Tissue-specific gene expression of prolactin receptor in the acute-phase response induced by lipopolysaccharides

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Although prolactin (PRL) was originally identified as a lactotrophic hormone secreted by the pituitary gland, accumulating evidence has implicated PRL in a strikingly diverse array of physiological functions (5). A large body of literature supports a PRL role as a stimulatory factor of the immune-hematopoietic system (8, 11, 39, 49, 57). Although controversy still exists regarding the absolute requirement of PRL in the immune function (6, 21, 29), renewed interest has arisen on PRL administration (33) and a regulatory effect of PRL on the production of inflammatory mediators (3, 12, 34, 38, 48, 57).

Immune challenges that perturb the stability of the internal milieu can be regarded as stressors. In this context, tissue injury, infection, and inflammation elicit an orchestrated host defense reaction known as the acute-phase response (APR). The APR is characterized by profound immune, neuroendocrine, and metabolic changes that are considered crucial for reestablishing homeostasis in the host (1, 23, 28, 40, 52). During the APR, the adaptive immune response is suppressed, and a rapid elevation of natural immune defense mechanisms takes place. The early systemic release of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and interferon-γ (IFN-γ), stimulate the hypothalamo-pituitary-adrenal axis and the central nervous system, resulting in increased glucocorticoid and catecholamine levels (1, 40). The complex changes occurring during the APR can potentially lead to sepsis and multiple organ failure (1, 22, 54). During the APR, PRL actions have been considered to be suppressed (1, 28); however, in this context the role of PRL and its receptors (PRLRs) remains incompletely characterized.

Injection of lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, has long been used as a model to mimic the APR. Using this approach, previous studies have shown variable results on plasma levels of PRL (9, 16, 35, 47, 51). However, regardless of the possible changes in circulating levels of PRL, the extent of PRL effects during the APR will ultimately depend on the presence of PRLR in the target tissues. In this regard, changes in PRL binding activity to hepatic cell membranes have been previously shown after LPS injection (9, 27). In female rats, intraperitoneal LPS injection decreased the specific binding of PRL to hepatic cell membranes but did not affect its binding to membranes of the mammary gland (27). In lactating mice, LPS injection induced a rapid increase followed by a decrease of PRL binding sites in hepatic membranes (9).

Recently, we have shown that a mixture of TNF-α, IL-1β, and IFN-γ induced the expression of the PRLR in primary cultures of rat pulmonary fibroblasts in vitro (12). Because the same proinflammatory cytokines are elevated during the APR, in the present report we have investigated the in vivo expression of PRLR in various tissues of the mouse during the LPS-induced APR.

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Materials and Methods.

Materials. LPS form Escherichia coli serotype 0111:B4 was purchased from Sigma (St. Louis, MO). Rat pituitary PRL was from the National Hormone and Pituitary Program (NHP). Anti-PRLR monoclonal antibody U5 (MA1-610, Clone U5) was purchased from Sigma (St. Louis, MO). Rat pituitary PRL was from the Recommended Requirements. Mice were housed in the animal research facility in caging conditions and received food and water ad libitum. All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

LPS-induced inflammation. Mice were intraperitoneally injected with either sterile saline or LPS at a sublethal dose of 10 μg/kg body wt and killed 12–14 h later by intraperitoneal pentobarbital sodium injection (200 mg/kg). Blood was extracted by cardiac puncture and drawn into heparin-containing tubes, and plasma was collected after 12–14 h later by intraperitoneal pentobarbital sodium. Blood was extracted by cardiac puncture and drawn into heparin-containing tubes, and plasma was collected after. Blood was extracted by cardiac puncture and drawn into heparin-containing tubes, and plasma was collected after. Blood was extracted by cardiac puncture and drawn into heparin-containing tubes, and plasma was collected after.

Cytokine levels in plasma. Plasma concentrations of TNF-α, IL-1β, and IFNγ were measured using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Whole lysate proteins were denatured in 4X electrophoresis buffer and boiled for 5 min. Samples (100 μg total protein/lane) were resolved on 4–20% Tris-HCl Ready Gel Precast Gel (Bio-Rad) and electroblotted onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were blocked in 3% bovine serum albumin (BSA; Fisher Scientific)-phosphate buffered saline (PBS)-0.5% Tween-20 (Sigma).

Real-time PCR. Total RNA from tissues was purified using TRIzol reagent, and first-strand complementary DNA was generated using 5 μg of total RNA and Superscript II system; both procedures were performed according to the manufacturer’s instructions (Invitrogen Life Technologies, Carlsbad, CA). Primers were synthesized as custom primers (Life Technologies; Table 1). PCR reactions were set up in 50-μl volumes, consisting of 2.5 μl of 1:1,000 SYBR Green (Molecular Probes, Eugene, OR), 2.5 μl of 10XPCR buffer, 2 μl of 50 mM MgCl₂, 1 μl of 10 mM deoxy-NTPs, 0.2 μM of each primer, 5 μl of template DNA, and 1 unit of Platinum Taq DNA polymerase (Invitrogen Life Technologies). Real-time PCR was performed using a GeneAmp 5700 Sequence Detector (Applied Biosystems, Foster City, CA). For all PRLR sets of primers, a denaturing step at 94°C for 10 min was followed by 3 cycles of 94°C denaturing for 30 s, annealing at 60°C for 45 s, 72°C extension for 30 s; 3 cycles of 94°C denaturing for 30 s, annealing at 58°C for 45 s, 72°C extension for 30 s; and 40 cycles of annealing at 56°C for 45 s, 72°C extension for 30 s. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a denaturing step at 94°C for 10 min was followed by 25 cycles of 94°C denaturing for 30 s, annealing at 58°C for 45 s, and 72°C extension for 30 s. Data were collected and analyzed using GeneAmp 5700 SDS Software (Applied Biosystems). The relative concentration of PRLR mRNA was measured as the number of cycles of PCR required to reach threshold fluorescence and normalized against that of an internal standard gene (GAPDH), according to the procedures recommended by Applied Biosystems.

Western blot analysis of PRLR in liver lysates. Liver tissues were homogenized in RIPA buffer (50 mM Tris, pH 7.4, 0.5% NP-40, 0.2% sodium deoxycholate, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 mM sodium orthovanadate, and 1 mM NaF). Protein quantitation was performed using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Whole lysate proteins were denatured in 4X electrophoresis buffer and boiled for 5 min. Samples (100 μg total protein/lane) were resolved on 4–20% Tris-HCl Ready Gel Precast Gel (Bio-Rad) and electroblotted onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were blocked in 3% bovine serum albumin (BSA; Fisher Scientific)-phosphate buffered saline (PBS)-0.5% Tween-20 (Sigma).

Table 1. Sequence of primers used to amplify PRLR and GAPDH transcripts

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>PRLR common-forward-A (41)</td>
<td>5′-GATCAGCAATCGCAGCAAGAAA-3′</td>
</tr>
<tr>
<td>PRLR common-forward-B (13)</td>
<td>5′-AACAGGCTCACTGGTCTATAA-3′</td>
</tr>
<tr>
<td>Long PRLR reverse (13)</td>
<td>5′-GATAAAATCTAGTGTGAAATCTTCA-3′</td>
</tr>
<tr>
<td>S1 PRLR reverse (13)</td>
<td>5′-GAAGAAGAATCTGACGAGAAC-3′</td>
</tr>
<tr>
<td>S2 PRLR reverse (13)</td>
<td>5′-GTCAGCCACTCCAGAACCTCC-3′</td>
</tr>
<tr>
<td>GAPDH forward (13)</td>
<td>5′-TGACACAAGAGACTGTTAG-3′</td>
</tr>
<tr>
<td>GAPDH reverse (13)</td>
<td>5′-GATAGGAGGGATAGTGGTTC-3′</td>
</tr>
</tbody>
</table>

PRLR, prolactin receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; S1, -2, and -3, short PRLR isoforms. Nos. in parentheses refer to references. To amplify the common sequence to all receptors, PRLR common-forward-A was used in combination with PRLR common-reverse sequence. To distinguish between the 4 PRLR isoforms a PRLR common-forward-B was used in combination with the specific sequences for each PRLR isoform.

Fig. 1. Downregulation of prolactin (PRL) receptor (PRLR) expression in hepatic tissue after ip LPS injection. A: relative quantitation of mRNA for PRLR. AU, arbitrary units. Real-time PCR was performed using primers to amplify a common sequence to all PRLRs. Values of PRLR expression were normalized to the expression of GAPDH. Data are expressed as means ± SE. Statistical significance of the data was analyzed by Student’s t-test (P = 0.025). Data are representative of 3 independent experiments with 4 mice in each group. B: Western blot detection of PRLR in total liver lysates by use of anti-PRLR monoclonal antibody U5. An immunoreactive protein of ~40 kDa was clearly observed in lysates from control (Cont) animals, and a significant reduction in its intensity was observed in lysates from LPS-treated mice. Each lane corresponds to individual control of LPS-injected mice. Actin detection is shown as loading control.
Aldrich) for 1 h and probed with 1 μg/ml anti-PRLR monoclonal antibody U5 or anti-actin goat polyclonal antibody (sc-1616, Santa Cruz Biotechnology) in 3% BSA-PBS-0.5% Tween-20 at 4°C overnight. Membranes were washed three times with PBS 0.5%-Tween-20 and incubated for 1 h at room temperature with horseradish peroxidase-conjugated protein A (Santa Cruz Biotechnology) diluted 1:5,000 in 3% BSA-PBS-0.5% Tween-20. Membrane was washed, incubated with West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 min, and visualized using Super RX film (Fuji Photo Film, Düsseldorf, Germany).

Immunohistochemistry. Mice were deeply anesthetized with intraperitoneal pentobarbital sodium (200 mg/kg) and perfused via the left ventricle with PBS followed by 10 ml of 1% paraformaldehyde. Tissues were extracted and further fixed in 1% paraformaldehyde for 1 h, dehydrated, included in paraffin, and sectioned following standard procedures. Tissue sections were deparaffinized and hydrated through xylene and graded alcohol series. Antigen unmasking was performed using Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was blocked by incubating sections with 3% hydrogen peroxide (Sigma-Aldrich) in tap water for 5 min. Blocking was performed using an Avidin/Biotin Blocking Kit (Vector Labs). Immunodetection was performed using the Vector M.O.M. Immunodetection Kit (Vector Labs), designed to minimize background staining when using monoclonal antibodies on mouse tissues. Adjacent sections in the same slide were incubated with either 10 μg/ml anti-PRLR monoclonal antibody U5 (MA1-610, Affinity Bioreagents) or 10 μg/ml control IgG-1 (DakoCytomation), which corresponds to the same isotype of IgGs as the U5 antibody. Additional sections, where primary antibodies were omitted and the secondary antibody was either absent or present, were used as additional controls.

Fig. 2. PRLR immunoreactivity in hepatic tissues from control and LPS-injected mice. Immunohistochemical staining using anti-PRLR monoclonal antibody U5 or mouse IgG-1. Although in tissues from control mice the hepatocytes appear clearly individualized by PRLR staining in the cell membrane, in tissues from the LPS-injected mice this demarcation is almost lost, partially remaining in isolated cells (arrows). Nuclear PRLR immunoreactivity is observed in sections from both control and LPS-injected mice. Tissue sections incubated with control mouse IgG-1 as primary antibody are shown for negative control of staining. Microphotographs were taken using ×40 and ×100 (inset) objective lenses.

Fig. 3. Relative levels of mRNA for PRLR in different tissues of control mice. Real-time PCR was performed using primers to amplify a common sequence to all PRL receptors. Results are expressed as percentage of maximum expression found in prostate tissue. Values for PRLR expression were normalized to expression of GAPDH. Sem. Ves., seminal vesicle.
control slides. Vectastain ABC and DAB Substrate Kits were used according to the manufacturer’s instructions (Vector Labs). Toning of diaminobenzidine (DAB)-stained sections was performed with DAB-enhancing solution (Vector Labs). Finally, sections were counterstained with hematoxylin (Vector Labs) and mounted with the aqueous-based mounting medium Crystal Mount (Biomed, Foster City, CA). Immunostained sections were examined and digitally photographed under a Zeiss Axioskop 2 microscope and AxioCAM HRC camera (Zeiss, Jena, Germany).

PRL levels in plasma. PRL levels in plasma were evaluated using the Nb2 cell bioassay as previously described (53). Nb2 cells were grown in high-glucose DMEM supplemented with 10% horse serum, 1% FBS, 50 U/ml penicillin-streptomycin, and 10^{-4} M β-mercaptoethanol. The Nb2 cell cycle was synchronized by incubation for 24 h in high-glucose DMEM supplemented with 10% horse serum, 1% FBS, 50 U/ml penicillin-streptomycin, and 10^{-4} M β-mercaptoethanol (GIBCO Life Technologies, Carlsbad, CA). Bioassay was performed in the same medium composition but in the absence of FBS. Cells were cultured in 96-well plates in the presence of 0.2% plasma samples. Relative concentrations of PRL were determined from a standard curve generated using rat PRL. Cell number was determined after 48 h using a Coulter Particle Counter Z1 (Beckman-Coulter, Fullerton, CA).

Statistical analysis. Results were analyzed with Student’s t-test for independent samples with Statistica for Windows 5.0 (Statsoft). Differences of $P < 0.05$ were regarded as statistically significant. All values are reported as means ± SE.

RESULTS

Hepatic PRLR expression is downregulated during LPS-induced APR. To determine whether acute inflammation is accompanied by changes in PRLR expression, we investigated the effect of intraperitoneal LPS injection on the levels of hepatic PRLR transcripts. As assessed by real-time PCR using primers to amplify the common sequence to all PRLRs, the relative expression of PRLR mRNA in hepatic tissues from LPS-injected mice was dramatically reduced to 0.1-fold the relative expression of PRLR mRNA in hepatic tissues from control mice (Fig. 1). The Nb2 cell cycle was synchronized by incubation for 24 h in high-glucose DMEM supplemented with 10% horse serum, 1% FBS, 50 U/ml penicillin-streptomycin, and 10^{-4} M β-mercaptoethanol (GIBCO Life Technologies, Carlsbad, CA). Bioassay was performed in the same medium composition but in the absence of FBS. Cells were cultured in 96-well plates in the presence of 0.2% plasma samples. Relative concentrations of PRL were determined from a standard curve generated using rat PRL. Cell number was determined after 48 h using a Coulter Particle Counter Z1 (Beckman-Coulter, Fullerton, CA).

Statistical analysis. Results were analyzed with Student’s t-test for independent samples with Statistica for Windows 5.0 (Statsoft). Differences of $P < 0.05$ were regarded as statistically significant. All values are reported as means ± SE.

Relative expression of PRLR mRNA in control tissues. As measured by real-time PCR, the relative expression of transcripts for PRLR in several control tissues is shown in Fig. 3. High expression was observed in ventral prostate, seminal vesicle, testicle, and liver tissues, whereas lower levels of expression were observed in lung, heart, thymus, kidney, and spleen tissues.

Tissue-specific regulation of PRLR expression during LPS-induced APR. Next, we investigated whether or not the suppressive effect of LPS injection on PRLR expression also occurs in tissues other than liver, where PRLR transcripts are detected. A dramatic suppressive effect of LPS injection on PRLR mRNA levels was found in prostate, seminal vesicle, heart, and kidney tissues (fold expression ≤0.2; Fig. 4). A significant reduced expression was also observed in the lung (0.6-fold), whereas in the testis a reduction was observed but values were not statistically significant (Fig. 4). Furthermore, we studied whether or not similar changes in PRLR expression...
occur in immune system-related tissues, such as thymus and spleen. In contrast to the suppression observed in the liver and most other tissues analyzed, LPS injection induced a 2.3-fold increase in PRLR mRNA levels in the thymus, whereas no significant changes were observed in the spleen (Fig. 5).

Proportion of transcripts for PRLR isomers was not altered by LPS injection. Several forms of PRLR arise from alternative initiation sites of transcription and gene splicing. In mice, one long and three short forms (S1, S2, and S3) have been described (15, 46). We investigated the levels of mRNA for each of the four PRLR isoforms in liver and thymus tissues (Fig. 6). Relative quantitation of mRNA levels in the liver showed that mRNA for the long and S3 PRLRs were the most abundant, with minor expression of the S1 and S2 PRLRs. In the thymus, most PRLR transcripts corresponded to the long PRLR, whereas S2 and S3 PRLRs are expressed in very low amounts, and no expression of the S1 isoform was detected (Fig. 6). Although in LPS-treated mice the expression of total PRLR transcripts was decreased in the liver and increased in the thymus, the relative proportion of the transcripts for the different receptor isoforms remained the same in both tissues (Fig. 6).

**DISCUSSION**

In vitro, a mixture of TNF-α, IL-1β, and IFNγ has been shown to induce the expression of PRLR in primary cultures of rat pulmonary fibroblasts (12). Because these proinflammatory cytokines are elevated during the in vivo LPS-induced APR, we undertook a global approach to evaluate the expression of PRLR in several tissues of mice subjected to intraperitoneal LPS injection. Our results show that PRLR mRNA levels, as measured by real-time PCR, were dramatically suppressed in the liver after intraperitoneal LPS injection. A reduced expression of PRLR in LPS-injected mice was confirmed by Western blot analysis. Furthermore, a reduction in PRLR immunoreactivity associated with the plasma membrane was observed in liver sections from LPS-injected mice. PRLR immunoreactivity was observed in the nucleus of hepatocytes from both mouse groups. Although unexpected, the presence of PRLR in the cell nucleus has been previously demonstrated by Gadd and Clevenger (24) and may have functional significance at the level of gene transcription regulation. The downregulation of PRLR in the liver of LPS-injected mice is supported by previous studies showing that the intraperitoneal injection of LPS resulted in the reduction of specific binding sites for PRL in hepatic membranes from mice and rats (9, 27).

To further characterize the regulation of PRLR expression during the APR, we investigated whether or not the profound changes observed in the liver were paralleled by similar changes in other organs. The expression of PRLR transcripts as

**Table 2. Plasma concentration of TNF-α, IL-1β, and IFNγ**

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>TNF-α, pg/ml</th>
<th>IL-1β, pg/ml</th>
<th>IFNγ, pg/ml</th>
</tr>
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<tbody>
<tr>
<td>Control (n = 8)</td>
<td>9.9±1.8</td>
<td>8.5±1.8</td>
<td>4.5±0.4</td>
<td></td>
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<tr>
<td>LPS injected (n = 8)</td>
<td>40.5±6.5*</td>
<td>56.5±3.7*</td>
<td>18.1±6.1*</td>
<td></td>
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</table>

Data are expressed as means ± SE. Statistical significance of the data was analyzed by Student’s t-test (*P < 0.05).
measured by real-time PCR was also suppressed in prostate, seminal vesicle, kidney, heart, and lung tissues from LPS-treated mice. In contrast, PRLR mRNA levels in the thymus were increased, and no significant changes were observed in the spleen. Although changes in PRLR mRNA levels may be followed by similar changes at the level of PRLR protein synthesis, we have analyzed this correlation only in hepatic tissues, and it is possible that PRLR mRNA may be translated with varying efficiency in different tissues. Nonetheless, our results demonstrate a complex tissue-specific regulation of PRLR expression in the context of the APR. These findings are in agreement with previous studies showing that the regulation of PRLR expression is tissue specific, and it involves the differential activation of multiple promoters in the PRLR gene. For instance, PRLR expression in the spleen (7, 15, 25, 30, 31, 42, 46).

Furthermore, we analyzed the expression of the mRNA for the different PRLR isoforms. In mice, one long and three short forms of the PRLR have been described (15, 46), which arise from the alternative splicing of a common transcript. Although all receptors can bind the ligand, the activation of PRLR-signaling pathways varies according to the receptor isoform (2, 4, 10, 14, 36, 44, 50). Because distinct PRLR isoforms may lead to different physiological responses, we investigated whether or not LPS administration could affect the proportion of expression of each receptor isoform. Although the proportion of transcripts for the PRLR isoforms differed when liver and thymus were compared, no changes were observed in the relative proportion of transcripts in control and LPS-injected tissues in either hepatic or thymic tissues. In this regard, our results suggest that the mechanism involved in the regulation of PRLR expression during the APR is common to all PRLR transcripts and may occur at the level of gene transcription rather than during the posttranscriptional processing of the primary transcript.

Previously, we showed that PRLR expression by pulmonary cells in vitro can be induced by a combination of proinflammatory cytokines, including TNF-α, IL-1β, and IFNγ (12). However, the results shown herein indicate that, although plasma levels of TNF-α, IL-1β, and IFNγ were elevated in LPS-treated mice, the expression of PRLR in the lung was downregulated. The difference between in vitro and in vivo results may be explained by the influence of other key mediators (i.e., IL-6, catecholamines, and glucocorticoids) that, although present during the APR in vivo, were not considered in our previous in vitro study. In addition, the influence of the temporal changes occurring during the APR in vivo should be considered. In vitro and in vivo research is in process to investigate the influence and temporal interactions of the key inflammatory mediators of the APR on the regulation of PRLR expression by different cell types.

Previously, PRL functions during the APR have been regarded as being suppressed (1, 28). Although PRL functions could be considered suppressed by the reduction of its receptor in several tissues, this is not the case in all organs, being increased in the thymus and preserved in the spleen. The physiological significance of the specific changes in PRLR expression in different tissues warrants further investigation. This process may be common to other stressful situations that induce the APR, including trauma-hemorrhage, surgery, and burn injury. In this regard, several studies have been performed to determine PRL binding activity on hepatic membranes after different experimental insults. Studies have shown that ovariectomy, thyroidectomy, and hypophysectomy induced a diminution in hepatic binding sites for lactogenic hormones (26, 32), suggesting that the surgical trauma itself may trigger the mechanism. Several other stress-related stimuli, including subcutaneous inflammation, skin incision, and cold exposure, can also suppress PRL binding to hepatic membranes (27). Recently, Chaudry and colleagues have shown that, in a model of trauma-hemorrhage, the expression of the long PRLR was upregulated in the thymus (43) but downregulated in hepatocytes (56).

During the APR, thymic involution occurs mainly due to glucocorticoid-induced thymocyte apoptosis (28). The expression of PRLR in the thymus during the APR is in support of the previously described protective role of PRL on thymocytes. Several in vitro studies have demonstrated that PRL can function as a survival factor for thymocytes, antagonizing the apoptotic effect of glucocorticoids (20, 34, 55). This concept is strongly supported by a recent study showing that elevated PRL can suppress glucocorticoid-induced thymocyte apoptosis in vivo (33). A role of PRL in maintaining the immune function under acute inflammation is further supported by the observation that, contrary to what is observed in most tissues analyzed, PRLR expression in the spleen is not suppressed. In recent studies using in vivo models of stress, the importance of PRL as a survival and proliferative factor for splenocytes was shown (19, 33). In PRL−/− mice subjected to burn injury, the splenic T cell proliferative response to mitogens is attenuated (19), and elevated PRL can suppress splenocyte apoptosis in mice treated with glucocorticoids (33). In summary, our results demonstrate the tissue-specific regulation of PRLR expression during the LPS-induced APR. We hypothesize that, during the APR, the unique regulation of PRLR expression constitutes a finely orchestrated mechanism to redirect PRL signaling to organs of the immune system while suppressing the signal in most other tissues.

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