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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