VIP contribution to the decidualization program: regulatory T cell recruitment

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Abstract
During early pregnancy, the human uterus undergoes profound tissue remodeling characterized by leukocyte invasion and production of proinflammatory cytokines, followed by tissue repair and tolerance maintenance induction. Vasoactive intestinal peptide (VIP) is produced by trophoblast cells and modulates the maternal immune response toward a tolerogenic profile. Here, we evaluated the contribution of the VIP/VPAC to endometrial renewal, inducing decidualization and the recruitment of induced regulatory T cells (iTregs) that accompany the implantation period. For that purpose, we used an in vitro model of decidualization with a human endometrial stromal cell line (HESC) stimulated with progesterone (P4) and lipopolysaccharide (LPS) simulating the inflammatory response during implantation and human iTregs (CD4+CD25+FOXP3+) differentiated from naïve T cells obtained from peripheral blood mononuclear cells of fertile women. We observed that VIP and its receptor VPAC1 are constitutively expressed in HESCs and that P4 increased VIP expression. Moreover, in HESC VIP induced expression of RANTES (CCL5), one of the main chemokines involved in T cell recruitment, and this effect is enhanced by the presence of P4 and LPS. Finally, assays of the migration of iTregs toward conditioned media from HESCs revealed that endogenous VIP production induced by P4 and LPS and RANTES production were involved, as anti-RANTES neutralizing Ab or VIP antagonist prevented their migration. We conclude that VIP may have an active role in the decidualization process, thus contributing to recruitment of iTregs toward endometrial stromal cells by increasing RANTES expression in a P4-dependent manner.

Key Words
- VIP
- early pregnancy
- human endometrial stromal cells
- decidualization

Introduction
Endometrial receptivity, embryo implantation and the maintenance of pregnancy is a sequence of intricate events that requires a coordinated interaction between the endometrial epithelial and stromal cells, the maternal immune system and the blastocyst (Dey et al. 2004, Gellersen et al. 2007, Stoikos et al. 2008).

In this context, during early pregnancy, the uterus undergoes profound remodeling and leukocyte invasion...
associated with the production of proinflammatory factors (Dimitriadis et al. 2010, Pérez Leirós & Ramhorst 2013). Under the influence of progesterone (P4), endometrial stromal cells differentiate into epithelioid decidual cells and secrete diverse mediators, which contribute to the generation of a local immune-privileged site supporting the nidation of a semiallogenic fetus (Gellersen & Brosens 2003, Mesiano et al. 2011).

The decidualization of the stromal cells also occurs in the absence of pregnancy and the declining P4 levels trigger several effects, such as the expression of pro-inflammatory cytokines, chemokines and matrix metalloproteinases, and activate a sequence of events leading to menstruation (Salamonsen & Woolley 1999, Catalano et al. 2007). Moreover, decidualization in humans is apparent 10 days after ovulation, indicating that P4 is not the primary trigger of this differentiation process. In fact, initiation of the decidual process is dependent on elevated levels of cAMP (Teklenburg et al. 2010a), indicating that local factors could be involved in the activation of adenylate cyclase in stromal cells.

As menstruation and early pregnancy are inflammatory conditions that cause a degree of physiological tissue injury, the exposure of the uterus to a threatening stimulus at a dose below the threshold for tissue injury will provide tolerance against a more severe subsequent insult (King & Critchley 2010, Teklenburg et al. 2010b).

During the process of tissue renewal associated with the menstrual cycle, uterine cells undergo apoptosis necessary for the removal of cellular debris. Implantation and early placentation represent still another period of high tissue turnover and renewal. During the early stage of implantation, trophoblast cells break the epithelial lining of the uterus in order to adhere, then invade endometrial tissue and replace endothelial cells, generating apoptotic bodies of the trophoblast that will contribute to the induction of a tolerogenic microenvironment (Abrahams et al. 2004a). Therefore, implantation involves a tight homeostatic control provided by immune cells selectively recruited and/or expanded depending on the subpopulation during the early stages of gestation and the contribution of redundant molecules able to trigger multiple tolerogenic programs (Gomez-Lopez et al. 2010, Pérez Leirós & Ramhorst 2013). In this context, the modulation of chemokines and their receptors selectively controls the recruitment of different leukocyte populations (Bromley et al. 2008, Fraccaroli et al. 2009a). During the implantation period, in particular, the β-chemokine CCL5 (RANTES) is locally produced by the human endometrium and, interestingly, it has the potential to act in an autocrine manner by the differential expression of its receptors CCR1, CCR3 and CCR5 (Ramhorst et al. 2006, 2007). In addition, RANTES is produced by human endometrial T lymphocytes, CD4+ and CD8+ cells, and its production is increased in the presence of physiological concentrations of P4 (Ramhorst et al. 2006).

One of the main effects of RANTES is the induction and the recruitment of regulatory T cells (Tregs; Fraccaroli et al. 2009b). The specialized population of Tregs is essential for preventing a maternal immune response against paternal antigens. Basically, natural Tregs (nTregs) (derived from the thymus) that constitutively express CD25 can be distinguished from inducible Tregs, CD4+CD25+ FOXP3+ cells that are induced from CD4+CD25− precursors in the peripheral lymphoid organs (induced regulatory T cells (iTregs); Guerin et al. 2009). Prior to implantation, the seminal fluid can drive the expansion of iTregs (Robertson et al. 2013) and then the continuous release of placental antigens into the maternal circulation would maintain a Treg population targeted specifically against paternal antigens (Aluvihare et al. 2004). Previously, we described the development of an in vitro differentiation model of iTregs from naïve CD45RA+CCR7+ cells obtained from peripheral blood mononuclear cells (PBMCs) isolated from fertile women. We observed that trophoblast cells not only contributed to their differentiation in a TGFβ-dependent pathway, but also secreted chemokines, such as RANTES, MCP1 (CCL2) and IL8, which were capable of selectively recruiting them (Ramhorst et al. 2012).

Vasoactive intestinal peptide (VIP) is a pleiotropic peptide with embryotrophic, smooth-muscle-relaxing, prosecretory and immunomodulatory effects (Ekström et al. 1983, Spong et al. 1999, Gonzalez-Rey et al. 2007, Leceta et al. 2007, Couvineau & Laburthe 2012). VIP was shown to downregulate inflammatory factors and inhibit antigen-specific Th1-driven immune responses switching to a tolerogenic profile with the generation or expansion of Treg cells (Gonzalez-Rey et al. 2007, Leceta et al. 2007). In addition, among several mediators released locally, we have proposed a role for VIP at the early maternal–placental interface with immunosuppressant and trophic effects (Fraccaroli et al. 2009c, Pérez Leirós & Ramhorst 2013. Certainly, using an in vitro model of trophoblast and maternal leukocyte interaction, VIP showed a Th1-limiting and Treg-promoting response that would favor early pregnancy outcome. VIP also decreased the production of inflammatory mediators after culture of PBMCs from fertile women with trophoblast cells, while it increased TFGβ and IL10 production (Fraccaroli et al. 2009c).
Taking into account the findings that endometrial stromal cells are exposed to an inflammatory response that preconditions the uterus during the peri-implantation period and that VIP mediates protolerogenic responses, we evaluated the contribution of the VIP/VPAC system to endometrial renewal, induction of decidualization and the recruitment of iTregs that accompany the implantation period. In the present study, we used an in vitro model of decidualization with a human endometrial stromal cell line (HESC) stimulated with P4 and lipopolysaccharide (LPS) simulating the inflammatory response during implantation.

Materials and methods

Human endometrial stromal cells

Immortalized HESC described by Krikun et al. (2004) were maintained in DMEM–F12 media supplemented with 10% FCS and 2 mM glutamine. For the different assays, HESCs were cultured in 24-well plates until they reached 70% confluence. Different combinations of VIP (10⁻⁷ M), LPS (100 ng/ml), P4 (10⁻⁶ M), the physiological concentration reported at the feto-maternal interface (Van Voorhis et al. 1989) and VIP antagonist (ANT, 10⁻⁵ M) were added for 24 h.

Conditioned media

HESCs were cultured in DMEM–F12 media supplemented with 10% FCS, overnight supernatants were collected and maintained at −20 °C until use.

Decidualization

HESCs were cultured in 24-well plates containing DMEM–F12 media supplemented with 10% FCS in the presence of VIP (10⁻⁶–10⁻⁸ M), or medroxyprogesterone acetate (MPA) (10⁻⁸ M)–dibutyryl cAMP (2.5×10⁻³ M) for 8 days, changing half of the culture media every 48 h, and then used in the assays described below.

Peripheral blood mononuclear cells

PBMCs were isolated from fertile, non-pregnant women who had two or more previous normal pregnancies without any miscarriage. The ‘Investigation and Ethics Committee’ from the Argentine Society of Gynecological and Reproductive Endocrinology (SAEGRE) approved this study and all the patients provided their written consent to participate in it. PBMCs were isolated from heparinized peripheral blood by density gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech). Cells were extensively washed and resuspended in RPMI 1640 media (Life Technologies) supplemented with 10% human AB serum, 2 mM glutamine and 1% penicillin–streptomycin.

In vitro differentiation of iTregs

In vitro differentiation was performed as described previously (Ramhorst et al. 2012). Briefly, naïve CD4 T cells were isolated from PBMCs of fertile women by negative depletion using the Easy Sep Kit and following the manufacturer’s recommendations. The recovered naïve CD4 T cells were cultured in plates precoated with anti-CD3 (10 μg/ml, BD-Pharmigen, Franklin Lakes, NJ, USA) + anti-CD28 (1 μg/ml, BD-Pharmigen) and maintained in media supplemented with IL2 (2 ng/ml, Peprotech, Rocky Hill, NJ, USA) and recombinant TGFβ (10 ng/ml, R&D Systems, Minneapolis, MN, USA). Media were changed every 48 h, and after 5 days of culture, we obtained 26 ± 4% of CD4⁺ FOXP3⁺ cells.

Real-time PCR

We evaluated the expression of chemokines, IL8, MCP1 and RANTES, as well as VIP and its receptors, VPAC1 and VPAC2, and the decidualization markers, KLF13 and IGF-binding protein 1 (IGFBP1, also known as placental protein 12), in HESCs under different combinations of stimuli. After 24 h of stimulation, total RNA was isolated using TRIzol reagent (Life Technologies) following the manufacturer’s recommendations, cDNAs were generated from 1 μg RNA using a MMLV reverse transcriptase, RNasin (RNase inhibitor) and oligo (dT) kit (Clontech) and stored at −20 °C for batch analysis. 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 μM forward and reverse primers (described in Table 1), 1 U Taq polymerase in a DNA Thermocycler (PerkinElmer/Cetus, Boston, MA, USA) and 1:30 000 dilution of SybrGreen. The PCR programs used were an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 s, 20 s at a melting temperature specified in Table 1 and 20 s at 72 °C. SybrGreen fluorescence was measured at the end of each cycle. A final elongation at 72 °C for 10 min was also performed. Real-time PCR was performed on a Bio-Rad iQ5 Real-time PCR system. Results were expressed as arbitrary units normalized to GAPDH expression.

Flow cytometry analysis

Intracellular staining for FOXP3 detection

The flow cytometric analysis was performed according to the
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Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’–3’)</th>
<th>Product length (bp)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIP</td>
<td>F: TACAGGCCACCTTCTGCTCT</td>
<td>198</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>R: CAAGAGTTTCAAGGAAGTCACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPAC1</td>
<td>F: CCCCTGGTGGTAGCTGGTG</td>
<td>100</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>R: GAGACCTGACCCGCCGTGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPAC2</td>
<td>F: CCAAGTGTCCGGCGCAACG</td>
<td>114</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>R: GCTGATGGAAAACGGGCAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL8</td>
<td>F: CCAACACAGAAATATTGTAAGACG</td>
<td>163</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>R: CACTGGCCATCTTACACTGATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP1</td>
<td>F: CAGCGACAAAGTTCGCGCAACG</td>
<td>146</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>R: GAGTGAAGTGTTCAAGCTTCCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>F: TGCTGCTTTGCTACATGG</td>
<td>95</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>R: AAGACGACTGCTGGGTCCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLF13</td>
<td>F: TTCGTTGAGTCTTCTTGAGGTCGG</td>
<td>169</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>R: TGGACCCCTGGATTCTGCTTGG</td>
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</tr>
</tbody>
</table>

We evaluated the migration of the FOXP3+ cells using different conditioned media (CM) as stimuli obtained from HESCs cultured in the presence of P4, LPS, VIP or VIP antagonist. An anti-RANTES neutralizing antibody (1 μg/ml, R&D Systems) was added during the assay to evaluate the role of chemokines. After differentiation, the naïve T cells were seeded in 8 μm inserts (4 × 10^4 cells/insert) (BD Falcon Cell culture inserts), which then were set in a 24-well plate containing the CM from HESCs cultured under different conditions. After 24 h, the cells were recovered from the lower compartment and the frequencies of FOXP3+ cells were quantified by FACS analysis. As a positive control, we used 20% human serum. The results are expressed as the folds of increase with respect to the positive control.

**Statistical analysis**

The significance of the results was analyzed by Student’s t-test and ANOVA with the Bonferroni post-test for parametric analysis of HESC samples. The Mann–Whitney U test was used for the analysis of non-parametric samples from maternal PBMCs. We used the GraphPad Prism5 software (GraphPad, San Diego, CA, USA) and a value of *P*<0.05 was considered significant.

**Results**

Endometrial stromal cells express the VIP/VPAC system and P4 modulates its expression

First, we evaluated the expression of VIP and its receptors VPAC1 and VPAC2 in HESC. As depicted in Fig. 1A, VIP and VPAC1 are constitutively expressed in stromal cells. As P4 has modulatory effects on endometrial cell differentiation and function at early pregnancy, we evaluated whether it affected the VIP/VPAC system expression in HESC. For that purpose, HESCs were cultured at 70% of confluence in the absence or presence of P4 (10^-6 M), and we observed that P4 significantly increased VIP expression in HESCs, while VPAC1 was not modulated, as determined by RTqPCR (Fig. 1A). This result was confirmed by generating a P4 concentration-response curve and determining median intensity of fluorescence by flow cytometry, and we observed that P4 significantly increased intracellular production of VIP with a peak at 10^-6 M (Fig. 1B). The MIF of VIP in HESC treated with different P4 concentrations is also shown as a representative histogram. VPAC2 expression was not detected in HESCs under these conditions.
VIP induces chemokine expression

Our next objective was to determine the effect of VIP on the expression of chemokines involved in leukocyte recruitment toward endometrial stromal cells. In addition, we evaluated the effect of LPS as a proinflammatory stimulus. Hence, the expression of the chemokines RANTES, involved in T cell recruitment, IL8 (CXCL8), involved in neutrophil recruitment and MCP1, one of the main chemokines involved in monocyte/macrophage recruitment, were evaluated by RTqPCR in HESCs stimulated or not with VIP (10^{-7} M) and LPS (100 ng/ml). As shown in Fig. 2A, B and C, LPS increased the expression of RANTES, IL8 and MCP1. VIP by itself did not have a significant effect on production of cytokines by HESCs; however, the combination of LPS and VIP further enhanced LPS-induced expression of RANTES.

P₄ induces RANTES expression through a VIP pathway

As RANTES expression was further increased in the presence of VIP and LPS, and P₄ induced production of endogenous VIP, we investigated whether RANTES expression was modulated by endogenous VIP in HESCs. Therefore, HESCs were cultured in the absence or presence of P₄, LPS (100 ng/ml) and VIP antagonist to evaluate the relevance of the endogenous VIP. We observed that VIP-induced RANTES expression in the presence of LPS was prevented by VIP antagonist (Fig. 3A). P₄ also induced RANTES expression and this was far more pronounced in the presence of LPS. VIP antagonist prevented the increase in RANTES expression mediated by P₄ and LPS, indicating that RANTES induction involved a VIP-mediated pathway (Fig. 3A).

We then investigated the modulation of one transcription factor involved in RANTES expression, the Kruppel-like factor 13 (KLF13; Song et al. 2002, Pabona et al. 2010). HESCs were cultured in the absence or presence of P₄, VIP, LPS and VIP antagonist, and then KLF13 expression was evaluated by RTqPCR. As shown in Fig. 3B, P₄ and VIP in the presence of LPS significantly increased KLF13 expression. VIP antagonist only prevented the increase in KLF13 expression induced by VIP and LPS.
Endometrial stromal cells specifically recruit iTregs through RANTES production

Our next step was to determine if HESCs have the ability to attract iTregs. Human Treg cells were differentiated from naive CD45RA<sup>+</sup>CCR7<sup>+</sup> cells obtained from PBMCs obtained from fertile women and cultured with IL2 and TGFβ over 5 days, as described in the Materials and methods section. We then performed migration assays using a multi-chamber system. In-vitro-differentiated iTregs were seeded onto 8 µm pore inserts, allowing cell migration toward the CM used as a chemotactic stimulus in the lower compartment. After 24 h, cells were recovered from the lower compartment and FOXP3 expression was quantified by FACS analysis. As shown in Fig. 4A, the CM from HESCs increased the frequency of FOXP3<sup>+</sup> cells to levels similar to the migration observed in the presence of human serum (positive control). However, when the migration assay was performed in the presence of CM from HESCs cultured in the presence of VIP antagonist, the recruitment of iTregs to the lower compartment was prevented (Fig. 4A). Moreover, addition of anti-RANTES...
neutralizing Ab to the CM from HESC treated with P₄ and LPS was also able to prevent migration of iTregs (see Fig. 4A). Figure 4B shows representative dot plots with the percentages of FOXP3⁺ cells. We did not observe changes in the migration rate of the FOXP3⁻ population under the same conditions mentioned above indicating that RANTES participates in the specific recruitment of iTregs toward HESCs (Fig. 4C).

VIP induces decidualization of endometrial stromal cells

Based on the hypothesis of a potential contribution of VIP to the decidualization program, we investigated the direct effects of VIP on endometrial stromal cells. Therefore, HESCs were cultured in the absence or presence of VIP (10⁻⁷ M), P₄ (10⁻⁶ M), VIP antagonist (ANT, 10⁻⁵ M) and LPS (100 ng/ml)) in the lower compartment in the presence or absence of anti-RANTES (αRANTES) neutralizing antibody. Migration was evaluated by flow cytometry as the total number of FOXP3⁺ cells. (A) The results are expressed as the folds of increase with respect to the positive control (AB human serum) from three independent experiments using different maternal PBMCs for FOXP3⁺ cells (A) and FOXP3⁻ cells (B) (Mann–Whitney U test, *P<0.05). (C) Representative dot plot profiles with the percentages of FOXP3⁺ cells.

We observed that HESCs cultured with 10⁻⁷ and 10⁻⁶ M VIP displayed significantly increased IGFBP1 expression (Fig. 5A). The modulation in IGFBP1 was accompanied by morphological changes that characterize the decidualization process as depicted in Fig. 5B.

As endometrial stromal cells after decidualization increased chemokine production and VIP induced the marker of decidualization IGFBP1 in HESCs, we wondered if VIP was also able to increase the expression of RANTES after cell differentiation. HESCs were decidualized in the presence of VIP (10⁻⁶–10⁻⁸ M), and after 8 days, we observed a significant increase in RANTES expression (see Fig. 5C). This increase was also accompanied by a significant increase in the expression in HESCs of KLF13, which is a RANTES transcription factor besides being a decidualization marker (see Fig. 5D).

Taken together, these results indicate that VIP might participate in the decidualization process not only by the induction of decidualization markers, but also by...
increasing RANTES production, which mediates the recruitment of iTregs.

**Discussion**

In humans, the decidualization process involves the transformation of stromal fibroblasts into epithelioid decidual cells and the recruitment of immune cells critical for decidual development in an early inflammatory microenvironment; thus, multiple regulatory mechanisms are required to maintain the local immune homeostasis (Wilcox et al. 1999, Terness et al. 2007, Chaouat et al. 2010, Yoshinaga 2010).

In line with the strict regulation that Treg cells have on the control of the effector immune responses throughout pregnancy (Aluvihare et al. 2004, Guerin et al. 2009, Ramhorst et al. 2012, Robertson et al. 2013), we analyzed the contribution of the neuropeptide VIP to the decidualization program, reflected by the increase in decidualization markers and by the recruitment of iTregs toward endometrial stromal cells, as a local regulator of the inflammatory response during implantation. For that purpose, we used the HESC cultured under different stimuli and iTregs differentiated from naïve CD45RA<sup>+</sup>CCR7<sup>+</sup> cells obtained from PBMCs of fertile women as an in vitro model of interaction. HESCs were cultured in the presence of an effective concentration of P<sub>4</sub> (10<sup>–6</sup>–10<sup>–8</sup> M) and LPS (100 ng/ml) as an inflammatory stimulus that modulated chemokine production but did not affect cell viability (Abrahams et al. 2004b).

The results presented in this study indicating that the neuropeptide VIP, with potent anti-inflammatory and immunomodulatory effects, could contribute to the decidualization process inducing endometrial stromal cell expression of decidualization markers and the selective recruitment of iTregs toward HESCs by increasing production of RANTES under the effect of P<sub>4</sub> in the presence of a inflammatory microenvironment.
Our conclusions are based on several observations: first, HESCs express VIP and its constitutive receptor VPAC1, and \( P_4 \) has the ability to increase VIP production. Secondly, expression of RANTES, one of the main chemokines involved in T cell recruitment, was induced by VIP in the presence of LPS, and its induction was mediated by \( P_4 \). Finally, the assay of migration of iTregs toward CM from HESCs revealed that the endogenous VIP production induced by \( P_4 \) and LPS stimulation could selectively attract them through production of RANTES, as the anti-RANTES neutralizing Ab or VIP antagonist prevented the migration of iTregs.

VIP might be one of the first mediators that induces decidualization through its interaction with the VPAC1 receptor and triggering cAMP signaling in HESCs to increase the expression of IGFBP1 and KLF13, both markers of decidualization accompanied by morphological changes characteristic of decidualized cells. In fact, KLF13 is not only a decidualization marker, but also a transcription factor that binds to the RANTES promoter, which is necessary to mediate RANTES transcription (Song et al. 2002). This mechanism could explain how endogenous VIP regulates RANTES expression in HESCs, thus contributing to the selective recruitment of iTregs that might allow the control of tissue damage during embryo implantation. In this context, Nancy et al. (2012) recently reported that genes encoding chemokines are subject to epigenetic silencing in decidual stromal cells to restrain the attraction of Th1 and T cytotoxic profiles as a strategy to prevent potential tissue damage. In brief, the decidualization program involves many regulatory molecules that play functional roles, such as insulin-like growth factors, interleukin 1, 6, 10 and TGF\( \beta \) families, the neuropeptide VIP, chemokines such as RANTES with their receptors and adhesion molecules that generate a network to control implantation processes such as trophoblast adhesion, invasion and the selective recruitment of maternal leukocyte subpopulations (Salamonsen & Woolley 1999, Dimitriadis et al. 2010, Terness et al. 2007, Yoshinaga 2010, Fraccaroli et al. 2011).

Interestingly, spontaneous decidualization of stromal cells occurs in the absence of pregnancy. It was proposed that cyclic endometrial decidualization followed by menstruation ‘preconditions’ uterine tissues for a hyper-inflammatory response and oxidative stress that is in turn accompanied by deep trophoblast invasion during early pregnancy (Brosens et al. 2009, Teklenburg et al. 2010a,b). Therefore, the ability of the human endometrium to generate an adequate decidual response based on successive inflammatory events might contribute to the sensitization of the uterine tissues. Under this hypothesis of repeated inflammatory events, it is conceivable that tight immune homeostatic control prior to implantation is required (Kim et al. 2009, Weiss et al. 2009). In this context, the ability of HESCs to selectively recruit iTregs might contribute to maintenance of immune homeostasis at early stages of implantation.

Finally, even though research in the past few years have provided a better understanding of trophoblast-endometrial interactions during the initial stage of implantation by means of various human cell experimental approaches, the identification of biomarkers with clinical utility for patients with implantation failures is still an objective to be achieved.

Declaration of interest
The authors declare that they have no financial nor any other potential conflict of interest.

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Author contribution statement
C P L and R R designed the study and wrote the manuscript. E G carried out all the experiments with HESC cells and decidualization, the differentiation of iTregs the co-cultures, and the migration assays. D P and M A helped with all the experiments with HESC cells and decidualization, the differentiation and RT-PCRs data analyses and interpretation. G M supervised the study and discussed the results. All authors read and approved the final manuscript.

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