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Interaction between endothelial cell-derived extracellular vesicles and monocytes: A potential link between vascular thrombosis and pregnancy-related morbidity in antiphospholipid syndrome

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ABSTRACT

Antiphospholipid syndrome (APS) is an autoimmune disease driven by a wide group of autoantibodies primarily directed against phospholipid-binding proteins (antiphospholipid antibodies). APS is defined by two main kinds of clinical manifestations: vascular thrombosis and pregnancy-related morbidity. In recent years, *in vitro* and *in vivo* assays, as well as the study of large groups of patients with APS, have led some authors to suggest that obstetric and vascular manifestations of the disease are probably the result of different pathogenic mechanisms. According to this hypothesis, the disease could be differentiated into two parallel entities: Vascular APS and obstetric APS. Thus, vascular APS is understood as an acquired thrombophilia in which a generalised phenomenon of endothelial activation and dysfunction (coupled with a triggering factor) causes thrombosis at any location. In contrast, obstetric APS seems to be due to an inflammatory phenomenon accompanied by trophoblast cell dysfunction. The recent approach to APS raises new issues; for instance, the mechanisms by which a single set of autoantibodies can lead to two different clinical entities are unclear. This review will address the monocyte, a cell with well-known roles in haemostasis and pregnancy, as a potential participant in vascular thrombosis and pregnancy-related morbidity in APS. We will discuss how in a steady state the monocyte-endothelial interaction occurs *via* extracellular vesicles (EVs), and how antiphospholipid antibodies, by inducing endothelial activation and dysfunction, may disturb this interaction to promote the release of monocyte-targeted procoagulant and inflammatory messages.

1. Introduction

Antiphospholipid antibodies (aPL) are a wide and heterogeneous group of autoantibodies directed against anionic phospholipids [1], phospholipid-binding proteins, or protein-phospholipid complexes [2]. Experimentally, aPL have been confirmed to be a cause of vascular thrombosis and adverse pregnancy outcomes, although the pathogenic mechanisms, as well as the exact antigenic specificities of the autoantibodies responsible for these phenomena, are still a subject of research.

The classical approach to antiphospholipid syndrome (APS), a clinical entity defined by the persistent presence of aPL along with the occurrence of vascular thrombosis and pregnancy-related morbidity [3], has been revisited in recent years. As the first insight into the pathophysiology of the disease, thrombosis and placenta infarction were suggested as the direct causes of obstetric disturbances [4], and aPL were thought to trigger a non-inflammatory hypercoagulable state [3]. In opposite to this view of APS, other authors have concluded that: a) In fact, thrombosis is not a common finding among patients with obstetric APS [5], b) Foetal loss and foetal growth restriction in animal models of APS can be attributed to inflammatory causes [6], and c) the favourable effect of heparin in murine models of aPL-driven pregnancy-related morbidity does not rely on its anticoagulant function, rather than on its

Abbreviations: APS, Antiphospholipid syndrome; aPL, Antiphospholipid antibodies; EVs, Extracellular vesicles; m/lEVs, Medium/large extracellular vesicles; sEVs, Small extracellular vesicles; TF, Tissue factor.

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capability to block the complement activation [7]. These findings, along with other research results from *in vitro* experiments [8], suggest that obstetric APS is not the direct consequence of a hypercoagulable state, but reflects other unrelated pathogenic mechanisms.

Not only the understanding of obstetric APS has been revisited, but also the conception of vascular APS. It is accepted that aPL can lead to thrombophilia through direct interaction with platelets, plasma proteins, and surface molecules such as heparan sulfate and annexin A5 [9–13]. However, there is increasing evidence of the prominent role that the activation of endothelial cells, monocytes and neutrophils also play in the course of aPL-driven thrombosis [14–18]. Therefore, the non-inflammatory feature of vascular APS, as the classification criteria stated [3], should be reconsidered [19].

To explain the causes underlying both types of clinical manifestations of APS, different effects have been attributed to aPL. Among these effects, recently it has been described a relationship between the presence of aPL and an increased quantity of extracellular vesicles (EVs) from monocytes, platelets and endothelial cells, in the plasma of patients with APS and cell cultures stimulated with aPL [20–22]. EVs are lipid bilayer fragments derived from cells that cannot divide by definition [23]. These fragments, notably those released directly from the cell membrane and sized between 0.1 and 2 μm (here referred to as medium/large EVs (m/lEVs)), exhibit a putative procoagulant activity [24].

Previously, we reviewed and discussed how EVs could explain the behaviour of vascular APS by means of procoagulant mechanisms involving direct activation of the coagulation cascade [25]. Nevertheless, our current results [26], and those of other authors [27–29], suggest that m/lEVs released in the presence of aPL exhibit a dampened direct coagulation activity. Furthermore, as outlined above, the procoagulant mechanisms by themselves are not suitable to explain the obstetric clinical manifestations of APS. In contrast, there is evidence describing that endothelial cell-derived EVs can pose a procoagulant and inflammatory message addressed to other cells, thereby performing as indirect mediators of vascular thrombosis and pregnancy-related morbidity. For instance, it has been shown that EVs released by endothelial cells stimulated with anti-β2-glycoprotein-I (aβ2GPI) antibodies (one of the best-described aPL) act as amplifiers of dysfunction and activation among unstimulated endothelial cells [30].

Although different cells can be recipients of EVs released by activated endothelium in the presence of aPL, the monocytes are receiving particular interest. Monocytes are well-known for their roles in haemostasis, clearance of apoptotic bodies, placentation, pregnancy and labour [31–35]. These characteristics make them essential for understanding the mechanisms by which aPL can lead to vascular thrombosis and pregnancy-related morbidity. The interaction between activated endothelium and monocytes *via* EVs, and its potential impacts on haemostasis and pregnancy, will be addressed in the present review.

2. Monocytes in haemostasis

Monocytes are myeloid cells of the mononuclear phagocyte system. It was argued during the 1960s that the most relevant function of monocytes should be the renewal of tissue macrophage populations, given their known capability to migrate into tissues and differentiate into this cell type [36]. It is now known that resident, tissue-specific, embryonic-derived macrophages have the potential to self-renew their populations, so new functions have been explored for bone marrow-derived monocytes produced in postnatal life (recently reviewed by [37]). Thus, monocytes cannot be considered cells in a transient state that require differentiation into macrophages or dendritic-like cells to fulfil specific functions. While it is clear that classical monocytes are able to transmigrate through the endothelium and carry out this differentiation, depending on the subpopulation to which they belong, these cells by themselves can fulfil other tasks. For instance, monocytes can perform as scavengers of apoptotic bodies [32], promoters of inflammation through the secretion of cytokines such as tumour necrosis factor-alpha (TNFα), interleukin (IL) 1β (IL-1β) and IL-6 [38], or they may patrol the blood vessel lumina and support tissue repair through pro-angiogenic functions [39].

Besides their role in the innate immune response and tissue repair, monocytes are actively involved in haemostasis. The paradigm of the activation of the coagulation cascade *in vivo* states that only when the integrity of endothelium is lost, tissue factor (TF) in the vascular wall is exposed for its interaction with platelets and plasma proteins, leading to the onset of primary and secondary haemostasis [40]. According to this idea, the absence of TF in cells that are in direct contact with plasma proteins of the coagulation cascade would ensure that blood did not undergo spontaneous clot activation. However, in later years, when collagen-coated sheets completely devoid of TF were perfused with normal human blood to assess the platelet adhesion phenomenon, immunostaining analysis of the resulting aggregates revealed the presence of abundant TF. The only feasible source of this TF could be blood ("blood-borne TF" in the author's words) [41]. Following the separation of various components of whole blood by centrifugation, density gradient, and immunoadsorption (platelets, granulocytes, mononuclear cells and monocytes), it was evident that the main sources of blood-borne TF are monocytes and m/lEVs [31].

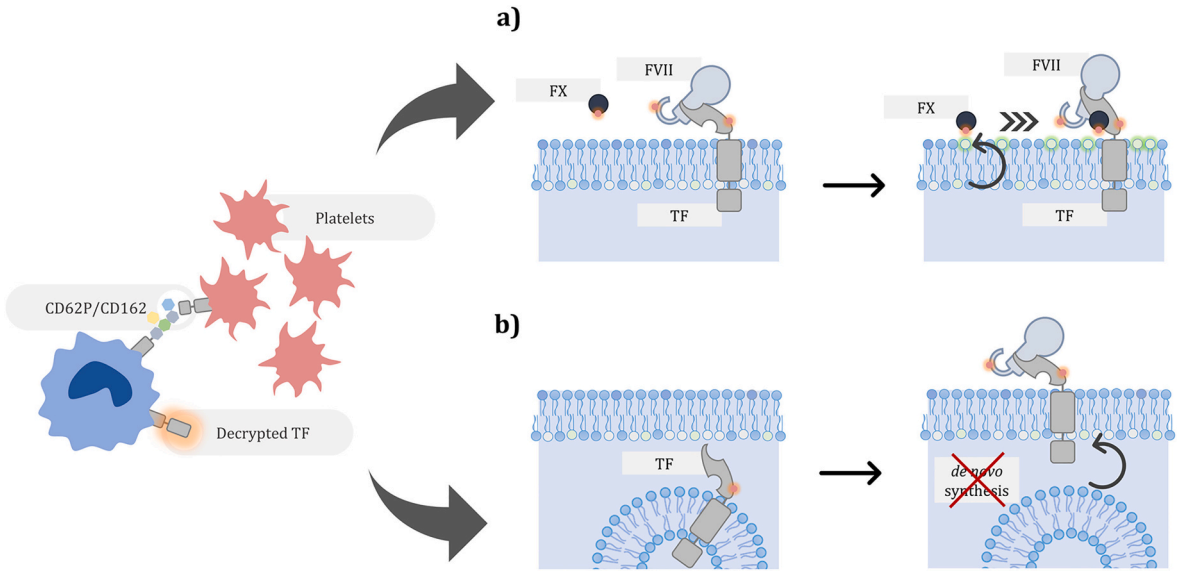
Since monocytes are in direct contact with the plasma components of the coagulation cascade and express TF, there must be mechanisms to restrict these cells from spontaneously triggering clot formation. In fact, most of the TF present on the monocyte membrane is encrypted and inactive, and its activity increases several-fold after an inflammatory or mitogenic stimulus in whole blood [31]. The functional consequences of TF encryption and decryption are clear, yet the molecular details of this process are still a subject of research. While it is thought that decryption may be due to changes in the molecular structure of TF, also, following certain stimuli, there is a rapid increase in membrane exposure of this protein, which is independent of *de novo* synthesis [42,43] (Box 1).

As described above, stimuli leading to TF decryption must be provided to monocytes in whole blood. The explanation given for this requirement is that the decryption process is triggered by interaction with CD62P (also known as P-selectin), which is an adhesion molecule presented to monocytes mainly by platelets [43,44]. The interaction between platelets and monocytes is bidirectional and has been described to be related to hypercoagulable states [45]. Recently, Hottz et al. described that platelets from critically ill COVID-19 patients activate monocytes from healthy individuals through interaction with CD62P, then upregulating the expression of TF in the first 2 h of co-culture. Reciprocally, monocytes enhance the activation of platelets, leading to the secretion of platelet-derived growth factor and soluble CD62P [45]. Critically ill COVID-19 patients have higher amounts of platelet/monocyte complexes in their blood compared to healthy volunteers, which is consistent with the increased risk of thrombosis that these patients exhibit [45]. Surprisingly, not only platelets, but also CD62P bound to 2 μm-sized polystyrene beads, is effective in increasing the TF exposed by monocytes [43], so it is feasible to hypothesise that EVs may perform a similar role.

Analysis of a large group of patients with a history of thrombosis showed that an increased blood monocyte count is related to a high risk of venous thrombosis, as opposed to the number of other leukocytes [46]. Moreover, the significance of monocytes, and their interaction with the activated (but undamaged) endothelium, has been proven in animal models of thrombosis [47]. In a murine model attempting to recapitulate deep vein thrombosis induced by blood flow restriction, von Brühl et al. demonstrated that, even in the absence of endothelial disruption, leukocytes roll and adhere massively to the luminal surface of the affected blood vessel. This interaction between endothelium and leukocytes is mediated by CD62P and precedes the activation of the coagulation cascade in a TF-dependent manner [47]. Although it is known that activated endothelium also expresses TF and that, in fact, the largest proportion of endothelium-recruited leukocytes are neutrophils, using a bone marrow transplant-based chimeric model and a

Box 1
Encryption and decryption of tissue factor.

Although the major proportion of the TF of monocytes is found on their surfaces, it represents less than a quarter of the total TF activity that can be reached from the cell lysate. From this fact arises the theory that TF remains encrypted on the surface of monocytes [44]. The latent TF activity can be unlocked by stimulating monocytes in the presence of platelets in a CD62P-dependent process. **a)** TF decryption may be the result of changes in the conformation or orientation of the extracellular domain of the protein upon exposure to anionic phospholipids on the surface of monocytes. These changes would enable the coupling of FX (the substrate of TF in the Xase complex). This hypothesis has been supported by the finding that the extracellular domain of TF remains bound to the cell membrane *via* anionic phospholipids, even in its soluble form (and in complex or not with FVII). The specific amino acid residues involved in this interaction are in close proximity to the exosite presumed to be responsible for the binding of FX [42]. **b)** Also, as soon as 5 min after platelet activation in whole blood, platelets enhance the exposure of membrane TF in monocytes. Rapid surface exposure of TF occurs even in cells in which *de novo* synthesis of proteins is blocked, demonstrating that TF comes from a pool that is ready to be mobilised upon cell stimulation [43].



recombinase-based conditional allele, it was found that TF from monocytes is the main contributor to the activation of the coagulation cascade in deep vein thrombosis [47] (Fig. 1).

In addition to promoting thrombus formation under certain conditions, monocytes also fulfil other functions in haemostasis. For example, it has been shown that, through a TF-dependent mechanism, activated monocytes participate in the contraction of the clot, once it has been formed, thereby reducing its volume [48]. Furthermore, monocytes can expose a dimeric form of factor XIII (FXIII) on their membrane upon stimulation with IL-10 and IL-4. This enzyme is a transglutaminase that provides stability to the clot and protects it from fibrinolytic degradation [49].

3. Monocytes in pregnancy

Healthy pregnancy involves changes in the maternal immune response, comprising both inflammatory and tolerogenic effector mechanisms, adjusted according to a specific timing (reviewed by [50]). Not surprisingly, the monocyte population undergoes changes over the course of pregnancy: The percentage of classical monocytes decreases progressively, probably due to the recruitment of a substantial number of these cells to the foetoplacental barrier for their differentiation into macrophages. As compensation, intermediate monocytes increase in number, while the proportion of non-classical monocytes remains unchanged [51]. This gradual reorganisation of monocyte subpopulations is accompanied by changes in their phenotype, denoting increased activation, differentiation, and adhesion potential. In this regard, classical monocytes show an increased expression of the adhesion molecule CD54. In intermediate monocytes, expression of the lipopolysaccharide (LPS) co-receptor CD14 and the C—C chemokine receptor type 2 (CCR2) is up-regulated. Finally, the integrin CD11b, the fragment crystallizable-gamma receptor I (FcγRI/CD64), and the granulocyte-macrophage colony-stimulating factor receptor CD116 are expressed at increased levels on both classical and intermediate monocytes [51]. Despite these changes, and probably due to a tolerance phenomenon upon a significant activation, the responsiveness of peripheral blood monocytes to toll-like receptor (TLR)-4, TLR7 and TLR8 ligands is progressively reduced, which is reflected, for instance, in a dampened capacity of these cells to secrete TNFα and IL-6 upon stimulation with LPS [51,52].

Besides the changes described above, there are several phenomena in early and late normal pregnancy that involve cytokine- and adhesion molecule-mediated communication between monocytes and other cells, such as trophoblast cells, stromal cells of the decidua, or smooth muscle cells of the myometrium. During early pregnancy, decidua becomes the environment for direct interaction between extravillous trophoblast cells and the maternal immune cells. First-trimester trophoblast cells express and secrete the chemokine C-X-C motif ligand 16 (CXCL16),

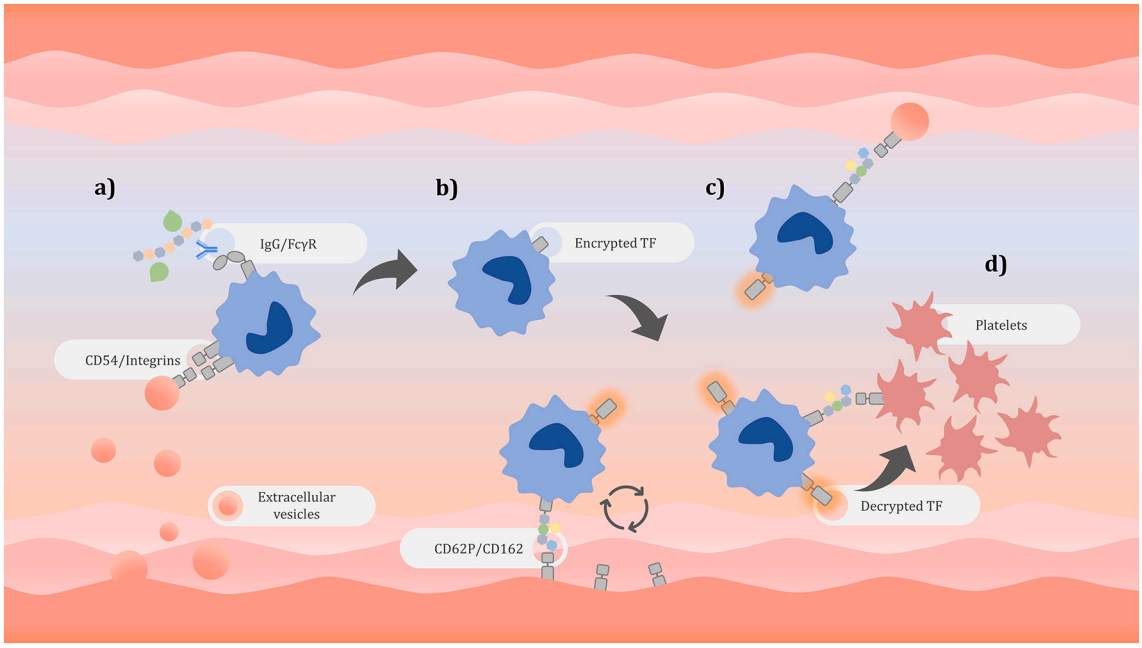


Fig. 1. Mechanisms by which monocytes are involved in haemostasis. a) Monocytes can be activated by different stimuli such as immune complexes (through their interaction with Fc receptors), or endothelial cell-derived EVs that carry adhesion molecules (through their interaction with integrins). b) In response to these stimuli, monocytes increase their expression of TF. Although monocytes are a major source of blood-borne TF, the vast majority of this membrane protein remains encrypted and inactive. c) Interaction of CD162 with its ligand, CD62P, rapidly increase TF exposure and TF-dependent coagulation activity in monocytes, even in the absence of *de novo* protein expression (decryption). CD62P can be presented by platelets, synthetic particles (and probably EVs), or activated endothelium. In the latter case, monocytes roll across the vessel surface and are deposited along with other leukocytes to initiate thrombus formation. d) Increased TF activity leads to thrombin production, which in turn stimulates platelets *via* protease-activated receptors. IgG, Immunoglobulin G; FcγR, crystallisable fragment γ receptor; TF, tissue factor.

whose receptor, C-X-C chemokine receptor type 6 (CXCR6), is expressed by monocytes, thereby attracting them to the decidua [33]. Dendritic cells derived from the recruited monocytes undergo functional reprogramming by decidual stromal cells in a process mediated by IL-1β and granulocyte colony-stimulating factor. Accordingly, the cytokine profile secreted by monocyte-derived dendritic cells is modified, increasing their secretion of IL-1β, IL-6 and IL-10, and decreasing their secretion of IL-8 and TNFα. As a result of those changes, the capability of these cells to prime lymphocytes towards a Th2 profile is improved [53]. Another major site of interaction between peripheral blood monocytes and the placenta is the apical surface of the syncytiotrophoblast that composes the chorionic villi. Close physical contact between monocytes and villous trophoblast cells occurs through interaction between the membrane-bound form of the chemokine C-X3-C motif ligand 1 (CX3CL1/fractalkine) (expressed by trophoblast cells), and its receptor, C-X3-C chemokine receptor type 1 (CX3CR1) (expressed by monocytes) [35]. More recently, the same interaction system was shown to work in extravillous trophoblast cells [54]. Finally, it has been described the capability of monocytes to induce contraction of myometrial myocytes through direct interaction, in a process involving IL-6 and IL-8 [34].

The implications of monocyte-specific changes during pregnancy, as well as the interactions between these and trophoblast cells, are not yet completely understood. However, the relevance of these processes is clear once they are disturbed in the context of diseases such as pre-eclampsia [55,56]. This scenario is of particular interest in the study of APS, as pre-eclampsia is one of the clinical manifestations involved in obstetric APS [3].

Monocytes from women with pre-eclampsia exhibit increased TLR4 expression and increased endogenous activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [57]. Consequently, these monocytes show enhanced gene expression of inflammasome- and TLR4 pathway-related proteins such as NOD-like receptor pyrin domain-containing-3 (NLRP3), caspase 1, IL-1β and TNFα [56]. In general, these features are in line with a systemic inflammatory response. Indeed, compared to monocytes from normotensive pregnant women, monocytes from women with pre-eclampsia respond less vigorously to LPS *ex vivo*, demonstrating the development of a tolerance phenomenon towards inflammatory stimuli [57]. Unfortunately, it is not possible to conclude whether the aforementioned monocyte activation is a cause or a consequence of pre-eclampsia based on the design of this research.

A potential mechanism of pregnancy-related morbidity in which monocytes have been involved is the production of anti-angiogenic factors such as the soluble vascular endothelial growth factor receptor 1 (sVEGFR1/sFlt-1). This molecule has been specifically linked to the development of pre-eclampsia [58] and is suspected to impair the placentation process by suppressing uterine spiral artery remodelling [59] and reducing the number of foetal capillaries [60]. In a transgenic mouse model with an inducible tet-based allele encoding human sFlt-1, expression of this protein during early pregnancy (between 7.5 days- and 10.5 days-*post coitum*, corresponding to the placentation process) triggered foetal growth restriction [60]. This finding was accompanied by severe defects in the formed placenta (18.5 days *post coitum*). Hence, in the labyrinthine region (an area similar to the chorionic villi of the human placenta, responsible for nutrient exchange), isolated endothelial cells were found instead of well-formed foetal capillaries [60]. Now, so far it has been described how the interaction between platelets and monocytes enhances the coagulation activity of monocytes. Also, the presence of aggregates between platelets and monocytes has been found in patients with pre-eclampsia. Unlike monocytes from pregnant controls, these aggregates release sFlt-1. *Ex vivo* assays have confirmed that thrombin-activated platelets induce sFlt-1 production by monocytes [61].

Activation of complement is another trigger that has been involved in the production of sFlt-1 by monocytes in the context of pregnancy-related morbidity. As will be discussed below, this fact is promissory in the study of APS, as findings in animal models with obstetric manifestations induced by passive transfer of aPL suggest the participation of complement in the pathophysiology of the disease. In a model of

immune-mediated recurrent pregnancy loss arising from the mating of two inbred mouse strains, Girardi et al. found deposits of complement protein C3 and abundant monocyte infiltrates in the decidua [62]. These findings were overlaid with a reduction in free vascular endothelial growth factor during pregnancy. Ultimately, the authors found that anaphylatoxin C5a induces monocyte infiltration into the decidua and production of sFlt-1 by such cells, which triggers foetal resorption [62]. In earlier years, the same research group reported that pregnancy-related morbidity induced in mice by the infusion of immunoglobulin G (IgG) from aPL-positive patients is mediated by C5a and its receptor [6]. Furthermore, using the same animal model, they demonstrated that a synthetic molecule similar to low molecular weight heparins, which recapitulates the anticoagulant capacity of the pentasaccharide structure but lacks its other effects, fails to prevent foetal resorption, suggesting that the protective capability of heparin in APS depends on its complement inhibitory properties rather than its antithrombotic properties [7].

Disruption of the CX3CL1/CX3CR1 system has also been implicated in the development of pregnancy-related morbidity. As mentioned earlier, this system mediates the interaction between monocytes and villous and extravillous trophoblast cells. Analysis of placentas from patients with early-onset pre-eclampsia shows increased gene expression and increased amount of CX3CL1 protein in trophoblast cells and endothelial cells [55]. In fact, the higher the expression of CX3CL1 in placental tissue at the time of delivery, the higher the proportion of adverse clinical findings related to pre-eclampsia [63]. Also, the villi of first-trimester placentas are known to upregulate CX3CL1 expression upon stimulation with TNFα [55]. Overexpression of CX3CL1 in extravillous trophoblast leads to increased adhesion of monocytes, as well as increased secretion of inflammatory cytokines such as IL-8, chemokine C—C motif ligand 19 (CCL19) and chemokine C—C motif ligand 13 (CCL13) by the co-culture [54] (Fig. 2).

4. Extracellular vesicles and monocytes

A hallmark of aPL-stimulated animal models is the development of

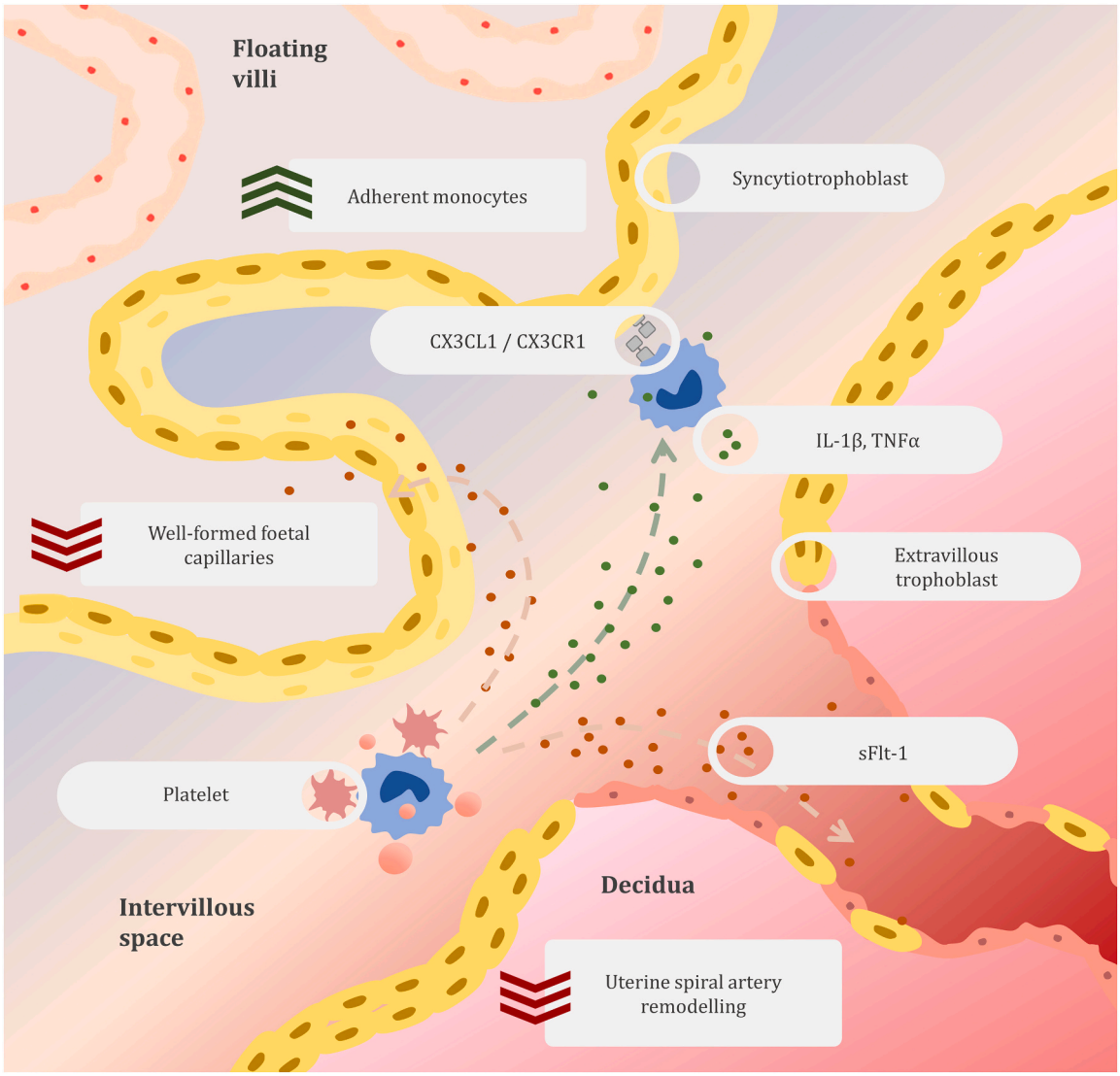


Fig. 2. Monocyte-mediated mechanisms of pregnancy-related morbidity. Activated monocytes, *e.g.*, upon interaction with activated platelets, or with immune complexes formed by EVs and immunoglobulin G, express increased amounts of CX3CR1, and secrete inflammatory cytokines such as IL-1β, TNFα, and the anti-angiogenic factor sFlt-1. TNFα induces overexpression of CX3CL1 on the apical surface of chorionic villi resulting in enhanced adhesion of monocytes and the development of an inflammatory environment. sFlt-1 disrupts normal placentation by reducing foetal capillary formation and suppressing uterine spiral artery remodelling. IL-1β, interleukin 1β; TNFα, tumour necrosis factor-α; sFlt-1, soluble vascular endothelial growth factor receptor; CX3CL1, chemokine C-X3-C motif ligand 1; CX3CR1, C-X3-C motif chemokine receptor type 1.

widespread endothelial activation and dysfunction. After passive transfer of IgG from APS patients, murine blood vessels become less responsive to acetylcholine-mediated relaxation, endothelial nitric oxide synthase (eNOS) gene expression is downregulated, TF expression is upregulated, there is a drop in the ratio of phosphorylated to non-phosphorylated eNOS, plasma levels of soluble *E*-selectin/CD62E and sVCAM-1/CD54 increase, and endogenous thrombin potential rises [15]. If IgG infusion is coupled with a second stimulus (such as LPS or iron chloride), antibodies and complement protein C3 are deposited in the vascular beds within a few minutes. Consequently, thrombi become apparent in murine blood vessels, *e.g.*, in mesenteric capillaries [2,64].

In addition to upregulating its expression of TF and adhesion molecules such as CD106, CD54 and CD62E, aPL-activated endothelium releases an increased number of m/lEVs [22]. As mentioned above, m/lEVs are one of the main sources of TF [31,65]. Moreover, EVs increase the total surface area of anionic phospholipids available for the assembly of prothrombinase complexes and FX-activating complexes (Xase), so they are considered to be prothrombotic [66]. However, for reasons that will be discussed later, in the presence of aPL the direct coagulation activity of m/lEVs seems to be attenuated. Instead, m/lEVs released in the context of APS may provide a procoagulant and inflammatory message to other cells, namely monocytes.

Quiescent endothelium constantly delivers signals to monocytes *via* EVs. When monocytes are co-cultured with endothelial cells in an experimental setting that prevents direct contact between the two cell types, after 24 h, the monocytes have taken up fragments of the endothelial cell membrane [67]. The transfer of EVs-related material from endothelial cells to monocytes includes miRNAs that have the capability to polarise monocyte responses to LPS, preventing an inflammatory activation and favouring an immunomodulatory response [67]. Using an animal model of peritonitis in which leukocytes were challenged with LPS, Njock et al. described that EVs released by quiescent endothelium can reduce the gene expression of TNFα, IL-1β and inducible nitric oxide synthase (iNOS), and upregulate the expression of arginase 1, in peritoneal cells. These effects were attributed to the transfer of miRNA-10a, miRNA-126-3p and miRNA-181b, which are three components of quiescent endothelial cells that control NF-κB- and interferon regulatory factor 5 (IRF5)-related gene expression in monocytes [67].

Notably, the transfer of miRNA-126 may be involved in the regulation of haemostasis, not only because it restricts leukocyte adhesion to the endothelium by controlling the expression of molecules such as CD106 [68,69], but also because it constrains TF gene expression by binding to the 3′ untranslated region of the transcript encoding for this protein [70]. Transfection of monocytes with miRNA-126 reduces their coagulation activity at baseline and under stimulation with LPS [70]. Remarkably, miRNA profiling of small EVs (sEVs) released by endothelial cells upon stimulation with aβ2GPI antibodies *in vitro* shows a statistically significant decrease in the amount of loaded miRNA-126 [30]. sEVs constitute a subpopulation of EVs smaller than 0.1 μm that is presumed to be produced in multivesicular bodies.

In the same way that resting endothelium delivers immunomodulatory signals to monocytes, activated endothelium can deliver procoagulant and inflammatory messages to the same cells. For instance, endothelial cells stimulated with TNFα release m/lEVs and sEVs that carry the adhesion molecules of their respective cells of origin [71]. Because of this, EVs are able to interact with monocytes in a process that does not require the fusion of membranes [72]. Thus, the binding of CD54 (present on the surface of endothelial cell-derived EVs) to integrins (present on the surface of monocytes) has been shown to trigger an increment in TF gene expression and, finally, an enhancement of the TF-dependent coagulation activity of monocytes [72].

Endothelial cell-derived m/lEVs not only can perform as a procoagulant message but also as an inflammatory message. When endothelial cells are placed under stress conditions of hypoxia and serum starvation, released m/lEVs upregulate the expression of IL-1β, TNFα, IL-6 and IL-8 in monocytes [73]. Also, these vesicles promote in monocytes an improved adhesion potential to fibronectin surfaces and enhance their capacity to phagocytose oxidised lipoproteins, resulting in their differentiation into foam cells [73]. Such interactions are probably mediated by adhesion molecules and may be important in explaining the pathophysiology of thrombosis. For instance, patients with pulmonary thromboembolism, compared to healthy volunteers, exhibit a higher number of plasma endothelial cell-derived m/lEVs, CD62E-positive endothelial m/lEVs, and conjugates between m/lEVs and monocytes [74]. Endothelial cells stimulated *in vitro* with IgG from aPL-positive patients release a higher quantity of CD62E-positive m/lEVs compared to endothelial cells stimulated with IgG from healthy volunteers [22] (Fig. 3).

5. The hypothesis of immune complexes and the lupus anticoagulant paradox

A functional property of some aPL is their potential to extend clotting times *in vitro*. Autoantibodies that share this feature have been collectively referred to as "lupus anticoagulant". Although lupus anticoagulant was originally thought to result from the capability of antibodies directed against β2GPI (in complex with its antigenic protein) to occupy and block the anionic phospholipid sites necessary for the assembly of the complexes of the coagulation cascade [75,76], this theory has been challenged in recent years [77]. For this reason, new mechanisms to explain the lupus anticoagulant phenomenon are currently being explored [78].

The "lupus anticoagulant paradox" (as it has been named by other authors [79]) arises from the finding that detection of lupus anticoagulant-like antibodies better predicts a potential first episode of thrombosis, or adverse pregnancy outcomes, than the isolated assessment of any single aPL with a given antigenic specificity [80–82]. The reason why the functional properties of autoantibodies to behave as anticoagulants *in vitro* relate to their potential to trigger thrombosis and pregnancy-related morbidity *in vivo* remains unclear. The interaction between monocytes and endothelial cell-derived EVs may explain this phenomenon.

Some authors have reported that aPL-positive patients exhibit a high proportion of phosphatidylserine-negative m/lEVs [21,83]. In fact, although patients with aPL have greater levels of plasma m/lEVs compared to healthy volunteers, this is not mirrored by a greater amount of anionic phospholipids available for thrombin formation [28], nor by an increased TF-like activity associated with m/lEVs [27]. Recently, we have shown that the coagulation activity of m/lEVs released *in vitro* by endothelial cells stimulated with IgG from aPL-positive patients is attenuated by direct interaction with lupus anticoagulant-like autoantibodies [26]. In parallel, Mobarrez et al. identified that patients with systemic lupus erythematosus and the presence of aβ2GPI antibodies have a decreased number of β2GPI-positive m/lEVs [29]. These results suggest that m/lEVs in the presence of lupus anticoagulant-like antibodies may probably form immune complexes in which both phosphatidylserine and β2GPI in the surface of vesicles are hidden. The relationship between such immune complexes, vascular thrombosis, and pregnancy-related morbidity could be explained by how immune complexes interact with monocytes.

One of the proposed roles of β2GPI is to facilitate EVs clearance by phagocytes [85]. β2GPI has a phosphatidylserine-binding domain (domain 5) that allows the protein to tag EVs [77], whilst its domain 1 is recognised by low-density lipoprotein receptors [86]. In this regard, aβ2GPI antibodies (especially the more pathogenic ones, *i.e.* those targeting domain 1 [2]) would hinder the clearance of EVs. Beyond the consequent accumulation of EVs, it is known that recognition of immune complexes comprising IgG through Fc receptors (FcR) can induce monocyte activation, leading to the secretion of inflammatory cytokines and triggering the onset of the coagulation cascade [87,88].

Besides APS, another disease in which antibody-mediated thrombosis occurs, and which exemplifies the relationship between immune

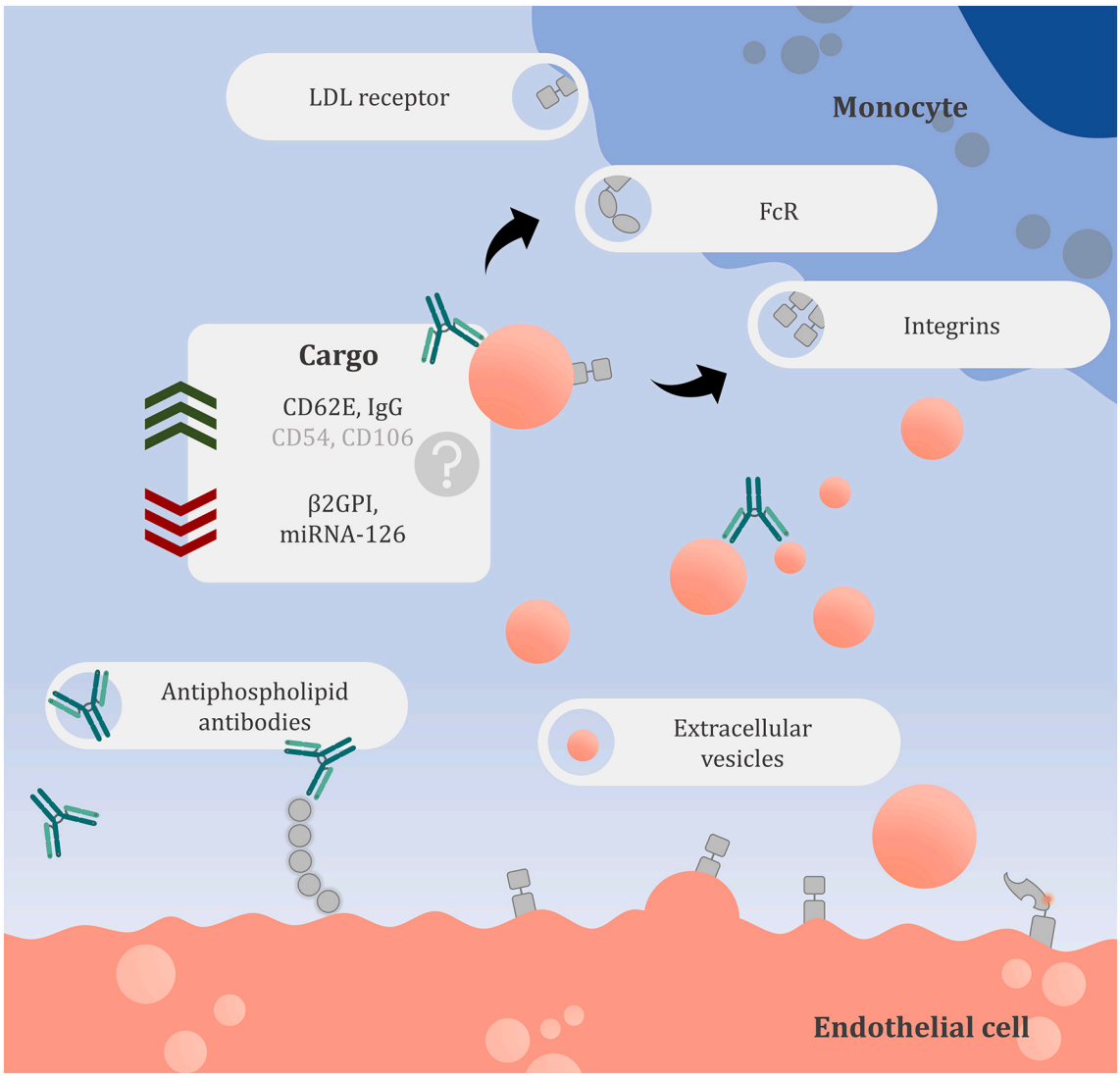


Fig. 3. Potential mechanisms of interaction between endothelial cell-derived EVs and monocytes in antiphospholipid syndrome. Antiphospholipid antibodies lead to a state of endothelial activation and dysfunction that is accompanied by the release of EVs. In this context, analysis of EVs has revealed increased expression of CD62E and decreased expression of β2GPI and phosphatidylserine. Also, endothelial cell-derived sEVs from aPL-stimulated endothelial cells carry a decreased amount of miRNA-126. Other adhesion molecules of endothelial origin such as CD106 and CD54 might be present in these structures. According to the described features, EVs would interact with monocytes by binding to integrins or Fc receptors, while their clearance mediated by low-density lipoprotein receptors would be impaired. IgG, immunoglobulin G; miRNA, micro-RNA; FcR, Fc receptors; β2GPI, β2-glycoprotein-I; LDL receptor, low-density lipoprotein receptor.

response and haemostasis, is heparin-induced thrombocytopenia. In this disease, heparin administration induces the production of antibodies that recognise complexes between platelet factor 4 and heparin or cell membrane glycosaminoglycans [89]. Clot activation in heparin-induced thrombocytopenia requires the presence of monocytes. Immune complexes comprising platelet factor 4, heparin, and IgG, are recognised by the FcγRIIA on monocytes, then inducing thrombin generation *via* the TF pathway. Thrombin, in turn, activates platelets, which leads to their coating with FX and increases their clotting activity [87]. The case of heparin-induced thrombocytopenia may help to better understand APS and other diseases characterised by immuno-thrombosis.

Specifically, the potential of immune complexes comprising m/lEVs and autoantibodies to activate monocytes in the context of autoimmune diseases has already been addressed by other authors. Burbano et al. isolated immune complexes composed of platelet-derived m/lEVs and IgG from patients with systemic lupus erythematosus. These complexes activated monocytes *in vitro*, as demonstrated by increased expression of CD64 and CD69 markers, and secretion of inflammatory cytokines such as IL-1β, TNFα, IL-6 and IL-8 [88]. The same research group *in vitro* opsonised m/lEVs from platelets by incubating them with IgG from patients with seropositive rheumatoid arthritis. The resulting complexes upregulated in monocytes the expression of CX3CR1 [38].

6. Conclusions

New approaches to APS propose the existence of independent pathogenic mechanisms underlying the vascular and obstetric manifestations of the disease [90]. First, obstetric manifestations are not presumed to attend to placental infarction [5,6,8]. Secondly, although vascular APS has been explained by a non-inflammatory hypercoagulable state, emerging evidence demonstrates the involvement of leukocytes and endothelial cells in the development of thrombosis [16,18]. Amidst both clinical manifestations, the monocyte may constitute an important link.

In haemostasis, monocytes are an important source of blood-borne TF [31], bidirectionally interacting with platelets to induce clot activation [45], and then participating in clot contraction and stabilisation [48,49]. In pregnancy, the monocyte population undergoes changes that denote increased activation, although its responsiveness to

inflammatory stimuli decreases [51]. Employing cytokines or, in some cases, by direct contact, monocytes interact with villous and extravillous trophoblast cells [54,91], as well as with stromal cells of the decidua and myometrial myocytes [34,53]. Disturbances in these processes can underlie pregnancy-related morbidity [61,63].

Monocytes are thought to continuously receive signals from quiescent endothelium *via* EVs, which modulate their functions [67]. When endothelial cells are activated, such signals are turned into procoagulant and inflammatory messages that modify the phenotype and coagulation activity of monocytes [72]. Specifically, in APS, aPL have been identified as inducing a state of widespread endothelial dysfunction and activation [15]. Thus, endothelial cell-derived EVs released upon stimulation with aPL show features that suggest that they may activate monocytes, as the expression of adhesion molecules [22], the reduction in their cargo of key miRNAs involved in coagulation control [30], and the presumed formation of immune complexes with lupus anticoagulant-like autoantibodies [21,26,29]. Further research should address how the interaction between monocytes and EVs from aPL-activated endothelium links vascular thrombosis and pregnancy-related morbidity in APS.

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Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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