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Expansion of CD4$^+$CD25$^+$and FOXP3$^+$ Regulatory T Cells during the Follicular Phase of the Menstrual Cycle: Implications for Human Reproduction[1]

Lourdes Arruvito,*   Marianela Sanz,*   Alison H. Banham,[†]   and Leonardo Fainboim[2]*[‡]

Regulatory T cells (Tregs) are thought to affect the severity of various infectious and autoimmune diseases. The incidence of autoimmune disease is higher in fertile women than in men. Thus, we investigated whether Treg numbers were modulated during the menstrual cycle by sex hormones. In fertile nonpregnant women, we detected an expansion of CD4$^+$CD25$^+$FOXP3$^+$ Tregs in the late follicular phase of the menstrual cycle. This increase was tightly correlated with serum levels of estradiol and was followed by a dramatic decrease in Treg numbers at the luteal phase. Women who have had recurrent spontaneous abortions (RSA) showed similarly low numbers of Tregs at both the follicular and luteal phases, comparable to numbers we observed in postmenopausal women. In addition to decreased numbers, Tregs from women with RSA were also functionally deficient, as higher numbers were required to exert a similar magnitude of suppression to CD4$^+$CD25$^+$FOXP3$^+$ cells from fertile women. Consequently, reproductive failure might result from the inability of Tregs in women with RSA to expand during the preimplantatory phase combined with their lower functional capacity. Additionally, the modulation of Treg numbers we observed in fertile women suggests that the stage of the menstrual cycle should be taken into account when Treg numbers are investigated clinically. *The Journal of Immunology,* 2007, 178: 2572–2578.

P henotypically, human CD4$^+$CD25$^+$ regulatory T cells (Tregs)[3] are broadly comparable to their murine counterparts; they constitutively express CD25, glucocorticoid-induced TNF receptor family-related gene, intracellular CTLA-4, and the transcription factor forkhead box P3 (FOXP3), which is the established marker for Tregs in mice and humans (1).

There is clear evidence that when Tregs are depleted through mutation of the *FOXP3* gene, both mice and humans develop severe autoimmune disease (2, 3). Tregs control the size of the peripheral T cell pool, modulate immune responses to infection or to the presence of tumors, and contribute to inducing tolerance after solid organ transplantation. They also participate in the maintenance of immunological self-tolerance by suppressing immune responses mediated by autoreactive T cells that lead to autoimmune disease. In this context, Tregs are implicated in controlling several autoimmune diseases, such as type I diabetes (4), multiple sclero-

sis (5, 6), and rheumatoid arthritis (7). Additionally, Tregs were reported to be defective in autoimmune polyglandular syndrome type II (8), autoimmune hepatitis (9), and in patients with primary biliary cirrhosis (10). In patients with metastatic melanoma, both the number and suppressor effects of Tregs were increased (11). Supporting their role in response to viral infections, Treg numbers are higher in patients who progressed to the chronic phase of hepatitis C virus infection (12). However, apart from situations where there is almost total absence of these cells, some of the most crucial evidence for their participation in maintaining tolerance is their role in mediating maternal tolerance of the fetus (13–16).

Pregnancy constitutes a major challenge to the maternal immune system, because the persistence of paternal alloantigens must be tolerated while defenses against pathogens are maintained. Although localized mechanisms contribute to fetal protection from immune attack, maternal alloreactive lymphocytes persist. The hormone estradiol, which increases during pregnancy, might provide one explanation for enhanced maternal Treg development during gestation. The influence of estrogens on the incidence and course of autoimmune diseases has been clearly established in murine models of systemic lupus erythematosus (17), autoimmune encephalomyelitis (18–19), and multiple sclerosis (20). Additionally, estrogen is known to drive expansion of the CD4$^+$CD25$^+$ regulatory T cell compartment and promote suppressive function by inducing Treg proliferation (21–23). In the present study, we found that the Treg population expanded during the follicular phase of the menstrual cycle. Although the estrogen levels in women with recurrent spontaneous abortions (RSA) and healthy nonpregnant women were similar, Tregs from women with RSA were reduced in both number and functionality, indicating that Treg expansion requires additional stimuli that might be altered in RSA.

Patients and Methods

Subjects

We evaluated Tregs in samples of peripheral blood obtained from four different groups: group I included 75 women with a history of three or more

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[3] Abbreviations used in this paper: Treg, regulatory T cell; FOXP3, factor forkhead box P3; RSA, recurrent spontaneous abortion; SI, suppressive index; E2, estradiol; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

consecutive RSA that occurred before week 12 of gestation. In these subjects we previously excluded any infectious, endocrine, and anatomic disease that might have caused the abortion (mean age 30.13 years, range 22–45 years). Group II included 60 fertile women (who had at least one successful pregnancy and no previous abortions) whose menstrual cycles were regular (mean age 34.19 years, range 22–42 years). None of the women from group I or II was taking oral contraceptives.

In group III we included 55 postmenopausal women, who had entered menopause at least 2 years earlier and were not using hormone replacement therapies (mean age 57 years, range 46–67 years). Their estrogen levels were <20 pg/ml, and the progesterone levels were <0.5 ng/ml. Group IV comprised 52 men (mean age 34.17 years, range 27–44 years). All subjects were off any therapy at the time of the study. 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

PBMCs were isolated from 10 ml of heparinized peripheral blood through a 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). Negative control samples were incubated with an isotype-matched Ab. 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$^+$ population was expressed as a percentage of all CD4$^+$ T lymphocytes.

Intracellular staining for detecting endogenous FOXP3

The flow cytometry analysis was performed according to the method described by Roncador et al. (24). In brief, 1×10^6 cells were fixed in 1 ml of PBS with 1% paraformaldehyde containing 0.05% Tween 20. After an overnight incubation at 4°C, cells were treated twice with 0.5 ml of RNase-free DNase at 100 U/ml (Promega). Staining steps were performed for 1 h at room temperature. Cells were incubated with a mouse anti-human-FOXP3 IgG mAb (clone 236A/E7, provided by A.H.B.) and washed with PBS supplemented with 3% heat-inactivated FCS (Natocor), 0.50% Tween 20, and 0.05% azide. FOXP3 mAb binding was detected using Alexa Fluor-488 goat anti-mouse-IgG (Molecular Probes) and washed as described above. Cell surface staining was then performed using the mAb Cy-Chrome-anti-human-CD4 (BD Pharmingen) and PE-anti-human-CD25 (BD Biosciences) for 20 min at room temperature followed by washing in PBS. Cells were analyzed using a FACSCalibur cytometer (BD Biosciences).

Suppression assay

CD4$^+$CD25$^+$ Tregs were purified using the Dynal CD4$^+$CD25$^+$ Treg kit (Dynal Biotech) according to the manufacturer's protocols. In brief, in the first step, PBMCs were incubated with Abs against B cells, NK cells, monocytes, CD8$^+$ T cells, erythrocytes, and CD45RA cells, which were depleted by mixing with Dynabeads (immunomagnetic negative selection). In the second step, CD25 Dynabeads were added to purified CD4$^+$ cells to positively isolate the regulatory CD4$^+$CD25$^+$ T cells. After positive selection, CD4$^+$CD25$^+$ cells were detached by adding DETACHaBEAD. Approximately 2×10^8 PBMCs were isolated from 200 ml of heparinized peripheral blood through a density gradient centrifugation using the Ficoll-Hypaque technique (Amersham Biosciences) and were then used to purify the Treg cells. We conducted seven experiments in which the purity of isolated Tregs was between 83 and 90%. We obtained similar yields both in fertile women and in women with RSA. After isolation, 1×10^6 purified CD4$^+$CD25$^+$ cells were stained with anti-FOXP3 mAb 236A/E7. The expression of FOXP3, determined in three different samples of purified CD4$^+$CD25$^+$ cells, was between 65 and 77%. APCs were isolated from PBMCs depleted of CD3$^+$ cells by negative immunomagnetic selection (Dynabeads M-450 CD3 pan T; Dynal Biotech). A MLR was performed by mixing 3×10^4 APCs with 3×10^4 CD4$^+$CD25$^-$ cells from the same female donor and stimulating them with 5×10^4 mitomycin-treated PBMCs from two fully HLA-mismatched allogeneic male donors per well. Autologous controls were included by mixing 3×10^4 APCs with 3×10^4 CD4$^+$CD25$^-$ cells. The suppressor activity was measured by adding the following ratios of CD4$^+$CD25$^+$ cells compared with CD4$^+$CD25$^-$ cells to the MLR: 1:1 (e.g., 3×10^4 CD4$^+$CD25$^-$ and 3×10^4 CD4$^+$CD25$^+$) or 1:0.5, 1:0.25, and 1:0.125, in 200-μl final volumes. Cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS, 200 mM L-glutamine (Sigma-Aldrich), and gentamicin for 5 days. Cell proliferation was measured by adding 1 μC of [^{3}H]thymidine (PerkinElmer Life and Analytical Sciences) for the last 18 h of culture. In addition, CD4$^+$CD25$^+$ cells mixed with APC and stimulator cells were also cultured for the same period of time to assess their state of anergy (ratio CD25$^-$/CD25$^+$ = 0:1), and the response was always lower than that in the autologous control. CD4$^+$CD25$^-$ cells cultured without Tregs were used as a control to measure baseline proliferation before suppression by Tregs (ratio CD25$^-$ /CD25$^+$ = 1:0).

The percentage of suppression was calculated as follows:

$$\frac{[(\text{MLR plus ratio of CD25}-:\text{CD25}+) - (\text{autologous control})]}{(\text{MLR without CD4}+\text{CD25}+) - (\text{autologous control})} \times 100$$

The percentage of suppression was plotted against CD4$^+$CD25$^-$/CD4$^+$CD25$^+$ cell ratios, and a regression line was calculated. The I$_{50}$ was determined as the ratio that produced 50% suppression [suppressive index (SI) = 100 − I$_{50}$].

Hormone levels

Estradiol (E2) was quantified in the serum of subjects from groups I, II, and III by radioimmunoassay (Coat-A-Count estradiol-6; Diagnostic Products Corporation (DPC)). Progesterone was also quantified in the serum of the same three groups by a chemiluminescent method (Immulite; DPC).

Statistical analysis

We performed statistical analyses using GraphPad Prism version 3.0 for Windows (GraphPad). Paired t test with equal variance was used to analyze paired samples. When comparing two groups, significant differences were determined by two-tailed unpaired t test. Pearson's correlation was used to analyze correlations between the levels of estradiol and Tregs during the menstrual cycle. The Mann-Whitney U test was used to evaluate possible differences in CD4$^+$CD25$^+$ function between women with RSA and fertile women. The p values were considered significant if they were <0.05. The data had a normal distribution.

Results

Estrogen levels are associated with Treg numbers

Peripheral blood samples from a single fertile donor were collected at days 3, 9, 12, 19, and 24 in two consecutive menstrual cycles. At each point we quantified the number of CD4$^+$ cells, the proportion of CD4$^+$CD25$^+$ and CD4$^+$CD25^{high} cells within the CD4$^+$ population, the frequency of CD4$^+$ cells expressing the FOXP3$^+$ protein, and the serum levels of E2 and progesterone (Table I). The presence of a biphasic cycle was confirmed when progesterone level was higher than 8 ng/ml. Fig. 1 depicts a representative staining profile of FOXP3$^+$ vs CD4$^+$ vs CD25$^+$ FACS at days 3, 12, 19, and 24, which corresponds to one menstrual cycle investigated in a fertile woman. This particular cycle shows the highest percentage of FOXP3$^+$ cells at day 12.

This preliminary observation was confirmed by investigating the frequency of CD4$^+$CD25$^+$, CD4$^+$CD25^{high}, and FOXP3$^+$ cells during the late follicular phase (days 9–13) or the luteal phase (days 20–24) in 31 normal healthy fertile women (group II). During the late follicular phase, the frequency of CD4$^+$CD25$^+$ cells was 21.76 ± 1.45%, which decreased significantly to 16.43 ± 0.70% in the luteal phase; $p < 0.0001$. Analysis of the frequency of the CD4$^+$CD25^{high} subset within the CD4$^+$ population, which is known to have suppressive activity and to contain most of the FOXP3$^+$ cells (24) also showed an increase in the late follicular phase compared with the luteal phase (0.83 ± 0.07% vs 0.61 ± 0.07%; $p = 0.01$). Similarly, the proportion of FOXP3$^+$ cells within the CD4$^+$ population also increased at the late follicular phase in comparison with the luteal phase (6.14 ± 0.39% vs 3.77 ± 0.42%; $p = 0.0003$). To exclude the possibility that values

Table I.   *Comparative frequencies of CD4$^+$CD25$^+$, CD4$^+$CD25^{high}, and FOXP3$^+$ cells and sex hormone levels during the menstrual cycle*

	Day 3[a]	Day 9	Day 12[a]	Day 19	Day 24
First menstrual cycle					
Absolute number CD4$^+$/μl[b]	900.0	974.0	1150.0	1102.0	957.0
CD4$^+$CD25$^+$ frequency, %[c]	13.0	22.0	26.0	15.0	11.0
CD4$^+$CD25^{high} frequency, %[d]	0.97	1.3	1.4	1.0	0.89
FOXP3 expression, %[e]	3.8	8.3	9.6	7.3	4.1
Estradiol level, pg/ml	30.0	146.0	219.0	106.0	31.0
Progesterone level, ng/ml	0.25	0.20	0.89	11.70	0.43
Second menstrual cycle					
Absolute number CD4$^+$/μl[b]	953.0	870.0	910.0	890.0	960.0
CD4$^+$CD25$^+$ frequency, %[c]	9.9	11.0	17.0	10.0	18.0
CD4$^+$CD25^{high} frequency, %[d]	0.78	0.86	1.12	0.95	1.27
FOXP3 expression, %[e]	2.7	4.3	8.2	4.0	5.0
Estradiol level, pg/ml	31.0	74.0	173.0	82.0	118.0
Progesterone level, ng/ml	0.20	0.20	0.22	12.3	13.90

[a] Days of the menstrual cycle.
[b] Absolute count of CD4$^+$ cells acquired from complete blood.
[c] Frequency of peripheral blood CD4$^+$CD25$^+$ T lymphocytes expressed as percentage of CD4$^+$ T lymphocytes.
[d] Frequency of peripheral blood CD4$^+$CD25^{high} T cell subset expressed as percentage of CD4$^+$ T lymphocytes.
[e] Frequency of FOXP3 expression in peripheral blood CD4$^+$ T lymphocytes.

obtained in fertile women were associated with previous pregnancies, we compared our data with those of a panel of 10 healthy women without prior pregnancies. These data also showed Treg expansion during the late follicular phase, having values comparable to those of fertile women (CD4$^+$CD25$^+$: 21.71 $\pm$ 2.8% and FOXP3$^+$: 5.4 $\pm$ 0.8%). Thus, both female control groups had similar frequencies of Tregs during the menstrual cycle.

In this large cohort of healthy women, we also confirmed the strong positive association between the proportion of CD4$^+$CD25$^+$, CD4$^+$CD25^{high}, and FOXP3$^+$ cells within the CD4$^+$ population and estrogen level ($R^2 = 0.76$, $p < 0.0001$; $R^2 = 0.60$, $p < 0.0001$, and $R^2 = 0.78$; $p < 0.0001$, respectively). Data also showed a positive correlation between age and FOXP3 expression ($R^2 = 0.48$, $p < 0.0001$) and age and E2 level ($R^2 = 0.40$, $p = 0.0001$; Fig. 2). There was no correlation between progesterone level and the Treg pool (data not shown). Five women with very low levels of progesterone, indicative of the absence of ovulation, were excluded from this analysis.

Decreased Treg frequencies in women with RSA compared with fertile women

In both mice and humans it has been reported that an expansion of Treg cells is necessary to mediate maternal tolerance to the fetus (13–16). It was thus of interest to investigate the normal behavior of Tregs during the menstrual cycle of women who had 3 or more recurrent spontaneous abortions. In particular, we explored whether the Treg pool expanded during the menstrual cycle of these women. We analyzed the frequency of Tregs within the CD4$^+$ population during the follicular phase and luteal phase of 36 women with RSA from group I. The frequencies of CD4$^+$CD25$^+$, CD4$^+$CD25^{high}, and FOXP3$^+$ cells at the late follicular phase were 15.95 $\pm$ 1.4%, 0.54 $\pm$ 0.06%, and 2.91 $\pm$ 0.40%, respectively. During the luteal phase the values observed were 14.45% $\pm$ 0.46, 0.62% $\pm$ 0.09, and 3.99% $\pm$ 0.32, respectively.

Neither the Treg pool nor age correlated with E2 level (data not shown). In contrast, similar to results from fertile controls, we found a positive association between age and FOXP3 expression in women with RSA ($R^2 = 0.6$, $p < 0.0001$; Fig. 2, *F* and *G*)

In comparison with fertile women, those with RSA showed significantly limited expansion of Tregs at the late follicular phase: 15.95 $\pm$ 1.44% vs 21.76 $\pm$ 1.45%, $p = 0.0073$; 0.54 $\pm$ 0.06% vs 0.83 $\pm$ 0.07%, $p = 0.004$; 2.91 $\pm$ 0.40% vs 6.14 $\pm$ 0.39%, $p <$

0.0001 for CD4$^+$CD25$^+$, CD4$^+$CD25^{high}, and FOXP3$^+$, respectively (Fig. 3).

At the beginning of the present study, because we were unaware that Tregs were modulated during the menstrual cycle, we investigated the frequency of Tregs in patients and controls only at the luteal phase (days 20–24). With this approach we recruited a higher number of controls ($n = 60$) and RSA patients ($n = 75$). In these samples, the frequencies of CD4$^+$CD25$^+$, CD4$^+$CD25^{high}, and FOXP3$^+$ in RSA patients compared with fertile controls were 15.24 $\pm$ 0.36% vs 17.62 $\pm$ 0.47%, $p = 0.0001$; 0.77 $\pm$ 0.06% vs 0.99 $\pm$ 0.05%, $p = 0.007$; 3.16 $\pm$ 0.39% vs 4.36 $\pm$ 0.42%, $p = 0.046$, respectively.

Men have comparable frequencies of Tregs to women at the late follicular phase of their cycle

It was of interest to compare the relative frequency of Tregs in men with those in fertile and postmenopausal women (Fig. 4). The frequency of CD4$^+$CD25$^+$, CD4$^+$CD25^{high}, and FOXP3$^+$ cells within the CD4$^+$ population from men ($n = 52$) was 19.63 $\pm$ 0.68%, 0.69 $\pm$ 0.06%, and 7.18 $\pm$ 0.77%, respectively. This is not significantly different from the frequencies in fertile women at the late follicular phase. In contrast, the frequencies of CD4$^+$CD25$^+$ and CD4$^+$CD25^{high} in postmenopausal women were 15.45% $\pm$ 0.35 and 0.57% $\pm$ 0.08, which are comparable to those detected in women with RSA (FOXP3 frequencies were not analyzed in postmenopausal women).

Differential suppressor capacity of Tregs from fertile women and those with RSA

In several autoimmune diseases, in addition to their well-known female prevalence, it was recently demonstrated that Treg populations in these patients are functionally defective (4–10). The hallmark of Tregs is their ability to suppress immune responses by inhibiting the proliferation and cytokine secretion of effector T cells (1). After we found that Tregs from women with RSA were numerically deficient, we also explored their functionality in comparison with Tregs from fertile women. Thus, we measured their ability to inhibit proliferation of CD4$^+$CD25$^-$ effector cells in response to paternal allostimulation in an MLR. As described in *Patients and Methods*, fixed numbers of purified effectors CD4$^+$CD25$^-$ (3×10^4) cocultured with the same number of autologous APCs were stimulated with 5×10^4 allogeneic cells. To
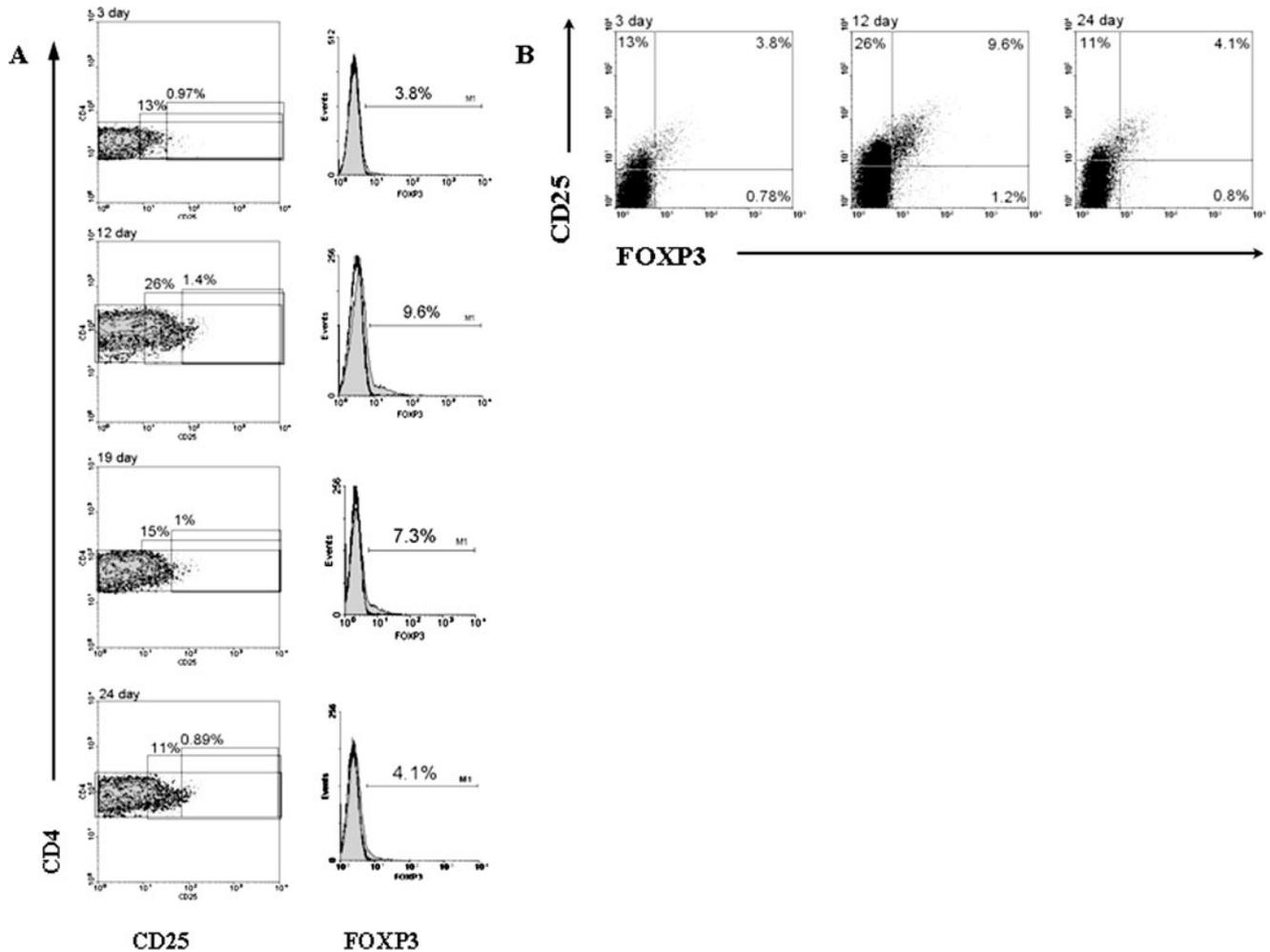
FIGURE 1. Representative FACS profiles comparing $CD4^+CD25^+FOXP3^+$ populations during the menstrual cycle. *A*, Intracellular FOXP3 and $CD4^+CD25^+$ surface staining expression observed at days 3, 12, 19, and 24 during the menstrual cycle of a fertile woman. Data show the frequency of the $CD4^+CD25^+FOXP3^+$ population expressed as a percentage of all $CD4^+$ T lymphocytes. *B*, Representative FACS dot plots showing the expansion of $FOXP3^+$ Tregs during the menstrual cycle.

establish their suppressor activity, different ratios of $CD4^+CD25^+$ cells were added to a fixed number of $CD4^+CD25^-$ target cells: ratio 1:1 (3×10^4), ratio 1:0.5 (1.5×10^4 Tregs), ratio 1:0.25 (0.75×10^4 Tregs), and ratio 1:0.12 (0.325×10^4 Tregs). In seven independent experiments, we compared the suppressor activity of Tregs from fertile women or those with RSA, on the proliferation of the $CD4^+CD25^-$ cells stimulated by paternal allogeneic cells. In each experiment, we compared the suppressive effect of purified $CD4^+CD25^+$ obtained from a fertile woman vs purified $CD4^+CD25^+$ from a woman with RSA. In all experiments, addition of 15,000 Treg cells obtained from fertile women reached the maximum level of inhibition with an I_{50} between 15 and 23% and an SI that ranged between 85 and 77%. In contrast, RSA patients had an I_{50} between 43 and 54% and an SI that ranged between 46 and 57% (median $\pm$ SEM 79.5 $\pm$ 1.06 vs 53 $\pm$ 1.5; $p < 0.0006$; Fig. 5*A*). Results from one representative experiment, depicted in Fig. 5*B*, show that higher numbers of Tregs were required from women with RSA to reach similar levels of MLR inhibition.

We next explored whether the functional deficit is specific to Tregs or T effectors. We performed three criss-cross experiments in which patient and control Treg cells were cocultured with the autologous $CD4^+CD25^-$ effector T cells from either fertile women or those with RSA. Regulatory T cells from fertile women similarly suppressed the proliferative response of target

$CD4^+CD25^-$ T cells derived from both controls and RSA patients (suppression $\geq 75\%$). However, Tregs from women with RSA showed significantly lower suppression of the proliferative response of target cells from either fertile women or women with RSA (suppression $\geq 43\%$; $p < 0.02$). Hence, Tregs from women with RSA are both numerically and functionally deficient and might contribute to reduced immunosuppression during the course of an alloimmune response (Fig. 6).

Discussion

We demonstrated that Treg frequencies within the $CD4^+$ population undergo profound changes during the menstrual cycle that could affect female immunoregulation. We demonstrated that Treg numbers increased during the follicular phase of the menstrual cycle. Although our results contrast with a recent study that failed to detect changes in numbers of Tregs during the menstrual cycle (22) that study not only did not measure the presence of FOXP3 but also looked at the presence of Tregs at days 6–9 vs 20–24 of the menstrual cycle. The present study clearly demonstrates that Tregs reach their maximum numbers during the late follicular phase (days 9–13). The expansion we detected in the late follicular phase was tightly correlated with an increase in the serum level of estrogen. Also observed in fertile women was a dramatic reduction in the Treg frequency during the luteal phase. The preovulatory

FIGURE 2. Correlation between the frequencies of Tregs with estrogen level at the late follicular phase. The frequency of CD4$^+$CD25$^+$ (A), CD4$^+$CD25^{high} (B), and FOXP3$^+$ (C) cells within the CD4$^+$ population obtained from each female donor was determined by flow cytometry between days 9 and 12 of the menstrual cycle. These frequencies were compared with the level of estradiol present on the same day. Data indicate a strong positive association between the frequency of all three markers of Tregs and estrogen level as analyzed by Pearson correlation coefficient and coefficient of determination. D, Positive association between age and FOXP3 expression in healthy fertile women. E, Positive association between age and estradiol level in healthy fertile women. F, Positive association between age and FOXP3 expression in women with RSA. G, Absence of correlation between age and estradiol level in women with RSA.
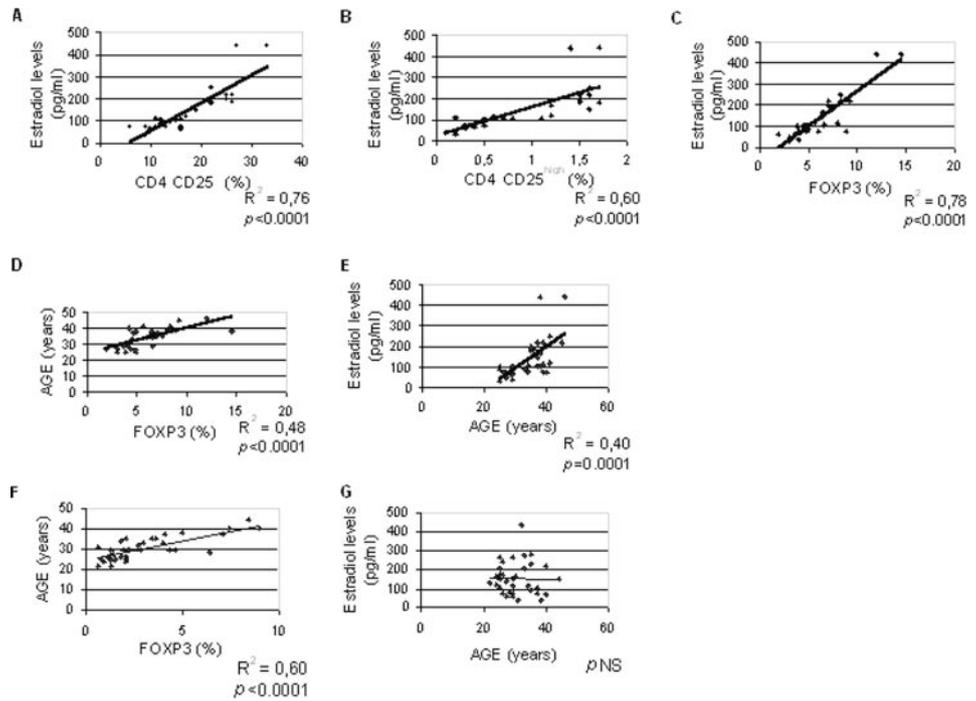
increase in Tregs might reflect a requirement for induction of immune tolerance to facilitate a successful implantation.

When we investigated the frequency of Tregs in patients and controls with samples obtained only at the luteal phase, Treg frequencies differed from the ones observed in the smaller group. We should stress that data from the smaller group were obtained from the same donor at the follicular phase and luteal phase. Additionally, in the smaller group we excluded individuals without a clear biphasic cycle, thus, we considered these data more accurate.

In mice, an expansion of regulatory T cells, which also seems to begin before implantation, mediates maternal tolerance to the fetus (25) and estrogen has been implicated as the driving force for this expansion (21, 23). Conversely, the depletion of CD4$^+$CD25$^+$ T cells after implantation leads to an early failure of gestation (26). Interestingly in a murine model of spontaneous abortion, the abortion-prone mice showed deficient Treg activity that could be treated by adoptive transfer of Tregs from normal pregnant but not nonpregnant female mice, to prevent abortion (26). In humans, three independent studies have described both the systemic and decidual expansion of CD4$^+$CD25$^+$FOXP3$^+$ Treg populations in the first two trimesters of pregnancy (14–16). Also, the proportion of Tregs is reported to be much lower in deciduas of women with RSA (15). In the present study we demonstrate that women with RSA had similar Treg frequencies in the CD4$^+$ population at both the late follicular and luteal phases of the menstrual cycle. These findings indicate that women with RSA did not show the Treg expansion during the follicular phase that we observed in fertile women. In addition to a decreased Treg frequency, our results also suggest that Tregs from women with RSA are functionally deficient, because higher numbers are required to exert a suppressive effect comparable to that of CD4$^+$CD25$^+$ cells from fertile women. Anergy, a hallmark of CD4$^+$CD25$^+$ Tregs was clearly demonstrated both in women with RSA and in fertile women stimulated by allogeneic cells in an MLR. We also demonstrated that the decrease in Treg function observed in women with RSA was an intrinsic functional defect in the CD4$^+$CD25$^+$ Treg subset rather than reduced susceptibility of their T-effector cells to Treg-mediated suppression. A previous study suggested that CD4$^+$CD25$^+$

Tregs completely suppressed the proliferation of Th1 clones with only a partial inhibition of the proliferation of Th2 clones (27). This inhibition of the suppressor effect on Th2 cells seems to be mediated by IL-4 and IL-9. The receptors for these cytokines share a γ-chain with IL-2R but possess a distinct α-chain (28). During a normal pregnancy, a Th2 response acting through its anti-inflammatory capacity might contribute to the embryo's survival, which could be enhanced by the differential effects of CD4$^+$CD25$^+$ on Th1 and Th2 cells. However, studies from IL-4- and IL-10-deficient mice do not show disturbed pregnancy, suggesting that, in mice, Th2 cells are not essential for normal pregnancy (29).
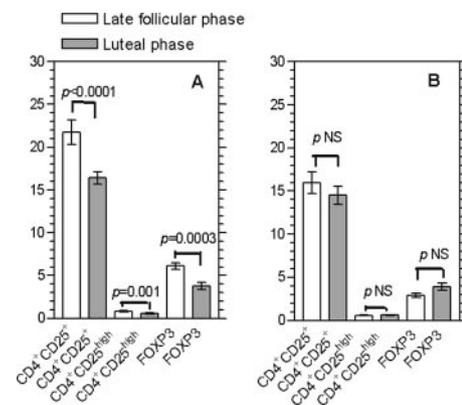

FIGURE 3. Lower level of Tregs in RSA patients compared with control women at the late follicular phase. The frequency of CD4$^+$CD25$^+$, CD4$^+$CD25^{high}, and FOXP3$^+$ cells within the CD4$^+$ population from controls ($n = 31$) and women with RSA ($n = 36$) was determined by flow cytometry between days 9 and 12 and days 20 and 24 of the menstrual cycle. A, Fertile women showed an expansion of Tregs in the late follicular phase followed by a dramatic decrease in Treg numbers in the luteal phase of the menstrual cycle. B, Women with RSA had similar numbers of Tregs at both phases. Data are presented as mean ± SEM and p value as analyzed by unpaired t test.
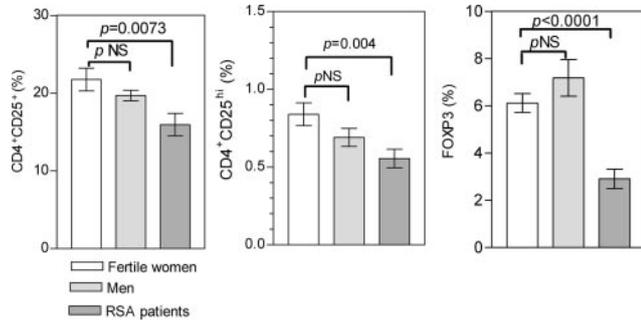
FIGURE 4. The frequencies of Treg in men are comparable to the frequencies of Treg in fertile women at the late follicular phase of their cycle. The frequency of CD4$^+$CD25$^+$, CD4$^+$CD25^{high}, and FOXP3$^+$ cells within the CD4$^+$ population from men, fertile women, or women with RSA was determined by flow cytometry. Values in both subsets of women represent data obtained between days 9 and 12 of the menstrual cycle. There were no significant differences in the percentage of CD4$^+$CD25$^+$, CD4$^+$CD25^{high}, cells and FOXP3$^+$ expression between men and fertile women. Data are presented as mean $\pm$ SEM and p value as analyzed by unpaired t test.

In the current study we observed that postmenopausal women, who would not require expansion of Treg to sustain a successful pregnancy, showed Treg frequencies that were similar to those detected in women with RSA. In men, testosterone was shown to play a critical role in maintaining the level of Tregs, without the profound changes in Treg frequencies we describe here in women under the cyclic influence of female sex hormones (30). As discussed above, the expansion of Tregs during the follicular phase that was observed in control women was highly correlated with the levels of serum E2. Treg expansion was not present in women with RSA, even though they had similar or even higher levels of E2.
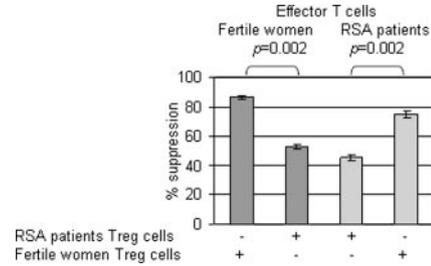
FIGURE 6. CD4$^+$CD25$^+$ T cells from RSA patients show low suppression of effector CD4$^+$CD25$^-$ T cells isolated from either women with RSA or fertile controls. In contrast, higher suppressor activity of Tregs from fertile women is observed both on CD4$^+$CD25$^-$ effector T cells from fertile controls and individuals with RSA. CD4$^+$CD25$^+$ cells from RSA patients and fertile women were purified, and MLR was performed as described in *Patients and Methods*. A total of 3×10^4 CD4$^+$CD25$^-$ effector cells/well from fertile women were cocultured with 1.5×10^4 autologous CD4$^+$CD25$^+$ cells (1st bar) or with the same number of Tregs from RSA patients (2nd bar). Conversely, 3×10^4 CD4$^+$CD25$^-$ effector cells from RSA patients were cocultured with 1.5×10^4 autologous CD4$^+$CD25$^+$ cells (3rd bar) or with CD4$^+$CD25$^+$ cells from fertile women (4th bar). One representative result of three independent experiments performed with different individuals is shown. Data were analyzed using the Mann-Whitney U test, $p = 0.002$.

Although fertile women showed a positive correlation between age and E2 level, the same correlation was not observed in women with RSA. These data suggest that E2 could play a role in the function of Treg in women with RSA. This effect seems not to be related to age, because controls and women with RSA showed a similar correlation between age and FOXP3 expression. Signaling through IL-2 is known to be required for peripheral expansion and
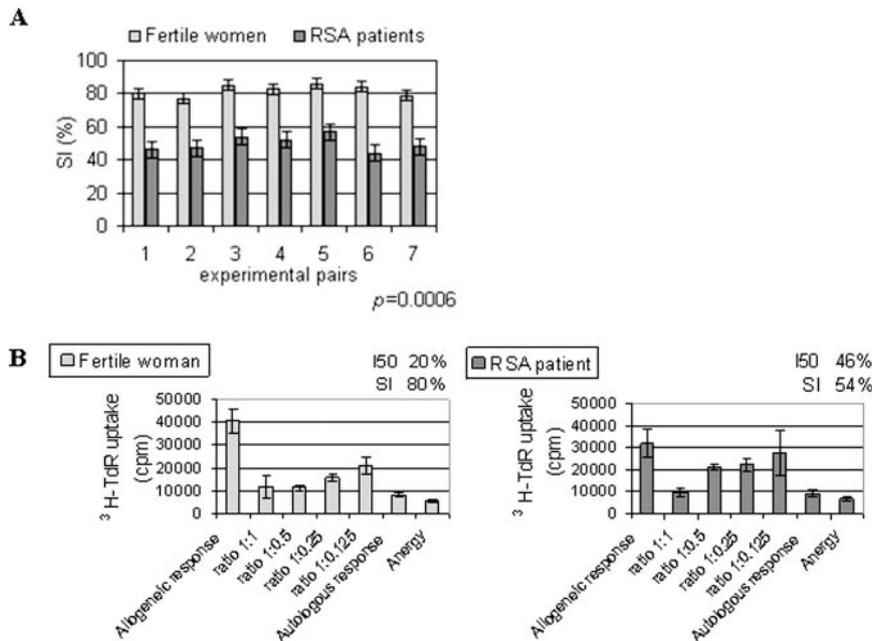
FIGURE 5. Differential suppressor capacity of CD4$^+$CD25$^+$ cells from fertile women and those with RSA. CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells were purified by using the Dynal CD4$^+$CD25$^+$ Treg kit, as described in *Patients and Methods*. APCs were isolated from PBMCs depleted of CD3 cells by negative immunomagnetic selection. MLR was performed as described. *A*, Seven independent experiments examined the number of CD4$^+$CD25$^+$ Treg cells from fertile women or women with RSA required to obtain maximum inhibition of the proliferation of CD4$^+$CD25$^-$ cells. The addition of 15,000 Tregs obtained from a fertile woman reached the maximum level of inhibition. Tregs from women with RSA required almost twice as many cells to reach similar levels of MLR inhibition. Data were analyzed by the Mann-Whitney U test, $p < 0.0006$. *B*, One representative experiment. I_{50} was determined as the ratio that produced 50% suppression. Suppressive index (SI) $= 100 - I_{50}$. Allogeneic response was determined as the ratio CD4$^+$CD25$^-$/CD4$^+$CD25$^+$ $= 1:0$. Anergy was determined as the ratio CD4$^+$CD25$^-$/CD4$^+$CD25$^+$ $= 0:1$.

suppressive activity of Tregs (31, 32). There is also a distinct IL-2 receptor signaling pattern in $CD4^+CD25^+$ Tregs compared with activated T lymphocytes in which the Janus/kinase/STAT pathway remains intact while downstream targets of the PI3K are not activated (33). In vitro studies suggested that the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) acts as a negative regulator of IL-2-mediated expansion of Tregs. Of potential relevance are findings that human PTEN expression is regulated by ovarian steroids, including estrogen, and that PTEN expression shows temporal and spatial changes during the menstrual cycle and during early pregnancy (34). According to our results, it remains to be elucidated whether Tregs from RSA patients are capable of a normal response to E2, and an impaired regulatory function of E2 in RSA patients might be further investigated. Similarly, additional studies should investigate the role of PTEN, IL-2, and related cytokines that are known to play a central role in the expansion and function of Tregs.

In summary, we conclude that a dysregulation in Treg frequency and functionality in women with RSA might contribute to their reproductive failure. Additionally, it is strongly recommended that the profound changes that occur in Treg frequencies during the normal menstrual cycle should be considered when number of Tregs is investigated in premenopausal women in a clinical setting.

Acknowledgments

Disclosures

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