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# Function of $T_{reg}$ Cells Decreased in Patients With Systemic Lupus Erythematosus Due To the Effect of Prolactin

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**Abstract:** Prolactin has different functions, including cytokine secretion and inhibition of the suppressor effect of regulatory T ($T_{reg}$) cells in healthy individuals. Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by defects in the functions of B, T, and $T_{reg}$ cells. Prolactin plays an important role in the physiopathology of SLE. Our objective was to establish the participation of prolactin in the regulation of the immune response mediated by $T_{reg}$ cells from patients with SLE. $CD4^+CD25^{hi}CD127^{-/low}$ cells were purified using magnetic beads and the relative expression of prolactin receptor was measured. The functional activity was evaluated by proliferation assay and cytokine secretion in activated cells, in the presence and absence of prolactin. We found that both percentage and function of $T_{reg}$ cells decrease in SLE patients compared to healthy individuals with statistical significance. The prolactin receptor is constitutively expressed on $T_{reg}$ and effector T ($T_{eff}$) cells in SLE patients, and this expression is higher than in healthy individuals. The expression of this receptor differs in inactive and active patients: in the former, the expression is higher in $T_{reg}$ cells than in $T_{eff}$ cells, similar to healthy individuals, whereas there is no difference in the expression between $T_{reg}$ and $T_{eff}$ cells from active patients. In $T_{reg}$:$T_{eff}$ cell cocultures, addition of prolactin decreases the suppressor effect exerted by $T_{reg}$ cells and increases IFNγ secretion. Our results suggest that prolactin plays an important role in the activation of the disease in inactive patients by decreasing the suppressor function exerted by $T_{reg}$ cells over $T_{eff}$ cells, thereby favoring an inflammatory microenvironment.

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**Abbreviations**: IFNγ = interferon gamma, PBMCs = peripheral blood mononuclear cells, PRL = prolactin, SLE = systemic lupus erythematosus, SLEDAI = systemic lupus erythematosus disease activity index, $T_{eff}$ = T effector cells, $T_{reg}$ = T regulatory cells.

## INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune rheumatic disease characterized by widespread inflammation, alteration in T cell activation, and overproduction of autoantibodies. This disease is most commonly observed in women. The course of the disease is characterized by remissions and exacerbation. The exacerbation of the disease has been linked to the activity of the immune system.[1] Autoreactive T cells assist autoreactive B cells and infiltrate into the target organs to promote inflammation via cytokine secretion, which causes damage. Thus, autoreactive T cells are key players in the pathogenesis of SLE.[2]

Hyperprolactinemia has been reported in several autoimmune diseases, including SLE.[3–6] Prolactin (PRL) can be synthesized in an extra-pituitary fashion by cells from the immune system, such as B and T cells, which also express the PRL receptor.[7,8] During an immune response, PRL promotes the proliferation, growth, activation, and differentiation of T cells[9,10] and intervenes in the expression of CD69 and CD154 by $CD4^+$ T cells.[11] In human $CD4^+$ T cell cultures activated with phorbol myristate acetate and subjected to PRL blockade by using an anti-PRL antibody, IL2 and IFNγ secretion is decreased, indicating a role for PRL in the regulation of cytokine secretion.[12] Furthermore, PRL can decrease the function of regulatory T ($T_{reg}$) cells[13] in healthy individuals. These studies show the importance of PRL in the regulation of the immune system.

The pathogenesis of SLE involves complex interactions between genetic and environmental factors and the adaptive and innate immune systems. The breakdown of immunologic self-tolerance results in the development of autoimmune diseases.[14,15] Other alterations could also be involved in regulating the immune response mediated by $T_{reg}$ cells. There are 2 types

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of $T_{reg}$ cells: natural $T_{reg}$ cells, which are generated in the thymus, and inducible $T_{reg}$ cells, which are generated in peripheral sites. Both cells exhibit the same $CD4^+CD25^{hi}CD127^{low/-}FoxP3^+$ phenotype.[16,17] $T_{reg}$ cells exert an inhibitory effect on $CD4^+CD25^-CD127^+$ conventional or effector T ($T_{eff}$) cells.[18] A numerical defect in $T_{reg}$ cells has been observed in autoimmune pathologies such as thyroiditis[19] and diabetes,[20] whereas in SLE, decreased[21–26] as well as normal[27–30] $T_{reg}$ cell numbers have been reported. Moreover, in SLE patients, conventional T cells exhibit reduced sensitivity to $T_{reg}$ cell inhibition.[22,31,32]

The objective of our work was to determine whether PRL participates in the regulation of the immune response mediated by $T_{reg}$ cells in patients with SLE. We found that both percentage and function of $T_{reg}$ ($CD4^+CD25^{hi}CD127^{-/low}FoxP3^+$) cells were decreased in SLE patients compared to healthy individuals. The expression of PRL receptor was found to be constitutive in both $T_{reg}$ and $T_{eff}$ cells in patients with SLE and this expression was increased compared to that in healthy individuals. PRL receptor expression varied among SLE patients; in inactive patients, the expression of the receptor was higher in $T_{reg}$ cells compared to $T_{eff}$ cells, similar to what was observed in healthy individuals. However, there was no difference in the expression of the receptor between $T_{reg}$ and $T_{eff}$ cells among active SLE patients. We also found that PRL affects the function of $T_{reg}$ cells. The addition of prolactin to $T_{reg}$:$T_{eff}$ cocultures decreased the suppressor effect in $T_{reg}$ cells and increased IFNγ secretion. These results suggest that PRL increases IFNγ secretion, favoring an inflammatory environment, and decreases the suppressor function of $T_{reg}$ cells; this, in addition to the decrease in the number of $T_{reg}$ cells, contributes to the expansion of autoreactive lymphocytes, favoring disease activation.

## METHODS

### Study Group

The Ethics Committee of Human Research of the Instituto Mexicano del Seguro Social (IMSS) and the Ethics and Research Committees of the Hospital General de México approved this study (2009-785-028). It was conducted according to the Declaration of Helsinki. Informed consent was obtained from all participants. The samples were obtained from 17 healthy women in the reproductive age (18–50 years) without menstrual disorders and with normal levels of serum prolactin (<20 ng/ml). Since $T_{reg}$ is a rare cell population, the cells from 1 patient are inadequate for all experiments; therefore, from a total of 68 patients with SLE (25–50 years of age), we used samples from an average of 13 patients with inactive lupus and 13 patients with active lupus for each experiment. All patients with SLE fulfilled the American College of Rheumatology (ACR) criteria for SLE.[33]

Disease activity was measured by SLEDAI (systemic lupus erythematosus disease activity index). Inactive lupus was considered when the SLEDAI value was equal to 0; lupus was considered to be active when the SLEDAI value was ≥4. The samples were obtained between 08:00 and 11:00 AM from the cubital vein.

### Prolactin

The human PRL used in this study was kindly provided by Dr. A.F. Parlow, from the National Hormone & Pituitary Program (NHPP; Harbor UCLA Medical Center, Los Angeles, CA).

### Antibodies

The following antibodies were used: mouse anti-human CD4-APC (OKT4), CD25-PE-Cy5 (BC96), CD127-FITC (eBioRDR5), FoxP3-PE (PCH101), and CD25-APC (BC96), all from eBioscience (San Diego, CA); mouse anti-PRL receptor (ECD, 1A2B1) from Invitrogen (Carlsbad, CA); and Biotin Rat Anti-Mouse IgG2b (R12-3) from BD Pharmingen (San Jose, CA). The biotinylated secondary antibody was detected using streptavidin–phycoerythrin–Cy5.5 from BD Biosciences (Mountain View, CA).

### $T_{reg}$ and $T_{eff}$ Cell Purification

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples by density centrifugation using Lymphoprep (Axis Shield, Oslo, Norway). $T_{reg}$ cells were isolated from PBMCs by using a $CD4^+CD25^+CD127^{dim/-}$ Regulatory T cell Isolation Kit II (Miltenyi Biotec, Bergish Gladbach, Germany), according to the manufacturer's instructions. The purity of the cells ranged from 93% to 97% (Supplemental Content 1, http://links.lww.com/MD/A610).

### Cell Culture and Proliferation Assays

Cells were cultured in AIM-V liquid medium (Gibco BRL, NY, New York) supplemented with 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco BRL). $T_{reg}$ cells ($CD4^+CD25^{hi}CD127^{low/-}$) were plated at a density of $4.0 \times 10^4$ cells/well in 96-well U-bottomed plates (Nunc, Roskilde, Denmark) with or without $8.0 \times 10^4$ $T_{eff}$ cells ($CD4^+CD25^-CD127^+$) and cultured in synthetic serum-free medium (AIM-V, Gibco BRL). We standardized the optimum ratio of $T_{reg}$:$T_{eff}$ cells required to generate a response by using a standard curve illustrating the following ratios: 0.5:1, 1:1, 2:1, and 4:1. The suppressor effect was observed under all conditions; thus, we decided to use a 0.5:1 $T_{reg}$:$T_{eff}$ cell ratio, on the basis of the percentage of circulating $T_{reg}$ cells and the feasibility of obtaining sufficient quantities for all tests.

$T_{reg}$ Suppression Inspector human (anti-CD2/CD3/CD28 beads; Miltenyi Biotec, Germany) was used for the functional characterization of human $T_{reg}$ cells by in vitro suppression assays in the presence and absence of 50 ng/ml human PRL (NHPP, Los Angeles, CA). The concentrations of Inspector and PRL were obtained using a dose–response curve. Cells were cultured for 5 days, and 1 μCi [$^3$H]-thymidine (Hartmann Analytical, Braunschweig, Germany) was added 18 hours before harvesting. Thymidine incorporation was determined using a liquid scintillation analyzer (Packard 1900 TR, Meriden, Connecticut), and the percentage of proliferation suppression was determined. All conditions were previously standardized and optimized.

### Cytokine Detection

Cell culture supernatants were collected on day 5, and cytokine levels were measured using a commercial BD Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit (IL2, IL4, IL10, IL6, TNF, IFNγ, and IL17A) by BD Biosciences.

### Real-Time PCR Assay

Total RNA was extracted from purified $T_{reg}$ and $T_{eff}$ cells by using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. RNA concentration was determined using UV spectrophotometry, and 1 μg of total RNA was used

to generate cDNA with SuperScript II reverse transcriptase (Invitrogen). The PRL receptor and β actin were then amplified by real-time PCR using a LightCycler TaqMan Master kit (Roche Diagnostic, Mannheim, Germany), hydrolysis probes, and primers designed by Roche Diagnostic; all reactions were performed according to the manufacturer's specifications. The primers and probes used are as follows: number 8 probe from the Universal Probe Library for PRL receptor determination, forward primer CTT TCC ACA TGA ACC CTG AAG and reverse primer GCA GAT GCC ACA TTT TCC TT, and number 64 probe from Universal Probe Library for β-actin determination, forward primer CCA ACC GCG AGA AGA TGA and reverse primer CCA GAG GCG TAC AGG GAT AG. Reactions were carried out in a final volume of 10 μl, and a LightCycler 1.5 instrument was used (Roche Diagnostic). The PCR conditions were as follows: 10 minutes at 95°C, followed by 45 cycles of 10 seconds at 95°C, 30 seconds at 59°C, and 1 seconds at 72°C, with a final cycle for 30 seconds at 40°C. The samples were normalized to β-actin gene expression. The relative expression of PRL and its receptor was calculated using the $2^{\Delta CT}$ formula.

## Cell Surface Staining and Flow Cytometry

To determine the percentage of peripheral blood $T_{reg}$ cells, PBMCs were incubated with fluorescently labeled antibodies (anti-CD4, CD25, CD127, and PRL receptor or unrelated antibody) for 20 minutes at 4°C in staining buffer (phosphate-buffered saline [PBS] with 0.5% bovine serum albumin [BSA] and 0.01% sodium azide). The cells were then washed and fixed in 2% PBS–paraformaldehyde (Sigma Aldrich, St. Louis, MO). Data were obtained using a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec, Auburn, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR).

## Intracellular Staining for FoxP3

After the superficial staining, the cells were fixed and permeabilized with the Foxp3 Staining Buffer Set (eBioscience) for 18 hours, and stained with fluorescent antibodies. After washing, the stained cells were assayed in a MACSQuant Analyzer 10 flow cytometer and the data were analyzed with FlowJo software.

## Statistical Analysis

Statistical analysis was performed using the SPSS package, version 20.0 (SPSS, Inc., Chicago, IL). Normality of the data was checked using the Kolmogorov–Smirnoff test, followed by an analysis using the relevant parametric or nonparametric test. The suppressor function among the groups was assessed using the Kruskal–Wallis test. Comparisons between individual groups were tested using the unpaired Mann–Whitney *U* or paired Wilcoxon matched-pairs test, at a significance level of $P < 0.05$.

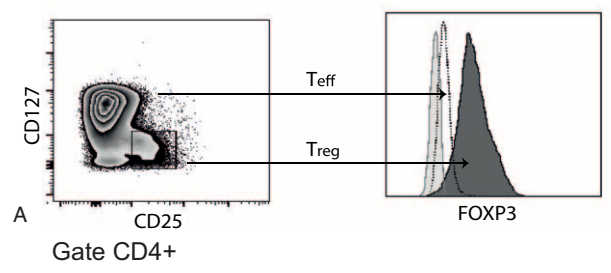
# RESULTS

## Percentage of $T_{reg}$ Cells

The percentage of $T_{reg}$ ($CD4^+CD25^{hi}CD127^{low/-}FoxP3^+$) cells was determined based on PBMCs from healthy individuals and SLE patients (active and inactive). We found that the percentage of $T_{reg}$ cells decreased in a statistically significant way ($P < 0.001$) in patients with active and inactive SLE, compared to healthy individuals ($\chi = 2.95\%$), but no difference was observed between the inactive ($\chi = 1.67\%$) and active ($\chi = 1.19\%$) patients, suggesting that the number of $T_{reg}$ cells is less in SLE patients (active and inactive) (Figure 1).

## PRL Receptor Expression in $T_{reg}$ and $T_{eff}$ Cells

Our results showed that $T_{reg}$ cells from SLE patients express the PRL receptor even in absence of stimuli and that both mRNA (relative expression) and protein (FMI = mean fluorescence intensity) expression by $T_{reg}$ cells from active and inactive SLE patients was higher than that in $T_{reg}$ cells from healthy individuals (Table 1). This result showed a statistically significant difference (Figure 2 A and B), although no statistically significant difference were observed between the active and inactive patients. We found that the expression of PRL receptor mRNA and protein in $T_{eff}$ cells from active and inactive SLE patients was higher than that in cells from healthy individuals, with a statistically significant difference (Figure 2C and D). There was no difference in the expression of PRL receptor between active and inactive patients. Moreover, the expression of PRL receptor was higher in $T_{reg}$ cells compared to $T_{eff}$ cells from patients with inactive SLE, similar to that observed in healthy individuals. However, in patients with active SLE, there was no difference in the expression of the receptor between $T_{reg}$ and $T_{eff}$ cells and the expression of the PRL receptor in $T_{eff}$ cells from patients with SLE was higher than in healthy controls (Figure 2).



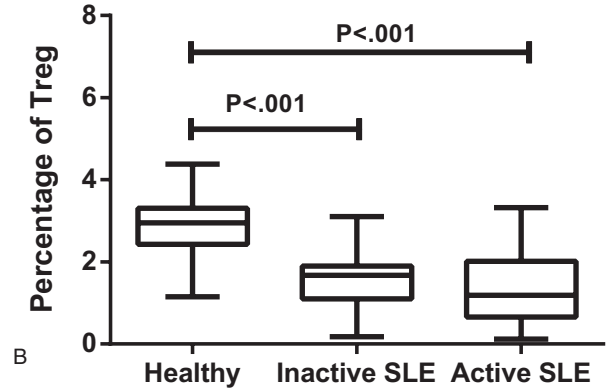




**FIGURE 1.** Percentage of $T_{reg}$ $CD4^+CD25^{hi}CD127^{-/low}FOXP3^+$ cells, PBMCs were stained with CD4, CD25, CD127, and FOXP3 antibodies, and the percentage of $T_{reg}$ cells was determined by flow cytometry. (A) Analysis strategy for determining the percentage of $T_{reg}$ cells from the $CD4^+$ gate. (B) Percentage of $T_{reg}$ cells in healthy individuals as well as patients with active and inactive SLE. The graph shows the median value; $P < 0.001$.

**TABLE 1.** Expression of Prolactin Receptor

| Prolactin Receptor | | Healthy ($\bar{x}$) | Inactive (SLE) ($\bar{x}$) | Active (SLE) ($\bar{x}$) |
|---|---|---|---|---|
| mRNA (relative expression) | $T_{reg}$ | 1.77 | 5.85 | 92.40 |
| | $T_{eff}$ | 0.01 | 1.67 | 22.47 |
| Protein (FMI) | $T_{reg}$ | 34.15 | 2347.73 | 6834.00 |
| | $T_{eff}$ | 34.44 | 1931.50 | 3202.00 |

PRL receptor expression in $T_{reg}$ and $T_{eff}$ subpopulations from healthy people and patients with active and inactive SLE.
FMI = mean fluorescence intensity, SLE = Systemic lupus erythematosus, $T^{eff}$ = effector T cells, $T^{reg}$ = regulatory T cells.

## PRL Function Regarding $T_{reg}$ Cell-Meditated Regulation

The suppressor capacity of $T_{reg}$ cells stimulated with "$T_{reg}$ Suppression Inspector human" (anti-CD2/CD3/CD28 beads) in the presence and absence of PRL was evaluated through in vitro cellular proliferation studies. The proliferation of $T_{eff}$ cells from healthy individuals is shown in Figure 3A. We observed that the addition of PRL did not exert any effect on the proliferation of these cells when $T_{reg}$ cells were added (coculture $T_{reg}$:$T_{eff}$), but the cells exerted suppressor activity over $T_{eff}$ cells by decreasing their proliferation in a significant manner ($P = 0.001$). The addition of PRL to this coculture interfered with the activity of $T_{reg}$ cells, reestablishing the proliferative capacity of $T_{eff}$ cells to levels similar to that of $T_{eff}$ cells in the absence of $T_{reg}$ cells. PRL did not affect the proliferation of $T_{eff}$ cells from patients with inactive SLE. The suppressor effect exerted by $T_{reg}$ cells over $T_{eff}$ cells was observed in most patients (Supplemental Content 2, http://links.lww.com/MD/A610). However, when considering the entire group, we did not find any statistically significant difference ($P = 0.08$) in the suppressor effect of $T_{reg}$ cells over $T_{eff}$ cells. Similar to healthy subjects, PRL does not increase the proliferation of $T_{eff}$ cells from inactive SLE patients. In $T_{reg}$:$T_{eff}$ coculture, the addition of PRL decreased the regulatory effect of $T_{reg}$ cells, thus causing an increase in the proliferation of $T_{eff}$ cells, with a statistically significant difference ($P = 0.001$; Figure 3B). In contrast, in cells from patients with active SLE, PRL activity increased the proliferation of $T_{eff}$ cells in a statistically significant manner ($P = 0.006$). The $T_{reg}$ cells from these patients did not have the capacity to exert their suppressor activity over the $T_{eff}$ cells, although the addition of PRL to the $T_{reg}$:$T_{eff}$ coculture tended to increase the proliferation of $T_{eff}$ cells, with no statistically significant difference ($P = 0.06$; Figure 3C). This result suggests that the function of $T_{reg}$ cells is no longer adequate under this condition (Table 2).

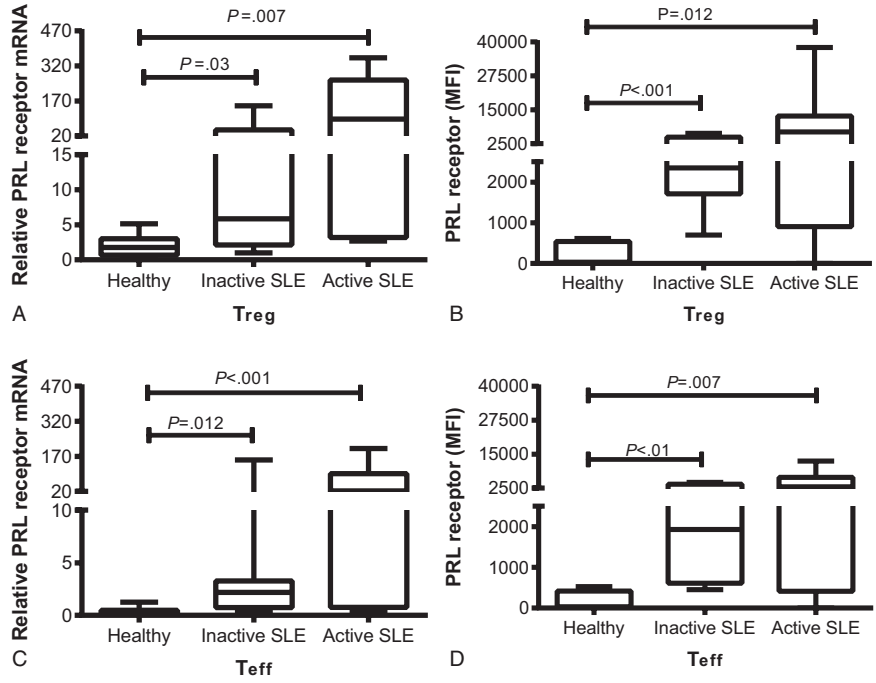



**FIGURE 2.** Expression of PRL receptor, $T_{eff}$ ($CD4^+CD25^-CD127^+$) and $T_{reg}$ ($CD4^+CD25^{hi}CD127^{-/low}$) cell subpopulations from healthy individuals and SLE patients were purified from the PBMCs by using magnetic beads. The relative mRNA expression of PRL receptor was determined in (A) $T_{reg}$ and (B) $T_{eff}$ cells by PCR-RT. Flow cytometry was used to determine the protein expression in (C) $T_{reg}$ and (D) $T_{eff}$ cells. The graph shows the median value.

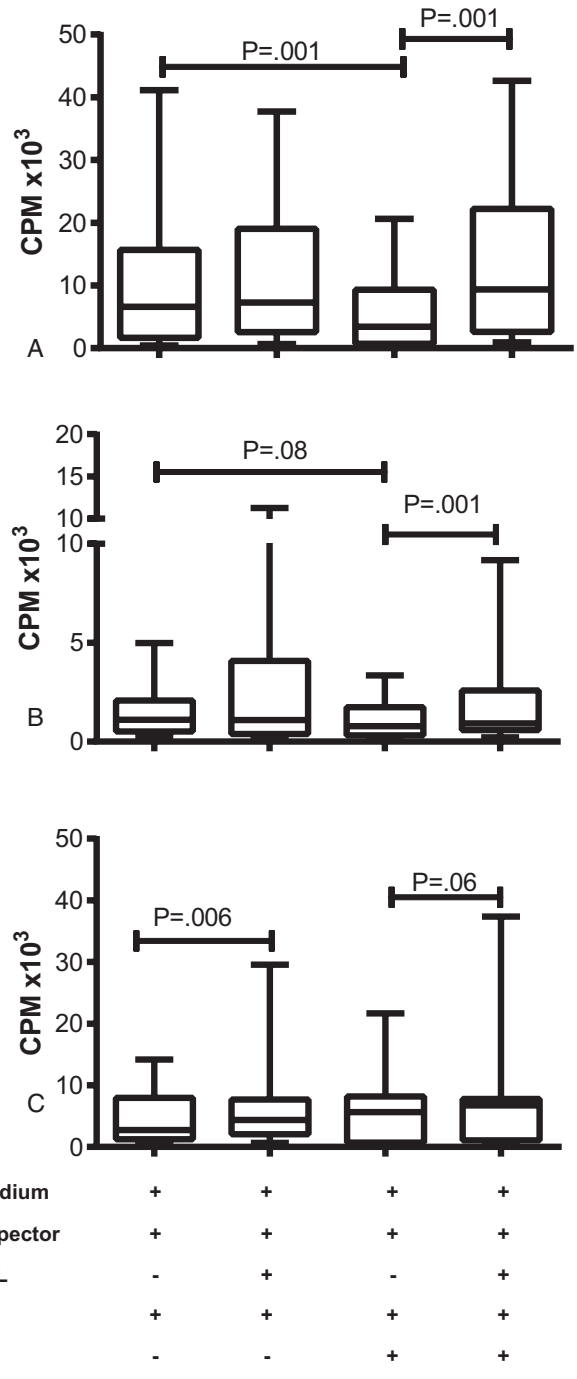



**FIGURE 3.** Effects of PRL on the functions of $T_{eff}$ and $T_{reg}$ cells, $T_{eff}$ ($CD4^+CD25^-CD127^+$) and $T_{reg}$ ($CD4^+CD25^{hi}CD127^{-/low}$) cells from healthy individuals and SLE patients were stimulated with "$T_{reg}$ Suppression Inspector human" (anti-CD2/CD3/CD28 beads) in the presence and absence of PRL (50 ng/ml). Cell proliferation was measured by incorporating [$^3$H]-thymidine in the cells from (A) healthy individuals, (B) inactive SLE patients, and (C) active SLE patients. The median value of 12 independent trials for each group is presented. The assays were performed in triplicate (statistical significance, $P \leq 0.05$).

## Cytokine Secretion by $T_{eff}$ Cells Cultured in the Presence and Absence of PRL

Cytokine concentrations were determined in $T_{eff}$ culture supernatants stimulated with "$T_{reg}$ Suppression Inspector human" (anti-CD2/CD3/CD28 beads) in the presence and absence of PRL. As shown in Figure 4A, we observed that IL10 secretion from $T_{eff}$ cells from patients with active or inactive SLE was decreased with respect to that from the cells from healthy individuals, with a statistically significant difference ($P = 0.05$), but there were no differences between inactive and active patients. The addition of PRL to the $T_{eff}$ cell culture did not modify the secretion pattern of IL10 when using cells from any of the 3 studied groups. No difference in the secretion of IL17A, TNF, or IFNγ was observed for the $T_{eff}$ cells from the 3 groups, and the addition of PRL did not affect IL17A and TNF secretion. However, PRL treatment increased IFNγ secretion from $Te_{ff}$ from inactive patients, with a statistically significant difference ($P = 0.01$) (Figure 4B); meanwhile, in active patients, only an increase was observed, without any statistically significant difference ($P = 0.08$; Table 3).

## Cytokine Secretion by $T_{reg}$ and $T_{eff}$ Cell Coculture in the Presence and Absence of PRL

Cytokine secretion was determined in the presence and absence of PRL by using $T_{reg:}T_{eff}$ cocultures from the 3 groups being studied. The addition of PRL to the $T_{reg:}T_{eff}$ coculture from healthy individuals significantly increased the secretion of IL10, TNF, and IFNγ, whereas IL17A secretion was unaffected. Meanwhile, PRL significantly increased IFNγ secretion in $T_{reg}$:$T_{eff}$ cocultures using cells from patients with inactive SLE ($P = 0.05$) and IL17A secretion increased in most patients. However, we did not find any statistically significant difference ($P = .07$) in case of the entire group; there was no difference in TNF and IL10 secretion. Cytokine secretion was not affected by the addition of PRL to the cocultures using cells from patients with active SLE (Figure 5, Table 4).

## DISCUSSION

Sex hormones such as PRL play an important role in the modulation of immune response, which depends on the type of cell expressing the PRL receptor.[7,34] Moreover, PRL has an immune-stimulating effect and promotes autoimmunity,[5] interfering with the tolerance of B cells[35] and increasing the production of antibodies.[5,36] We previously reported that the PRL receptor is constitutively expressed in the $T_{reg}$ cells of healthy individuals (females), whereas the expression increases in $T_{eff}$ cells in response to a stimulus.[13] The results of this study showed that compared to healthy individuals, the expression of PRL receptor was higher in the $T_{reg}$ and $T_{eff}$ cells from patients with SLE (females), with the receptor being expressed even in the absence of a stimulus. This expression tended to increase in cells from active patients compared to that from inactive patients, suggesting higher activity in the disease, along with higher expression of the receptor. Which occurs in B cells from mice that developed lupus (MRL, MRL/lpr), whereby the expression of the receptor increased with the manifestation of the disease.[37,38] In addition, the expression patterns of $T_{eff}$ and $T_{reg}$ cells differed between active and inactive patients. In inactive patients, the expression of the receptor was higher in $T_{reg}$ cells compared to $T_{eff}$ cells, a behavior similar to that observed in healthy individuals. However, there was no difference in the expression of the receptor between $T_{eff}$ and $T_{reg}$ cells from active patients, most likely because the $T_{eff}$ cells were

**TABLE 2.** Cell Proliferation

| | $T_{eff}$ ($\bar{x}$) | $T_{eff}$ + PRL ($\bar{x}$) | $T_{eff}$:$T_{reg}$ ($\bar{x}$) | $T_{eff}$:$T_{reg}$ + PRL ($\bar{x}$) |
|---|---|---|---|---|
| Healthy | $6.58 \times 10^3$ | $7.27 \times 10^3$ | $3.41 \times 10^3$ | $9.39 \times 10^3$ |
| Inactive (SLE) | $1.10 \times 10^3$ | $1.09 \times 10^3$ | $0.77 \times 10^3$ | $0.93 \times 10^3$ |
| Active (SLE) | $2.76 \times 10^3$ | $4.35 \times 10^3$ | $5.66 \times 10^3$ | $6.74 \times 10^3$ |

Different cell populations ($T_{eff}$, $T_{reg}$, and $T_{eff}$ $T_{reg}$ cocultivation) were activated with the inspector (anti-CD2/CD3/CD28 beads) in the presence and absence of PRL from healthy, active, and inactive SLE patients.

PRL = Prolactin, SLE = Systemic lupus erythematosus, $T_{eff}$ = effector T cells, $T_{reg}$ = regulatory T cells.

already active, increasing the expression of PRL receptor. This would be similar to the phenomenon in $T_{eff}$ cells from healthy individuals: when activated, the cells increase the expression of the receptor to a level higher than that in $T_{reg}$ cells.[13]

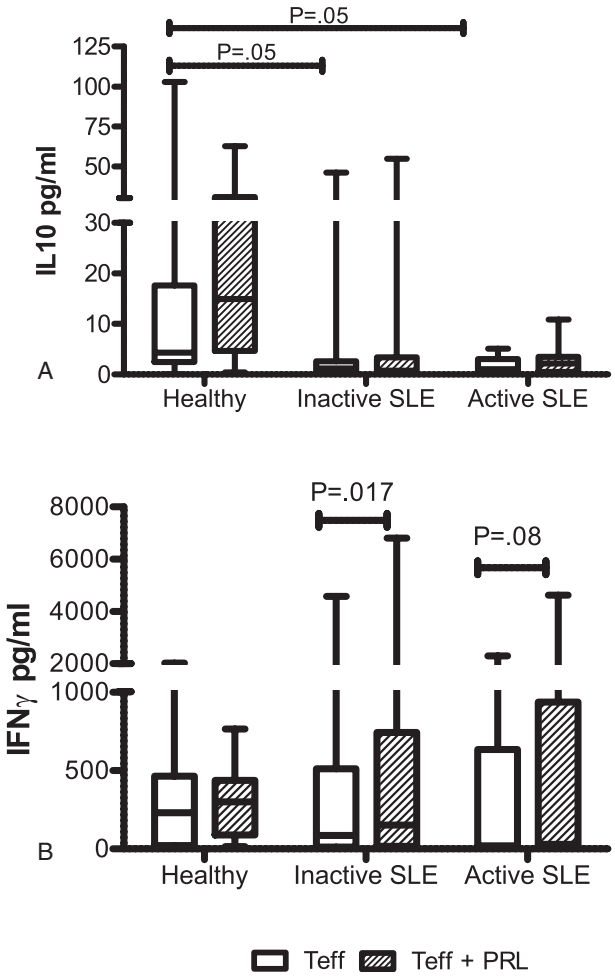



**FIGURE 4.** Cytokine secretion profile of $T_{eff}$ in the presence and absence of PRL, $T_{eff}$ cells from healthy persons and SLE patients were stimulated with "$T_{reg}$ Suppression Inspector human" (anti-CD2/CD3/CD28 beads) in the presence and absence of PRL. The secretion of (A) IL10, and (B) IFNγ was determined by CBA. The median value is presented for each group (statistical significance, $P \leq 0.05$).

$T_{reg}$ cells are a component of one of the peripheral tolerance mechanisms, which fail in autoimmune diseases such as SLE; therefore, these cells are important in the pathogenesis of the disease.[2] However, available data on the number and function of $T_{reg}$ cells in SLE are contradictory, and the definitive role of $T_{reg}$ cells in SLE remains unclear.[29] Therefore, we decided to explore, the percentage of $T_{reg}$ cells in patients with active and inactive SLE, and the role played by PRL in the regulatory function of these cells ex vivo. A statistically significant decrease was found in the percentage of $T_{reg}$ ($CD4^+CD25^{hi}CD127^{-/low}$ $FOXP3^+$) cells from patients with SLE, both active and inactive, compared to that in healthy individuals, supporting the findings of previous studies.[22,24,39,40] Additionally, the suppressor function exerted by $T_{reg}$ cells over $T_{eff}$ cells depends on the stage of the disease. In patients with inactive SLE, we observed 2 behaviors; first, $T_{reg}$ cells did not present any defects in their suppressor activity (majority of the patients), and second, $T_{reg}$ cells did not present a suppressor function in another group of patients (minority of the patients). Although the patients are clinically inactive, their immune system is probably active, and therefore, $T_{reg}$ cells no longer exert their suppressor effect, as observed in active patients where we did not observe $T_{reg}$ suppressor function, as has been reported. The decrease in the number and function of $T_{reg}$ cells in SLE patients favors the activation of autoreactive clones, and thus, disease manifestation.[26,40,41]

Because $T_{reg}$ cells from SLE patients express high levels of PRL receptor, we studied whether an interaction with its PRL receptor could affect the suppressor effect of $T_{reg}$ cells, especially those from inactive patients, possessing suppressor function. In these patients, PRL blocked the suppressor effect of $T_{reg}$ cells on $T_{eff}$ cells, a behavior similar to healthy individuals.[13] The loss of suppressor effect cannot be attributed to the notion that PRL increases the proliferation of $T_{eff}$ cells, because the addition of PRL to the $T_{eff}$ cell culture did not increase the proliferation of these cells. It might be due to the presence of proinflammatory cytokines (IFNα, IFNγ, and TNF),[42–45] as their presence in the culture reduces the suppressor effect of $T_{reg}$ cells. It is also known that PRL promotes the secretion of cytokines such as IFNγ, IL2, IL12, and TNF.[12,46,47] Our results showed an increase in IFNγ levels in the cocultures incubated with PRL ($T_{reg}$:$T_{eff}$ of inactive patients), and although an increase in IL17 levels was observed in these cultures, it was not statistically significant. The increase in IFNγ levels by the addition of PRL was also observed in $T_{eff}$ cell cultures (expressing PRL receptor), which makes us hypothesize that interaction of PRL with its receptor on $T_{eff}$ cells increases IFNγ secretion, and that the presence of this cytokine in the culture decreases the suppressor function of $T_{reg}$ cells in patients with inactive SLE, because this cytokine is known to inhibit the generation and/or

**TABLE 3.** Cytokine Secretion by $T_{eff}$ Cells

| Cytokine (pg/ml) | Cultures ($T_{eff}$) | Healthy ($\bar{x}$) | Inactive (SLE) ($\bar{x}$) | Active (SLE) ($\bar{x}$) |
|---|---|---|---|---|
| IL10 | With PRL | 5.97 | 1.31 | 1.10 |
| | Without PRL | 10.57 | 0.64 | 2.10 |
| | *P*= | 0.79 | 0.37 | 0.28 |
| IL17 | With PRL | 10.10 | 12.13 | 4.44 |
| | Without PRL | 10.03 | 12.29 | 10 |
| | *P*= | 0.72 | 0.07 | 0.99 |
| TNF | With PRL | 13.65 | 13.49 | 9.14 |
| | Without PRL | 15.78 | 18.98 | 11.42 |
| | *P*= | 0.59 | 0.22 | 0.51 |
| IFNγ | With PRL | 229.74 | 85.22 | 18.76 |
| | Without PRL | 299.99 | 150.90 | 29.26 |
| | *P*= | 0.33 | 0.01* | 0.08 |

$T_{eff}$ cells from healthy, active, and inactive SLE patients were activated with the inspector (anti-CD2/CD3/CD28 beads) in the presence and absence of PRL for 5 days.

IFNγ = Interferon gamma, IL = Interleukin, PRL = Prolactin, SLE = Systemic lupus erythematosus, $T_{eff}$ = effector T cells, TNF = Tumor Necrosis Factor.

* Statistically significant difference.

function of $T_{reg}$ cells.[44,48,49] It is also possible that IFNγ is secreted by $T_{reg}$ cells, as reported in patients with type I diabetes and rheumatoid arthritis, diseases in which $T_{reg}$ cells that secrete proinflammatory cytokines as IFNγ and IL17.[50–52] Unfortunately because of the low number of $T_{reg}$ cells purified from patients, we could not verify whether PRL favors IFNγ secretion in these cells. It will be interesting to show whether PRL favors the presence of $T_{reg}$ IFNγ-secreting cells, especially because this has been reported for other autoimmune diseases.[50–52]

Our results show that both $T_{reg}$ and $T_{eff}$ cells in women with inactive SLE constitutively express the PRL receptor, and therefore, an increase in serum PRL levels will favor the interaction of PRL and its receptor and, in turn, the malfunctioning of the Treg cells, probably because of presence of IFNγ. This malfunction, added to the decrease in the cell

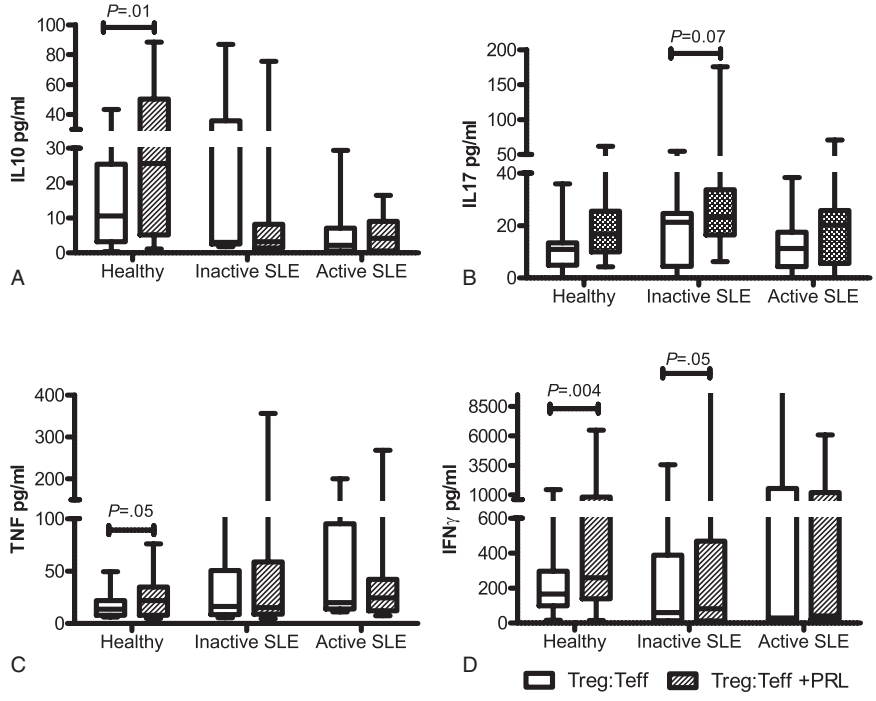



**FIGURE 5.** Cytokine secretion profile in $T_{reg}$:$T_{eff}$ coculture in the presence and absence of PRL, $T_{reg}$:$T_{eff}$ cocultures using cells from healthy persons and SLE patients were stimulated with "$T_{reg}$ Suppression Inspector human" (anti-CD2/CD3/CD28 beads) in the presence and absence of PRL. The secretion of (A) IL10, (B) IL17, (C) TNF, and (D) IFNγ was determined by CBA. The median value is presented for each group (statistical significance, $P \leq 0.05$).

**TABLE 4.** Cytokine Secretion by $T_{eff}$:$T_{reg}$ Cocultures

| Cytokine (pg/ml) | Cocultures ($T_{reg}$:$T_{eff}$) | Healthy (x̄) | Inactive (SLE) (x̄) | Active (SLE) (x̄) |
|---|---|---|---|---|
| IL10 | With PRL | 10.56 | 2.25 | 2.15 |
| | Without PRL | 25.60 | 2.16 | 4.2 |
| | *P*= | 0.01* | 0.17 | 0.23 |
| IL17 | With PRL | 10.88 | 21.32 | 11.23 |
| | Without PRL | 16.80 | 23.03 | 20.08 |
| | *P*= | 0.19 | 0.07 | 0.48 |
| TNF | With PRL | 13.46 | 16.16 | 19.90 |
| | Without PRL | 21.80 | 15.06 | 24.46 |
| | *P*= | 0.05* | 0.22 | 0.37 |
| IFNγ | With PRL | 165.29 | 59.53 | 28.65 |
| | Without PRL | 258.89 | 95.67 | 39.33 |
| | *P*= | 0.004* | 0.05* | 0.72 |

$T_{eff}$ cells were cocultured with $T_{reg}$ cells (healthy, active, and inactive SLE patients), and were activated with the inspector (anti-CD2/CD3/CD28 beads) in the presence and absence of PRL for 5 days.

IFNγ = Interferon gamma, IL = Interleukin, PRL = Prolactin, SLE = Systemic lupus erythematosus, $T_{eff}$ = effector T cells, TNF = Tumor Necrosis Factor, $T_{reg}$ = regulatory T cells.

* Statistically significant difference.

number, will contribute to the expansion of autoreactive T-lymphocytes, favoring disease activation. In patients with active SLE, different from those with inactive SLE, PRL increased the cellular proliferation of $T_{eff}$ cells. Thus, PRL in active patients could help in maintaining the disease active by favoring the proliferation of $T_{eff}$ cells among those that are autoreactive.

It is worth mentioning that in our study, we did not use antigen-presenting cells (APCs); only $T_{reg}$ cells were coincubated with $T_{eff}$ cells to observe the suppressor effect of $T_{reg}$ cells. Other models using APCs as a suppressor of the function of $T_{reg}$ cells have been reported. In this sense, it has been proposed that the APCs can block $T_{reg}$ cell activity via overproduction of pro-inflammatory cytokines such as IFNα.[42] It would be interesting to determine whether APCs express PRL receptor, and whether PRL favors the secretion of IFNα and other inflammatory cytokines, thereby aiding the malfunction of $T_{reg}$ cells in SLE patients.

## CONCLUSIONS

Our results showed that $T_{reg}$ cells from patients with SLE differed from those from healthy individuals with regard to number and function. In inactive patients, PRL acts on $T_{eff}$ cells, which constitutively express the receptor, increasing IFNγ secretion and encouraging an inflammatory microenvironment and $T_{reg}$ cell malfunction. The decrease in the number of T reg cells and their malfunction can contribute to the expansion of autoreactive T-lymphocytes to favor disease activation. Additionally, in active patients, PRL increases the proliferation of inspector-stimulated $T_{eff}$ cells, which can further aid the $T_{eff}$ cells to be more resistant to regulation by $T_{reg}$ cells. It will be interesting to study whether PRL decreases the function of different subpopulations of $T_{reg}$ cells and whether this decrease occurs because PRL favors the plasticity of $T_{reg}$ cells toward a Th1 profile.

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