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human reproduction ORIGINAL ARTICLE *Early pregnancy*

Trophoblast cells inhibit neutrophil extracellular trap formation and enhance apoptosis through vasoactive intestinal peptide-mediated pathways

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STUDY QUESTION: Do human trophoblast cells modulate neutrophil extracellular trap (NET) formation, reactive oxygen species (ROS) synthesis and neutrophil apoptosis through mechanisms involving vasoactive intestinal peptide (VIP)?

SUMMARY ANSWER: Trophoblast cells inhibited NET formation and ROS synthesis and enhanced neutrophil apoptosis through VIP-mediated pathways in a model of maternal–placental interaction.

WHAT IS KNOWN ALREADY: Immune homeostasis maintenance at the maternal–placental interface is mostly coordinated by trophoblast cells. Neutrophil activation and NET formation increases in pregnancies complicated by exacerbated pro-inflammatory responses. VIP has anti-inflammatory and immunosuppressant effects and is synthesized by trophoblast cells.

STUDY DESIGN, SIZE, DURATION: This is a laboratory-based observational study that sampled circulating neutrophils from 50 healthy volunteers to explore their response *in vitro* to factors derived from human trophoblast cells.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Peripheral blood neutrophils were isolated from healthy volunteers and tested *in vitro* with first trimester trophoblast cell line (Swan-71 and HTR8) conditioned media (CM) or with VIP. The effect of VIP and trophoblast CM on NET formation was assessed by co-localization of elastase and DNA by confocal microscopy, DNA release and elastase activity measurement. Neutrophil apoptosis was determined by flow cytometry or fluorescence microscopy. ROS formation was assessed by flow cytometry with a fluorescent probe. VIP silencing was performed by siRNA transfection. For phagocytosis of apoptotic neutrophils, autologous monocytes were sampled, and engulfment and cytokines were assessed by flow cytometry and ELISA.

MAIN RESULTS AND THE ROLE OF CHANCE: Trophoblast CM and 10 nM VIP promoted neutrophil deactivation by preventing phorbol myristate acetate–induced NET formation and ROS synthesis while they increased neutrophil spontaneous apoptosis and reversed the anti-apoptotic effect of lipopolysaccharide (all $P < 0.05$ versus control). The effects of trophoblast CM were prevented by a VIP antagonist or when VIP knocked-down trophoblast cells were used ($P < 0.05$ versus control). Neutrophils driven to apoptosis by trophoblast CM could be rapidly engulfed by monocytes without increasing IL-12 production.

LARGE SCALE DATA: Not applicable.

LIMITATIONS, REASONS FOR CAUTION: The mechanisms of neutrophil deactivation by trophoblast VIP are based on the results obtained with neutrophils drawn from peripheral blood of healthy individuals interacting with trophoblast cell lines *in vitro*. These studies were designed to investigate biological processes at the cellular and molecular level; therefore, they have the limitations of studies *in vitro* and it is not possible to ascertain if these mechanisms operate similarly *in vivo*. We tested 50 neutrophil samples from healthy volunteers that have a

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normal variability in their responses. Cell lines derived from human trophoblast were used, and we cannot rule out a differential behavior of trophoblast cells in contact with neutrophils *in vivo*.

WIDER IMPLICATIONS OF THE FINDINGS: Results presented here are consistent with an active mechanism through which neutrophils in contact with trophoblast cells would be deactivated and silently cleared by decidual macrophages throughout pregnancy. They support a novel immunomodulatory role of trophoblast VIP on neutrophils at the placenta, providing new clues for pharmacological targeting of immune and trophoblast cells in pregnancy complications associated with exacerbated inflammation.

STUDY FUNDING/COMPETING INTERESTS: This work was funded by the National Agency of Sciences and Technology (PICT 2011-0144, 2014-0657 and 2013-2177) and University of Buenos Aires (UBACyT 20020130100040BA, 20020150100161BA and 20020130100744BA). The authors declare no competing interests.

Key words: trophoblast cells / neutrophils / vasoactive intestinal peptide / neutrophil extracellular traps / neutrophil apoptosis / phagocytosis of apoptotic cells

Introduction

The recruitment and functional shaping of different leukocyte populations at the early maternal–placental interface is orchestrated by trophoblast cells ensuring normal placentation and immune homeostasis maintenance (Racicot et al., 2014). Consistently, in pregnancies complicated by preeclampsia, an exacerbated pro-inflammatory response arises in the mother early in pregnancy with a vasculopathy that threatens both maternal and fetal survival in the third trimester (Sacks et al., 1998; Redman and Sargent, 2010).

In contrast to macrophages and natural killer cells recruited to the pregnant uterus from the first weeks, neutrophils are barely found until the second trimester where a novel pro-angiogenic decidual neutrophil population has been identified (Amsalem et al., 2014). However, the highest neutrophil influx to the pregnant uterus occurs near term, associated with the pro-inflammatory response that prepares the uterus for delivery (Gomez-Lopez et al., 2014). Chemokine (C-X-C motif) ligand 8 (CXCL8 or IL-8), a chemokine released at high levels by trophoblast cells, is one of the major cytokines that induce NETosis, a distinct form of active cell death characterized by the release of decondensed chromatin into the extracellular space with granular and cytoplasmic proteins associated (Brinkmann et al., 2004; Papayannopoulos and Zychlinsky, 2009). Neutrophils forming NETs do not appear to display "eat-me" signals like apoptotic cells, which would prevent their silent clearance by phagocytic cells (Kaplan and Radic, 2012). In fact, when neutrophils undergoing NETosis are faced to M2 alternative differentiated macrophages, a pro-inflammatory mediator release is observed (Nakazawa et al., 2016). NETs have been linked to the promotion of thrombosis as scaffolds for fibrin and von Willebrand factor (Fuchs et al., 2010), and to the pathogenesis of lupus nephritis (Hakkim et al., 2010) among other disorders. Trophoblast microvesicles derived from placental syncytium in normal pregnancies induce NET formation *in vitro*, and both IL-8 and trophoblast microvesicles were proposed as potential triggers of NET formation in preeclampsia (Gupta et al., 2005). On the basis that circulating neutrophils from normal pregnant versus non-pregnant women synthesize higher levels of reactive oxygen species (ROS) (Tsukimori et al., 1993; Sacks et al., 1998; Aly et al., 2004) and that preeclamptic placenta revealed massive presence of NETs largely localized within the intervillous space (Gupta et al., 2005), it was proposed that neutrophils become activated during their circulation through the intervillous space (Walsh and Wang, 1995; Mellembakken et al., 2002; Hahn et al., 2012). However, evidence of placental-derived local factors that can modulate neutrophil activation and NET formation in normal pregnancies is still lacking.

The vasoactive intestinal peptide (VIP) is a pleiotropic polypeptide with vasodilating, pro-secretory and anti-inflammatory effects binding upon high-affinity VPAC1 and VPAC2 receptors (Waschek, 2013). In human pregnancy, VIP is synthesized by cytotrophoblast and syncytiotrophoblast cells of the first and third trimester placenta as well as the first trimester trophoblast-derived cell lines Swan-71 and HTR8 (Marzioni et al., 2005; Vota et al., 2016). VIP modulates trophoblast cell function and their interaction with monocytes and macrophages to promote immunosuppressive apoptotic cell phagocytosis (Paparini et al., 2015; Vota et al., 2016). VPAC1 receptors are expressed on neutrophils and VIP inhibits neutrophil cytotoxicity and superoxide anion generation (Palermo et al., 1996; Harfi et al., 2004). On this basis, we hypothesized that trophoblast cells modulate the functional profile of neutrophils through VIP-mediated pathways to prevent NET formation, induce neutrophil apoptosis and contribute to their silent clearance. By means of an *in vitro* model of maternal–placental interaction, we explored the effects of trophoblast conditioned media (CM) and VIP on NET formation, ROS synthesis and neutrophil apoptosis. We evaluated their ability as well to modulate apoptotic neutrophil phagocytosis by autologous monocytes. Our results demonstrate that VIP from trophoblast cells inhibits neutrophil activation and NET formation and accelerates neutrophil apoptosis. Moreover, neutrophils undergoing apoptosis in the presence of CM were more rapidly phagocytosed without pro-inflammatory cytokine release, suggesting a regulatory network mediated by trophoblast cells to prevent neutrophil activation throughout pregnancy.

Materials and Methods

Blood samples

Peripheral blood samples were obtained from 50 healthy volunteers who were not under pharmacological treatment for at least 10 days before sampling. Studies were approved by the Argentine Society of Clinical Investigation Board and Ethical Committee (Ref. SAIC 46/14). All healthy donors provided written informed consent for sample collection and subsequent analysis.

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Neutrophil and monocyte isolation

Neutrophils were isolated by Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) gradient centrifugation and Dextran (MP Biomedicals, Santa Ana, CA, USA) sedimentation. Contaminating erythrocytes were removed by hypotonic lysis, and neutrophils were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Natocor, Villa Carlos Paz, Argentina). The purity was checked by flow cytometry using an anti-CD14 antibody (BD Pharmingen, San Diego, CA, USA) to guarantee a monocyte contamination <0.5% and that forward scatter/side scatter parameters of the neutrophil population were compatible with those of non-activated cells. Cells were used immediately after isolation. Monocytes were purified by Percoll (GE Healthcare) gradient (>80% purity) as done earlier (Paparini *et al.*, 2015).

Trophoblast-derived cell cultures

Swan-71 and HTR8/SVneo human first trimester cell lines were kindly given by Dr Gil Mor (Yale University, New Haven, CT, USA). To obtain trophoblast CM, cells were cultured for 20 h in RPMI 2% FBS with or without 50 nM VIP antagonist (Bachem, CA, USA).

VIP silencing

Swan-71 cells were transfected with 50–100 nM VIP siRNA (Santa Cruz Biotechnology, Dallas, TX, USA): Lipofectamine RNAimax (Life Technologies, Grand Island) complex as previously described (Vota *et al.*, 2016). Twenty-four hours post-transfection, the media were changed to DMEM-F12 with 2% FBS for 48 h and then collected and stored at −20°C until used. siRNA with a scramble sequence was used as a control.

Elastase activity and DNA determinations

After neutrophil stimulation with phorbol myristate acetate (PMA) for 210 min, cells were treated for 30 min with 1 U/ml micrococcal nuclease (Roche Diagnostics, Mannheim, Germany), and supernatants were collected to determine elastase activity and DNA concentration (Ramos *et al.*, 2016). Neutrophil elastase activity was measured spectrophotometrically at

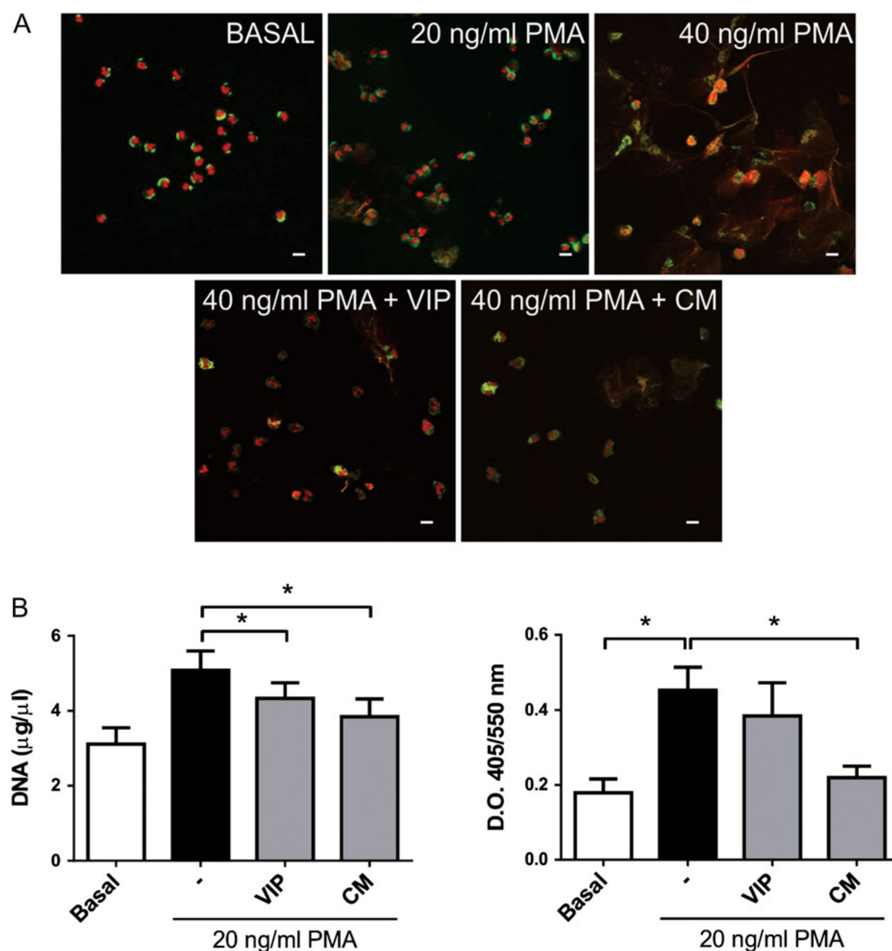

Figure 1 Trophoblast conditioned media (CM) and vasoactive intestinal peptide (VIP) inhibit neutrophil extracellular trap (NET) formation. Neutrophils were incubated with phorbol myristate acetate (PMA) and VIP or CM to analyze NET formation, DNA and elastase release as described in Materials and Methods. (**A**) Neutrophils (1×10^6) were placed on coverslips and stimulated or not (Basal) with 20 or 40 ng/ml PMA, 10 nM VIP or Swan-71 trophoblast cell CM, and NETs were detected by propidium iodide (PI) staining of DNA (red) and anti-elastase and secondary DyLight-488 antibody (green). Merged representative images of at least three experiments with different neutrophil donors are shown. Scale bars = 10 µm. (**B**) Neutrophils (1×10^6) were incubated for 4 h with PMA and 10 nM VIP or CM and released DNA, or elastase activity were measured in the supernatants. Results are mean ± SEM (*$P < 0.05$; $n = 5$, Wilcoxon test).

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405/550 nm, using the specific peptide substrate N-Methoxysuccinyl-Ala-Ala-Pro-Val (Sigma-Aldrich, St. Louis, MO, USA). DNA concentration was determined fluorometrically with Sybr Gold (Invitrogen).

NETosis and confocal microscopy

Glass cover slides and Lab-Tek chambers were pretreated with Poly-L-Lysine (Sigma-Aldrich), and then neutrophils were seeded. Four hours post-PMA stimulation, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and NETs were detected using propidium iodide (PI) to visualize DNA and an anti-elastase antibody (Calbiochem, Billerica, MA, USA) with a secondary DyLight-488 (Jackson ImmunoResearch). In one set of experiments, at 150 min post-PMA stimulation, cells were stained with PI (Ramos *et al.*, 2016) and neutrophils undergoing NETosis, apoptosis and viable cells were identified by examining nuclei morphology and differential interference contrast signal. Viable neutrophils were identified by a multilobulated nucleus, which stained brightly with PI; apoptotic cells were characterized by their highly condensed nuclear material into one to three very brightly PI-stained nuclear bodies and cytoplasmic vacuolation (Taylor *et al.*, 2007), while NETotic neutrophils were recognized by their decondensed nuclei occupying practically the whole cell volume and a reduced intensity of PI signal (Brinkmann *et al.*, 2013). Images were obtained using a FluoView FV1000 confocal microscope (Olympus, Tokyo) equipped with a Plapon 60X/1.42 objective and analyzed with Olympus FV10-ASW software (Olympus, France).

ROS production

Neutrophils were incubated at 37°C with PMA (Sigma-Aldrich) and VIP or CM. Then 5 μM 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) (Sigma-Aldrich) was added for another 15 min and cells analyzed by flow cytometry.

Apoptosis assay

Neutrophils were incubated for 6 or 18 h in RPMI/2% FBS, with VIP, CM and/or lipopolysaccharide (LPS) 055:B5 (Sigma-Aldrich), and apoptosis was assessed by flow cytometry and fluorescence microscopy. Cells were stained with Annexin V FITC/PI for flow cytometry where 10 000 events were acquired and results analyzed using FlowJo software (Ashland, OR, USA). Alternatively, cells were stained with ethidium bromide and acridine orange, and 300 cells in each condition were counted by fluorescence microscopy.

Phagocytosis assay and assessment of phagocytic cell profile

Apoptotic neutrophils were obtained after 18 h incubation in RPMI 1640 or CM and stained with carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies, Buenos Aires, Argentina). The percentage of apoptosis was 50% as determined by flow cytometry. Monocytes were challenged with apoptotic neutrophils in a 1:10 ratio. After phagocytosis, monocytes were stained with anti-CD14, and CD14/CFSE-positive cells were analyzed

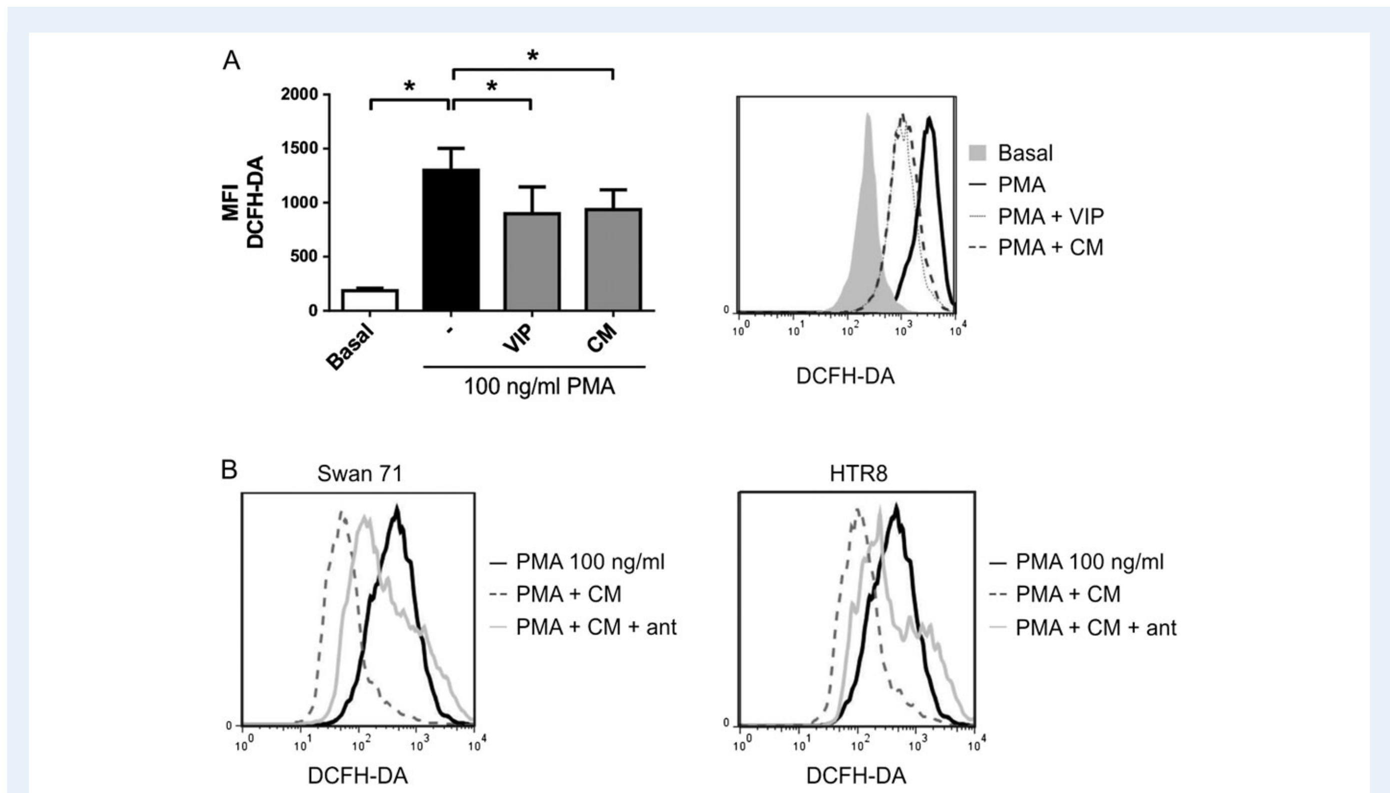

Figure 2 Swan-71 and HTR8 trophoblast CM and VIP reduce reactive oxygen species (ROS) formation. Neutrophils were stimulated with PMA, and ROS production was quantified by flow cytometry using 2′,7′-dichlorofluorescin diacetate as indicated in Materials and Methods. (**A**) Neutrophils (2×10^5) were incubated for 45 min with 100 ng/ml PMA and 10 nM VIP or CM, and mean fluorescence intensity was assessed. Values are mean ± SEM (*$P < 0.05$; $n = 6$, Kruskal–Wallis test). Representative histograms are shown. (**B**) Neutrophils (2×10^5) were stimulated with PMA and CM from either Swan-71 or HTR8 trophoblast cells in the presence or absence of a VIP receptor antagonist. Representative histograms of two independent experiments are shown.

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by flow cytometry as previously described (Paparini *et al.*, 2015). Supernatants from CD14+ cells were collected after phagocytosis, and IL-12 and IL-10 concentrations were determined by ELISA (OptEIA, BD Biosciences, Franklin Lakes, NJ, USA).

Table I VIP antagonist inhibits the effect of trophoblast CM.

	ROS formation	DNA release
PMA	1299 ± 204	5.1 ± 0.5
PMA + CM	935 ± 184[a]	3.9 ± 0.5[a]
PMA + CM + VIP antagonist	1410 ± 200[b]	5.1 ± 0.4[b]

Neutrophils were stimulated with phorbol myristate acetate (PMA) in the presence or absence of trophoblast conditioned media (CM) and vasoactive intestinal polypeptide (VIP) receptor antagonist (50 nM) to determine reactive oxygen species (ROS) production or DNA release as indicated in Materials and Methods. Values are mean ± SEM (n = 3). [a]P < 0.05 versus PMA; [b]P < 0.05 versus PMA + CM (Wilcoxon test).

Statistical analysis

The significance of the results was analyzed by the Mann–Whitney test or the Wilcoxon test for nonparametric samples. When multiple comparisons were necessary, the Kruskal–Wallis test was used. Differences between groups were considered significant at P < 0.05 using the GraphPad Prism4 software (GraphPad, San Diego, CA, USA).

Results

CM and VIP inhibit NET formation

Neutrophils in maternal circulation, especially at the intervillous space, are exposed to pro-inflammatory stimuli that could rapidly induce ROS synthesis and NET formation with deleterious consequences. Based on the immunoregulatory effects of trophoblast cells and VIP, we first explored their effect on PMA-induced NET formation. Figure 1A upper panel shows the characteristic structure of NETs released by neutrophils treated with PMA for 4 h in which DNA co-localized with elastase either extracellularly or inside cells before they were released. NETosis

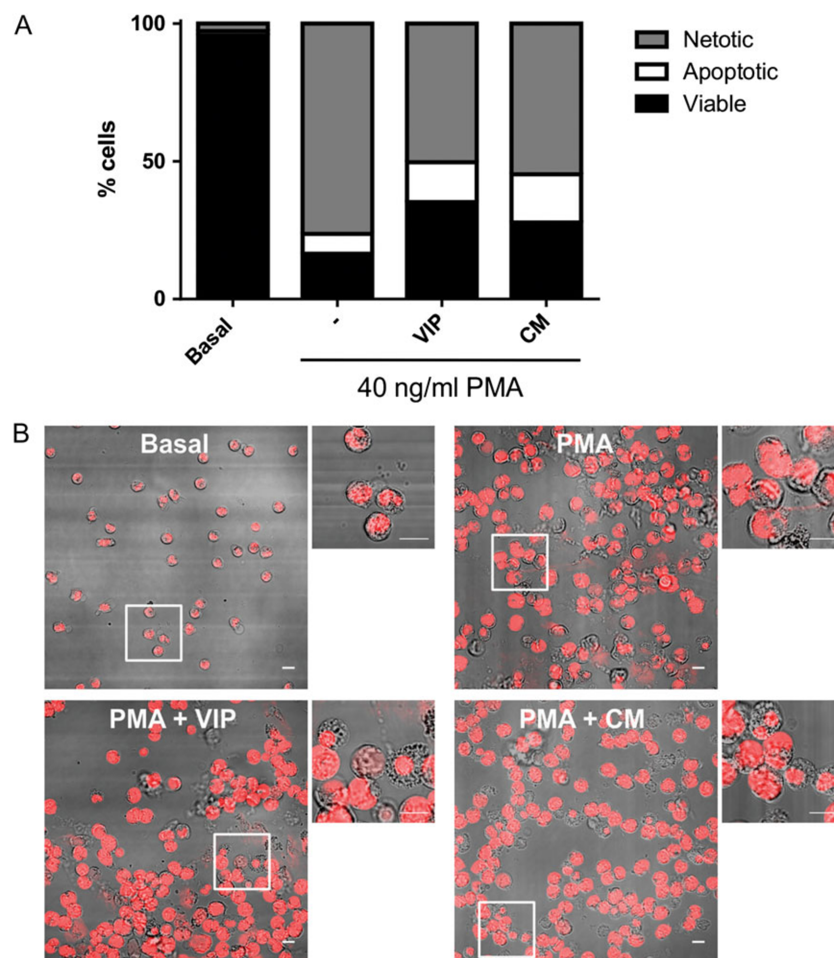

Figure 3 Trophoblast CM and VIP diminish NETosis and increase apoptosis. (**A**) Neutrophils (1 × 10^6) were incubated with PMA in the presence of 10 nM VIP or Swan-71 CM for 150 min on Lab-Tek chambers as indicated in Materials and Methods. Cells were stained with PI and the percentage of netotic, apoptotic and viable cells was determined. A representative experiment out of six run similarly is shown. (**B**) PI-stained neutrophils in representative images of one out of six independent experiments depicted in (A) are shown. Cropped images correspond to selected areas. Scale bars = 10 µm.

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levels were reduced when PMA was added to neutrophils in the presence of 10 nM VIP or Swan-71 trophoblast cell CM. The inhibitory effects of VIP and CM on NETosis were also assessed by measuring DNA levels in the supernatants of neutrophils exposed to PMA (Fig. 1B). CM reduced elastase release by PMA-stimulated neutrophils and, although not significant, a decrease of elastase was seen with 10 nM VIP alone (Fig. 1B).

CM and VIP inhibit neutrophil ROS formation

ROS synthesis has a major role in NET formation, so we explored the effect of VIP and trophoblast CM on ROS synthesis by neutrophils. Figure 2A shows that 10 nM VIP and CM from Swan-71 trophoblast cells inhibit PMA-induced ROS formation. The effect displayed by CM from Swan-71 cells was further assessed using another first trimester cytotrophoblast cell line (HTR8). The antagonist of VIP receptors reversed the inhibition of CM on ROS formation (Fig. 2B), and a similar effect was observed for NETosis as determined by DNA release (Table I). The effect of the VIP receptor antagonist on CM is consistent with the presence of VIP in CM of both trophoblast cell lines, as determined by ELISA (205 ± 10 and 172 ± 51 pg/ml for Swan-71 and HTR8, respectively) and previously shown for both cell lines (Fraccaroli et al., 2015; Vota et al., 2016).

NETosis inhibition by CM and VIP parallels an increase in neutrophil apoptosis

The inhibitory effect of CM or VIP on NETosis was also assessed after a 150 min PMA treatment, where neutrophils undergoing NETosis

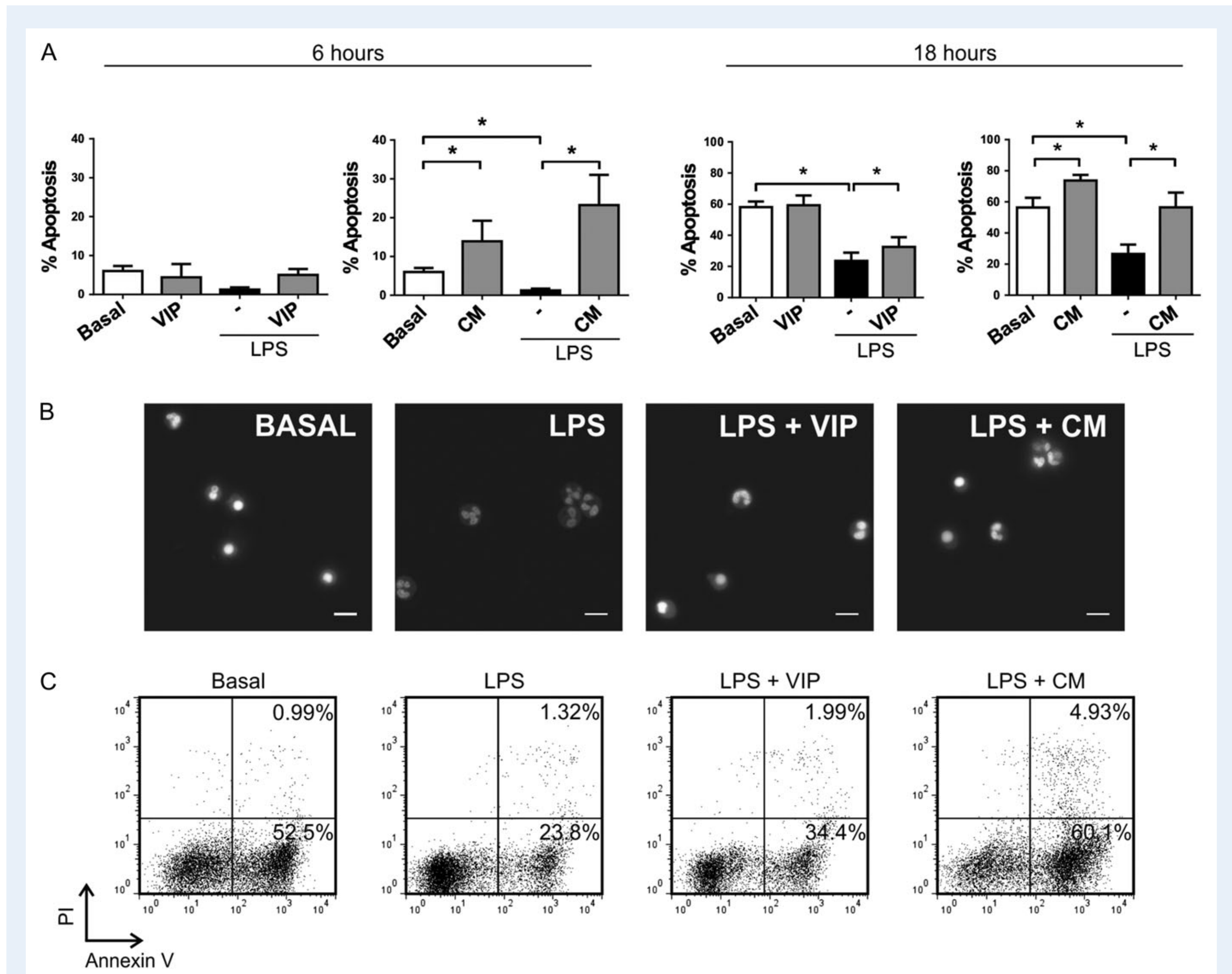

Figure 4 CM and VIP reverse the anti-apoptotic effect of LPS. (**A**) Neutrophils (5×10^5) were incubated for 6 or 18 h with 10 nM VIP or Swan-71 trophoblast CM in the presence or absence of 200 ng/ml lipopolysaccharide (LPS), and the percentage of apoptotic neutrophils was assessed as indicated in Materials and Methods. Values are mean ± SEM (*$P < 0.05$; $n = 7$, Kruskal–Wallis test). (**B**) Representative images of apoptotic neutrophils at 18 h in each condition as depicted in (A). Scale bars = 10 μm. (**C**) Neutrophils (5×10^5) were incubated for 18 h with 10 nM VIP or Swan-71 CM in the presence or absence of 200 ng/ml LPS, and the percentage of apoptotic neutrophils was assessed with Annexin/PI staining. Representative dot plots of four independent experiments are shown.

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presented intracellular chromatin decondensation but still preserved plasma membrane integrity, and could be clearly distinguished from apoptotic and viable nuclei. In this condition, a lower number of NETotic cells was paralleled by an increase in apoptotic and viable cells (Fig. 3A and B).

VIP and CM reverse the anti-apoptotic effect of LPS

We next examined the ability of VIP and CM to accelerate spontaneous neutrophil apoptosis and to interfere with the anti-apoptotic effect of LPS. LPS reduced by >50% the number of neutrophils undergoing spontaneous apoptosis and CM reversed the LPS effect, increasing the number of apoptotic neutrophils at 6 and 18 h (Fig. 4A–C). VIP reversed the LPS effect at 18 h. Of note, in the absence of LPS, CM but not VIP alone accelerated spontaneous neutrophil apoptosis at 6 and 18 h (Fig. 4A).

CM from VIP knocked-down cells fail to promote neutrophil apoptosis

To further test the role of VIP synthesized by trophoblast cells on neutrophil apoptosis, we studied spontaneous apoptosis of neutrophils incubated for 18 h with CM from scramble siRNA (control) or from VIP knocked-down trophoblast cells at two VIP siRNA level conditions (50 and 100 nM). We previously demonstrated a >60% reduction in VIP at the protein level in trophoblast cells with 100 nM siRNA (not shown) (Vota *et al.*, 2016). As shown in Fig. 5, CM from scramble siRNA transfected cells (control) enhanced neutrophil apoptosis, whereas CM from VIP-silenced cells did not modify apoptotic neutrophil number when compared to basal levels, suggesting a facilitating effect of endogenous trophoblast VIP on neutrophil apoptosis.

CM promote silent phagocytosis of apoptotic neutrophils

To assess the potential relevance of the deactivating and pro-apoptotic effects of CM on neutrophils for homeostasis maintenance, we analyzed its effect on apoptotic neutrophil phagocytosis by autologous monocytes (Fig. 6A). Monocytes plated overnight were then faced for 40 min to either neutrophils undergoing spontaneous apoptosis (basal) or apoptosis promoted by CM. Enhanced phagocytosis was observed when neutrophils were exposed to CM compared with neutrophils undergoing spontaneous apoptosis at either 6 or 18 h (Fig. 6B), being almost 40% higher at 18 h (Fig. 6C). Moreover, the production of the pro-inflammatory cytokine IL-12 did not increase upon phagocytosis of neutrophils cultured in the presence of CM, and only a trend (not significant) of an increase in IL-10 was observed (Fig. 6D).

Discussion

Neutrophils circulating through the intervillous space can become activated by placental oxidation metabolites, placental microvesicles and IL-8, among other stimuli. So far, local regulatory mechanisms that restrain their activation before they return to the maternal systemic circulation are still unclear. Results presented here support that VIP synthesized by trophoblast cells might have this role by preventing neutrophil activation and NET formation while increasing neutrophil apoptosis and their clearance by professional phagocytes. This conclusion is based on the following observations: First, VIP and trophoblast CM inhibited PMA-induced NET formation. The effect of trophoblast CM on NETosis was prevented by the VIP receptor antagonist. Second, VIP inhibited PMA-induced ROS formation, and the effect was mimicked by trophoblast CM containing VIP in the absence but not in the presence of an antagonist of VIP receptors. Third, VIP and CM enhanced neutrophil apoptosis or reversed the anti-apoptotic effect of LPS. The pro-apoptotic effect of CM was diminished by silencing VIP in trophoblast cells. Finally, neutrophils cultured in the presence of CM were more efficiently engulfed by autologous monocytes without pro-inflammatory cytokine production.

Neutrophils are short life span cells subjected to a complex network of cell survival and death signals (Gabelloni *et al.*, 2013). They undergo rapid spontaneous apoptosis in the absence of survival stimuli, and they are efficiently removed by phagocytic cells, such as macrophages or dendritic cells, with anti-inflammatory cytokine release as a key

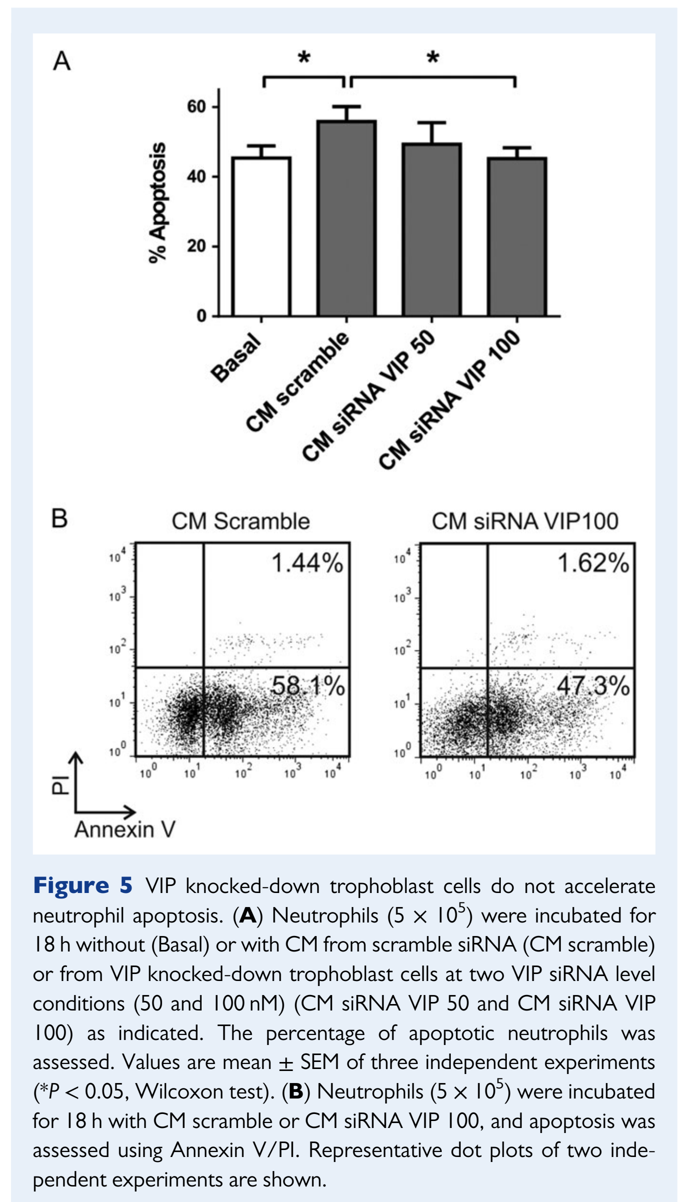

Figure 5 VIP knocked-down trophoblast cells do not accelerate neutrophil apoptosis. (**A**) Neutrophils (5×10^5) were incubated for 18 h without (Basal) or with CM from scramble siRNA (CM scramble) or from VIP knocked-down trophoblast cells at two VIP siRNA level conditions (50 and 100 nM) (CM siRNA VIP 50 and CM siRNA VIP 100) as indicated. The percentage of apoptotic neutrophils was assessed. Values are mean ± SEM of three independent experiments (*$P < 0.05$, Wilcoxon test). (**B**) Neutrophils (5×10^5) were incubated for 18 h with CM scramble or CM siRNA VIP 100, and apoptosis was assessed using Annexin V/PI. Representative dot plots of two independent experiments are shown.

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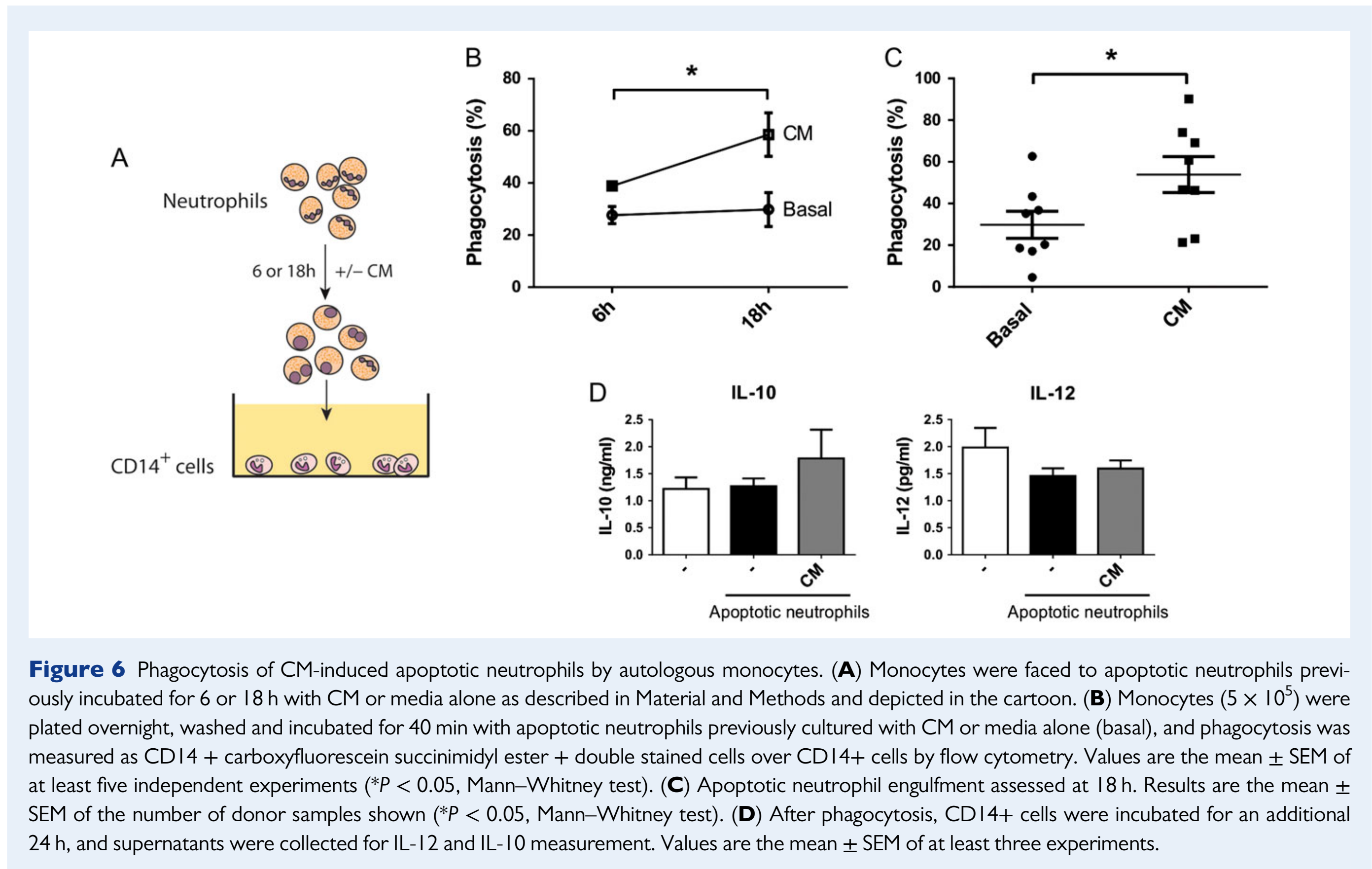

Figure 6 Phagocytosis of CM-induced apoptotic neutrophils by autologous monocytes. (**A**) Monocytes were faced to apoptotic neutrophils previously incubated for 6 or 18 h with CM or media alone as described in Material and Methods and depicted in the cartoon. (**B**) Monocytes (5×10^5) were plated overnight, washed and incubated for 40 min with apoptotic neutrophils previously cultured with CM or media alone (basal), and phagocytosis was measured as CD14 + carboxyfluorescein succinimidyl ester + double stained cells over CD14+ cells by flow cytometry. Values are the mean ± SEM of at least five independent experiments (*$P < 0.05$, Mann–Whitney test). (**C**) Apoptotic neutrophil engulfment assessed at 18 h. Results are the mean ± SEM of the number of donor samples shown (*$P < 0.05$, Mann–Whitney test). (**D**) After phagocytosis, CD14+ cells were incubated for an additional 24 h, and supernatants were collected for IL-12 and IL-10 measurement. Values are the mean ± SEM of at least three experiments.

mechanism in the resolution of inflammation. In contrast, neutrophils forming NETs induce M2 alternative activated macrophages to release pro-inflammatory mediators (Nakazawa *et al.*, 2016). Several cytokines including IL-8, tumor necrosis factor (TNF) and interferon (IFN)-γ can signal to induce NETosis (Brinkmann *et al.*, 2004; Kaplan and Radic, 2012). Both trophoblast microvesicles and IL-8 release in high amounts by trophoblast cells were described as potential triggers of NET formation in preeclampsia (Gupta *et al.*, 2005). Thus, it is conceivable that placental microvesicles or the high levels of IL-8 trigger NETosis in circulating neutrophils at the intervillous space and that anti-inflammatory signals emerge locally to prevent massive NET formation. Also, high levels of TNF and IFN-γ are detected in pregnancies complicated by strong pathogenic or damage stimuli (Hamilton *et al.*, 2012). These signals would increase neutrophil activation and impair pregnancy outcome if not timely overcome by a potent local modulation.

Our results add new evidence on VIP as a factor synthesized by trophoblast cells to modulate nearby neutrophil function. In this regard, a deactivating effect of trophoblast cells on neutrophil oxidant production capacity was demonstrated through an elegant design using single-cell assays to assess cell–cell interactions (Petty *et al.*, 2006). ROS plays a central role in initiating the NETosis programme, as demonstrated in patients with chronic granulomatous disease (Papayannopoulos and Zychlinsky, 2009; Remijsen *et al.*, 2011). Likewise, NETosis is inhibited by ROS scavengers (Metzler *et al.*, 2011). The fact that VIP inhibited both ROS synthesis and NETosis when added together with a potent stimulus like PMA and at a range of concentrations present in trophoblast CM strongly supports that trophoblast VIP could down-modulate neutrophil activation *in vivo*.

Moreover, here we showed that VIP and trophoblast CM accelerated neutrophil spontaneous apoptosis and reversed the anti-apoptotic effect of LPS. In support of a role of this neuropeptide in the pro-apoptotic effect of trophoblast CM, neutrophil apoptosis decreased when neutrophils were incubated with VIP knocked-down trophoblast cells. VIP did not modify caspase 3 activity in human neutrophils (Djanani and Kahler, 2002), but it accelerated neutrophil apoptosis determined with an ATP viability assay in responsive subjects (Abdalla, 2010). It is noteworthy that 10 nM VIP alone was not as potent as CM to promote apoptosis and to inhibit NETosis, strongly suggesting that additional factors released by trophoblast cells are also involved. Accordingly, knocking-down VIP in trophoblast cells not only resulted in a reduced pro-apoptotic effect on neutrophils but also in an impaired trophoblast migration and interaction with monocytes (Vota *et al.*, 2016), pointing to the convergence of trophoblast VIP with other trophoblast mediators to maintain homeostasis throughout pregnancy. Finally, CM increased the amount and the rate of apoptotic neutrophil engulfment by monocytes without IL-12 release, reinforcing the anti-inflammatory nature of the effects elicited by trophoblast-derived factors. The present novel mechanism mediated by VIP might provide new clues for immune and trophoblast cell pharmacological targeting in pregnancy. Consistently, VIP treatment increased anti-inflammatory mediator expression and improved pregnancy outcome in the CBAxDBA resorption-prone mouse model (Gallino *et al.*, 2016). The inhibition of NET formation by trophoblast VIP and its priming effect on neutrophil apoptosis might reflect an active mechanism to deactivate and remove neutrophils, thus contributing to the synergy of local signals induced at the placenta to maintain immune homeostasis.

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Authors' roles

G.C., F.S. and D.P. carried out neutrophil functional characterization, trophoblast co-cultures and phagocytosis. D.V. transfected trophoblast cells and obtained CM. A.T. obtained and analyzed confocal images. R.R., A.T. and C.P.L. designed the whole work, analyzed the results and prepared the manuscript.

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Conflict of interest

None declared.

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