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Progesterone and VIP cross-talk enhances phagocytosis and anti-inflammatory profile in trophoblast-derived cells

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ABSTRACT

Trophoblast cells produce several inmmuneregulators like the Vasoactive Intestinal Peptide (VIP) and P4 targeting multiple circuits, and also display an intese phagocytic ability allowing embryo implantation in a tolerogenic context. Here, we explored whether P4 and VIP- crosstalk modulates trophoblast cell function, focus on the phagocytic ability and the immune homeostasis maintenance. P4 enhanced the phagocytosis in trophoblast-derived cells quantified by the engulfment of latex-beads or eryptotic erythrocytes. P4 and VIP modulated the balance of anti/pro-inflammatory mediators, increasing TGF-β expression, with no changes in IL-1, IL-6, or nitrites production. This modulation was accompained by transcription factor expression changes that could turn on tolerogenic programs represented by increased PPAR-γ and decreased IRF-5 expression. Finally, P4 stimulated VPAC2 expression in trophoblast cells and VPAC2 over-expression enhanced phagocytosis mimicking P4-effect. Therefore, P4 and VIP network enhances the phagocytic ability of trophoblast-derived cells, through a mechanism involving VPAC2 accompained with an anti-inflammatory context.

1. Introduction

Early pregnancy implies deep tissue remodelling accompanied by a sterile inflammatory response and redundant immunoregulatory circuits to sustain immune homeostasis (Mor et al., 2011) (Mor and Cardenas, 2010, Maybin et al., 2011).

The trophoblast, an epithelial cell of fetal origin that forms the physical barrier between the mother and developing conceptus, becomes a component of the host immune system during pregnancy. Trophoblast invasion and migration is controlled by components of the trophoblast itself and the maternal microenvironment, through molecular and cellular interactions (Aluvihare et al., 2004; Guerin et al., 2009; Leber et al., 2010; Ramhorst et al., 2012; Saito et al., 2010; Terness et al., 2007).

Similarly to professional phagocytosis (macrophages, dendritic cells and granulocytes) other cells display phagocytic ability controlled by *in vivo* mediators, like endothelial cells (Rengarajan et al., 2016), enterocytes (Moyes et al., 2007) and trophoblast cells. Particularly, trophoblast cells display intense phagocytic activity during the peri implantation period and internalize maternal components, such as uterine epithelial and decidual cells, that are present along the invasion pathway of the trophoblast. A role in providing nutrition, iron uptake for fetal hemopoiesis and space for the early embryonic development is generally attributed to this activity (Contractor and Krakauer, 1976; Bevilacqua et al., 2010).

Trophoblast resembles the macrophage in some aspects since they share many characteristics such as phagocytosis, syncytialization, invasiveness, the expression of PRR and the ability to produce pro or anti-inflammatory mediators upon stimulation (Guilbert et al., 1993) (Mellor et al., 2002). In fact, trophoblast cells share with macrophages the expression of several genes and they have been suggested to function as a component of the innate immune system during pregnancy (Guleria and Pollard, 2000). In the placental-maternal context, macrophages acquire a predominant alternative activation profile contributing to the production of suppressor cytokines and the synthesis of wound healing mediators (Heikkinen et al., 2003) (Mor and Koga, 2008) (Houser et al., 2011), instead of the classical activation associated with inflammatory mediators (Mosser and Edwards, 2008).

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The tissue remodelation during the peri implantation period generates apoptotic cells that should be quickly and efficiently removed to maintain tissue homeostasis, embryo development and immuno-regulation (Korns et al., 2011). Macrophages exert a profound influence on the resolution of inflammation, through the secretion of anti-inflammatory cytokines such as TGF-β and IL-10 that inhibit inflammatory mediator production indicating that apoptotic cell signaling can modulate adaptative immune responses (Mosser and Edwards, 2008).

In addition, progesterone (P4) is critical for the establishment and the maintenance of pregnancy, both by its endocrine and immunological effects. The genomic actions of progesterone are mediated by the intracellular P4 receptors, PR-A and PR-B, which act as transcription factors, although non-genomic effects of P4 have been reported (Kowalik et al., 2013). Regarding the immunoregulatory properties of P4, Menzies and colleagues demonstrated its ability to selectively regulate the expression of different genes associated with alternative macrophage activation in bone marrow cells isolated and differentiated from male BALB/c mice (Menzies et al., 2011).

An interesting point is that dose-dependent stimulation of P4 release the vasoactive intestinal peptide (VIP) was reported in human trophoblast primary cultures (Marzioni et al., 2005). VIP displays potent immunomodulatory and trophic effects upon binding VPAC1 or VPAC2 receptors coupled to stimulatory G protein and adenylate cyclase activation on adult and embryonic cells (Waschek, 2013). Particularly during pregnancy, VIP treatment at day 6.5 of gestation of two resorption prone mouse models, the non obese diabetic mice and the CBAxDBA mice, improved pregnancy outcome, increased the number of implanted embryos and the expression of alternatively activated macrophages and regulatory T cell markers (Gallino et al., 2016) (Hauk et al., 2014). In fact, first trimester human placental trophoblasts cell lines (Swan-71 and HTR8) express VIP/VPAC receptor system and VIP priming enhances apoptotic cell engulfment by macrophages involving thrombospondin-1/αvβ3 portal formation (Paparini et al., 2015b).

On the basis that P4 and VIP contribute to the immunosuppressive microenvironment at the maternal-placental interface, we evaluated the immunomodulatory effects of P4 and VIP on trophoblast cell function focusing on their phagocytic ability and whether they modulate anti-inflammatory mediators. In the present work, we showed that P4 and VIP network enhances the phagocytic ability of trophoblast-derived cells, through a mechanism involving VPAC2 induction in response to P4 treatment, and inducing the expression of anti-inflammatory mediators.

2. Material and methods

2.1. Trophoblast cell line cultures

Two trophoblast cell lines from human first trimester pregnancies were used: the HTR-8/SVneo cell line (HTR-8) was derived from transformed extravillous trophoblast and Swan-71 cell line, derived by telomerase-mediated transformation of a 7-weeks cytotrophoblast isolate described by (Straszewski-Chavez) (Straszewski-Chavez et al., 2009). Both cell lines were a gift from Dr. Gil Mor from Yale, University. Cells were cultured in 24-well flat bottom polystyrene plates in Dulbeco's modified Eagle's medium and Nutrient Mixture F-12 (DMEM-F12) (Life Technologies, Grand Islands, NY, USA) containing 25 mM HEPES and supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM Glutamine (Sigma-Aldrich) and 100 U/ml streptomycin-100 μg/ml penicillin solution (Life Technologies, Grand Islands, NY, USA).

Trophoblast-derived cells at 80% confluence (2×10^5 cells/well) were cultured in the absence/presence of P4 or VIP during 24 h, then performed the phagocytosis assays and cells were recovered for FACS or RT-PCR analysis.

2.2. Trophoblast-derived cell transfection

Transfection assays were performed using the X-tremeGENE HP DNA (Roche, Mannheim, Germany) reagent and the VPAC2 plasmid or its corresponding empty vector (CDNA Resource Center Bloomsburg University, USA) as described (Vota et al., 2016). Briefly, the transfection reagent:plasmid complex pre incubated for 15 min at room temperature were added drop wise to the cells. 48 h post transfection, phagocytosis assays were carried out to evaluate the effect of VPAC2 receptor over-expression compared to Empty Vector (EV, pCDNA 3.1+) transfected cells. VPAC2 mRNA overexpression was confirmed by qRT-PCR as described Vota et al., 2016 (data not shown) (Vota et al., 2016).

2.3. Phagocytosis assays

To evaluate the phagocytic ability of trophoblast-derived cells we used two approaches, the phagocytosis of latex beads-FITC or eryptotic red blood cells (RBC).

- *Phagocytosis of latex beads-FITC:* Swan-71 cells were cultured in 24-well plates in absence/presence of P4 (10^{-6} M, representing the concentration at the maternal-fetal interface (Van Voorhis et al., 1989), or VIP (10^{-7} M, associated with immunomodulatory effects) during 24 h and then FITC-latex beads (0.8 μm, School of Chemistry, Udelar, Uruguay) were added in a 1:100 ratio (trophoblast-derived cell:beads). Incubations were performed at 37 °C with 5% CO_2 for 4 h and trophoblast-derived cells were harvested to quantify the fluorescent particles engulfment by FACS analysis using FlowJo 7.6 software. To exclude non-phagocytic binding of the beads to trophoblast-derived cells, the same assay was carried out at 4 °C. Results are expressed as the percentage of FITC positive cells compared to the trophoblast-derived cell autofluorescence.
- *Phagocytosis of eryptotic RBC:* Swan-71 cells were cultured in absence/presence of P4 (10^{-6} M) or VIP (10^{-7} M) during 24 h, washed with PBS and resuspended in fresh medium to performed the phagocytosis assays. Human RBC from healthy donors were obtained as previously described (Vota et al., 2013), labeled with the fluorescent compound CFSE and incubated with the Calcium Ionophore A23187 for 20 h at 37 °C to induced eryptosis with phosphatidyl serine (PS) exposure (RBC PS exposure (%): Basal: 21 ± 1.2; A23187: 49 ± 6.2; $p < 0.05$, $n = 3$). Then CFSE-stained eryptotic RBC and Swan-71 cells were co-cultured in a 100:1 ratio at 37 °C with 5% CO_2 for 4 h. Non-phagocyted RBC were lysed with cold destilated water for 30 s and after washing trophoblast-derived cells with PBS, they were recovered by TrypLe® (Gybco, Invitrogen, Argentina) and the engulfment was quantified by FACS analysis as previously depicted.

2.4. Flow cytometry analysis

Swan-71 cells after 24 h cells were recovered by TrypLe treatment (Gybco, Invitrogen, Argentina). For intracellular VIP detection Stop Golgi was added to the medium in the last 4 h of culture following manufacturer's instructions (Becton Dickinson, San José, CA), to promote intracellular accumulation. Then cells were recovered and, after washing with FACS solutions (PBS-2% FBS), cells were incubated with the fixation/permeabilization buffer for 30 min stained with mAb anti-VIP-FITC-conjugated (Abcam, CA). Cells were finally washed with FACS solution. Twenty thousand events were acquired in a FACSAria II cytometer and results were analyzed using

FlowJo 7.6 Software. Negative control samples were incubated in parallel with an irrelevant, isotype-matched Ab. Results are expressed as fold increase of the mean fluorescence intensity (MFI) of the marker of interest relative to negative MFI control.

2.5. VIP, VPAC receptors, transcription factors and pro/anti inflammatory mediators detection

The expression of VIP and VPAC receptors, transcription factors and pro/anti inflammatory mediators was determined by RT-PCR. Briefly, total RNA was isolated following manufacturer recommendations with Trizol reagent (Life Technologies, Grand Island, NY, USA), cDNAs were generated from 1 μg or RNA using a MMLV 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 MgCl2; 0.1 mM forward and reverse primers of VIP, VPAC1, VPAC2, TGF-β, IL-1, IL-6, PPARγ, IRF-5, KLF-4 and GAPDH as internal control (described in Table 1) and 1 U Taq polymerase in a DNA Thermocycler (PerkinElmer/Cetus, Boston, MA, USA). 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 GAPDH expression.

2.6. Immunofluorescence microscopy

Swan-71 cells were grown over a glass slide until they reached 70% confluence then the phagocytosis assays of latex beads-FITC were performed in absence/presence of P4 10^{-6} M, as depicted in 2.3. Cells were washed with PBS, fixed with cold methanol during 20 s and then nucleus were staining with DAPI (Cell Signaling, Danvers, MA, USA) during 10 min in darkness. Cells were mounted with 20% glycerol in PBS. Photographs were acquired using a IX71® Olympus inverted fluorescence microscope (Olympus, Center Valley, PA, USA) and Micro-Manager Software.

2.7. Statistical analysis

The significance of the results was analyzed by Student's *t*-test for parametric samples. When multiple comparisons were necessary ANOVA of two way factors were used with Dunnett's post-hoc test.

Table 1

Primers	Sequence	Product (bp)
GAPDH	*sense* 5'–TGATGACATCAAGAAGGTGGTGAAG–3′ *antisense* 5'–TCCTTGGAGGCCATGTAGGCCAT –3′	240
VIP	*sense* 5'–TAC AGG GCA CCT TCT GCT CT–3′ *antisense* 5'–CAA GAG TTT ACT GAA GTC ACT–3′	198
VPAC1	*sense* 5'–CCC CTG GGT CAG TCT GGT G–3′ *antisense* 5'–GAG ACC TAG CAT TCG CTG GTG–3′	100
VPAC2	*sense* 5'–CCA GAT GTC GGC AAC G–3′ antisense 5'–GCT GAT GGG AAA CAC GGC AAA C–3′	114
TGF-β1	*sense* 5'–TGA ACC GGC CTT TCC TGC TTC TCA TG–3′ antisense 5'–GCG GAA GTC AAT GTA CAG CTG CCG C–3′	152
IL-6	*sense* 5'–CAG ATT TGA GAG TAG TGA GGA AC–3′ antisense 5'–CGC AGA ATG AGA TGA GTT GTC–3′	195
IL-1β	*sense* 5'–TGA TGG CTT ATT ACA GTG GCA ATG–3′ *antisense* 5'–GTA GTG GTG GTC GGA GAT TCG–3′	140
PPAR-γ	*sense* 5'–CAG ATC CAG TGG TTG CAG–3′ *antisense* 5'–GTC AGC GGA CTC TGG ATT–3′	458
IRF-5	*sense* 5'–CCA ACA CCC CAC CAC CCT TC–3′ *antisense* 5' –AGA ACA TCT CCA GCA GCA GTC G–3′	132
KLF-4	*sense* 5'–CGC TGC TCC CAT CTT TCT CC–3′ *antisense* 5'–CCG CCG CCA GGT CAT AGG–3′	159

Differences between groups were considered significant at $P < 0.05$ using the GraphPad Prism4 software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Progesterone promotes the phagocytic ability of trophoblast-derived cells

In a first step, to evaluate whether P4 and VIP modulate the trophoblast phagocytic ability, Swan-71 cells were cultured in the absence or presence of P4 or VIP during 24 h and then FITC-latex beads were added for 4 h. As depicted Fig. 1A, P4 significantly increased the engulfment of latex beads by trophoblast cells quantified by FACS analysis. Fig. 1B shows representative dotplots in the absence or presence of P4, at 37 °C and 4 °C. Additional, phagocytic ability was also quantified in HTR-8 cells in the absence or presence of 10^{-6} M P4 as in Swan-71 cells.

The present results were confirmed using another phagocytic assay based in the phagocytosis of eryptotic RBC-CFSE and also quantified by FACS analysis (Fig. 1C).

3.2. P4 and VIP promote anti-inflammatory marker profile on trophoblast-derived cells upon phagocytosis

Since in the placental-maternal context, macrophages activation post phagocytosis is associated with the production of immunosuppressive cytokines and the reduced production of pro-inflammatory mediators, we evaluated whether P4 and VIP modulate the balance of pro/anti-inflammatory mediators in trophoblast cells upon phagocytosis. For that purpose, after the phagocytosis assay, Swan-71 were washed to remove the latex-beads, and then recovered to evaluate TGF-β, IL-1, IL-6 expression and nitrites production. VIP increased TGF-β expression while did not modulate IL-1 nor IL-6 expression (Fig. 2A).

On the other hand, after the phagocytosis of eryptotic RBC, P4 and VIP significantly increased TGF-β expression while they did not modulate IL-1 or IL-6 expression (Fig. 2B).

3.3. P4 and VIP modulate the balance of transcription factors associated with an anti-inflammatory response in trophoblast-derived cells after phagocytosis

Macrophage different activation profiles are associated with the modulation of characteristic transcription factors as PPARγ (its expression and/or activity is essential for the acquisition of an alternative activation and suppressor functions), KLF-4 (Kruppel-like factor 4, that regulates the expression of key transcription factors during embryonic development) and IRF-5 (INF response factor 5, a transcription factor involved in the induction of interferons IFN-α and IFN-β and inflammatory cytokines upon virus infection) (Bouhlel et al., 2007) (Odegaard et al., 2007). Therefore, we investigated whether the P4 and VIP modulate the expression of these transcription factors in trophoblast cells after phagocytosis. Fig. 3A, shows that PPARγ significantly increased in the presence of P4 while IRF-5 significantly decreased (Fig. 3B). KLF-4 was not modulated under the present conditions (data not shown). These results suggest that after phagocytosis trophoblast-derived cells activate a regulatory program associated with the production of anti-inflammatory mediators instead and prevent a pro-inflammatory activation profile.

3.4. P4 induces an increase of VIP and VPAC2 expression on trophoblast-derived cells

Since P4 enhances phagocytosis by trophoblast cells and VIP contributes to the production of anti-inflammatory mediators, we

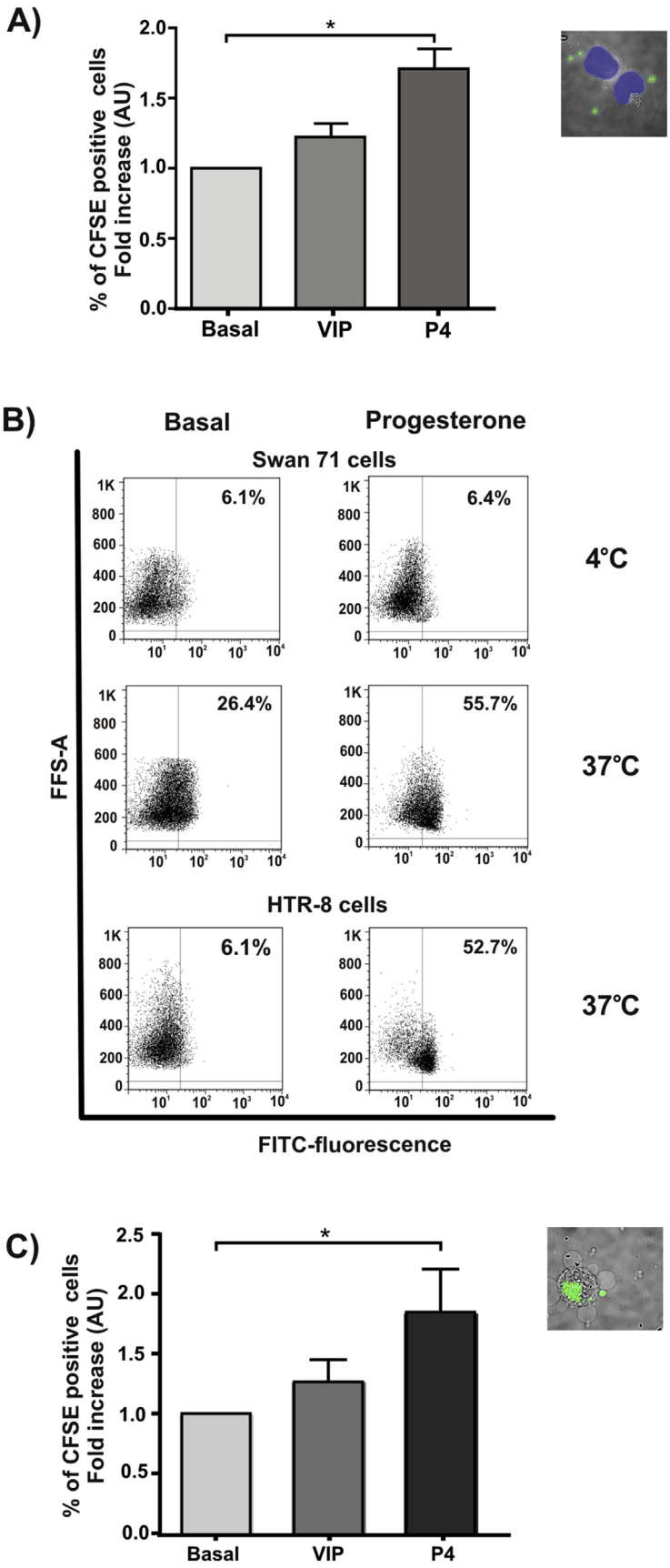

Fig. 1. Progesterone increases the phagocytic ability on trophoblast-derived cells. Swan-71 cells were cultured in the absence or presence of 10^{-6} M P4 or 10^{-7} M VIP during 24 h, then cells were washed with PBS and: **A)** FITC-latex beads were added to the culture cells for 4 h, cells were harvested and the engulfment was quantified by FACS analysis. Results represent the fold increase of positive cells respect to basal cells expressed as Mean ± S.E.M. (Anova-Dunnett's *P < 0.05, n = 4). The right picture inserted shows a

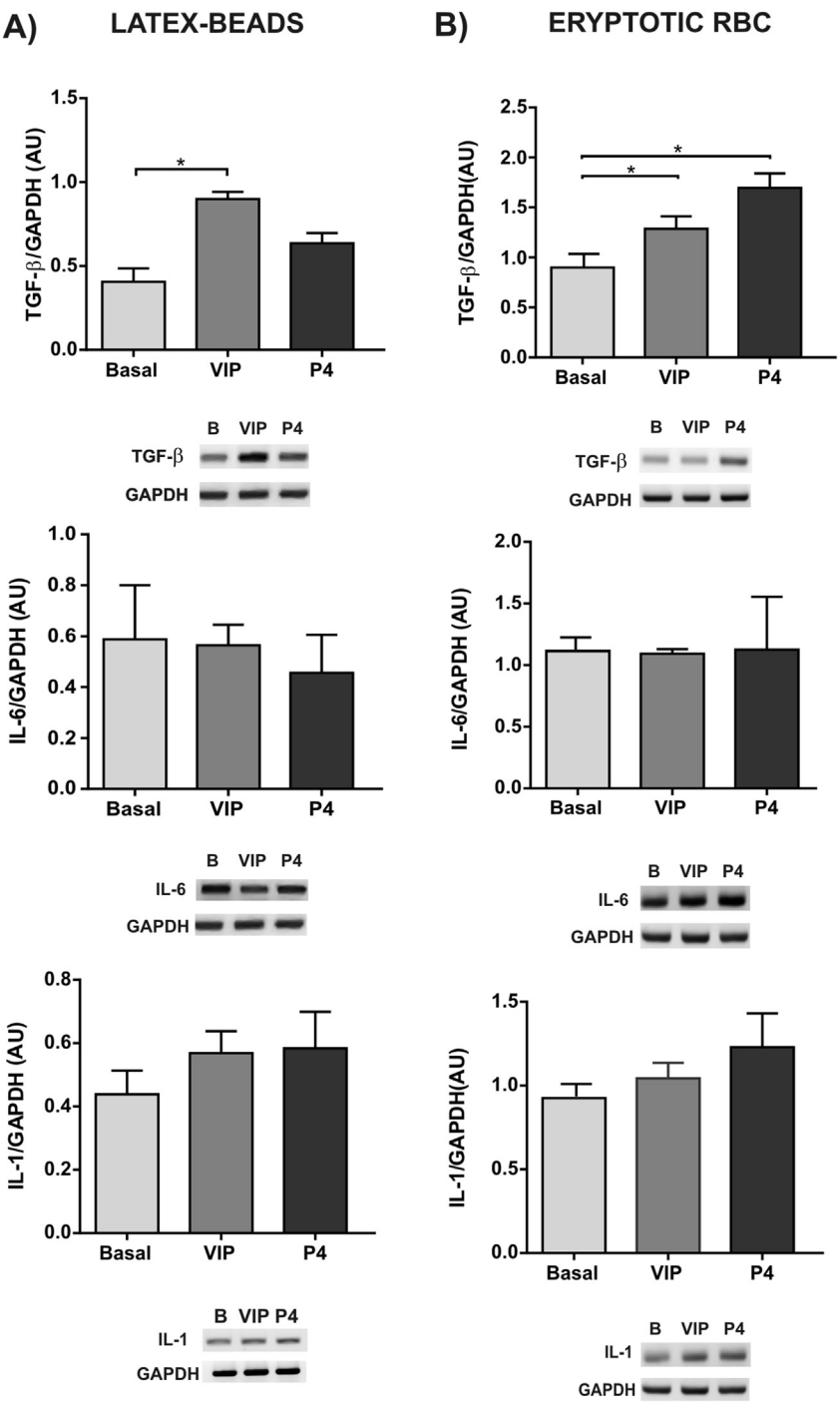

Fig. 2. P4 and VIP promote anti-inflammatory mediators production on trophoblast cells upon phagocytosis. Swan-71 cells were cultured in the absence or presence of P4 during 24 h, then **A)** FITC-latex beads or **B)** eryptotic RBC cells-CFSE were added for 4 h. After the phagocytosis, Swan-71 were washed and the expression of TGF-β, IL-1 and IL-6 was evaluated by RT-PCR (Anova-Dunnett's $^*P \leq 0.05$).

investigated P4 effect on the expression of VIP and its high affinity receptors (VPAC1 and VPAC2). Therefore, Swan-71 cells were cultured in the absence/presence of P4 during 24 h and the VIP/VPAC receptor system was evaluated by RT-PCR. P4 significantly increased the expression of VIP in trophoblast cells evaluated by RT-PCR and quantified by intracellular immunostaining and FACS analysis (Fig. 4A and B). Fig. 4B also shows representative dot plots with the % of VIP positive cells in the absence of presence of P4. Regarding VIP receptor expression, we observed a significant increase in VPAC2 receptor expression in Swan-71 cells after P4

trophoblast-derived cell phagocyting FITC-latex beads. **B)** FACS plots are representative from 4 experiments with Swan-71 cells and include the phagocytic assay controls at 4 °C. Additional, phagocytic ability was also quantified in HTR-8 cells in the absence or presence of 10^{-6} M P4 as in Swan-71 cells. **C)** Eryptotic RBC were stained with CFSE, cultured with Swan-71 cells for 4 h, cells were harvested and the engulfment was quantified by FACS analysis. Results represent fold increase of positive cells respect to basal cells expressed as Mean ± S.E.M. (Anova-Dunnett's $^*P < 0.05$, $n = 3$). The right picture inserted shows a trophoblast-derived cell phagocyting an eryptotic RBC stained with CFSE.

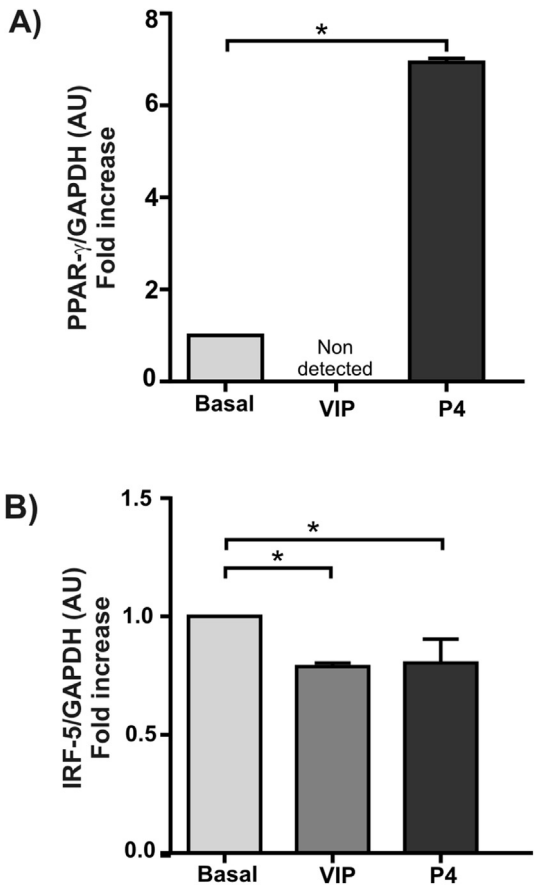

Fig. 3. P4 and VIP modulate the balance of transcription factors associated with an anti-inflammatory response in trophoblast-derived cells after phagocytosis. Swan-71 cells were cultured in the absence or presence of P4 during 24 h and after the phagocytosis assay we evaluated the mRNA expression of **A)** PPAR-γ and **B)** IRF-5 by RT-PCR. Bands were semi-quantified with ImageJ® and intensity expressed in arbitrary units (AU) relative to GAPDH. Values represent the Fold increase mean ± S.E.M of at least 3 experiments (Student T test *P < 0.05).

treatment while VPAC1 was not modulated in the present settings (Fig. 4C).

3.5. *VPAC2 over-expression enhances the phagocytic ability of trophoblast-derived cells*

On the basis that P4 significantly increases VPAC2 receptor expression in Swan-71 cells and that VPAC2 expression is induced at high levels on pregnant uteri treated *in vitro* with P4 in murine pregnancy models (Hauk et al., 2014), we next explored the effect of VPAC2 over-expression on the phagocytosis process. Transfection assays carried out with a VPAC2-plasmid resulted in over-expression of this receptor subtype with high efficiency as indicated by co-transfection experiments with a green fluorescence protein (GFP) plasmid and by qRT-PCR as previously described (Vota et al., 2016). Post-transfection cells were subjected to phagocytosis assays with FITC-latex beads for 4 h. As shown in Fig. 5A, VPAC2 over-expression increased the phagocytosis of FITC-latex beads compared with cells transfected with the empty vector as control (EV). Fig. 5B shows representative dot plots of experiments shown in Fig. 5A on Swan-71 cells transfected with VPAC2-plasmid or EV after the phagocytosis assay.

To further support the present results, we performed the over expression of VPAC2 in HTR-8 cells, another trophoblast-derived cell line. As depicted in Fig. 5C, HTR-8 cells over-expressing VPAC2 significantly increased their phagocytic ability of latex-beads as observed with P4, supporting that VPAC2-increased expression is associated with a higher phagocytic ability in first trimester trophoblast derived cells.

4. Discussion

Maternal-placental interface is a unique system in which trophoblast unit mediates and coordinates several processes, controls the inflammatory response and conditions the adaptive response (Pérez Leirós and Ramhorst, 2013). Trophoblast cells represent an ancestral macrophage regarding gene expression (Guilbert et al., 1993) (Bevilacqua et al., 2010), an also mediate several processes that contribute to the immune and tissular homeostasis (Svensson et al., 2011). In the present work we demonstrated that P4 enhances the phagocytic ability inducing the production of anti-inflammatory mediators associated with specific transcription factors expression in trophoblast cells accompained by a modulation of VPAC2 expression.

We based this conclusion in several observations; first, P4 in trophoblast-derived cells enhanced the phagocytosis ability quantified by two approaches, using latex-FITC beads and eryptosis of RBC. Second, P4 and VIP modulated the balance of anti/pro-inflammatory mediators, reflected by the increase in TGF-β expression, with no changes in IL-1, IL-6, or nitrites production. In fact, this modulation was accompained by transcription factor expression changes that could turn on tolerogenic programs represented by increased PPARγ and decreased IRF-5 expression. Finally, P4 stimulated VPAC2 expression in trophoblast cells and VPAC2 over-expression lead to enhanced phagocytosis of latex-beads mimicking the effect of progesterone. The phagocytic capacity of trophoblast cells was higher with P4 than with VPAC2 overexpression suggesting that additional processes and mediators induced by P4 have a role in this effect.

Particularly, the invasion pathway of trophoblast cells during the implantation involves a deep remodelation and clearance of apoptotic bodies (Bevilacqua et al., 2010) (Peng et al., 2010) (Abrahams et al., 2004). Trophoblast cells display intense phagocytic activity during the peri implantation period since this process is involved in nutrition, hemopoiesis, tissue remodelation and immune response (Albieri et al., 2005) (Bevilacqua et al., 2010). The phagocytic ability of trophoblast cells throughout gestation is directly related with the hemochorial placentation and the removal of cellular debris in different species. In babbon, phagocytic cytotrophoblast cells differentiate between days 23–25 of gestation and in humans necrotic decidual cells were found in phagosomal vacuoles within cytotrophoblast cells. These mechanisms are also especially relevant in rodents since no other phagocytic cells have been found at the endometrial-trophoblast boundary (Bevilacqua et al., 2010). On the other hand, trophoblast selectively recruit macrophages to the maternal-placental interface; therefore their main contribution is during the late phase of the implantation process. In addition, macrophages represent about 20% of the maternal leukocytes at the interface highlighting the contribution of the trophoblast's phagocytic ability into tissue remodulation. However, the molecular mechanisms involved in the phagocytosis and its modulatory effects on trophoblast cells are still unclear.

Here, we could observe that Swan-71 cells treated with VIP and P4, have the ability to increase the expression of TGF-β after the phagocytosis, In addition, P4 selectively increased PPAR-γ and reduced IRF-5 expression, as macrophages do when activated in an alternative profile. In this sense, the activation of macrophages in an alternative profile has been associated with enhanced phagocytosis of apoptotic cells preventing the release of intracellular contents that can contribute to inflammation and autoimmunity

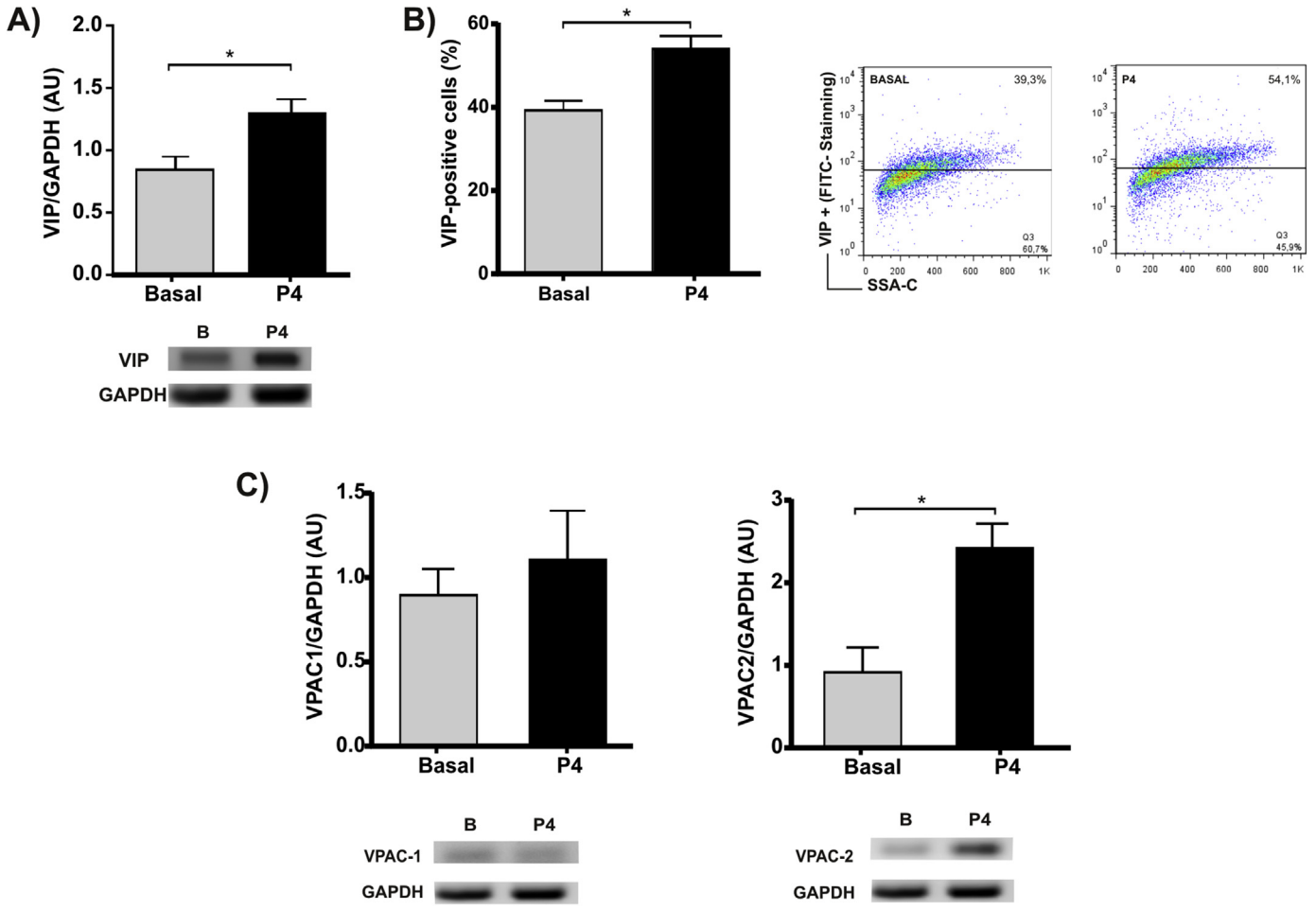

Fig. 4. P4 induces an increase in VIP and VPAC2 expression on trophoblast-derived cells. Swan-71 cells were cultured in the absence/presence of P4 during 24 h and **A)** VIP were evaluated by RT-PCR and **B)** confirmed by intracellular staining and FACS analysis. Results are expressed as the % of positive cells and the in the right panel picture show representative dotplots from one of 3 experiments. **C)** VPAC1 and VPAC2 mRNA expression were evaluated by RT-PCR. Bands were semi-quantified with ImageJ® and intensity expressed in arbitrary units (AU) relative to GAPDH. Values represent mean fold increase ± S.E.M of at least 3 experiments (Student T test *P < 0.05).

(Schif-Zuck et al., 2011). Collectively, these macrophages exhibit increased expression and/or activity of the nuclear receptors, PPAR-γ and PPAR-δ, essential to their acquisition of alternative activation profiles, associated with increased expression of arginase, and anti-inflammatory cytokines (Bouhlel et al., 2007; Odegaard et al., 2007). In addition, previous data show that P4 decrease inflammatory mediators, as down-modulate NOS2, and arginase I enzyme activity in murine macrophages in a dose-dependent manner (Menzies et al., 2011).

Our results support that P4 induces an increase in the phagocytosis capacity of first trimester trophoblast cells associated with an increase in VPAC2 expression. As the unit of the placenta, trophoblast cells represent a complete system and an immunological privilege site. On one hand, trophoblast cells display immunoregulatory properties associated with a selective expression of different molecules, as HLA molecules; whereas other trophoblast cells display hormonal regulation. Particularly the P4 concentrations used in the present work are similar to those reported at the placental-maternal interface, and even higher than reported in serum levels during pregnancy (Van Voorhis et al., 1989). A higher expression of VPAC2 on ex vivo monocytes has been associated to impaired phagocytosis in a chronic pro-inflammatory context (Hauk et al., 2014) supporting the notion that although comparable in some aspects, professional phagocytes like monocytes and trophoblast cells might differ in their functional profile under physiological or pathological conditions. On the other hand it has been proposed that the levels of cAMP condition an increase of phagocytosis in macrophages (Makranz et al., 2006). High cAMP intracellular concentrations were found as negative regulators of phagocytosis in microglia and macrophages whereas phagocytosis was enhanced at lower normal operating cAMP levels. Taken together these observations confirm the complex nature of the phagocytic process in non-professional phagocytic cells, as well as the role of VPAC2 expression and the possible association with cAMP levels, in different cell types as a still open point for research.

In fact, the conclusions are based on *in vitro* experiments with two trophoblast derived cells and we cannot rule out a differential behavior of trophoblast cells targeted *in vivo* with P4, or in primary cultures from first trimester trophoblast cells.

Regarding VIP effects, previous results demonstrated that VIP produced by trophoblast cells could have an active role in the immunoregulatory processes operating in the maternal-placental interface by contributing to the induction of regulatory T cells through a mechanism involving TGF-β1 (Fraccaroli et al., 2009) (Fraccaroli et al., 2015).

In the CBA/J x DBA/2 mating with high resorption rate associated with a failure in the tolerogenic maternal response, VIP-treatment at day 6.5 of gestation, prevents embryo resorption, increases the number of viable embryo and modulates the phagocytosis of maternal macrophages. In addition, VIP treatment *in vivo* increased

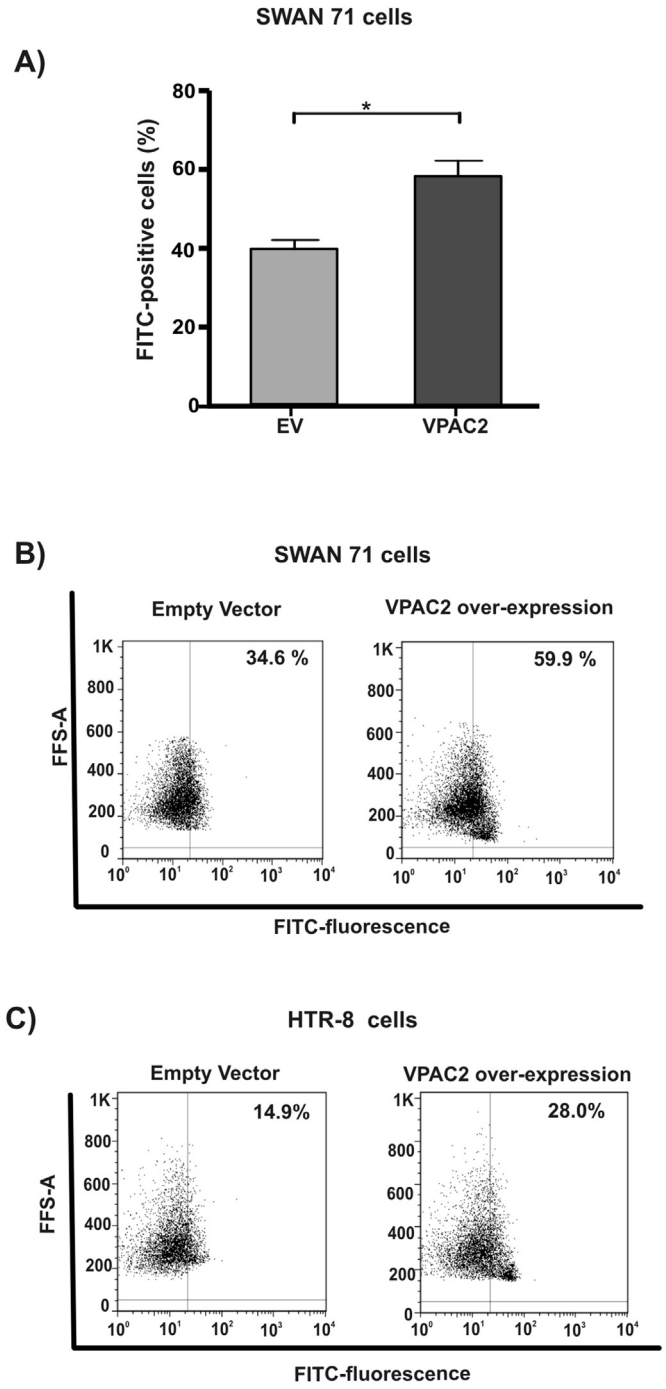

Fig. 5. VPAC2 over-expression induces the phagocytosis on trophoblast-derived cells. Swan-71 were co-transfected with a VPAC2 plasmid to induce over-expression and a GFP plasmid to evaluate the transfection efficiency (data not shown). Post-transfection cells were subjected to phagocytosis assays with FITC-latex beads for 4 h. The empty vector (EV) was used as negative transfection control. **A)** The results are expressed as the mean ± S.E.M. of FITC-positive cells (Student T-test *P < 0.05; n = 3). **B)** Dot plots representative of 3 experiments with similar results are shown. **C)** HTR-8 cells were transfected or not with a VPAC2 plasmid to induce over-expression, washed with PBS and then FITC-latex beads were added to the culture cells for 4 h and the engulfment was quantified by FACS analysis; figure shows a representative experiment.

the frequency of F4/80+ IL-10-producing cells suggesting their activation in an alternative profile (Gallino et al., 2016). Likewise, VIP priming of two first trimester cell lines (Swan-71 and HTR-8) induces thrombospondin-1 expression and enhances the phagocytosis of apoptotic cells by macrophages through αvβ3 portal formation (Paparini et al., 2015a).

Understanding the mechanisms and local factors that regulate trophoblast function and the immunoregulatory mediators network might help to understand the physiological mechanisms that sustain tissue homestasis in a tolerogenic microenvironment throughout pregnancy.

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Authors' roles

The contribution of M.A. D.V. E.G. L.S. L.G. and V.H. to this work included all the experiments on trophoblast cells, RT–PCR, fluorescence microscopy assays, Flow cytometry analysis, and phagocytosis experiments; C.P.L. and R.R. designed the whole study, discussed the results and prepared the manuscript.

Conflict of interest

The authors declare no commercial or financial conflict of interest.

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