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Mammary Gland Involution as an Immunotherapeutic Target for Postpartum Breast Cancer

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Abstract Postpartum mammary gland involution has been identified as tumor-promotional and is proposed to contribute to the increased rates of metastasis and poor survival observed in postpartum breast cancer patients. In rodent models, the involuting mammary gland microenvironment is sufficient to induce enhanced tumor cell growth, local invasion, and metastasis. Postpartum involution shares many attributes with wound healing, including upregulation of genes involved in immune responsiveness and infiltration of tissue by immune cells. In rodent models, treatment with non-steroidal anti-inflammatory drugs (NSAIDs) ameliorates the tumor-promotional effects of involution, consistent with the immune milieu of the involuting gland contributing to tumor promotion. Currently, immunotherapy is being investigated as a means of breast cancer treatment with the purpose of identifying ways to enhance anti-tumor immune responses. Here we review evidence for postpartum mammary gland involution being a uniquely defined 'hot-spot' of pro-tumorigenic immune cell infiltration, and propose that immunotherapy should be explored for prevention and treatment of breast cancers that arise in this environment.

Keywords Macrophages · Immunotherapy · Microenvironment · Chemoprevention

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Abbreviations

ATP	adenosine triphosphate
arg-1	arginase 1
AMP	adenosine monophosphate
BMI	body mass index
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
CK	cytokeratin
COX-2	cyclooxygenase-2
CSF-1	colony stimulating factor-1
CSF-1R	colony stimulating factor-1 receptor
CTLA-4	cytotoxic T-lymphocyte antigen 4
CXCL	chemoattractant chemokine (C-X-C motif) ligand
ECM	extracellular matrix
EGF	epidermal growth factor
FDA	Food and Drug Administration
FGF	fibroblast growth factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
ICE	interleukin-1β converting enzyme
IFNγ	interferon gamma

IL	interleukin
iNOS	inducible nitric oxide synthase
Inv	Involution
Lac	lactation
LBP	lipopolysaccharide binding protein
LRP1	low density lipoprotein-related protein 1
LPC	lysophosphatidylcholine
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein 1_2
MHC	major histocompatibility complex
MMPs	matrix metalloproteinases
MMTV	mouse mammary tumor virus
MSC	myeloid suppressor cell
NK	natural killer
NOD	non-obese diabetic
NSAIDs	non-steroidal anti-inflammatory drugs
PD-L1	programmed death ligand 1
PD-1	programmed cell death protein 1
PGE_2	prostaglandin E_2
Preg	Pregnant
PyMT	polyoma virus middle T antigen
Reg	Regressed
SCID	severe combined immunodeficiency
STAT3	signal transducer and activator of transcription 3
TGF-β	transforming growth factor beta
TNFα	tumor necrosis factor alpha
Treg	regulatory T cell
uPA	urokinase-type plasminogen activator
UTP	uridine-5'-triphosphate
VEGF	vascular endothelial growth factor
Vir	Virgin

Introduction

In the breast, epithelial cells are the source of milk and the target of oncogenic transformation; thus, understandably, the field of mammary gland biology is epithelial cell-centric. However, in the last decade, stromal-epithelial interactions, including immune cell interactions, have been recognized as key to physiologic and pathologic breast development. In all organs, including breast, the percent of tissue composed of immune cells is high. Data obtained from immunohistochemical analyses and genetic models that permit lineage tracing estimate a minimum of 10–30 % of cells within "epithelial" organs as being of immune origin [1, and references therein]. Immunohistochemical analyses for epithelial and immune cell lineage markers demonstrate that this is also true for human breast tissue (Fig. 1). Historically, the role of immune cells in the mammary gland was thought to be restricted to immune-surveillance, particularly during lactation and postpartum involution due to increased risk for mastitis. More recently, a paradigm shift has occurred and immune cells are being studied as obligate partners in normal tissue development. The mammary gland field has led in this area, in large part due to the pioneering macrophage work from the laboratory of Jeffery Pollard [2]. One advantage of the mammary gland as a model to study the roles of immune cells in normal development is that the majority of mammary development occurs postnatally, over the course of weeks in rodents, which is in contrast to fetal organ development that occurs within hours and days. Roles for immune cells in mammary gland development have been identified in pubertal duct elongation [3, 4], estrous cycle regulation [5, 6], gland expansion during pregnancy [7], cell death during weaning-induced involution [8], and adipocyte repopulation after weaning [9]. In this review, our objective is to explore immune cells in the postpartum involuting mammary gland as potential targets for the prevention and treatment of postpartum breast cancers.

Postpartum Breast Cancer

Following pregnancy, women experience a transient increase in breast cancer risk that peaks approximately 5 to 6 years postpartum and may persist for up to 30 years [10–14]. Over time, the increased risk following pregnancy diminishes, such that a crossover in risk occurs and women who have had their first birth below age 35, have a lower breast cancer risk than age-matched women who have never given birth [10, 12, 15–17]. The phenomenon of a transient increase in breast cancer risk postpartum followed by protection was first described by Janerich and Hoff in the early 1980s and is referred to as the "dual effect" of pregnancy [17]. Breast cancer diagnosis in the postpartum period has been identified as an independent risk factor for poor outcomes [12, 18]. Stensheim et al. reported 11 year survival rates of 33 % for breast cancer cases diagnosed within 7 months postpartum, compared to 69 % for non-pregnancy-associated cases [19]. Surprisingly, this same study found that survival rates in cases diagnosed during pregnancy were not significantly different from nulliparous cases [19]. These data are further supported by subsequent studies from Johansson et al. reporting similar results for 10 year survival rates [20]. More recently, Callihan et al. reported a breast cancer diagnosis within 5 years of a recent pregnancy independently associated with a 2.8-fold increased risk for metastasis and a 2.7-fold increase in mortality as compared to nulliparous cases [21]. This study was unique in the large number of cases with known reproductive histories (n=619), and thus indicates that postpartum breast cancer carries a significantly worse prognosis when diagnosed within 5 years postpartum, an assertion also supported by earlier,

albeit lower powered studies [22–24]. One potential mediator underlying the poor prognosis of breast cancer diagnoses following pregnancy is postpartum mammary gland involution [12, 25].

Postpartum Mammary Gland Involution

In virgin rodents, the mammary gland consists of a rudimentary, epithelial ductal network embedded within a stroma comprised of adipocytes, extracellular matrix, fibroblasts, immune cells, blood vessels, and lymphatics. Upon pregnancy, the epithelium extensively proliferates to meet the demand of lactation (Fig. 2). Recent lobular analysis of human breast tissue has revealed a >10-fold increase in epithelial content in the lactating breast [26]. Following lactation, or pregnancy if lactation does not occur, the mammary gland undergoes the process of postpartum involution to return to a state morphologically resembling the relatively simple ductal network of the pre-pregnant gland (Fig. 2). Though involution has been predominantly characterized in rodent models, support for postpartum involution similarly eliminating lactationally-competent lobules in women is demonstrated by the observation that the epithelial content in the breast following pregnancy becomes indistinguishable from that of nulliparous women within 18 months postpartum [26].

In rodents, postpartum involution is characterized by programmed death of the majority of alveolar epithelial cells, extracellular matrix remodeling, leukocyte infiltration, and adipocyte repopulation [27–30]. The involution process has been described as occurring in two phases—a reversible cell-death phase with maintenance of the lobuloalveolar structures, followed by a non-reversible, tissue-remodeling phase with additional cell death and lobuloalveolar loss [27]. Through teat-sealing experiments, the initial phase of involution has been found to be locally regulated by milk stasis and in mice, lasts approximately 48 h following cessation of lactation [31, 32]. Recently, it has been reported that cell death during the first phase of involution is lysosomally-mediated through signal transducer and activator of transcription 3 (STAT3) and activation of cathepsins B and L [33]. Somewhat surprisingly, in a separate study, macrophages were found to be essential for execution of cell death during involution, as distended milk-filled lumens and STAT3 activation were not sufficient to induce cell death following macrophage depletion [8]. How macrophages mediate epithelial cell death during early involution remains to be determined. The non-reversible tissue remodeling phase of involution is characterized histologically by loss of lobuloalveolar structures and adipocyte repopulation [27]. This phase is regulated by changes in systemic hormones and associated with downregulation of protease inhibitors and upregulation of proteases, including matrix metalloproteinases (MMPs) 2, 3, 9, and 11, interleukin-1β converting enzyme (ICE), and urokinase-type plasminogen activator (uPA) [28, 31, 34–37]. The complexity of weaning-induced mammary gland involution and its regulation are also evident from transcriptional activity analyses. In one murine study, nine temporal patterns of expression profiles were identified, each with distinct gene ontology pathways [29]. However, one unifying theme when comparing histological, immunological, biochemical and RNA expression profiling data across involution is evidence for tissue remodeling reminiscent of wound healing.

Similarities Between Physiologic and Pathology-Associated Tissue Remodeling

Physiologic tissue remodeling during postpartum involution shares multiple attributes with wound healing, a process known to be tumor promotional [28, 29, 38–43]. These attributes include expression of the inflammatory mediator

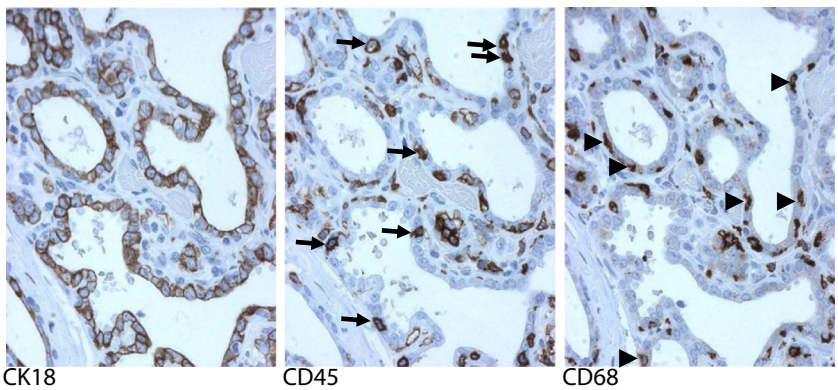

Fig 1 Serial sections of human involuting breast tissue demonstrate immune cells in very close proximity to breast epithelium. Representative immunohistochemical staining (brown) for the epithelial cell marker cytokeratin 18 (CK18), the general leukocyte marker CD45, and the macrophage marker CD68 are shown. CD45 and CD68 positive cells within the breast epithelium are indicated with arrows and arrowheads, respectively. Modified with permission from O'Brien et al. [39]

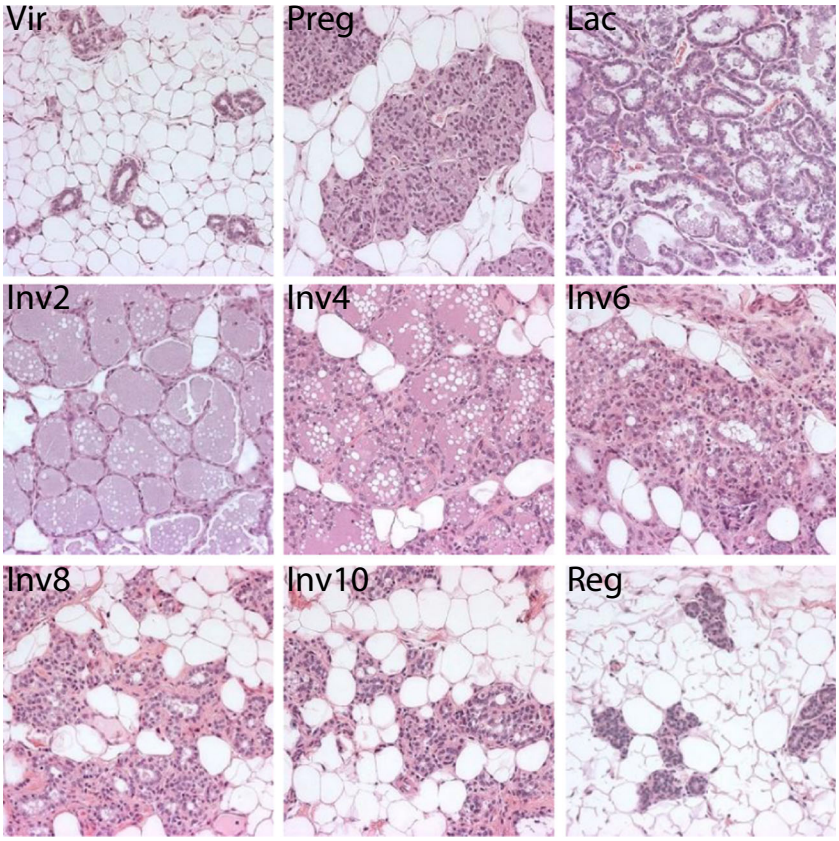

Fig 2 Epithelial regression and adipocyte repopulation during postpartum involution in the rat mammary gland. Hematoxylin and eosin staining of rat mammary tissue generated as described [39]. Vir = virgin, Preg = pregnancy day 18, Lac = lactation day 10, Inv2–10 = 2–10 days post-wean, Reg = Regressed (4 weeks post-wean)

cyclooxygenase-2 (COX-2), elevated protease activity as described above, release of bioactive extracellular matrix fragments, deposition of fibrillar collagen and significant leukocytic infiltration [29, 38–42, 44]. Based on the similarities between wound healing and postpartum involution, the involution-hypothesis was proposed to account for the increased metastasis and poor prognosis of breast cancers diagnosed following pregnancy [25]. In support of this hypothesis, orthotopic injection of human tumor cells into mammary glands of immunocompromised mice on involution day one leads to increased tumor growth, local invasion, and metastatic seeding as compared to tumor cells injected into mammary glands of virgin mice [18, 45]. Here we review data implicating the immune microenvironment in promoting mammary tumors in the postpartum period [46].

Immune Milieu of the Postpartum Involuting Mammary Gland

During postpartum involution, increased expression of immune-related genes and leukocyte infiltration is observed in the absence of overt inflammatory insult [47]. Provocative evidence for immune cell involvement in postpartum involution was provided by microarray analyses demonstrating upregulation of waves of immune-related genes throughout the involution period in weaning-induced murine models. Within the first 12 h post-weaning, upregulation of acute-phase response genes were observed, such as STAT3, lipopolysaccharide-binding protein (LBP), cluster of differentiation (CD) 14, and inflammatory mediators, including interleukin (IL)-1α, IL-1β, and IL-13 [29, 38]. In addition, the neutrophil chemoattractant chemokine (C-X-C motif) ligand (CXCL) 1 and the neutrophilic-granulocyte marker leucine-rich α_2-glycoprotein (LRG) were also upregulated during early involution, peaking 2 days post-wean [29, 38]. These neutrophil-associated gene expression data were corroborated by histological analysis demonstrating neutrophil influx with early involution [29]. This initial wave of pro-inflammatory gene expression and neutrophil infiltrate was followed by increased expression of monocyte and macrophage chemoattractants including chemokine (C-C motif) ligand (CCL) 6, CCL7, CCL8, and CXCL14, followed by upregulation of macrophage-specific antigens themselves, including colony stimulating factor-1 receptor (CSF-1R), CD68, and low density lipoprotein-related protein (LRP1), all of which peaked at 72–96 h post-wean [29, 38]. Consistent with upregulation of macrophage chemoattractants and antigens, macrophage infiltration into the involuting gland has been reported in numerous studies in mice and rats, with peak macrophage influx occurring mid- to late-involution [29, 39, 48]. Furthermore, increased numbers of eosinophils, mast cells, and

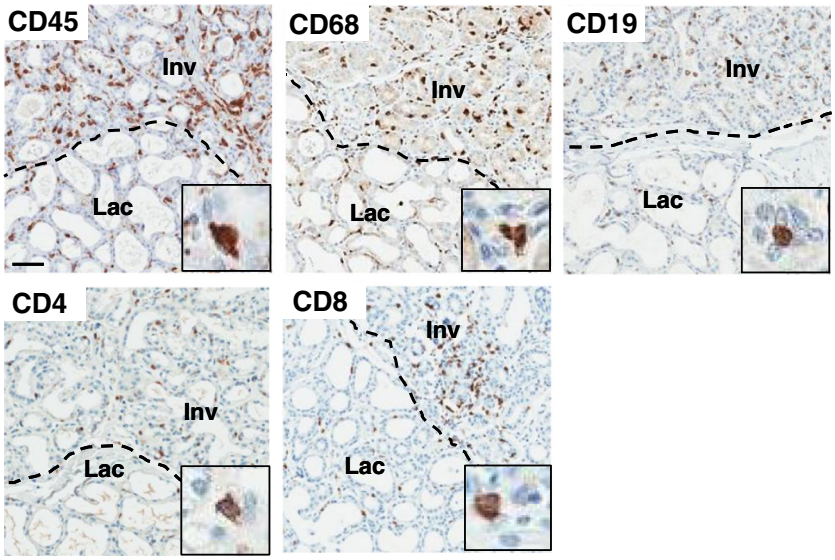

Fig 3 Postpartum involuting lobules have increased immune cell infiltrate. Immunohistochemical staining for the general leukocyte marker CD45, the macrophage marker CD68, the B cell marker CD19, and the T cell markers CD4 and CD8 in actively involuting (Inv) and lactating (Lac) human breast tissue lobules. Scale bar = 100 μM; inset = representative positively-stained cell at 6× magnification of the larger image

plasma cells have been described in the involuting mammary glands of rodents [29, 49, 50].

In women, extensive characterization of immune cell infiltration during postpartum breast involution has been hindered by lack of tissue; however, data to date are consistent with involvement of immune cells during involution in women as observed in rodents. Immunohistochemical staining for the general leukocyte antigen CD45 is markedly increased in involuting breast lobules (Fig. 3), and is significantly increased in breast tissue in the first 12 months postpartum [26, 39]. Within this 12 month postpartum window, macrophages are also significantly increased, as determined by positivity for CD68, a marker highly expressed on macrophages (Fig. 3) [26, 39]. In addition, we observe increased presence of CD4-, CD8-, and CD19-positive cells, consistent with the presence of effector T and B cells within involuting lobules of the postpartum breast (Fig. 3).

Of the immune cells present in the involuting rodent mammary gland, macrophages are the most well-characterized. During maturation, monocytes mature into a spectrum of macrophage phenotypes in response to the surrounding cytokine milieu. The two ends of this spectrum include the classically-activated and alternatively-activated macrophages, also variably referred to as M1 or M2, respectively [51–53]. Classically-activated macrophages are promoted by tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), IL-1β, and lipopolysaccharide (LPS), and can be identified by inducible nitric oxide synthase (iNOS), IL-1, and IL-12 expression. These macrophages have pro-inflammatory and antigen presentation properties, thus likely function in immunosurveillance. In contrast, IL-4, IL-10 and IL-13 promote alternatively activated macrophages, which can be identified by expression of arginase-1 (arg-1), mannose receptor, FIZZ1, Ym1, IL-1RA, and IL-10 [51–53]. Alternatively-activated macrophages function in immunosuppression, wound repair and tissue remodeling [51, 54]. In the context of cancer, classically-activated macrophages are thought to function predominantly in tumor cell elimination, while alternatively-activated macrophages have tumor-promotional attributes and share many properties with tumor-associated macrophages [52, 55]. Importantly, macrophages in the involuting mammary gland have been characterized as having an alternatively-activated phenotype based on arg-1 and mannose receptor expression [39]. Known inducers of alternative macrophages, IL-4, IL-10, and IL-13, are also increased in the involuting gland [39, 56], data consistent with promotion of alternative activation.

It is anticipated that elucidating how the immune microenvironment of the involuting gland is established and maintained will reveal immunotherapeutic targets for postpartum breast cancer. However, regulation of immune cell infiltration, differentiation and activation in the postpartum involuting mammary gland remains largely unexplored. Possible mediators of the immune milieu attributes include COX-2 expression by mammary epithelial cells, generation and clearance of apoptotic cells, extracellular matrix remodeling, adipocyte repopulation, and changes in systemic hormones.

COX-2 Expression by Mammary Epithelial Cells

In the mammary gland, normal mammary epithelial cells are the dominant cell type expressing the inflammatory mediator COX-2, and furthermore, epithelial cell expression of COX-2 increases during involution [44]. In mammary tumor models, COX-2 overexpression is sufficient to induce tumorigenesis and is associated with tumor cell migration and invasion [57–62]. COX-2 is an enzyme involved in formation of prostaglandins from arachidonic acid. Prostaglandin E_2 (PGE_2) is considered the dominant prostaglandin secreted in cancer and

has many pro-tumorigenic properties, acting on tumor cells themselves, as well as on the tumor microenvironment. In addition, COX-2 expression and synthesis of PGE_2 contribute to a tumor-promotional immune microenvironment. PGE_2 promotes tumor-associated macrophages by inhibiting pro-inflammatory cytokines, such as IL-12, required for classical macrophage activation [63, 64]. Furthermore, PGE_2 has been implicated in recruitment and promotion of suppressive myeloid cells and regulatory T cells, both of which inhibit anti-tumor immunity [65]. In a murine model of postpartum breast cancer, COX-2 inhibition reduced tumor promotion during postpartum involution [62]. Moreover, NSAID treatment during postpartum involution reduced mammary PGE metabolite levels [66]. Altogether, these data implicate a role for mammary epithelial cell-derived COX-2 in establishing a pro-tumorigenic immune milieu during involution.

Generation and Clearance of Apoptotic Cells

The dominant feature of postpartum involution is death and removal of the lactationally-competent mammary epithelium. While macrophages from the involuting gland are capable of engulfing apoptotic cells, temporal morphometric analyses and gene knockout of the apoptotic cell receptor MerTK identifies mammary epithelial cells as the primary phagocyte [48, 67]. Insights from previous studies in other non-involution systems have demonstrated that apoptotic cell clearance contributes to local immune suppression required for maintenance of tissue homeostasis and prevention of autoimmunity. Upon binding to apoptotic cells, professional phagocytes, such as macrophages, have been shown to promote a locally suppressive environment through production of TGF-β, PGE_2, and IL-10 [68–70]. Consistent with mammary epithelial cells similarly promoting local immune suppression during involution, mammary epithelial cells secrete TGF-β and, in vitro, are able to suppress pro-inflammation cytokine production upon engulfment of apoptotic cells [71]. Furthermore, STAT3 expression by mammary epithelial cells contributes to the immune milieu in the involuting gland, and is implicated in poor prognosis in breast cancer [49, 72]. In addition to contributing to an immunosuppressive environment, phagocytic mammary epithelial cells may also directly contribute to tumor promotion by functioning like tumor-associated macrophages, as suggested by elevated production of the pro-angiogenic factor vascular endothelial growth factor (VEGF) [73, 74].

While studies characterizing the immune suppressive environment of apoptotic cell clearance have primarily focused on the role of phagocytes in this clearance, the apoptotic cells themselves also contribute directly to the surrounding immune milieu. Apoptotic cells promote recruitment of macrophages and other phagocytes to areas of cell death by releasing cellular constituents such as lysophosphatidylcholine (LPC), adenosine triphosphate (ATP), and uridine-5′-triphosphate (UTP) which bind to specific receptors on phagocytic cells [75–77]. Following leukocyte recruitment, apoptotic cells induce immune suppressive gene responses in local leukocytes by releasing adenosine monophosphate (AMP), transforming growth factor-beta (TGF-β), and PGE_2 [78, 79]. Furthermore, PGE_2 from apoptotic cells also suppresses local T cell function [80, 81]. Given the vast amount of apoptosis occurring in the involuting mammary gland, it is anticipated that the apoptotic cells themselves, as well as the phagocytic mammary epithelial cells responsible for their clearance, contribute to an immune suppressive milieu which facilitates tumor progression [46].

Extracellular Matrix

The tissue-remodeling phase of postpartum mammary gland involution results in numerous changes in extracellular matrix (ECM) abundance, organization, and proteolysis, and ECM from involuting rat mammary glands has demonstrated pro-tumor activity. Culturing of MDA-MB-231 human breast tumor or D2.OR murine mammary tumor cells on ECM isolated from involuting rat mammary glands leads to disruption of cell-cell adhesion junctions, loss of apical-basal polarity and induction of front-back polarity, resulting in formation of more invasive cells as compared to cells cultured on matrix isolated from nulliparous rat mammary glands [28, 66]. Furthermore, in orthotopic xenograft models, tumor cells co-injected with involution-mammary ECM metastasize at high rates [28]. These data indicate that involution-mammary ECM is sufficient to enhance tumor growth and metastasis in breast cancer models, with increased abundance of collagen and tenascin-C, radial alignment of collagen, and proteolysis of collagen, fibronectin and laminin all implicated as mediators [28, 39, 42, 62, 66, 82]. While these ECM changes likely have direct pro-tumorigenic effects on tumor cells themselves, the role of ECM proteins in influencing immune complexity of the involuting mammary gland cannot be discounted. ECM has roles in immune cell recruitment, activation, and function [83, 84]. Of potential relevance to the protumorigenic environment in the postpartum involuting mammary gland, some collagen and laminin fragments are chemotactic for neutrophils and macrophages [39, 85, 86]. Collagen has also been found to regulate tumor cytotoxicity by macrophages [85, 87]. Treatment of lung alveolar macrophages with native and synthetic collagen peptides enhance cytotoxicity by macrophages against both normal and transformed cells [85]. Conversely, culturing macrophages on collagen fiber-coated plates impaired their ability to kill target cells [87]. Tenascin-C is another involution-associated ECM protein that may modify leukocyte function during involution, as tenascin-C has been found to inhibit T cell activation in multiple models [88–92]. Interestingly, in rodent models, the tumor-promotional properties of ECM isolated from involuting glands can be ameliorated by NSAID treatment during involution [66].

Specifically, mammary gland collagen and tenascin-C deposition are reduced by NSAID treatment [66, 62], raising the hypothesis that epithelial cell-derived COX-2 activity during involution promotes changes in the ECM, which in turn alter leukocyte function.

In addition to the ability of ECM proteins to directly influence recruitment of immune cells and their subsequent activation, ECM can also serve as a reservoir for cytokines. Many ECM molecules have glycosaminoglycan side chains that interact with and sequester cytokines (as reviewed by [93]), including fibroblast growth factor (FGF), IFNγ, TNFα, IL-8, CCL12, IL-4, and TGF-β [94–96]. Upon ECM cleavage and remodeling during mammary gland involution [40, 42, 97], cytokines may be released from ECM, permitting them to act upon local immune cells. For example, upon protease activation (thrombospondin, MMP2, MMP9), engagement of integrin binding (αvβ6), or mechanical stress upon the ECM [98], active TGF-β fragments are released from ECM stores [98–100]. TGF-β inhibits T cell cytotoxicity [101], macrophage effector function [102], neutrophil activation [103], and NK cell activity [104]. In addition, TGF-β can augment immunosuppression by down-regulating major histocompatibility complex (MHC) expression and enhancing Treg function [105, 106]. IL-4 levels may also be regulated by the ECM during involution as IL-4 also binds glycosaminoglycan side chains [96]; thus, ECM remodeling and cleavage during postpartum involution may release IL-4 into the local environment, and notably, IL-4 levels are increased in the mammary gland during involution [39].

Another mechanism by which ECM proteolysis appears to modulate immune cell function is through release of bioactive fragments (matrikines) that have different activities than the intact molecule. The anti-angiogenic factors endostatin, a 20kD C-terminal fragment of collagen XVIII, and tumstatin, a fragment of collagen IV α3 chain, serve as classic examples of matrikines [107, 108]. It has been found that specific ECM matrikines alter immune cell chemotaxis, phagocytosis, differentiation, activation, and cytokine production. Fibronectin fragments, which are generated during mammary gland involution [28], can enhance phagocytosis and oxidative burst in monocytes and macrophages [109–111]. In addition, fibronectin matrikines can increase macrophage production of the protease MMP9, and the pro-inflammatory cytokines IL-1, IL-6, and TNFα [109, 112, 113]. Laminin fragments, which are also generated during mammary gland involution [28], can function as matrikines by attracting macrophages and enhancing expression of the proteases MMP9 and MMP14, as well as TNFα [114, 115].

Adipocyte Repopulation

In rodent models, the adipocyte content of the mammary gland changes dramatically across the pregnancy-lactation-involution cycle. In the nulliparous mouse mammary gland, adipocytes occupy approximately 97 % of the tissue volume; however, the adipocyte content of the lactating gland is only ~10 % [116]. During involution, adipocyte re-population occurs such that adipocyte content in the fully regressed gland is comparable to that of pre-pregnancy [116]. While the relationship between adipocytes and immune cells during postpartum involution is yet to be investigated, the role of adipocytes in obesity-associated inflammation indicates that adipocytes are worth considering as potential contributors to the immune profile of the involuting gland. During involution, adipocytes may have roles in both macrophage attraction and activation; adipocytes can make CCL2 (also known as monocyte chemoattractant protein 1 (MCP-1)), which is upregulated during postpartum involution, though the cell type responsible for CCL2 expression is unknown [39, 117, 118]. In addition, adipocytes influence macrophage activation through production of leptin [119, 120]. Leptin has been demonstrated to promote a combination of alternative and classical activation phenotypes in macrophages, inducing mannose receptor expression as well as the pro-inflammatory cytokines TNF-α, IL-6, IL-1β, and IL-1RA [119]. Leptin can also serve as a chemoattractant for monocytes and macrophages [121]. Importantly, leptin expression is increased in mammary adipocytes and ductal epithelium throughout involution [122]. Cumulatively, these data support a role for adipocyte regulation of macrophage activation during postpartum involution.

Hormones

In a model of postpartum breast cancer, treatment with the aromatase inhibitor letrozole led to reduced tumor growth of estrogen receptor negative tumors, indicating estrogen may act on stromal cells to contribute to tumor promotion [45]. Co-injection of tumor cells and bone marrow isolated from estrogen-treated NOD/SCID mice into nulliparous hosts was sufficient to promote tumor growth, providing support for estrogen acting on cells of hematopoietic origin [45]. Additional support for a potential role for estrogen in modulating the immune profile of the mammary gland comes from studies demonstrating changes in macrophage number and phenotype in the mammary gland across the estrous cycle and with estradiol and progesterone treatment [5, 6]. Further, in a wound-healing model, estradiol and progesterone were found to promote an alternative phenotype in macrophages [123]. However, it should be noted that estradiol can also promote a pro-inflammatory phenotype in macrophages [124]. Mast cell number in the mammary gland has also been found to fluctuate throughout the estrous cycle and during involution in rats [50]. Furthermore, in response to estrogen and progesterone, mast cells upregulate chemokine receptors, maturation markers, and degranulate [125, 126]. While changes in T and B cells have not been reported in

the mammary gland during the estrous cycle, there is considerable evidence indicating these cells can also be directly regulated by estrogen [127–132] and as such, should be considered as potential targets of hormone regulation in the postpartum mammary gland.

Immune Mediators of Breast Cancer Promotion

The immune environment of the involuting mammary gland is anticipated to contribute to poor outcomes in postpartum breast cancer. In breast cancer patients, increased macrophage infiltration into primary tumors correlates with tumor cell proliferation and significant decreases in relapse-free and overall survival [133–137]. In addition, high levels of the macrophage chemoattractant and growth factor colony stimulating factor 1 (CSF-1) and the chemoattractant CCL2 associate with breast cancer metastasis and poorer outcomes [138, 139]. Notably, CCL2 significantly increases in the mammary gland during involution [39]. Direct evidence supporting a role for macrophages in breast cancer progression comes from murine studies. For example, in the MMTV-PyMT mammary carcinoma model, macrophage depletion through CSF-1 deletion slows tumor progression and significantly decreases metastasis, while overexpression of CSF-1 increases macrophage infiltration in primary tumors and elevates rates of metastasis [140]. Subsequently, it was reported that $CD4^+$ T cells indirectly regulate metastasis in this model by inducing a pro-tumorigenic phenotype in CD11b(+)Gr1(−)F4/80(+) macrophages via IL-4 secretion [141]. Moreover, therapeutic depletion of macrophages through delivery of CSF-1 receptor (CSF-1R) antagonists, in combination with taxol-based chemotherapy, significantly improves outcomes for mice harboring mammary carcinomas by reducing metastasis through $CD8^+$ T cell-dependent mechanisms [142].

In addition to macrophages, there is convincing evidence that myeloid suppressive cells (MSCs) and regulatory T cells (Tregs) play important roles in breast cancer promotion. In breast cancer patients, elevated numbers of MSCs correlate with increased clinical stage and metastasis, and are also associated with a poorer response to chemotherapy [143, 144]. Similarly, increased numbers of Tregs within the tumor and peripheral blood correlates with disease progression and worse outcomes for breast cancer patients [145–147]. MSCs and Tregs contribute to tumor progression predominantly by suppressing anti-tumor immunity in both innate and adaptive immune cells. One example of adaptive immune suppression by MSCs is suppression of T-cell activation and proliferation, occurring in part through increased MSC production of arginase and iNOS that depletes local L-arginine levels [148]. L-arginine is required for expression of the T cell receptor associated ζ chain, a receptor essential for T cell activation and subsequent proliferation; thus, L-arginine depletion by

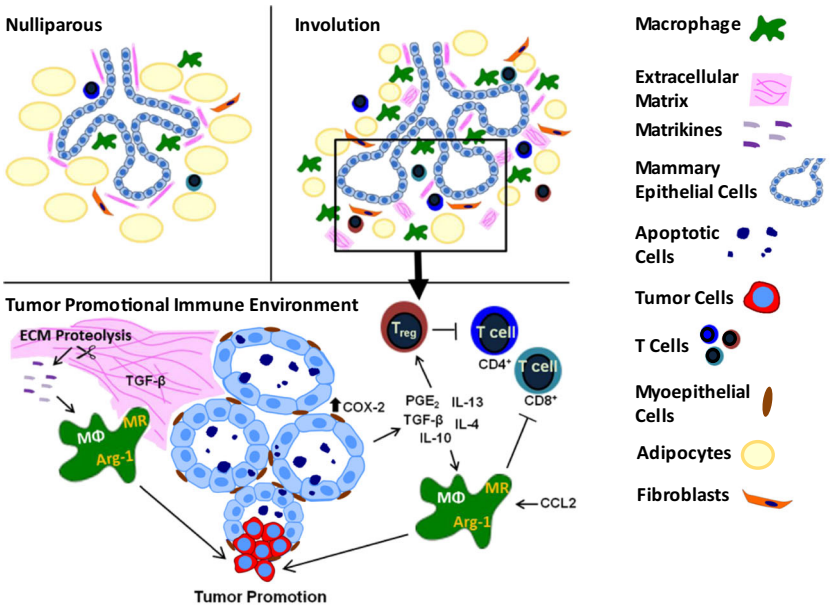

Fig 4 Macrophages as orchestrators of a tumor-promotional immune environment during postpartum mammary involution. Alternatively activated macrophages, characterized by mannose receptor and arg-1 expression, increase in the mammary gland during involution. Involution macrophages are anticipated to contribute to tumor promotion directly through the production of growth factors, such as epidermal growth factor (EGF) [176], and indirectly by suppressing anti-tumor immunity. Targeting macrophage recruitment and activation may be one way to alleviate macrophage-induced tumor promotion during postpartum involution. Involution macrophage recruitment and activation are anticipated to be promoted by ECM components, cytokines, growth factors, and prostaglandins, all of which represent potential immunotherapeutic targets directed toward involution macrophages

MSCs (and macrophages) contributes directly to T cell suppression [142, 148, 149].

Potential Immunotherapeutic Targets During Postpartum Involution

The influx of leukocytes into the postpartum involuting mammary gland, and the known roles for immune cells in breast cancer progression, raises the question of whether immunotherapy during normal mammary involution could block incidence or progression of postpartum breast cancer. The goal of immunotherapy is to activate a patient's own immune system to elicit an anti-tumor response that detects and eliminates cancer cells. Cancer immunotherapies include vaccines, adoptive cell transfer, and targeting tumor-associated macrophages and immune checkpoint pathways (Reviewed in [150]). Here, we discuss the potential of targeting involution macrophages and immune checkpoints, as well as the use of NSAIDs, in postpartum breast cancer.

Involution macrophages have been proposed to contribute to the poor prognosis of postpartum breast cancer [25], and as such represent one target for immunotherapy directed at blocking the tumor-promotional attributes of the involuting gland (Fig. 4). Targeting tumor-associated macrophage recruitment and activation has had therapeutic success in pre-clinical mammary cancer models. For example, reducing macrophage recruitment into murine mammary tumors by blocking CSF-1 or CSF-1R decreases tumor growth and metastasis and increases sensitivity to chemotherapy [142, 151, 152]. These data indicate that targeting CSF-1/CSF-1R may be beneficial in postpartum breast cancer as well. However, CSF-1R is not expressed on all macrophage populations in the involuting mammary gland [39], indicating that anti-CSF-1/CSF-1R treatment may need to be combined with additional therapies when targeting involution. Notably, levels of the macrophage chemoattractant CCL2 greatly increase in the involuting gland and precede the macrophage influx [39], identifying CCL2 as an additional target with immunotherapeutic potential. Blocking CCL2 levels in models of non-small cell lung cancer was found to effectively reduce local immunosuppression and enhance development of vaccine-mediated anti-tumor immunity [153]. Another way to target involution macrophages in postpartum breast cancer would be to "re-educate" macrophages away from a tumor-promotional, alternatively-activated phenotype toward classical activation with increased anti-tumor attributes. In murine mammary carcinoma models, inducing classical activation in tumor-associated macrophages with GM-CSF treatment or by targeting STAT3 expression has proven successful at reducing tumor growth, angiogenesis, and metastasis [154, 155]. Candidate cytokines to target for macrophage "re-education" in the involution gland include IL-4, IL-13, IL-10, and TGF-β (Fig. 4) [39, 56, 156, 157]. Given the role of resident macrophages in the execution of cell death during postpartum involution [8], successful immunotherapies targeting involution macrophages will be those which tip the balance of the immune microenvironment toward one of anti-tumor immunity, while allowing involution to progress unabated.

In addition to promoting anti-tumor immunity through macrophages, targeting immune checkpoints is another way to relieve immunosuppression in the tumor microenvironment. Immune checkpoints are negative regulators of the immune system that modulate the extent of immune responses and function in maintaining self-tolerance [158]. Antibodies targeting the immune checkpoint molecules cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed death ligand 1 (PD-L1) are finding success in the clinic. Ipilimumab, a monocolonal antibody against CTLA-4, has been FDA approved since 2011 for metastatic melanoma and has been successfully combined with other immunotherapies [159]. Immune checkpoint modulation is currently being evaluated in breast cancer, with early studies showing promise [160].

As an alternative approach to targeting specific cell types or molecules, targeting the immune environment with general anti-inflammatory agents may also be effective in the involuting mammary gland. NSAID treatment limited to the window of involution has previously been identified as a potential strategy for prevention and treatment of postpartum breast cancer through ECM- and tumor cell-mediated mechanisms [62, 66]. Effects on the immune cells were not evaluated in these studies, as they utilized orthotopic xenograft models in immunocompromised mice. However, given the function of NSAIDs as anti-inflammatory agents, it is likely that the immune microenvironment is also affected and warrants further investigation.

Limitations and Future Questions

In rodents, characterization of the immunologic programs activated during postpartum involution has utilized forced-weaning models to synchronize involution programs throughout the entire gland. While there are multiple advantages of these models, including the ability to perform molecular analyses under controlled conditions, it is important to acknowledge potential limitations as the field of postpartum breast cancer research progresses. In most circumstances, forced-wean models do not fully recapitulate the onset of involution in women, which more frequently occurs through a gradual-weaning process. Recently it has been proposed that abrupt weaning, as used in the postpartum breast cancer models described here, may

be associated with an increased risk of developing breast cancer, while gradual weaning may protect against breast cancer [161]. This hypothesis has yet to be tested; however, epidemiologic data are consistent with gradual involution contributing to tumor promotion, as postpartum women, regardless of lactation history, are at increased risk for early onset breast cancer with poor prognosis [21]. Characterization of involution in women may provide some insight, as gradual involution is associated with lobule by lobule regression with hallmarks of a tumor-promotional environment associated with involuting lobules, but not adjacent lactational lobules [26, 39]. Since local interactions between tumor cells and the microenvironment are shown to be sufficient to promote metastasis [162], it is anticipated that tumor cells present within involuting lobules may be promoted in this environment independent of whether the neighboring lobules are lactating or involuting.

An additional question to address moving forward is the role of lactation. Multiple meta-analyses using both case–control and large cohort studies support a role for lactation in reducing overall breast cancer risk [18, 163]. Briefly, these meta-analyses revealed modest reduction in breast cancer risk with any lactation [164], reduction in risk with prolonged lactation [165], and/or no correlation between lactation and reduction in breast cancer risk [166]. The results of these studies may be confounded by the difficulties associated with obtaining accurate lactation history. In addition, breast cancer is a complex disease with significant differences in onset and/or severity based on tumor biologic subtype, as well as patient race, age, body mass index (BMI), age at first birth, time since last child birth, and menopausal status [21]. Thus, more refined analyses are necessary for additional insight into the role of lactation in breast cancer risk as well as disease prognosis. Several recent studies have taken such an approach.

In a case–control study of Tanzanian pre-menopausal women, prolonged lactation was associated with modest risk reduction (OR 0.98, 95 % CI 0.97–0.99) [167]. Furthermore, in the Carolina Breast Cancer Study, a cohort of primarily African American women, and the Black Women's Health Study, lack of breastfeeding was associated with significant increases in risk for basal-like and hormone receptor negative breast cancers, respectively [168, 169]. An additional study, from a cohort of pre-menopausal primarily white women, revealed that risk for triple negative tumors was similarly increased in women who did not breastfeed [170]. In addition, Stuebe et al. revealed that breastfeeding was protective in premenopausal women with a family history that included a first-degree relative with breast cancer [171]. In contrast, some studies have reported that breast cancers diagnosed during lactation exhibit aggressive phenotypes and poor survival rates [19, 172]. However, in these studies the lactation group was defined as less than 2 years or less than 6 months postpartum, respectively, and no data were presented to indicate whether the women were lactating at the time of diagnosis. Thus, the data are likely confounded by inclusion of women who are post-lactational and at higher risk for more aggressive tumors and poor survival rates [21]. Recently, several studies have more specifically examined the role that breastfeeding may play in promoting more aggressive disease phenoytpes. In one study, breastfeeding for more than 12 months was associated with *increased* risk for triple negative tumors compared to luminal A tumors in women of Mexican descent [173]. Furthermore, a study of Swedish women revealed that excessive milk production during breastfeeding and breastfeeding for >12 months was associated with a two-fold increased risk for early breast cancer events, defined as new, local, regional, or distant recurrence in primary breast cancer patients [174]. While these studies are in contrast to data from a transgenic rodent model of continuous lactation, which revealed that the lactogenic microenvironment protected against mammary tumor growth and lung metastasis [175], more recent data support a role for mammary adipose stromal cells obtained from lactating mammary glands in breast tumor promotion [116]. Cumulatively, these studies highlight the need for additional animal models to address the role of lactation and involution in mammary tumor promotion. Furthermore, longitudinal prospective studies on the effects of lactation and weaning on breast cancer risk with women grouped by race, age at diagnosis, BMI, parity status, menopause status, and tumor biologic subtype may shed light on the roles for lactation and involution in breast cancer risk.

Conclusion

The increased rate of metastasis and poor prognosis of postpartum breast cancer are anticipated to be due, in part, to the pro-tumorigenic immune milieu of the involuting mammary gland. While exposure to gestational hormones and lactation may contribute to risk and poor prognosis of breast cancers diagnosed in the postpartum period, therapies targeted to the postpartum window have clear benefits. For example, strategies targeting pregnant or lactating women have the undesirable consequence of cross-targeting the developing fetus or infant. However, the postpartum involution window is unencumbered by these potential problems. The dramatic upregulation of immune-associated genes and influx of immune cells into the involuting gland indicate that immunotherapeutic strategies may be particularly effective. Future work should be directed toward investigating the efficacy of immunotherapies directed toward the window of postpartum mammary involution as preventive and therapeutic agents for postpartum breast cancers.

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