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A potential tolerogenic immune mechanism in a trophoblast cell line through the activation of chemokine-induced T cell death and regulatory T cell modulation

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BACKGROUND: Successful implantation is followed by a local pro-inflammatory and Th1 response, subsequently controlled by Th2. Regulated upon activation, normal T cell expressed and secreted (RANTES) promotes a Th1 response and is implicated as a physiologic tolerogenic factor; therefore, we studied its potential role in the trophoblast–maternal leukocyte dialog.

METHODS: We performed co-cultures of immortalized trophoblast cell line (Swan 71) and peripheral blood mononuclear cells (PBMCs) from fertile women ($n = 23$) or with recurrent spontaneous abortions ($n = 18$, RSA). After 24 and 48 h, supernatant and cells were analyzed by enzyme-linked immunosorbent assay, fluorescence-activated cell sorting, Western blot and apoptosis assay. To investigate the physiological effects at peripheral level, we co-cultured maternal and paternal PBMCs with conditioned media from Swan cells and progesterone.

RESULTS: Following interaction of maternal PBMCs and trophoblast cells, RANTES production increased ($P < 0.05$) and was accompanied by low levels of interferon γ, interleukin-12 p70 and high levels of tumor necrosis factor-α, nitrites and leukemia-inhibitory factor. RANTES production resulted in elevated apoptosis of potentially deleterious maternal CD3+ lymphocytes, accompanied by a decrease in the proliferative maternal response. During fetal–maternal dialog, the anti-RANTES antibody significantly reduced the frequency of CD4+CD25+Foxp3+ cells ($P < 0.05$) and was associated with trophoblast cell survival. However, co-cultures of Swan cells and RSA-PBMCs displayed a differential RANTES kinetics, lower levels of regulatory T cells (Tregs) and CD3+annexin-V+cells, accompanied by higher levels of apoptotic trophoblast cells.

CONCLUSIONS: RANTES promotes an adequate pro-implantatory microenvironment that influences trophoblast cell survival and modulates the balance of maternal Treg/T effector lymphocytes in favor of maternal tolerance.

Key words: chemokines / recurrent spontaneous miscarriages / tolerance and pregnancy / T cell

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Introduction

A successful pregnancy is the result of several complex interactions between the receptive uterus and the mature blastocyst under hormonal stimulation (Saito, 2000; Thellin et al., 2000). Nowadays, the most accepted hypothesis sustains that the ability of trophoblastic antigens to induce a natural and tolerogenic maternal response involves regulatory T cells, cytokines, chemokines and galectin-1 derived from the feto-placental tissue (Aluvihare et al., 2004; Blois et al., 2007; Terness et al., 2007). Recent data suggest that, during the early phase of pregnancy, a successful implantation occurs in a pro-inflammatory microenvironment and Th1-type response followed by a Th2 shift in order to control endocrine and immune interactions (Fest et al., 2000; Clark et al., 2002; Chaouat et al., 2004). For example, several cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 induce leukemia-inhibitory factor (LIF) expression in the stroma and epithelial cells and through their receptors provide paracrine signals to both embryonic tissues and uterine epithelium during implantation (Song and Lim, 2006). In addition, progesterone stimulates a Th2-type response, diminishes inflammatory cytokines and suppresses allogeneic response (potentially deleterious response) allowing fetal survival (Miayaura and Iwata, 2002; Beagley and Gockel, 2003).

Chemokines are another important component of this intricate network by regulating the normal T cell trafficking and the inflammatory process (Rossi and Zlotnik, 2000; Lutther et al., 2001; Moser and Loetscher, 2001). Particularly, CCL5 or regulated upon activation, normal T cell expressed and secreted (RANTES) is considered as a pro-inflammatory chemokine part of the Th1-type response which contributes to a tolerogenic response at immune-privileged sites in murine models and might function as an important modulator of alloantigen-specific T cell responses during normal pregnancy (Faunce and Stein-Streilen, 2001,2002; Hornung et al., 2001a,b). In this sense, RANTES is able to suppress the allogeneic response in a dose-dependent manner in fertile women, inducing apoptosis of alloreactive CD45R0+ leukocytes through modulation of Bcl-2 protein levels and a caspase-dependent and independent mechanism (Ramhorst et al., 2004). In addition, successful pregnancy is accompanied by an increase in RANTES serum levels whereas it was found diminished in patients with recurrent spontaneous abortions (RSA) (Ramhorst et al., 2004). At the local level, progesterone significantly increased intracellular RANTES expression in CD4+ and CD8+ endometrial T-infiltrated lymphocytes. Moreover, after treatment with recombinant RANTES and interaction with CCR5 (one of RANTES receptors), we observed a decrease in CXCR4-mRNA (a receptor chemokine associated with a Th2-response) that correlated with an increase in T-bet expression (the main transcription factor involved in Th1-response development) on Ishikawa cells, a human endocervical cell line (Ramhorst et al., 2006,2007).

Since implantation can be seen as a 'controlled inflammatory process' and pregnancy success depends on alloantigen recognition and the development of a Th1 response, we hypothesize that early RANTES production during the feto-maternal cross-talk could limit the initial Th1-effector response, contributing to tolerogenic maternal responses essential for a successful pregnancy. In the present work, we evaluated the role of RANTES in the trophoblast–maternal leukocyte microenvironment as a modulator of the maternal balance of T effector/T regulatory cells, and possible alterations in RANTES production or function implicated in recurrent miscarriage.

Materials and Methods

Patients

'RSA patients' were defined as women with a history of three or more consecutive pregnancy losses before Week 12 of gestation after excluding any infectious, endocrine or anatomic disease that might have caused the miscarriage. Criteria for exclusion of patients were: (i) occurrence of anti-phospholipid antibodies, (ii) hepatitis B or C infection, (iii) human immunodeficiency virus (HIV) infection and (iv) genetic causes.

'Control fertile women' were defined as women who had two or more previous normal pregnancies without any miscarriage. The Investigation and Ethics Committee at the Hospital de Clínicas José de San Martín has approved this study and all patients provided their written consent to participate in the study.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) from RSA patients ($n = 18$), fertile women ($n = 23$) and the husbands of fertile women ($n = 5$) were isolated from heparinized peripheral blood by density gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). Cells were extensively washed and resuspended in RPMI 1640 (Life Technologies Grand Island, NY, USA) supplemented with 10% human serum, glutamine and penicillin–streptomycin.

Co-cultures

The trophoblast cell line (Swan 71 cell line, derived by telomerase-mediated transformation of a 7 week cytotrophoblast isolate described by Straswski-Chavez) (Aplin et al., 2006) was cultured in 24 flat-bottom polystyrene plates (Becton Dickinson, Franklin Lakes, NJ, USA) in complete Dulbecco's modified Eagle's medium (DMEM), 10% FCS (Gibco, Invitrogen, Argentina). At 60% confluence, PBMCs from RSA or fertile women were added (5×10^5 cells/well). At different time periods, the recovered cells, including those in suspension or adherent cells after trypsinization treatment (1%, Gibco, Invitrogen), were used for flow cytometry, apoptosis assays or Western blot analysis. To test RANTES specificity on apoptosis induction of CD3+ lymphocytes or trophoblast cell line, cultures were also performed in the presence of the anti-RANTES neutralizing monoclonal antibody (mAb) (R&D System, MN, USA).

Allogeneic stimulation

Maternal PBMCs ('responder cells') were resuspended in complete RPMI-1640 (1×10^5 cells/well). PBMCs from the husband were resuspended in complete RPMI-1640 (1×10^5 cells/well) and treated with mitomycin C (Sigma, St Louis, MO, USA) for 30 min at 37°C to inhibit paternal DNA synthesis by cross-linking DNA at guanine and adenine residues, disrupting base pairing ('stimulator cells'). The mixture of 'responder' and 'stimulator' cells was incubated in a U-shaped microtitre plate (Corning) in the presence of 10% human AB serum.

Co-cultures were performed also in the absence or presence of serum-free conditioned media (SFCM), progesterone (10^{-5} M, Sigma) and anti-RANTES Ab (10 µg/ml, R&D System) in several combinations. After 72 h, cells were pulsed with 1 µCi/well of methyl-^{3}H-thymidine [^{3}H]TdR (NEN, Boston, MA, USA) during the last 18 h of cell culture, and then harvested on glass fiber filters using a Packard Filtermate cell harvester (Packard Instruments, LaGrange, IL, USA). Incorporated

radioactivity was measured in a liquid scintillation β-counter (Packard Instruments). Since the thymidine incorporation by paternal PBMCs is prevented, the obtained counts per minute (cpm) reflects only maternal T lymphocyte proliferation in response to paternal antigens.

Tests were conducted in triplicate wells and results were expressed as mean cpm $\pm$ SEM of *n* experiments, as indicated.

Cytokine quantification

Interferon (IFN)γ, TNFα and IL-12 p70 were assayed by enzyme-linked immunosorbent assay (ELISA) in supernatant collected from the co-cultures performed in the presence of maternal PBMCs at different time periods. ELISA test was performed according to the manufacturer's instructions (Endogen and Becton Dickinson) and results were expressed as picogram per milliliter.

Nitrites determination

Nitrite levels were determined in supernatants obtained as described above for cytokine measurements using the Griess method with *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDA) and sulfanilamide (Ding *et al.*, 1988). Results were expressed as micromole nitrites/liter during the different time periods mentioned (0, 24 and 48 h).

Western blot assays

Cells recovered from co-culture were analyzed by Western blot for LIF and Foxp3 protein expression. Cells were extensively washed with phosphate-buffered saline (PBS), then cell pellet was mixed gently with 1 ml ice-cold lysis buffer (PBS containing 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 142.5 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.2) with freshly added protease inhibitor cocktail (0.2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 0.7 μg/ml pepstatin and 1 μg/ml leupeptin) and incubated for 1 h on ice. Samples were finally centrifuged at 12 000*g* for 20 min at 4°C and the supernatant fluids, representing the whole cell protein lysates, were stored at −70°C until use. Protein concentration was estimated by using the micro-BCA™ Protein Assay reagent kit (Pierce, Rockford, IL, USA). Equal amounts of protein were diluted in sample buffer and resolved on a separating polyacrylamide slab gel (15% for LIF and 10% for Foxp3). After electrophoresis, the separated proteins were transferred onto nitrocellulose membranes and probed with a 1:500 dilution of the anti-iNOS mAb (Becton Dickinson), 2 μg/ml anti-LIF monoclonal Ab (R&D Systems, Palo Alto, CA, USA) or 1:500 anti-Foxp3 Ab (eBioscience, San Diego, USA). Blots were then incubated with a 1:3000 dilution of a horse-radish peroxidase-conjugated anti-mouse immunoglobulin (Ig) G for LIF, or with a 1:3000 anti-goat IgG for Foxp3; and developed using an enhanced chemiluminescence detection kit (Amersham, Uppsala, Sweden). Equal loading and absence of protein degradation were checked by Ponceau S staining (Sigma). The immunoreactive protein bands were analyzed with a Fotodyne Image Analyzer® (Fotodyne, Inc., Hartland, WI, USA). Results were expressed as relative densitometric values using the Image Quant software, relative to β-actin protein.

Flow cytometric analysis

Intracellular staining for RANTES
To assess RANTES production by trophoblast cell line or T lymphocytes, co-cultures were incubated for 24 and 48 h at 37°C in a 5% CO_2 atmosphere and in the presence of Brefeldin A (10 μg/ml Sigma) for the last 4 h to promote the intracellular accumulation of secretory proteins. After washing, cells were fixed in 4% paraformaldehyde in PBS-2% FCS for 20 min at room temperature. Cells were then washed and permeabilized

with 0.5% (w/v) saponin (Sigma) in PBS for 30 min at room temperature. Permeabilized cells were washed and incubated for 30 min with anti-RANTES Ab. Cells were again washed, then incubated in the presence of anti-mouse IgG-fluorescein isothiocyanate (FITC) conjugated Abs in the presence of 0.5% saponin (Sigma). Finally, cells were washed with PBS-2% FCS to allow membrane closure.

Intracellular staining for Foxp3
The flow cytometry analysis was performed essentially as described (Arruvito *et al.*, 2007). In brief, only maternal PBMCs (1×10^6) suspensions were recovered after co-culture with Swan 71 cells and fixed in 1 ml PBS with 1% paraformaldehyde containing 0.05% Tween 20. After an overnight incubation at 4°C, cells were treated twice with 0.5 ml of RNase free DNase at 100 U/ml (Promega). Staining steps were performed for 1 h at room temperature. Cells were incubated with mouse anti-Foxp3 IgG mAb (clone 236A/E7 provided by L. Fainboim) and washed with PBS supplemented with 3% heat inactivated FCS, 0.5% Tween 20 and 0.05% azide. Foxp3 mAb binding was detected by using Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) and washed as described above. Cell surface staining was then performed using the mAb Cy Chrome anti-human CD4 and phycoerythrin (PE) anti-human CD25 (BD Pharmigen) for 20 min at room temperature followed by washing in PBS.

Ten thousand 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 Ab. Results for positive cells are expressed as a percentage of RANTES positive cells or inside the electronically gated CD4-positive cell population for T regulatory analysis.

Apoptosis assays

The frequency of apoptotic cells was assessed by double staining using PE-conjugated anti-human CD3 mAb (Becton Dickinson, San José, CA, USA) and an FITC-conjugated-annexin-V following manufacturer's recommendations (Immunotech, Marseille, France). Results were analyzed using the WinMDI software® and positive cells are expressed as a percentage of double-positive CD3 and annexin-V population and the quadrant was set using irrelevant isotype-specific Abs.

RT–PCR for RANTES and its receptors

Determination of RANTES and its receptors (CCR5, CCR3 and CCR1) was performed by RT–PCR on the trophoblast cell line. Swan 71 extract using the primers previously described (Douglas *et al.*, 2000). Briefly, total RNA was isolated using the Trizol reagent (Life Technologies). Complementary DNAs (cDNAs) were generated using a commercial kit (Clontech, Palo Alto, CA, USA) and stored at −20°C for batched analyses. cDNA samples were amplified with specific PCR primers and *Taq* polymerase in a DNA Thermocycler (PerkinElmer/Cetus, Boston, MA, USA). PCR products were electrophoresed through a 2% ethidium bromide-stained agarose gel and visualized by transillumination. All results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. As a positive control, we used PBMCs from a normal healthy donor polyclonally stimulated with phytohemaglutinin for 36 h.

Statistical analysis

The significance of the results was analyzed by Student's *t*-test, and Mann–Whitney test for comparison of non-parametric samples, using the GraphPad Prism4 software (GraphPad, San Diego, CA, USA). A value of $P < 0.05$ was considered significant.

Results

RANTES is expressed by trophoblast cells and significantly increases during the dialog with maternal leukocytes

Hormonal and embryonic signals specifically modulate endometrial chemokines during the peri-implantation phase (Caballero-Campo et al., 2002). Then, to investigate RANTES production by trophoblast cells, we used the first trimester trophoblast cell line Swan 71 treated with Brefeldin A to inhibit secretion of intracellular proteins followed

by labeling with anti-RANTES mAb. Flow cytometry analysis revealed that $30 \pm 2.5\%$ of Swan 71 cells was positive for RANTES staining under basal conditions (Fig. 1A). This result was confirmed by RT–PCR, reflecting that trophoblastic cell line expressed RANTES mRNA (Fig. 1B).

In order to evaluate RANTES production during the feto-maternal dialog, we performed co-cultures of trophoblast cells and PBMCs isolated from fertile women or RSA patients. As shown in Fig. 1C, trophoblast cells stimulated by the presence of PBMCs from fertile women displayed a 14% increase in the frequency of RANTES producer cells after 24 h, decreasing to the observed basal levels after 48 h of
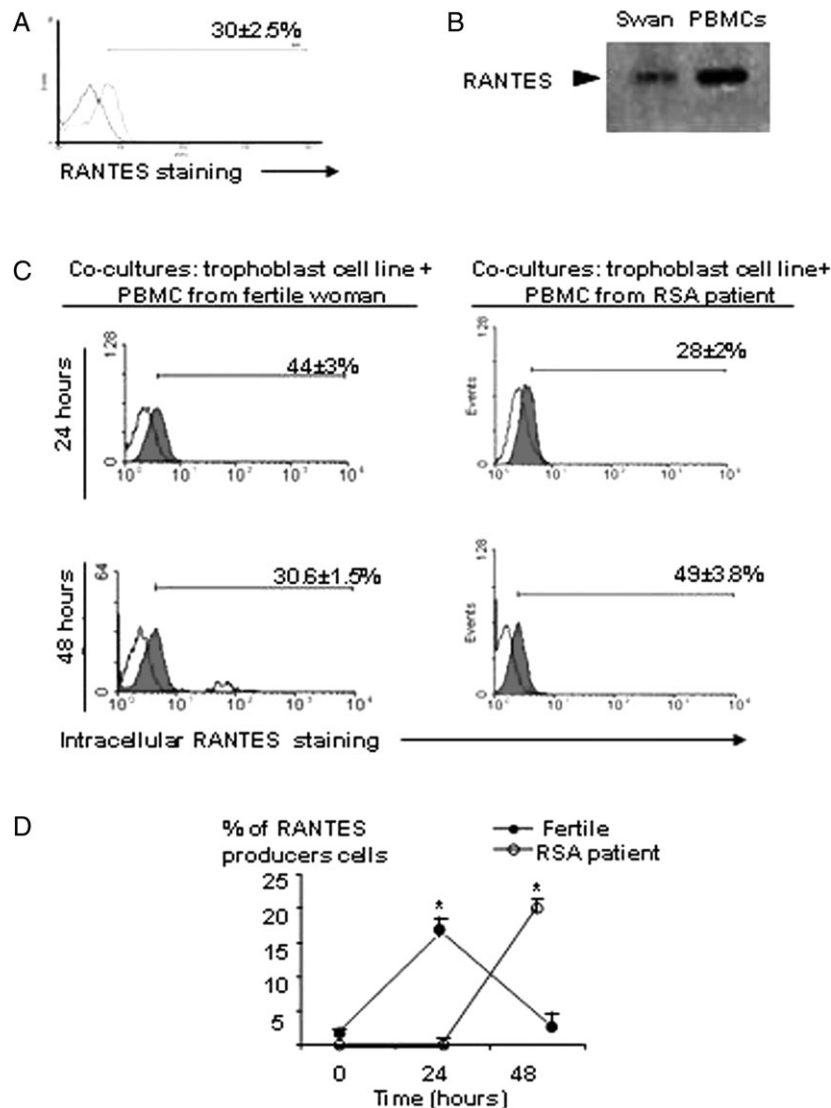

Figure 1 Trophoblast cell line expresses RANTES and is modulated by maternal PBMCs stimulation.

(**A**) Trophoblast cell line, under basal conditions, were permeabilized and intracellularly stained with anti-RANTES mAb (Swan 71 cells: $30 \pm 2.5\%$ positive for RANTES staining). (**B**) Swan 71 cells were recovered and RANTES expression was investigated by RT–PCR. (**C**) Swan 71 cell line at 60% confluence in a 24-well flat-bottom plate was cultured in the presence of PBMCs from fertile or RSA women. At 0, 24 and 48 h of co-culture, Swan 71 cells were recovered and stained for intracellular RANTES detection. Figures show representative cytometric profiles and data are expressed as percentage of intracellular-RANTES positive cells. Results are representative of independent experiments using five different fertile and five RSA women. (**D**) Kinetics of RANTES production in co-cultures from RSA patient and fertile women. The points correspond to mean $\pm$ SEM. Percentage of RANTES producer cells minus the percentage obtained for the basal condition (time 0 h: $30 \pm 2.5\%$) ($*P < 0.05$, Student's t-test versus basal production).

co-culture. When trophoblast cells were incubated in the presence of RSA-PBMCs, we also observed an increase in the number of RANTES positive Swan 71 cells, but only after 48 h of culture. Figure 1D shows flow cytometry profiles of culture kinetics in the presence of maternal PBMCs from both groups (*P* < 0.05 Mann–Whitney test). These data suggest that even though RANTES levels reached in both culture conditions were similar, fertile and RSA women displayed differential time-production window kinetics, evidenced by a delay in RANTES production under RSA-PBMCs stimulation.

Trophoblast–maternal leukocyte cross-talk occurs in a pro-inflammatory microenvironment

In order to characterize the trophoblast–maternal leukocyte microenvironment as an approach to the feto-maternal dialog, we investigated pro-inflammatory markers potentially associated with successful implantation. Trophoblast cells and maternal PBMCs from RSA or fertile women were co-cultured and cytokine secretion quantified by ELISA. As depicted in Fig. 2A, TNFα and IL-12 p70 (functional subunit)

levels significantly increased in the trophoblast–maternal PBMC culture supernatant after 24 h (*P* < 0.05, Student's *t*-test for each cytokine). However, IFNγ secreted levels were significantly higher in trophoblast cell line incubated in the presence of RSA-PBMCs in comparison with those obtained from fertile women (*P* < 0.05, Mann–Whitney test). Moreover, similar increases of TNFα levels after 24 h were observed in supernatants from co-cultures in the presence of PBMCs from both groups of women. Since TNFα is a potent inducer of LIF expression, a pleiotrophic glycoprotein essential for implantation in endometrial stromal cells (Song and Lim, 2006), we investigated LIF expression in the trophoblast–maternal PBMCs dialog. As depicted in Fig. 2B, LIF expression, evaluated by Western blot, significantly increased in the mixture of trophoblast and PBMC cells after 48 h of co-culture for both groups under study (*P* < 0.05 Student's *t*-test). LIF protein expression at 0 h was similar to 24 h for both groups (data not shown). Figure 2B lower panel shows the semiquantification of the immunoreactive bands expressed in arbitrary units relative to β-actin protein expression.

In addition, quantification of nitrite accumulation, a pro-inflammatory marker with angiogenic effect, revealed similar
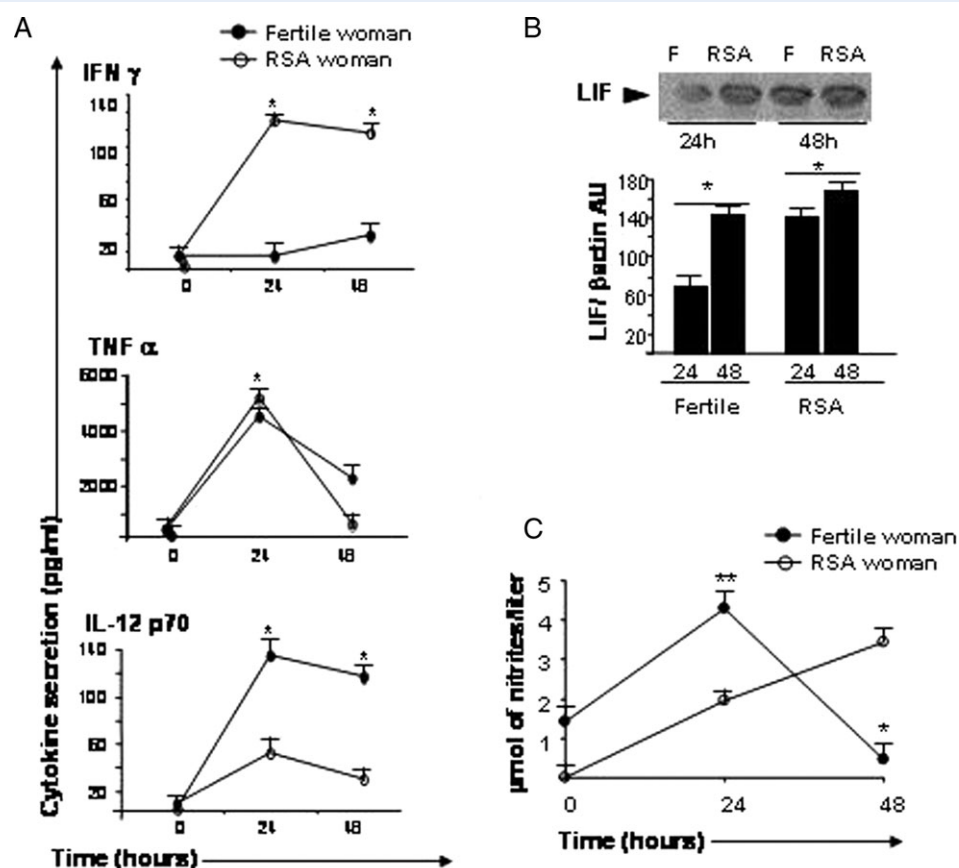
Figure 2 Modulation of pro-inflammatory markers in response to maternal leukocyte stimulation.

Swan 71 cell line at 60% confluence in a 24-well flat-bottom plate were cultured in the presence of PBMCs from fertile or RSA women. At 0, 24 and 48 h of co-culture, supernatants and cells were collected. (**A**) IFNγ, TNFα and IL-12 p70 were quantified in the supernatants by ELISA. Results are mean ± SD expressed as pictogram per milliliter (*P* < 0.05 versus time 0, Student's *t*-test). (**B**) Swan 71 cells and PBMCs were harvested and analyzed by Western blot for LIF expression. Immunoreactive protein is shown in the upper panel and the semiquantification expressed as arbitrary units relative to β-actin expression in lower panel (*P* < 0.05 Student's *t*-test). (**C**) Nitrite levels were determined in the supernatants using the Griess method. Results were expressed as micromole nitrites per liter during the mentioned time periods (*P* < 0.001 versus time 0, Student's *t*-test). Results are representative of five independent experiments using five different fertile and five RSA women.

kinetics to that observed for RANTES production, displaying a peak in fertile women at 24 h of culture ($P < 0.001$ Student's t-test, Fig. 2C).

These data suggest that in fertile women, the increase in RANTES production in a short time window is also accompanied with a micro-environment characterized by low levels of IFNγ and the increase of IL-12 p70, TNFα, LIF expression, as well as of nitrites production, reaching their maximal production at 24 h and decreasing following 48 h of maternal PBMC stimulation, except for LIF which could be induced by TNFα.

RANTES modulates maternal T regulatory cells during the trophoblast–maternal leukocyte cross-talk

Since human CD4+CD25+ regulatory T cells mediate feto-maternal tolerance and a subpopulation expresses CCR5 (one of RANTES receptors) with highly suppressive function (Kallikourdis et al., 2007), we studied Foxp3 modulation in maternal PBMCs after tropho-blast cell line interaction and the effect of RANTES on this dialog. PBMCs from fertile or RSA women were cultured with Swan 71 in the absence or presence of anti-RANTES neutralizing Ab, and Foxp3 expression was analyzed by Western blot. After 48 h of culture, Foxp3 expression significantly increased in fertile PBMC com-pared with 24 h, and with RSA-PBMCs at the same 48 h incubation time ($P < 0.05$, Mann–Whitney test). Interestingly, the anti-RANTES neutralizing Ab was able to reduce Foxp3 expression in RSA and fertile PBMCs (Fig. 3A). These data were confirmed by studying the modu-lation of CD4+CD25+Foxp3+ population by triple staining and fluorescence-activated cell sorter analysis. RSA patients displayed lower frequency of CD4+CD25+Foxp3+ cells in comparison with fertile women ($3.0 \pm 0.2\%$ versus $5.5 \pm 0.5\%$ $P < 0.05$, Mann–Whitney test), and also the anti-RANTES neutralizing Ab decreased these percentages ($1.0 \pm 0.2\%$ and $2.1 \pm 0.5\%$, respectively, $P < 0.05$ Mann–Whitney test) (Fig. 3B). These results suggest that RANTES might be implicated in the increase of CD4+CD25+ Foxp3+ population trophoblast–maternal leukocyte dialog, although other compensatory mechanisms could be involved.

RANTES induces apoptosis of maternal alloactivated T lymphocytes during the trophoblast–maternal leukocyte cross-talk

Since RANTES is produced by trophoblast cells and could induce apoptosis of alloreative leukocytes, we investigated maternal T cell or trophoblast cell susceptibility to RANTES-induced apoptosis. Thus, after the interaction of maternal PBMCs with Swan 71 cells, we recovered both populations and analyzed the frequency of annexin-V+ cells on T lymphocyte or trophoblast cells. The flow cyto-metry analysis revealed that PBMCs from fertile women displayed a significant increase in the frequency of CD3+ annexin-V+ cells in a time-dependent manner. However, the frequency of apoptotic T cells in RSA patients was significantly lower than in fertile women for each time point of culture. Figure 4A shows representative dot plot kinetics for double positive CD3 and annexin-V cells in both groups of women and Fig. 4B the percentages for each point studied.
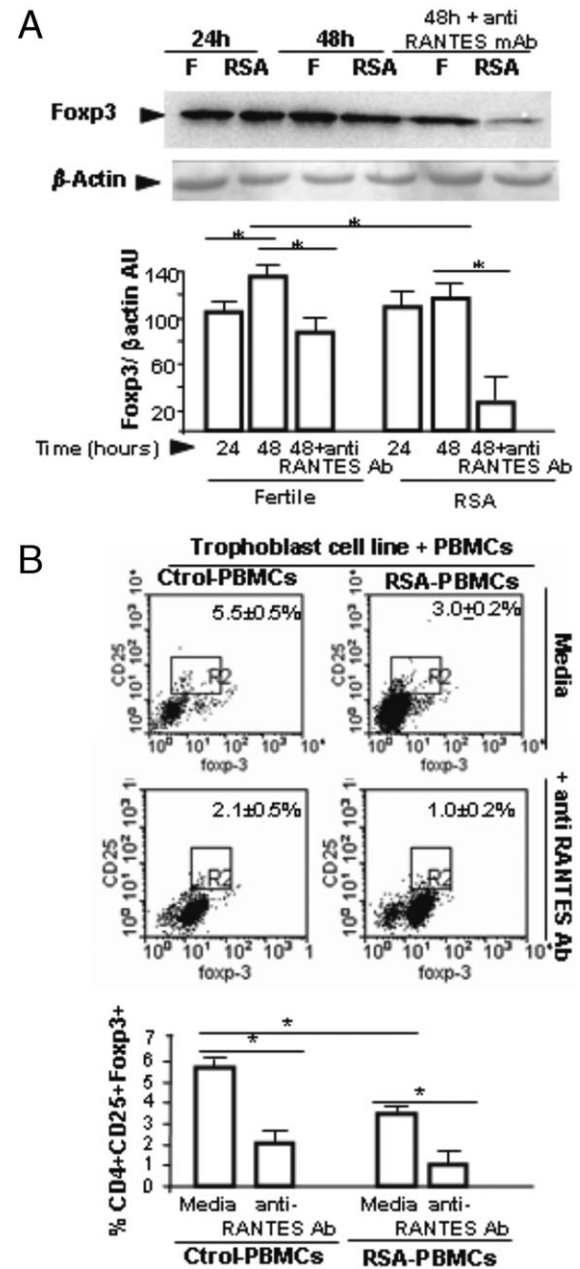

Figure 3 Foxp3 immunomodulation in the trophoblast cells and maternal leukocytes dialog.

(**A**) Swan 71 cell line was cultured with PBMCs from fertile or RSA women, in the absence or presence of anti-RANTES neutralizing Ab. At 24 and 48 h of co-culture, maternal PBMCs were recovered, harvested and analyzed by Western blot for Foxp3 expression. Representative immunoreactive bands are shown in the upper panel and the semi quantification expressed as arbitrary units relative to β-actin using three different RSA and three fertile women in the lower panel. Results are representative of independent experiments (*$P < 0.05$ Mann–Whitney test). (**B**) After 48 h of culture, suspension cells were recov-ered and triple stained for surface CD4+CD25+ and intranuclear Foxp3+ and analyzed by fluorescence-activated cell sorter. Figure shows representative dot plots and the frequency of the CD4+CD25+Foxp3+ population expressed as a percentage of CD4+ cells (*$P < 0.05$ Mann–Whitney test).
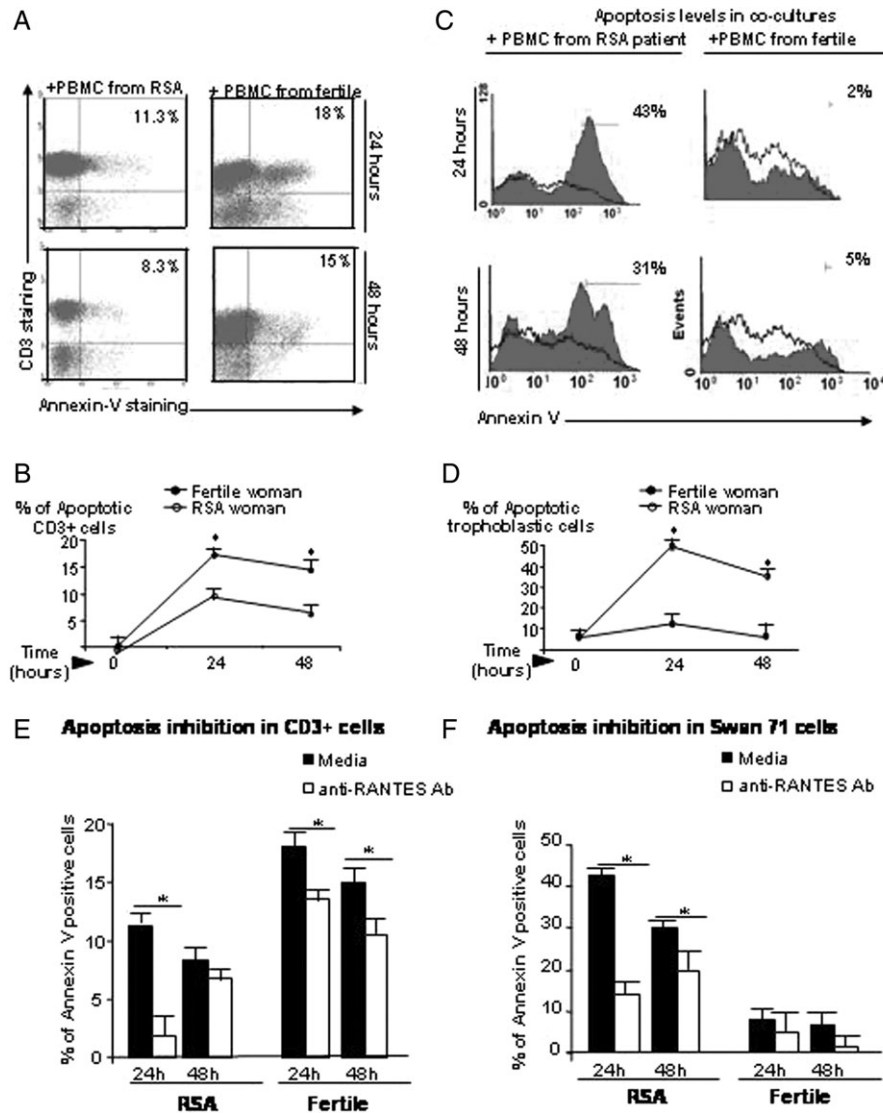
Figure 4 Apoptosis levels in maternal–trophoblast dialog.

Swan 71 cells were cultured in the presence of PBMCs from fertile or RSA women for the indicated periods and analyzed for apoptosis levels by annexin-V and CD3 staining. The analysis was performed inside the electronically gated CD3+ cells (**A**) or trophoblast cell line (**B**). Figure shows representative cytometric profiles and percentages represent positive annexin-V cells inside the T or trophoblast cell gate. Representative kinetics of the apoptosis levels in RSA or fertile-co-cultures (*$P <$ 0.05, Mann–Whitney test) are shown in (**C**) and (**D**). Apoptosis inhibition: co-cultures were performed in the absence or presence of the anti-RANTES neutralizing mAb for the indicated periods and analyzed for apoptosis reversion levels in CD3+ population (**E**) and trophoblast cell line (**F**). Percentages represent the frequency of annexin-V+ cells inside the electronically gated trophoblast or CD3+ cells (*$P <$ 0.05, Student's *t*-test). Results are representative of five RSA patients and five fertile women.

When the analysis was performed for trophoblast cells, we found low apoptosis levels after interaction with fertile PBMCs; however, there was a significant increase in trophoblast apoptotic cells after co-culture with RSA-PBMCs (Fig. 4C and D).

To determine the specificity of RANTES pro-apoptotic effect on maternal T or trophoblast cell line, co-cultures were performed in the presence of anti-RANTES Ab. The anti-RANTES neutralizing Ab was able to significantly decrease the percentage of apoptotic CD3+ cells in fertile co-culture (Fig. 4E) and decrease trophoblast cell death in RSA co-culture (Fig. 4F).

Trophoblast cell line does not express CCR5 and CCR1 RANTES receptors

Taking into account that Swan 71 cells express RANTES and can trigger apoptosis through CCR5 (one of the RANTES receptors) inter-action, we seek to determine their susceptibility to RANTES-induced apoptosis. Therefore, we investigated the expression of the three known RANTES receptors (CCR1, CCR3 and CCR5) in trophoblast cell line by RT–PCR. As described in Fig. 5A, Swan 71 cells only expressed CCR3 suggesting that, through the restriction of RANTES
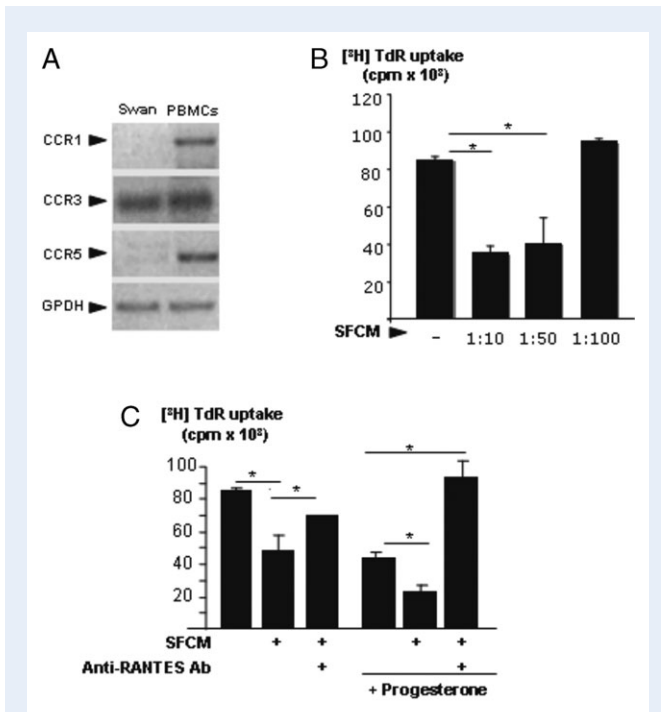
Figure 5 (**A**) Expression of RANTES receptors (CCR5, CCR3 and CCR1) in Swan 71 cells.

Results were normalized to GAPDH expression. As a positive control, we used PBMCs from a normal healthy donor polyclonally stimulated with phytohema-glutinin for 36 h. RANTES suppressor effect on maternal alloactivated leuko-cytes. Fertile PBMCs and mitomycin-treated stimulator paternal PBMCs were co-cultured (**B**) in the presence/absence of several dilutions of SFCM obtained from Swan 71 cell line, (**C**) in the presence/absence of SFCM (dilution 1:50), anti-RANTES neutralizing mAb and progesterone (10^{-5}M). After 72 h, methyl-^{3}H-thymidine ([^{3}H]TdR) was added for 18 h and uptake was determined using a β-scintillation counter. Results are expressed as mean cpm ± SD of triplicate determinations (*$P < 0.05$, Student's t-test) for one representative experiment of five different fertile couples.

receptors, trophoblast cells could be resistant to the apoptosis induced by RANTES.

RANTES secreted by trophoblast cells specifically suppresses maternal alloactivated T cell proliferation

To study the relevance of RANTES produced by trophoblast cells as a physiologic mechanism to suppress alloactivated maternal T lympho-cytes, we performed mixed lymphocyte reaction (MLR) with maternal and paternal PBMCs in the presence of SFCM obtained from the first trimester trophoblast Swan 71 cell line. After 72 h of culture, SFCM (at dilutions 1:50 and 1:10) was able to significantly suppress maternal alloresponse ($P < 0.05$ Student's t-test) (Fig. 5B). To study the contri-bution of RANTES to trophoblastic-induced immunosuppression, we also performed MLR in the absence or presence of anti-RANTES neu-tralizing Ab. The SFCM significantly suppressed alloactivated lympho-cytes and this effect was partially prevented by the anti-RANTES neutralizing Ab ($P < 0.05$ Student's t-test, Fig. 5C). Since progesterone is the main hormone at feto-maternal interface with suppressor effects, and in a previous work, we demonstrated that progesterone induced an

increase in RANTES production in endometrial CD4 and CD8 lympho-cytes, we investigated a potential interaction between RANTES and progesterone at a systemic level. We could observe an additive effect on the suppression of maternal response of SFCM and progesterone at a physiologic concentration. Moreover, the anti-RANTES Ab specifi-cally prevented the reduction in the lymphocyte-thymidine uptake (Fig. 5C). These results suggest that RANTES could be a natural mech-anism at the feto-maternal dialog able to specifically suppress the pro-liferation of the maternal allolymphocytes in response to paternal stimulation in the presence of high-progesterone doses.

Discussion

The appropriate generation of a pro-inflammatory response is thought to be a prerequisite for successful implantation (Rugeles and shearer, 2004; Abrahams et al., 2005). However, elevated leukocyte infiltration and inappropiate activation may be an underlying cause of pregnancy complications and failures, attributed to an exacerbated Th1 response (effector maternal response) responsible for tissue damage and fetal resorption (Fest et al., 2000; Aluvihare et al., 2004; Terness et al., 2007).

Results presented herein provide experimental evidence that RANTES may play an important role during early implantation in an adequate pro-inflammatory microenvironment by increasing regulat-ory T lymphocytes, inducing apoptosis of maternal alloactivated T cells, allowing trophoblast survival and promoting maternal tolerance to fetal antigens.

We have demonstrated that a first trimester trophoblast cell line constitutively secretes RANTES and its production increased within 24 h after maternal PBMC−trophoblast dialog, reflecting, like other CC chemokines, its ability to be expressed as an early gene under pro-inflammatory cytokine stimulation (Nelson and Krensky, 2001). In fact, increased RANTES production in this dialog is associated with pro-inflammatory cytokines like TNFα, low dose of IFNγ and IL-12 (necessary for uterine vascular modification), nitrites production (related to uterine quiescence and angiogenesis) and LIF expression: characterizing a pro-implantatory microenvironment (Ashkar et al., 2000; Paria et al., 2002; Abrahams et al., 2005).

This inflammatory context is a hallmark of a normal implantation and could be later autocontrolled by RANTES through the modulation of the T effector/regulatory T cell (Tregs) lymphocyte balance. First, increased RANTES levels result in elevated apoptosis of potentially deleterious CD3+ lymphocytes correlating with a significant decrease in the proliferative response. In accordance with these data, Mellado et al. reported that melanoma-inducible RANTES production, through the interaction with CCR5, activates an apoptotic pathway in tumor infiltrating lymphocytes, rendering tumor cells virtually invis-ible to the immune system (Mellado et al., 2001).

Trophoblast cell line did not express CCR5 making them resistant to apoptosis induced by RANTES, reflecting a potential mechanism by which RANTES could selectively induce apoptosis of alloreactive maternal lymphocytes. However, other mediators and apoptotic path-ways could be involved. These results are in agreement with previous data showing that cytotrophoblast cells did not express CCR1 and CCR5 (HIV-1 receptor for the M-tropic isolates). Hence by this mech-anism, there is no vertical HIV transmission between mother and fetus (Douglas et al., 2000) and this might also be implicated in the

resistance of trophoblast cells to the apoptosis induced by RANTES during trophoblast–maternal leukocyte dialog.

Second, with the anti-RANTES neutralizing Ab, we demonstrated that RANTES has the ability to modulate the frequency of T regulatory cells during the maternal PBMCs–trophoblast cell dialog, clearly reducing Foxp3 expression and the frequency of CD4+CD25+Foxp3+. In fact, Kallikourdis et al. recently demonstrated that a subpopulation of Tregs expresses CCR5 with high suppressor activity in a murine model (Kallikourdis et al., 2007); however, there is no evidence of them in human so far.

Under pathological conditions, RSA patients displayed an altered time window of RANTES production that could affect later events in the implantation phenomena. But the most interesting finding is the high frequency of apoptotic trophoblast cell line that follows the interaction with maternal RSA–PBMCs. This effect correlated with low levels of apoptotic maternal CD3+ lymphocytes, supporting that RSA patients might have an altered mechanism allowing the survival of T cells activated by fetal antigens that could compromise the success of pregnancy. In fact, RSA patients showed a significant decrease in Tregs after trophoblastic stimulation in comparison with fertile women, suggesting the inability of Tregs in RSA patients to expand during the preimplantatory phase. Previous reports by Arruvito et al. demonstrated that RSA patients have a decreased number of Tregs and lower capacity to suppress maternal response to paternal antigens (Arruvito et al., 2007). Consequently, reproductive failure might result from the combination of their lower frequency and suppression function.

Finally, high levels of progesterone and paternal alloantigens may both be important local regulatory factors during and after the process of implantation (Rugeles and shearer, 2004). Data presented here show that RANTES specifically suppresses alloactivated maternal T cells, even in the presence of progesterone. Previous results showed that progesterone induces RANTES production in CD4+ and CD8+ infiltrated-endometrial lymphocytes (Ramhorst et al., 2006). Therefore, the high levels of progesterone present during normal human pregnancy, particularly at the maternal–fetal interface, would be predicted to promote RANTES production to levels that may be required for the local induction of a tolerogenic immune response.

Although research over the past decade investigated the molecular mechanisms leading to immune tolerance, the definitive cellular and molecular mechanisms underlying the embryo–uterine interactions remain to be resolved. Results presented herein provide the experimental evidence that RANTES may play an important role during feto-maternal dialog allowing trophoblast cell survival and a maternal tolerogenic response.

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