Progesterone promotes immunomodulation and tumor development in the murine mammary gland

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ABSTRACT

Background Clinical studies have linked usage of progestins (synthetic progesterone [P4]) to breast cancer risk. However, little is understood regarding the role of native P4, signaling through the progesterone receptor (PR), in breast tumor formation. Recently, we reported a link between PR and immune signaling pathways, showing that P4/PR can repress type I interferon signaling pathways. Given these findings, we sought to investigate whether P4/PR drive immunomodulation in the mammary gland and promote tumor formation.

Methods To determine the effect of P4 on immune cell populations in the murine mammary gland, mice were treated with P4 or placebo pellets for 21 days. Immune cell populations in the mammary gland, spleen, and inguinal lymph nodes were subsequently analyzed by flow cytometry. To assess the effect of PR overexpression on mammary gland tumor development as well as immune cell populations in the mammary gland, a transgenic mouse model was used in which PR was overexpressed throughout the entire mouse. Immune cell populations were assessed in the mammary glands, spleens, and inguinal lymph nodes of 6-month-old transgenic and control mice by flow cytometry. Transgenic mice were also monitored for mammary gland tumor development over a 2-year time span. Following development of mammary gland tumors, immune cell populations in the tumors and spleens of transgenic and control mice were analyzed by flow cytometry.

Results We found that mice treated with P4 exhibited changes in the mammary gland indicative of an inhibited immune response compared with placebo-treated mice. Furthermore, transgenic mice with PR overexpression demonstrated decreased numbers of immune cell populations in their mammary glands, lymph nodes, and spleens. On long-term monitoring, we determined that multiparous PR-overexpressing mice developed significantly more mammary gland tumors than control mice. Additionally, tumors from PR-overexpressing mice contained fewer infiltrating immune cells. Finally, RNA sequencing analysis of tumor samples revealed that immune-related gene signatures were lower in tumors from PR-overexpressing mice as compared with control mice.

Conclusion Together, these findings offer a novel mechanism of P4-driven mammary gland tumor development and provide rationale in investigating the usage of antiprogestin therapies to promote immune-mediated elimination of mammary gland tumors.

BACKGROUND

Breast cancer is the most common cancer occurring in women in the USA and is currently the second most common cause of cancer-related death in women.[1 2] Of the four molecular subtypes of breast cancer, the majority are of the luminal A subtype, defined by estrogen receptor (ER) and/or progesterone receptor (PR) status and lack of human epidermal growth factor receptor 2 (HER2) amplification.[1] While ER's role in promoting the development of hormone receptor (HR)-positive breast cancers has been studied extensively,[3–6] the roles that PR and progesterone (P4) play are not completely understood. Importantly, however, numerous studies have suggested that PR and progestins (synthetic P4) play an important role in breast cancer development and growth. A cohort study published in 2000 demonstrated that postmenopausal women treated with a hormone replacement therapy regimen containing estrogen plus progestin had an increased risk of developing breast cancer when compared with women treated with estrogen alone.[7–9] In addition, breast cancer risk has been shown to increase just

after pregnancy, when circulating P4 levels are highest compared with any other time in a woman's life.[1] Use of hormonal birth control, most of which are progestin-based, has also been associated with a slightly increased risk of developing breast cancer.[1 10]

In the past 10 years, cancer immunotherapies, such as immune checkpoint inhibitors (ICIs), have demonstrated success in treating multiple types of cancer.[11] Despite these successes, responses to ICIs in breast cancer have been modest, with triple negative breast cancers (ie, those lacking expression of ER, PR, and HER2) demonstrating the best response rates of any subtype.[11–13] Results from trials investigating ICI therapy in HR+ breast cancer have been disappointing, with overall response rates ranging from 5% to 12%.[14 15] HR+ breast cancers (ie, luminal A) lack immunogenicity due a low mutation burden,[16] making these cancers less responsive to ICI treatment.[17–19] Additionally, luminal breast tumors lack immune cell infiltration, a negative predictor for response to ICI therapy.[20–22]

P4 is a steroid hormone that serves many functions, one of which is to suppress the immune system at various stages of human development and aging, especially during pregnancy. PR is expressed in reproductive organs as well as in multiple immune cell subtypes in humans.[23–28] Numerous studies have demonstrated P4's ability to suppress cells of the innate and adaptive immune systems in the context of pregnancy or with P4 administered at pregnancy-level concentrations. In these studies, P4 has been shown to decrease the activity of natural killer (NK) cells,[24] macrophages (Mφs),[29–31] dendritic cells,[31–34] CD4+ and CD8+ T cells,[35–41] likely in an effort to maintain maternal tolerance towards the developing fetus.[42] P4 has also been shown to increase numbers of regulatory T cells (Tregs) in pregnant mice, contributing towards the immunosuppression needed during pregnancy.[35 37–40 42] Though these studies have demonstrated the ability of P4 (pregnancy-level concentrations) and PR to suppress bone-marrow derived and splenic immune cells, no one has yet examined whether P4 has similar immune-modulatory effects in mammary tissues.

The immunomodulatory effects of P4 and PR have been shown to play a role in regulating several pathological and physiological processes, including infection and pregnancy. Numerous studies have implicated P4 and progestins with increased susceptibility to several bacterial and viral infections, including influenza A, HIV, and herpes simplex virus type 1.[28 43–51] Similar immunomodulatory changes are seen during pregnancy in mice, which have been demonstrated to protect against pregnancy loss and preterm labor.[37 52–56]

Given P4's role in suppressing the immune response during pregnancy and increasing susceptibility to viral and bacterial infections, we questioned whether P4 might have immune modulatory effects in the murine mammary gland and whether this contributes to the development of mammary tumors. Herein, we provide the first evidence in murine models that both the presence of P4 and the overexpression of PR promote an immunosuppressed microenvironment in the mammary gland and lead to increased development of mammary gland tumors. These findings establish a rationale to further investigate whether targeting PR in patients with breast cancer can increase antitumor immune responses, either alone or in combination with immunotherapies.

METHODS

Antibodies and reagents

Antibodies used for flow cytometry are listed in online supplemental table 1. For western blots, a PR antibody that detects mouse PR was used (Cell Signaling, cat #8757).

Immunohistochemistry (IHC)

A PR antibody (Cell Signaling #8757) was used for immunohistochemical staining according to the following procedure: 4-micron paraffin sections were mounted on Tanner adhesive slides and baked for 60 min at 60°C then deparaffinized. Epitope retrieval was performed in Biocare Decloaking Chamber (pressure cooker), under pressure for 5 min, using pH 6.0 citrate buffer followed by a 10 min cool-down period. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min followed by incubation with PR (1:500) primary antibody for 30 min (PR), followed by Mach 2 HRP Polymer (Biocare Medical) for 30 min and DAB+chromogen (Dako) for 5 min. IHC staining was performed using the IntelliPATH FLX Automated Stainer at room temperature. A light hematoxylin counterstain was performed, following which the slides were dehydrated, cleared, and mounted using permanent mounting media. All IHC images were captured on a microscope at ×40 magnification. Scoring of IHC slides was performed by a board-certified pathologist specializing in breast tissues.

Exogenous P4 treatment study

For P4 treatment studies, 12-week-old ovariectomized FVB/n mice were purchased from the Jackson Laboratory (strain #001800). At 13–15 weeks of age, P4 or placebo pellets (30 mg/30-day release, Innovative Research of America) were surgically implanted subcutaneously in the necks of the mice. Twenty-one days later, mice were sacrificed, and mammary glands were isolated for tissue digestion and subsequent immunophenotyping via flow cytometry.

PR transgenic mouse model

The PR transgenic mouse model, initially created by Shyamala et al,[57] was resurrected from frozen embryos deposited at the Mutant Mouse Resource and Research Center (strain 032089-UCD) by the Lange and Hagan Labs.[57] For immunophenotyping of mammary glands, inguinal lymph nodes, and spleens in 6-month-old transgenic mice and controls, mice were bred and aged for 6 months before they were sacrificed for tissue collection.

For long-term tumor studies, mice were bred and aged for approximately 2 years. During the aging period, mice were bred three times to mimic normal steroid hormone levels during cycling and pregnancy. Only mice that became pregnant three times were included in subsequent analyses. Mice developed tumors at an average age of 23 months, and animals that developed tumors were sacrificed when tumors reached 15 mm in length or width or when required due to other health conditions. On sacrifice, tumors were collected and sectioned for immunophenotyping by flow cytometry, IHC, and RNA quantitation. Whole spleens were harvested at the time of sacrifice for immunophenotyping via flow cytometry.

Mammary gland whole mounts
Whole mounts of mammary glands (#3 or #4) were stained with carmine-red as described previously[58–60].

Flow cytometry
Tissue digestion of tumors for flow cytometry
After sacrificing the mice, tumors were excised and one-fourth of each tumor sample was taken for tissue digestion for flow cytometry. Each tumor sample was processed as outlined in Irey et al.[61] Briefly, tumor samples were minced using surgical scissors, and the resulting tumor slurry was suspended in 10 mL of 1× phosphate-buffered saline (PBS) and incubated on ice. Liberase (Roche) and DNAse I (Alfa Aesar) were added to the tumor slurry and samples were then incubated on a shaker at 37°C and were rotated at 80 rpm for 30 min. Tumor samples were spun down for 5 min at 200 G and the supernatant was aspirated. The samples were then resuspended in 10 mL of DMEM (Dulbecco's Modified Eagle Medium) with 10% fetal bovine serum (FBS) and were passed through a 70-micron cell strainer. The tumor cells were then pelleted, supernatant was aspirated, and 2–5 mL of ACK Red Blood Cell Lysis Buffer (Gibco) was added to each sample. Samples were then vortexed and incubated at room temperature for 5 min. PBS (1×) was added to quench the reaction and samples were spun down at 200 G for 5 min. Tumor cells were suspended in 5 mL of 1× PBS containing 0.5% FBS and 1 mM EDTA and were transferred into 5 mL round-bottom glass tubes. Cell counting and staining were then performed.

Tissue digestion of mammary glands, inguinal lymph nodes, and spleens for flow cytometry
After sacrificing mice, the inguinal lymph nodes, fourth mammary glands, and spleens were excised and placed in Hanks balanced salt solution (HBSS, Gibco). Each organ was processed as defined by Betts et al.[62] Briefly, organs were minced over ice using surgical scissors. Collagenase type II and collagenase type IV were added to the minced organs as well as DNAse I. Tissues were digested on a shaker at 37°C for 30 min rotating at 150 rpm. EDTA (20 mM) in PBS was then added to quench the reaction. The organ slurries were filtered through a 70-micron cell strainer and were washed with HBSS. The samples were

centrifuged at 500 G for 5 min and the supernatant was aspirated. Pellets were suspended in ACK Lysing Buffer (Gibco) and were incubated at room temperature for 5 min. HBSS was added to quench the reaction, and cells were pelleted by centrifugation. Cells were suspended in 5 mL of 1× PBS containing 0.5% FBS and 1 mM EDTA and transferred into 5 mL round-bottom glass tubes. Cell counting and staining were subsequently performed.

Staining for immunophenotyping

RNA sequencing
Library generation and sequencing
RNA (1 µg) was used for cDNA library construction at Novogene using an NEBNext Ultra RNA Library Prep Kit for Illumina (cat# E7420S; New England Biolabs, Ipswich, Massachusetts, USA) according to the manufacturer's protocol. Briefly, mRNA was enriched using oligo(dT) beads, which was followed by two rounds of purification, and was fragmented randomly by adding fragmentation buffer. The first-strand cDNA was synthesized using random hexamers primer, after which a custom second-strand synthesis buffer (Illumina), dNTPs, RNase H and DNA polymerase I were added to generate the second strand (ds cDNA). After a series of terminal repair, poly-adenylation, and sequencing adaptor ligation, the double-stranded cDNA library was completed following size selection and PCR enrichment. The resulting 250–350 bp insert libraries were quantified using a Qubit V.2.0 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and quantitative PCR. Size distribution was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Qualified libraries were sequenced on an Illumina Nova 6000 Platform using a paired-end 150 run (2×150 bases). The required reads were generated from each library.

Analysis: Gene Ontology (GO) and Gene Set Enrichment Analysis (GSEA)
RNA sequences were aligned against mouse genome mm10 by STAR V.2.7.3, and RNA expression was calculated by RNA-sequencing (RNA-Seq) by expectation maximization. The differentially expressed genes were found by using DESeq2 to compare RNA expression between controls and PR-overexpressing samples. Based on the statistics from the DESeq2 analysis, the significant HallMark pathways and GO pathways were found by GSEA.[63]

Analysis: subtype analysis
For the subtype analysis, the human PAM50 data and the mouse RNA-Seq data were merged by 47 homolog genes. The merged data were normalized by using the R limma package, and the batch effects between PAM50 and mouse data were removed by using SVA package. The mouse samples were assigned into the PAM50 subtypes by using support vector machine (e1071 package).

Analysis: CIBERSORT (Cell Type Identifcation by Estimating Relative Subsets of RNA Transcripts)

Immune cell populations were inferred using CIBER-SORTx.[64] The ImmuCC murine signature matrix was used as a reference.[65] CIBERSORTx was run in absolute mode with 1000 permutations with quantile normalization disabled. Statistics and plotting were done in R.

Cell lines and treatments

E0771 (isolated and modified as in Sugiura and Stock,[66] Crosby *et al*,[67] Johnstone *et al*,[68] and Dunham and Stewart[69]) were maintained in DMEM with 10% FBS. E0771-vec and E0771-mPR cell lines were generated through lentiviral transduction using mouse progesterone receptor (mPR) or empty control vectors using pLenti-CMV-Hygro (Addgene# 17454, cloning details available on request) backbone. Stably transduced cell lines were selected using hygromycin (250 µg/mL). Cells were treated with the following reagents where indicated: R5020 (10 nM, Sigma).

Western blot analysis

Western blot analysis was performed as previously described[70 71] using a PR antibody that detects mPR (Cell Signaling, cat #8757).

Luciferase assays

E0771 cell line variants were transiently transfected with a PRE-luciferase reporter construct (described in Faivre and Lange[72]) and pRL-TK, a *Renilla* luciferase construct used for normalizing transfection efficiency. Forty-eight hours following transfection, cells were treated with 10 nM R5020 or EtOH control for 18 hours. Luciferase assays were performed as previously described[72] using the Dual-Luciferase Reporter Assay (Promega). Relative luciferase units were normalized to *Renilla*±SD.

Quantitative RT-PCR

RNA isolation, cDNA generation, and qRT-PCR were performed as previously described.[70 71]

E0771 tumor growth experiments

P4 and placebo pellets (30 mg, 30 day; Innovative Research of America) were implanted into 8-week old C57BL/6 mice obtained from the Jackson Laboratory (strain #000664). Seven days later, E0771-vec and E0771-mPR cells (1×10^6 cells per tumor) were injected into the mammary glands of the mice. Twenty-one days later, mice were sacrificed; tumors were removed and minced; and a single suspension of tumor cells was generated according to the same protocol used previously. Immune cell populations in each tumor were then analyzed via flow cytometry following the protocol outlined earlier.

Statistical analysis

For tumor-free interval and overall survival comparison between the groups, Kaplan-Meier analyses followed by log-rank tests were carried out. Differences in the biomarkers between the two groups were assessed using two-sample t-tests or the Mann-Whitney test, depending on whether the data satisfied normality assumption or not. The statistical tests used are also described in the figure legends. Statistical software R or Prism V.6 (GraphPad Software) were used for all the analyses. All data presented are reported as mean±SD.

RESULTS

P4 treatment alters immune cell populations in the murine mammary gland

Based on P4's known immune-suppressive roles in pregnancy and infection, we questioned whether P4 treatment had immunomodulatory effects in the murine mammary gland. To test this question, P4 pellets (30 mg/pellet, 30-day release) were implanted subcutaneously into ovariectomized FVB/n mice for 21 days in order to mimic non-pregnant, premenopausal concentrations of P4.[73] Subsequently, the mice were sacrificed, and digested mammary glands were immunophenotyped via flow cytometry. Mice treated with P4 exhibited immune cell changes in the mammary gland indicative of immunosuppression, as compared with mice treated with placebo. We found that the percent of CD4+CD25hi T cells, likely Tregs, was significantly increased with P4 treatment (figure 1A, left). When populations of innate immune cells were examined, we observed a significant decrease in the number of CD45+CD3–CD11c+CD8+ dendritic cells (cDC1s), the subset of classical dendritic cells critical for tumor cell recognition by CD8+ T cells, in the mammary glands of the mice treated with P4 compared with controls (figure 1A, right). The percentage of B cells (CD45+CD3-CD19+), T cells (CD45+CD3+), Mφs (CD45+CD3-F480+ cells), NK cells (CD45+CD3–NK1.1+), and CD11b+CD11c+ cells (likely dendritic cells [DCs]) were unchanged with P4 treatment (online supplemental figure 29). No overt signs of hyperplasia were observed in mammary gland whole mounts from these mice following the 21-day course of P4 treatment (online supplemental figure 30). Together, modulation of cDC1s and Tregs suggests that P4 treatment changes the immune microenvironment of the mammary gland towards immunosuppression.

Mammary glands, associated lymph nodes, and spleens of PR-overexpressing mice show early immunomodulation

After observing immunomodulation induced by P4 treatment (the ligand) in the murine mammary gland, we sought to investigate whether PR (the receptor) overexpression would lead to similar changes. To accomplish this task, we used the PR/Gal4 transgenic mouse.[57] This mouse carries transgenic overexpression of the full-length PR isoform (PR-B; the isoform with the highest activity in the mammary gland),[74] under control of the Gal4_UAS promoter (not tissue specific).[57] To verify overexpression of PR, several mice from each group were sacrificed at 6 months of age, and isolated mammary glands were analyzed for PR expression via IHC. IHC results showed
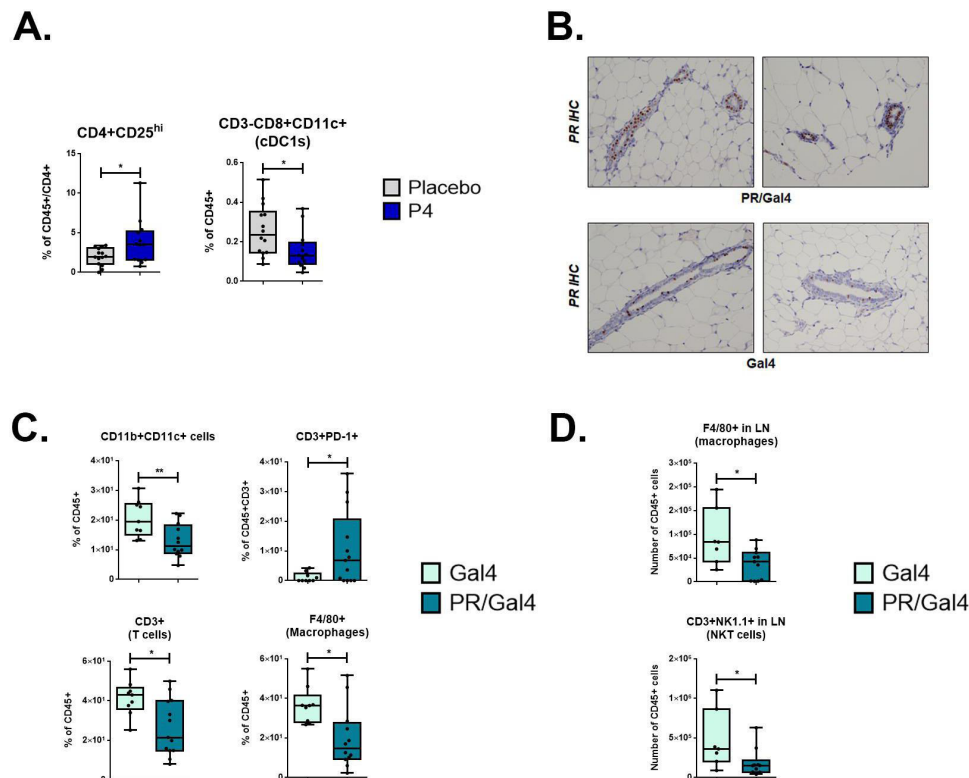
Figure 1 P4 treatment and PR overexpression alters immune cell populations in the murine mammary gland. (A) FVB/n mice were implanted with P4 (30 mg) (n=13) or placebo (n=14) pellets. Twenty-one days later, mice were sacrificed and the right and left mammary glands (#4) were excised; inguinal lymph nodes were removed; and the remaining mammary tissue was digested to single-cell suspension. Immunophenotyping was performed by flow cytometry with respective antibodies. An unpaired two-sample student's t-test was used to compare the means between treatment groups. (B) Representative IHC images depicting PR staining in the mammary glands from two representative 6-month-old virgin PR-overexpressing (PR/Gal4) and control (GAL4) mice. (C) Cohorts of PR/Gal4 (n=11) and GAL4 (n=7) mice were aged for 6 months and were subsequently sacrificed. Left and right mammary glands (#4) and excised inguinal lymph nodes (D) were collected from the mice; tissue digestion was performed; and immunophenotyping was performed via flow cytometry. Changes in the percentages of various immune cell types observed in the mammary glands of 6-month-old PR/Gal4 mice compared with control (GAL4) mice are shown. (D) Numbers of macrophages and NKT cells present in the excised inguinal lymph nodes (LN) of 6-month-old PR/Gal4 and Gal4 mice. A two-sample t-test with Welch's correction was used (C,D) to compare means between groups. *P<0.05, **P<0.01. IHC, immunohistochemistry; P4, progesterone; PR, progesterone receptor; NKT, natural killer T cells.

mammary glands from PR/Gal4 mice displayed ≥80% PR+ cells in the ductal epithelium, as compared with <10% in the control Gal4 mice (figure 1B). Whole mount mammary gland images from Gal4 and PR/Gal4 mice display normal architecture and no overt signs of hyperplasia at 20 weeks of age (~5 months, online supplemental figure 31).

To determine how PR overexpression may modulate the immune microenvironment, locally (within the mammary gland) and systemically, PR/Gal4 and Gal4 control mice were sacrificed at 6 months of age and their mammary glands, inguinal lymph nodes (draining lymph node imbedded in the mammary gland), and spleens were collected for immunophenotyping via flow cytometry. The mammary glands of the 6-month-old PR-overexpressing (PR/Gal4) mice showed decreased numbers of both innate and adaptive immune cells. The percentages of CD11b+CD11c+ cells and F480+ Mφs were significantly decreased in the mammary glands of the PR/Gal4 mice, as compared with Gal4 controls (figure 1C).

Additionally, the percentage of T cells (CD45+CD3+) in the mammary gland was significantly decreased in PR/Gal4 mice compared with Gal4 controls (figure 1C). While CD4+ and CD8+ T cell populations did not differ between genotypes, NKT (natural killer T) cells were decreased in PR/Gal4 mice but failed to reach statistical significance (p=0.06, online supplemental figure 32). Although NKT cells are the most likely major contributor to this overall change in CD3+ cells (as the difference between NKT cells in Gal4 and PR/Gal4 mice is trending but not statistically significant), it is possible that more rare CD3+ cell types, such as γδ (gamma-delta) T cells or the newly emerging CD3+ macrophage population (not included in this immune phenotyping panel),[75] may also contribute to this change observed in overall CD3+ cells. In addition to decreased CD3+ cells, the mammary glands of mice overexpressing PR contained a significantly increased percentage of PD-1+ T cells compared with controls (figure 1C), suggesting potential T cell exhaustion. Together, these results suggest that the
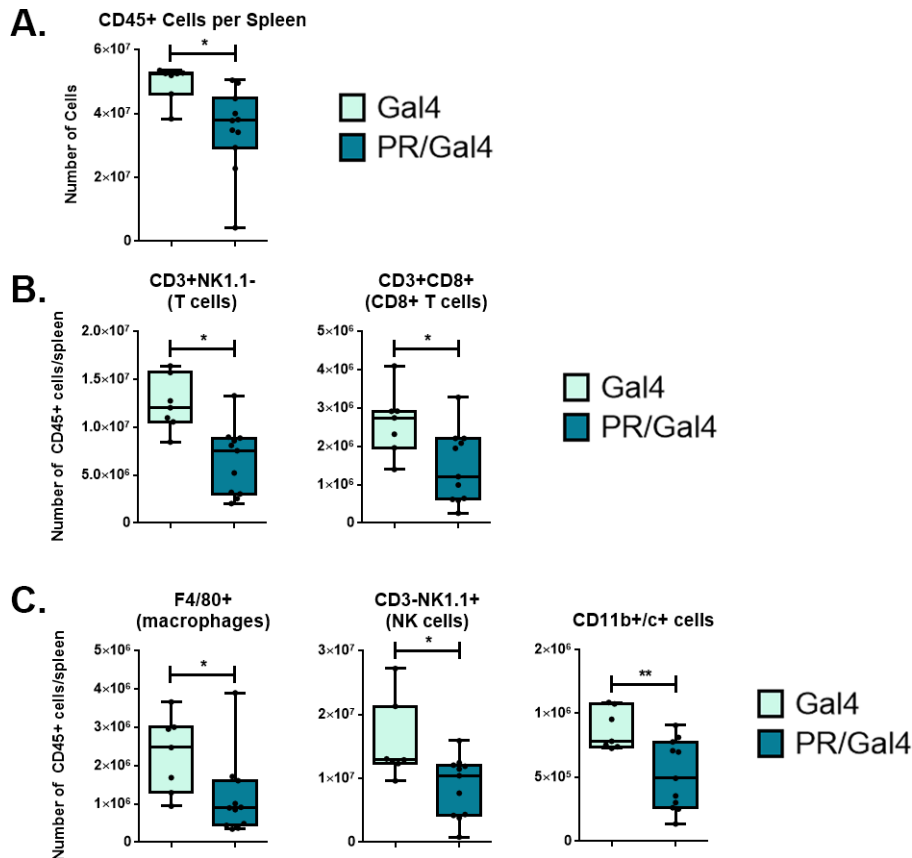
Figure 2 PR overexpression suppresses various immune cell populations present in the spleens of 6-month-old mice. Cohorts of PR-overexpressing (PR/Gal4, n=11) and control (GAL4, n=7) mice were aged for 6 months and were subsequently sacrificed. Spleens were collected from the mice; tissue digestion was performed; and immunophenotyping was performed via flow cytometry. (A) Number of immune cells present per spleen of 6-month-old PR-overexpressing (PR/Gal4) and control (GAL4) mice. (B) Adaptive immune cell populations present in the spleens of 6-month-old mice that were impacted by PR overexpression. (C) Innate immune cell populations present in the spleens of 6-month-old mice that were impacted by PR overexpression. For all data represented, two-sample t-test with Welch's correction was used to compare means between groups. *P<0.05, **P<0.01. NK, natural killer; PR, progesterone receptor.

mice overexpressing PR may have fewer T cells present in the mammary gland as well as an exhausted phenotype (due to increased PD-1 expression) in the T cells that are present. In the inguinal lymph nodes of the PR/Gal4 mice, there were significantly fewer Mφs and NKT cells compared with age-matched Gal4 mice (figure 2D, all populations shown in online supplemental figure 33). Collectively, these results suggest that mice overexpressing PR have significantly fewer innate and adaptive immune cells present in their mammary glands at 6 months of age, which could provide an environment more conducive to growth of mammary gland tumors.

To assess the effect of PR overexpression on systemic immunity, immunophenotyping was performed on cells isolated from the spleens of 6 month old PR/Gal4 and Gal4 mice. Examination of total numbers of immune cells present in the spleen revealed that PR/Gal4 mice had significantly fewer total immune cells (CD45+) compared with Gal4 mice (figure 2A). Interestingly, PR/Gal4 mice also had fewer total live cells per spleen (online supplemental figure 35), which suggests PR expression may have broad impacts on the development

or migration of murine immune cells in the spleen (and possibly elsewhere in body). Of note, when numbers of total live cells and total immune cells in the spleen were compared among older PR-overexpressing and Gal4 mice (described further), there was no significant difference found between groups for either cell population.

On examination of individual types of immune cells present in the spleens, numbers of adaptive immune cells were also decreased in the spleens of PR/Gal4 mice, as the number of T cells (CD3+) and CD8 +T cells (CD3+CD4–CD8+) were significantly decreased compared with Gal4 mice (figure 2B). The number of B cells, however, did not significantly differ between the two groups (online supplemental figure 34). Numbers of innate immune cells per spleen were also significantly decreased in the PR/Gal4 mice compared with controls, including Mφs (CD45+F480+), NK cells, and CD11b+CD11c+ DCs (figure 2C). Cumulatively, these data suggest that PR overexpression results in decreased numbers of numerous types of immune cells both locally (in the mammary gland and associated lymph node) and systemically (in the spleen), which may suppress immune
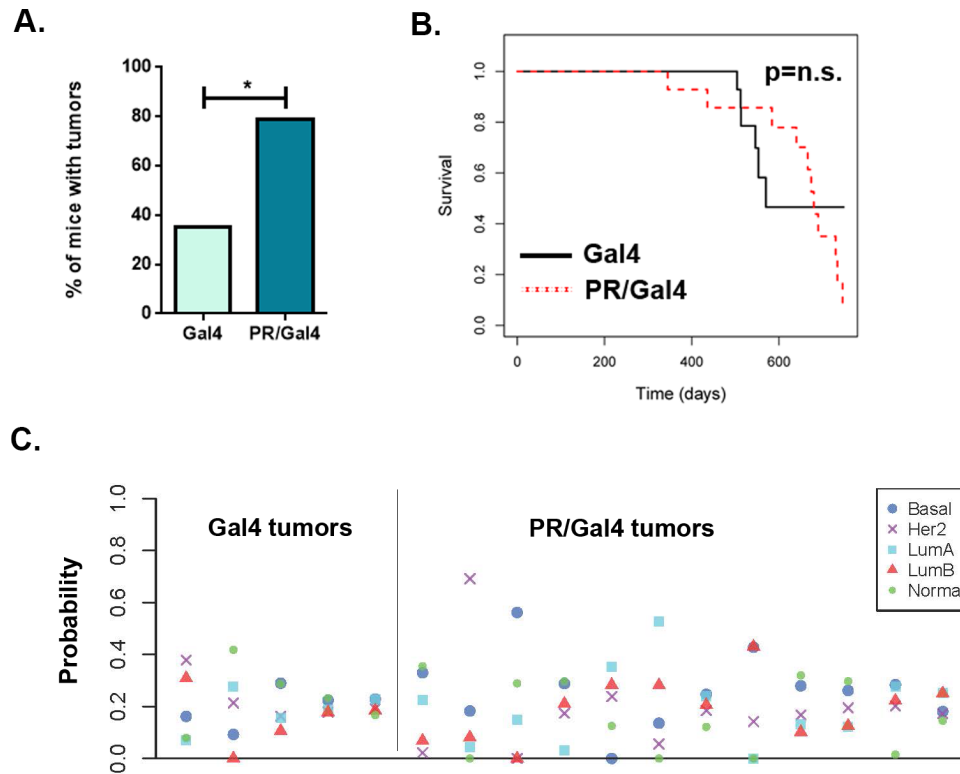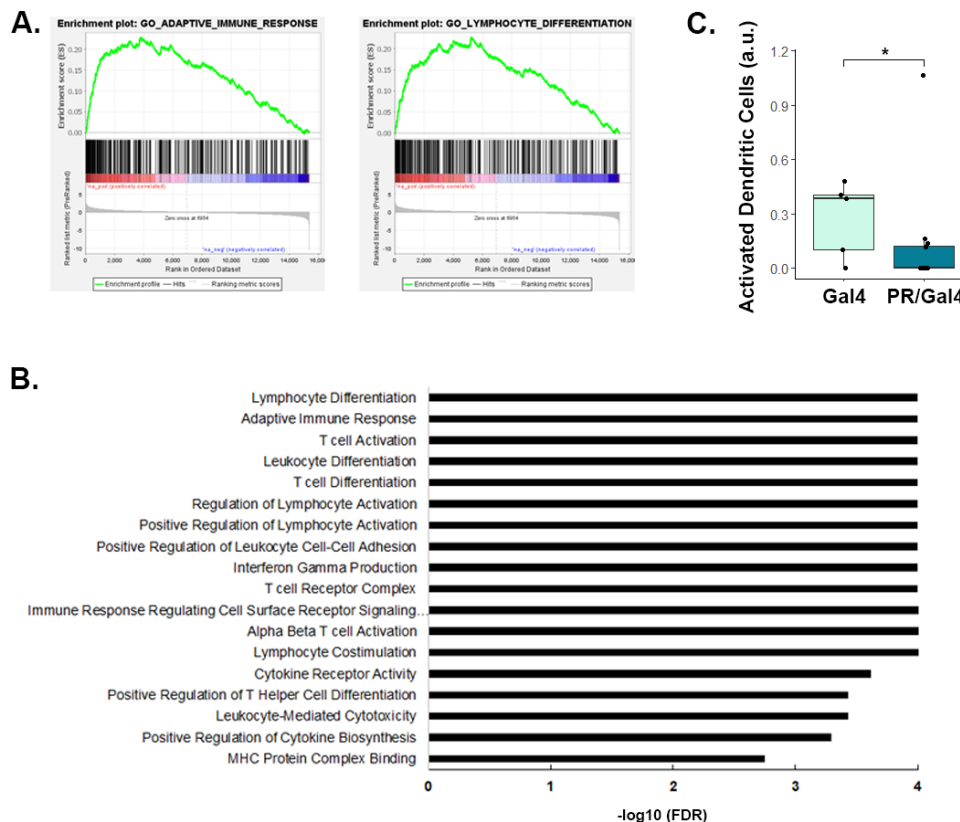
Figure 3 PR/Gal4 mice developed significantly more mammary gland tumors than control mice. Cohorts of PR-overexpressing (PR/Gal4, n=17) and control (GAL4, n=14) mice were aged until tumor development (~2 years of age on average). During the aging period, mice were bred three times to mimic normal cycling levels of hormone throughout the lifetime. (A) Percentages of PR-overexpressing (PR/Gal4) and control (GAL4) mice that developed tumors by 750 days of age. The difference in the tumor rates between the groups was assessed using two-sample test of proportions. (B) Kaplan-Meier plot depicting overall survival of PR-overexpressing (PR/Gal4) mice and control (GAL4) mice through 750 days of age. No significant differences were observed between the groups, as determined using a log-rank test. (C) Following RNA sequencing of tumor samples (5 Gal4 tumors and 12 PR/Gal4 tumors), subtype analysis was performed by merging the human PAM50 data and the mouse RNA sequencing data by 47 homolog genes. Merged data were subsequently normalized and samples were assigned to PAM50 subtypes. The graph represents the probability of each PAM50 subtype for representative tumor samples from five control (GAL4) mice and 12 PR-overexpressing (PR/Gal4) mice. *P<0.05. n.s., not significant; PR, progesterone receptor.

responses and support the development of mammary gland tumors.

Increased incidence of mammary gland tumors in PR/Gal4 mice

We then sought to investigate whether the immunomodulation we observed with either P4 treatment or PR overexpression would correlate with increased formation of murine mammary gland tumors. To test this question, we assayed tumor formation in multiparous, aged PR/Gal4 and Gal4 mice. Multiparity was achieved by breeding mice three times during their aging period in order to mimic hormone levels achieved during normal cycling as well as pregnancy. Although tumor formation in younger mice (previously aged up to 1 year) was not previously reported in this model,[57] multiparous mice in both groups developed mammary gland tumors during the 2-year follow-up period. Notably, significantly more PR/Gal4 mice developed mammary gland tumors compared with Gal4 mice (figure 3A). Although the PR-overexpressing mice developed significantly more mammary gland tumors, there was no significant effect on overall survival (figure 3B). Assessment of tumor histology was performed following (H&E) staining, which revealed the majority of the tumors were adenocarcinomas with squamous metaplasia (online supplemental table 2). Interestingly, as is the case with the majority of murine mammary gland models,[76] all tumors in both groups were ER-negative/PR-negative (by IHC and RT-qPCR, data not shown), despite arising from a majority-PR+ mammary gland (see figure 1B). In order to molecularly classify the tumors that developed in the PR/Gal4 mice versus controls, we performed RNA-Seq on tumor RNA isolated at the time of sacrifice. Subtype probability analysis was performed on RNA-Seq data obtained from the majority of tumors using genes from the PAM50 gene signature, the 50-gene signature used to characterize the intrinsic subtypes of human breast cancer.[77 78] The analysis revealed that murine tumors arising from either genotype did not classify well into the human PAM50 subtypes (figure 3C), indicative of the tumor heterogeneity that occurs in spontaneous murine tumor models.

Figure 4 Differences in expression of immune-related gene signatures among tumors from PR/Gal4 versus control mice. Cohorts of PR-overexpressing (PR/Gal4, n=17) and control (GAL4, n=14) mice were aged until tumor development (~2 years of age on average). During the aging period, mice were bred three times to mimic normal cycling levels of hormone throughout the lifetime. On sacrifice, sections of each tumor were flash frozen and RNA samples were isolated for subsequent RNA sequencing analysis (5 Gal4 tumors and 12 PR/Gal4 tumors). (A) Representative enrichment plots from GO pathway analysis using GSEA. (B) Select GO gene sets enriched in tumor samples from control (GAL4) mice are represented. The graph represents the log of the p value for each gene set. The complete list of the enriched GO gene sets can be found in online supplemental table 3. (C) CIBERSORT analysis was performed on RNA sequencing data obtained from tumor samples of PR-overexpressing (PR/Gal4) and control (GAL4) mice. The graph represents the immune cell population that was found to significantly differ between groups (activated dendritic cells) by one-tailed Wilcoxon test. *P<0.05. GO, Gene Ontology; GSEA, gene set enrichment analysis; PR, progesterone receptor; FDR, false discovery rate; MHC, major histocompatability complex.

Tumors from PR/Gal4 mice express gene sets associated with immunosuppressed microenvironments and contain decreased numbers of infiltrating immune cells

Gene expression analysis from the RNA-Seq data revealed that while only two genes (Igsf1 and Krt10) were found to be differentially expressed between tumors from PR/Gal4 and Gal4 mice, GSEA analysis[63 79] revealed that numerous immune-related gene sets were enriched in tumors of Gal4 mice, as compared with PR/Gal4 mice (figure 4A,B); these gene signatures were lost in PR/Gal4 mice. Of the 266 gene sets that were significantly (False Discovery Rate [FDR] ≤0.05) enriched in tumors from Gal4 mice, 130 were gene sets involved in immune cell development, function, and regulation, including activation of lymphocytes, leukocyte differentiation, cytokine biosynthesis, and interferon receptor signaling (figure 4A,B). Representative GSEA plots and gene sets are displayed in figure 4A. The entire list of gene sets enriched in tumors from Gal4 mice is presented in online supplemental table 3. These data suggest that the tumors arising in Gal4 mice display RNA expression

patterns indicative of more active immune cell responses. In turn, the PR/Gal4 tumors display less immunogenic gene signatures. Finally, CIBERSORT (Cell Type Identification by Estimating Relative Subsets of RNA Transcripts) analysis, which uses gene expression data to estimate the abundance of various types of immune cells from a mixed cell population,[64 65] revealed that there was a significantly decreased activated dendritic cell signature in the tumor samples of the PR/Gal4 mice compared with Gal4 mice (figure 4C). Together, these results suggest that the tumors of PR/Gal4 mice exhibit an immune-suppressed microenvironment compared with tumors from control mice, demonstrating both fewer numbers and activity of immune cells.

In order to interrogate the specific cell types within the immune microenvironment of the tumors that developed in PR/Gal4 and Gal4 mice, we performed immunophenotyping via flow cytometry. Before assessing various populations of infiltrating immune cells, we compared tumor weights and absolute numbers of immune cells. Though the tumors from PR/Gal4 mice tended to be larger in size
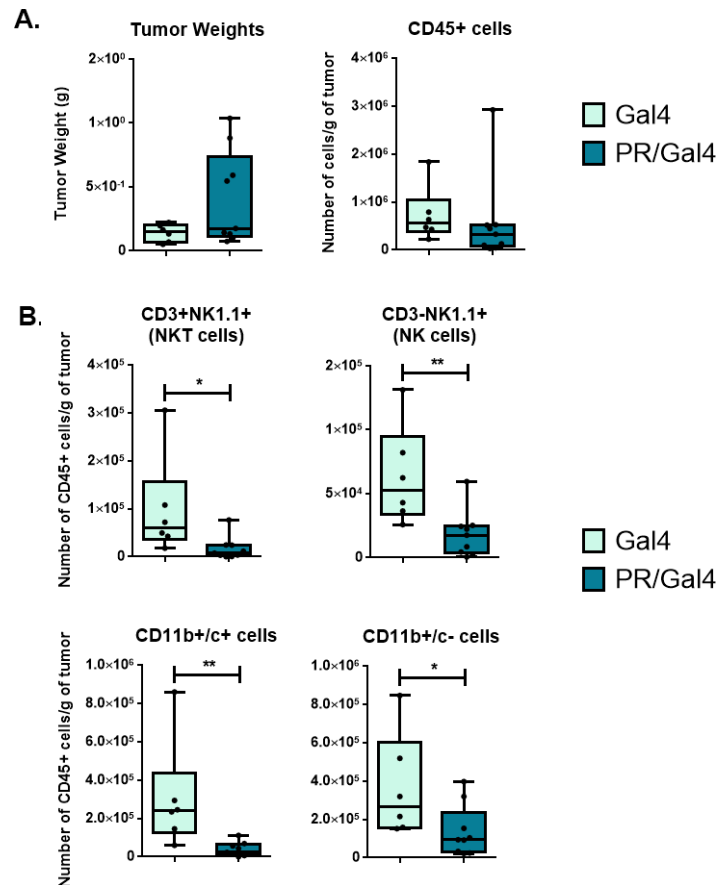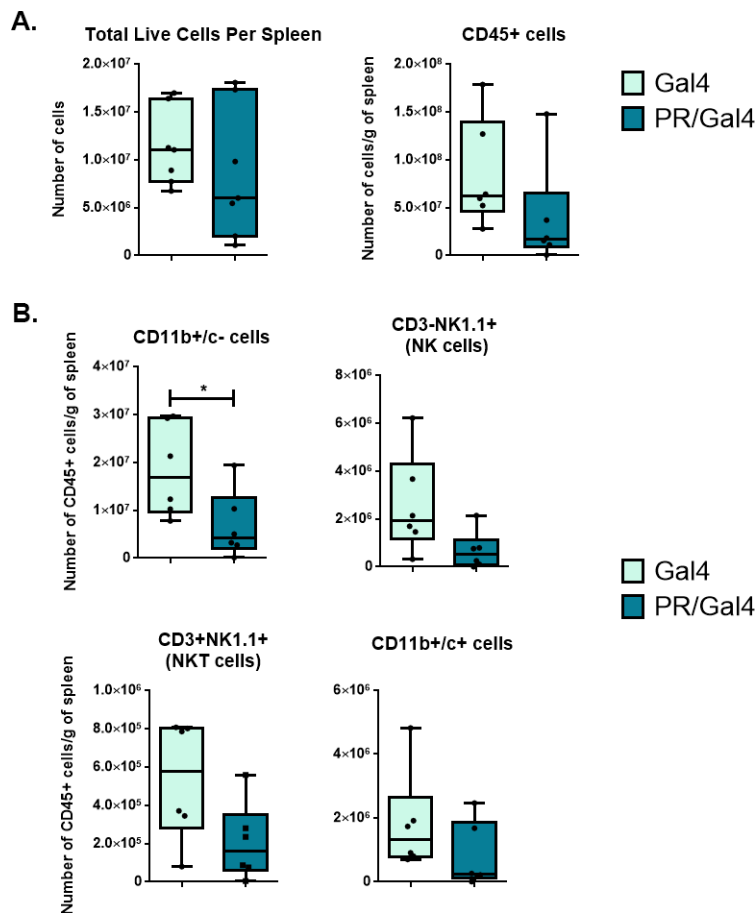
Figure 5 Tumors of PR-overexpressing mice contain fewer infiltrating immune cells compared with controls. Cohorts of PR-overexpressing (PR/Gal4, n=10) and control (GAL4, n=7) mice were aged until tumor development (~2 years of age on average). During the aging period, mice were bred three times to mimic normal cycling levels of hormone throughout the lifetime. On sacrifice, tissue digestion was performed on sections of each tumor and a single-cell suspension was obtained, which was used for immunophenotyping by flow cytometry. (A) Comparison of average tumor weights and number of total infiltrating immune cells (CD45+) per gram of tumor among PR-overexpressing (PR/Gal4) and control (GAL4) mice. (B) Changes in numbers of various immune cell population observed between tumor samples of PR-overexpressing (PR/Gal4) and control (GAL4) mice. For all data represented, a Mann-Whitney test was used to compare groups. *P<0.05, **P<0.01. NK, natural killer; PR, progesterone receptor.

than those of the control mice, these data did not reach statistical significance (figure 5A). In addition, the total number of immune cells (CD45+) per gram of tumor did not differ between tumors of PR-overexpressing and control mice (figure 5A). Interestingly, while the total number of CD45+ cells present did not differ between tumors isolated from PR/Gal4 and Gal4 mice, several individual immune cell types were found to be decreased in the tumors from PR/Gal4 mice. When examining cell types of the adaptive immune system, we found that tumors from PR/Gal4 mice contained significantly lower numbers of NKT cells (CD45+CD3+NK1.1+) per gram of tumor cell compared with Gal4 mice (figure 5B). Numbers of B cells and T cells were not found to differ among the two groups (data not shown). The immunophenotyping results also showed that numbers of several types of innate immune cells differed between the groups of mice. Tumors from PR/Gal4 mice contained significantly fewer NK cells (CD45+CD3–NK1.1+) per gram of tumor compared with controls (figure 5B). In

addition, tumors from PR/Gal4 mice contained significantly fewer CD11b+/CD11c– cells, a population representative of many other types of leukocytes involved in innate immune responses, including Mφs, granulocytes, and monocytes. When antigen-presenting cell types were examined, we found less CD11b+CD11c+cells (likely DCs) per gram of tumor in PR/Gal4 mice compared with controls (figure 5B). All together, these data demonstrate that there was decreased infiltration of both adaptive and innate immune cell types in tumors from PR/Gal4 mice.

Spleens from tumor-bearing PR-overexpressing mice showed decreased numbers of innate and adaptive immune cells

To determine whether overexpression of PR led to systemic suppression of the immune system to promote tumor development, we performed immunotyping on the spleens of PR-overexpressing mice and control mice that developed mammary gland tumors using flow cytometric analysis. When examining the total number of live cells and immune cells (CD45+) in the spleens of

Figure 6 Comparison of immune cell populations in the spleens of PR-overexpressing and control mice that developed mammary gland tumors. Cohorts of PR-overexpressing (PR/Gal4, n=10) and control (GAL4, n=7) mice were aged until tumor development (~2 years of age on average). During the aging period, mice were bred three times to mimic normal cycling levels of hormone throughout the lifetime. On sacrifice, spleens were collected from each animal; tissue digestion was performed; and a single-cell suspension was obtained, which was used for immunophenotyping by flow cytometry. (A) Total number of live cells and immune cells (CD45+) isolated from spleens of PR-overexpressing (PR/Gal4) and control (GAL4) mice that developed mammary gland tumors. (B) Numbers of various immune cell populations per gram of spleen present in the spleens of PR-overexpressing (PR/Gal4) and control (GAL4) mice that developed mammary gland tumors. For all data represented, a Mann-Whitney test was used to compare groups. *P<0.05. NK, natural killer; PR, progesterone receptor.

tumor-bearing mice, no significant differences were found between groups (figure 6A). Analysis of specific immune cell populations revealed that numbers of CD11b+CD11c− innate immune cells (population representative of many leukocytes involved in innate immune responses, including Mφs, granulocytes, NK cells, and monocytes) were found to be significantly decreased in the spleens of tumor-bearing PR/Gal4 mice compared with spleens of tumor-bearing controls (figure 6B). Several other types of immune cells were found to be decreased in the spleens of the PR-overexpressing mice, including NK cells, NKT cells, and CD11b+CD11c+ cells, though not to a statistically significant degree (figure 6B). These data reveal that in addition to having decreased numbers of various types of immune cells infiltrating their tumors compared with controls, the PR-overexpressing mice also contain decreased numbers of innate immune cells in their spleens after developing mammary gland tumors. Overall, these data suggest that overexpression of PR may have tumor-promoting, immune inhibitory effects both

locally in the tumor microenvironment and systemically. This suggests a novel mechanism by which the steroid hormone P4 and its receptor may promote mammary gland tumorigenesis.

Syngeneic PR-positive mammary gland tumors exhibit immunosuppressive microenvironments and increased tumor growth following treatment with P4

To determine if the phenotypical changes observed with P4 treatment and PR expression are due to activation of canonical PR signaling in tumor cells that translates into functional immunosuppression and subsequent development/promotion of mammary tumors, we generated mammary tumor lines expressing mouse PR, along with control lines, and determined their ability to grow in vivo with and without P4. For these studies, we used an ER/PR-negative murine mammary gland tumor cell line, E0771, derived from a spontaneous mammary gland tumor in a C57BL/6 mouse.[66–69] Our initial studies determined that mPR is expressed and active in these cells,
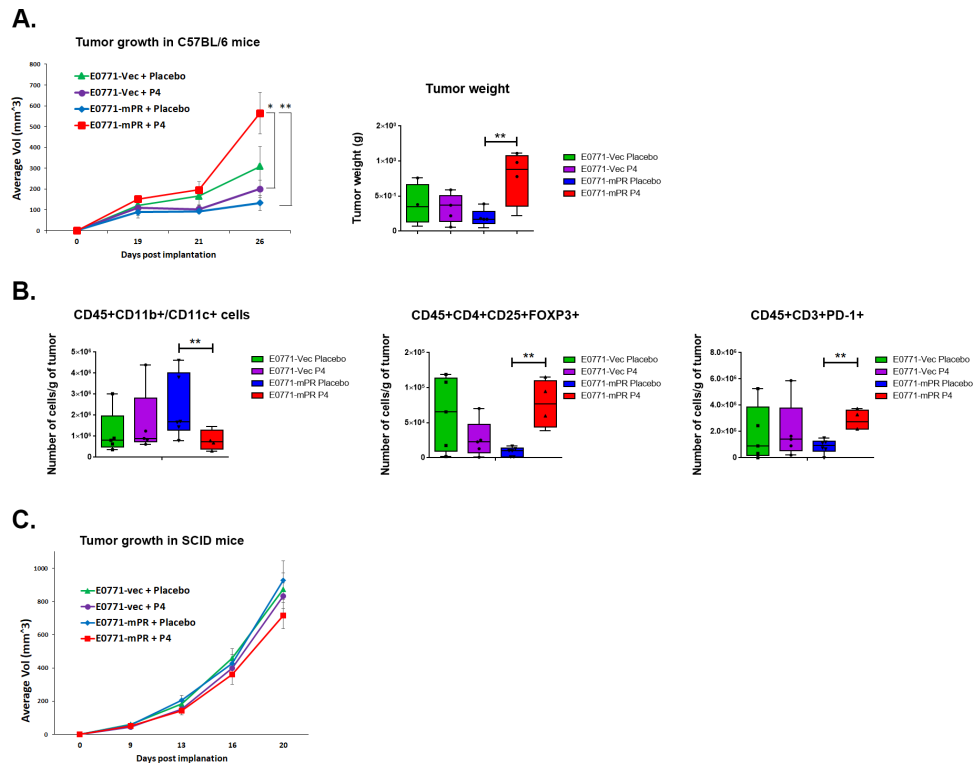
Figure 7 P4/PR promote immunosuppression and growth of syngeneic PR+ mammary tumors. (A) Left: E0771-vec and E0771-mPR (expressing mPR) cells were injected into the mammary fat pads of C57BL/6 mice pretreated with placebo or P4 (30 mg, 30 days) pellets. Right: tumor weights at the time of sacrifice. (B) Flow cytometry was performed on digested tumors using respective antibodies. (C) Tumor experiments were performed as in A, except cells were injected into the mammary fat pads of SCID(severe combined immunodeficiency)-beige mice. Significance between groups was determined using a two-sided t-test. *P≤0.05, **P≤0.01. mPR, mouse progesterone receptor; P4, progesterone; PR, progesterone receptor.

confirmed through western blotting, luciferase assays, and assessments of PR-target genes after P4 treatment (online supplemental figure 36). Having confirmed their activity, we determined the impact of tumor-expressed PR signaling on growth and tumor development. E0771 cell line variants were injected into immune competent C57BL/6 mice pretreated with placebo or P4 pellets (as in figure 1). Mice bearing E0771-mPR tumors pretreated with P4 developed the largest tumors, indicating that the combination of PR and P4 promotes the growth of mammary tumors (figure 7A). Flow cytometry from digested tumors revealed that P4-treated PR+ tumors exhibited lower numbers of cDC1s, higher numbers of Foxp3+ Tregs, and increased PD-1+ T cells (indicative of exhausted T cells) when compared with the other groups/treatments (per gram of tumor, figure 7B). These data are consistent with effects observed following P4 treatment in a normal mammary gland and PR expression in a transgenic mammary gland (see figure 1). These differences in immune cell composition were not observed in PR-negative tumors treated with P4 (E0771-vec cells), suggesting that PR signaling in tumor cells altered local immune infiltrates through PR-mediated immune-suppressive signaling.[70 71 80] To determine if the P4-promoted tumor growth observed in the syngeneic/immunocompetent mice (C57BL/6) was indeed mediated through immunosuppression, E0771 cell line variants were grown in immunocompromised SCID (severe combined immunodeficiency)-beige mice, which lack adaptive T cells and B cells, and are deficient for NK cells. Critically, we observed no differences in tumor growth between the cell lines or treatment conditions in SCID-beige mice (figure 7C). Cumulatively, these data demonstrate that PR-expressing tumor cells promote immunosuppressive changes in the mammary gland leading to significantly enhanced growth mediated by the presence of innate and adaptive immunity.

DISCUSSION

In this study, we present evidence that both treatment with P4 and overexpression of PR result in changes that may inhibit immune responses in the murine mammary gland, inguinal lymph nodes, and spleen. We also report for the first time that mice overexpressing PR develop an increased number of mammary gland tumors compared with control mice. In addition, tumors of PR-overexpressing mice exhibit fewer infiltrating lymphocytes and decreased immune gene expression signatures compared with tumors of control mice. Moreover, we show that the effects of PR and P4 on the immune system and subsequent tumor growth are likely mediated through direct actions/released factors from PR-positive tumor cells, as PR-negative tumor cells did not elicit a similar immune

response. The findings presented in this study provide insight relevant to the future development of improved immune-based therapies for clinical treatment of breast cancers.

On evaluation of infiltrating immune cells present in tumors isolated from PR/Gal4 mice and E0771-mPR tumors, we found significantly decreased levels of several types of infiltrating immune cells compared with control tumors, including NKT cells, NK cells, CD11b+CD11c– innate immune cells, CD11b+CD11c+ DCs, and T cells. These findings may have broad implications for patients with breast cancer, as tumor-infiltrating immune cell populations have been shown to hold prognostic value and may predict response of breast tumors to immunotherapies. Previous studies have demonstrated that the immune makeup of the tumor microenvironment has an impact on the response to immunotherapeutic agents, including anti-PD-1 and anti-CTLA4 monoclonal antibodies.[81 82] Studies in human breast cancer have shown that the presence of tumor-infiltrating lymphocytes (TILs) predicts response to treatment as well as a reduced risk of relapse in patients with breast cancer, regardless of the subtype of breast cancer.[83 84] Our findings reveal a potential PR-dependent mechanism for the decreased number of TILs seen in HR+ breast cancers compared with other subtypes.[21 85] If increased PR signaling directly results in decreased infiltrating immune cells, as our results suggest, this also highlights a potential avenue to increase immune infiltrates in tumors by targeting PR with the use of antiprogestins. This strategy could be used to increase response to immune-based therapies, as numerous studies have shown that decreased numbers of TILs is associated with poor response to immune checkpoint blockade in patients with breast cancer.[14 84 86–88] Given the poor response rates to immunotherapy demonstrated thus far in HR+ breast cancers, this strategy could offer invaluable benefit to patients in the future.

Preclinical studies in mice have demonstrated the importance of NK cells in controlling growth of mammary gland tumors and cancer stem cells.[89 90] In addition, clinical studies have provided evidence that protein and mRNA expression of NK cell receptor ligands (NKG2D ligands) on human breast tumors were associated with prolonged relapse-free survival as well as overall survival.[91 92] Finally, recent studies have identified that NK cells, in addition to T cells, are key mediators of responses to immunotherapies targeting PD-1.[93] NKT cells, which provide a bridge between the innate and adaptive immune systems, have also been shown to be important in antitumor immune responses via studies in mice.[94–96] Recent studies have shown that activation of NKT cells resulted in increased tumor-specific immune responses against murine mammary gland tumors[97] and that presence of functional NKT cells in the tumor may be crucial in determining response to glycolipid-based immunotherapies.[98 99] Together, these findings highlight that decreased levels of tumor-infiltrating NK cells and NKT cells, as seen in the PR-overexpressing mice, may

have detrimental effects on immune-mediated tumor cell killing and overall survival.

DCs play a crucial early role in the cancer immunity cycle[100] by digesting and presenting tumor antigens in order to stimulate the adaptive immune system. Several studies have demonstrated this important role of DCs by showing that their depletion results in loss of T cell priming and immune-mediated tumor elimination.[101 102] Furthermore, the presence of DCs has been shown to be essential for CD8+ T cell activation following treatment with immunotherapies.[103] Studies in other tumor types such as non-small cell lung cancer have demonstrated that increased density of mature DCs in tumors was associated with a favorable clinical outcome.[104] Overall, these studies provide evidence that DCs are key in driving antitumor responses and highlight the detrimental impact decreased numbers of DCs, as seen in the tumors of PR-overexpressing mice, may have on antitumor immune responses.

In addition to displaying changes in number of immune cells in their mammary glands, the PR-overexpressing mice also exhibited changes in immune cell populations present in the spleen, both at 6 months of age (several months before tumor development) and at the time of sacrifice with tumors present (See figures 2 and 6). Though the systemic modulation of immune cell populations may be a contributing factor to the development of mammary gland tumors in the mice, we hypothesize that both systemic changes as well as local changes in immune cell populations in the mammary gland contribute to tumor development, as tumors were only found in the mammary glands of these mice. Local changes in the mammary gland induced by multiple gestations, such as mammary gland reorganization and increased levels of P4, may have further contributed to local changes in the mammary gland environment promoting tumor development.

Our findings show select immune populations that change in the mammary glands of mice treated with P4, PR-overexpressing mice, or PR-positive tumors. However, one caveat to the interpretation of these data is that we did not evaluate putative changes in myeloid-derived suppressor cells (MDSCs). MDSCs have an emerging role in promoting tumor immune evasion, immunosuppression, and subsequent growth in many types of cancers, including breast (reviewed in De Cicco et al[105]). MDSC characterization requires a combination of extensive phenotypical and functional markers, many of which overlap with non-immunosuppressive neutrophils and monocytes.[106] As such, it can be difficult to distinguish MDSCs from other cell types in an immune-poor microenvironment such as the mammary gland. For these reasons, and to keep our immune phenotyping as broad as possible, we did not include MDSCs in the analysis presented here. Therefore it's possible that, in addition to the cell types we showed herein to be affected by PR/P4, MDSCs could also contribute to P4-mediated immunosuppression.

Surprisingly, despite arising from mammary glands with transgenic PR overexpression reaching nearly 80%, the tumors that developed in the PR/Gal4 mice (and Gal4 controls) are ER/PR-negative tumors. Although the majority of spontaneous tumor models in mice are ER/PR-negative,[76] it raises interesting questions about what role PR plays in driving increased tumor incidence in this mouse model. First, the immune-related gene signature differences between the PR/Gal4 and Gal4 tumors suggest that PR expression in the transgenic mammary gland serves to remodel the immune system to support tumor development, more so than to drive oncogenic events within the tumor cell. In this case, PR expression in the tumor cells (vs the normal mammary gland and/or stroma) may be a secondary factor contributing to tumorigenesis. Second, differences in biology between the human and mouse mammary gland and the role that PR plays in each may serve to explain the lack of PR expression in the tumors. Finally, PR expression may be lost over the course of tumorigenesis. In these studies, PR expression was only analyzed in end-stage tumors. Therefore, additional studies would be needed to determine how PR expression changes over the course of tumorigenesis in the Gal4/PR model.

The findings presented in this study provide novel insights into PR's role in promoting breast cancer development via immunomodulation, but several follow-up experiments are merited in order to develop a more comprehensive understanding of PR-induced immunomodulation and how it promotes mammary cancer. First, studies should be done in order to evaluate the impact of PR expression or P4 treatment on the activation or effector function of immune cell types in the mammary gland, as several studies have noted changes in the number of activated immune cells induced by PR/P4.[39 42] While our studies showed changes in the number or percentage of various types of immune cells present due to P4 treatment or PR overexpression in murine tissues, knowing how P4/PR impacts the activity of these cells would provide valuable insight regarding the functional status of the immune cells present. In addition to examining markers of activation, further studies are required to evaluate the effect of P4 treatment and PR expression on the distribution of subtypes of immune cells, including dendritic cells, Mφs, NK cells, and granulocytes. Finally, future studies are necessary in order to examine which immunomodulatory effects of P4 are mediated by PR and which are mediated by the glucocorticoid receptor (GR), as a handful of studies have provided evidence that PR induces immunosuppressive effects as a result of its interaction with GR.[50 107]

Here, we report that P4 treatment or PR overexpression induces immunomodulation in the murine mammary gland that promotes the development of mammary gland tumors. These findings establish a rationale for targeting PR in breast cancer as a mechanism to promote antitumor immunity. In addition, these findings may explain, in part, the decreased response of HR+ breast cancers to ICIs and provide a rationale for investigating whether combining antiprogestins with ICIs may enhance the response to these agents in breast cancer.

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