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Role of NOD2 in antiphospholipid antibody-induced and bacterial MDP amplification of trophoblast inflammation

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ABSTRACT

Women with antiphospholipid antibodies (aPL) are at high risk for pregnancy complications, such as preeclampsia. We previously demonstrated that aPL recognizing β_2GPI promote an extravillous trophoblast pro-inflammatory, anti-migratory and anti-angiogenic profile similar to that seen in preeclampsia. Since preeclampsia in the absence of aPL may have an underlying infectious element, women with aPL may be at increased risk for preeclampsia or other adverse outcomes if an infection is present. Our objective was to determine the impact the common bacterial component, muramyl dipeptide (MDP), has on trophoblast responses to aPL. Herein, we report that bacterial MDP amplifies trophoblast IL-1β expression, processing, and secretion in the presence of aPL through activation of NOD2. In the absence of MDP, NOD2 also mediates anti- β_2GPI antibody-induced trophoblast IL-1β and VEGF secretion. Additionally, we report a role for extravillous trophoblast vimentin as a novel danger signal that contributes to the aPL-induced trophoblast IL-1β production. Together our data indicate that NOD2 mediates trophoblast inflammatory and angiogenic responses to aPL alone, and mediates trophoblast inflammation in the presence of bacterial MDP. These findings suggest that a bacterial infection at the maternal-fetal interface may exacerbate the impact aPL have on trophoblast inflammation and, thus, on pregnancy outcome.

1. Introduction

Obstetric antiphospholipid syndrome (APS) occurs in women with aPL that target the placental trophoblast and uterine endothelium [1]. Adverse outcomes such as recurrent pregnancy loss (RPL), preeclampsia or intrauterine growth restriction (IUGR) are common complications in women with aPL [2]. However, it is currently impossible to predict which pregnancies are destined for which adverse outcome. While systemic APS is a pro-thrombotic disorder, obstetric APS arises from inflammation at the maternal-fetal interface; and placental insufficiency associated with reduced trophoblast invasion and limited uterine spiral artery remodeling [3]. This pathology is caused by aPL recognizing beta$_2$ glycoprotein I (β_2GPI) and preferentially binding to the placental trophoblast early in pregnancy, leading to placental dysfunction and impaired placentation [1,4].

In vitro, aPL recognizing β_2GPI trigger human first trimester extravillous trophoblast cells to produce elevated levels of pro-inflammatory IL-1β and IL-8 [5,6]; inhibit spontaneous trophoblast migration [7]; increase trophoblast anti-angiogenic sEndoglin secretion [8]; and disrupt trophoblast-endothelial interactions in a model of spiral artery transformation [9]. Thus, aPL promotes an extravillous trophoblast pro-inflammatory, anti-migratory and anti-angiogenic profile similar to that seen in preeclampsia [10]; which may explain the decidual inflammation and impaired placentation seen in histological samples from aPL-positive patients [3]. The mechanisms by which this arises are, in part, through activation of innate immune signaling pathways. aPL induce the trophoblast to secrete IL-1β and IL-8 via activation of Toll-like receptor 4 (TLR4) [5,6]. Downstream of TLR4, IL-1β secretion is mediated by the induction of endogenous uric acid, which in turn activates the NOD-like receptor (NLR), NLRP3, leading to inflammasome activation,

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and subsequent IL-1β [5,6]. In parallel and downstream of TLR4, IL-8 secretion is mediated by the induction of miR-146a-3p, which in turn activates the RNA sensor, TLR8 [11].

While environmental exposures like infection may contribute to the origin of APS [12], infection may also contribute to the end-organ damage in these diseases. Indeed, such a scenario has been proposed for systemic APS where vascular thrombosis requires aPL and a second hit, such as bacterial LPS [1,13,14]. Furthermore, in addition to aPL, infection represents another risk factor for preeclampsia [15,16]. Bacterial and viral infections have been associated with preeclampsia [17,18]; and increase a woman's risk for preeclampsia by 2-fold [17]. Based on this, a woman with APS may be at increased risk for developing preeclampsia in the presence of an infection.

In this study we report that the peptidoglycan-derived peptide muramyl dipeptide (MDP), that is common to all bacteria, synergistically augmented human trophoblast IL-1β production in the presence of aPL through activation of the NLR, NOD2. Additionally we report a role for trophoblast vimentin as a novel danger signal and endogenous mediator of NOD2 activation in response to aPL that contributes to trophoblast IL-1β and VEGF production in the absence of an infection.

2. Materials and methods

2.1. Reagents

The NOD2 agonist, bacterial muramyl dipeptide (MDP) was purchased from Invivogen, and based on previous studies was used at a concentration 1 μg/ml [19].

2.2. Trophoblast cell lines

The human first trimester extravillous trophoblast telomerase-transformed cell line, Sw.71 [20], was used. As an extravillous trophoblast cell, they express the mesenchymal marker vimentin [20]; similarly to primary extravillous trophoblast cells [21–24]. Sw.71 cells were also stably transfected with the pDeNy plasmid containing a human NOD2-dominant negative (DN) (Invivogen).

2.3. Antiphospholipid antibodies

This study used the aPL, IIC5, which is a mouse IgG1 anti-human β_2GPI monoclonal antibody (mAb). Like patient-derived polyclonal aPL, IIC5 binds β_2GPI when immobilized on a negatively charged surface such as the phospholipids, cardiolipin, phosphatidyl serine or irradiated polystyrene, and thus behaves as both an anti-cardiolipin and anti-β_2GPI antibody (Ab) [25]. IIC5 reacts specifically with an epitope within domain V of β_2GPI [26]. Furthermore, IIC5 has pronounced lupus anticoagulant activity and thus is a “triple positive” aPL [27]. IIC5 is an appropriate model for human aPL since they share similar epitopes; IIC5 can block human polyclonal aPL binding to β_2GPI [28]. Moreover, IIC5 binds to human first trimester extravillous trophoblast [5,29], and alters their function in a similar fashion to patient-derived polyclonal aPL-IgG [6], and polyclonal IgG aPL recognizing β_2GPI [5,8]. Mouse IgG1 clone 107.3 (BD Biosciences) was used as an isotype control. Trophoblast were treated with aPL or the IgG isotype control at 20 μg/ml [5–8,11]. In addition, patient-derived total IgG containing aPL (aPL-IgG) was used at 500 μg/ml. This aPL-IgG was isolated from the sera of a patient with APS, which was characterized as having high-titer aPL (> 140 GPL U), thromboses, and/or pregnancy losses, with confirmed β_2GPI activity [6]. Normal human IgG negative for aPL served as a control (Sigma).

2.4. Western blot

Western blot analysis was performed as described [6]. Pro-IL-1β (31 kDa) was detected using the rabbit anti-human polyclonal antibody (#2022) from Cell Signaling Technology (Danvers, MA). NLRP3 was detected using the rabbit anti-human mAb (D2P5E) from Cell Signaling Technology. HSP90 was used as internal control and was detected using the mouse mAb (sc-13119) from Santa Cruz Biotechnology (Santa Cruz, CA). Images were recorded and semi-quantitative densitometry performed using the Gel Logic 100 and Kodak MI software (Eastman Kodak, Rochester, NY) and normalized to HSP90.

2.5. RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Trophoblast cell RNA was extracted using TRIzol and expression of IL1B mRNA was measured by qRT-PCR using the KAPA SYBR FAST qPCR kit (Kapa Biosystems, Wilmington, MA). GAPDH was used as an internal control.

2.6. Measurement of cytokines, angiogenic factors, uric acid, and caspase-1 activity

Trophoblast culture supernatants were analyzed for IL-1β, VEGF, PlGF, sEndoglin, and sFlt-1 using ELISA kits from R&D Systems (Minneapolis, MN); and for IL-8 using an ELISA kit from Enzo Life Sciences (Farmingdale, NY). For the measurement of uric acid, supernatants were analyzed using the QuantiChrom assay kit from BioAssay Systems (Hayward, CA). Caspase-1 activity was measured using the caspase-glo 1 inflammasome assay from Promega (Madison, WI).

2.7. Trophoblast migration

Trophoblast migration was measured using a two-chamber colorimetric assay from EMD Millipore (Billerica, MA) as described [7,11].

2.8. Statistical analysis

Each treatment experiment was performed at least three times. All analyses were performed in duplicate or triplicate. All data are reported as mean ± standard error of the mean (SEM) of pooled experiments. The number of independent experiments that data were pooled from are indicated in the figure legends as “n=”. Statistical significance was set at $p < 0.05$ and determined using Prism Software (Graphpad, Inc; La Jolla, CA). For normally distributed data, significance was determined using either one-way analysis of variance (ANOVA) for multiple comparisons or a *t*-test. For data not normally distributed, significance was determined using a non-parametric multiple comparison test for multiple comparisons or the wilcoxon matched-pairs signed rank test.

3. Results

3.1. Bacterial MDP synergistically augments aPL-induced trophoblast IL-1β secretion

As previously reported [5,8], treatment of trophoblast cells with aPL (anti-β_2GPI mAb) significantly increased the secretion of: (A) IL-1β; (B) IL-8; (C) PlGF; (D) VEGF; and (E) sEndoglin compared to the no treatment (NT) control, while the IgG control had no effect on these factors (Fig. 1). Also as previously reported [8], aPL did not significantly alter trophoblast sFlt-1 production (Fig. 1F).

Treatment of trophoblast with bacterial MDP alone had no effect on inflammatory IL-1β secretion under NT or IgG control conditions (Fig. 1A). However, MDP in combination with aPL significantly and synergistically augmented IL-1β secretion by 2.0 ± 0.3 fold when compared to aPL alone, and by 6.3 ± 1.1 fold when compared to the NT control (Fig. 1A). Treatment of trophoblast with bacterial MDP alone significantly increased inflammatory IL-8 secretion by 1.7 ± 0.3 fold compared to the NT control (Fig. 1B) as previously reported [19].

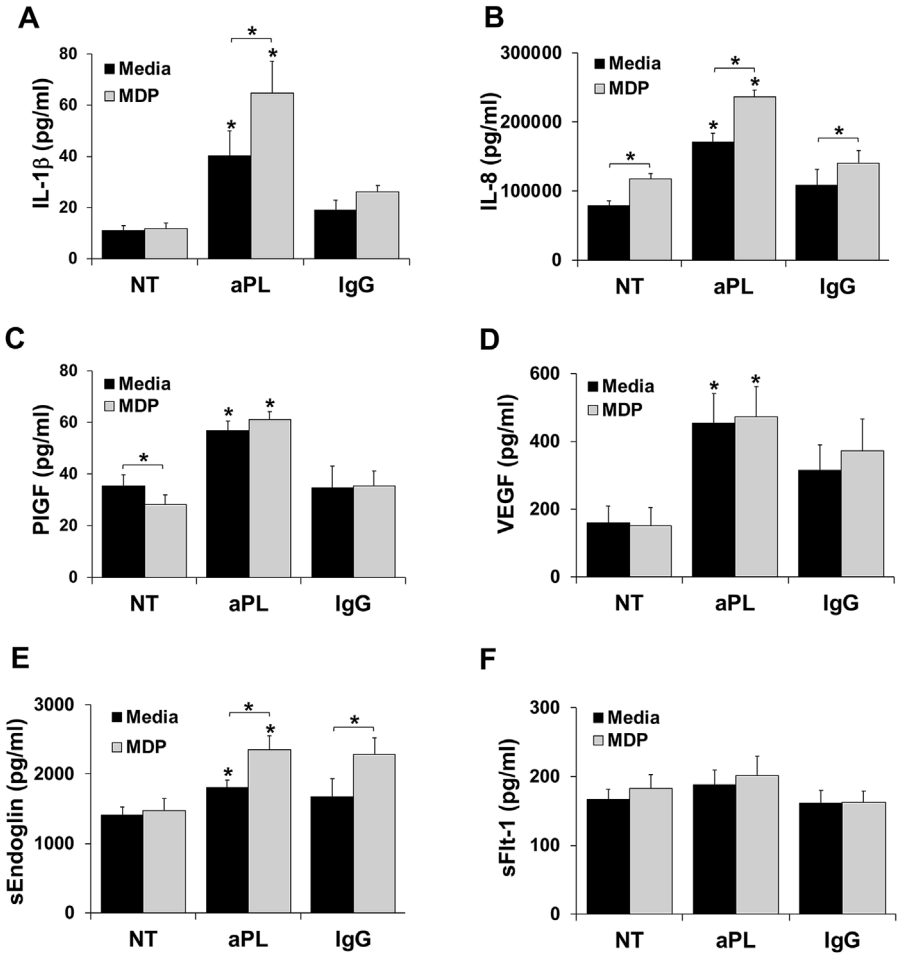

Fig. 1. Bacterial MDP synergistically augments anti-β2GPI mAb-induced trophoblast IL-1β. Sw.71 cells were treated with no treatment (NT), anti-β2GPI mAb (aPL) or mouse IgG control (IgG) in the presence of media or MDP (n = 9–11). After 72 h supernatants were collected and measured for: A) IL-1β; B) IL-8; C) PlGF; D) VEGF; E) sEndoglin; and F) sFlt-1. $p < 0.05$ relative to the NT control under each condition (media or MDP) unless otherwise indicated.

Similarly, in the presence of control IgG, MDP significantly increased IL-8 secretion by 1.5 ± 0.4 fold when compared to the NT control (Fig. 1B). MDP, in combination with aPL, also significantly augmented IL-8 secretion by 1.5 ± 0.2 fold when compared to aPL alone, and by 3.6 ± 0.6 fold when compared to the NT control (Fig. 1B). This augmentation was an additive effect of the combined MDP and aPL on trophoblast IL-8 secretion.

Bacterial MDP alone significantly reduced trophoblast pro-angiogenic PlGF secretion compared to the NT control (Fig. 1C), but had no effect on the secretion of pro-angiogenic VEGF (Fig. 1D). However MDP, in combination with either aPL or control IgG, had no effect on the secretion of (C) PlGF; or (D) VEGF (Fig. 1).

Treatment of trophoblast with bacterial MDP alone had no effect on anti-angiogenic (E) sEndoglin; or (F) sFlt-1 secretion compared to the NT control (Fig. 1). MDP in combination with aPL, significantly augmented sEndoglin secretion by 1.3 ± 0.1 fold compared to aPL alone; and by 1.7 ± 0.2 fold compared to the NT control (Fig. 1E). This indicted a synergistic effect. However, in the presence of control IgG, MDP also significantly augmented sEndoglin secretion by 1.6 ± 0.0 fold compared to IgG alone, indicating this was a non-specific effect (Fig. 1E). Bacterial MDP in combination with aPL or control IgG did not significantly alter trophoblast sFlt-1 secretion (Fig. 1F).

As previously reported [7], treatment of trophoblast with aPL significantly reduced their ability to spontaneously migrate by 44.2 ± 7.4% compared to the NT and IgG controls. MDP, either alone or in combination with aPL or control IgG, had no effect on trophoblast migration (data not shown).

As shown in Fig. 2, our findings were validated using patient-derived aPL-IgG with β_2GPI activity. Similar to previous studies using either aPL-IgG [6] or purified patient-derived anti-β_2GPI aPL [5], treatment of trophoblast cells with aPL-IgG significantly increased the secretion of IL-1β by 2.3 ± 0.6 fold compared to the no treatment (NT) control, while the human IgG control had no effect (Fig. 2A). MDP in combination with aPL significantly and synergistically augmented IL-1β secretion by 1.4 ± 0.2 fold when compared to aPL alone, and by 3.21 ± 0.8 fold when compared to the NT control, supporting our data using the anti-β_2GPI mAb (Fig. 2A). Also, similar to previous studies using purified patient-derived anti-β_2GPI aPL [5], aPL-IgG, but not human control IgG, significantly increased trophoblast secretion of (E) sEndoglin and (F) sFlt-1, while (B) IL-8, (C) PlGF and (D) VEGF were not altered (Fig. 2). Furthermore, similarly to our findings using the anti-β_2GPI mAb aPL, MDP had no specific or significant effect on trophoblast production of these factors under aPL-IgG conditions (Fig. 2). Thus, MDP specifically augmented trophoblast aPL-induced IL-1β secretion.

3.2. Bacterial MDP does not modulate endothelial responses to aPL

To see whether the effects of bacterial MDP on trophoblast responses to aPL were unique to this cell type, we examined another cell found at the maternal-fetal interface. Human endometrial endothelial cells (HEECs) can also be a target for aPL and generate a response that is distinct from the trophoblast [30]. aPL increased HEEC secretion of anti-angiogenic sFlt-1 and pro-angiogenic VEGF and PlGF; while

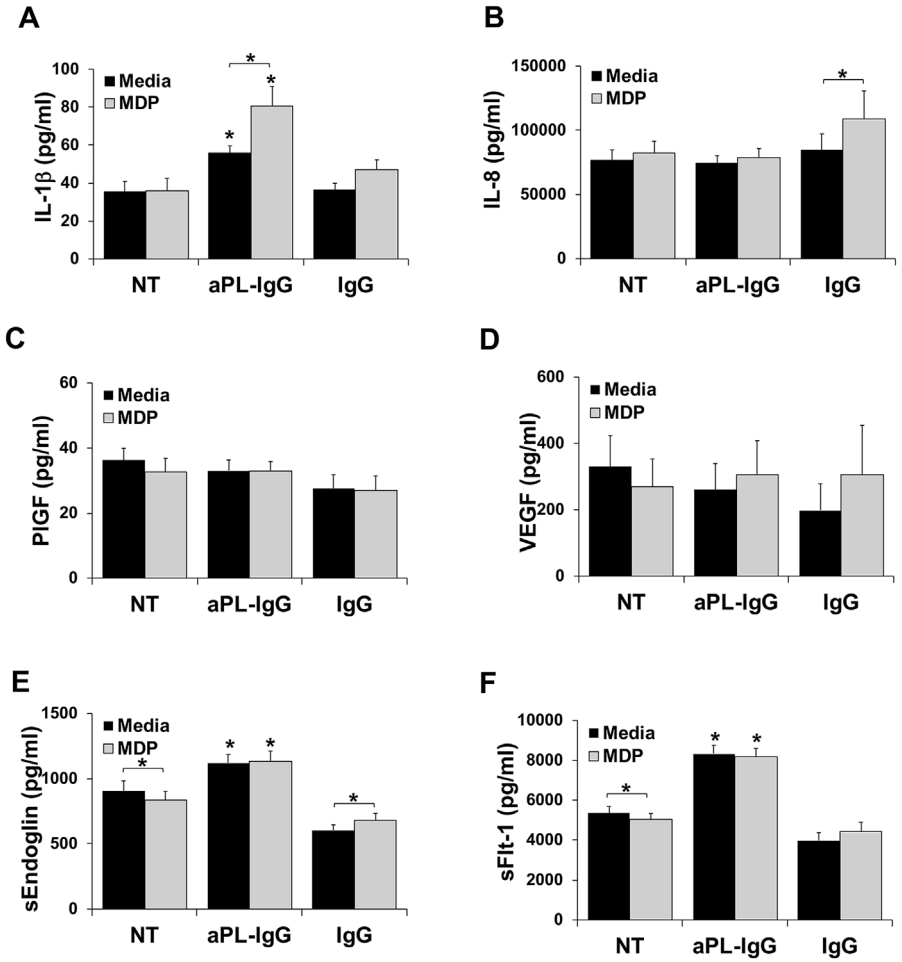

Fig. 2. Bacterial MDP synergistically augments patient aPL-IgG-induced trophoblast IL-1β. Sw.71 cells were treated with no treatment (NT), aPL-IgG or human IgG control (IgG) in the presence of media or MDP (n = 8). After 96h supernatants were collected and measured for: A) IL-1β; B) IL-8; C) PlGF; D) VEGF; E) sEndoglin; and F) sFlt-1. $p < 0.05$ relative to the NT control under each condition (media or MDP) unless otherwise indicated.

inhibiting basal secretion of the chemokines MCP-1, G-CSF and GRO-α (Supplemental Fig. 1) [30]. Bacterial MDP alone significantly increased HEEC secretion of (B) IL-8; and (D) GRO-α, confirming functional NOD2 in these cells [31] (Supplemental Fig. 1). However, MDP had no effect on the ability of aPL to inhibit HEEC secretion of (C) MCP-1, (D) GRO-α, or (E) G-CSF; nor did MDP alter the ability of aPL to upregulate HEEC secretion of (F) sFlt-1, (H) PlGF or (I) VEGF (Supplemental Fig. 1). Furthermore, MDP had no effect on HEEC (A) IL-1β, (B) IL-8, or (G) sEndoglin in the presence of aPL (Supplemental Fig. 1).

3.3. Bacterial MDP augments trophoblast pro-IL-1β expression under aPL conditions

Having determined that bacterial MDP synergistically augmented aPL-induced IL-1β secretion by the trophoblast, we sought to determine which upstream mediators of this response were involved. We previously reported that aPL induce trophoblast IL-1β secretion through the TLR4-mediated induction of endogenous uric acid, which in turn activates the NLRP3 inflammasome to process basal expressed pro-IL-1β into its active form [6]. As shown in Fig. 3, and as previously reported [6], aPL alone significantly increased trophoblast production of (A) uric acid, and (B) caspase-1 activity compared to the NT control, while the IgG control had no effect. MDP, either alone or in combination with aPL, had no effect on trophoblast (A) uric acid production or (B) caspase-1 activity (Fig. 3). MDP did also not alter trophoblast uric acid production or caspase-1 activity in the presence of patient-derived aPL-IgG (data not shown).

Since, combination MDP and aPL did not affect uric acid which activates NLRP3, and caspase-1 which mediates IL-1β processing, we next evaluated the expression of NLRP3, which mediates caspase-1 activity by assembling the inflammasome components; and expression of pro-IL-1β which feeds the inflammasome pathway. As shown in Fig. 3C, aPL significantly increased trophoblast NLRP3 by 1.2 ± 0.1 fold compared to the NT control, while MDP alone had no effect. Combination MDP and aPL significantly decreased NLRP3 expression compared to aPL alone by 32.8 ± 3.5%, (Fig. 3C).

As shown in Fig. 3D, and as previously reported [6], trophoblast cells expressed high levels of pro-IL-1β under no treatment (NT) conditions, and MDP treatment did not affect this. aPL significantly reduced trophoblast expression of pro-IL-1β by 59.8 ± 14.5% compared to the NT control, demonstrating processing into active secreted IL-1β [6]. However, in the presence of aPL and MDP, trophoblast expression of pro-IL-1β was reduced by only 31.9 ± 11.2% when compared to the NT control, and was significantly 1.7 ± 0.2 fold higher than under aPL alone conditions (Fig. 3D). We also found this to be the case using patient-derived aPL-IgG. As shown in Fig. 3E, aPL-IgG reduced trophoblast expression of pro-IL-1β by 34.3 ± 13.4% compared to the NT control. However, in the presence of aPL and MDP, trophoblast expression of pro-IL-1β was a similar level to the NT control, and was significantly 1.7 ± 0.7 fold higher than under aPL alone conditions. This data suggests that both induction of pro-IL-1β expression and elevated processing may be occurring under combination MDP and aPL conditions. To further confirm this, we evaluated IL1B mRNA levels. As shown in Fig. 3F, aPL alone significantly increased trophoblast IL1B

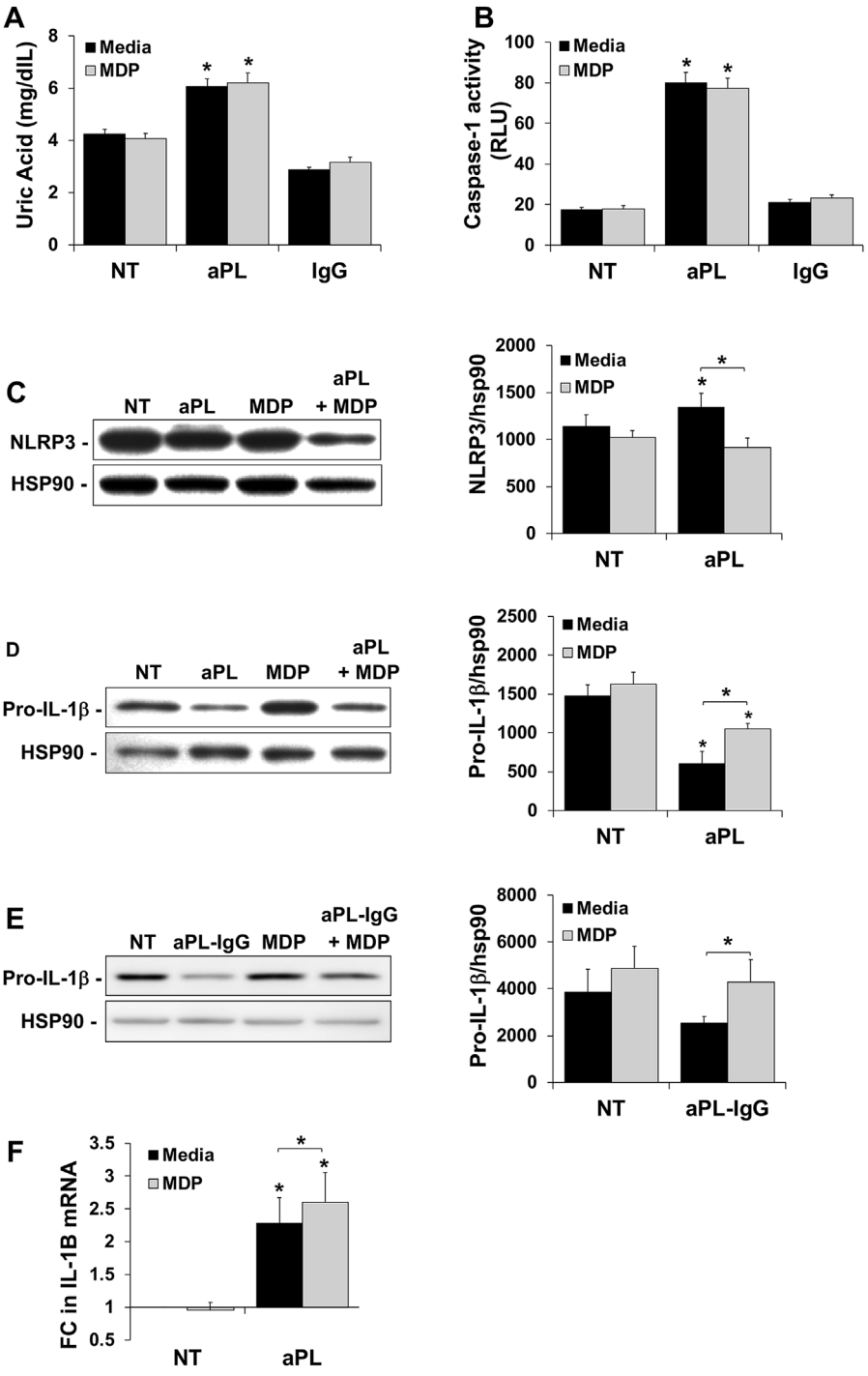

Fig. 3. MDP augments trophoblast pro-IL-1β expression under aPL conditions. Sw.71 cells were treated with no treatment (NT), anti-β2GPI mAb (aPL), human-derived aPL-IgG, or IgG control (IgG) in the presence of media or MDP. After 48 h RNA was collected and after 72–96 h cell-free supernatants and cell lysates were collected. Supernatants were measured for: A) uric acid (n = 10); and B) caspase-1 activity (n = 9). Cell lysates were evaluated for C) NLRP3; and D-E) pro-IL-1β by Western blot. Blots are from one representative experiment. Bar charts show C) NLRP3; and D-E) pro-IL-1β levels as determined by densitometry after normalization to HSP90 (n = 3–4). F) RNA was measured for IL1B mRNA levels by qRT-PCR (n = 7). FC = fold change. $p < 0.05$ relative to the NT control under each condition (media or MDP) unless otherwise indicated.

mRNA levels by 2.3 ± 0.4 fold compared to the NT control, while MDP alone had no effect on basal IL1B mRNA. MDP in combination with aPL significantly augmented trophoblast IL1B mRNA by 1.1 ± 0.0 fold compared to aPL alone and by 2.6 ± 0.5 fold compared to the NT control (Fig. 3F).

3.4. aPL-induced trophoblast IL-1β in the presence and absence of bacterial MDP is mediated by NOD2

To further understand the mechanism by which bacterial MDP augmented trophoblast aPL-induced expression and processing of IL-1β, leading to its augmented secretion, the role of the MDP sensor NOD2 [19] was examined. For this, wildtype trophoblast and trophoblast stably expressing a NOD2-DN were compared. As shown in Fig. 4A, the reduction in basal trophoblast pro-IL-1β expression under combination MDP and aPL conditions was significantly reversed by 1.7 ± 0.5 fold in the presence of the NOD2-DN to near basal levels. Similarly, IL-1β secretion in response to combination MDP and aPL was significantly reduced by 53.2 ± 17.5% by the presence of the NOD2-DN (Fig. 4B). Interestingly, in the absence of MDP, the NOD2-DN significantly reduced the ability of aPL alone to induce trophoblast IL-1β secretion by 46.9 ± 17.9%, indicating that aPL could itself lead to the NOD2 activation (Fig. 4B). This dependency on NOD2 was not seen at the pro-IL-1β level (Fig. 4A).

The induction of uric acid under aPL conditions, either with or without MDP, was not significantly different between the wildtype and

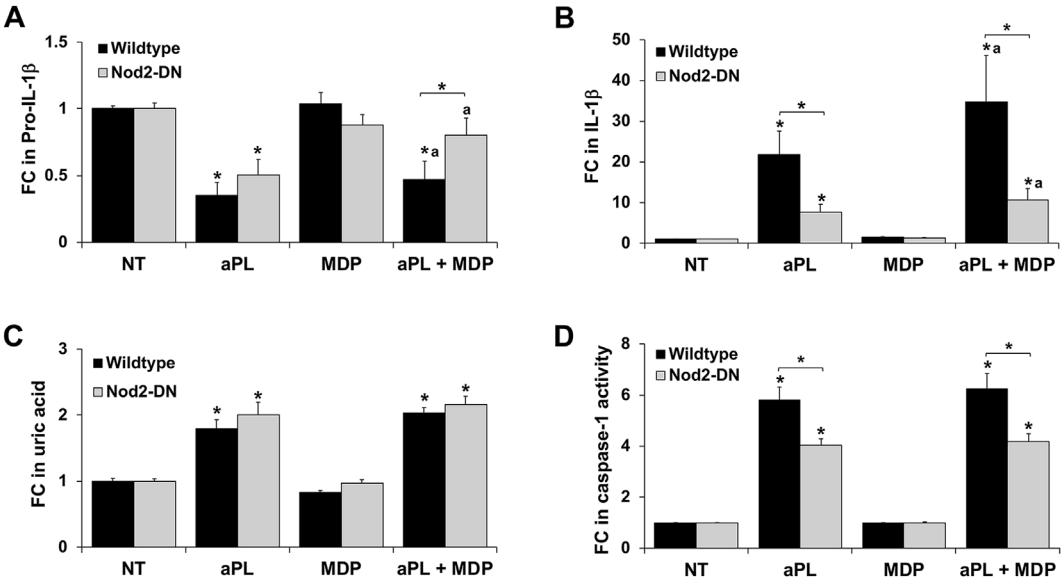

Fig. 4. MDP augments aPL-induced trophoblast IL-1β via NOD2 activation. Wildtype Sw.71 and Sw.71 cells expressing a NOD2-DN were treated with no treatment (NT), aPL, MDP, or aPL + MDP (n = 5). After 72 h supernatants and cell lysates were collected. A) Cell lysates were evaluated for pro-IL-1β by Western blot and densitometry. Supernatants were measured for: B) IL-1β; C) uric acid; and D) caspase-1 activity. $^{*}p < 0.05$ relative to the NT control for each cell (wildtype or NOD2-DN), and $^{a}p < 0.05$ relative to aPL for each cell (wildtype or NOD2-DN), unless otherwise indicated. FC = fold change.

NOD2-DN trophoblast (Fig. 4C). However, similarly to IL-1β secretion, caspase-1 activity in response to combination MDP and aPL was significantly reduced by 26.2 ± 11.4% by the presence of the NOD2-DN (Fig. 4D). Furthermore, in the absence of MDP, the NOD2-DN significantly reduced the ability of aPL alone to induce trophoblast caspase-1 activity by 24.4 ± 11.9% (Fig. 4D). This indicates that caspase-1 activity is regulated by aPL via NOD2, and not by combination MDP and aPL, further supporting the data in Fig. 3B.

3.5. aPL-induced trophoblast VEGF is dependent on NOD2 activation

Having found a role for NOD2 in mediating aPL-induced trophoblast IL-1β secretion, as well as mediating the MDP augmentation of aPL-induced IL-1β, we examined whether NOD2 mediated any other aPL-associated responses. Wildtype trophoblast responded to MDP alone by secreting significantly 1.5 ± 0.2 fold more IL-8 compared to the no treatment (NT) control, and this was significantly and completely inhibited by the presence of the NOD2-DN (Fig. 5A). In the absence of MDP, aPL-induced IL-8 secretion was significantly augmented by 1.2 ± 0.1 fold by the presence of the NOD2-DN when compared to the wildtype cells (Fig. 5A). However, the presence of the NOD2-DN significantly, but partially, inhibited IL-8 secretion in response to combination MDP and aPL by 23.2 ± 11.9%, indicating that the contribution of NOD2 was in response to the MDP only (Fig. 5A).

As shown in Fig. 5B, the presence of the NOD2-DN had no effect on sEndoglin secretion under NT or aPL conditions when compared to the wildtype cells. However, the presence of the NOD2-DN significantly increased sEndoglin in response to MDP alone; and significantly inhibited combination MDP and aPL-induced sEndoglin secretion (Fig. 5B).

As shown in Fig. 5C MDP alone significantly reduced trophoblast PlGF secretion by and this was significantly reversed by the presence of the NOD2-DN. The induction of PlGF under aPL conditions, either with or without MDP, was not significantly different between the wildtype and NOD2-DN trophoblast cells (Fig. 5C). While the presence of the NOD2-DN had no effect on VEGF secretion under NT and MDP conditions when compared to the wildtype cells, the NOD2-DN significantly inhibited combination MDP and aPL induced sEndoglin secretion by 28.7 ± 9.4% (Fig. 5D). Furthermore, when compared to the wildtype cells, the NOD2-DN significantly inhibited aPL induced sEndoglin secretion in the absence of MDP by 32.3 ± 8.0%, suggesting that aPL-induced VEGF is in part NOD2-mediated (Fig. 5D).

3.6. aPL activate trophoblast NOD2 through the novel danger signal, vimentin

Vimentin, a major intermediate filament protein, has recently been shown to be involved in host responses to bacteria having inflammatory properties [32]. In particular vimentin has been reported as acting like a danger signal or damage associated molecular pattern (DAMP) by interacting with, and activating, cytosolic NOD2 at the cell surface [33]. Since we found, in the absence of MDP, that aPL-induced trophoblast inflammatory IL-1β and pro-angiogenic VEGF secretion are partly mediated by NOD2, we postulated that vimentin may be an intermediate in this pathway. To test this, trophoblast were treated with withaferin-A (WFA), a plant steroidal lactone that inhibits vimentin-NOD2 interactions [33]. As shown in Fig. 6A, the presence of WFA significantly inhibited trophoblast IL-1β secretion under NT and aPL conditions by 11.8 ± 5.6% and 31.0 ± 5.8%, respectively. The presence of WFA had no effect on trophoblast (B) pro-IL-1β expression; (C) uric acid production; or (D) caspase-1 activity in response to aPL or under NT conditions (Fig. 6). The presence of WFA also had no inhibitory effect on trophoblast VEGF secretion in response to aPL on under NT conditions, but did slightly and significantly augment aPL-induced VEGF (Fig. 6E).

4. Discussion

Obstetric APS includes RPL, and later gestational complications such as preeclampsia and IUGR. However, not all patients with aPL will have an adverse outcome [34], and if they do exhibit a pregnancy complication, it is currently impossible to predict which that will be. This is particularly relevant to later gestational complications such as preeclampsia, since early treatment with heparin, alone or in combination with low dose aspirin, has only been fully characterized in its ability to increase the live birth rate for women with aPL [34]. Since infection represents another risk factor associated with preeclampsia [17,18], this study sought to test the hypothesis that a woman with APS

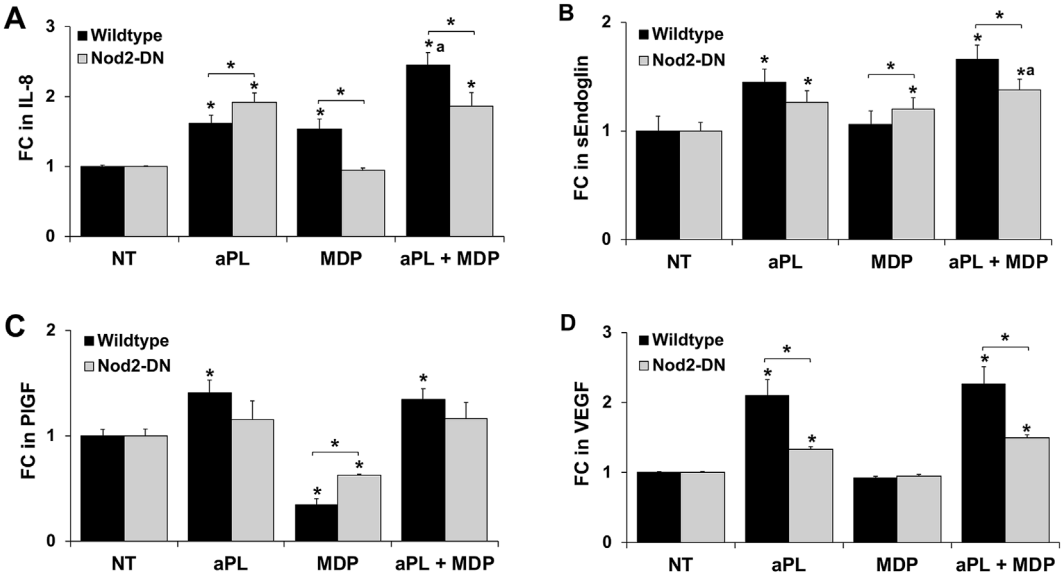

Fig. 5. aPL-induced trophoblast VEGF is dependent on NOD2 activation. Wildtype Sw.71 and Sw.71 cells expressing a NOD2-DN were treated with no treatment (NT), aPL, MDP, or aPL + MDP (n = 4–5). After 72 h supernatants were collected and measured for: A) IL-8; B) sEndoglin; C) PlGF; and D) VEGF. $^{*}p < 0.05$ relative to the NT control for each cell (wildtype or NOD2-DN), and $^{a}p < 0.05$ relative to aPL for each cell (wildtype or NOD2-DN), unless otherwise indicated. FC = fold change.

may be at increased risk for developing preeclampsia in the presence of an infection. To test this, we employed an *in vitro* model in which aPL recognizing β_2GPI induces a pro-inflammatory, anti-migratory and anti-angiogenic profile in human extravillous trophoblast cells [5–9]; a profile also common to preeclampsia [10]. Herein, we report that the common bacterial component, MDP, synergistically augmented aPL-induced trophoblast IL-1β production through NOD2 activation. Additionally, we report for the first time, a role for extravillous trophoblast vimentin as a novel danger signal and endogenous mediator of NOD2 activation in response to aPL, that contributes to the trophoblast inflammatory and angiogenic profile in the absence of an infection.

An infection or microbiome may be present locally at the maternal-fetal interface [35,36], or may disseminate to the placenta from a distant site [37,38]. The concept that an infection may contribute to aPL-induced end-organ damage has been described in models of systemic APS where bacterial LPS primes the endothelium for aPL-triggered vascular thrombosis [1,13,14]. This second hit from an infectious component has been reported as facilitating β_2GPI binding to systemic endothelial cells, and thus facilitating aPL binding [1,13,14]; whereas such priming is not needed for uterine and placental localization of β_2GPI [1]. Thus, while our studies further support the concept that an infection contributes to aPL-induced pathology, the mechanism by which an infection may modulate the placental trophoblast to aPL may be different from systemic events.

aPL induce human first trimester extravillous trophoblast to secrete IL-1β and IL-8 via activation of TLR4 [5,6], most likely because β_2GPI, which shares molecular mimicry with LPS, provides the bridge [12,39]. This aPL-induced extravillous trophoblast pro-inflammatory response may explain the decidual inflammation seen in histological samples from aPL-positive patients [3]. To our knowledge there have been no studies that have directly examined the production of inflammatory mediators by trophoblast cells in pregnant women with aPL. This would be particularly difficult to study since the extravillous trophoblast invades into the decidua and are, therefore, not accessible to researchers unless a placental bed biopsy is taken. However, the production of increased inflammatory mediators by extravillous trophoblasts in response to aPL is consistent with the increased numbers of inflammatory cells reported to be present in the placental bed and superficial deciduae of women with aPL [3,40,41]. Indeed inflammation of the decidua is one of the five key features that comprise the histopathological fingerprint of aPL in pregnant women [3].

Downstream of TLR4, aPL-induced trophoblast IL-1β secretion is mediated by the induction of endogenous uric acid, which in turn activates the NLRP3 inflammasome and subsequent IL-1β processing [5,6]. In this current study we report that bacterial MDP synergistically augmented aPL-induced trophoblast IL-1β secretion via NOD2 activation, and this augmentation was mediated upstream of uric acid production and inflammasome/caspase-1 activity. Such synergism between the NOD2 and TLR4 signaling pathways, leading to augmented IL-1β and other cytokines has been reported in immune cells [42–44], and we previously demonstrated that TLR4 activation increases trophoblast NOD2 expression [45].

To further understand how this synergism between MDP and aPL may be regulated in the trophoblast, we investigated upstream of the inflammasome. Our data indicates that, in combination with aPL, MDP through NOD2, upregulates pro-IL-1β expression at both the mRNA and protein level; which feeds the inflammasome pathway. This is in keeping with a study showing a role for NOD2 in mediating the induction of pro-IL-1β [46]. A reduction in NLRP3 expression under combination aPL and MDP conditions may be due to proteasomal degradation of NLRP3 acting as a compensatory mechanism in response to the augmented IL-1β production, in order to control inflammasome activity [47]. This synergistic augmentation of the aPL-driven inflammatory response by MDP was specific to IL-1β since the augmented IL-8 production was an additive effect. Furthermore, MDP had no specific effects on the trophoblast pro-angiogenic, anti-angiogenic, or anti-migratory responses to aPL. While MDP synergistically augmented aPL-induced sEndoglin secretion, also in a NOD2-dependent manner, this response was non-specific as sEndoglin was also augmented under MDP and control IgG conditions.

In studying the role of NOD2 in the ability of MDP to modulate trophoblast responses to aPL, we made some unexpected discoveries. We found that NOD2 activation by MDP alone reduced basal secretion of PlGF. More surprisingly, we found that aPL, in the absence of MDP, induced trophoblast IL-1β and VEGF secretion, in part through activation of NOD2. Our current understanding, thus far, has been that aPL induce trophoblast IL-1β secretion though activation of the TLR4 and subsequent inflammasome pathways [5,6]; while elevated VEGF secretion is TLR4-independent and the signaling pathway regulating this response is currently unknown [8]. What our findings indicate is that

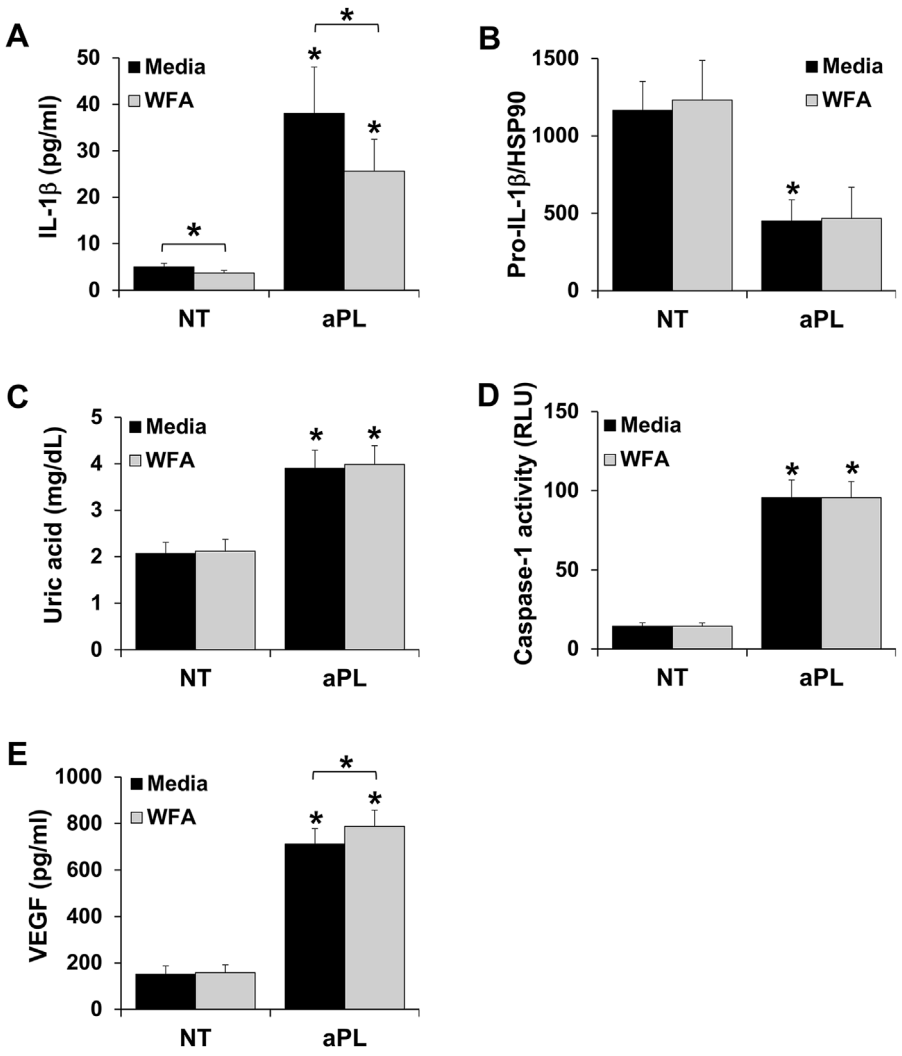

Fig. 6. The vimentin inhibitor WFA reduces aPL-induced trophoblast IL-1β secretion. Sw.71 cells were treated with no treatment (NT) or aPL in the presence of media or WFA (n = 8). After 72 h supernatants and cell lysates were collected and measured for: A) IL-1β secretion; B) pro-IL-1β expression; C) uric acid production; D) caspase-1 activity; and E) VEGF secretion. $p < 0.05$ relative to the NT control under each condition (media or WFA) unless otherwise indicated.

multiple innate immune pathways can be simultaneously activated in the trophoblast by aPL, thus contributing to the same and different responses; and NOD2 signaling is one of these pathways. Furthermore, the contribution of NOD2 to the aPL-induced IL-1β would explain why inhibition of TLR4 did not completely inhibit this aPL-driven response [5,6].

NOD2 is able to contribute to IL-1β production is a number of ways. NOD2 can directly interact with and activate caspase-1 [46,48]. NOD2 can complex with either NLRP1 [48] or NLRP3 [49], thus contributing to inflammasome function. Lastly, NOD2 can crosstalk with TLR4 signaling to regulate inflammation [50]. For example, in bone marrow derived macrophages, NOD2 signaling has been found to mostly limit TLR4-induced inflammation [50], and this could explain why the presence of the NOD2-DN augmented trophoblast aPL-induced IL-8 secretion. However, as already discussed, some TLR4-mediated inflammatory responses can be augmented by NOD2 [42–44,50]. Combination MDP and aPL did not alter the levels of caspase-1 activity when compared to aPL alone, but inhibition of NOD2 signaling similarly inhibited aPL-induced caspase-1 activity in both the presence or absence of MDP. Therefore, this indicated that in response to aPL alone, NOD2 is able to directly trigger caspase-1 activation. This is in contrast to the mechanism by which MDP amplifies aPL-induced IL-1β production, which appears to be via the upregulation of pro-IL-1β expression, which feeds the inflammasome-mediated pathway.

So far, our data supports trophoblast: 1) NOD2-TLR4 crosstalk to negatively regulate aPL-induced IL-8 secretion; 2) MDP/NOD2-mediated amplification of aPL-induced IL-1β processing and secretion; 3) aPL-induced caspase-1 activation and IL-1β secretion via NOD2; and 4) aPL-induced VEGF secretion via NOD2.

In terms of the ability of aPL to induce trophoblast IL-1β secretion via NOD2, we questioned whether there may be an endogenous intermediate in this pathway. We had already reported similar scenarios: aPL induction of the DAMP, uric acid, mediates NLRP3 activation and IL-1β production [6]; and aPL induction of miR-146a-3p mediates TLR8 activation and subsequent IL-8 production [11]. Recently, the major intermediate filament protein, vimentin, has been reported as acting as a DAMP by interacting with, and activating NOD2, to induce inflammatory responses [32,33]. While NOD2 is typically cytosolic, it can localize to the cell surface and be activated at the plasma membrane [51]. Indeed, vimentin interacts with cytosolic NOD2 at the cell surface, and the plant steroidal lactone, withaferin-A (WFA), can bind to vimentin, inhibiting vimentin-NOD2 interactions, leading to NOD2 re-localization to the cytosol [33]. In this current study we found that WFA partially inhibited aPL-induced IL-1β secretion, supporting a role for vimentin as a extravillous trophoblast DAMP and secondary signal for NOD2 activation. While villous trophoblast cells lack vimentin, first trimester extravillous trophoblast cells do express vimentin [21–24]. How aPL promote trophoblast vimentin-NOD2 interactions may be due

to the ability of certain aPL to bind vimentin/cardiolipin complexes [52]. Whether our anti-β_2GPI aPL, which has cardiolipin reactivity [25], can target vimentin/cardiolipin complexes is currently under investigation. In contrast, the NOD2-mediated caspase-1 activation and VEGF production in response to aPL was not inhibited by WFA, suggesting either another NOD2-activating intermediate is involved or vimentin activation of NOD2 occurs in conjunction with NOD2-TLR4 crosstalk and synergy.

The antibody we used in this study, IIC5, is a murine mAb that reacts with domain V of the β_2GPI molecule [26] (β_2GPI is comprised of five short consensus repeat domains - SCRs also called sushi domains). We have reported that this mAb binds to a similar epitope in β_2GPI as patient-derived aPL [28]. Recently, considerable attention has been focused on the role of aPL reactive with the first SCR domain of β_2GPI (DI), which some groups claim are more pathogenic than aPL reactive with the remainder of the β_2GPI molecule. However, careful examination of the evidence regarding anti-β_2GPI DI Abs led the task force on aPL to conclude "… it has clearly emerged that not all anti-β_2GPI detectable in APS patients target DI, with significant subpopulations reacting against other β_2GPI epitopes" [53]. Indeed, a systematic review found only 44.3 - 45.4% of patients with APS (either alone or with SLE) had domain I anti-β_2GPI Abs [54]; and while the presence of anti-β_2GPI DI Abs doubles the risk for thrombotic events, no studies have reported an association with pregnancy morbidities [54]. Another recent systematic review reported that the most important feature of pathogenic aPL in obstetric APS is triple positivity [55]; and the anti-β_2GPI mAb we used in this work is a triple positive (behaves as an anti-β_2GPI and anti-cardiolipin Ab, as well as showing lupus anticoagulant activity) [26,27]. Furthermore, we confirmed our findings that bacterial MDP synergistically augmented the anti-β_2GPI mAb-induced trophoblast IL-1β response using patient-derived polyclonal aPL.

In summary, we have found that bacterial MDP amplifies trophoblast IL-1β expression, processing, and secretion in the presence of aPL through activation of NOD2. In the absence of MDP, NOD2 also mediates aPL-induced trophoblast IL-1β and VEGF secretion, and negatively regulates aPL-driven IL-8 production. Additionally, we report a role for extravillous trophoblast vimentin as a novel danger signal that contributes to the aPL-induced trophoblast IL-1β production. Together our data indicate a role for NOD2 in mediating trophoblast inflammatory and angiogenic responses to aPL alone, and in mediating trophoblast inflammation in the presence of bacterial MDP. Our findings suggest that a bacterial infection at the maternal-fetal interface may exacerbate the impact aPL have on trophoblast inflammation and, thus, on pregnancy outcome. Our *in vitro* findings regarding the pathogenesis of obstetric APS and the novel role for NOD2 have the potential to impact more targeted approaches to treating patients; and further *in vivo* and human studies are warranted.

Author contributions

MJM and MCP performed the experiments. VMA, MJM, MCP, LCW and JES designed the study. VMA, MJM, and MCP analyzed the data and wrote the first draft. LWC provided the antibodies. MCP, MJM, JES, LWC and VMA revised the paper for important intellectual content.

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Appendix A. Supplementary data

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References

[1] C. Agostinis, S. Biffi, C. Garrovo, P. Durigutto, A. Lorenzon, A. Bek, et al., In vivo distribution of beta2 glycoprotein I under various pathophysiologic conditions, Blood 118 (2011) 4231–4238.
[2] D.P. D'Cruz, M.A. Khamashta, G.R. Hughes, Systemic lupus erythematosus, Lancet 369 (2007) 587–596.
[3] C.A. Viall, L.W. Chamley, Histopathology in the placentae of women with antiphospholipid antibodies: a systematic review of the literature, Autoimmun. Rev. 14 (2015) 446–471.
[4] M. Tong, C.A. Viall, L.W. Chamley, Antiphospholipid antibodies and the placenta: a systematic review of their in vitro effects and modulation by treatment, Hum. Reprod. Update 21 (2015) 97–118.
[5] M.J. Mulla, J.J. Brosens, L.W. Chamley, I. Giles, C. Pericleous, A. Rahman, et al., Antiphospholipid antibodies induce a pro-inflammatory response in first trimester trophoblast via the TLR4/MyD88 pathway, Am. J. Reprod. Immunol. 62 (2009) 96–111.
[6] M.J. Mulla, J.E. Salmon, L.W. Chamley, J.J. Brosens, C.M. Boeras, P.B. Kavathas, et al., A Role for Uric Acid and the Nalp3 Inflammasome in Antiphospholipid Antibody-Induced IL-1beta Production by Human First Trimester Trophoblast, PLoS One 8 (2013) e65237.
[7] M.J. Mulla, K. Myrtolli, J.J. Brosens, L.W. Chamley, J.Y. Kwak-Kim, M.J. Paidas, et al., Antiphospholipid antibodies limit trophoblast migration by reducing IL-6 production and STAT3 activity, Am. J. Reprod. Immunol. 63 (2010) 339–348.
[8] T.Y. Carroll, M.J. Mulla, C.S. Han, J.J. Brosens, L.W. Chamley, I. Giles, et al., Modulation of trophoblast angiogenic factor secretion by antiphospholipid antibodies is not reversed by heparin, Am. J. Reprod. Immunol. 66 (2011) 286–296.
[9] A.M. Alvarez, M.J. Mulla, L.W. Chamley, A.P. Cadavid, V.M. Abrahams, Aspirin-triggered lipoxin prevents antiphospholipid antibody effects on human trophoblast migration and endothelial cell interactions, Arthritis Rheum. 67 (2015) 488–497.
[10] B.C. Young, R.J. Levine, S.A. Karumanchi, Pathogenesis of preeclampsia, Annu. Rev. Pathol. 5 (2010) 173–192.
[11] S.M. Gysler, M.J. Mulla, M. Guerra, J.J. Brosens, J.E. Salmon, L.W. Chamley, et al., Antiphospholipid antibody-induced miR-146a-3p drives trophoblast interleukin-8 secretion through activation of Toll-like receptor 8, Mol. Hum. Reprod. 22 (2016) 465–474.
[12] M. Harel, A. Aron-Maor, Y. Sherer, M. Blank, Y. Shoenfeld, The infectious etiology of the antiphospholipid syndrome: links between infection and autoimmunity, Immunobiology 210 (2005) 743–747.
[13] F. Fischetti, P. Durigutto, V. Pellis, A. Debeus, P. Macor, R. Bulla, et al., Thrombus formation induced by antibodies to beta2-glycoprotein I is complement dependent and requires a priming factor, Blood 106 (2005) 2340–2346.
[14] E. Raschi, C.B. Chighizola, C. Grossi, N. Ronda, R. Gatti, P.L. Meroni, et al., beta2-glycoprotein I, lipopolysaccharide and endothelial TLR4: three players in the two hit theory for anti-phospholipid-mediated thrombosis, J. Autoimmun. 55 (2014) 42–50.
[15] K. Duckitt, D. Harrington, Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies, Bmj 330 (2005) 565.
[16] L. Andreoli, C.B. Chighizola, A. Banzato, G.J. Pons-Estel, G. Ramire de Jesus, D. Erkan, Estimated frequency of antiphospholipid antibodies in patients with pregnancy morbidity, stroke, myocardial infarction, and deep vein thrombosis: a critical review of the literature, Arthritis Care Res (Hoboken) 65 (2013) 1869–1873.
[17] L.O. Rustveld, Association between maternal infections and preeclampsia: a systematic review of epidemiologic studies, Matern. Child Health J. 12 (2008) 223.
[18] A. Conde-Agudelo, J. Villar, M. Lindheimer, Maternal infection and risk of preeclampsia: systematic review and metaanalysis, Am. J. Obstet. Gynecol. 198 (2008) 7–22.
[19] M.J. Costello, S.K. Joyce, V.M. Abrahams, NOD protein expression and function in first trimester trophoblast cells, Am. J. Reprod. Immunol. 57 (2007) 67–80.
[20] S.L. Straszewski-Chavez, V.M. Abrahams, A.B. Alvero, P.B. Aldo, Y. Ma, S. Guller, et al., Isolation and characterization of a novel telomerase immortalized first trimester trophoblast cell line, swan 71, Placenta 30 (2009) 939–948.
[21] Y. Loke, B. Butterworth, Heterogeneity of human trophoblast populations, in: T.J. Gill, T.G. Wegmann (Eds.), Immunoregulation and Fetal Survival, Oxford University Press, New York, 1987197e209.
[22] S. DaSilva-Arnold, J.L. James, A. Al-Khan, S. Zamudio, N.P. Illsley, Differentiation of first trimester cytotrophoblast to extravillous trophoblast involves an epithelial-mesenchymal transition, Placenta 36 (2015) 1412–1418.
[23] G. Aboagye-Mathiesen, J. Laugesen, M. Zdravkovic, P. Ebbesen, Isolation and characterization of human placental trophoblast subpopulations from first-trimester chorionic villi, Clin. Diagn. Lab. Immunol. 3 (1996) 14–22.
[24] M. Bilban, S. Tauber, P. Haslinger, J. Pollheimer, L. Saleh, H. Pehamberger, et al., Trophoblast invasion: assessment of cellular models using gene expression signatures, Placenta 31 (2010) 989–996.
[25] L.W. Chamley, B. Konarkowska, A.M. Duncalf, M.D. Mitchell, P.M. Johnson, Is interleukin-3 important in antiphospholipid antibody-mediated pregnancy failure? Fertil. Steril. 76 (2001) 700–706.
[26] C.R. Albert, W.J. Schlesinger, C.A. Viall, M.J. Mulla, J.J. Brosens, L.W. Chamley, et al., Effect of hydroxychloroquine on antiphospholipid antibody-induced changes in first trimester trophoblast function, Am. J. Reprod. Immunol. 71 (2014) 154–164.
[27] C.A. Viall, Q. Chen, P.R. Stone, L.W. Chamley, Human extravillous trophoblasts bind but do not internalize antiphospholipid antibodies, Placenta 42 (2016) 9–16.
[28] C.A. Viall, Q. Chen, B. Liu, A. Hickey, S. Snowise, J.E. Salmon, et al., Antiphospholipid antibodies internalised by human syncytiotrophoblast cause

aberrant cell death and the release of necrotic trophoblast debris, J. Autoimmun. 47 (2013) 45–57.
[29] L.W. Chamley, J.L. Allen, P.M. Johnson, Synthesis of beta2 glycoprotein 1 by the human placenta, Placenta 18 (1997) 403–410.
[30] Z.C. Quao, M. Tong, E. Bryce, S. Guller, L.W. Chamley, V.M. Abrahams, Low molecular weight heparin and aspirin exacerbate human endometrial endothelial cell responses to antiphospholipid antibodies, Am. J. Reprod. Immunol. 79 (2018).
[31] A.E. King, A.W. Horne, S. Hombach-Klonisch, J.I. Mason, H.O. Critchley, Differential expression and regulation of nuclear oligomerization domain proteins NOD1 and NOD2 in human endometrium: a potential role in innate immune protection and menstruation, Mol. Hum. Reprod. 15 (2009) 311–319.
[32] T.N. Mak, H. Bruggemann, Vimentin in bacterial infections, Cells 5 (2016).
[33] C. Stevens, P. Henderson, E.R. Nimmo, D.C. Soares, B. Dogan, K.W. Simpson, et al., The intermediate filament protein, vimentin, is a regulator of NOD2 activity, Gut 62 (2013) 695–707.
[34] C.B. Chighizola, L. Andreoli, M. Gerosa, A. Tincani, A. Ruffatti, P.L. Meroni, The treatment of anti-phospholipid syndrome: a comprehensive clinical approach, J. Autoimmun. 90 (2018) 1–27.
[35] K. Aagaard, J. Ma, K.M. Antony, R. Ganu, J. Petrosino, J. Versalovic, The placenta harbors a unique microbiome, Sci. Transl. Med. 6 (2014) 237ra65.
[36] D.B. Kell, L.C. Kenny, A dormant microbial component in the development of preeclampsia, Front. Med. (Lausanne) 3 (2016) 60.
[37] I. Cetin, P. Pileri, A. Villa, S. Calabrese, L. Ottolenghi, S. Abati, Pathogenic mechanisms linking periodontal diseases with adverse pregnancy outcomes, Reprod. Sci. 19 (2012) 633–641.
[38] F. Xie, Y. Hu, L.A. Magee, D.M. Money, D.M. Patrick, R.M. Brunham, et al., Chlamydia pneumoniae infection in preeclampsia, Hypertens. Pregnancy 29 (2010) 468–477.
[39] M. Sorice, A. Longo, A. Capozzi, T. Garofalo, R. Misasi, C. Alessandri, et al., Anti-beta2-glycoprotein I antibodies induce monocyte release of tumor necrosis factor alpha and tissue factor by signal transduction pathways involving lipid rafts, Arthritis Rheum. 56 (2007) 2687–2697.
[40] S. Stone, R. Pijnenborg, L. Vercruysse, R. Poston, M.A. Khamashta, B.J. Hunt, et al., The placental bed in pregnancies complicated by primary antiphospholipid syndrome, Placenta 27 (2006) 457–467.
[41] J.T. Van Horn, C. Craven, K. Ward, D.W. Branch, R.M. Silver, Histologic features of placentas and abortion specimens from women with antiphospholipid and antiphospholipid-like syndromes, Placenta 25 (2004) 642–648.
[42] S. Yang, R. Tamai, S. Akashi, O. Takeuchi, S. Akira, S. Sugawara, et al., Synergistic effect of muramyldipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture, Infect. Immun. 69 (2001) 2045–2053.
[43] M.A. Wolfert, T.F. Murray, G.J. Boons, J.N. Moore, The origin of the synergistic effect of muramyl dipeptide with endotoxin and peptidoglycan, J. Biol. Chem. 277 (2002) 39179–39186.
[44] M.G. Netea, G. Ferwerda, D.J. de Jong, T. Jansen, L. Jacobs, M. Kramer, et al., Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release, J. Immunol. 174 (2005) 6518–6523.
[45] M.J. Mulla, A.G. Yu, I. Cardenas, S. Guller, B. Panda, V.M. Abrahams, Regulation of Nod1 and Nod2 in first trimester trophoblast cells, Am. J. Reprod. Immunol. 61 (2009) 294–302.
[46] G. Ferwerda, M. Kramer, D. de Jong, A. Piccini, L.A. Joosten, I. Devesaginer, et al., Engagement of NOD2 has a dual effect on proIL-1beta mRNA transcription and secretion of bioactive IL-1beta, Eur. J. Immunol. 38 (2008) 184–191.
[47] A.A. Filardy, J. He, J. Bennink, J. Yewdell, B.L. Kelsall, Posttranscriptional control of NLRP3 inflammasome activation in colonic macrophages, Mucosal Immunol. 9 (2016) 850–858.
[48] L.C. Hsu, S.R. Ali, S. McGillivray, P.H. Tseng, S. Mariathasan, E.W. Humke, et al., A NOD2-NALP1 complex mediates caspase-1-dependent IL-1beta secretion in response to Bacillus anthracis infection and muramyl dipeptide, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 7803–7808.
[49] Q. Pan, J. Mathison, C. Fearns, V.V. Kravchenko, J. Da Silva Correia, H.M. Hoffman, et al., MDP-induced interleukin-1beta processing requires Nod2 and CIAS1/NALP3, J. Leukoc. Biol. 82 (2007) 177–183.
[50] H. Kim, Q. Zhao, H. Zheng, X. Li, T. Zhang, X. Ma, A novel crosstalk between TLR4- and NOD2-mediated signaling in the regulation of intestinal inflammation, Sci. Rep. 5 (2015) 12018.
[51] N. Barnich, J.E. Aguirre, H.C. Reinecker, R. Xavier, D.K. Podolsky, Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-{kappa}B activation in muramyl dipeptide recognition, J. Cell Biol. 170 (2005) 21–26.
[52] E. Ortona, A. Capozzi, T. Colasanti, F. Conti, C. Alessandri, A. Longo, et al., Vimentin/cardiolipin complex as a new antigenic target of the antiphospholipid syndrome, Blood 116 (2010) 2960–2967.
[53] M.L. Bertolaccini, O. Amengual, L. Andreoli, T. Atsumi, C.B. Chighizola, R. Forastiero, et al., 14th international congress on antiphospholipid antibodies task force. Report on antiphospholipid syndrome laboratory diagnostics and trends, Autoimmun. Rev. 13 (2014) 917–930.
[54] M. Radin, I. Cecchi, D. Roccatello, P.L. Meroni, S. Sciascia, Prevalence and thrombotic risk assessment of anti-beta2 glycoprotein I domain I antibodies: a systematic review, Semin. Thromb. Hemost. 44 (2018) 466–474.
[55] S. De Carolis, S. Tabacco, F. Rizzo, A. Giannini, A. Botta, S. Salvi, et al., Antiphospholipid syndrome: an update on risk factors for pregnancy outcome, Autoimmun. Rev. 17 (2018) 956–966.