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A physiological role for inducible FOXP3$^+$ TREG cells Lessons from women with reproductive failure ☆

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Abstract We have previously shown a decreased frequency and function of Tregs in women suffering from recurrent spontaneous abortions (RSA). In the current study, we first investigated the expression of FOXP3 after T-cell activation. We observed that expression of FOXP3 in activated PBMCs was already present above baseline before any cell division, indicating that it was induced in cells that were previously negative for this transcription factor. Because RSA women showed a more limited expansion of FOXP3-positive cells, we next assessed the role of IL-2 signaling through STAT5, which is known to be required for generation of inducible Tregs (iTregs). We demonstrated not only that TGF-β and IL-2 were diminished but also that the IL-2–STAT-5 signaling axis was down regulated in RSA women. Finally, in addition to a limited FOXP3$^+$ cells expansion in vitro, iTregs from RSA women showed a strikingly lower suppressor activity.

Introduction

Regulatory T cells (Tregs) play an indispensable role in maintaining immunological unresponsiveness to self antigens and in suppressing excessive immune responses that are deleterious to the host [1]. Natural Tregs (nTregs) are produced in the thymus as a functionally mature subpopulation of T cells and comprise a population of cells enriched in CD4$^+$CD25$^+$ T cells that express the transcription factor forkhead box P3 (FOXP3). FOXP3 expression is necessary and sufficient for Treg development and

Abbreviations: Treg, regulatory T cell; nTreg, natural regulatory T cell; iTreg, inducible regulatory T cell; iPBMC-Treg, inducible PBMC–Treg cells; FOXP3, forkhead box P3; RSA, recurrent spontaneous abortion.

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function, and it has become a reliable marker for the Treg lineage. These thymus-derived natural Treg cells retain a stable phenotype following export into the periphery. There, they can become activated by specific antigens and acquire some of the phenotypic properties of effector memory T cells, such as the capacity to migrate into inflamed peripheral tissues, while maintaining FOXP3 expression and their suppressive function. Early in 1980, we demonstrated that regulatory or suppressor T cells can be induced as part of an adaptive response [2], and more recently, it was also shown that induced Tregs (iTregs) can be generated in peripheral lymphoid organs by IL-2 and TGF-β from $CD4^+CD25^-$ precursors [3]. These iTregs share a similar phenotype with nTregs, and IL-2 and TGF-β seem to have a nonredundant role in the maintenance and survival of both types of Tregs [4–6]. Little is known about the physiological role of inducible Tregs during the normal immune response. Putative conditions in which iTregs may play a central role can be associated with the expansion of Tregs observed in the preimplantation phase of the menstrual cycle [7] and during pregnancy [8–12]. Similarly, the high frequency of Tregs observed in patients with cancer [13–20] or during the course of chronic infections like HIV or HCV [21–24] could be representative of the expansion of iTregs.

Pregnancy constitutes a major challenge to the maternal immune system, as it must tolerate the persistence of paternal alloantigens. Although localized mechanisms contribute to fetal evasion from immune attack, maternal alloreactive lymphocytes persist [12,25,26]. During the menstrual cycle, Tregs from fertile women are expanded in tight correlation with their serum levels of estradiol. However, despite normal serum levels of this hormone, women who have had recurrent spontaneous abortions (RSA) not only failed to increase Tregs but also showed a decreased suppressor function of their Tregs [7].

IL-2 regulates T-cell function by activating the JAK–STAT signaling pathway, and it has been reported that IL-2 signaling is required for peripheral expansion and suppressive activity of Tregs [27,28]. Human studies that have monitored Treg cells during immune reconstitution in cancer or in lymphopenic patients receiving IL-2 therapy detected a homeostatic peripheral expansion of $CD4^+CD25^+FOXP3^+$ cells [29]. In addition, it was shown that IL-2 is essential for the TGF-β induction of $FOXP3^+$ Treg cells from naive $CD4^+$ T cells [30]. A recent study that determined the relationship between IL-2 and FOXP3 demonstrated that the transcription factor and IL-2 expression are mutually exclusive, especially upon restimulation [31,32]. However, many of the molecular factors that regulate the development and homeostasis of both types of Treg cells remain to be fully elucidated.

In the present study, we first investigated the cause of the diminished expansion of Tregs we previously detected during the menstrual cycle of women with RSA [7] by focusing on the earliest TCR activation events of Tregs. In particular, we assessed the role of IL-2 signaling through STAT5, which is known to participate in the generation of Tregs [33]. We also investigated the rationale for our previous finding that in addition to the diminished expansion, Tregs from RSA patients showed decreased suppressor activity.

Patients and methods

Subjects

We evaluated samples of peripheral blood from two different groups collected at the late follicular phase (days 9–12) of the menstrual cycle. Group 1 included 40 fertile women (who had at least one successful pregnancy and no previous abortions) with regular menstrual cycles (mean age 34.19 years, range 22–42 years). Group 2 included 40 women with a history of three or more consecutive RSAs that occurred before week 12 of gestation (mean age 30.13 years, range 22–45 years). The study excluded those women suffering autoimmune diseases or carrying autoantibodies, infectious, endocrine, and anatomic pathology. None of the women from this study was taking oral contraceptives. This investigation was approved by the "Investigation and Ethics Committee at the Hospital de Clínicas José de San Martín," and informed consent was obtained from all subjects enrolled.

Cell isolation

Peripheral blood mononuclear cells were isolated from 30 ml of heparinized peripheral blood through density gradient centrifugation using the Ficoll-Hypaque technique (Amersham Biosciences). Cells were washed twice in PBS (Sigma Aldrich) before their use in the following studies.

Flow cytometry

PBMCs were depleted of monocytes by adherence to plastic Petri dishes for 1 h at 37 °C and were stained with FITC- or PE-labeled mAb specific for CD4, CD25, CD14, CD45, and CD8 (BD Biosciences). Depletion was performed to avoid the nonspecific binding of mouse monoclonal antibodies to human cell surface antigen present in this population. Negative control samples were incubated with an isotype-matched antibody. Cells were analyzed in a FACSCalibur cytometer using WinMDI software (BD Biosciences). Dead cells were excluded by forward and side scatter characteristics. Statistical analyses are based on at least 30,000 events gated on the population of interest. As the overall numbers of $CD4^+$ cells remained constant, the frequency of the $CD4^+CD25^+FOXP3^+$ population was expressed as a percentage of all $CD4^+$ T lymphocytes.

Intracellular staining for detecting endogenous FOXP3

For intracellular staining, cells were first stained with mAb Cy-Chrome–anti-human–CD4 and FITC–anti-human–CD25 (BD Biosciences) simultaneously for 20 min at RT, followed by washing in PBS. Afterwards, cells were fixed and permeabilized followed by intracellular staining with PE-labeled anti-human FOXP3 (clone 259D/C7) according to the manufacturer's instructions (BD Biosciences), and cells were analyzed immediately.

Intracellular staining for P-STAT-5

Cells (1×10^6) were starved for 2 h in serum-free medium. After this period, cells were either stimulated with 100 ng/ml recombinant IL-2 cytokine (R&D) or vehicle for 15 min at 37 °C. Cells were then fixed with BD™ Cytofix Buffer (BD Biosciences) for 10 min at 37 °C, and then permeabilized by adding BD™ Phosflow Perm Buffer III (BD Biosciences) for

30 min on ice. Cells were washed twice with PBS and stained with the Alexa Fluor 647 mouse anti-STAT-5 (pY694) mAb for 30 min at RT. For subset analysis, cells were also incubated with the appropriate surface marker (anti-CD4 PE mAb) as a final step in the protocol. Cells were then washed and analyzed on a BD FACSAria II flow cytometer. The same samples were stimulated with decreasing concentrations of IL-2 (100 ng/ml, 1 ng/ml, and 0.01 ng/ml), and FOXP3 expression was determined for each condition.

Generation of inducible PBMC–Treg cells

PBMC were isolated from whole blood using centrifugation over a Ficoll-Hypaque density gradient. To induce the expansion of inducible PBMC-Treg cells, total PBMCs were activated at 1×10^6 cells/ml in 24-well plates (Greiner Bio-One) with 1 μg/ml PHA (Sigma Aldrich) in complete RPMI 1640 medium (Sigma Aldrich) containing 10% heat-inactivated FBS (Natocor), 200 mM L-glutamine (Sigma Aldrich) and gentamicin. Culture medium was completely changed and supplemented with IL-2 (5 ng/ml, R&D Systems) for 72 h. Cells were then cultured in a 37 °C humidified incubator with 5% CO_2 for 5 days. FOXP3 analyses were performed every 24 h or as indicated by flow cytometry, and the supernatants were collected for cytokine quantification. In similar experiments, prior to culture, freshly isolated PBMCs were first labeled with 10 μM 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes), as recommended. To investigate the role of IL-2 role in the maintenance of iPBMC–Treg cells, in similar experiments, cells were cultured for 9 days, and to prevent overcrowding in long-term cultures, wells were examined and split every 3 days into medium supplemented with this cytokine.

Suppression assay

We performed suppression assays using inducible Tregs ("iPBMC-Treg cells" indicates that they were obtained after 5 days of culture, unless otherwise specified) as suppressor cells.

An MLR was performed by mixing 5×10^4 resting PBMCs from the same female donor and stimulating them with 5×10^4 mytomicin-treated PBMCs from HLA-mismatched allogeneic male partner per well. Because of the relevance of HLA matching in the generation of Treg [34], we included only those couples with a strong MLR showing at least one or two HLA-DR mismatches.

The suppressor activity was measured by adding the following ratios of iPBMC–Treg cells to the MLR: 1:1 (e.g., 5×10^4 resting PBMCs and 5×10^4 iPBMC–Treg cells), 2:1, 5:1, 10:1, or 20:1, in 200-μl final volumes. Cells were cultured in complete medium for 5 days. Cell proliferation was measured by adding 1 μCi of [^{3}H]-thymidine (PerkinElmer Life and Analytical Sciences) for the last 18 h of culture. In addition, iPBMC–Treg cells mixed with stimulator cells were also cultured for the same period of time to assess their state of anergy, and the response was always lower than that in the autologous control. The percentage of suppression was calculated as follows:

$$\frac{[(\text{MLR plus ratio of iPBMC–Treg}) - (\text{autologous control})]\times 100}{(\text{MLR without iPBMC–Treg}) - (\text{autologous control})}$$

Cytokine quantification

Levels of IFN-γ were determined in 24-h and 9-day culture supernatants using the appropriate Endogen reagents. Human IL-2 and IL-6 levels in cell culture supernatants or in serum from fertile women and RSA patients were determined by ELISA using Ready-SET-GO! as recommended (eBioscience). TGF-β1 was measured in cell culture supernatants using BioSource equipment.

Immunoblotting

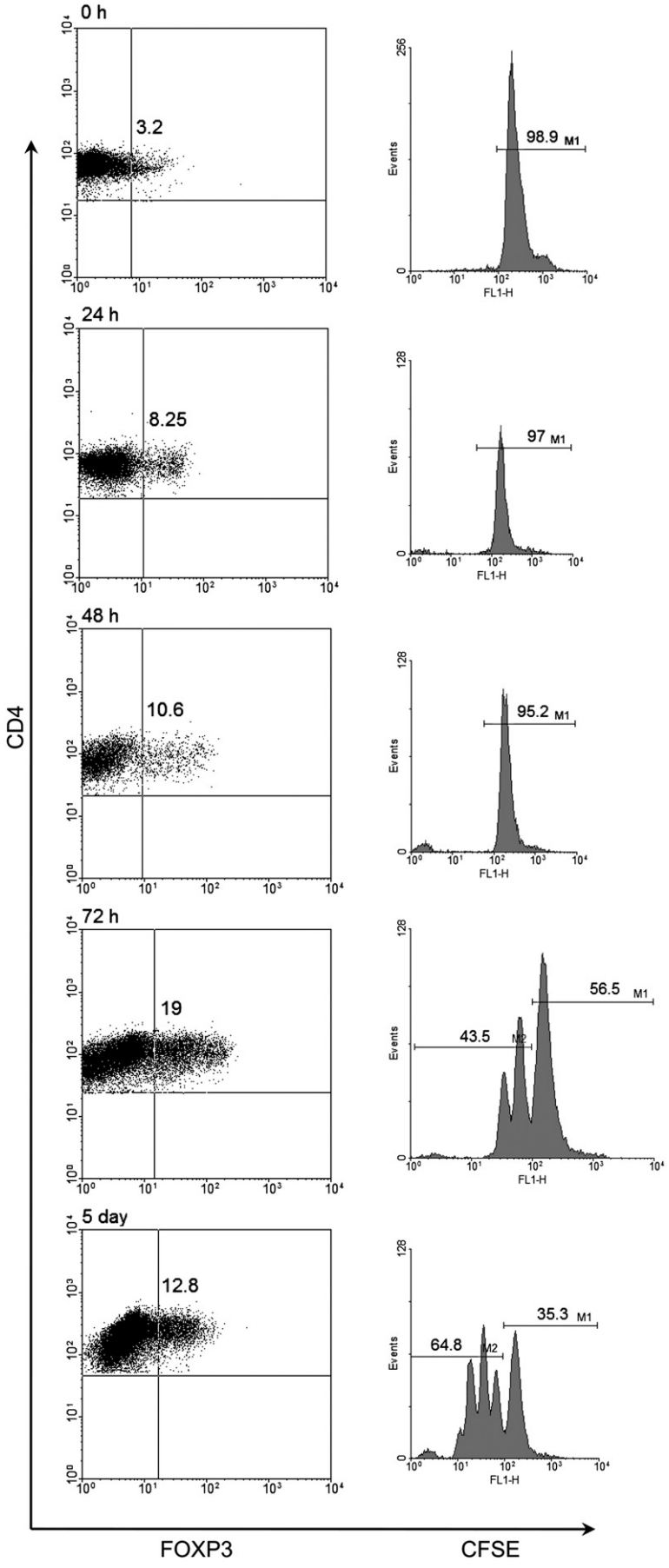
Cells were starved for 2 h in serum-free medium, after which, 3.5×10^6 cells were stimulated with 1 ng/ml or 0.1 ng/ml recombinant IL-2 cytokine (R&D) or vehicle for 15 min at 37 °C. After pelleting, cells were solubilized with 1% Triton X-100, 100 mM HEPES, pH 7.4, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF, and 0.035 trypsin inhibitory units/ml aprotinin. Homogenates were centrifuged at 15,000×*g* at 4 °C for 30 min to remove insoluble material. Protein concentrations of supernatants were determined by the BCA method (BCA protein assay kit; Thermo Scientific). An aliquot of solubilized cells was diluted in Laemmli buffer, boiled for 5 min, and stored at −20 °C until electrophoresis.

Samples (10 or 4 μg of solubilized protein) were resolved by SDS–PAGE under reducing conditions using the Bio-Rad Mini Protean apparatus (Bio-Rad Laboratories). Electrotransfer of proteins from the gel to PVDF membranes was performed for 45 min at 0.1 mA/membrane (constant) using a semidry transfer apparatus in buffer containing 25 mM Tris, 192 mM glycine, 20% (vol./vol.) methanol, pH 8.3, containing 0.03% SDS. To reduce nonspecific binding, membranes were incubated for 2 h at room temperature in T-TBS blocking buffer (10 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.02% Tween 20) containing 3% BSA. The membranes were then incubated overnight at 4 °C with αP-STAT5 (Tyr 694/699, Millipore; 1:1000), αSTAT5b (C17 Santa Cruz Biotech; 1:20,000), or αactin (Sigma-Aldrich; 1:1000). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences) using pre-flashed Kodak XAR film. Band intensities were quantified by Gel Pro Analyzer 4.0 software (Media Cybernetics).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 3.0 for Windows (GraphPad software). Paired *t*-test with equal variance was used to analyze paired samples. When comparing two groups, significant differences were

Figure 1 In vitro inducible PBMC–Treg cells were derived from FOXP3− precursors. CFSE-labeled PBMCs (1×10^6 cells/ml) were stimulated with PHA for 5 days in culture. At different time points, proliferation was measured based on CFSE signal, and the frequency of $CD4^+FOXP3^+$ cells was analyzed by flow cytometry. A FACS profile from one representative donor ($n=3$) shows the frequencies of $FOXP3^+$ cells observed at 0, 24, 48, and 72 h and at day 5 of cell culture. The expansion of inducible PBMC–Treg cells reached its peak level at 72 h; at that time, according to CFSE labeling, cells had entered the first division.


0 h
3.2
98.9 M1
24 h
8.25
97 M1
48 h
10.6
95.2 M1
72 h
19
56.5 M1
43.5 M2
5 day
12.8
35.3 M1
64.8 M2
CD4
FOXP3
CFSE
Events
FL1-H

determined by two-tailed unpaired *t*-test. The Mann–Whitney test was used to evaluate possible differences in function between women with RSA and fertile women. When comparing six groups (Fig. 5D), significant differences were determined by one-way ANOVA followed by the Newman–Keuls post-test. *P* values were considered significant if they were less than 0.05. The data had a normal distribution (Kolmogorov–Smirnov test).

Results

Differential kinetics of IL-2-induced FOXP3 expression in fertile women and in women with RSA

We first investigated the expression of FOXP3 after T-cell activation. One representative experiment ($n=3$) of a fertile woman (Fig. 1) showed that increased expression of FOXP3 in activated PBMCs was already present above baseline after 24 h of PHA activation. The frequency of FOXP3 positive cells increased from 3.2% at time 0 h to 10.6% at 48 h. It is important to note that this increase was observed before any cell division could be detected by CFSE staining (division = 0), indicating that the expression of this transcription factor was induced in cells that were previously negative for the expression of FOXP3. The expansion of inducible FOXP3$^+$ PBMC–Tregs reached its peak level at 72 h, at the time cells had entered their first division (19.0%). In this representative experiment, the expression of FOXP3 started to decrease after 72 h. Those activated PBMCs showing the induction of a relatively high frequency of FOXP3 will be referred to as inducible PBMC–Tregs (iPBMC-Tregs).

We next compared the induction of FOXP3 in PHA-activated PBMCs from five fertile women with five women suffering from RSA. In the fertile control group, at $t=0$, when cells could be ascribed to a homogeneous single CFSE peak (division = 0), the constitutive expression of FOXP3 had a mean ± SEM value of 5.4% ± 1.56. At 24 h, the FOXP3 expression increased to a mean ± SEM value of 9.25% ± 2.35, which increased to 13.75% ± 3.54 at 48 h when all cells remained undivided. At 72 h, in concordance with the first cell division, the expression of FOXP3 reached a mean value of 19.2% ± 2.2, which persisted after day 5 when cells had entered a new cell division (Fig. 2). Thereafter, without IL-2 addition, the frequency of FOXP3$^+$ cells declined progressively, returning to nearly baseline levels after 5 days in culture (data not shown).

PBMC–Tregs (iPBMC–Tregs) in RSA women showed a different kinetics. Expression of FOXP3 reached its peak at 48 h, (t = 0; 4.6% ± 1.2, t = 24 h; 6.0% ± 2.3; t = 48 h; 13.0% ± 4.0), but in contrast with the profile of fertile women, at 72 h (the time of the first PBMC cellular division), the expression of FOXP3 started to decrease (t = 72 h, 12.67% ± 2.6; t = day 5, 10.0% ± 2.5). Thus, we next investigated whether addition of IL-2 could restore the different kinetics observed in controls and RSA patients. After 72 h of PHA activation, PBMCs from control and RSA patients were supplemented with the addition of IL-2 every three days, and the expression of FOXP3 was assesses at day 9. In fertile women, the mean ± SEM frequency of CD4$^+$CD25$^+$FOXP3$^+$ cells was 21.36% ± 2.1 vs. 6.29% ± 0.47 at t = 0 ($p<0.0001$, n = 34). In contrast the addition of IL-2 to PHA-activated PBMCs from 32 RSA women showed a more limited expansion of FOXP3+ cells (12.38% ± 1.8 vs. 4.9% ± 0.4 at t = 0;

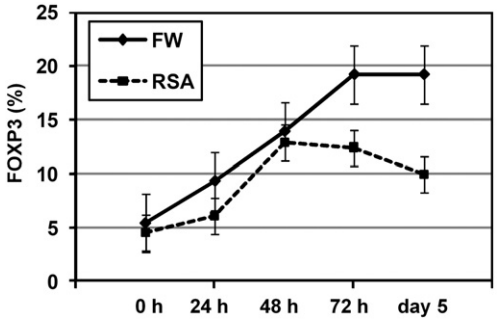


Figure 2 Differential kinetics of induced FOXP3 expression in fertile women and in women with RSA. The frequency of CD4$^+$ FOXP3$^+$ cells within the CD4$^+$ population from controls ($n=5$) and women with RSA ($n=5$) was determined by flow cytometry at different time points. Fertile women (continuous line) showed an expansion of FOXP3 expression at 72 h of culture followed by a slow decrease over the following days. In contrast, RSA women (dotted line) reached peak expression of FOXP3 at 48 h, followed by a decrease at 72 h, when PBMCs began their first cellular division. Data are presented as mean ± SEM.

$p=0.0003$, Fig. 3), suggesting that RSA patients may have a deficient intracellular response to IL-2 stimulus.

Diminished levels of IL-2 and TGF-β and increase secretion of IL-6 in RSA women

It was reported [31,32] that addition of exogenous IL-2 to anti-CD3-activated PBMCs at t = 0 did not accelerate FOXP3 expression, but did sustain its presence. We observed that, in contrast with fertile controls, RSA women did not increase FOXP3 expression after 48 h of activation. This observation raised the question of whether the secretion of IL-2 by RSA

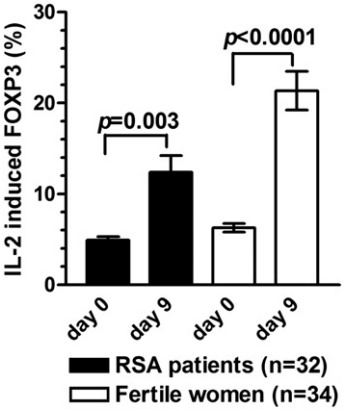


Figure 3 RSA women showed a limited expansion of IL-2-induced Tregs. After the first 72 h of culture, PHA-activated PBMCs were supplemented every 3 days with IL-2, and expression of FOXP3 was assessed at day 9. The frequency of CD4$^+$FOXP3$^+$ cells within the CD4$^+$ population was determined by flow cytometry. Fertile women showed a significant expansion of iPBMC–Treg cells in response to IL-2, which contrasted with a more limited expansion in RSA women. Data are presented as mean ± SEM, and the *p* value was analyzed by unpaired *t*-test.

patients could be impaired. Comparison of the serum levels of IL-2 in fertile (n=20) and RSA women (n=24), showed a significant decrease of expression in RSA patients (24.0±5.3 pg/ml vs. 56.42±13.76 pg/ml in controls; $p=0.02$). Inversely, IL-6, a cytokine reported to downmodulate Tregs [35], was significantly increased in 35 RSA women (7.2±0.7 pg/ml vs. 5.3±0.5 pg/ml in 35 fertile controls; $p=0.03$, Fig. 4A).

In addition of the evaluation of serum levels, we measured the secretion of IL-2, IL-6, TGF-β and IFN-γ in the supernatants of 24 h activated-PBMCs from 30 fertile women and 30 RSA patients. As depicted in Fig. 4B, the levels of IL-2 and TGF-β1 were significantly diminished in RSA women (IL-2: 17.72±5.2 pg/ml vs. 64.72±10.78 pg/ml in controls, $p=0.003$; TGF-β1: 247.0±44.62 pg/ml vs. 580.4±106.7 pg/ml in controls, $p<0.005$).

Confirming the sera results, IL-6 levels were significantly increased in supernatants from the activated PBMCs of RSA women (6795.0±621.4 pg/ml vs. 3892.0±710 pg/ml in controls; $p=0.004$). Additionally, RSA women also produced larger amounts of IFN-γ (3408.0±825 pg/ml vs. 1082.0±310 pg/ml, $p=0.009$).

Role of IL-2 and TGF-β in the generation of human iTreg

After detection of decreased levels of IL-2 and TGF-β in RSA patients, we investigated the role of both cytokines over the expansion of iTregs. There is now considerable evidence indicating a crucial nonredundant role for TGF-β and IL-2 in the generation of iTregs [36]. However, few of these experiments were performed in humans. Thus we performed five independent experiments comparing the effects of the addition of IL-2 and TGF-β to resting human PBMC depleted of CD4$^+$CD25$^-$ cells. Cells were cultured during 5 days at 2×10^6 cells/well in a 24-well plates coated with anti-CD3 (10 μg/ml, clone UCHT1, Immunotech) and soluble anti-CD28 (1 μg/ml, BD Bioscience), or with PHA, and were resuspended in free-serum culture medium. The single addition of IL-2 (5 ng/ml) or TGF-β (10 ng/ml) induced the expression of FOXP3$^+$ cells (22.3%±3.6 and 37%±6.4 for IL-2 and TGF-β respectively). When compared with the effect of the addition of IL-2, the simultaneous addition of IL-2 and TGF-β did not increase the levels obtained by the single addition of TGF-β (35.3%±4.8). These results indicate that IL-2 did not have and additive effect over TGF-β on the induction of iTregs.

Lower P-STAT-5 levels in RSA patients

As described above, the addition of IL-2 to long term culture of PBMCs from RSA patients did not achieve the levels of FOXP3$^+$ observed in controls. We did not find differences in the MFI expression of the α-chain of the IL-2R (data not shown). Therefore, we explored if besides a lower production of IL-2, RSA patients may have a defective response to the IL-2 signaling. Upon IL-2 binding to its receptor (IL-2R), associated

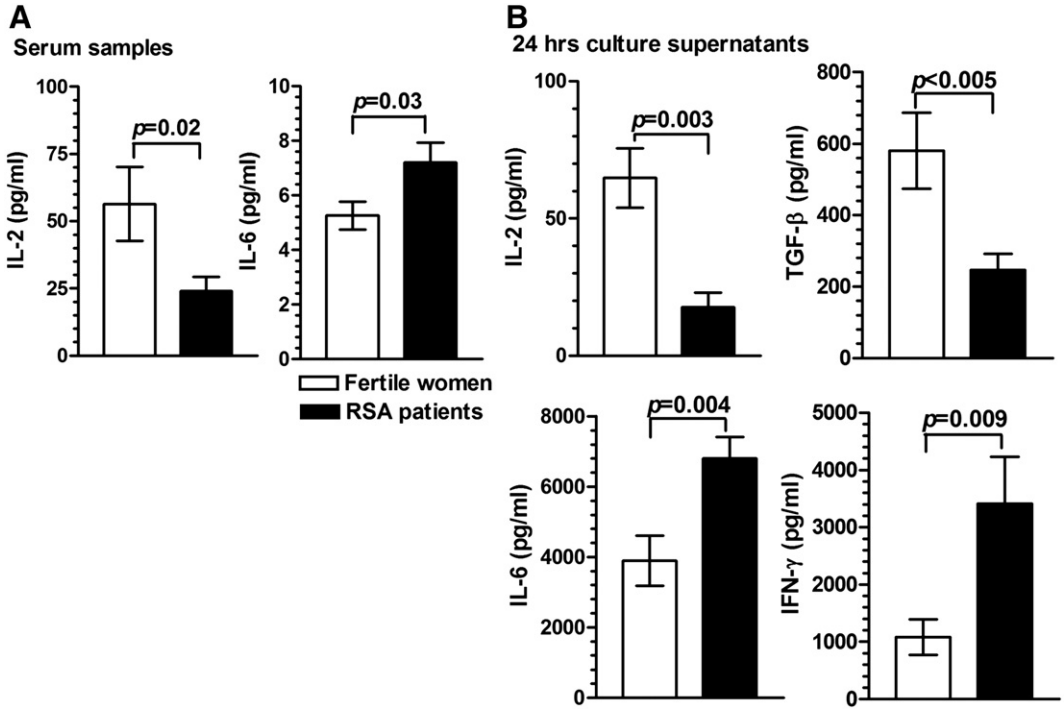


Figure 4 Women with RSA showed decreased levels of IL-2 and TGF-β and increased secretion of IL-6. (A) Serum levels of IL-2 and IL-6 were measured by ELISA. RSA patients ($n=24$) showed a significant decrease of IL-2 level with respect to fertile women ($n=20$, $p=0.02$). In comparison with controls ($n=35$), the serum of 35 RSA women showed a significant increase of IL-6 levels ($p=0.03$). (B) Levels of IL-2, IL-6, TGF-β, and IFN-γ were also measured by ELISA in 24 h supernatants of PHA-stimulated PBMCs from fertile women ($n=30$) and RSA women ($n=30$). Significantly higher levels of IFN-γ and IL-6 levels were found in supernatants from RSA patients, which also showed lower levels of IL-2 and TGF-β. Data are presented as mean±SEM, and the p value was analyzed by unpaired t-test.

Janus-activated kinases (JAK) 1 and 3 become activated. These tyrosine kinases, in turn, phosphorylate the cytoplasmic domains of the IL-2Rβ and IL-2Rγ subunits of the IL-2R creating docking sites for intracellular proteins, including STAT-5. After is recruited to the receptor complex and become phosphorilated, STAT-5 dimerizes and translocates to the cell nucleus where it directs the transcription of multiple IL-2-responsive genes. Because STAT5 regulates FOXP3 expression [33], we analyzed whether this transcription factor plays a role in the differential expression of FOXP3 observed in RSA patients and controls. With this purpose, we investigated the frequency of $CD4^+$ P-STAT-5^+ cells in response to IL-2 by comparing the flow cytometry response of PBMCs of fertile women ($n=10$) and RSA patients ($n=11$) to 15 min of stimulation in the absence or in the presence of decreasing concentrations of IL-2. Although the frequency of $CD4^+$P-STAT-5^+ cells in response to IL-2 usually shows individual variability, the stimulus with 100 ng/ml of IL-2 (maximum stimulus) demonstrated a significantly limited expression in RSA patients (70.25%±4.9 vs. 50.82%±6.0 in RSA, $p=0.01$; Fig. 5A). In addition, the strong correlation between levels of P-STAT-5 with the dose of IL-2 used as stimulus suggested that the decreased phosphorylation of this transcription factor may be due to an intrinsic deficiency in STAT-5 activation (Fig. 5B). Immunoblotting also confirmed decreased STAT-5 phosphorylation. The preliminary dose–response curves using IL-2 concentrations ranging from 100 ng/ml to 0.01 ng/ml demonstrated that 1 ng/ml and 0.1 ng/ml are the lowest doses that are able to induce phosphorylation (data not shown). Accordingly, to evaluate phosphorylation, cells from fertile and RSA women were treated with these two IL-2 concentrations or with vehicle alone. While in the absence of stimulation, no STAT-5 phosphorylation was observed in either group; RSA women showed lower STAT-5 phosphorylation levels in response to 1 ng/ml of IL-2 (35.33%±13.30 vs. 100%±16.67 in fertile controls, $p=0.001$, $n=6$; Fig. 5C). Although STAT-5 protein content also showed lower levels, this difference did not reach statistical significance (62.17%±20.42 vs. 100%±7.19, $n=6$, $p=$ns). To exclude the possibility that lower phosphorylation status could be related to the lower protein content, we calculated the P-STAT-5/STAT-5 ratio. For the higher dose of IL-

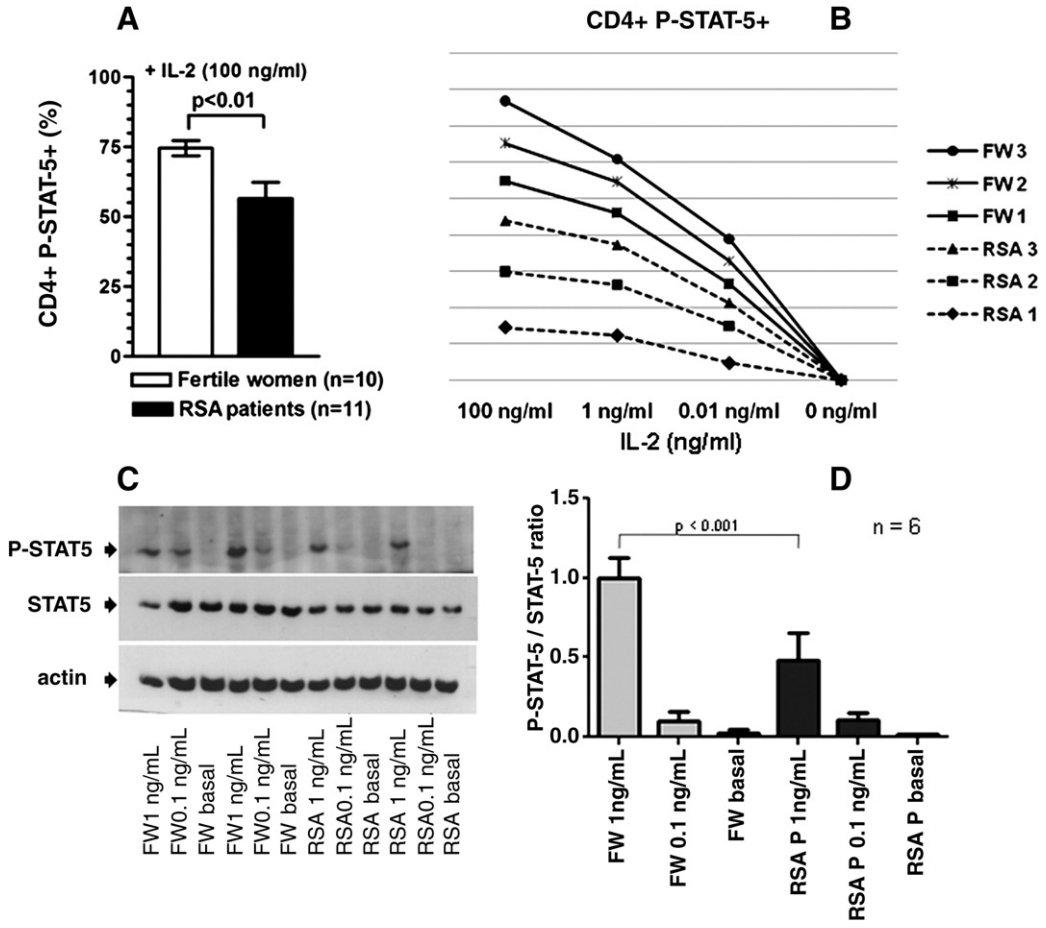


Figure 5 Lower P-STAT-5 levels in RSA patients. (A) The frequency of $CD4^+$ P-STAT-5^+ cells in response to maximum IL-2 stimulus (100 ng/ml) in 11 RSA patients was compared with the response of 10 fertile women by flow cytometry. A deficient phosphorylation of STAT-5 was observed in RSA patients ($p<0.01$). (B) Similar to (A), but showing the response to increasing concentrations of IL-2. Data correspond to three fertile women and three RSA patients. (C) Immunoblotting from PBMCs obtained from fertile women (FW) and RSA patients (RSA) that were untreated (basal stimulation) or treated with 1 ng/ml or 0.1 ng/ml of IL-2. (i) STAT-5 phosphorylation at Tyr 694/699; (ii) STAT-5 protein content; (iii) actin content. (D) P-STAT5/STAT5 ratio. Statistical difference was assessed by one-way ANOVA followed by Newman–Keuls post-test. Results represent six different individuals from each group (control or patient) as assessed in three independent experiments. Representative blots are shown ($n=2$ per blot).

2, the ratio was 0.48±0.17 for RSA women vs. 1.00±0.13 ($p<0.001$; Fig. 5D) for fertile controls. These results confirmed the lower activation of STAT-5 induced by IL-2 in RSA patients. Actin was used as a protein load control and showed similar results as those obtained for STAT-5 protein content (76.18%± 9.14 vs. 100%±3.14, $n=6$, $p=$ns), suggesting a protein imbalance in the patients.

Inducible PBMC–Treg cells from fertile women showed higher suppressor activity

The supernatants of iPBMC–Treg cells obtained from RSA patients after 9 days exhibited larger amounts of IFN-γ when compared with those obtained from fertile controls (10550.0± 3081 pg/ml vs. 2242.0±1057 pg/ml, $p<0.001$, $n=17$). We speculate that these results might arise from a higher suppressor activity present in the iPBMC–Treg cells obtained in healthy controls. This assumption is supported by the observation that iPBMC–Treg cells from RSA women showed a decreased expansion of their FOXP3+ cells (Fig. 2). Therefore, we set up an MLR to measure the ability of iPBMC–Tregs from RSA and control women to inhibit the proliferation of resting PBMCs from the same donor in response to paternal allostimulation. As described in the Patients and methods, fixed numbers of resting PBMCs (5×10^4) were stimulated with 5×10^4 allogeneic cells. To establish their suppressor activity, different ratios of iPBMC–Treg cells were added to a fixed number of resting PBMCs as follows: ratio 1:1 (5×10^4 iPBMC–Treg), ratio 2:1 (2.5×10^4 iPBMC–Treg), ratio 5:1 (1×10^4 iPBMC–Treg), ratio 10:1 (0.5×10^4 iPBMC–Treg), and ratio 20:1 (0.25×10^4 iPBMC–Treg). Each experiment compared the suppressive effect of iPBMC–Tregs from fertile women to that of iPBMC–Tregs obtained from a woman with RSA (Fig. 6; $n=4$). Control iPBMC–Tregs showed a strong suppressor activity upon paternal allostimulation, which remained high even at ratios of resting effectors PBMCs/iPBMC–Treg of 10:1, decreasing at higher ratios. In contrast, a marked reduction of the suppressor activity of iPBMC–Tregs was observed in RSA women. In those patients, a small suppressor activity was only detected at the ratios of 1:1 or 2:1, which disappeared or even became negative at higher ratios of resting effectors PBMCs/ iPBMCs–Treg.

Discussion

In the present study, we investigated the cause of the diminished expansion of Tregs we had detected during the menstrual cycles of women with RSA [7] using a model we previously described, which allowed us to obtain iPBMC–Tregs by polyclonal activation [2]. In line with previous reports [31,32], we demonstrated that the expression of FOXP3 on PBMCs can be detected above baseline before any cell division occurs, indicating that PBMCs activation induces the expression of FOXP3 in cells that did not previously express this transcription factor. Accordingly, we defined these cells as FOXP3+ Tregs induced in activated PBMCs or iPBMC–Tregs. The results described in the present study provide insight to understand the differential behaviour of Tregs in fertile women when compared with RSA patients. RSA patients showed not only decreased serum levels of IL-2 but also showed a limited secretion of this cytokine and TGF-β upon PBMC activation. It is well known that iTregs sharing phenotype with nTregs can be generated in peripheral lymphoid organs by IL-2 and TGF-β from $CD4^+CD25^-$ precursors [3]. Therefore, diminished levels of this cytokines can explain the limited expansion of the iTregs in RSA patients. As reviewed by Horwitz et al. [36], there is now considerable evidence indicating a crucial nonredundant role for TGF-β and IL-2 in the generation of iTregs. However, most experiments were performed in mice and only few

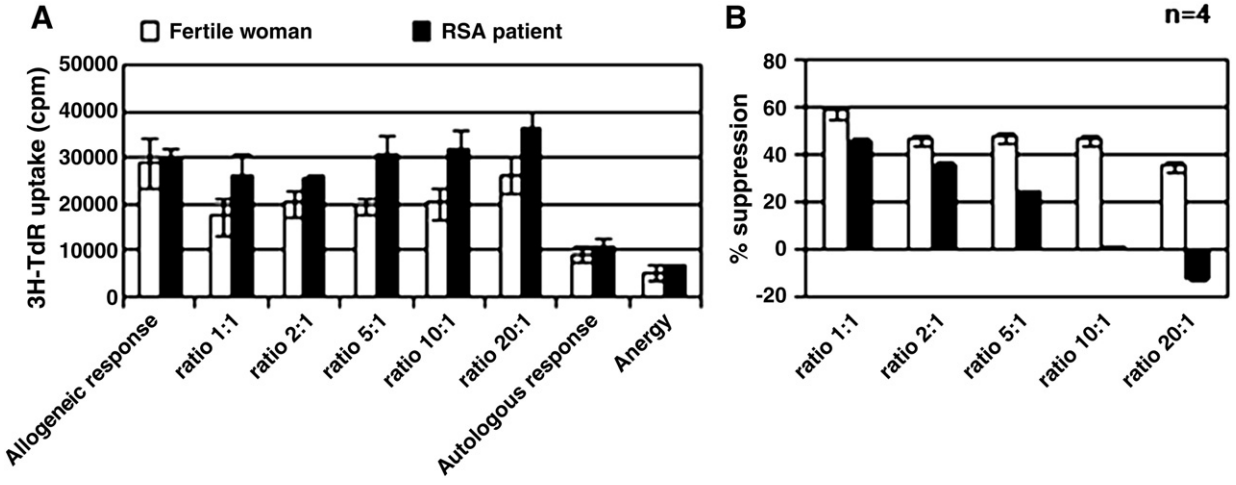


Figure 6 Differential suppressor capacity of inducible PBMC–Treg cells from fertile women and those with RSA. (A) One representative result of four independent MLR experiments performed with different donors. Each experiment compared MLR performed by mixing resting PBMCs with paternal allogeneic PBMCs. The suppressor activity was evaluated by mixing different ratios of iPBMC–Tregs with autologous resting PBMCs obtained from fertile or RSA patients. (B) Four independent experiments examined the percent of suppression obtained at each ratio of resting autologous PBMCs/iPBMC–Tregs. In fertile women, suppressor activity of iPBMC–Tregs remained high up to ratio 10:1, decreasing afterwards. In contrast, suppression induced by iPBMC–Tregs from RSA patients was only present at high ratios. Data are presented as mean±SEM and were analyzed by the Mann–Whitney *U* test.

studies have evaluated the role of TGF-β and IL-2 in the induction of human iTreg cells. Our results indicate that, in addition to a defect in the IL-2 signaling, the decreased secretion of TGF-β could also contribute to the diminished expansion of iTreg we observed in RSA women.

From reports in both mice [37] and humans [33], it has been shown that STAT-5 activation is required for the development of FOXP3$^+$ Tregs. In line with a putative role that involved the IL-2–STAT-5 axis, we also demonstrated that RSA patients showed a limited expression of CD4$^+$ P-STAT-5 cells. Because the levels of P-STAT-5 in controls and patients showed an association with the dose of IL-2 used as stimulus, we assumed that the decreased expression of P-STAT-5 represents an intrinsic defect in STAT-5 expression and/or STAT5 activating capacity that might be added to the observed diminished level of IL-2 detected in RSA patients.

We knew from our early report that inducible Treg cells have a potent suppressor activity [7]. We have now confirmed this potent suppressor activity of iPBMC–Tregs in fertile women, which contrasted with a weak suppressor capacity of iPBMC–Tregs from RSA patients. The suppressor activity detected in fertile control women was clearly demonstrated even at ratios of resting effectors PBMCs/iTregs of 10:1. In contrast, RSA patient suppressor activity was detected only at ratios of 1:1 or 2:1 and disappeared or even became negative for higher ratios. These results are in line with the previously reported functional suppressor deficiency we detected using ex vivo experiments in RSA patients. These results are indicative not only of the limited capacity of RSA patients to induce FOXP3$^+$ Tregs but also of quantitative and qualitative deficiencies in these cells, as reported in our previous study.

Although there has been some disagreement on whether in vitro TGF-β-generated FOXP3$^+$ iTreg cells are as good suppressor cells as nTreg cells, there is consensus that in vivo–generated iTreg cells are effective suppressors [38]. Therefore, although difficult to prove in humans, our in vitro results tempted us to suggest that low in vivo levels of TGF-β could also contribute to the low expansion of iTreg cells we previously reported in vivo along the menstrual cycle.

The present study also showed that IL-6, which has been reported to modulate Tregs [35], was found to be increased in RSA patients. IL-6 has been implicated in the pathogenesis of many autoimmune and chronic inflammatory diseases [39]. In autoimmune diseases such as systemic lupus erythematosus, IL-6 produced by DCs has been reported to block Treg activity [35,40]. However, despite the role assigned to IL-6 in Tregs, little is known concerning the mechanism by which IL-6 modulates this cell type. A recent report demonstrated that IL-6 limits the activity of virus-specific Tregs, thereby facilitating the activity of virus-specific memory CD4$^+$ cells. In this same study, IL-6$^{-/-}$ mice showed impairment of the memory but not the primary response [41]. In mice, IL-6 *trans*-signaling via the soluble IL-6R abrogates both the induction of FOXP3 and its suppressive function in naïve mouse CD4$^+$CD25$^-$ T cells [42]. In line with this hypothesis, we have recently demonstrated an increase in IL-6 and the soluble IL-6R in RSA patients, which is associated with a decrease in soluble gp130, a component which is known to inhibit the *trans*-signaling [43]. In addition, it was reported that activation of naïve CD4$^+$ T cells or isolated nTregs with IL-2 and TGF-β downregulated IL-6 receptor expression and its signaling pathway and caused them to become resistant to Th17 conversion [44].

We can conclude that the deficiency in Treg cells detected in RSA patients results from a diminished induction of inducible Tregs caused by a poor expansion of FOXP3$^+$ cells, mediated by a low production of TGF-β and by the involvement of IL-2 and its signaling through STAT-5. In addition, IL-6 seems to play an important role in abrogating the functional capacity of inducible Treg cells. Therefore, its increased levels in RSA patients may contribute to the impairment of Tregs function during pregnancy. We think that the findings reported in the present study may have relevance in relation to an eventual benefit to either counselling or treatment options for RSA women. For example, use of paternal immunotherapy in the treatment of RSA is a controversial issue. However, in our previous report [40], we demonstrated that the imbalance in the components of the *trans*-signaling and also the frequency of Tregs can be modified by the use of the paternal immunotherapy. Because elevated levels of IL-6 seem to play a role in the pathogenesis of RSA, the use of commercial available anti-IL-6R could be considered as a potential maternal immunotherapy in patients with reproductive failures. In addition, we are currently exploring several transcription factors that may be responsible for the defects we described in the present study on the pathway of the IL-2 signalling, which could be eventually modulated by immunotherapy.

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