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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