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Aspirin-Triggered Lipoxin Prevents Antiphospholipid Antibody Effects on Human Trophoblast Migration and Endothelial Cell Interactions

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Objective. **Antiphospholipid antibodies (aPL) interfere with several physiologic functions of human trophoblasts, including reducing their ability to migrate, decreasing their production of angiogenic factors, and inducing an inflammatory response. This may provide the underlying mechanism by which aPL responses lead to recurrent pregnancy loss or preeclampsia in women with obstetric antiphospholipid syndrome (APS). Although treatment with heparin may reduce the rate of recurrent pregnancy loss, the risk of preeclampsia remains high. Therefore, alternative treatments are needed for the management of pregnant patients with APS. Since aspirin-triggered lipoxins (ATLs) have immune and angiogenic modulatory properties, the objective of this study was to determine the effects of the ATL 15-epi-lipoxin A_4 on the function of aPL-altered human trophoblasts in the first trimester of pregnancy.**

Methods. **A first-trimester human trophoblast cell line (HTR8) was treated with mouse anti-human β_2-glycoprotein I monoclonal antibodies (aPL) in the presence or absence of the ATL 15-epi-lipoxin A_4. Trophoblast migration and interactions with endometrial endothelial cells were measured using Transwell and coculture assays. Trophoblast secretion of cytokines and angiogenic factors was measured by enzyme-linked immunosorbent assay.**

Results. **Treatment of HTR8 cells with ATL reversed the aPL-induced decrease in trophoblast migration, an effect that appeared to be regulated through restoration of interleukin-6 production. Using a model of spiral artery transformation, aPL and sera from APS patients with pregnancy morbidity disrupted trophoblast–endothelial cell interactions, and treatment with ATL restored the stability of the cocultures. In contrast, ATL treatment did not resolve the proinflammatory and antiangiogenic responses of trophoblasts induced by aPL.**

Conclusion. **These findings indicate that ATLs may have some benefits in terms of preventing the effects of aPL on trophoblast function, which raises the possibility of the use of ATLs as an adjuvant therapy in women with aPL.**

Antiphospholipid syndrome (APS) is characterized by the presence of antiphospholipid antibodies (aPL) and is associated with clinical manifestations of thrombosis in different vascular territories, blood disorders, including thrombocytopenia and hemolytic anemia, and/or adverse pregnancy outcomes. Women with persistent aPL are at high risk for early- and late-gestation fetal losses, intrauterine growth restriction (IUGR), placental insufficiency, and preeclampsia (1). The aPL are a heterogeneous population of autoantibodies that recognize phospholipid-binding proteins, such as annexin V, proteins C and S, prothrombin, and β_2-glycoprotein I (β_2GPI) (2). However, it is now appreciated that aPL reacting with β_2GPI are the most pathologic autoantibodies in obstetric APS (3). More-

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over, aPL-associated adverse pregnancy outcomes are primarily caused by inflammatory processes and placental insufficiency, rather than thrombotic events at the maternal–fetal interface (1,4–6).

Normal placentation begins with the blastocyst adhering to the uterine wall. This is followed by placental cytotrophoblast cells invading and infiltrating the pregnant endometrium (decidua). The trophoblast migrates and invades deep into maternal decidua, differentiating into extravillous trophoblast cells, which then proceed to invade into, and remodel, the maternal spiral arteries, ultimately replacing the decidual endothelium and destroying the vascular smooth muscle. This transformation of the maternal vasculature results in increased and continuous blood flow into the placental intervillous space (7). Thus, appropriate placental angiogenesis and interactions between the invading trophoblast and the endothelium of the uterine spiral arteries are critical for proper placentation. Pathologically limited spiral artery transformation resulting from shallow trophoblast invasion into the decidua or reduced numbers of transformed spiral arteries are features that are seen in women with preeclampsia, those with IUGR, and those with obstetric APS (4,7).

Although β_2GPI can bind to the surface of cells under stress when negative phospholipids are exposed, the placental trophoblasts bind β_2GPI under normal physiologic conditions. Moreover, the trophoblasts synthesize their own β_2GPI, making the placenta a major target of anti-β_2GPI antibodies (3,8). Subsequently, it has been demonstrated in experimental systems that aPL directed against β_2GPI disrupt normal trophoblast function, and thereby trigger adverse pregnancy outcomes (3,6,9,10).

Currently, pregnant women with APS are treated with low molecular weight heparin (LMWH), either alone or in combination with low-dose aspirin (1). Although this treatment approach has been reported to increase the live birth rate in APS patients who have experienced multiple miscarriages, the incidence of late-gestation obstetric complications, such as preeclampsia, placental insufficiency, and IUGR, remains high (1). In addition, clinical and experimental studies have produced contradictory results regarding the effectiveness of LMWH and aspirin in preventing aPL-associated adverse pregnancy outcomes (1,5,11–15), and in being able to reverse the detrimental effects of aPL on trophoblast function in vitro (6,9,10,16,17). Therefore, there is a need to find alternative treatments for the management of APS in pregnant patients.

Classically, aspirin reduces inflammation by inhibiting the cyclooxygenase 1 (COX-1) enzyme, thus blocking arachidonic acid conversion into prostaglandins and thromboxanes (18). More recently, an alternative mechanism for aspirin-mediated resolution of inflammation has been described. Low-dose aspirin, through transcellular biosynthesis of arachidonic acid, can acetylate COX-2 to retain and redirect its catalytic activity, leading to the production of 15(R)-hydroxyeicosatetraenoic acid, which is then converted by 5-lipoxygenase into aspirin-triggered lipoxins (ATLs) (19). ATLs act to reduce and resolve inflammation through their immune and angiogenic modulatory properties. In endothelial cells, ATLs can reduce the production of reactive oxygen species, can differentially regulate neutrophil and monocyte chemotaxis, and, in immune cells, can inhibit NF-κB activity and proinflammatory cytokine production (19).

One limitation of endogenous lipoxins is their rapid metabolism and inactivation, which may explain why aspirin treatment in some clinical scenarios, such as aPL-associated adverse pregnancy outcomes, is controversial. However, stable ATL analogs have longer-lasting biologic activity and, therefore, may constitute a better therapeutic option (20). Since ATLs have been shown to be beneficial in experimental models of inflammatory diseases such as colitis, peritonitis, and glomerulonephritis (21–23), the aim of this study was to determine the effects of the ATL 15-epi-lipoxin A_4 on basal and aPL-modulated human trophoblast cell function in the first trimester of pregnancy, using a well-characterized in vitro system in which aPL, but not a nonspecific IgG antibody, had a demonstrated capacity to reduce cell migration, induce trophoblast proinflammatory cytokines, and alter angiogenic factor production (6,9,10,24).

PATIENTS AND METHODS

Cell culture. The first-trimester human extravillous trophoblast cell line HTR8 (25) was a kind gift from Dr. Charles Graham (Queens University, Kingston, Ontario, Canada). The cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone), 10 m*M* HEPES, 0.1 m*M* minimal essential medium with nonessential amino acids, 1 m*M* sodium pyruvate, and 100 n*M* penicillin/streptomycin (Gibco). The human endometrial endothelial cell (HEEC) line (26) was a kind gift from Dr. Gil Mor (Yale School of Medicine, New Haven, CT). HEEC cells were maintained in endothelial basal growth medium 2 (EBM-2; Lonza) supplemented with 10% FBS.

Antiphospholipid antibodies. Two mouse IgG1 anti-human β_2GPI monoclonal antibodies, ID2 and IIC5 (27), were used in this study. These antibodies were produced by one of us (LWC). ID2 and IIC5 bind β_2GPI in the same manner as

Table 1. Clinical and laboratory characteristics of the study subjects*

Parameter	NHS (n = 7)	PM alone (n = 8)	PM/VT (n = 7)
Age, mean ± SEM years	36.1 ± 2.4	33 ± 1.8	37.1 ± 2.2
Previous fetal losses, mean (range) no.			
≤10 weeks' gestation	0	1.0 (1–4)	2.0 (2–5)
>10 weeks' gestation	0	0.75 (1–2)	1.4 (1–5)
Preeclampsia at <34 weeks, no.	0	0	4
Arterial/venous thrombosis, no.	0	0	7
LAC positive, no.	0	1	7
Anti-β_2GPI, mean ± SEM SGU	0	42.3†	49.81 ± 11.02
aCL ELISA			
Commercial ACA Kit, mean ± SEM GPL units/ml	0	4.56 ± 2.1	59.69 ± 12.72
In-house ELISA, mean OD in patients/mean OD in positive controls (% of control)	0.109/2.25 (4.84)	1.10/1.66 (66.2)	1.42/2.16 (65.7)
Other aPL, no. (%) positive			
Anti-GPI	0	6 (75)	3 (42)
Anti-GPG	0	4 (50)	2 (28.6)
Anti-GPS	0	3 (37.5)	1 (14.3)
Anti-GPA	0	2 (25)	3 (42.8)
Anti-GPE	0	1 (12.5)	1 (14.3)

* NHS = normal healthy human serum; PM = pregnancy morbidity in women with antiphospholipid antibodies (aPL); PM/VT = pregnancy morbidity plus vascular thrombosis in women with aPL; LAC = lupus anticoagulant; anti-β_2GPI = anti–β_2-glycoprotein I; SGU = standard IgG anti-β_2GPI antibody units; aCL = anticardiolipin antibodies; ELISA = enzyme-linked immunosorbent assay; GPL = IgG phospholipid; anti-GPG = anti–glycoprotein G; anti-GPS = anti–glycoprotein S; anti-GPA = anti–glycoprotein A; anti-GPE = anti–glycoprotein E.
† One patient in this group was positive for anti-β_2GPI antibodies.

human aPL when immobilized on a suitable negatively charged surface, such as on the phospholipids cardiolipin or phosphatidylserine or on irradiated polystyrene (28). Moreover, ID2 and IIC5 react specifically with epitopes within domain V of β_2GPI (24). The binding of both ID2 and IIC5 to β_2GPI is inhibited by patient-derived aPL (29), and both antibodies have been shown to alter trophoblast function in a manner similar to that of patient-derived polyclonal aPL upon binding to first-trimester trophoblast cells, whereas a mouse IgG1 isotype control was found to have no effect (6,9). Mouse IgG1 clone 107.3 (BD PharMingen) served as the isotype control for this study.

Analysis of patient serum. Sterile serum samples were obtained from women with APS whose condition was characterized by the presence of aPL along with pregnancy morbidity and vascular thrombosis (PM/VT group; n = 7). In addition, serum samples were obtained from a group of women with aPL and pregnancy morbidity alone (PM alone group; n = 8) whose clinical features fulfilled the international consensus (Sapporo) criteria for APS (30). Serum samples were also obtained from normal healthy women (NHS group; n = 7). All patients attended the Recurrent Pregnancy Loss Program at the Reproduction Group, University of Antioquia (Medellín, Colombia). The collection of their serum was approved by the Ethics Review Committee of University of Antioquia, and written consent was obtained from all subjects.

In each serum sample, anticardiolipin antibodies (aCL) were detected at least 3 times at high optical density using an in-house enzyme-linked immunosorbent assay (ELISA), and were also detected using a commercial aCL ELISA (ACA Elisa Kit; BioSystems). Lupus anticoagulant (LAC) was measured using an in-house dilute Russell's viper venom time test. Anti-β_2GPI antibodies were detected using a commercial ELISA (BioSystems).

Women in the PM/VT group were found to be positive for aCL using both the in-house ELISA and the commercial ELISA. In the PM alone group, the commercial aCL ELISA did not detect elevated aCL levels, whereas aCL positivity was detected using the in-house test, and other aPL (antiglycoprotein antibodies anti-GPG, anti-GPE, anti-GPI, anti-GPA, and anti-GPS) were also detected. Although this antibody profile in women with PM alone did not fit the international laboratory criteria for APS, we included these women in our study since we believe it is important to evaluate the relationship of other aPL present (i.e., noncriteria aPL [31]) in patients who experience pregnancy morbidity. Indeed, although the presence of these other antibodies is not considered an indication of classic APS, treatment with heparin and aspirin leads to successful pregnancy outcomes in 64% of these patients (32).

The clinical and laboratory features of the patients in the PM/VT and PM alone groups are further described in Table 1. In particular, the PM/VT group had high positivity for LAC, IgG aCL, and IgG anti-β_2GPI, which is consistent with the observations in previous studies (33). In addition, they were also positive for other negatively charged phospholipids detected by an in-house ELISA. The severity of gestational morbidity in this PM/VT group of patients was higher than in the PM alone group; they had more pregnancy losses than women with PM alone, and had other complications associated with APS, such as preeclampsia. The inclusion of a group of patients with gestational morbidity without thrombosis was important, since patients with a single occurrence of gestational morbidity may have low titers of conventional aPL and also may have antibodies to other negatively charged phospholipids.

Trophoblast migration assay. The spontaneous migratory property of HTR8 cells was measured using a Transwell

2-chamber migration assay, as previously described (10). For this assay, the lower chamber was a 24-well tissue culture plate (BD Falcon) containing 800 μl of Opti-MEM. HTR8 trophoblast cells (1×10^5 cells in 200 μl) were left untreated (untreated controls) or treated with aPL (ID2 or IIC5) or 10% patient serum in the presence or absence of the ATL 15-epi-lipoxin A_4 (100 n*M*; Cayman Chemical). The cells were then seeded into cell culture well inserts with an 8-μm–pore size membrane (Millipore). After 48 hours, the inserts were removed and trophoblast cell migration across the membrane was determined using the QCM 24-Well Colorimetric Cell Migration Assay (Chemicon International). Briefly, migrated cells were stained, collected, and lysed according to the manufacturer's instructions. The resulting colored mixture was transferred to a 96-well plate and optical densities were read in triplicate at 560 nm. The relative percentage of cell migration was determined by comparing the optical density to that in control wells (set at 100%) (10).

Trophoblast–endothelial cell cocultures. A 3-dimensional in vitro system was used to study trophoblast–endothelial cell interactions (26). HEEC cells were stained with the red fluorescent linker dye PKH-26 (Sigma), seeded into 24-well tissue culture plates (2×10^5 cells/well) over undiluted growth factor–reduced Matrigel (BD Biosciences), and then cultured in 250 μl of supplemented EBM-2 medium overnight until tube-like structures were observed, as previously described (26). The medium was then removed and HTR8 cells, stained with the green fluorescent linker dye PKH-67 (Sigma), were seeded (1×10^5 cells/well) in 500 μl of Opti-MEM and left untreated (untreated controls) or treated with aPL (ID2 or IIC5) at 20 μg/ml, an IgG1 isotype control (20 μg/ml), or 10% sterile patient serum in the presence or absence of the ATL 15-epi-lipoxin A_4 at 100 n*M*. Studies using fluorescence microscopy have shown that over an incubation time of 48 hours, HTR8 cells are seen to invade the Matrigel, migrate toward the HEEC cells, and begin to take on a tube-like formation (26). Therefore, the trophoblast–endothelial cell coculture was incubated for 48 hours. Four fields per well were recorded by fluorescence microscopy (Observer Z1; Carl Zeiss) using OpenLab software (Perkin Elmer), and the number of tubes per field was determined.

Measurements of cytokines, tissue inhibitor of metalloproteinases 2 (TIMP-2), and angiogenic factors. HTR8 trophoblast cells were treated in serum-free Opti-MEM with ID2 or IIC5 aPL (each at 20 μg/ml) in the presence or absence of the ATL 15-epi-lipoxin A_4 (at 100 n*M*); medium-only served as the negative (untreated) control. After 72 hours, cell-free supernatants were collected and stored at −80°C until analyzed. ELISAs were used to measure the supernatant concentrations of the proinflammatory chemokine interleukin-8 (IL-8), the promigratory cytokine IL-6 (10,24), the proangiogenic factor placenta growth factor (PlGF), the antiangiogenic factors soluble endoglin and soluble tyrosine kinase Flt-1 (sFlt-1), and the antimigratory factor TIMP-2 (R&D Systems) (24). Since 15-epi-lipoxin A_4 was dissolved in ethanol, the effect of this vehicle on the HTR8 cells was tested. Treatment of HTR8 cells with the ethanol vehicle control had no effect on cell viability or cytokine/angiogenic factor production. Similarly, 15-epi-lipoxin A_4 at 100 n*M* had no effect on cell viability (results not shown).

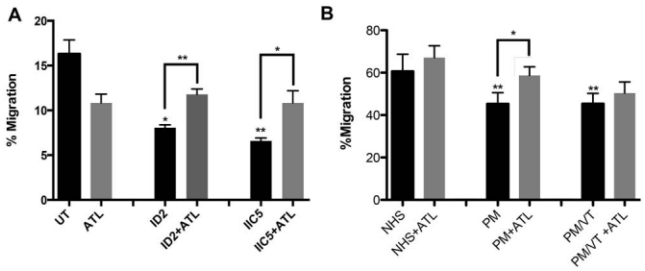

Figure 1. Effects of aspirin-triggered lipoxin (ATL) on antiphospholipid antibody (aPL)–mediated modulation of trophoblast cell migration. **A,** HTR8 cells (n = 5) were treated for 48 hours with the ATL 15-epi-lipoxin A_4 (100 n*M*) alone or in the presence of the aPL ID2 or IIC5 (20 μg/ml each), or were left untreated (UT) as controls. **B,** HTR8 cells were treated with sera from normal healthy women (NHS), women with aPL and pregnancy morbidity alone (PM), or women with aPL and pregnancy morbidity plus vascular thrombosis (PM/VT) (n = 3), all in the presence or absence of ATL. Bars show the mean ± SEM change in percentage of cell migration relative to control wells (set at 100%). $* = P < 0.01$; $** = P < 0.001$ versus controls or as otherwise indicated.

Statistical analysis. Experiments were performed at least 3 times, in triplicate. Results are expressed as the mean ± SEM of pooled experiments. Comparisons between groups were performed using either one-way analysis of variance with Bonferroni correction for multiple comparisons or Student's paired *t*-test, with data analysis using Prism software (GraphPad Software). *P* values less than 0.05 were considered statistically significant.

RESULTS

Reversal of the aPL-induced decrease in trophoblast migration following treatment with ATL. Investigators in our group have previously demonstrated that the anti-β_2GPI antibodies ID2 and IIC5 can significantly impair first-trimester human trophoblast cell migration (10). In this study, following treatment of human trophoblasts with the ATL 15-epi-lipoxin A_4, we found that the ATL had no significant effect on basal trophoblast migration. In contrast, ATL treatment partially, but significantly, reversed the reduction in cell migration that had occurred in response to the ID2 and IIC5 aPL (Figure 1A).

Having observed this beneficial effect of ATL, we next sought to validate the clinical relevance of these results by determining the effects of human serum, obtained from women with APS, on trophoblast cell migration in the presence or absence of 15-epi-lipoxin A_4. For these experiments, HTR8 cells were treated with serum from normal healthy female donors (NHS), women with aPL and pregnancy morbidity alone (PM

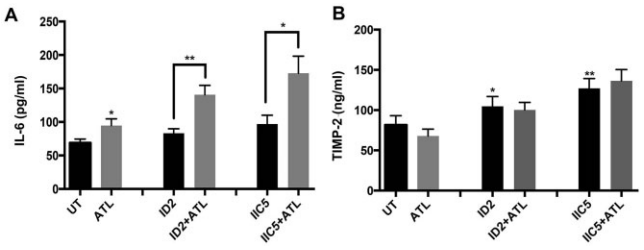

Figure 2. Effects of ATL on aPL modulation of trophoblast production of interleukin-6 (IL-6) and tissue inhibitor of metalloproteinases 2 (TIMP-2). HTR8 cells were treated for 72 hours with the ATL 15-epi-lipoxin A_4 (100 n*M*) alone or in the presence of the aPL ID2 or IIC5 (20 μg/ml each), or were left untreated as controls. Supernatants were analyzed for levels of **A,** IL-6 (n = 7) and **B,** TIMP-2 (n = 4). Bars show the mean ± SEM. $* = P < 0.05$; $** = P < 0.001$ versus untreated controls or as otherwise indicated. See Figure 1 for other definitions.

serum), and women with aPL and pregnancy morbidity plus vascular thrombosis (PM/VT serum). When compared to the effects of NHS control serum, trophoblast migration was significantly reduced in the presence of both PM serum and PM/VT serum, by a mean ± SEM 22.19 ± 4.78% and 22.70 ± 2.64%, respectively. Treatment with ATL in the presence of the PM serum completely restored the levels of trophoblast migration to basal levels, but treatment with ATL had no effect on the reduced migration caused by the presence of the PM/VT serum (Figure 1B).

Promotion of trophoblast secretion of the promigratory cytokine IL-6 following treatment with ATL. In previous studies, we demonstrated a role for suppressed production/signaling of the promigratory IL-6 and elevated secretion of the antimigratory TIMP-2 in mediating the aPL-induced inhibition of trophoblast migration (10,24). Therefore, we sought to determine the effects of 15-epi-lipoxin A_4 on these pro- and antimigratory factors. As shown in Figure 2A, treatment of human trophoblast cells with ATL significantly increased the trophoblast secretion of IL-6, both alone and in the presence of ID2 or IIC5 aPL. In contrast, ATL treatment had no effect on basal TIMP-2 secretion and did not alter the up-regulated response of TIMP-2 in the presence of either ID2 or IIC5 (Figure 2B).

Restoration of trophoblast–endothelial cell interactions by ATL following aPL-mediated disruption. Since aPL appear to limit trophoblast migration and this can be restored by treatment with 15-epi-lipoxin A_4, we further sought to examine this effect in a more physiologically representative model. During normal pregnancy, trophoblasts invade and remodel the uterine spiral arteries, and thereafter the cells take on an endothelial cell–like phenotype and begin to replace the resident endothelial cells; in patients with APS, this process is attenuated (4). This spiral artery transformation can be modeled in vitro using a HEEC cell line that, in Matrigel, forms tube-like structures that resemble vessels (26). When trophoblast cells are then introduced into the culture, they invade these tube-like structures and colocalize with, and eventually replace, the endothelial cells (26).

This trophoblast–endothelial cell interaction can be seen in the images of untreated control cells presented in Figure 3A, with HEEC cells labeled red and trophoblast cells labeled green. After 48 hours under basal conditions, the trophoblasts had migrated into, and completely integrated with, the endothelial cell vessel-like tubes, resulting in mostly colocalization (areas of yellow fluorescence in Figure 3A). However, it was also possible to see some of the endothelial cells being displaced (red-labeled cells) and to see areas of only trophoblast cells (green-labeled cells) (Figure 3A). When compared to that in untreated cells and IgG isotype–treated control cells, the presence of the anti-β_2GPI antibodies ID2 or IIC5 appeared to disrupt trophoblast–endothelial cell interactions and tube stabil-

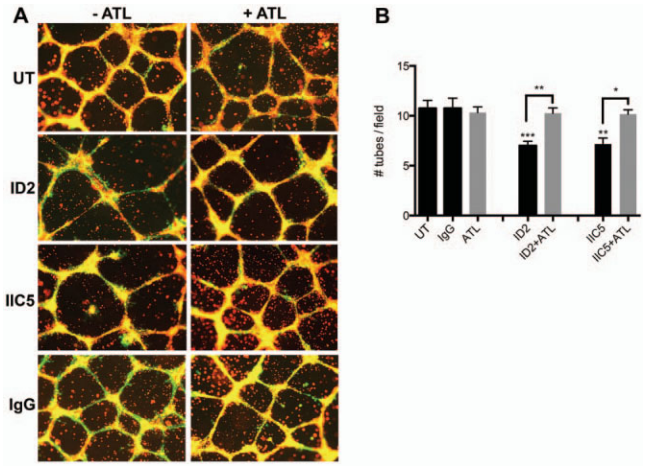

Figure 3. Effects of aPL and ATL on trophoblast–endothelial cell interactions. Human endometrial endothelial cells (red) were plated on Matrigel overnight to allow tube formation, after which HTR8 trophoblast cells (green) were added and were left untreated or treated with the aPL ID2 or IIC5 (20 μg/ml each) or an IgG isotype control (20 μg/ml), all in the presence or absence of ATL (100 n*M*). The cocultures were then incubated for 48 hours. **A,** Representative fluorescence microscopy images from 1 experiment are shown. Original magnification × 5. **B,** Results are shown quantitatively as the mean ± SEM number of tubes per field from 3 pooled experiments. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$ versus untreated controls or as otherwise indicated. See Figure 1 for definitions.

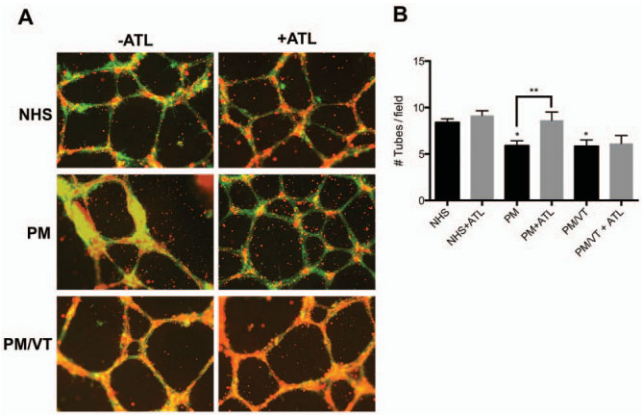

Figure 4. Effects of ATL on trophoblast–endothelial cell interactions after alteration by human serum from patients with antiphospholipid syndrome. Human endometrial endothelial cells (red) were plated on Matrigel overnight to allow tube formation, after which HTR8 trophoblast cells (green) were added and were treated with NHS, PM serum, or PM/VT serum, all in the presence or absence of ATL (100 n*M*). The cocultures were then incubated for 48 hours. **A,** Representative fluorescence microscopy images from 1 experiment are shown. Original magnification × 5. **B,** Results are shown quantitatively as the mean ± SEM number of tubes per field from 6 pooled experiments. * = $P < 0.05$; ** = $P < 0.01$ versus NHS controls or as otherwise indicated. See Figure 1 for definitions.

ity (Figure 3A), resulting in a significant decrease in the number of tube-like structures by a mean ± SEM 31.39 ± 5.96% and 32.06 ± 5.06%, respectively (Figure 3B). This disruption was significantly and completely reversed by the presence of ATL, which had no effect in cell cultures without the antibodies (Figures 3A and B).

The impact of APS patient serum on these trophoblast–endothelial cell interactions was then tested. Similar to the effects of the anti-β_2GPI antibodies, when compared to NHS, the presence of PM serum and PM/VT serum disrupted the trophoblast–endothelial cell interactions and tube stability (Figure 4A), resulting in a significant decrease in the number of tube-like structures by a mean ± SEM 29.98 ± 5.82% and 27.41 ± 8.60%, respectively (Figure 4B). Similar to our data on cell migration (shown in Figure 1B), the presence of the ATL 15-epi-lipoxin A_4 significantly and completely reversed the effect of the PM serum on the trophoblast–endothelial cell interactions and tube counts, whereas ATL treatment was unable to restore the tube stability that had been disrupted in response to the PM/VT serum (Figures 4A and B).

Lack of effect of ATL on aPL-induced changes in trophoblast secretion of IL-8 or angiogenic factors. In parallel to impaired trophoblast migration, we have previously reported that the anti-β_2GPI antibodies ID2 and IIC5, but not an isotype control antibody, can induce first-trimester human trophoblast secretion of the proinflammatory chemokine IL-8, in a Toll-like receptor 4 (TLR-4)–dependent manner (6), and can increase the secretion of the angiogenic factors PlGF and soluble endoglin, without increasing the production of sFlt-1 (9). Therefore, our last objective was to determine whether the ATL 15-epi-lipoxin A_4 could reverse these aPL-mediated effects on trophoblast function. As shown in Figures 5A–D, ATL alone had no effect on HTR8 trophoblast basal secretion of IL-8, PlGF, sFlt-1, or soluble endoglin. In the presence of the ID2 or IIC5 aPL, the secretion of IL-8 and PlGF was significantly increased in HTR8 cells when compared to that in untreated control cells (Figures 5A and B). Although secretion of soluble endoglin was also increased in the presence of both ID2 and IIC5 when compared to untreated controls, this was only significant in the presence of IIC5 (Figure 5D). Neither aPL had an effect on sFlt-1 release (Figure 5C).

The presence of ATL had no effect on the ability of the aPL to up-regulate trophoblast secretion of the proinflammatory chemokine IL-8 (Figure 5A), the proangiogenic factor PlGF (Figure 5B), or the antiangiogenic factor soluble endoglin (Figure 5D). Moreover, in the presence of the ID2 or IIC5 aPL, ATL treatment

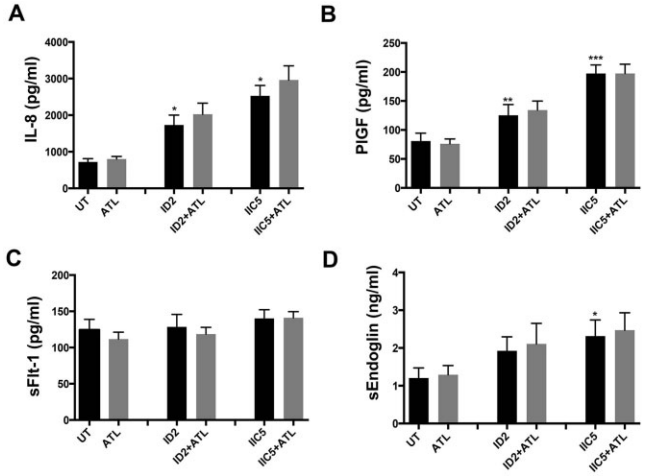

Figure 5. Effects of ATL on aPL-mediated modulation of trophoblast secretion of interleukin-8 (IL-8) and angiogenic factors. HTR8 cells were treated for 72 hours with the ATL 15-epi-lipoxin A_4 (100 n*M*) alone or in the presence of the aPL ID2 or IIC5 (20 μg/ml each), or were left untreated as controls. Cell-free supernatants were assessed for levels of **A,** IL-8, **B,** placenta growth factor (PlGF), **C,** soluble tyrosine kinase Flt-1 (sFlt-1), and **D,** soluble endoglin. Bars show the mean ± SEM (n = 4). * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ versus untreated controls. See Figure 1 for other definitions.

had no effect on the persistent sFlt-1 release by HTR8 cells (Figure 5C).

DISCUSSION

Antiphospholipid antibodies recognizing β_2GPI have deleterious effects on the functions of first-trimester human trophoblasts by reducing cell migration, inducing a potentially unfavorable proinflammatory cytokine profile, and altering the angiogenic factor milieu (6,9,10). While pregnant women with APS are routinely treated with LMWH, either alone or in combination with aspirin, there are conflicting data regarding the clinical efficacy of this regimen (1,5,11–15) and whether such treatment is able to reverse the detrimental effects of aPL on human trophoblast function (6,9,10,16,17). This highlights the need for additional investigations into novel therapeutic strategies for the management of APS-associated pregnancy morbidities. In the current study, we investigated the ATL 15-epi-lipoxin A_4 as a potential therapeutic approach for resolving the negative effects of aPL on placental function. Herein, we report for the first time that treatment with this ATL reversed the ability of aPL to reduce the migration of first-trimester human trophoblasts. We also showed, for the first time, using an in vitro model of spiral artery transformation, that ATL treatment completely restored the trophoblast–endothelial cell interactions that were disrupted by aPL. Furthermore, we validated our findings using sera from women with aPL and pregnancy morbidities.

In addition to the classic mechanism of blocking COX-2 to inhibit prostaglandin and thromboxane production, aspirin also acetylates COX-2, triggering a 15-epimeric form of endogenous lipoxins. Lipoxins are bioactive eicosanoids generated by transcellular lipoxygenation of arachidonic acid. Among them, lipoxin A_4 and its analogs exhibit an inflammatory proresolving effect (20). In vitro, ATLs have been shown to prevent lipopolysaccharide (LPS)–induced cytokine production in immune cells, endothelial cells, microglia, and myometrium (34–37), to prevent LPS-induced hyperpermeability in endothelial cells (38), and to exert antiangiogenic effects on hypoxic human umbilical vein endothelial cells (39). Furthermore, we have previously demonstrated that lipoxin A_4 reduces the adhesion of human neutrophils to endothelial cells induced by plasma from women with preeclampsia (40). Indeed, deficiency in endogenous lipoxin A_4 has been correlated with the development of preeclampsia (41), and administration of lipoxin A_4 alleviates adverse pregnancy outcomes in rat models of preeclampsia and IUGR (41–43). We therefore sought to evaluate the actions of the ATL 15-epi-lipoxin A_4 on first-trimester human trophoblast function in the presence of aPL.

Previous work from our group has demonstrated a number of effects of anti-β_2GPI antibodies on first-trimester human trophoblast function. Our studies using a human trophoblast cell line have previously demonstrated that anti-β_2GPI antibodies increase the secretion of the proinflammatory chemokine IL-8, alter angiogenic factor production, and reduce cell migration (6,9,10,24). In the current study, we demonstrated a pronounced effect of the ATL on human trophoblasts in terms of being able to reverse the effects of the aPL on cell migration. Using both the anti-β_2GPI antibodies ID2 and IIC5 and serum samples from patients with aPL and either pregnancy morbidity alone (PM serum) or pregnancy morbidity plus vascular thrombosis (PM/VT serum), we observed a significant reduction in spontaneous trophoblast cell migration. The addition of ATL partially reversed the effect of the anti-β_2GPI antibodies, and completely reversed the reduced trophoblast migration induced by the PM serum. In contrast, ATL treatment had no effect on the inhibited trophoblast migration induced by the PM/VT serum.

The reason that this ATL was unable to reverse the effects of the PM/VT serum on trophoblast migration is unclear. However, it is possible that, since these patients have both pregnancy morbidities and thrombosis, the fine specificities of the aPL in the sera from these women are distinct from those in the sera from women with obstetric APS who do not have thrombotic disease (PM alone). Indeed, aPL have long been known to be very heterogeneous (31,44), and more recently it has been suggested that the pathogenic antibodies primarily react with an epitope in domain I of β_2GPI (45). However, although the primary mechanism of pathology in APS outside of pregnancy is thrombotic, thrombosis is very clearly not a major contributor to the pathogenesis of obstetric APS (46). It is widely accepted that, in obstetric APS, aPL interact directly with the placental trophoblast, and thus it is highly likely that the epitope specificity of aPL that are pathogenic during pregnancy is different from the specificity of aPL that induce thrombosis. Indeed, there is now evidence to suggest that domain I antibodies are not the major pathogenic aPL in obstetric APS (for review, see ref. 47). Moreover, binding of aPL to domain V of β_2GPI, similar to the ID2 and IIC5 aPL, may be more important for pregnancy morbidity in APS patients (24,48).

Thus, our results support the concept that aPL

reactive with domain I of β_2GPI may not be the primary disease-causing antibodies in women with obstetric APS. Consequently, aPL in PM/VT serum may trigger additional signals in the trophoblast that can regulate cell migration, and these may not be regulated by ATLs. Therefore, the management of women with APS may need to be different because of their clinical manifestations (32). Women with aPL and pregnancy morbidity alone could start a low-dose aspirin regimen to reduce the risk of pregnancy losses, but in women with pregnancy morbidity and vascular thrombosis, the addition of heparin seems clearly indicated (49). Thus, for women with pregnancy morbidity and vascular thrombosis, the induction of lipoxins by treatment with aspirin may not be enough to resolve the deleterious effects induced by aPL, and the inclusion of heparin treatment may be necessary to counteract the additional effects on the trophoblast due to the thrombotic status of these patients.

Previously, we have shown that anti-β_2GPI antibodies reduce trophoblast migration by preventing endogenous IL-6–induced STAT-3 activation, which normally occurs in an autocrine/paracrine manner, and by increasing TIMP-2 production (10,24). Moreover, the antimalarial drug hydroxychloroquine partially antagonizes aPL-induced inhibition of trophoblast migration by promoting IL-6 production (24). In this study, we found that ATL in the presence of the anti-β_2GPI antibodies promoted IL-6 production, but did not prevent aPL-induced TIMP-2 secretion. Thus, similar to the effects of hydroxychloroquine, ATL may be able to restore trophoblast migration in the presence of aPL or human PM serum through increased production of IL-6 (24). This is in contrast to findings showing that ATL inhibits IL-6 production and reduces STAT-3 activation (35,37,50). Although little is known about the effects of ATL on the process of migration, in endothelial cells it can reduce vascular endothelial growth factor (VEGF)–induced migration (51). We observed no effect on trophoblast VEGF production in the presence of ATL and aPL (results not shown).

Having established the ability of ATL to modulate trophoblast migration in the setting of aPL, we next sought to investigate this in a more physiologically representative model. Using an in vitro model of spiral artery transformation (26), we showed, for the first time, that anti-β_2GPI antibodies, as well as the serum from PM and PM/VT patients, disrupt the normal interactions between HEEC cells and trophoblast cells, resulting in decreased tube formation. In this system, the adequate invasion of the trophoblast into the endothelial tubes maintains their structure over time (26). Thus, it appears that the ability of aPL to reduce trophoblast migration may provide the mechanism responsible for the tube disruption. Indeed, similar to our findings regarding cell migration, the presence of the ATL completely restored the coculture stability in the presence of the anti-β_2GPI antibodies and the PM serum, but not in the presence of the PM/VT serum.

Finally, contrary to our expectations, 15-epi-lipoxin A_4 was unable to resolve the aPL-induced inflammatory IL-8 response. This ATL also had no effect on the ability of the anti-β_2GPI antibodies to upregulate trophoblast secretion of the proangiogenic factor PlGF or the antiangiogenic factor soluble endoglin. This was surprising, since aPL-induced trophoblast inflammation is mediated by TLR-4 (6), and previous studies in other systems have shown that an ATL (at the same dose as used in the present study [100 n*M*]) attenuated LPS-induced IL-8 production (34,35,37).

In conclusion, the ATL 15-epi-lipoxin A_4 restored aPL inhibition of trophoblast migration and trophoblast–endothelial cell interactions, although it could not resolve the proinflammatory and antiangiogenic responses of the trophoblasts to the presence of aPL. Therefore, while further research is warranted, our studies indicate that ATLs may have some benefits for preventing the effects of aPL on trophoblast function. This raises the possibility of using ATLs as an adjuvant therapy for women with aPL at risk of developing obstetric APS.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Abrahams had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Alvarez, Chamley, Cadavid, Abrahams.
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