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Antiphospholipid antibodies induce a pro-inflammatory response in first trimester trophoblast via the TLR4/MyD88 pathway

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Abstract

Problem—Women with antiphospholipid antibodies (aPL) are at risk for recurrent miscarriage, preeclampsia and preterm labor. aPL target the placenta directly by binding to Beta₂-Glycoprotein I (β₂GPI) expressed on the surface of trophoblast cells. The objective of this study was to determine the effects of aPL on trophoblast function and the mechanisms involved.

Method of study—First trimester trophoblast were treated with anti-β₂GPI monoclonal antibodies and patient-derived aPL, after which cell survival and function was evaluated.

Results—We report that anti-β₂GPI antibodies trigger an inflammatory response in trophoblast, characterized by increased secretion of IL-8, MCP-1, GRO-α and IL-1β, and that this occurs in a TLR-4/MyD88-dependent manner. At high concentrations, these antibodies also induce caspase-mediated cell death. This was attenuated upon disabling of the MyD88 pathway, suggesting that anti-β₂GPI-induced inflammatory mediators compromise trophoblast survival by acting in an autocrine/paracrine manner. Enhanced IL-8, GRO-α and IL-1β secretion also occurred when trophoblast were incubated with antibodies from patients with antiphospholipid syndrome. Heparin, which acts as a pro-survival factor in human trophoblast, attenuated the anti-β₂GPI antibody-mediated cell death, and also the pro-inflammatory response, but only at high concentrations.

Conclusions—These findings demonstrate that aPL triggers a placental inflammatory response via the TLR-4/MyD88 pathway, which in turn compromises trophoblast survival. Thus, the TLR-4/MyD88 pathway may provide a new therapeutic target to improve pregnancy outcome in antiphospholipid syndrome patients.

Keywords

Apoptosis; Autoantibodies; Human; Inflammation; Toll-like receptor

Introduction

The presence of antiphospholipid antibodies (aPL) negatively impacts on a woman's chance of reproductive success. This heterogeneous family of autoantibodies are found in patients with primary antiphospholipid syndrome (APS) which may occur in isolation (primary APS) or in the presence of other autoimmune rheumatic disease (secondary APS); most commonly in patients with systemic lupus erythematosus (SLE) ¹. Pregnant women with aPL are at high risk for thrombosis, recurrent pregnancy loss, as well as other pregnancy complications associated with impaired placental function, such as fetal growth restriction and preeclampsia ¹⁻⁵. While treatment with aspirin and heparin from early pregnancy significantly increases the live birth rate in recurrent miscarriage patients with APS, the incidence of severe late obstetric complications, including preeclampsia, intrauterine growth restriction and prematurity, still remains high ^{6,7}.

Most aPL in APS and SLE are detected by the anti-cardiolipin ELISA or lupus anticoagulant test ¹. However, it has become increasingly clear that pathogenic aPL actually recognize epitopes on phospholipid-binding proteins rather than on the phospholipids themselves. The most important of these is the plasma glycoprotein Beta₂-Glycoprotein I (β_2 GPI), which is a major antigen of aPL ². Trophoblast cells synthesize their own β_2 GPI and express this protein on the cell surface under normal physiological conditions ⁸. Furthermore, exogenous β_2 GPI can bind to the surface of viable trophoblast cells ⁹. *In vivo*, β_2 GPI localizes to the surface of the extravillous trophoblast cells that invade the decidua, and to the syncytiotrophoblast cells that are in direct contact with maternal blood ^{8,10}. Thus, the placenta is a target for anti- β_2 GPI Abs.

As aPL are one of the most common causes of acquired hypercoagulability in the general population, pregnancy complications in patients with APS are often attributed to thrombotic events at the maternal-fetal interface. This notion is seemingly supported by the success of anticoagulant treatment, and more specifically heparin, in preventing fetal loss in affected patients. However, intravascular or intervillous blood clots are rarely found on histological examination of miscarriage samples from APS patients ¹¹. Instead, the process of placenta formation is compromised, evidenced by reduced endovascular trophoblast invasion, limited spiral artery transformation ^{11,12}, and profound influx of inflammatory immune cells, such as neutrophils, at the maternal-fetal interface ¹³⁻¹⁵. These observations suggest that aPL may primarily impact on trophoblast function rather than causing a direct insult on the fetomaternal vasculature. Indeed, several *in vitro* studies have shown that aPL compromise proliferation, invasion and differentiation of term trophoblast and choriocarcinoma cell lines ^{9,16-22}, although the underlying mechanisms are unknown. Furthermore, early pregnancy loss is the commonest pregnancy complication associated with APS, yet the effects of aPL on first trimester trophoblast function and survival have not been studied.

This study demonstrates for the first time that anti- β_2 GPI Abs elicit a dose-dependent response in human first trimester trophoblast cells. High anti- β_2 GPI Ab concentrations induce cell death and apoptosis, whereas lower concentrations markedly enhance trophoblast production of IL-8, MCP-1, GRO- α , and IL-1 β in a Toll-like receptor 4/myeloid differentiation factor 88 (TLR4/MyD88)-dependent manner. Interestingly, disabling of the TLR4/MyD88 pathway not only attenuated this pro-inflammatory response but also partially protected trophoblast to cell death induced by anti- β_2 GPI Abs. Furthermore, the effect of anti- β_2 GPI Abs on trophoblast cytokine and chemokine production was recapitulated with purified Abs from sera of affected patients, indicating that adverse pregnancy outcome in APS may be primarily caused by an excessive placental inflammatory response, leading to trophoblast cell death and tissue injury.

Materials and Methods

Reagents and antibodies

Camptothecin (CPT) and heparin were both purchased from Sigma (St Louis, MO). The mouse antibody for β_2 GPI was purchased from Abnova (Walnut, CA), and the rabbit anti- β -actin polyclonal antibody was purchased from Sigma. Specific signals for Western blot were detected using a peroxidase-conjugated horse anti-mouse secondary antibody (Vector Laboratories).

Antiphospholipid antibodies

These studies utilized two mouse IgG1 anti-human β_2 GPI monoclonal Abs (mAb), designated ID2 and IIC5, which were produced by one of us (LWC), under sterile conditions, and were filter-sterilized prior to use. The antibodies, ID2 and IIC5 were cloned from mice immunized with purified human β_2 GPI. They have been previously characterized²⁰, and like human aPL, they bind β_2 GPI, but only when it is immobilized on a suitable negatively charged surface, such the phospholipids, cardiolipin or phosphatidyl serine, or irradiated polystyrene²³. In addition, polyclonal IgG purified from the serum of 18 patients with different clinical manifestations of APS, fulfilling APS criteria²⁴, under long term follow up at University College London Hospital (UCLH) were studied. All subjects signed consent forms approved by the local ethics committees at UCLH. IgG from the nine subjects was purified by protein G sepharose affinity columns (Amersham Biosciences, Sweden) and then passed through Detoxi-Gel™ Endotoxin removing columns coated with immobilised polymyxin B (Pierce, Perbio, UK) and subsequently determined to be endotoxin free (<0.06 endotoxin units/ml) by the *Limulus* Amoebocyte Lysate assay (Sigma, UK). The polyclonal APS-IgG was purified from stored (−80) serum samples which were taken close to the time of the APS related clinical event and confirmed to have current anti-cardiolipin/anti- β_2 GPI activity, as shown in Table I. Anti- β_2 GPI activity was measured using HCAL (Sapporo Standard, used at 25 μ g/ml), and anti-cardiolipin activity, was determined using cardiolipin calibrators (APS Diagnostics laboratory, Galveston, TX). The patient samples were then divided into 3 groups: 1) PM⁺/VT[−]: patients who have had previous episodes of pregnancy morbidity (PM), but no history of venous thrombosis (VT) (n=6); 2) PM[−]/VT⁺: patients who have had VT, but not PM (n=6); and 3) PM⁺/VT⁺: patients who have had both VT and PM (n=6). In the VT/PM group, three samples were obtained from close to the PM event, two were close to the VT event, and one sample was obtained from a patient who had recently experienced a pulmonary embolus two weeks post APS related PM. All patient clinical and serological information is shown in Table I.

First trimester trophoblast cell line

The human first trimester extravillous trophoblast cell line, HTR8, was used in these studies. The HTR8 cells were immortalized by SV40²⁵, and was a kind gift from Dr Charles Graham (Queens University, Kingston, ON, Canada). HTR8 cells were cultured in RPMI 1640 (Gibco) which was supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 10mM Hepes, 0.1mM MEM non-essential amino acids, 1mM sodium pyruvate, 100nm penicillin/streptomycin (Gibco). Cells were maintained at 37°C/5% CO₂.

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 Bridgeport Hospital. The use of patient samples was approved under both Yale University's HIC and Bridgeport Hospital's IRB. Tissue specimens were washed with cold Hanks Balanced Salt Solution (Gibco) to remove excess blood. Cells were scraped from the membranes, transferred to trypsin-EDTA (Invitrogen, Carlsbad, CA) digestion buffer and incubated at 37°C for 40 minutes with shaking. The mixture was then

passed through a nylon strainer and then layered over Lymphocyte Separation Media (ICN Biomedicals, Inc., Aurora, OH) and centrifuged at 2000rpm for 25 minutes. The cellular interface containing the trophoblast cells was collected and resuspended in D-MEM with D-valine (Caisson Labs, North Logan, UT) supplemented with 10% normal human serum (Gemini Bio-Products, Woodland, CA) and cultured at 37°C/5% CO₂. Purity of the trophoblast cells was determined by monitoring cytokeratin 7 expression.

Transfection of trophoblast cells with MyD88 and TLR4 dominant negatives

HTR8 cells were transiently transfected with either the pDeNy plasmid containing the human MyD88 dominant negative (MyD88-DN), or the pZERO plasmid containing the human HA-tagged TLR4 Δ TIR (Invivogen, San Diego, CA). The TLR4 Δ TIR acts as a dominant negative since the TIR domain has been deleted. Thus, TLR4 Δ TIR (TLR4-DN) can compete with endogenous TLR4 for ligand binding, but cannot transduce a signal²⁶. Transfection was performed as previously described²⁷. Briefly, cells were transfected overnight with 2 μ g of DNA using Fugene 6 (Roche Diagnostics, Indianapolis, IN) before a treatment experiment was performed. Transfection was monitored by performing Western blot analysis on cell lysates for the HA-tag using the mouse anti-HA-tag mAb from Invivogen.

Western blot analysis

For analysis of proteins, Western blot analysis was performed. Proteins, diluted to 20 μ g with gel loading buffer were boiled for 5 minutes and resolved under reducing conditions on 12% SDS-PAGE gels and then transferred onto PVDF paper (PerkinElmer, Boston, MA). Membranes were blocked with 5% fat-free powdered milk (FFPM) in PBS/0.05% Tween-20 (PBS-T). Following washes with PBS-T, membranes were incubated overnight at 4°C with primary antibody in PBS-T/1% FFPM. Following this incubation, membranes were washed as before and then incubated with the appropriate secondary antibody conjugated to peroxidase (Vector Labs) in PBS-T/1% FFPM. Following washes with PBS-T and then with distilled water, the peroxidase-conjugated antibody was detected by enhanced chemiluminescence (PerkinElmer). β -actin was used as internal control, in addition to Ponceau Red, to validate the amount of protein loaded onto the gels. Images were recorded using the Gel Logic 100 (Kodak) and Kodak MI software.

Immunohistochemistry

The binding of the anti- β_2 GPI mAbs, ID2 and IIC5, to the trophoblast was determined by immunocytochemistry. In short, trophoblast cells, previously adhered to glass slides, were fixed with 4% paraformaldehyde. They were then blocked with 10% horse serum in PBS for 1 hour at room temperature. Following three washes with PBS, samples were incubated overnight at 4°C with either ID2 or IIC5 (40 μ g/ml). Mouse IgG1 served as a negative control. After three washes with PBS, specific staining was detected by incubating with a peroxidase-conjugated horse anti-mouse antibody (1:500 dilution) for 1 hour followed by a five minute incubation with DAB substrate (Vector Laboratories). Cells were then counterstained with haematoxylin (Sigma) before dehydration with ethanol and Histo-solve (Shandon Inc., Pittsburgh, PA). Slides were then mounted with Permount (Fisher Scientific, Pittsburgh, PA) and visualized by light microscopy.

Cell viability assay

Trophoblast cell viability was determined using the CellTiter 96™ viability assay (Promega, Madison, WI). Trophoblast cells were plated in wells of a 96 well plate at 1×10^4 cells per well in growth media and cultured until 70% confluent after which the media was then replaced with the reduced serum medium, Opti-MEM (Gibco) and cultured for another 4 hours prior to treatment. Following treatment, the CellTiter 96™ substrate was added to all wells and

following a 1–4 hour incubation at 37°C, optical densities were read at 490nm. All samples were assayed in triplicate and cell viability was presented as a percentage relative to the untreated control.

Caspase activity assay

The effect of the anti- β_2 GPI Abs on trophoblast caspase activity was determined using the Caspase-Glo™ assay (Promega, Madison, WI). Briefly, 10 μ g of whole cell lysates were incubated at room temperature in the dark for 1 hour with either the caspase 3 substrate, the caspase-8 substrate or the caspase-9 substrate. Following incubation, luminescence was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The amount of luminescence detected as relative light units was proportional to caspase activity.

Cytokine studies

Trophoblast cells were treated with or without the anti- β_2 GPI Abs. Following a culture of 72 hours, the cell-free supernatants, also termed conditioned media (CM), were collected by centrifugation at 400g for 10 minutes and stored at –80°C until analysis was performed. The concentrations of IL-8 were evaluated by ELISA (Assay Designs, Ann Arbor, MI). Subsequently, multiplex analysis was performed on the supernatants using the BioPlex assay (BioRad) for the following cytokines/chemokines: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, GRO- α , IFN γ , MCP-1, MIP-1 α , RANTES and TNF α . Detection and analysis were performed using the Luminex 100 IS system (Upstate Biotechnology, Charlottesville, VA).

Statistical analysis

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

Results

Anti- β_2 GPI antibodies induce trophoblast cell apoptosis

Since anti- β_2 GPI Abs have been shown to impair choriocarcinoma cell proliferation²⁰, the first objective of this study was to determine whether the two anti- β_2 GPI Abs, ID2 and IIC5, also impact on first trimester trophoblast cell viability, using HTR8 cells as a model cell system. To validate this model, we first evaluated the expression of β_2 GPI by the HTR8 trophoblast cells, and assessed whether these cells would bind the two anti- β_2 GPI Abs. As shown in Figure 1A, Western blot analysis confirmed the expression of β_2 GPI in HTR8 cells. Moreover, both ID2 and IIC5 were able to bind to the trophoblast cells, as evidenced by positive immunoreactivity (Figure 1B). Thus, the effect of these antibodies on HTR8 cell viability was evaluated next. As shown in Figure 2A, neither antibody had an adverse effect on trophoblast viability at 5, 10, or 20 μ g/ml. If anything, proliferation was modestly enhanced at the lower two doses. However, at a concentration of 40 μ g/ml, both antibodies significantly reduced cell viability after 48 hours of incubation, indicative of cell death (Figure 2A). While ID2 and IIC5 (40 μ g/ml) significantly reduced trophoblast viability, the mouse IgG1 (IgG) mAb isotype control at the same concentration had no effect (Figure 2A and B). Camptothecin, which is cytotoxic to trophoblast cells, was used a positive control. It is noteworthy that ID2 was modestly more potent at reducing trophoblast cell viability than IIC5. Since heparin has been shown to protect human trophoblast against a variety of cytotoxic stimuli by enhancing heparin-binding growth factor activation of the phosphoinositide 3-kinase/AKT survival pathway²⁸, we examined if simultaneous treatment with heparin would also antagonize cell death induced

by these anti- β_2 GPI Abs in first trimester trophoblast. As expected, the loss in cell viability induced by ID2 and IIC5 was significantly attenuated when cells were treated heparin at 100ng/ml (Figure 2C). Higher heparin concentrations had no additional inhibitory effect (data not shown). In order to determine whether the decrease in trophoblast cell viability in response to the anti- β_2 GPI Abs was the result of apoptosis, the activity of various caspases was measured. As shown in Figure 2D, following treatment of the trophoblast cells with ID2 and IIC5 (40 μ g/ml) for 48 hours, there was a significant increase in the activities of caspase-8, caspase-9 and caspase-3. Interestingly, ID2 induced significantly more caspase-3 activity than IIC5 whereas the reverse was true for activation of caspase-8 and caspase-9 ($p < 0.05$).

Anti- β_2 GPI antibodies induce a trophoblast inflammatory response

Although not overtly cytotoxic, we speculated that lower concentrations of anti- β_2 GPI Abs may, still adversely affect trophoblast function; for instance by altering cellular cytokine or chemokine production. To test this hypothesis, we first monitored the effects of ID2 and IIC5 on IL-8 production, an inflammatory mediator constitutively expressed by primary first trimester trophoblast and HTR8 cells²⁹. As shown in Figure 3A, exposure of HTR8 cells to low concentrations of the anti- β_2 GPI Abs (1 – 20 μ g/ml) significantly increased IL-8 secretion in a dose-dependent manner. After 72 hours of treatment, trophoblast IL-8 production in response to ID2 or IIC5 (20 μ g/ml) was increased by 12.2-fold and 6.6-fold, respectively, whereas the mIgG isotype control had no effect (Figure 3B). Since heparin is also known to have anti-inflammatory properties^{30–32}, we examined if heparin also protects first trimester trophoblast against the inflammatory cytokine response induced by anti- β_2 GPI antibodies. While, the presence of heparin at 100ng/ml had no effect on IL-8 production upon treatment with ID2 or IIC5 (data not shown), at the higher dose of 10 μ g/ml, heparin significantly reduced the anti- β_2 GPI Ab-induced IL-8 response (Figure 3C).

We next extended our search for other cytokines and chemokines upregulated or induced by the anti- β_2 GPI Abs by performing multiplex analysis on the trophoblast culture supernatants. This screen allowed us to analyze the following cytokines/chemokines: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, GRO- α , IFN γ , MCP-1, MIP-1 α , RANTES and TNF α . This screen revealed that, in addition to IL-8, ID2 and IIC5 (20 μ g/ml) also significantly increased the secretion of MCP-1, GRO- α , and IL-1 β , by the HTR8 cells (Figure 4A). The anti- β_2 GPI Abs had no significant effect on the remaining cytokines and chemokines tested. Furthermore, there was a significant increase in the secretion of these same cytokines and chemokines when primary trophoblast cultures were treated with the same concentration of ID2 or IIC5 (Figure 4B).

Anti- β_2 GPI antibodies upregulate trophoblast cytokine and chemokine production via the TLR-4/MyD88 pathway

The next objective was to elucidate the mechanisms by which anti- β_2 GPI Abs induce a pro-inflammatory response in human trophoblast. A recent study in monocytes reported that anti- β_2 GPI Abs may recruit the innate immune receptor, TLR4, upon interaction with the β_2 GPI³³. Since first trimester trophoblast cells express functional TLR4³⁴, we questioned whether this signaling pathway is involved in the trophoblast response to the anti- β_2 GPI Abs. Thus, HTR8 cells were transfected with an expression vector that encodes for a dominant-negative TLR4 mutant (TLR4-DN) before treatment with ID2 and IIC5 (20 μ g/ml). The TLR4-DN lacks the TIR (Toll-IL-1R) domain and therefore competes with the endogenous receptor for ligand binding but cannot signal. As shown in Figure 5A, expression of TLR4-DN in trophoblast cells significantly inhibited the ability of the anti- β_2 GPI Abs to upregulate IL-8, MCP-1, GRO- α and IL-1 β secretion.

TLR4 is known to signal via the adapter protein MyD88, but can also transduce signals in a MyD88-independent manner³⁵. Therefore, in order to determine if the TLR4-mediated trophoblast responses to the anti- β_2 GPI Abs were occurring through MyD88, HTR8 cells were transiently transfected to express a dominant-negative MyD88 mutant (MyD88-DN). As shown in Figure 6, the ability of ID2, as well as IIC5, to upregulate IL-8, MCP-1, GRO- α and IL-1 β secretion was significantly attenuated in trophoblast cells expressing MyD88-DN when compared to the vector control cells. Together, the results establish that anti- β_2 GPI Abs induce a pro-inflammatory response in human trophoblast cells by activating the TLR-4/MyD88 pathway.

Anti- β_2 GPI antibody-induced inflammation triggers trophoblast cell death

Inflammatory mediators, such as IL-1 β , TNF α and IFN γ , are known to induce trophoblast cell death^{36,37}. As anti- β_2 GPI Abs markedly upregulated IL-1 β production in first trimester trophoblast cells, we hypothesized that this inflammatory response may feed back onto the trophoblast cells in an autocrine/paracrine manner to induce this cell death. To test this conjecture, conditioned media (CM) collected from untreated trophoblast cultures (CM/NT) or cells incubated for 72 hours with 20 μ g/ml ID2 (CM/ID2) or IIC5 (CM/IIC5) were diluted to 50% in serum-free medium and then added to fresh trophoblast cultures. As shown in Figure 7A, while the supernatant from the untreated cells (CM/NT) did induce some trophoblast cell death, this response was significantly less than that induced by the conditioned media from cells treated with ID2 or IIC5 (CM/ID2; CM/IIC5). In addition, trophoblast cells exposed to CM/ID2 and CM/IIC5 had significantly higher levels of active caspase-3 than cells exposed to either CM/NT or medium alone (NT), confirming that apoptosis was being induced by the trophoblast CM (Figure 7B). In order to confirm that the apoptotic response was mediated indirectly via the antibody-induced inflammatory factors, trophoblast cells were transiently transfected to express the MyD88-DN after which cells were treated with ID2 or IIC5 at 40 μ g/ml for 48 hours. The presence of the MyD88-DN significantly reduced that amount of anti- β_2 GPI Ab-induced trophoblast cell death (Figure 7C).

Patient-derived aPL enhance trophoblast cytokine/chemokine production

Having found that the two mouse anti- β_2 GPI mAbs were able to upregulate first trimester trophoblast cytokine and chemokine production, we next sought to validate the clinical relevance of these results by determining the effects of polyclonal IgG derived from patients with APS on trophoblast cell function. For this experiment, polyclonal APS-IgG were purified from the serum of APS patients with a history of pregnancy morbidity, venous thrombosis, or both (PM⁺/VT⁻; PM⁻/VT⁺; or PM⁺/VT⁺, respectively). HTR8 cells were incubated with the APS-IgG at 12.5 μ g/ml for 72 hours, after which the supernatants were collected, pooled on the basis of patient group, and then evaluated by multiplex analysis. As with ID2 and IIC5, the APS-IgG from all three clinical groups significantly upregulated trophoblast secretion of IL-8, GRO- α , and IL-1 β , although not MCP-1 (Figure 8). Interestingly, the pro-inflammatory trophoblast response tended to be more pronounced upon incubation of polyclonal APS-IgG purified from patients with a history of pregnancy morbidity. Specifically, polyclonal APS-IgG from the PM⁺/VT⁺ group induced significantly more trophoblast IL-8 and GRO- α secretion than the IgG from the PM⁻/VT⁺ group. Similarly, polyclonal APS-IgG from the PM⁺/VT⁻ group induced significantly more trophoblast IL-8 production than the IgG from the PM⁻/VT⁺ group (Figure 8).

Discussion

Women with aPL are at high risk of early pregnancy loss and late pregnancy complications, such as preeclampsia and prematurity, as well as vascular thrombosis. However, the pathogenesis of these pregnancy-associated complications in patients with APS remains

largely undefined. Both clinical and experimental observations suggest that pregnancy failure in these patients may involve an inflammatory response at the maternal-fetal interface, as well as disruption of normal trophoblast survival and function. Yet, to date, the mechanisms involved have not been studied in any depth, and little is known about the effect of aPL on human first trimester trophoblast cells. In this study we demonstrate, for the first time, that anti- β_2 GPI Abs can modulate first trimester trophoblast cytokine and chemokine production, which in turn compromises cell viability.

Animal models have supported a role for aPL in the pathogenesis of pregnancy failure and pregnancy complications. Several groups have shown that either immunization of animals with β_2 GPI or the passive transfer of aPL to mice promotes fetal resorption, fetal death, reduced litter sizes and IUGR^{38–41}. Moreover, one study showed that while the passive transfer of human anti- β_2 GPI I Abs to β_2 GPI^{+/+} mice triggered fetal loss, β_2 GPI^{-/-} mice were resistant to this antibody-induced effect, highlighting the importance of β_2 GPI as a major antigen in APS⁴². Exposure of pregnant mice to human aPL results in complement activation and elevated levels of TNF α at the maternal-fetal interface, which are key players in subsequent pregnancy failure^{13,43}. In order to better understand how aPL might impact human placental function, and thus pregnancy outcome, a number of *in vitro* studies have evaluated the effects of aPL on trophoblast cell function. These studies have shown, using either anti- β_2 GPI Abs or aPL Ab containing IgG, that these autoantibodies can inhibit trophoblast expression and secretion of human chorionic gonadotrophin; reduce trophoblast proliferation; decrease trophoblast fusion and differentiation; alter their expression of cell adhesion molecules; and limit trophoblast chemotaxis and invasiveness^{9,16–22}. While important, these studies have been limited by their use of term trophoblast cells or malignant choriocarcinoma cell lines and the lack of mechanistic analysis of the aPL Ab response.

In this study, we have characterized the effects of anti- β_2 GPI Abs on trophoblast function and survival and identified the molecular pathway involved. Initially, we tested the effects of two anti- β_2 GPI mAbs, ID2 and IIC5, on trophoblast survival and apoptosis. At high concentrations, the anti- β_2 GPI Abs caused trophoblast cell death through the induction of caspase-mediated apoptosis. In support of our findings, Orney *et al.* have shown that first trimester placental villi explants cultured in the serum of patients with SLE/APS and recurrent pregnancy loss exhibit increased levels of apoptosis⁴⁴, an observation confirmed by Bose *et al.* using first trimester placental explants cultures⁴⁵. Notably, ID2 activated significantly more caspase-3 but less caspase-8 and caspase-9, when compared to IIC5. Since caspase-8 and caspase-9 are upstream of caspase-3, these findings suggest that kinetics of the apoptotic response may differ between these antibodies, with ID2 activating the caspase pathway more rapidly than IIC5. This is further supported by the observation that ID2 has slightly more detrimental effects on trophoblast cell viability than IIC5. This suggests that these two anti- β_2 GPI Abs may recognize different, or perhaps overlapping, epitopes²². Indeed small differences in antibody fine specificity can result in diverse biological effects^{46,47}.

Since lower concentrations of the anti- β_2 GPI Abs had no effect on trophoblast cell death, and even seemed to promote proliferation, we next investigated their effects on trophoblast function. It is known that trophoblast cells constitutively produce chemokines that are important for crosstalk with local immune cells⁴⁸, and that in the presence of bacterial and viral components trophoblast generate an inflammatory cytokine/chemokine response^{26,29,48}. Therefore, we explored if anti- β_2 GPI Abs affect the cytokine/chemokine expression profile of placental cells. Indeed, lower concentrations of the anti- β_2 GPI Abs significantly increased IL-8, MCP-1, IL-1 β and GRO α secretion by first trimester trophoblast cells. This response is not a result of LPS contamination of the antibody preparation, as very high doses of LPS (50–100 μ g/ml) are required to induce an inflammatory response in the trophoblast⁴⁸. Moreover,

the cytokine/chemokine profile triggered by LPS is very different from that induced by anti- β_2 GPI Abs²⁹.

When effects of polyclonal IgG, derived from patients with different clinical manifestations of APS, were tested on trophoblast cells, we found the response was similar to that observed with the monoclonal anti- β_2 GPI Abs. Polyclonal APS-IgG from patients with and without a history of pregnancy mortality significantly increased trophoblast cell secretion of IL-8, GRO- α , and IL-1 β . Furthermore, this inflammatory response was more pronounced upon incubation with polyclonal APS-IgG purified from patients with a history of pregnancy morbidity. In particular, the strong increase in IL-8 secretion may account for the increased recruitment of inflammatory immune cells, such as neutrophils, at the maternal-fetal interface⁴⁸. In agreement, in both clinical samples from women with APS and experimental models of APS-complicated pregnancies, the maternal-fetal interface is characterized by a neutrophil infiltrate^{13,15,43,49}. Having found anti- β_2 GPI Abs perturb the cytokine/chemokine profile of placental cells, we next investigated the underlying signaling mechanisms. In a recent study using monocytes, Sorice *et al.* demonstrated that β_2 GPI recruits the innate immune receptor TLR4 in the presence of anti- β_2 GPI Abs³³. This finding suggests molecular mimicry between β_2 GPI and bacterial components that can be potentiated by aPL⁵⁰. We and others have shown that trophoblast cells express functional TLR4 and its signaling adapter protein MyD88^{34,51}. Using dominant negative mutants for TLR4 and MyD88, we demonstrate that the inflammatory trophoblast response triggered by anti- β_2 GPI Abs is dependent upon activation of the TLR4/MyD88 pathway. This finding is in keeping with studies in endothelial cells, in which anti- β_2 GPI Abs activate NF κ B via MyD88⁵². In addition, in a mouse model of aPL Ab-induced thrombosis, TLR4 was shown to play a role in thrombus formation and endothelial damage⁵³.

Our next objective was to establish if the anti- β_2 GPI Ab-dependent cytokine/chemokine response, characterized by induction of IL-8, MCP-1, GRO- α , and IL-1 β , plays a role in subsequent trophoblast cell death. The pro-inflammatory cytokine, IL-1 β , has recently been shown to induce trophoblast apoptosis³⁶, raising the possibility that the anti- β_2 GPI Abs-induced inflammatory response induces trophoblast cell death in an autocrine/paracrine fashion. To test this hypothesis, trophoblast cells were exposed to the conditioned media from cells treated with or without the anti- β_2 GPI Abs. The conditioned media from anti- β_2 GPI Ab-treated trophoblast cells, which contains higher levels of IL-1 β , significantly induced more trophoblast cell death and apoptosis than the conditioned media from unstimulated trophoblast cells. These results are indicative of an indirect effect of a soluble factor, rather than direct effect of any residual antibodies in the conditioned media. Although anti- β_2 GPI Ab were likely still present in the trophoblast conditioned media, they were now diluted to a concentration, at best 10 μ g/ml, insufficient to elicit a cell death response. Moreover, the anti- β_2 GPI Ab-induced trophoblast cell death was inhibited in the presence of the MyD88-DN, the adapter protein that is used by both TLR4 and IL-1R⁵⁴. Therefore, TLR4/MyD88-mediated induction of IL-1 β likely contributes to enhanced trophoblast cell death in APS. Indeed, IL-1 β has been shown to induce beta cell apoptosis via MyD88⁵⁵.

Since women with APS are at high risk for pregnancy loss and late gestational complications, heparin is administered with the intention to block the pro-thrombotic events associated with aPL. However, heparin is also known to have anti-inflammatory properties^{30–32} and reportedly binds directly to β_2 GPI, thereby antagonizing β_2 GPI-phospholipid interactions⁵⁶. This study further confirms that heparin blocks the anti- β_2 GPI Ab-induced trophoblast cell death, in agreement with others⁴⁵. Indeed, we have recently reported that heparin inhibits first trimester trophoblast apoptosis triggered by the cytokines TNF α and IFN γ , as well as the TLR-2 agonist, peptidoglycan, by activating pro-survival pathways²⁸. We now also show that heparin inhibits the effects of these antibodies on the trophoblast cytokine/chemokine response, albeit only at

a concentration much higher than needed for cytoprotection. Since Girardi *et al.*, found *in vivo* that heparin did not alter the binding of aPL to the trophoblast³¹, our findings suggest an anti-inflammatory role for heparin. Indeed, a recent study suggests that heparin can bind TLR4 and block dendritic cell activation⁵⁷. Thus, while the anti-inflammatory properties of heparin may contribute to improved pregnancy outcome in pregnant women with APS, our observations also raise the possibility that its efficacy could be enhanced by simultaneously and specifically disabling the TLR4/MyD88 pathway.

In summary, we have found that anti- β_2 GPI Abs modulate human first trimester trophoblast cytokine/chemokine production, a response mediated by the TLR4/MyD88 pathway. Our findings suggest that early pregnancy loss and late obstetric complications in APS may arise from anti- β_2 GPI Abs acting on first trimester trophoblast cells, thereby triggering placental inflammation and cell death.

Abbreviations

aPL, antiphospholipid antibody; APS, antiphospholipid syndrome; β_2 GPI, Beta₂-Glycoprotein I; CPT, camptothecin; NT, no treatment; PM, pregnancy morbidity; SLE, systemic lupus erythematosus; TLR, Toll-like receptor; VT, venous thrombosis.

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Figure 1. Expression of β_2 GPI by first trimester trophoblast cells

A) Lysates of the first trimester trophoblast cells (HTR8) and, as a positive control, placental tissue, were analyzed for β_2 GPI expression by Western blot. The trophoblast cells were positive β_2 GPI.

B) First trimester trophoblast cells (HTR8) were assessed for ID2 and IIC5 binding by immunocytochemistry. Cells were incubated with ID2 or IIC5 (40 μ g/ml) followed by a peroxidase-conjugated horse anti-mouse antibody (magnification $\times 60$). Note the strong positive immunoreactivity of the trophoblast cells for ID2 and IIC5, localized to both the cytoplasm and plasma membrane. Control cells (Neg; magnification $\times 40$) showed no staining.

Figure 2. Effects of anti-β₂GPI Abs on trophoblast cell viability and apoptosis

(A) First trimester trophoblast cells (HTR8) were incubated with ID2, IIC5 or the mouse IgG1 isotype control (IgG) at 0, 5, 10, 20 and 40 μg/ml for 48 hours. Cell viability was then determined using the CellTiter 96 assay. Line graph shows the percentage cell viability relative to the untreated control (0 μg/ml). Treatment with ID2 or IIC5 at the high dose of 40 μg/ml significantly reduced trophoblast cell viability when compared to the untreated control (**p* < 0.05, ***p* < 0.001). This figure is representative of at least three independent experiments performed in triplicate. Significance was determined using ANOVA.

(B) First trimester trophoblast cells (HTR8) were incubated with no treatment (NT); ID2 (40 μg/ml); IIC5 (40 μg/ml); mouse IgG (40 μg/ml), as a negative isotype control (IgG); or CPT, (4 μM) as a positive control for cell death, for 48 hours. Cell viability was then determined using the CellTiter 96 assay. Bar chart shows the percentage cell viability relative to the NT control **p* < 0.05, ***p* < 0.001. Significance was determined using ANOVA. Data are pooled from five individual experiments.

(C) First trimester trophoblast cells (HTR8) were incubated with NT, ID2 (40 μg/ml); IIC5 (40 μg/ml) or mIgG (IgG; 40 μg/ml) in the presence and absence of heparin (100 ng/ml) for 48 hours. Cell viability was then determined using the CellTiter 96 assay. Bar chart shows the percentage cell viability relative to the NT control unless otherwise indicated and significance was determined using ANOVA. The presence of heparin significantly reduced the amount of cell death induced by ID2 and IIC5 (**p* < 0.01, ***p* < 0.001), as determined using a paired *t*-test. Data are pooled from three individual experiments.

(D) Trophoblast cells (HTR8) were incubated with either no treatment (NT), ID2 (40 μ g/ml) or IIC5 (40 μ g/ml). After 48 hours, caspase-3; caspase-8; and caspase-9 activation was determined. Barchart shows caspase activity in relative light units (RLU) relative to the NT control (* p <0.001). Significance was determined using ANOVA. Data are pooled from three individual experiments.

Figure 3. Effects of anti- β_2 GPI Abs on trophoblast IL-8 secretion

Trophoblast cells (HTR8) were treated with ID2 and IIC5 (0 – 20 μ g/ml) or the mouse IgG isotype control (mIgG) (20 μ g/ml) for 72 hours after which cell-free supernatants were collected and analyzed for IL-8 by ELISA. (A) Line graph shows that both ID2 and IIC5 treatment upregulated trophoblast production of IL-8 in a dose dependent manner (* p <0.05, ** p <0.001). (B) Bar chart shows that while ID2 and IIC5 at 20 μ g/ml significantly increased trophoblast IL-8 secretion relative the no treatment (NT) control (* p <0.05, ** p <0.001), the mIgG had no effect. Significance was determined using ANOVA. (C) Trophoblast cells (HTR8) were incubated with NT, ID2 (20 μ g/ml) or IIC5 (20 μ g/ml) in the presence and absence of heparin (10 μ g/ml) for 72 hours after which cell-free supernatants were collected and analyzed for IL-8 by ELISA. Bar chart shows that while ID2 and IIC5 significantly increased trophoblast IL-8 secretion relative the no treatment (NT) control (* p <0.05; ** p <0.001), as determined using ANOVA, the presence of heparin significantly inhibited this response as determined by paired t -test (* p <0.05; ** p <0.001). Data are representative of at least three independent experiments.

Figure 4. Effects of anti- β_2 GPI Abs on trophoblast cytokine and chemokine production

(A) HTR8 cells and (B) primary trophoblast cells were treated with either no treatment (NT), ID2 (20 μ g/ml), IIC5 (20 μ g/ml) or mouse IgG1 (mIgG; 20 μ g/ml) for 48 hours after which cell-free supernatants were collected and analyzed by multiplex analysis. Barcharts show changes in the secretion of i) IL-8; ii) MCP-1; iii) GRO α ; and iv) IL-1 β relative to the NT control (* p <0.05; ** p <0.001). Data are representative of at least three independent experiments. Significance was determined using ANOVA.

Figure 5. Effects of a TLR4-DN on the modulation of trophoblast cytokine/chemokine production by anti-β₂GPI Abs

Trophoblast cells (HTR8) were transiently transfected to express the TLR4-DN. Following transfection with either the TLR4-DN or the vector control, trophoblast cells were treated with either no treatment (NT), ID2 (20μg/ml), or IIC5 (20μg/ml) for 48 hours after which cell-free supernatants were collected and analyzed by multiplex analysis. Barcharts show changes in the secretion of i) IL-8; ii) MCP-1; iii) GROα; and iv) IL-1β. * $p < 0.05$; ** $p < 0.001$ relative to the NT control was determined using ANOVA. Significant differences between the TLR4-DN and vector control were determined by paired t -test (* $p < 0.05$; ** $p < 0.001$). Data are a representative of at least three independent experiments.

Figure 6. Effects of a MyD88-DN on the modulation of trophoblast cytokine/chemokine production by anti-β₂GPI Abs

Trophoblast cells (HTR8) were transiently transfected to express the MyD88-DN. Following transfection with either the MyD88-DN or the vector control, trophoblast cells were treated with either no treatment (NT), ID2 (20 μg/ml), or IIC5 (20 μg/ml) for 48 hours after which cell-free supernatants were collected and analyzed by multiplex analysis. Barcharts show changes in the secretion of i) IL-8; ii) MCP-1; iii) GROα; and iv) IL-1β. * $p < 0.05$ and ** $p < 0.001$ relative to the NT was determined using ANOVA. Significant differences between the MyD88-DN and vector control were determined by paired t -test (* $p < 0.05$; ** $p < 0.001$). Data are a representative of at least three independent experiments.

Figure 7. Effects of anti- β_2 GPI Abs induced inflammation on trophoblast cell death

First trimester trophoblast cells (HTR8) were incubated with no treatment (NT) or the CM from trophoblast cells treated with NT (CM/NT), ID2 at 20 μ g/ml (CM/ID2) or IIC5 at 20 μ g/ml (CM/IIC5). After 96 hours, cell viability was determined using the CellTiter 96 assay and after 72 hours caspase-3 activity was determined using the caspase-Glo assay. Barcharts shows (A) the differences in trophoblast cell death; and (B) the differences in trophoblast caspase-3 activity, relative to the NT, as determined using ANOVA (* p < 0.05, ** p < 0.001). Statistical differences between the treatment groups was determined by a paired t-test (* p < 0.05, ** p < 0.001). Data are pooled from three independent experiments.

(C) Trophoblast cells (HTR8) were transiently transfected to express the MyD88-DN. Following transfection, trophoblast cells were treated with either no treatment (NT), ID2 (40 μ g/ml), or IIC5 (40 μ g/ml) for 48 hours, after which cell viability was determined using the CellTiter 96 assay. Barcharts show the differences in trophoblast cell death relative to the NT control. * p < 0.05 and ** p < 0.001 relative to the NT control was determined using ANOVA. Statistical differences between the MyD88-DN and vector control were determined by a paired t-test (* p < 0.05, ** p < 0.001). Data are pooled from three independent experiments.

Figure 8. Effects of patient derived aPL on trophoblast cytokine/chemokine production
Trophoblast cells were incubated with either no treatment (NT) or polyclonal IgG aPL (12.5μg/ml) from patients with PM⁺/VT⁻ (n=6); PM⁻/VT⁺ (n=6) or PM⁺/VT⁺ (n=6). After 72 hours, the supernatants were collected, pooled on the basis of patient group, and then evaluated by multiplex analysis. Bar charts show the effects of aPL on trophoblast secretion of i) IL-8; ii) MCP-1; iii) GROα; and iv) IL-1β after 72 hours. **p*<0.05 relative to the NT control, as determined using ANOVA.

Table 1

Summary of clinical data from patients with APS^a

Age	Ethnicity	Other Disease	Live Births	PM History	VT History	Clinical aCL	Clinical anti- β_2 GPI	Clinical LA	Lab [*] aCL (GPLU)	Lab [*] anti- β_2 GPI (% HCAL activity)	Other autoAbs	Treatments
VT1	46	Caucasian	0	-	5x TIA	+	NT	-	50	>50%	-	Warfarin; Diclofenac
VT2	39	Asian	0	-	CAPS; Pulmar thrombosis	-	+	+	>96	>50%	ANA ENA DNA	Aspirin; Alendronate; Prednisolone; Losartan; Atorvastatin; Morphine Sulphate; Oramorph
VT3	25	Caucasian	1	-	1x PE	-	+	-	85	>50%	ANA ENA DNA	Warfarin; Aspirin; Hydroxychloroquine; Calcium; Thyroxine
VT4	60	Caucasian	0	-	1x CVA	+	+	+	>96	>100%	-	Warfarin; Aspirin
VT5	72	Caucasian	2	-	1x DVT	+	+	+	90	>100%	ANA DNA	Warfarin
VT6	35	Caucasian	0	-	1x CVA	+	NT	+	70	>100%	ANA DNA	Warfarin; Azathioprine
PM1	51	Caucasian	1	1x 2 nd trimester PL; 1x death in utero due to rhesus incompatibility	-	+	+	+	>96	>100%	ANA	Aspirin
PM2	50	Caucasian	3	1x 2 nd trimester PL	-	+	+	+	>96	>100%	ANA DNA	Aspirin; Rituximab; Insulin
PM3	60	Caucasian	1	3x 2 nd trimester PL; 1x LB 36/52 weeks (Pre-eclampsia & IUGR)	-	+	NT	+	>90	>100%	-	Aspirin; Amlodipine; Enalapril
PM4	64	Caucasian	0	5x 1 st trimester PL; 1x 2 nd trimester PL	-	-	+	+	50	>100%	-	Aspirin; Hydroxychloroquine
PM5	45	Oriental	0	2x 2 nd trimester PL; 1x ectopic pregnancy	-	+	NT	+	<30	-	ANA ENA DNA	Prednisolone; Azathioprine
PM6	47	African-Caribbean	2	1x LB delivered at 5/12 months (low birth weight); 1x ectopic pregnancy	-	-	-	+	50	-	ANA ENA DNA	Hydroxychloroquine; Diclofenac
VT/PM1	51	Caucasian	2	1x LB at 32/52 weeks	1x PE	+	NT	+	>90	>100%	ANA	Warfarin; Aspirin; Nifedipine
VT/PM2	52	Caucasian	0	3x 1 st trimester PL; 1x 2 nd trimester PL	1x DVT	-	NT	+	90	100%	ANA	Warfarin; Telmisartan
VT/PM3	40	Caucasian	0	1x IUD 23/52 weeks due to placental thrombosis	1x CVA	+	NT	+	>96	>100%	-	Aspirin
VT/PM4	52	Caucasian	2	1x LB at 29/52 weeks (preeclampsia); 1x blighted ovum	1x PE; 2x CVA	+	+	+	>96	>100%	-	Warfarin

Age	Ethnicity	Other Disease	Live Births	PM History	VT History	Clinical aCL	Clinical anti- β_2 GPI	Clinical LA	Lab aCL (GPLU)	Lab anti- β_2 GPI (% HCAL activity)	Other autoAbs	Treatments
VT/PM5 34	Caucasian	-	0	1 $\times$ 1 st trimester PL, 2 $\times$ 2 nd trimester PL	1 $\times$ DVT	+	+	+	70	>100%	-	Warfarin
VT/PM6 51	Caucasian	-	3	1 $\times$ 2 nd trimester PL (twins)	3 $\times$ CVA; 1 $\times$ DVT; 1 $\times$ PE	+	NT	+	90	>100%	-	Warfarin

^a* Purified IgG at 500 μ g/ml was tested for anticardiolipin and anti- β_2 GPI activity using cardiolipin calibrators and HCAL, respectively. Values are shown in GPLU and as a percentage of HCAL activity. Serum lupus anticoagulant activity was measured using either dRVVT or aPPT. All positive ANA are $\geq$ 1/80.

Abbreviations: $\alpha\beta_2$ GPI: anti- β_2 GPI antibodies; aCL: anticardiolipin antibodies; ANA: antinuclear antibodies; CAPS: catastrophic APS; CNS: central nervous system; CVA: cerebrovascular accident; DNA: anti-DNA antibodies; DVT: deep vein thrombosis; ENA: extractable nuclear antigens; HT: hypertension; IDDM: insulin-dependent diabetes mellitus; IUD: intrauterine death; IUGR: intra-uterine growth restriction; PE: pulmonary embolism; PL: pregnancy loss PM: Pregnancy Mortality; ITP: idiopathic thrombocytopenic purpura; LA: lupus anticoagulant; LB: live birth; SLE: systemic lupus erythematosus; TIA: transient ischaemic attack; VT: vascular thrombosis.