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Potential immunomodulatory role of VIP in the implantation sites of prediabetic NOD mice

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Running title: VIP and embryonic resorption in NOD mice

Abstract

Among several factors known to modulate embryo implantation and survival, uterine quiescence and neovascularization, maternal immunotolerance through the Th1/Th2 cytokine balance towards a Th2 profile, local regulatory T cell activation and high levels of progesterone were assigned a prominent role.

Vasoactive intestinal peptide (VIP) is a neuroimmunopeptide that has anti-inflammatory effects, promotes Th2 cytokines and $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cell activation whereas it stimulates exocrine secretion, smooth muscle relaxation and vasodilatation favoring uterus quiescence. The goal of the present work was to explore the participation of VIP in the implantation sites of normal and pregnant prediabetic NOD females, a mouse strain that spontaneously develops an autoimmune exocrinopathy similar to Sjögren's syndrome. Our results indicate a reduction in litter size from the 3^{rd} parturition onwards in the NOD female lifespan with increased resorption rates. Progesterone systemic levels were significantly decreased in pregnant NOD mice compared with BALB/c mice, although the allogeneic response to progesterone by spleen cells was not impaired. VIP receptors VPAC 1 and VPAC 2 were expressed at the implantation sites and VIP induced LIF and Treg marker expression in both strains, however, a reduced VIP expression was found in NOD implantation sites.

We conclude that the reduced birth rate at 16 week-old NOD mice with a Th1 systemic cytokine profile involves resorption processes with a lower expression of VIP at the sites of implantation which acts as a local inducer of pro-implantatory LIF and regulatory T cell activation.

Introduction

During pregnancy, immune and neuroendocrine regulation of the maternal-fetal "dialogue" is central to both implantation and the development of the placenta. Several factors modulate embryo implantation and survival thus promoting maternal immunotolerance, uterine quiescence and neovascularization.

The Th1/Th2 cytokine shift towards a Th2 profile was shown as a favoring factor for fetus survival (Raghupathy 1997; Piccinni *et al.* 1998; Hanzlikova *et al.* 2009). In line with this, patients with Th1 autoimmune diseases such as multiple sclerosis and rheumatoid arthritis improve during pregnancy (Nelson & Ostensen 1997; Cutolo 2000; Olsen & Kovacs 2002). Also, reports showed that the incidence of fetal loss is not increased in autoimmune patients with rheumatoid arthritis while a significantly higher frequency of spontaneous abortion was found before the disease onset in a retrospective study of patients with Sjögren´s Syndrome (Siamopoulou-Mavridou *et al.* 1988). However, recent reports show that rather than a global Th2 bias, most cytokine production appears regulated in the feto-maternal interface during early pregnancy to maintain a relative balance (Halonen *et al.* 2009).

Among various immunomodulatory factors that participate in the establishment and progression of gestation, progesterone has a prominent role by shifting Th1/Th2 cytokines to a Th2 profile (Szekeres-Bartho 2002). Also, high levels of progesterone prolong the survival of allogeneic skin grafts in hamster uteri (Moriyama & Sugawa 1972) while stimulation by fetal antigens induces the expression of progesterone receptors (Chiu *et al.* 1996). Allorecognition of paternal antigens can also increase the production of growth factors and hormones essential for embryonic and fetal development as leukaemia inhibitory factor (LIF) among others (Rugeles & Shearer 2004). Similarly, $CD4^+CD25^+$ regulatory T cells (Treg) are known to have an essential role in the induction of maternal tolerance preventing spontaneous abortion (Aluvihare *et al.* 2004; Saito *et al.* 2007). A decreased number of decidual Treg cells were reported in the mouse model of abortion CBA/J x DBA/2

(Zenclussen *et al*. 2005) whereas $CD4^+CD25^+$ T cell increase was stated from days 2-3 of gestation independently of the allogeneic or syngeneic nature of pregnancy (Aluvihare *et al.* 2004). Also, CTLA4Ig gene transfer was recently shown to improve pregnancy outcome by expanding the $CD4^+CD25^+$ regulatory T cell population (Li *et al*. 2009). Finally, LIF has also been involved in graft acceptance and alloantigen driven tolerance, whereas Tregs release high levels of LIF (Metcalfe *et al.* 2005; Zenclussen *et al.* 2006).

Vasoactive intestinal peptide (VIP) mediates a wide variety of nervous, immune and developmental functions. As a neuropeptide of the peripheral nervous system it stimulates exocrine secretion and vasodilatation (Ekström *et al.* 1983; Inoue *et al*. 1985). Interestingly, VIP contributes to smooth muscle relaxation and vasodilatation favoring uterus quiescence (Clark *et al.* 1981; Jovanovic *et al.* 1998). As an immunopeptide, it promotes anti-inflammatory and Th2 cytokine responses in various models of inflammatory response and autoimmune disease (Leceta *et al.* 2007; Gonzalez Rey & Delgado 2007). VIP has been also proposed as an inducer of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs), helping to maintain immunotolerance in different animal models including NOD mice (Rosignoli *et al.* 2006; Gonzalez Rey & Delgado 2007). Finally, VIP participates in the maternal regulation of embryonic growth in rodents during the early postimplantation period and the blockade of VIP function induced growth retardation and microcephaly (Gressens *et al.* 1994; Spong *et al.* 1999, Rangon *et al.* 2006).

The non obese diabetic (NOD) mouse model of Sjögren´s syndrome is an invaluable tool to study the outcome of pregnancy before the onset and during the autoimmune response. NOD mice at the prediabetic stage spontaneously develop an autoimmune exocrinopathy with a systemic Th1 cytokine response resembling Sjögren´s syndrome. A deep exocrine dysfunction precedes a mild mononuclear infiltration of the glands which can be partly explained by multiple immune regulatory defects (Rosignoli *et al* 2005; Anderson & Bluestone 2005; Piccirillo *et al.* 2005).

Among these defects, NOD mice present a lower number of Tregs, although they retain their suppressive capacity since depletion accelerates the progression of the autoimmune response (Pop *et al.* 2005). Regarding reproductive tissues, we have previously reported a decreased response to VIP in the uterus of normally cycling 16 weeks old prediabetic NOD mice, simultaneously to the increase of Th1 cytokines in serum (Roca *et al.* 2006).

The goal of the present work was to monitor the reproductive score of prediabetic NOD females focusing on the potential regulation by VIP of local modulatory factors at the implantation sites. We provide evidence of a reduced birth rate from the 3rd litter onwards at 16 weeks of age that is associated with increased resorption processes, decreased serum progesterone and decreased expression of VIP at the sites of implantation, which acts locally as an inducer of pro-implantatory factors LIF and Treg activated cells.

Results

Litter size in NOD mice lifespan and embryonic resorption profile

Figure 1A shows a significant decline in the litter size of NOD mice from the 3rd parturition onwards. It is worth noting that the first reduction in litter size is around the 18th week of mothers' age. These females had been mated at 16 weeks of age coinciding with the onset of the systemic Th1 cytokine response previously described in these mice (Roca *et al*. 2006). A more profound failure in reproductive score is registered at the diabetic stage that occurs about the 30th week in our breeding conditions. Control BALB/c mice litter sizes (Figure 1B) show that there is no decline up to the 4th gestation and even not further (28 weeks of age, data not shown). To investigate whether this decline in offspring at the 3rd gestation was an effect of multiple gestations or it also occurred at the first pregnancy, we mated virgin NOD females of 16 weeks of age and obtained similar results (NOD mice born/mother, mean ± S.E.M.= 5,0 ± 0,8). Also, since diabetes is known to impair pregnancy in this

strain, we measured glucose serum levels in pregnant 16 weeks old NOD mice, either in their first gestation or in the third one. Glucose levels did not differ either between NOD mice or compared to normal BALB/c pregnant mice (Table 1). In order to explore whether an implantation failure or a resorption process underlies this lower offspring score, we mated 16 weeks old female NOD mice (first mating) with male NOD mice. On the 9th day after the vaginal plug was seen, female mice were sacrificed, post implantation embryos were counted and separated for histological studies. As it can be seen in Figure 2A, healthy embryos were macroscopically different from those in process of resorption. Histological studies revealed a conserved muscular layer, infiltrating immune cells in the decidualized tissue (a) and hemorrhages (b). In the lower panel, an incipient infiltration of aligned mononuclear cells can be seen as well as decidual cells detaching from the villi (c). The rate of resorbed embryos vs. total embryos was 32% (60 resorbed/187 evaluated) (Figure 2B), higher than the normal rate reported for control mouse strains (Zenclussen *et al.* 2006). Similar resorption rates were obtained in mothers NOD at 3rd gestation (not shown).

Serum levels of progesterone and estradiol and systemic alloreactivity

Since progesterone and estradiol play key roles in the physiology of reproduction, we measured their levels in the serum of pregnant NOD and BALB/c mice. Compared with BALB/c mice, significantly lower progesterone levels were found in pregnant NOD mice serum even if they had no signs of embryo resorption (Figure 3A). In fact, progesterone levels were even lower in NOD mice with more than 4 resorption sites. However, no differences were seen in the estradiol levels in pregnant NOD mice compared to NOD mice with more than 4 resorption sites or with BALB/c mice (Figure 3A, left pannel). On the hypothesis that an exacerbated splenocyte alloresponse not properly regulated by progesterone might have a role in the increased resorption rate, we measured the maternal immune response to paternal

antigens by splenocytes in pregnant NOD and BALB/c mice at day 9 of gestation. When taking into consideration the proliferation rate, no significant difference was found between the two strains. Also, progesterone was able to inhibit the response to the same extent in both mice strains, suggesting that although progesterone levels are diminished in NOD mice, spleen cells present a similar response to paternal antigens and progesterone regulation compared with normal mice cells (Figure 3B).

VIP and VPAC receptors expression

VIP has smooth muscle relaxation effects and induces proTh2-proTreg profiles consistent with the maintenance of uterine quiescence and immuno-tolerogenic mechanisms, on one hand, and it has been also involved in fetal growth, on the other. Thus, we investigated the expression levels of VIP and VPAC receptors VPAC 1 and VPAC 2 mRNA in NOD and BALB/c implantation sites. As shown in figure 4A, there was a decrease in VIP mRNA levels at the implantation sites of NOD mice compared with BALB/c mice. NOD mice with more than 4 resorption sites were also tested for VIP expression and the levels were significantly reduced compared to NOD mice with normal embryos. To quantify VIP mRNA expression, real time RT-PCR was performed and the above results were further confirmed (Figure 4B). In contrast, there were no detectable differences in mRNA levels of VPAC 1 or VPAC 2 receptors between normal NOD and BALB/c implantation sites (Figure 4C).

Effect of VIP on pro-implantatory factors.

Since VIP has been associated with induction of Tregs and we have described a lower response to VIP in uteri of non pregnant female NOD mice, we investigated the functionality of VIP receptors by exploring the ability of exogenous VIP to induce LIF expression and Foxp3 major differentiation marker of CD4+CD25+ Treg in the implantation sites. Hence, explants of healthy implantation sites from NOD and BALB/c mice were cultured for 24 h in the presence or absence of 100 nM VIP, and

the expression of Foxp3 and LIF was assessed by western blot. We observed that VIP significantly increased Foxp3 and LIF expression in implantation sites from NOD and BALB/c mice (Figure 5A). To further analyze the effect of VIP on Treg population we performed triple staining protocols to identify Treg population (CD4-FITC, CD25-APC, Foxp3-PE) in NOD and BALB/c mice healthy implantation sites. Figure 5B shows a representative dot plot for NOD and BALB/c mice in basal and VIP stimulated conditions. No detectable differences in the frequency of Tregs in basal conditions were seen between NOD and BALB/c mice, and also, VIP slightly increased the frequency of this population to the same extent in both mice strains.

Discussion

Pregnancy is a tightly regulated process where systemic and local mechanisms act in synchronicity to allow the maternal immune system to tolerate the fetus. A unique situation takes place when autoimmunity underlies the course of pregnancy. Certainly, the outcome of pregnancy may be affected by the autoimmune context and while pregnancy was shown to ameliorate various autoimmune diseases, it can also worsen the outcome of others (Waldorf & Nelson 2008). Therefore, a more deep insight into the mechanisms of maternal-fetal interaction in normal and autoimmune conditions might help to improve the current/available treatments. Several reports describe the effect of established autoimmune disease on pregnancy and its effect on disease, nevertheless, few retrospective reports focused on the outcome of pregnancy before the clinical manifestations of an autoimmune disease. This situation was analyzed in Sjögren's disease, more frequently diagnosed in elder women, and a higher frequency of recurrent spontaneous abortions was reported (Siamopoulou-Mavridou *et al.* 1988).

The aim of the present work was to analyze the reproductive score of prediabetic NOD females as a model of Sjögren's syndrome focusing on the potential role of VIP as a local immunomodulatory factor at the implantation sites. Our results indicate a decline of birth rate in NOD mice paralleling the development of the systemic Th1 cytokine response, with increased resorption rates, decreased systemic progesterone and decreased expression of VIP at the sites of implantation. VIP appears to act locally as an inducer of pro-implantatory factors LIF and Treg activated cells. These conclusions are supported by the following evidences presented: First, a reduction in litter size was recorded from the 3rd parturition onwards only in NOD mice. This occurred at an age of the mother when Th1 cytokines such as TNF-α are increasing in their serum. Second, progesterone systemic levels are significantly decreased in pregnant NOD mice compared with BALB/c mice, although the response to progesterone by spleen cells is not impaired.

Third, a significant reduction in VIP mRNA levels was found locally in NOD implantation sites with normal expression of VIP receptors, VPAC 1 and VPAC 2. These receptors are responsive to exogenous VIP as it was able to increase the expression of two pro-implantatory markers, Foxp3 and LIF, in healthy implantation sites and to increase the frequency of Treg population.

The decrease in offspring around the 16^{th} -18^{th} week of age parallels not only the onset of the systemic Th1 cytokine response (Roca *et al.* 2006) but also the decline in salivary flow rate characteristic of Sjögren's syndrome-like stage in NOD mice and it also clearly precedes the hyperglycemia of the type 1 diabetic stage in NOD mice. (Rosignoli *et al.* 2005), since 16 weeks old pregnant NOD mice are normoglycemic. In addition, we found that the resorption rate in this singeneic pregnancy model was significantly higher than the 3-10% resorption rate reported for allogeneic and singeneic pregnancy in control mouse strains. NOD resorption rates shown here are similar to allogeneic pregnancy in NOD/C57BL/6 of comparable age (Formby *et al.* 1987; Lin *et al.* 2008) and comparable with the resorption rates reported for the immunologic abortive model CBA/2 x DBA/J (Zenclussen *et al.* 2006).

Embryonic resorption has been associated with systemic responses such as a Th1 cytokine profile (Chaouat *et al.* 1990) and low progesterone levels (Elson & Jurkovic 2004). At the local level, unusually high levels of nitric oxide synthesis are responsible of resorption in an acute inflammation model in mice (Ogando *et al*. 2003; Aisemberg *et al.* 2007). Similarly, a low number of Tregs at implantation sites parallels resorption in the abortive mouse model (Aluvihare *et al.* 2004; Zenclussen *et al.* 2006). As we showed here, progesterone levels were decreased in the sera of pregnant NOD mice, and this reduction was even greater when resorbed embryos were counted at day 9 of gestation. Progesterone plays a key role in the regulation of gestation due to endocrine as well as immunological effects. Progesterone was found necessary for NK cells homing to the uterus mediating angiogenesis and neovascularization in human pregnancy (Ancelin *et al*. 2002). In line with this, we can

speculate that a reduction in progesterone serum levels could in turn impair NK cells homing to the uterus. Interestingly, a lower uterine NK cell number was observed in the decidua basalis of diabetic NOD mice females, along with reduced expression of vascular cell adhesion molecule (VCAM)-1 and aberrant expression of cell adhesion molecule (MAdCAM)-1 in deciduas (Burke *et al.* 2007). In the pregnant NOD mouse model of Sjögren's syndrome, we have recently shown that macrophages from mothers at 16 weeks of age and at day 9 of gestation present a lower basal production of IL-12 and nitric oxide than macrophages of age matched-non pregnant NOD mice (Larocca *et al*. 2008). Moreover, this 'silenced' condition of pregnant NOD macrophages could be partly mimicked in non pregnant NOD macrophages by incubating cells with progesterone. This result suggests that progesterone or progesterone/estradiol ratio, among other hormonal changes during gestation, is responsible for the anti-inflammatory macrophage profile. Interestingly, no significant differences were seen in estradiol serum levels in pregnant NOD mice, either with or without signs of resorption. Thus, in addition to the reduced progesterone levels, the relative ratio progesterone/estradiol is also decreased. Regarding Sjögren's syndrome patients, no significant differences were observed in the levels of estrogens and progesterone in sera between patients and controls although a higher estrogen/ progesterone relative ratio was reported (Taiym *et al.* 2004). It is worth noting that not only the appropriate levels of circulating hormones can influence the progression of gestation, but also the expression and signaling through their receptors. To further analyze this, we explored the allogeneic response of maternal splenocytes to paternal antigens and the inhibitory effect of progesterone. Though progesterone levels were reduced in pregnant NOD mice, the allogeneic response was similar in NOD and control mice. Moreover, progesterone added to the cultures inhibited the response to the same extent in both cultures confirming that progesterone receptors and signaling seem appropriate.

Regarding locally acting homeostatic signals, we have previously reported a reduced nitric oxide and increased prostaglandin E_2 synthesis in the uterus of NOD mice with a concomitant development of a Th1 cytokine profile (Roca *et al.* 2006). Both signals are known to impair the progression of gestation. Also, other authors have reported on aberrant endometrial features in diabetic pregnant NOD mice, where vascular defects (limited spiral artery development) due to a decreased NK cells activity, resulted in increased murine fetal loss (Burke *et al.* 2007).

On the knowledge that VIP has anti-inflammatory effects and promotes Th2/Treg profiles in several models of Th1 disease, while it showed an embryotrophic effect at days 9 to 12 of gestation in rodents, we investigated the presence of VIP in the implantation sites. Local expression of VIP mRNA was assessed at the implantation sites of NOD mice although at lower levels compared with control mice. VIP receptors VPAC 1 and VPAC 2 were also expressed at the maternal-embryonic interface suggesting that VIP could specifically act by a local/paracrine mechanism. The level of receptor expression was similar for both subtypes and for control and NOD healthy sites. Also, functionality of VIP receptors locally expressed was assessed by the addition of exogenous VIP to the media culture which induced a significant increase of Foxp3 and LIF expression and a trend to increase Tregs frequency. In rodent models of embryo implantation and growth, VIP levels increase in the deciduas at the early phases post-implantation and it has been assigned a role as a neural growth factor for the embryos (Gressens *et al.* 1998; Spong *et al.* 1999). Moreover, a reduction in the levels of VIP could lead to growth retardation and microcephaly (Gressens *et al.*1994). We have recently reported on the expression of VIP and VPAC 1 receptor in a human trophoblast cell line (Fraccaroli *et al*. 2009). By means of an experimental approach to the human fetal-maternal interface, we showed the participation of endogenous VIP in the fetal-maternal interaction with a pro-implantatory role by increasing the expression of Treg markers and LIF (Fraccaroli *et al*. 2009). Other authors have reported on the ability of VIP to modulate

hCG and progesterone in human trophoblast cultures (Marzioni *et al.* 2005) and to be selectively concentrated in the uterine vasculature, where its levels have been reported to be 2.5 fold greater than in maternal blood (Ottensen *et al.* 1982).

Finally, VIP has been recently proposed as an inducer of $CD4^+CD25^+Foxp3^+$ regulatory T cells in vivo in the prediabetic NOD mice model (Rosignoli *et al. 2*006). During pregnancy, a systemic expansion of $CD25^+$ Tregs has been shown and the lack of this subset leads to gestation failure (Aluvihare *et al.* 2004; Zenclussen *et al.* 2006; Saito *et al.* 2007). A lower level of systemic Tregs was reported in diabetic NOD mice (Pop *et al.* 2005), however a role for these cells in NOD pregnancy has not been clarified yet.

The reduced expression of VIP at the sites of implantation of NOD mice confirm and extend the observations on the potential pro-implantatory role of VIP in the human maternal fetal dialogue recently reported by means of an *in vitro* approach (Fraccaroli *et al.* 2009). Further studies are needed to address the mechanisms underlying the potential role of VIP as a modulatory factor and the perspectives for its application to therapy of pregnancy failures.

Materials and Methods

Animals: NOD and BALB/c female and C57Bl6J males were bred and maintained at the Central Animal Care facility of the School of Exact and Natural Sciences, University of Buenos Aires. They were maintained on a 12:12 h light–dark schedule. Each mouse was considered mature at the age of 9-10 weeks. Normally cycling NOD and BALB/c mice were mated and day 0 was taken as the day when the vaginal plug was seen. Mice were fasted overnight with water *ad libitum* before sacrificed and tissues and blood were obtained and processed immediately after. Mice were routinely tested for blood glucose levels (Wiener Lab., Rosario, Argentina) and considered pre-diabetic as their values of serum glucose on two occasions over a 24-hour period did not significantly differ from those of control mice (1.0 ± 0.1 g/l, n=27). In our breeding conditions, NOD mice diabetes onset is around the 30th week of age and none of the pregnant animals used throughout were diabetic. Also, confirming previous reports (Roca *et al.* 2006), NOD mice sera were also assayed for TNF-α levels showing a significant increase of this cytokine at 16 week-old NOD mice before mating compared with age-matched control mice (TNF-α pg/ml, NOD 230±11*, BALB/c 100±5; * P<0.05 vs BALB/c, n=7). All studies were conducted according to standard protocols of the Animal Care and Use Committee of the School of Exact and Natural Sciences, University of Buenos Aires.

Immunohistochemistry

Uteri from NOD and BALB/c mice were fixed in 4% paraformaldehyde overnight at 4ºC. The tissues were embedded in paraffin wax and sections of 4 µm were cut and placed on silanized glass slides. Haematoxylin-eosin staining was performed as described elsewhere (Roca *et al.* 2004).

Progesterone determination

Progesterone was quantified by specific radioimmunoassay using rabbit antiserum (Sigma Chemical Co., St. Louis, MO, USA). Briefly, progesterone was extracted from sera with ethyl ether and repeated freeze/thaw cycles. (Abraham *et al.* 1971) The organic phase was dried in vacuum, resuspended in radioimmunoassay buffer and measured immediately. Tests were conducted in duplicate and results were expressed as mean ± S.E.M (ng/ml).

Estradiol determination

Estradiol was quantified by specific radioimmunoassay Coat a Count Estradiol (Siemmens, Los Angeles, USA) according to manufacturers instructions. Briefly, the serum samples and the calibrators were incubated with 125I-labeled estradiol, in the antibody-coated tubes provided by the manufacturer for 3 hours at room temperature. After decantation, the tubes were measured inmediatly. Tests were conducted in duplicate and results were expressed as mean ± S.E.M (ng/ml).

Allogeneic stimulation.

Spleens from pregnant NOD and BALB/c mice, and from C57Bl6J male were removed aseptically and single-cell suspensions were prepared**.**

NOD and BALB/c splenic cells (Responder cells) were resuspended in complete RPMI-1640 (1 x 10^5 cells/well). Male C57Bl6J splenocytes resuspended in complete RPMI-1640 (1 x 10^5 cells/well) were treated with mitomycin C (0.5 ng/ml, Sigma, St. Louis, MO) during 30 minutes at 37°C to inhibit paternal DNA synthesis (stimulator cells). The mixture of responder and stimulator cells was incubated in a U-shape microtitre plate (Corning) at 37°C in a humidified atmosphere of 5% CO2 in the presence or absence of progesterone (10^{-5} M, Sigma, St. Louis, MO)

After 72 hours, cells were pulsed with 1 µCi/ well of methyl-[3H]-thymidine [3H]TdR (NEN, Boston, MA) during the last 18h of cell culture, and then harvested on glass fiber filters using a Packard Filtermate cell harvester (Packard Instruments,

LaGrange, IL). Incorporated radioactivity was measured in a liquid scintillation β-counter (Packard Instruments). Tests were conducted in triplicate and results were expressed as mean cpm ± S.E.M.

Immunoblotting detection of Foxp3 and LIF

Implantation sites explants were excised out, washed twice and incubated for 24 hs at 37°C in RPMI 1640 medium supplemented with 10% FCS (Life Technologies, Rockville, MD) in the presence or absence of VIP (10^{-7} M, Neosystem, France). After incubation, explants were homogenized at 4ºC in 50 mM Tris-HCl buffer pH 7.5 with 0.15 % Triton X-100 and protease inhibitors as previously reported for exocrine tissues and uterus (Rosignoli & Perez Leiros 2002; Roca *et al.* 2006). Once centrifuged at 5000xg 10 min at 4ºC, supernatants were frozen at –80ºC until used and an aliquot of each sample was separated for protein determination. Extracts (50-100 μg protein/lane), positive controls and molecular weight standards (Amersham Pharmacia Biotech Inc, NJ, USA) were subjected to 10% or 15% SDS-PAGE for Foxp3 (MW: 50 KD, Clone: FJK-16s, eBioscience, USA) and LIF (MW: 40 KD, Clone: 9824.11, R&D, MN, USA) respectively, transferred to nitrocellulose membranes (Amersham Pharmacia Biotech Inc, NJ, USA) and revealed with ECL substrate reagent (Pierce Biotechnology, Woburn, MA, USA). The immunoreactive protein bands were analyzed with a Fotodyne Image Analyzer® (Fotodyne, Inc., Hartland, WI). Results were expressed as relative densitometric values by means of the Image Quant software relatives to β-actin expression.

Flow Citometry analysis

Regulatory T cells were identified using the *Mouse Regulatory T cell Staining Kit* (PE Foxp3 clone: FJK-16s, FITC CD4 clone: RM4-5, APC CD25 clone: PC61.5, eBioscience, USA) according to the manufacturer protocol. Implantation sites explants were excised out, washed twice and incubated for 24 h at 37°C in RPMI

1640 medium supplemented with 10% FCS (Life Technologies, Rockville, MD) in the presence or absence of VIP (10-7 M, Neosystem, France). After incubation, explants were mechanically disrupted with a tissue homogenizer, and cellular suspension was centrifuged at 2000g 5 min at 4ºC and pellets were resuspended. The prepared cells were stained for surface molecules CD4-FITC (0,125 µg/test) and CD25-APC (0,06 µg/test) in 100 µl staining buffer. The tests were incubated for 30 min 4°C, and then washed twice (2ml Staining buffer) centrifuged at 2000g 5 min 4°C and decanted. Pellet was resuspended with 1ml Fix/Perm Buffer and incubated for 30 m 4°C in the dark. After washing twice (2ml Perm buffer) and centrifuged at 2000g 5 min 4°C , supernatants were decanted. Intracellular staining for Foxp3 was assed using Foxp3-PE antibody (0,5 µg/test) in 100 µl Perm Buffer and incubated for 30 min 4°C in the dark. After washing twice (2ml Perm buffer) and centrifuged at 2000g 5 min 4°C , supernatants were decanted and pellets resuspended in Flow Cytometry Staining Buffer for analysis. 100.000 events were acquired in a FACSCalibur cytometer® and results were analyzed using the WinMDI software®. Negative control samples were incubated in parallel with an irrelevant, isotype-matched antibody. Results for $CD25^{+}Foxp3^{+}$ cells are inside the electronically gate performed by CD4 positive staining and on viable cell population, to avoid nonspecific uptake of Abs by dead cells.

RT-PCR for VIP and VPAC receptors detection

Total RNA isolation and reverse transcription was performed using TRIZOL (Invitrogen, USA) and Ready-to-Go T primed First Strand Kit (Amersham Pharmacia Biotech Inc, NJ, USA) as previously described. (Rosignoli *et al*. 2004). The cDNA was then amplified using the specific primers for VIP, VPAC 1, VPAC 2 and GAPDH as internal control. Primers are described in Table 2 and PCR conditions are as follows: VIP, 95°C 10 min, 31 cycles of 96°C 45 s, 57°C 45 s, 72°C 1 min, and 72°C 10 min, for VPAC 1/VPAC 2, 94°C 10 min, 35 cycles of 94°C 45 s, 55°C 45 s, 72°C

90 s and 72°C 10 min. Finally, PCR products and molecular markers were fractionated on 2% agarose gels and visualized by staining with ethidium bromide. Densitometry was performed and the results were expressed as arbitrary units normalized to GAPDH expression.

Real-Time RT-PCR assays for VIP mRNA expression were performed in the same conditions as RT-PCR. Briefly, for a final volume of 25 µl, 2 µl of cDNA, 0.20 mM dNTPs, 0.25 µM specific primers, 3 mM MgCl2, 2 U Taq DNA polymerase, and 1:30,000 dilution of Sybr Green were added to the reaction mix. Real-Time PCR reactions were performed in a DNA Engine Opticon (MJ Research Inc.). PCR products were quantified in the Opticon Sowtware® and normalized to endogenous GAPDH. Each assay included a DNA minus control and a standard curve performed with serial dilutions of control cDNA. All samples were run in duplicate and the experiment was repeated three times with independently isolated RNA.

Statistics

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. Differences between groups were considered significant at $P<0.05$.

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641 **Figure legends**

642 *Figure 1: Reduced litter size in NOD mice*.

643 NOD (A) and BALB/c (B) mice were singeneically mated during lifespan beginning at
644 8 weeks and offspring was recorded as well as the mother´s age at the time of
645 parturition. Values represented are the mean ± S.E.M. of 20 females. *P<0.05 vs.
646 NOD 1st litter.

647

648 *Figure 2: Increased resorption rate in prediabetic NOD mice*

649 A) Uteri from pregnant NOD mice were processed for histological studies and
650 haematoxylin-eosin staining. Sections shown are representative of four other slices
651 analyzed similarly, 250X. The arrows indicate: (a) mononuclear infiltrates, (b)
652 hemorrhages, (c) detached cells. B) Resorption rate was calculated as the number of
653 resorbed embryos over total embryos counted.

654

655 *Figure 3: Decreased progesterone serum levels.*

656 A) Freshly isolated serum from each animal, NOD mice with healthy implantation
657 sites (NS, black bars), NOD mice showing sites with signs of resorption (RS, striped
658 bars) and BALB/c mice (empty bars), were individually processed and progesterone
659 and estradiol levels were determined by RIA as described in Materials and Methods.
660 Values are the mean ± S.E.M. of six separate animals. **P<0.01 vs. BALB/c, a
661 P<0.05 vs. NOD NS. B) Single-cell suspensions were prepared from pregnant NOD
662 and BALB/c mice spleens and co-cultured with male C57Bl6J splenocytes, previously
663 treated with mitomycin C (0.5 ng/ml) in the presence or absence of Progesterone (10^{-5} M).
664 After 72 hours, cells were pulsed with 1 µCi/well of methyl-[3H]-thymidine
665 [3H]TdR and then harvested. Tests were conducted in triplicate and results were
666 expressed as mean cpm ± S.E.M. *P<0.05 vs. basal.

667

668 *Figure 4: Decreased VIP mRNA levels in normal implantation sites of NOD mice*

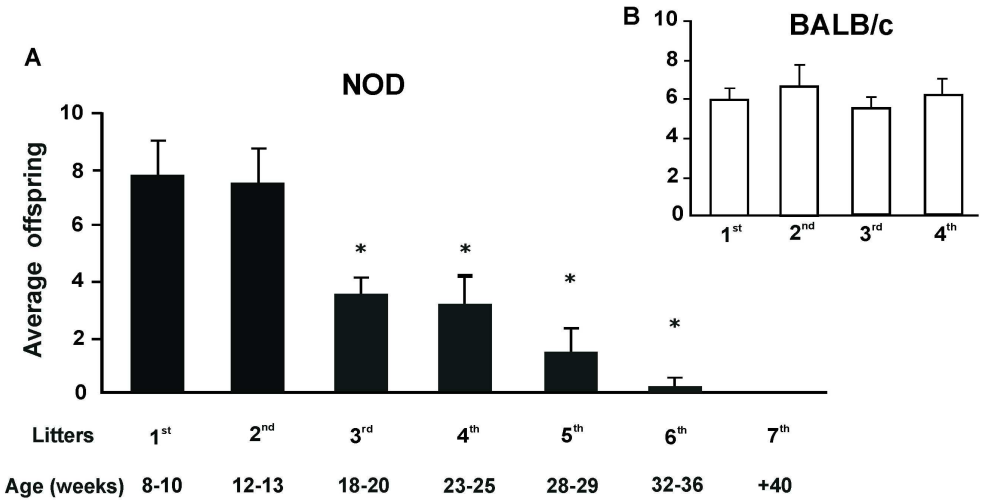
669 A) VIP mRNA expression was evaluated by RT-PCR from normal implantation sites
670 (NS, black bars) of NOD mice, NOD sites with signs of resorption (RS, grey bars)
671 and BALB/c mice normal sites (empty bars) as described in Materials and Methods.
672 Agarose gels shown are representative of three others. Values indicate the mean
673 intensity relative to GAPDH of each band in arbitrary units (AU) and represent the
674 mean ± S.E.M. for three separate experiments. *P<0.05 vs. BALB/c. a P<0.05 vs.
675 NOD NS.
676 B) VIP mRNA expression was quantified by real time RT-PCR from NOD mice
677 normal sites (NS, black bars), NOD mice sites with signs of resorption (RS, grey
678 bars) and BALB/c mice normal sites (empty bars) as described in Materials and
679 Methods. Values indicate the mean intensity relative to GAPDH of each test in
680 arbitrary units (AU) and represent the mean ± S.E.M. for three separate experiments.
681 *P<0.05 vs BALB/c. a P<0.05 vs NOD NS
682 C) $VPAC_1$ and $VPAC_2$ mRNA expression was evaluated by RT-PCR from normal
683 implantation sites of NOD mice (black bars), and BALB/c mice (empty bars) as
684 described in Materials and Methods. Agarose gels shown are representative of three
685 others. Values indicate the mean intensity relative to GAPDH of each band in
686 arbitrary units (AU) and represent the mean ± S.E.M. for three separate experiments.
687
688 *Figure 5: Effect of VIP on pro-implantatory factors.*
689 The effect of VIP on the expression of Foxp3 and LIF in normal implantation sites of
690 NOD and BALB/c mice was assessed by immunoblotting, after a 24hs culture in
691 presence or absence of VIP (10^{-7}M) as described in Materials and Methods. A) Blots
692 shown are representative of five others. Bars on the right side indicate the mean
693 intensity relative to β-actin expression of each band in arbitrary units and represent
694 the mean ± S.E.M. of five blots. B: Basal values, VIP-treated (gray bars), *P<0.05 vs.
695 basal.

B) VIP effect on Tregs frequency was assessed in viable implantation sites from NOD and BALB/c mice by FACS analysis as described in Materials and Methods. Dot plots presented are representative of two other experiments run similarly.

Table 1: Glucose levels in 16 weeks old pregnant NOD mice

Freshly isolated serum from each pregnant animal either NOD or BALB/c was individually processed and glucose levels were determined as described in Materials and Methods. Values are the mean ± S.E.M. of six separate determinations.

Table 2: Primer sequences



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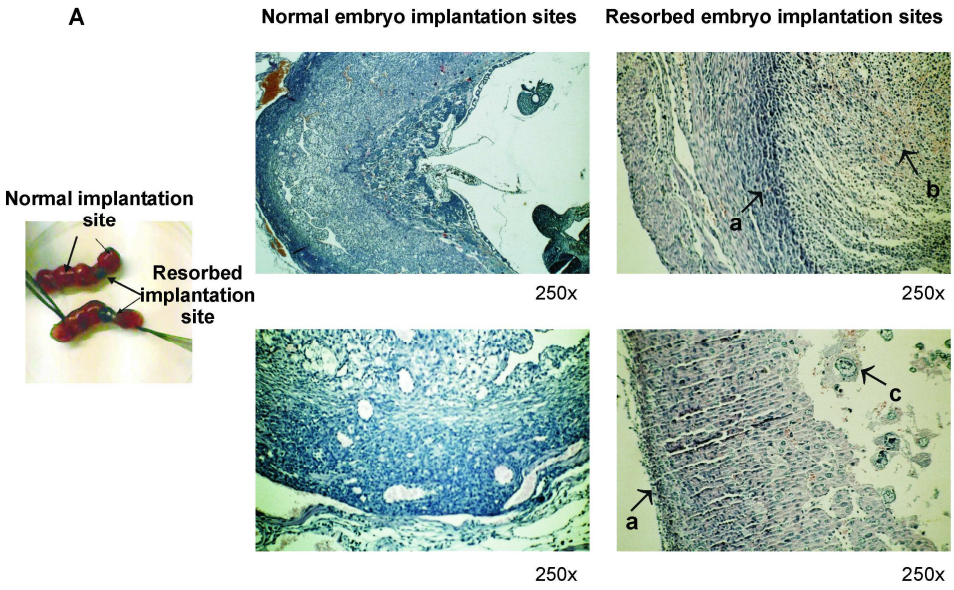

B

Resorption rate: **(Resorbed embryos/ total embryos)**	**60 / 187 (32%)**

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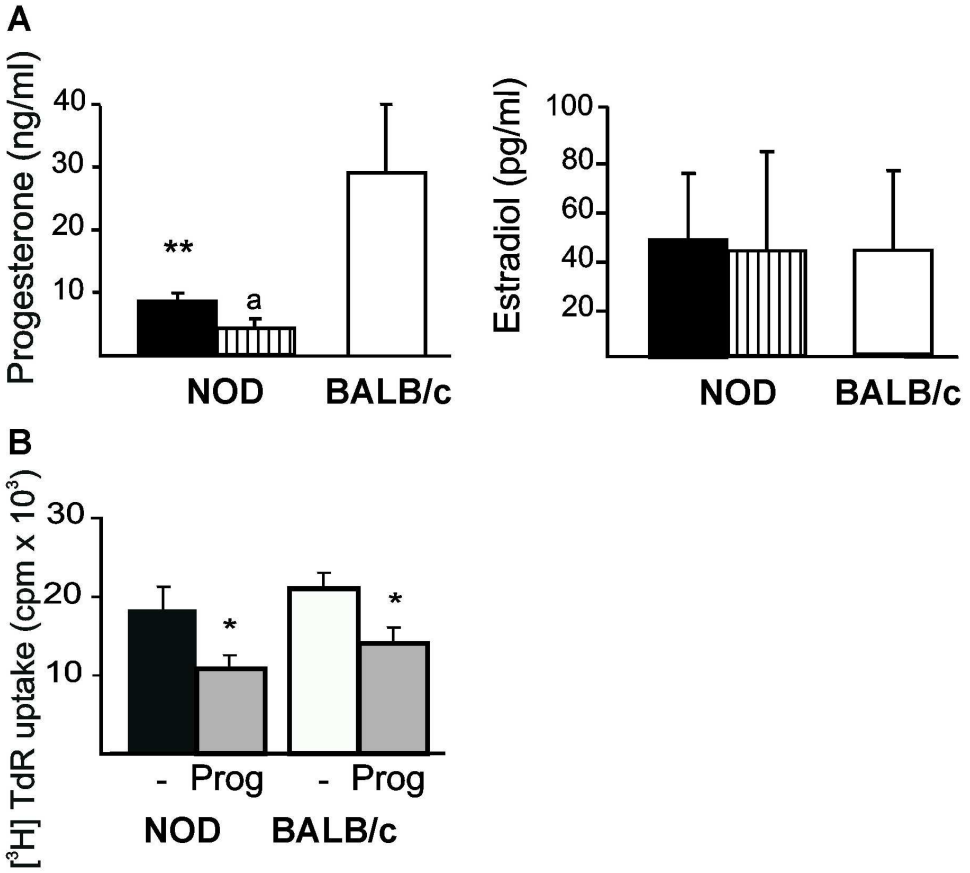

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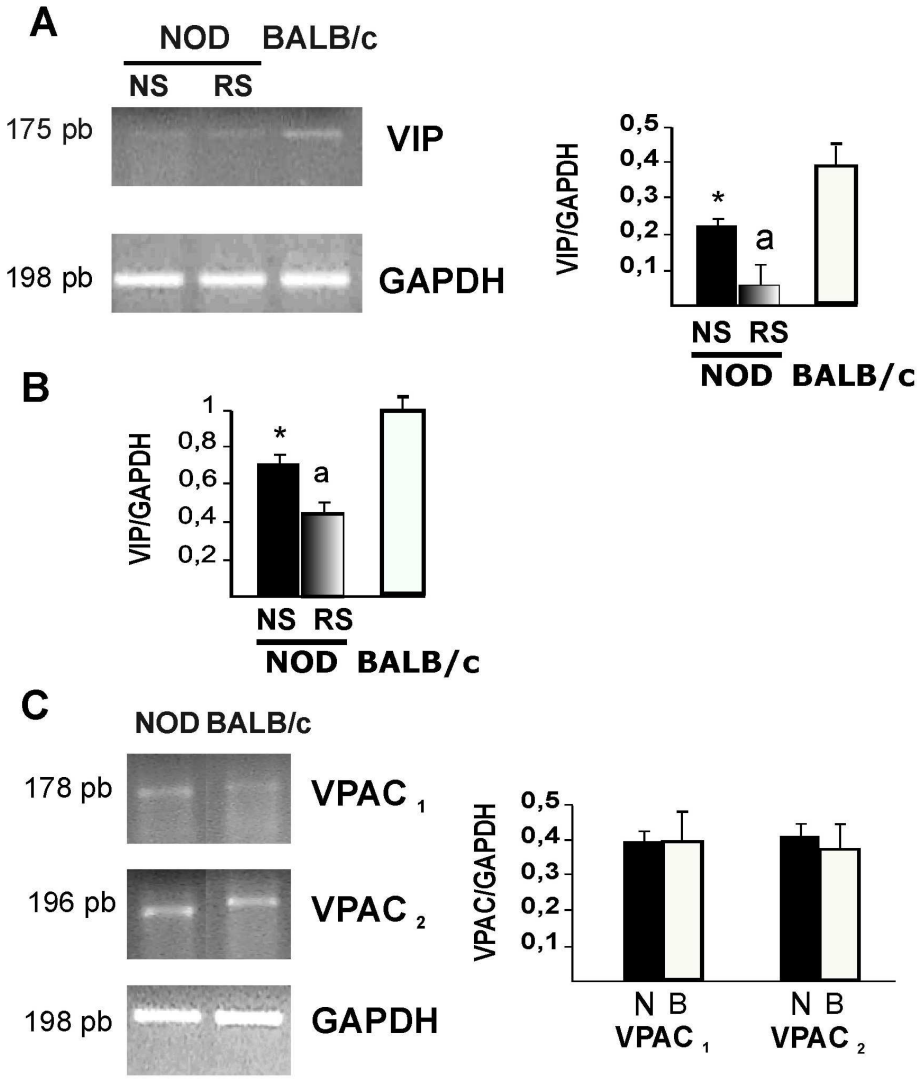

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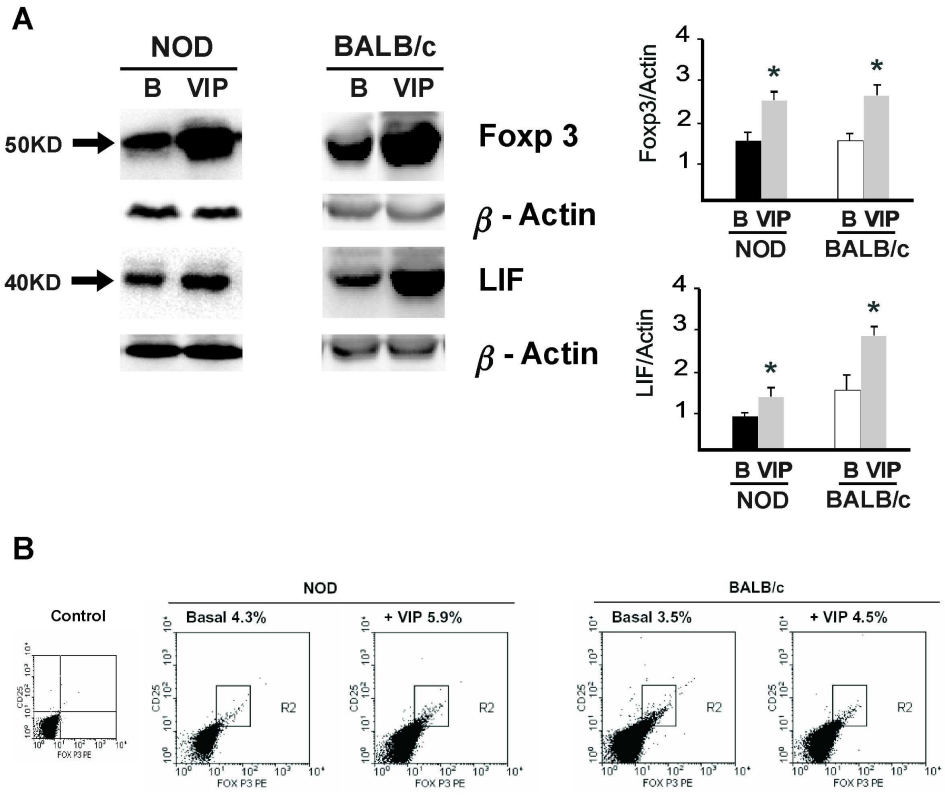

158x137mm (400 x 400 DPI)

Table 1: Glucose serum levels in pregnant 16 weeks old NOD mice

	NOD 1st gestation	NOD 3rd gestation	BALB/c
Glucose (g/l)	0,8 ±0,2	0,75±0,1	1,0±0,1

Table 2: primers sequences

VIP	sense: 5´TTC ACC AGC GAT TAC AGC AG 3´ antisense: 5´TCA CAG CCA TTT GCT TTC TG 3´
VPAC1	sense: 5´GTG AAG ACC GGC TAC ACC AT 3´ antisense: 5´TGA AGA GGG CCA TAT CCT TG 3´
VPAC2	sense: 5´ CCA AGT CCA CAC TGC TGC TA 3´ antisense: 5´ CCT CGC CAT CTT CTT TTC AG 3´
GAPDH	sense: 5´TGA TGA CAT CAA GAA GGT GGT GAA G 3´ antisense: 5´TCC TTG GAG GCC ATG TAG GCC AT 3´