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Immunomodulatory effects of chemokines during the early implantation window

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1. ABSTRACT

Successful implantation requires a functionally normal embryo at the blastocyst stage and a receptive endometrium as well as adequate communication between them throughout the implantation process. This cross-talk is highly regulated by a number of different kinds of molecules. Particularly, chemokines---small polypeptides that attract specific leukocyte subsets by binding to cell-surface receptors--- are also required to maintain immune-privileged sites as the feto-maternal interface. Chemokines expression involves an interdependent network with the absence of a single chemokine affecting the expression of multiple other chemokines, we have chosen to focus on just two representative examples: RANTES (regulated on normal T cell expressed and secreted) and MCP-1 (Monocyte chemo-attractant protein). Here, we present updated information on their expression levels and regulation on three different levels: 1) systemic effects on maternal allogeneic response; 2) local effects on endometrial cells; and 3) during an early stage of the feto-maternal dialogue. For each of the three levels, we analyzed data from both fertile women and patients having experienced recurrent spontaneous abortions as representative of physiological and pathological situations respectively.

2. IMMUNOMODULATORS OF THE FETO-MATERNAL CROSS-TALK

A successful pregnancy is the result of several complex interactions that take place between a receptive uterus and a mature blastocyst under hormonal stimulation (1, 2). The current accepted hypothesis argues that the ability of trophoblastic antigens to induce a natural and tolerogenic maternal response necessary to allow proper implantation and fetal development involves regulatory T cells (Tregs), cytokines, and chemokines as well as galectin-1 derived from the feto-placental tissue (3-5).

Recent data suggests that during the early phase of pregnancy a successful implantation occurs in a pro-inflammatory microenvironment and in the presence of a Th1-type response (the immune effector cellular response) followed by a shift to a tolerogenic response characterized by the production of Th2-type cytokines (associated with humoral responses) and suppressor cytokines produced by maternal Treg cells. In short, the initial inflammatory/Th1 responses allow the embryo to implant while the shift to a Th2/tolerogenic response controls endocrine and immune interactions that ensure successful implantation (6-8).

The pro-inflammatory cytokines produced by macrophages at the feto-placentary unit, such as IL-1, TNF-alpha, IL-6, and LIF (leukemia inhibitor factor), participate in processes that include endometrial cyclical development, endometrial trophoblast interaction, and endometrial tissue regeneration (9,10). Moreover, these pro-inflammatory cytokines are interconnected, maintaining an intricate network essential for implantation. For example, TNF-alpha and IL-1 induce LIF expression in the stroma while epithelial cells provide paracrine signals through their receptors to both embryonic tissues and uterine epithelium during implantation (11). In fact, the ability of cytotrophoblast to synthesize and secrete IL-1β correlates with its invasive capacity. IL-1β modulates trophoblast invasion by altering the ratio of matrix metalloproteinases to tissue inhibitors of metalloproteinases (TIMP) and by promoting angiogenesis by VEGF expression. It also contributes to uterine remodeling and the transformation of the spiral arteries that will support the oxygen demand of the developing fetus (10).

While a pro-inflammatory Th1 microenvironment may seem to represent the normal situation in implantation and early pregnancy, it is also clear that an excess of these cytokines may be deleterious (12,13). As regards, trophoblast-macrophage interactions contribute to the normal progression of gestation by modulating the Th1/Th2 switch and properly removing apoptotic bodies to prevent an inflammatory reaction that could result in the failure of the pregnancy (14). It has also been proposed that macrophages are "educated" by trophoblast cells to create their own survival environment by the release of cytokines and chemokines (15). Thus trophoblast invasion and uterine remodeling are the main sources of the apoptotic bodies that are cleared by macrophages and that prevent inflammation through IL-10 and TGF-beta production. Thus a physiological mechanism of the immune response homeostasis is used at the feto-maternal interface in order to balance the pro/anti-inflammatory mediators in favor of controlling the initial pro-implantatory response during the implantation window. Additionally, progesterone stimulates a Th2-type response, decreases inflammatory cytokines, and suppresses maternal T lymphocytes activated in response to fetal antigens (allogeneic response), all in order to promote fetal survival (16, 17).

The present data reflects a fine tuning of the pro/anti-inflammatory mediators and the maternal T effector/T regulatory responses. While Th2 and Treg cells may be able to restore the balance, too many Th2/Tregs cytokines generally do not constitute a healthy situation. TGF-beta in particular can inhibit the trophoblast growth and invasion necessary for implantation through the inhibition of MMP-9 production and hCG secretion by human trophoblast (18).

3. PRO-INFLAMMATORY MEDIATORS IN THE PERI-IMPLANTATION WINDOW

Chemokines (short for chemo-attractant cytokines) are an important component of the intricate network that regulates the normal T-cell trafficking and the inflammatory process (19-21). This family of small chemo-attractant-peptides is not only involved in leukocyte migration, but is also implicated in both angiogenesis and the process of cell activation.

Chemokines and their receptors are divided into two families based on structural and genetic considerations. These are the CXC family (alpha-chemokines) and the CC family (beta-chemokines); they are distinguished by the cysteins in conserved positions. Chemokines can also be divided into two larger categories according to their expression: inflammatory chemokines induced upon T-cell activation and constitutive chemokines that fulfill housekeeping functions and participate in constitutive leukocyte trafficking (21,22). The switch from receptors for constitutive chemokines to receptors for inflammatory chemokines follows T-cell activation and results in a polarization that results in changes in the migratory properties of these cells (19, 20, 23). Moreover, chemokines can act via multiple receptors (they belong to the super-family of the G-protein-coupled receptors) and the distinctive functions in immune Th1 and Th2 responses correlate with their distinctive cytokine secretion patterns and with the expression of different chemokine receptors. These receptors transmit information from the extra-cellular environment and are named in accordance with the structure of their particular ligand (CXC or CC). Thus leukocytes modulate their trafficking by the preferential expression of CCR or CXCR receptors, including naïve, effector, and memory T cells. Recent data focuses on the ability of the chemokines to attract CD4+CD25+ Tregs selectively. Wysocki *et al.* in particular has shown that in murine model Tregs suppress graft-versus-host disease (GVHD); moreover, Treg cells that lacked expression of the chemokine receptor CCR5 have been shown to be far less effective in preventing lethality from GVHD (24). For example, the survival rate of irradiated recipient animals when given transplants supplemented with CCR5-/-Tregs was significantly decreased and GVHD scores were increased in comparison with animals receiving wild-type. This deficient function of CCR5-/-Tregs correlated with the impaired accumulation of these cells in the liver, lung, spleen, and mesenteric lymph nodes more than one week after transplantation. CCR5 is therefore required for proper Treg function, and this indicates that Treg cells, in addition to their previously defined role in inhibiting effector T-cell expansion in lymphoid tissues during GVHD, are important when later recruited to both lymphoid tissues and GVHD target organs because they help prolong survival following allogeneic bone marrow transplantation (24).

As with the graft-versus-host disease in the feto-maternal dialogue, the recognition of paternal antigens by maternal T lymphocytes can generate a deleterious allogeneic response and embryo resorption. However, allo-recognition may recruit Tregs to the uterus, playing an essential role in preventing fetal rejection by the maternal immune system. Kallikourdis *et al.* has demonstrated that Treg cells can be divided according to the expression of CCR5 into a highly suppressive CCR5+ subpopulation and a far less suppressive CCR5-

subpopulation. This would suggest that the former group represents the effector arm of regulatory T cells (25). The most intriguing data, however, reveals that the accumulation of CCR5+ regulatory T cells at this site would seem to be enhanced by the presence of allo-antigen, in contrast to the systemic expansion of Treg cells during pregnancy that appears to be alloantigen-independent (25). Thus the interaction of chemokines and their promiscuous receptors is responsible for the accumulation of those Treg cells that are activated by paternal antigens and go further to induce a tolerogenic response.

Interestingly, these molecules are involved in leukocyte trafficking and homing not only in the course of normal physiological processes, but also in the course of pathological events such as inflammation and endothelium damage (26).

As a representative example, RANTES (regulated on activation, normal T cell expressed and secreted) or CCL5 is associated with a physiological mechanism based on its well-established chemo-attractant properties that allow the migration of trophoblast cells and spermatozoa (27). Thus even RANTES is produced and secreted by several tissues, such as endometrial and endometriotic stromal cells, and is associated with pro-inflammatory and Th1 immune responses (28, 29). It is further known that the RANTES concentration level and bioactivity levels are elevated in the peritoneal fluid of women with endometriosis (30, 31). Elevated concentration levels of RANTES in such patients correlated positively with the stage of disease, suggesting that RANTES may be involved in leukocyte recruitment into the endometrium, even when endometrial tissue is present at ectopic sites. Moreover, prolonged treatment with medroxyprogesterone acetate improves the pain associated with endometriosis, and the clinical effectiveness of this drug has been attributed to the inhibition of RANTES gene transcription (32).

The association between chemokines and reproductive pathological effects was also evidenced in animal models such as the CBA/J x DBA/2 murine model. In this model, increased number of fetal losses were associated with an exacerbated inflammatory response (33). In fact, the placentas of primiparous CBA/J females tended to produce high levels of RANTES, correlating with a highly exacerbated Th1 response. Furthermore, the deleterious effect was abrogated after multiple pregnancies. This fact would support the hypothesis that the recognition of paternal antigens (allorecognition) may also confer beneficial effects (34).

4. CHEMOKINES: DO THEY CONTRIBUTE TO A PRO-INFLAMMATORY OR A TOLEROGENIC RESPONSE?

Until recently, the chemokines responsible for directing leukocyte migration during both immune homeostasis and inflammation were thought to be well characterized; yet there is in fact an overall lack of information about the role of such chemokines in the induction of peripheral tolerance. A recent wave of evidence has indicated that several chemokines, particularly β-chemokines, regulate the immune response and display immunomodulatory effects that contribute to a tolerogenic state (35-36).

Interestingly, the induction of peripheral tolerance via immune-privileged sites, such as the eye, requires splenic co-localization of NKT cells and CD1d+ tolerogenic APCs (antigen-presenting cells); both of these are needed for the generation of the tolerogenic response. CD1d-stimulated NKT cells produce chemokines, such as RANTES, that are able to recruit Treg cells (28, 37, 38). In fact, a RANTES blockade *in vivo* prevented the accumulation in the spleen and the generation of Treg cells that suppress Th1 immunity. Thus, through RANTES production, CD1d-restricted NKT cells provide critical signals that orchestrate the accumulation of cells needed for tolerance induction (37,39).

Additional evidence by Wang *et al.* showed that women with recurrent spontaneous abortions (RSA) were resistant to HIV infection after allo-immunization treatment with paternal mononuclear cells (40). Having gained insight into the molecular mechanism involved in this natural resistance, Wang was able to demonstrate that after paternal allo-immunization, $CD8^+$ cells acquired the capacity to up-regulate the secretion of RANTES, MIP-1 alpha (CCL3), and MIP-1beta (CCL4) with immunoregulatory properties. Thus, *in vivo* as well as *in vitro* allo-stimulation of human mononuclear cells would appear to generate soluble factors that inhibit HIV transmission by beta-chemokines in uninfected cells that support the immunoregulatory properties of these chemokines (40).

5. COULD RANTES BE A PHYSIOLOGICAL FACTOR THAT CONTRIBUTES TO FETO-MATERNAL TOLERANCE, ALLOWING FETAL SURVIVAL?

RANTES, classically considered a part of Th1-type reactions, may act as an important modulator of allo-antigen-specific T-cell responses during normal pregnancy. However, progesterone, present in high amounts in maternal serum and at the maternal-fetal interface, suppresses Th1 responses, decreases inflammatory cytokines and cytotoxic T-lymphocyte activity while stimulates Th2-response (16,41,42).

Given that the implantation window is considered an inflammatory process that must be followed by a Th2 shift in order to control the endocrine and immune systems, we hypothesized that failures in RANTES production or regulation could lead to pregnancy loss and that progesterone or allo-antigens could modulate its production. In order to answer this question we focused on three levels: 1) systemic effects on maternal allogeneic response; 2) local effects on endometrial cells and on endometrial infiltrated T lymphocytes; and 3) during early stage of the feto-

maternal dialogue. For each of these levels, we analyzed the physiological and pathological conditions involved.

5.1. RANTES as a novel immunomodulator of the maternal allogeneic response

Recent data supports the hypothesis that allo-antigen recognition in the uterus confers an advantage on the developing fetus. The increases in LIF, hCG, and SDF-1 that occurs with allo-activation may be feto-protective (43). Nevertheless, increased leukocyte infiltration and inappropriate activation may be an underlying cause of pregnancy complications and failures that have instead been attributed to an exacerbated Th1-response as this promotes tissue damage and fetal resorption (4-6).

Successful pregnancy is accompanied by an increase in RANTES serum levels; such an increase has not been observed, however, in patients with RSA, suggesting that an adequate allo-recognition can induce RANTES production (44).

RANTES is able to specifically suppress the maternal allogeneic response to paternal antigens in a dose-dependent manner. The suppressor activity of RANTES seems to be mediated by a mechanism that involves the induction of the apoptosis of activated T cells and the modulation of the Bcl-2 protein levels. Likewise, a blockade of the proliferative response and the induction of apoptosis induced by the sera of fertile women were prevented with an anti-RANTES mAb, supporting the notion that RANTES has a specific role to play as a novel suppressive factor of the allogeneic response.

Many apoptotic stimuli, such as Fas ligand, TNF-alpha, and TRAIL deliver death signals through the recruitment of a series of aspartic, acid-specific proteases known as caspases (45), while other stimuli function independently of caspase activation (46). In the maternal-paternal alloresponse, treatment of mixed lymphocyte cultures with a broad range caspase inhibitor (ZVAD-fmk) only slightly overcame the cell growth inhibition triggered by RANTES, suggesting that this beta-chemokine transduces inhibitory/death signals through both caspase-independent and dependent mechanisms. In addition, Mellado *et al*. has observed a chemokine-dependent mechanism by which melanoma tumor cells shield themselves from immune reactions. In this model, RANTES production by infiltrating $CD8^+$ cells activated an apoptotic pathway involving cytochrome c release into the cytosol and activation of caspase-9 and –3 (47).

Peripheral maternal lymphocytes thus produce higher levels of RANTES in response to allo-stimulation. Moreover, the presence of high levels of progesterone during normal human pregnancy, particularly at the maternal-fetal interface, promotes RANTES production to the levels that may be required for the local induction of a tolerogenic response. Under pathological conditions, RSA patients do not induce RANTES secretion in response to fetal antigens, as do fertile women. This could indicate intrinsic maternal immunologic deficiencies and/or possible breaks in maternal immune tolerance.

This experimental evidence reveals the specific ability of RANTES to down regulate maternal T-cell responses to fetal antigens and suggests that this ability may be relevant for fetal-tolerance induction and could potentially be used to avoid recurrent miscarriage.

5.2. Induction of maternal tolerance to fetal alloantigens by RANTES production in endometrial T lymphocytes

In order to focus on RANTES-local effects we evaluated endometrial samples obtained from fertile women during the secretory phase of the menstrual cycle. The analysis of CD4+ and CD8+ endometrial T-infiltrated lymphocytes cultured in the presence of physiological progesterone concentrations revealed an increase in intracellular RANTES expression.

High progesterone levels may induce differential responses in the periphery and in the endometrium. These responses would in turn promote a Th1-type response in the endometrium that may be essential for implantation. Moreover, Ishikawa cells (a human endocervical cell line, representative of endometrial cells) treated with recombinant RANTES displayed a Th1-pattern response correlated with an increase in T-bet expression (the main nuclear transcription factor involved in Th1- immune response development) (48).

The pre-implantation endometrium is able to produce RANTES and has the potential to respond to it in an autocrine manner. RANTES mRNA is expressed in the pre-implantation endometrium in both fertile women and RSA patients; however, the effects of RANTES in this microenvironment may be largely dependent on the differential expression of the various RANTES receptors (CCR1, CCR3, and CCR5). The majority of the fertile women expressed at least two different RANTES receptors while the majority of the RSA patients expressed only one RANTES receptor. This may have important implications for women with a history of RSA, given that they exhibit an altered potential for autocrine response to RANTES (48).

5.3. RANTES produced by trophoblast-maternal leukocyte dialogue modulates maternal T-cell response inducing a tolerance to fetal antigens

Finally, in order to focus on RANTES-immunoregulatory properties in the feto-maternal cross-talk, we performed co-cultures between trophoblast-cell-line [Swan 71 cell line, derived by the telomerase-mediated transformation of a 7- week cytotrophoblast isolate described by Straswski-Chavez (49)] and maternal PBMCs. Trophoblast cells constitutively secrete RANTES; moreover, the production of RANTES was found to increase within 24 hours of the maternal-PBMCs trophoblast dialogue. As with other CC chemokines, this reflects RANTES ability to be expressed as an early gene under pro-inflammatory cytokine stimulation (50).

In fact, increased RANTES production in this dialogue is associated with pro-inflammatory cytokines such as TNF-alpha, a low dose of IFN-gamma and IL-12

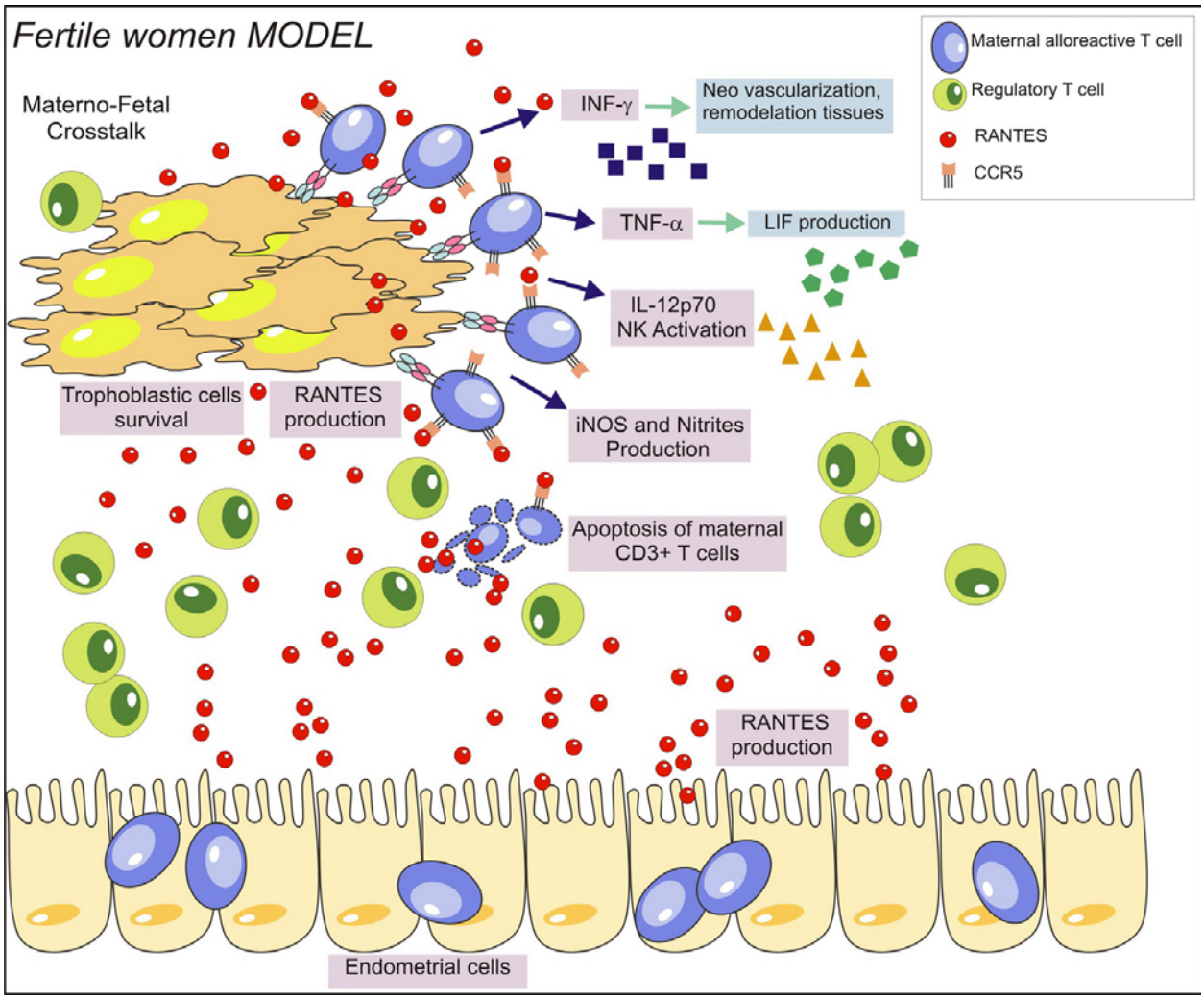

Figure 1. Model of RANTES-immunoregulatory effects in the feto-maternal cross talk under physiological conditions. Trophoblast cells constitutively secrete RANTES, and its production appeared to increase after the maternal-PBMCs trophoblast dialogue, accompanied by pro-inflammatory cytokines such as TNF-alpha, a low dose of IFN-gamma and IL-12 (necessary for uterine vascular modification), nitrite production (related to uterine quiescence and angiogenesis), and LIF expression; characterizing a *pro-implantatory microenvironment*. This inflammatory context is a hallmark of normal implantation and could be later auto-controlled by RANTES through the modulation of the *T effector/Tregs lymphocyte balance:* First of all, an increase in RANTES results in the elevated apoptosis of potentially deleterious CD3+ lymphocytes. Second, RANTES has the ability to modulate the frequency of T regulatory cells during the maternal PBMCs-trophoblast cell dialogue, as evidenced by an increase in the frequency of CD4+CD25+Foxp3+. Interestingly, the trophoblast-cell-line did not express CCR5, making these cells potentially resistant to apoptosis induced by RANTES and reflecting a potential mechanism whereby RANTES could selectively induce apoptosis of alloreactive maternal lymphocytes.

(necessary for uterine vascular modification), nitrite production (related to uterine quiescence and angiogenesis), and LIF expression, as these characterize a pro-implantatory microenvironment (51-53). This inflammatory context is a hallmark of normal implantation and could later be auto-controlled by RANTES through the modulation of the *T effector/Tregs balance* (54).

First, the increase in RANTES results in increased apoptosis of potentially deleterious CD3+ lymphocytes correlating with a significant decrease in the maternal T cell-proliferative response. Interestingly, the trophoblast-cell-line did not express CCR5, making it potentially resistant to RANTES-induced apoptosis and reflecting a possible mechanism by which RANTES could selectively induce the apoptosis of alloreactive maternal lymphocytes as a way of controlling an exacerbated alloresponse. In fact, cytotrophoblast cells did not express CCR1 and CCR5 (HIV-1 receptor for the M-tropic isolates), thus there is no vertical HIV transmit via this mechanism between mother and fetus (55) (see Figure 1).

Second, RANTES has the ability to modulate the frequency of T regulatory cells during the maternal

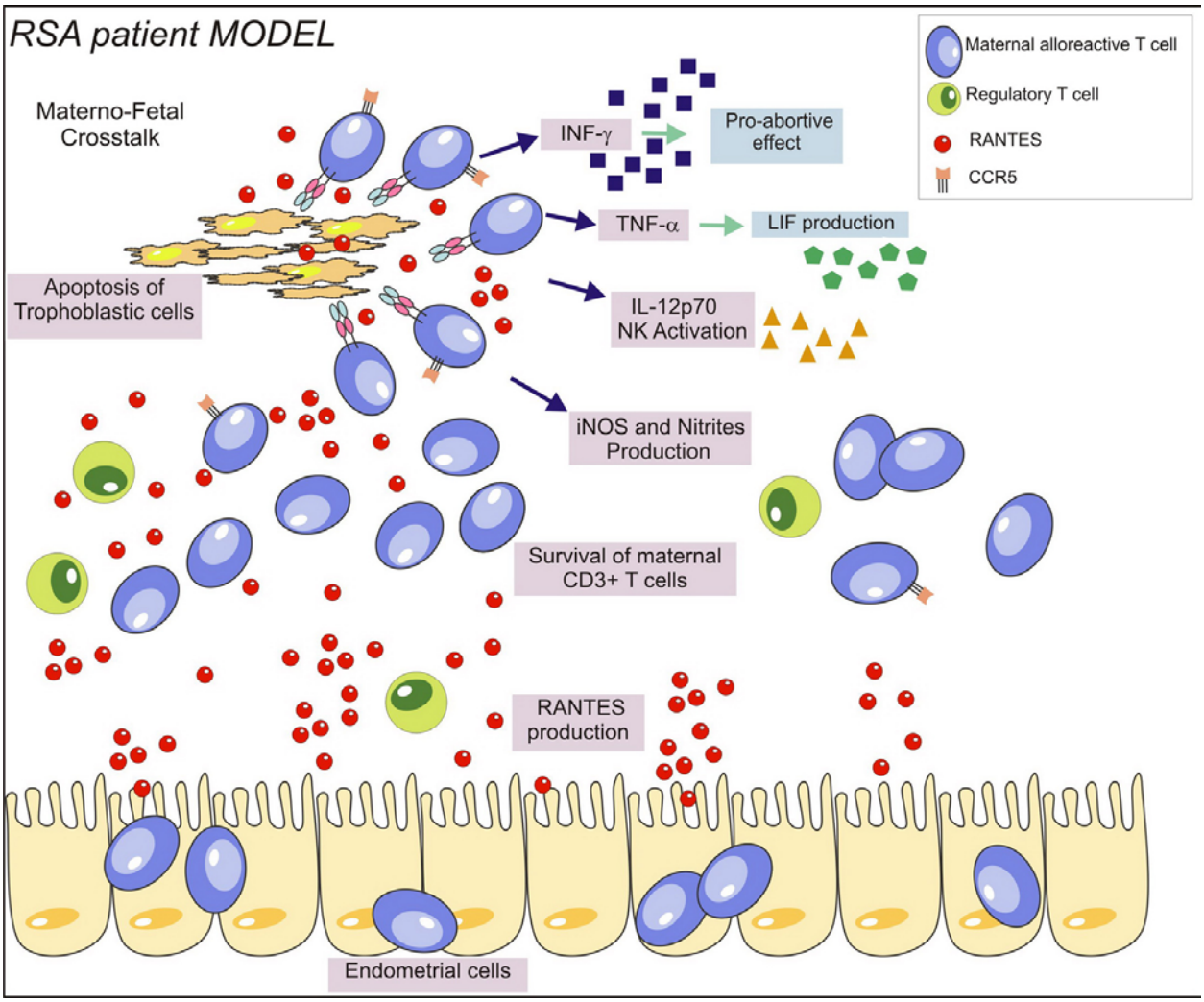

Figure 2. Model of RANTES-immunoregulatory effects in the feto-maternal cross-talk under pathological conditions. During the dialogue between trophoblast cells and PBMCs from RSA patients there is an altered time window of RANTES production correlating with a high frequency of apoptotic trophoblast-cell-line that follows the interaction with maternal RSA-PBMCs. Intriguingly, this cross-talk also reflects low levels of apoptotic maternal CD3+ lymphocytes. This supports the hypothesis that RSA patients may have an altered mechanism allowing the persistence of allo-activated T cells attempting to fetal survival. This phenomenon is also associated with a decrease in Tregs and suggests that in RSA patients Tregs are unable to expand during the pre-implantatory phase. Finally, even RANTES mRNA is expressed in the pre-implantation endometria in both fertile women and in RSA patients with the latter having a differential expression of RANTES receptors.

PBMCs-trophoblast cell dialogue, as evidenced by the reduction of Foxp3 expression (forxed box p3: the transcription factor associated with regulatory T-cell population) and also in the frequency of CD4+CD25+Foxp3+ (characteristic Tregs phenotype) in the presence of the anti-RANTES neutralizing Ab (see Figure 1).

Under pathological conditions, co-cultures of trophoblast cells and PBMCs from RSA patients displayed an altered time window of RANTES production. This correlated with a high frequency of apoptotic trophoblast-cell-line that follows the interaction with maternal RSA-PBMCs (54). These co-cultures did reflect low levels of apoptotic maternal CD3+ lymphocytes, thus supporting the hypothesis that RSA patients may have an altered mechanism that allows an exacerbate maternal allo-response potentially deleterious to fetal survival. In fact, the RSA patients showed a significant decrease in the number of Treg cells after trophoblastic stimulation in comparison with the fertile women. This suggests that Treg cells in RSA patients cannot expand during the pre-implantatory phase (54) (Figure 1B). Previous reports by Arruvito *et al.* demonstrated that RSA patients have a decreased number of Treg cells combined with a diminished capacity to suppress maternal response to paternal antigens (56). Consequently, reproductive failure may result from the combination of their reduced frequency and their suppressive function (see Figure 2).

Table 1. Primer sequences used for PCR

	Upstream **sequence 5'-3'**	**Downstream sequence 5'-3'**
GAPDH	TGA TGA CAT CAA GAA GGT GGT GAA G	TCC TTG GAG GCC ATG TAG GCC AT
CCR1	GCC TGA AAC AGC TTC C	AGA AGG TGA ACG AGA GG
CCR3	GTT GGT CAT AAT TCG GAG CCT CC	AAA GCT GAT ACC AGA GCA CTG ATG G
MCP-1	**R and D System kit primer pair**	**R and D System kit primer pair**

Experimental evidence indicates that RANTES may play an important role during early implantation in an adequate pro-inflammatory microenvironment by increasing regulatory T lymphocytes, inducing apoptosis of maternal allo-activated T cells, allowing trophoblast survival, and promoting maternal tolerance to fetal antigens.

6. CHEMOKINE NETWORK DURING FETO-MATERNAL DIALOGUE

As previously stated, it has been proposed that the expression of chemokines involves an interdependent network with the absence of a single chemokine affecting the expression of many others. Hence, the loss of a single chemokine can disrupt the entire chemokine network not just at the site of acute inflammation, but even in an isolated inflammatory cell, such as the macrophage (57). In particular, monocyte chemo-attractant protein (MCP-1), a member of the CC chemokine super-family, plays a critical role in the recruitment and activation of leukocytes during acute inflammation. Ferreira *et al*. demonstrated that the loss of MCP-1 expression affected the chemokine network by comparing the mRNA expression profiles of MCP-1(-/-) and wild type mice during the acute inflammatory phase of excisional wounds (58). Utilizing a mouse cDNA array, the authors observed both significant up-regulation and down-regulation of several chemokines and chemokine-receptors in acute wounds.

After taking into consideration the following facts: 1) a pro-inflammatory microenvironment contributes to a successful implantation; 2) macrophages are one of the strongest sources of pro/anti-inflammatory mediators, and 3) RSA patients displayed a differential time-kinetic in RANTES production when compared to fertile women, the question arises, *Could the chemokine network be disrupted in patients with recurrent spontaneous abortions?*

In order to answer this question, we focused on the pre-implantation level and on the feto-maternal dialogue. First, we evaluated MCP-1 and its receptor expression in pre- implantation endometrial tissue obtained from both RSA patients and fertile women.

RSA patients were defined as women with a history of two or more consecutive pregnancy losses prior to the twelfth week of gestation without any infectious, endocrine, or anatomic disease to trigger the abortion. The criteria for the exclusion of patients were: 1) occurrence of anti-phospholipid antibodies, 2) hepatitis B or C infection, 3) human immunodeficiency virus infection, and 4) balanced translocation. The *control group of women* were fertile women who had had two or more previous normal pregnancies without miscarriage and who had consulted the Gynecology Unit for barrier methods of contraception. The "Investigation and Ethics Committee at the Hospital de Clínicas José de San Martín" gave its approval for this study, and each patient gave her written consent to participate. Secretory-phase endometrial biopsies were obtained from 8 RSA patients and 8 fertile women. The total RNA was extracted from the tissue samples, and RT-PCR was performed using primers amplifying CCR1, CCR3, and MPC-1 (Table 1).

As depicted in Figure 3A, all the fertile women expressed MCP-1 while none of the 8 RSA patients expressed it. Moreover, even when endometrial cells from the RSA patients expressed CCR1 and CCR3, the mRNA-levels were lower than those observed in the fertile women. Figure 3A upper panel presents the expression profiles of both groups of women under study. Gels were scanned, densitometry was performed, and the results were expressed as the media of the arbitrary units relative to GAPDH expression (lower panel). These findings show that the pre-implantation endometrium from fertile women can produce MCP-1 as well as induce an autocrine response. Those women experiencing RSA, however, exhibited a strong reduction in MCP-1 and its receptors.

In order to focus on the early events during the feto-maternal dialogue, trophoblast-cell-lines (Swan 71 cells) were cultured in 24 flat-bottom polystyrene plates in complete DMEM 10% FCS. At 60% of confluence, PBMCs from RSA or fertile women were added (5×10^5 cells/well). After 48 hours of co-culture, the supernatants were collected and MCP-1 secretion was quantified by ELISA (R&D System, Minneapolis, USA). As depicted in Figure 3B, MCP-1 levels significantly increased in the trophoblast-fertile PBMC culture supernatant in comparison with those performed with RSA-PBMCs (p less than 0.05, Mann-Whitney test).

The present data indicates that MCP-1 is expressed by the endometrial cells of fertile women and can be auto-regulated through interaction with CCR1 and CCR3. Moreover, PBMCs from fertile women significantly increase MCP-1 secretion after trophoblast cell interaction. Under pathological conditions, the endometria from RSA patients showed a reduction in MCP-1 expression correlated with a diminished secretion during the feto-maternal cross-talk. When considered as a whole, this data suggests that the increase in RANTES production is accompanied by MCP-1 production in fertile women, while in RSA patients the chemokine network is altered at both the endometrial and implantation levels.

7. SUMMARY AND PERSPECTIVE

Early pregnancy presents a challenge to a variety of cells and molecules in both the mother and the embryo.

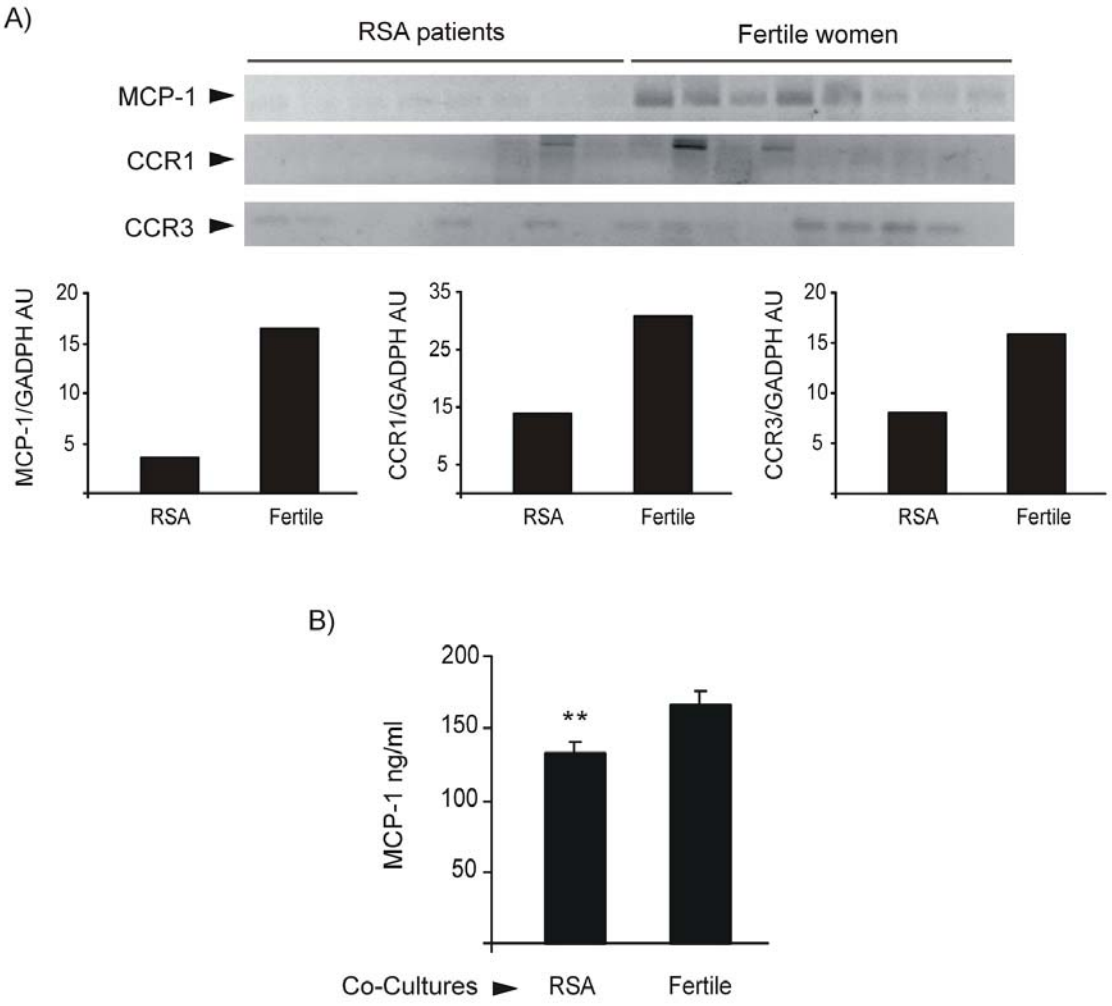

Figure 3. A: Expression of MCP-1 and its receptors in endometrium from RSA and fertile women. mRNA for MCP-1 and its receptors (CCR1 and CCR3) were semi-quantified in the pre-implantation endometrium isolated from fertile women and RSA patients by RT-PCR. The upper panel shows the expression profiles of 8 RSA patients and 8 fertile women. Gels were scanned, densitometry was performed, and the results were expressed as the media of the arbitrary units relative to GAPDH expression. B: Expression of MCP-1 in response to maternal leukocyte stimulation. Swan 71 cell line at 60% of confluence in a 24-well flat-bottom plate, were cultured in the presence of PBMCs from fertile or RSA women ($5x10^5$ cells). At 48 hours of co-culture, supernatants were collected, and the amount of MCP-1 was quantified in the supernatants obtained by ELISA (R and D System, Minneapolis, USA). The results are mean ± SD expressed as pg/ml (* p less than 0.05 Mann-Whitney test). Lastly, the results are representative of 5 independent experiments using different 5 fertile and 5 RSA women.

However, the definitive mechanisms that underly the interactions that take place between these maternal and embryonic cells/molecules are not yet sufficiently understood. The facts suggest that RANTES may play an important role during the feto-maternal dialogue and so contribute to trophoblast cell survival and to a maternal tolerogenic response allowing for the survival of the semi-allogeneic fetal graft.

Investigation of the molecular mechanisms leading to immune tolerance at the maternal-fetal interface will contribute not only to an improved understanding of successful pregnancy, but will also help to identify possible failure mechanisms in patients who have suffered recurrent spontaneous abortions. In this regard, the use of microarray technology in co-cultures of human trophoblast cell lines with maternal leukocytes (as those referred to above) or the possibility of culturing uterine-decidual explants from pregnant murine models may help in screening drug targets and mechanisms.

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