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HYDROXYCHLOROQUINE PREVENTS ANTIPHOSPHOLIPID ANTIBODY-INDUCED INHIBITION OF TROPHOBLAST MIGRATION

A Thesis Submitted to the

Yale University School of Medicine

In Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

By

Caroline R. Albert

2015

ABSTRACT

HYDROXYCHLOROQUINE PREVENTS ANTIPHOSPHOLIPID ANTIBODY-INDUCED INHIBITION OF TROPHOBLAST MIGRATION

Women with antiphospholipid syndrome (APS) are at high risk of recurrent pregnancy complications. Antiphospholipid antibodies (aPL) target the trophoblast by binding beta$_2$-glycoprotein I (β_2GPI) and alter human first trimester trophoblast function by: triggering a pro-inflammatory cytokine response; modulating angiogenic factor secretion; and inhibiting cell migration. While patients with APS are often treated with hydroxychloroquine (HCQ), and it is safe to use during pregnancy, little is known about the effects of HCQ on aPL-associated adverse pregnancy outcomes. Therefore, the objective of this study was to test the hypothesis that HCQ prevents the effects of aPL on human first trimester trophoblast function. A human first trimester trophoblast cell line was treated with or without anti-human β_2GPI monoclonal antibodies in the presence or absence of HCQ. Using ELISA, culture supernatants were analyzed for: pro-inflammatory cytokines IL-8 and IL-1β; pro-angiogenic factors VEGF and PlGF; anti-angiogenic factors sFlt-1 and soluble Endoglin; pro-migratory cytokine IL-6; and tissue inhibitors of metalloproteinase 1 and 2 (TIMP1 and TIMP2). Cell migration was measured using a colormetric two-chamber assay. The aPL-induced upregulation of trophoblast IL-8, IL-1β, PlGF, and sEndoglin secretion was not significantly altered by the presence of HCQ. The presence of HCQ partially, yet significantly, reversed the aPL-induced inhibition of trophoblast cell migration and secretion of pro-migratory cytokine IL-6. aPL-induced upregulation of trophoblast TIMP secretion appears to inhibit cell migration. HCQ was unable to completely prevent aPL-induced TIMP secretion, and this

may explain why migration was only partially restored. HCQ reversed the aPL-induced inhibition of IL-6 secretion and partially limited the ability of aPL to reduce trophoblast cell migration. Our data indicate the possibility that some form of combination therapy that includes HCQ may be beneficial to pregnant APS patients and warrants further investigation.

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INTRODUCTION

Classification of Antiphospholipid Syndrome

Antiphospholipid syndrome (APS), an autoimmune systemic disease characterized by circulating antiphospholipid antibodies (aPL), places women at risk of adverse pregnancy outcomes, such as recurrent pregnancy loss and late gestational complications including preeclampsia and associated premature delivery, HELLP syndrome (hemolytic anemia, elevated liver enzymes, and low platelet counts), and intrauterine growth restriction (1). Complications such as preeclampsia are also known to increase health risks for the mother later in life, including hypertension and cardiovascular disease (2). While health consequences for the neonate are known to be related to low birth weight and prematurity, several studies have suggested potential negative long-term cognitive and psychological outcomes, including autism spectrum and other neurodevelopmental disorders (3, 4).

Methods of treatment for APS during pregnancy typically involve low molecular weight heparin (LMWH), either alone or in combination with aspirin (5, 6). In some studies, this treatment has been shown to significantly increase the live birth rate in APS patients who have experienced multiple miscarriages. However, the incidence of severe late obstetric complications, including preeclampsia, intrauterine growth restriction and prematurity, remains high (5, 6). In addition, clinical and experimental studies have produced contradictory results regarding the effectiveness of heparin in preventing adverse

pregnancy outcomes in APS (7-10). Therefore, the development of improved strategies for the prevention of pregnancy complications in APS is a pressing concern.

APS was recognized as a unique form of autoantibody-induced thrombophilia in the early 1980s (11). The true frequency of APS is unknown, but it is estimated that the incidence is around 5 new cases per 100,000 persons per year, and the prevalence is around 40-50 cases per 100,000 persons (12). The male-to-female ratio is about 1:3.5, and the mean age of onset is 35 years (13). APS is specifically characterized by recurrent thrombosis and pregnancy complications. Other manifestations include cardiac valvular disease renal thrombotic microangiopathy, thrombocytopenia, hemolytic anemia, and cognitive impairment (11). In fact, APS is the most common cause of acquired thrombophilia in the general population. Up to twenty percent of patients who have had a stroke at less than fifty years of age have been found to have circulating aPL (11). APS may occur as a primary disease, or may occur as secondary to another autoimmune disease, including systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjogren's syndrome, and certain malignancies (14, 15). In fact, forty percent of patients diagnosed with SLE with have circulating aPL (11).

Diagnosis of APS must involve at least one clinical criterion and one laboratory criterion. Clinical criteria involve either vascular thrombosis or obstetric morbidity. Vascular thrombosis must be confirmed by Doppler study, imaging, or histopathology. Obstetric morbidity must include at least one unexplained fetal demise beyond ten weeks of gestation (with a morphologically normal fetus), at least one premature birth due to

eclampsia/preeclampsia/placental insufficiency, or at least three unexplained consecutive and spontaneous miscarriages before ten weeks gestation (11, 15, 16). Laboratory criteria include the presence of anticardiolipin antibodies, anti-beta2-glycoprotein I antibodies, or lupus anticoagulant (15).

Pathogenesis of Systemic and Obstetric Antiphospholipid Syndrome

The pathogenesis of APS involves acquired antibodies (aPL), which are IgG, IgM, or IgA subtypes that react against negatively charged phospholipids. Such antibodies include, as discussed above, anticardiolipin antibodies, anti-beta$_2$-glycoprotein I antibodies, and lupus anticoagulant (14). The main autoantigen for APS is beta$_2$-glycoprotein I (β_2GPI), a protein localized to the plasma membrane that mediates aPL binding to target cells such as platelets, monocytes, endothelial cells, and trophoblasts (14). These antibodies increase thrombus formation in both the arterial and venous circulation. Prothrombotic effects of aPL involve inhibition of the fibrinolytic system and activation of the coagulation cascade, activation of the complement system, and inhibition of annexin A5, a potent anticoagulant protein on endothelial cell surfaces (14).

Despite the association of APS with thrombosis and the success of anticoagulants such as heparin in preventing pregnancy loss, recent studies have shown that intravascular or intravillous blood clots are not typically found in placental samples from APS patients (17). Rather, clinical and experimental studies suggest that the primary causes for adverse pregnancy events are inflammatory processes, including cytokine production, complement deposition, and immune cell activation (18-22). Furthermore, studies

indicate that there is placental dysfunction and insufficient placentation in APS-complicated pregnancies (17, 23, 24). Placental dysfunction in APS appears to be related to reduced migration of trophoblast cells and trophoblast invasion, as well as limited uterine spiral artery transformation (17, 23, 24).

Antiphospholipid antibodies are able to specifically target the placenta by binding β_2GPI (25-27). β_2GPI is a phospholipid-binding protein that binds with high affinity to the negatively charged phospholipids on the inner cell membrane. Typically, cells only bind β_2GPI under pathologic and apoptotic conditions, as this is the only time that the negatively charged inner phospholipids will be exposed to the outer membrane. However, the trophoblast is unique because it constitutively expresses β_2GPI on its cell surface under normal circumstances, due to the high levels of proliferation and differentiation (25, 28). In addition, the trophoblast synthesizes β_2GPI itself, increasing the levels of cell surface expressed and circulating β_2GPI in the local environment. β_2GPI has been shown to be expressed on the surface of extravillous trophoblast cells that invade the decidua of the uterus, as well as on the syncytiotrophoblast cells (28). The placenta is, therefore, a major target for circulating aPL, which recognize β_2GPI.

Physiology of Placentation

Normal placentation occurs at six or seven days after conception, with the adhesion of the blastocyst to the uterine wall and the trophoectoderm giving rise to the placental trophoblast cells. The trophoblast layer of the placenta consists of an inner cytotrophoblast, which contains the stem cells that proliferate and differentiate. The

second layer is the syncytiotrophoblast, which is formed from the differentiation and fusion of cytotrophoblast cells. The cytotrophoblast cells invade the decidua, the proliferative layer of the uterus present during pregnancy. Cytotrophoblasts eventually differentiate into extravillous trophoblast cells, which migrate from the trophoblast layer to invade the entire endometrium and the inner third of the myometrium (28, 29). These cells remodel the maternal spiral arteries, resulting in increased blood flow to the placenta.

The innate invasiveness of cytotrophoblasts is driven by both autocrine and paracrine signals. Signals like interleukin-6 (IL-6) produced by the trophoblast can drive migration through activation of the STAT3 pathway (24, 30, 31). Similarly, factors produced by the decidua, such as leukemia inhibitory factor (LIF) and IL-11, can also promote trophoblast invasiveness (31, 32). Trophoblast invasiveness is also related to the secretion and balance of proteinases such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (29). MMPs, a family of zinc-dependent endopeptidases, function by degrading and remodeling components of the extracellular matrix. Several human MMPs have been identified, including collagenases, gelatinases, and membrane-associated MMPs (33). TIMPs belong to a family of endogenous extracellular proteins that function as MMP inhibitors by directly binding MMPs or by activating nuclear transcription factors that control the expression of MMPs (33). Several studies have indicated that the levels of MMP2, MMP9, TIMP1, and TIMP2 are particularly important for trophoblast invasion (34, 35). Abnormally low levels of MMP2 and MMP9 and abnormally high levels of TIMP1 and TIMP2 have been implicated in adverse pregnancy

outcomes such as preeclampsia and maternal hypertension (33, 34). The normal invasion of the decidua and remodeling of the maternal vasculature are impaired in disease states such as preeclampsia, preterm birth, and miscarriage (36). In these cases, there is inadequate remodeling of the maternal vasculature, shallow invasion of the decidua by trophoblast cells, and increased inflammation at the maternal-fetal interface (23, 28).

Effects of Antiphospholipid Antibodies on the Trophoblast

Many studies have investigated the potential mechanisms by which aPL affect trophoblast function by examining placental specimens from patients with APS or using murine models of APS in pregnancy (19, 21, 22). These studies have noted the existence of necrosis and acute and chronic inflammation in placental tissue from APS patients (22). In addition, they have implicated aPL-induced upregulation of complement C5 levels, neutrophil response, and tissue factor production as mediators of fetal injury in APS, leading to increased inflammation in the decidua and to subsequent miscarriages (19, 21). Many *in vitro* studies have examined the effects of aPL on term trophoblast cells. The presence of aPL has been shown to be associated with overall decreased viability of term trophoblast cells, with decreased trophoblast proliferation and increased trophoblast cell death (37). In addition, studies have noted that term trophoblast cells exposed to aPL demonstrate decreased syncytialization and decreased giant cell formation (38-40). Term trophoblast cells exposed to aPL have also been shown to produce reduced levels of syncytiotrophoblast hormones such as hCG (37, 41, 42). Several studies have shown the presence of aPL is associated with decreased term trophoblast cell invasion, although it should be noted that term trophoblast cells have

decreased invasive capacity than earlier gestational trophoblast cells (38, 43). Finally, term trophoblast cells exposed to aPL have been shown to have increased levels of prothrombotic thromboxane and decreased levels of antithrombotic annexin V (44, 45).

Such studies are helpful in elucidating the effects of aPL in the late gestational and term trophoblast. However, aPL are always present in patients and are therefore present at the time of implantation and early in pregnancy. Moreover, obstetric APS includes placental insufficiency and preeclampsia, which are thought to be established early in pregnancy due to impaired early placental implantation and differentiation. Therefore, an investigation of the effects of aPL on first trimester trophoblast cells is critical. Indeed, studies from the laboratory have begun to examine the effects of aPL on human first trimester trophoblast function and some of the mechanisms involved (20, 24, 46). aPL trigger human first trimester trophoblast cells to produce elevated levels of pro-inflammatory cytokines and chemokines, such as IL-8, IL-1β, through activation of the innate immune receptor, Toll-like receptor 4 (TLR4) (20). aPL have also been shown to alter first trimester trophoblast function by inhibiting trophoblast migration (24). This inhibition of migration has been linked to an aPL-induced downregulation of IL-6, a cytokine that normally promotes cell migration through the phosphorylation and activation of STAT3 (31). Indeed, aPL have been shown to decrease levels of both IL-6 and phosphorylated STAT3 (24). In addition, aPL have been shown to modulate the trophoblast angiogenic factor profile by increasing pro-angiogenic vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF), as well as increasing anti-angiogenic soluble Endoglin (sEndoglin) secretion (46). Finally, aPL have been

implicated in the modulation of MMPs and TIMPs. Specifically, aPL have been shown to be associated with downregulation of MMP-2 and MMP-9 in primary cultures obtained from first trimester placental samples, in a first trimester trophoblast cell line, and in a human choriocarcinoma cell line (47, 48). An additional study has shown that aPL downregulate MMP-2 and MMP-9 in term trophoblast cells (43).

The identification of therapeutic interventions that can prevent such trophoblast responses to aPL may translate clinically to improved pregnancy outcomes in APS patients. Indeed, this *in vitro* model has been used to assess the potential impact of heparin, aspirin, aspirin-triggered lipoxin, and pravastatin on trophoblast responses to aPL (9, 20, 24, 46, 49, 50). These studies have reported that LMWH may have some benefits, specifically in terms of partially reversing the upregulation of pro-inflammatory cytokines IL-8 and IL-1β (9). LMWH has also been shown to have some benefit in reversing the downregulation of MMP-2 (51). However, LMWH has also been shown to increase levels of anti-angiogenic factor soluble fms-like tyrosine kinase-1 (sFlt-1) (46). This could potentially have an adverse effect on pregnancy, as increased levels of sFlt-1 have been associated with preeclampsia, and overexpression of sFlt-1 in mice leads to preeclampsia-like symptoms (52-54). Aspirin-triggered lipoxin has been shown to have some benefit in restoring IL-6 levels and cell migration (50). Due to these limited benefits, there is a need to evaluate the efficacy of alternative therapeutics, especially those that may help reverse the aPL-induced modulation of trophoblast angiogenic factor secretion and impaired trophoblast migration.

Mechanism of Action of Hydroxychloroquine

Patients with SLE and APS are often treated with the anti-malarial drug hydroxychloroquine (HCQ), which has been shown to be beneficial in their management (55, 56). Quinines were first recognized as anti-malarial agents over hundreds of years ago, with their first use traditionally attributed to Incan descendants in Peru in 1630, who used a bark powder to treat a febrile illness (57). During World War II, the widespread use of quinines as malarial prophylaxis for soldiers led to the discovery that soldiers' rashes and inflammatory arthritis improved as well, leading to the first trial showing antimalarials were effective in the treatment of SLE (57). In more recent times, such antimalarials have become established agents for treating rheumatic diseases such as SLE, Sjogren's syndrome, chronic Q fever, and skin diseases (55).

In addition to having anti-thrombotic effects, HCQ has anti-inflammatory and immunomodulatory properties, which recent studies have sought to characterize (55, 56, 58, 59). Chloroquines are lipophilic, and thus cross cell membranes readily (56). As weak bases, chloroquines accumulate in lysosomes, increase the pH of the intracellular compartment, and impair the function of phagocytosis that normally requires lysosomal acidification for protease functioning. (60). In addition, chloroquines have been shown to decrease the production of inflammatory cytokines, including IL-1, IL-2, IL-6, IL-17, IL-22, interferon-alpha and gamma, and tumor necrosis factor-alpha (57, 61, 62). HCQ is also proposed to inhibit the effects of prostaglandins by inhibiting phospholipase A2 (57).

More recently, HCQ has been shown to affect TLR signaling (56). Specifically, antimalarials such as HCQ are thought to inhibit the activation of intracellular TLRs, such as TLR3, TLR7, TLR8 and TLR9 (59, 63). Such TLRs normally bind nucleic acids (bacterial and viral) into late endosomes and early lysosomes, but are also known to bind endogenous genetic material, which perhaps evolved as a response to damaged tissue (62, 63). Such TLRs are implicated in the pathogenesis of autoimmune diseases such as APS and lupus, due to the presence of antibodies directed at nucleic material. Chloroquines, which target microsomes and disrupt the acidic pH as described above, are therefore postulated to block such TLR interaction with nucleic acids (59). However, an alternative pathway has also been proposed, in which chloroquines inhibit TLR activation by directly binding to nucleic acids and masking their TLR-binding epitope, rather than functioning by inhibiting endosomal proteolysis as previously thought (58). A further proposed mechanism suggests that HCQ may function by preventing the phosphorylation and activation of mitogen-activated protein kinases, thus preventing the downstream effects of lipopolysaccharide-induced inflammatory response via TLR4 (64). Studies from our group have demonstrated that aPL induce trophoblast inflammation via TLR4 (20). Moreover, recent evidence suggests that trophoblast fusion and differentiation are inhibited by aPL via a TLR4-mediated pathway, and that HCQ is able to reverse this effect (40).

Safety of Hydroxychloroquine

HCQ is known to be a very safe medication when taken at therapeutic doses, but toxicity is recognized at doses above the safety margin (65). Toxic manifestations include

cardiovascular events such as hypotension, decreased myocardial function, cardiac arrhythmias, and cardiac arrest (57, 65). It is also known to have CNS toxicity, causing confusion, convulsions, and coma. At typical oral doses used for rheumatic disease, the main side effects of chloroquines are nausea, headache, and pruritus. Irreversible retinopathy and ototoxicity are possible at high daily doses, typically above 250 mg per day (66). Currently, the American Academy of Ophthalmology recommends a baseline screening ophthalmologic exam to all patients beginning hydroxychloroquine, followed by annual screening after five years of medication use (62).

HCQ is known to cross the placenta, with fetal levels shown to be equivalent to maternal levels (67). However, HCQ is considered safe to use during pregnancy, with no evidence of fetal toxicity or congenital abnormalities (62, 67-70). Studies have specifically examined the association of HCQ exposure *in utero* with fetal ocular toxicity and found no evidence (71). However, little is known about the effects of HCQ on aPL-induced adverse pregnancy outcomes. Recent studies have reported that HCQ reduces the binding of aPL-β_2GPI complexes to phospholipid bilayers and protects the anticoagulant protein annexin A5 from disruption by aPL in the term trophoblast, again by reduced antibody binding (72-74). Such studies suggest that HCQ might be able to prevent many of the effects of aPL on the trophoblast cell that are downstream effects of aPL binding to β_2GPI.

Since circulating aPL are present in women with APS at the time of implantation, the objective of this study was to investigate the impact that HCQ has on human first

trimester trophoblast cell responses to aPL. The ultimate goal of this study was to determine whether HCQ could be used as a therapy to prevent adverse pregnancy outcomes in women with APS. The specific hypotheses of this project were:

1. HCQ can prevent the TLR4-mediated pro-inflammatory cytokine response triggered by aPL
2. HCQ can prevent the aPL-induced modulation of pro- and anti-angiogenic factor secretion
3. HCQ can prevent the aPL-induced inhibition of IL-6 production and cell migration
4. aPL modulate the expression of MMPs and TIMPs, and this can be reversed by HCQ

METHODS

Trophoblast Cell Line

The human first trimester trophoblast cell line, SVneo transformed HTR8, was used in all experiments. The cell line (HTR8) was a gift from Dr. Charles Graham (Queen's University, Kingston, ON, Canada). This cell line was established from explant cultures of first trimester (7 weeks of gestation) chorionic villi. The cells were immortalized through transfection with cDNA encoding the simian virus-40 large T antigen (75). This cell line has been well characterized and shown to express various cell markers characteristic of extravillous invasive trophoblast cells (76). As extravillous trophoblast cells are responsible for invasion of the decidua and transformation of the spiral arteries, this cell line is an excellent *in vitro* model for studies of mechanisms regulating the

human first trimester trophoblast growth, migration, and invasion that is associated with normal placentation.

HTR8 cells were grown in sterile 75 cm^2 polystyrene cell culture flasks at 37°C/5% CO_2. The culture media used was RPMI-1640 (Gibco; Grand Island, NY), supplemented with 10% fetal bovine serum (Hyclone; South Logan, UT), 10 mM Hepes, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate, and 100 nm penicillin/streptomycin (Gibco). The cells were grown until confluent, at which point they were split and plated for experiments or maintained in continued culture.

The cells were split through the use of 3-5 mL of 0.05% Trypsin followed by activation with a brief incubation at 37°C until the cells had detached. An equal volume of culture media was added to inactivate the trypsin and the solution was transferred to a sterile 10 mL polypropylene tube and centrifuged at room temperature at 3000 rpm for 10 minutes. The pellet was resuspended in growth media and the cells were counted and either plated for future experimentation or seeded into T75 for further propagation.

Antiphospholipid Antibodies

The aPL used in all experiments were the mouse IgG1 anti-human β_2GPI mAbs, ID2 and IIC5. These antibodies were produced by Larry W. Chamley (Department of Obstetrics and Gynecology, University of Auckland, New Zealand), and have been previously characterized (77). ID2 and IIC5 bind β_2GPI in the same manner as human aPL when immobilized on a suitable negatively charged surface, such as the phospholipids,

cardiolipin or phosphatidyl serine, or irradiated polystyrene (78). ID2 and IIC5 have been shown to alter trophoblast function similarly to patient-derived polyclonal aPL upon binding to first trimester trophoblast cells (20, 46).

Cell Viability Assay

Trophoblast cell viability was determined using the CellTiter 96 viability assay (Promega, Madison, WI), as previously described (79). Cells ($1x10^4$) were seeded into wells of a 96-well plate in growth media and incubated overnight. The media was then replaced with serum-free OptiMEM (Invitrogen; Carlsbad, CA), and cells were incubated for another 4 hours. Cells were then treated with HCQ (Sigma Aldrich) at concentrations of 0, 0.01, 0.1, 1, 10 and 100 µg/ml. HCQ was reconstituted in endotoxin-free water and subsequent dilutions made using OptiMEM prior to treatment. After 48 hours, the CellTiter 96 substrate (Promega) was added to all wells and incubated for 2 hours at 37°C. Optical densities were read at 490 nm. All samples were assayed in triplicate, and cell viability was presented as a percentage of the untreated control (0µg/ml).

Cytokine, Angiogenic Factor, and TIMP Secretion

Trophoblast cells ($1x10^5$) were seeded into 35 mm tissue culture dishes in growth media and allowed to adhere overnight. After cells were 70% confluent, the growth media was replaced with serum-free OptiMEM (Invitrogen) and cells incubated for another 4 hours. Cells were then treated with or without aPL (ID2 or IIC5) at 20µg/ml in OptiMEM (Invitrogen) in the presence and absence of HCQ at 1µg/ml. After 72 hours, cell-free supernatants were collected by centrifugation at 400g for 10 minutes and stored at -80˚C

until analysis was performed. At the time of analysis, the supernatants were thawed on ice. The concentrations of interleukin 8 (IL-8), interleukin 1β (IL-1β), interleukin 6 (IL-6), vascular endothelial growth factor (VEGF), placenta growth factor (PlGF), soluble fms-like tyrosine kinase-1 (sFlt-1), soluble Endoglin (sEndoglin), tissue inhibitor of metalloproteinase 1 (TIMP1) and tissue inhibitor of metalloproteinase 2 (TIMP2) were measured by ELISA (R & D Systems; Minneapolis, MN).

Cell Migration

To assess trophoblast migration, a two chamber colorimetric assay was used, as previously described (24). An 8µm pore size cell culture insert served as the top chamber (BD Biosciences, Franklin Lakes, NJ, USA), while the lower chambers were wells of a 24-well tissue culture plate (BD Falcon, Franklin Lakes, NJ, USA). The lower chamber was filled with 800µL of OptiMEM while the top chamber was seeded with 1×10^5 cells HTR8 cells suspended in 200µL of treatments. Following a 48-hour incubation, the 8µm inserts were removed and trophoblast migration across the membrane was determined using the QCM 24-Well Colorimetric Cell Migration assay according to the manufacturer's instructions (Chemicon International, Temecula, CA). Briefly, migrated cells were stained, collected, and lysed. The resulting colored mixture was transferred to a 96-well plate and optical densities read in triplicate at 560 nm. A 100% migration control consisted of the starting number of cells (1×10^5). Observed optical density values were measured using a BioRad plate reader (Hercules, CA, USA), and were expressed as a percentage of the starting total number of cells.

Statistical Analysis

All experiments were performed at least three times. Data from individual experiments were pooled and expressed as mean ± standard error of the mean (SEM). Statistical significance was set at $p<0.05$ and was determined by one-way ANOVA using Prism software (GraphPad Software, Inc., La Jolla, CA).

I produced the data for the all of the cell viability studies, cytokine and angiogenic factor studies, and migration studies, with the exception of the data for the TIMP1 and TIMP2 studies, which were produced by Mr. Schlesinger. With the help of Dr. Abrahams, I performed the statistical analysis.

RESULTS

HCQ decreases trophoblast viability at high doses

The first objective of this study was to determine the concentration range of HCQ that has no trophoblast cytotoxicity. Figure 1 shows that the two highest HCQ doses tested, 10μg/ml and 100μg/ml, significantly reduced trophoblast cell viability when compared to the untreated control (0μg/ml). At all others concentrations tested, there was no significant effect on cell viability (Figure 1). Therefore, for all subsequent experiments, the highest non-cytotoxic dose of 1μg/ml HCQ was used, which is consistent with previous reports studying the effects of HCQ on the trophoblast (72-74).

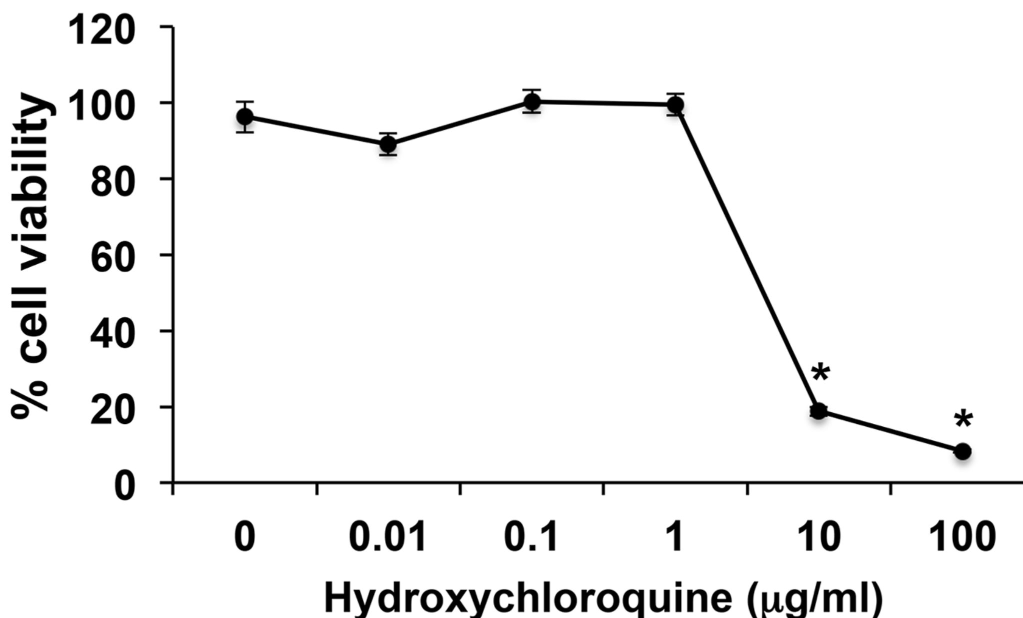

Figure 1: High levels of HCQ reduce trophoblast cell viability. The first trimester trophoblast cell line (HTR8) was incubated with no treatment (0μg/ml) or HCQ at 0.01, 0.1, 1, 10 or 100μg/ml for 72hrs. Chart shows cell viability as a percentage of the 0μg/ml HCQ control. Data are from three independent experiments. *p<0.0001 relative to the control.

HCQ has no effect on aPL-induced trophoblast secretion of pro-inflammatory cytokines or chemokines

Having established the HCQ dose, we first sought to determine the effect of HCQ on aPL-induced trophoblast inflammation (9, 20, 49). Treatment of trophoblast cells with ID2 or IIC5 induced a significant increase in the secretion of IL-8 when compared to the no treatment (NT) control. The presence of HCQ had no significant effect on aPL-induced trophoblast IL-8 production (Figure 2A). Similarly, treatment of trophoblast cells with ID2 induced a significant increase in secretion of IL-1β and this was unaffected by the presence of HCQ (Figure 2B). HCQ alone had no effect on basal trophoblast IL-8 or IL-1β secretion (Figure 2).

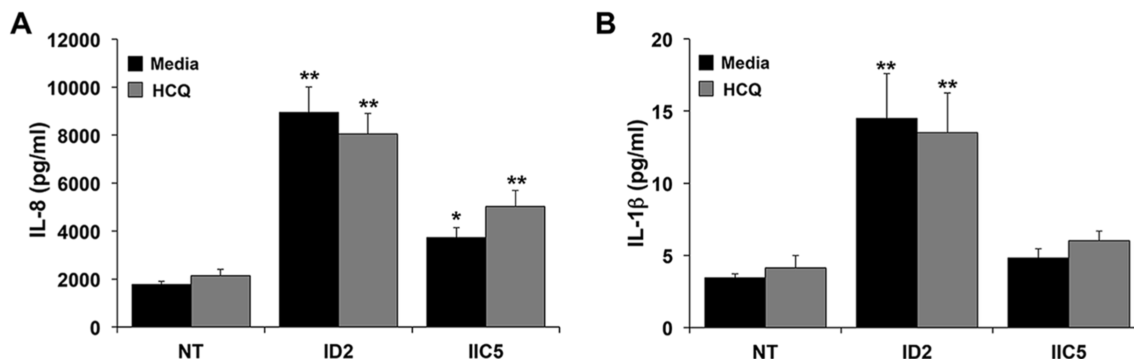

Figure 2: HCQ has no effect on aPL-induced trophoblast secretion of pro-inflammatory cytokines. HTR8 cells were incubated with either no treatment (NT) or the anti-β_2GPI mAbs, ID2 or IIC5 (20µg/ml), in the presence of media or HCQ (1µg/ml) for 72hrs. Supernatants were assayed for (A) IL-8 and (B) IL-1β. Data are from nine independent experiments. **p*<0.05; ***p*<0.001 relative to the NT control.

HCQ has no effect on aPL-induced modulation of trophoblast angiogenic factor production

We next investigated the effect of HCQ on aPL-induced modulation of trophoblast angiogenic factor secretion (9, 46, 49). HCQ alone had no effect on basal trophoblast secretion of VEGF (Figure 3A), PlGF (Figure 3B), sEndoglin (Figure 3C), or sFlt-1 (Figure 3D). Treatment of trophoblast cells with ID2 or IIC5 induced a significant increase in the secretion of VEGF (Figure 3A). The presence of HCQ significantly lowered the ID2-induced VEGF secretion, but had no effect on IIC5-induced VEGF (Figure 3A). Treatment of trophoblast cells with ID2 or IIC5 significantly increased trophoblast secretion of PlGF, and this was unaffected by HCQ (Figure 3B). Neither ID2 nor IIC5 in the presence or absence of HCQ had any effect on trophoblast sFlt-1 secretion

(Figure 3C). ID2 significantly increased trophoblast secretion of sEndoglin, and this was unaffected by the presence of HCQ (Figure 3D).

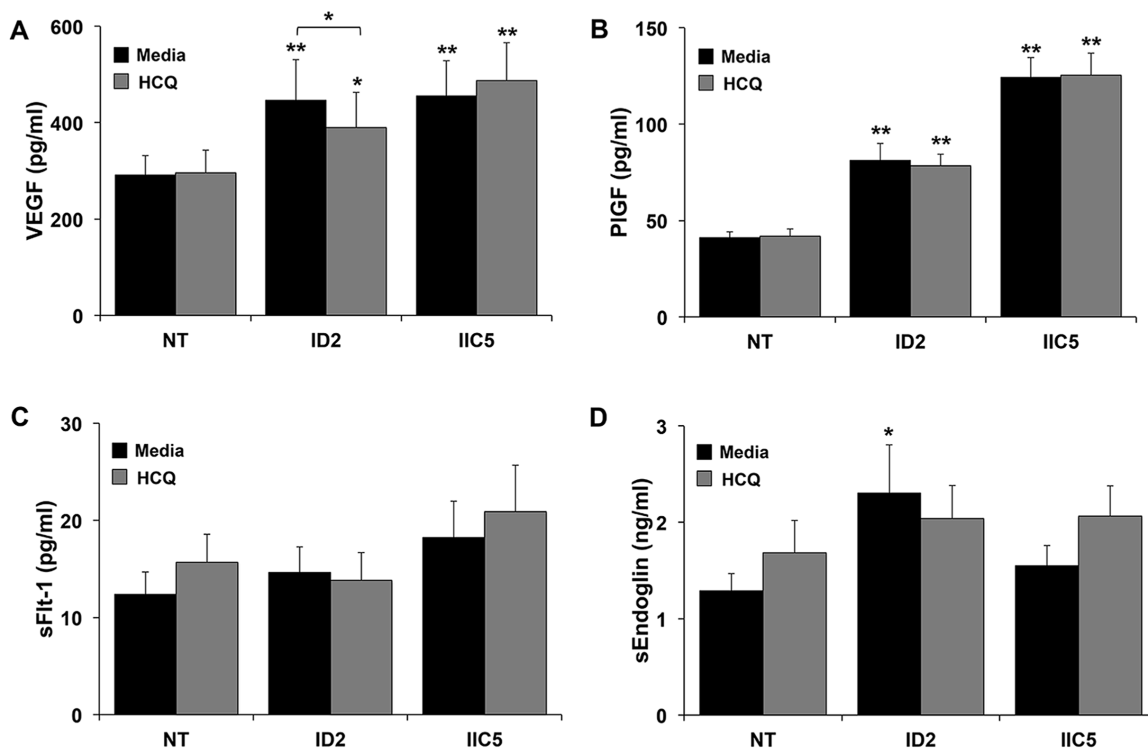

Figure 3: Effect of HCQ on aPL modulation of trophoblast angiogenic factor production. HTR8 cells were incubated with either no treatment (NT) or the anti-β_2GPI mAbs ID2 or IIC5 (20µg/ml) in the presence of media or HCQ (1µg/ml) for 72hrs. Supernatants were assayed for (A) VEGF, (B) PlGF, (C) sFlt-1, and (D) sEndoglin. Data are from nine independent experiments. *$p<0.05$; **$p<0.001$ relative to NT unless otherwise indicated.

HCQ limits aPL-induced inhibition of trophoblast IL-6 secretion and cell migration

We previously demonstrated that first trimester trophoblast cell production of IL-6 plays a role in driving the cell's spontaneous migration through activation of STAT3, and that treatment of first trimester trophoblast cells with aPL decreases constitutive IL-6 secretion and cell migration (24). Treatment of trophoblast cells with ID2 or IIC5 reduced

IL-6 secretion, although not to the level of significance. However, IL-6 secretion in the ID2 or IIC5 treatment groups was significantly increased in the presence of HCQ when compared to ID2 or IIC5 treatment in the absence of HCQ (Figure 4A). HCQ had no significant effect on basal trophoblast IL-6 secretion, although there was an increase noted. Treatment of trophoblast cells with ID2 or IIC5 significantly reduced trophoblast migration by 65.9±32.0% and 65.6±18.2%, respectively, when compared to the NT control. The presence of HCQ had no effect on basal trophoblast migration. However, HCQ significantly reversed ID2 and IIC5-inhibition of trophoblast migration, although migration did not return to the level of pretreatment controls (Figure 4B).

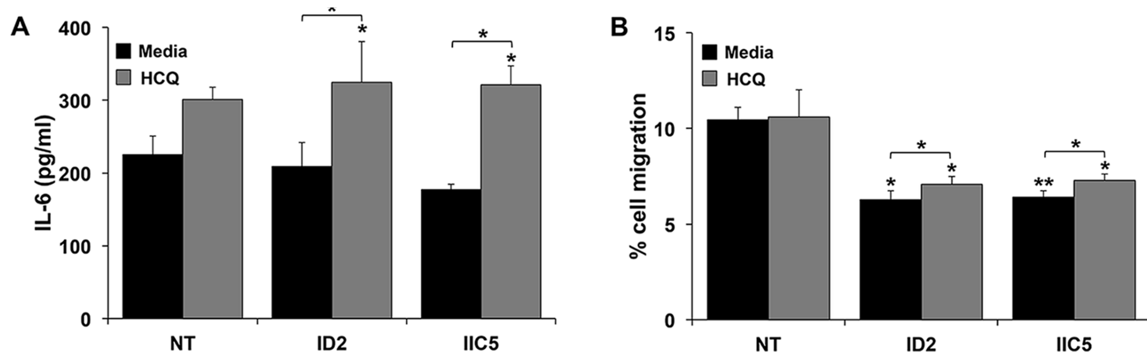

Figure 4: Effect of HCQ on aPL-induced inhibition of trophoblast IL-6 secretion and migration. HTR8 cells were incubated with either NT or the anti-β_2GPI mAbs, ID2 or IIC5 (20μg/ml) in the presence of media or HCQ (1μg/ml). (A) After 72hrs supernatants were assayed for IL-6. Data are from five independent experiments. *$p<0.05$ relative to the NT control unless otherwise indicated. (B) After 48hrs, cell migration was measured. Data are from four independent experiments. *$p<0.05$; **$p<0.001$ relative to the NT control unless otherwise indicated.

aPL increase secretion of TIMPs and this is partially reversed by HCQ

Trophoblast migration and invasion have been shown to be partially governed by the balance between trophoblast production of the matrix metalloproteinases (MMPs),

MMP2 and MMP9, and the tissue inhibitors of matrix metalloproteinases (TIMPs), TIMP1 and TIMP2 (80). While treatment of trophoblast cells with aPL had no effect on the expression levels of MMP2 or MMP9 (data not shown), treatment with ID2 or IIC5 significantly increased secretion of TIMP1 and TIMP2 when compared to the NT and IgG controls (Figure 5A). Furthermore, treatment of trophoblast cells with recombinant (r) TIMP2 significantly reduced trophoblast migration when compared to the NT control (Figure 5B). rTIMP2 in combination with rTIMP1 significantly reduced trophoblast migration when compared to both the NT control and rTIMP1 alone (Figure 5B). As shown in Figure 6, treatment of trophoblast cells with either ID2 or IIC5 significantly increased the secretion of (A) TIMP1 and (B) TIMP2, when compared NT control. The presence of HCQ significantly inhibited ID2-induced secretion of TIMP1 (Figure 6A). The presence of HCQ significantly increased IIC5-induced TIMP2 secretion (Figure 6B).

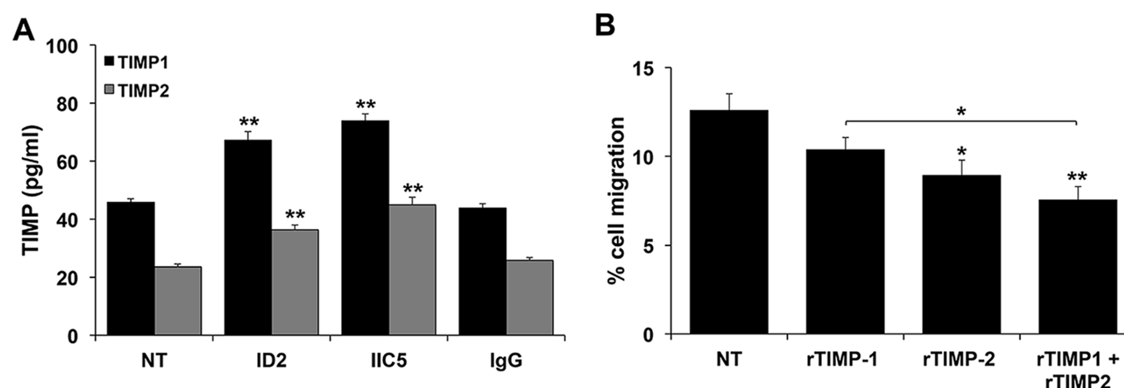

Figure 5: aPL upregulate TIMP1 and TIMP2 secretion, which reduces trophoblast migration. (A) HTR8 cells were incubated with no treatment (NT), the anti-β_2GPI mAb ID2 or IIC5 (20μg/ml), or an IgG control (20μg/ml) for 72hrs, after which supernatants were assayed for TIMP1 and TIMP2. Data are from four independent experiments; **p<0.001 relative to the NT control. (B) HTR8 cells were incubated with either NT, recombinant TIMP1 (rTIMP1) at 1ng/ml, recombinant TIMP2 (rTIMP2) at 1ng/ml, or rTIMP1 and rTIMP2 at 1ng/ml for 48hrs after which cell migration was measured. Data are from five independent experiments. *p<0.05; **p<0.001 relative to NT control unless otherwise indicated.

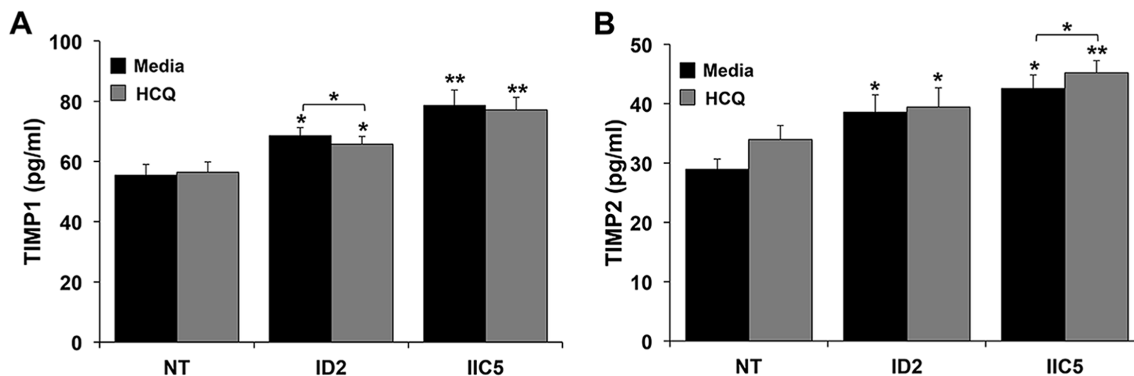

Figure 6: Effect of HCQ on aPL-induced trophoblast TIMP1 and TIMP2 secretion. HTR8 cells were incubated with either no treatment (NT) or the anti-β_2GPI mAb ID2 or IIC5 (20μg/ml), in the presence of media or HCQ (1μg/ml) for 72hrs. Supernatants were assayed for (A) TIMP1 (n=9) and (B) TIMP2 (n=6). **p*<0.05; ***p*<0.001 relative to the NT control unless otherwise indicated.

DISCUSSION

We hypothesized that HCQ would prevent the aPL-triggered release of pro-inflammatory cytokines, modulation of angiogenic factors, and inhibition of cell migration. We specifically hypothesized that HCQ would protect cell migration by preventing aPL-induced inhibition of pro-migratory factor IL-6 and pro-migratory MMPs, as well as preventing aPL-induced upregulation of anti-migratory TIMPs. Our data show that HCQ did not prevent the TLR4-mediated pro-inflammatory cytokine response triggered by aPL. HCQ was also unable to prevent the aPL-induced modulation of pro- and anti-angiogenic factor secretion. However, HCQ was able to prevent the aPL-induced inhibition of IL-6 production and cell migration. The presence of aPL had no effect on

the expression of MMPs, but increased the expression of both TIMP1 and TIMP2. HCQ inhibited the aPL-induced secretion of TIMP1, but not TIMP2.

Women with APS continue to face substantial risk for complications during pregnancy, even when treated with the standard of care, heparin and/or aspirin. Furthermore, several studies have disputed the effects of heparin on aPL-induced abnormal trophoblast cell function and have called its efficacy into question (5-10, 81). Thus, further research into the potential management of APS-related pregnancy complications is critical. While the drug hydroxychloroquine has been used to treat APS and related autoimmune disorders, such as lupus, for decades, little is known about its effects on pregnancy outcome in patients with aPL (55, 56). Therefore, using an *in vitro* system, we sought to determine the ability of HCQ to alter aPL-mediated modulation of first trimester trophoblast function, specifically, cell migration and the secretion of inflammatory cytokines and angiogenic factors. In this study, we report that HCQ was able to partially limit aPL-induced inhibition of trophoblast migration, possibly through the modulation of IL-6 production.

APS increases the risk of many serious adverse pregnancy outcomes, including recurrent pregnancy loss and late gestational complications of preeclampsia, HELLP syndrome, premature delivery, and intrauterine growth restriction. Historically, APS was thought to cause pregnancy complications through prothrombotic events within the placenta (16). However, during the last decade, obstetric APS has been recognized as a distinct clinical

entity from vascular APS, given that patients with APS can display vascular thrombosis but no obstetric complications, or vice-versa (13).

Recent clinical and experimental studies suggest that the primary causes for adverse pregnancy events are inflammatory processes, namely cytokine production, complement deposition, and immune cell activation (18, 20, 21). Studies also indicate that placental dysfunction and insufficient placentation play a major role in APS-complicated pregnancies (17, 23, 24). This insufficient placentation is related in part to impaired migration of trophoblast cells and trophoblast invasion, as well as limited uterine spiral artery transformation (17, 23, 24). Because aPL are found in maternal circulation as early as the first trimester, and early impairment of placentation is likely a factor in the pathology of the obstetric complications of APS, we chose to conduct our investigation with an *in vitro* model using human first trimester trophoblast cells (13).

Recent studies have shown that the immunomodulatory properties of HCQ may be explained by an inhibition of TLR signaling, possibly through interference with downstream mitogen-activated protein kinase (MAPK) activation and by dampening the expression of TLRs and their associated signaling molecules (56, 58, 59, 82). HCQ has been suggested to inhibit the functioning of endosome-bound TLRs that degrade nucleic acids and are implicated in autoimmune diseases such as APS and lupus (59). Additional recent evidence suggests that the fusion and differentiation of trophoblast cells are blocked by aPL via a TLR4 pathway, and that HCQ is able to reverse this effect (40). Based on this, we postulated that HCQ might prevent the TLR4-dependent trophoblast

inflammatory cytokine response induced by aPL (20). Moreover, recent studies have demonstrated that HCQ is able to reduce the binding of aPL-β_2GPI complexes to phospholipid bilayers and protect the anticoagulant protein annexin A5 from disruption by aPL in term trophoblast cells (72-74). In our initial experiments, we found that high doses of HCQ (10µg/ml and 100µg/ml) negatively affected cell viability. Therefore, a dose of 1µg/ml was selected for use in further experiments, which correlated with concentrations previously used (72-74).

We found that HCQ alone had no significant effect on basal trophoblast secretion of inflammatory cytokines or angiogenic factors. Moreover, HCQ had no effect on aPL-induced IL-8 and IL-1β production, suggesting that the ability of HCQ to inhibit TLR4 signaling may not be applicable to the trophoblast (82). Alternatively, it could be a reflection of the short period of exposure of the trophoblast to HCQ. While an *in vitro* exposure to HCQ can inhibit activation of the endosomal TLRs (58), the ability of HCQ to down-modulate the TLR4 signaling pathway has only been shown *in vivo* after a long term exposure of 6 months (82). Our data also suggest that HCQ does not prevent anti-β_2GPI mAbs binding to first trimester trophoblast cells. This is in contrast to what has been reported for the effects of HCQ on aPL-β_2GPI complexes binding to lipid bilayers and for aPL binding to term trophoblast, even though for these studies as well as our own, aPL and HCQ were delivered to the cells at the same time (72-74). While HCQ had no effect on aPL-induced PlGF or sEndoglin secretion, we did observe a slight, but significant, reduction in VEGF secretion induced by one of the anti-β_2GPI mAbs, ID2.

This is in keeping with a study showing that that chloroquine reduces epidermal VEGF expression (83).

While HCQ did not have an overt effect on the angiogenic factor profile, nor did it prevent aPL-induced trophoblast inflammation, we did observe an effect on the aPL-mediated reduction in IL-6 secretion and cell migration. Conflicting reports of the effects of HCQ on cell migration exist, but its effects on migration in the trophoblast have not been extensively studied (84, 85). It has previously been reported that treatment of trophoblast cells with aPL reduced basal IL-6 secretion, STAT3 phosphorylation, and trophoblast migration (24). Moreover, trophoblast migration has been shown to be inhibited by blocking the IL-6-to-IL-6 receptor interaction, suggesting this to be the mechanism by which aPL limit cell migration (24). In this current study, HCQ was able to reverse the effect of aPL on IL-6 production. However, the aPL-induced reduction in cell migration was only partially reversed by the presence of HCQ. This finding suggests that while HCQ might have a beneficial effect on aPL-induced inhibition of trophoblast migration, another factor in addition to IL-6 may be involved in its regulation, and this factor is not altered by HCQ.

Trophoblast migration and invasion can be regulated by the balance between the cell's production of the matrix metalloproteinases, MMP2 and MMP9, and the tissue inhibitors of matrix metalloproteinases, TIMP1 and TIMP2 (80). Therefore, we sought to determine whether aPL disrupted this balance of MMPs and TIMPs in the trophoblast, and whether the presence of HCQ provided any protection. Unlike previous reports that have shown

aPL to reduce MMP2 and MMP9 expression in trophoblast cells, we found MMP2 and MMP9 expression to be unchanged after exposure to either of the anti-β_2GPI mAbs (43, 47, 48).

Instead, our data show for the first time that aPL upregulate both TIMP1 and TIMP2 secretion, and that the presence of recombinant TIMP2 either alone, or in combination with TIMP1, can limit trophoblast migration, similarly to treatment with aPL or disruption of IL-6 signaling (24). However, the presence of HCQ was not able to entirely prevent ID2-induced TIMP1 secretion and had no protective effect on aPL-induced TIMP2 secretion. Our findings are supported by previous data that chloroquine promotes endothelial cell migration (84), and are inconsistent with previous reports that chloroquine increases TIMP1 in serum levels of patients with lupus (85). Our studies indicate that the beneficial effects of HCQ on IL-6 and TIMP1 secretion, but its inability to affect TIMP2 secretion, may explain why HCQ was able to improve but not wholly reverse the inhibition of trophoblast migration by aPL. These data are mirrored by a recent study examining the effects of an anti-inflammatory product of aspirin, aspirin-triggered lipoxin (ATL), on HTR8 cell function (50). This study found that ATL was able to reverse both the aPL-induced downregulation of IL-6 and the inhibition of cell migration, reinforcing the importance of IL-6 levels in migration of trophoblast cells, previously shown to be related to the STAT3 pathway (24). This study also found that aPL induced an upregulation of TIMP2, supporting our data. The upregulation of TIMP2 was not reversed by ATL. This evidence, in combination with our study, could suggest

that there are multiple mechanisms by which aPL inhibit trophoblast migration, and that drugs such as HCQ and ATL target some but not all of these pathways.

In summary, our research demonstrates that HCQ was able to partially but significantly reverse the aPL-induced inhibition of trophoblast migration. Our research showed that HCQ was able to reverse the aPL-induced down-regulation of promigratory cytokine IL-6, and was able to partially reverse the aPL-induced upregulation of TIMP1. However, HCQ was found to have no effect on aPL-induced upregulation of pro-inflammatory cytokines or aPL-induced modulation of angiogenic factors. Our study did not reveal any overt negative effects of HCQ on basal trophoblast function. Together, our data indicate that HCQ may be useful as a therapeutic agent to target pregnancy complications in women with APS. Moreover, the potential benefit of HCQ on trophoblast migration, but not on the inflammatory or angiogenic profile, suggests that a combination therapy that includes HCQ may offer greater protection to women with APS during pregnancy.

It is important to note that these *in vitro* studies may not perform as a comprehensive model of the *in vivo* condition of APS during pregnancy. Our results show that HCQ has no negative effect on basal trophoblast function, and there is a broad fund of research demonstrating the safety of HCQ in pregnancy, as it is often used to treat APS and lupus as an anti-inflammatory and immunomodulatory drug. Therefore, it may be useful to examine whether HCQ can produce any benefits during pregnancy in the APS population as a clinical trial. In cases with APS patients at high risk for obstetrical complications, it could also be argued that HCQ may be a safe and viable option for treatment. However,

further experimentation is necessary to determine the optimal combination of drug therapy in this complex condition.

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