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Novel Mechanisms Of Trophoblast Responses To Antiphospholipid Antibodies And Therapeutics In Obstetric Antiphospholipid Syndrome

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NOVEL MECHANISMS OF TROPHOBLAST RESPONSES TO ANTIPHOSPHOLIPID ANTIBODIES
AND THERAPEUTICS IN OBSTETRIC ANTIPHOSPHOLIPID SYNDROME

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ABSTRACT

Obstetric antiphospholipid syndrome (APS) is a systemic autoimmune disorder characterized by circulating antiphospholipid antibodies (aPL) and an increased risk of recurrent pregnancy loss and preeclampsia. Current treatments, such as low molecular weight heparin (LMWH) may maintain pregnancy but do not reduce the risk of these late gestational complications. aPL trigger placental inflammation by activating trophoblast Toll-like receptor 4 (TLR4), leading to cytokine production. Since some microRNAs (miRs) regulate TLR responses, this study sought to determine the functional role of aPL-induced miR expression in regulating trophoblast inflammation. Since vitamin D deficiency is common in APS patients, the effect of vitamin D on trophoblast function in the setting of aPL and LMWH was also evaluated. Thus, the human first trimester trophoblast cell line (HTR8) and primary trophoblast cultures were incubated with or without aPL in the presence or absence of vitamin D and/or LMWH. miR expression was profiled using RT-qPCR, and cytokine secretion was measured by ELISA. miR function was assessed by transfection of specific miR mimics or inhibitors. Indeed, treatment of trophoblast cells with aPL significantly upregulated expression of miR-146a-5p, miR-146a-3p, miR-155, and miR-210 when compared to controls. Furthermore, miR-146a-5p and miR-146a-3p upregulation by aPL was TLR4 dependent. Functional studies demonstrated that miR-146a-3p directly promoted trophoblast IL-8 secretion by activating the RNA sensing receptor, TLR8. Vitamin D reduced aPL-induced miR-146a-5p, IL-8 and LMWH-induced sFlt-1 release, but did not reduce aPL-induced miR-146a-3p. These findings suggest that aPL induce trophoblast IL-8 production through upregulation of miR-146a-3p and subsequent activation of TLR8. Our findings also suggest that that vitamin D can dampen this inflammatory response downstream of miR-146a-3p. Thus, further study of trophoblast miR function in the context of aPL and the therapeutic potential of vitamin D for obstetric APS is warranted.

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To Melissa and Julie,

Who made life in the lab a little easier and a lot more fun

To Dr. Abrahams,

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foundation for my life in medicine and science

To my family,

Whose unending love and support
have made my dreams reality

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INTRODUCTION

Antiphospholipid syndrome (APS) is a systemic autoimmune disorder characterized by a pro-thrombotic state and significant pregnancy morbidity. The disease is more common in women than men, and can present in isolation as primary systemic APS, or as secondary systemic APS in the context of another autoimmune disease such as systemic lupus erythematosus (SLE) (1). It is also the most common cause of acquired thrombophilia in the general population (2). Pregnant patients may develop obstetric APS, which increases the risk of both recurrent pregnancy loss (RPL) and late gestational complications such as preeclampsia, placental insufficiency and fetal growth restriction (1,3). Indeed, between 7 and 25% of RPL is attributable to APS (4), and nearly 50% of fetuses born to patients with APS are premature (5). Although the exact prevalence of APS is unknown, it is estimated to be between 40-50 cases per 100,000 persons (6). Diagnosis of obstetric APS requires one or more of the following: unexplained death of a morphologically normal fetus beyond 10 weeks; preeclampsia necessitating delivery before the 34th week; or three or more consecutive abortions before the tenth week of gestation (7).

Antiphospholipid antibodies and β_2-glycoprotein I

In addition to these clinical manifestations, the diagnosis of APS requires detection of persistently high titers of antiphospholipid autoantibodies (aPL) in the circulation (7). aPL are a heterogeneous population of autoantibodies that recognize anionic phospholipid-binding proteins rather than phospholipids themselves (8,9). The prevalence of aPL has been difficult to assess due to heterogeneous patient populations and inconsistent laboratory protocols, however it is estimated that aPL are present in 1-4% of normal controls (10), 15.5% of women with RPL, and as high as 30% of women with fetal losses after 20 weeks (4). While anti-cardiolipin and lupus anticoagulant antibodies are included

in the diagnostic criteria, antibodies that target β_2-glycoprotein I (β_2GPI) are considered to be the most pathologic in obstetric APS (11-14).

β_2-glycoprotein I, also known as apolipoprotein H, is a highly abundant, highly glycosylated single chain polypeptide that circulates at a plasma concentration of 200µg/mL (15). The protein consists of five sushi domains of approximately 60 amino acids, and aPL that react with domains I and V are of particular interest in obstetric APS (8,16,17). *In vivo,* β_2GPI exists in two distinct conformations with different immunogenicities (18). The circular, or unbound form of β_2GPI is not recognized by autoantibodies due to folding that hides the epitopes recognized by aPL. However, when bound to anionic surfaces, such as phospholipids, cardiolipin or phosphatidyl serine, β_2GPI undergoes a conformational change that exposes critical regions, thereby allowing anti-β_2GPI antibodies to bind (18).

Anionic phospholipids are rarely physiologically exposed on the outer surface of the cell membrane, and β_2GPI-autoantibodies do not bind free β_2GPI in serum (18). However, because the placenta undergoes extensive proliferation and remodeling, anionic phospholipids become exposed on the trophoblast surface, allowing exogenous β_2GPI to bind the trophoblast under physiologic conditions (9,19,20). Furthermore, the trophoblast constitutively express β_2GPI, independently making the placenta a target of aPL (21). However, the direct implication of β_2GPI in aPL-mediated pregnancy loss was highlighted by Roberston *et al.* using a mouse model; by passively immunizing both wildtype and β_2GPI-null pregnant mice with human aPL, it was demonstrated that functioning β_2GPI is not required for the maintenance of pregnancy, but is necessary for the development of aPL-induced pregnancy loss (14). Thus, β_2GPI plays a critical role in the pathophysiology of obstetric APS.

Antiphospholipid antibodies, inflammation and trophoblast function

Given that systemic APS is a pro-thrombotic disease, adverse pregnancy outcomes associated with APS were initially attributed to clotting at the maternal-fetal interface. This notion was initially supported by the apparent beneficial effect of heparin in reducing fetal loss in APS patients (22-24). However, evidence of placental thrombosis on histologic examination of APS miscarriage samples is uncommon (25). More frequently, disruptions in placentation are identified, such as reduced endovascular trophoblast invasion and impaired maternal spiral artery transformation (25,26). Evidence of inflammation is also common in these samples, and numerous groups have identified inflammatory processes as mediators of aPL-induced pregnancy loss (27-31). Thus, pregnancy complications associated with APS may due to placental dysfunction and inflammation, rather than intravascular thrombosis. Indeed, this hypothesis has been supported by both *in vitro* and animal studies using aPL. For example, in mice exposed to high levels of human aPL, elevated pro-inflammatory TNFα levels are found both systemically and in decidual tissue where aPL localize, resulting in increased fetal resorption (28). Importantly, blockade of this TNFα response reduces fetal resorption to baseline levels. Furthermore, in concurrence with histologic findings (25), both monoclonal and patient-derived aPL have been shown to reduce trophoblast migration and invasion *in vitro* (32,33). Taken together, these data suggest that inflammation and altered trophoblast function due to aPL are instrumental in APS pathogenesis.

Antiphospholipid antibodies and Toll-like receptors

While the mechanisms underlying these processes were initially unclear, the observation made by Raschi *et al.* identifying similarities between endothelial cell responses to aPL and bacterial lipopolysaccharide (LPS) suggested interactions between β_2GPI and the prototypical innate immune receptor, Toll-like receptor 4 (TLR4) (34). Toll-like receptors

are a group of highly conserved pattern recognition receptors that recognize a wide variety of ligands associated with pathogens, known as pathogen-associated molecular patterns (PAMPs), as well as some host-derived danger signals, known as damage-associated molecular patterns (DAMPs). PAMPs, such as LPS, are not expressed by humans, which allows for rapid and specific identification of microbes by immune cells. DAMPs are endogenous molecules that, following tissue injury, are released from their physiologic compartments and become exposed to and recognized by the immune system. TLRs recognizing either PAMPs or DAMPs subsequently trigger innate immune responses critical to both immediate defense against pathogens and development of adaptive immunity. The 10 known human TLRs share the same type I transmembrane glycoprotein structure, each consisting of extracellular and transmembrane domains and an intracellular signaling domain. Physiologically, TLR4 is responsible for sensing LPS expressed on the surface of gram-negative bacteria. When bound to LPS, TLR4 homodimerizes, allowing recruitment of the adapter protein myeloid differentiation factor 88 (MyD88) and subsequent activation of downstream signaling proteins interleukin-1 receptor-associated kinase 1 (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6), ultimately triggering nuclear factor κB (NF-κB) -dependent transcription of inflammatory genes (35).

In recent years, the ability of TLR4 to interact with endogenous ligands such as β_2GPI in a pathological context has been explored. In endothelial cells, which do not produce β_2GPI, serum-derived β_2GPI binds to cell surface receptors ApoER2, allowing autoantibodies to interact with β_2GPI. This results in endothelial cell activation and increased cell adhesion (36-38). Moreover, these aPL-mediated effects are dependent on TLR4/MyD88-dependent signaling (34,39,40). Similar results have been found in monocytes, where anti-β_2GPI antibodies induce co-localization of TLR4 and β_2GPI to

lipid rafts, leading to TLR4 activation and subsequent NF-κB-dependent gene transcription (41). This ability to activate TLR4 signaling is thought to be due to shared epitopes between β_2GPI and LPS. To that end, Blank *et al.* illustrated that mice immunized with bacterial products produce anti-β_2GPI antibodies capable of inducing fetal loss, suggesting that molecular mimicry results in production of anti-β_2GPI-antibodies that recognize epitopes common to β_2GPI and LPS (42). Since the trophoblast constitutively express β_2GPI, and are likely targeted by circulating aPL in the earliest stages of placentation, our group has worked to address the mechanisms by which aPL alter human first trimester trophoblast function.

Similar to the observations made in endothelial cell and monocyte systems, we described the ability of anti-β_2GPI antibodies to induce human first trimester trophoblast responses through both TLR4/MyD88 -dependent and -independent pathways (30). Specifically, it was demonstrated that anti-β_2GPI antibodies elicit TLR4/MyD88-dependent trophoblast secretion of inflammatory cytokines: interleukin-8 (IL-8), growth related oncogene-α, monocyte chemoattractant-1 and interleukin-1β (IL-1β)(30). Recent work by our lab has further shown that the IL-1β response in the trophoblast is dependent on TLR4-mediated uric acid production, which in turn activates the Nalp3/ASC inflammasome, leading to IL-1β processing and release (43). However, the IL-8 response was shown to be independent of the inflammasome, and the mechanisms by which the other cytokines are regulated remain unclear. Nonetheless, the observed upregulation of IL-8 and IL-1β by aPL provides a potential mechanism for the recruitment of neutrophils to the placenta observed in APS miscarriage samples (27). Furthermore, in parallel to these inflammatory responses, anti-β_2GPI antibodies modulate human first trimester trophoblast secretion of angiogenic factors, including upregulation of the pro-angiogenic factors, vascular endothelial growth factor (VEGF), placental growth factor

(PIGF), and the anti-angiogenic factor soluble endoglin (sENG) (44-47). Interestingly, this occurs via both MyD88-dependent (PIGF) and independent processes (VEGF, sENG). In addition, anti-β$_2$GPI antibody induces the downregulation of basal interleukin-6 (IL-6) production, which subsequently inhibits STAT3 activity resulting in decreased trophoblast migration *in vitro*. Furthermore, aPL upregulate trophoblast expression of the matrix metalloproteinase inhibitor TIMP-2, which further contributes to this inhibition of cell migration (45). Since precise regulation of cytokines, chemokines, and angiogenic factors is required for development of a functional maternal-fetal interface, even minor changes in the milieu may result in damaging inflammation and impaired invasion and vascular remodeling that lead to the pathological sequelae observed in APS.

Current APS therapies and the trophoblast

Currently, pregnant women with APS are treated with low molecular weight heparin (LMWH), either alone or in combination with aspirin (22,23,48). While this treatment approach might reduce the risk of RPL, the occurrence of late-term obstetrical complications such as preeclampsia remains high (22,23,48). To date, clinical and experimental studies have produced contradictory results regarding the effectiveness of LMWH and aspirin in preventing aPL-associated adverse pregnancy outcomes (48-54), and in being able to reverse the detrimental effects of aPL on trophoblast function *in vitro* (30,55-58). Studies from our group have shown that aspirin has no beneficial effects on aPL-modulation of trophoblast function (59). While successfully reducing aPL-mediated upregulation of IL-8 and IL-1β, LMWH has no beneficial effect on aPL-reduced trophoblast migration or altered angiogenic factor secretion. Moreover, LMHW induces a robust elevation of trophoblast sFlt-1 release, even in the absence of aPL (30,32,44,46). Similar elevations of sFlt-1 have been observed in placental villus explants (60-62) and in pregnant women treated with LMWH (61,62). Since sFlt-1 is a potent anti-angiogenic

factor that is thought to be a mediator of end-organ damage in preeclampsia (63,64), it is clear that LMWH may have both beneficial and deleterious effects on the trophoblast. This may explain the inability of LMWH to reverse the late gestational complications associated with APS, such as preeclampsia, despite its relative success in prolonging pregnancy. Undoubtedly, the mixed effects of LMWH highlight the need to identify alternative therapeutic targets and treatments for the management of obstetric APS.

Vitamin D, the immune system and the trophoblast

In recent years, the biologically active form of vitamin D, 1,25-dihydroxyvitamin D (vitamin D), has been found to have numerous immunomodulatory effects in addition to its classical role in calcium homeostasis. Vitamin D is a steroid hormone produced from endogenous and dietary precursors that controls gene regulation through interaction with the vitamin D receptor (VDR) (65). When vitamin D binds the VDR in the nucleus, the resulting dimer acts as a transcription factor to upregulate genes controlled by the vitamin D response element (65). Interestingly, the first evidence of vitamin D's ties to the immune system came from observations in patients with sarcoidosis, whose elevated vitamin D levels were attributed to increased synthesis in disease-associated macrophages (66,67). Since then, VDR expression has been identified in both immune and non-immune cells alike, including the trophoblast (68-70), and the placenta has been identified as a major site of vitamin D activation (71). Although conflicting data exist, it has been suggested that vitamin D broadly influences T-cell maturation and function, preferentially shifting T-cell cytokine responses away from cell-mediated immunity (Th1) towards humoral immunity (Th2), therefore potentially limiting tissue damage caused by inflammation (72). Furthermore, vitamin D deficiency has been directly implicated in the pathogenesis of APS since Vitamin D can inhibit anti-β_2GPI antibody-mediated tissue factor expression in endothelial cells (73). Recently,

interactions between TLRs and vitamin D have been demonstrated, including the upregulation of CYP27B1, the enzyme responsible for activating vitamin D, by TLR4 signaling (68,74). Moreover, treatment of murine placental explants with vitamin D results in attenuation of inflammatory cytokines induced by the TLR4 ligand LPS (71). Interestingly, population studies suggest vitamin D deficiency may be associated with APS and SLE (75,76), and also with adverse pregnancy outcomes such as preeclampsia (77-79) and recurrent pregnancy loss (80). Thus, vitamin D supplementation represents a promising adjunct for treating pregnant women with APS (73), particularly since aPL induced placental inflammation is TLR4-mediated (11,81).

Toll-like receptors and microRNAs

In the physiologic state, TLRs and their respective downstream signaling pathways must be tightly regulated to allow for a robust inflammatory response while ensuring appropriate attenuation in order to prevent subsequent tissue injury. One proposed mechanism of such precise regulation is through microRNAs (miRs). miRs are small, noncoding RNAs that regulate protein expression through post-transcriptional interaction with mRNA, either by suppressing translation, or reducing levels of target mRNA (82,83). miRs are highly conserved among mammalian species and can target hundreds of distinct mRNAs, thus expression of most genes is likely under some degree of control by miRs. Indeed, many miRs have been identified that both regulate and are induced by TLR signaling (82). Therefore, miRs are well poised to act in a negative feedback mechanism of control over TLR genes. Indeed, recent studies have revealed a number of miRs that specifically target elements of the TLR4 signaling pathway; these include miR-9, which targets NF-κB (84); miR-146a-5p (formerly designated miR-146a), which targets IRAK1 and TRAF6 (85); and miR-155, which targets MyD88 (86). While these studies were conducted in other cell types, altered miR expression has been identified in

trophoblast exposed to hypoxia, environmental toxins or LPS, and in placentas from pathological pregnancies (87-89). Interestingly, vitamin D has been shown to alter levels of miR-146a-5p and miR-155 in the serum (90), and thus likely modulates intracellular levels as well. To date, little is known about the role of miRs in trophoblast function, and no information exists on whether aPL-induced responses may be regulated by miRs. Thus, aPL may indeed be exerting its effects on trophoblast function through modulation of miR expression, leading to changes in the TLR4/MyD88 signaling pathway. **Therefore, my central hypothesis is that aPL decrease trophoblast miR expression, allowing activation of the TLR4/MyD88 pathway and the subsequent alteration of trophoblast function, and that vitamin D reverses these effects**. The mechanisms by which aPL affect trophoblast function still remain largely unclear, and current therapies do not reduce the risk of severe late gestational pregnancy complications. Therefore, a better understanding of the pathophysiology of obstetric APS is needed to identify novel targets for pharmacotherapy. The overall goals of this study were to: 1) characterize the effects of aPL on trophoblast miR expression and function; and to 2) investigate the effects of vitamin D on trophoblast function and miR expression in the setting of aPL and LMWH. Thus, the specific objectives of this project were:

1. To determine the effects of aPL on miR expression in first trimester trophoblast cells.

2. To determine the functional role of miRs in the regulation of trophoblast responses to aPL.

3. To determine the potential of therapeutic vitamin D to reverse aPL-mediated effects on trophoblast function and miR expression.

MATERIALS AND METHODS

Reagents

The low molecular weight heparin (LMWH) preparation, Lovenox (Enoxaparin), was obtained from Aventis Pharmaceuticals Inc. (Bridgewater, NJ, USA). Active vitamin D (1,25-Dihydroxyvitamin D_3) was obtained from Sigma-Aldrich, (St Louis, MO, USA), reconstituted in ethanol and filter sterilized prior to use. The TLR4 antagonist, LPS from *R. sphaeroides* (LPS-RS) was purchased from Invivogen (San Diego, CA).

Antiphospholipid antibodies

The current study utilized two mouse IgG1 anti-human β_2GP1 monoclonal antibodies (aPL), designated ID2 and IIC5, which were produced by one of our collaborators (L.W. Chamley), under sterile conditions and filter sterilized prior to use. The antibodies were cloned from mice immunized with purified human β_2GPI, and have been previously characterized (91). Like patient-derived polyclonal aPL, ID2 and IIC5 bind β_2GPI when it is immobilized on a negatively charged surface such as phospholipids, cardiolipin, phosphatidyl serine or irradiated polystyrene (92). Moreover, ID2 and IIC5 react specifically with epitopes within domain V of β_2GPI (45), and have been shown to bind to human first trimester trophoblast cells and alter their function in a similar fashion to patient-derived polyclonal aPL (30,43,44). A mouse IgG1 monoclonal antibody (BD Biosciences, San Jose, CA) was used as an isotype control.

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 by Yale University's Human Research Protection

Program. Tissue specimens were washed with cold Hanks Balanced Salt Solution (Gibco) to remove excess blood, minced, transferred to trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) digestion buffer and incubated at 37°C for 40 min with shaking. The mixture was then passed through a nylon strainer and then layered over Lymphocyte Separation Media (ICN Biomedicals, Inc., Aurora, OH, USA) and centrifuged at 400 × g for 25 min. The cellular interface containing the trophoblast cells was collected and resuspended in D-MEM with D-valine (Caisson Labs, North Logan, UT, USA) supplemented with 10% normal human serum (Gemini Bio-Products, Woodland, CA, USA) and cultured at 37°C/5% CO2 as previously described (30,44,47).

Human first trimester trophoblast cell lines

The human first trimester extravillous trophoblast cell line, HTR8, was used in these studies. The HTR8 cells were immortalized by SV40 (93) and were a kind gift from Dr. Charles Graham (Queens University, Kingston, ON, Canada). HTR8 cells were cultured in RPMI 1640 (Gibco, Carlsbad, CA, USA), which was supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT, USA), 10 mm Hepes, 0.1 mm MEM non-essential amino acids, 1 mm sodium pyruvate, and 1000U/ml/1000ug/ml penicillin/streptomycin (Gibco). Cells were maintained at 37°C/5% CO_2 and passaged between 80%-100% confluency by trypsinization, centrifugation and resuspension. The TLR8 dominant negative (TLR8-DN) trophoblast cells were previously produced from HTR8 cells stably transfected with the pZERO plasmid containing the TLR8-ΔTIR gene (Invivogen). Without the TIR domain, the TLR8 protein maintains ligand affinity but is unable to induce signaling cascades. 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 24hrs, after which selection for stable

expression was performed using 250µg/mL of puromycin (Invivogen). The TLR8-DN trophoblast cell-line has been previously characterized (94).

Trophoblast cell treatment experiments and transfections

For all experiments, confluent cells were trypsinized, plated into 60mm dishes at 2.0×10^5 cells/mL and allowed to adhere overnight. Cells were then treated with or without the aPL ID2, IIC5 or an IgG1 isotype control (20µg/mL) in serum-free Opti-MEM media (Gibco). For vitamin D and LPS-RS experiments, cells were pre-treated with either 10nM active vitamin D or 10µg/mL LPS-RS 1 hour prior to treatment with aPL. Isolated primary trophoblast cells were treated with no treatment (NT) or with the aPL, ID2 (20µg/ml) in the presence or absence of LMWH (100µg/ml), vitamin D (10nM), or a combination of both LMWH and vitamin D. Dosages of vitamin D and LMWH were determined from previous studies (30,44,46,58,69), and assessed for toxicity using the Celltiter 96™ viability assay prior to use. Neither the treatments nor the ethanol control had any effect on cell viability (data not shown).

To assess miR function, cells were transfected with 100nM of either an anti-miR scramble sequence control or specific inhibitors of miR-146a-5p, miR-146a-3p, miR-155, or miR-210 (Anti-miR, Life Technologies, Grand Island, NY), using siPORT NeoFX transfection reagent (Invitrogen, Grand Island, NY). Similarly, cells were transfected with a specific miR-146a-5p precursor, miR-146a-3p precursor, or a scramble sequence control at 200nM (Pre-miR, Life Technologies, Grand Island, NY). Transfection efficiency in excess of 99% was assured using Cy-3 labeled anti-miR scramble sequence control and direct visualization under fluorescence microscopy.

Migration studies

Trophoblast migration was assessed using the two-chamber QCM Colorimetric Cell Migration Assay (EMD Millipore, Billerica, MA) as previously described (32). Briefly, HTR8 trophoblast cells were transfected using specific inhibitors of miR-155, or miR-210 or a scramble control as above. After 24h, transfected cells were trypsinized and 1×10^5 cells in 200µL were placed into cell culture inserts with 8µm pores in the presence or absence of aPL (20µg/mL). The inserts were then placed into 24-well culture plates, with each well containing 800µL of either OptiMEM (NT), or aPL (20µg/mL). 1×10^5 cells in 1mL were also placed directly into the 24-well plate to serve as a 100% migration control. After 48h of treatment, migrated cells were stained and lysed according to the manufacturer's protocol. Stained lysates were then transferred to a 96-well plate in triplicate and optical densities were measured using the iMArk Microplate Absorbance Reader (BioRad, Hercules, CA) and compared to the 100% migration control.

Cytokine and angiogenic factor studies

After 72hrs in treatment, trophoblast cell-free culture supernatants were collected by centrifugation at 1500 × g for 10 min at 4°C stored at -80°C. The pro-inflammatory cytokines, IL-8, IL-6 and IL-1β; the pro-angiogenic factors, VEGF and PlGF; and the anti-angiogenic factors, sENG and sFlt-1 were measured in the supernatants by ELISA (Enzo Life Sciences, Farmingdale, NY, USA or R&D Systems, Minneapolis, MN, USA) following the manufacturers' protocols directly.

RNA isolation and quantitative real-time qPCR

After 6-48h in culture, trophoblast cellular RNA was extracted using TRIzol (Life Technologies). Briefly, supernatants were removed and 1 mL of TRIzol was added to adherent cells for 5 min on ice. Cells were then scraped and transferred to 1.5 mL tubes,

vortexed, and combined with 200µL chloroform. Samples were vortexed, incubated at RT for 5 min and then centrifuged at 13000 × g for 15 min at 4°C. The aqueous phase was then collected, combined with 500 µL isopropanol and allowed to precipitate overnight at -20°C. RNA was then pelleted by centrifugation at 13000 × g for 10 min at 4°C and resuspended in RNAse free water. Total RNA concentration was then measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). The expression of miR-9, miR-146a-5p, miR-146a-3p, miR-155, miR-210, and let-7c were assessed using the Taqman MicroRNA Assay (Life Technologies) and normalized to the endogenous control snU6. Briefly, 10ng of total RNA was reverse transcribed using Taqman reverse transcriptase kit in a reaction volume of 15µL containing 3µl of primers specific for miR-9, miR-146a-5p, miR-155, miR-210, and let-7c using the following thermal cycling conditions: 16°C for 30min, 42°C for 30min, 85°C for 5min. For miR-146a-3p, 250ng of total RNA were reverse transcribed as above, and then pre-amplified using Taqman PreAmp master mix kit (Life Technologies) for 10 cycles according to the manufacturer's protocol. The resulting cDNA was then amplified using Taqman Universal PCR Master Mix II with UNG (Life Technologies) and specific primers for target miRs under the following conditions: 50°C for 2min, 95°C for 10min, 95°C for 15sec and 60°C for 60sec. Data were analyzed using the Δ-Δ CT method and plotted as fold change (FC) in the expression of gene of interest normalized to the endogenous control.

Statistical analysis

Experiments were performed a minimum of three times and assayed in duplicate. Data were then pooled and are expressed as mean ± SEM. Statistical significance ($p<0.05$) was determined by Student's t-test or analysis of variance (ANOVA) where appropriate using Prism software (Graphpad Software inc, La Jolla, CA).

Statement of contribution

The author was responsible for execution of all cell culture maintenance and experiments, including plating, transfection, treatment and sample processing as described above, with the exception of: isolation and treatment of primary cell cultures, a portion of the ELISA data, and the generation and characterization of the TLR8-DN HTR8 cell line (94), which were completed by lab members Melissa Mulla, Meredith Stuhlman, and Julie Potter, respectively. All remaining data presented herein were generated by the author.

RESULTS

aPL upregulate trophoblast expression of miR-146a-5p, miR-155, and miR-210

Since the effects of aPL on trophoblast expression of miRs have yet to be studied, the first objective of this study was to characterize first trimester trophoblast expression of miRs in response to aPL. To accomplish this, we utilized a well-characterized *in vitro* system consisting of a human first trimester trophoblast cell line (HTR8) and the mouse anti-human β_2GPI monoclonal antibody, IIC5 (aPL). Previous work from our lab has shown that aPL induce a pro-inflammatory response in the trophoblast by binding β_2GPI and activating the TLR4/MyD88 pathway (30). Therefore, a panel of five miRs known to be involved in TLR signaling was selected for initial analysis; miR-146a-5p, miR-155, and miR-9 are known to target IRAK/TRAF6, TAB2 and NF-κB/p50 of the TLR4 signaling pathway, respectively (82). The panel also included two miRs known to affect trophoblast function; miR-210, which inhibits trophoblast migration and is induced by TLR3, and Let-7c, which inhibits trophoblast IL-6 secretion and is induced by TLR2 (95-98). Given that these miRs are also known to be induced by TLR signaling and inhibit migration, we postulated that treatment with aPL would alter their expression in the trophoblast. First, we conducted a preliminary time course study evaluating miR expression in the HTR8 trophoblast cell line in response to aPL between 6-48h. As shown in Figure 1A, miR-146a-5p was significantly elevated at 48h ($p < 0.05$). miR-155 and miR-210 exhibited a similar trend, but were not significantly elevated (Figure 1A). Having established that 48h was the optimal time point, further experiments were performed using full controls. As shown in Figure 1B, after 48h of treatment, aPL induced a significant upregulation of trophoblast miR-146a-5p, miR-155 and miR-210, when compared to the no treatment (NT) control. In contrast, no significant changes in expression of miR-9 or let-7c in response to aPL were identified. No significant alteration in miR-146a-5p, miR-155, miR210, miR-9 or Let-7c expression levels was observed in

response to the mouse IgG1 isotype control, suggesting that the responses observed were specific to aPL (Figure 1B).
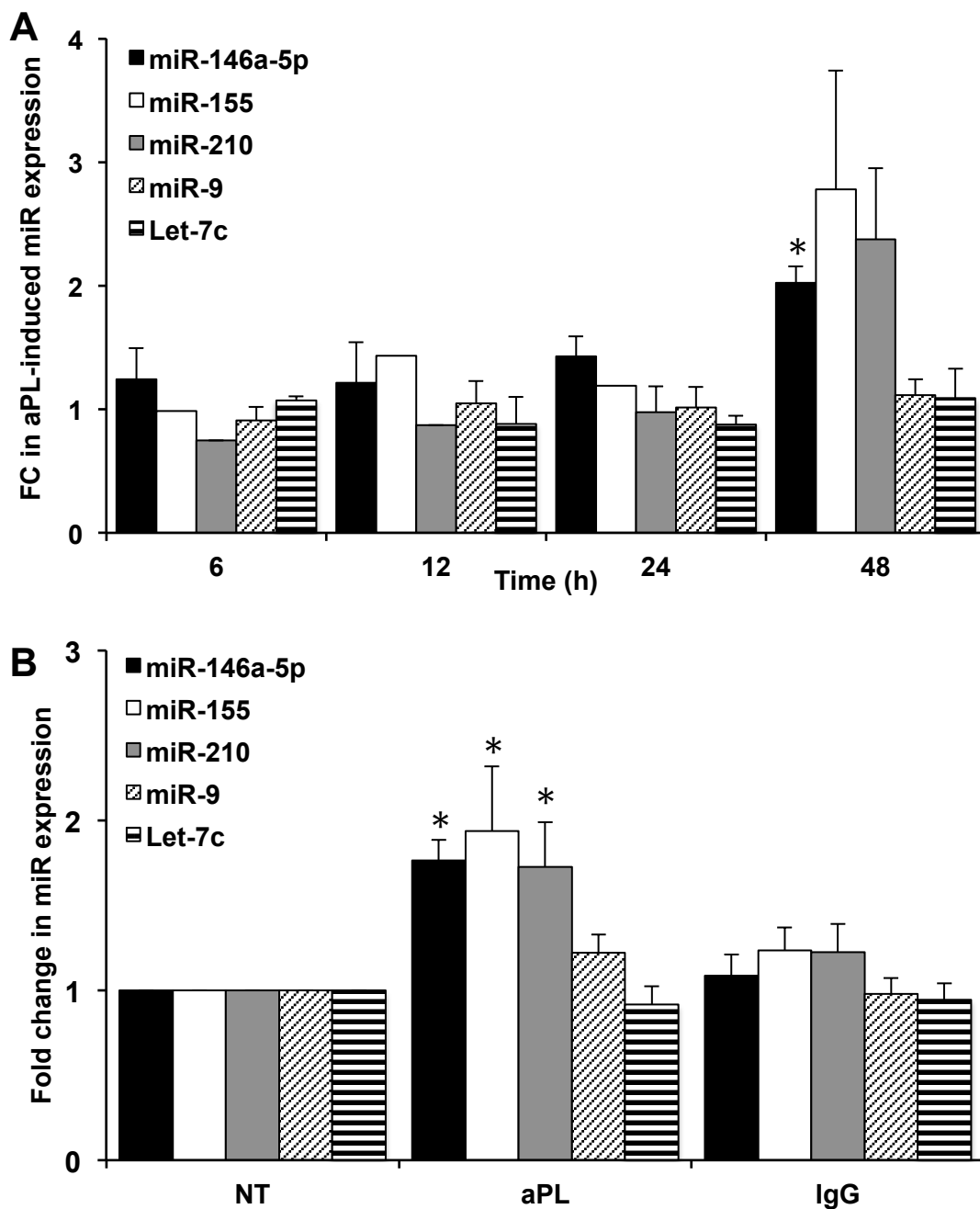
Figure 1. aPL induce cellular expression of miR-146a-5p, miR-155 and miR-210, but not miR-9 or Let 7c.
Trophoblast cells (HTR8) were treated with either no treatment (NT), aPL (20µg/mL) or IgG isotype control (20µg/mL). After 6-48h (A) or 48h (B), cellular RNA was isolated and analyzed for miR-146a-5p, miR-155, miR210, miR-9 and Let-7c expression by qPCR using snU6 as an internal control. Data were analyzed by ANOVA and expressed as fold change (FC) relative to the NT control. A: n=1-4, B: n=10. *p<0.05.

aPL-induced trophoblast expression of miR-146a-5p, but not miR-155 or miR-210, is TLR4 dependent

Since aPL induce a pro-inflammatory response in the trophoblast through TLR4 (30) and a number of miRs can be induced after TLR activation (82), the next objective of this study was to determine the role of TLR4 in the observed induction of trophoblast miR expression by aPL. To test this, trophoblast cells were treated with aPL in the presence or absence of the TLR4 competitive antagonist LPS-RS. As shown in Figure 2, addition of LPS-RS to the culture resulted in a significant attenuation of aPL-induced miR-146a-5p expression from a 1.9 fold to 1.4 fold increase ($p<0.05$), suggesting that this response is TLR4 mediated. aPL-induced expression of miR-155 and miR-210 was not significantly altered by LPS-RS (Figure 2).
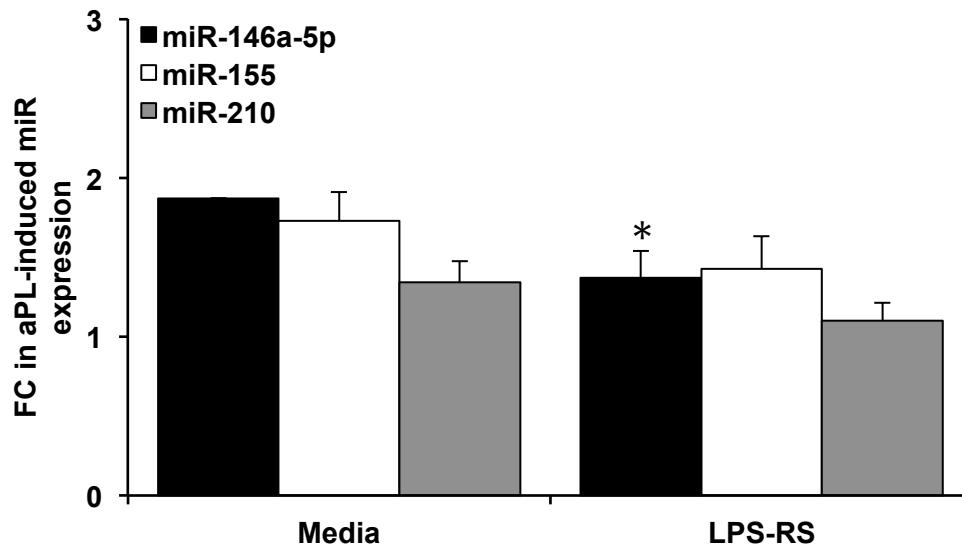
Figure 2. aPL-induced miR-146a-5p expression is inhibited by LPS-RS
Trophoblast cells (HTR8) were treated with either media or LPS-RS (10µg/mL) and then incubated with or without aPL (20µg/mL) for 48h. Cellular RNA was isolated and analyzed for miR-146a-5p, miR-155 and miR210 by qPCR using snU6 as an internal control. Data were analyzed by student's t-test and expressed as fold change (FC) relative to the untreated control. n=4, *$p<0.05$.

aPL upregulate trophoblast expression of miR-146a-3p in a TLR4 dependent manner

Given that only miR-146a-5p expression was found to be TLR4-mediated, we decided to focus the remainder of our studies on miR-146a expression and function. In this regard, we evaluated both the dominant miR-146a-5p form, as well as its isomiR, miR-146a-3p (formerly miR-146a*). Initially thought to be responsible solely for ensuring accurate processing of the dominant miR, isomiRs, or miR*s, have been reexamined in a functional context after being found to be evolutionarily conserved and to have regulatory activity (99,100). Recent studies suggests that despite expression levels often 20-200 fold lower than their miR counterparts, isomiRs can associate with the RNA-induced silencing complex and interact with mRNA, further suggesting that they may serve a functional role (100,101). Thus, we next examined the effects of aPL on trophoblast miR-146a-3p expression. Similarly to miR-146a-5p (Figure 1B), miR-146a-3p expression was significantly upregulated 7.5 fold in response to aPL (p<0.05) compared to the no treatment (NT) control (Figure 3A). The mouse IgG1 isotype control had no effect on miR-146a-3p expression. Furthermore, aPL-induced miR-146a-3p expression was significantly reduced from 12.3 to 6.8 fold in the presence of LPS-RS (Figure 3B; p<0.05), suggesting that miR-146a-3p is regulated by TLR4, similar to miR-146a-5p.
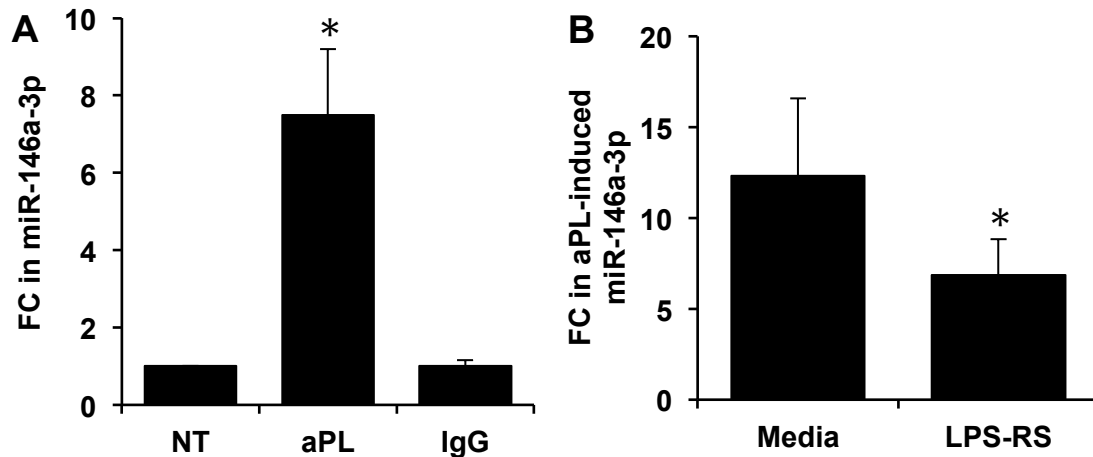
Figure 3. miR-146a-3p expression is induced by aPL and this is inhibited by LPS-RS. (A) Trophoblast cells (HTR8) were treated with either no treatment (NT), aPL (20µg/mL) or IgG isotype control (20µg/mL). (B) Trophoblast cells (HTR8) were treated with or without aPL (20µg/mL) in the presence of media or LPS-RS (10ug/ml). After 48h, cellular RNA was isolated and analyzed for miR-146a-3p by qPCR using snU6 as an internal control. Data are expressed as fold change (FC) relative to the NT control. (A) n=8; *$p<0.05$ as determined by ANOVA; (B) n=7; *p<0.05 as determined by Student's t test.

Inhibition of trophoblast miR-146a-3p reduces aPL-induced IL-8 secretion

The end result of TLR activation is marked by production of pro-inflammatory cytokines and chemokines, which are transcribed by RNA polymerase II-sensitive genes (102). miRs are also transcribed by RNA polymerase II and thus can be induced by TLR signaling (103). Therefore, expression of miRs can be both a product and a target of TLR signaling. Since miR-146a-5p is known to inhibit IRAK1/TRAF6 of the TLR4 signaling pathway, we postulated that inhibition of aPL-induced miR-146a-5p expression in the trophoblast would result in unrestrained TLR4 signaling and a further increase in TLR4 end-function responses to aPL. Currently, no information exists on the functional role of miR-146a-3p, although it has been implicated the development of thyroid cancer (104). Thus, we transfected HTR-8 trophoblast cells with specific inhibitors to miR-146a-5p and miR-146a-3p in the presence or absence of aPL, and subsequently measured IL-

8 secretion, well-established by our lab as a marker of aPL-mediated TLR4 signaling (30). Interestingly, transfection of trophoblast cells with a miR-146a-3p inhibitor resulted in a significant reduction in aPL-induced trophoblast IL-8 secretion from 3.6 fold to 2.1 fold, as compared to the miR scramble control ($p<0.05$) (Figure 4A). However, there was no significant effect of miR-146a-5p inhibition on aPL-induced trophoblast IL-8 secretion (Figure 4B).
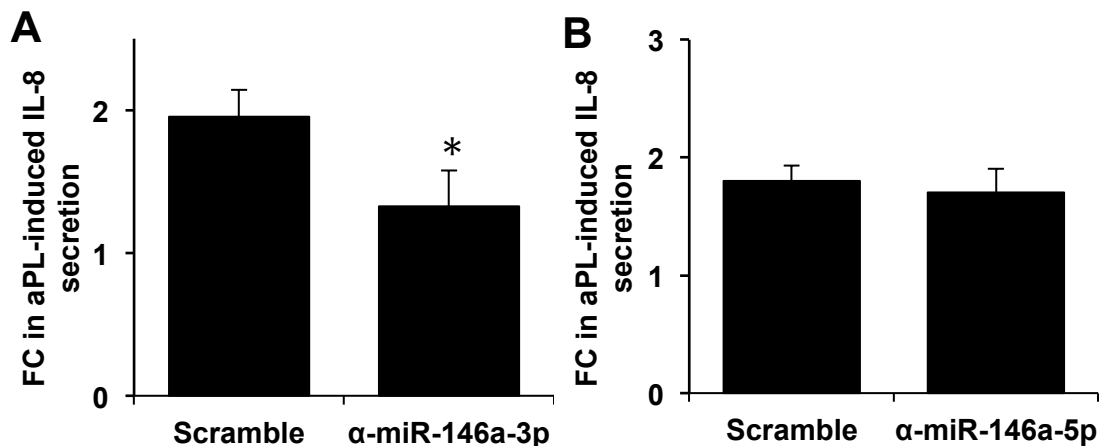

Figure 4. Inhibition of miR-146a-3p decreases aPL-mediated trophoblast IL-8 secretion. Trophoblast cells (HTR8) were transfected with a miR scramble control and either (A) a miR-146a-3p inhibitor (α-miR-146a-3p; 100nM) (n=10), or (B) a miR-146a-5p inhibitor (α-miR-146a-5p; 100nM) (n=4). Following transfection, cells were treated with or without aPL (20μg/mL). After 72h, cell-free supernatants were collected and measured for IL-8 secretion by ELISA. Data were analyzed by Student's t-test and expressed as fold change (FC) relative to the untreated control; $*p<0.05$.

aPL-induced expression of miR-146a-3p induces trophoblast IL-8 secretion through activation of TLR8

Recent data suggest that isomiRs can have widely varying targets and both synergistic and antagonistic functions within the same cell (101,105). Thus, it remains plausible that aPL-induced miR-146a-5p and miR-146a-3p act on different targets within the trophoblast. In recent years, some miRs have been found to act outside of the canonical role of mRNA degradation and translational repression by directly interacting with proteins to alter function (106). Indeed, this evidence provides the foundation for a novel

hypothesis suggesting that certain miRs may act in a non-classical fashion by binding and activating protein receptors. One protein receptor capable of binding small single-stranded RNAs (ssRNA) like miRs is TLR8. TLR8 is an endosomal TLR expressed by both immune cells (107) and first trimester trophoblast (108), and is typically responsible for the detection of ssRNA derived from viruses such as influenza and human immunodeficiency virus (107). Similar to TLR4, TLR8 recruits MyD88 upon activation and initiates a signaling cascade carried by IRAK1 and TRAF6, ending in transcription of NF-κB dependent genes (35). Thus, TLR8 is capable of inducing robust inflammatory responses. Indeed, others have recently identified the ability of miR-21 and miR-29a to bind and activate TLR8, resulting in NF-κB induction (109). Moreover, let-7 has been associated with activation of TLR7, a predominantly murine RNA-sensing TLR closely related to human TLR8 (110). Given our observation that miR-146a-3p inhibition reduces trophoblast IL-8 in response to aPL, we postulated that aPL trigger this inflammatory response through the upregulation of miR-146a-3p and subsequent activation of TLR8 within the same cells. To test this, we compared the IL-8 secretory responses of wildtype and TLR8-DN trophoblast cells to both aPL and exogenous miR-146a-3p. As shown in Figure 5A, treatment of wildtype trophoblast cells with aPL resulted in a 2.1 fold increase in IL-8 secretion ($p<0.05$) and this was significantly and almost completely inhibited by the presence of the TLR8-DN. Similarly, in the absence of aPL, transfection of wildtype trophoblast cells with the miR-146a-3p mimic resulted in a significant 1.6 fold increase in IL-8 secretion compared to the miR scramble control ($p<0.05$) (Figure 5B), and this response was completely eliminated in the presence of the TLR8-DN (Figure 5B). These results suggest that aPL may induce IL-8 secretion through miR-146a-3p-dependent activation of TLR8.
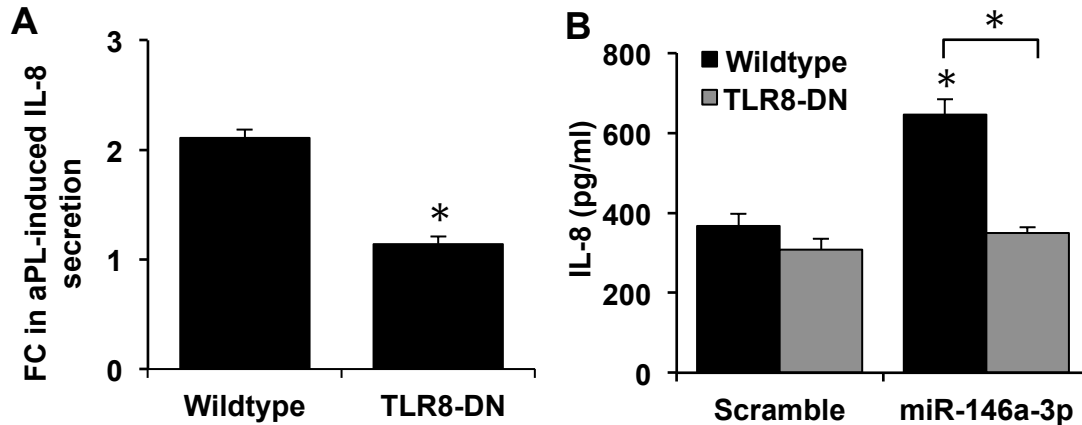
Figure 5. aPL and miR-146a-3p induce trophoblast IL-8 secretion in a TLR8 dependent manner. Wildtype and TLR8-dominant negative (TLR8-DN)-expressing trophoblast (HTR-8) were (A) treated with or without aPL (20µg/mL) (n=3); or (B) transfected with either a miR scramble control or miR-146a-3p precursor (200nM) (n=4). After 72h cell-free supernatants were measured for IL-8 by ELISA. Data were analyzed (A) by student's t test and expressed as fold change (FC) compared to the untreated control *p<0.05; or (B) by ANOVA; *p<0.05 vs. the scramble control unless otherwise indicated.

aPL-mediated inhibition of trophoblast migration is independent of miR-155 and miR-210

aPL are known to reduce trophoblast migration (32), which is thought to contribute to the pathogenesis of APS (32,81). Indeed, recent studies by our group showed that aPL reduce trophoblast invasion and interactions with endothelial cells using a model of spiral artery transformation (111). Moreover, this aPL-mediated reduction in trophoblast migration is TLR4-independent (32). Recently, miR-155 and miR-210 have been shown to be dysregulated in placentae from pathologic pregnancies (112,113), and overexpression of trophoblast miR-155 and miR-210 reduces cell migration (89,96). Since we found that aPL upregulate expression of both miR-155 and miR-210 independent of TLR4, we hypothesized that aPL may regulate trophoblast migration through these miRs. Thus, we investigated the effects of miR-155 and miR-210 inhibition on trophoblast migration in the presence of aPL using a two-chamber colorimetric migration assay. As previously reported (32), exposure to aPL significantly reduced

trophoblast migration by 70.0% (Figure 6A) and 60.0% (B) as compared to the untreated

scramble control (p<0.05). However, transfection of specific inhibitors of miR-155 or

miR-210 had no effect on this aPL-mediated inhibition of migration (Figure 6).
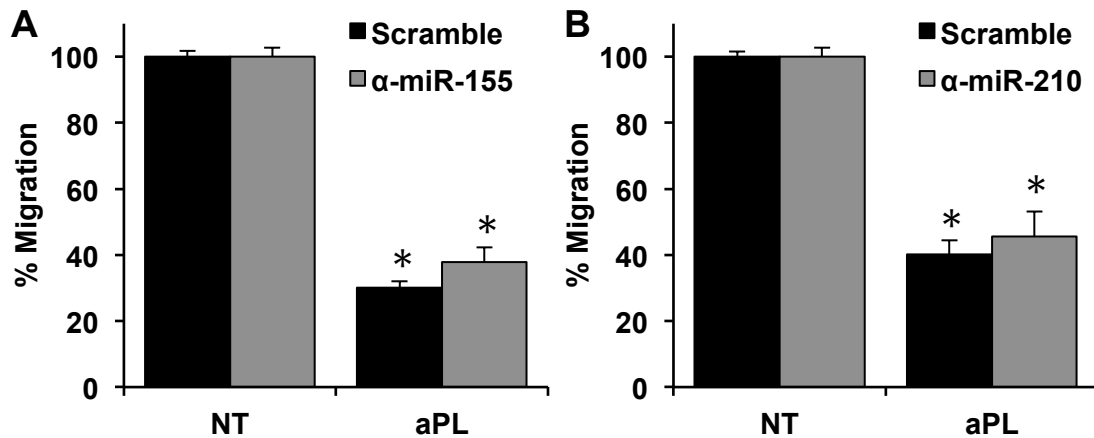
Figure 6. aPL-inhibited trophoblast migration is independent of miR-155 and miR-210 . Trophoblast cells (HTR8) were transfected with a miR scramble control or either (A) a miR-155 inhibitor (α-miR-155; 100nM) (n=3), or (B) a miR-210 inhibitor (α-miR-210; 100nM) (n=4). Cells were then placed in the upper chamber of a two chamber migration assay and were treated with either no treatment (NT) or aPL (20μg/mL). After 48h in culture, migration across an 8μm pore was determined. Data are expressed as % migration normalized to the NT control, which was then set to 100%. *p<0.05 as determined by ANOVA.

Vitamin D reduces aPL-induced trophoblast IL-8 secretion

Since LMWH, the current therapy for pregnant APS patients, does not reduce the risk of

late gestational complications, there remains a serious need for discovery of new

therapeutic targets and treatments for obstetric APS. Vitamin D, an immunomodulatory

hormone, has been implicated in the pathogenesis of APS and is commonly deficient in

APS patients (73), and thus represents a possible candidate for treating or preventing

APS. Therefore, in the translational arm of this study, we sought to examine the effects

of vitamin D exposure on human first trimester trophoblast responses to aPL. As

previously reported (30,45,46), exposure of HTR8 cells to aPL for 72h resulted in a

significant increase in IL-8 and IL-1β secretion (Figure 7). The presence of vitamin D

significantly reduced aPL-induced IL-8 secretion by 24.9 ± 4.6% (Figure 7A; p<0.05). In

contrast, vitamin D had no significant effect on the ability of the aPL to upregulate

trophoblast HTR8 secretion of IL-1β (Figure 7B). Neither vitamin D alone (Figure 7), nor

the ethanol control (data not shown) had any effect on HTR8 IL-8 or IL-1β secretion.
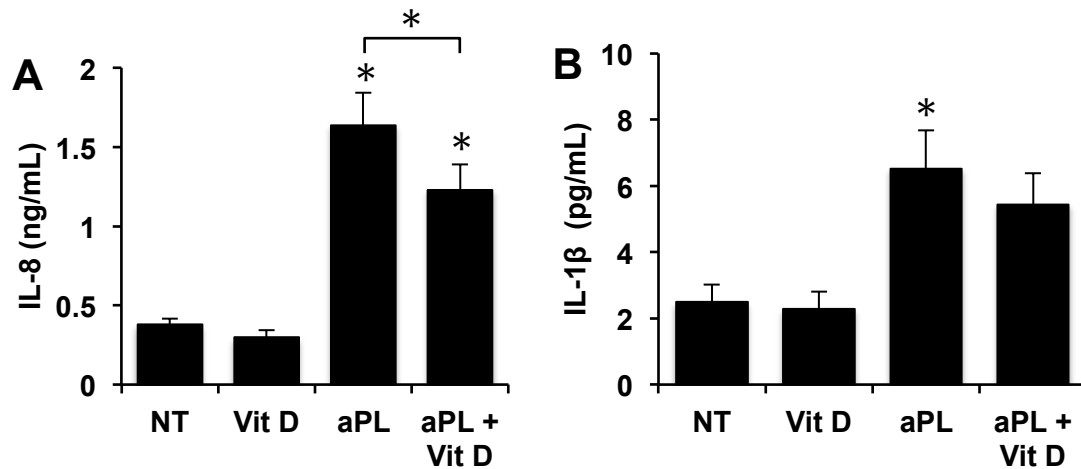
Figure 7. Effect of aPL and vitamin D on a trophoblast cell line secretion of IL-8 and IL-1β. HTR8 trophoblast cells were incubated with no treatment (NT), active vitamin D (Vit D), aPL or both aPL + Vit D. Supernatants were measured for IL-8 and IL-1β by ELISA. *p<0.05 relative to the NT control unless otherwise indicated as determined by ANOVA.

In previous studies we have found that the aPL, ID2, also modulates HTR8 angiogenic

factor production. Specifically, ID2 increases HTR8 secretion of the pro-angiogenic

factor PIGF and the anti-angiogenic factor sEndoglin, while either decreasing or having

no effect on anti-angiogenic sFlt-1. This aPL (ID2) also had either no effect or a mild

stimulatory impact on HTR8 secretion of the VEGF (44-46). As shown in Figure 8, the

presence of vitamin D had no significant effect on the secretion of (A) sEndoglin, (B)

sFlt-1, (C) PIGF or (D) VEGF in the presence of the aPL. Vitamin D did, however,

significantly reduce basal HTR8 secretion of sFlt-1 by 26.7±7.8% (Figure 8B).
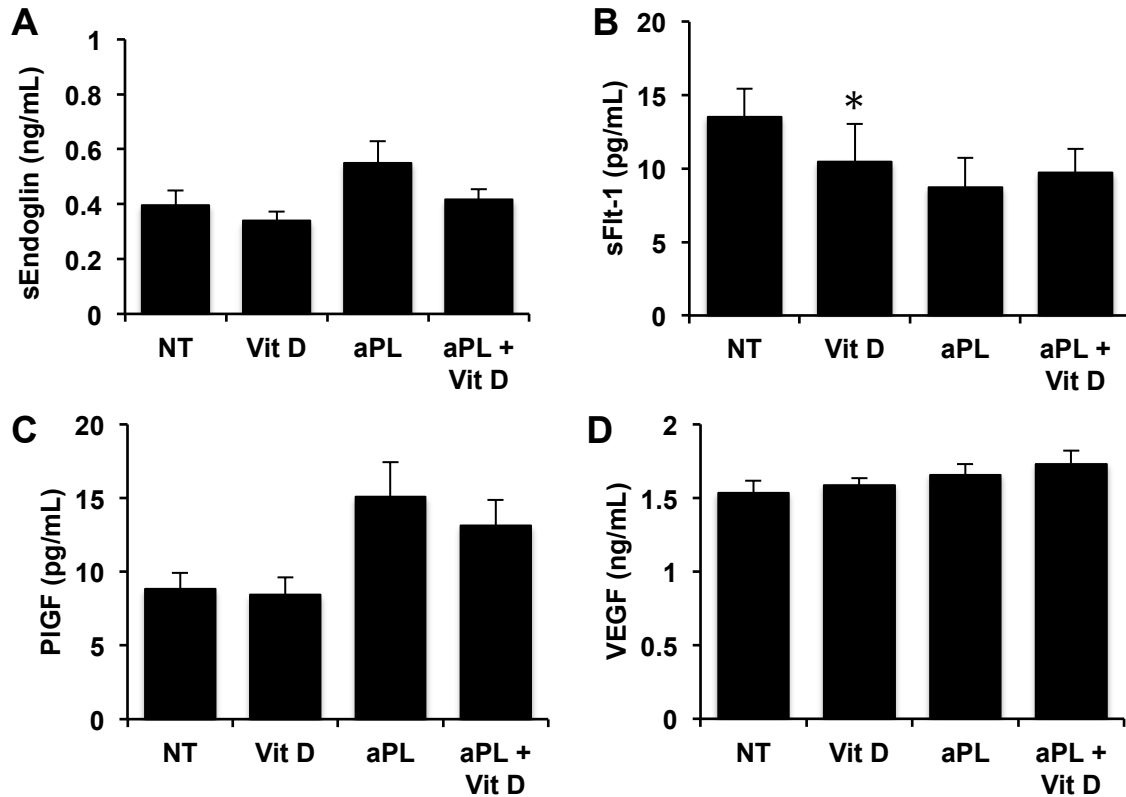
Figure 8. Effect of aPL and vitamin D on a trophoblast cell line secretion of angiogenic factors. HTR8 trophoblast cells were incubated with no treatment (NT), Vit D, aPL or aPL + Vit D. Supernatants were analyzed for the anti-angiogenic factors, sEndoglin and sFlt-1 and the pro-angiogenic factors, PlGF and VEGF by ELISA. *p<0.05 relative to the NT control as determined by ANOVA.

Vitamin D inhibits trophoblast aPL-induced miR-146a-5p expression

Vitamin D has recently been found to have numerous immunomodulatory effects, and the trophoblast express the vitamin D receptor and key enzymes required for activating and deactivating vitamin D (69). Since miR-146a-3p drives trophoblast IL-8 secretion in response to aPL (Figure 5), we postulated that vitamin D may inhibit trophoblast IL-8 secretion through regulation of miR-146a-3p and miR-146a-5p. As shown in Figure 9A, vitamin D significantly reduced aPL-induced miR-146a-5p expression from 2.15 fold to 1.23 fold (p<0.05). While a similar trend was observed with respect to miR-146a-3p, the effect of vitamin D did not reach significance. Vitamin D alone did not affect miR-146a-5p or miR-146a-3p expression (Figure 9).
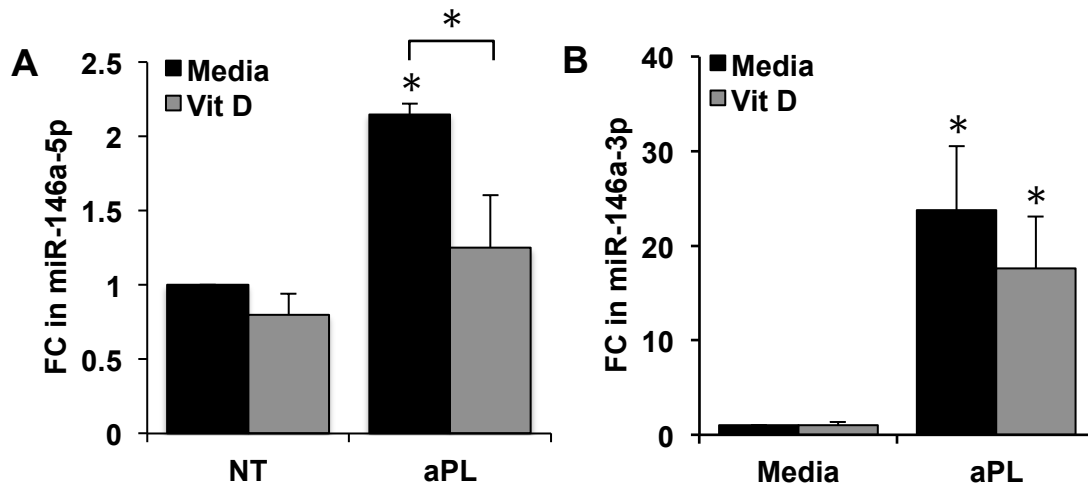
Figure 9. Vitamin D reduces aPL-mediated miR-146a-5p expression. Trophoblast cells were treated with either no treatment (NT) or aPL (20µg/mL) in the presence or absence of Vitamin D (10nM). After 48h in culture, RNA was isolated and analyzed for (A) miR-146a-5p and (B) miR-146a-3p via qPCR using snU6 as an internal control. Data were analyzed by ANOVA and expressed as fold change (FC) compared to the NT control. n=4, p*<0.05.

Vitamin D and LMWH inhibit aPL-induced inflammation in primary trophoblast cells

While LMWH is the standard of care for pregnant APS patients, it has been shown to have both beneficial and deleterious effects on human trophoblast responses to aPL (44,46,58). Thus, we next sought to examine the effects of LMWH and vitamin D, alone or in combination, on aPL-mediated trophoblast responses. We previously reported that pravastatin exerts different effects on trophoblast responses to aPL when using either the HTR8 cell line or primary first trimester trophoblast cells (47). Based on this, and since we had only tested the effects of aPL in the presence of LMWH using the HTR8 cell line (44,46,58), we moved to a primary human first trimester trophoblast culture system. As shown in Figure 10A, and consistent with previous reports, aPL significantly increased primary trophoblast IL-8 secretion (30). In contrast, in the presence of vitamin D and/or LMWH, aPL did not significantly increase IL-8 secretion when compared to the

no treatment (NT) control (Figure 10A). Furthermore, neither treatment alone or in combination significantly reduced IL-8 secretion compared to aPL alone (Figure 10A). Thus, combination of vitamin D and LMWH did appear to reduce aPL-induced IL-8 secretion to near baseline levels (Figure 10A). The levels of IL-1β secreted from primary trophoblast cell in the presence of aPL were significantly reduced by LMWH and vitamin D both alone and in combination by 77.3±7.1%, 99.8±0.2% and 95.3±4.2%, respectively (Figure 10B).
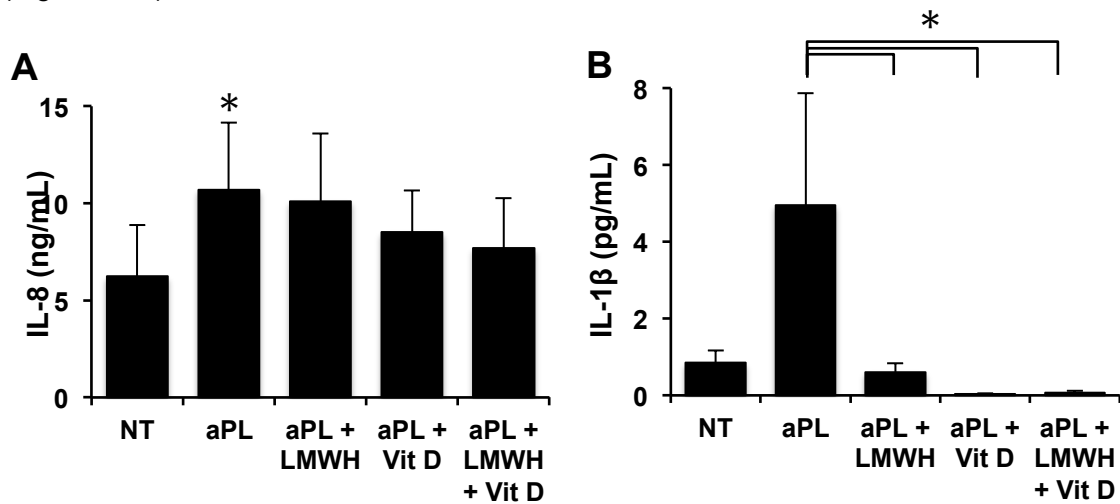
Figure 10. Effect of LMWH and Vitamin D on aPL-induced secretion of IL-8 and IL-1β by primary trophoblast cells. Primary human first trimester trophoblast cells were incubated with no treatment (NT), aPL, or aPL with LMWH, Vit D, or a combination of LMWH + Vit D. Supernatants were analyzed for IL-8 and IL-1β by ELISA. *p<0.05 relative to the NT control unless otherwise indicated, as determined by ANOVA.

Vitamin D reduces LMWH-induced sFlt-1 release from primary first trimester trophoblast cells

We next sought to evaluate the impact of LMWH and vitamin D, alone and in combination on primary trophoblast angiogenic factor production in the presence of aPL. Shown in Figure 11A aPL had no effect on sEndoglin secretion by primary trophoblast (44), and this was not altered by the presence of LMWH, vitamin D or both compounds. Similarly, aPL had no effect on VEGF secretion when compared to the NT control

(Figure 11D). However, aPL in the presence of vitamin D, either alone or in combination with LMWH, did significantly elevate VEGF secretion when compared to the NT control and when compared to cell treated with aPL and LMWH (Figure 11D). aPL significantly increased primary trophoblast PIGF secretion and this was not altered by LMWH or vitamin D (Figure 11C). Lastly, aPL in the presence of LMWH elevated sFlt-1 release by 2.4±0.4 fold when compared to aPL alone, and this was significantly reduced to near baseline levels when vitamin D was also present (Figure 11B).
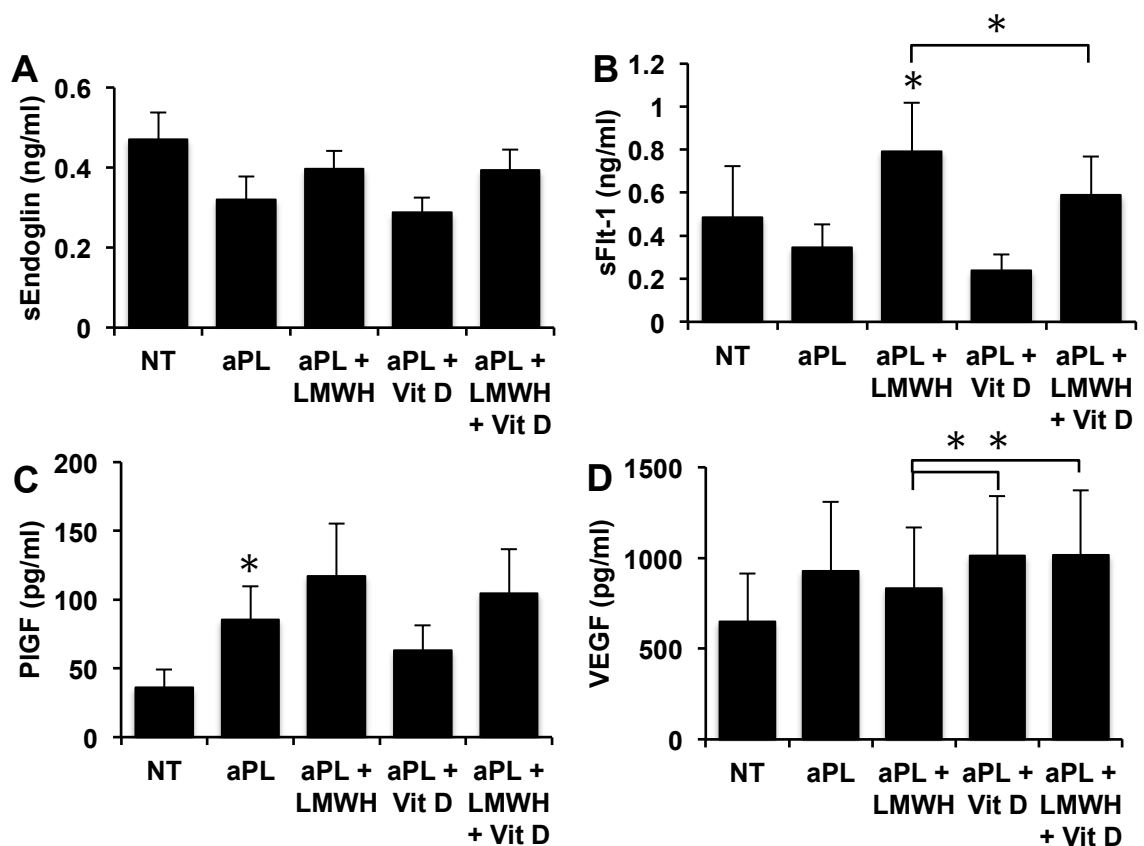
Figure 11. Effect of LMWH and Vitamin D on aPL-induced modulation of angiogenic factors by primary trophoblast cells. Primary human first trimester trophoblast cells were incubated with no treatment (NT), aPL, or aPL with LMWH, Vit D, or a combination of LMWH + Vit D. Supernatants were analyzed for the anti-angiogenic factors, sEndoglin and sFlt-1 and the pro-angiogenic factors, PIGF and VEGF by ELISA. *p<0.05 relative to the NT control unless otherwise indicated as determined by ANOVA.

DISCUSSION

Women with obstetric APS are at increased risk of both early pregnancy loss and late gestational pregnancy complications such as preeclampsia and fetal growth restriction, in addition to the risk of vascular thrombosis (1,3). It is now well established from both clinical and experimental observations that these complications are caused by inflammatory processes and altered trophoblast function at the maternal-fetal interface (27-30,32,43,114). While the mechanisms underlying aPL-mediated trophoblast dysfunction have come to light in recent years, much remains unclear and current therapies remain unable to prevent dangerous complications related to APS. Therefore, further elucidation of the mechanisms by which aPL cause placental dysfunction is needed in order to develop therapeutic strategies to prevent both fetal and maternal complications of APS.

Since aPL are present in the circulation before conception, it is likely that alteration of trophoblast function occurs at the earliest stages of pregnancy. For this reason, we focused our study on the effects of aPL on first trimester trophoblast, using both primary cultures and a human trophoblast cell line that has been well-characterized by our lab and others (30,44,115). The anti-β2GPI monoclonal antibodies (aPL) used in these studies have also been shown to behave similarly to patient-derived polyclonal IgG with anti-β2GPI activity (30,32,43,44), therefore providing a valid and robust experimental system in which to analyze both mechanism and function.

It is now known that anti-β2GPI antibodies are capable of binding the trophoblast and inducing functional changes through TLR4 and their downstream signaling pathways (30). Classically, the end result of TLR activation is the production of inflammatory cytokines that function to recruit immune cells responsible for fighting infection (35).

Indeed, trophoblast cells have been shown to produce cytokine and chemokine responses capable of recruiting immune cells (116) and evidence of neutrophil infiltrate has been seen in APS miscarriage samples (27,116), as well as in a mouse model of aPL-induced pregnancy loss (29). Thus, it appears the trophoblast plays a key role in both placental innate immune-like responses and communication with the maternal immune system. Moreover, aberrant activation of the trophoblast may give rise to an altered immune microenvironment at the maternal-fetal interface, resulting in pregnancy complications. Since excessive inflammation can damage tissues, tight regulation of immune responses is critical to prevent infection, while maintaining placental and immune function that is capable of supporting pregnancy.

Since their discovery, miRs have emerged as important regulators of gene expression. Indeed, individual miRs can target hundreds of genes, and many miRs that target TLR signaling elements have been discovered (82). Moreover, their dynamic, highly regulated nature makes them well suited to a level of control over the intensity, timing and duration of intracellular signaling that is required for mounting an immune response. It is now known that miRs both target and are induced by TLR signaling, and many miRs have been directly implicated in trophoblast function (89,96,112). To date, however, little is known about how exposure to aPL impacts miR expression and function, and no information exists as to whether miRs regulate responses to aPL in the trophoblast. Herein, we describe for the first time that aPL, through TLR4, induce the expression of trophoblast miR-146a-3p, which in turn drives IL-8 secretion through a novel, TLR8-dependent mechanism. Notably, we add to a small, yet growing body of literature that supports the existence of biologically functional isomiRs.

Since aPL induce trophoblast inflammation through activation of the TLR4/MyD88 signaling pathway (30), we began our studies by examining a small panel of miRs known to be involved in the regulation of TLR signaling. Indeed, we found that miR-146a-5p, miR-155, and miR-210 were all significantly upregulated by exposure to aPL. We also found that miR-146a-3p, the less abundant and thus understudied isomiR of miR-146a, was also upregulated in response to aPL. The response was specific to these miRs, as aPL did not significantly alter expression of miR-9 or let-7c. Furthermore, the most significant upregulation of these miRs occurred at the 48h time point. While this is late in comparison to miR responses in other systems, trophoblast responses to aPL are typically slow, with end function changes being seen at 72h (30,114). By treating trophoblast cells in the presence of aPL with the TLR4 competitive antagonist LPS-RS, we observed a significant partial inhibition of aPL-induced miR-146a-5p and miR-146a-3p, suggesting that aPL-induced expression of both miR-146a isomiRs is TLR4-mediated. Similar trends were observed with respect to miR-155 and miR-210, however these did not reach statistical significance. These observations are in keeping with current literature, with miR-146a-5p and miR-155 being two of the most commonly induced miRs by TLRs and also the best characterized (82,117). Moreover, in other systems, induction of miR-146a-5p and miR-155 has been shown to be dependent on NF-κB activation, the final step in TLR4 signaling (117). In a landmark study, Taganov et al. were the first to describe TLR-induced miRs and showed that miR-146a-5p expression is regulated by NF-κB and induced by a number of TLR-stimulating microbial components such as Poly(I:C), LPS and peptidoglycans, as well as by pro-inflammatory cytokines (85). Importantly, through in silico and reporter gene studies, they illustrated that miR-146a-5p targets IRAK1 and TRAF6, two adapter proteins critical to TLR and cytokine signaling. Taken together, these data support a role for TLR-induced miRs as part of a feedback loop that controls TLR-dependent signaling.

In an effort to better understand the functional role of aPL-induced miR expression, we utilized a transfection system to introduce specific miR inhibitors and mimics into HTR8 cells. Since miR-146a-5p and miR-146a-3p upregulation illustrated TLR4 dependence, we initially focused our functional studies on these miRs. End function changes were then analyzed by measurement of IL-8 secretion, a well-characterized product of TLR4 signaling in response to aPL (30,32). With respect to miR-146a-5p, transfection of a specific inhibitor did not result in a change in aPL-induced IL-8 secretion, whereas the addition of a miR-146a-3p inhibitor resulted in a significant decrease in aPL-mediated IL-8 secretion compared to the scramble control. Taken together, these experimental data suggest that miR-146a isomiRs are not functioning classically in the trophoblast in the context of aPL exposure, since the inhibition of miR-146a-5p would be expected to result in unrestrained TLR4 signaling activity and thus increased IL-8 secretion. An alternative explanation suggests that aPL-induced TLR4 activation exceeds the ability of endogenous miR-146a-5p to negatively regulate the pathway, such that further, experimental inhibition of miR-146a-5p activity is not detectable. However, the reduction in IL-8 secretion by miR-146a-3p inhibition provides strong evidence that miR-146a-3p is functionally active in the trophoblast as part of a pro-inflammatory mechanism, clearly distinct from the canonical role of miR-146a-5p in the negative feedback of TLR4 signaling (85,118). It is well-known that miR expression and function can be highly tissue-specific (119), and recent data suggests that miRs, such as miR-155, and their respective isomiRs can have opposing effects on TLR-induced inflammation, with one isomiR promoting inflammatory signaling and the other dampening it (105). While miRs serving as positive regulatory molecules is a relatively novel concept, a number of groups have identified miRs that induce inflammation through a novel mechanism by binding to and activating TLRs themselves. First described by Fabbri et al., the endosomal, single-stranded RNA-sensing receptor TLR8 can bind miR-21 and miR-29a,

resulting in activation of inflammatory signaling (109). They illustrated for the first time that miRs can reach TLR-containing endosomes and associate with TLR8 in monocytes and dendritic cells, resulting in functional activation of the receptor (109).

Thus, we decided to test the hypothesis that aPL-induced miR-146a-3p aberrantly activates TLR8 in the trophoblast. To accomplish this, we compared the IL-8 secretion of wildtype and TLR8-DN HTR8 cells treated with aPL or a miR-146a mimic. Indeed, our results demonstrate that both aPL-mediated and miR-146a-3p-mediated trophoblast IL-8 secretion is dependent on TLR8, as the presence of the TLR8-DN (94) resulted in complete inhibition of the IL-8 response. This suggests that aPL may induce inflammation in the trophoblast by upregulating expression of miR-146a-3p through TLR4, which subsequently binds and activates TLR8, leading to increased secretion of IL-8. This finding is in keeping with recent studies reporting that miR-146a promotes TNFα and TGF-β1 production in endothelial cells (120), and that aPL-induced inflammation in monocytes is dependent on TLR8 (121). Furthermore, Printz et al. have shown that aPL-mediated induction of inflammatory cytokines in monocytes is dependent on RNAs, as introduction of RNAses into the culture medium abolishes all inflammatory effects of aPL (122). Interestingly, these reports also noted that aPL upregulates both expression of TLR8 as well as its translocation to the endosome, where it becomes functional. Indeed, this sensitization effect in combination with increased miR-146a-3p expression may synergistically facilitate miR-146a-3p-mediated TLR8 activation. Taken together, these results suggest a novel mechanism of aPL-induced trophoblast inflammation, and further supports the concept that miRs may induce functional change through endogenous activation of ssRNA-sensing TLRs, like TLR8. To our knowledge, we demonstrate for the first time a functional role of the isomiR

miR-146a-3p, reinforcing the need to address both mature forms of a miR, regardless of abundance, when studying miRs in a physiological context.

Since decidual invasion and migration are impaired in APS pregnancies (27), and aPL are known to reduce trophoblast migration independently of TLR4 (30), we sought to evaluate whether aPL-mediated regulation of TLR4-independent miRs were responsible for these effects. Since we demonstrated that miR-155 and miR-210 were upregulated in response to aPL independently of TLR4, and these miRs have been independently shown to negatively regulate trophoblast migration (95,96), we transfected trophoblast cells with inhibitors to miR-155 and miR-210 and observed the effects on migration in the context of aPL. In our study, there was no rescue of migration by inhibition of either miR-155 or miR-210 alone (Figure 6) or in combination (data not shown), suggesting that aPL impair trophoblast migration independently of these miRs. However, work in HTR8 cells by another group has shown that miR-155 targets cyclin D1, a protein involved for controlling proliferation and migration (95). In their study, overexpression of miR-155 resulted in decreased expression levels of cyclin D1 and an associated inhibition of migration. miR-210 has been shown by others to be upregulated by LPS and subsequently downregulate iron-sulfur cluster scaffold homologue, leading to decreased trophoblast invasion (123). Thus, while it remains plausible that miR-155 and miR-210 play a role in regulating trophoblast migration and invasion, aPL-mediated inhibition of these functions likely occur through alternate mechanisms.

Without treatment, women with APS are at high risk of pregnancy loss and late gestational obstetric complications, including preeclampsia. Despite conflicting data from clinical trials, pregnant women with APS are routinely treated with LMWH (51,54,124). While this therapy might be successful in maintaining a viable pregnancy, it does not

appear to reduce the risk of associated late gestational complications (22,23,48). Heparin therapy was introduced under the assumption that recurrent pregnancy loss in APS patients was due to thrombosis, however, it has become clear that aPL-associated adverse pregnancy outcomes are primarily pro-inflammatory rather than pro-thrombotic (27-30,125), and that heparin might be acting through mechanisms other than anticoagulation (30,46,52). Indeed, these extended actions of LMWH may even have detrimental effects by significantly increasing placental and circulating levels of sFlt-1 (44,46,60-62), a potent anti-angiogenic factor that is elevated in preeclampsia (63) and promotes preeclamptic symptoms in animal models of pregnancy (64,126). Thus, there remains a need for the development and/or identification of therapeutics that can reduce both the recurrent pregnancy loss and the late gestational complications associated with APS, either alone or in combination with the current therapeutic approaches.

Vitamin D, a steroid hormone now recognized for its immunomodulatory properties, has been shown to be deficient in APS patients compared to controls (73). Vitamin D deficiency may also be associated with adverse pregnancy outcomes such as preeclampsia and recurrent pregnancy loss (77-80). Indeed, the placenta is a major site for conversion of vitamin D into its active form, 1,25-Dihydroxyvitamin D3 (71). Furthermore, vitamin D has been directly implicated in the pathogenesis of APS by its ability to inhibit anti-β2GPI antibody-mediated tissue factor expression (73). Taken together, these data support a role for vitamin D in the treatment of pregnant APS patients. In the translational arm of this study, we show for the first time that active vitamin D is able to both regulate aPL-mediated inflammation and mitigate LMWH-induced sFlt-1 release in human first trimester trophoblast.

For this part of the study, both the HTR8 trophoblast cell line and primary human first trimester trophoblast cultures were utilized. Previous work from our group has revealed a number of effects of aPL on human first trimester trophoblast function using both cellular models; in particular the increased secretion of the pro-inflammatory cytokines/chemokines, IL-8 and IL-1β; and altered angiogenic factor production (30,43-47). Thus, we first sought to assess the effects of vitamin D alone on aPL-mediated trophoblast responses using HTR8 cells. Since we previously reported that pravastatin exerts different effects on trophoblast responses to aPL when using either the HTR8 cell line or primary trophoblast cells (47), we also investigated the impact of vitamin D both alone and in combination with LMWH on aPL-mediated effects using primary cultures.

Indeed, we found that treatment of HTR8 cells with vitamin D alone attenuated aPL-mediated IL-8 secretion. In the primary cultures, LMWH inhibited aPL-induced IL-1β secretion, confirming previous studies in the HTR8 cell line (46). Moreover, vitamin D either alone, or in combination with LMWH, completely inhibited the aPL-induced IL-1β response, while combination LMWH and vitamin D appeared to attenuate the IL-8 response. This suggests that vitamin D both alone and in combination with LMWH may be acting to regulate aPL-induced inflammatory signals in the trophoblast. Studies in human lymphocytes and a melanoma cell line have demonstrated the ability of vitamin D to downregulate IL-8 release by repressing NF-κB signaling (127,128) and active vitamin D has been shown to suppress decidual natural killer cell cytokine production (129). Moreover, studies in mice have shown that vitamin D, through activation of the vitamin D-activating enzyme 1α-hydroxylase (CYP27B1), can reduce LPS-induced placental inflammation. This suggests that vitamin D can regulate TLR4-mediated inflammatory responses (71). Since aPL induce human trophoblast IL-8 and IL-1β production through

activation of the TLR4 signaling pathway (30,43), vitamin D may be acting to suppress trophoblast inflammation induced by aPL in this manner.

It is also possible that vitamin D exerts its anti-inflammatory activity through the regulation of miRs. Indeed, the vitamin D receptor is regulated by miRs and vitamin D has been shown to alter miR expression in numerous cell types (130-132), although the effects of vitamin D on trophoblast miR expression have yet to be studied. Since aPL-mediated miR-146a-3p expression promotes trophoblast IL-8 secretion, we postulated that vitamin D reduces the aPL-induced IL-8 response through downregulation of miR-146a-3p. To that end, we investigated the effects of vitamin D on trophoblast expression of both miR-146a isomiRs, and found that treatment with vitamin D reduces aPL-induced miR-146a-5p expression. While a similar trend was observed, miR-146a-3p expression was not significantly inhibited by vitamin D. Thus, our results do not support the hypothesis that vitamin D decreases trophoblast inflammation through downregulation of miR-146a-3p. Rather, our data suggests that vitamin D may reduce aPL-mediated IL-8 secretion and miR-146a-5p expression. Thus, vitamin D may inhibit TLR8 function and miR-146a-5p may have an as yet undetermined function in the trophoblast. Indeed, a recent study in monocytes showed that vitamin D suppresses TLR8-mediated IL-1β and TNFα expression in response to the TLR8 ligands, ssRNA and CL075 (133). While further study is warranted, these data provide further support for a role of vitamin D in regulating responses to aPL, and that vitamin D may have a direct impact on trophoblast TLR4 and TLR8 pathways.

In addition to inflammatory factors, we also examined the ability of vitamin D to modulate aPL-mediated changes in angiogenic factor secretion. Proper placentation depends on appropriate vascular development and remodeling, which is controlled by both maternal-

and placental-derived angiogenic factors (134). These factors are tightly regulated in normal pregnancy, and thus aberrant expression of angiogenic factors may negatively impact placentation and subsequent pregnancy outcomes. Indeed, pro- and anti-angiogenic factors are dysregulated in APS patients (135-137), as well as locally and systemically in pregnant women with preeclampsia (63,138-141). Furthermore, the combination of low vitamin D levels with sFlt-1/PlGF ratios has been shown to provide better prediction of severe preeclampsia than either marker alone (142). Herein, we show that aPL significantly upregulated secretion of sEndoglin and PlGF in the HTR8 cell line, and PlGF in the primary trophoblast cells as previously reported (44), reinforcing the potential of aPL to dysregulate placental angiogenic factor secretion. While vitamin D did not have any overt impact on aPL-mediated modulation of angiogenic factors in the trophoblast cell line, we noted that it significantly reduced sFlt-1 release.

In order to assess the potential of vitamin D to be used as an adjuvant therapeutic for pregnant APS patients, we tested the effects of vitamin D both alone and in combination with standard of care treatment, LMWH, on primary trophoblast function in the presence of aPL. Again, vitamin D either alone or in combination with LMWH did not have any overt impact on aPL-mediated modulation of angiogenic factors in the primary trophoblast. However, vitamin D did appear to promote trophoblast VEGF secretion, and recent studies in endothelial progenitor cells have shown that vitamin D can promote VEGF expression (143). In this study, we reconfirmed previous findings that LMWH significantly promotes sFlt-1 release from the trophoblast (44,46), as well as from placental villus explants (60-62). Moreover, the presence of vitamin D significantly inhibited this LMWH-induced sFlt-1 release to near-baseline levels. Since studies have implicated sFlt-1 in the pathogenesis of preeclampsia (63,64,126), this finding supports

the potential of vitamin D to be used in addition to LMWH to help prevent negative effects of LMWH administration.

The mechanism by which LWMH affects sFlt-1 release and how vitamin D regulates this process remains unclear. There is evidence that sFlt-1 is induced by EGFR1 pathway transactivation in endothelial cells (144), while others suggest that circulating sFlt-1 levels are controlled by regulated release from extracellular stores (62). Thus, vitamin D may be counteracting the effects of LMWH through multiple mechanisms. One possible mechanism is through parallel antagonism of pro-angiogenic pathways, evidenced by the ability of vitamin D to upregulate VEGF production through vitamin D-mediated VEGF gene promotion (145). Alternatively, vitamin D may work through regulation of extracellular enzymes responsible for sFlt-1 release.

Thus, our findings suggest a beneficial role for vitamin D supplementation in pregnant patients with APS, as its effects both alone and in combination with LMWH counteract the inflammatory effects of aPL on the trophoblast. While vitamin D alone reduces aPL-mediated functional changes, the capacity to reduce LMWH-mediated sFlt-1 release is even more promising, given the direct implications of elevated sFlt-1 in the pathogenesis of preeclampsia. Altogether, this work supports further investigation into the role that vitamin D plays in both trophoblast function, and in the development and treatment of obstetric APS.

The primary limitation affecting the generalizability of this study is the use of the HTR8 trophoblast cell line and monoclonal anti-β2GPI antibodies, without support from primary cultures and/or patient-derived aPL. While the use of this experimental system provides a clean platform for functional analysis, the results cannot be readily applied to an in vivo

setting. It is now known that the immortalization technology used to produce the HTR8 cell line can introduce mutations that may cause untoward, non-physiologic effects, calling into question data gained from experiments using these cells. However, our lab has directly compared the aPL-mediated responses between HTR8 cells and primary cultures in previous work, finding them to behave similarly (30,114). Thus, while experiments using an alternative first trimester trophoblast cell line, such as Sw.71 or 3A, as well as primary cultures and patient-derived aPL would reinforce the data described here, we believe that our experimental system provides an excellent foundation for the study of signaling pathways in the trophoblast and that our findings warrant further study in more extensive explant or murine models of APS. Furthermore, while illustrated in other trophoblast cell lines (69), we recognize that the presence of the vitamin D receptor and associated activating enzymes in HTR8 cells has not yet been confirmed. While our observations suggest that the vitamin D machinery is functional within this cell line, confirmation of VDR and CYP27B1 expression as well as functional studies using either siRNA knockdown or dominant negative signaling elements will further strengthen our results. Another limitation to this work is the reliance on synthetic miR mimics and inhibitors to assess miR function. The commercial mimics and inhibitors used in this study are broadly used and provide excellent evidence of the functional roles of miRs in a given system, however it is difficult to establish the biological relevance of a miR *in vivo* as they are naturally highly dynamic regulators of expression and have the capacity to affect multiple targets within a single cell. Thus, while our data strongly suggests a functional role of miR-146a, further work is warranted in determining its exact relevance in the trophoblast and the development of APS.

In summary, we present here new insights into the mechanisms by which aPL induce inflammation and altered function in the trophoblast, and also the ability of vitamin D to

partially reverse these negative effects. We also describe further evidence of a novel mechanism by which TLR8 is activated by an endogenous miR, miR-136a-3p, and that in our system this is induced by aPL. More broadly, we contribute to the growing literature on miR and isomiR function, especially within the trophoblast. This work provides the foundation for further study into the concept of miRs as ligands of TLRs and protein receptors, and the biochemical evaluation of how this process occurs, so that other miRs with similar functions may be discovered more efficiently through *in silico* analysis. Finally, this study provides insight into the pathophysiology of APS and further confirms the inflammatory and autoimmune nature of the disease. Imminent work from our lab will seek to examine miR-146a levels within the serum and placentae of patients with APS, which may serve both clinically as a potential diagnostic or prognostic biomarker for APS and associated gestational complications, as well as pathophysiologically, providing further implication of miR-146a in the development of APS. Indeed, miR-146a is only the first of many miRs to be involved in the pathophysiology of APS and the placenta, and future study into the function of miRs is bound to lead to a better understanding of APS, the immune system and human physiology.

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