ORIGINAL ARTICLE

Vasoactive Intestinal Peptide Induces an Immunosuppressant Microenvironment in the Maternal–Fetal Interface of *Non-Obese Diabetic* Mice and Improves Early Pregnancy Outcome

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Problem
Impaired pregnancy in non-obese diabetic (NOD) mice was related to limited vascular remodeling and autoimmune background. Vasoactive intestinal peptide (VIP) has anti-inflammatory and immunosuppressant effects, so we explored its ability to modulate the immune microenvironment at the early maternal–placental interface and improve pregnancy in NOD mice.

Method of study
Implantation sites were isolated from pregnant NOD mice at gestational day 9.5 and were incubated with VIP for evaluation of cytokine or transcription factor expression by RT-PCR, immunoblotting, and immunohistochemistry. Alternatively, pregnant mice were injected with VIP at day 6.5 and studied at day 9.5.

Results
VIP and VPAC receptors were detected in viable implantation sites. VIP immunostaining was found predominantly on trophoblast giant cells. The *in vitro* treatment of viable implantation sites with VIP increased IL-10, TGF-β, and Foxp3 expression. Sites with resorption processes presented lower VIP expression, reduced suppressant markers, and increased IL-17 and RORγT expression compared with viable sites and VIP reduced RORγT expression. Pregnant mice treated with VIP at day 6.5 presented an even distribution of viable implantation sites with an increased expression of IL-10, TGF- β, and Foxp3.

Conclusion
VIP induces an immunosuppressant profile at the early maternal–placental interface of NOD mice and improves pregnancy outcome.

Introduction

The pregnant uterus at the implantation and early post-implantation stages undergoes intense tissue remodeling and leukocyte invasion with proinflammatory mediator release.[1,2] Selective recruitment of maternal immune cells expressing suppressant and anti-inflammatory functional profiles and the local production of hormones and polypeptides contribute to modulate the inflammatory response and maintain immune homeostasis at the early maternal–placental interface. In line with this, a deficient control

of the inflammatory response at early pregnancy stages has been associated with recurrent pregnancy loss and preeclampsia.[2–6] Regulatory T-cells (Treg) and alternatively activated M2 macrophages appear to have a central regulatory role at this stage. Treg cells increase from days 2 to 3 of gestation independently of the allogeneic or syngeneic nature of pregnancy and contribute to the predominant suppressant profile that characterizes the post-implantation period.[7,8] They actively participate in tolerance induction to prevent spontaneous abortion.[7] Consistently, a decreased number of decidual Treg cells were reported in the CBA/JxDBA/2 mouse mating characterized by high resorption rates.[9–11] On the other hand, macrophages bearing a predominant M2 alternative activation phenotype are mostly found early after post-implantation favoring wound healing processes and providing suppressor/regulatory signals during the silent clearance of apoptotic cells.[12–14]

Pregnancy in the non-obese diabetic (NOD) strain of mice, a model of type 1 diabetes, presents some pathological features both in the prediabetic and diabetic stages. In the prediabetic stage, NOD females have the unique characteristic of developing exocrine gland dysfunction[15–17] along with a decline in litter size and increased resorption rates associated with defects in the activity of NK cells[18] and $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T-cells (Treg).[19,20] Accordingly, CTLA4Ig gene transfer improved pregnancy outcome by expanding the $CD4^+$ $CD25^+$ regulatory T-cell population.[21] Moreover, at day 9 of gestation in NOD mice, a switch from a predominant M1 inflammatory phenotype to an M2 alternative activated phenotype was found in peritoneal macrophages.[22,23]

Vasoactive intestinal peptide (VIP) is a 28 aa endogenous polypeptide with actions at multiple levels and has been proposed as a promising therapeutic agent based on its anti-inflammatory and neuroprotective effects.[24] It promotes exocrine gland secretion[25] and hormone secretion by pituitary gland and trophoblast cells,[26–28] and smooth muscle relaxation in pregnant uterus,[29] and it regulates embryonic growth.[30,31] On the other hand, VIP has potent anti-inflammatory and protolerogenic properties in mouse models of inflammation through its action on VPAC receptors on macrophages and $CD4^+$ T-cells.[32–34] In prediabetic NOD mice, VIP reduced prostaglandin synthesis and increased nitric oxide synthase activity in non-pregnant uterus consistent with a uterine quiescent effect.[35] In pregnant NOD mice, VIP increased the expression of leukocyte inhibitory factor (LIF) in viable implantation sites and $CD4^+$ $CD25^+$ $Foxp3^+$ Treg frequency to a similar extent than normal mice.[20] Peritoneal macrophages from pregnant NOD mice treated *in vitro* with VIP synthesized high levels of IL-10 and reduced Th1 cytokines contributing to a predominant M2 alternatively activated phenotype.[23]

We hypothesized that a local release of VIP at the early post-implantation and placentation stage contributes to the anti-inflammatory and suppressant microenvironment required for immune homeostasis and fetal growth and that a failure in this mechanism in NOD pregnancy could be overcome by treating mice with the peptide. We present evidence to indicate that VIP treatment improves NOD early pregnancy outcome through a mechanism that involves the targeting of VPAC receptors at the early maternal–placental interface leading to a local induction of IL-10, TGF-β, and Foxp3 synthesis and the reduced expression of proinflammatory signals.

Materials and methods

Mice mating and Treatments

Normally cycling NOD mice of 15–16 weeks of age were mated (syngeneic mating), and the appearance of vaginal plug was considered as gestational day 0.5. Mice were bred and maintained on a 12:12 hr light–dark schedule in the Central Animal Care facility at the School of Exact and Natural Sciences, University of Buenos Aires (FCEyN-UBA). NOD mice at days 8.5 or 9.5 of gestation were used for studies on implantation sites and their cultures and pregnancy outcome evaluation. NOD mice blood glucose levels were registered and their values on two occasions over a 24-hr period did not differ from normal mice control values (NOD: 1.0 ± 0.1 g/L, $n = 37$), thus considered normoglycemic prediabetic. NOD mice treatment with VIP was carried out in the morning of day 6.5 of pregnancy by means of one i.p. injection of 0.1, 1, 2, or 10 nmol VIP in 200 μL of PBS or the same volume of PBS alone. Different end points of early pregnancy outcome were analyzed macroscopically as the number of implanted viable embryos, resorption process ongoing, distribution of the embryos along the horns, and correspondence of gestational day with embryo size and weight. Implantation sites were excised and analyzed for the expression of VIP, VPAC receptors, cytokines,

transcription factors, and histological features. All studies were approved by the Animal Care and Use Committee of the FCEyN-UBA.

Uterus and Implantation Site Isolation for VIP/VPAC Expression and Function

Implantation sites were excised at day 8.5 or 9.5 of pregnancy, embryos discarded, and the uterine tissues were either fixed in 4% paraformaldehyde for immunohistochemistry, or processed for RT-PCR and Western blot assays immediately or after 6 or 24 hr of incubation in RPMI 1640 medium (Invitrogen, Life Technologies, Buenos Aires, Argentina) with 10% FBS in 24-well plates at 37°C and 5% CO2 with VIP (1-100 nM) (Polypeptide labs, France), progesterone (1 μM) (Sigma Chemical Co, St Louis, MO, USA) in the presence or not of a specific VIP antagonist (Nts 6-11/VIP 7-28 hybrid; Sigma Chemical Co) in a final volume of 1 mL. Sites with viable embryos and sites with incipient resorption processes at gestational day 9.5 were assessed under magnifying glass, quantified to calculate resorption rate, and photographed to show embryo distribution along horns among other gestation outcome signs as previously described.[20]

VIP/VPAC, Foxp3, RORγT, and Cytokines Detection

Vasoactive intestinal peptide and VPAC receptors, transcription factors, and cytokine expression were determined by RT-PCR. Total RNA isolation and reverse transcription were performed as previously described[20] using TRIzol® reagent (Invitrogen, Life Technologies), Moloney murine leukaemia virus reverse transcriptase and RNase inhibitor (Promega, WI, USA), oligo-dTs (Biodynamics, Buenos Aires, Argentina), and dNTPs (Embiotec, Buenos Aires, Argentina). Specific primers for VIP, VPAC 1, VPAC 2, TGF-β, IL-17, IL-10, Foxp3, RORγT, and GAPDH as internal control were used for cDNA amplification. Primers and conditions are indicated in Table I and previously shown.[20,36] PCR products and DNA size markers were fractionated on 2% agarose gels, visualized with ethidium bromide staining, and band density was expressed in arbitrary units normalized to GAPDH. For quantitative RT-PCR determinations, 2 μL of cDNA, 0.20 mM dNTPs, 0.25 μM specific primers, 3 mM $MgCl_2$, 2 U Taq DNA polymerase, and 1:30,000 dilution of SYBR Green were added to the reaction mix in a final volume of 25 μL, and reactions were performed in a DNA Engine Opticon (MJ Research Inc., Quebec, Canada). PCR products were quantified in the Opticon Software®, Quebec, Canada and normalized to endogenous GAPDH. Each assay included a DNA minus control and a standard curve performed with serial dilutions of control cDNA.

Western Blot and Immunohistochemistry

The expression and localization of VIP by immunohistochemistry and Foxp3 protein levels by Western blot in implantation sites were determined as previously described.[20,35] Briefly, for Western blot assays, implantation sites isolated from NOD mice at day 9.5 of gestation were homogenized in 50 mM Tris–HCl buffer pH 7.5 with 0.15% Triton-X-100 and protease inhibitors at 4°C, subjected to 10% SDS–PAGE and Foxp3 revealed by immunoblotting (clone: FJK-16s, eBioscience, San Diego, CA, USA) and chemiluminescent reagent Amersham ECL (GE Healthcare, Wauwa-

Table I Primers used in RT-PCR Assays. Oligonucleotide Primers were Designed Using the Online tool Primer3® (Whitehead Institute for Biomedical Research)

Primers	Forward	Reverse
GAPDH	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTAGGCCAT
VIP	TTCACCAGCGATTACAGCAG	TCACAGCCATTTGCTTTCTG
$VPAC_1$	GTGAAGACCGGCTACACCAT	TGAAGAGGGCCATATCCTTG
$VPAC_2$	GTGAAGACCGGCTACACCAT	TGAAGAGGGCCATATCCTTG
IL-10	GTTGCCAAGCCTTATCGGAAATG	CACTCTTCACCTGCTCCACTG
TGF-β	GACTCTCCACCTGCAAGACCA	TTGGGGGACTGGCGAGCCTT
Foxp3	GGCCCTTCTCCAGGACAGA	GCTGATCATGGCTGGGTTGT
IL-17	CTCCAGAAGGCCCTCAGACTAC	AGCTTTCCCCTCCGCATTGACACAG
RORγT	CACGGCCCTGGTTCTCAT	CAGATGTTCCACTCTCCTCTTCTCT

tosa, WI, USA). The immunoreactive protein bands were analyzed with a Fotodyne Image Analyzer® (Fotodyne, Inc., Los Angeles, CA, USA). Band densitometry was performed with the Image Quant software, and results were expressed relative to β-actin expression. In some experiments, implantation sites either viable or undergoing resorption were incubated for 24 hr in RPMI 1640 supplemented with 10% FBS as indicated above in the presence of 10 nM VIP to assess Foxp3 protein expression by Western blot. For immunohistochemistry studies, implantation sites were fixed in 4% paraformaldehyde, sections rehydrated in buffer, and after endogenous peroxidase activity, quenched slices were incubated with 1:20 anti-VIP antibody (Abcam, Cambridge, UK) or anticytokeratin antibodies and then biotinylated secondary antibodies and immunoperoxidase staining kit Ldab2 (DAKO, Carpinteria, CA, USA).

Statistical analysis

Statistical significance of differences was determined by the two-tailed t-test for independent populations. When multiple comparisons were necessary, the Student–Newman–Keuls test was used after analysis of variance. For *in vivo* treatment experiments, the Mann–Whitney test was used. Differences between groups were considered significant at $P < 0.05$.

Results

VIP and Functional VPAC Receptor Expression in NOD Mice Viable Implantation Sites

We first studied the expression of VIP in viable implantation sites. As shown in Fig. 1a, pregnancy highly induced the expression of VIP at day 9.5,

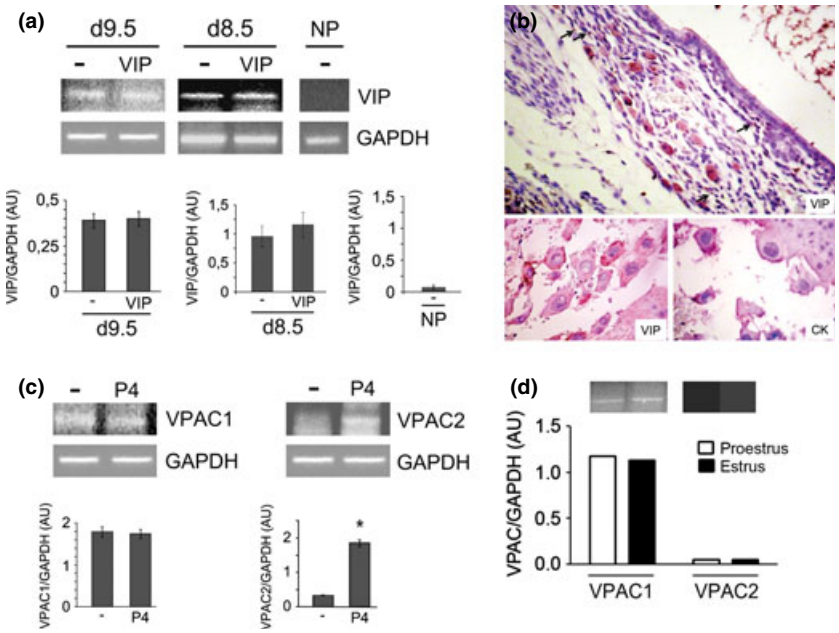

Fig. 1 Vasoactive intestinal peptide and VPAC expression in viable implantation sites of NOD mice and non-pregnant uterus. Uteri from 16-week-old NOD mice either pregnant or non-pregnant were excised out for the assessment of VIP/VPAC system expression as described in Materials and Methods. (a) Implantation sites at day 9.5 (day 9.5), deciduae at day 8.5 (day 8.5), or non-pregnant uteri at proestrus (NP) were evaluated for the expression of VIP by RT-PCR after 6 hr of culture at 37°C in the presence of VIP (100 nM) or non-treated (-). Bands were semiquantified with ImageJ® and intensity expressed in arbitrary units (AU) relative to GAPDH. Values represent mean ± S.E.M of at least three experiments. (b) Implantation sites at day 9.5 were removed and immediately fixed in paraformaldehyde for immunohistochemistry studies as indicated in Materials and Methods. Cells immunostained for cytokeratin (CK) or VIP (VIP) are shown at original magnification ×250 and ×400. Representative of at least four different implantation sites analyzed similarly. Arrows point to small VIP-positive/cytokeratin-negative decidual cells. (c) Implantation sites from NOD mice at day 9.5 were processed for VPAC1 and VPAC2 expression by RT-PCR after 6 hr of culture at 37°C with progesterone (P4) (1 uM) or non-treated (–). Bands were semiquantified as in (a) and AU represent mean ± S.E.M. of at least three experiments. *$P < 0.05$ versus non-treated. (d) Uterine tissue from non-pregnant mice at proestrus or estrus stage was evaluated for VPAC1 and VPAC2 expression by RT-PCR. Bands were semiquantified and AU of a representative experiment of three different mice at estrus and proestrus is shown.

while VIP expression was undetectable in non-pregnant uterus. On the knowledge that VIP induces the transcription of several genes through cAMP-responsive elements (CRE) that are present in the promoter region of its own gene,[37] we incubated implantation sites with VIP and, as it can be seen in Fig. 1a, it could not further induce VIP expression in day 9.5 implantation sites. VIP was also highly expressed in decidua isolated from implantation sites at day 8.5, and a subtle increase was observed after VIP treatment (Fig. 1a). Microphotographs in Fig. 1b indicate that trophoblast cells were the predominant cell type positive for VIP immunostaining in the viable implantation sites at day 9.5. Some of these cytokeratin-positive and VIP-positive trophoblast cells were lying adjacent to maternal blood vessels at the interface between the placenta and the deciduas. Small round scattered cells with VIP immunostaining were also found in the mesometrial deciduae (Fig. 1b arrows).

The expression of VIP high-affinity receptors VPAC1 and VPAC2 was assessed next in day 9.5 viable implantation sites. VPAC1 and VPAC2 subtypes of VIP receptors were expressed in pregnant uterus (Fig. 1c). VPAC2 receptors were induced by pregnancy as they were absent in non-pregnant uterus (Fig. 1c and d). Moreover, implantation sites were incubated with 1 μM progesterone and assayed for VPAC1 and VPAC2 expression. Fig. 1c shows that progesterone added *in vitro* to implantation sites had a further inducing effect only on VPAC2 expression.

VIP Effect on Cytokine and Transcription Factor Expression in Implantation Sites

We next analyzed if VIP added *in vitro* to the cultured implantation sites could modulate the immune profile of implantation site microenvironment through VPAC receptors. Fig. 2a shows that both 100 and 10 nM VIP induced IL-10, TGF-β, and Foxp3 expression with a maximum at 10 nM of the peptide. A lower VIP concentration (1 nM) tested on implantation sites showed no significant effect compared with basal expression values of all three suppressant mediators (not shown). Fig. 2b shows that the effect of 10 nM VIP was specific of VPAC receptors and comparable to that of progesterone, a known immunomodulatory hormone at implantation sites.

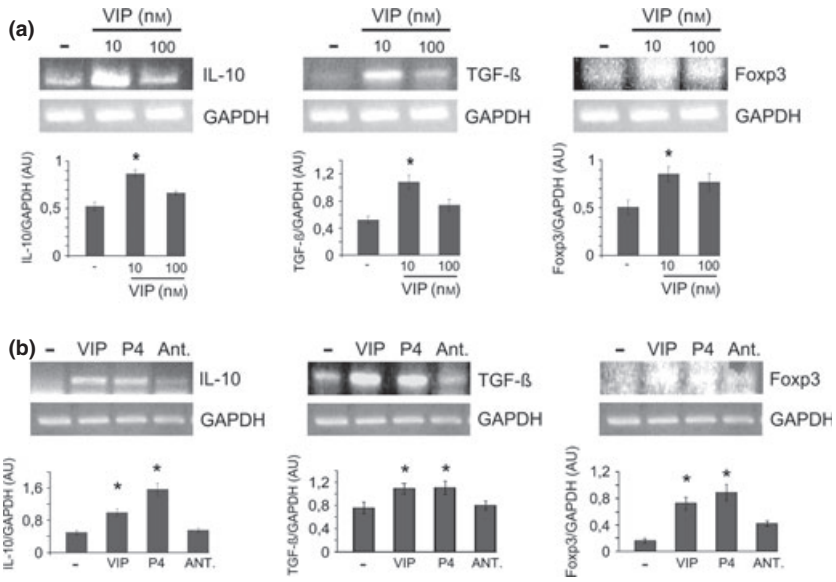

Fig. 2 Effect of VIP on immune suppressant marker expression at viable implantation sites of NOD mice. Implantation sites from NOD mice at gestational day 9.5 were isolated and treated *in vitro* with VIP or progesterone. IL-10, TGF-β, and Foxp3 expression was determined by RT-PCR as indicated in Materials and Methods. Bands were semiquantified with ImageJ® and intensity expressed in AU relative to GAPDH. (a) IL-10, TGF-β, and Foxp3 expression was determined after 6 hr of culture in the absence or presence of VIP (10 or 100 nM). Values are mean ± S.E.M. of at least three experiments. *$P < 0.05$ versus non-treated. (b) Immunosuppressant marker expression was determined in the implantation sites after 6 hr culture at 37°C with VIP (10 nM), progesterone (P4) (1 μM), VIP antagonist (ANT, 1 μM), or non-treated and then their extracts processed as indicated in Materials and Methods. Values are mean ± S.E.M. of at least three experiments. *$P < 0.05$ versus non-treated.

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Our next question was whether the implantation sites with incipient resorption processes were able to synthesize cytokines or transcription factors, and also, whether they could be induced through VIP receptor stimulation. Fig. 3a shows that sites with resorption processes express lower levels of IL-10, TGF-β, and Foxp3 as compared with viable sites, with a similar VPAC1 and VPAC2 receptor basal expression determined by RT-PCR. VIP expression level quantified by real-time RT-PCR was reduced in sites with resorption compared with viable sites (VIP/GAPDH $2^{-\Delta Ct}$ resorption site: 0.40 ± 0.05; viable site: 0.75 ± 0.06; $P < 0.05$; $n = 10$) similar to previously reported in this NOD strain.[20] The lower VIP expression within the sites with resorption processes was concomitant with an increase in IL-17 and RORγT expression compared with viable sites (Fig. 3c). Exogenously added VIP to the implantation sites reduced RORγT expression in both viable and resorption sites (Fig. 3c), but it could not induce suppressant factors IL-10, TGF-β, and Foxp3 in implantation sites undergoing resorption processes (Fig. 3d). To further assess the *in vitro* effect of VIP on one of these markers' expression in both viable and resorption sites at the protein level, we incubated sites with VIP 10 nM for 24 hr and determined Foxp3 expression by Western blot. Fig. 3e shows increased expression of Foxp3 induced by VIP only in viable sites.

Early Pregnancy Outcome and Expression of Local Factors After VIP Treatment

Finally, we explored whether the treatment of NOD pregnant mice *in vivo* with VIP early after implantation could modify any of the most common end points of early pregnancy complications such as the reduced number of implanted viable embryos, the uneven distribution of the embryos along the horns, or the lack of correspondence of gestational day with embryo size. We treated NOD mice with VIP at day 6.5 of pregnancy on the basis that implantation has already occurred, and therefore, the immunomodulatory action of the peptide would only affect post-implantation processes such as the critical regulation of the inflammatory response leading to normal placentation and fetal growth. Fig. 4a shows that mice injected with 2 nmol VIP presented an increase in the number of implantation sites with viable embryos ($P = 0.14$ $n = 8$, Mann–Whitney). Fig. 4b shows an uneven distribution of implanted embryos along both horns, some of them with a reduced size, and a higher number of sites with resorption in the control PBS group, and how this was restored in mice of the VIP-injected group. The profile of cytokines and transcription factor expression at the implantation sites of 2 nmol VIP-injected vs. PBS-injected pregnant mice was analyzed. Fig. 4c shows the expression of different markers in implantation sites of mice that were injected at gestational day 6.5 with 2 nmol VIP and then analyzed their implantation sites at day 9.5 by RT-PCR. An increased expression of Foxp3, IL-10, and TGF-β without changes in RORγT can be observed at day 9.5 implantation sites of one representative pregnant NOD mice (Fig. 4c). Particularly, 4 of 8 NOD mice (50%) injected with VIP showed increased levels of all Foxp3, IL-10, and TGF-β, whereas 6 of 8 (66%) were positive for both Foxp3 and IL-10 expression induction after the *in vivo* treatment. Regarding VIP dosing, 0.1 nmol VIP was ineffective and 1 nmol had results comparable to the 2 nmol dose shown, including the adequate correspondence of embryo size and gestational age. However, when 10 nmol VIP was injected i.p. at day 6.5 in NOD mice, a reduced number of viable embryos (<6) was quantified in 4 of 5 pregnant mice at day 9.5, as well as sites with signs of hemorrhage and the loss of embryos implanted all along one of the horns (Fig. 4d), suggesting the narrow dose window for this peptide to improve early pregnancy outcome.

Discussion

Regulatory immune cells are timely recruited and differentiated to control the inflammatory response induced by the implantation process and the intense tissue remodeling at the early maternal–placental interface. Here, we analyzed the immunoregulatory role of VIP in prediabetic pregnant NOD mice where embryo resorption was related to the autoimmune background as well as to a deficient NK and regulatory T-cell local response.[18–20,38] Results shown indicate that VIP/VPAC system is expressed and functional in NOD mouse viable implantation sites, with the polypeptide synthesized mostly by cells in the decidua, particularly trophoblast giant cells. The mechanism of VIP to improve pregnancy outcome in this mouse pregnancy model involves the induction of immunosuppressant marker synthesis and the reduced expression of inflammatory markers. The immunomodulatory effect of VIP through VPAC

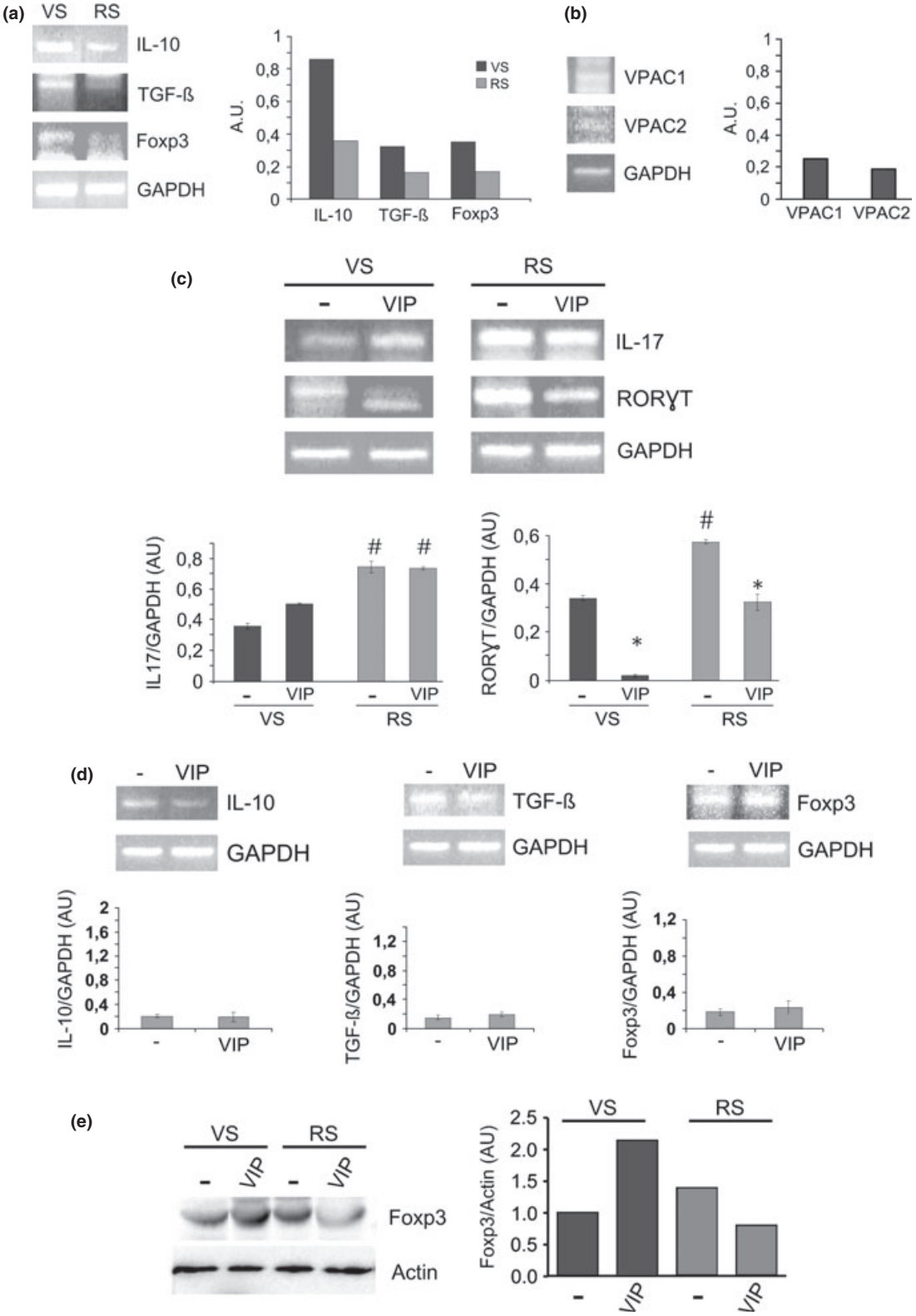

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Fig. 3 Inflammatory profile at implantation sites undergoing resorption. Implantation sites with incipient resorption processes were identified at day 9.5 of gestation and isolated to analyze the expression of suppressant markers as described in Materials and Methods. (a) IL-10, TGF-β, and Foxp3 expression was determined by RT-PCR in viable sites (VS) or sites with signs of resorption (RS) from the same pregnant mouse. The gels and the values of arbitrary units are representative of 10 different NOD mice analyzed in separate experiments. (b) VPAC1 and VPAC2 expression by RT-PCR at sites with signs of resorption. A representative gel and the semiquantification in AU of three different experiments are shown. (c) RORγT and IL-17 expression was assessed by RT-PCR in viable implantation sites (VS) or sites with signs of resorption (RS) incubated for 6 hr at 37°C with VIP (10 nM). Values of AU are mean ± S.E.M. of at least 3 experiments. $^{*}P < 0.05$ versus non-treated; $^{\#}P < 0.05$ versus corresponding bar in VS. (d) IL-10, TGF-β, and Foxp3 expression was determined by RT-PCR in sites with signs of resorption (RS) after 6 hr incubation with or without 10 nM VIP as described in Materials and Methods. Values are mean ± S.E.M. of three experiments. (e) Foxp3 expression was determined by Western Blot in viable sites (VS) or sites with signs of resorption (RS) after 24-hr incubation at 37°C with or without 10 nM VIP as described in Materials and Methods. Gels shown and their corresponding values of arbitrary units are representative of three experiments.

receptors was observed either by *in vitro* treatment of implantation site cultures with VIP or by *in vivo* treating pregnant mice with a pulse of the peptide at gestational day 6.5. In line with a physiological effect of locally synthesized endogenous VIP, implantation sites undergoing resorption processes showed lower VIP expression along with a lower expression of suppressant cytokines and transcription factor Foxp3, and a higher expression of IL-17 and RORγT. Moreover, the increased expression of local suppressant mediators induced by VIP injection at day 6.5 also improved early pregnancy outcome in NOD mice, with more viable embryos implanted, their sizes corresponding to gestational age and evenly distributed along the horns at day 9.5.

Increased resorption rates in prediabetic NOD mice and decrease in offspring around the 16th week of age of the NOD mothers preceding the hyperglycemic stage were reported.[18,20] Lower frequency and function of Treg cells,[19,20] lower uNK cell number and impaired vascular remodeling with aberrant decidua basalis expression of cell adhesin molecules,[18] and a higher expression of complement component C1q in uterine lymphocytes at gestational day 9.5 compared with pregnant BALB/c mice were described.[39]

VIP was reported to contribute to uterine quiescence with a vasodilating effect on human uterine arteries[29] and by reducing prostaglandin production and increasing nitric oxide synthase activity in murine uterus.[35] A role for VIP from maternal origin with a peak expression in decidual cells between gestational days 8 and 12 was proposed in the regu-

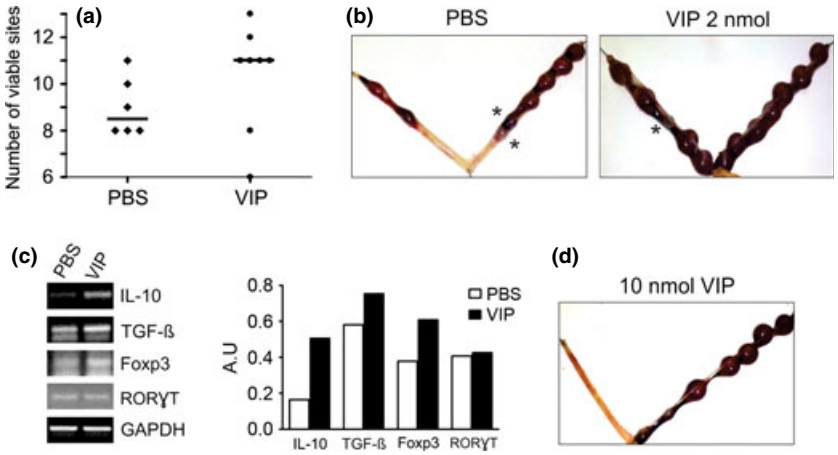

Fig. 4 Effect of VIP treatment on NOD pregnancy outcome. Pregnant NOD mice were injected at gestational day 6.5 with 2 nmol VIP (i.p.) or with PBS and different parameters were determined. (a) VIP (2 nmol/200 μL PBS i.p.) or PBS (200 μL) was injected to pregnant NOD mice at day 6.5, and sites with viable embryos were counted at day 9.5 of gestation. Values are median ± S.E. of viable sites counted in 8 mice from each group. (b) Representative photograph of implanted embryos along both horns in the PBS- and VIP (2 nmol)-treated groups. Photograph is representative of at least six mice in each group with similar results. (c) IL-10, TGF-β, Foxp3, and RORγT expression was assessed by RT-PCR in implantation sites of NOD mice that have been injected at day 6.5 with 2 nmol VIP or PBS. Images and arbitrary units of a representative experiment out of at least three others are depicted. (d) VIP at 10 nmol/200 μL PBS was injected i.p. to pregnant NOD mice at day 6.5, and sites with viable embryos were assessed at day 9.5 of gestation. Representative of four pregnant mice treated.

lation of embryonic growth in rodents[30,40,41] and the blockade of VIP function induced growth retardation and microcephaly.[31,42,43] Finally, an anti-inflammatory and tolerogenic effect of VIP at the maternal–placental interface was demonstrated in the human immune–trophoblast cell interaction, where it reduces nitric oxide and pro-inflammatory cytokine production and induces STAT3 activation and the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ cells.[44,45]

Results presented here indicate that cells located to the decidua synthesize VIP within the viable implantation site *in vivo,* suggesting that endogenous VIP probably acts in an autocrine/paracrine manner. Various cell types could be targeted by the polypeptide to induce its own synthesis as well as to promote anti-inflammatory/suppressant cytokine expression. Among them, trophoblast cells,[27,28] macrophages, and $CD4^+$ T-cells[32,34] are known to be responsive to the peptide. Previous experiments performed by flow cytometry indicate that VIP increased $CD4^+$ $CD25^+$ $Foxp3^+$ cells in the implantation sites of both NOD and normal BALB/c mice.[20] In addition, a phenotype shift of peritoneal macrophages to a predominant $F4/80^+$ $IL\text{-}10^+$ cell population induced by VIP was also seen during pregnancy in the NOD strain.[23] An alloantigen-independent expansion of maternal $CD4^+$ $CD25^+$ $Foxp3^+$ cell pool during pregnancy with suppressing responses through IL-10 and TGF-β has been demonstrated.[7,8] Also, elevated Foxp3 mRNA levels after adoptive transfer of Treg cells were shown to prevent fetal loss in an abortion-prone strain.[9–11] These observations point to the relevance of this master gene modulation to promote the immune tolerant microenvironment required for placentation and fetal growth in normal pregnancy. On the other hand, TGF-β has been implicated in either suppressant or pro-inflammatory responses in different models and in the NOD mouse model of diabetes depending on the cytokine microenvironment and the different subpopulations of T-cells and macrophages targeted.[46–48] NOD resorption sites showed low expression of TGF-β or IL-10, but there was a high increase in IL-17 and RORγT. This observation and the fact that VIP-induced expression of TGF-β in viable sites was paralleled by a reduction in RORγT are consistent with TGF-β expression mostly reflecting an immunosuppressant profile at the implantation sites of NOD mice.

The dosing of VIP used here for *in vivo* mice treatments (1–2 nmol/mouse i.p. at day 6.5) and its effect on various end points of adequate early pregnancy outcome is consistent with VIP as a contributing factor to the generation and maintenance of the maternal–placental interaction. In fact, it could be targeting different cell types with local vasodilating and trophic effects along with the immunomodulatory effects observed here. Moreover, our results support its role as a factor synthesized by decidual immune cells in a narrow temporal window[30,31,41] and by trophoblast cells lining the deciduae during early post-implantation stages in rodents. The fact that a lower expression of VIP was found in viable versus resorption sites from NOD mice described previously[20] and herein and the observation that a single injection of VIP to NOD mothers at day 6.5 was enough to modulate pregnancy outcome providing a quiescent and immunosuppressant milieu for embryo growth strongly suggest that maternal VIP can be supplemented as a pulse dose to circumvent this deficit of pregnant NOD mice. In this regard, it is noteworthy that a comparable dose applied to normal pregnant mice results in the loss of most embryo as previously discussed by other authors[30,31,40,42] and observed in our laboratory in normal BALB/c mice syngeneic pregnancies (unpublished results). The observation that a higher dose of VIP (10 nmol) resulted in the loss of implanted embryos in the NOD model shown here is also suggestive of a narrow dose range of the peptide required for therapeutic effects, which becomes toxic at higher doses. Finally, the effect of VIP was comparable to that of progesterone in the induction of suppressant markers. Consistently, progesterone treatment reduced the allogeneic response of pregnant NOD mouse splenocytes,[20] and it had an inhibitory effect on pro-inflammatory mediators in LPS-treated macrophages of NOD mice[49] as well as in normal murine peritoneal macrophages primed *in vivo* with LPS.[50] VIP was shown to induce progesterone release by human trophoblast cells.[28] Interestingly, lower serum levels of progesterone were detected in pregnant and non-pregnant NOD mice compared with standard strains,[20,51,52] supporting a role of VIP-progesterone deficit in the poor pregnancy score of this strain. Finally, we observed that the expression of VPAC2 receptors in the implantation sites was induced by pregnancy in NOD mice and that the treatment of viable implantation sites with progesterone further increased VPAC2 expression *in vitro*. In line with this observation, pregnancy was shown to up-regulate VPAC2 expression

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levels in the pituitary, suggesting the involvement of this priming effect in the neuroendocrine actions of VIP.[26]

Conclusions

VIP promoted a local anti-inflammatory and immunosuppressant microenvironment in implantation sites and improved pregnancy outcome in NOD mice characterized by a deficient regulation of the inflammatory response. The local expression of a functional VIP/VPAC system in viable sites and the narrow effective dose range suggest that VIP acting in autocrine and paracrine circuits might have a role as an endogenous regulator of the inflammatory response at the early post-implantation stage. These results also suggest that VIP dosing probably needs adjustment in other models of pregnancy impairment with different immunopathogenic mechanisms.

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Competing interests

The authors declare that they have no competing interests.

References

1 Gomez-Lopez N, Guilbert L, Olson DM: Invasion of the leukocytes into the fetal-maternal interface during pregnancy. *J Leukoc Biol* 2010; 88:625–633.
2 Mor G, Cardenas I: The immune system in pregnancy: a unique complexity. *Am J Reprod Immunol* 2010; 63:425–433.
3 Redman CW, Sargent IL: Immunology of pre-eclampsia. *Am J Reprod Immunol* 2010; 63:534–543.
4 Dekel N, Gnainsky Y, Granot I: Inflammation and implantation. *Am J Reprod Immunol* 2010; 63:17–21.
5 Girardi G, Yarlini D, Thurman JM, Holers VM, Salmon JE: Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. *J Exp Med* 2006; 203:2165–2175.
6 Kwak-Kim J, Yang K, Gilman-Sachs A: Recurrent pregnancy loss: a disease of inflammation and coagulation. *J Obstet Gynaecol Res* 2009; 35:609–622.
7 Aluvihare VR, Kallikourdis M, Betz AG: Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol* 2004; 5:266–271.
8 Saito S, Shima T, Nakashima A, Shiozaki A, Ito M, Sasaki Y: What is the role of regulatory T cells in the success of implantation and early pregnancy? *J Assist Reprod Genet* 2007; 24:379–386.
9 Zenclussen AC, Gerlof K, Zenclussen ML, Sollwedel A, Bertoja AZ, Ritter T, Kotsch K, Leber J, Volk H-D: Abnormal T-cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced CD4 + CD25 + T regulatory cells prevents fetal rejection in a murine abortion model. *Am J Pathol* 2005; 166:811–822.
10 Zenclussen AC: Adaptive immune responses during pregnancy. *Am J Reprod Immunol* 2013; 69:291–303.
11 Teles A, Zenclussen AC, Schumacher A: Regulatory T cells are baby's best friends. *Am J Reprod Immunol* 2013; 69:331–339.
12 Abrahams VM, Kim YM, Straszewski SL, Romero R, Mor G: Macrophages and apoptotic cell clearance during pregnancy. *Am J Reprod Immunol* 2004; 51:275–282.
13 Fest S, Aldo PB, Abrahams VM, Visintin I, Alvero a, Chen R, Chavez SL, Romero R, Mor G: Trophoblast-macrophage interactions: a regulatory network for the protection of pregnancy. *Am J Reprod Immunol* 2007; 57:55–66.
14 Nagamatsu T, Schust DJ: The contribution of macrophages to normal and pathological pregnancies. *Am J Reprod Immunol* 2010; 63:460–471.
15 Delaleu NNC, Peck AB, Jonsson R: Sjögren's syndrome: studying the disease in mice. *Arthritis Res Ther* 2011; 13:217.
16 Rosignoli F, Roca V, Meiss R, Leceta J, Gomariz RP, Pérez LC: Defective signalling in salivary glands precedes the autoimmune response in the non-obese diabetic mouse model of sialadenitis. *Clin Exp Immunol* 2005; 142:411–418.
17 Jonsson MV, Delaleu N, Brokstad K, Berggreen E, Skarstein K: Impaired salivary gland function in NOD mice: association with changes in cytokine profile but not with histopathologic changes in the salivary gland. *Arthritis Rheum* 2006; 54:2300–2305.
18 Burke SD, Dong H, Hazan AD, Croy BA: Aberrant endometrial features of pregnancy in diabetic NOD mice. *Diabetes* 2007; 56:2919–2926.
19 Lin Y, Xu L, Jin H, Zhong Y, Di J, Lin QD: CXCL12 enhances exogenous $CD4^+$ $CD25^+$ T cell migration and prevents embryo loss in non-obese diabetic mice. *Fertil Steril* 2009; 91:2687–2696.
20 Roca V, Calafat M, Larocca L, Ramhorst R, Farina M, Franchi AM, Pérez Leirós C: Potential immunomodulatory role of VIP in the implantation sites of prediabetic nonobese diabetic mice. *Reproduction* 2009; 138:733–742.
21 Li W, Li B, Fan W, Geng L, Li X, Li L, Huang Z, Li S: CTLA4Ig gene transfer alleviates abortion in mice by expanding CD4 + CD25 + regulatory T cells and inducing indoleamine 2,3-dioxygenase. *J Reprod Immunol* 2009; 80:1–11.
22 Larocca L, Calafat M, Roca V, Franchi AM, Pérez C: VIP limits LPS-induced nitric oxide production through IL-10 in NOD mice macrophages. *Int Immunopharmacol* 2007; 7:1343–1349.
23 Larocca L, Hauk V, Calafat M, Roca V, Fraccaroli L, Franchi A, Ramhorst R, Leirós CP: Modulation of macrophage inflammatory profile in pregnant nonobese diabetic (NOD) mice. *Mol Cell Endocrinol* 2011; 333:112–118.

24 Couvineau A, Laburthe M: VPAC receptors: structure, molecular pharmacology and interaction with accessory proteins. *Br J Pharmacol* 2011; 166:42–50.

25 Ekström J, Mansson B, Tobin G: Vasoactive intestinal peptide evoked secretion of fluid and protein from rat salivary glands and the development of supersensitivity. *Acta Physiol Scand* 1983; 119:169–175.

26 Bjøro T, Sand O, Ostberg BC, Gordeladze JO, Torjesen P, Gautvik KM, Haug E: The mechanisms by which vasoactive intestinal peptide (VIP) and thyrotropin releasing hormone (TRH) stimulate prolactin release from pituitary cells. *Biosci Rep* 1990; 10:189–199.

27 Deutsch PJSY, Sun Y, Kroog GS: Vasoactive intestinal peptide increases intracellular cAMP and gonadotropin-alpha gene activity in JEG-3 syncytial trophoblasts. *J Biol Chem* 1990; 265:10274–10281.

28 Marzioni D, Fiore G, Giordano a, Nabissi M, Florio P, Verdenelli F, Petraglia F, Castellucci M: Placental expression of substance P and vasoactive intestinal peptide: evidence for a local effect on hormone release. *J Clin Endocrinol Metab* 2005; 90:2378–2383.

29 Jovanovic SGL, Grbovic L, Jovanovic A: Pregnancy does not alter the response of uterine arteries to vasoactive intestinal polypeptide. *Mol Hum Reprod* 2000; 6:361–368.

30 Hill JM, McCune SK, Alvero RJ, Glazner GW, Henins KA, Stanziale SF, Keimowitz JR, Brenneman DE: Maternal Vasoactive Intestinal Peptide and the Regulation of Embryonic Growth in the Rodent. *Measurement* 1996; 97:202–208.

31 Passemard S, Sokolowska P, Schwendimann L, Gressens P: VIP-induced neuroprotection of the developing brain. *Curr Pharm Des* 2011; 17:1036–1039.

32 Gomariz RP, Arranz A, Juarranz Y, Gutierrez-Cañas I, Garcia-Gomez M, Leceta J, Martínez C: Regulation of TLR expression, a new perspective for the role of VIP in immunity. *Peptides* 2007; 28:1825–1832.

33 Gonzalez-Rey E, Delgado M: Vasoactive intestinal peptide and regulatory T-cell induction: a new mechanism and therapeutic potential for immune homeostasis. *Trends Mol Med* 2007; 13:241–251.

34 Delgado M, Munoz-Elias EJ, Martinez C, Gomariz RP, Ganea D: VIP and PACAP38 modulate cytokine and nitric oxide production in peritoneal macrophages and macrophage cell lines. *Ann N Y Acad Sci* 1999; 897:401–414.

35 Roca V, Larocca L, Calafat M, Aisemberg J, Meiss R, Franchi AM, Pérez Leirós C: Reduced nitric oxide synthase and cyclo-oxygenase activity in the uterus of non-obese diabetic mice. *Reproduction (Cambridge, England)* 2006; 132:931–938.

36 Hauk V, Calafat M, Larocca L, Fraccaroli L, Grasso E, Ramhorst R, Perez Leirós C: Vasoactive intestinal peptide/vasoactive intestinal peptide receptor relative expression in salivary glands as one endogenous modulator of acinar cell apoptosis in a murine model of Sjögren's syndrome. *Clin Exp Immunol* 2011; 166:309–316.

37 Tsukada T, Fink JS, Mandel G, Goodman RH: Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP. *J Biol Chem* 1987; 262:8743–8747.

38 Lin Y, Ren L, Wang W, Di J, Zeng S, Saito S: Effect of TLR3 and TLR7 activation in uterine NK cells from non-obese diabetic (NOD) mice. *J Reprod Immunol* 2009; 82:12–23.

39 Li C, Wang W, Wang H, Zhong Y, Di J, Lin Y: Proteomic analysis of proteins differentially expressed in uterine lymphocytes obtained from wild-type and NOD mice. *J Cell Biolchem* 2009; 108:447–457.

40 Hill JM, Cuasay K, Abebe DT: Vasoactive intestinal peptide antagonist treatment during mouse embryogenesis impairs social behavior and cognitive function of adult male offspring. *Exp Neurol* 2007; 206:101–113.

41 Spong CY, Lee SJ, Cune SKMC, Gibney G, Abebe DT, Alvero R, Brenneman DE, Hill JM: Maternal regulation of embryonic growth: the role of vasoactive intestinal peptide. *Endocrinology* 1999; 140:917–924.

42 Gressens P, Marret S, Hill JM, Brenneman DE, Gozes I, Fridkin M, Evrard P: Vasoactive intestinal peptide prevents excitotoxic cell death in the murine developing brain. *J Clin Invest* 1997; 100:390–397.

43 Rangon C-M, Dicou E, Goursaud S, Mounien L, Jégou S, Janet T, Muller J-M, Lelièvre V, Gressens P: Mechanisms of VIP-induced neuroprotection against neonatal excitotoxicity. *Ann N Y Acad Sci* 2006; 1070:512–517.

44 Fraccaroli L, Alfieri J, Larocca L, Calafat M, Roca V, Lombardi E, Ramhorst R, Pérez Leirós C: VIP modulates the pro-inflammatory maternal response inducing tolerance to trophoblast cells. *Br J Pharmacol* 2009; 156:116–126.

45 Fraccaroli L, Grasso E, Zeitler E, Lombardi E, Gogorza S, Etchepareborda JJ, Nagle C, Cortelezzi M, Pérez LC, Ramhorst R: Modulation of maternal LIF producers T cells by trophoblast and paternal antigens. *Am J Reprod Immunol* 2010; 65:133–145.

46 Sallusto F, Zielinski CE, Lanzavecchia A: Human Th17 subsets. *Eur J Immunol* 2012; 42:2215–2220.

47 Veldhoen M, Stockinger B: TGFbeta1, a "Jack of all trades": the link with pro-inflammatory IL-17-producing T cells. *Trends Immunol* 2006; 27:358–361.

48 Nikoopour E, Schwartz JA, Huszarik K, Sandrock C, Krougly O, Lee-Chan E, Singh B: Th17 polarized cells from nonobese diabetic mice following mycobacterial adjuvant immunotherapy delay type 1 diabetes. *J Immunol* 2010; 184:4779–4778.

49 Larocca L, Roca V: Neuroimmune-Endocrine Interactions during Early Pregnancy in an Autoimmune Context: Focus on Macrophage Activation. *NeuroImmunoModulation* 2008; 15:84–90.

50 Kohmura Y, Kirikae T, Kirikae F, Nakano M, Sato I: Lipopolysaccharide (LPS)-induced intra-uterine fetal death (IUFD) in mice is principally due to maternal cause but not fetal sensitivity to LPS. *Microbiol Immunol* 2000; 44:897–904.

51 Durant S, Christeff N, Coulaud J, Nunez EA, Dardenne M, Homo-Delarche F: Basal concentrations of various steroids in the nonobese diabetic (NOD) mouse and effect of immobilization stress. *Autoimmunity* 1998; 28:249–258.

52 Homo-Delarche F: Neuroendocrine immuno-ontogeny of the pathogenesis of autoimmune disease in the nonobese diabetic (NOD) mouse. *ILAR J* 2004; 45:237–258.