

# New Actors for the Immunological Mechanisms Involved in the Materno-Fetal Tolerance

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**Abstract:** Tolerance to the semi-allogeneic fetal graft by the maternal immune system is the consequence of a wide panel of mechanisms that may be interconnected. In the present work we discuss the participation of new actors that might contribute to the materno-fetal tolerance and their involvement in the failure of a successful pregnancy occurring in patients with recurrent spontaneous abortions (RSA). The presence of blocking factors (BF) of the maternal allogeneic response in the sera of fertile women is usually associated with a successful pregnancy. In this context, Galectin-1 (a galactoside-binding protein expressed at sites of T-cell activation and immune privilege) and RANTES (regulated on activated normal T cells, expressed and secreted) are able to immunosuppress the allogeneic response. This effect induces a significant increase of the apoptosis of CD45RO<sup>+</sup> cells and is accompanied by caspases activation and Bcl-2 down regulation.

Moreover, trying to identify reliable markers for RSA diagnosis, we investigated some activation markers like CD69 (an early activation marker), SLAM (signaling lymphocytic activation molecule) and the pattern of Th1/Th2 cytokine expression in the preimplantation endometrium and in the peripheral blood from fertile and RSA women.

Finally, as a treatment for RSA patients, we evaluated the effectiveness of the alloimmunization, with paternal leukocyte and investigated the immunomodulation induced by this treatment on RANTES, and the subpopulations of CD3<sup>+</sup>CD69<sup>+</sup> and CD3<sup>+</sup>SLAM<sup>+</sup> T cells in RSA patients.

**Keywords:** Tolerance, Galectin-1, RANTES, CD69, SLAM, immunotherapy.

## INTRODUCTION

Successful pregnancy is an immunological paradox because the fetus is not rejected despite the presence of paternal major histocompatibility complex antigens. Maternal leukocytes in close contact with the fetal tissue might form a local network supporting the cellular and humoral immunity that contributes to maintain pregnancy [1-3]. It has also been suggested that the immunologic success of the fetus during pregnancy might be based on a bidirectional interaction between the mother and the fetus [4].

One hypothesis sustains, that during normal pregnancy a shift from a Th1- to a Th2 polarised immune response contributes to the survival of the fetus [5-7] and Th2 cytokines, such as TGF- $\beta$ , IL-4 and IL-10, are also able to prevent immunopathology [8,9]. Little is known about the pattern of cytokines secreted by infiltrating endometrial lymphocytes during the preimplantation stage. However, Th2 cytokines secretions are detected at the fetomaternal interface during human and murine pregnancy [10,11]. In contrast, high levels of Th1 cytokines, particularly IFN- $\gamma$  are found in patients with recurrent spontaneous abortions (RSA) [14,15]. To complete the picture of the fetomaternal interface during normal pregnancy, it was also described that maternal decidua is infiltrated by NK and T cells expressing activation markers [16].

Several investigators have demonstrated the relationship between the production of mixed lymphocyte reaction (MLR) blocking factors (BF) with a successful pregnancy [17-20]. The levels of MLR BF increase with consecutive pregnancies, which constitutes a major evidence of the maternal immune recognition at the systemic level [3, 21].

Research over the last few years suggested that BF might represent antibodies that are able to "block" the maternal recognition of the fetal antigens. Maternal antibodies recognizing alloantigens present on paternal T-cell lymphocytes detected by flow cytometry cross match correlates with blocking activity evidence in the MLR [22]. However, 20% of the RSA patients with a positive cross match were unable to block the MLR, suggesting that BF might represent additional soluble factors different from antibodies. Hence, the identity and nature of these MLR BFs remain elusive and prompt the identification of new BFs able to suppress the vigorous maternal immune response.

## INDUCTION OF ALLOGENIC T CELL-HYPORESPONSIVENESS BY GALECTIN-1-MEDIATED APOPTOTIC AND NON-APOPTOTIC MECHANISMS

In this context, Galectin-1 (Gal-1) is a member of a growing family of animal lectins which are highly conserved throughout the evolution sharing sequence similarities in the carbohydrate recognition domain [23,24]. Gal-1, which is expressed in activated T cells, induces apoptosis of immature thymocytes and activated, but not resting T cells, by crosslinking of T-cell surface glycoproteins [25-28,32]. The

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immunomodulatory and anti-inflammatory effects of this carbohydrate-binding protein have been also validated *in vivo* in experimental models of autoimmunity and inflammation [33-37]. This new wealth of information suggests that Gal-1 could be used as a selective immunosuppressive agent to shut-off T-cell effector functions under several pathological conditions. The investigation of Gal-1-function in the normal allogeneic response revealed that this protein specifically induced a dose-dependent inhibition of the allogeneic T-cell proliferative response and relies on its carbohydrate-binding properties [38].

The study of the mechanisms implicated in Gal-1-induced inhibition of the allogeneic response revealed a time-dependent increase of subdiploid DNA content showed by propidium iodide staining, when cells were allostimulated in the presence of Gal-1. In addition, treatment with this sugar-binding protein resulted in 25% of early apoptotic CD45R0<sup>+</sup> lymphocytes and 28% of cells positive for late apoptotic cells. This immunosuppressor effect was accompanied by a Bcl-2 modulation [39]. During the course of an allostimulation, Bcl-2 expression reached its maximal levels within 24 h and declined only after 72 h of cell culture. However, when Gal-1 was added to the alloreactive lymphocytes, Bcl-2 expression also peaked after 24 h, but immediately decreased in a time-dependent manner suggesting that Gal-1 delivers intracellular inhibitory signals through Bcl-2. The use of a broad caspase inhibitor (ZVAD-fmk) prevented Gal-1 induced suppression, indicating that Gal-1 involved a caspase-dependent pathway [38].

Another interesting finding is that, Gal-1 is differentially expressed during the allogeneic T-cell response. The absence of this protein at the initiation of the cell culture and its substantial increase during allostimulation, suggest that this protein might play a key role during the effector phase of the alloimmune response.

Induction of partial tolerance in alloreactive lymphocytes by a lectin-dependent process is clearly suggestive of a natural mechanism to extend allograft survival and achieve homeostasis in immune privileged tissues such as testis, placenta and the eye. The specific ability of Gal-1 to downregulate T-cell responses, suggests that this galactoside-binding protein might be a relevant inductor of the fetomaternal-tolerance.

#### **IDENTIFICATION OF RANTES AS A NOVEL IMMUNOMODULATOR OF THE MATERNAL ALLOGENEIC RESPONSE**

In search of novel BF's we investigated the role of chemokines, a family of chemoattractant cytokines involved in leukocyte migration, angiogenesis and cell activation. These low molecular weight-proteins can be broadly divided into two categories: inflammatory chemokines induced after T cell activation, and constitutive chemokines, which fulfill housekeeping functions and participate in constitutive leukocyte trafficking [40,41]. The switch from receptors for constitutive chemokines to receptors for inflammatory chemokines follows T-cell activation and results in a polarization, which determines changes in migratory properties of these cells [42-44]. Moreover, chemokines can act via multiple receptors; eg. RANTES ("regulated on

activated normal T-cell expressed and secreted") activates CCR1, CCR3, and CCR5 [45]. The distinctive functions in immune responses of type 1 (Th1) and type 2 (Th2) cell subsets correlate with their distinctive cytokine secretion patterns and with different chemokine receptor expression. Th1 cells preferentially express CCR5 and CCR1, both receptors for RANTES that are expressed in peripheral CD45R0<sup>+</sup> memory T-cells [46]. Recent studies demonstrated that RANTES derived from NKT cells, plays a critical role for the generation of CD8<sup>+</sup> T regulatory cells during peripheral tolerance induction. In this murine model, RANTES seems to act by suppressing Th1 and Th2 effector cells [47,48]. The requirement for RANTES in tolerance was also demonstrated by *in vivo* studies where blockage of RANTES prevented the generation of CD8<sup>+</sup> T regulatory cells [48].

In accordance, studies performed in alloreactive lymphocytes cultured in the presence or absence of increasing concentrations of recombinant RANTES significantly suppress the allogeneic response in a dose-dependent manner [49]. This effect was abrogated by the anti-RANTES neutralizing antibody. In addition, an MLR performed in the presence of serum containing BF's together with a neutralizing anti-RANTES mAb, also showed a significant reversion of MLR inhibition, suggesting that RANTES might be an important BF of the MLR. In contrast, anti-RANTES mAb was not able to increase proliferative response when pre-incubated with a non-blocking serum. This finding might support the assumption that the absence of RANTES might represent one of the factors associated with the lack of BF in RSA patients. Moreover, the addition of the specific anti-RANTES mAb decreased the subdiploid DNA content induced by RANTES to levels even lower than those obtained with medium alone, indicating that the mAb was also able to neutralise endogenous RANTES. Investigation of the mechanisms involved in RANTES suppression revealed that, like Gal-1, RANTES also delivers intracellular inhibitory signals through a Bcl-2-dependent pathway and involved caspase-independent mechanisms [49].

These data clearly suggest that RANTES is not only a novel blocking factor, but it is also involved into the fetomaternal cross talk. In fact, the quantification of RANTES levels in sera from RSA and fertile women measured by ELISA showed that RANTES was significantly decreased in RSA patients. Moreover, RANTES levels in women with no previous pregnancies were also significantly lower compared to fertile women, suggesting that successful pregnancy is accompanied by an increase in RANTES serum production [49].

#### **THE EXPRESSION OF CD69 AND SLAM IN ENDOMETRIAL AND PERIPHERAL T-CELLS REPRESENT USEFUL MARKERS FOR RSA PATIENTS**

Immunotolerance to fetal antigens might involve either the participation of the whole maternal immune system or by the direct contact of maternal and fetal cells that occurs at the placenta level [4]. The leukocytes present in the human endometrium have different characteristics across the menstrual cycle. As an example, CD3<sup>+</sup> lymphocytes

predominate in the proliferative and early secretory phases of the menstrual cycle, while endometrial granulated lymphocytes (EGLs) are the most abundant endometrial leukocyte population in the late secretory phase and first trimester of pregnancy [50,51]. According to several reports, a differential characteristic of human endometrium leukocytes is the expression of the early activation marker CD69 in T cells and EGLs [8,52]. CD69 seems to be the earliest inducible cell surface glycoprotein, which is synthesized *de novo* and expressed by activated T and B-lymphocytes and natural killer (NK) cells [53]. By using a FACS method of intracellular staining, it was demonstrated that CD69 expression was completely restricted to the intracellular compartment. According to this evidence, intracellular expression of this marker in endometrial T cells from RSA patients is significantly higher compared to fertile women ( $68.2 \pm 12\%$  vs.  $23.7 \pm 22\%$ ) [54]. Similarly, peripheral blood lymphocytes from RSA patients also show a significant increase of CD3<sup>+</sup> cells expressing intracellular CD69 compared to fertile women ( $20 \pm 9.5\%$  vs.  $2.1 \pm 3.8\%$ ). This observation correlates with the elevated numbers of CD69<sup>+</sup> cells detected in deciduas from spontaneous abortion cases compared with the apparently normal pregnancy controls [10].

As this subpopulation is also increased in RSA's endometrium, we can speculate that the increased presence of CD3<sup>+</sup>CD69<sup>+</sup> cells in endometrium is a result of a preferential migration of this population into the uterus from peripheral blood. In fact, they might arrive to the endometrium across particular homing receptors. However, CD3<sup>+</sup>CD69<sup>+</sup> peripheral blood T cells subset do not co-express others activation markers like CD25 [54]. Therefore, the expression of CD3<sup>+</sup>CD69<sup>+</sup>T cells in an immunologically privileged site might be associated with a yet unknown immunoregulatory function, such as a receptor for soluble or cell-associated ligands, involved in cellular proliferation and expansion control.

The CD3<sup>+</sup>CD69<sup>+</sup> population detected in RSA patients was evenly represented in CD4<sup>+</sup>, CD8<sup>+</sup>. Cell sorting studies to investigate whether and to what extent these cells play a role in the failure of the pregnancy revealed that CD69<sup>+</sup>CD3<sup>+</sup> cells were unable to produce INF  $\gamma$  in response to polyclonal stimulation [54]. This observation is particularly interesting in light of a recent report indicating a contribution of INF  $\gamma$  to initiation of uterine vascular modification, decidual integrity and uterine natural killer cells maturation during normal murine pregnancy [55]. Consequently, the increase of the CD3<sup>+</sup>CD69 subset in RSA women, might not only represent a useful marker, it might also indicate their attempt to regulate an undesirable immune response.

Further investigation of the T cells activation status during the secretory phase of the menstrual cycle, focuses on SLAM. This type I glycoprotein surface expression is rapidly upregulated on T cells upon activation (also on B, NK and dendritic cells) [56]. Signaling through SLAM induces IFN-  $\gamma$  expression and redirects Th2 responses to Th1/Th0 phenotypes. Therefore, SLAM is considered a key regulator of the T cell cytokine patterns [57,58]. We found in preimplantation endometrium of RSA patients a significant increase in the expression of SLAM protein [59]. Increased

levels of SLAM were also detected in peripheral blood T cells from RSA patients. Thus, SLAM expression might represent an additional useful marker for RSA. On the other hand, SLAM expression and intracellular CD69 expression did not correlate, supporting the hypothesis that the increased intracellular expression of CD69 observed in the T cells from RSA patients might be more related to an immune-regulatory function rather than representative of an activated state [59].

#### MODULATION OF RANTES, CD69 AND SLAM AFTER PATERNAL IMMUNIZATION OF RSA PATIENTS

Alloimmunization as a treatment for RSA, was controversial due to the lack of enough controls to evaluate its effectiveness. Clinical trials have both supported [60] and refuted [61] this method of immunotherapy for the treatment of RSA. In 1994 a world-wide collaborative study demonstrated that this treatment may be useful for RSA patients [62]. A study performed in our institution indicated that RSA-patients under treatment with paternal leukocyte have an 89% live birth rate after immunotherapy [63]. This value was not significantly different from the live births observed in the non-immunized RSA women "control group". However, the absolute difference in livebirth rates between treatment and control group was 18%, suggesting one more time that this treatment may be useful for RSA patients [63]. Immunotherapy with paternal leukocytes has proven to induce the production of BFs [62,63]. Moreover, recent studies have shown that this treatment upregulates CD8<sup>+</sup> T-cell-derived RANTES, MIP-1 and MIP-1 chemokines with immunosuppressive properties [64]. However, it was also reported that BFs were equally detected in couples with either success or failure in reaching a live birth, so BF may represent an epiphenomenon. These data evidence the need to continue the search for new reliable markers for a successful pregnancy.

In this context, the above mentioned "potential markers" for RSA patients: RANTES, CD3<sup>+</sup>CD69<sup>+</sup>, CD3<sup>+</sup>SLAM<sup>+</sup> were followed up in RSA women alloimmunized with paternal leukocytes. RANTES production increases after immunotherapy, in a way that resembles what occurs after a successful pregnancy, suggesting that both situations are associated with alloimmune stimulation. Several studies have described the role of the  $\gamma$ -chemokines as regulators of the immune response [67-65]. In particular, Wang et al [64] have shown that after paternal alloimmunization, CD8<sup>+</sup> cells up-regulate the secretion of the immunoregulatory chemokines RANTES, MIP-1 and MIP-1.

The frequency of CD3<sup>+</sup>CD69<sup>+</sup> and CD3<sup>+</sup>SLAM<sup>+</sup> cells was significantly reduced after immunotherapy with a progressive decrease along the different immunotherapy-doses. Therefore, immunization with paternal leukocytes might down-regulate the frequency of CD69<sup>+</sup> and SLAM on CD3<sup>+</sup> T cells and RANTES sera-levels from RSA women to values similar to those observed in fertile women [59].

#### CONCLUSIONS

Peripheral tolerance, like immune privilege has been proposed to be an active process that employs evolutionarily

conserved mechanisms such as apoptosis, anergy, or T cell regulation to induce cell death and immune tolerance.

Recent studies demonstrated that alloantigen-driven T-cell death mediated by Fas ligand and TNF- may not be essential for the induction of allograft acceptance and suggested that alternative apoptotic pathways might participate in alloantigen-driven T-cell apoptosis [68]. In this sense, we have found that Gal-1 and RANTES have immunosuppressive effects on the allogeneic response through apoptotic and non-apoptotic mechanisms and might also represent an alternative BF induced during successful pregnancy [38,49]. The specific ability of both molecules to downregulate T-cell responses suggests that it might be relevant for fetal-tolerance induction and could be potentially used to avoid recurrent miscarriage. In addition, women with successful pregnancies or following paternal immunization displayed increased levels of RANTES indicating its potential use for the follow up of RSA patients.

Interestingly, allogeneic leukocyte immunotherapy reduced this T cell expansion returning it to levels which were similar to the ones detected in non-pregnant healthy females [69]. Within this context, we analyzed the frequency of CD69<sup>+</sup> and SLAM<sup>+</sup> T-cells both in the endometrium and in the peripheral blood of RSA women in their role to

regulate a putative expanded effector T cell population. This idea is supported by the significant decrease of CD3<sup>+</sup>CD69<sup>+</sup> in response to allogeneic leukocyte immunotherapy, mirroring a similar reduction of the T cell clonal expansion induced by this treatment in RSA women [69].

All the data presented above point to a RSA "pathological model-pattern" defined by low levels of RANTES in serum and high frequency of intracellular CD69 and SLAM on endometrial and peripheral T cells, during the secretory phase of the menstrual cycle (see Fig. 1). In addition, after paternal immunotherapy, RSA patients modulate both, RANTES sera levels and the frequency of CD3<sup>+</sup>CD69<sup>+</sup> and CD3<sup>+</sup>SLAM<sup>+</sup> subsets to those values observed in fertile women. In summary, allopaternal immunotherapy in RSA patients induces a high rate of livebirth and also the modulation of the herein-described novel potential markers for RSA.

Investigation of the molecular mechanisms leading to immune tolerance and homeostasis will contribute to delineate novel therapeutic strategies to prevent immunologically-mediated recurrent spontaneous abortions. Further studies will be required to assess the clinical, diagnostic and therapeutic applications of our results.

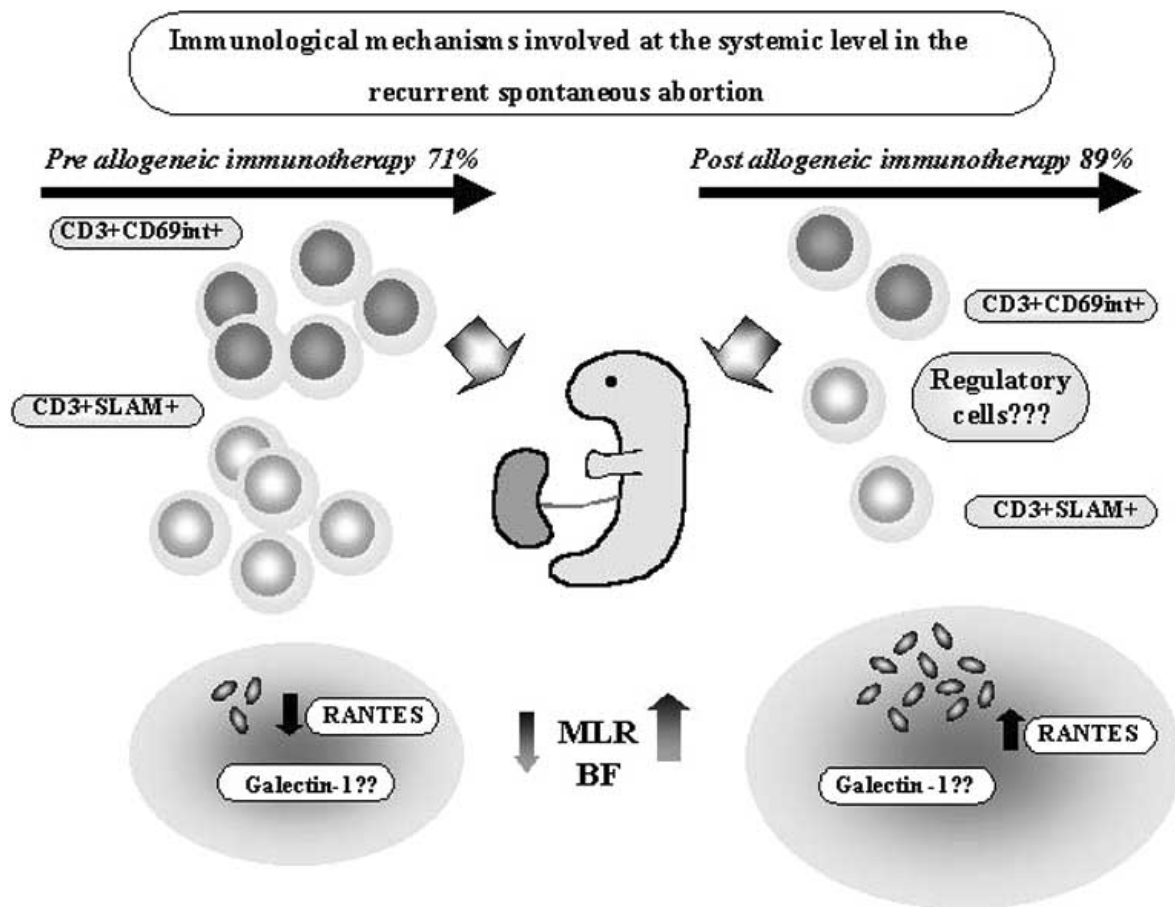


Fig. (1).

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