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_J Immunol_ 2013; 191:2082-2088; Prepublished online 24 July 2013;
doi: 10.4049/jimmunol.1301128
http://www.jimmunol.org/content/191/5/2082

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Prolactin Is Not Required for the Development of Severe Chronic Experimental Autoimmune Encephalomyelitis

Massimo Costanza,* Silvia Musio,* Mhamad Abou-Hamdan,* Nadine Binart, † and Rosetta Pedotti*

Predominance of multiple sclerosis (MS) in women, reductions of disease flares during pregnancy, and their increase in the postpartum period have suggested a hormonal influence on MS activity. The hormone prolactin (PRL) has long been debated as a potential immune-stimulating factor in several autoimmune disorders, including MS and its animal model experimental autoimmune encephalomyelitis (EAE). However, to date, no data clearly ascribe a pathogenic role to PRL in these diseases. Using PRL receptor–deficient (Prlr−/−) and PRL-deficient (Prl−/−) mice, we show that PRL plays a redundant role in the development of chronic EAE. In Prlr−/− and Prl−/− mice, EAE developed with a delayed onset compared with littermate control mice, but with full clinical severity. In line with the clinical outcome, T cell proliferation and production of IFN-γ, IL-17A, and IL-6 induced by myelin Ag were delayed in Prlr−/− and Prl−/− mice. Ag-specific IgG Ab responses were not affected by PRLR or PRL deficiency. We also show that mouse lymph node cells and purified CD4+ T cells express transcript for Prl, but not for Prl. These results reveal that PRL does not play a central role in the development of chronic EAE and optimal Th1 and Th17 responses against myelin. Moreover, they also rule out a possible contribution of PRL secreted by immune cells to the modulation of autoreactive T cell response in this model. The Journal of Immunology, 2013, 191: 2082–2088.

Multiple sclerosis (MS) is an inflammatory demyelinating disorder of the CNS that affects 2.5 million people worldwide and represents the leading cause of neurologic disability in the young adult population (1). In MS, myelin-reactive CD4+ Th1 and Th17 cells are generally believed to drive an immune-mediated attack against components of the myelin sheath, leading to demyelination and axonal damage (2). However, the pathologic mechanisms underlying the development of MS are still incompletely understood.

In recent years, several pieces of evidence have suggested that sex-related factors might influence both incidence and progression of MS (3). Epidemiologic studies have shown that MS affects more frequently women than men, with a female/male ratio ranging from 2:1 to 3:1, depending on geographic areas (3). Pregnancy also importantly affects the clinical course of MS. Relapse rate significantly declines during the third trimester of pregnancy but considerably increases in the first 3 mo after delivery, if compared with prepregnancy rates (4, 5). Similarly to human disease, pregnancy suppresses clinical symptoms of experimental autoimmune encephalomyelitis (EAE), an animal model for MS (6, 7), and induction of chronic EAE in the postpartum period results in enhanced mortality and slightly worsened severity (7). Among sex-related factors, hormones have been hypothesized to play an important role in regulating MS and EAE (3). Prolactin (PRL) is a 199-aa peptide hormone mainly secreted by lactotrophic cells of the anterior pituitary gland and by other sources including immune cells (8). The best established functions of PRL are related to mammary gland development and regulation of lactation and female reproduction (9). Mean PRL serum levels are slightly but significantly higher in women (~2–20 μg/l) than in men (~2–10 μg/l) (10). PRL secretion increases during pregnancy and peaks postpartum in association with breastfeeding. In parallel to its reproductive functions, a large body of literature has argued for a role of PRL in the regulation of both cell-mediated and humoral immune responses (11–13). PRL binds to a single-pass transmembrane receptor (PRLR), belonging to the class I cytokine receptor superfamily, which includes receptors for IL-2, IL-6, GM-CSF, and leptin (8). In vitro studies have shown that PRL sustains survival, proliferation, and differentiation of T cell precursors (14), and modulates CD4+ T cell expression of T-bet, a key transcription factor for the differentiation of Th1 cells (15). PRL has also been reported to stimulate the maturation of monocyte-derived dendritic cells (16). In mouse models of systemic lupus erythematosus (SLE), a multigorgan Ab-dependent autoimmune disease, PRL administration increases autoreactive immune responses (17, 18), by breaking B cell tolerance and enhancing titers of autoreactive Abs (18). In human SLE, higher serum levels of PRL correlate with greater disease severity, and treatment of SLE patients with bromocriptine (BCR), a dopamine D2 agonist that inhibits PRL secretion, reduces disease activity (19).

In MS, hyperprolactinemia has been largely debated (20). Early studies in rat EAE showed that serum PRL levels increase during the induction phase of disease (21), and treatment with BCR improves clinical signs of EAE (21, 22), suggesting a detrimental role for PRL in EAE. However, stimulation of D2 dopaminergic receptors on immune cells can modulate their functions, and BCR has been shown to directly suppress human T cell proliferation.
independently of PRL (23). Moreover, BCR might not allow a complete depletion of PRL, as other extratypic sites of PRL secretion have been suggested (9). Given these limitations, a clear indication about the role of PRL in MS and EAE is still lacking.

In this study, using two mouse models with gene-targeted deletions of either PRL receptor (Prlr<sup>−/−</sup>) or its ligand (Prl<sup>−/−</sup>), we show that in absence of PRLR or PRL, EAE develops with delayed onset as compared with wild-type (WT) littermate controls, but with full clinical severity. Prlr<sup>−/−</sup> and Prl<sup>−/−</sup> mice exhibited reduced T cell proliferation and production of IFN-γ, IL-17A, and IL-6 against myelin peptide as compared with WT controls, at an early time point of EAE (7 d postimmunization [p.i.]), but not at a later one (10 d p.i.). Serum concentration of anti–myelin oligodendrocyte glycoprotein (MOG) aa 35–55 (anti-MOG35–55) IgG Abs was not affected by PRLR or PRL deficiency. Lastly, in lymph node (LN) cells and CD4<sup>+</sup> T cells of naive and EAE mice, we found expression of transcript for Prl<sub>r</sub>, but not for Prl.<br><br>**Materials and Methods**

**Mice**

Prlr<sup>−/−</sup> mice were backcrossed for >12 generations into 129P2/Ola background (24), Prl<sup>−/−</sup>, B6.129S2(Cg)-Prl<sub>tm1Hmn</sub>/J mice backcrossed for 10 generation into C57BL/6 background were purchased from Jackson Laboratories (25). Heterozygous pairs of each strain were bred to obtain Prlr<sup>−/−</sup> and Prl<sup>+/−</sup> or Prlr<sup>−/−</sup> and Prl<sup>−/−</sup> mice. Genotyping was performed by PCR in Prlr<sup>−/−</sup> and Prl<sup>+/−</sup> as previously described (24) and in Prlr<sup>−/−</sup> and Prl<sup>−/−</sup> as recommended by the vendor. C57BL/6 mice were from Charles River. Mice were bred and maintained under pathogen-free conditions at the animal facility of the Foundation IRCCS Neurological Institute Carlo Besta. Age-matched female 8- to 12-wk-old mice were used in all EAE experiments. Both knockout strains bear an H-2b haplotype, which confers susceptibility to MOG<sub>35–55</sub>-induced EAE. However, Prlr<sup>−/−</sup> mice were backcrossed into C57BL/6 background, which is known to be more susceptible to EAE development than the 129 background, into which Prlr<sup>−/−</sup> mice were backcrossed (26, 27). All procedures involving animals were approved by the Institute Ethical Committee and performed in accordance to institutional guidelines and national law (DL116/92), and animals were approved by the Institute Ethical Committee and performed in accordance to institutional guidelines and national law (DL116/92), and carried out according to the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC).

**Peptide synthesis and EAE induction**

MOG<sub>35–55</sub> (MEGVYWRSPFSRVRHLYRNGK) and control peptide (rat P0: DGDFAIVKFTKVLDDTGYH) were synthesized using a standard 9-fluorenylethoxycarbonyl chemistry on a 433A automated peptide synthesizer (Applied Biosystems) and purified by HPLC. The purity of each peptide was >95% as assessed by analytical reverse-phase HPLC. EAE was induced as previously described (28). In brief, MOG<sub>35–55</sub> peptide was dissolved in PBS to a concentration of 2 mg/ml and emulsified with an equal volume of IFA supplemented with 8 mg/ml heat-killed Mycobacterium tuberculosis H37Ra (Difco). Mice were injected s.c. in their flanks with 0.1 ml of the peptide emulsion (for a total of 100 µg MOG<sub>35–55</sub> and 400 µg M. tuberculosis/ mouse) and, on the same day and 48 h later, were injected i.v. with 0.2 ml containing 200 µg Bordetella pertussis toxin (List Laboratories) dissolved in PBS. Mice were assessed daily for neurologic signs of EAE according to the following five-point scale: 0, healthy; 1, tail weakness or paralysis; 2, paraparesis (incomplete paralysis of one or two hind limbs); 3, paraplegia extending to the thoracic hind limbs/pels/jug; 4, forelimb weakness or paralysis with hind limbs paraplegia or paraplegia; 5, moribund or dead animal.

**T cell activation assay and cytokine analysis**

Draining LN cells (LNCs) were isolated from immunized mice 7–10 d after EAE induction and cultured in vitro with MOG<sub>35–55</sub>. Con A (1 µg/ml; positive control), rat P0 (negative control), or medium alone. Cells were cultured in 96-well U-bottom plates at a density of 2 × 10<sup>5</sup> cells/well in 200 µl RPMI 1640 (EuroClone) supplemented with 1-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), HEPES buffer (0.01 M), and 10% FCS (enriched RPMI 1640). After 48 h of incubation at 37˚C in 5% CO<sub>2</sub>, cultures were pulsed with 0.5 µCi [3H]thymidine per well for 18 h, and proliferation was measured from triplicate cultures on a beta counter (PerkinElmer). Supernatants from parallel cultures were tested for production of IFN-γ, IL-6, IL-10 (anti-mouse OptEIA ELISA Set; BD Pharmingen) and IL-17A (Mouse IL-17 Duoset; R&D Systems) by ELISA, according to manufacturer’s protocols. Results are shown as mean of duplicates; SEM were always within 10% of the mean. Untouched CD4<sup>+</sup> T cells were magnetically purified by negative selection from suspensions of splenocytes from naive C57BL/6 mice according to manufacturer’s protocol (CD4<sup>+</sup> T cell Isolation Kit II; Miltenyi). Cell purity (>95%) was confirmed by flow cytometry. CD4<sup>+</sup> T cells (2 × 10<sup>6</sup> cells/well) were cultured in 96-well plates in 200 µl enriched RPMI 1640 with anti-CD3 (1 µg/ml) and anti-CD28 (5 µg/ml) Abs (BD Pharmingen) or alone and harvested after 48 h at 37˚C with 5% CO<sub>2</sub> for gene expression analysis of Prl and Prlr, and measurement of PRL in supernatants.

**Measurement of MOG<sub>35–55</sub>-specific IgG response**

Blood was collected from the tail vein of immunized mice before and 5 wk after the induction of EAE. MOG<sub>35–55</sub>-specific IgG, IgG1, IgG2a, IgG2b, and IgG3 Abs were measured by ELISA as described elsewhere (28). In brief, 96-well plates (Immunol; Thermo Labsystems) were coated overnight at 4˚C with 0.1 ml MOG<sub>35–55</sub> diluted in 0.1 M NaHCO<sub>3</sub> buffer (pH 9.5) at a concentration of 0.010 mg/ml. The plates were blocked with PBS/10% FCS (blocking buffer) for 2 h. Samples were diluted in blocking buffer at 1/100, and Ab binding was tested by the addition of peroxidase-conjugated monoclonal goat anti-mouse IgG, IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotechnology Associates), each at a 1:5000 dilution in blocking buffer. Enzyme substrate was added, and plates were read at 450 nm on a microplate reader.

**Real-time PCR**

Gene expression analysis was performed ex vivo on LNCs or magnetically purified CD4<sup>+</sup> T cells (purity >95% by flow cytometry; CD4<sup>+</sup> T cell Isolation Kit II, Miltenyi) from LNCs of naive or immunized C57BL/6 mice 7 d after immunization, and on in vitro–stimulated CD4<sup>+</sup> T cells from naive mice. Total RNA was extracted with RNasy Mini Kit (Qiagen) and reverse transcribed with Quantitec Reverse Transcription Kit (Qiagen) according to manufacturer’s guidelines. Real-time PCR was performed on 7500 Fast Real-time PCR system (Applied Biosystems). Prlr detection was performed with Fast Universal Master Mix (Applied Biosystems) and the following primer/probe sets (Applied Biosystems): Prlr forward: 5′-TGGACATACTGGAAAGAGAGGAGAAGGA-3′, Prlr reverse: 5′-TGCTTTGCTAAAGAGGACGAATTTG-3′, Gapdh forward: 5′-TGCACCACAACTGCTTAG-3′, Gapdh reverse: 5′-GGATGACGGAGTGATGTCC-3′ (29). Prlr primer pairs have been designed to amplify exons 4–5, which are common to the four described isoforms of PRLR. Expression of target genes was quantified by the comparative threshold cycle method, and Gapdh was used as housekeeping gene. Data are presented as percentage of the housekeeping gene Gapdh ± SD.

**Measurement of PRL in supernatants and sera**

PRL was measured in supernatants of cultured CD4<sup>+</sup> T cells and sera of naive or immunized C57BL/6 mice by ELISA (Mouse Prolactin Duoset; R&D Systems), according to manufacturer’s instructions. Serum PRL concentrations were tested during priming (i.e., day 7 p.i., onset, and acute phases of MOG<sub>35–55</sub>-induced EAE. Sera collected from naive Prlr<sup>−/−</sup> mice, which display hyperprolactinemia (9), and naive Prl<sup>−/−</sup> mice were used as positive and negative controls, respectively.

**Statistical analysis**

For clinical data, Mann–Whitney U test was used to compare results between two groups. For all other analyses, unpaired Student t test, two tails, was used to compare results between two groups. Analysis was performed by SPSS software. In all tests, *p < 0.05 was considered statistically significant.

**Results**

EAE develops with delayed onset but full clinical severity in absence of PRLR or PRL.

To investigate the role of PRL in CNS autoimmunity, we induced EAE in female PRLR-deficient (Prlr<sup>−/−</sup>) mice and WT littermates (Prl<sup>+/−</sup> (H-2b haplotype) by immunization with MOG<sub>35–55</sub> in CFA. Prlr<sup>−/−</sup> mice developed EAE with delayed onset but similar...
MOG35–55 peptide in CFA, and mice were scored daily for clinical signs of PRL-deficient (effect of PRL on CNS autoimmunity, we next induced EAE in terminal 16-kDa fragment of full-length 23-kDa PRL has been prolactinemic (data not shown). Moreover, in recent years, an N-terminal 16-kDa fragment of full-length 23-kDa PRL has been shown to increase serum titers of anti-DNA Abs and IgG deposits in glomeruli (18). We therefore evaluated in our models whether PRL had any impact on the production of autoreactive IgG Abs. We did not find significant differences in titers of IgG specific for MOG35–55 in sera from immunized Prlr−/−, Prl−/−, and their respective control mice (Fig. 4A, 4B). The analysis of IgG subclasses revealed an increase, albeit not statistically significant, of serum Ag-specific IgG1 and IgG2a in Prlr−/− mice as compared with Prlr+/+ mice (mean OD of IgG1: 0.408 ± 0.145 in Prlr+/+ versus 0.749 ± 0.237 in Prlr−/− mice; p = 0.216; mean OD of IgG2a: 0.519 ± 0.102 Prlr+/+ versus 0.826 ± 0.175 in Prlr−/− mice; p = 0.122). No differences in titers of IgG subclasses between Prlr−/− and controls were detected (Fig. 4B). These data indicate that the PRL/PRLR axis is dispensable for the production of MOG35–55–specific IgG Abs during chronic EAE.

LNCs and CD4+ T cells express mRNA for Prlr but not for Prl

To gain insight into why Prlr−/− and Prl−/− mice displayed a delay of EAE onset and development of Th1 and Th17 autoimmune responses, we induced chronic EAE in C57BL/6 mice to MOG35–55 stimulation. LNCs from Prlr−/− mice harvested 7 d after the induction of EAE displayed a significantly reduced proliferation in response to peptide stimulation if compared with Prlr+/+ littersmates (Fig. 2A). Moreover, we found a significantly decreased production of IFN-γ, IL-17A, IL-6, and IL-10 in peptide-stimulated LNCs of Prlr−/− as compared with Prlr+/+ littersmates (Fig. 2A). These findings could reflect a decreased frequency of MOG35–55–reactive T cells in LNs of Prlr−/− mice compared with Prlr+/+ mice, and/or a reduced potential of autoreactive cells from Prlr−/− mice to proliferate and secrete proinflammatory cytokines in response to MOG35–55. Because we observed that in absence of PRLR the onset of EAE was delayed, we performed the same analysis at a later time point during EAE priming (day 10 p.i.), when Prlr+/+ control mice displayed first clinical symptoms of disease, whereas Prlr−/− mice were still disease free. At this time point, we observed that LNCs from Prlr−/− mice were fully responsive to MOG35–55, showing even higher proliferation and increased production of IFN-γ, IL-17A, IL-6, and IL-10 (Fig. 2B) as compared with Prlr+/+ control mice. In line with data obtained with PRLR-deficient strain, LNCs from Prlr−/− mice displayed significantly reduced proliferation (Fig. 3A) and production of IFN-γ, IL-17A, IL-6, and IL-10 (Fig. 3B) in response to MOG35–55 as compared with WT controls at day 7, but not at day 10 after EAE induction.

These results reveal that the absence of PRLR or PRL does not impair the development of Th1 and Th17 responses against MOG35–55, but induces a delay in the generation of these responses. This delay appears consistent with the delayed appearance of clinical symptoms of EAE observed in Prlr−/− and Prl−/− mice.

Prlr−/− and Prl−/− mice display normal serum titers of anti-MOG35–55 IgG Abs

Several lines of evidence have suggested that PRL might importantly modulate B cell functions. In a mouse model of SLE, PRL has been shown to increase serum titers of anti-DNA Abs and IgG deposits in glomeruli (18). We therefore evaluated in our models whether PRL had any impact on the production of autoreactive IgG Abs. We did not find significant differences in titers of IgG specific for MOG35–55 in sera from immunized Prlr−/−, Prl−/−, and their respective control mice (Fig. 4A, 4B). The analysis of IgG subclasses revealed an increase, albeit not statistically significant, of serum Ag-specific IgG1 and IgG2a in Prlr−/− mice as compared with Prlr+/+ mice (mean OD of IgG1: 0.408 ± 0.145 in Prlr+/+ versus 0.749 ± 0.237 in Prlr−/− mice; p = 0.216; mean OD of IgG2a: 0.519 ± 0.102 Prlr+/+ versus 0.826 ± 0.175 in Prlr−/− mice; p = 0.122). No differences in titers of IgG subclasses between Prlr−/− and controls were detected (Fig. 4B).

These data indicate that the PRL/PRLR axis is dispensable for the production of MOG35–55–specific IgG Abs during chronic EAE.

Table I. EAE in Prlr−/− and Prl−/− mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Incidence (%)</th>
<th>EAE Onset (d)</th>
<th>Peak Disease Severity</th>
<th>Cumulative Disease Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prlr+/+</td>
<td>100 (29/29)</td>
<td>12.5 ± 0.7</td>
<td>3.7 ± 0.2</td>
<td>59.8 ± 6.0</td>
</tr>
<tr>
<td>Prlr−/−</td>
<td>100 (14/14)</td>
<td>15.1 ± 1.2*</td>
<td>3.6 ± 0.3</td>
<td>60.7 ± 10.3</td>
</tr>
<tr>
<td>Prl+/+</td>
<td>100 (27/27)</td>
<td>11.0 ± 0.4</td>
<td>4.5 ± 0.1</td>
<td>89.5 ± 3.5</td>
</tr>
<tr>
<td>Prl−/−</td>
<td>100 (15/15)</td>
<td>13.5 ± 1.4**</td>
<td>4.2 ± 0.3</td>
<td>82.5 ± 7.6</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM.

*p = 0.035 versus Prlr+/+ WT mice by Mann–Whitney U test.

**p = 0.045 versus Prlr+/+ WT mice by Mann–Whitney U test.

Th1 and Th17 autoimmune responses are delayed in absence of PRLR or PRL

Because Th1 and Th17 responses play a key role in CNS inflammation of EAE, we wanted to explore whether peripheral T cell responses against myelin were affected by PRLR deficiency. For this purpose, we isolated draining LNs from Prlr+/+ and Prlr−/− mice during the priming phase of EAE, and examined in vitro the proliferative recall response and cytokine production in response.
with MOG35–55 and measured serum PRL concentrations. In contrast with a previous study reporting an increase in serum PRL levels in rat EAE (21), we observed a reduction of serum PRL concentrations in mice with EAE compared with naive mice (Fig. 5). Although PRL is mainly secreted centrally by the pituitary, PRL has been suggested to be secreted also peripherally in lymphoid organs (30). However, previous work has failed to detect mRNA for Prl in mouse spleen, thymus, LN, and bone marrow (31). More recent work reported Prl mRNA expression in mouse thymocytes and in freshly isolated or mitogen-stimulated splenocytes (30). Moreover, immunoreactivity for PRL in some splenic CD4+ T cells has been described (32), although this work did not clarify whether CD4+ cells were the sources of PRL or were binding PRL deriving from other sources. To evaluate any possible contribution of PRL produced and secreted locally in secondary lymphoid organs to the development of autoreactive T cell responses, we first harvested LNs from naive C57BL/6 mice (H-2b haplotype) and assessed ex vivo by real-time PCR the expression of Prl transcript in total LNCs and in magnetically purified CD4+ T cells. We did not find expression of Prl mRNA in either LNCs or CD4+ T cells of these mice (Fig. 6A). Next, to evaluate the possibility that the transcription of Prl gene is

FIGURE 2. Delayed Th1 and Th17 responses against myelin in PRLR-deficient mice. LNCs were isolated from draining (axillary and inguinal) LNs of Prlr+/+ and Prlr−/− mice at day 7 (A) or 10 (B) after EAE induction and stimulated in vitro with MOG35–55 or medium alone. Proliferation rate was assessed by [3H]thymidine incorporation after 48 h of culture. Data represent the mean cpm ± SEM of triplicate cultures of cells pooled from three to four mice per group. Cytokine production was determined in supernatants of parallel cultures by ELISA (means ± SEM, from duplicate wells). Data are representative of two independent experiments each including three to four mice per group. *p < 0.05 by Student t test.

FIGURE 3. PRL-deficient mice display delayed Th1 and Th17 responses against myelin. LNCs were isolated from draining (axillary and inguinal) LNs of Prlr+/+ and Prlr−/− mice at day 7 (A) or 10 (B) after EAE induction and stimulated in vitro with MOG35–55 or medium alone. Proliferation rate was assessed by [3H]thymidine incorporation after 48 h of culture. Data represent the mean cpm ± SEM of triplicate cultures of cells pooled from three to four mice per group. Cytokine production was determined in supernatants of parallel cultures by ELISA (mean ± SEM, from duplicate wells). Results are representative of two independent experiments each including three to four mice per group. *p < 0.05 by Student t test.
induced in immune cells during EAE, we repeated the analysis in LNCs and purified CD4+ T cells harvested from C57BL/6 mice during the priming phase of MOG35–55–induced EAE. We failed to detect \textit{Prl} transcript also in these in vivo activated cells (Fig. 6A). To further test the hypothesis of an induction of \textit{Prl} gene transcription upon immune stimulation, we activated in vitro CD4+ T cells purified from naive C57BL/6 mice with anti-CD3 and anti-CD28 Abs, and analyzed mRNA expression of \textit{Prl} by real-time PCR and PRL protein secretion in culture supernatants by ELISA. PRL was undetectable at both transcript (Fig. 6C) and protein levels (data not shown) also after stimulation. These data are consistent with results reported by Clevenger and colleagues (33) showing that a murine Th cell line, either resting or stimulated with IL-2 or Con A, does not express PRL at either mRNA or protein level. Taken together, our findings indicate that PRL concentrations do not increase during EAE, and that neither T cells nor LNCs express and secrete PRL, thus ruling out CD4+ T cells as a possible source of local PRL that could contribute to the development of autoreactive T cell responses in EAE. Conversely, in line with previous findings (8), we found expression of \textit{Prlr} mRNA in both LNCs and purified CD4+ T cells isolated from C57BL/6 naive mice or mice with EAE. Interestingly, we observed a reduction of \textit{Prlr} transcript during the priming phase of EAE (Fig. 6B). CD4+ T cells activated in vitro with anti-CD3 and anti-CD28 Abs displayed a downregulation of \textit{Prlr} mRNA 48 h after stimulation in comparison with unstimulated cells (Fig. 6C). It must be taken into consideration that expression of \textit{Prlr} gene at the mRNA level might be different from that of PRLR at the protein level, because of complex regulatory processes controlling the final expression of protein in a cell. Nevertheless, given the observed downregulation of \textit{Prlr} mRNA in CD4+ T cells after in vitro stimulation, it is possible that downregulation of \textit{Prlr} transcript in mice immunized for EAE reflects the activation state of CD4+ T cells during the disease.

\textbf{Discussion}

Collectively, this study provides evidence that PRL plays a redundant role in the development of chronic EAE, and that PRL
locally produced in lymphoid organs is unlikely to exert an immune-modulating effect on immune cells during EAE in this model. Even though we observed a delay in the onset of EAE symptoms, which was associated with a delay in the establishment of anti-MOG peptide Th1 and Th17 responses in LN s, the overall severity of EAE was indistinguishable between Prlr−/−, Prl−/−, and corresponding WT littermates. This finding suggests that the PRL/PRLR axis can be readily compensated by other factors in the development of chronic EAE. The results obtained in Prlr−/− and Prl−/− mice are in contrast with earlier studies proposing that PRL exerts a detrimental effect in CNS autoimmunity (21, 22). However, in these previous studies, PRL functions were evaluated in rat EAE by the use of BCR, whose immune-modulating effects are not solely related to PRL depletion (23), whereas our results were obtained in Prlr−/− and Prl−/− mice. Importantly, Prlr−/− mice display a normal composition of lymphocyte subsets in primary and secondary lymphoid organs (34). Similarly, Prl−/− mice have normal myelopoiesis and primary lymphopoiesis (25). Our findings are consistent with the study of Bouchard and colleagues (34) showing that Prlr−/− mice mount an effective immune response to several types of stimuli, such as infection with an intracellular pathogen (i.e., Listeria monocytogenes) and infection with an allogeneic tumor cell line. In this work, it was also demonstrated that Prlr−/− mice develop a normal specific Ig response after immunization with a non-self Ag (34). In line with these results, we show that IgG response against the self-Ag MOG35–55 was not affected by PRLR or PRL deficiency.

In the MS field, PRL has gained renewed interest in recent years, because two studies showed that exclusive breastfeeding (a hyperprolactinemic physiologic condition) reduces the risk for postpartum relapses (35, 36), albeit other articles reported discordant results (5, 37, 38). Further, another article has demonstrated that PRL mediates proliferation of oligodendrocyte progenitor cells during pregnancy and promotes myelin repair in a spontaneously remyelinating model of chemical (lyssolecithin)-induced focal demyelination, suggesting PRL as a potential therapeutic agent for MS (39). By studying EAE, an immune-mediated model of demyelination, in PRLR- and PRL-deficient mice, we had the possibility to evaluate the net effect of PRL on disease development between the potential immune-stimulating (detrimental) and myelogenic (beneficial) properties of this hormone. Our data indicate that physiologic levels of PRL do not impact crucially on the clinical course of chronic EAE and on autoreactive immune response against myelin underlying the disease. Moreover, we did not observe in absence of PRL or PRLR a worsening of EAE severity, which could have been expected in the hypothesis of a key role for PRL in myelin repair. However, the robustness of the EAE model and the severe clinical phenotype developed by PRL-deficient, PRLR-deficient, and control mice might have well masked possible clinical effects related to the lack of PRL or PRLR in disease recovery. Lastly, we cannot rule out that a physiopathologic or pharmacologically induced hyperprolactinemic state might impact more importantly on EAE and on self-reactive T cell responses. In fact, pregnancy and breastfeeding are characterized by high hyperprolactinemia (~100–800 ng/ml), and by several other physiologic adaptations, which further complicate the interpretation of the possible role of PRL on disease activity. Thus, further investigation is required to understand whether hyperprolactinemia of pregnancy and breastfeeding has any effect on the development and progression of EAE and MS.

Disclosures
The authors have no financial conflicts of interest.

References


