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Brigitte Bouchard,* Christopher J. Ormandy, † James P. Di Santo, ‡ and Paul A. Kelly 2*

Prolactin (PRL), 3 which is a peptide hormone synthesized and secreted primarily by the anterior pituitary gland, regulates the growth and differentiation of the mammary gland and the ovary (reviewed in Ref. 1). PRL has also been classified as a cytokine and signals through specific membrane receptors that are also members of the cytokine receptor superfamily and are devoid of intrinsic tyrosine kinase activity. Upon ligand binding and dimerization of the PRL receptor (PRLR), signal transduction occurs through the recruitment of cytoplasmic molecules to the PRLR, including the tyrosine kinase Janus kinase 2 (JAK2) and the STAT family members STAT-1, -3 and -5 (reviewed in Ref. 2). Because it is known that 1) PRL shares similarity to other known cytokines, 2) the PRLR is a member of the cytokine receptor superfamily, and 3) most members of the cytokine/cytokine receptor system have important roles in hematopoietic development, the idea that PRL/PRLR signaling could have biological effects outside of the reproductive system and possibly during hemopoiesis has emerged.

Other earlier studies using in vivo models of pituitary depletion suggested a role for PRL in lymphohemopoietic development. Hypophysectomized rats present symptoms of immunosuppression such as lymphopenia and impaired humoral and cellular Ag responses (3). Bromocryptine, a dopamine receptor agonist, causes PRL depletion and thereby mimics hypophysectomy. Administration of bromocryptine is also associated with a decreased Ab response following immunization with SRBCs (4). The effects of hypophysectomy or bromocryptine on certain immune responses can be reversed by exogenous PRL administration, implying a role for PRL in immune regulation. However, these models do not allow for complete PRL depletion, because a number of extrapituitary sites of PRL synthesis have also been described. In addition, multiple endocrine pathways are potentially altered in these experimental models, which renders their interpretation difficult.

Subsequent in vitro models have indicated that PRL can induce lymphocyte proliferation, although these findings are controversial (reviewed in Ref. 5). In addition, PRL has been shown to increase the expression of CD25, of the erythropoietin receptor, and of its own receptor. In NK cells, PRL increases DNA synthesis and cytotoxicity against specific target cells (6). PRL inhibits T cell apoptosis under certain conditions and enhances Ab production by mature B cells (7). Finally, a series of publications have indicated a role for nuclear PRL in the T cell response to IL-2 (8). Overall, the body of literature on PRL and its effects on the immune system has suggested several important immunomodulatory roles for this cytokine; however, some of them appear to be contradictory, particularly with regard to the in vitro studies, which indicate either an immunostimulatory or an immunosuppressive role for PRL (reviewed in Ref. 5).

Recently, renewed attention has been given to the potential actions of PRL on the immune system. Montgomery et al. identified a PRL-like molecule that is produced and secreted by lymphocytes...
(9, 10). These authors suggested that lymphocytes might therefore use PRL in an autocrine fashion. Indeed, in all species analyzed, the expression of the PRLR appears almost ubiquitous, and several studies have aimed at determining the level of expression of the PRLR on immunocompetent cells. Classical approaches involving radiolabeled ligand binding assays have indicated that the number of PRLR molecules present on the lymphocyte cell surface is quite low, and often at the limits of detection (~300 receptors per cell (11)). In contrast, levels of IL-2R or IL-7R approach 10^5 receptors per lymphoid cell. More recently, lymphocyte PRLRs have been evaluated using biotinylated ligands or specific mAbs in FACS analysis (12). These studies demonstrated the expression of PRLR on all lymphoid subsets, but did not allow the exact number of PRL binding sites on these different cell populations to be assessed.

Our laboratory has recently generated a PRLR-deficient mouse model by gene-targeting techniques. In this mouse strain, the PRLR has been invalidated in all tissues. Using immunoprecipitation of PRLR followed by immunoblotting, we could not detect PRLR proteins in extracts from liver and bone cells from knockout (KO) animals (13, 14). In addition, no significant binding of PRLR-specific ligands (growth hormone or PRL) was detected in liver microsome preparations from PRLR^-/- mice. Therefore, these mice are useful to analyze any potential role of PRLR signaling in developmental processes. In this report, we have assessed the role of PRL/PRLR pathways in lymphocyte activation and maturation.

Materials and Methods

Animals

Mice carrying a null mutation in the PRLR on a mixed (129Ola × C57BL/6) background have been described previously (13) and were maintained in a specific pathogen-free animal facility. C57BL/6 PRLR^-/- mice were obtained by backcrossing the PRLR mutation for eight generations onto the C57BL/6 background followed by intercrossing to obtain homozygous mutant mice. Simultaneous identification of the wild-type (WT) or mutated genomic PRLR loci was performed by a three-primer PCR using the following primers were used: WT forward, 5'-GAAGAGCAAGATCTCAAGAAC-3'; Neo forward, 5'-CCAGTCCCTTCCCGCTTCAGT-3'; Neo reverse, 5'-GAAGAGCAAGATCTCAAGAAC-3'. Conditions for the PCR were 5' denaturation at 94°C followed by 35 cycles consisting of 45 s at 94°C, 1 min at 57°C, and 45 sec at 72°C. Using these conditions, the WT locus generates a 350-bp fragment, whereas the mutant allele gives a 455-bp fragment at 94°C, 1 min at 57°C, and 45 sec at 72°C. The primers were designed to distinguish the wild-type allele from the mutated allele.

Cell isolation and in vitro proliferation analysis

Organs were removed aseptically, and single-cell suspensions were prepared in sterile medium using a mesh filter. After lysis of contaminating erythrocytes by hypotonic NH4Cl, cells were resuspended and counted; viability was determined using trypan blue dye exclusion. For mitogenic assays, splenocytes were cultured at 2 × 10^6 cells/well in flat-bottom, 96-well plates in RPMI 1640 medium supplemented with 10% FCS with or without Con A (2.5 µg/ml) and cytokines (IL-2 or IL-7 at 20 ng/ml) for 3 days. For mixed lymphocyte reaction, 2500 rad-irradiated allogeneic (from BALB/c mice, H-2d) or syngeneic (from C57BL/6 mice, H-2b) spleen cells were used as stimulator cells and cocultured with equal numbers (10^5) of responder cells in round-bottom, 96-well plates for 5 days. In all experiments, cells were pulsed with 1 µCi of [3H]thymidine during the final 8 h of culture.

Immunofluorescence analysis

Cells were resuspended in PBS/1% FCS/0.01% sodium azide (washing buffer). Abs against the splenic cell surface Ags were used for immunofluorescence analysis (all from PharMingen, San Diego, CA) as FITC, PE, or tricolor conjugates: CD4, CD8, CD24 (heat-stable Ag), CD25 (IL-2Rα), CD43, CD45R (B220), CD69, TCRαβ, IgM, IgD, and NK1.1. Staining combinations are indicated in the figure legends. Cells (5 × 10^5) were initially incubated for 20 min at room temperature with anti-FcRII/III Ab (2.4G2) to block nonspecific reactivity. Incubation was then performed with specific Abs for 20 min on ice before extensive washing. Labeled cells were analyzed on a FACScan flow cytometer using Lysis II software (Becton Dickinson, Mountain View, CA).

Determination of serum Ig levels and Ag-specific Ig responses

For total serum Ig concentrations, 8- to 10-wk-old mice were analyzed. Whole blood was collected from retroorbital plexus, and plasma was stored at -20°C before analysis. Serum Ig isotypes were determined by ELISA as described previously (15). For T cell-dependent Ig responses, mice were injected i.p. with alum-precipitated 4-hydroxy-3-nitrophenyl acetyl (NP)·chicken γ-globulin (CG) (NP-CG) (100 µg per mouse). After 7 and 14 days, Ig isotypes and concentrations were determined for NP-CG-binding Abs, respectively, by a direct plate binding assay as described previously.

Tumor cell cytotoxicity assays

Mice were injected with poly(I:C) (100 µg in 1 ml normal saline) at 48 h before sacrifice. Unfractionated splenocytes from PRLR^-/- or their normal littermates were cocultured with 5000 3H-labeled YAC-1 target cells at varying E:T ratios for 4 h at 37°C with 5% CO2. Radioactivity was measured in the supernatants, and specific lysis was determined according to the following formula: % lysis = ([cpm free cpm] / [max cpm - control cpm]). For in vivo tumor rejection studies, mice (H-2b) were injected i.p. with 10^3 cells from the allogeneic tumor line P815 (H-2d). The animals were then monitored daily for the appearance of tumors, and survival over a period of 60 days was assessed.

Listeria monocytogenes (L.m.) infection

Groups of five PRLR^-/- or control mice were injected i.v. with 5 × 10^6 or 10^7 cfu of L.m. as described previously (16); survival was monitored for a period of 6 wk. For early innate responses to L.m., mice were injected i.v. with 3 × 10^6 cfu, and the total L.m. burden in the liver and spleen were determined on day 2 postinfection by plating whole organ homogenates on tryptic soy broth plates (16).

Results

To identify a role for the PRL signaling pathway in the development or function of the immune system, mice harboring a null mutation in the PRLR were analyzed. The study was initially performed on mice on the mixed (129Ola × C57BL/6) background. Subsequently, the PRLR mutation was backcrossed for eight generations onto the C57BL/6 background. PRLR^-/- mice were then intercrossed to generate control (PRLR^-/-) and PRLR^-/- mice, and a similar analysis was performed. Disruption of the PRLR gene is not associated with any differences in growth: PRLR^-/- mice develop normally, and there have been no deaths due to disease or any apparent illness in mice ≤13 mo of age (data not shown).

The spleen and thymus of adult (6- to 8-wk-old) PRLR^-/- mice were similar in weight and cellularity to those of WT control mice (60 ± 8 × 10^6 cells vs 50 ± 12 × 10^6 cells, respectively, for spleen WT and PRLR^-/-; 87 ± 11 × 10^6 cells vs 98 ± 15 × 10^6 cells, respectively, for thymus WT and PRLR^-/-). Histological examination of these tissues, as well as of the intestinal epithelia, failed to reveal obvious structural abnormalities (data not shown).

B cell development in PRLR^-/- mice

Total numbers of nucleated bone marrow (BM) cells were similar in both PRLR^-/- and PRLR^-/- mice (20.2 ± 4 × 10^6 cells vs 22.4 ± 5 × 10^6 cells, respectively, for WT and PRLR^-/-), as were the percentages of B220^+ lymphoid cells (Fig. 1A). We found that the percentages of immature B220^+IgM^+ pro/pre-B cells were not significantly decreased in PRLR^-/- mice compared with controls. The early B cells expressed normal patterns of CD43 and CD24 (heat-stable Ag) (data not shown). More mature IgM^+ B cells in the BM were also normally represented (Fig. 1A). These results demonstrate that the absence of PRLR has no major deleterious effects on the generation of B cell precursors in the BM.
Mature, peripheral B220⁺ B cells were found at similar frequencies in the spleen and lymph nodes of PRLR⁻/⁻ and control animals (Fig. 1B). These cells expressed normal levels of IgM and IgD. Moreover, the ratio of newly generated B cells (IgM⁺⁺/IgD⁺⁻) and mature, recirculating B cells (IgM⁺⁻/IgD⁺⁺) was unchanged in the absence of the PRLR. IgM⁺⁺/IgD⁺⁻ B cells did not appear to be activated as evidenced by their lack of CD25 and CD69 expression (data not shown).

T cell development in PRLR⁻/⁻ mice

Thymocyte differentiation was not impaired in PRLR⁻/⁻ mice in that the percentages of CD4⁻ CD8⁻ double-negative, CD4⁺ CD8⁺ double-positive, CD4⁺ single-positive, and CD8⁺ single-positive thymocytes were similar to control mice (Fig. 2A). The expression of TCR β-chain was normal (Fig. 2B), suggesting that PRLRs do not play a major role in pre-TCR or TCR selection pathways. With regard to early thymocyte development, earlier reports suggested that PRL decreased thymic cellularity in vivo (17) and induced a preferential development of mature CD4⁺ thymocytes (18). We examined early T cell precursors from PRLR⁻/⁻ mice by analyzing CD44 and CD25 expression on CD3⁻ CD4⁻ CD8⁻ thymocytes. No differences in the differentiation of pre- or pre-T cells could be discerned between PRLR⁻/⁻ and control thymocytes (Fig. 2C). Finally, no age-related changes in thymocyte development were noted when comparing newborn, 3- to 4-wk-old, or 6- to 8-wk-old PRLR⁻/⁻ and control mice (data not shown).

Mature CD4⁺ and CD8⁺ TCRαβ-expressing cells were found in the spleen and lymph nodes of adult PRLR⁻/⁻ mice at normal frequencies (Fig. 3A). TCRγδ⁺ cells were also present normally among PRLR⁻/⁻ splenocytes (data not shown). Previous reports demonstrated that CD25 expression was increased in the presence of PRL (19). In contrast, we found a normal constitutive expression of CD25 on a small subpopulation of peripheral splenic CD4⁺ Dlow and mature, recirculating B cells (IgMlow/IgDhigh) was unchanged in the absence of the PRLR. IgMhigh/IgDlow B cells did not appear to be activated as evidenced by their lack of CD25 and CD69 expression (data not shown).
T cells (20) from control and PRLR^{-/-} mice (Fig. 4A). Taken together, these data rule out any essential role for PRLRs in normal T cell development.

Ig levels and specific Ab responses

Previous reports have indicated that PRL depletion by hypophysectomy or by treatment with bromocryptine can provoke marked abnormalities in humoral immune responses (4). To analyze any effect of PRLR deficiency on B cell Ig production, sera from 8-wk-old PRLR^{-/-} and control mice were analyzed for total Ig levels. No significant differences were observed in the levels of IgM, all IgG subclasses, IgA, or IgE (data not shown). We subsequently sought to determine whether PRLR^{-/-} mice could generate normal Ag-specific Ig responses in vivo. WT or mutant mice were immunized with NP-CG to elicit T-dependent Ig responses. NP-specific IgM and switched IgG levels were detected in both types of mice (Fig. 5). Ab responses of WT and mutant mice were quantitatively and kinetically indistinguishable. Thus, in contrast to previous reports, absence of PRLR signaling does not appear to perturb normal B cell Ig responses.
T cell responses to mitogens and alloantigens

The secretion of a PRL-like molecule by lymphocytes has led to the speculation that PRL could act as an autocrine factor for lymphocyte growth (9). Clevenger et al. have further presented a model in which PRL signaling via its receptor plays a role in the mitogen-induced CD25 expression necessary for IL-2 mediated proliferation (21). To evaluate a role for PRLR signaling in T cell mitogen responses in vitro, we stimulated splenocytes (or lymph node cells) from control or PRLR\(^{2/2}\) mice with Con A and examined the patterns of CD25 expression. No qualitative or quantitative differences in the levels of induction of CD25 could be observed between these mice (Fig. 4B). The proliferation of Con A-activated splenocytes was increased by the addition of exogenous cytokines (including IL-2 or IL-7), demonstrating that PRLR-deficient lymphocytes are fully competent to proliferate in this setting (Fig. 6). These results call into question the role of the proposed PRL/PRLR autocrine loop during in vitro mitogen-induced proliferation.

Purified splenocytes from control or PRLR\(^{2/2}\) mice (H-2b) were cocultured with irradiated, allogeneic splenocytes from BALB/c mice (H-2d) or with syngeneic spleen cells (H-2b); proliferative responses were measured. As shown on Fig. 7, both PRLR\(^{2/2}\) and control animals demonstrated normal allogeneic responses and only background levels of proliferation against syngeneic cells.

NK cell development and antitumor responses

NK and NK-T cell development were examined in PRLR\(^{-/-}\) mice on the C57BL/6 background. Compared with control animals, PRLR\(^{-/-}\) mice demonstrated normal percentages of liver NK1.1\(^{+}\) CD3\(^{-}\) NK cells and NK1.1\(^{+}\) CD3\(^{+}\) NK-T cells (Fig. 3B). After in vivo NK cell priming with poly(IC), unfractionated splenocytes were tested for their ability to lyse the NK-sensitive target cell YAC-1. Both control and PRLR\(^{-/-}\) splenocytes were capable of specifically lysing YAC-1 cells (35% specific lysis vs 32% specific lysis at a 100:1 E:T ratio, respectively, for PRLR\(^{-/-}\) and controls, see Fig. 8). In vivo antitumor responses were examined after an i.p. injection of allogeneic P815 mastocytoma cells. PRLR\(^{-/-}\) animals successfully eliminated tumor cells, with no evidence of palpable lesions over the length of the experiment (>60 days; data not shown).
Response to L.m.

Infection with L.m. serves as the prototypic model for immune responses against intracellular pathogens (reviewed in Ref. 22). Immunity to L.m. in normal animals proceeds in two stages: the initial innate immune response involving macrophages, NK cells, and neutrophils followed by a sterilizing adaptive immune response requiring T and B cells. In contrast, infection of immunodeficient animals can result in either 1) rapid, lethal dissemination of L.m. in a matter of days or 2) chronic listeriosis with mortality after 2–3 wk. Because earlier reports have implicated a role for PRL signaling in the response to L.m. (23), we compared sublethal (3 × 10^3 cfu, i.v.) L.m. infection in PRLR−/− and control animals. No deaths of PRLR KO or control mice were observed during the period of 6 wk after L.m. infection (data not shown). PRLR−/− mice showed normal innate (day 2) responses to L.m., because total liver or spleen cfu burdens were similar to control mice (4.1 ± 0.6 × 10^3 cfu/liver and 4.5 ± 0.5 × 10^4 cfu/spleen vs 4.0 ± 0.9 × 10^4 cfu/liver and 4.5 ± 0.6 × 10^5 cfu/spleen, respectively, for PRLR−/− and controls). These results rule out an essential role for PRL signaling in normal immune responses to L.m.

Discussion

Previous experiments have attributed a rather wide array of functions to PRL with respect to the development of immunity (reviewed in Ref. 5). However, the immunomodulatory properties ascribed to PRL have not always been consistent: in some instances, PRL acted to enhance immune responses (24), whereas other reports showed PRL to be immunosuppressive (25–28). The difficulty in reconciling these differences probably stems from the use of different model systems. In this report, we have directly addressed the role of PRL signaling in the development and function of the immune system by analyzing mice with a deletion in the PRLR gene. Our results exclude any essential role for the PRLR in lymphocyte differentiation. PRLR−/− mice develop mature functional B, T, and NK cells and demonstrate the capacity to generate diverse types of immune responses in vitro as well as in vivo. A recent report on PRL-deficient animals (29) has indicated that PRL is not required for normal hemopoiesis. Our results together with the data from Horseman et al. (29) directly show that the PRL/ PRLR ligand receptor system is not essential for normal immune system development. Our data further demonstrate that PRLR signaling is not required for normal immunity and call into question the numerous reports attributing a major role for PRL in immunoregulation.

Such discrepancies are illustrated by comparing our data with initial reports using hypophysectomized animals in which the Ig production in response to T-dependent Ags such as SRBCs is abrogated (3). From these initial studies, it is not clear whether the defect directly affects B cells or is due to an indirect effect on T cell help. In contrast, our study clearly establishes that PRLR−/− animals exhibit a normal B cell response to T-dependent Ags.

A second example involves the reported role of PRL in normal immunity to L.m. Bernton et al. reported that hypoprolactinemia resulting from bromocryptine treatment of mice was associated with increased susceptibility to L.m within 7 days postinfection (23). In this report, the authors further suggested that impaired production of macrophage-activating factors (such as T cell-derived IFN-γ) could explain these observations. In support of this hypothesis, the authors indicated that bromocryptine-treated mice had an impaired proliferative response to the T cell mitogen Con A. Using our PRLR KO model, we have reassessed the role of PRL signaling in immunity to L.m. In contrast to Bernton et al., we find that the resistance of PRLR−/− mice to sublethal L.m. infection is similar to that of control mice. The fact that PRLR−/− mice have normal day 2 liver and spleen L.m burdens indicates that PRL is not required for generating macrophage effector functions, which are the hallmark of innate responses to this pathogen. Normal day 2 responses also indicate that NK cell-derived IFN-γ production has occurred at that time. T cells are only required during the adaptive later phases of L.m. infection (reviewed in Ref. 22), and normal T cell function in the absence of PRLRs is suggested by the long-term survival of infected PRLR−/− mice. Finally, it should be emphasized that an important role for PRL in normal Con A-induced T cell proliferation is unlikely, because PRLR−/− lymphocytes respond to this mitogen in vitro similarly to control cells (Fig. 5).

The discrepancies between numerous previous reports (5) and the data presented in this study can probably be best accounted for by the differences between the experimental models. As stated above, hypophysectomized animals or bromocryptine-treated mice present pituitary deficiencies encompassing several endocrine pathways. In contrast, our KO model allows direct evaluation of the role of PRL in the immune system. In a similar manner, Montecino-Rodriguez et al. have shown that the B cell defects in Snell-Dwarf mice are most likely due to the absence of thyroid hormone (30), whereas previous reports had left undetermined the exact participation of other pituitary hormones in this defect (31, 32).

An alternative hypothesis for the absence of the immune system alterations in PRLR KO mice would be the existence of another receptor for PRL. Following this scenario, PRL signals would still be possible in the absence of the known PRLR, being transmitted through this alternative receptor. Possible candidates for this “second receptor model” include the growth hormone receptor (GHR), the placental lactogen (PL) receptor, or another as yet unidentified PRLR-like molecule. A role for GHR in transmitting PRL signals appears very unlikely, because in all species analyzed, the GHR is unable to bind PRL (33, 34). We are currently generating double KO animals, deficient for both GHRs and PRLRs, to further analyze the interactions between these two signaling systems. Although a potential PL binding site has been suggested (35, 36), a specific PL receptor has not been identified to date. Finally, despite efforts from several laboratories, experimental evidence for the existence of a second PRLR has not been obtained. The existence of another PRLR would also imply a differential regulation and expression in hemopoietic cells, but not in the mammary gland or the ovary and uterus, where deletion of the known PRLR gene leads to strong phenotypes (13).

As for most members of the cytokine receptor superfamily, the PRLR signaling cascade activates members of the JAK-STAT family. Still, cytokine specificity does not appear to be controlled by JAK-STAT molecules. For example, JAK2, which is activated by PRL, is also involved in signaling through a large number of other cytokine receptors, including the erythropoietin receptor, IFN-γR, IL-3R, GM-CSF receptor, IL-5R, thrombopoietin receptor, and GHR. Additional pathways have also been described for the PRLR, such as the ras/rad/mitogen-activated protein kinase pathway; however, these pathways are also not restricted to the PRLR (37, 38). The existence of these overlapping signaling mechanisms strongly suggests the occurrence of redundancy between members of the cytokine receptor superfamily. However, our previous observations using PRLR KO mice have shown that...
PRL is essential for reproduction and lactation, and that compensatory mechanisms do not take place in these systems (13). In contrast, our current data demonstrate that if PRL has an immunomodulatory role, its function is completely compensated. This is not the case for all cytokines, because recent reports have shown that deficiencies in IL-4 or IL-7 can have deleterious effects on the immune system (39, 40) without any possible compensation. Moreover, recent literature strongly suggests that JAK proteins have nonredundant roles during the induction of biological effects in response to specific cytokines (41). Finally, it would be tempting to speculate that the PRLR could signal using a pleiotropic system, in which the reproductive and lactating systems would rely on the JAK-STAT pathway, whereas alternative molecules would be recruited in immunocompetent cells. However, this hypothesis seems to contradict the body of literature defining the essential role of the JAK-STAT cascade, even in the lymphoid tissues (42, 43).

Initial in vitro analysis of the various cytokine/cytokine receptor systems had suggested that redundancy existed for this family of molecules, and their fine specificities remained unknown. The establishment of genetically defined in vivo models has allowed investigators to reassess the biological role of various cytokines and to better evaluate their physiological functions. Using our KO model, we have clearly established that PRL is not essential for the proper development and function of the mouse immune system.

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