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Effects Of Antiphospholipid Antibodies On Trophoblast-Mediated Angiogenesis And Vascular Remodeling

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EFFECTS OF ANTIPHOSPHOLIPID ANTIBODIES ON TROPHOBLAST-MEDIATED ANGIOGENESIS AND VASCULAR REMODELING

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Women with antiphospholipid antibody syndrome (APS) are at increased risk for miscarriage and preeclampsia, which are associated with diminished trophoblast invasion and limited spiral artery transformation. Antiphospholipid antibodies (aPL) specifically target the placenta by binding trophoblast beta$_2$glycoprotein I (β_2GPI). Thus, we determined whether aPLs alter trophoblast angiogenic factor production, endothelial tube formation, and trophoblast-endothelial cell interactions, and evaluated the effect of low molecular weight heparin (LMWH) on these responses. Human first trimester trophoblast produced significantly more VEGF, PlGF and sEng following exposure to aPL. This occurred independently of TLR4 and in both MyD88-dependent and independent manners. LMWH was unable to reverse the effects of the aPL on trophoblast VEGF and sEng production, and enhanced PlGF secretion. Strikingly, sFlt-1 secretion in the untreated and aPL-treated cells was increased in the presence of LMWH. However, aPL did not alter endothelial tube formation or trophoblast-endothelial cell interactions, nor did LMWH have an impact. In conclusion, this study demonstrates that aPL modulate the normal production of trophoblast angiogenic factors, that LMWH cannot reverse this effect, and LMWH may even enhance it. However, this altered angiogenic factor profile may not directly impact trophoblast-endothelial interactions/remodeling, suggesting that other mechanisms may be involved.

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Introduction

The Antiphospholipid Antibody Syndrome (APS) is a systemic autoimmune disorder characterized by venous and arterial thrombophilia, thrombocytopenia, premature stroke and cardiovascular problems [1]. Women with APS are at high risk for recurrent pregnancy loss in the first or second trimester, preterm labor, intrauterine growth restriction (IUGR), systemic and pulmonary hypertension and preeclampsia [2, 3]. Heparin has been used successfully to maintain the pregnancy in these women but has not been shown to prevent or reduce the risk of late stage complications like preeclampsia [3, 4]. In fact, beyond the use of heparin, aspirin [4-6] and occasionally IVIG [7, 8], treatment for APS remains limited. Due to this need for further understanding of the pathogenesis of these complications in order to improve therapeutic measures, APS, especially obstetric APS remains an area of prolific study [9].

APS can be categorized as primary or secondary. It is considered to be primary in patients with recurrent venous or arterial occlusions, recurrent fetal loss, or thrombocytopenia in the presence of antiphospholipid antibodies (aPLs) but not other features of distinct autoimmune disorders. Whereas secondary APS is thrombophilia associated with the presence of aPLs, as well as a chronic inflammatory or autoimmune disorder such as Rheumatoid Arthritis (RA) or systemic lupus erythematosus (SLE) [10]. The presence of aPLs, usually identified as lupus anticoagulant (LA) and anticardiolipin antibodies (ACA), in patients with thrombosis is not necessarily pathognomonic of APS given that there may be transient elevations [11]. For standardization and consistency, there are established guidelines to make the diagnosis of APS, known as the Sapporo Classification Criteria. The Sapporo Classification Criteria consist of predetermined

clinical and laboratory criteria, with definitive APS involving the presence of at least one clinical and one laboratory criteria [11, 12].

The current clinical criteria include: arterial, venous, or small vessel thrombosis, in any organ or tissue; and pregnancy morbidity consisting of 1) one or more unexplained deaths of a morphologically normal fetus at or beyond the 10^{th} week of gestation, or 2) one or more premature births of morphologically normal neonate at or before the 34^{th} week of gestation because of severe preeclampsia, eclampsia or severe placental insufficiency; or 3) three or more unexplained consecutive spontaneous abortions before the 10^{th} week of gestation. The updated laboratory criteria include: medium to high serum titers (greater than 40 GPL/MPL or > 99^{th} percentile) of IgG or IgM isotype of ACA on two or more occasions, at least 12 weeks apart measured by standardized ELISA; LA present in plasma on two or more occasions at least 12 weeks apart detected by the guidelines of the International Society of Thrombosis and Haemostasis; and Anti-beta$_2$ glycoprotein-I (anti-β_2GPI) antibody of IgG or IgM isotype in serum or plasma (>99^{th} percentile), present on two or more occasions 12 weeks apart, measured by standardized ELISA [12].

As mentioned above, in APS, the antiphospholipid antibodies implicated are LA and ACA [13]. Antiphospholipid antibodies are defined as autoantibodies produced against phospholipids (a class of lipids containing a negatively charged phosphate group). However, studies from various research groups have shown that antiphospholipid antibodies in APS do not react with cardiolipin or other phospholipids (PLs) directly but rather with serum proteins that bind to phospholipids, including beta$_2$glycoprotein-I

(β_2GPI), prothrombin, and proteins C and S [14, 15]. The cardiolipin binding serum protein recognized by ACAs is β_2GPI and it is the main pathogenic target for aPLs [16].

β_2GPI is a fifty kilodalton, largely glycosylated phospholipid binding protein. It is a member of the complement control family and as such contains five homologous domains, aka sushi domains [17]. While studies have demonstrated varied results, domain V is consistently thought to function to anchor β_2GPI to PLs and various cell surface receptors such as annexin II, Toll-like receptors (TLRs), and other negatively charged molecules such as heparin, DNA, low density oxidized lipoproteins and apoptotic cells [17, 18]. Its physiologic role is not well understood but it is thought to be a natural anticoagulant [19] and important for apoptotic cell clearance [20]. In APS, β_2GPI binds to cardiolipin and upon binding reveals an epitope(s) that is recognized by the ACAs [15, 21]. Domain I is generally agreed to be the major harbor of the epitope for binding pathogenic aPLs [22, 23], although domain IV has also been implicated [24]. The pathogenic antibodies in APS can thus be described as anti-β_2GPI autoantibodies [25] as they recognize and bind to β_2GPI.

Anti-β_2GPI antibodies are a heterogeneous group of polyclonal antibodies, with differing fine specificities [14, 26]. Studies have suggested that different epitopes on β_2GPI are recognized by different antibodies, with only some of these populations having pathological significance [14]. This may in part explain the clinical heterogeneity of APS. It has also been found that small changes in epitopes can have largely different functional/biological effects in terms of antibody binding and pathogenicity [27, 28].

Patients with APS are at risk for pregnancy complications [2]. This is because the placenta is a major target of aPLs. To understand why this is so, one must first look at normal placentation. After fertilization, the zygote undergoes a series of cleavages to form the blastocyst, which then implants into the decidua (pregnant endometrium). The blastocyst is surrounded by an outer layer of cells (the trophoectoderm), which develops into the placenta. Placental trophoblast cells are responsible for the ability of the blastocyst to implant. There are 3 stages of implantation: initial adhesion of the blastocyst to the uterine wall, followed by prolonged contact between the blastocyst and uterine epithelium. Lastly, there is invasion and infiltration of the endometrium by cytotrophoblasts (inner layer of trophoblasts). As the cytotrophoblasts invade deep into the maternal decidua they differentiate into extravillous trophoblasts, which proceed to invade into and remodel the uterine spiral arteries, turning them into low resistance, high capacitance vessels [29].

The trophoblast is an unusual cell type as it expresses cell membrane anionic PLs on their cell surface which can bind exogenous β_2GPI under normal physiologic conditions. The reason this is unusual is that PLs, being components of cell membranes, are not exposed under normal conditions in most cell types-rather, are seen in pathologic or apoptotic conditions. This expression of PLs on the trophoblast cell surface occurs because there is a high level of proliferation associated with this remodeling of the tissue [29, 30]. More importantly the placenta is a major target for anti-β_2GPI antibodies because the trophoblast synthesizes its own β_2GPI as demonstrated by mRNA transcripts isolated from normal human placental villous tissue, and translocates it to its cell surface [31]. In fact, *in vivo*, there is evidence of β_2GPI localized to the surface of the extravillous

trophoblast cells that invade the decidua, and to the syncytiotrophoblast cells that are in direct contact with maternal blood [31, 32]. Given this targeting of the placenta, it is no surprise that one sees obstetric complications with antiphospholipid antibodies.

Of the later obstetric complications associated with APS, preeclampsia is the most common and it can have devastating effects on both mother and fetus [33, 34]. Preeclampsia is a systemic disorder of pregnancy associated with new onset proteinuria and hypertension [35]. It is well established that the placenta is central to the problem as the only cure is delivery of the placenta, and in women with molar pregnancies (characterized by absence of the fetus), preeclampsia still occurs [33]. Clinically, preeclampsia is associated with pathologic signs of placental hypoperfusion and ischemia, leading to vascular endothelial stress. It is hypothesized that preeclampsia is caused by abnormal placentation especially early abnormalities in placental vascular remodeling [35] and in fact the placentae from women with severe preeclampsia are characterized by shallow invasion of cytotrophoblasts and impaired spiral artery remodeling with accompanying inflammatory infiltrate [36-38].

Normal placentation is dependent on the processes of vasculogenesis, angiogenesis and vascular transformation. Vasculogenesis is the formation of blood vessels by *in situ* differentiation of endothelial cells, which then join to form patent tubes [39]. Angiogenesis is the sprouting of new vessels from pre-existing vessels [40]. Vasculogenesis begins early, at about gestational week three and then gives way to angiogenesis at a still unknown time point. By the third trimester, angiogenesis is the main mechanism for growth of the placenta. Vascular transformation is the process

whereby the spiral arteries are invaded by extravillous trophoblasts, which disrupt the smooth muscle of the spiral arteries. This transforms them into high flow, low resistance vessels [41]. Partial failure of this process has been implicated in the pathogenesis of pregnancy complications associated with insufficient placentation, such as preeclampsia [42].

Angiogenic factors are produced by the placenta [43] to modulate these essential processes. Vascular endothelial growth factor (VEGF), and placental growth factor (PlGF) are both related proteins produced by the placenta, and act as pro-angiogenic factors and endothelial cell mitogens [44]. Studies have also shown that heterodimers of VEGF and PlGF act as pro-angiogenic factors [45, 46]. Both VEGF and PlGF interact with fms like tyrosine kinase receptor 1 (Flt-1), whereas VEGF and the VEGF-PlGF heterodimer can also bind the kinase domain receptor (KDR) [45].

The placenta also produces circulating anti-angiogenic factors to modulate the systemic and local effects of VEGF and PlGF, and thereby control angiogenesis [47]. One such factor is a soluble version of the VEGF receptor Flt-1 (sFlt-1). Initially described by Kendall and Thomas, it is a truncated version of Flt-1 lacking downstream signaling, and which binds and sequesters VEGF and PlGF and thus acts as an antagonist to both pro-angiogenic factors [47]. Another anti-angiogenic factor produced is soluble Endoglin (sEng). Surface Endoglin (Eng) is a membrane bound receptor expressed on the surface of endothelial cells and placental syncytiotrophoblasts, which binds transforming growth factor beta (TGF-β). TGF-β is a potent angiogenic factor, which acts to upregulate VEGF in early gestational trophoblasts [48]. sEng is a truncated, soluble form of Eng also without downstream signaling which acts to antagonize TGF-β [49].

In both preeclampsia and APS there is dysregulation of angiogenic factor production. In preeclampsia, studies have observed a downregulation of circulating VEGF, and PlGF and a marked increase in sFlt1 and sEng [33], to the extent that the levels of sFlt-1, sEng and PlGF used together are more strongly predictive of preeclampsia than other biomarkers [50]. However, placental VEGF levels have also been shown to be increased in preeclampsia [51]. Patients with APS are also found to have altered angiogenic factor levels, namely elevated PlGF and VEGF [52-54] and *in vitro* studies show decreased VEGF in endothelial cells [55]. However, there continues to be uncertainty as to relevance of these altered levels to the pathogenesis of the disease.

Women in APS are at increased risk for thrombosis, which is thought to be triggered by aPLs [56]. Indeed there exist several mechanisms proposed by which aPLs cause thrombosis [57-60]. It was thus thought that the primary mechanism underlying APS mediated pregnancy morbidity was thrombotic episodes occurring at the maternal-fetal interface [5, 61]. The use of the anticoagulant heparin to prolong pregnancy and prevent recurrent pregnancy loss [6, 62-67] seemed to corroborate this theory as well. However, in a study by Sebire et al., 135 products of conception from early pregnancy failures were examined histologically- 31 from women with APS, 50 from women without, 34 with aneuploidy and 20 control cases at 6-14 weeks gestation- and it was found that there were no increases in the proportion of cases with thrombotic events in the APS positive group compared with the others. In fact, what was most often seen in APS positive samples was defective endovascular trophoblast invasion and reduced transformation of the spiral arteries [68], much like what is seen in clinical preeclampsia. In addition, one study has noted that the decidua of these patients become infiltrated by

inflammatory immune cells, such as macrophages [69]. This suggests that histologically there appears to be more of a pro-inflammatory/insufficient trophoblast invasion profile rather than a pro-thrombotic one. The etiology of aPL-mediated pregnancy loss is likely multifactorial, and there exists within the literature multiple proposals and models to explain this phenomenon. What unifies all these different theories is that they have recently moved beyond the simple infarction/ thrombosis notion and all propose various ways the aPLs themselves change or alter the environment at the maternal-fetal interface and in many cases propose a pro-inflammatory mechanism [70].

Animal models have supported a role for aPLs in the pathogenesis of pregnancy failure and pregnancy complications. There have been studies from several groups which have shown that either immunization of animals with β_2GPI or the passive transfer of aPLs to mice promotes resorption of the fetus, fetal demise, reduced litter sizes and IUGR [71-73]. Furthermore, a study by Robertson et al. showed that passive transfer of human anti-β_2GPI I Abs to β_2GPI+/+ mice triggered fetal loss and that mice deficient in β_2GPI (β_2GPI−/−) were resistant to this loss [74]. This study highlighted the pivotal role of β_2GPI in APS. Salmon and Girardi also showed in their *in vivo* mouse models that antiphospholipid antibodies are able to activate both classical and alternative complement pathways which then propagate injury to the developing fetus by recruiting inflammatory neutrophils and overwhelming the trophoblast's normal inhibitory mechanisms [30, 75].

Their proposed mechanism of action of aPLs is that they activate the classical complement pathway, which leads to the generation of C3a and C5a, as well as a rapid increase in levels of decidual and systemic TNF-α. Inflammatory cells are recruited and

they accelerate the local alternative pathway activation. This creates a pro-inflammatory amplification loop, which enhances C3 activation and deposition, generates more C3a and C5a, and in turn causes further influx of inflammatory cells into the placenta. This results in death or damage to the developing fetus [75-77]. Treatment with unfractionated or low molecular weight heparin protects pregnancies from aPL-induced damage even at sub-therapeutic doses that do not prevent coagulation [78].

The major limitation of this model is that the antibodies used to study this effect was used in concentrations greater than a thousand fold what was used in other mouse APS models[71-73], which may bias the system towards a complement model. Also, they injected the antibodies halfway through the pregnancy, while other studies immunized the mice with the antibodies prior to mating [71-73]. Immunization prior to mating is more true to real life conditions where the autoantibodies are circulating prior to conception and implantation. By injecting halfway through pregnancy, the model is not being true to reality, and the impact of the circulating antibodies on the development of the placenta is less likely to be appreciated.

Another proposed mechanism of APS mediated pregnancy failure is through a direct effect of the aPLs on trophoblast placental biology. Di Simone et al. found that polyclonal patient derived aPLs reduce placental human chorionic gonadotropin (hCG) and placental placental lactogen (hPL) secretion by the trophoblasts, and completely blocks trophoblast invasiveness and fusion [79, 80]. Katsuragawa also found that monoclonal aPLs decrease hCG and hPL secretion by the trophoblasts and also completely blocks trophoblast invasiveness [81]. Quenby et al. demonstrated that the

mouse monoclonal aPLs ID2 and IIC5 can inhibit the differentiation of extravillous trophoblasts and giant multinuclear cell formation *in vitro* thus possibly affecting subsequent uteroplacental development [26]. Finally, Di Simone et al. also demonstrated through the use of patient derived aPLs that aPLs are able to change the expression of trophoblast adhesion molecules [82]. The cumulative effect of the aPLs then is that once bound they interfere with trophoblast adhesion, invasion, and maturation and result in defective placentation [26, 79-82]. There are two main limitations to these studies- one they used term trophoblast cells and choriocarcinoma cell lines which do not act in the same way as first trimester trophoblasts; secondly none of these studies have demonstrated any mechanism of action.

Recent studies support the hypothesis that the morbidity in patients suffering from APS can be attributed to enhanced cytokine release through the activation of certain toll-like receptors (TLRs). TLRs are known to be crucial in the early detection of pathogen associated molecular patterns (PAMPS) which subsequently leads to the activation of the adaptive immune system through the MyD88 signaling cascade (except TLR3) [83]. Studies have shown that aPLs are able to activate TLRs, particularly TLR4 through homology of β_2GPI to microbial structures [84, 85]. In particular, our group demonstrated, using *in vitro* studies, that aPLs induce an inflammatory response in first trimester trophoblasts by triggering increased cytokine/chemokine production, notably increased interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), growth regulated oncogene-alpha (GRO-α) and IL-1β, via the TLR4/MyD88 pathway [86]. This triggering of a TLR4-mediated pro-inflammatory phenotype by the binding of aPLs is consistent with a study done by Sorice which showed that in monocytes anti-β_2GPI

binding causes β_2GPI and TLR4 to co-localize in lipid rafts and induce interleukin-1 receptor associated kinase (IRAK) phosphorylation and nuclear factor-κB (NF-κB) translocation and a subsequent release of TNF-α and TF, respectively [87]. Raschi, utilizing human endothelial cells, also showed that both human anti–β_2GPI IgM monoclonal antibodies and polyclonal affinity-purified anti-β_2GPI IgG antibodies are able to induce a pro-inflammatory phenotype in endothelial cells, most likely via the involvement of toll-like receptors [88]. Our lab has also demonstrated that aPLs result in decreased migration of the trophoblasts via diminished interleukin-6 (IL-6) production leading to decreased STAT3 activity [89] and that this occurs in a TLR4/MyD88-independent manner. These studies suggest that these effects of aPL on the trophoblast might lead to inflammation and reduced trophoblast invasion and, possibly leading to a defect in placentation, much like is seen *in vivo*.

Heparin, aspirin, prednisone and immunoglobulin have all been used for years in the management of Obstetric APS. Prednisone was the initial therapy studied, however it was found that there was increased maternal morbidity in the form of preeclampsia and gestational diabetes and increased preterm births with its usage [62, 90, 91]. Immunoglobulin (IVIG) in three trials was found to have inferior results or to make no difference compared to heparin and aspirin, and is now relegated to usage in women with thrombocytopenia. The current gold standard of care is heparin alone, aspirin alone or heparin/aspirin together depending on the patient profile. In a woman with aPLs without history of previous clot, or pregnancy loss, low dose aspirin (LDA) alone is deemed sufficient. In women, with history of previous thromboembolism, therapeutic doses of unfractionated heparin (UFH) or low molecular weight heparin (LMWH) are used.

Finally, in women with medium titers of ACAs, anti-β_2GPI antibodies or LA, and who have a history of 2 or more early pregnancy losses, fetal deaths or >1 preterm births, low dose aspirin along with prophylactic doses of UFH or LMWH is the standard of care [5].

However, it should be pointed out that while LDA is deemed sufficient in low risk women, there have been that studies that have demonstrated that aspirin alone is not as beneficial as the heparin/aspirin combination[65, 66]. Also, while heparin is the accepted gold standard, there have been a few studies that have disputed its efficacy [6, 64, 92], and heparin therapy has also been linked to harmful side effects such as osteoporosis in the mother [92, 93].

While the precise mechanism by which heparin is beneficial in preventing pregnancy loss is unknown, there are several proposed theories. The first and obvious theory for the role of heparin is its ability to anticoagulate, which it does through its action as a cofactor for the endogenous anticoagulant antithrombin [94]. This was the rationale for its original use, as the pregnancy loss was thought to be due to thrombotic events at the placenta. However, given that other mechanisms have been proposed for aPL-induced pregnancy loss, it stands to reason that the efficacy of heparin may be due to additional properties and not just its anticoagulant property. Also, while effective at maintaining a successful pregnancy, heparin therapy does not decrease the risk of patients developing complications such as preeclampsia, IUGR, and prematurity [3, 4], which may mean that there are other mechanisms of action involved as well in these complications.

Besides its anticoagulant property, heparin also has other properties that may explain its efficacy in the treatment of obstetric APS. It can physically inhibit the binding of aPLs through its interference with the β_2GPI-phospholipid interaction [95], which may only be of minor benefit given that the placenta expresses β_2GPI on its surface prior to exposure of aPLs. Secondly, heparin has been shown to have anti-inflammatory properties. Girardi et al. who demonstrated the role of complement in the pathogenesis of APS-mediated pregnancy losses, also demonstrated with a mouse model diminished pregnancy losses in mice treated with heparin at sub-therapeutic levels for anticoagulation. Furthermore, their *in vivo* and *in vitro* models showed that heparin blocks complement activation but does not stop aPL deposition, thus ruling out the interference theory [78].

Studies from our lab showed that at high concentrations, heparin can block the TLR4-mediated inflammatory response produced by first trimester trophoblasts [86], and that heparin is able to inhibit first trimester trophoblast apoptosis by activating the epidermal growth factor receptor survival pathway [9] and blocking aPL mediated trophoblast cell death [86].

Lastly, heparin is thought to play a role in the facilitation of implantation. Di Simone et al. suggested that women with APS experience loss through inhibition of placental development and embryonic implantation in early pregnancy and showed in this same study that heparin was able to increase significantly *in vitro* trophoblast differentiation and invasion [96, 97]. In a similar fashion, Di Simone et al. also

demonstrated LMWH restoring *in vitro* trophoblast invasiveness and differentiation in cells treated with aPLs in a dose dependent manner [79].

While these properties of heparin are able to explain its efficacy in preventing pregnancy loss, heparin is not able to reverse all the effects of the aPLs. For instance, our group has found that heparin is unable to reverse the diminished migration of trophoblasts [89]. And while Di Simone's group demonstrated increased invasion of the trophoblast in the presence of heparin, a study by Ganapathy et al. found no impact of heparin on trophoblast invasiveness and that in the presence of hepatocyte growth factor, heparin may in fact diminish it [98]. This demonstrates that further studies are needed to fully investigate the effects of heparin on the trophoblast. These conflicting findings may also begin to explain why heparin is able to mitigate early pregnancy loss but not the later complications associated with obstetric APS.

In summary, over the years, with more research and improving laboratory techniques, the theories behind the pathogenesis of APS and the mechanisms of aPL mediated pregnancy morbidity have evolved from simple infarction to more complicated concepts of direct aPL-induced inflammation involving the innate immune system. As placental samples from APS patients are filled with an inflammatory infiltrate and show shallow placentation, if aPLs are thought to play a direct role in pregnancy failure then the mechanisms involved may primarily impact placental function and any impairment of the vasculature at the maternal fetal interface may be a consequence of this. Thus further work should be concentrated on first trimester trophoblasts as these cells are responsible for placentation and vascular remodeling. Since data already show that pro and anti-

angiogenic factor dysregulation occurs in APS and its complication preeclampsia, the next step, as will be explored by our study, is to determine the effects of the antibodies on first trimester trophoblast angiogenic factor production, and trophoblast-endothelial interaction/remodeling.

Despite this evolution in the knowledge of the pathogenesis, the available therapy remains limited. The current therapeutic gold standard of APS is LMWH. More information is needed to further our understanding as to how it actually works to mitigate the effects of the antibodies; and in this case if it is able to mitigate all their effects, particularly the effects if any on the angiogenic factor profile of first trimester trophoblasts and trophoblast mediated vascular remodeling. This study also seeks to shed light on this particular aspect of heparin therapy.

Hypothesis

Previous studies from our lab have found that aPLs alter trophoblast-generated inflammation and constitutive trophoblast IL-6 production and migration. We propose that aPLs also alter the balance of pro- and anti-angiogenic factor production by the first trimester trophoblast, which is critical for promoting successful placentation and vasculature remodeling, and for maintaining normal pregnancy. More specifically, we hypothesize that aPLs will reduce VEGF and PlGF and increase sFLt-1 and sEng production by the trophoblast and reduce trophoblast-endothelial interactions. Furthermore, we propose that LMWH will abrogate the effects of the antibodies on

angiogenic factor production by the first trimester trophoblasts and on trophoblast-endothelial interactions.

Specific Aims

1. To evaluate the effects of anti-β_2GP1 antibodies on trophoblast angiogenic factor production and the mechanisms involved.
2. To qualitatively observe the effects of anti-β_2GP1 antibodies on trophoblast endothelial cell interactions.
3. To evaluate the effects of heparin on the impact of anti-β_2GP1 antibodies on the trophoblast angiogenic factor production, and trophoblast endothelial cell interactions.

Materials and Methods

Reagents and antibodies

The low molecular weight heparin (LMWH), Lovenox (Enoxaparin sodium injection), was obtained from Aventis Pharmaceuticals Inc. (Bridgewater, NJ). The mouse IgG isotype control (Clone 107.3) was obtained from BD Pharmingen (Franklin Lakes, NJ).

The LMWH was utilized at a concentration of 10 μg/ml based on prior work in our group which showed it to be the optimal dose at which we saw an effect on trophoblast cytokine production [86, 89].

Antiphospholipid antibodies

These studies utilized two mouse IgG1 anti-human β_2GPI monoclonal Abs (mAbs), designated ID2 and IIC5, which were produced by our collaborator Dr Chamley under sterile conditions. The antibodies, ID2 and IIC5 were cloned from mice immunized with purified human β_2GPI. They have been previously characterized [99] and like human aPLs, they bind β_2GPI, but only when it is immobilized on a suitable negatively charged surface, such the phospholipids, cardiolipin or phosphatidyl serine, or irradiated polystyrene [100]. Both ID2 and IIC5 can bind to first trimester trophoblast cells [86].

Polyclonal IgG purified from the serum of 15 patients with different clinical manifestations of APS, fulfilling APS criteria [12], under long term follow up at University College London Hospital (UCLH) were also studied and obtained from our collaborators Ian Giles and Anisur Rahman. Details of aPL purification have been previously described [86]. The patient samples were divided into 3 groups: 1) PM^+/VT^-: patients who have had previous episodes of pregnancy morbidity (PM), but no history of venous thrombosis (VT) (n=6); 2) PM^-/VT^+: patients who have had VT, but not PM (n=4); and 3) PM^+/VT^+: patients who have had both VT and PM (n=5). All aPL exhibited anti-cardiolipin/anti-β_2GPI activity.

The antibodies were utilized at a concentration of 20 μg/ml based on dose response studies performed in prior work by our group [86]. In these studies, 20 μg/ml was the highest dose at which we saw both viable cells and optimal cytokine upregulation.

Isolation of primary trophoblast cells from first trimester placenta

First trimester placentas (7-12 weeks gestation) were obtained from elective terminations of normal pregnancies performed at Yale-New Haven Hospital. The use of patient samples was approved under Yale University's HIC in full and collected by our collaborator, Dr Anna Sfakianaki. Tissue specimens were washed with cold Hanks Balanced Salt Solution (Gibco) to remove excess blood. Cells were scraped from the membranes, transferred to trypsin-EDTA (Invitrogen, Carlsbad, CA) digestion buffer and incubated at 37°C for 40 minutes with shaking. The mixture was then passed through a nylon strainer and then layered over Lymphocyte Separation Media (ICN Biomedicals, Inc., Aurora, OH) and centrifuged at 2000 rpm for 25 minutes. The cellular interface containing the trophoblast cells was collected and resuspended in D-MEM with D-valine (Caisson Labs, North Logan, UT) supplemented with 10% normal human serum (Gemini Bio-Products, Woodland, CA) and cultured at 37˚C/5% CO_2. This isolation and culture of primary trophoblast cells was performed by Melissa Mulla.

Cell lines

We used the SV40 transformed HTR8 cells (hereafter referred to as H8) first trimester trophoblast cell line. This cell line was a gift from Dr Charles Graham (Queens University, Kingston, Ontario, Canada). The H8 cells were maintained in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 10mM Hepes, 0.1mM MEM non-essential amino acids, 1mM sodium pyruvate, 100nm penicillin/streptomycin (Gibco). Cells were maintained at 37˚C/5% CO_2. Maintenance and culture of cell lines was performed by Melissa Mulla.

Transfection of trophoblast cell line with MyD88 and TLR4 dominant negatives

The H8 cells were stably transfected with either the pDeNy plasmid containing the human MyD88 dominant negative (MyD88-DN), or the pZERO plasmid containing the human HA-tagged TLR4ΔTIR (Invivogen, San Diego, CA). The TLR4ΔTIR acts as a dominant negative since the TIR domain has been deleted. Thus, TLR4ΔTIR (TLR4-DN) can compete with endogenous TLR4 for ligand binding, but cannot transduce a signal. Briefly, cells were transfected overnight with 2μg of DNA using Fugene 6 (Roche Diagnostics, Indianapolis, IN). Following transfection, cells were allowed to recover in growth media for 24 hours, after which selection for stable expression was performed. Cells expressing the TLR4ΔTIR (TLR4-DN) or the MyD88-DN were selected for using 1μg/ml of puromycin and 250μg/ml of zeocin, respectively (Invivogen). Thus, only transfected cells, which had resistance to these antibiotics, were maintained in culture. Occasionally, functionality of dominant negative cell lines was assessed by checking for aPL induced upregulation of IL-8 as previously demonstrated [86], which the transfected cells would not display. Untransfected cells served as the wildtype control. Cell transfection was performed by Melissa Mulla.

Angiogenic Factor Production

Trophoblast cells were treated with or without the anti-β_2GPI mAbs, or patient-derived aPL in Opti-MEM® (Invitrogen; Carlsbad, CA). The mouse IgG1 isotype antibody was also used to treat the cells as a control. Following a culture of 24, 48 and 72 hours, the cell-free supernatants, were collected by centrifugation at 400*g* for 10 minutes and stored at -80˚C until analysis was performed. The concentrations of vascular endothelial growth

factor (VEGF), placenta growth factor (PlGF), soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble Endoglin (sEng) were evaluated by ELISA according to the manufacturer's instructions (R & D Systems; Minneapolis, MN).

LMWH and Angiogenic Factor Production

Trophoblast cells were incubated with no treatment (NT), ID2 or IIC5 (20 μg/ml) either without (Media) or with LMWH (10 μg/ml) for 72 hours. Following a culture of 72 hours, the cell-free supernatants, were collected by centrifugation at 400*g* for 10 minutes and stored at -80˚C until analysis was performed. The concentrations of vascular endothelial growth factor (VEGF), placenta growth factor (PlGF), soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble Endoglin (sEng) were evaluated by ELISA according to the manufacturer's instructions (R & D Systems; Minneapolis, MN).

Endothelial Tube Formation

Immortalized human endometrial endothelial cells (HEECs) were obtained as a kind gift from the Mor lab. The cells were maintained in EMB-2 growth media purchased from Cambrex (East Rutherford, NJ, USA) supplemented with an additional 8% fetal bovine serum (FBS) purchased from Gemini Bio-Products (Woodland, CA, USA).

Undiluted Matrigel™ was plated into 12-well tissue culture plates at 300 μL/well and polymerized for 30 min at 37°C. HEECs were then plated at 1.0×10^5 in 250 μL of Opti-Mem® (Invitrogen; Carlsbad, CA). Formation of tube-like structures was monitored by light microscopy and captured by using OpenLab Image Analysis software.

Antibodies and Endothelial tube Formation

Undiluted Matrigel™ was plated into 12-well tissue culture plates at 300 µL/well and polymerized for 30 min at 37°C. HEECs were plated at 1.0×10^5 in 250 µL of Opti-Mem® (Invitrogen; Carlsbad, CA) and either left untreated or treated with a mouse IgG1 isotype control, ID2 or IIC5 at 20µg/ml directly or were plated at 1.0×10^5 in 250 µL conditioned media (CM) obtained from H8 trophoblasts treated as above (untreated, ID2, IIC5, IgG1). Formation of tube-like structures was monitored by light microscopy and captured by using OpenLab Image Analysis software.

LMWH and Endothelial tube Formation

Undiluted Matrigel™ was plated into 12-well tissue culture plates at 300 µL/well and polymerized for 30 min at 37°C. HEECs were plated at 1.0×10^5 in 250 µL of Opti-Mem® (Invitrogen; Carlsbad, CA) and either treated with NT, ID2 or IIC5 at 20µg/ml, or NT, ID2 or IIC5 at 20µg/ml in the presence of 10 µg/ml LMWH. Formation of tube-like structures was monitored by light microscopy and captured by using OpenLab Image Analysis software. Image collection was performed by Melissa Mulla.

Co-culture studies

The HEECs were stained with green fluorescent linker dye PKH67, were seeded into a 12-well tissue culture plate containing Matrigel™ and cultured 2–4 hours in 250 µL Opti-Mem® (Invitrogen; Carlsbad, CA) until vessel formation occurred. Upon vessel formation, Opti-Mem® media was removed and first trimester trophoblast cells, stained with red fluorescent linker dye PKH26, were seeded at 8.0×10^4 cells/well in 500 µL of

Opti-Mem®. The co-culture was incubated over a period of 24–72 hours. During this incubation, cell migration was tracked by fluorescent microscopy, and captured using OpenLab Image Analysis software.

Statistical analysis

Experiments were performed at least three times. Data are expressed as mean ± standard deviation (S.D.) of pooled experiments. Statistical significance ($p< 0.05$) was determined using either the one-way ANOVA with the Bonferroni correction for multiple comparisons, or the paired student's *t*-test.

Results

Anti-β2GP1 antibodies modulate trophoblast angiogenic factor production

Since previous studies from our group have indicated that aPLs induce trophoblast inflammation and reduce trophoblast migration by altering the cell's basal cytokine/chemokine profile [86], our first objective was to look at what effects, if any, these antibodies have on the angiogenic factors produced by first trimester trophoblasts.

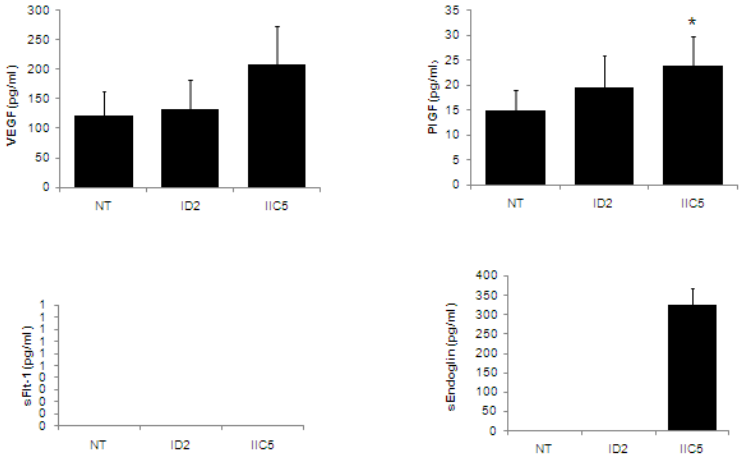

Figure 1. Effects of Anti-β$_2$GPI Abs on angiogenic factor production by a first trimester trophoblast cell line after 24 hours incubation. *The human first trimester trophoblast cell line, HTR8, was incubated with no treatment (NT), ID2 or IIC5 (20 µg/ml) for 24 hours, after which cell-free supernatants were collected and analyzed for VEGF, PlGF, sFlt-1, and sEng (sEndoglin) by ELISA. Bar charts show changes in angiogenic factor production relative to the NT control (*p≤0.05). Significance was determined using ANOVA. Data are pooled from at least seven individual experiments.*

Figure 1 shows angiogenic factor production after treatment of the human first trimester extravillous trophoblast cell line, H8, with or without the anti-β_2GPI mAbs. When compared to the untreated control after 24 hours, treatment of the H8 cells with ID2 had no significant effect of the basal production of VEGF, PlGF, or sEng. Treatment with IIC5 cells, on the other hand, induced increases in VEGF, PlGF and sEng, with only

the increase in PlGF being significant ($p \leq 0.05$). sFlt-1 levels were undetectable at this time point.

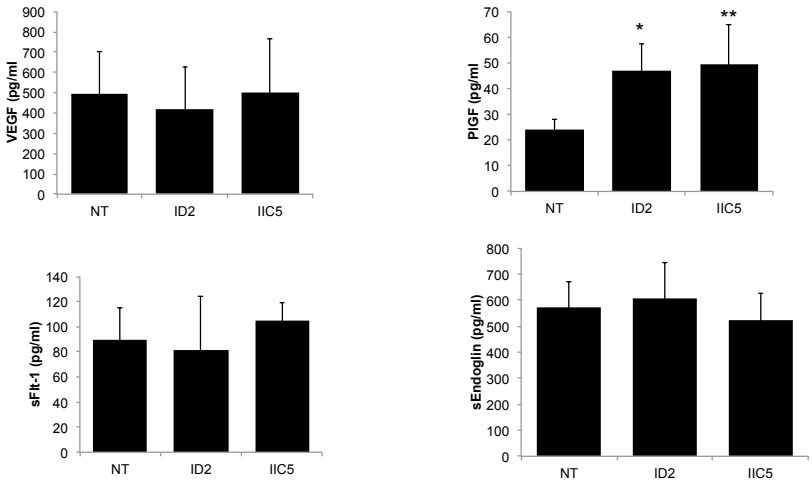

Figure 2. Anti-β_2GPI mAbs modulate angiogenic factor production by a first trimester trophoblast cell line at 48 hours incubation. *The human first trimester trophoblast cell line, HTR8, was incubated with no treatment (NT), ID2 or IIC5 (20 µg/ml) for 48 hours, after which cell-free supernatants were collected and analyzed for VEGF, PlGF, sFlt-1, and sEng (sEndoglin) by ELISA. Bar charts show changes in angiogenic factor production relative to the NT control (*p≤0.01; **p≤0.001). Significance was determined using ANOVA. Data are pooled from at least seven individual experiments.*

Figure 2 shows the levels of angiogenic factors produced after incubation of the trophoblast with or without the aPL cells for 48 hours. Unlike at 24 hours, sFlt-1 levels were much increased and could be measured by ELISA. Cells treated with ID2 showed a significant increase in PlGF ($p<0.01$) production, while the production of sFlt-1 and sEng were similar to the NT control. Cells treated with IIC5 showed a significant increase in PlGF ($p<0.001$) while the production of the other angiogenic factors were unchanged or insignificant. At 72 hours, however, is where we observed the majority of significant changes in the levels of pro- and anti-angiogenic factors produced by the HTR8 cells (Figure 3). Cells treated with ID2 showed a statistically significant increase in PlGF

($p<0.001$), and sEng ($p <0.001$) and a decrease in sFlt-1 ($p<0.01$), with no significant change in VEGF levels compared to the NT control. Cells treated with IIC5 showed a significant increase in VEGF ($p <0.001$), PlGF ($p<0.001$) and sEng ($p<0.001$), with no significant change in sFlt-1 levels. Overall, both antibodies were able to significantly increase PlGF and sEng, with differential responses seen for VEGF and sFLt-1, particularly a notable downregulation of sFlt-1 by ID2. The mouse IG1 isotype control was not affected by either ID2 or IIC5 (Figure 3).

Figure 4 shows the effects on the mAbs on primary trophoblast cells. Overall, figure 4 shows an upholding of the trends found using the HTR8 cell line with some differences. The antibodies, like with the HTR8 cells, cause an increase in VEGF, PlGF and sEng, although only IIC5 upregulated sEng. However, unlike the cell line, both ID2 and IIC5 caused an upregulation of sFlt-1 production, although this was not a large response.

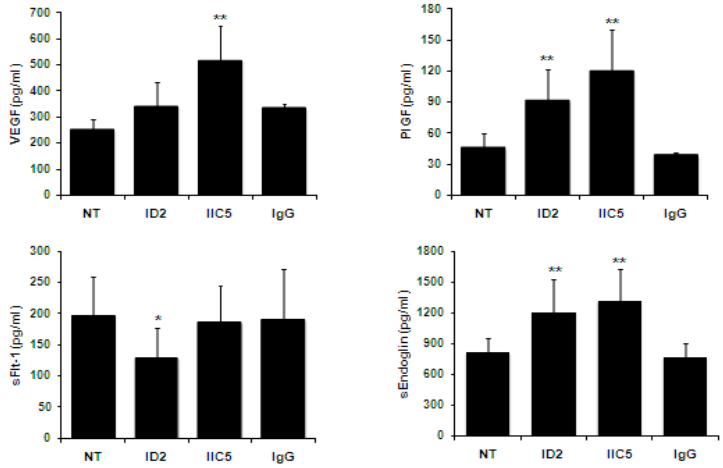

Figure 3. Effect of Anti-β₂GPI monoclonal Abs on angiogenic factor production by a first trimester trophoblast cell line*. The human first trimester trophoblast cell line, HTR8, was incubated with no treatment (NT), ID2 or IIC5 (20 µg/ml) or the mouse IgG1 isotype control (IgG) (20 µg/ml) for 72 hours, after which cell-free supernatants were collected and analyzed for VEGF, PlGF, sFlt-1, and sEng (sEndoglin) by ELISA. Bar charts show changes in angiogenic factor production relative to the NT control (*p<0.01; **p<0.001). Significance was determined using ANOVA. Data are pooled from at least five individual experiments.*

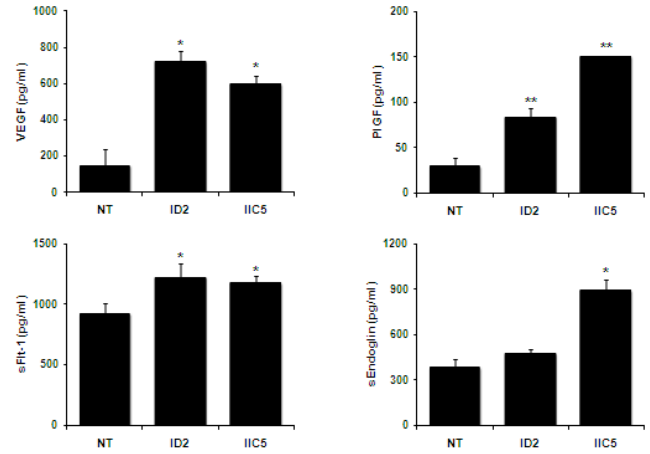

Figure 4. Anti-β₂GPI mAbs modulate angiogenic factor production by primary first trimester trophoblast cells at 72 hours incubation. *Primary isolated first trimester trophoblast cells incubated with either no treatment (NT), ID2 or IIC5 (20 µg/ml) for 72 hours, after which cell-free supernatants were collected and analyzed for VEGF, PlGF, sFlt-1, and sEng(sEndoglin) by ELISA. Barcharts show changes in angiogenic factor production relative to the NT control (*p<0.05; **p<0.001). Significance was determined using ANOVA. Data are representative of at least three individual experiments.*

To further validate the response of the first trimester trophoblast cells to the anti-β_2GPI antibodies, H8 cells were treated with aPLs derived from patient sera. These patient derived aPLs were polyclonal IgG antibodies obtained from patients with either a history of venous thrombosis (PM^-/VT^+), pregnancy morbidity (PM^+/VT^-), or both (PM^+/VT^+). These antibodies have been previously characterized and have been shown to also alter cytokine/chemokine production by first trimester trophoblast [86].

Figure 5 shows the effect of the patient derived aPLs on angiogenic factor production. Patient derived aPL from all three patient groups caused a significant increase in VEGF levels, much like ID2 and IIC5, but did not demonstrate an effect on PlGF. sEng was significantly increased only with aPL from patients in the PM^+/VT^- group. Interestingly, sFlt-1 was increased by aPL derived from PM^-/VT^+ and PM^+/VT^+ patient groups but unchanged by aPL from patients in the PM^+/VT^- group.

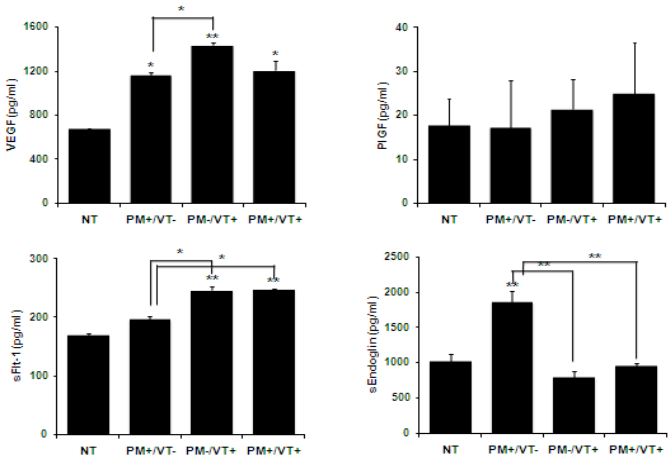

Figure 5. Effects of patient-derived aPL on trophoblast angiogenic factor production. *Trophoblast HTR8 cells were incubated with either no treatment (NT) or polyclonal IgG aPL (12.5 µg/ml) from patients with PM^+/VT^- (n=6); PM^-/VT^+ (n=4) or PM^+/VT^+ (n=5). After 72 hours, the supernatants were collected, pooled on the basis of patient group, and then evaluated by ELISA for VEGF, PlGF, sEng (sEndoglin) or sFlt-1. Bar charts show significant differences relative to the NT control, unless otherwise specified, as determined using ANOVA (*p<0.05, **p<0.001).*

Anti-β2GPI induced changes in the angiogenic factor profile are TLR4-independent, while the aPL-induced increase in PlGF and sEng are MyD88 dependent.

Having determined that the anti-β2GPI antibodies are able to modulate the angiogenic factor production of first trimester trophoblasts, we set out to elucidate the mechanism behind these changes. Prior studies from our group have demonstrated the role of the TLR4/MyD88 pathway in the upregulation of inflammatory cytokines [86]. Based on this, it was decided to investigate whether similarly the TLR4/MyD88 pathway is also responsible for the changes seen in the angiogenic factor production, notably the increases seen in PlGF, VEGF, and sEng.

To determine the mechanism responsible, the HTR8 trophoblast cells were stably transfected to express aTLR4 dominant negative (TLR4-DN). These and untransfected (wildtype) cells were either left untreated or treated with anti-β2GPI antibodies, ID2 and IIC5. Figure 6 shows the levels of angiogenic factors produced by the wildtype and TLR4-DN trophoblast cells in response to the aPL for 72 hours. The TLR4-DN cells displayed the same angiogenic factor profile in response to the aPL as the wild type cells. Thus, PlGF, VEGF and sEng were increased in both ID2 and IIC5 treated cells in both WT and TLR4-DN. sFlt-1 levels were decreased in ID2 treated cells in both WT ($p<0.01$) and TLR4-DN, with no change seen with the IIC5 antibody. Overall, there were no significant differences between the levels of cytokines produced in WT versus TLR4-DN cells with the exception of PlGF, which was significantly increased by the ID2 antibody but not the IIC5 ($p=0.02$).

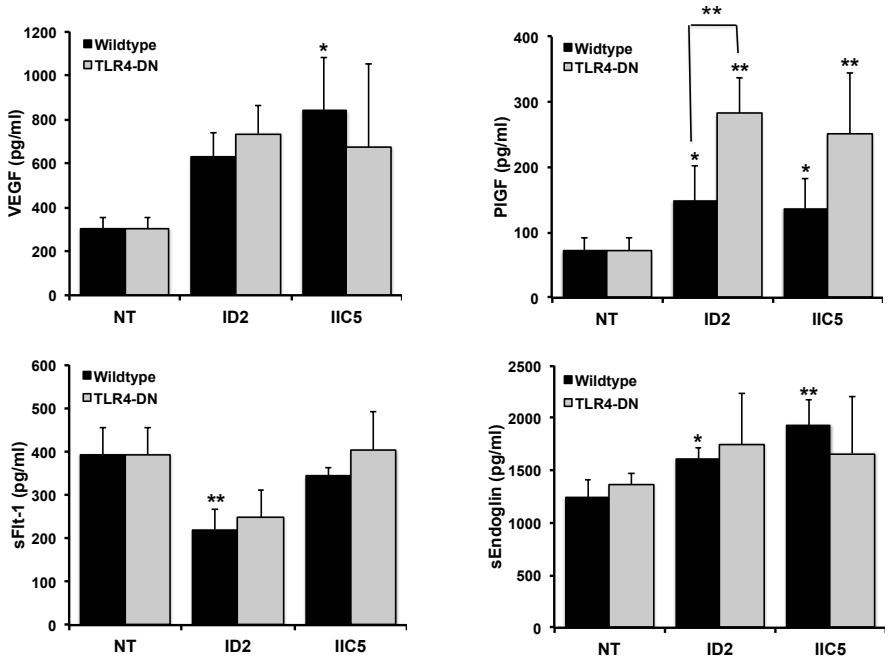

Figure 6. Anti-β_2GPI Abs modulate trophoblast angiogenic factor production in a TLR4-independent manner*. Wildtype trophoblast HTR8 cells, or HTR8 cells stably transfected to express the TLR4-DN were incubated with either: no treatment (NT), ID2 (20 µg/ml), or IIC5 (20 µg/ml) for 72 hours, after which cell-free supernatants were collected and analyzed for VEGF, PlGF, sFlt-1, and sEng (sEndoglin). *p<0.05 and **p<0.01 relative to the NT control, as determined using ANOVA Significant differences between the wildtype and TLR4-DN cells were determined by paired t-test (**p<0.02). Data are pooled from at least three individual experiments.*

Similarly, to determine whether or not the MyD88 pathway was involved, a trophoblast cell line (HTR8) stably transfected with a MyD88 dominant negative (myD88-DN) or the wildtype cells were either left untreated or treated with ID2, and IIC5 for 72 hours. As shown in Figure 7, compared to the WT cells where there is a significant increase in PlGF, in the MyD88-DN cells we see a marked reversal of this effect in both ID2 and IIC5 treated cells ($p < 0.05$ and $p < 0.01$ respectively). Similar results are also seen for sEng levels for both ID2 and IIC5 ($p < 0.05$ for both).

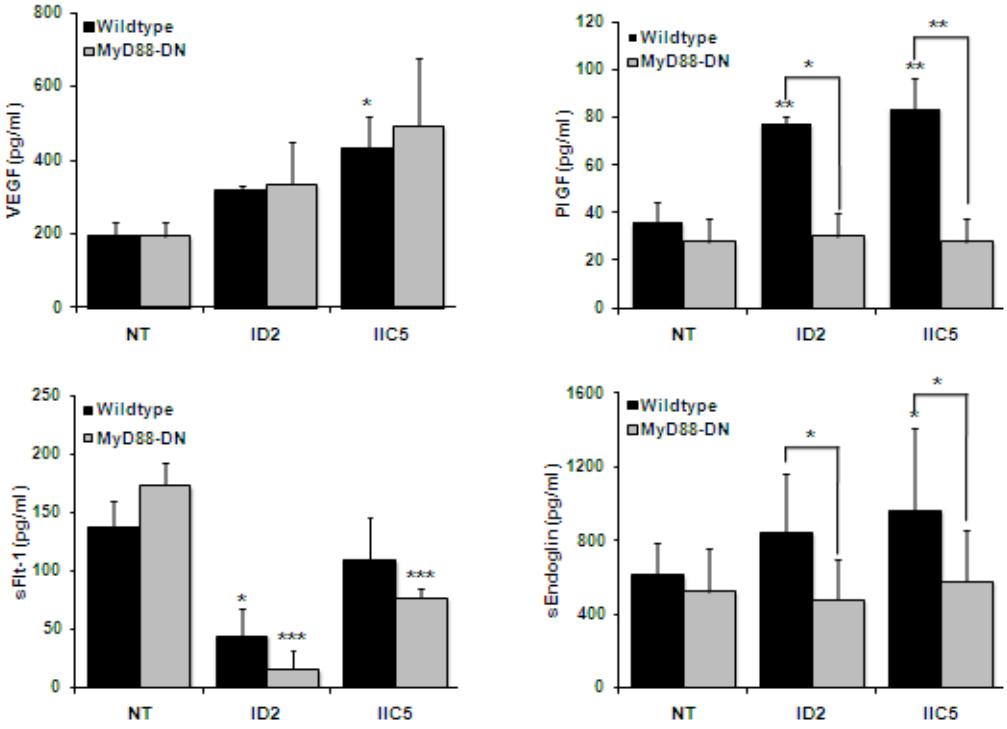

Figure 7. Anti-β$_2$GPI Abs modulate trophoblast angiogenic factor production in a MyD88-dependent and -independent manner*. Wildtype trophoblast HTR8 cells, or HTR8 cells stably transfected to express the MyD88-DN were incubated with either: no treatment (NT), ID2 (20 µg/ml), or IIC5 (20 µg/ml) for 72 hours, after which cell-free supernatants were collected and analyzed for VEGF, PlGF, sFlt-1, and sEng (sEndoglin). Barcharts show significant changes relative to the NT control (*p<0.05; **p<0.01; ***p<0.001), as determined using ANOVA. Significant differences between the wildtype and MyD88-DN cells were determined by paired t-test (*p<0.05; **p<0.01). Data are pooled from three individual experiments.*

No reversal of the increase in VEGF by ID2 and IIC5, or the decrease in sFlt-1 is seen in the MyD88-DN cells.

Anti-β2GP1 antibodies do not significantly alter qualitative endothelial tube formation or trophoblast-endothelial cell interactions

Prior work from the Mor group described the ability of immortalized human endometrial endothelial cells (HEECs) to differentiate in Matrigel ™ (a proprietary brand of artificial extracellular matrix) and form tubes resembling blood vessels [101], and also effectively illustrated that in the absence of Matrigel ™ the endothelial cells remained in monolayers [102]. Matrigel ™ because of this property allows for an effective *in vitro* model to be developed for studying trophoblast endothelial cell interactions. Figure 8 confirms this prior work and demonstrates the formation of tubes in Matrigel ™ by telomerase immortalized HEECs. In the figure we see, single cell HEECs as well as HEECs grouped and differentiated into forming connected vessel like structures with apparent lumens.

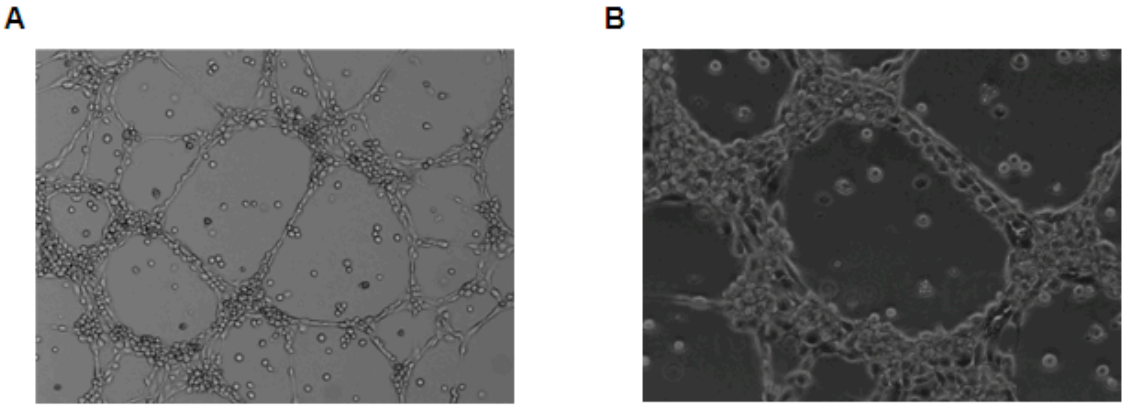

Figure 8. Endothelial Tube Formation. *Light microscopy images after 6 hours of incubation of immortalized human endometrial endothelial cells (1.0 x 10^5) grown in Matrigel ™ coated cells (A) 5x magnification (b) 10 x magnification.*

Although a snapshot is seen in this image, it should be explicitly stated that the vessel formation occurs in a network that spans more than one layer of the Matrigel ™.

Given that our particular interest is in the effects of the anti-β_2GPI antibodies on angiogenic factor poduction by first trimester trophoblasts, we decided to use this model system to further explore the effects of the antibodies on vessel formation by the endothelial cells. Previous studies have demonstrated that aPLs are in fact able to affect angiogenesis in human endometrial cells [55] *in vitro* and we wanted to confirm this in our own model.

Firstly, we looked at whether or not the antibodies were directly deleterious to endothelial cell tube formation by adding the aPLs ID2 IIC5 or an IgG1 isotype control at 20 µg/ml directly to the HEECs in the Matrigel ™ and observing the qualitative effects on the vessel like structures.. These are illustrated in Figure 9A. Compared to the NT cells, the vessels formed by the HEECs directly incubated with the aPLs did not demonstrate much difference. One can still appreciate tubular structures that are interconnected, and although from gross inspection it appears the ID2, IIC5 and IgG1 vessels are less structurally intact, it does not seem to be a significant, nor specific (there is a similar effect in the IgG isotype) change. Thus, it seems that the antibodies do not significantly directly affect endothelial cells in a specific manner.

Secondly, we looked at whether or not the change in angiogenic factor profile produced by the trophoblasts in response to the antibodies would have an effect on endothelial cell tube formation. This was done by treating the HEECs in the Matrigel ™ with conditioned media from trophoblasts incubated four 72 hrs with NT, ID2 IIC5 and IgG1 isotype control, and then visualizing under light microscopy the qualitative effects on the tubular structures formed. Based on Figure 9B, the HEECs treated with conditioned media appear structurally more interconnected and stable than those treated

directly with antibodies. In terms of differences amongst the various conditioned media, although the morphology of the ID2 treated cells looks different from the IgG and NT and the IIC5 looks less well formed than the NT and IgG, these differences are not striking. All the HEECs regardless of treatment show large, interconnected tubular structures with areas of cell confluence at the connection points. It seems then that despite the modulation in angiogenic factor profile of the trophoblasts by the antibodies, this does not translate to a readily observable difference in quality of the vessels formed by the endothelial cells.

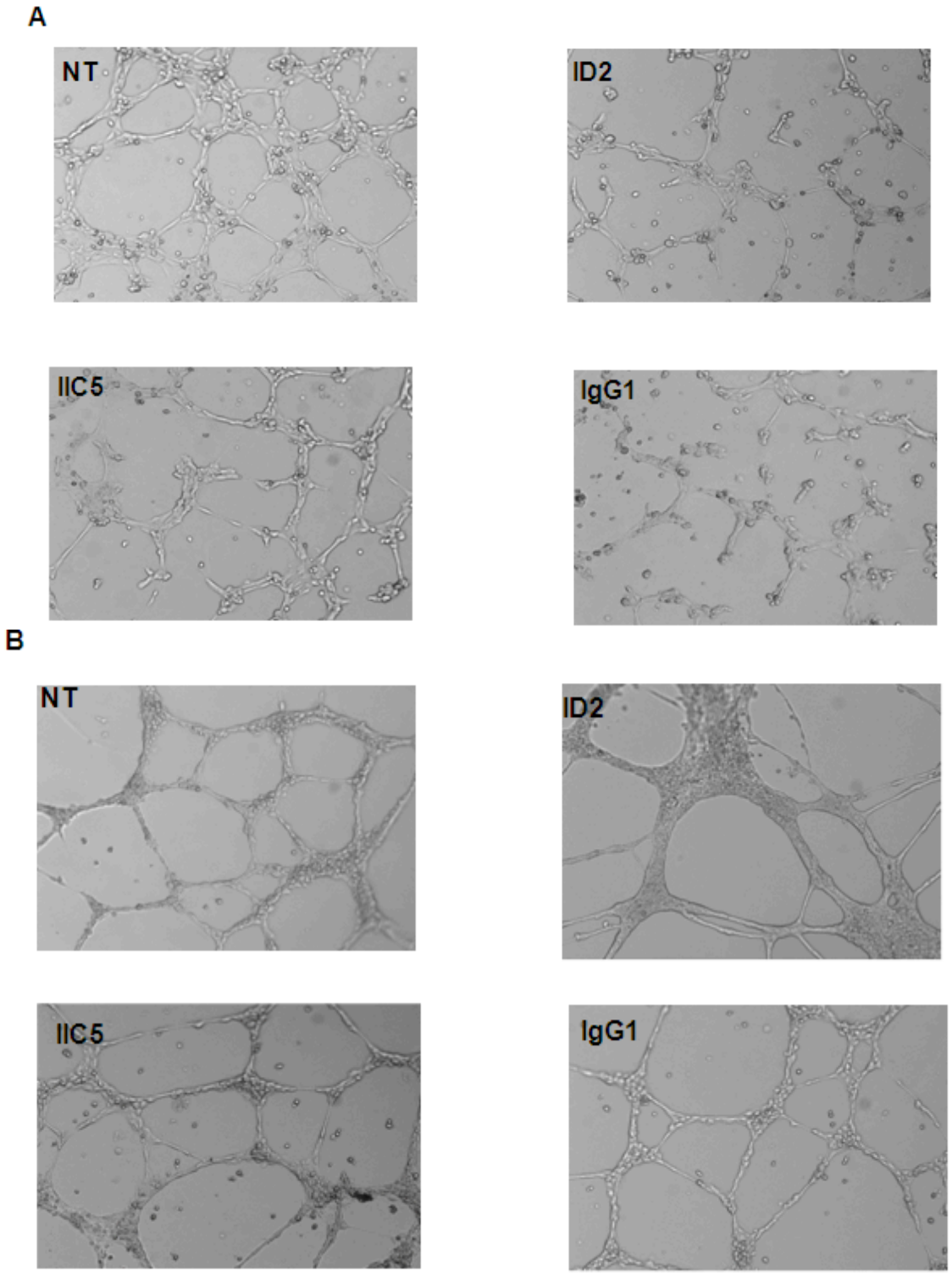

Figure 9. Effects of Anti-β$_2$GPI Antibodies on Endothelial cell tube formation. *Light microscopy images after 6 hours of incubation of human endometrial endothelial cells (HEECs) in* ***(A)*** *media treated with 20 µg/ml antibodies added directly and* ***(B)*** *in media treated with conditioned media from trophoblasts treated with 20 µg/ml antibodies for 72 hrs. All images are at 5x magnification.*

Prior work from the Mor group also demonstrated that differentiation of HEECs causes the upregulation of the secretion of chemokines which promote trophoblast migration [102]. We then wondered whether trophoblast-endothelial interaction would be affected in the presence of the anti-β_2GPI antibodies. We set out to explore this by using labeled endothelial cells (green) and trophoblasts (red), in the Matrigel ™ model. HEECS and trophoblasts were co-cultured in Matrigel ™, and their interaction was visualized using fluorescence microscopy. Figure 10 demonstrates the normal trophoblast-endothelial cell interaction over time in the absence of the antibodies. In figure 10 we see that that the trophoblasts (red) begin to migrate towards differentiated endothelial cells (green) after 1 hour (Fig. 10a), and by two hours have reached the walls of the tubes and distributed themselves on top and in between (Fig. 10b). By 4 hours, we observe greater numbers of trophoblasts interacting with the vessel like structures (Fig. 10c) and by 19 hours, we see that the two cell types have come together, and appear as yellow under double color montage (Fig. 10d) due to co-localization.

HEECs and trophoblasts were also co-cultured in the presence of ID2, IIC5 and IgG1 antibodies. Figure 11 shows the trophoblast-endothelial interactions after 19 hours for untreated (NT) cells and cells treated with anti-β_2GPI antibodies. Compared to NT, cells treated with ID2 and IIC5 also showed HEECs differentiated into vessels (green), with trophoblasts (red) lining the walls of the tubes, with small areas where HEECs and trophoblasts came together (yellow). Although there appears to be greater areas of yellow in the ID2 and NT, than the IIC5 and IgG1, these areas do not appear to be significantly greater and overall the levels of interaction between the HEECs and the trophoblasts appeared to be similar.

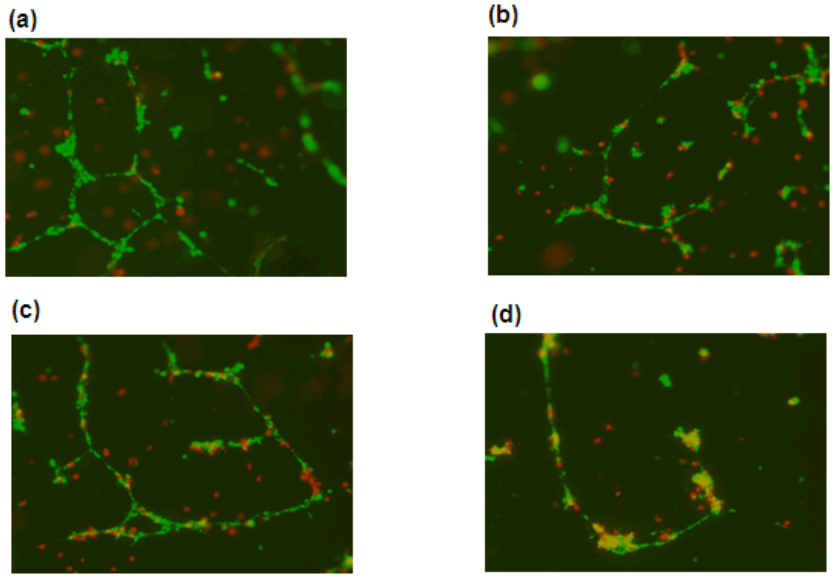

Figure 10. Trophoblast-Endothelium Interaction. *Sequential distribution of trophoblast and human endometrial endothelial cells in the three-dimensional Matrigel ™ system can be observed by labeling each cell type with a different fluorescent dye. (a) After incubation for 1 hour, trophoblast cells (red) are migrating towards the walls of the tubes formed by the endothelial cells (green). (b) After incubation for 2 hours, the trophoblast cells (red) have reached the walls of the tubes (green) and distribute themselves on top and between. (c and d) After 4 hours and 19 hours incubation respectively, composite picture of red fluorescently labeled trophoblast cells and green endothelium revels areas of yellow color product of the combination of red and green. All images are shown 5× magnification.*

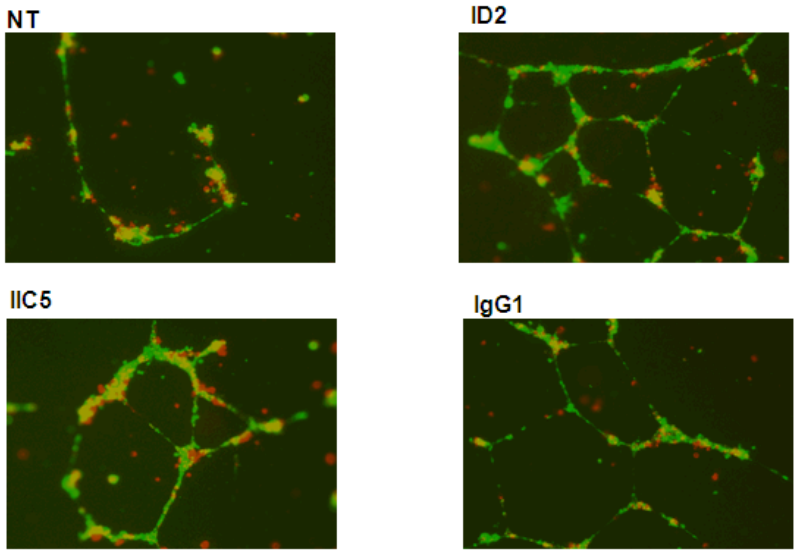

Figure 11. Effects of Anti-β_2GPI mAbs on trophoblast endothelial cell interaction in 3-D Matrigel™ toward differentiated immortalized human endometrial endothelial cells. *Composite pictures of red fluorescently labeled trophoblast cells and green endothelium reveals areas of yellow color in the NT, ID2, IIC5 and IgG1 after 19 hrs incubation. All images at 5x magnification.*

Low molecular weight heparin does not reverse the modulation of trophoblast angiogenic factor production by first trimester trophoblasts and does not change endothelial tube formation

Obstetric APS is routinely treated with low molecular weight heparin (LMWH) and this has been shown to maintain the viability of the pregnancy [5]. For that reason we wondered whether or not LMWH could reverse the modulation of the first trimester trophoblast angiogenic factor profile caused by anti-β_2GPI antibodies.

Compared to the cells in media, cells treated with LMWH showed continued upregulation of VEGF and PlGF by ID2 and IIC5 (Figure 12). For PlGF, however, in the presence of LMWH the upregulation was significantly greater for the NT, ID2 and IIC5 ($p<0.01$, $p<0.01$ and $p<0.05$ respectively). Conversely, while LMWH also did not

change the upregulation of sEng with ID2, the IIC5 induced upregulation was markedly reduced ($p < 0.05$). The most striking effect of the LMWH is where levels of sFlt-1 are concerned. There we see upregulation of levels of sFlt-1 in NT, ID2 and IIC5, with the most marked upregulation being in the NT ($p < 0.0001$), followed by the ID2 ($p<0.01$). LMWH then is unable to reverse the modulation of the angiogenic factor profile induced by anti-β_2GPI antibodies in first trimester trophoblasts, and may actually make it worse.

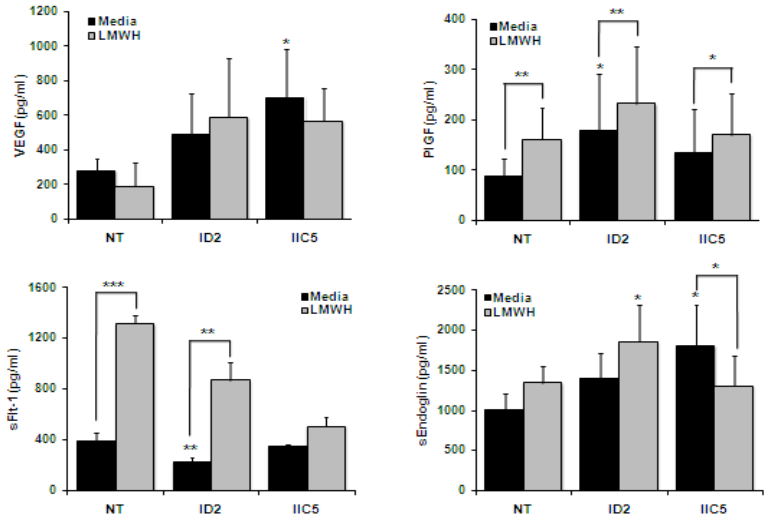

Figure 12. Effect of LMWH on anti-β_2GPI Ab-modulation of trophoblast angiogenic factor production. *Trophoblast HTR8 cells were incubated with no treatment (NT), ID2 or IIC5 (20 µg/ml) either without (Media) or with LMWH (10 µg/ml) for 72 hours, after which cell-free supernatants were analyzed for VEGF, PlGF, sFlt-1, and sEng (sEndoglin). Bar charts show changes in angiogenic factor production relative to the NT control (*p<0.05; **p<0.01; ***p<0.001) as determined using ANOVA. Significant differences between the presence and absence of LMWH were determined by paired t-test (*p<0.05; **p<0.01; ***p<0.001). Data are pooled from at least three individual experiments.*

Given these findings, particularly that LMWH increase trophoblast sFlt-1, a pathological marker of preeclampsia and APS, we questioned if we would be able to see

a difference in endothelial tube formation in the presence of trophoblast CM from cells treated with LMWH. Figure 13B shows HEECs treated with NT, ID2 and IIC5 conditioned media in the presence of LMWH, while 13A shows the comparison of HEECs treated with conditioned media from NT, ID2 and IIC5 all in the absence of LMWH. One can see from gross inspection that there are no obvious differences.

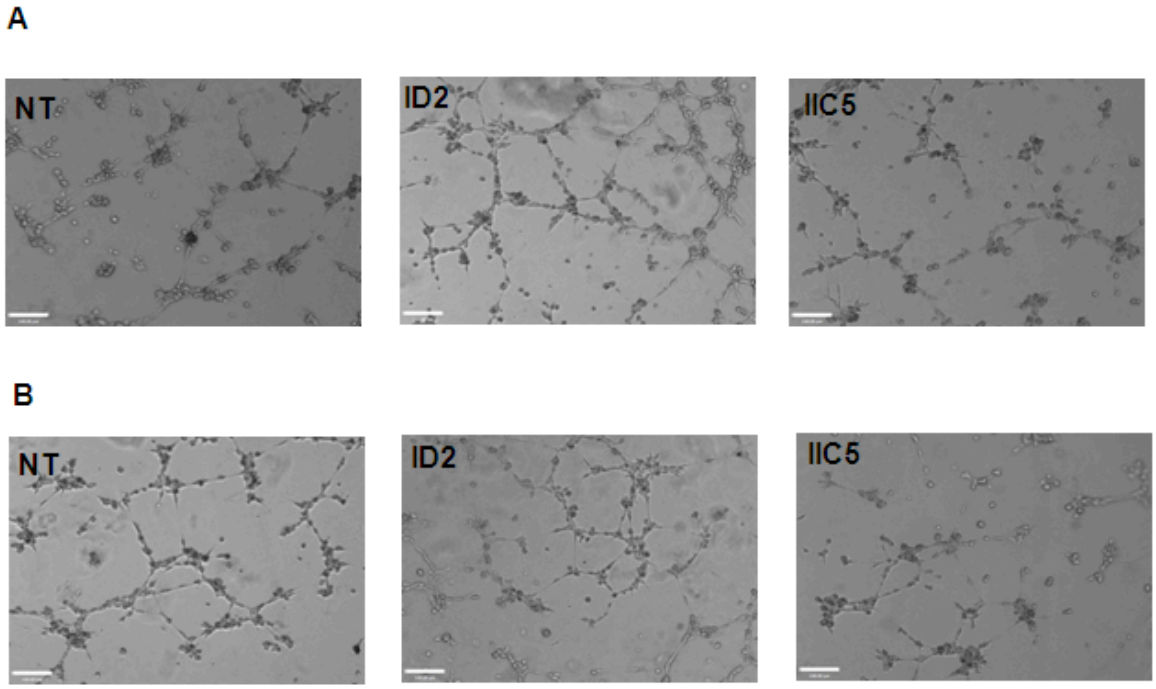

Figure 13. LMWH and the Effects of Antibodies on Endothelial cell tube formation. ***A***. *Light microscopy images after3 hours of incubation of human endometrial endothelial cells (HEECs) in media treated with conditioned media from trophoblasts treated with 20 µg/ml antibodies (ID2 and IIC5) for 72 hrs.* ***B*** *Light microscopy images after3 hours of incubation of human endometrial endothelial cells (HEECs) in media treated with conditioned media from trophoblasts treated with 20 µg/ml antibodies (ID2 and IIC5) for 72 hrs and 10 µg/ml low molecular weight heparin (LMWH).*

Compared to Figure 13A, however, there does not seem to be very much difference. In both experiments at 3 hours incubation, there is little tubular formation and

cell interconnection. It may be argued that the cells with LMWH especially the NT and IIC5 appear to structurally better, and have more vessel-like structures than the cells without; however, given the design of the experiment it is difficult to say how significant that difference is. There is at least no striking difference overall between the cells treated with LMWH and the ones without, so despite the alteration in angiogenic factor profile produced by heparin alone, it does not seem to translate to affecting endothelial tube formation.

Discussion

The main objective of this project was to look at the effects of anti-β_2GPI antibodies on angiogenic factor production of first trimester trophoblasts. Previous experiments studying the effects of aPL on trophoblasts have looked at third trimester cells or choriocarcinoma cell lines (see Introduction). However, given our interest in its possible effects on vascular remodeling and angiogenesis, both of which occur early in placentation [40], we felt it best to focus our study on first trimester cells.

Normally in the placenta there is production of pro and anti-angiogenic factors VEGF, PlGF [43, 48, 103], and sFlt-1 [47] respectively. In preeclampsia, which is a late stage complication of obstetric APS, there is a dysregulation of the angiogenic factors produced normally. Studies have found a downregulation of the normally high levels of VEGF and PlGF, and a marked increased in the levels of sFlt-1 and sEng [33, 49], (although Gu et al. report an increase in PlGF as well [104]). This dysregulation of the trophoblast angiogenic factor profile with its shift to a more anti-angiogenic state is

thought to result in widespread endothelial dysfunction [105] and subsequently preeclampsia. Also partial failure of vascular transformation of the spiral arteries to high flow, low resistance vessels [41], has been implicated in the pathogenesis of preeclampsia [42].

In our studies we looked at the effects of aPL on trophoblast angiogenic factor secretion at 24, 48 and 72 hours, and found that while the antibodies were able to modulate the trophoblasts' angiogenic factor profile at 72 hrs, there were little significant effects at 24 and 48 hours. Indeed, at 24 hrs, sFlt-1 was not even measurable by ELISA. It is not entirely clear why maximal effect occurs at 72 hours, however, this is consistent with prior experiments done in our lab where the levels of cytokine and chemokines being investigated were greatest at 72 hours [86, 89]. This effect could perhaps be due to an accumulation of the factors being measured in culture media, thus making it easier to detect at the later time point (72 hours).

At 72 hours, we found aPL-induced modulation of pro and anti-angiogenic factors. Specifically we observed an increase in VEGF, PlGF and sEng with no effect or a slight down regulation in sFlt-1 in the H8 cells. When we tested primary trophoblast cell culture isolated from first trimester placental tissue cell cultures, we observed a similar increase in VEGF, PlGF and sEng but also found an increase in sFlt-1, although this was not a large effect.

Thus we show that aPLs result in a dysregulation of angiogenic factor levels. Given our knowledge of the role of these factors in early angiogenesis (see Introduction), we could speculate on the effects of this shift in production. The marked increases in VEGF and PlGF without a concomitant increase in sFlt-1 (a decoy receptor that normally

acts to control the effects of these two pro-angiogenic factors [47]) could lead to disordered angiogenesis. An increase in sEng (which acts as a decoy receptor for TGF-β and blocks its binding and downstream signaling, its NOS mediated vasodilation, and its activation of eNOS [49]) could be detrimental in two ways. One, it blocks the angiogenic effects of TGF-β and two, it prevents further TGF-β induced upregulation of VEGF [48]. This marked increase in sEng is highly implicated in the pathogenesis of preeclampsia, where the levels were found to correlate with disease severity and fall after delivery [49]. So the unchecked, disordered angiogenesis caused by increased VEGF and PlGF and the anti-angiogenic effect of sEng increase caused by the antibodies overall could lead to impaired/dysregulated angiogenesis.

Our finding that anti-β_2GPI Abs can directly modulate trophoblast angiogenic factor production is supported by studies using other cells types. aPLs have been shown to reduce endometrial endothelial cell VEGF production [55], and increase monocyte expression of VEGF and Flt-1 [52]. In terms of sFlt-1 and VEGF release by the trophoblast cell line, we did observe differences in the magnitude of response generated by the two anti-β_2GPI mAbs, which may be explained by their recognition of different or overlapping epitopes [106]. Indeed, in previous studies we have found their potency to differ [107].

When compared to patient derived antibodies, we also saw slightly differing responses but overall the results were consistent. aPL from all 3 groups increased VEGF but had no effect on PlGF. sEng was significantly increased only with aPL from patients in the PM^+/VT^- group. Interestingly, sFlt-1 was increased by aPL derived from PM^-/VT^+ and PM^+/VT^+ patient groups but unchanged by aPL from patients in the PM^+/VT^- group.

This is in keeping with observations that aPL can upregulate VEGF expression on monocytes, and that monocyte and circulating VEGF levels are increased in APS patients, either with or without a history of thrombosis [52-54]. Although the anti-β_2GPI mAbs increased trophoblast PlGF secretion, and elevated PlGF levels are found in APS patients [54] , none of the patient-derived aPL upregulated trophoblast PlGF production. Interestingly only the polyclonal APS-IgG from patients with a history of pregnancy mortality (PM^+/VT^-) significantly increased trophoblast release of sEng, and only the aPL from patients with a history of VT, with or without pregnancy mortality (PM^-/VT^+, PM^+/VT^+), increased trophoblast sFlt-1 production. Although Flt-1 levels are increased in monocytes from APS patients, both with and without thrombosis [52], our findings suggest that trophoblast sEng dysregulation may be specific to patients with a history of pregnancy mortality, while sFlt-1 upregulation may be associated with a history of thrombosis. Since the anti-β_2GPI mAbs did not markedly upregulate sFlt-1 release by the trophoblast, their fine specificity may more closely resemble aPL that are associated with pregnancy complications.

The effects of the antibodies on the angiogenic factor profile could be a primary or direct effect of the antibodies on the trophoblasts or it could be secondary to ischemia/hypoxia. In normal placentation, cytotrophoblasts undergo a program of pseudovasculogenesis by acquiring endothelial markers VE-cadherin and $alpha_5beta_3$ ($\alpha_5\beta_3$) integrin [36]. This is impaired in preeclampsia [37]. Impaired placentation and accompanying ischemia are thought to be the primary events leading to elaboration of soluble factors in circulation [108] in preeclampsia. aPLs also regulate the expression of trophoblast adhesion molecules. They cause a decrease in $alpha_1$ (α_1) [109] and an

increase in alpha$_5$ (α_5) integrins, a decrease in VE-cadherin and an increase in E-cadherin [82]. This overall may result in inadequate trophoblast invasion and subsequent impaired placentation and ischemia.

While hypoxia could be responsible for the increased VEGF levels [110], it would not account for the other changes seen. Hypoxia would upregulate sFlt-1 [111-113] and decrease PlGF levels [104, 114], in trophoblasts, whereas in this study sFlt-1 levels were essentially unchanged and PlGF was markedly upregulated. Also, hypoxia has been shown to not be responsible for sEng induction in the villous trophoblast [113].

Finally, our study design runs the risk of studying the cells in hyperoxic, not hypoxic, conditions relative to *in vivo*. The placenta develops initially in a very oxygen poor environment with ambient pO_2 of <20 mm Hg [115], and in our experiment, the trophoblasts are incubated in room air (20% oxygen or about 160 mm Hg). It stands to reason then that the effect of the mAbs is not due to hypoxia, but is most likely due to a direct effect of the antibody binding.

We then set out to elucidate the mechanism of aPL-induced dysregulation of the first trimester trophoblast angiogenic factor profile. Based on work in our group, the aPL induced inflammatory response in trophoblasts occurs in a TLR-4/MyD88 dependent way [107]. This finding is supported by studies showing the role of toll-like receptors [116, 117], and the MyD88 pathway [88] in aPL induced signaling/activation. Given this involvement of toll-like receptors and the fact that angiogenesis can be regulated through inflammatory mechanisms [118, 119], we wondered whether or not TLR4 and MyD88 are similarly responsible for the modulation of angiogenic factors induced by the antibodies. We used TLR4-DN and MyD88-DN cell lines, treated with or without ID2

and IIC5, and measured the levels of angiogenic factors with that of wild type. There were no significant differences between the levels of cytokines produced in WT versus TLR4-DN cells. These results indicate that the increase in PlGF, VEGF, sEng and the decrease in sFlt-1 are independent of TLR4. For the MyD88-DN experiment, compared to the WT cells there is a marked reversal of the increase in PlGF and sEng in the MyD88-DN cells, whereas no reversal of the increase in VEGF or the decrease in sFlt-1 is seen. This indicates that the anti-β_2GPI mAbs induced change in PlGF and sEng, but not VEG and sFlt-1, was found to be dependent upon functional MyD88, which may implicate another toll-like receptor [83].

Other possible mechanisms include, in the case of the MyD88 dependent changes, the activation of another TLR or the activation of another receptor utilizing MyD88. In the case of the MyD88 independent changes, this may involve the activation of another TLR, particularly TLR3 which is the only TLR to signal independently of MyD88 [83], or another signaling pathway altogether.

The cytoplasmic portion of TLRs shows high similarity to that of the Interleukin-1 receptor family [83], so it is possible a receptor in the IL-1 Receptor family may be involved. In fact, synoviocytes from patients with RA, when stimulated with IL-1β express more PlGF [120]. IL-1β also was found to stimulate VEGF and sFlt-1 by human oviductal epithelial cells and stromal fibroblasts [121]. Blocking IL-1 in macrophages has also been found to reduce VEGF levels significantly [122]. However, it is unlikely that IL-1β is involved, since aPL-induced IL-1β production is TLR4-mediated and prior experiments in our group have found that recombinant IL-1β does not modulate trophoblast PlGF or sEng production (data not shown).

Other candidate receptors/pathways for the aPL-mediated change in cytokine levels include the cAMP/protein kinase A pathway and the proteinase-activated receptor 2 pathway. The cAMP/protein kinase A pathway has been shown to upregulate PlGF expression in trophoblasts [123]. This experiment was done using the BeWo choriocarcinoma cell line and placental villous explants. This is less likely, however, as the cAMP/protein kinase A pathway does not utilize MyD88 [124] and the upregulation of PlGF is dependent on MyD88. Release of sFlt-1 was stimulated in human umbilical vein endothelial cells through activation of proteinase-activated receptor 2 (PAR-2) via Epidermal Growth Factor receptor transactivation [125]. This is plausible but less likely, as while the proteinase-activated receptor 2 does not directly utilize MyD88, studies have shown cooperativity between TLRS and PARs [126, 127], with one study implicating MyD88 as the responsible molecule [127]; and the sFlt-1 release is independent of MyD88. Also, given that this experiment was performed in endothelial cells, rather than trophoblasts, it is uncertain how applicable this is across cell types.

For the MyD88 independent upregulation of VEGF and sFlt-1, we turn to information on the MyD88 independent cascade to suggest possible mechanisms. In the MyD88-independent pathway, LPS stimulation leads to activation of the transcription factor interferon regulatory factor 3 (IRF-3), and thereby induces interferon-beta (IFN-β). IFN- β, in turn, activates STAT1, leading to the induction of several IFN-inducible genes [128-130]. TLR3 and TLR4 have been shown to utilize the MyD88 independent/IRF-3 pathway [131]. Since upregulation of VEGF and sFlt-1 is independent of TLR4, and since TLR3 has been implicated in upregulating sFlt-1 in trophoblasts [132], and VEGF in other cell types [133], TLR3 is more likely to be involved.

It is important to highlight the fact that the aPL-induced changes in the angiogenic factor profile of first trimester trophoblast cells was found to occur independently of TLR4. This is because, given the fact that the inflammatory response and the angiogenic response appear to share some regulatory machinery (MyD88), it would seem that both responses are tightly linked. However, the fact that TLR4 is not involved makes it clear that the angiogenic response induced by the aPLs is not a consequence of the inflammatory response. The aPL-mediated inflammatory response is TLR4 dependent as is IL-1β production (an inflammatory mediator) in the trophoblast (refer to above paragraph on IL-1 receptor family).

Having found that the antibodies produced a shift in the angiogenic factor profile, we looked at whether or not they had an effect on endothelial tube formation and trophoblast-endothelial cell interactions. In our studies neither the treatment with the antibodies directly nor with the trophoblast conditioned media resulted in any significant changes in endothelial tube formation. This is in contrast to an experiment done by Di Simone et al., where antiphospholipid antibodies were shown to decrease the total length and number of tubes formed in Matrigel™ by endothelial cells [55]. We are limited by the fact that we did not quantitatively assess the differences in tube length and number as Di Simone's group, and so do not have an objective measure of tube formation. Overall in our experiment though, morphologically the tubes appeared to be similar despite treatment.

Given that experiments in our group and others have shown that aPLs limit trophoblast migration and invasion [89, 109] and given that we saw a shift in angiogenic factor profile, and that aPLs cause decrease in adhesion factors produced by trophoblasts

[82], we expected to see a difference in the level of trophoblast-endothelial cell interactions in the treatment groups. We expected less trophoblasts to be present and for the ones present to align less. However, we saw similar levels of interaction and alignment between HEECs.

The lack of effect of the antibodies on the trophoblast-endothelial cell interaction in our system is curious. Given that we utilized a similar model system (i.e. the Matrigel™ matrix model) to that of the other groups, the results should have been reproducible. In reality, even the NT cells in our Matrigel™ set up did not behave as previously described by Mor's group [102]. In their set-up, the trophoblasts migrated towards endothelial cells in Matrigel™, aligned on top of the endothelium within 4–8 hours and achieved complete replacement of the endothelium by 72–96 hours. In our experiment, we never saw complete replacement of the endothelium. Our cells also were dead by 24 hours with the tubular structures collapsing entirely. It is possible that this difference is due to the change in growth media. Mor's group used EBM growth media with 10% FBS, whereas we utilized Opti-Mem® serum free media to diminish the possibility of the growth factors and angiogenic factors present in FBS [134] altering the effects of the Abs on the cells.

Another reason we could be seeing these results is that Di Simone et al. in her experiment used polyclonal patient derived aPLs, while we used monoclonal mouse antibodies. While in our experiment the polyclonal patient derived and monoclonal mouse model aPLs behaved overall similarly and gave us consistent results with our cytokine expression, they did not behave exactly the same. Our polyclonal patient derived aPLs gave somewhat different results depending on the subset of patients derived from,

which was most likely due to variability in epitope recognition [27, 28]. This variability in epitope recognition might be enough to give such different outcomes in the HEECs Matrigel™ experiment.

Our data are likely to be a reflection of the choices and conditions we utilized for our *in vitro* model. In light of our results, perhaps these choices were not the best way to go forward. This experiment would bear repeating with different conditions, in order to determine why our model did not behave as expected given our previous data. Perhaps we could do a head to head comparison of both EBM and Opti-Mem® media and a head to head comparison of mAbs versus patient derived polyclonal Abs to determine if there is a real difference. Future experiments should also involve a method of quantifying the tubules to ensure a more objective comparison with existing studies in the literature.

The current standard of treatment for obstetric APS is aspirin alone, LMWH alone, or a combination of both depending on the patient profile [5]; however, while effective at maintaining a successful pregnancy, heparin therapy does not decrease the risk of patients developing late stage complications such as preeclampsia, IUGR, and prematurity [3, 4].

This experiment sought to investigate the effects of LMWH on the aPL-induced modulation of trophoblast angiogenic factor profile. The cells treated with LMWH showed continued upregulation of VEGF and PlGF by ID2 and IIC5, with very significant increases in the levels of PlGF even in the NT cells. Conversely, while LMWH also did not change the upregulation of sEng with ID2, the IIC5 induced upregulation was markedly reduced. The most striking effect of the LMWH is where levels of sFlt-1 are concerned. There we see upregulation of levels of sFlt-1 in NT, ID2

and IIC5, with the most marked upregulation being in the NT, followed by the ID2. We found then that LMWH was unable to reverse the modulation of the angiogenic factor profile induced by anti-β_2GPI antibodies in first trimester trophoblasts, and in the case of sFlt-1 and PlGF may actually make it worse. While sFlt-1 elevation has been associated with adverse pregnancy complications [50] and the fact that heparin markedly elevates sFlt-1 levels might imply it wouldn't be beneficial therapy, one could also argue that the marked upregulation of the pro-angiogenic factor PlGF by heparin may counteract this anti-angiogenic response as the PlGF would bind to sFlt-1. However, further studies are required to determine the clinical impact of this.

As mentioned in the introduction, the rationale for the use of heparin in APS is as an anticoagulant. However, given that there is an inflammatory component as well to aPL-mediated morbidity, it is thought that heparin has other mechanisms through which it acts. Besides being an anticoagulant, heparin is known to act on the activity of angiogenic growth factors [135]. Heparin can also act in an anti-inflammatory capacity [136, 137], and studies in our group have shown that heparin is able to inhibit the TLR-4 dependent aPL-induced inflammatory response [86]. In *in vivo* studies where aPLs were shown to trigger complement, heparin was also shown to inhibit this activation [78]. Heparin can also inhibit apoptosis in first trimester trophoblasts by activating the epidermal growth factor receptor (EGFR) survival pathway [9] and can block aPL mediated cell death [86, 138].

While these properties of heparin are able to explain its efficacy in preventing pregnancy loss, heparin is not able to reverse all the effects of the aPLs. For instance, our group has found that heparin is unable to reverse the decreased IL-6 secretion and

diminished migration of trophoblasts [89]. Moreover, a study by Ganapathy et al. found no impact of heparin on trophoblast invasiveness and that in the presence of hepatocyte growth factor, heparin may in fact diminish it [98]. Furthermore, our current study shows heparin is unable to mitigate the aPL-mediated shift in angiogenic factor profile and may in fact enhance it through its very marked upregulation of sFlt-1 and PlGF even in untreated cells.

The mechanism behind these changes is not clearly understood. Given that these changes occurred in NT cells, it is unlikely that it is due to a synergistic effect of LMWH with the antibodies, or due to the antibodies themselves. It is most likely due to heparin. Indeed, Flt-1 is known to contain a heparin-binding domain [139] and heparin may, therefore, physically promote sFlt-1 release from the cells. Therefore, LMWH treatment may actually exacerbate certain pathological hallmarks of preeclampsia, such as elevated sFlt-1 levels.

Given that heparin shifted the angiogenic factor production of the trophoblasts by markedly elevating sFlt-1 levels, we again wondered whether or not this effect would translate to a qualitative difference in endothelial tube formation. Our experiment, however, showed no obvious differences between HEECs treated with NT, ID2 and IIC5 conditioned media in the presence of LMWH and HEECs treated with NT, ID2 and IIC5 conditioned media without heparin. It is unclear why this did not translate to significant qualitative changes in the vessels, but it is consistent with our earlier finding of the antibodies, despite causing a change in the angiogenic factor profile, not having a significant effect on vessel formation.

To conclude, our group has found that not only are aPLs responsible for inducing an inflammatory response in first trimester trophoblasts and reducing migration as shown in prior studies [86, 89], but as this study shows, they also are able to change the pro- and anti-angiogenic factors normally produced by first trimester trophoblasts and shift it towards a more anti-angiogenic profile. This response occurs both dependently and independently of MyD88 signaling and is separate from the inflammatory response. LMWH is unable to reverse this aPL-induced alteration in angiogenic factor production, and even enhanced certain aspects of the response (i.e. sFlt-1). Our study also found that the aPLs are not able to significantly alter endothelial tube formation or trophoblast-endothelial cell interactions. Neither did LMWH have an impact on the formation of endothelial tubes. These findings, in combination with our previous studies [86, 89] may signify that the limited spiral artery transformation and shallow placentation seen in preeclampsia and APS is due to more than just the shift in first trimester angiogenic factor production caused by the antibodies. Further investigation is required to elucidate the other mechanisms that may be involved.

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