Prolactin down-regulates CD4⁺CD25^{hi}CD127^{low/-} regulatory T cell function in humans

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Abstract

Among its many functions, prolactin (PRL) participates in immune responses and promotes the activation, differentiation and proliferation of T cells. However, the mechanisms by which PRL regulates regulatory T (T_{reg}) cells are still unknown. Our goal was to determine whether PRL plays a role in T_{reg} function. We measured the expression of PRL and its receptor in T_{reg} and effector T (T_{eff}) cells from 15 healthy individuals. We also evaluated the functional activity of T_{reg} cells by examining proliferation and cytokine secretion in cells activated with anti-CD3/CD28 in the presence or absence of PRL. We report that T_{reg} cells constitutively expressed PRL receptor, whereas T_{eff} cells required stimulation with anti-CD3/CD28 to induce PRL receptor expression. Expression of PRL was constitutive in both populations. We found that the addition of PRL inhibited the suppressor effect (proliferation) mediated by T_{reg} cells *in vitro*, reducing suppression from 37.4 to 13% when PRL was added to co-cultures of T_{reg} and T_{eff} cells ($P < 0.05$). Cultures treated with PRL favoured a Th1 cytokine profile, with increased production of TNF and IFN γ . We report for the first time that PRL receptor expression was constitutive in T_{reg} cells but not in T_{eff} cells, which require stimulation to induce PRL receptor expression. PRL inhibited the suppressive function of T_{reg} cells, apparently through the induced secretion of Th1 cytokines.

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Introduction

Prolactin (PRL) is a lactogenic hormone that is synthesised and secreted by the pituitary gland. It has been associated with more than 300 different functions, with its functionality depending on the type of cells that express PRL receptor (Kelley *et al.* 2007). The PRL receptor belongs to the haematopoietic receptor superfamily; interaction between PRL and its receptor activates the Jak–Stat pathway, which is also commonly activated during cytokine signalling (Bole-Feysot *et al.* 1998, Kelley *et al.* 2007). PRL can also be synthesised in an extra-pituitary way by B and T cells from the immune system, which also express PRL receptor (Bole-Feysot *et al.* 1998). During an immune response, PRL promotes the proliferation and differentiation of T cells (Carreño *et al.* 2005) and influences the expression of CD69 and CD154 in CD4⁺T cells (Chávez-Rueda *et al.* 2005, 2007). In addition, when CD8⁺T cells are co-incubated with PRL and phytohaemagglutinin, CD69 expression increases (Takizawa *et al.* 2005). In CD4⁺T cell cultures activated with phorbol myristate acetate and incubated with an anti-PRL antibody,

reduced secretion of IL2 and IFN γ is observed, indicating a role for PRL in the regulation of cytokine secretion (Chávez-Rueda *et al.* 2005). These results demonstrate the importance of PRL in the immune system. In B cells, PRL is involved in the induction of tolerance (Saha *et al.* 2009). We hypothesised that PRL could participate in the regulation of the immune system via regulatory T (T_{reg}) cells.

T_{reg} cells help maintain peripheral tolerance and exert a suppressive effect on the activation, proliferation and function of other cells in the immune system (Le & Chao 2007). There are two types of T_{reg} cells: natural T_{reg} cells, which are generated in the thymus, and inducible T_{reg} cells, which are generated in the periphery; both exhibit the same CD4⁺CD25^{hi}CD127^{low/-}FoxP3⁺ phenotype (Liu *et al.* 2006, Seddiki *et al.* 2006, Le & Chao 2007). T_{reg} cells are unable to proliferate or secrete IL2 after their TCR is stimulated; instead, they exert an inhibitory effect on CD4⁺CD25⁻CD127⁺ conventional or effector T (T_{eff}) cells. T_{reg} cells inhibit T_{eff} cell proliferation and IL2 production (Thornton & Shevach 1998) through a contact-dependent mechanism and/or the secretion of

anti-inflammatory cytokines, including TGF β (Nakamura *et al.* 2001) and IL10 (Asseman *et al.* 1999). It has been proposed that T_{reg} cells prevent the development of autoimmune diseases that have been associated with high levels of PRL (De Bellis *et al.* 2005), including systemic lupus erythematous (Scheinecker *et al.* 2010), type 1 diabetes (Salomon *et al.* 2000) and inflammatory bowel disease (Maloy *et al.* 2003, Mottet *et al.* 2003).

Therefore, we decided to investigate the participation of PRL in T_{reg} cell function. Our results indicate that T_{reg} cells constitutively express PRL receptor, whereas in T_{eff} cells, PRL receptor expression requires stimulation. We observed that when PRL is added to co-cultures of T_{reg} and T_{eff} cells, it reverses the T_{reg} suppressive effect on T_{eff} cells and promotes a Th1 cytokine profile.

Materials and methods

Study group

The ethics committee on Human Research of the Instituto Mexicano del Seguro Social (IMSS) approved this study, and it was conducted according to the Declaration of Helsinki with the informed consent of all participants. The samples studied were obtained from 15 healthy women of reproductive age (25–45 years) without menstrual disorders and with normal levels of serum PRL (<20 ng/ml). The samples were obtained from the cubital vein between 0800 and 1100 h after overnight fasting or a light morning meal consisting of fruit and tea.

Prolactin

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

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, USA); mouse anti-human CD28 (37407) from R&D Systems (Minneapolis, MN, USA); mouse anti-human CD3 (HIT3a) from BD Biosciences (San Jose, CA, USA); mouse anti-PRL receptor (ECD, 1A2B1) from Invitrogen and biotin rat anti-mouse IgG2b (R12-3) from BD Pharmingen (San Jose, CA, USA). The biotinylated secondary antibody was detected with streptavidin-phycoerythrin-Cy5.5 from BD Biosciences (Mountain View, CA, USA).

CD4⁺T cell purification and sorting of T_{reg} and T_{eff} populations

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples by density centrifugation using Lymphoprep (Axis Shield, Oslo, Norway). CD4⁺T cells were isolated from PBMCs using a CD4⁺T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Enriched CD4⁺T cells were stained with anti-CD4, -CD127 and -CD25 for 30 min at 4 °C. CD4⁺CD25^{hi}CD127^{low/-} (T_{eff}) cells and CD4⁺CD25^{hi}CD127^{low/-} (T_{reg}) cells were sorted using a FACS Aria (BD Biosciences) supported by BD Biosciences FACSDiva software. The purity of the sorted cells ranged between 96 and 98%.

Cell culture and proliferation assays

Cells were cultured in AIM-V liquid medium (Gibco BRL) supplemented with 50 units/ml penicillin and 50 μ g/ml streptomycin (Gibco). T_{reg} cells (CD4⁺CD25^{hi}CD127^{low/-}) were plated at 1.0×10^4 cells/well in 96-well U-bottom plates (Nunc, Roskilde, Denmark) in the presence or absence of 2.0×10^4 T_{eff} cells (CD4⁺CD25^{hi}CD127⁺) and cultured in synthetic serum-free medium (AIM-V, Gibco). We determined the optimum ratio of T_{eff} : T_{reg} cells using a dose-response curve with the following ratios: 1:0.5, 1:1, 1:2 and 1:4. The suppressive effect was observed with all ratios tested. We decided to use a ratio of 1:0.5 T_{eff} : T_{reg} because, given the percentage of circulating T_{reg} cells, it would be feasible to obtain enough cells to perform all the experiments.

Based on a dose-response curve, we decided to use 2.5 μ g/ml soluble anti-CD3 and 1 μ g/ml soluble anti-CD28 antibodies to specifically activate T cells in the presence or absence of 50 ng/ml human PRL (NHPP). The concentrations of antibodies and PRL were also determined based on dose-response curves. Cells were cultured for 5 days, and 1 μ Ci [³H]thymidine (Hartmann Analytical, Braunschweig, Germany) was added 18 h before harvesting. Thymidine incorporation was determined using a liquid scintillation analyser (Packard 1900 TR, Meriden, CT, USA), and the suppression of proliferation was calculated as a percentage. All conditions had previously been standardised and optimised.

Cytokine detection

Cell culture supernatants were collected on day 2, and Th1 (TNF, IFN γ and IL2), Th2 (IL4, IL5 and IL10) and Th17 (IL17A) cytokine levels were measured using a commercially available BD Cytometric Bead Array (CBA); TGF β was measured by ELISA (R&D Systems).

Real-time PCR assay

Total RNA was extracted from purified T_{reg} and T_{eff} cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The RNA concentration was determined using u.v. spectrophotometry. To generate cDNA, 1 μ g of total RNA was used with SuperScript II reverse transcriptase (Invitrogen). The PRL receptor, PRL and β -actin were then amplified by real-time PCR using a LightCycler TaqMan Master kit (Roche Diagnostic) and hydrolysis probes and primers designed by Roche Diagnostic; all reactions were performed according to the manufacturer's specifications. The following primers and probes were used: probe number 18 from the Universal Probe Library for PRL determination, forward primer AAA GGA TCG GCC ATG GAA AG and reverse primer GCA CAG GAG CAG GGT TTG AC; probe number 8 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 probe number 64 from the Universal Probe Library for β -actin determination, forward primer CCA ACC GCG AGA AGATGA and reverse primer CCA GAG GCG TAC AGG GAT AG. The reactions were carried out in a final volume of 10 μ l using a LightCycler 1.5 instrument (Roche Diagnostic). The PCR conditions were as follows: 10 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 30 s at 59 °C and 1 s at 72 °C, followed by 1 cycle of 30 s at 40 °C. The samples were normalised to β -actin gene expression. The relative expression of PRL and its receptor were calculated using the $2\Delta C_T$ formula.

Cell surface staining and flow cytometry

T_{reg} and T_{eff} cells were incubated with fluorescently labelled antibodies (anti-CD4, -CD25, -CD127 and -PRL receptor) for 20 min at 4 °C in staining buffer (PBS with 0.5% BSA and 0.01% sodium azide). The cells were then washed and fixed in 2% paraformaldehyde (Sigma-Aldrich). Data were acquired using a FACSaria flow cytometer (BD Biosciences) and analysed with FlowJo software (Tree Star, Ashland, OR, USA).

Detection of PRL in the culture supernatants

PRL concentrations in culture supernatants from T_{eff} and T_{reg} cells were measured using the Nb2 lymphoma cell assay. The cells were kept at 37 °C in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, 50 units/ml penicillin and 50 μ g/ml streptomycin. The cells were arrested in the early G1 phase of the cell cycle by pre-incubation (24 h) in medium with 1% FBS. Afterwards, the cells were distributed in flat-bottom 96-well plates at a density of 2×10^5 cells/well,

and the culture supernatants from T_{eff} and T_{reg} cells (1×10^6 cells/well) stimulated with anti-CD3/CD28 were added to reach a final volume of 200 μ l, without FBS addition. After 72 h at 37 °C with 5% CO₂, the presence of viable cells was assessed by measuring incorporated [³H]thymidine.

Statistical analysis

Statistical analyses were performed by the statistical software package SPSS, version 15.0 (SPSS, Inc., Chicago, IL, USA). The experimental data were analysed using standard statistical tests (e.g. mean value, s.d., Student's *t*-test and ANOVA), and the results were expressed as the mean $\pm$ s.d. The threshold for significance was set at $P < 0.05$.

Results

CD4⁺T cells were isolated by negative selection from healthy women PBMCs with purity >90%. The cells were then stained with fluorochrome-conjugated monoclonal antibodies, and T_{reg} (CD4⁺CD25^{hi}CD127^{low/-}) and T_{eff} (CD4⁺CD25⁻CD127⁺) cells were sorted using a FACSaria; a purity higher than 98% was obtained for each population. In addition, FoxP3 expression analysis showed that only the sorted T_{reg} cell population was FoxP3⁺ (Fig. 1).

When the relative mRNA expression of PRL receptor was examined in unfractionated CD4⁺T cells, we found it to be constitutively expressed; this cell population was used as a control to calculate the relative expression of PRL receptor mRNA in purified cell subsets. Next, PRL receptor mRNA expression was examined in the sorted T_{reg} and T_{eff} cell populations. We found that while T_{reg}

Figure 1 Purification of CD4⁺CD25^{hi}CD127^{low/-}FoxP3⁺ (T_{reg}) and CD4⁺CD25⁻CD127⁺FoxP3⁻ (T_{eff}) cell populations. CD4⁺T cells from the peripheral blood of healthy women were isolated by negative selection; T_{reg} and T_{eff} cells were purified by fluorescence-activated cell sorting using monoclonal antibodies directed against human CD4, CD25 and CD127. A sample of these populations was used to determine Foxp3 expression. The cell subsets obtained had a purity >98%.

cells constitutively expressed PRL receptor mRNA (0.8 ± 0.4), PRL receptor mRNA expression was not detected in T_{eff} cells (Fig. 2A). We also evaluated expression at the protein level by flow cytometry, and again, we detected PRL receptor expression in T_{reg} cells (349.5 ± 80.75 mean fluorescence intensity (MFI)) but not in T_{eff} cells (Fig. 2B and C). To investigate whether PRL receptor mRNA expression in T_{eff} cells could be induced, cells were stimulated with anti-CD3/CD28 antibodies for different periods of time before the mRNA expression was analysed. T_{eff} cells up-regulated the expression of PRL receptor mRNA as early as 2 h after stimulation (0.77 ± 0.55); this expression increased at 4 h post-stimulation (2.3 ± 0.41). However, at 6 h (1.83 ± 0.28) after stimulation, PRL receptor mRNA expression decreased without the difference observed being statistically significant (Fig. 2D). At the protein level, we found that T_{eff} cells up-regulated the expression of the PRL receptor 2 h after stimulation (505 ± 17 MFI); this expression was maintained

throughout the time course (Fig. 2E). Together, these results suggest that PRL receptor mRNA expression in T_{eff} cells is inducible. As mentioned, T_{reg} cells constitutively expressed PRL receptor mRNA; this gene was overexpressed at 2 h post-stimulation (3.9 ± 1.39) and tended to be down-regulated at 4 and 6 h after stimulation (2.9 ± 1.31 and 0.76 ± 0.15 , respectively; Fig. 2F). We also observed an increase in the expression of the protein at 2 h post-stimulation (781 ± 104.5 MFI), and these levels were maintained at 4–6 h (698.7 ± 175.6 and 625.7 ± 226.7 MFI; Fig. 2G).

Using the same approach, we found that, unlike PRL receptor, PRL mRNA was constitutively expressed in all cell populations examined (T_{reg} and T_{eff} cells). PRL mRNA expression in T_{eff} cells was significantly higher compared with T_{reg} cells (10.52 ± 3.61 vs 4.71 ± 0.68 , respectively, $P < 0.05$; Fig. 3A). We also stimulated T_{eff} and T_{reg} cells with anti-CD3/CD28 at different times to evaluate the expression of this gene, and we found that PRL mRNA remained constant over time in T_{eff} and T_{reg}

Figure 2 PRL receptor expression in T_{reg} and T_{eff} cells. Using real-time PCR, the expression of PRL receptor mRNA was measured in purified T_{reg} and T_{eff} cells. In all cases, we used $CD4^+$ T cells as a reference population. (A) Relative PRL receptor mRNA expression in T_{reg} and T_{eff} cells under basal conditions. (D) Kinetic expression of PRL receptor mRNA in T_{eff} cells stimulated with anti-CD3 ($2.5 \mu\text{g/ml}$) and anti-CD28 ($1 \mu\text{g/ml}$). (F) Kinetic expression of PRL receptor mRNA in T_{reg} cells stimulated with anti-CD3 ($2.5 \mu\text{g/ml}$) and anti-CD28 ($1 \mu\text{g/ml}$). Using flow cytometry, we determined the expression of PRL receptor in purified T_{reg} and T_{eff} cell populations (shown as the mean fluorescence intensity). (B) PRL receptor expression in T_{reg} and T_{eff} cells under basal conditions. (C) Cytometric analysis of PRL receptor expression in T_{reg} and T_{eff} cells under basal conditions. (E) Kinetic expression of PRL receptor in T_{eff} cells stimulated with anti-CD3 ($2.5 \mu\text{g/ml}$) and anti-CD28 ($1 \mu\text{g/ml}$). (G) Kinetic expression of PRL receptor in T_{reg} cells stimulated with anti-CD3 ($2.5 \mu\text{g/ml}$) and anti-CD28 ($1 \mu\text{g/ml}$). * $P < 0.05$.

Figure 3 PRL expression in T_{reg} and T_{eff} cells. Using real-time PCR, the presence of PRL mRNA was measured in purified T_{reg} and T_{eff} cells. (A) Relative PRL mRNA expression in T_{reg} and T_{eff} cells under basal conditions ($*P < 0.05$). (B) Kinetic expression of PRL mRNA in T_{eff} cells stimulated with anti-CD3 (2.5 μ g/ml) and anti-CD28 (1 μ g/ml). (C) Kinetic expression of PRL mRNA in T_{reg} cells stimulated with anti-CD3 (2.5 μ g/ml) and anti-CD28 (1 μ g/ml).

cells (Fig. 3B and C). We used Nb2 cell bioassays to evaluate the secretion of PRL into culture supernatants from T_{eff} and T_{reg} cells stimulated with anti-CD3/CD28. We were able to detect secreted PRL in the supernatants from cultures of both T cell subsets 18 h after stimulation (T_{eff} : 11.30 pg/ml and T_{reg} : 9.41 pg/ml).

Next, the suppressive function of T_{reg} cells was analysed *in vitro*. Figure 4 shows the mean $\pm$ s.d. of 15 independent cellular proliferation assays, each one performed in triplicate. When T_{eff} cells were stimulated with anti-CD3/CD28, the cells proliferated and incorporated [3 H]thymidine ($67.0 \times 10^3 \pm 10 \times 10^3$ c.p.m.). The addition of PRL to this culture resulted in a slight increase in cell proliferation ($81.32 \times 10^3 \pm 6 \times 10^3$ c.p.m.); however, this was not statistically significant ($P = 0.08$). As expected, T_{reg} cells did not proliferate when they were stimulated with anti-CD3/CD28, and the addition of PRL had no further effect. In the suppression assay, when T_{reg} and T_{eff} cells were

co-incubated at a ratio of 0.5:1 T_{reg} to T_{eff} cells, we observed a 37.4% decrease in proliferation, which was statistically significant ($42.4 \times 10^3 \pm 8.9 \times 10^3$ vs $67.0 \times 10^3 \pm 10 \times 10^3$ c.p.m.; $P < 0.05$). However, when PRL was added to the T_{reg} : T_{eff} co-culture, we only observed a 13% decrease in proliferation ($59.0 \times 10^3 \pm 8.8 \times 10^3$ vs $67.0 \times 10^3 \pm 10 \times 10^3$ c.p.m.), suggesting that PRL restored the proliferative capacity of the T_{eff} cells. Furthermore, if we compare the proliferation of T_{eff} cells alone against the co-culture (T_{eff} : T_{reg} cells) when both cultures were treated with PRL, no significant differences were found; therefore, the T_{reg} cells had no suppressive effect in the co-culture with PRL.

We examined the presence of Th1 and Th2 cytokines in supernatants from cells cultured under the following conditions: T_{eff} cells stimulated with anti-CD3/CD28 in the presence or absence of PRL; T_{reg} cells stimulated in the presence or absence of PRL; and T_{reg} : T_{eff} (0.5:1) co-cultures of stimulated cells in the presence or absence of PRL. We did not detect IL4, IL6, IL10, IL17A or TGF β in any of the conditions tested (data not shown). Although we did detect IL2, the differences between the conditions were not statistically significant. Figure 5A shows the concentration of each cytokine.

T_{eff} cells stimulated with anti-CD3/CD28 in the absence or presence of PRL secreted IFN γ (31.25 ± 2.93 and 36.9 ± 10.0 pg/ml, respectively), but the difference between the conditions was not statistically significant. In contrast, in the T_{reg} : T_{eff} co-cultures, IFN γ production was reduced 24.48% compared with stimulated T_{eff} cells cultured alone (23.60 ± 8.05 vs 31.25 ± 2.93 pg/ml; $P < 0.05$). The concentration of IFN γ produced in the T_{reg} : T_{eff} cells co-culture increased in the presence of PRL (51.4 ± 14.84 pg/ml), as shown in Fig. 5B.

The TNF concentration was similar in the absence or presence of PRL (6.7 ± 1.3 vs 6.48 ± 1.48 pg/ml). The TNF concentration decreased when stimulated T_{eff} cells were co-incubated with the T_{reg} cells (4.4 ± 0.7 pg/ml) and increased in the presence of PRL (6.7 ± 0.3 pg/ml); the difference between the TNF concentrations in co-cultures treated with or without PRL was statistically significant ($P < 0.05$), as shown in Fig. 5C. These results suggest that PRL inhibits the suppressive function of T_{reg} cells through the induction of Th1 cytokines.

Discussion

Several studies have demonstrated that PRL participates in immune responses by interacting with its receptor, which is expressed by a wide variety of immune cells (Bole-Feysot *et al.* 1998, Kelley *et al.* 2007). PRL influences the proliferation and activation of T cells and stimulates the secretion of cytokines

Figure 4 Treg cells function in the presence or absence of PRL. Treg (CD4⁺CD25^{hi}CD127^{low/-}) and Teff (CD4⁺CD25⁻CD127⁺) cell populations purified from PBMCs from healthy women were stimulated with anti-CD3 (2.5 µg/ml) and anti-CD28 (1 µg/ml) in the presence or absence of PRL (50 ng/ml). Cellular proliferation was determined on the fifth day of culture by measuring the incorporation of [³H]thymidine and reported as c.p.m. The mean values ± s.d. from 15 independent assays performed in triplicate are shown. *P<0.05.

(Chávez-Rueda *et al.* 2005, Takizawa *et al.* 2005, Tomio *et al.* 2008). In this study, we demonstrated for the first time that Treg cells (CD4⁺CD25^{hi}CD127^{low/-}) constitutively expressed PRL receptor, whereas resting Teff cells (CD4⁺CD25⁻CD127⁺) did not. However, stimulation of Teff cells with anti-CD3/CD28 induced PRL receptor expression, which increased over time. These results introduce the possibility of using PRL receptor expression as an activation marker for Teff cells. The difference in PRL receptor expression between Teff and Treg cells suggests a different role for PRL in these populations. It appears that PRL can signal at any time in Treg cells and therefore could be involved in early activation events in these cells. In contrast, Teff cells require activation to express PRL receptor; thus, PRL in Teff cells most likely acts to increase their proliferation, as has been reported in studies with PBMCs, T and B cells (Chávez-Rueda *et al.* 2005, Xu *et al.* 2010). However, it is also possible that PRL could be involved in the activation of Teff cells.

Lymphocytes have previously been shown to constitutively express the gene for PRL (Montgomery 2001). Our results showed that both Teff and Treg cells constitutively expressed PRL mRNA; this is different from PRL receptor expression, which is only constitutively expressed in Treg cells. In addition, we showed that PRL mRNA expression did not increase in Teff or Treg cells when they were activated with anti-CD3/CD28; these results differed from a previously

published report in which the authors observed an increase in PRL mRNA expression in T cells (Gerlo *et al.* 2006). This difference could be accounted for in several ways. For instance, different activation pathways were used; we stimulated T cells with anti-CD3/CD28, whereas they used cAMP. They also conducted their studies using a mixed population of T cells, including both CD4⁺ and CD8⁺ cells, whereas we used purified CD4⁺ Treg and Teff cells. Despite these differences, we agree with their finding that T cells express PRL mRNA. Moreover, we found that CD4⁺ Teff cells expressed more PRL mRNA than Treg cells, although the protein level detected in culture supernatants was similar in both subsets. Therefore, both Teff and Treg cells could be the source of PRL in CD4⁺T cells. These results suggest that the PRL secreted by either Teff or Treg cells may act in an autocrine manner to form a feedback loop in the cells that produced it. However, any possible autocrine effect is dependent on the expression of PRL receptor in Teff and Treg cells, which varies depending on the activation status and time post-stimulation.

It is well known that Treg cells function to regulate the response of Teff cells by mediating a decrease in Teff cell proliferation; our *in vitro* findings are consistent with this phenomenon (Sakaguchi *et al.* 2009). Knowing that Treg cells constitutively express PRL receptor and that Teff cells up-regulate PRL receptor expression following stimulation, we investigated the effect of PRL on cell proliferation.

We observed a tendency towards increased Teff cell proliferation after co-incubation with PRL and anti-CD3/CD28; these results are consistent with a previously observed additive effect of PRL in PBMCs co-incubated with a mitogen (Chávez-Rueda *et al.* 2005). However, this effect was not observed in Treg cells because Treg cells do not proliferate (Annunziato *et al.* 2002). When Teff and Treg cells were co-incubated, our results confirmed previous observations of a decrease in Teff cell proliferation (Sakaguchi *et al.* 2009). Interestingly, when PRL was added to the Treg:Teff cell co-cultures, the suppressive effect exerted by the Treg cells was weaker (comparing the proliferation of the co-cultured cells with the proliferation of Teff cells activated in the presence or absence of PRL). Our results suggest that PRL down-regulates Treg cell function because the addition of PRL to the Treg:Teff co-culture restored the proliferative capacity of the Teff cells, and the levels of proliferation were comparable to those of Teff cells alone. If we compared the proliferation of Teff cells with the co-culture (Treg:Teff) when both had been treated with PRL, no statistically significant difference was found, which indicates that no suppression occurred in the co-culture with PRL. Moreover, we found a statistically significant difference between the co-cultures in the presence and absence of PRL. This reinforces our hypothesis that PRL

*P<0.05

Figure 5 PRL alters cytokine secretion in T_{eff}:T_{reg} cell co-cultures. Purified T_{reg} and T_{eff} cells were stimulated with anti-CD3 (2.5 µg/ml) and anti-CD28 (1 µg/ml) in the presence or absence of PRL (50 ng/ml). The secretion of cytokines in each of the culture conditions was determined using CBA. (A) IL2 secretion, (B) IFNγ secretion, and (C) TNF secretion. *P<0.05.

down-regulates T_{reg} cell function to prevent their suppressive activity. One possible explanation is that PRL could favour the expression of co-stimulatory molecules or change the cytokine secretion patterns of T_{eff} cells, similar to what we have previously described in PBMCs (Chávez-Rueda *et al.* 2005); these changes would exert a protective effect on T_{eff} cells, which would probably make them less susceptible to T_{reg} cell-mediated suppression. Another possibility is that

PRL could interact with the T_{reg} cells, which constitutively express PRL receptor, to modify their regulatory function (Sojka *et al.* 2008). Given that T_{reg} cells may suppress the response of T_{eff} cells through cell–cell contact and/or the secretion of cytokines, particularly IL10 or TGFβ (Annunziato *et al.* 2002, Buckner 2010), we examined the role of PRL in T_{eff} regulation by measuring the secretion of different cytokines associated with Th1, Th2 and Th17 CD4⁺ T cell profiles. Our results show that the secretion of IFNγ and TNF increased when PRL was added to the T_{reg}:T_{eff} co-cultures and that this increase correlated with an increase in cellular proliferation. These results suggest that PRL favours a Th1 cytokine profile, thus inhibiting the suppressive function of T_{reg}. This hypothesis is further supported by reports that increased concentrations of TNF decreased the regulatory function of T_{reg} cells (Valencia *et al.* 2006, Nagar *et al.* 2010). IFNγ also inhibits the function of T_{reg} cells in collagen-induced arthritis murine models (Kelchtermans *et al.* 2005, Wood & Sawitzki 2006). Interestingly, IFNγ inhibits the generation of T_{reg} cells *in vivo* (Caretto *et al.* 2010), and PRL is important for IFNγ secretion (Chávez-Rueda *et al.* 2005).

The key findings of this study were as follows: a) PRL receptor was constitutively expressed in T_{reg} cells but not in T_{eff} cells, which required stimulation for expression; b) PRL was secreted by both T_{eff} and T_{reg} cells; c) the suppressive effect of T_{reg} cells in co-cultures was reduced in the presence of PRL; and d) Th1 cytokines, which have previously been shown to have an inhibitor effect on T_{reg} cells, were produced after PRL treatment. Based on these results, it is possible that T_{reg} and T_{eff} cells respond to PRL with different kinetics during the T cell activation process, favouring the secretion of Th1 cytokines and thus inhibiting the activity of T_{reg} cells.

Our results may explain the relationship between hyperprolactinaemia and disease activity in lupus (Blanco *et al.* 1999, Jara *et al.* 2008, 2009, Nociti *et al.* 2010) because, by down-regulating the suppressive activity of T_{reg} cells, PRL could favour the activation of T_{eff} cells and thus perpetuate the disease. This hypothesis is supported by the fact that a functional deficiency has been shown in T_{reg} cells (Crispin *et al.* 2003, Dejaco *et al.* 2006, Mudd *et al.* 2006), and there is evidence to suggest that this deficiency is not an inherent defect in the T_{reg} cells of patients with lupus but is the result of changes in other mechanisms (Tower *et al.* 2011), which could involve PRL. Together, with the fact that PRL has been shown to modulate the immune response (Chávez-Rueda *et al.* 2005, 2007, De Bellis *et al.* 2005), these findings suggest that PRL could modulate the regulatory function of T_{reg} cells through its receptor to favour a Th1 response and activate T_{eff} cells. Such activation may then influence the clinical course of patients with lupus.

Based on the results described here, we suggest that PRL inhibits the function of T_{reg} cells by inducing a Th1 cytokine profile. However, further research into the effects of PRL and its mechanism of action will be required.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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