Wound healing-like immune program facilitates postpartum mammary gland involution and tumor progression

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Abstract

Women diagnosed with breast cancer within 5 years postpartum have poor survival rates. The process of postpartum mammary gland involution, whereby the lactating gland remodels to its pre-pregnant state, promotes breast cancer progression in xenograft models. Macrophage influx occurs during mammary gland involution, implicating immune modulation in the promotion of postpartum breast cancer. Herein, we characterize the postpartum murine mammary gland and find an orchestrated influx of immune cells similar to that which occurs during wound healing. Further, the normal involuting gland may be in an immunosuppressed state as discerned by the transient presence of Foxp3+ regulatory T cells and IL-10+ macrophages with T cell suppressive function. To determine the influence of the postpartum immune microenvironment on mammary tumor promotion, we developed an immune-competent model. In this model, mammary tumors in the involution group are six-fold larger than nulliparous group tumors, have decreased CD4+ and CD8+ T cell infiltrates and contain a greater number of macrophages with the ability to inhibit T cell activation. Targeting involution with a neutralizing antibody against the immunosuppressive cytokine IL-10 reduces tumor growth in involution group mice but not in nulliparous mice, implicating the involution microenvironment as the primary target of αIL-10 treatment. Relevance to women is implicated, as we find post-lactational human breast tissue has transient high IL-10+...
and Foxp3+ immune cell infiltrate. These data show an immune modulated microenvironment within the normal involuting mammary gland suggestive of immunosuppression, that when targeted reduces tumor promotion, revealing possible immune-based strategies for postpartum breast cancer.

**Keywords**
Macrophages; myeloid derived suppressor cells; regulatory T cells; postpartum breast cancer; immune modulation

**Introduction**

Postpartum mammary gland involution is a multifaceted tissue remodeling process that returns the lactation-competent gland to a quiescent state ready to respond to new gestational signals \(^1\), \(^2\). Postpartum involution is characterized by extensive death of the mammary secretory epithelium followed by repopulation of the gland with a fibrous and adipocyte rich stroma. While involution is a physiologically normal, developmentally orchestrated tissue-remodeling process, it shares striking similarities with pathologically induced wound-healing and tumor-promotional microenvironments \(^3\)–\(^5\). Consistent with tumor promotion, in a SCID xenograft model we reported that breast cancer cells exposed to the postpartum involution microenvironment have increased growth, invasion and metastasis compared to nulliparous hosts \(^5\). Of potential relevance, young women diagnosed with breast cancer in the postpartum window have a near three-fold increased risk of recurrence and death compared to nulliparous women, even after adjusting for patient age, cancer stage and breast cancer subtype, identifying a postpartum diagnosis as an independent risk factor for metastasis \(^6\)–\(^8\). Based on these observations, we predict that further understanding of the immune infiltrate during postpartum mammary gland involution will elucidate novel mechanisms of tumor progression and identify potential therapeutic targets for the prevention and treatment of postpartum breast cancer.

Weaning-induced involution in the murine mammary gland occurs in two distinct phases. The early, reversible stage (days 1–3) is characterized by accumulation of milk, distended lumen and extensive death of the secretory mammary epithelium and the second, irreversible stage (days 3–8), with adipocyte repopulation and stromal restructuring \(^1\), \(^2\). Molecular profiling of the murine mammary gland involution has provided gene signatures consistent with acute phase, innate and adaptive immune responses; however, the cellular basis for these signatures remains largely unknown \(^4\), \(^9\). During involution, mammary epithelial cells themselves express transcripts traditionally associated with immune cells \(^10\), \(^11\), and acquire phagocytic capability \(^12\), making it difficult to assign immune-like gene signatures to specific cell types.

With regard to the role of the immune system during postpartum involution, little data exist for the adaptive arm; however, innate immune cells have been partially characterized. Granulocytes infiltrate the mouse gland on the first day of involution, implicating their role in the early cell death phase \(^13\). Recently, resident macrophages in the mammary gland were found to be essential in early involution, as deletion of colony stimulating factor-1 receptor...
positive cells during involution inhibited mammary epithelial cell death and restructuring of the gland back to its pre-pregnant state\textsuperscript{14}. While the mechanism by which macrophages facilitate postpartum involution is currently unknown, the absence of early-phase mammary epithelial cell death is consistent with a role for resident tissue macrophages, rather than the macrophages that infiltrate in the second, remodeling phase. Resident mammary macrophages have yet to be phenotypically characterized, however, the second-phase infiltrating macrophages express low iNOS, high arginase-1 and the mannose receptor\textsuperscript{4}, consistent with alternative activation or M2 polarization\textsuperscript{15}. Importantly, the alternatively-activated macrophage phenotype correlates with tumor promotion in breast cancer patients\textsuperscript{16}, and with metastasis in rodent models\textsuperscript{17}.

To better understand the role of the immune system during postpartum mammary gland involution, as well as its potential influence on tumor cells exposed to this microenvironment, we characterized immune cells across involution and developed an immunocompetent mouse model of postpartum breast cancer. During gland involution, we find immune cell infiltrates consistent with acute, proliferative, and resolution phases of wound healing providing further evidence for similarities between the physiologic process of postpartum involution and wound healing. Moreover, we demonstrate for the first time that postpartum mammary tumors have a significant decrease in CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell infiltrate and an increase in macrophages with T cell suppressive activity. Further, targeting involution with a neutralizing antibody against the immunosuppressive cytokine IL-10 reduces the tumor promotional microenvironment of involution. Our findings are consistent with the postpartum mammary gland involution having a microenvironment that favors tumor growth, in part through wound-healing like programs, which may account, in part, for the poor prognosis of women diagnosed with postpartum breast cancer.

**Materials and Methods**

**Preclinical mouse models and tissue collection**

University of Colorado IACUC approved all mouse procedures. For specific reproductive stages, Balb/c female mice (Jackson Laboratories) were bred with male C57Bl/6 male mice (Jackson Laboratories) in ventilated micro-isolator cages, with 12 hour light/dark cycles. Two days post-parturition litter sizes were standardized to 6–7 pups. Weaning was initiated at lactation days 10–14 (denoted L10). Mice were anesthetized with Isofuran (VetOne) and injected with 20,000 D2A1 cells in 20µl PBS into the right and left 4\textsuperscript{th} mammary gland fat pads of involution day 1 or age-matched nulliparous group mice. The D2A1 cell line was a generous gift from Dr. Ann Chambers (Ontario, Canada) and was maintained as previously described\textsuperscript{18}. For our treatment model, we treated involution mice by intraperitoneal injection with 0.2mg rat anti-IL-10 (JES5-2A5, BioXCell) or isotype control rat IgG (HRPN, BioXCell) every two days beginning on day 8 of lactation, L10, INV2, INV4, INV6 and ending on day 8 of involution, with same injection schedule in age-matched nulliparous controls. Tumor burden was evaluated by caliper measurement twice weekly following tumor cell injection, and calculated as width \times length \times length \times 0.5. Mice were sacrificed at 3-weeks post-injection for tissue and blood isolation. Lymph node-free mammary gland 4–5 (tumor free mice) or tumor tissue was collected for flow cytometry, immunohistochemistry.
and western blot analysis. Primary blood leukocytes were collected by density gradient cell separation of whole blood using Ficoll then analyzed by flow cytometry. Blood granulocytes were uniformly removed across reproductive groups due to Ficoll separation.

**Tissue digestion and immune cell enrichment**

Single cell suspensions were prepared from mammary gland or tumor dissection by dicing tissue into 1mm fragments, followed by digestion for 2hours at 37°C in 1mg/mL collagenase (Sigma) and 10µg/mL hyaluronidase (Sigma) in HBSS while rotating. Spleens were diced into 1mm fragments and digested for 20mins at 37°C in 1mg/mL collagenase. Digestion mixtures were quenched using RPMI containing 10% FBS and filtered through a 70µm nylon strainer (BD Biosciences). Total cell digest of mammary gland and spleen were stained with trypan blue and total viable cell count determined by hemocytometer analysis. T cells were negatively selected from spleens using the T cell enrichment set (BD Biosciences), and CD11b$^+$ cells were positively selected from mammary glands or tumors, using CD11b magnetic particles (BD Biosciences) per manufacturer’s instructions. The purity of isolation for all cell types was >90%, as evaluated by flow cytometry.

**Leukocyte co-culture assay**

Spleens were harvested from mice and T cells enriched as described above. CD11b$^+$ cells were isolated from mammary glands or tumors as described above. Autologous splenic CD3$^+$ T cells (8×10$^5$ cells) were co-cultured 1:1 with CD11b$^+$ cells (8×10$^5$ cells) in RPMI 1640 (Invitrogen) containing 10% FBS and 1% penicillin-streptomycin (HyClone) in a tissue culture grade 96-well plate. Mouse T-activator CD3/CD28 Dynabeads (Invitrogen) were used to activate T cells with and without CD11b$^+$ cells and after 96 hours T cells were analyzed by flow cytometry for CD3, CD4, CD8, CD25, and CD69. Percentage T cell activation co-cultured with CD11b$^+$ cells was normalized against % activation by T cells stimulated with CD3/CD28 Dynabeads (Supplementary Fig. S3B and S6B) and was calculated as fold change.

**Human tissues**

Breast specimens from pre-menopausal women age 20–45 who underwent clinically indicated biopsies or surgical procedures were obtained from a Colorado Multiple Institution Review Board approved protocol. Adjacent normal mammary tissues, confirmed free of pathology by a clinical pathologist, were grouped by reproductive categories of nulliparous (n=7), ≤1 month (n=6), >1–≤6 months (n=4), >6–≤12 months (n=5), >12–≤18 months (n=5), >18–≤24 months (n=7), >2–≤3 years (n=6), >3–≤6 years (n=8), >6–≤10 years (n=6), and >10 years postpartum (n=5).

**Statistical analysis**

All data were analyzed using the Student’s t test and are expressed as mean +/- Standard error of the mean (SEM) unless otherwise noted. Differences were considered significant when the P values were <0.05. Welch correction was used when variances between the groups were significantly different.
**Results**

**Dynamic influx of immune cells during postpartum mammary gland involution**

To characterize the immune cell composition of the murine mammary gland during postpartum involution, we utilized flow cytometry on whole mammary gland digests. Dendritic cells, granulocytes, macrophages, CD4+ T cells, CD8+ T cells, and regulatory T cells were investigated in nulliparous (Nullip), lactation day 10 (L10), weaning-induced involution (INV) days 1–4, 6, 8, and 28, and 12 weeks post-weaning regressed (Reg) mice (Supplementary Fig. S1). Dendritic cells were recruited early to the mammary gland, peaking on day 2 of involution (Fig. 1A). Granulocytes, positive for early myeloid lineage markers CD11b and GR1high, remained constant throughout the reproductive cycle (Fig. 1B). Macrophages, dual positive for the immature myeloid lineage marker GR1 and the differentiation macrophage marker F4/80, increased during mid-involution days 6–8 (Fig. 1B), in agreement with published data4,13. T helper and cytotoxic T cells increased during mid-involution (Fig. 1C). Further, Foxp3+ regulatory T cells (Tregs) increased eight-fold on involution day 6 (Fig. 1D). These patterns of immune cell influx were mostly resolved by involution day 28, with continued elevation in macrophages and CD4 T cells, and fully resolved by 12 weeks postpartum. Taken together, these data demonstrate that the postpartum mammary gland utilizes immune programs to involute. Importantly, the early influx of dendritic cells followed by macrophages and T cells is a temporal pattern of immune cell infiltration consistent with classical wound healing patterns19.

**Involuting mammary gland immune profile is not apparent in the circulation**

To determine whether the dynamic influx of immune cells into the involuting mammary gland coincides with changes in systemic immune cell populations, we analyzed blood samples corresponding to the reproductive stages described above. The dynamic changes in immune cell populations observed in the involuting mammary gland were not mirrored in the blood (Supplemental Fig. 2). Dendritic cells increased two-fold over nulliparous levels by day 4 of involution, and this increase was delayed with respect to the peak levels observed in the mammary gland (Supplemental Fig. 2A). Circulating monocytes, positive for both GR1 and F4/80, increased modestly over nulliparous levels, and preceded the increase in macrophage levels seen in the mammary gland (Supplemental Fig. 2B). Surprisingly, given the significant T cell subset changes within the mammary gland itself, systemic CD4+, CD8+, and Treg levels during involution were not significantly altered (Supplemental Fig. 2C–D). These data indicate that the immune cell influx observed in the mammary gland is largely independent of circulating immune cell levels, and likely driven by local, mammary specific events.

**Involution macrophages exhibit markers of immune suppression**

Tumor microenvironments enriched in macrophages predict poor outcomes in breast cancer20,21. Macrophages present in the involuting mammary gland have been previously described by immunohistochemistry as alternatively activated/M2 macrophages, as they express arginase-1 (Arg-1) and the mannose receptor (MRC1)4. To further characterize the macrophage programing state and their function during normal involution, we isolated CD11b+ cells from nulliparous and day 4 and 6 involuting mouse mammary glands and
evaluated them for classical M1 and M2 markers. The isolated CD11b+ cells were found to be approximately 90% positive for GR1 and F4/80, data consistent with a macrophage phenotype (Supplemental Fig. S3A). Both nulliparous and involution CD11b+ cells yielded similar levels of the M2 marker Arg-1 and the M1 marker iNOS (Fig. 2A). However, the involuting CD11b+ cells compared to nulliparous mammary gland derived CD11b+ cells expressed significantly higher levels of the M2 marker MRC1 and the inflammatory cytokine Allograft Inflammatory Factor-1 (AIF-1) (Fig. 2A). During tissue injury, resolution-macrophages have been shown to express both M1 and M2 markers23; suggesting involution macrophages may have a similar programing state to macrophages present during wound healing. As an independent assessment of tissue macrophages within the involuting gland, tissue sections from day 4 involuting mammary glands were stained for AIF-1 and Ly6c (Fig. 2B), a marker of GR1+ monocytes that have been shown to influx into inflamed tissues24. The presence of Ly6C+ cells within the involuting mammary gland is consistent with an immature macrophage phenotype25,26.

Given the role for alternatively activated/M2 macrophages in tumor induced immune suppression4,21, we evaluated for markers of immune suppression in macrophages isolated from the normal involuting gland. Approximately 30% of the involuting CD11b+, F4/80+, GR1int/low macrophages identified by flow cytometry were positive for the cytokine IL-10 (Fig. 2C); with intracellular IL-10 levels peaking during the remodeling phase at involution days 6–8. Macrophage expression of IL-10 is consistent with a reparative programing state of wound healing27, where it contributes to local immune suppression28. To test the ability of these CD11b+ cells to suppress T cell activation, we utilized an ex vivo assay where autologous splenic T cells in the presence of stimulatory CD3/CD28 beads were co-cultured with mammary gland CD11b+ cells. In this assay, CD11b+ cells isolated from involution day 4 and 6 mice significantly inhibited the upregulation of T cell activation markers CD69 and CD25, and reduced T cell IFN-γ levels in the media compared to CD11b+ cells isolated from nulliparous mammary glands (Fig. 2D–E and Supplemental Fig. S3B). We did not observe an increase in apoptotic T cells when T cells were co-cultured with CD11b+ cells (Supplemental Fig. S4). CD11b+ cells isolated from involuting mammary glands express high levels of the IFN-γ receptor compared to nulliparous CD11b+ cells (Fig. 2F); suggesting involution CD11b+ cells may deplete IFN-γ from the co-cultures. The IFN-γ receptor has previously been shown to be expressed by suppressive monocytic-myeloid derived suppressor cells (MO-MDSCs)29 and in our study CD11b+ cells enriched from mammary gland tumors also express the IFN-γ receptor (Fig. 2F). In summary, the phenotype and function of involution CD11b+ cells are similar to immature myeloid cells (iMCs) or MO-MDSC, previously described in the literature as CD11b+GR1int/lowLy6c+Ly6glow cells that express low levels of F4/8025,26,30.

Postpartum, immune competent model elucidates a role for macrophages in tumor promotion

The influx of immature macrophages during normal mammary gland involution led us to hypothesize that the immune microenvironment of the involuting gland favors the growth of breast cancer. To begin to address this question, we injected murine mammary D2A1 carcinoma cells into the mammary fat pads of Balb/cJ mice that were nulliparous or at day 1
of weaning-induced involution. In this immune competent model, D2A1 cells formed invasive carcinomas that were negative for the estrogen receptor, progesterone receptor and Her 2 neu (Supplemental Fig. S5A). Beginning at 1.5 weeks post-tumor cell injection, the involution group mice had significantly larger tumors compared to the nulliparous group (Fig. 3A). By three weeks post-injection, the involution group mice had an average tumor size that was six-fold greater than the nulliparous group. Additional tumor studies taken out to 5 weeks post-injection show the involution group have a continued tumor growth advantage over nulliparous (Supplemental Fig. S5B).

Macrophage infiltration in human breast cancer correlates with recurrence in lymph-node positive patients\textsuperscript{20} and overall poor clinical outcomes\textsuperscript{31, 32}. To investigate if involution group tumors have increased macrophage content or changes in other immune cell profiles, we characterized the mammary mouse tumors for immune cell infiltrates. Tumors arising in the involution microenvironment had higher levels of total leukocytes (CD45\textsuperscript{+}), with the vast majority (93\%) of the leukocytes being CD11b\textsuperscript{+} cells (Fig. 3B). The CD11b\textsuperscript{+} were further characterized as GR1\textsuperscript{−}F4/80\textsuperscript{+} and GR1\textsuperscript{int/lo}F4/80\textsuperscript{+}, and both populations were present in significantly higher levels within tumors from the involution group compared to the nulliparous group (Fig. 3C and Supplemental Fig. S5C). Granulocytes (CD11b\textsuperscript{+}GR1\textsuperscript{high}) made up a small percentage of the total CD11b\textsuperscript{+} cells within the tumor, and were not significantly different between groups (Fig. 3C). Since effector T cells are instrumental in orchestrating immune rejection of a tumor, we next characterized infiltrating T cells in the primary tumors as well as in the peripheral blood. Analysis of the primary tumors and peripheral blood showed that involution group had significantly lower numbers of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells both within the tumor and systemically (Fig. 3D–F and Supplemental Fig. S5C). When comparing the ratio of macrophages to CD8\textsuperscript{+} T cells present within the tumors to that observed in the normal gland, we observed a nineteen-fold increase in macrophages over CD8\textsuperscript{+} T cells within nulliparous tumors and a seventy-fold increase in the involution tumors (p=0.009). The large increase in macrophages and decrease in infiltrating CD8\textsuperscript{+} T cells in the involution group is indicative of involution tumor macrophages suppressing effector T cell infiltration, and possibly function.

Further characterization of tumor infiltrating macrophages show that CD11b\textsuperscript{+} cells isolated from involution tumors had similar expression of Arg-1, AIF-1, and iNOS compared to nulliparous group CD11b\textsuperscript{+} cells; however, involution CD11b\textsuperscript{+} cells had significantly higher levels of the M2 marker, MRC1 (Fig. 4A). Enriched CD11b\textsuperscript{+} cells from mammary tumors were approximately 75\% GR1\textsuperscript{int/lo}F4/80\textsuperscript{+} and 15\% GR1\textsuperscript{int}F4/80\textsuperscript{+} by flow cytometry (Supplemental Fig. S6A). Functionally, we found these involution tumor infiltrating CD11b\textsuperscript{+} cells suppressed effector T cell responses more than CD11b\textsuperscript{+} cells isolated from nulliparous tumors, as analyzed by CD25, CD69 and IFN-\gamma levels (Fig. 4B–C and Supplementary Fig. S6B). In summary, these murine data show that postpartum mammary tumors are characterized by immune cell infiltrates that display markers previously associated with immune suppression; observations consistent with the transient presence of macrophages in the involuting mammary gland durably influencing the tumors that arise during this unique developmental window.
IL-10 depletion inhibits postpartum mammary gland tumor growth

The cytokine IL-10\(^+\) has been implicated in tumor promotion through immunosuppression\(^{33-35}\), and the infiltration of IL-10\(^+\) macrophages during involution identifies this cytokine as a potential mediator of postpartum tumor promotion. To determine whether IL-10 influences tumor promotion, we systemically depleted the cytokine using an IL-10 neutralizing monoclonal antibody (mAb) in nulliparous and involution groups with treatment being limited to 10 days. Compared to isotype IgG controls, involution group mice treated with an IL-10 mAb had significantly decreased tumor size (Fig. 5A). In contrast, treatment of nulliparous group mice with αIL-10 did not affect tumor size, consistent with the tumor promotional effect of IL-10 being specific to the postpartum involution microenvironment. Importantly, while αIL-10 treatment ended on day 8 of involution, a delay in tumor growth persisted to study end, at 3 weeks post weaning. Characterization of intratumoral CD4 and CD8 T cells at study end showed that αIL-10 treated involution group mice had significantly higher levels of infiltrating T cells compared to involution IgG treated mice (Fig. 5B and C). There was no significant change in CD4 and CD8 T cells between nulliparous αIL-10 and IgG-group tumors (data not shown), suggesting that αIL-10 is specifically targeting the involution microenvironment. The demonstrated efficacy of this involution-limited treatment further supports postpartum involution as a window of opportunity for targeted therapies.

IL-10\(^+\) and Foxp3\(^+\) immune cells infiltrate the involuting human postpartum breast

To investigate potential relevance of immune modulation in human postpartum breast involution, we evaluated young women’s adjacent normal breast tissue for IL-10\(^+\) and Foxp3\(^+\) cells by immunohistochemistry. We observed significant increase in IL-10\(^+\) cells during active breast involution, ≤1 month post-lactation (Fig. 6A). In a single involuting case, we found ~50% of the IL-10\(^+\) cells were CD68\(^+\), 25% were CD4\(^+\), 15% were CD19\(^+\) and <2% were CD8\(^+\) (Fig. 6B and Supplemental Fig. S7A–C). These data indicate infiltration of IL-10\(^+\) subsets of macrophages as well as T and B cells. Involution breast tissue also contained an elevated number of Foxp3\(^+\) immune cells that co-stained for CD4, suggesting the presence of regulatory T cells (Fig. 6C and Supplemental Fig. S7D). The increase in IL10\(^+\) and Foxp3\(^+\) immune cells in the human breast specifically during postpartum involution is suggestive of an immune suppressed microenvironment.

Discussion

We show for the first time that murine postpartum mammary gland involution is characterized by an orchestrated influx of immune cells that mirror classic wound healing. Moreover, mammary tumors exposed to the involution microenvironment are infiltrated by immature macrophages that inhibit T cell activation ex vivo and involution tumors have a growth advantage that is IL-10 dependent. These data are consistent with the transient wound healing programs of involution creating a pro-tumorigenic immune microenvironment within the tumor that persists beyond the narrow window of postpartum mammary gland remodeling. Further, we show potential relevance to women as increased IL-10\(^+\) and Foxp3\(^+\) immune cell infiltrate are observed in the human postpartum involuting breast. This demonstrated link between mammary gland involution and wound healing-like...
immune environment provides compelling evidence to investigate immune based mechanisms for the poor prognosis of postpartum breast cancer.

In previous studies, we and others report similarities between normal mammary gland weaning-induced involution and wound healing microenvironments, including deposition and remodeling of fibrillar collagen, increased matrix metalloproteinase activity, elevated M2-like macrophages, and T helper 2 (Th2)-associated cytokines IL-4, IL-13, TGF-β, as well as gene expression profiles consistent with immune infiltrate\textsuperscript{3, 4, 9, 13, 36–38}. While these markers are consistent with wound healing, evidence for involvement of immune cells classically associated with wound healing and resolution have been lacking. Here we identify the immune cell composition across postpartum mammary gland involution and demonstrate resemblance with the inflammation, proliferation, and remodeling/resolution phases associated with classic wound healing\textsuperscript{19, 39}. Wound healing begins with an initial infiltration of phagocytic neutrophils and antigen presenting dendritic cells, which prevent infection and initiate an adaptive immune response, respectively\textsuperscript{19, 39}. Similarly, we find the first immune cells to enter the gland post weaning are dendritic cells. Next, in both wounds and mammary gland involution, this initial inflammatory response is followed by macrophage and lymphocyte infiltration, which in wounds are known to augment the inflammatory phase and the transition to the proliferative phase\textsuperscript{19, 39}. Finally, during wound healing, transition to wound-resolution occurs with the loss of immune cells, in part through programmed death\textsuperscript{40}. We find complete resolution in the involuting mammary gland occurring between 4 and 12 weeks post weaning. Importantly, when apoptotic cells are phagocytized by macrophages, macrophages are stimulated to an immune-suppressive phenotype, characterized by TGF-β and IL-10 expression\textsuperscript{23, 27, 41–43}. We show a similar immune-suppressed mechanism may occur during mammary gland involution, as the monocytic cells present in involuting tissue display markers of alternative activation, express the classically immunosuppressive cytokine IL-10 and are capable of inhibiting effector T cell activation markers \textit{ex vivo}. Further, we find an eight-fold increase in regulatory T cells during mid to late involution. Tregs provide a checkpoint against inflammation during wounding by suppressing multiple cell types including CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, B cells, natural killer (NK), NKT cells, and antigen presenting cells\textsuperscript{44}. Thus, the observed increases in macrophages with T cell suppressive function and regulatory T cells during involution are further suggestive of classic wound resolution.

The association between wound healing and tumor promotion has been recognized as a potential therapeutic target for several decades\textsuperscript{45}. Relevance in breast cancer has recently been obtained, as mammary gland biopsies in rodents promotes lung metastasis through recruitment of immune cells\textsuperscript{46} and a skin-wound adjacent to a mammary tumor stimulates tumor progression\textsuperscript{47}. These studies support a potential for tumor promotional effects of wound healing programs associated with normal mammary gland involution. Hormone regulation of immune cell infiltrate during breast involution remains a provocative but uninvestigated area. Studies have shown biologically-active estrogens and androgens in breast extracellular matrix (ECM); ECM that promotes invasiveness, tumorigenicity, and metastases in rodent models\textsuperscript{48}. Further, in rodent models, circulating estrogens are
responsible for recruiting ERα+ bone marrow cells that promote mammary tumor growth\textsuperscript{49, 50}.

In order to investigate the potential role of the involution immune cell milieu in breast cancer progression, we developed an immune-competent murine model of postpartum breast cancer. In this model, we demonstrate that tumor size is increased six-fold in the involution microenvironment compared to nulliparous hosts. Additionally, mammary tumors in the postpartum group have high levels of immature macrophages and reduced cytotoxic T cell levels, an immune cell profile predictive of poor prognosis in human breast cancer patients\textsuperscript{20, 21}. Further, we show potential relevance in women, as normal adjacent breast tissue from postpartum women within 1 month of cessation of lactation have infiltration of IL-10\textsuperscript{+} and Foxp3\textsuperscript{+} expressing immune cells compared to all other reproductive stages characterized. Of note, expression of these classic immune suppression markers in the normal involuting gland is confined to a relatively narrow developmental window; however, our data show that postpartum tumors display an immune profile consistent with tumor progression beyond this developmental window. Such persistence could be explained by the establishment of a positive feedback loop within the tumor. Evidence that a transient inflammatory signal can initiate a positive feedback loop resulting in a persistent, inflammatory autocrine-loop within tumor cells has been recently described\textsuperscript{51}.

We further demonstrate that neutralizing the immunosuppressive cytokine IL-10 during involution decreases growth of postpartum tumors, demonstrating a causal relationship between tumor progression and IL-10 as well as the feasibility of targeting this interaction. Cytokine IL-10 depletion during involution resulted in increased T cell infiltration to tumors, data consistent with loss of immune suppression. While the correlation of immune cell infiltrate and poor prognosis in breast cancer patients has been well studied\textsuperscript{17, 20, 31, 32}, evaluation of the immune and cytokine presence specifically in postpartum breast cancers is lacking and deserves further attention. Of note, in our mouse model, αIL-10 treatment did not decrease tumor levels to that observed in nulliparous hosts, indicating additional pro-tumorigenic targets exist during postpartum involution. Supportive of this, fibular collagen has also been identified as a stromal mediator of postpartum tumor progression, and COX-2 inhibition during involution blocked both fibular collagen deposition and tumor progression in this model\textsuperscript{5}.

In summary, our study demonstrates the dynamic composition of immune cells during normal mammary gland involution and suggests the window of mammary gland involution as a localized immune-suppressed state. We find murine postpartum tumors to be characterized by low T-cell infiltrate and the presence of immature macrophages with T cell suppressive activity. These data justify future research investigating the role of immune suppression in postpartum breast cancers and raise the possibility of using immune therapies for the prevention and treatment of these poor prognostic breast cancers.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgements

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References


Novelty and Impact Statements

Young women diagnosed with postpartum breast cancer have poor prognosis for unknown reasons, and in all age groups regardless of reproductive history, breast cancers characterized by high macrophage number and low T cell number have poor outcomes. Here we link a transient immune suppressed microenvironment of postpartum involution with tumor monocyte infiltrate and low T cells, implicating immune suppression in the poor prognosis of young women’s postpartum breast cancer.
Figure 1. Evidence for wound-like immune cell influx during mammary gland involution
Flow cytometry analysis of immune cells in mammary gland digests from mice at distinct reproductive stages: nulliparous (Nullip), lactation day 10 (L10), involution (INV) days, 1, 2, 3, 4, 6, 8, and 28, and 12 weeks postpartum regressed (Reg). (A) Dendritic cells (CD11c+MHC-II+), (B) granulocytes (CD45+CD11b+GR1highF4/80−), macrophages (CD45+CD11b+ GR1int/loF4/80+), (C) CD4+ T cells (CD3+CD4+), CD8+ T cells (CD3+CD8+), and (D) regulatory T cells (Treg) (CD3+CD4+CD25+FOXP3+). Mean values ±SEM (n=3–4/mice/group), *P<0.05 vs nullip.
Figure 2. Macrophages from the involuting mammary gland display markers of immune suppression

(A) Representative immunoblot of CD11b+ cell lysates isolated from Nullip, INV D4, and INV D6 mammary glands for macrophage markers: Arg-1, iNOS, MRC1, AIF-1, and GAPDH. Protein expression normalized to GAPDH by densitometry (right panel). Mean arbitrary units ±SEM (n=5–6 mice/group), *P<0.02 vs nullip. (B) Immunohistochemistry of INV D4 mammary gland tissue for macrophage markers AIF-1 and Ly6c. Scale bar, 120µm. (C) IL-10+ macrophages (CD45+CD11b+GR1int/loF4/80+) in the mammary gland. Mean
values ±SEM (n=3–4 mice/group), *P<0.03 vs nullip. (D) CD25+ (Di) and CD69+ (Dii) T cells following stimulation and co-culture with CD11b+ cells from nullip, INV D4 or INV D6 mammary glands, data normalized to stimulated T cells. Fold change ±SEM (n=5 mice/group analyzed in duplicate), *P<0.0001 vs nullip (E) CD11b+ cells from INV D4 and INV D6 mammary glands suppress IFN-γ production in mixed leukocyte assays. Left, media only; middle, T cells stimulated with CD3/CD28 beads; and right stimulated T cells co-cultured with CD11b+ cells. Fold change ±SEM (n=5/group analyzed in duplicate), *P<0.0001 vs Nullip CD11b+. (F) Representative immunoblot of CD11b+ cell lysates isolated from Nullip, INV D4, INV D6 mammary glands, Nullip and INV tumors for IFN-γ receptor (IFN-γR) and GAPDH.
Figure 3. Postpartum involution increases tumor growth and immune cell infiltration

(A) Average tumor volume from Nullip and INV group mice ±SEM (n=10 mice/group), *P<0.001 vs nullip. (B) Percent leukocyte (CD45*) and myeloid cells (CD45*CD11b*) is increased in INV group tumors. Mean % of total cells ±SEM (n=5 mice/group), *P<0.01 vs nullip. (C) Mean frequency of GR1\text{int/lo} F4/80*, GR1\text{hi} F4/80*, and GR1 F4/80+ shows elevated macrophages in INV tumors ±SEM (n=5 mice/group), *P<0.003 vs nullip. (D) Mean frequency of CD4+ T cells (CD3+CD4+) and CD8+ T cells (CD3+CD8+) shows decreased infiltration in INV tumors ±SEM (n=5 mice/group), *P<0.005 vs nullip. Mean frequency of circulating (E) monocytes and (F) CD4+ and CD8+ T cells ±SEM (n=5 mice/group), *P<0.04 vs nullip.
**Figure 4. Tumor infiltrating macrophages from involution group tumors suppress T cell activation**

(A) Representative immunoblot of CD11b+ cells isolated from Nullip or INV group tumors for: Arg-1, iNOS, MRC1, AIF-1, and GAPDH (left panel). Protein expression normalized to GAPDH by densitometry (right panel) shows INV tumor macrophages enriched for M2 marker, MRC1. Mean arbitrary units ±SEM (n=5–6 mice), *P<0.004 vs nullip.

(B) CD25+ and CD69+ T cells following stimulation and co-culture with CD11b+ cells from Nullip and INV tumors, data normalized to stimulated T cells. Fold change ±SEM (n=5–6 mice/group analyzed in duplicate), *P<0.05 vs nullip.

(C) CD11b+ cells from INV group tumors suppress IFN-γ production in mixed leukocyte assays. Fold change ±SEM (n=5/group analyzed in duplicate), *P<0.0001 vs Stim vs nullip.
Figure 5. Postpartum mammary gland tumor growth is delayed by IL-10 depletion

(A) Primary tumor growth of nullip and INV group mice treated with αIL-10 neutralizing antibody or rat IgG1 isotype control with treatment schedules depicted with arrows. Average tumor volume ±SEM (n=5–12 mice/group), *P<0.04 vs INV IgG, ** P<0.0001 vs INV IgG. Mean frequency of CD4+ (B) and CD8+ (C) T cells are elevated in αIL-10 INV group tumors ±SEM (n=5–6 mice/group), *P<0.05 vs INV IgG.
Figure 6. Human postpartum involution breast tissue shows increased IL-10+ and Foxp3+ cell infiltration

Quantitative immunohistologic assessment of (A) IL-10 and (C) Foxp3 in histologically normal, adjacent human breast tissue obtained from premenopausal women: nulliparous (NBP), ≤1 month, >1–≤6 months, >6–≤12 months, >12–≤18 months, >18–≤24 months, >2–≤3 years, >3–≤6 years, >6–≤10 years, and >10 years postpartum. Mean % positive area ±SEM, *P<0.02 vs NBP. Immunohistochemistry of (B) dual stain for CD68 (green) and IL-10 (red) and (D) Foxp3. Pictures represent normal adjacent breast tissue obtained from a women ≤1 month post-lactation. Black arrows represent single stained cells and open arrows represent dual stained cells. Scale bar, 10µm.