Acetylcholine contributes to control the physiological inflammatory response during the peri-implantation period

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Abstract

Background: Maternal antigen-presenting cells attracted to the pregnant uterus interact with trophoblast cells and modulate their functional profile to favour immunosuppressant responses. Non-neuronal cholinergic system is expressed in human cytotrophoblast cells and in immune cells with homeostatic regulatory functions.

Aim: The aim of this work was to evaluate whether non-neuronal acetylcholine conditions maternal monocyte and DC migration and activation profiles.

Methods: We used an in vitro model resembling maternal–placental interface represented by the co-culture of human trophoblast cells (Swan-71 cell line) and monocytes or DC.

Results: When cytotrophoblast cells were treated with neostigmine (Neo) to concentrate endogenous acetylcholine levels, monocyte migration was increased. In parallel, high levels of IL-10 and decreased levels of TNF-α were observed upon interaction of maternal monocytes with trophoblast cells. This effect was synergized by Neo and was prevented by atropine, a muscarinic acetylcholine receptor antagonist. Similarly, trophoblast cells increased the migration of DC independently of Neo treatment; however, enhanced IL-10 and MCP-1 synthesis in trophoblast–DC co-cultures with no changes in TNF-α and IL-6 was observed. In fact, there were no changes in HLA-DR, CD86 or CD83 expression. Finally, trophoblast cells treated with Neo increased the expression of two antigen-presenting cells attracting chemokines, MCP-1, MIP-1α and RANTES through muscarinic receptors, and it was prevented by atropine.

Conclusions: Our present results support a novel role of acetylcholine synthesized by trophoblast cells to modulate antigen-presenting cell migration and activation favouring an immunosuppressant profile that contributes to immune homeostasis maintenance at the maternal–foetal interface.

Keywords: dendritic cells, monocytes, non-neuronal acetylcholine, trophoblast cells.
Pregnancy involves different immunological stages with a pro-inflammatory or anti-inflammatory predominant profile depending on the stage of gestation analysed (Mor 2008, Dekel et al. 2010). A successful implantation occurs in a regulated pro-inflammatory microenvironment that allows tissue remodelling and angiogenesis at the maternal–placental interface. After implantation, the predominant pro-inflammatory microenvironment is modulated to an anti-inflammatory/tolerogenic profile required for foetal growth (Mor & Cardenas 2010).

The control of the pro/anti-inflammatory microenvironment implies several regulatory and tolerogenic circuits at the site of foetal antigen exposure that might operate all coordinated to sustain gestation (Aluvihare et al. 2005, Blois et al. 2007, Terness et al. 2007, Leber et al. 2010). In this sense, trophoblast cells coordinate the selective recruitment of maternal immune cells, such as antigen-presenting cells (Huang et al. 2008, Fraccaroli et al. 2009a,b, Dekel et al. 2010, Gomez-Lopez et al. 2010, Harris 2011).

Particularly, decidual macrophages and dendritic cells (DC) are recruited towards the foeto-maternal interface following a chemokine gradient where they are conditioned by trophoblast cells to express an anti-inflammatory profile contributing to immune homeostasis maintenance through immunosuppressant cytokine synthesis (Laskarin et al. 2007, Plaks et al. 2008, Dekel et al. 2010). In fact, human DC upon interaction with first trimester trophoblast cells was able to increase the frequency of CD4+CD25+Foxp3 cells with suppressor ability (Salamone et al. 2012). Regarding human maternal monocytes, they are recruited to the decidua and differentiated to macrophages with a predominant ‘alternative’ activation profile characterized by the clearance of apoptotic cells and the release of immunosuppressant mediators (Abrahams et al. 2004, Renaud & Graham 2008, Nagamatsu & Schust 2010). DC and macrophage functional plasticity is modulated by cytokines, chemokines and neurotransmitters through autocrine and paracrine circuits under the control of trophoblast cells (Abrahams et al. 2004, Mor et al. 2006, Fest et al. 2007, Renaud & Graham 2008, Nagamatsu & Schust 2010, Harris 2011, Pérez Leirós & Ramhorst 2013, Grasso et al. 2014).

Regarding the neurotransmitters, non-neuronal acetylcholine (ACh) is synthesized and released mainly by cytotrophoblast cells of human placenta where it regulates placental blood flow, facilitates amino acid transport and hormone release (Olubadewo & Rama Sastry 1978, Bhuiyan et al. 2006, Wessler & Kirkpatrick 2008, Grando et al. 2012, Wessler et al. 2012).

Previous data have shown that macrophages express choline acetyltransferase (ChAT) and synthesize ACh which inhibits the production of pro-inflammatory cytokines (Borovikova et al. 2000, Wessler & Kirkpatrick 2008). Likewise, human DC express M3, M4 and M5 muscarinic ACh receptors (mAChRs), ChAT and acetylcholinesterase (AChE), and both ACh and the acetylcholine mimetic drug carbachol modulate DC activation and cytokine production (Salamone et al. 2011). However, the role of ACh as a modulator of the maternal immune response during pregnancy is unknown.

Taking into account that trophoblasts condition the profile of the antigen-presenting cells and ACh can modulate their cytokine production, here, we investigated whether ACh produced by trophoblast cells conditions the migration and activation profile of antigen-presenting cells. For that purpose, we used an in vitro model resembling maternal–placental interface represented by the in vitro culture of human trophoblast cells (Swan-71 cell line) and maternal monocytes or DC.

**Materials and methods**

**Blood samples**

Blood samples were processed from healthy fertile women, defined as women who had two or more previous normal pregnancies without any miscarriage in their clinical records, were non-smokers and who were not under pharmacological treatment for at least 10 days before the day of sampling. Blood was obtained by puncture of the forearm vein, and it was drawn directly into heparin containing plastic tubes. Studies were approved by the ‘Academia Nacional de Medicina Review Board’ and Ethical Committee. All healthy donors provided written informed consent for sample collection and subsequent analysis.

**Monocyte isolation and differentiation to dendritic cells**

Peripheral blood mononuclear cells were isolated from individual subjects, and CD14+ cells were separated by positive selection with CD14+ micromagnetic beads (Miltenyi Biotec., Bergisch Gladbach, Germany). Cell population purity (95%) was checked by fluorescent-activated cell sorting (FACS) analysis. DC were obtained from monocytes (10⁶ cells mL⁻¹) in the presence of 20 ng mL⁻¹ IL-4 and 20 ng mL⁻¹ GM-CSF for 5 days as described (Salamone et al. 2011, 2012). Differentiated immature DC (99%) were checked by FACS analysis using anti-CD1a mAb.

**Co-cultures**

Trophoblast cells (Swan-71 cell line), derived by telomerase-mediated transformation of a 7-week cytotrophoblast isolate (Aplin et al. 2006, Straszewski-Chavez et al. 2007) are conditioned by trophoblast cells to express an anti-inflammatory profile depending on the stage of gestation.
et al. 2009), were kindly given by Dr Gil Mor (Yale University, New Haven, USA) and cultured in 24-well flat-bottom polystyrene plates in complete DMEM: F12/10% FCS (Life Technologies, Buenos Aires, Argentina) or with 2% FCS (DMEM 2%) to prepare conditioned media (CM). At 60% confluence, adherent trophoblast cells were cultured with DC or Mo (5 × 10^5 cells per well) as described previously (Salamone et al. 2012) (in the presence or absence of 20 μM Neo (acetylcholinesterase inhibitor) and 100 nM atropine (AT) or 10 nM carbachol (Carb) during 24 h. Trophoblast cell viability was not affected in the presence of 20 μM Neo. DC co-cultures were performed in the presence of IL-4 and GM-CSF to maintain DC differentiated profile.

Collection of trophoblast cell conditioned media

Swan 71 cells were cultured in 24-well flat-bottom polystyrene plates in complete DMEM 2% FCS overnight to obtain CM in the absence or presence of 20 μM neostigmine, 100 nM AT or 10 nM Carb. To assess that, adherent trophoblast cells were not removed during the CM collection procedure, RNA levels were determined, and they were below the detection limit. CM were collected and were all stored at –20 °C until use.

RT-PCR

Expression of ChAT, AChE, muscarinic receptors (mAChR) and chemokines (MCP-1, MIP-1α and RANTES) was determined by RT-PCR as previously described (Fraccaroli et al. 2009b, Salamone et al. 2011). Briefly, total RNA was isolated (Life Technologies, Grand Island, NY, USA), reverse transcription was performed (Clontech, Palo Alto, CA, USA), and cDNA fragments were amplified using 0.1 μM of each primer (sense and antisense) (Table 1) and 1 U Taq polymerase in a DNA Thermocycler (PerkinElmer/ Cetus, Boston, MA, USA). PCR products were fractioned on 2% ethidium bromide-stained agarose gels, visualized by transillumination and scanned. Densitometry was performed using IMAGEJ software (http://rsb.info.nih.gov/ij/download.html), and results were expressed as arbitrary units normalized to GAPDH expression.

Flow cytometry analysis

Cells were stained with the following mAbs: FITC- or PE-conjugated mAbs directed to CD1a, CD14, CD83, CD86, HLA-DR, TNF-α and IL-10. Ten thousand events were acquired in a FACS Calibur® cytometer, and results were analysed using WinMDI 2.9 software (http://facs.scripps.edu/software.html).

| Table 1 Primer sequences and annealing temperature (M1–M5 primers were previously published by Kylie J. Mansfield, British Journal of Pharmacology (2005) 144, 1089–1099) |
|---------------------------------------------|
| Primer sequences | Annealing T (°C) |
|------------------|------------------|
| AChE Sense 5′-AATTTTGCCCGCACAGGGG-3′ | 55 |
| AChE Antisense 5′-GCTCGGTGAGGTTG-3′ | |
| ChAT Sense 5′-GGAGATGTTCTGCTGATG-3′ | 56 |
| ChAT Antisense 5′-GGAGGTTAAGCCTAGGTGCA-3′ | |
| M1 Sense 5′-GCTCCCATTATACTGAGCG-3′ | 56 |
| M1 Antisense 5′-CAACGCAGGCGGAGAAATG-3′ | |
| M2 Sense 5′-GATGGGCTTGAACAGCAACA-3′ | 56 |
| M2 Antisense 5′-GCTGCTTATCTATCACACTAT-3′ | |
| M3 Sense 5′-CAGGAGCAGATGGGAGGAAAC-3′ | 56 |
| M3 Antisense 5′-AGGTAAGATGCGGTGCTC-3′ | |
| M4 Sense 5′-TCCAAATGAGGTCCGATAGCAG-3′ | 56 |
| M4 Antisense 5′-AGGAGCAGGCGGAGGAAATG-3′ | |
| M5 Sense 5′-GGACTATAGTTCCGATGTTG-3′ | 56 |
| M5 Antisense 5′-GGGACTATAGTTCCGATGTTG-3′ | |
| MCP-1 Sense 5′-CAGCGACACTGTCACATGG-3′ | 64 |
| MCP-1 Antisense 5′-GACTGATGGTGTTCAGGTCG-3′ | |
| MIP-1α Sense 5′-CTTACAGCTTCAGAAGAC-3′ | 62 |
| MIP-1α Antisense 5′-TGAGAGGTTCAAGA-3′ | |
| RANTES Sense 5′-TGCTGCTTTGCTCTACATT-3′ | 64 |
| RANTES Antisense 5′-AACGACGTCTGCTGTTG-3′ | |
| GAPDH Sense 5′-TACACAGACTAAAGGAAGG-3′ | 62 |
| GAPDH Antisense 5′-TTACAGCTTCAGAAGC-3′ | |

Measurement of cytokine production

DC were cultured or not with trophoblast cells in the presence of 20 μM Neo and 100 nM AT for 24 h at 37 °C. As an additional control, trophoblast cell secretion was quantified under basal conditions for each mediator. Then, culture supernatants were collected, centrifuged and analysed for the presence of TNF-α, IL-6, IL-10 and MCP-1 by ELISA (e-Bioscience, Los Angeles, CA, USA).

Migration assays

Migration assays were performed in 24-transwell plates across 5-μm polycarbonate membranes (Costar, Corning Incorporated, NY, USA). DC (2 × 10^5 cells) were re suspended in DMEM containing 2% FCS and placed on the upper chamber. The lower chamber contained 600 μL of DMEM 2% FCS supplemented with 20 ng mL⁻¹ IL-4 and 20 ng mL⁻¹ GM-CSF, or CM in the presence or not of Neo, which were
equally supplemented with IL-4 and GM-CSF. After incubation for 4 h at 37 °C, cells in the lower chamber were recovered and counted with a FACS Calibur. Migration of monocytes was similar to DC except that it was performed for 2 h and each well contained 600 μL of DMEM 2% FCS, CM in the presence or not of Neo and atropine.

**Western blotting**

Trophoblast cells (2 x 10⁶) were cultured for 24 h at 37 °C, washed with PBS supplemented with proteases inhibitors and pellets immediately frozen in dry ice. Samples were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Sigma-Aldrich, St. Louis, MO, USA) and blotted with the indicated antibodies overnight at 4 °C and specific bands revealed with ECL (Amersham Biosciences, Wauwatosa, WI, USA) using mouse monoclonal IgG anti-ChAT (Chemicon International, ON, Canada), goat polyclonal IgG anti-AChE, washed and incubated with FITC-conjugated secondary polyclonal antibodies (Sigma-Aldrich). The breast cancer cell line MCF-7 and DC were employed as positive controls for AChE and ChAT expression.

**Fluorescence microscopy**

Trophoblast cells (5 x 10⁶) were grown over glass slides and cultured for 24 h at 37 °C. Cells were washed with PBS, fixed with methanol and permeabilized using PBS-1% BSA-0.5% saponin buffer during 15 min. Slides were incubated overnight at 4 °C with mouse IgG mAb anti-ChAT or goat polyclonal IgG anti-AChE, washed and incubated with FITC-conjugated secondary antibodies anti-mouse or anti-goat IgG for 2 h. DAPI staining (Cell Signaling, Danvers, MA, USA) was performed for 10 min in darkness, and microphotographs were acquired using a IX71 Olympus inverted fluorescence microscope (Olympus, Center Valley, PA, USA) and MICRO-MANAGER Software (Micro-Manager 1.4, San Francisco, CA, USA). Negative control was performed in the absence of anti-ChAT or anti-AChE Ab.

**Statistical analysis**

The significance of the results was analysed by Student’s t-test or Mann–Whitney test for nonparametric samples. 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 using the GRAPHPad PRISM4 software (GraphPad, San Diego, CA, USA).

**Results**

**ACh produced by trophoblast cells induces the migration of maternal monocytes and dendritic cells**

Taking into account, the relevance of DC and macrophages in the generation of the matero-placental interface and that they are able to respond to ACh stimulation, we evaluated the ability of first-trimester trophoblast cell CM to modulate maternal monocytes and DC migration and the effect of acetylcholine. For that purpose, migration assays were performed using CD14+ cells or DC in the presence of CM from trophoblast cells cultured with or without neostigmine (Neo), an acetylcholinesterase inhibitor that increases endogenous ACh levels, or atropine, a competitive inhibitor of muscarinic ACh receptors. As shown in Figure 1a, CM of first-trimester trophoblast cells induced a higher monocyte migration rate than DMEM medium alone. On the other hand, when trophoblast cells were grown in the presence of 20 μM Neo, there was an increased monocyte migration and the effect was blocked by atropine.

In migration assays with DC, we observed a marked increase of DC migration when they were exposed to trophoblast cell CM compared with DMEM 2% FCS alone (Fig. 1b). The treatment of

**Reagents and antibodies**

Endotoxin-free reagents and plastic materials were used in all experiments. RPMI-1640 and PBS were purchased from HyClone Laboratories (Logan, UT, USA). DMEM was from Life Technologies, Buenos Aires, Argentina. Foetal calf serum (FCS) and penicillin/streptomycin were purchased from Invitrogen Life Technologies (Grand Island, NY, USA). Twenty-four-well flat-bottom polystyrene plates, recombinant human interleukin-4 (IL-4), fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-CD1a, CD14, CD83, CD86, HLA-DR, IL-10, TNF-α and control isotype-matched Abs were from BD Pharmingen (San Diego, CA, USA). Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), atropine and carbachol were from Sigma-Aldrich, and neostigmine was from Phadapharma Laboratories (Buenos Aires, Argentina). Anti-ChAT antibody (MAB5350) was from Chemicon International and anti-AChE antibody (N-19) b (sc-6431) was from Santa Cruz Biotechnology. Secondary polyclonal IgG labelled with HRP was from Sigma-Aldrich and ECL detection kit from Amersham Biosciences.
trophoblast cells with Neo could not further increase DC migration compared with trophoblast cells alone, which produced enough chemokine levels for maximum migration capacity of DC. Atropine (100 nM) had no effect on DC migration induced by trophoblast (Tb) cell CM obtained in the presence or absence of 20 μM Neo and supplemented with 20 ng mL−1 IL-4 and 20 ng mL−1 GM-CSF as indicated in Materials and methods. Cells recovered from the lower chamber were counted with a FACS Calibur. Basal migration values were obtained with DMEM 2% FCS (Media). Results shown are x ± SEM of three independent experiments with different DC samples. **P < 0.01 Mann–Whitney.

**Figure 1** ACh produced by trophoblast cells induces the migration of maternal monocytes and dendritic cells. (a) Mo (2 × 10⁵ cells) were placed on the upper 5-μm transwell chamber, and the lower chamber contained conditioned media (CM) from trophoblast (Tb) cells in the presence or absence of 20 μM Neo and 100 nM atropine. Migration was allowed for 2 h at 37 °C, and basal migration was assessed with DMEM 2% FCS (Media). Cells recovered from the lower chamber were counted with a FACS Calibur. Values shown are x ± SEM of different monocyte samples. *P < 0.05; **P < 0.01 Mann–Whitney. (b) Migration of DC (2 × 10⁵ cells) was performed for 4 h at 37 °C in 5-μm transwell chambers with trophoblast (Tb) cell CM obtained in the presence or absence of 20 μM Neo and supplemented with 20 ng mL−1 IL-4 and 20 ng mL−1 GM-CSF as indicated in Materials and methods. Cells recovered from the lower chamber were counted with a FACS Calibur. Basal migration values were obtained with DMEM 2% FCS (Media). Results shown are x ± SEM of three independent experiments with different DC samples. **P < 0.01 Mann–Whitney.

**Figure 2** ACh modulates the cytokine production from monocyte after the interaction with trophoblast cells. Monocytes were co-cultured with trophoblast cells (Mo + Tb) or not (Mo) in the presence or absence of 20 μM Neo and 100 nM AT for 24 h at 37 °C. Cells were analysed for TNF-α and IL-10 synthesis by FACS. Results are expressed as the percentage of CD14+TNF-α+ and CD14+IL-10+ cells and are representative of at least four different monocyte samples run in duplicates. *P < 0.05; **P < 0.01 Mann–Whitney. Dot plots representative of four experiments with different monocyte samples are also shown.

ACh modulates the cytokine production from monocyte after the interaction with trophoblast cells

We next investigated whether acetylcholine modifies the activation profile of monocytes attracted towards trophoblast cells (Thaxton & Sharma 2010). Maternal monocytes were co-cultured with trophoblast cells in

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the absence or presence of 20 μM Neo and atropine, and after 24 h, IL-10 production and TNF-α production were quantified by FACS. Figure 2 shows increased IL-10 production while decreased TNF-α in CD14+ cells after interaction with trophoblast cells, and this effect was potentiated in the presence of Neo. The effect of Neo was prevented by atropine.

**ACh conditions DC profile during their interaction with trophoblast cells**

As the physiological inflammatory response during the implantation should be shifted towards a tolerogenic one, we investigated ACh ability to condition maternal DC to a tolerogenic profile. Then, maternal DC were co-cultured with trophoblast cells in the presence of Neo for 24 h, and then, cytokine release, activation and maturation markers were quantified. As shown in Figure 3a trophoblast–DC interaction increased the levels of IL-10 and MCP-1 production. In parallel, Neo increased the levels of IL-10 and MCP-1 through mAChR in these co-cultures but did not modulate IL-6 or TNF-α production. In fact, the expression of HLA-DR, CD86 and CD83, markers of DC activation and maturation, showed no changes when DC were co-cultured with trophoblast cells in the presence or absence of Neo (Fig. 3b), suggesting that DC in the presence of Neo might be in an immature state producing IL-10.

**Trophoblast cells increase chemokine expression through ACh stimulation**

Taking into account that ACh produced by trophoblast cells increase the migration of monocytes and DC, we analysed the expression of monocyte and DC chemoattractant proteins, MCP-1, MIP-1α and RANTES and its modulation by acetylcholine. Trophoblast cells treated with 20 μM neostigmine increased the expression of the three chemokines at the same extent than elicited by the muscarinic ACh receptor agonist carbachol (10 nM), and the effect was inhibited by the, competitive inhibitor of muscarinic ACh receptors, atropine (Fig. 4a).

Finally, to confirm the endogenous ACh production, we explored ChAT and AChE expression in the Swan-71 cells by RT-PCR, Western blot and immunofluorescence microscopy. As shown in Figure 4b, both enzymes are expressed in cytrophoblast cells at mRNA and protein level and they were both localized in the cytoplasm as revealed by immunofluorescence. In addition, muscarinic acetylcholine receptor expression was assessed in Swan-71 cells. Figure 4c shows the expression of M1, M2, M3 and M4 subtypes of mAChRs, with a predominant M4 subtype expression.

The present results suggest that endogenous ACh production by trophoblast cells induces monocyte and DC migration increasing MCP-1, MIP-1α and RANTES expression.

**Discussion**

Successful embryo implantation occurs followed by a local physiological and sterile inflammatory response, subsequently redirected towards a tolerogenic predominant profile. Consistently, a deregulated persistent inflammatory response during early placentation has been associated with pregnancy complications such as spontaneous recurrent abortion and pre-eclampsia (Girardi et al. 2006, Dekel et al. 2010, Kwak-Kim et al. 2010, Redman & Sargent 2010). By means of co-cultures of a human first-trimester trophoblast cell line with monocytes or DC from fertile women, here, we present evidence to support that non-neuronal ACh from trophoblast cells can modulate the migration and condition the activation of CD14+ cells and DC profile. These conclusions are based on two main observations. First, through endogenous ACh production, trophoblast cells modulate monocyte and DC migration and particularly in monocytes, it is prevented by atropine. Besides, human trophoblast cell line Swan-71 increased the expression of antigen-presenting cell attracting chemokines (MCP-1 and MIP-1α) and RANTES which can also interact with CCR5 as MIP-1α. Second, the interaction of trophoblast cells with antigen-presenting cells in vitro conditions their activation profile. DC displayed an increase in the synthesis of anti-inflammatory IL-10 and the chemokine MCP-1 by endogenous ACh with no changes in TNF-α and IL-6 in trophoblast–dendritic cell co-cultures. In parallel, the contact with trophoblast cells and neostigmine, CD14+ cells modulate their marker profile to a predominant immunosuppressant phenotype, an effect that is prevented by atropine.

Antigen-presenting cells secrete angiogenic factors that induce vascular growth in receptive deciduas and shape the cytokine profile at the materno-placental interface (Dominguez et al. 2005, Dekel et al. 2010). They have to migrate to the interface, and consistently, several factors synthesized by trophoblast cells were proposed to increase DC, monocyte and macrophage migration as well as their differentiation to an immunosuppressant predominant response, the immune profile characteristic of midgestation (Fest et al. 2007, Huang et al. 2008, Fraccaroli et al. 2009a,b, Gomez-Lopez et al. 2010, Salamone et al. 2012, Grasso et al. 2014). Interestingly, non-neuronal ACh released by placental trophoblast cells varies with gestational age with a maximum at 20–22 weeks in humans and decreases thereafter (Sastry & Janson 1997, Tayebati et al. 1997,
1998, Bhuiyan et al. 2006), coinciding with the pattern of immunosuppressant predominant microenvironment at the maternal–placental interface (Mor & Cardenas 2010). Our results are in line with this observation, showing that acetylcholine released by cytotrophoblast cells not only facilitates monocyte migration but it also modulates monocyte/macrophage functional phenotype to favour an immunosuppressant milieu essential for homeostasis maintenance. This promoting effect of non-neuronal cholinergic system on cell migration has

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Figure 3 ACh conditions DC profile during their interaction with trophoblast cells. DC cultures or DC-Tb co-cultures were carried out alone or in the presence of 20 μM neostigmine (Neo) or with 20 μM Neo and 100 nM AT (Neo + AT) as described in Materials and methods and supernatants collected for (a) IL-10, TNF-α, MCP-1 or and IL-6 determination by ELISA. Values represent x ± SEM of at least three experiments. *P < 0.05; **P < 0.01 Mann–Whitney. (b) DC cultured with 20 μM Neo (DC + Neo) or co-cultured with trophoblast cells in the presence or not of Neo (DC + Tb) and (DC + Tb + Neo) were analysed by FACS for the expression of HLA-DR, CD86 and CD83. The values represent the MFI and are expressed as x ± SEM. One representative of three others run similarly.
been previously shown in other cell types such as keratinocytes and granulocytes (Wessler & Kirkpatrick 2008). Our results indicate that the treatment of trophoblast cells with Neo could not further increase DC migration compared with trophoblast cells alone. Considering that chemokine expression levels did increase with neostigmine, this result strongly suggests that functional chemokine levels released by Tb cells were enough for maximum migration capacity of DC.

Here, we have identified monocyte and DC attracting chemokines MCP-1, MIP-1α and RANTES as key molecules which synthesis and release by trophoblast cells was synergized by non-neuronal acetylcholine. MCP-1 is a well-known chemotactant of monocytes and macrophages often released with pro-inflammatory cytokines that activate macrophages in an inflammatory activation profile (Mosser & Edwards 2008, Biswas et al. 2012, Sica & Mantovani 2012). As an
enhanced pro-inflammatory reaction would be deleterious for placentation and adequate foetal growth, it is conceivable that trophoblast endogenous acetylcholine had a dual role by inducing MCP-1 release to attract monocytes and dendritic cells, and in parallel, it would favour immunosuppressant cytokine production and phenotype expression on attracted cells, as derived from our present results.

The modulatory effect of acetylcholine on dendritic cell functional profile through muscarinic ACh receptors has been documented, and it appears strongly dependent on their maturation status (Liu et al. 2010, Salamone et al. 2011). In fact, cholinergic stimuli added to mature DC prevented HLA-DR expression and TNF-α production (Salamone et al. 2011). This observation supports the ability of trophoblast cells to condition or ‘educate’ DC to express a tolerogenic profile that contributes to immune homeostasis maintenance at the maternal–foetal compartment (Salamone et al. 2012). Our present results confirm the trophoblast–DC interaction as a permanent regulatory source for immunosuppression and provide new evidence on how endogenous acetylcholine modulates this interaction.

In line with a boosting effect of endogenous acetylcholine in the normal human maternal–placental interaction, microarray studies of term placenta from women treated with choline showed a decrease in the placental and circulating levels of the anti-angiogenic fms-like tyrosine kinase-1 (sFLT1), proposed as placental and circulating levels of the anti-angiogenic women treated with choline showed a decrease in the action, microarray studies of term placenta from this interaction.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

DP, EG and GC performed all the experiments on trophoblast cells and co-cultures, obtained and characterized blood monocytes, carried out RT-PCR, Western blotting, immunocytochemistry assay and migration of antigen-presenting cells; SG and GS differentiated and characterized DC from human blood samples, performed FACS analysis and ELISA; WS obtained blood samples and assessed clinical data; CPL, RR and GS designed the whole study, discussed the results and prepared the manuscript.

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