Research Article

Prolactin Increases the Frequency of Follicular T Helper Cells with Enhanced IL21 Secretion and OX40 Expression in Lupus-Prone MRL/lpr Mice

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1. Introduction

The neuroendocrine and immune systems are closely interrelated, as the secretory products of the neuroendocrine system can act on the immune system and vice versa [1]. One example involves hormones that can regulate the immune system [2, 3], such as prolactin (PRL) secreted by the pituitary gland, and extrapituitary immune system cells, such as T cells [4, 5], B cells, antigen presenting cells (APCs) [6], natural killer cells [7, 8], and monocytes/macrophages [9]. The immunostimulatory functions of PRL have been previously described. PRL favors the differentiation of thymocytes [10], increasing the expression of CD69 and CD25 in activated CD8+ T cells [11]. In CD4+ T cells, autocrine PRL is important for maintaining the expression of CD69 and CD40L and the secretion of IL2 and IFN-γ [5]. In a CD4+ T cell line, PRL induced T-bet transcription through phosphorylation of JAK2 and STAT5 [12]. In addition, hyperprolactinemia has been detected in many patients with different autoimmune diseases [13–15], including systemic lupus erythematosus (SLE), where it has been associated with disease activity [16, 17], with the concentration of anti-dsDNA antibodies [18],
anemia, and all types of serositis [19]. SLE is a chronic autoimmune disease characterized by the presence of autoantibodies targeting DNA, RNA, histones, RNP, Ro, La, etc. [20]. These antibodies are from the IgG isotype, which form immune complexes that are deposited in any organ, causing damage. The prevalence of SLE is approximately ninefolds higher in women than in men, and it increases after puberty and decreases after menopause [21]. There are well-established experimental models mimicking many aspects of SLE, such as the MRL/lpr mouse strain [22]. Raising serum PRL levels in this strain, we demonstrated that the concentration of IgG isotype anti-dsDNA autoantibodies increased, resulting in earlier and more severe manifestations of the disease [23, 24].

In the different mouse models that develop SLE, there is an increase in the spontaneous formation of germinal centers (GCs), which correlates with the beginning of the production of autoantibodies [25, 26]. GCs provide a proper microenvironment for the activation, somatic diversification, and affinity maturation of autoreactive B cells, which occur before the production of autoantibodies [27, 28]. GC formation depends on the presence of follicular T helper cells (T\textsubscript{FH}), a specialized subpopulation of CD4\textsuperscript{+} T cells. T\textsubscript{FH} cells are characterized by their expression of CXCR5, ICOS, PD1, CD154, and transcription factor BCL6, in addition to secreting IL21 [29–33]. An increase in the frequency of circulating T\textsubscript{FH} is reported in patients with SLE, having a positive correlation with autoantibody titer and disease activity [34–37]. Meanwhile, it has been observed that the clinical manifestations of the disease decrease upon inhibiting the expression of the IL21 receptor in mouse models [38]. Therefore, dysregulation of the T\textsubscript{FH} response contributes to the production of pathogenic autoantibodies and, therefore, to the promotion of autoimmune diseases mediated by autoantibodies such as SLE [39].

Taking into account all aforementioned findings, we designed this study to determine the contribution that PRL has to the differentiation and activation of T\textsubscript{FH} cells in the MRL/lpr mouse strain. We found that T\textsubscript{FH} cells express the long isoform of the PRL receptor and promoted STAT3 phosphorylation. Furthermore, PRL favors the dysregulation of T\textsubscript{FH} cells by increasing both their absolute number and their activation.

2. Materials and Methods

2.1. Mice. All studies were approved by the Animal Care Committee of the Instituto Nacional de Enfermedades Respiratorias “Ismael Cosio Villegas” and the Hospital de Pediatría, Centro Médico Nacional Siglo XXI, IMSS (protocol numbers R-2016-785-050 and R-2017-785-114), and all mouse measurements were in accordance with the approved guidelines established by Mexico (Norma Oficial Mexicana NOM-062-ZOO-1999) and the NIH Guide for the Care and Use of Laboratory Animals. MRL/MpJFASlpr (MRL/lpr) mice were purchased from the Jackson Laboratory (Maine, USA), and C57BL/6 mice were purchased from the Instituto Nacional de Ciencias Médicas y Nutrición (CDMX Mexico). Mice were housed in a pathogen-free barrier facility and were provided with sterile food and water ad libitum.

2.2. Prolactin Hormone. We used murine recombinant PRL (National Hormone and Peptide Program, NIH).

2.3. Antibodies. All cells were labeled with the viability dye Ghost Red (Tonbo Bioscience, USA). The antibodies used for cell culture were as follows: anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51) from In VitroGen, USA; anti-IFN-γ (clone XMG12) and anti-IL4 (clone 11B11) from BioLegend, USA; anti-TGFβ from Peprotech, USA; cytokines IL6 and IL21 from Miltenyi Biotec, Germany. The antibodies used for staining were as follows: anti-mouse PRL receptor APC (clone T6, Novus Biologicals, USA); anti-CD4 PE/Cy5 (clone GK1.5), anti-Ki-67 Alexa 488 (clone 16A8), anti-IL21 biotin (clone 7H20-I19-M3), and PE-conjugated streptavidin from BioLegend, USA; anti-CD44 PE/Cy5 (clone IM7), anti-CD62L PE, (clone MEL-14), anti-BCL6 PE (clone BCL-DWN), anti-CXCR5 PE/Cy7 (clone SPCRL5), and anti-PD1 APC (clone J43) from eBioscience, In VitroGen, USA; anti-ICOS VioGreen (clone 7E17G9), anti-OX40 PE (clone REA625), and anti-AKT PE (clone REA677) from Miltenyi Biotec, Germany; and anti-STAT1 PE (clone A15158B), anti-STAT3 PE (clone 13A3-1), and anti-STAT5 PE (clone SRBC2X) from BioLegend.

2.4. Induction of High Prolactin Levels and Assessment of SLE Manifestations. MRL/lpr and C57BL/6 female mice (9-weeks-old) were subcutaneously injected with (i) 200 μg of metoclopramide (Sigma-Aldrich, US) in 100 μL of PBS, (ii) 0.6 mg/kg of bromocriptine (Santa Cruz Biotechnology, USA) in 100 μL of PBS, (iii) 100 μL of PBS, or (iv) no treatment for 6 weeks. Urinary protein levels were assessed semiquantitatively using reagent strips for urinalysis (Mission, USA). Serum samples obtained at the beginning and at the end of the experiments were kept at −35°C until they were assayed for anti-dsDNA antibodies as we have previously reported [23, 24].

2.5. Serum IL21 Concentration. For the detection of IL21 in sera, the commercial Legend Max Mouse IL21 ELISA kit (BioLegend, USA) was used according to the supplier’s instructions. For each determination, 50 μL of serum was used. The plate was read in the ELISA reader (Dynatech MR5000) at 450 nm.

2.6. Purification of T\textsubscript{nat} and T\textsubscript{FH} Cells from the Spleen. Eighteen-week-old mice were euthanized, and spleen cells were collected with cold RPMI supplemented with 2% FBS and 2 mM EDTA (IBI Scientific, USA). Red blood cells were depleted with lysis buffer (Sigma-Aldrich, USA) and incubated with anti-CD4 MicroBeads (for T CD4 cells, Miltenyi Biotec); they were selected with the magnetically activated cell sorting (MACS) system (Miltenyi Biotec, Germany) through positive selection using LS columns (Miltenyi Biotec). Single-cell suspensions of CD4\textsuperscript{+} T cells were incubated with fluorescently labeled antibodies specific for CD44, CD62L, CXCR5, and PD1 in staining buffer (PBS with 0.5% BSA) for 20 min at 4°C. Further, the cells were incubated with
DAPI to select living cells (DAPI+), washed, and T naïve (CD44+CD62L+), and T FH cells (CXCR5+PD1+) were isolated. Cell sorting was performed using a FACS Influx Sorter (BD Biosciences). The purity of sorted cells ranged from 95% to 98%.

2.7. RT-PCR for Prolactin Receptor Isoforms. To determine the expression of PRL receptor isoforms, T naïve, and T FH cells from 18-week-old MRL/lpr mice were purified by sorting with a BD Influx Cytometer. Real-time PCR was performed using the following primers synthesized by Integrated DNA Technologies (IDT, USA): 

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\beta\text{-actin (housekeeping control)}: 5'-GAGGAGCTCTGGTCAAACA-3' \quad (\text{left}) \quad \text{and} \quad 5'-\text{CAGTAAATGCCACGAACGAA-3'} \quad (\text{right}).
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To determine the PRL receptor isoforms, three primers were used: common 5′−AGGCGAGCACATGGATACTGGAG−3′ (left), long isoform 5′−AGCCATTCTTCAGACTTGGCCTT−3′ (right), and short isoform 5′−TTGTATTTGGCAGAGCCAGT−3′ (right). The samples were run in the LightCycler II thermal cycler (Roche, Germany) under the following conditions: one cycle at 95°C for 15 min, 40 cycles at 95°C for 10 s, 61°C for 30 s, and 72°C for 30 s, and one cycle at 72°C for 30 s. The relative expression was analyzed using the 2−ΔΔCt method. The murine breast cancer cell line Ephi4 1424 was used as a positive control for the expression of the long and short PRL receptor isoforms.

2.8. Prolactin Receptor Expression (Protein). CD4+ T cells from 9- and 18-week-old mice were isolated from the spleen with the CD4+ T Cell Isolation Kit (Miltenyi Biotec, Germany) and stained with anti-mouse PRL receptor, anti-CD4, anti-CD44, and anti-CD62L for naïve T cells or anti-CD4, anti-CXCR5, and anti-PD1 T FH cells.

2.9. Purification of T naïve. Nine-week-old mice were euthanized and spleen cells were collected with cold RPMI, and blood cells were depleted with lysis buffer. Naïve T cells were isolated from the spleen using a CD4+ naïve T cell (T naïve) Isolation Kit (BioLegend, USA), following the manufacturer’s instructions.

2.10. T FH Differentiation. T naïve cells were differentiated to T FH cells in the presence of the following antibodies and cytokines: anti-CD3, 2.5 μg/mL; anti-CD28, 5 μg/mL; anti-IFN-γ, 10 μg/mL; anti-IL4, 10 μg/mL; anti-TGFβ, 20 μg/mL; IL6, 10 ng/mL; IL21, 10 ng/mL; and with or without 50 ng/mL of PRL for 48 h at 37°C and 5% CO₂.

2.11. Flow Cytometry of In Vitro Differentiated T FH Cells. For OX40 expression, differentiated T FH cells in vitro or splenocytes from mice that underwent different treatments were stained with Ghost Red (viability), anti-CD4, anti-CXCR5, anti-PD1, and anti-OX40. For intracellular IL21, cells were incubated with 1x Cell Stimulation cocktail (Invitrogen, USA) and 1x Protein Transport Inhibitor cocktail (Invitrogen, USA) for 5 h at 37°C and 5% CO₂. Cells were stained with Ghost Red, as well as anti-CD4, anti-CXCR5, and anti-PD1 antibodies. To stain for intracellular proteins (BCL/6, Ki-67, and IL21), cells were fixed and permeated using a Foxp3/transcription factor staining buffer set (eBioscience, USA) or an Intracellular Fixation and Permeabilization Buffer Set (eBioscience, USA) for the latter two. All FACS data were acquired with an MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec, Germany) and analyzed using the FlowJo software (Tree Star, USA).

2.12. Analysis of STATS and AKT Phosphorylation. T FH cells differentiated in vitro were left to rest for 8 h in medium, then T FH and T naïve cells were incubated with PRL (50 ng/mL) for 30 min and fixed with 1x BD Phosflow Lyse/5 Fx Buffer (BD Biosciences, USA) for 10 min. Cells were permeabilized with Perm Buffer III from BD Phosflow (BD Biosciences) to determine STAT3, STAT1, STAT5, and AKT phosphorylation. Cells were washed with FACS buffer and incubated at 4°C for 30 min with the antibodies for flow cytometry analysis. Data were acquired using an MACSQuant Analyzer 10 cytometer (Miltenyi Biotec) and analyzed with FlowJo software (Tree Star, USA). To confirm STAT3 activation, T FH and T naïve cells were preincubated for 30 min in basal medium alone or with 10 mM of the STAT3 inhibitor (Statc, Cell Signaling Technology, USA).

2.13. Statistical Analysis. The Shapiro–Wilk normality test was used to determine the distribution of data. The results were expressed as the mean and standard deviation. Differences between groups were determined using the ANOVA test. A p value < 0.05 was considered significant; statistical analysis of the data was performed using the SPSS Statistics 27 software.

3. Results

3.1. T FH Cells Increase in Mice That Develop Lupus. Murine models of SLE spontaneously increase the formation of GC, which, however, has not been further explored. Seeking for an explanation for this observation and given that the increased GC formation correlates with prodromal SLE features, we measured the percentage of T FH cells in splenocytes of 9- and 18-week-old mice, in which the disease activity was determined by measuring the concentration of anti-dsDNA antibodies (IgG) and proteinuria. The 18-week-old MRL/lpr mice showed significantly elevated serum concentrations of anti-dsDNA antibodies (9.7 ± 3.97 μg/mL) and proteinuria (100 ± 7.56 mg/dL) compared with 9-week-old mice (anti-dsDNA 0.82 ± 1.18 μg/mL; proteinuria 4.29 ± 6.50 mg/dL). We did not or we barely detected anti-dsDNA antibodies and proteinuria in the control strain (C57BL/6) (Figures 1(a) and 1(b)).

We found that the 18-week-old MRL/lpr mice had a significantly higher percentage of T FH (6.35% ± 1.98%) compared with the 9-week-old MRL/lpr mice (0.71% ± 0.43%) and C57BL/6 mice (9 weeks old, 1.22% ± 0.90%; 18 weeks old, 0.88% ± 0.08%) (Figures 1(c) and 1(d)). A similar behavior was observed with the cell absolute numbers; 18-week-old MRL/lpr mice had a higher number of T FH cells (2.16 ± 0.97 × 10⁶ cells/spleen) compared with 9-week-old MRL/lpr mice (0.40 ± 0.24 × 10⁶ cells/spleen) and C57BL/6 mice (9 weeks old, 0.26 ± 0.19 × 10⁶ cells/spleen; 18 weeks
Figure 1: Continued.
old, 0.11 ± 0.01 × 10^6 cells/spleen) (Figure 1(e)). Therefore, in lupus-prone MRL/lpr mice, the increased formation of GCs may be at least partially explained by the increased formation of T<sub>FH</sub> cells. Indeed, T<sub>FH</sub> cell numbers correlated with autoantibody concentrations and with age (Figures 1(f) and 1(g)).

3.2. T<sub> naïve</sub> and T<sub>FH</sub> Cells Express the Long Isoform of the PRL Receptor. To explore whether the formation of T<sub>FH</sub> cells may be influenced by PRL, we determined the expression pattern of the PRL receptor between lupus-prone and control mice, reporting the expression of the PRL receptor as the fold change in T<sub>FH</sub> cells with respect to that of 9-week-old T<sub> naïve</sub> cells. We did not find an increase in the PRL receptor expression in T<sub>FH</sub> cells of 9- and 18-week-old C57BL/6 mice. On the contrary, the MRL/lpr strain showed augmented expression, both at 9 (2.85 ± 0.56-fold change) and at 18 weeks of age (3.87 ± 0.33-fold change), with T<sub>FH</sub> cells of the 18-week-old mice exhibiting the greatest expression (Figures 2(a)–2(c)); we have previously made a similar observation in B cell splenocytes [24]. We observed that both T<sub> naïve</sub> and T<sub>FH</sub> cells of MRL/lpr mice only express the long isoform of the PRL receptor (Figure 2(d)).

3.3. Prolactin Increases the Absolute Number of T<sub>FH</sub> OX40<sup>+</sup> Cells and IL21-Secreting Cells. We previously reported in MRL/lpr mice that pharmacologically raising serum PRL levels with metoclopramide exacerbates the clinical manifestations of SLE, with an increase in autoantibody concentration, as well as proteinuria [23, 24]. To determine whether PRL could affect the number of T<sub>FH</sub> cells, as well as their activation in vivo, we treated MRL/lpr mice with metoclopramide (to increase PRL levels), bromocriptine (to decrease PRL levels), or PBS (Figure 3(a)). We found that the absolute number of splenocytes spontaneously increased with age, as it was observed even in MRL/lpr mice treated with PBS (16 weeks 235.36 ± 78.21 × 10<sup>6</sup> cells). Still, this increase was more significant in mice treated with metoclopramide (348.84 ± 52.71 × 10<sup>6</sup> cells), while the numbers of splenocytes in the bromocriptine condition (138.80 ± 25.95 × 10<sup>6</sup> cells) were closer to the 9-week baseline (92.27 ± 12.45 × 10<sup>6</sup> cells) (Figure 3(b)). A similar observation was made for the absolute numbers of CD4<sup>+</sup> T cells and T<sub>FH</sub> cells, as well as for activated T<sub>FH</sub> IL21<sup>+</sup> cells and T<sub>FH</sub> IL21<sup>+</sup> cells. For all these populations, the highest absolute numbers were from mice treated with metoclopramide and the lowest for the bromocriptine condition. CD4<sup>+</sup> T cells are composed of the following: metoclopramide 63.58 ± 6.15 × 10<sup>6</sup> cells, PBS 44.74 ± 18.71 × 10<sup>6</sup> cells, and bromocriptine 24.57 ± 1.22 × 10<sup>6</sup> cells (Figure 3(c)). T<sub>FH</sub> populations are composed of the following: metoclopramide (T<sub>FH</sub> 7.69 ± 2.66; T<sub>FH</sub> OX40<sup>+</sup> 2.35 ± 0.60; T<sub>FH</sub> IL21<sup>+</sup> 0.14 ± 0.07 × 10<sup>6</sup> cells), PBS (T<sub>FH</sub> 3.77 ± 2.72; T<sub>FH</sub> OX40<sup>+</sup> 0.03 ± 0.43; T<sub>FH</sub> IL21<sup>+</sup> 0.05 ± 0.01 × 10<sup>6</sup> cells), and bromocriptine (T<sub>FH</sub> 2.20 ± 0.59; T<sub>FH</sub> OX40<sup>+</sup> 0.70 ± 0.11; T<sub>FH</sub> IL21<sup>+</sup> 0.03 ± 0.01 × 10<sup>6</sup> cells) (Figures 3(d)–3(f)). Therefore, the increased number of splenocytes observed in each condition mirrors the numbers of each of these CD4 populations that participate in GC formation. We did not observe differences in the absolute numbers of these cells between PBS treated or untreated MRL/lpr mice.

We determined the expression of BCL6 in T<sub>FH</sub> cells, observing an increase only in mice treated with metoclopramide (Figure 3(g)). Furthermore, the serum levels of IL21 were also more elevated in mice treated with metoclopramide (Figure 3(h)). Meanwhile, we did not observe changes in the numbers of these populations in C57BL/6 mice (Figure S1, Supplementary Materials).

3.4. Prolactin Does Not Affect the Survival or Differentiation of T<sub>FH</sub> Cells. PRL has been reported to increase survival in immature B cells of mice that develop SLE [40]. We determined whether PRL could favor the survival and/or
Figure 2: PRL receptor expression on \( T_{\text{naive}} \) and \( T_{\text{FH}} \) cells. Splenocytes from 9- and 18-week-old C57BL/6 and MRL/lpr mice were stained with anti-CD4, anti-CXCR5, and anti-PD1 for \( T_{\text{FH}} \) cells (as shown in Figure 1(c)) and with anti-CD4, anti-CD44, and anti-CD62L for \( T_{\text{naive}} \) cells, then cells were stained with an anti-PRL receptor antibody. (a) Demonstration of the gating strategy for the flow cytometry analysis of \( T_{\text{naive}} \) cells. Doublets were excluded by gating on FSC-H×FSC-A, lymphocytes were identified on the basis of their scatter properties (FSC-A×SSC-A plot), and live cells were gated in the Ghost Red\(^-\). The gate of CD4\(^+\) \( T_{\text{naive}} \) was selected (CD62L\(^-\), CD44\(^-\)). (b) Expression of the PRL receptor is reported as the fold change in receptor expression with respect to PRL receptor expression in \( T_{\text{naive}} \) cells. (c) Representative histogram of PRL receptor expression in MRL/lpr mouse cells. The measurement was carried out in duplicate in six mice per group. Pooled data are presented as the mean ± SD; \(* * * p < 0.001\) using ANOVA. (d) \( T_{\text{naive}} \) and \( T_{\text{FH}} \) cells from 18-week-old MRL/lpr mice were purified by Sort, and the isoform of the PRL receptor was determined by real-time (RT-) PCR. The murine breast cancer cell line EpH4 1424 was used as a positive control for the expression of the long and short PRL receptor isoforms (not shown). Two different experiments were performed; in each experiment, a pool of cells isolated from three mice was used.
differentiation of T\textsubscript{FH} cells as a mechanism to explain their increased numbers. For this, we isolated CD4 T\textsubscript{naive} cells from 9-week-old C57BL/6 and MRL/lpr mice and induced T\textsubscript{FH} differentiation in culture. We did not find differences in the percentage of T\textsubscript{FH} cells differentiated without PRL (C57BL/6 9.26% ± 3.50%; MRL/LPR 13.12% ± 3.26%) and with PRL (C57/BL6 8.56% ± 2.35%; MRL/lpr 12.50% ± 4.41%) (Figures 4(a) and 4(b)) nor did we find a difference in the expression (mean fluorescence intensity, MFI) of BCL6, ICOS, and CXCR5 (Figure 4(c)); although a greater differentiation to T\textsubscript{FH} was observed in cells from MRL/lpr mice compared with cells from C57BL/6 mice. When we determined the survival of TFH cells after the differentiation assay, we did not find significant differences between the percentage of live differentiated T\textsubscript{FH} cells without PRL (C57BL/6 38.69% ± 4.83%; MRL/lpr 45.49% ± 4.72%) and with PRL.

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**Figure 3:** Metoclopramide increases the absolute number of T\textsubscript{FH} populations in MRL/lpr mice. Nine-week-old MRL/lpr mice were treated with metoclopramide (meto), bromocriptine (bromo), or PBS or were left without intervention (left column marked by age in weeks) for 6 weeks. (a) Flow chart of treatment strategy. At the end of the treatment, cells were labeled with anti-CD4, anti-CXCR5, anti-PD1, anti-\textsuperscript{OX40}, anti-BCL6, or anti-IL21 antibodies. The graphs show the absolute number of (b) splenocytes, (c) CD4\textsuperscript{+} T cells, (d) T\textsubscript{FH} cells, (e) \textsuperscript{OX40}\textsuperscript{+} T\textsubscript{FH} cells, and (f) \textsuperscript{IL21}\textsuperscript{+} T\textsubscript{FH} cells. (g) Expression of BCL6 in T\textsubscript{FH} cells. (h) IL21 concentration in serum. For the determination of T\textsubscript{FH} IL21\textsuperscript{+}, the cells were stimulated with stimulation cocktail (PMA ionomycin) for 5 h and then stained. MFI: mean fluorescent intensity. Eight mice per condition were used. Pooled data are presented as the mean ± SD; **p < 0.001, *p < 0.01, and *p < 0.05 using ANOVA.
(C57BL/6 36.62% ± 4.87%; MRL/lpr 45.33% ± 5.33%) (Figure 4(d)). Additionally, the cells differentiated to $T_{FH}$ from MRL/lpr mice presented slightly better survival than those from C57BL/6 mice. We did not observe differences in the percentages of proliferating cells (Figure 4(e)).

3.5. Prolactin Activates $T_{FH}$ Cells. To assess whether $T_{FH}$ cells were more active upon PRL treatment, we measured the expression of OX40 and IL21, both molecules serving as activation markers of $T_{FH}$ cells. We found that the $T_{FH}$ cells differentiated in the presence of PRL presented a statistically

![Graph showing differentiation and survival of $T_{FH}$ cells in the presence of PRL.](image)

**Figure 4:** Differentiation and survival of $T_{FH}$ cells in the presence of PRL. $T_{naive}$ cells from 9-week-old C57BL/6 and MRL/lpr mice were purified by MACS and differentiated to $T_{FH}$ in the presence and absence of PRL for 48 h, before staining with a viability marker (Ghost Red) and anti-Ki-67, anti-CD4, anti-CXCR5, anti-BCL6, and anti-ICOS antibodies. The surface CXCR5⁺PD1⁺ population represents $T_{FH}$ cells. (a) Percentage of differentiation to $T_{FH}$ in vitro. (b) Zebra plot of one representative experiment. (c) Expression of BCL6, CXCR5, and ICOS (MFI) in $T_{FH}$ cells. (d) Percentage of $T_{FH}$ cell survival. (e) Percentage of proliferation (Ki-67⁺). Six different experiments were performed; each experiment was done in triplicate. Pooled data are presented as the mean ± SD; **$p < 0.01$ and *$p < 0.05$ using ANOVA.
Figure 5: Continued.
significant increase in MRL/lpr mice, determined by both expression (MFI) and percentage of OX40 (1359.88 ± 172.05 MFI; 82.85% ± 4.20%), compared with the condition without PRL (1138 ± 76.87 MFI; 70.87% ± 4.07%), likewise, for IL21, with PRL (97.36 ± 4.00 MFI; 11.90% ± 1.12%) versus without PRL (87.43 ± 1.70 MFI; 9.27% ± 0.61%). On the other hand, we did not observe any difference in the C57BL/6 mouse cells (Figures 5(a)–5(d)). Moreover, T_{FH} cells derived from MRL/lpr mice expressed more OX40 and IL21 than cells derived from C57BL/6 mice at baseline.

3.6. Prolactin Promoted STAT3 Phosphorylation in T_{FH} Cells.

It is known that the long PRL isoforms signal through the JAK-STAT and PI3K-AKT pathways [41, 42]. We determined the signaling components associated with the PRL receptor upon activation with recombinant PRL in T_{FH} cells differentiated in vitro. We measured STAT1, STAT3, STAT5, and AKT phosphorylation via flow cytometry. We found that PRL induced phosphorylation of STAT3 (pSTAT3) only in T_{FH} cells derived from MRL/lpr mice and confirmed this PRL activity with an inhibitor of STAT3 (Stattic). The level of pSTAT3 was measured as a fold change (with respect to T_{FH} cells treated with medium) and percentage of positive cells (medium: 1.00-fold change, 8.46% ± 1.00%; PRL: 1.74-fold change, 11.02% ± 1.33%; Stattic: 1.01-fold change, 7.74% ± 1.06%) (Figure 5(e)). In addition, the PRL activity was more prominent in MRL/lpr T_{FH} cells, since the inhibitor significantly reduced pSTAT3 only in the lupus-prone strain. We did not observe pSTAT3 in T_{FH} cells from C57BL/6 mice or in T_{naive} cells from any mice. In addition, PRL did not induce STAT1, STAT5, and AKT phosphorylation in MRL/lpr mice (Figure S2, Supplementary Materials).

4. Discussion

The endocrine system produces hormones that regulate different systems, one of them being the immune system [43]. The bidirectional interactions between the endocrine and immune systems play critical roles in the maintenance of homeostasis. Disturbing mutual communication between these systems might initiate or exacerbate the development of a wide variety of diseases, such as autoimmune thyroid disease [44], rheumatoid arthritis [45], Sjögren syndrome [46], and SLE [47]. Patients with SLE, as well as experimental model mice of the disease (MRL/lpr, NZB/W), show an increase in serum PRL levels associated with the activity of
the disease and/or the concentration of IgG autoantibodies [17, 18, 24]. Furthermore, the activity of lupus has also been associated with an increase in T<sub>FH</sub> cells [36, 48, 49], a subset of helper CD4<sup>+</sup> T cells that play a crucial role in the generation of antibodies. Indeed, dysfunctional T<sub>FH</sub> cells can activate autoantibody-producing B cells that cause SLE [50]. Although these studies support that PRL in autoantibody-producing B cells that cause SLE [50]. In di

In this study, we present new evidence of the importance of PRL in the development of SLE by increasing the absolute number of T<sub>FH</sub> cells, the activation of T<sub>FH</sub> cells, and IL21 secretion in lupus-prone mice. This could favor an uncontrolled response of GCs, faulty tolerance, and an increase in the production of autoantibodies implicated in the pathogenesis of the disease. It has been observed in the B6.MRL-Faslp (B6.lpr) and BXD2 strains, the increase in T<sub>FH</sub> correlates positively with total IgG concentration in serum, as well as with anti-dsDNA antibody levels [51, 52]. We demonstrated here that lupus-prone MRL/lpr mice also presented a positive correlation between the absolute number of T<sub>FH</sub> cells and the concentration of anti-DNA IgG isotype autoantibodies, as well as a correlation with age. Increased serum PRL levels in these SLE-developing mice are associated with disease exacerbation [24, 53, 54]. Previously, we have also reproduced the exacerbation of the disease by pharmacologically raising serum PRL concentrations with metoclopramide [23, 24]. Here, this same treatment induced an increase in the absolute number of CD4<sup>+</sup> T cells and T<sub>FH</sub> cells. Conversely, treating mice with an antagonist of the secretion of PRL (bromocriptine) decreased the absolute number of these cells with respect to mice treated with PBS or without treatment; this behavior was only observed in lupus-prone mice. This increase in T<sub>FH</sub> cells may be due to an increase in the differentiation of T<sub>FH</sub> cells, as the expression of BCL6, the master transcription factor of T<sub>FH</sub> cells [55], was increased only in mice treated with metoclopramide. This increase in the absolute number of T<sub>FH</sub> cells could give us at least a partial explanation for the association between high levels of PRL and the increase in autoantibodies of the IgG isotype in patients or mice with SLE, as the uncontrolled accumulation of T<sub>FH</sub> cells might activate autoreactive B cells to produce excessive autoantibodies that cause autoimmune responses [50, 56].

In different reports, it has been shown that PRL is an important factor for both survival and proliferation of different cell types [57, 58]. It has been demonstrated that PRL is an important factor for both the survival and proliferation of early T-cell precursors, such as CD25<sup>+</sup>CD4<sup>+</sup> CD8<sup>+</sup> double negative cells [10], as well as for the protection of thymocytes from glucocorticoid-induced apoptosis [59]. However, in this work, the prosurvival effects of PRL were not observed in T<sub>FH</sub> cells differentiated from mature T<sub>naive</sub> cells, as happens in the immature B cells of these mice [40]. In addition, there was no effect on the differentiation and proliferation of T<sub>FH</sub> cells, despite the fact that T<sub>naive</sub> and T<sub>FH</sub> cells expressed the PRL receptor; however, receptor expression was lower in T<sub>naive</sub> cells. Furthermore, we did not find evidence of STAT3 activation in T<sub>naive</sub> cells, as this kinase was not phosphorylated upon PRL treatment, explaining why the effect of PRL on T<sub>naive</sub> cells and their differentiation to T<sub>FH</sub> was not observed. The increase in the absolute number of T<sub>FH</sub> cells and the expression of BCL6 (MFI) in vivo may be rather due to an indirect effect of PRL. PRL could be acting on other cells that are helping T<sub>FH</sub> cells to differentiate. For example, it is known that B cells (follicular and marginal zone) express the PRL receptor and that this expression increases when PRL concentrations rise [24]. On the other hand, it has been reported that IL6 secreted by B cells is important for the differentiation of T<sub>FH</sub> cells [25]; thus, it will be important to demonstrate, in future tests, if PRL can increase IL6 secretion in B cells, thus favoring the differentiation of T<sub>FH</sub> cells.

It could also be due to the effect that PRL may have on other hormones that also influence specific components of the immune responses, such as the thyroid-stimulating hormone (TSH). The elevated TSH levels increased the mitogen-induced proliferative response of mouse lymphocytes [60], as well as the percentage of CD4<sup>+</sup> T cells [61]. Furthermore, serum levels of TSH correlate positively with those of PRL [62], and 11.6% of patients with SLE present elevated levels of TSH [63]. This suggests that in our in vivo tests, the increase in the number of T<sub>FH</sub> cells in the mice treated with metoclopramide may be due both to an indirect effect of PRL on other cells and to the effect of other hormones such as TSH on CD4<sup>+</sup> T cells. Therefore, it will be important to study the effect of TSH on T<sub>FH</sub> cells.

It is probable that the effect of PRL directly occurs in cells that are already differentiated and/or activated where the expression of the receptor is greater. The stimulatory roles of PRL in the in vitro activation of T cells and B cells have been previously reported [5, 64]. In addition, PRL promotes differentiation into CD4<sup>+</sup> T-bet<sup>+</sup> T cells [12], CD4<sup>+</sup> Eomes<sup>+</sup> T cells [6], and NK cells [8]. T<sub>FH</sub> cells have a higher expression of the receptor with respect to T<sub>naive</sub> cells. This expression increases with age and with the manifestations of the disease in mice that develop SLE, as seen for T cells from patients with SLE, where the T cells express higher levels of the receptor than T cells from healthy subjects [65, 66].

In this work, we demonstrated that T<sub>FH</sub> cells exclusively express the long isoform of the PRL receptor, finding that PRL could participate in signaling through STAT3 in these cells. An extensive body of evidence links STAT3 with autoimmune diseases. Most of this evidence is related to the capacity of STAT3 to influence the differentiation of lymphoid cells, such as Th17 and Treg CD4<sup>+</sup> T cells [67]. Statick has also been used to delay the onset of disease in MRL/lpr mice, reducing the levels of clinical hallmarks of SLE, such as nephritis, renal and skin lesions, proteinuria, and serum autoantibodies [68, 69]. This increase in the phosphorylation of STAT3 when incubating T<sub>FH</sub> cells with PRL could explain the role of PRL in increasing the percentage (in vitro) and absolute number (in vivo) of IL21-secreting T<sub>FH</sub> cells (T<sub>FH</sub> IL21<sup>+</sup>). This is consistent with the observations that the increased IL21 mRNA expression in CD4<sup>+</sup> T cells from SLE patients is dependent on the activation of STAT3 [70, 71] and that STAT3 directly binds the IL21 promoter [72, 73]. Furthermore, in mice treated with metoclopramide, the
serum levels of IL21 were increased. IL21 serves as a “helper” cytokine to stimulate B cells through interacting with IL21R. IL21 enhances murine B-cell proliferation, IgG class switching, and plasmablast differentiation [74, 75]. Therefore, the increase in IL21 in lupus-prone mice could favor the generation of autoreactive plasma cells and the increase in autoantibodies.

Another effect of PRL on T FH cells was an increase of T FH OX40 + cells. OX40 is transiently induced following TCR engagement after antigen (Ag) recognition. Many factors are involved in the kinetics of OX40 expression, including IL21 [76]. IL21 acts in an autocrine way in T FH cells [77]; thus, the PRL-dependent increase in the percentage and number of T FH IL21 + cells, as well as the serum levels of IL21, could favor an increase in the percentage of activated T FH cells (OX40+). However, it has also been reported that STAT3 plays a direct regulatory role in OX40 mRNA expression in CD4+ T cells [78]. Similarly, STAT3 enhances T cell survival by upregulating OX40, BCL2, and Fas ligand [76]. Therefore, the PRL-mediated increase of OX40 on T FH cells could be a direct effect or mediated through IL21. Furthermore, an increased percentage of OX40-expressing CD4+ T cells was found in SLE patients, in which it was an indicator of disease activity [79], and the OX40L-OX40 axis was also found to contribute to lupus pathogenesis by promoting the generation of T FH cells [80]. Therefore, PRL influences the immune system in SLE exacerbating the activity of the disease by increasing the number of OX40 T FH cells and activating the OX40-OX40L axis.

5. Conclusions

Collectively, our data suggest that PRL acts on T FH cells that express the long isoform of the receptor and could participate in signaling through STAT3. We also observed an increase in the number and activation of T FH cells that may favor the formation of GC, interfere with tolerance, and facilitate the generation of autoreactive plasma cells and the secretion of autoantibodies. Therefore, in future studies, it will be important to assess the influence of PRL on GCs, as well as the interaction of B cells and T FH in an environment featuring high levels of PRL, to better understand the role of PRL in GC formation and to define the most important steps in the pathogenesis of SLE that could be targeted by antagonistic molecules (Figure 6).

Data Availability

All data included in this study are available upon request by contact with the corresponding author.

Ethical Approval

All studies were approved by the Animal Care Committee of the Instituto Nacional de Enfermedades Respiratorias “Ismael Cosio Villegas” and the Hospital de Pediatría, Centro Médico Nacional Siglo XXI, IMSS (protocol number R-2016-785-050 and R-2017-785-114); all experiments were performed in accordance with approved guidelines established
by Mexico (NOM-062-ZOO-1999) and the NIH Guide for the Care and Use of Laboratory Animals.

**Conflicts of Interest**
The authors declare no conflict of interest.

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**Supplementary Materials**
Figure S1: absolute number of T<sub>FH</sub> cells in C57BL/6 mice. Figure S2: PRL does not activate AKT, STAT1, and STAT5 in MRL/lpr mice. (Supplementary Materials)

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