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# RESEARCH PAPER

# VIP modulates the pro-inflammatory maternal response, inducing tolerance to trophoblast cells

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**Background and purpose:** Successful embryo implantation is followed by a local pro-inflammatory and Th1 response, subsequently controlled by a Th2 response. Vasoactive intestinal peptide (VIP) has anti-inflammatory effects and promotes tolerogenic/Th2 responses while favouring embryonic development. We investigated the potential regulatory role of VIP on human trophoblast cells, maternal pro-inflammatory responses and trophoblast-maternal leukocyte interactions.
**Experimental approach:** We tested VIP effects directly on a trophoblast cell line (Swan 71 cells) and after co-culture with maternal peripheral blood mononuclear cells (PBMCs) as models of the feto-maternal dialogue. We also co-cultured maternal and paternal PBMCs to test effects of endogenous VIP on maternal alloresponses.
**Key results:** Swan 71 cells express $VPAC_1$ receptors and VIP induced their proliferation and the expression of leukaemia inhibitor factor, a pro-implantatory marker. After interaction with trophoblast cells, VIP increased Foxp3, the proportion of CD4+CD25+Foxp3+ cells within maternal PBMCs and transforming growth factor β expression. Also, during the trophoblast-maternal PBMCs interaction, VIP reduced pro-inflammatory mediators [interleukin (IL)-6, monocyte chemoattractant protein 1, nitric oxide], while increasing IL-10. Trophoblast cells produced VIP which dose-dependently suppressed allomaternal responses, accompanied by reduced expression of the T cell transcription factor, T-bet.
**Conclusions and implications:** Vasoactive intestinal peptide induced pro-implantatory markers and trophoblast cell proliferation, while controlling the initial pro-inflammatory response, by increasing maternal regulatory T cells and anti-inflammatory cytokines. As an autocrine regulatory peptide VIP might contribute to fetal survival through two mechanisms; a direct trophic effect on trophoblast cells and an immunomodulatory effect that favours tolerance to fetal antigens.
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**Keywords:** VIP; early pregnancy; tolerance and pregnancy; human implantation

**Abbreviations:** LIF, leukaemia inhibitor factor; MCP-1, monocyte chemoattractant protein 1; PBMC, peripheral blood mononuclear cell; SFCM, serum-free conditioned media; TGF β, transforming growth factor β; VIP, vasoactive intestinal peptide

## Introduction

A successful pregnancy is the result of several complex interactions between the receptive uterus and the mature blastocyst under hormonal stimulation (Saito, 2000; Thellin *et al.*, 2000). For most cells, but especially for trophoblast cell growth and survival, it is essential to maintain uterine quiescence and neovascularization while promoting maternal immunotolerance. It is now hypothesized that during the early phase of pregnancy an inflammatory Th1 microenvironment sustains trophoblast invasion and tissue remodeling followed by a shift to Th2-type responses that would control endocrine and immune interactions, for embryo growth and development (Fest *et al.*, 2000; Clark *et al.*, 2002; Chaouat *et al.*, 2004). In line with this, trophoblastic antigens induce a natural and tolerogenic maternal response that involves regulatory T cells, cytokines, chemokines and galectin-1 derived from the feto-placental tissue (Aluvihare *et al.*, 2004; Blois *et al.*, 2007; Terness *et al.*, 2007). At the feto-maternal interface, allorecognition requires a finely regulated equilibrium. On one side, HLA alloantigen recognition in the uterus enhances the production of growth factors and hormones essential for embryonic and fetal development as the leukaemia inhibitor factor (LIF), stromal cell-derived growth

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factor-1, ribonucleases and human chorionic gonadotropin (Rugeles and Shearer, 2004; Larocca *et al.*, 2008). Such a microenvironment appears to control the first stages of gestation for both implantation and uterine receptivity (Chaouat *et al.*, 2007). On the other hand, alloreactive T cells represent the major barrier for successful allograft acceptance (Sherman and Chattopadhyay, 1993; Auchincloss and Sachs, 1998). Instead of an antigen-specific response where 0.1% of T cells are able to be activated, in allorecognition, 10% of T cells can differentiate to effector lymphocytes. This powerful response demonstrates that several tolerogenic mechanisms might be involved in the regulation of potentially deleterious, maternal T cells, activated in response to fetal antigens.

Vasoactive intestinal peptide (VIP) is a neuropeptide that promotes secretion in glandular epithelium (Ekstrom *et al.*, 1983; Inoue *et al.*, 1985) and contributes to smooth muscle relaxation and vasodilation in the uterus (Clark *et al.*, 1981; Jovanovic *et al.*, 1998). Through its action on specific receptors on macrophages, T and B lymphocytes, VIP has potent anti-inflammatory effects and, similar to progesterone, it promotes Th2 cytokine profiles (Gonzalez-Rey *et al.*, 2007). It has shown strong anti-inflammatory properties in several models of chronic autoimmune and immune-mediated inflammatory diseases (Henson *et al.*, 2001; Killedar *et al.*, 2006; Pregi *et al.*, 2006). VIP interacts with Toll-like receptor-mediated responses (Gutiérrez-Cañas *et al.*, 2006). Particularly in the non-obese diabetic (NOD) mouse model of type 1 diabetes, the anti-inflammatory and disease-protective effects of VIP were associated with a decrease of Th1 cytokines in serum and an increase of Th2 and the regulatory T cell markers, Foxp3 and transforming growth factor (TGF) β in the pancreas (Rosignoli *et al.*, 2006). In addition to its neural and immune regulatory properties, VIP participates in the maternal regulation of embryonic growth (Gressens *et al.*, 1998; Spong *et al.*, 1999). VIP was recently used to induce tolerogenic denditric cells with capacity to prevent acute graft-versus host disease following bone marrow transplant in a murine model (Chorny *et al.*, 2006). Thus, dendritic cells differentiated in the presence of VIP impaired allogeneic haplotype-specific responses of donor CD4+ cells by inducing regulatory T cells.

As implantation can be seen as a controlled inflammatory process and a successful pregnancy depends on an equilibrated alloantigen recognition, we hypothesized that VIP might contribute to fetal survival by limiting the initial Th1 response thus favouring the induction of a maternal tolerant state. In the present work, we tested the direct effects of VIP on a trophoblast cell line and, with trophoblast-immune cell co-cultures, the ability of VIP to modulate the inflammatory and allogeneic maternal immune response at the systemic and local feto-maternal levels. We also intended to dissect its potential functional role in each counterpart of this active cross-talk.

## Methods

*Patients*

Control fertile women were defined as women who had two or more previous normal pregnancies without any miscarriage. The Investigation and Ethics Committee at the Hospital de Clínicas José de San Martín approved this study and all ($n = 15$) patients provided their written consent to participate in the study.

*Peripheral blood mononuclear cells (PBMCs)*

Peripheral blood mononuclear cells from fertile women and their partners were isolated from heparinized peripheral blood by density gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). Cells were extensively washed and resuspended in RPMI 1640 (Life Technologies Grand Island, NY, USA) supplemented with 10% human serum, glutamine and penicillin-streptomycin.

*Co-cultures*

Trophoblastic cells (Swan 71 cell line, derived by telomerase-mediated transformation of a 7 week cytotrophoblast isolate described by Straswski-Chavez) (Aplin *et al.*, 2006) were cultured in 24 flat bottom polystyrene plates (Becton Dickinson, Franklin Lakes, NJ, USA) in complete DMEM 10% fetal calf serum (FCS) (Gibco, Invitrogen, Argentina). At 60% of confluence VIP was added ($10^{-7}$ mol·L$^{-1}$, BioSystem, France) and LIF expression was quantified.

For the quantification of the proliferative response in the presence of VIP, Swan 71 cells at 60% of confluence in a 96 flat bottom plate were cultured for 72 h and cells were pulsed for 18 h with 1 μCi per well of methyl-[$^3$H]-thymidine ([$^3$H]TdR; NEN, Boston, MA, USA). Then, trophoblast cells were lysed and harvested on glass fibre filters using a Packard Filtermate cell harvester (Packard Instruments, LaGrange, IL, USA). Incorporated radioactivity was measured in a liquid scintillation β-counter (Packard Instruments). Tests were conducted in triplicate and results were expressed as mean cpm ± SEM.

For co-cultures, trophoblast cells were cultured in the absence/presence of PBMCs from fertile women ($5 \times 10^5$ cells per well) with or without VIP ($10^{-7}$ mol·L$^{-1}$). After 48 h of culture, maternal PBMCs were recovered and then used for flow cytometry, reverse transcription-polymerase chain reaction (RT-PCR) or Western blot analysis.

*Allogeneic stimulation*

Maternal PBMCs (responder cells) were resuspended in complete RPMI-1640 ($1 \times 10^5$ cells per well). PBMCs from male partners (paternal PBMCs) resuspended in complete RPMI-1640 ($1 \times 10^5$ cells per well) were treated with mitomycin C (0.5 ng mL$^{-1}$, Sigma, St. Louis, MO, USA) for 30 min at 37°C, to inhibit paternal DNA synthesis by cross-linking DNA at guanine and adenine residues, disrupting base pairing (stimulator cells). Mixtures of responder and stimulator cells were incubated in a U-shape microtitre plate (Corning), in the presence of 10% human AB serum.

Cells were co-cultured also in the absence or in the presence of serum-free conditioned media (SFCM) and a VIP receptor antagonist (Peninsula-Bachem Inc, San Carlos, CA, USA, $10^{-5}$ mol·L$^{-1}$) in several combinations. The antagonist peptide, (H-Lys-Pro-Arg-Arg-Pro-Tyr-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-$NH_2$), a hybrid of neurotensin (6–11) and VIP (7–28) is a

competitive antagonist of VIP receptors on glial cells and in the secretory and vasodepressor actions of VIP (Gozes *et al.*, 1989). This VIP antagonist did not antagonize the vasodepressor effect induced by PACAP (Wheeler *et al.*, 1997). After 72 h, cells were pulsed with 1 μCi per well of [$^3$H]TdR during the last 18 h of cell culture, and then harvested on glass fibre filters using a Packard Filtermate cell harvester (Packard Instruments, LaGrange, IL, USA). Incorporated radioactivity was measured in a liquid scintillation β-counter (Packard Instruments). As [$^3$H]TdR incorporation by paternal PBMCs was prevented (by the mitomycin treatment), the cpm obtained reflected only maternal T lymphocyte proliferation in response to paternal antigens. Tests were conducted in triplicate and results were expressed as mean cpm ± SEM.

To study the modulation of the expression of a T cell transcription factor, T-bet, during the allogeneic response, co-cultures were performed also in the absence or in the presence of SFCM, VIP antagonist ($10^{-5}$ mol·L$^{-1}$) and VIP ($10^{-7}$ mol·L$^{-1}$) as positive control. After 48 h and cells were recovered and analysed by Western blot for T-bet expression.

*Cytokine and chemokine quantification*

Interleukin (IL)-6, IL-10 and monocyte chemoattractant protein 1 (MCP-1) were assayed by enzyme-linked immunosorbent assay (ELISA) in supernatant collected from the co-cultures performed in the presence of maternal PBMCs during 48 h. ELISAs were performed according to the manufacturer's instructions (Endogen and Becton Dickinson, Franklin Lakes, NJ for IL-6 and IL-10) and (R&D System MN, USA for MCP-1). Results were expressed in pg mL$^{-1}$.

*Nitrite determination*

Nitrite concentration was determined in supernatants obtained as described above for cytokine and chemokine measurements using the Griess method with N-(1-naphthylethylenediamine dihydrochloride and sulphanilamide (Ding *et al.*, 1988) Results were expressed as μmol·L$^{-1}$ of nitrite synthesized during the period mentioned.

*Western blot assays*

After co-culture, cells were analysed by Western blot for Foxp3, TGFβ and T-bet expression. Cells were extensively washed with phosphate-buffered saline (PBS), then cell pellet mixed gently with 1 mL ice-cold lysis buffer (PBS containing 5 mmol·L$^{-1}$ EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 142.5 mmol·L$^{-1}$ KCl, 5 mmol·L$^{-1}$ $MgCl_2$, 10 mmol·L$^{-1}$ HEPES, pH 7.2) with freshly added protease inhibitor cocktail (0.2 mmol·L$^{-1}$ PMSF, 0.1% aprotinin, 0.7 μg mL$^{-1}$ pepstatin and 1 μg mL$^{-1}$ leupeptin) and incubated for 1 h on ice. Samples were finally centrifuged at 12 000× *g* for 20 min at 4°C and the supernatant fluids, representing the whole cell protein lysates, were stored at −70°C until use. Protein concentration was estimated by using the micro-BCA™ Protein Assay reagent kit (Pierce, Rockford, IL, USA). Equal amounts of proteins were diluted in sample buffer and separated on SDS-polyacrylamide gels (10% for Foxp3 and T-bet or 15% for TGFβ). After electrophoresis, the separated proteins were transferred onto nitrocellulose membranes and probed with antibodies against Foxp3 (1:500; eBioscience, San Diego, USA) or against TGFβ (1:500; R&D System MN, USA) or against T-bet (1:500; Santa Cruz Biotechnol, CA, USA). Blots were then incubated with a 1:3000 dilution of a horseradish peroxidase (HRP)-conjugated anti-goat IgG for Foxp3 and T-bet or anti-rabbit IgG for TGFβ; and developed using an enhanced chemoluminescence detection kit (Amersham, Uppsala, Sweden). Equal loading and absence of protein degradation were checked by Ponceau S staining (Sigma, St. Louis, MO, USA). The immunoreactive protein bands were analysed with a Fotodyne Image Analyzer® (Fotodyne, Inc., Hartland, WI, USA). Results were expressed as relative densitometric values by means of the Image Quant software, relative to β-actin expression.

*Flow-cytometric analysis*

*Intracellular staining for LIF detection* To assess LIF production by the trophoblast cell line, cells were cultured in the absence or presence of VIP ($10^{-7}$ mol·L$^{-1}$) during 24 h at 37°C in a 5% $CO_2$ atmosphere and in the presence of Brefeldin A (10 μg mL$^{-1}$ Sigma, St. Louis, MO, USA) for the last 4 h to promote the intracellular accumulation of secretory proteins. After washing, cells were fixed in 4% paraformaldehyde in PBS-2% FCS for 20 min at room temperature. Then, cells were washed and permeabilized with 0.5% (w/v) saponin (Sigma, St. Louis MO, USA) in PBS for 30 min at room temperature. Permeabilized cells were washed and incubated for 30 min with anti-LIF antibody (BD Pharmigen, San José, CA, USA). Finally, cells were washed with PBS-2% FCS to allow membrane closure.

*Surface staining for LIF receptor detection* Swan 71 cells were cultured in the absence or presence of VIP ($10^{-7}$ mol·L$^{-1}$) at 60% of confluence were trypsinized with 1% trypsin (Invitrogen), washed and incubated for 30 min with anti-LIF receptor antibody (BD Pharmigen, San José, CA, USA). Finally, cells were washed with 2% PBS and analysed by fluorescence-activated cell sorter (FACS) analysis.

*Intracellular staining for Foxp3 detection* The flow cytometry analysis was performed essentially as described (Arruvito *et al.*, 2007). In brief, $1 \times 10^6$ cells were fixed in 1 mL PBS with 1% paraformaldehyde containing 0.05% Tween 20. After an overnight incubation at 4°C, cells were treated twice with 0.5 mL of RNase-free DNase at 100 U mL$^{-1}$ (Promega). Staining steps were performed for 1 h at room temperature. Cells were incubated with mouse anti-Foxp3 IgG monoclonal antibody (clone 236A/E7 provided by Dr L. Fainboim) and washed with PBS supplemented with 3% heat inactivated FCS, 0.5% Tween 20 and 0.05% azide. Foxp3 monoclonal antibody binding was detected by using Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) and washed as described above. Cell surface staining was then performed using the monoclonal antibodies Cy chrome anti-human CD4 and phycoerytrin (PE) anti-human CD25 (BD Pharmigen) for 20 min at room temperature followed by washing in PBS.

Ten thousand events were acquired in a FACSCalibur cytometer® and results were analysed using the WinMDI software®. Negative control samples were incubated in parallel with an irrelevant, isotype-matched antibody. Results for

**Table 1** Primer sequences used for PCR

| | *Primer* | *Sequence* |
|---|---|---|
| GAPDH | Upstream | TGA TGA CAT CAA GAA GGT GGT GAA G |
| | Downstream | TCC TTG GAG GCC ATG TAG GCC AT |
| VIP | Upstream | TAC AGG GCA CCT TCT GCT CT |
| | Downstream | CAA GAG TTT ACT GAA GTC ACT |
| $VPAC_1$ | Upstream | CCC CTG GGT CAG TCT GGT G |
| | Downstream | GAG ACC TAG CAT TCG CTG GTG |
| $VPAC_2$ | Upstream | TGG TTG GGT GTG GGC AG |
| | Downstream | GGA GAA ACC AGT CAG CTC CG |

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; VIP, vasoactive intestinal peptide.

positive cells are expressed as a percentage of LIF trophoblast-positive cells or inside the electronically gated CD4-positive cell population for analysis of regulatory T cells.

*RT-PCR for VIP and its receptors*

Expression of VIP and its receptors ($VPAC_1$ and $VPAC_2$; nomenclature follows Alexander *et al*., 2008) were performed by RT-PCR on extracts of the Swan 71 trophoblastic cells using the specific primers listed in Table 1. Briefly, total RNA was isolated using the Trizol reagent (Life Technologies, Grand Island, NY, USA). cDNAs were generated using a commercial kit (Clontech; Palo Alto, CA, USA) and stored at –20°C for batched analyses. For amplification of the resulting cDNA, 5 μL of the RT mixture were used. The sample volume was increased to 50 μL with the solution containing 50 mmol·$L^{-1}$ KCl, 10 mmol·$L^{-1}$ Tris (pH 8.3), 2 mmol·$L^{-1}$ $MgCl_2$, 0.1 μmol·$L^{-1}$ up- and downstream primers, and 1 U Taq polymerase in a DNA Thermocycler (PerkinElmer/Cetus, Boston, MA, USA). The primers and the thermal profile were selected with the software Primer-3. In the thermal cycler after a predenaturation step at 94°C for 5 min, we used denaturation step at 95°C for 30 s, an annealing step at 60°C for 30 s and elongation step at 72°C for 1 min for a total of 33 cycles. An additional extension step at 72°C for 10 min was carried out. PCR products were analysed by electrophoresis through a 2% ethidium bromide-stained agarose gel, visualized by transillumination and scanned. Densitometry was performed and the results were expressed as arbitrary units normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. As a positive control for VIP and VPAC we used the human neuroblastoma cell line, SH-SY5Y, cultured as previously described (Pregi *et al*., 2006).

*Statistical analysis*

Data are given as means ± SEM. The significance of the results was analysed by Student's *t*-test and Mann–Whitney, for non-parametric samples, using the GraphPad Prism4 software (GraphPad, San Diego, CA, USA). A value of $P < 0.05$ was considered significant.

## Results

*VIP induced, dose-dependently, pro-implantatory markers and proliferation in trophoblastic cells*

In order to evaluate potential embryotrophic effects of VIP, we first analysed VIP receptor expression in first trimester trophoblast cell line (Swan 71 cells). The results from RT-PCR for $VPAC_1$ and $VPAC_2$ receptor mRNA expression indicated that the trophoblastic cells constitutively expressed $VPAC_1$ receptors under basal conditions while $VPAC_2$ receptors were not detected (Fig. 1A). The control reference cells were human neuroblastoma SH-SY5Y cells. To assess the functional role of the $VPAC_1$ receptors, we quantified the nitrite production as a result of signaling via the $VPAC_1$ receptors on Swan 71 cells. In these cells, stimulated with LPS (10 μL $mL^{-1}$), incubation with VIP ($10^{-7}$ mol·$L^{-1}$) for 24 h, significantly decreased nitrite production as measured by the Griess method (basal production, 3.6 ± 0.5 μmol $L^{-1}$; with LPS, 9.8 ± 0.9 μmol $L^{-1}$ in the absence of VIP vs. 2.9 ± 0.5 μmol $L^{-1}$ in the presence of VIP, $P < 0.05$ Student *t*-test vs. LPS). This finding showed that Swan 71 cells not only express $VPAC_1$ receptors but that the receptors were also functional.

Next, to test effects of VIP on trophoblastic cells, Swan 71 cells were cultured in the absence or presence of VIP ($10^{-8}$ mol·$L^{-1}$ and $10^{-7}$ mol·$L^{-1}$). After 72 h, the thymidine incorporation reflected a significant increase in proliferation of the trophoblastic cells, in the presence of VIP ($10^{-7}$ mol·$L^{-1}$) (Fig. 1B). To investigate whether VIP could promote early pro-implantatory events in these first trimester trophoblast cells at a stage comparable to the implantation window, we evaluated the effect of VIP on the expression of pro-implantatory markers. One of the earliest factors involved in the maternal–fetal cross-talk that is up-regulated to favour a successful implantation is LIF. Western blot analysis revealed that after 24 h of culture, LIF expression increased in the presence of VIP ($10^{-7}$ mol·$L^{-1}$) (Fig. 1C). Moreover, staining for intracellular LIF expression or for surface LIF receptor expression and subsequent analysis by flow cytometry revealed that 15 ± 2% of trophoblast cells were able to produce LIF and 49 ± 10% showed LIF receptor expression in response to VIP ($10^{-7}$ mol·$L^{-1}$), suggesting a possible autocrine regulation (Fig. 1C,D).

Taken together, these data suggest that VIP could act as an embryotrophic factor inducing proliferation of trophoblast cells and increasing the production of LIF and expression of LIF receptor, thus favouring a pro-implantatory microenvironment.

*VIP increased the proportion of maternal T regulatory cells and TGFβ expression*

As we mentioned previously, VIP has clear anti-inflammatory effects and it could also regulate the immune response, promoting a tolerogenic response in several murine models through the modulation of the balance of maternal T cells between T effectors and regulatory T cells. As human CD4+CD25+ regulatory T cells mediate feto-maternal tolerance, we first focused on the ability of VIP to modulate maternal regulatory T cells and consequently the maternal response leading to tolerance to trophoblast antigens. Therefore, we performed co-cultures of trophoblastic cells and PBMCs isolated from fertile women (maternal PBMCs), as a model of the maternal-embryonic dialogue. We studied Foxp3 expression by maternal PBMCs after interaction with trophoblasts, in the absence or presence of VIP ($10^{-7}$ mol·$L^{-1}$). As shown in Figure 2A, Foxp3 expression

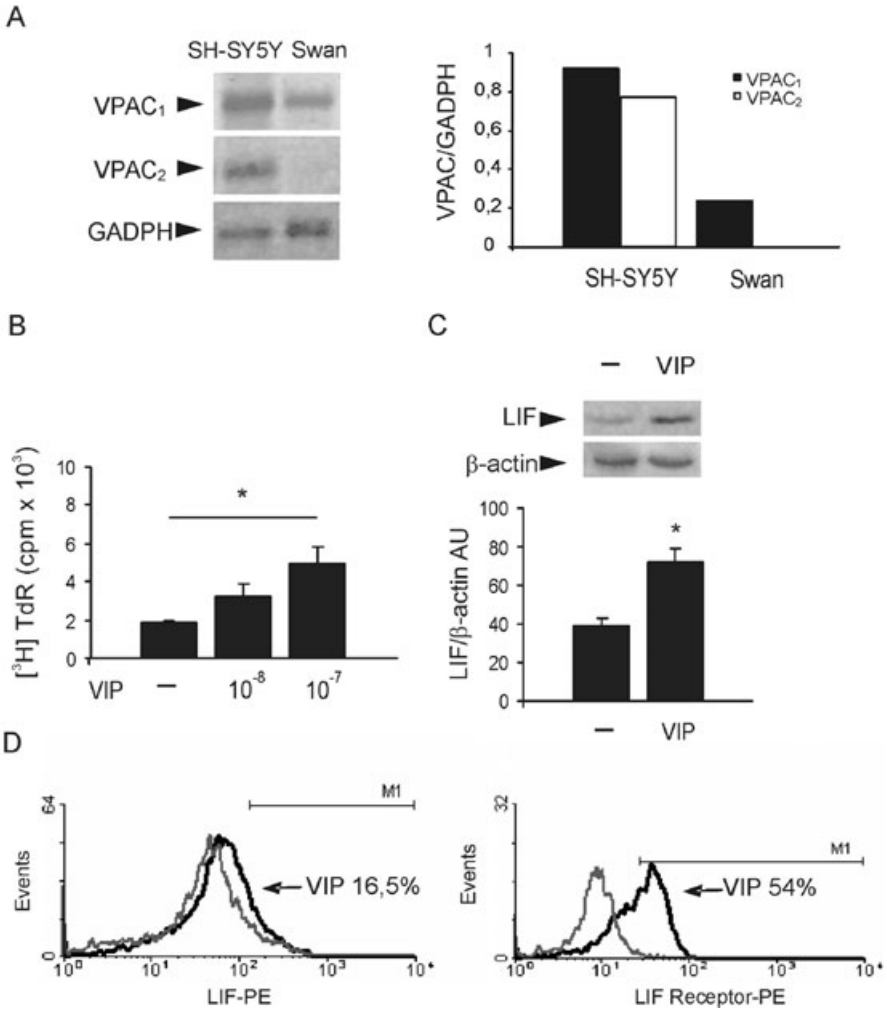


**Figure 1** Vasoactive intestinal peptide (VIP) induced pro-implantatory markers and trophoblast cell proliferation. (A): The trophoblast cell line (Swan 71 cells) at 60% of confluence in a 24 well flat-bottom plate, were cultured as described in Methods and analysed for VIP receptors, $VPAC_1$ and $VPAC_2$ by RT-PCR. The result shown is representative of five others run similarly; summary data are shown in the right-hand graph, as the density of the bands relative to GAPDH (means± SEM from five experiments; $*P < 0.05$, Student *t*-test). As a positive control we used SH-SY5Y human neuroblastoma cells. (B): Swan 71 cells were cultured as in A in the presence or absence of VIP ($10^{-7}$ mol·$L^{-1}$ and $10^{-8}$ mol·$L^{-1}$). After 72 h, [$^3$H]TdR was added for 18 h and uptake was determined using a β-scintillation counter. Results are expressed as mean cpm ± SEM of at least five independent experiments run in triplicate ($*P < 0.05$, Student *t*-test). (C): Swan 71 cells were cultured in the absence/presence of VIP ($10^{-7}$ mol·$L^{-1}$) and analysed by Western blot for LIF expression. Immunoreactive proteins are shown in the upper panel and the semi-quantification, relative to β-actin, in arbitrary units in the lower panel. Data are from a representative experiment, out of five ($*P < 0.05$ Mann–Whitney-test). (D): Cultured cells also were permeabilized and intracellularly stained with anti-LIF monoclonal antibody or surface stained for LIF-receptors. Figure shows one cytometric profile where data are expressed as % of LIF+ or LIF-receptor+ trophoblast cell line cultured in the presence of VIP versus those cultured in its absence. This profile is representative of three others with mean values (±SEM) of 15 ± 2% and 49 ± 10% for LIF and LIF-receptor respectively ($*P < 0.05$ vs. basal, Student *t*-test). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LIF, leukaemia inhibitor factor; PE, phycoerytrin; RT-PCR, reverse transcription-polymerase chain reaction.

dose-dependently increased in maternal PBMCs when cultured in the presence of VIP. These data were confirmed by assessing the size of the CD4+CD25+Foxp3+ cell population by triple staining and FACS analysis. The proportion of CD4+ CD25+Foxp3+ cells was increased in the presence of VIP, compared with those cultured without VIP. Figure 2B shows a representative dot plot of results from several different maternal PBMC donors, plotted in right-hand panel of Figure 2B. We also evaluated TGFβ expression by Western blot in maternal PBMCs and found that TGFβ expression was also increased by VIP in maternal PBMCs, after co-culture with trophoblastic cells (Fig. 2C).

*VIP modulates the balance of pro/anti-inflammatory mediators during the feto-maternal dialogue*

As VIP modulated the response of maternal PBMCs in favour of a tolerogenic response after PBMCs had contacted trophoblastic cells, we investigated if this modulatory effect could also be assessed in the feto-maternal microenvironment. Hence, we analysed the trophoblast-maternal leukocyte conditioned media to evaluate the modulation of pro/anti-inflammatory mediators associated with successful implantation. Trophoblast cells and maternal PBMCs were co-cultured for 48 h, in the absence or presence of VIP, the supernatant collected and cytokine secretion quantified by ELISA or nitrite

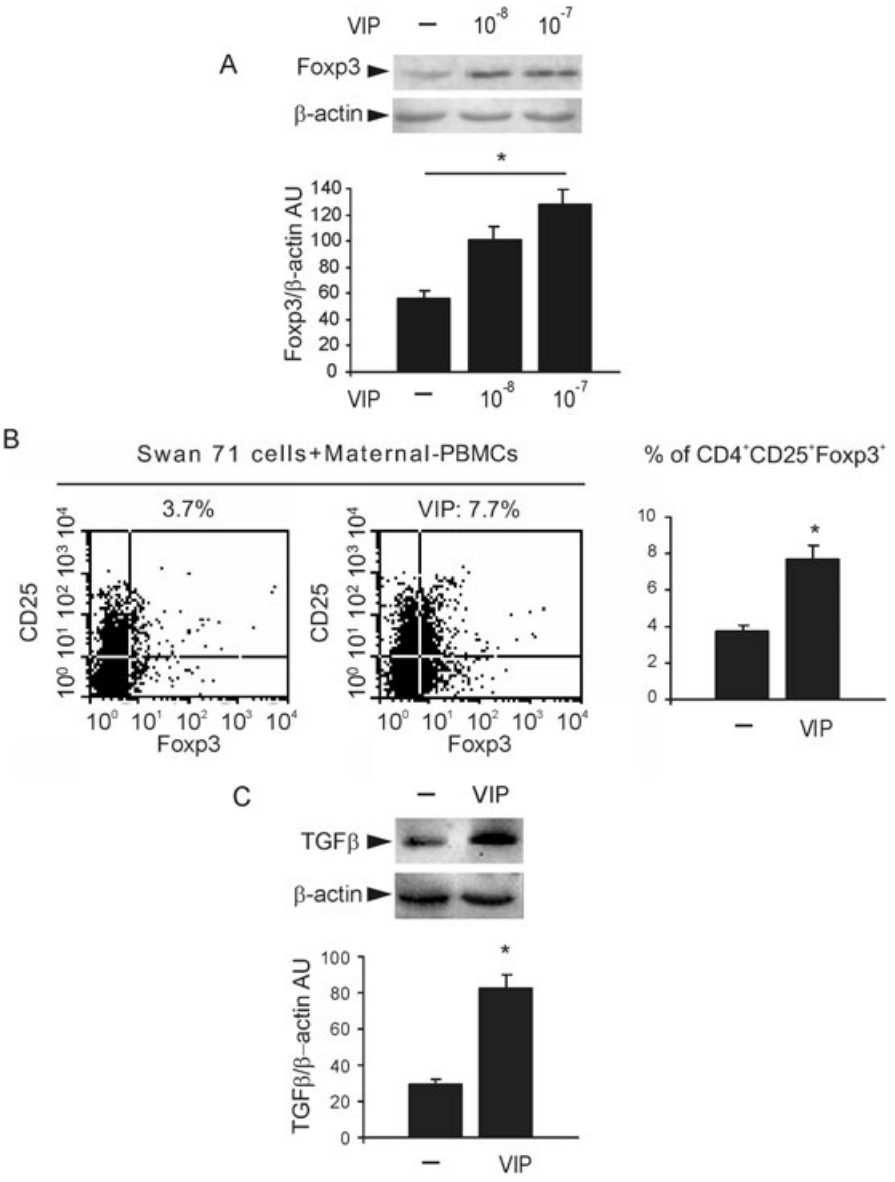


**Figure 2** Vasoactive intestinal peptide (VIP) increased the proportion of maternal T regulatory cells and TGFβ expression. Swan 71 cells were co-cultured with maternal PBMCs from fertile women, in the absence or presence of VIP ($10^{-7}$ mol·L$^{-1}$ and $10^{-8}$ mol·L$^{-1}$). (A) After 48 h of co-culture, the cells were analysed by Western blot for Foxp3 expression. Representative immunoreactive bands are shown and the semi-quantification expressed, relative to β-actin, in arbitrary units is shown below. Results are representative of four independent experiments using PBMCs from different fertile women (*$P < 0.05$, Mann–Whitney test). (B) After 48 h of culture, cells in suspension were recovered and triple stained for surface CD4, CD25 and intranuclear Foxp3 markers and analysed by FACS. The figure shows representative dot plots and the proportion of the CD4+CD25+Foxp3+ population is expressed as a percentage of CD4+ cells. Mean values ± SEM of four independent PBMC preparations are shown on the right side (*$P < 0.05$, Mann–Whitney test). (C) From the same co-cultures mentioned above, cells were analysed by Western blot for TGFβ expression. Representative immunoreactive bands are shown in the upper panel and the semi-quantification expressed, relative to β-actin, in arbitrary units in the lower panel. Results are representative of four independent experiments using different fertile women (*$P < 0.05$, Mann–Whitney test). FACS, fluorescence-activated cell sorter; PBMC, peripheral blood mononuclear cell; TGF, transforming growth factor.

production by Griess method. As shown in Figure 3A–C, IL-6, MCP-1 and nitrite levels significantly decreased while IL-10 was significantly increased, in the presence of VIP.

These data suggest that in fertile women, a VIP-mediated increase in the proportion of regulatory T cells correlates with a modulation of the balance of pro/anti-inflammatory markers in the media, by decreasing IL-6, MCP-1 and nitrites while increasing IL-10, as a result of the trophoblast-maternal cell cross-talk.

*VIP produced by trophoblast cells specifically suppressed allomaternal response*

Taking into account that exogenous VIP acted as an embryotrophic and tolerogenic factor modulating the maternal response in favour of tolerance to fetal antigens, we studied the relevance of VIP, produced by trophoblast cells, as a physiological mechanism that could contribute to the suppression of potentially deleterious, alloactivated, maternal T cells. First, we explored whether human trophoblast cells were able to

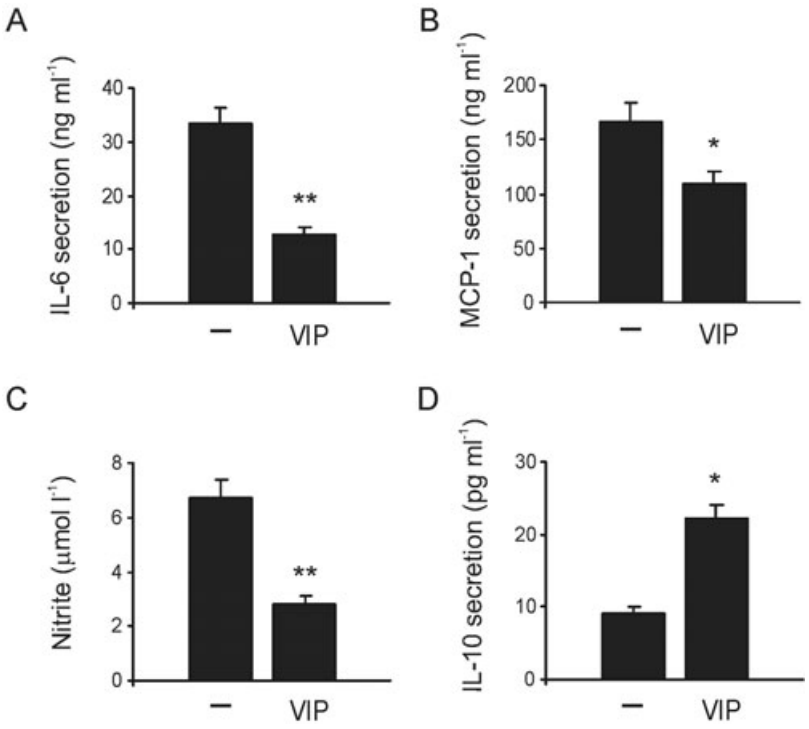


**Figure 3** Vasoactive intestinal peptide (VIP) modulated the balance of pro/anti-inflammatory mediators during the feto-maternal dialogue. Swan 71 cells at 60% of confluence in a 24 well flat-bottom plate were cultured in the presence of PBMCs from fertile women with or without VIP ($10^{-7}$ mol·L$^{-1}$). After 48 h of co-culture, supernatants and cells were collected. Concentrations of IL-6 (A), MCP-1 (B), nitrite (C) and IL-10 (D) in the supernatants were measured, by ELISA for the cytokines and by the Griess method for nitrite. Results are expressed as mean ± SEM of at least three independent experiments in triplicates with PBMCs from five different fertile women (*$P < 0.05$, **$P < 0.01$ vs. basal values, Student *t*-test). ELISA, enzyme-linked immunosorbent assay; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; PBMC, peripheral blood mononuclear cell.

produce VIP. For that purpose, primers and the thermal profile were selected with the software Primer-3 and RT-PCR for VIP was performed. As shown in Figure 4A, the analysis by RT-PCR of VIP mRNA expression revealed that Swan 71 trophoblast cells constitutively expressed VIP to the same extent as the control human neuroblastoma cells. Then, we investigated the contribution of this endogenous VIP to the suppression of allomaternal responses. For that purpose we performed a mixed lymphocyte reaction (MLR) with maternal and paternal PBMCs in the presence of SFCM obtained from the trophoblastic cells. To specifically determine maternal alloresponses, paternal PBMCs were pre treated with mitomycin C to prevent DNA synthesis. After 72 h of culture, SFCM (dilutions 1:50 and 1:10) was able to suppress dose-dependently the proliferation of maternal PBMCs (Fig. 4B). The contribution of endogenous VIP to trophoblastic-induced immunosuppression of the MLR was explored by conducting the same experiments in the presence of a VIP antagonist peptide. SFCM inhibition of alloactivated lymphocytes was prevented by the VIP antagonist peptide ($10^{-5}$ M) (Fig. 4C). Moreover, expression of the T cell transcription factor, T-bet, analysed by Western blot, also decreased in the MLR culture in the presence of SFCM (1:50) and the VIP antagonist peptide prevented this effect (Fig. 4D). As a positive control, exogenous VIP ($10^{-5}$ M) added to the mixed lymphocyte cultures also decreased T-bet levels (Fig. 4D). Taken together, these results suggest that trophoblast cells express VIP, that they can secrete it to the conditioned media and that endogenous VIP is able to specifically suppress potentially deleterious reactions of maternal Th1 lymphocytes against paternal alloantigens.

## Discussion

The appropriate generation of a pro-inflammatory response is thought to be a prerequisite for successful implantation of a fertilized ovum in the uterus (Rugeles and Shearer, 2004; Abrahams *et al.*, 2005). However, elevated leukocyte infiltration and inappropriate activation may be an underlying cause of complications and failures in pregnancy, attributed to an exacerbated inflammatory/Th1 response ultimately leading to tissue damage and embryo resorption (Bulmer and Sunderland, 1984; Chaouat *et al.*, 2002; Paria *et al.*, 2002).

Results presented herein provide experimental evidence that VIP could contribute to embryo survival in the trophoblast-maternal microenvironment through two relevant strategies: as an embryotrophic factor, VIP induced trophoblast cell proliferation and pro-implantatory markers. As a tolerogenic factor, VIP controlled the initial pro-inflammatory response increasing maternal regulatory T cells and suppressing effector Th1 cells and pro-inflammatory factors in the microenvironment, such as chemokines, cytokines and NO, thus promoting an overall balance that favours tolerance to fetal antigens.

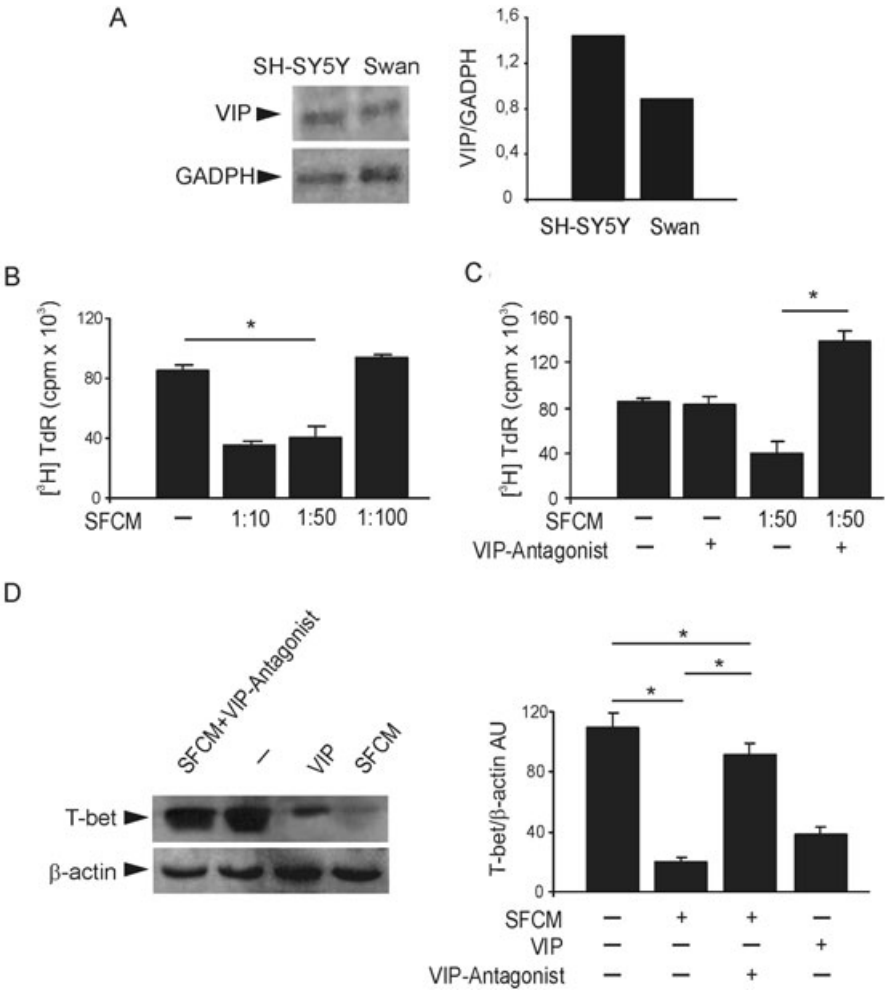


**Figure 4** (A) Vasoactive intestinal peptide (VIP) expression in Swan-71 cells. The mRNA and cDNA were obtained from trophoblast cell cultures, under basal conditions and VIP expression was measured by RT-PCR and normalized to GAPDH expression. As a positive control we used SH-SY5Y human neuroblastoma cells. Results shown are representative of three others run similarly. (B), (C) VIP produced by trophoblast cells specifically suppressed allomaternal responses. In (B), maternal PBMCs and mitomycin-treated, stimulator, paternal PBMCs were co-cultured in the presence/absence of increasing dilutions of serum-free conditioned media (SFCM) obtained from Swan 71 cells. In (C) the cells were incubated as in (B), but with SFCM (dilution 1:50) or the VIP antagonist ($10^{-5}$ mol·$L^{-1}$). After 72 h, [$^3$H]TdR was added for 18 h and uptake was determined using a β-scintillation counter. Results are expressed as mean cpm ± SEM of triplicate determinations (*$P < 0.05$, Student *t*-test) of three independent experiments using different fertile couples. (D) Maternal PBMCs and mitomycin-treated stimulator paternal PBMCs were co-cultured in the presence of SFCM (dilution 1:50). The VIP antagonist was added for 48 h and then cells were analysed by Western blot for T-bet expression. As a positive control for the effects of endogenous VIP on T-bet down-regulation, mixed lymphocytes cultures were treated with exogenous VIP ($10^{-5}$ mol·$L^{-1}$). Representative immunoreactive bands are shown in the upper panel and the semi-quantification expressed, relative to β-actin, in arbitrary units is shown in the lower panel. Results are representative of three independent experiments using different fertile couples (*$P < 0.05$, Mann–Whitney test). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription-polymerase chain reaction; SFCM, serum-free conditioned media.

Our conclusions are based on several observations: we have demonstrated that a first trimester trophoblast cell line (Swan 71) constitutively expressed both VIP and the VIP receptor $VPAC_1$, to the same extent as control, human neuroblastoma cells, suggesting a potential autocrine regulation. Expression of this subtype of VIP receptor has been also shown in human placenta (Sreedharan *et al.*, 1995). The $VPAC_1$ receptors were active, as the neuropeptide was able to induce trophoblast cell proliferation and to increase the expression of LIF and its receptor, both markers of a pro-implantatory milleu. In this sense, LIF is a key cytokine in the feto-maternal cross-talk. In fertile women, when the embryo invades the epithelium and reaches the endometrial stroma, it establishes an active dialogue by the expression of LIF receptors and the increase of LIF induced by several cytokines like TGFβ (Dimitriadis *et al.*, 2005; Piccinni, 2007). Moreover, LIF and LIF mRNA have been found expressed in the endometrial glands, throughout pre-implantation blastocyst development (Bulletti *et al.*, 2005; Song and Lim, 2006).

Regarding VIP tolerogenic effects on maternal response at the local level, we analysed VIP effects in co-cultures of trophoblastic cells and maternal PBMCs, as a model of the embryonic-maternal dialogue. VIP modulated maternal response after the interaction with trophoblast cells, reflected as an increased proportion of CD4+CD25+Foxp3+ regulatory T cells, accompanied by an increase in TFGβ expression. Tro-

phoblastic antigens induce a tolerogenic maternal response involving regulatory T cells, cytokines, chemokines and galectin-1 derived from the feto-placental tissue (Aluvihare *et al*., 2004; Blois *et al*., 2007; Terness *et al*., 2007). Similarly, VIP was able to modulate the subpopulation of regulatory T cells in several acute and chronic inflammatory processes (Mauri *et al*., 1996; Gomariz *et al*., 2001; Delgado *et al*., 2002; Abad *et al*., 2003; Armstrong *et al*., 2003). In line with this, we have previously demonstrated a potentiation of regulatory T cell function by VIP, through the up-regulation of Foxp3 and TFGβ in pancreas of diabetic NOD mice, which may lead to restoration of tolerance to pancreatic autoantigens (Rosignoli *et al*., 2006).

In addition, VIP could also modulate the balance of pro- and anti-inflammatory mediators, shown by a reduction in IL-6, MCP-1 and nitrite production and as an increase in IL-10 production. Particularly IL-6 appears to have different actions on different cell types and can act to either promote or suppress inflammatory responses. Kawano *et al*. (2004) have demonstrated that IL-1α stimulated endometrial cells to produce IL-6, IL-8 and macrophage colony-stimulating factor and these cytpokines were suppressed by IL-1RA in a dose-dependent manner. Moreover, in chemotaxis assays, IL-6 was an effective chemoattractive factor for human trophoblast cells (Domínguez *et al*., 2008). However, IL-6 can also have deleterious effects: it could enhance inflammatory responses under pathological conditions, as in rheumatoid arthritis (Deon *et al*., 2001). In this regard, in the feto-maternal dialogue, VIP could act as an additional control of IL-6 production, after the peri-implantation period and down-regulate the inflammatory response. On the other hand, VIP inhibited induction of NO synthase while stimulating IL-10 in mouse activated macrophages, under normal and pathological conditions (Larocca *et al*., 2007). Finally, an increase in IL-10 production together with decreased IL-6, as an example of a immunoregulatory loop, was also observed in other inflammatory models in mice and humans, such as murine macrophages stimulated with LPS (Delgado, *et al*., 1999) and in several models of autoimmunity (Delgado *et al*., 2001; Gonzalez-Rey *et al*., 2007; Arranz *et al*., 2008). Therefore, these results support a protective anti-inflammatory effect of VIP in favour of a tolerogenic maternal response.

On the other hand, maternal T cells activated in response to paternal alloantigens may be at the centre of a local regulatory mechanism during and after the process of implantation. As suggested by Rugeles *et al*. (2004), the allogeneic response represents a factor favouring successful implantation in humans; however, this response should be controlled to protect the surrounding cells from damage and consequently from implantation failure. Data presented here show that conditioned media from trophoblast cells suppressed alloactivated maternal T cells and reduced T-bet expression. The effect seems to be partly mediated by endogenous VIP, as it was specifically blunted by the VIP antagonist.

Control of the initial inflammatory response at the feto-maternal interface is a hallmark of a successful implantation and the anti-inflammatory and tolerogenic effects of VIP could be directly involved in this process. The effects of endogenous VIP produced by the trophoblast cell line on co-culture with PBMCs, support the participation of VIP in the induction and maintenance of maternal tolerance to fetal antigens. Interestingly, VIP acted as a survival factor for human prostate cancer cells by protecting them from immune surveillance increasing cell proliferation in an autocrine mechanism (Gutiérrez-Cañas *et al*., 2003). This tumour-immune privilege might be compared with trophoblast cell-immune interaction, as trophoblast cells are known to display multiple strategies to evade maternal immune attack. VIP-mediated immune regulation in the maternal–fetal dialogue shown here could be one of the mechanisms for semi-allograft fetal survival.

Given the action of VIP in the development of regulatory T cells and the efficacy of these cells to control inflammatory processes, this peptide emerges as a promising candidate for an effective treatment for early loss of pregnancy, such as recurrent spontaneous abortions or repetitive implantation failures after *in vitro* fertilization procedures. To our knowledge, this is the first report on regulatory T cell-promoting and anti-inflammatory effects of VIP in the human maternal–fetal dialogue. Similarly, the demonstration that VIP could induce proliferation and the expression of LIF and LIF receptors in trophoblastic cells suggests a potential role of the neuropeptide as an embryotrophic pro-implantatory factor.

Although research in the past few years has provided a better understanding of the molecular mechanisms leading to immune tolerance and homeostasis, the definitive cellular and molecular interactions underlying the embryo-uterine cross-talk remain to be resolved. Further studies are clearly required to assess the clinical, diagnostic and therapeutic applications of VIP in the human maternal–fetal interface, but the present observations might contribute to the design of novel therapeutic strategies to prevent fetal rejection.

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## Conflict of interest

Authors have no conflicts of interest.

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