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Progesterone via Its Receptor Antagonizes the Pro-Inflammatory Activity of Estrogen in the Mouse Uterus[1]

P_4 = Progesterone

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ABSTRACT

Populations of macrophages and neutrophils in the uterus are under the control of the female sex steroids estrogen and progesterone (P_4). Their influx is induced by estrogen, while P_4 can both stimulate and inhibit leukocyte influx depending on the timing of P_4 with respect to estrogen. Regulation of leukocytes has been implicated in changes in uterine immune responses during the estrous cycle, pregnancy, and implantation. This work demonstrates that P_4 given concurrently with estrogen to ovariectomized mice for 4 days antagonizes the ability of estrogen to recruit macrophages and neutrophils into the mouse uterus. Using progesterone receptor knockout (PRKO) mice, we show that this effect is dependent on progesterone receptors (PR). In the absence of PR, neutrophils recruited by estrogen were found to be degranulated, partially explaining the edema that is observed with long-term treatment of PRKO mice with estrogen and P_4. Populations of B lymphocyte cells were shown to be unchanged by estrogen and P_4 treatment in both wild-type and PRKO mice. The neutrophil chemotactic chemokine MIP-2 was examined for down-regulation by P_4 but was found to be unaffected by hormonal treatment. Together, these observations demonstrate that PR has a strong anti-inflammatory role in the mouse uterus when estrogen and P_4 are present together.

INTRODUCTION

Leukocytes, especially neutrophils and macrophages, form a significant but varying population of cells in the uterus of many species [1–5]. Numbers of these cells peak around estrus and reach their nadir around the time of diestrus [5]. Mating is also associated with a large influx of neutrophils and macrophages that closely resembles a pathological acute inflammatory response [2]. Decidualization and implantation, on the other hand, result in a loss of neutrophils, as well as a loss of macrophages from the vicinity of the embryo [3, 6–8]. This effect is thought to be due to the loss of uterine epithelium in the anti-mesometrial area of the uterus [3] concurrent with marginalization of macrophages by the proliferating decidual cells.

Although other factors have also been implicated, findings on the timing of leukocyte movements in the estrous cycle as well as studies involving artificial hormone manipulation have shown that estrogen and P_4 play central roles in recruitment and maintenance of leukocytes in the uterus [4, 5, 9]. Estrogen has pro-inflammatory effects in the uterus, where it causes an influx of neutrophils and macrophages, tissue edema, and proliferation of uterine epithelial cells [1, 9, 10]. At the same time, estrogen also causes a net induction of uterine progesterone receptors (PR), priming the uterus to respond to P_4. The increase in uterine PR is the combined result of an estrogen-dependent inhibition of epithelial PR expression and a redirection of PR expression to the stromal and myometrial compartments [11].

P_4, on the other hand, is classically thought to oppose the effects of estrogen in the uterus, where it blocks epithelial cell proliferation, induces proliferation of stromal cells, partially blocks the tissue edema caused by estrogen, and appears to have mixed effects on uterine leukocytes depending on the timing of administration with respect to estrogen [1, 12–15]. For example, treatment with P_4 followed by estrogen results in an increase in macrophage infiltration into the uterus over that observed with estrogen alone [1]. Examination of PR knockout (PRKO) mice has further shown that PR is required for nearly all aspects of female reproduction, including mammary gland development, sexual behavior, uterine decidualization, and ovulation (reviewed in [16]).

Initial studies in the PRKO mouse demonstrated that treatment with estrogen plus P_4 results in gross inflammation of the uterus that is not seen in the wild type [14], suggesting that P_4-liganded PR opposes the pro-inflammatory activity of estrogen in the mouse uterus. In this study we sought to define more carefully the anti-inflammatory role of PR in the mouse uterus by describing the effects of estrogen and P_4 treatment on the numbers and distribution of neutrophils, macrophages, and B lymphocytes. Comparison of wild-type and PRKO mice allows examination of the relative contributions of estrogen receptor and PR to uterine leukocyte numbers. Finally, expression of MIP-2, a chemokine capable of binding to the interleukin (IL)-8 receptor and attracting macrophages and neutrophils, is examined for estrogen and P_4 regulation.

MATERIALS AND METHODS

Reagents

Purified rat anti-mouse F4/80 antigen monoclonal antibody (clone A3–1) was purchased from Serotec (Oxford, England) [17]. Purified rat anti-mouse CD45R/B220 monoclonal antibody (clone RA3–6B2) [18], biotinylated goat anti-rat polyclonal antibody (cat. no. 12112D), and streptavidin-conjugated horseradish peroxidase were purchased from Pharmingen (San Diego, CA). Purified rat anti-mouse neutrophil monoclonal antibody (clone 7/4) [17] was purchased from Biosource (Camarillo, CA). Purified goat anti-mouse MIP-2 polyclonal antibody (cat. no. AF-452-NA) was purchased from R&D Systems (Minneapolis, MN). Biotinylated rabbit anti-goat polyclonal antibody (cat. no. BA-5000) was purchased from Vector Laboratories (Burlingame, CA). Polyclonal rabbit anti-lactoferrin antibody was acquired from Dr. Christina Teng (NIEHS, Research Triangle Park, North Carolina). Biotinylated goat anti-rabbit IgG (cat. no. BA-1000) was purchased from Vector Labs.

Goat serum was purchased from Gibco-BRL (Grand Island, NY) and heat inactivated at 56°C for 30 min before

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TABLE 1. Leukocyte numbers as percentages of total cells in the uterus following hormonal treatment.[a]

	Neutrophils			Macrophages			B cells		
Treatment	Control	Estrogen	E+P	Control	Estrogen	E+P	Control	Estrogen	E+P
Wild type	1 ± 0.3%	15 ± 2%	2 ± 0.6%	1 ± 0.3%	18 ± 3%	3 ± 1%	<1%	<1%	<1%
PRKO	1 ± 0.3%	16 ± 2%	14 ± 2%	2 ± 0.5%	19 ± 0.3%	20 ± 3%	<1%	<1%	<1%

[a] Mean ± SE (n = 3 animals).

use. Nonimmune rat and rabbit sera were purchased from Vector Labs. Trypsin-EDTA (0.05% trypsin in 0.54 mM EDTA) was purchased from Gibco-BRL. Diaminobenzidine (DAB) peroxidase substrate (Sigma Fast DAB tablet sets), P_4, E_2 (estradiol-17β), and sesame oil were purchased from Sigma Chemical Co. (St. Louis, MO). E_2 was dissolved in ethanol, then diluted in sesame oil to a concentration of 20 ng/μl. P_4 was dissolved directly in sesame oil at 55°C for 1 h at a concentration of 20 μg/μl. Histo-Clear was obtained from National Diagnostics (Atlanta, GA). Antigen Retrieval Citra was purchased from BioGenex (San Ramon, CA).

Animals

All mice were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. PRKO mice were generated in our laboratory as described previously [14]. Wild-type and PRKO mice were housed in the animal facility at Baylor College of Medicine (Houston, TX).

Hormone Treatments

Female wild-type and PRKO 8-wk-old mice were ovariectomized and then rested for 7 days. Mice were injected s.c. with 50 μl of sesame oil containing no hormone, 1 μg E_2, or 1 μg E_2 + 1 mg P_4 (E+P) daily for 4 days. Three mice were included in each treatment group. Mice were killed at 24 h after the final injection, and uteri were isolated. We have previously used these treatment conditions to demonstrate a significant anti-inflammatory activity of P_4 in the mouse uterus that is PR independent [14] and to localize changes in uterine PR that occur after E_2 and P_4 treatment [11].

Immunohistochemistry

Uteri were fixed in Bouin's fixative at room temperature for 7 h; they were then washed in 70% ethanol at room temperature overnight. After fixation, tissue was dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin for sectioning; 5-μm sections were cut and mounted on poly-L-lysine slides (Cel Tek Inc., Glenview, IL). Sections were deparaffinized in Histo-Clear and rehydrated in graded ethanol solutions. Peroxidase quenching was performed with 3% H_2O_2 in methanol for 10 min. For F4/80 staining, sections were incubated for 30 min with trypsin-EDTA at 37°C. All subsequent steps were carried out at room temperature. Sections were blocked with 10% goat serum in PBS for 30 min, followed by incubation with the primary antibody for 4 h. Sections were incubated without primary antibody or with nonimmune rabbit serum or rat serum as a control. Sections were then washed in PBS for 20 min followed by incubation with the biotinylated secondary antibody for 1 h. After a 10-min wash in PBS, sections were incubated for 30 min with streptavidin-conjugated peroxidase diluted 1:500 in PBS for 30 min. After a 10-min wash in PBS, the localization of the primary antibody was visualized with the imidazole-DAB reaction for 3–10 min, producing a brown-colored stain. Sections were then counterstained in hematoxylin, dehydrated through graded ethanol solutions, cleared in xylene, and mounted with Permount (Fisher Scientific, Pittsburgh, PA) for bright-field microscopy. Images were acquired digitally using a Zeiss Axioskop microscope (Carl Zeiss Inc., Thornwood, NY) coupled with a Hamamatsu C5810 CCD camera (Hamamatsu Corporation, Bridgewater, NJ) and were printed using a Codonics NP-1600 dye-sublimation printer (Condonics, Middleburg Heights, OH).

Cell Counting

The number of positively stained cells in a random field of 1000 cells was counted. The average number of positive cells was obtained by counting three separate fields per section. In the case of the macrophage stain, trypsin treatment rendered unstained cells difficult to distinguish. Thus, Image Tool software (UTHSCSA, San Antonio, TX, ftp://maxrad6.uthsca.edu) was used to calculate the total area of each field and then to count the number of positively stained cells in the field. Similar sections from the same mouse were also counted to determine the total number of cells per unit area. These two figures then allowed calculation of the number of positive cells as a percentage of total cells. Representative sections from each uterus were used in these studies; p values were calculated based on a one-tailed paired Student's t distribution.

RESULTS

Regulation of Uterine Leukocyte Populations by E_2 and P_4

To examine the effects of E_2 and P_4 on uterine leukocyte populations, 8-wk-old wild-type and PRKO mice were ovariectomized to eliminate endogenous hormones and then treated daily for 4 days with vehicle, E_2 alone, or E_2 and P_4 (E+P). Uteri were removed after 4 days, and 5-μm sections were analyzed immunohistochemically using anti-F4/80 (macrophage), anti-neutrophil, or anti-CD45R/B220 (B cell) antibodies (see *Materials and Methods*). The results of these analyses are shown in Figure 1 (neutrophils), Figure 2 (macrophages), and Figure 3 (B cells); quantitation of positively stained cells is summarized in Table 1.

Neutrophils were not present in any significant numbers in untreated ovariectomized wild-type or PRKO mice (Fig. 1, a and d; Table 1). Estrogen treatment resulted in a significant increase ($p < 0.001$) in neutrophil numbers that was observed equally in both wild-type and PRKO mice (Fig. 1, a vs. b, and d vs. e; Table 1). Neutrophil staining was predominantly localized within the stroma and myometrium. In addition, neutrophils were often seen traversing the uterine epithelium in PRKO mice and were occasionally found within the uterine lumen. Neutrophils were not seen

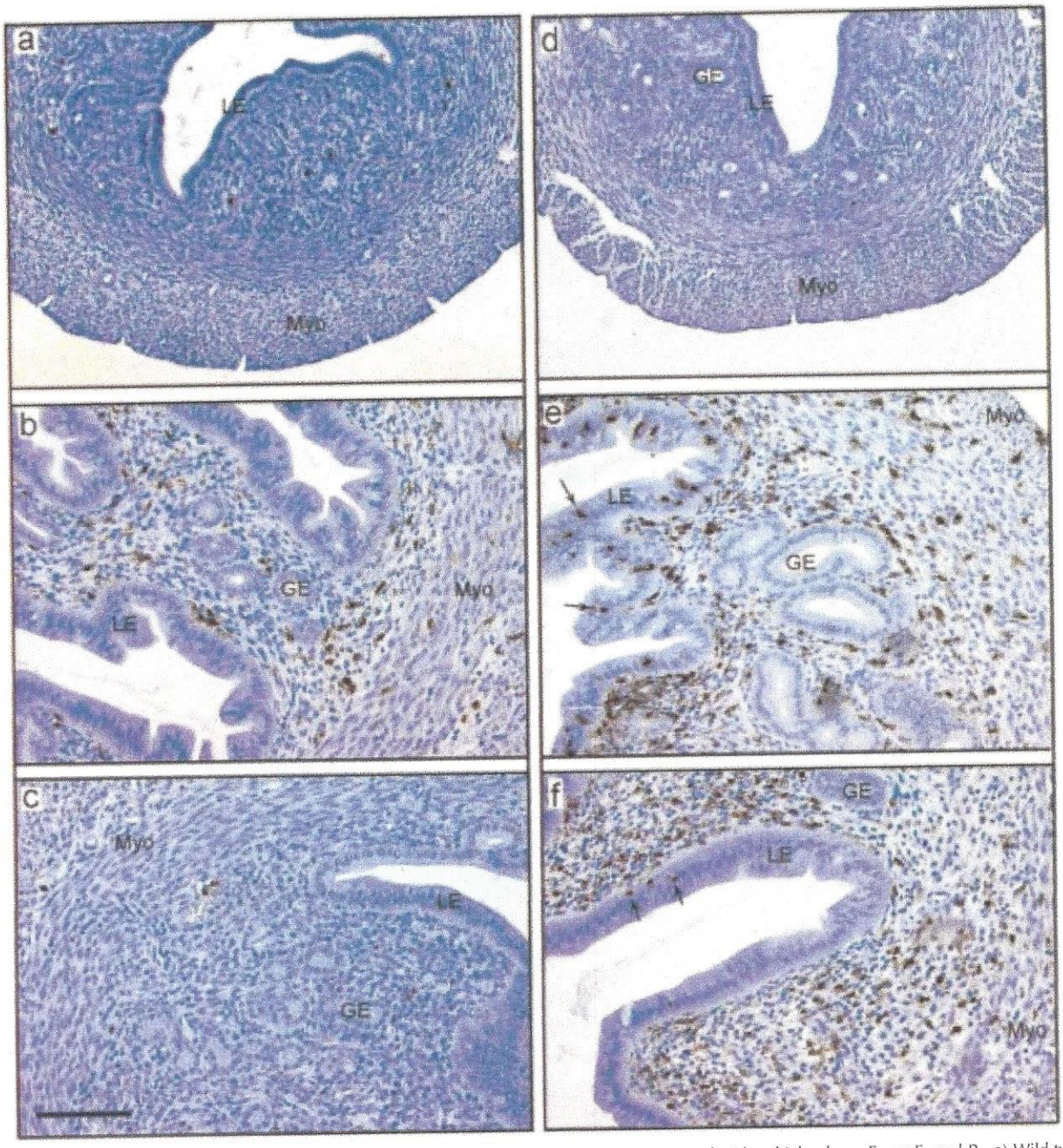

FIG. 1. Immunohistochemistry for neutrophils in the uterus of wild-type and PRKO mice treated with vehicle alone, E_2, or E_2 and P_4. **a**) Wild-type untreated; **b**) wild-type E_2 treated; **c**) wild-type E_2 and P_4 treated; **d**) PRKO untreated; **e**) PRKO E_2 treated; **f**) PRKO E_2 and P_4 treated. Bar = 100 μm. Arrows point to neutrophils migrating through the luminal epithelium. LE, Luminal epithelium; GE, glandular epithelium; Myo, myometrium.

traversing the epithelium or in the lumen of wild-type uteri. Treatment of mice with E+P resulted in a loss of neutrophil staining in wild-type animals, demonstrating that P_4 has a negative effect on estrogen-induced neutrophil immigration into the uterine stroma (Fig. 1c). PRKO mice, on the other hand, continued to have significantly larger numbers of uterine neutrophils than wild-type mice ($p < 0.001$) when treated with E+P (Fig. 1f; Table 1). This demonstrates that the negative effects of P_4 on neutrophil immigration into the uterus are mediated through the classical PR.

Macrophages were found to compose around 1% of the total cells in untreated wild-type and PRKO uterus (Fig. 2, a and d). Estrogen treatment resulted in a significant increase in macrophage numbers ($p < 0.001$) within the stromal and myometrial compartments, similar to that seen with neutrophils (Fig. 2, b and e; Table 1). In contrast to neutrophils, however, macrophages were not found penetrating the uterine epithelium, and no macrophage staining was noted within the uterine lumen. The increase in macrophage numbers was blocked by concurrent treatment with P_4 in

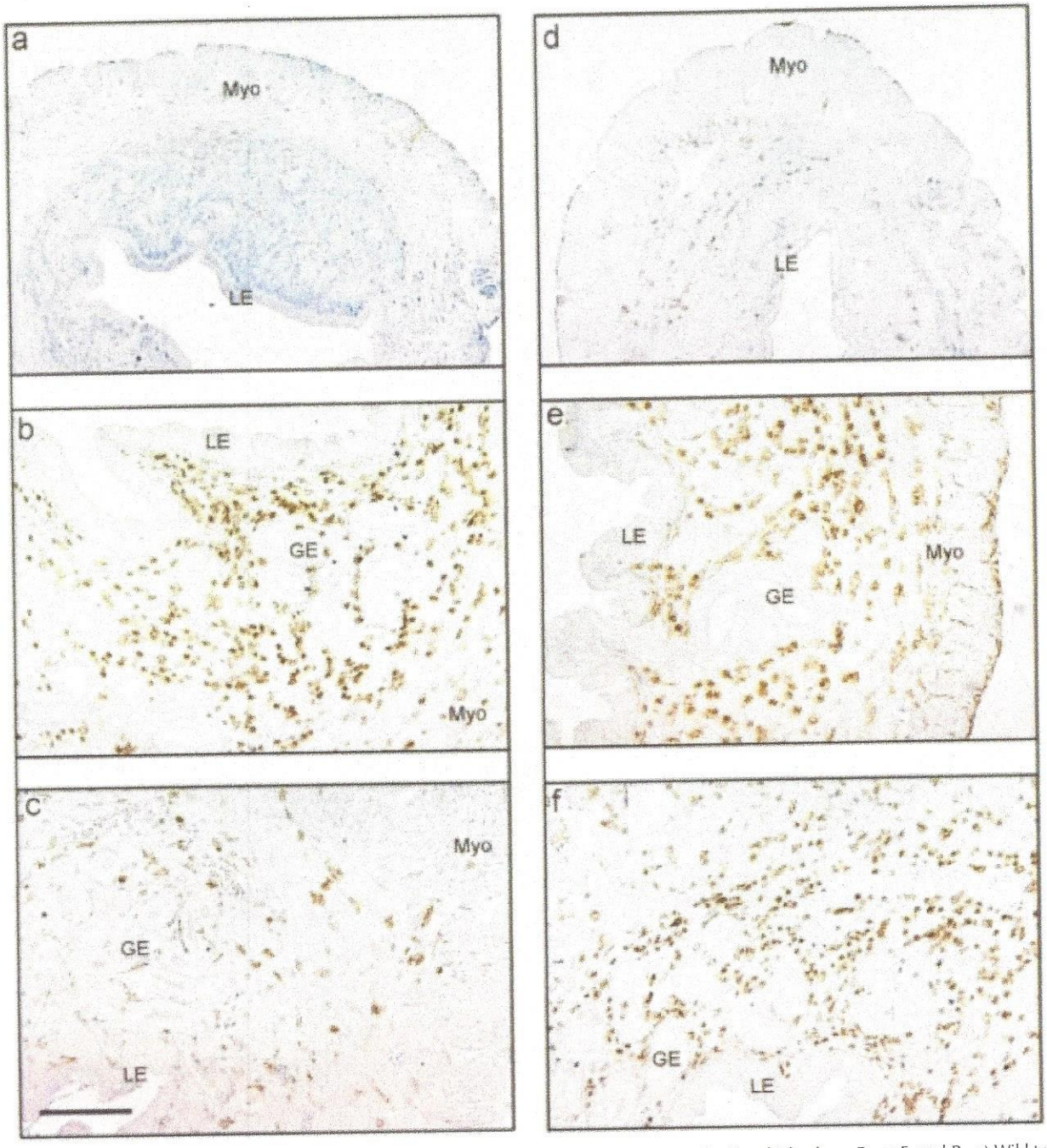

FIG. 2. Immunohistochemistry for macrophages in the uterus of wild-type and PRKO mice treated with vehicle alone, E_2, or E_2 and P_4. **a**) Wild-type untreated; **b**) wild-type E_2 treated; **c**) wild-type E_2 and P_4 treated; **d**) PRKO untreated; **e**) PRKO E_2 treated; **f**) PRKO E_2 and P_4 treated. Bar = 100 μm. LE, Luminal epithelium; GE, glandular epithelium; Myo, myometrium.

the wild-type uterus but not in the PRKO uterus (Fig. 2, c vs. d), again demonstrating a significant ($p < 0.001$) PR-dependent anti-inflammatory effect of P_4 in the uterus.

Staining for B lymphocytes demonstrated no significant changes between treatment groups, with all animals showing fewer than 1% of uterine cells staining positively for CD45R/B220 (Fig. 3). Thus, E+P treatment did not change B cell numbers as it did neutrophils and macrophages.

Activation of Invading Neutrophils

Previous studies in PRKO mice [14] have shown that estrogen treatment results in a gross inflammatory phenotype, including hyperemia and tissue edema. To determine whether this phenotype is associated with degranulation of the invading neutrophil population, neutrophil staining was compared with staining for lactoferrin (Fig. 4), a major con-

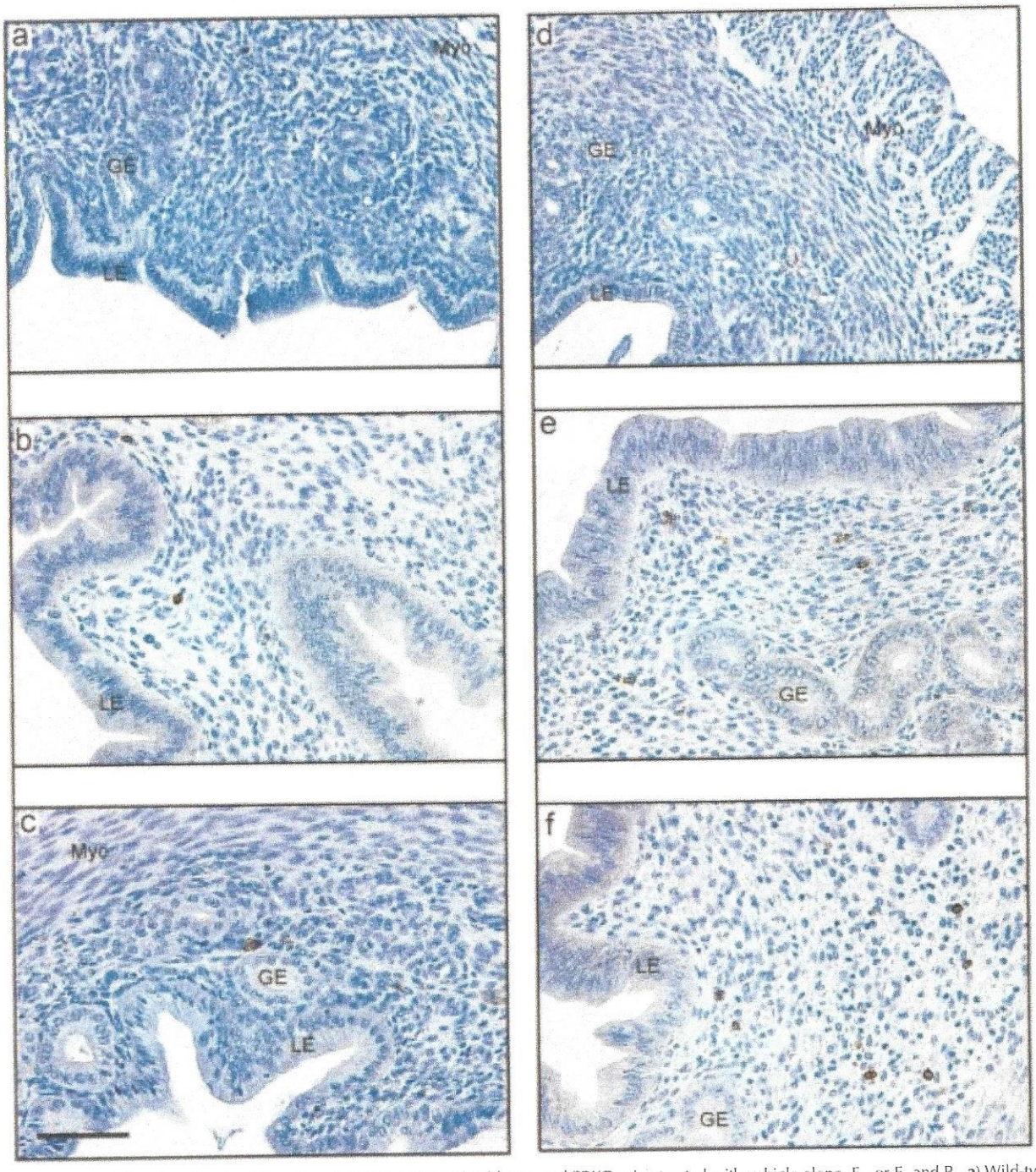

FIG. 3. Immunohistochemistry for B lymphocytes in the uterus of wild-type and PRKO mice treated with vehicle alone, E_2, or E_2 and P_4. **a**) Wild-type untreated; **b**) wild-type E_2 treated; **c**) wild-type E_2 and P_4 treated; **d**) PRKO untreated; **e**) PRKO E_2 treated; **f**) PRKO E_2 and P_4 treated. Bar = 100 μm. LE, Luminal epithelium; GE, glandular epithelium; Myo, myometrium.

stituent of neutrophil secondary granules [19, 20]. Although numerous neutrophils were present beneath the epithelium of estrogen-treated mice, no lactoferrin staining was observed within the stromal compartment, demonstrating a lack of secondary granules in the neutrophils. Lactoferrin staining of neutrophils in the neonatal spleen was included as a positive control (Fig. 4b, inset).

MIP-2 Expression Was Not Regulated by E_2 and P_4

The expression of the neutrophil-chemotactic chemokine MIP-2 was examined by immunohistochemistry in the uterus for its potential regulation by estrogen and P_4. MIP-2 expression was found to be localized to a very small number of cells (about four to five per cross section of the

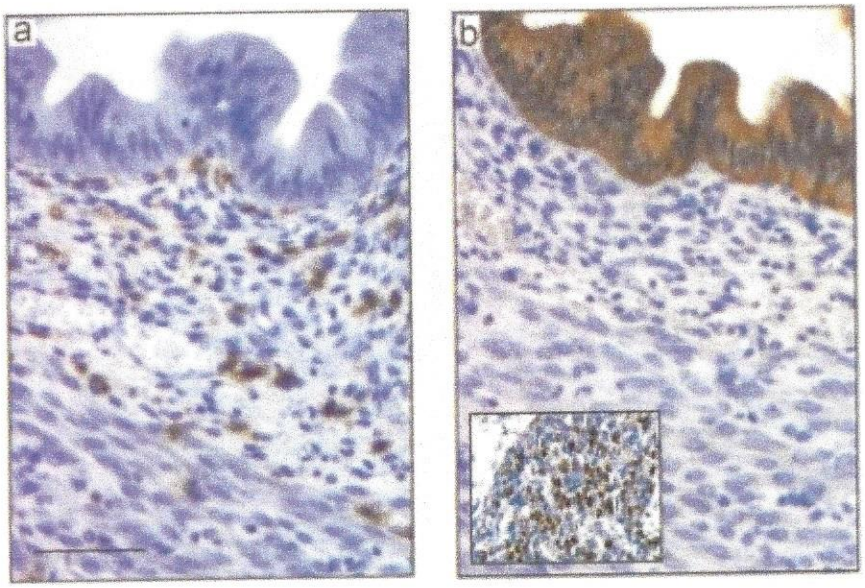

FIG. 4. Immunohistochemistry for neutrophils (**a**) and lactoferrin (**b**) within the same E_2-treated PRKO uterus. Note the large number of neutrophils present in this area of the uterus and the lack of lactoferrin staining, demonstrating the absence of secondary granules. Lactoferrin staining of the luminal epithelium serves as an internal positive control. Inset demonstrates neutrophil lactoferrin staining in the neonatal spleen under similar conditions as for an external positive control. Bar = 50 μm.

uterus) within the myometrium of estrogen-treated animals (data not shown). This pattern of expression was unchanged with the addition of P_4. Thus, on the basis of its scarcity of expression and lack of regulation by P_4, it does not appear that MIP-2 is downstream of estrogen and P_4 in regulating neutrophil numbers in the uterus.

DISCUSSION

The massive inflammation seen with E+P treatment in the PRKO uterus prompted us to examine more closely the regulation of leukocyte migration into the mouse uterus by estrogen and P_4. Our results show that estrogen treatment results in a massive influx of neutrophils and macrophages while numbers of B lymphocytes in the uterus are unaffected, demonstrating that the inflammation seen in the PRKO uterus is qualitatively similar to that seen and previously described in the wild-type mouse with estrogen treatment [1, 2]. Addition of P_4 antagonized the effects of estrogen on macrophage and neutrophil immigration, resulting in much lower numbers of these cells entering the uterus than seen with estrogen treatment alone. This effect was seen only in the wild type, demonstrating that it is PR dependent.

Pregnancy is associated with a shift toward humoral immunity and increased antibody production, and specific antibody production by B cells could be important to normal maintenance of the placental barrier [21–24]. In addition, estrogen is known to facilitate the secretion of IgA into the uterine lumen, while P_4 is known to inhibit this process [25–27]. We find, however, that B cell numbers in the uterus are unaffected by estrogen and P_4 treatment. This demonstrates that estrogen-induced uterine inflammation is specific to certain types of leukocytes as expected, and implies that B cells are activated during pregnancy to produce antitrophoblast antibodies in some other location, presumably the draining lymph nodes. This agrees with previous studies that have shown an increase in uterine draining lymph node size during pregnancy [28].

Estrogen induction of macrophage immigration into the mouse uterus has been previously observed [1] and was seen in this study as expected. P_4 treatment has also been reported to cause an influx of macrophages, with sequential P_4 followed by estrogen reported to result in an even larger number of uterine macrophages than seen with estrogen alone [1]. Our results, however, show that concurrent treatment of mice with E+P results in a PR-dependent antagonism of the ability of estrogen to attract macrophages into the uterus. This can be reconciled by considering the localization of uterine PR under each of these regimens. In the absence of estrogen, PR is localized to the uterine luminal epithelium [11]. After estrogen treatment, however, PR expression is turned off in the luminal epithelium and is strongly up-regulated in the stroma [11]. Thus, it is possible that the differing localization of PR following estrogen treatment could account for the differing effects of P_4 treatment on macrophage numbers in the uterus. That is, activation of stromal PR might antagonize the effects of estrogen on leukocyte migration while activation of luminal epithelial PR could potentiate those effects.

The reaction of the uterus to implantation has been said to resemble an inflammatory response, with local production of a number of inflammatory mediators and resultant influx of neutrophils [29–32]. Neutrophil numbers in the mouse uterus are high around the time of implantation, a time when P_4 levels are high. Subsequent studies showed that systemic depletion of neutrophils had no discernible effect on implantation, leaving the importance of neutrophil infiltration into the deciduum unclear [32]. In the current study, regulation of neutrophil accumulation in the uterus paralleled that of macrophages, with estrogen having a positive effect on uterine neutrophil numbers and P_4 having a PR-dependent negative effect. This leads to the question of why, if P_4-liganded PR inhibits neutrophil immigration into the uterus, neutrophil numbers are high around the time of implantation. It is possible that inflammation could in some ways be a harmful by-product of implantation that is curbed by the activity of liganded PR. In the absence of implantation, as in our experiments, the activation of PR is sufficient to completely prevent the estrogen-dependant influx of neutrophils; but in the presence of implantation it might serve only to curb the inflammation, and neutrophils could still be observed. That is, the estrogen-dependent implan-

tation event might result in uterine inflammation that is partially restrained by the anti-inflammatory activity of P_4, whereas in the absence of implantation, P_4 is capable of fully preventing estrogen-induced inflammation. The idea that PR restrains the pro-inflammatory effects of estrogen is supported by the observation that PRKO mice show gross uterine inflammation with massive fluid accumulation when treated with hormone combinations that are known to prime the uterus for implantation and decidualization [14].

The fact that neutrophils within the uterus did not stain positively for lactoferrin implies that activation and degranulation had already occurred. This further implicates neutrophils as being responsible for the gross inflammation and edema that are seen in PRKO mice following E+P treatment and further highlights the importance of PR as an anti-inflammatory agent in the uterus.

It has been previously observed that neutrophils actively cross the uterine epithelium in response to semen, whereas macrophages do not [33]. Although the presence of transforming growth factor β1 in the semen is thought to be responsible for stimulating inflammatory cell accumulation in the uterus [34], it has been hypothesized that there is a granulocyte-specific chemokine in the semen that is capable of mediating the differential effect of semen on neutrophils [33]. Although our study does not rule out the possibility of such a factor in the semen, it does demonstrate that estrogen treatment alone is sufficient to cause migration of neutrophils into the uterine lumen in PRKO mice, implying that neutrophil migration is at least partially controlled by an epithelial-derived factor. Further, it appears that production of this factor can be negatively affected by the presence of PR even in the absence of P_4, based on the differences we have observed in wild-type and PRKO mice after treatment with estrogen alone.

Although factors important in regulation of uterine macrophage populations have been extensively studied (reviewed in [33]), factors involved in regulating neutrophil immigration have received less attention. It has been shown that IL-8 expression in the rabbit uterus is down-regulated by P_4 [35], suggesting that this chemokine could be one of the targets for the anti-inflammatory activity of P_4. Unfortunately, there is no known mouse homologue to IL-8. Mice do, however, express a similar chemokine, known as MIP-2, that has been shown to bind to the IL-8 receptor on neutrophils and mediate similar functionality [36]. Examination of MIP-2 expression, however, showed only a very small number of positive cells per uterine cross section and no significant changes in expression following treatment with estrogen or P_4. This suggests that MIP-2 is not downstream of estrogen and P_4 in the regulation of uterine neutrophil immigration, although possible alterations in receptor levels or bioactivity prevent this from being completely ruled out.

Previous reports have shown that treatment with P_4 results in increased susceptibility to uterine infection and decreased resistance to intrauterine transplantation [37–39]. In this report, we demonstrate that PR is anti-inflammatory in the uterus when P_4 is given in concert with estrogen, resulting in decreased numbers of neutrophils and macrophages in the uterus. Based on these observations, PR could play a major role in modulating the immune status of the uterus in preparation for implantation.

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