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Modulation of macrophage inflammatory profile in pregnant nonobese diabetic (NOD) mice

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ABSTRACT

During normal early pregnancy circulating monocytes are recruited to the maternal–placental interface where they differentiate to macrophages expressing different functional phenotypes for the maintenance of tissue homeostasis. Pregnancy in the nonobese diabetic (NOD) mouse model presents some pathological features in the pre-diabetic stage. The aim of this work was to analyze the functional profile of peritoneal macrophages faced with inflammatory and phagocytic stimuli in early pregnant pre-diabetic NOD mice and their modulation by vasoactive intestinal peptide (VIP). Pregnant NOD mouse macrophages showed no basal NFκB activation, lower IL-12 and nitrites production compared with the macrophages from non-pregnant NOD mice. Their pro-inflammatory aberrant response to LPS and apoptotic cell challenge was reduced and VIP inhibited macrophage residual deleterious responses to apoptotic cells. A functional phenotype switch in macrophages during pregnancy in NOD mice and a promoting effect of VIP towards this regulatory phenotype would be in line with the central role of macrophages in the maternal–placental dialogue.

1. Introduction

During normal early pregnancy circulating monocytes are recruited to the maternal–placental interface where they differentiate to macrophages mainly expressing an immunosuppressant functional phenotype (Straszewski-Chavez et al., 2005; Gustafsson et al., 2008). Macrophages express a suppressant phenotype upon interaction with apoptotic cells for a rapid, efficient and non-inflammatory clearance of dying cells. This function is essential for the tissue remodeling processes that characterize the earliest stages of pregnancy (Straszewski-Chavez et al., 2005; Mor and Cardenas, 2010). Accordingly, macrophage phagocytic dysfunction seems to partly underlie endometriosis (Chuang et al., 2009) as well as pathological pregnancies especially those complicated with pre-eclampsia (Straszewski-Chavez et al., 2005; Mor et al., 2002). Moreover, some syncytial trophoblast cells (knots) and trophoblast cell debris reach circulation, where they can encounter monocytes thus amplifying the monocyte/trophoblast interaction (Huppertz, 2010; Abumaree et al., 2006). A higher risk of congenital malformations has been assessed in diabetic pregnancy than in normal, non-diabetic pregnancy (Eriksson, 2009). This teratogenicity might be partly related to macrophages localized to the uterus and the lymphoid organs as derived from results from a streptozotocin-induced diabetes model in ICR mice (Savion et al., 2004).

Pregnancy in the nonobese diabetic strain of mice (NOD), a model of type 1 diabetes, presents some pathological features both in the pre-diabetic and diabetic stages. In the prediabetic stage, NOD females have the unique characteristic of developing exocrine secretory dysfunction (van Blokland and Versnel, 2002; Anderson and Bluestone, 2005; Rosignoli et al., 2005; Jonsson et al., 2006) as well as a decline in litter size and increased resorption rates associated with local Treg and NK cells defective activity (Burke et al., 2007; Lin et al., 2008; Roca et al., 2009). Peritoneal macrophages of pre-diabetic nonpregnant NOD females present a dysregulated response to inflammatory and phagocytic stimuli with high levels of pro-inflammatory mediators and low production of IL-10 (O'Brien et al., 2002; Stoffels et al., 2004; Larocca et al., 2007).

Vasoactive intestinal peptide (VIP) is a neuroimmunopeptide with actions at multiple levels. It induces vasodilation and exocrine secretion (Ekström, 1989); it regulates embryonic growth (Hill et al., 1999) and it has potent anti-inflammatory properties in models of chronic inflammatory diseases through its action on VPAC receptors on macrophages and T cells (Gomariz et al., 2007; Gonzalez-Rey et al., 2007; Delgado et al., 1999).

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When injected to NOD females, VIP showed an efficient control of inflammation: it decreased diabetes incidence and insulitis score as well as plasmatic IL-12 levels; while it increased Foxp3 and TGF-β expression and plasmatic IL-10 levels (Rosignoli et al., 2006; Jimeno et al., 2010). VIP promoted a 'regulatory' phenotype in macrophages of normally cycling NOD mice, decreasing pro-inflammatory mediators and increasing IL-10 (Larocca et al., 2007). It also modulated the local expression of leukocyte inhibitory factor (LIF), Foxp3 and Treg cell activity at the implantation sites of pre-diabetic NOD mice and controls (Roca et al., 2009).

The goal of this work was to analyze in early pregnant pre-diabetic NOD mice the functional profile of peritoneal macrophages primed with inflammatory and apoptotic cell phagocytic stimuli and their modulation by VIP.

2. Materials and methods

2.1. Animals

Normally cycling NOD and BALB/c mice of 16 weeks of age were mated singenically and gestational day 0 was indicated by vaginal plug. NOD and BALB/c female mice were bred and maintained in the Central Animal Care facility at the School of Exact and Natural Sciences, University of Buenos Aires (FCEyN-UBA). Mice were maintained on a 12:12 h light–dark schedule and fasted overnight with water *ad libitum* before used. NOD mice either virgin or at the 9th day of gestation were used for macrophage isolation. NOD mice blood glucose levels were registered and their values on two occasions over a 24-h period did not differ from BALB/c values (NOD: 1.1 ± 0.1 g/l, $n = 27$; pregnant NOD: 0.8 ± 0.2 g/l), thus they were considered normoglycemic pre-diabetic. All studies were conducted according to standard protocols of the Animal Care and Use Committee of the FCEyN-UBA.

2.2. Peritoneal macrophages isolation and LPS treatment

Macrophages were obtained by washing the peritoneal cavity with ice-cold RPMI 1640 as reported (Larocca et al., 2007). Cells were resuspended in RPMI 10% fetal calf serum (FCS) (Life Technologies, MD) and seeded in 24-well plates (Corning Glass, Corning, NY) at 5×10^5 cells/well. After incubation at 37 °C for 2 h, monolayers (>95% macrophages by F4/80 flow cytometry staining) were stimulated with 10 μg/ml LPS-100 U/ml IFN-γ or with phagocytic stimuli as described below with or without 100 nM VIP (Neosystem, France) (Larocca et al., 2007).

2.3. Phagocytosis

Macrophages were co-cultured with singeneic thymocytes previously induced or not to apoptosis. Incubations were run at 37 °C in 24-well plates for the times indicated by placing a coverslip used for microscopy. VIP (100 nM) was added 30 min before the addition of apoptotic cells. Thymocytes obtained from singeneic mice of 21 days of age were thoroughly washed and induced to apoptosis with 1 nM dexamethasone for 4 h at 37 °C. This procedure yielded 30% Annexin V positive/propidium iodide (PI) negative staining of BALB/c and NOD thymocytes. Hematoxilin–Eosin staining was used for phagocytic index determination (O'Brien et al., 2002).

2.4. TUNEL

Macrophage were co-cultured, fixed in methanol and DNA fragmentation detected with DeadEnd™ Fluorometric TdT-mediated dUTP Nick-End Labeling (TUNEL) system assay (Apoptosis detection kit S7110, Chemicon Int, CA) and confocal analysis. Green labeling of DNA nicks by fluorescein-12-dUTP and red staining of chromatin with PI were detected by fluorescence microscopy. Six randomly chosen microscopic fields were captured with a 400× magnification.

2.5. NFκB activation

Confocal and Western blot analysis were used to analyze NFκB activation. Macrophages were incubated in 24 well plates for 15–90 min with LPS or apoptotic cells in the presence or absence of VIP. Once washed, cells were homogenized in 10 mM Hepes pH 7.9; 1 mM EDTA; 1 mM EGTA, 5 mM NaF, 5 mM $NaVO_4$, 1 mM DTT, 10 mM KCl, 0.5% NP-40 with protease inhibitors as described (Roca et al., 2009). After 15 min on ice samples were centrifuged at $8000 \times g$ for 15 min. Supernatants (cytosolic extracts) were fractionated in 12% SDS-PAGE gels and immunoblotted with rabbit polyclonal anti-IkB-α or goat polyclonal anti-Actin (Santa Cruz Biotechnology, CA) (Calafat et al., 2009). Bands were revealed with peroxidase-conjugated antibodies and enhanced chemiluminescence detection system (Pierce, Rockford, IL). Densitometry analysis of proteins was performed with ImageQuant®.

For confocal microscopy studies, macrophages incubated on coverslips were permeabilized with methanol at −20 °C, incubated with mouse p65 Ab (Santa Cruz Biotechnology) and FITC conjugated anti-mouse Ab (BD Pharmingen, CA), washed and stained with 0.5 μg/ml PI and observed at confocal microscope Olympus FV 300 coupled to Olympus BX61.

2.6. Flow cytometry analysis

2.6.1. F4/80 staining and intracellular staining for IL-10

Cells were incubated with 10 μg/ml Brefeldin A (Sigma, St. Louis, MO) for 4 h at 37 °C. After washing, cells were stained with FITC conjugated anti-F4/80 monoclonal Ab (eBioscience, San Diego, CA), fixed in 4% paraformaldehyde/PBS-2% FCS at room temperature and permeabilized with 0.5% saponin (Sigma, St. Louis, MO) in PBS for 30 min. Permeabilized cells were washed and incubated for 30 min with PE-conjugated anti-IL-10 monoclonal Ab (BD, San José, CA) or with the PE-conjugated IgG1 isotype. 10,000 events were acquired in a FACSAria cytometer® and results analyzed using the WinMDI software®. Results are expressed as a percentage of IL-10 positive cells inside the electronically gated F4/80 positive population.

2.7. Annexin V staining for early apoptosis detection

The frequency of apoptotic cells was assessed by double staining using PI and FITC-Annexin V following manufacturer's recommendations (Immunotech, France). Flow cytometry data were acquired and analyzed as above.

2.8. Cytokines and nitrites determination

Cytokines (IL-10 and IL-12) were determined in macrophage supernatants with a capture ELISA assay as described (Larocca et al., 2007; Roca et al., 2006). Microtitre plates (Corning Inc., New York) were coated with a capture monoclonal anti-mouse IL-10 or IL-12p70 antibody (BD, San José, CA) at 2 μg/ml at 4 °C. After blocking, supernatants were added and biotinylated monoclonal anti-IL-10 and IL-12 antibodies (BD, San José, CA) were added and revealed as stated (Larocca et al., 2007). Nitrite concentration was determined by the Griess method in macrophage supernatants obtained for cytokine measurements (Larocca et al., 2007, 2008).

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

3. Results

3.1. Basal and LPS-stimulated NOD macrophage functional profile at early pregnancy

Fig. 1 shows mediators released by NOD and BALB/c peritoneal macrophages isolated in resting and LPS-stimulated conditions in pregnant or normally cycling mice at diestrus stage. The 'classically activated' profile of non-stimulated macrophages characteristic of pre-diabetic NOD females, with increased production of nitrites and IL-12p70 even in basal conditions, was down-regulated at early pregnancy. Macrophages from NOD and BALB/c mice were then challenged *in vitro* with LPS + IFN-γ. Fig. 1 shows that at early pregnancy the ability of macrophages from NOD mice to produce IL-10 in response to LPS is restored reaching similar levels of macrophages from pregnant BALB/c mice (Fig. 1). Similar results for IL-10 were obtained by flow cytometry for NOD peritoneal macrophages, as well as for macrophages isolated from decidual tissue of NOD mice implantation sites which showed an F4/80+ IL-10+ phenotype similar to that seen for peritoneal macrophages (not shown). A strong reduction of nitrites production in response to LPS was also observed in pregnant NOD mouse macrophages, comparable to the levels in BALB/c mice (Fig. 1).

Since IL-12 and NO synthesis are closely related to a rapid NFκB activation and both mediators were increased in basal and LPS-stimulated conditions in NOD mouse macrophages, we then analyzed NFκB activation in macrophages in basal conditions and in response to LPS both from pregnant and non-pregnant mice. In line with the pro-inflammatory mediators released by macrophages from non-pregnant NOD mice in basal conditions, a basal traslocation of NFκB p65 to the nucleus was observed by confocal microscopy even though they had not been induced previous to

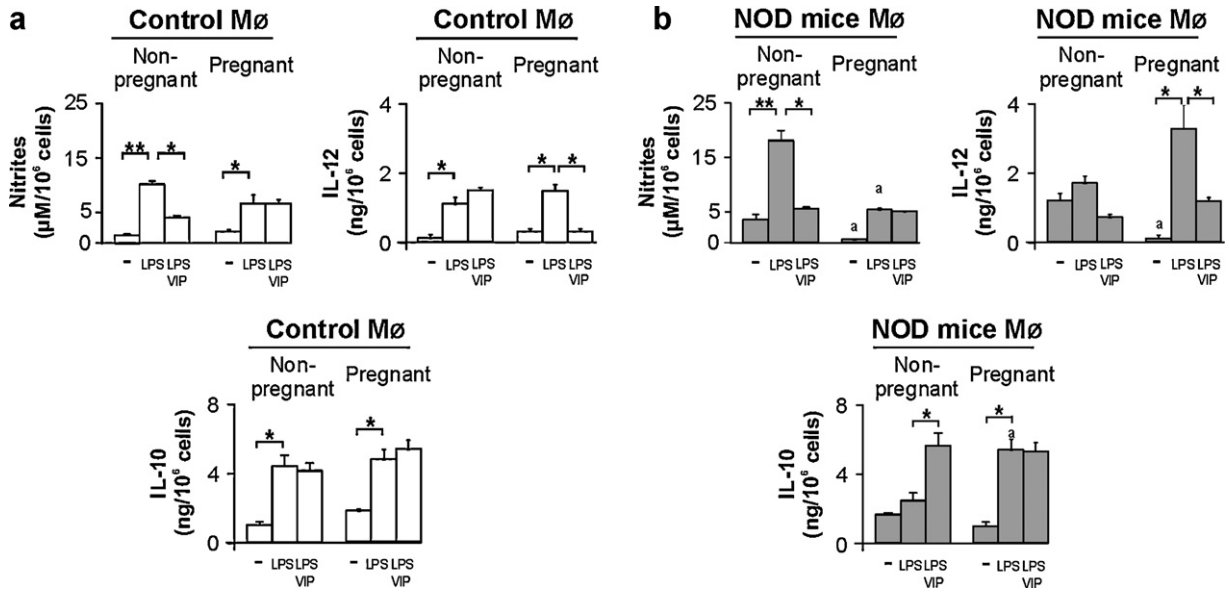

Fig. 1. Basal and LPS-stimulated functional profile of pregnant NOD mouse macrophages. Macrophages from non-pregnant or pregnant mice NOD or BALB/c mice of 16 weeks of age in their 9th day of gestation were isolated and plated for 24 h in 24-well plates at 5×10^5 cells/well in a final volume of 200 μl stimulated or not with 10 μg/ml LPS plus 100 U/ml IFN-γ in the presence or absence of 100 nM VIP. Cell-free supernatants were harvested for cytokines or nitrites determination as described in Section 2. (a) Control BALB/c mice macrophages; (b) NOD mice macrophages. Results shown are mean ± S.E. of at least 4 determinations for each condition and group of mice. $^{*}P < 0.05$; $^{**}P < 0.01$; (^{a}) $P < 0.05$ vs. non-pregnant in the same strain of mice.

their isolation (basal condition in Fig. 2a). Moreover, Fig. 2a and c also shows that macrophages from NOD mice fail to display a typical kinetic pattern of p65 traslocation to the nucleus/IkB disappearance-retrieval to the cytosol in response to LPS as seen for macrophages from control BALB/c mice. While subcellular localization of p65/IkB subunits of NFκB in macrophages from BALB/c control mice is consistent with a peak of maximal activation at 15 min, NFκB kinetics in NOD mouse macrophages is not clear, with p65 subunit already located to the nucleus at zero time (Fig. 2a). In contrast, macrophages from pregnant NOD mice showed no basal traslocation of p65 to the nucleus and, though they showed a slower kinetics of NFκB activation compared with normal nonpregnant mice (Fig. 2b and c). On the basis that VIP levels increase during pregnancy in the maternal–fetal interface and that it strongly regulates pro-inflammatory responses by acting on macrophages from normal mice, we assayed the effect of VIP on the macrophages of pregnant NOD mice to test whether it could reverse the observed inflammatory response and NFκB activation. As shown in Figs. 1 and 2, VIP reduced IL-12, inhibited NFκB activation and increased IL-10 similarly in macrophages of pregnant NOD and BALB/c mice.

3.2. Functional profile of NOD mouse macrophages co-cultured with apoptotic cells and the effect of pregnancy

Fig. 3 shows macrophages from NOD and control mice co-cultured with apoptotic cells (a) and their phagocytic index (b). Macrophage phagocytic avidity was reduced at gestational day 9 in both strains (Fig. 3b and c). Phagocytosis of apoptotic cells induced NFκB activation as reflected by p65 traslocation kinetics shown in Fig. 4a and b. Phagocytosis also induced nitrites and lack of IL-10 production only in the NOD. In macrophages from pregnant NOD mice, the immunosuppressant phagocytosis was restored with reduced nitrites, increased IL-10 levels and NFκB activation kinetics similar to what occurs in macrophages from pregnant BALB/c control mice, thus normalizing their aberrant activation in response to apoptotic cells. VIP had no effect on phagocytosis index in the macrophages from pregnant mice of either strain, but it reduced a residual aberrant nitrite production in macrophages from pregnant NOD mice (Figs. 3c and 4c).

4. Discussion

Here we provided new evidence supporting that macrophages from pregnant pre-diabetic NOD mice show most signals of a 'regulatory' phenotype when faced to inflammatory and phagocytic stimuli. VIP modulated residual pro-inflammatory response of pregnant NOD mice upon apoptotic cell stimulus. These conclusions are supported by three major observations: First, macrophages from pregnant NOD mice showed no NFκB basal activation and lower basal levels of nitrites and IL-12 compared with non-pregnant NOD mice cells. Second, the pro-inflammatory aberrant responses of macrophages to LPS or apoptotic cell challenge as well as NFκB activation kinetics to both stimuli were also normalized. Third, the increase of nitrites levels elicited by apoptotic cells only in macrophages from pregnant NOD mice was reduced by VIP.

Normally cycling NOD females at their pre-diabetic stage present high levels of IL-12, nitrites and TNF-α and lower levels of PGE2 and IL-10 in plasma and cultured peritoneal macrophages compared with younger NOD and BALB/c females (Rosignoli et al., 2006; Roca et al., 2006; Larocca et al., 2007). A dysregulated response to apoptotic cells and LPS was also reported in macrophages of pre-diabetic NOD females (Larocca et al., 2007; Stoffels et al., 2004; Mohammad et al., 2006; O'Brien et al., 2002). The switch of macrophages' profile isolated from pregnant mice showed here, either basally or when challenged with LPS, was comparable in both strains although in the NOD this modulation was more evident. Of note, the high levels of nitrites induced by LPS were drastically decreased in the macrophages from pregnant NOD mice reaching similar values as those seen in pregnant BALB/c mice. Nitric oxide has a dual effect on pregnancy: at low levels it favors uterine quiescence and vasodilation required for fetal growth, while at high concentrations as those released by LPS-stimulated macrophages, it is involved in embryo resorption (Ogando et al., 2003; Aisemberg et al., 2007).

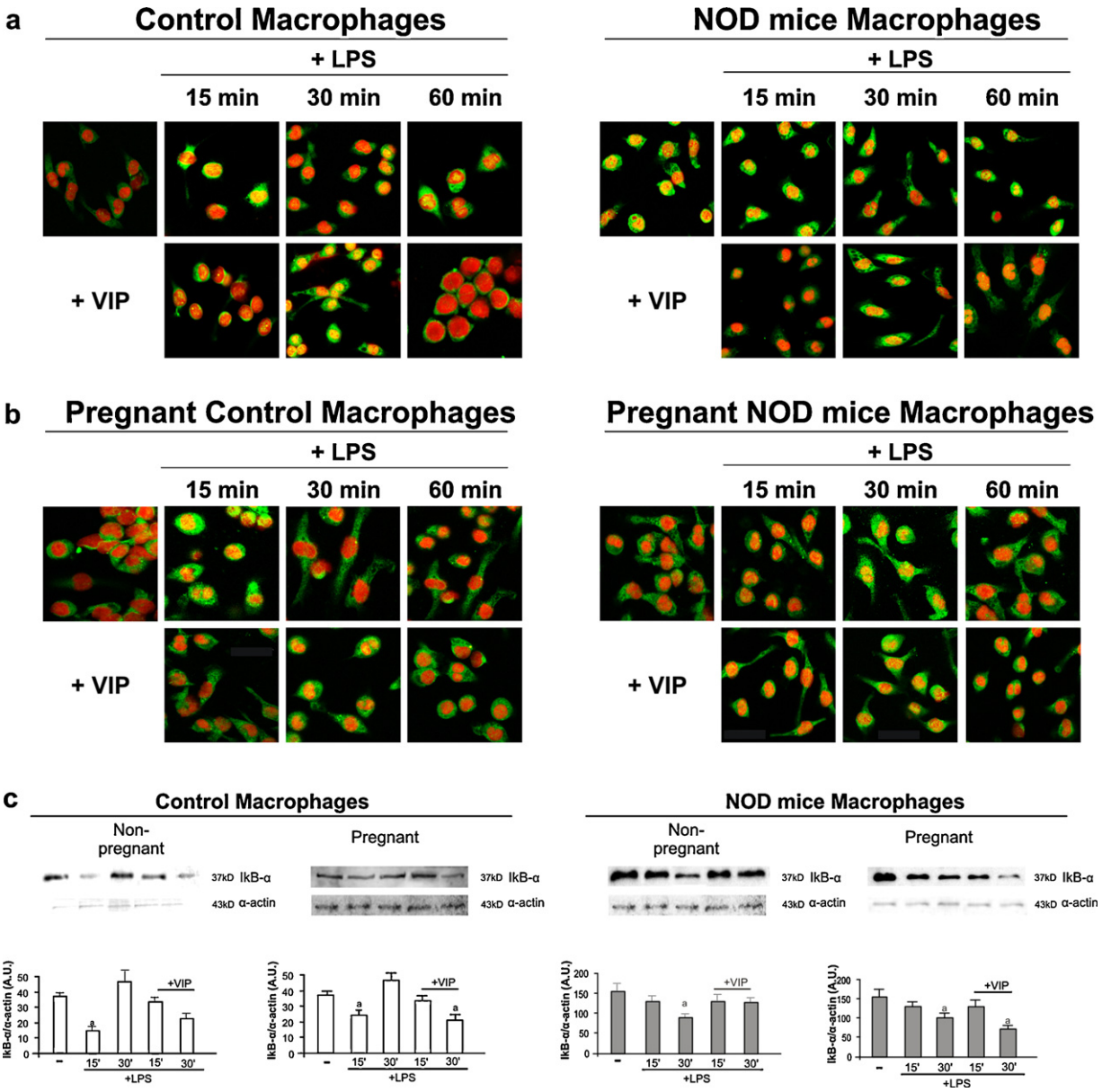

Fig. 2. NFκB activation kinetics in LPS-stimulated macrophages from pregnant NOD mice. NOD and control BALB/c macrophages isolated from 16 weeks old non-pregnant (a) or pregnant (b) mice at gestational day 9th were incubated in 24 well plates for the times depicted (15–60 min) alone or with 10 μg/ml LPS plus 100 U/ml IFN-γ (LPS) in the presence or absence of 100 nM VIP (lower row of each set) as indicated in Section 2. Merged images of confocal microscopy are shown: Cells cultured on coverslips were incubated with p65 primary Ab and FITC conjugated Ab (green fluorescence in cytosol or merged yellow in the nucleus) and propidium iodide (red staining of the nucleus). Microphotograph sets are representative of 5 others run with NOD and BALB/c (control) macrophage preparations (200×). (c) Macrophages cultured as in (a) and (b) were homogenized and cytosolic extracts were fractionated in 12% SDS-PAGE gels (50 μg/lane) for IkB-α assays. Blots shown are representative of 5 experiments run similarly and results shown for IkB-α band intensity for each condition are means ± S.E. in arbitrary units relative to actin for independent NOD and BALB/c samples. ([a]) $P < 0.05$ vs. basal in the corresponding strain of mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The pro-inflammatory aberrant response elicited by apoptotic cells on macrophages was also normalized with a strong reduction of nitrites and increased IL-10 levels paralleled by a normalization of NFκB activation kinetics during early pregnancy in NOD mice. Convincing evidence suggests that a controlled innate immune response and phagocytic function leaded by uNK cells and macrophages is central to the maintenance of tissue homeostasis at early pregnancy (Straszewski-Chavez et al., 2005). While uNK cells have been assigned a role in spiral arteries transformation, macrophages appear to have multiple roles. A deleterious non-regulated inflammatory response of macrophages against pathogens and necrotic cells, or a non-silent phagocytosis of apoptotic bodies might underlie a loss of homeostasis at the maternal–placental interface. Moreover, it has been proposed that macrophages act as supporting cells for trophoblast invasion and transformation of spiral arteries in normal pregnancies, while in complicated pregnancies they rather hamper this function by inducing inappropriate trophoblast apoptosis or even by undergoing apoptosis themselves (Straszewski-Chavez et al., 2005; Mor and Abrahams, 2003; Fest et al., 2007; Nagamatsu and Schust, 2010). Peritoneal macrophages studied here are the main pool of phagocytic cells differentiated in a compartment surrounding the maternal placental interface. Whether identical monocyte subpopulations replenish tissue compartments and, particularly, these neighboring compartments, is still unclear (Soehnlein and Lindbom, 2010; Mosser and Edwards, 2008). However, a predominant IL-10+ phenotype was also seen in macrophages isolated from explants of deciduas at viable implantation sites from pregnant

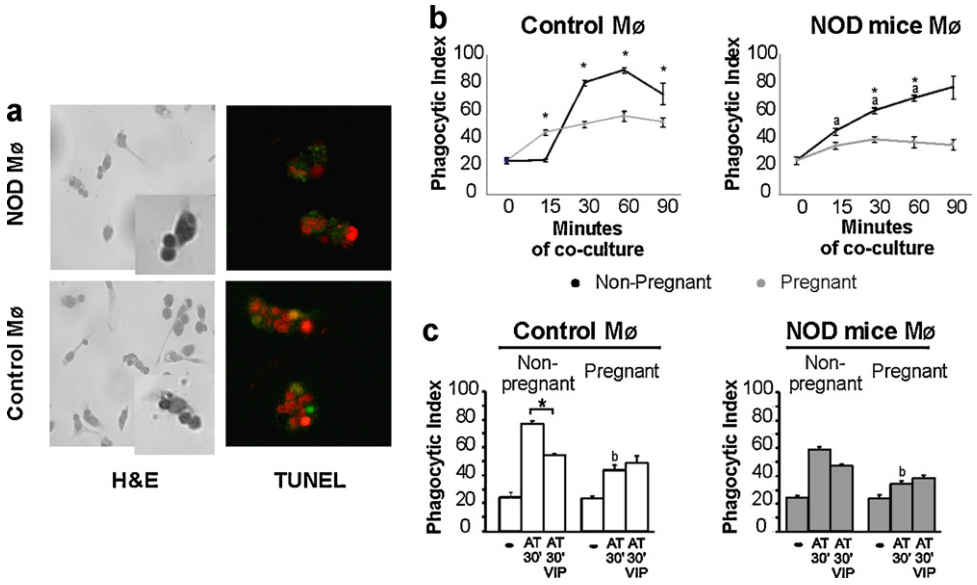

Fig. 3. Phagocytic function of NOD macrophages co-cultured with apoptotic cells. (a) NOD and BALB/c (control) macrophages co-cultured for 30 min with singeneic thymocytes previously induced to apoptosis and stained with Hematoxilin–Eosin (H&E) or fixed in methanol and DNA fragmentation was assessed by TUNEL assay (TdT-mediated dUTP Nick-End Labeling) and confocal analysis (TUNEL). (b) NOD macrophages (NOD mice M∅) and BALB/c macrophages (control M∅) isolated from 16 weeks old non-pregnant or pregnant mice at gestational day 9th were co-cultured for 15–90 min with apoptotic thymocytes (1:5 relationship) on coverslips placed inside 24-well plates and then used for microscopy. Zero times correspond to macrophages co-cultured with viable non-induced thymocytes. Results are means ± S.E. of 3 experiments respectively. * $P < 0.05$ vs. same concentration in same strain; ([a]) $P < 0.05$ vs. BALB/c. (c) NOD and BALB/c (control) macrophages from 16 weeks old non-pregnant or pregnant mice at gestational day 9th were co-cultured for 30 min with apoptotic thymocytes (AT) on coverslips placed inside 24-well plates as in (b) in the absence or presence of VIP (100 nM final concentration) added to co-cultures 30 min before the addition of apoptotic cells. Basal values (−) correspond to macrophages co-cultured with viable non-induced thymocytes. Results are means ± S.E. of at least 3 experiments for each condition. * $P < 0.05$; ([b]) $P < 0.05$ vs. non-pregnant of same strain.

NOD mice. Moreover, a role of macrophages as linking cells of innate and adaptive immunity is also involved in maternal–placental interface. As antigen presenting cells macrophages express co-stimulatory signals that favor immunotolerance to paternal antigens through the induction of Treg cells (Nagamatsu and Schust, 2010). Pre-eclampsia is a human disease related to a poor placentation with signs of hypoxia and defects in local apoptotic and vascular remodelling events associated to an impaired function of macrophages and NK cells (Nagamatsu and Schust, 2010). Diabetes increases 12 fold the risk of pre-eclampsia, and hyperglycemia is one of the major determinants but not the unique feature involved in intrauterine growth restriction, premature birth or neural defects, among other complications of gestation in diabetic mothers (Evers et al., 2004). Increased resorption rates in pre-diabetic NOD mice have been recently reported (Burke et al., 2007; Lin et al., 2008; Roca et al., 2009). The decrease in offspring around the 16–18th week of age of the mothers paralleled the onset of the systemic Th1 response and clearly preceded the hyperglycemia of the diabetic stage. Consistent with a predominant role of CD4+CD25+Foxp3+ regulatory T cells at early stages of pregnancy, higher resorption rate in NOD females was associated with lower Treg cells at viable implantation sites (Roca et al., 2009). A lower uNK cell number was observed in the decidua basalis of diabetic NOD mice females, as well as a lower or aberrant decidual expression of cell adhesion molecules (Burke et al., 2007). Interestingly, a higher expression of complement component C1q, known to modulate apoptotic cell clearance by phagocytes, was found in a proteomic analysis of uterine lymphocytes of pregnant NOD mice at gestational day 9.5 compared with pregnant BALB/c mice (Li et al., 2009). Pregnancy hormones have been shown to modulate the course of the autoimmune response in nonpregnant NOD females (Homo-Delarche, 2004). To this aim it was necessary a cocktail of several pregnancy hormones since progesterone alone, although the most relevant hormone to pregnancy, it was not enough stimulus to modulate the immune response. Working with pregnant NOD mice on day 9th we have obtained similar results, as progesterone treatment reduced the allogenic response of pregnant NOD mouse splenocytes (Roca et al., 2009). In line with this, the pro-inflammatory response of LPS-stimulated macrophages from nonpregnant NOD mice was inhibited by progesterone (Larocca et al., 2008). However, the anti-inflammatory effect of progesterone interacting *in vitro* with macrophages from NOD females did not stimulate IL-10 levels (Larocca et al., 2008). Thus, consistent with the concerted role of progesterone with other hormones during pregnancy, progesterone does not completely mimic the effect induced by pregnancy as a whole in modulating the classically activated profile into a regulatory profile at day 9th of pregnancy with the increased levels of IL-10 shown here. Progesterone also induced suppressive effects on normal murine peritoneal macrophages when primed *in vivo* with LPS (Kohmura et al., 2000). Even though progesterone appeared not sufficient to induce a regulatory functional profile in macrophages from NOD mice, it might have a role in decreasing basal inflammatory signals *in vivo* as reflected by lower nitrites and NFκB activation in pregnant females. The lower levels of progesterone detected in nonpregnant NOD mice compared with standard strains (Durant et al., 1998; Homo-Delarche, 2004) as well as in pregnant NOD mice with no changes in estrogen levels compared with BALB/C pregnant mice (Roca et al., 2009) support a predominant role for this hormone in the effects.

Macrophages are plastic cells that react upon microenvironmental inputs with a spectrum of functional phenotypes recently redefined as a continuum of 'inflammatory', 'regulatory' and 'wound healing' macrophages (Mosser and Edwards, 2008). Pregnancy somehow 'homogenized' peritoneal macrophage phenotypes to a predominant suppressant phenotype in both strains. However some interesting differences were found between control and NOD macrophages: While macrophages from pregnant BALB/c mice were not responsive with nitrites to the challenge of apoptotic bodies, NOD macrophages did produce this deleterious mediator, though in lower levels than macrophages from nonpregnant mice. And this lower modulatory effect of pregnancy on nitrites in the NOD was not seen when the stimulus was inflam-

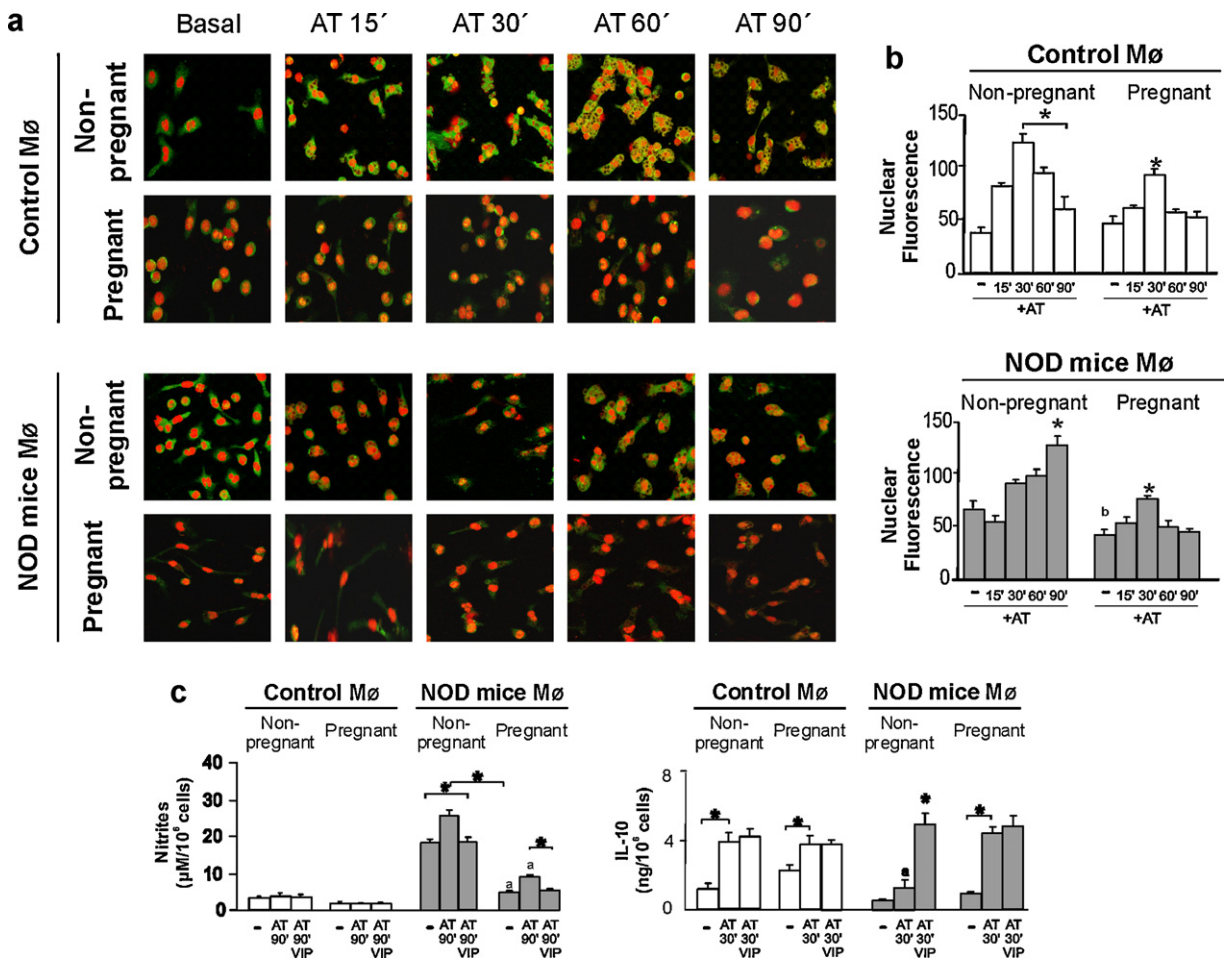

Fig. 4. Activation profile of NOD macrophages co-cultured with apoptotic cells. (a) NOD and control BALB/c macrophages isolated from 16 weeks old normally cycling or pregnant mice at gestational day 9th were incubated in 24 well plates for the times depicted (15–90 min) with non-induced thymocytes (basal) or with apoptotic thymocytes (1:5) (AT 15′-AT90′) as indicated in Section 2. Merged images of confocal microscopy are shown: Cells cultured on coverslips were incubated with p65 primary Ab and FITC conjugated Ab and propidium iodide as in Fig. 2. Microphotograph sets are representative of 4 others run with NOD and BALB/c (control) macrophage preparations (200×). (b) The bars represent the means ± S.E. of nuclear fluorescence intensity evaluated at the times shown in (a) and basal (−) with ImageJ®. $P < 0.05$ vs. basal or indicated bar; ([b]) $P < 0.05$ vs. nonpregnant. (c) Macrophages from NOD and BALB/c (control) non-pregnant or pregnant mice in their 9th day of gestation were isolated and plated as indicated in Fig. 1 and monolayers obtained were co-cultured with non-apoptotic thymocytes (−) or with apoptotic thymocytes (1:5) for 90 min (AT 90′) in the presence or absence of 100 nM VIP. After washing out non-ingested cells macrophages were cultured for 24 h and cell-free supernatants were harvested for cytokine or nitrites determination as described in Section 2. Results shown are mean ± S.E. of at least 4 determinations for each condition and group of mice. *$P < 0.05$; ([a]) $P < 0.05$ vs. same condition in BALB/c.

matory and not phagocytic. A lower phagocytic function associated to higher inflammatory signals in the NOD could underlie some of the placental abnormalities shown in NOD early pregnancy (Burke et al., 2007; Roca et al., 2009).

Results presented here support the role of VIP in the switch from 'inflammatory' to 'regulatory' macrophages in NOD mice during early pregnancy. In addition to its effects on uterine quiescence and immune response (Jovanović et al., 1998; Roca et al., 2006; Gomariz et al., 2007), VIP also participates in the maternal regulation of embryonic growth in rodents during the early post-implantation period (Hill et al., 1999; Spong et al., 1999) and modulates human immune-trophoblast cell dialogue favoring a Th2/Treg functional profile (Fraccaroli et al., 2009). Regarding NOD mice, VIP induced Treg cells in NOD females helping to maintain immunotolerance and increasing Treg/Th17 ratio (Rosignoli et al., 2006; Jimeno et al., 2010). An increase of decidual VIP expression is induced by pregnancy in BALB/c and NOD mice; and the incubation of decidual explants of NOD and normal mice with exogenous VIP increased Foxp3. However, lower levels of VIP and Foxp3 were found at the implantation sites of NOD vs. control mice (Roca et al., 2009). Taken together previous and present results in the pre-diabetic NOD pregnancy model, it is tempting to speculate that the regulatory effect of VIP on macrophages from NOD mice could provide a suitable microenvironment for local Treg induction.

The transient switch from 'inflammatory' to 'regulatory' phenotype in peritoneal macrophages from NOD mice during pregnancy, as well as the promoting effects of VIP towards this macrophage phenotype by reducing the residual nitrite response of pregnant NOD mouse macrophages are in line with the central role of macrophages in maternal–placental dialogue. These results also support that VIP-mediated mechanisms may operate to facilitate macrophage function in the early establishment of this dialogue. The possibility that mechanisms involving macrophage plasticity changes have a role in diabetic gestation and its common gestational complications is being currently explored.

5. Conclusions

Consistent with the central role of macrophages in the maternal–placental dialogue, a functional macrophage phenotype switch occurs at early gestation in pre-diabetic NOD mice when faced to inflammatory and apoptotic cell stimuli. VIP further promotes this macrophage phenotype in NOD mice.

Competing interest

The authors declare that they have no competing interests.

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