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ORIGINAL RESEARCH

Trophoblast cells primed with vasoactive intestinal peptide enhance monocyte migration and apoptotic cell clearance through αvβ3 integrin portal formation in a model of maternal–placental interaction

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STUDY HYPOTHESIS: Is apoptotic cell phagocytosis by monocytes modulated by pathways elicited by vasoactive intestinal peptide (VIP) action on trophoblast?

STUDY FINDING: Targeting trophoblast cells with VIP induces monocyte migration, polarization to anti-inflammatory phenotypes and apoptotic trophoblast cell clearance which involves increased αvβ3 integrin expression on phagocytic cells and binding to thrombospondin 1.

WHAT IS KNOWN ALREADY: Monocytes recruited to the maternal–placental interface interact with trophoblast cells and differentiate to alternatively activated macrophages involved in the silent clearance of apoptotic cells. Vasoactive intestinal peptide (VIP) is an immunomodulatory polypeptide synthesized at the human placenta that can target both trophoblast cells and monocytes/macrophages. Integrin αvβ3 and thrombospondin 1 are involved in the formation of a phagocytic portal for the immunosuppressant clearance of apoptotic cells.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: This is a laboratory-based study studying monocytes isolated from peripheral blood of healthy women ($n = 33$) and their interaction *in vitro* with first trimester trophoblast cell lines. Peripheral blood monocytes were isolated from healthy volunteers by Percoll gradient and tested in co-culture settings with first trimester trophoblast cell lines (Swan 71 and HTR8) or with trophoblast cell conditioned media obtained in the presence or absence of 10 or 100 nM VIP. The effect of VIP-conditioned media on monocyte migration was assessed through transwell systems and monocyte/macrophage phenotype was determined by flow cytometry. Phagocytosis of apoptotic cells and the mechanisms involved in phagocytic portal formation were assessed by flow cytometry, confocal microscopy, immunological blockade and RT–PCR.

MAIN RESULTS AND THE ROLE OF CHANCE: Exposing cells to 100 nM VIP increased the migration of monocytes toward trophoblast cell conditioned media (VIP conditioned medium) ($P < 0.05$ versus conditioned media from cells not exposed to VIP) and contributed to the monocytes acquiring an anti-inflammatory profile with increased CD39 and IL-10 expression ($P < 0.05$). Phagocytosis of apoptotic trophoblast cells by monocytes and monocyte-differentiated macrophages was increased by VIP conditioned medium ($P < 0.05$ versus media conditioned in the absence of VIP or direct addition of 100 nM VIP). The boosting effect of VIP conditioned medium on phagocytosis involved increased expression and re-localization of αvβ3 integrin on phagocytic cells along with enhanced expression of thrombospondin 1 on trophoblast cells.

LIMITATIONS, REASONS FOR CAUTION: The conclusions are based on *in vitro* experiments with monocytes drawn from peripheral blood of healthy individuals and trophoblast cell lines and we were unable to ascertain that these mechanisms operate similarly *in vivo*. We cannot rule out a differential behavior of either trophoblast cells targeted *in vivo* with VIP, or primary cultures of first trimester trophoblast cells assayed *in vitro*.

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WIDER IMPLICATIONS OF THE FINDINGS: The results presented provide new clues for immune and trophoblast cell pharmacological targeting in pregnancy complications of immunopathologic nature.

STUDY FUNDING/COMPETING INTEREST(S): This work was funded by the National Agency of Sciences and Technology ANPCyT (PICT 2011-0144), National Research Council CONICET (PIP 602/2012) and University of Buenos Aires (UBACyT 20020130100040BA) to C.P.L. The authors have no conflicts of interest to disclose.

Key words: vasoactive intestinal polypeptide / monocytes / apoptotic cell phagocytosis / thrombospondin 1 / apoptotic trophoblast cells / maternal–placental interface

Introduction

Monocytes are recruited, in large numbers, to the pregnant uterus in the first trimester of pregnancy where they differentiate to a predominant M2 alternative activation profile and contribute to the silent clearance of apoptotic cells generated during the intense vascular remodeling process of placentation (Abrahams *et al.*, 2004; Renaud and Graham, 2008; Nagamatsu and Shust, 2010; Svensson-Arvelund *et al.*, 2015). Monocyte migration and differentiation is coordinated by trophoblast cells ensuring the switch of the pro-inflammatory microenvironment characteristic of implantation and early placentation to an anti-inflammatory/tolerogenic predominant profile required for fetal growth (Mor and Cardenas, 2010). Consistently, a deregulated persistent inflammatory response at early placentation associates with pregnancy complications (Girardi *et al.*, 2006; Kwak-Kim *et al.*, 2009; Redman and Sargent, 2010; Brown *et al.*, 2014) whereas macrophages expressing an M1 predominant phenotype were shown to decrease cytotrophoblast invasiveness *in vitro* (Renaud *et al.*, 2005). Thrombospondin 1 (TSP-1) and αvβ3 integrin were described as part of the intricate network of 'eat-me' signals and phagocytic receptors that form the phagocytic portal, an engulfing gate within the macrophage for the immunosuppressant clearance of apoptotic cells (Savill *et al.*, 1992; Stern *et al.*, 1996; Hughes *et al.*, 1997). However, their involvement in apoptotic trophoblast cell clearance mediated by monocytes/macrophages at the human maternal–placental interface has not been described so far.

Vasoactive intestinal peptide (VIP) is an endogenous polypeptide with regulatory actions at multiple levels through targeting immune, neuronal and endocrine interactions (Harmar *et al.*, 2012). Its binding to high affinity VIP receptors (VPAC) on monocytes and macrophages induces anti-inflammatory and immunosuppressant effects (Delgado *et al.*, 1999; Arranz *et al.*, 2008; Carrión *et al.*, 2011). VIP deserves special interest at the maternal–placental interface since it modulates trophoblast function (Marzioni *et al.*, 2005) as well as immunosuppressant mediator release and Treg cell induction in both murine pregnancy models and *in vitro* settings with human peripheral blood mononuclear cells (Fraccaroli *et al.*, 2009; Larocca *et al.*, 2011; Hauk *et al.*, 2014a). Immunosuppressant factors, namely interleukin 10 (IL-10), indoleamine 2,3-dioxygenase, prostaglandin E2, among others, are induced and locally released at high levels at the end of the first trimester and have been proposed as key mediators to promote the switch from a predominant pro-inflammatory to an anti-inflammatory microenvironment required for fetal growth (Mor and Cardenas, 2010; Perez Leirós and Ramhorst, 2013).

On this basis, we hypothesized that VIP acting on trophoblast regulated mechanisms might promote monocyte migration and differentiation into regulatory phenotypes as well as increases phagocytosis of apoptotic cells involving typical phagocytic bridging structures. Here by means of co-culture designs with purified monocytes from individual donors and two first trimester trophoblast cell lines (Swan 71 and HTR8) as an *in vitro* model of maternal–placental interaction, we present evidence to demonstrate that targeting trophoblast cells with VIP increases monocyte migration toward trophoblast conditioned media and enhances apoptotic trophoblast cell phagocytosis by monocytes through thrombospondin 1 and αvβ3 integrin portal formation. Moreover, and particularly relevant for homeostasis maintenance at placentation, VIP also promoted CD39 and IL-10 expression on monocytes without increasing pro-inflammatory marker expression.

Materials and Methods

Blood samples

Blood samples were taken from healthy volunteers, all women of reproductive age ($n = 33$), who had received no pharmacological treatment for at least 10 days before the day of sampling. Blood was obtained by puncture of the forearm vein, and it was drawn directly into heparin containing plastic tubes. 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.

Monocyte isolation and differentiation to macrophages

Peripheral blood mononuclear cells were isolated from individual subjects by Ficoll-Hypaque (GE Healthcare, Sweden) and CD14+ cells were separated by Percoll gradient (GE Healthcare, Sweden), both according to the manufacturer's protocol. Cell population purity (>80%) was checked by flow cytometry analysis with CD14 labeling as previously (Hauk *et al.*, 2014b). For granulocyte-macrophage colony-stimulating factor (GM-CSF) differentiated macrophages, monocytes (5×10^5 cells) were incubated with 100 ng/ml GM-CSF (Immunotools, APBiotech, Argentina) in *Roswell Park Memorial Institute medium* (RPMI) 1640 HyClone Laboratories (Logan, UT, USA) for 5 days as described (Borge *et al.*, 2015).

Trophoblast cell conditioned media

The two trophoblast cell lines from human first trimester pregnancies used here were the Swan 71 cell line, derived by telomerase-mediated transformation of a 7-week cytotrophoblast isolate (Aplin *et al.*, 2006; Straszewski-Chavez *et al.*, 2009) and the HTR8 cell line, derived from transformed extravillous trophoblast. Both were kindly given by Dr Gil Mor (Yale University, New Haven, USA). To obtain conditioned media (CM), Swan 71 or HTR8 cells were cultured for 20 h in 24-well flat-bottom polystyrene plates with Dulbecco's modified Eagle's medium (DMEM:F12) containing 2% (v/v) fetal calf serum (FCS) (Life Technologies, Buenos Aires, Argentina) in the absence (CM) or presence of 10 or 100 nM VIP (Polypeptide, France) (CM

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VIP 10/100 nM). VIP did not modify the viability of trophoblast cells tested by the colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) and trypan blue. Trophoblast cell CM were collected and stored at −20°C until used. To assess that adherent trophoblast cells were not removed during the CM collection procedure, RNA levels were determined and they were below the detection limit.

CD14+ cell cultures and co-cultures

Monocytes were cultured in 24-well polystyrene plates (5×10^5 cells/well) in DMEM:F12 2% FCS in the presence or not of 100 nM VIP, or with CM, CM VIP 10 nM or CM VIP 100 nM for 20 h. In some experiments, co-cultures were carried out with monocytes (5×10^5 cells/well) and 60% confluent Swan 71 trophoblast cells in 24-well flat-bottom polystyrene plates in complete DMEM:F12/10% FCS (Life Technologies) with or without 10 or 100 nM VIP for 20 h. All experiments were run in duplicates unless otherwise stated. Trophoblast cell viability was not affected by VIP during the incubation times used.

RT–PCR and RT–qPCR

Chemokines: Monocyte chemotactic protein-1 (MCP-1), Macrophage Inflammatory Protein-1α (MIP-1α), Interleukin 8 (IL-8) and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) and thrombospondin-1 expression were determined by RT–PCR and RT–qPCR respectively, as previously described (Grasso *et al.*, 2014; Hauk *et al.*, 2014b). Briefly, total RNA was isolated with Trizol reagent following manufacturer recommendations (Life Technologies, Grand Island, NY, USA), cDNAs were generated from 1 μg of RNA using a Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, RNAsin RNAse inhibitor and oligodT kit (Promega Corporation, Madison, WI, USA) and stored at −20°C for batch analysis. The sample volume was increased to 25 μl with the solution containing 50 mM KCl; 10 mM Tris (pH 8.3); 1.5 mM $MgCl_2$; 0.1 mM forward and reverse primers (described in Table I) and 1 U Taq polymerase (Invitrogen, Life Technologies, Grand Island, NY, US) in a DNA Thermocycler (PerkinElmer/Cetus, Boston, MA, USA). The primers and the thermal profile were selected with PrimerBlast software (www.ncbi.nlm.nih.gov/ tools/primer-blast/). The corresponding PCR programs are described in Table I. PCR products were electrophoresed through a 2% ethidium bromide-stained agarose gel, visualized by transillumination and scanned. Densitometry was performed using ImageJ 1.47 software (NIH, USA) and results expressed as arbitrary units normalized to Glyceraldehyde-3-phosphate dehydrogenase expression.

For RT–qPCR master mix (Biodynamics, Buenos Aires, Argentina) was used according to the manufacturer's recommendation and 1:30 000 dilution of SybrGreen. Real-time PCR was performed on a Bio-Rad iQ5 Real-time PCR system. The relative gene expression levels were determined using the threshold cycle (CT) method ($2^{-\Delta\Delta CT}$ method) with reference to the endogenous GAPDH control.

Flow cytometry analysis and ELISA

Monocytes/macrophages were cultured with VIP, CM or co-cultured with Swan-71 cells and after 20 h cells were recovered by TrypLe treatment (Invitrogen Life Technologies, Grand Island, NY, USA) and stained with Fluorescein Isothiocyanate (FITC-), Phycoerythrin (PE-), Phycoerythrin-Cyanine5 (PECy5-) or Allophycocyanin (APC-) conjugated mAbs directed to CD14, CD86, Human Leukocyte Antigen-DR (HLA-DR), Interleukin (IL-12), Tumor Necrosis Factor alpha (TNF-α), CD39, CD16 and IL-10 (BD Pharmingen, San Diego, CA, USA) or CD40 (Immunotools, AP Biotech, Argentina). For intracellular cytokine detection Stop Golgi was added to the medium in the last 4 h of co-culture following manufacturer's instructions (Becton Dickinson, San José, CA, USA) to promote intracellular accumulation. Then cells were recovered and, after washing with Phosphate-Buffer Saline (PBS)-2% FCS, they were stained with mAb anti-superficial molecules, washed, fixed and permeabilized with the Fix/Perm kit as manufacturer recommended (Becton Dickinson). Permeabilized cells were incubated for 30 min with IL-10, IL-12 or TNF-α. Cells were finally washed with PBS-2% FCS. When RANTES synthesis was assayed on trophoblast cells 50 000 Swan71 cells were stained with anti-RANTES Ab (BD Pharmingen).

Table I **Primer sequences and annealing temperature used in RT–PCR reactions.**

Macrophage Inflammatory Protein-1α (MIP-1α)		
Sense	5′-TTCAGACTTCAGAAGGACAC-3′	Annealing T(°C) 62
Antisense	5′-TGAGCAGGTGACGGAATG-3′	
Monocyte chemotactic protein-1 (MCP-1)		
Sense	5′-CAGCAGCAAGTGTCCCAAAG-3′	Annealing T(°C) 64
Antisense	5′-GAGTGAGTGTTCAAGTCTTCGG-3′	
Regulated on Activation, Normal T cell Expressed and Secreted (RANTES)		
Sense	5′-TGCTGCTTTGCCTACATTGC-3′	Annealing T(°C) 64
Antisense	5′-AAGACGACTGCTGGGTTGG-3′	
Interleukin 8 (IL-8)		
Sense	5′-CCAACACAGAAATTATTGTAAAGC-3′	Annealing T(°C) 62
Antisense	5′-CACTGGCATCTTCACTGATTC-3′	
Thrombospondin 1(TSP-1)		
Sense	5′-CCTGATGGAGAATGCTGTC-3′	Annealing T(°C) 52
Antisense	5′-CGGTTGTTGAGGCTATCG-3′	
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)		
Sense	5′-TGATGACATCAAGAAGGTGGTGAAG-3′	Annealing T(°C) 62
Antisense	5′-TCCTTGGAGGCCATGTAGGCCAT-3′	

5′-3′oligonucleotide primers indicated below were designed using the online tool Primer3® (Whitehead Institute for Biomedical Research).

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Ten thousand events were acquired in a FACS Aria II cytometer® (Becton Dickinson) and results were analyzed using FlowJo software (http://www.flowjo.com/). Results were expressed as the percentage of the respective population and the quadrant was set using irrelevant isotype specific Ab and were expressed as MFI or double positive cell frequencies by flow cytometry. Particularly for monocyte profile, positive cells were determined inside the electronically gated CD14 positive cell population previously selected in Forward Scatter (FSC) versus Side Scatter (SSC). Enzyme-Linked ImmunoSorbent Assay (ELISA) was performed to determine IL-8 and MCP-1 levels in CM in the presence or not of 100 nM VIP in triplicates as previously described (Paparini et al., 2015).

Migration assays

Migration assays were performed in 24 *Transwell* plates across 5 μm polycarbonate membranes (Costar, Corning Incorporated, NY, USA). Experiments were run in triplicates. Monocytes (2×10^5 cells) were re-suspended in DMEM containing 2% FCS and placed on the upper chamber. The lower chamber contained 600 μl of DMEM 2% FCS (basal condition), 10 or 100 nM VIP or CM or CM-VIP (10 and 100 nM) as indicated. After incubation for 2 h at 37°C, CD14+ cells in the lower chamber were recovered and counted with a FACS Aria II cytometer® (Becton Dickinson).

Apoptosis of trophoblast cells and neutrophils

Swan 71 cells were treated with 3 μM camptothecin during 16 h as previously described (Sóñora et al., 2014). Apoptotic Swan 71 were collected and stained with Carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies, Buenos Aires) following manufacturer's recommendations. Briefly, 3 μM of CFSE/10^6 cells during 10 min in RPMI without FCS. Excess of CFSE was eliminated by serial washes with DMEM:F12 10% FCS. Neutrophils were obtained after Ficoll-Hypaque gradients and subsequent Dextran purification (Fuxman Bass et al., 2008; Gabelloni et al., 2013). Apoptotic neutrophils were obtained by spontaneous apoptosis after 16 h incubation in RPMI 1640 and stained with as above. The percentage of apoptosis was 40–50% for trophoblast cells and 50% for neutrophils determined by annexin-propidium iodide staining and flow cytometry detection.

Phagocytosis assays

Monocytes (5×10^5) conditioned for 20 h with DMEM:F12 2% FCS in the presence or not of VIP (10 or 100 nM) or CM, or CM-VIP (10 or 100 nM) were challenged with apoptotic trophoblast cells or neutrophils in a 3:1 or 10:1 ratio with respect to monocytes, respectively. After 2 h (apoptotic trophoblast cells) or 40 min (apoptotic neutrophils) of phagocytosis, monocytes were collected, stained with CD14 and the percentage of CD14/CFSE double positive cells was analyzed by flow cytometry as described (Hauk et al., 2014b). To analyze the effect of the antibody against thrombospondin 1 on phagocytosis, monocytes conditioned with CM (VIP 100 nM) were incubated for 30 min with 0.1 ng/ml anti-thrombospondin 1 antibody (Abcam, MA, USA) and then challenged with apoptotic cells as above. Results were analyzed by flow cytometry.

Confocal microscopy

Monocytes (2×10^5) were plated and cultured over glass slides with CM (VIP 100 nM) for 20 h at 37°C. Conditioned monocytes were challenged or not with apoptotic CFSE+ Swan 71 cells during 2 h. Cells were washed with PBS, fixed with methanol and permeabilized using PBS 1% Bovine Serum Albumin (BSA) 0.5% saponin buffer during 15 min. Slides were incubated overnight at 4°C with anti-αvβ3 integrin antibody (Abcam), washed and incubated with rhodamine triC-conjugated secondary antibody anti-mouse IgG for 2 h. Microphotographs were acquired using an FV 300® Olympus coupled to non-inverted fluorescence microscope Olympus BX61 (Olympus, Center Valley, PA, USA) and Fluoview Software FV-ASW 4.1 Viewer. Negative control was performed in the absence of anti-αvβ3 integrin Ab and images shown were at 120×.

Statistical analysis

The significance of the results was analyzed by Student's *t* test or Mann-Whitney test for nonparametric samples. When multiple comparisons were necessary ANOVA of two way factors or the Wilcoxon test were used with *post hoc* tests Bonferroni or Holm-Sidak. Differences between groups were considered significant at $P < 0.05$ using the GraphPad Prism4 software (GraphPad, San Diego, CA, USA).

Results

VIP boosts monocyte migration toward trophoblast cells

Based on the prominent role of monocyte recruitment and differentiation to macrophages with an M2 alternative activation profile in a trophoblast-coordinated manner during early pregnancy, we first analyzed the effect of VIP to modulate monocyte migration toward trophoblast cells. As shown in Fig. 1A Swan 71 trophoblast cell conditioned media increased monocyte cell migration and the effect was further induced when trophoblast cells were pretreated overnight with 100 nM VIP. VIP alone did not induce monocyte migration at any concentration tested pointing to trophoblast-derived factors involved. To identify the mechanism mediated by VIP-induced trophoblast cell factors, its effect on chemokine expression in trophoblast cells was determined. Figure 1B shows that MCP-1, MIP-1α, IL-8 and RANTES mRNA expression was rapidly induced in trophoblast cells by VIP providing suitable signals for monocyte migration. To ascertain whether the observed chemokine mRNA induced expression was related to functional levels of monocyte attracting chemokines, we assayed IL-8 and MCP-1 levels by ELISA and RANTES by flow cytometry. IL-8 concentration in CM was 12.7 ± 1.3 ng/ml and did not further increase confirming previous observations that trophoblast cells produce high functional levels of this chemokine (Fest et al., 2007). MCP-1 levels in CM increased from 0.97 ± 0.1 ng/ml to 1.6 ± 0.1 ng/ml when the trophoblast cells were stimulated with 100 nM VIP ($P < 0.05$, $n = 3$). RANTES synthesis in Swan 71 trophoblast cells was also up-regulated with VIP as indicated by a 31.7% and a 1.2 fold increase of MFI and frequency of positive cells, respectively.

VIP-induced trophoblast factors favor an anti-inflammatory profile on monocytes

Monocytes migrate toward trophoblast cells and they are susceptible to differentiation through binding of soluble and contact factors (those involved in the direct interaction) in a trophoblast-coordinated manner. On this basis, we next analyzed the effect of VIP, trophoblast cells and trophoblast conditioned media on cytokines and other markers expressed by CD14+ cells. We analyzed the expression of surface markers of activation on monocytes (CD40, HLA-DR, CD86) as well as two pro-inflammatory cytokines (IL-12p40, TNF-α) and two anti-inflammatory markers (IL-10 and CD39). Figure 2 shows that neither conditioned media (CM) nor contact factors on trophoblast cells alone or pre-incubated with VIP increased the expression of CD40, HLA-DR, CD86, IL-12p40 or TNF-α. In fact, IL-12p40 and TNF-α actually decreased their expression in some conditions with

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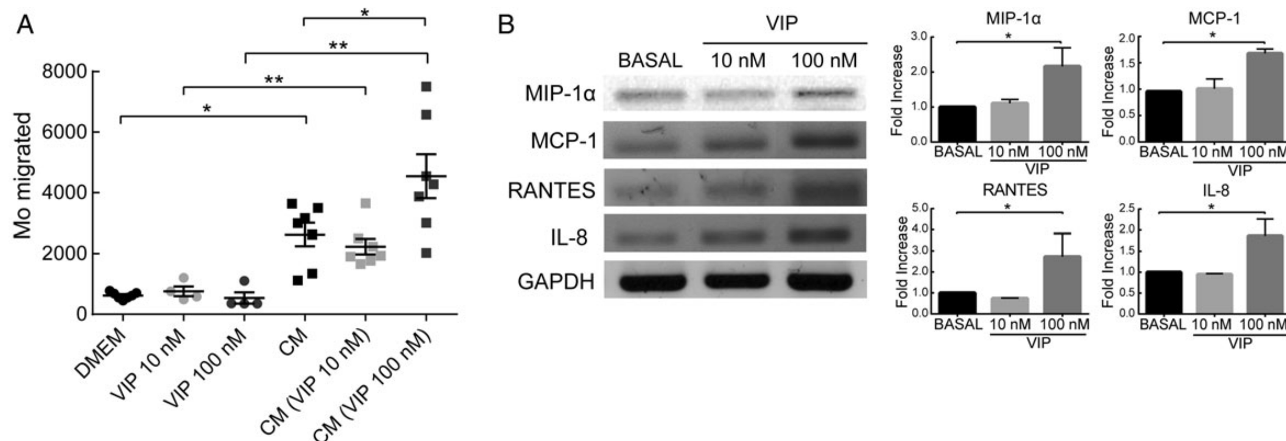

Figure 1 Addition of vasoactive intestinal peptide (VIP) to trophoblast cells induces monocyte migration toward trophoblast conditioned media. (**A**) Monocytes (Mo; 2×10^5 cells) were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum (FCS) and placed on the upper chamber of transwell systems. The lower chamber contained 600 µl of DMEM 2% FCS (basal condition) with 10 nM VIP, 100 nM VIP, trophoblast conditioned media (CM) or CM obtained in the presence or absence of 10 and 100 nM VIP (CM VIP). After 2 h at 37°C cells in the lower chamber were collected and analyzed by flow cytometry. Each point per condition represents an independent experiment. Results of monocytes migrated are expressed as mean ± SEM of the experiments shown. *$P < 0.05$, **$P < 0.01$. (**B**) Swan 71 cells were plated and treated or not (BASAL) with VIP (10 or 100 nM) during 20 h. Trophoblast cells were harvested and the expression of MCP-1, MIP-1α, RANTES and IL-8 mRNAs normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed by RT–PCR as indicated in Materials and Methods. Values represent fold increase as mean ± SEM of at least three experiments. *$P < 0.05$.

CM or CM with VIP. In contrast, the contact of monocytes with trophoblast cells or with trophoblast cell conditioned media induced the expression of IL-10 and CD39 and VIP further promoted this anti-inflammatory phenotype expression. Moreover, VIP also decreased the expression of CD16 on monocytes in the same settings when compared with CM and with basal values (% CD14+ and CD16+: Basal: 16.4 ± 2.1; CM: 27.5 ± 3.6; CM-VIP100 nM: 8.7 ± 1.7; $P < 0.05$). To determine whether the effect was attributable only to the Swan 71 first trimester trophoblast cell line, we also tested the effect of HTR8 cell line conditioned media with or without VIP on CD14+ cell differentiation. Table II shows that the modulatory effect of VIP on monocyte profile (CD86, CD40, HLA-DR and CD39) was also observed with HTR8 cell conditioned media.

VIP-elicited factors increase apoptotic cell phagocytosis by monocytes

Phagocytosis of apoptotic cells by macrophages with immunosuppressant mediator release is critical at placentation to overcome the inflammatory response triggered by potential intracellular leakage and cell debris. To explore whether conditioned medium from trophoblast cells incubated with VIP could modulate apoptotic cell phagocytosis by monocytes, we used two different apoptotic cells, trophoblast cells and neutrophils. Monocytes were pretreated overnight with trophoblast conditioned media prepared in the absence or presence of VIP (10 or 100 nM) and then incubated with apoptotic cells in a 3:1 (trophoblast cells:CD14+ cells) or a 10:1 (neutrophils:CD14+ cells) relationship, the latter was used as the standard phagocytotic assay of apoptotic cells. Figure 3A and B shows that trophoblast conditioned media was effective to increase apoptotic cell phagocytosis and that VIP preconditioning further increased phagocytosis compared with trophoblast conditioned media. The effect of the conditioned media prepared with VIP was seen either with apoptotic trophoblast cells or neutrophils, although VIP was more potent to induce apoptotic neutrophil phagocytosis since 10 nM was effective only in neutrophils but not in trophoblast cells. VIP alone added overnight to phagocytic cells did not modify apoptotic cell engulfment supporting the promoting role of trophoblast cell derived factors in the effect.

VIP-trophoblast conditioned media increases monocyte-derived macrophage phagocytosis

Finally, on the knowledge that GM-CSF differentiates monocytes to macrophages favoring the expression of a predominant inflammatory profile (Ambarus *et al.*, 2012), we next explored whether the modulatory effect of VIP-induced factors was also seen in GM-CSF differentiated macrophages. As seen with monocytes, macrophages conditioned with trophoblast media in the presence of VIP increased IL-10 production with a trend to diminish IL-12 and no changes in CD86 (Fig. 4A). Furthermore, fig. 4B shows a higher number of CD14+CFSE positive cells induced by VIP-trophoblast treatment, confirming that the increased phagocytosis displayed by monocytes was conserved after differentiation to macrophages.

The effect of VIP on phagocytosis involves TSP-1 and αvβ3 integrin expression and bridging

To identify potential bridging molecules induced by VIP in monocytes and trophoblast cells that could be responsible for the higher phagocytotic effect observed, we first explored the expression of TSP-1 and αvβ3 integrin. Figure 5A shows that the incubation of monocytes overnight with

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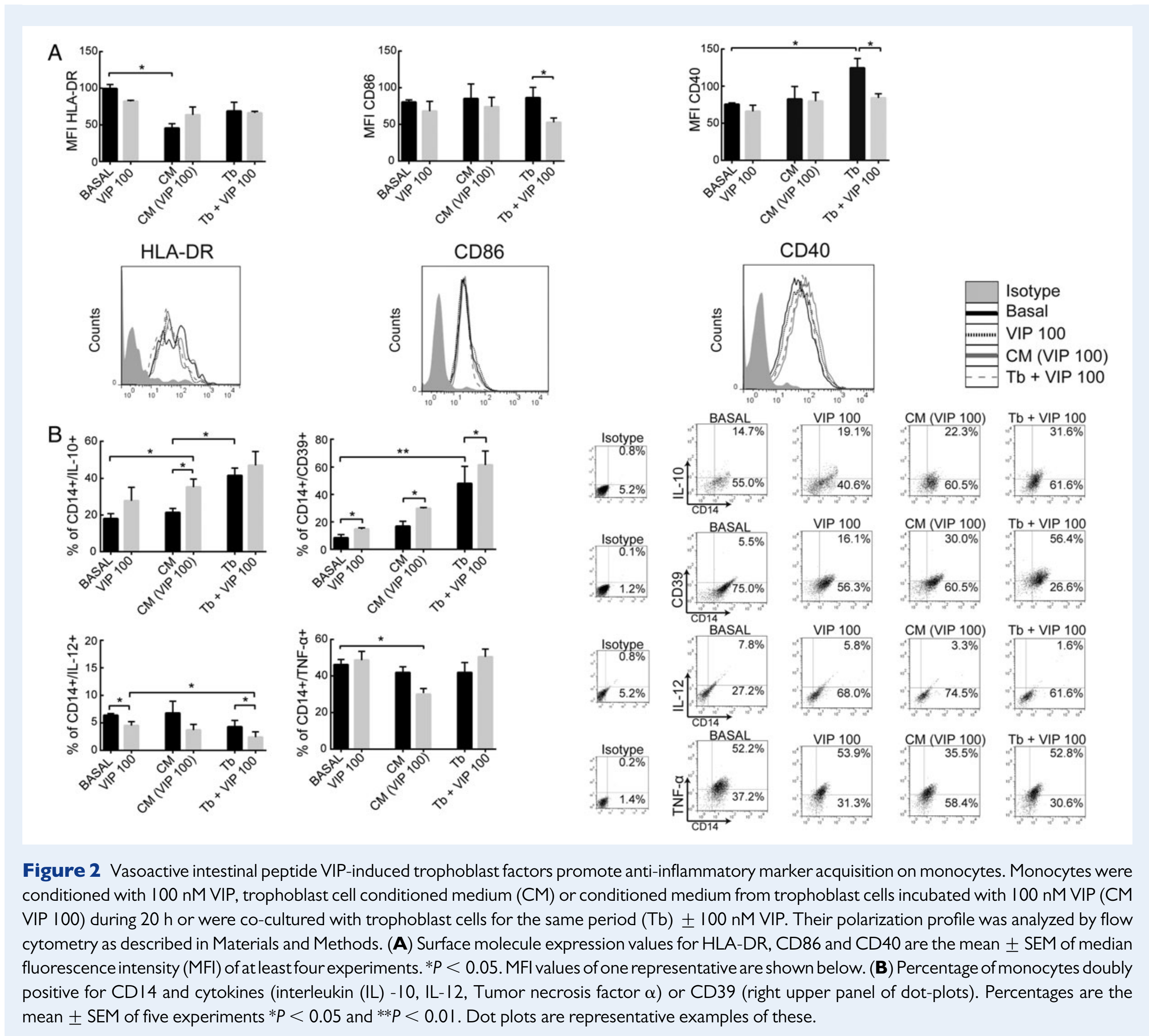

Figure 2 Vasoactive intestinal peptide VIP-induced trophoblast factors promote anti-inflammatory marker acquisition on monocytes. Monocytes were conditioned with 100 nM VIP, trophoblast cell conditioned medium (CM) or conditioned medium from trophoblast cells incubated with 100 nM VIP (CM VIP 100) during 20 h or were co-cultured with trophoblast cells for the same period (Tb) ± 100 nM VIP. Their polarization profile was analyzed by flow cytometry as described in Materials and Methods. (**A**) Surface molecule expression values for HLA-DR, CD86 and CD40 are the mean ± SEM of median fluorescence intensity (MFI) of at least four experiments. $*P < 0.05$. MFI values of one representative are shown below. (**B**) Percentage of monocytes doubly positive for CD14 and cytokines (interleukin (IL) -10, IL-12, Tumor necrosis factor α) or CD39 (right upper panel of dot-plots). Percentages are the mean ± SEM of five experiments $*P < 0.05$ and $**P < 0.01$. Dot plots are representative examples of these.

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Table II Effect of vasoactive intestinal peptide (VIP) and HTR8 cell conditioned media on monocyte marker expression.

	Median fluorescence intensity			% CD14+ CD39
	HLA-DR	CD86	CD40	
BASAL	94.6 ± 8.4	91.2 ± 20.1	78.3 ± 1.8	11.4 ± 0.8
VIP 100 nM	82.2 ± 12.2	64.2 ± 12.2	55.1 ± 7.0	18.6 ± 0.9*
CM	47.3 ± 16.4*	83.5 ± 6.7	67.8 ± 22.9	21.8 ± 1.0*
CM VIP 100 nM	57.7 ± 20.4	70.3 ± 4.1	57.1 ± 19.6	29.9 ± 4.1**,#

Surface molecules Human Leucocyte Antigen (HLA)-DR, CD40, CD86 and CD39 were determined on monocytes by cytometry as medium fluorescent intensity (MFI) or the percentage of double positive (CD14+ and CD39+) cells after incubation with 100 nM VIP or with conditioned medium obtained from HTR8 cells cultured without (CM) or with 100 nM VIP (CM VIP 100 nM)) during 20 h. Results are expressed as mean ± SEM from at least five experiments. $*P < 0.05$, $**P < 0.01$ versus BASAL; $^{\#}P < 0.05$ versus CM.

either VIP alone (100 nM) or trophoblast conditioned media increased the expression of αvβ3 integrin on phagocytic cells with the highest effect obtained with VIP-trophoblast conditioned media. Moreover, the re-localization of αvβ3 integrin on monocyte membrane upon contact with apoptotic cells was also observed. On the other hand, VIP increased TSP-1 expression on trophoblast cells in a concentration-

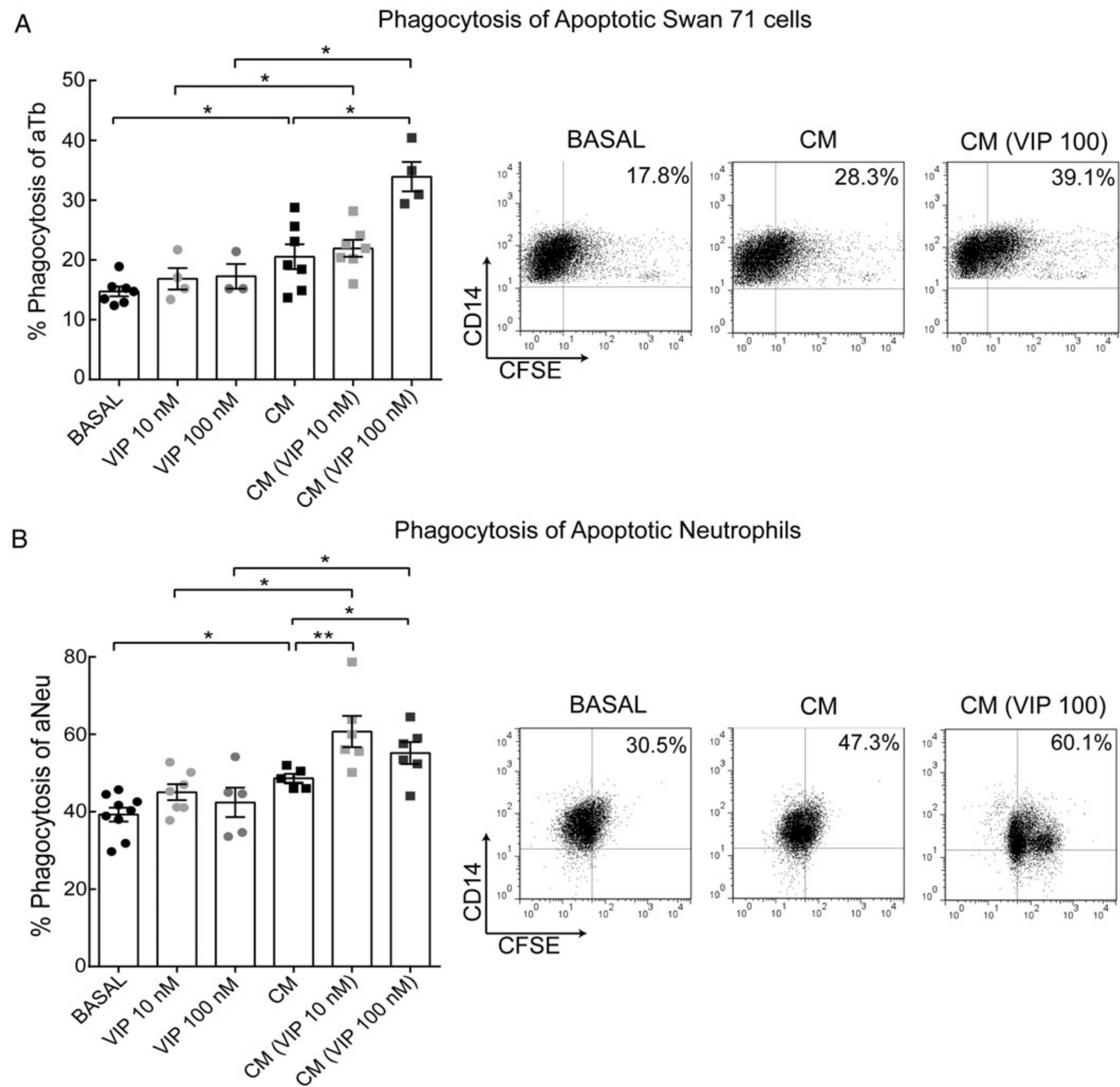

Figure 3 Conditioned media from trophoblast cells incubated with vasoactive intestinal peptide (VIP) enhance apoptotic cell phagocytosis by monocytes. Monocytes (5×10^5) incubated with Dulbecco's modified Eagle's medium (DMEM): F12 2% fetal calf serum (BASAL) or with 10 and 100 nM VIP or trophoblast cell conditioned medium (CM) or conditioned medium from trophoblast cells incubated with 10 or 100 nM VIP (CM VIP 10 or 100 nM) during 20 h were challenged with (**A**) apoptotic trophoblast cells (aTb) (3:1) during 120 min or (**B**) with apoptotic neutrophils (aNeu) (10:1) during 40 min both stained with CFSE. Monocytes were collected and stained with CD14 and the percentage of CD14/CFSE positive cells (right upper panel) was analyzed by flow cytometry. Each point per condition represents an independent experiment. Values are mean ± SEM of the number of experiments indicated. *$P < 0.05$ and **$P < 0.01$. Representative dot plots are shown.

dependent manner (Fig. 5B). Next, we analyzed whether TSP-1 induced upon stimulation with VIP was involved in the effect. The immunological blockade of TSP-1 with a monoclonal antibody completely prevented the effect of VIP on apoptotic cell phagocytosis, consistent with the enhanced αvβ3 integrin expression and the requirement of TSP-1 to form the phagocytic portal (Fig. 5C).

Discussion

Macrophages represent 20–30% of immune cells at the maternal–placental interface during placentation and display an M2 phenotype which might contribute to homeostasis maintenance favoring tissue remodeling and immunosuppressant phagocytosis of apoptotic cells (Gustafsson *et al.*, 2008; Nagamatsu and Shust, 2010). Their functional plasticity is modulated through autocrine and paracrine mechanisms under the control of trophoblast cells (Svensson *et al.*, 2011). Here we analyzed the mechanisms underlying apoptotic trophoblast engulfment by human monocytes and macrophages, the phagocytic portal formation and the effect of VIP in the trophoblast–macrophage interaction. Evidence presented here indicates that VIP acting on trophoblast cells promoted monocyte migration, polarization to anti-inflammatory phenotypes and increased phagocytosis of apoptotic cells. Moreover, our

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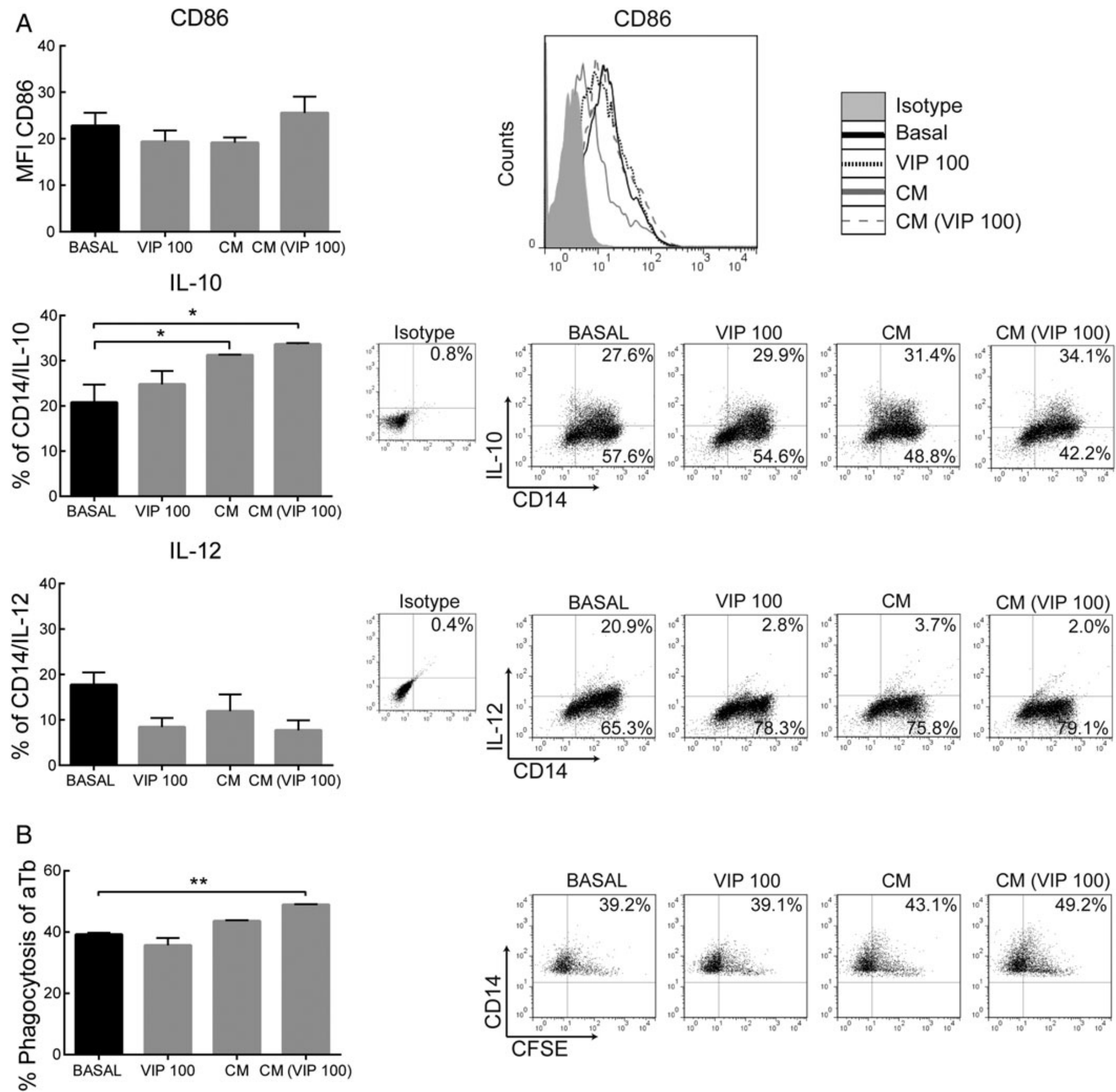

Figure 4 VIP-trophoblast factors modulate monocyte-derived macrophage function. Macrophages differentiated during 5 days with GM-CSF were conditioned with or without 100 nM VIP, CM or CM (VIP 100 nM) during 20 h. (**A**) Macrophage activation profile was analyzed by flow cytometry. MFI of CD86 and the percentage of double positive cell (right upper panel) of CD14+ IL-10+ and CD14+IL-12+ values are expressed as mean ± SEM of at least three experiments. One representative histogram and dot plots are shown. (**B**) Macrophages were conditioned and then challenged with CFSE+ apoptotic trophoblast cells during 120 min and the percentage of CD14/CFSE positive cells analyzed by FACS. Values are representative of three experiments run similarly.

results support that VIP mechanism involves the induction of thrombospondin 1 on trophoblast cells and αvβ3 integrin expression on phagocytic cells. These conclusions are based on the following observations: First, VIP promoted monocyte chemoattractant protein expression and monocyte migration toward trophoblast conditioned media. Second, CD14+ cells increased the expression of CD39 and IL-10 and decreased the expression of pro-inflammatory markers when exposed to trophoblast cells preconditioned with VIP. The effect was confirmed in monocyte-derived macrophages. Third, apoptotic trophoblast cells were rapidly engulfed by CD14+ cells in the presence of VIP-conditioned media which was able to significantly increase αvβ3 integrin expression on phagocytic cells. The effect also required the up-regulation and binding of TSP-1 since a monoclonal antibody anti-TSP-1 inhibited apoptotic trophoblast cell phagocytosis.

TSP-1 has been proposed among soluble bridging molecules that bind apoptotic cells and facilitate their engulfment through integrin family molecules expressed on phagocytic cells (Savill *et al.*, 1992; Hughes *et al.*, 1997). As an initial step of apoptotic cell phagocytosis, its expression is up-regulated as well as the expression of αvβ3 integrin counterpart molecules on phagocytic cells (Nakaya *et al.*, 2008; Ortiz-Masia *et al.*, 2012). Here we showed that VIP can rapidly induce TSP-1 expression on trophoblast cells and that monocytes primed with

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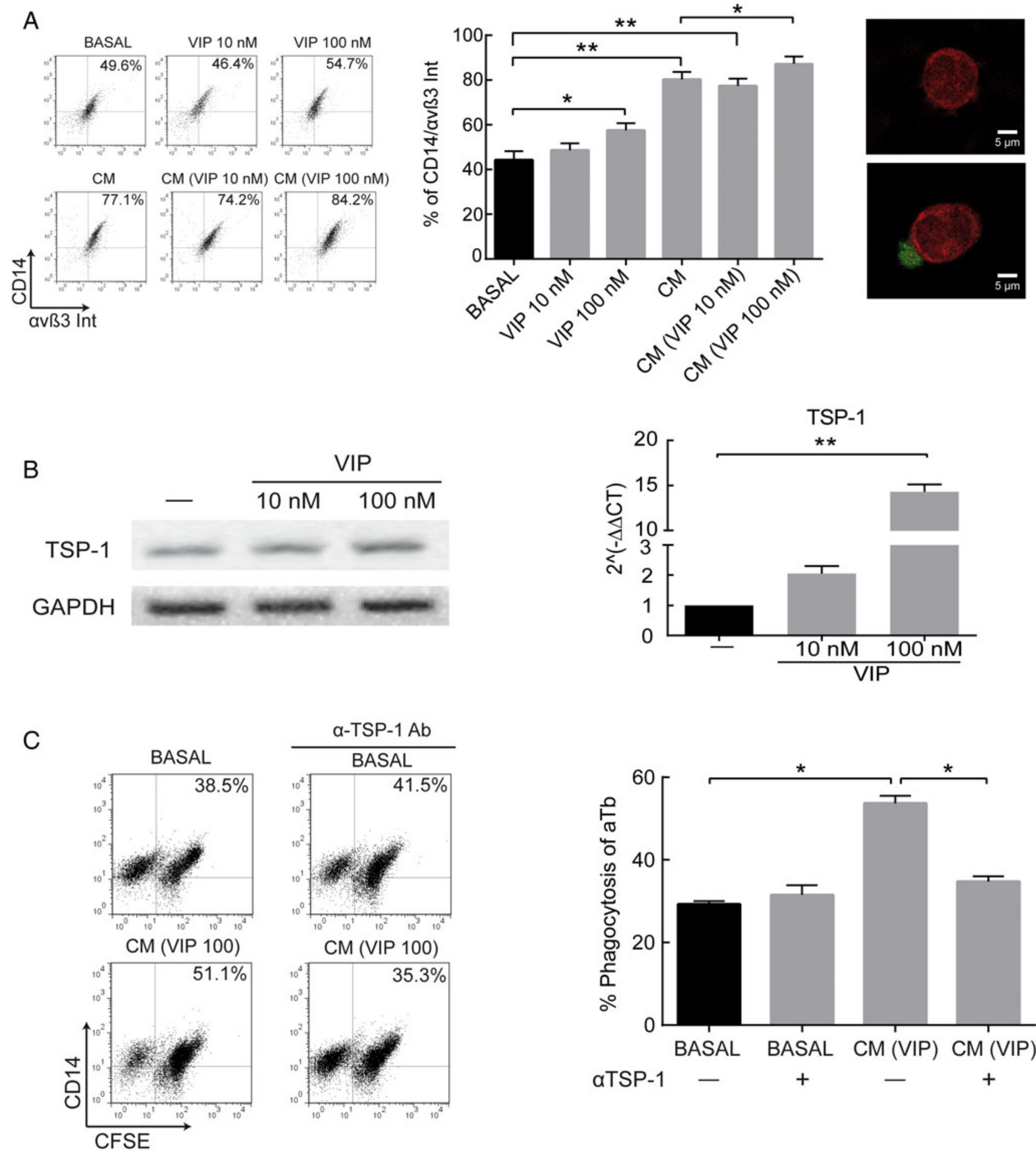

Figure 5 αvβ3 integrin and thrombospondin 1 are involved in vasoactive intestinal peptide (VIP)-promoted phagocytosis. (**A**) Monocytes were incubated with or without 10 or 100 nM VIP or conditioned media of trophoblast cells (CM) or conditioned media from trophoblast cells cultured with VIP (CM VIP 10 or 100 nM) during 20 h and analyzed the expression of αvβ3 integrin by flow cytometry as described in Materials and Methods. The percentages of CD14/ αvβ3 integrin positive cells were expressed as mean ± SEM. *$P < 0.05$. Left panel, one representative dot plot of three determinations is shown. Right panel, localization by confocal microscopy of αvβ3 integrin (red) in the absence (upper) or presence (lower) of CFSE-labeled apoptotic trophoblast cell (green). Representative microphotograph 120× of at least three experiments. (**B**) Thrombospondin 1 (TSP-1) expression was determined by RT–PCR in trophoblast cells cultured in the absence or presence of 10 or 100 nM VIP. Results are expressed as fold increase mean ± SEM normalized to GAPDH. *$P < 0.05$ and a representative gel of at least 4 is shown. (**C**) Monocytes conditioned in the absence (BASAL) or presence of CM (VIP 100 nM) or not (BASAL) for 20 h were incubated with CFSE+ apoptotic trophoblast cells (aTb) in the presence of anti-TSP-1 antibody for 20 min and after 120 min of phagocytosis the percentage of double positive cells (right upper panel) was determined by flow cytometry. Results are expressed as mean ± SEM of three experiments. *$P < 0.05$. Representative dot plots.

VIP-trophoblast cell conditioned media displayed higher apoptotic cell engulfment than non-primed monocytes. Moreover, we showed that phagocytosis was prevented by neutralizing TSP-1 with a specific antibody. On the other hand, VIP alone or VIP-modified conditioned media both increased αvβ3 integrin expression on phagocytic cells. Taken together these results support a role for VIP in TSP-1/αvβ3

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integrin phagocytic portal formation to contribute to apoptotic trophoblast cell uptake by monocytes. It is an interesting to note that in our experiments VIP does not induce apoptosis on trophoblast cells but it did induce TSP1 expression which would contribute to the clearance of nearby apoptotic cells by macrophages. Trophoblast cells coordinate the recruitment of maternal monocytes through the synthesis of several chemokines and provide signals to polarize them to anti-inflammatory phenotypes that favor the immunosuppressant microenvironment characteristic of the second and most of the third trimester of human pregnancy (Abrahams *et al.*, 2004; Straszewski-Chavez *et al.*, 2005; Mor *et al.*, 2006; Fest *et al.*, 2007; Nagamatsu and Shust, 2010; Grasso *et al.*, 2014). Our results are consistent with VIP as a local modulator of monocyte function at both steps during placentation: on their recruitment and on the subsequent acquisition of an immunosuppressant phenotype. VIP on trophoblast cells induced mRNA expression of four different monocyte-chemoattractant molecules as MCP-1, MIP-1α, IL-8 and RANTES. Functional IL-8 levels were present at high levels in the CM as previously shown (Fest *et al.*, 2007) whereas MCP-1 and RANTES were further induced by priming trophoblast cells with VIP. Certainly, increased chemokine production by VIP paralleled a higher monocyte migration toward conditioned media from trophoblast cells. Besides, monocytes in direct contact with trophoblast cells in the presence of VIP or treated with VIP-conditioned media increased CD39 and IL-10 expression whereas IL-12, TNF-α, CD86, HLA-DR and CD40 were not induced or were even decreased upon interaction. Interestingly, VIP diminished CD16 expression on monocytes consistent with a transition to a predominant *classical* monocyte profile with enhanced phagocytic capacity (Ziegler-Heitbrock *et al.*, 2010). The proportion of $CD14^{++}CD16^{+}HLA\text{-}DR^{+}$ monocyte subset is significantly increased in women with pre-eclampsia compared with normal pregnancies and also between severe and mild pre-eclampsia outcomes (Tang *et al.*, 2015). On the light of this observation, the effects of VIP shown here for HLA-DR and CD16 on CD14+ cells point to its immunomodulatory potential in a trophoblast cell context to promote *classical* over *non-classical* and *intermediate* monocyte subsets. Moreover, monocyte-derived macrophages were also conditioned by trophoblast and VIP derived factors. In this regard, it is noteworthy that VIP-trophoblast cell factors were strong enough to modulate GM-CSF-differentiated macrophages, more resembling the classically activated macrophages of the decidua found in pregnancy complications (Svensson *et al.*, 2011).

Overall, we have demonstrated the modulation of monocyte migration, polarization and phagocytosis of apoptotic cells by VIP in a trophoblast-coordinated manner and we have addressed a mechanism on how VIP enhances the phagocytic clearance of trophoblast apoptotic cells through TSP-1/$\alpha v\beta 3$ integrin phagocytic portal formation.

The mechanisms of apoptotic trophoblast cell clearance by monocytes and the proteins involved in portal formation are based on the results obtained with monocytes drawn from peripheral blood of healthy individuals with trophoblast cell lines. Two cell lines derived from human first trimester trophoblast were used since primary first trimester trophoblast cells are not readily available here, so we cannot rule out their differential behavior.

VIP concentrations in serum during pregnancy are 2–5 pM and increase to 10 pM at labor (Ottesen *et al.*, 1982). VIP levels are similarly higher in human umbilical cord blood. In this regard, considering the high concentrations that the locally synthesized polypeptides could reach nearby, and the potential deleterious effects of high VIP plasma concentrations, it is unlikely that these plasma levels of VIP reflect their local levels at the interface. In fact, trophoblast cells were shown to express VIP receptors and to respond to VIP with hormonal synthesis *in vitro* at a range concentration between 0.1 and 300 nM (Marzioni *et al.*, 2005) using settings comparable to those used here. Besides, VIP in a similar concentration range elicited immunomodulatory effects both in designs *in vitro* with human cells and *in vivo* mouse pregnancy models (Fraccaroli *et al.*, 2009; Perez Leirós and Ramhorst, 2013; Hauk *et al.*, 2014a).

Finally, trophoblast cells express TPS-1 and this factor was proposed to be involved in T cell modulation (Dong *et al.*, 2008). Our recent reports on iTreg differentiation by VIP involving trophoblast paracrine and autocrine interactions (Fraccaroli *et al.*, 2015) and the present observations on professional phagocytic cells contributing to an immunosuppressant microenvironment add more insight into the mechanisms of immune homeostasis maintenance during early pregnancy and provide new clues for immune and trophoblast cell pharmacological targeting in pregnancy complications of immunopathologic nature.

Authors' roles

The contribution of D.P., E.G., D.V. and V.H. to this work included all the experiments on trophoblast cells and co-cultures, monocyte characterization, RT–PCR, fluorescence microscopy assays, Flow cytometry analysis, migration and phagocytosis experiments; G.C. obtained and characterized homologous purified neutrophil population and carried out apoptosis induction and phagocytosis assays; R.R. and C.P.L. designed the whole study, discussed the results and prepared the manuscript.

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Conflict of interest

The authors declare no commercial or financial conflict of interest.

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