels for chemokine receptors where the ligands were present in the decidua. Decidual migration was mediated via IL-8 (CXCL8), as anti-IL-8 antibodies could antagonize the effect of decidual culture medium on PMN migration *in vitro*.

It was furthermore determined that these decidual neutrophils (dN) were pro-angiogenic, expressing increased levels of VEGF-A, arginase-1 (ARG1) and CCL^{-}2, and that in co-culture experiments they promoted angiogenic sprouting by uterine microvascular endothelial cells (UtMEC). Once again, IL-8 appeared to play a crucial role in promoting this novel chemotactic phenotype.[39]

The precise mechanism leading to the generation of these decidual neutrophils is currently unclear, other than it involves the migration of normal circulatory polymorphonuclear granulocytes into this placental tissue via the action of chemokines such as IL-8 (CXCL8). It is possible that other placentally-derived factors may contribute to this phenomenon, including syncytiotrophoblast microparticles, as these have been shown to activate PMN and induce NETosis.[9]

PP13, immune diversion and spiral artery modification

A feature of early onset preeclampsia (ePE), defined by the manifestation of symptoms prior to 34 weeks of gestation, is failure of adequate modification of the maternal spiral arteries by fetal invasive extravillous trophoblast cells.[40] In the procedure the maternal endothelium is replaced by trophoblast cells, which adopt an endothelial-like phenotype, resulting in much wider blood vessels and a concomitant slow even flow of maternal blood to the underlying fetal tissues.[34,41] In cases with ePE or intra-uterine growth restriction (IUGR), such failure results in highly pulsatile high pressure blood flow, leading to inadequate oxygenation or delivery of nutrients to the fetal tissues, thereby contributing to the underlying pathology of these disorders.

In the context of neutrophil migration into the decidua, an intriguing observation was made with regard to PP13 (placental protein 13; galectin-13) expression, which is reduced in cases with ePE, early in gestation before the onset of symptoms.[42] PP13 is a small glycan-binding protein uniquely produced by the placenta which may be a key regulator of maternal immune responses.[40,43-45] It is mainly produced by the syncytiotrophoblast on the maternal-fetal interface throughout

pregnancy from where it is secreted into the maternal circulation.[43,44] There, it may be downregulating maternal immune responses against fetal tissues due to its capability of inducing the apoptosis of activated T cells.[44] Interestingly, the gene encoding PP13 (*LGALS13*) has emerged in anthropoid primates as member of a primate-specific galectin-gene cluster on Chromosome 19.[44,46] These data collectively suggested that PP13 may have a unique role during placentation and the immunoregulation of pregnancy in anthropoid primates, and may provide be part of a novel pathway of maternal-fetal immune tolerance evolved in these species to promote deep hemochorial placentation during their long gestation.[44,46,47]

Interestingly, a recent immune-histological examination indicated that PP13 was chiefly produced by the syncytiotrophoblast (STB) of chorionic villi in the first trimester, but also with sporadic occurrence in trophoblast cells of modified decidual spiral arteries.[48] Interestingly, extracellular PP13 was detected in the decidua in so-called zones of necrosis (ZONEs). These ZONEs were associated with regions of necrotic or apoptotic cell death. Furthermore, these ZONEs are associated with the influx of numerous immune cells, including CD45RO memory T cells, CD68+ macrophages, CD57+ large granular lymphocytes and PMN. The presence of the latter could be indicative of an inflammatory response.

A key event in the formation of these ZONEs, was the deposition of PP13, which occurred prior to leukocyte influx, particularly that of PMNs.[48] It is of considerable interest that these ZONEs were located in tissues surrounding converted maternal spiral arteries, and also occurred in close proximity to decidual veins. This material was not associated with trophoblasts, but rather appeared in the form of dense aggregates.

The number of these ZONEs was found to increase during gestation, peaking at 7 to 8 weeks of gestation. It was also determined that their number or intensity correlated with the degree of spiral artery modification, being virtually absent in cases with low levels of circulatory PP13. This is particularly interesting as low maternal serum concentrations of PP13 has been associated with an increased risk for the development of PE, especially the early-onset form.[40,42,49]

Due to the regulated appearance of these ZONEs and their close association with the degree of spiral artery modification, it has been proposed that they act as decoy sites of inflammation, drawing maternal immune effector cells away from the sites being altered by invasive trophoblast action.

The action of PP13 in this system is quite complex, relying on a multimeric secreted form, which is transported via the decidual veins into the tissues surrounding the arteries requiring modification, where they form pro-inflammatory aggregates.[48] These PP13 aggregates have been shown to be pro-inflammatory *in vitro*, triggering the release of interleukin – 1beta (IL-1β) and IL-6 from buffy coat lymphocytes.

The concept of decidual diversionary sites of inflammation to facilitate spiral artery modification is intriguing in the view of recent findings, which indicate that aberrant systemic inflammation hinders or abrogates this process in rat model systems.[50]

Knowledge about neutrophil migration from animal systems – new lessons from the rat

Although scholars of human reproduction have long neglected animal models, particularly murine or rodent-based systems, due to the intrinsic differences in placentation, a number of recent studies have highlighted the need to peer across this ideological fence.[51-53] On the one hand, this is due to both human and rodent placentation being hemochorial systems, whereby the maternal blood is in direct contact with fetal tissues.[54] On the other hand, due to the ease whereby these systems can be manipulated using gene knock-out technologies.[52,53]

The use of a murine system was very useful in delineating the mechanism evoked by anti-phospholipid antibodies (aPL) in triggering fetal demise in anti-phopholipid syndrome (APS) patients.[19,55-58] In a key set of studies using discrete knockout mutants of members of the complement family, Girardi and Salmon showed that PMN infiltration into the decidua was very prominent in APL treated mice.[59,60] This effect could be antagonized using inhibitors or knock-out mutants for complement components C3, C5a or the coagulation promoting tissue factor (TF).[19,55-57] Interestingly, the deleterious effect of aPL could also be abolished by antibody mediated neutrophil depletion. The underlying cascade was determined to be activation of complement C5a by aPL antibody binding to the trophoblast. The binding of C5a to the C5a receptor (C5aR) triggered neutrophil activation via the TF/PAR2 (protease activated receptor 2) system, leading to the generation of toxic ROS molecules, thereby inducing placental damage and subsequent fetal demise.[19]

aPL have been implicated in arterial and venous thrombosis frequently observed in patients with APS.[61] A recent report showed that aPL from patients with APS can stimulate neutrophils to produce neutrophil extracellular traps (NETs), a possible mechanism for thrombi formation. Yalavarthi and colleagues[62] compared serum, plasma and isolated neutrophils from patients with primary APS and healthy volunteers. APS patients showed

higher levels of NETs, while neutrophils from patients showed higher spontaneous release of NETs *in vitro*. Moreover, β-2-glycoprotein 1 (β_2GP1) is also bound to the cell surface of neutrophils, and β_2GP1-specific antibodies stimulate NET formation; this effect was shown to be dependent on ROS production and on TLR4 activation. Furthermore, *in vitro* stimulation of neutrophils with purified aPL or with serum from patients with APS potentiated NET formation and thrombin production.

Although NETs were first identified as a defense mechanism against microbial pathogens,[7] it is now widely accepted that they can activate platelets and the coagulation cascade, serving as a scaffold for the assembly of thrombi. This data suggests that NETs present in the circulation can contribute to thrombotic events leading to excessive placental damage and consequent fetal loss (Fig. 3).

By examining the CBA/J x DBA/2 mouse model for spontaneous fetal loss, it was once again determined that C5a and TF played key roles, but this instance led to the production of the anti-angiogenic factor sFlt-1 (soluble fms-like tyrosine kinase – 1) by macrophages, which

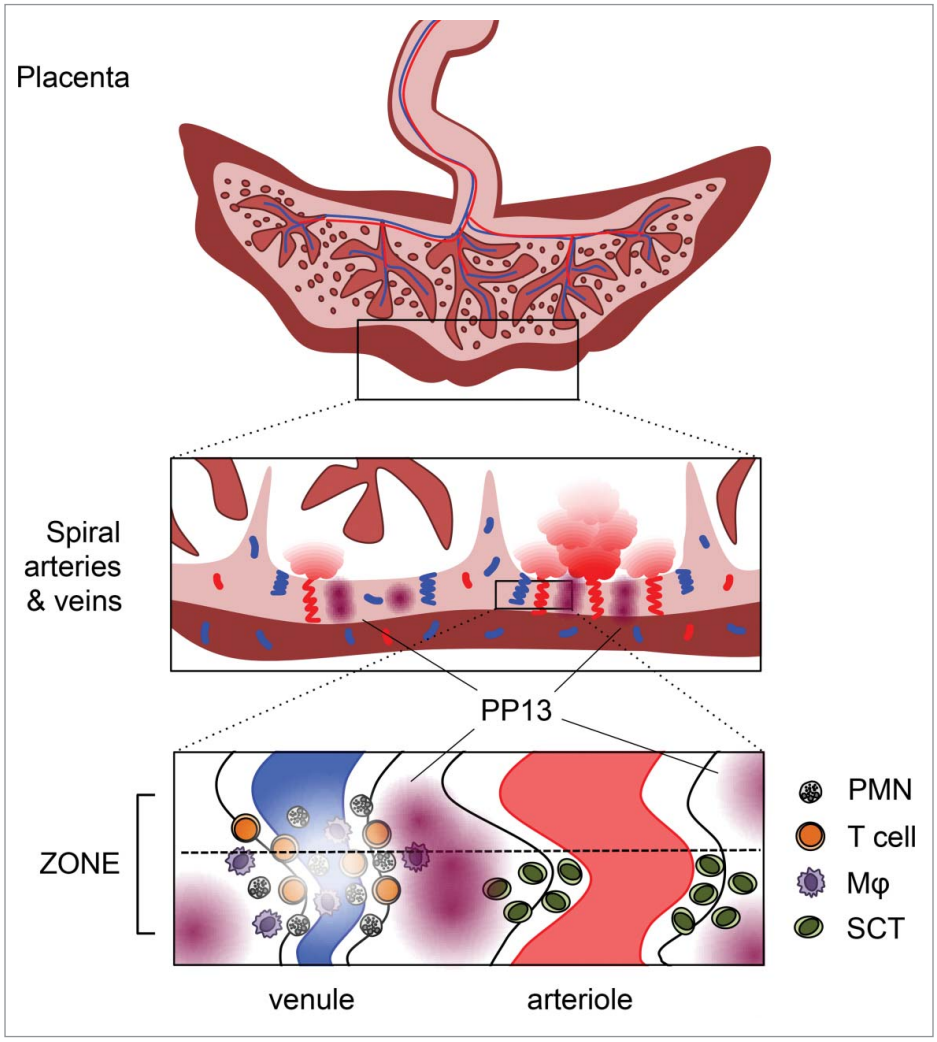

Figure 2. Immune diversion model, spiral artery modification and PP13 Upper panel: The hemochorial human placenta is nourished by maternal blood that is injected into the intervillus space via the uterine spiral arterioles (red decidual vessels). Products of syncytiotrophoblast secretion are released into the intervillus space and, along with blood, are returned to the maternal circulation through the decidual basalis veins (blue decidual vessels). Middle panel: Decidual veins are filled with placental protein 13 (PP13) while PP13 and associated neutrophils transudate to the region. Lower panel: PP13 shows intense deposition consistent with early and active ZONE formation, and other areas of end-stage ZONEs. Neutrophils follow an inverse pattern with the least intense staining in the early ZONEs and the most intense in the endstage ZONEs. Combining this data suggests that syncytiotrophoblast-secreted PP13 exits the intervillus space via the decidual basalis veins (blue) where it binds to the endothelial cells, traverses the veins to be deposited into the surrounding decidua, precipitates, and induces a ZONE consisting of activated T cells, macrophages, and neutrophils. At the same time, invasive trophoblasts migrate to and invade the maternal spiral arterioles (red) without interference from potentially cytotoxic elements of maternal immune surveillance. Figure adapted from Kliman et al.[48]

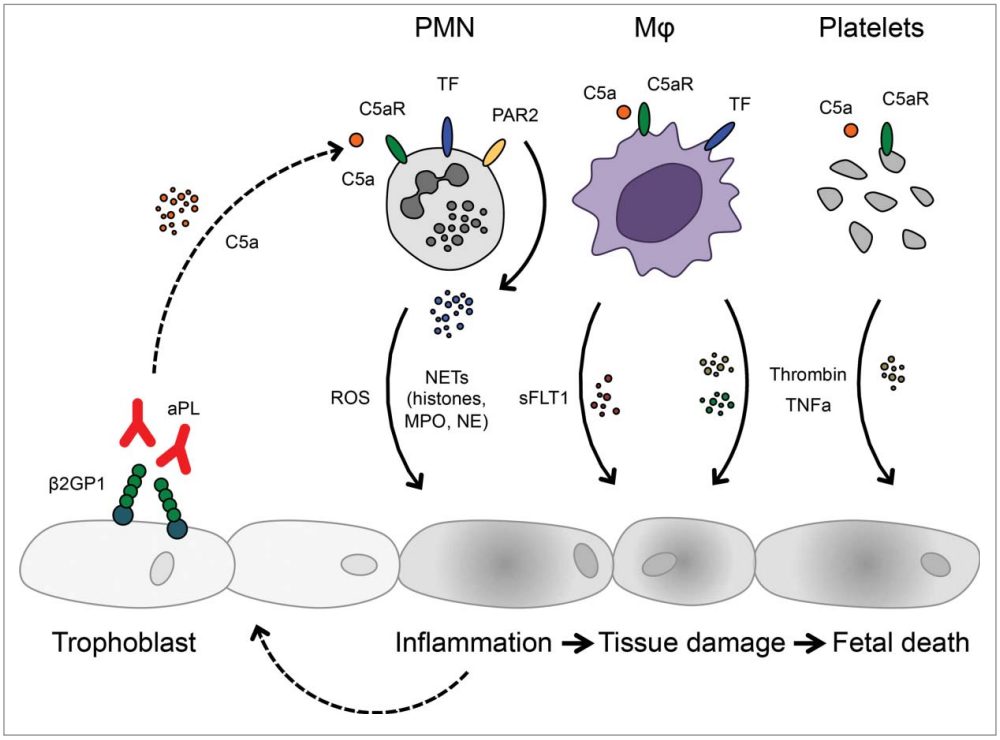

Figure 3. Mechanism of antiphospholipid (aPL) antibody–induced fetal damage. aPL antibodies are directed to the placenta where they activate the classical pathway of the complement cascade which leads to the expression of potent anaphylatoxins, C5a in particular. C5a is a neutrophil, monocyte and platelet activator, which furthermore stimulates the release of inflammatory mediators, including reactive oxygen species (ROS), proteolytic enzymes, histones, cytokines and chemokines, as well as additional complement and coagulation factors. Tissue factor (TF) expression on monocytes enhances the release of antiangiogenic molecule sFlt-1. sFlt-1 impairs trophoblast proliferation, reduces placental blood flow, induces oxidative stress, and increases TF expression on trophoblasts. This creates a proinflammatory amplification loop at sites of leukocyte infiltration that generates additional C5a. This results in enforced neutrophil influx, inflammation within the placenta, and ultimately, fetal injury. Either fetal growth restriction or even death in utero ensues depending on the extent of the damage. PMN: neutrophil, Mϕ: monocyte/macrophage. Figure adapted from Girardi et al.[51] and Redecha et al.[49]

adversely affected placental development.[51] Of interest is that this model also shows many traits associated with preeclampsia, such as albuminuria and endotheliosis, and could be pharmacologically treated with pravastatin.[51,63]

In a very recent study of the rat utero-placental unit investigating the localization of discrete innate immune effector cell populations, it was determined that uterine NK (uNK) cells were present, as expected, in the perivascular region of the mesometrial triangle adjacent to the uterine artery.[64] Of interest is that uNK cells co-localized with areas of TNFα and INFγ expression, indicative of a potential role in modulating trophoblast invasion. Previous studies have indicated that uNK cells play a crucial role in regulating the extent of trophoblast invasion and modification of spiral arteries, with TNFα limiting the extent of trophoblast migration.[35] PMNs, on the other hand, were located directly at the fetal-maternal interface, or directly in the spiral artery lumen in the mesometrial triangle.[64] In this instance, PMNs were found to be associated with regions of IL-10 expression, which would be indicative of an immune dampening condition. IL-10 could, however, also play a role in regulating trophoblast behavior, as previous studies have indicated that IL-10 could antagonize the action of TNFα, and facilitate trophoblast invasion.[64]

Although not yet elucidated in detail, this study does suggest that the location of PMNs in the utero-placental unit may play a subtler role than merely combatting infection, but may be crucial to ensure successful placentation by modulating trophoblast invasion and differentiation.[64]

Defective spiral artery modification, hypertension and poor pregnancy outcome – do PMNs play a role?

Defective placentation in combination with hypertension and poor pregnancy outcome is a hallmark of preeclampsia.[34,65] To examine this association in more detail, researchers have made use of the inbred BPH/5 murine model system for preeclampsia.[66] In this mouse,

pregnancy is characterized by the development of hypertension, proteinuria and endothelial dysfunction late in gestation. The pups are frequently growth restricted, and litter sizes may be reduced in comparison to wild type mice.

Previous investigations have indicated that placental sizes were reduced in pregnant BPH/5 mice, and that this involved aberrant trophoblast invasion of the proximal decidual zone.[67,68] Furthermore, the maternal decidual arteries were not modified by trophoblast cells to the same extent as in wild type mice, resulting in increased vascular resistance, detectable by pulse wave Doppler ultrasound analysis. Consequently, this BPH/5 murine model system shares several features in common with the human form of preeclampsia, in that placental dysfunction occurs in association with hypertension and endotheliosis, leading to poor pregnancy outcome.[66]

In a recent more detailed examination of this murine system, it was determined that PMN infiltration was at least 2-fold greater in BPH/5 mice, than in control C57BL/6J mice.[66] This was particularly evident in the ectoplacental cone at day E8.5 of gestation. An examination of placental homogenates indicated that the chemokine CXCL1, also termed neutrophil activating protein 3 (NAP-3), was present in significantly higher concentrations in BPH/5 than in C57 mice, indicating that this chemoattractant may be responsible for increased PMN infiltration. To discern whether excessive PMN infiltration contributed to defective placentation and poor pregnancy outcome in this model, they were depleted by treatment with either anti-GR1 (myeloid differentiation antigen Gr-1) or anti-Ly6G antibodies. These studies showed that depletion of PMNs with either antibodies lead to a reduction in fetal resorption, and an increase in both fetal and placental mass. These changes were reflected in altered placental development, including an increase in placental disc size, and most notably a change in maternal spiral artery modification, as these increasingly became transformed by trophoblast cells, thereby losing their smooth muscle actin phenotype.

Akin to the human form of preeclampsia, placental deficiency in BPH/5 mice is associated with an imbalance in angiogenic factors, most notably VEGF (vascular endothelial growth factor). Intriguingly, it was observed that plasma and placental VEGF concentrations were significantly elevated in anti-GR1 neutrophil depleted mice. Furthermore, it was observed that co-culture of isolated PMN with the trophoblast cell line HTR8/SVNeo lead to a significant reduction in VEGF production.

Since the complement system has been implicated in preeclampsia, and in mediating activation of PMN in murine model systems of spontaneous or aPL antibody induced fetal loss, this aspect was examined in BPH/5 mice. This data indicated that C3 complement deposition in the ectoplacental cone preceded PMN infiltration. As expected, blocking of the complement cascade lead to a decrease in fetal resorption and an increase in fetal and placental mass. Under these conditions, decreased PMN infiltration into the decidua was noted, which was accompanied by increased spiral artery modification.[66]

The mode of action whereby PMNs contribute to the pathology witnessed in pregnant BPH/5 mice was determined to involve the production of TNFα. The first indicator for such an involvement was the presence of increased concentrations of TNFα in BPH/5 placentae. In co-culture experiments using the human trophoblast cell line HTR8/SVNeo, it was observed that both murine and human PMNs produce prodigious quantities of TNFα under such conditions.[66] By treating pregnant BPH/5 mice with Etanercept (also known as Enbrel), a TNFα inhibitor used in the therapy of auto-inflammatory diseases such as rheumatoid arthritis, it was observed that this lead to a vast improvement of the underlying pathology.[66] This included decrease in fetal resorption, increase in fetal and placental mass, and increase in spiral artery modification. It is also noteworthy that an increase in placental VEGF production was noted following this therapeutic intervention.

As the pathology of this experimental system bears a striking resemblance to that of preeclampsia in humans, it begs to question whether it would be useful to treat at-risk pregnancies with anti-inflammatory biologics such as Etanercept.[47]

Sphingolipids – a key regulatory element of innate immune cell activity at the feto-maternal interface

Sphingolipids, also known as glycosylceramides, are important components for a series of signaling molecules, ranging from sphingosine which displays anti-apoptotic activities, to ceramide which is pro-apoptotic.[69] One of the bioactive sphingolipid metabolites is sphingosine 1-phosphate (S1P), produced by the action of 2 distinct kinases, sphingosine kinase 1 and 2 (Sphk1 and Sphk2).

Previous studies on mice in which both the Sphk1 and Sphk2 genes had been deleted, revealed that these suffered embryonic lethality in utero, while mice in only one of the kinase gene knockouts (Sphk1$^{-/-}$ or Sphk2$^{-/-}$) were functionally normal.[70,71] Of interest in the discourse of this review was the phenotype of mice with a heterozygous knock-out genotype (Sphk1$^{-/-}$ Sphk2$^{+/-}$) suffered from reproductive failure. Analysis of these mice indicated that the S1P pathway was highly

active during pregnancy. This was particularly evident by the death of decidual cells, reduced proliferation of stromal cells and massive breakdown maternal blood vessels in this tissue.

In a more recent detailed analysis of these mice, it was observed that the levels of the CXCL1 and CXCL2 chemokines were significantly increased in the decidua of such animals.[70] This was reflected by a massive influx and activation of PMNs in the decidua and uterus, coupled to a decrease of dNK cells in these tissues. Since uNK cells are prosed to play a key role in spiral artery modification, their absence could explain this defect in this murine system. As expected, depletion of neutrophils by application of Gr-1 antibodies lead to an amelioration of symptoms, including reduced fetal resorption.

In an examination of 1st trimester human decidual cells, it was observed that inhibition of the sphingosine kinase system lead to an increased production of CXCL1 and CXCL8 (IL-8), indicating that anomalies of this system could promote PMN infiltration via chemokine production.[70]

Although there is no clear data concerning the involvement of sphingolipids in pregnancy-related disorders such as preeclampsia, it is worth noting that S1P can have pronounced affects on vascular tone. In this manner, an imbalance in S1P synthesis could contribute to endothelial damage associated with preeclampsia. On the other hand, increased levels of ceramide have been noted in preeclampsia, which could affect trophoblast survival and turn-over by promoting autophagy.

What can we learn from tumor-associated neutrophils?

Solid tumors and the placenta have been suggested to share a number of common features, including tissue invasion, angiogenesis and immune modulation. For this reason an examination of tumor infiltrating PMNs may yield interesting clues as to PMNs in the placenta.

The tumor microenvironment plays an important role in the development and progression of cancer. It is characterized by a state of chronic inflammation enriched by the infiltration of immune cells and stromal cells, which promote tumorigenesis and metastasis.[72] Tumor associated neutrophils (TANs), depending on the microenvironment, play dual roles in exerting pro-inflammatory or anti-inflammatory functions.[73] Like the pro-tumor macrophages (M2), neutrophils exhibit a pro-tumor neutrophil (N2) phenotype.[74,75] These N2 TANs behave pro-tumoral by the activation of TGFβ released from the tumor microenvironment. Blockade of the TGFβ receptor by small molecule inhibitors reversed the N2 neutrophil phenotype to anti-tumor (N1) neutrophils (Fig. 4). PMNs have been shown to promote angiogenesis and

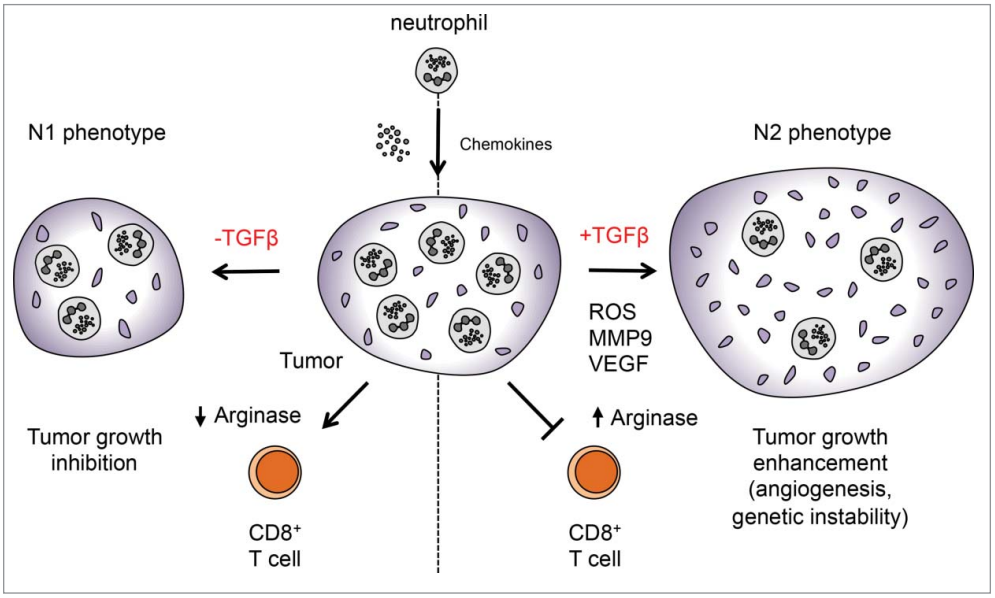

Figure 4. Tumor associated granulocytes. Chemokines expressed by tumor cells and tumor-associated macrophages (TAM) promote the recruitment of circulating neutrophils into the tumors. Neutrophils promote genetic instability, possibly through generation of ROS and stimulate angiogenesis through the production of matrix metalloproteinase 9 (MMP9) and vascular endothelial growth factor (VEGF). Transforming growth factorβ (TGFβ) forces neutrophils to obtain a polarized, pro-tumoral N2 phenotype, which is characterized by high levels of arginase production. On the other hand, inhibition of TGFβ promotes neutrophil maturation toward an N1 phenotype. This is associated with higher cytotoxic activity, higher capacity to generate H_2O_2, higher expression of tumor necrosis factor a (TNFα) and lower expression of arginase and intercellular adhesion molecule 1 (ICAM1), CD8+ T cell activation increases in the presence of N1 neutrophils, which leads to an effective antitumor effect. Figure adapted from Mantovani et al.[14]

neovascularization by secreting matrix metaloproteases (MMPs) and chemokines, which in turn activate the release of VEGF.[76] Neutrophil elastase, a major proteolytic enzyme released from the azurophilic granules of activated neutrophils has been shown to bind to the cell surface of cancer cells and gets internalized in a clathrin pit dependent endocytosis process, and mediates the proliferation of cancer cells.[77] In a mouse model of lung carcinoma, neutrophil elastase knockout significantly reduced the tumor progression. Treating a lung cancer cell line with neutrophil elastase has induced the proliferation of the cells by activating the PI3K/PDGFR (phophoinositide 3-kinase/ platelet derived growth factor receptor) pathway. Interestingly the activation of PI3K/PDGFR in proliferation was due to the rapid hydrolysis of IRS1 (insulin receptor substrate-1), a key adaptor molecule for the p85 subunit of PI3Kinase; hence the p85 subunit binds to PDGFR to induce proliferation. Mice overexpressing neutrophil elastase also have reduced levels of IRS1 *in vivo*, supporting cancer progression.[73] NETs are also involved in the development of deep vein thrombosis. Neutrophils along with platelets induce the formation of thrombi in blood vessels activating the endothelium.[78] G-CSF secreted by tumor cells activates neutrophils to generate NETs, allowing distant cancer cells to metastasize.[79]

Contribution of placental microparticles and exosomes

The placenta plays a key role in the modulation of the immune system, in order to fine-tune the attraction, education and response of the innate and adaptive immune cells during each stage of pregnancy. It secretes a series of both local and systemic soluble factors, which are essential for the normal maternal hemostatic status. Furthermore, the placenta produces a broad variety of extracellular vesicles (EVs) that participate in the regulation of the inflammatory profile during pregnancy.[80,81] EVs are released in large quantities from the syncytiotrophoblast layer and include microparticles (0.2–1 um) and exosomes (40–150 nm).

Syncytiotrophoblast-derived microparticles (STBMs) are able to stimulate monocytes and B cells toward pro-inflammatory cytokine production, triggering activation of neutrophils to generate superoxide radicals (ROS) and NETs.[9,82-84]

On the other hand, exosomes are involved in T-cell apoptosis via the expression of Fas ligand. Moreover, they have also been shown to carry the immune modifying MHC class I chain related protein A and B, which can down-regulate NKG2D on PBMCs that is associated with reduced activity.[85-88] The effects of placental exosomes on PMNs still remain to be explored, but it is recently reported that exosomes from human macrophages and dendritic cells produced chemotactic eicosanoids and induced granulocyte migration.[89] Rab27a-dependent secretion of exosomes permits a mobilization of a subpopulation of neutrophils required for local tumor growth.[90] The human placenta can be viewed as a tumor due to its rapid growth and can most likely utilize similar molecular and cellular mechanisms for growth and survival.

In general, STBMs may activate immune effector mechanisms, while exosomes lead toward an anti-inflammatory state. It is speculated that the physiological range of the STBMs/exosomes ratio is disturbed in various pregnancy complications and might reach >1 due to the overproduction of STBMs.[91] At present, multiple studies have investigated the levels of STBMs in pre-eclampsia. Although STBM abundance during pre-eclampsia is still under debate, an important discrimination between early-onset and late-onset disease seems to exist.[92,93] It has become clear, however, that in response to cellular stress, condition changes are evident not only in the abundance of syncytiotrophoblast-derived EVs, but in their molecular composition too. STBMs derived from preeclamptic placentae exhibit increased tissue factor activity and over 25 proteins with significantly higher expression were identified compared to healthy controls.[94,95] In exosomes isolated from 2nd and 3rd -trimester serum samples of patients with pre-eclampsia, Syncytin-2 was found to be significantly reduced.[96]

The role of EVs in regulating the maternal immune profile remains to be elucidated but it is clear that changes in this profile reduces the ability of the placenta to properly coordinate the activity and the inflammatory status of the involved immune cells.

Do placental/uterine pmns contribute to parturition?

A considerable line of investigations on PMNs in the systemic circulation and in uterine tissues revealed that these immune cells have multifaceted roles during parturition, either at term or preterm, both in humans and in other mammals.

In systemic circulation of women in term and preterm parturition, the number, activation state and migratory capacity of PMNs are increased compared to non-laboring women.[97-100] As described both in humans and in experimental animals, these activated PMNs are attracted into uterine tissues during labor due to the local increase in chemokine (e.g. IL-8) expression,[97,100-110] where they release cytokines and MMPs to contribute to

the orchestration of local inflammation and tissue remodeling during labor and to uterine involution in the post-partum period.[111-115] Of interest, the timing of PMN tissue-migration and the function of tissue-resident PMNs may vary according to the compartment in the uterine cavity.[100]

PMNs infiltrate the human cervix only postpartum. This was evidenced by a similar number of cervical PMNs in women not in labor with unripened and ripened cervices,[116] and by the increased number of cervical PMNs in women after spontaneous vaginal delivery at term compared to non-laboring women.[116] As a molecular basis for this phenomenon, microarray studies revealed that inflammation-related genes do not emerge as differentially regulated with the ripening,[117] only with the shortening of the cervix,[118] and the overexpression of genes involved in neutrophil chemotaxis (e.g., *IL8*) occur only with cervical dilatation and labor at term.[108] Human data is supported by experimental evidence in mice showing that the numbers of PMNs do not change significantly during pregnancy, only in the post-partum period following an increase in the cervical expression of the neutrophil chemoattractant *Cxcl1*, and that these cervical PMNs have increased myeloperoxidase activity.[119] Therefore, in spite of earlier thoughts on PMNs participating in cervical ripening,[106,111,112] recent evidence in humans and rodents support that PMNs rather play an important role in postpartum tissue repair[116,119,120]

PMNs infiltrate the human myometrium during term labor, where they are attracted by the local increase in chemokine expression. In fact, the potent neutrophil chemoattractant IL-8 is the most highly upregulated chemokine in the human myometrium in term labor as shown by high dimensional studies.[109,110] In accord with human data, rodent models of term as well as sterile and infectious preterm parturition have also revealed strong PMN infiltration into the myometrium during labor, and provided evidence that it mainly happens in the post-partum period, when PMNs may have an important role in uterine involution.[100,115] Of interest, a very recent *in vitro* study has provided mechanistic insights into this sequence by demonstrating that the mechanical stretch of the myometrium near term induces the secretion of chemokines (e.g. IL-8, CXCL1), which activate peripheral leukocytes including PMNs, and increase their adhesion to myometrial vascular endothelial cells and transendothelial migration into the myometrium.[121]

In the human chorioamniotic membranes, the number of PMNs rise modestly during term labor in the absence of infection or inflammation.[122,123] This is consistent with the relatively low increase in the expression of neutrophil chemoattractant molecules (e.g. IL-8) in the chorioamniotic membranes and the choriodecidua following term labor[103,124] These findings in humans are substantiated by the observations on the increased PMN numbers in the decidua in term labor and postpartum in mice.[104] Of importance, PMNs are recruited in large numbers into the chorioamniotic membranes upon infection and inflammation (i.e., histological chorioamnionitis).[125] Indeed, the abundance of PMNs in the decidua significantly increases in women with preterm labor associated with chorioamnionitis, while it is not the case in term and preterm labor without inflammation of the membranes.[123] This is consistent with the strongly elevated IL-8 concentrations in the amniotic fluid in women with infectious preterm labor compared to those with term labor[126]. After migrating into the decidua, PMNs also assault the chorion and then the mesodermal layer of the chorioamnion,[127,128] and their activation and apoptosis are in line with the sequence of inflammatory responses in histological chorioamnionitis.[129] In accord with findings in humans, there is an increased decidual influx of PMNs following intrauterine administration of LPS (lipopolysaccharide) in rodent models of infectious preterm labor.[104,130] This PMN influx during labor follows the increased decidual expression of chemokines (e.g. Cxcl1), and occurs most prominently post-partum.[104] Since decidual PMNs release pro-inflammatory mediators and MMPs, they were suggested to participate in the degradation of the extracellular matrix of the fetal membranes, the rupture of the membranes during term and preterm labor, and the postpartum involution in the decidua[104,113,114,131,132] (Fig. 5).

Of note, the depletion of PMNs in animal models did not prevent LPS-induced preterm birth, suggesting that PMNs do not act as causative agents in infectious preterm labor[100,119,130] However, PMN-depletion prior to LPS administration still reduced the pro-inflammatory responses evidenced by IL-1-β expression in uteroplacental tissues of mice,[130] which is remarkable since the systemic administration of IL-1-β itself is capable of inducing preterm birth in animal models.[133] These results may collectively suggest that PMNs are important but not essential components of the terminal pathway in infectious and inflammation-induced preterm birth.

PMN and their contribution to preeclampsia and recurrent fetal loss – what is the way forward?

A diverse body of evidence currently serves to link overt or aberrant PMN activation with the development of PE.[6] These range from the original observations made by the Redman and Sargent group on excessive PMN activation in cases with PE,[134] which was greater than in matching cases with sepsis, to our own observations on the presence of NETs in affected placentae.[9] In addition,

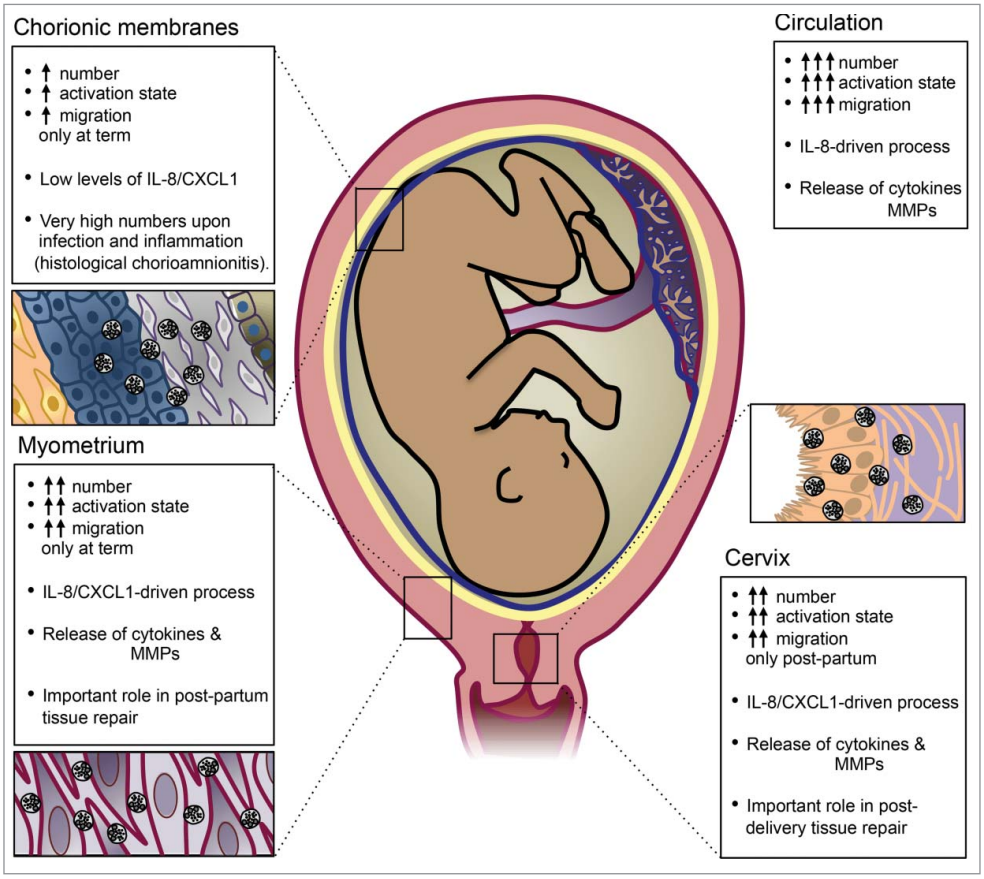

Figure 5. Contribution of PMNs to parturition. Neutrophils have multifaceted roles during parturition, either at term or preterm, and are attracted from the systemic circulation to the uterus by a process which is driven by IL-8 (top right) where they release cytokines and MMPs to contribute to labor and post-partum wound sealing and healing. PMNs infiltrate the human cervix only postpartum, again by chemotaxis to IL-8 and CXCL1 and play rather an important role in post-partum tissue repair (bottom right). PMNs infiltrate the human myometrium during term labor by similar conditions, i.e., cyto- and chemokine gradient-driven (bottom left). PMN numbers rise in the human chorioamniotic membranes modestly during term labor, which is consistent with the relatively low increase in the expression of IL-8 in the chorioamniotic membranes and the choriodecidua following term labor. PMNs are recruited in large numbers into the chorioamniotic membranes upon infection and inflammation (i.e., histological chorioamnionitis (top left). Figure adapted from Romero et al.[118]

deficient PP13 production may inadequately subvert PMN activity, thereby leading to inadequate modification of the maternal spiral arteries.[45,48]

Of considerable interest is the translation of animal model data suggesting that the interplay between the complement system and PMN may play a key role in the development of both PE[51,135] and RFL.[19] This has paved the way for the use of novel biologics targeting complement[136] or TNFα activity as therapies.[66] As such, the treatment of these disorders may finally enter the 21st century, making full use of cutting edge innovations.[47,137]

What remains to be discerned is a better understanding of how the underlying etiology contributes to PMN activation, and how the latter is involved in the disease pathology. This should focus on the fundamental etiological differences between early and late onset PE,[41] and include why such facets of obesity[138] or air pollution[139,140] contribute solely to the latter form of PE.

The recent finding that aPL can induce NETosis[62] begs the question whether this mechanism is active in RFL or in lupus induced PE-like conditions. This finding also suggests that PMN activation by aPL may involve both the complement system, as well as direct interaction by the PMN with the aPL antibodies. A clearer understanding of these 2 routes will assist in tailoring therapeutic options.

A final query of considerable interest is whether a direct link exists between RFL and PE. This is based on the observation that a high proportion of RFL cases successfully treated with heparin develop PE.[6] In these instances it will be very interesting to gain insight into the potential involvement of PMN in order to devise means of limiting aberrant activation.

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. Exciting times indeed for those interested in novel aspects of neutrophil biology.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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